

## LSD is on a New High

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**Background/Introduction:** The 1960s became the first decade of recreational psychedelic use for lysergic diethylamide (LSD). By the 1970s, legal restrictions slowed availability while publicity of horrifying experiences dissuaded its popularity. A new generation of enthusiasm arose in the 2010s with synthetic 2C phenethylamine derivatives such as 25I-NBOMe, but reports of fatal toxicity soon dampened its spread. Swiftly and quietly, LSD and its derivative 1P-LSD have been reintroduced within the past couple of years to cause a resurgence of law enforcement interventions and hospital visits due to LSD intoxication. In this presentation, we present a series of LSD-positive cases showing an increasing trend of abuse and explore the concentrations found in blood and urine specimens.

**Objective:** After completing this activity, attendees will be able to analyze new trends in LSD use and interpret the concentration ranges typically found in blood or urine of human performance toxicology casework.

**Method:** Blood and/or urine specimens were submitted by military law enforcement and screened by a CEDIA-based LSD immunoassay for presumptive positive identification at a 0.5 ng/mL cutoff. All LSD positive screens were further characterized by liquid chromatography tandem mass spectrometry for confirmation using a linear calibration model spanning 0.05 ng/mL to 2.0 ng/mL for LSD and 0.25 ng/mL to 10 ng/mL for 2-oxo-3-hydroxy-LSD.

**Results:** In the past couple of years, 27 LSD positive cases have been confirmed in our laboratory. The first case arrived in late 2014, with 8 submissions throughout 2015 and 14 cases in 2016, reaching a peak with 5 confirmed cases last November alone. Average blood LSD concentrations centered around 1 ng/mL, with a minimum of 0.22 and a maximum of 2.2 ng/mL. The average urine concentrations clustered at 2.4 ng/mL, but ranged from 0.05 up to 15 ng/mL. Several of the submitted cases contained both blood and urine, which allowed for a correlation plot to study the relative ratios. This study showed that urine specimens tend to represent ten-fold values above corresponding blood concentrations.

**Conclusion/Discussions:** In modern times, forensic toxicology laboratories have been racing to keep abreast of emerging novel psychoactive substances. Although LSD seems to be a drug of the past, it has been making a comeback in our casework which signals a need for renewed vigilance in screening and confirmation of blood or urine specimens. Tests in our laboratory also show that LSD immunoassays have poor cross-reactivity with the metabolite 2-oxo-3-hydroxy LSD, so we are developing a sensitive tandem mass spectrometry based panel to actively screen for recent LSD use when parent LSD may be depleted. This presentation raises awareness about the return of LSD in casework and provides a reference range for blood and urine concentrations to consider when developing screening or confirmation methods.

**Keywords:** LSD, Hallucinogen, Screening

## Detection of U-47700 in Blood and Drug Paraphernalia from Postmortem Cases

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**Background/Introduction:** U-47700 (*trans*-3,4-dichloro-*N*-(2-(dimethylamino)cyclohexyl)-*N*-methylbenzamide), also known as U4, is a US Schedule I synthetic opioid which was originally synthesized by the pharmaceutical firm, Upjohn, in the 1970s. U-47700 acts as a selective agonist of the  $\mu$ -opioid receptor and was demonstrated to have approximately 7.5 times the potency of morphine in animal models. The drug was intended to treat severe pain; however, it never progressed to human trials. Due to the public availability of the patent which detailed its synthesis, chemists in clandestine laboratories in China and elsewhere began to produce it; hence it found its way into the illicit drug market. Several deaths in the US and Europe have been attributed to U-47700 both alone, and in combination with other drugs.

**Objective:** To present postmortem cases from the Miami-Dade County Medical Examiner Department in which U-47700 was detected, either in blood or drug paraphernalia.

**Methods:** Whole blood specimens were extracted using mixed-mode solid phase extraction columns. Initial screening was performed using an Agilent gas chromatograph coupled to a mass selective detector (GC-MSD) Forensic Toxicology Analyzer, SP1 7890-0458 with Deconvolution Reporting Software (DRS). Confirmatory analysis was conducted using a Thermo Scientific Dionex Ultimate 3000 RSLC ultra high-performance liquid chromatograph coupled to a Bruker Daltonics AmaZon Speed™ ion trap mass spectrometer (UHPLC-Ion Trap-MS<sup>n</sup>) equipped with Toxtyper™ software. Drug paraphernalia (spoon residue and powder exhibits) was dissolved in 1 mL of methanol and subjected to analysis using an Agilent 5973 GC-MSD.

**Results:** U-47700 was detected in a total of eight postmortem cases dating from September 2016 to March 2017. In five of those cases it was detected in whole blood. In three cases, U-47700 was detected in drug paraphernalia only. U-47700 was detected in both blood and drug paraphernalia in one case, however it's important to note that drug paraphernalia was not available for testing in the other four positive blood cases. Seven cases involved white males between the ages of 23 years to 41 years with a mean age of 31 years and a median age of 29 years. The remaining case involved a 65 year old white female. Cause and manner of death has been ruled in seven of the eight cases, and are shown in the table below. The most recent case is still listed as pending toxicology. All cases involve two or more drugs, including fentanyl, fentanyl analogues, heroin and cocaine.

Case	Cause of Death	Manner
1	Acute Combined Drug Toxicity (Fentanyl, Carfentanil, Para-fluoroisobutyryl fentanyl, Morphine and U-47700)	Accident
2	Acute Combined Drug Toxicity (Heroin, Carfentanil and U-47700)	Accident
3	Acute Combined Drug Toxicity (Carfentanil, Fentanyl, probable Heroin, U-47700, Diphenhydramine and Cocaine)	Accident
4	Acute Bronchopneumonia due to Acute Combined Drug Toxicity (Carfentanil, Para-fluoroisobutyryl fentanyl, Diphenhydramine and U-47700)	Accident
5	Acute Combined Drug Toxicity (Cocaine, Morphine, Carfentanil, Fentanyl and Tramadol)	Accident
6	Acute Combined Drug Toxicity (Carfentanil, Fentanyl and Tramadol)	Accident
7	Acute Heroin and Carfentanil Toxicity	Accident

**Conclusion/Discussion:** Based upon the cases received in Miami-Dade County, U-47700 is present only in combination with other drugs, most commonly fentanyl, fentanyl analogues, and heroin. There were no cases that involved U-47700 alone, and all were ruled as accidental poly-drug overdoses. It is interesting to note that all the completed cases also contained carfentanil. Due to the constant evolution of synthetic drugs in the illicit market, it is imperative that toxicology laboratories adapt their testing protocols in order to keep up in this ever-changing field. The Miami-Dade County Medical Examiner Department Toxicology Laboratory is currently in the process of developing a method to quantify U-47700 by LC-MS/MS.

**Keywords:** U-47700, Synthetic Opioids, Postmortem

## Rapid Field Testing of Nicotine in E-Liquids

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**Background/Introduction:** Electronic cigarettes operate by vaporizing flavored solutions, called e-liquids, for inhalation by the user. Some regulatory issues related to e-liquids include mislabeling of e-liquids by vendors to avoid taxes on nicotine, the sale of nicotine-containing e-liquids to minors, and the addition of drugs of abuse to e-liquids. No method is currently available for analysis of drugs in e-liquids in the field.

**Objective:** The purpose of the project was the development of an accurate field test capable of analyzing e-liquids for drugs, using nicotine as a model. The field test was required to be rapid, accurate, safe, and simple.

**Methods:** Since e-liquids containing nicotine have a uniformly higher pH compared to nicotine-free e-liquids, a microchemical color test was developed as a presumptive assay. The effects of nicotine on the pH of e-liquids was assessed at variable nicotine concentration. Bromothymol blue, chlorophenol red, methyl red, and bromocresol purple were evaluated as pH indicators. A test strip was developed with the most effective and obvious visible color change, bromocresol purple, by immersing white cotton strips into a bromocresol purple solution, and then dried. A portable ultraviolet visible spectrophotometer design was also developed. Ultraviolet-visible absorbance spectra of commercial e-liquids and a nicotine standard were obtained. Existing technologies and components were combined into a portable device for the spectrophotometry of e-liquids on site, and a program for the identification of the nicotine peak in the collected spectra was developed.

**Results:** Proposed are two devices for the rapid detection of nicotine in e-liquids in a safe and straightforward manner. The bromocresol purple strips developed were tested against available commercial e-liquids and control solutions of nicotine and propylene glycol. Twenty seven commercial e-liquids were tested with varying nicotine concentrations including: 0 mg/mL, 6 mg/mL, 8 mg/mL, 10 mg/mL, 12 mg/mL, 16 mg/mL, 18 mg/mL. Control solutions of nicotine were made from 99% pure nicotine stock mixed with propylene glycol, and had concentrations of 1 mg/mL, 3 mg/mL, and 6 mg/mL. Compared to earlier attempts using different pH indicators, the bromocresol purple strips were more accurate for all e-liquids tested.

UV-Visible absorbance spectra of available e-liquids and control solutions were obtained. The program developed for the portable device analyzed these spectra. The program correctly identified nicotine in all e-liquid samples when present. Testing showed that the majority of ingredients in e-liquids does not impact the absorbance peak of nicotine.

**Conclusion/Discussions:** The problem was ultimately approached in two separate manners, a pH indication test strip and a portable ultraviolet-visible spectrophotometer. The pH test strip provides a manner of indication that is cheaper and easier to use. The test strips provide an indirect method for analyzing e-liquids by testing for the change in pH caused by nicotine. The spectrophotometer provides a more costly approach to the problem with a very accurate indication of the presence of nicotine by analyzing absorbance spectra of e-liquids.

**Keywords:** Nicotine, E-Liquids, Field Test

## Analysis of Cocaine Analogues Impregnated into Textiles by Raman Spectroscopy

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**Background/Introduction:** Cocaine trafficking in the form of textile impregnation has been encountered more commonly over time. Raman spectroscopy has been a popular testing method used for *in situ* screening of cocaine in textile and other matrices. Effective quantitative analysis of cocaine in these matrices using Raman spectroscopy is lacking.

**Objective:** The aim of this study was to develop a simple quantification method using Raman spectroscopy for the analysis of cocaine and cocaine analogues in various types of textiles.

**Methods:** The textiles used were a white towel, brown shirt, red singlet, check flannelette shirt, purple woollen jumper and blue denim jeans, covering both natural and synthetic fibres. A cocaine analogue, atropine, was used for impregnation studies based on regulation compliance consideration. Textile pieces (2 cm × 2 cm) were impregnated with 20% (w/v) atropine in methanol. After overnight drying, swatches were placed in plastic vial, where 250 µL of the internal standard (10% KSCN in water, w/v) was added and the solution was made up to 1 mL using 0.5M H<sub>2</sub>SO<sub>4</sub>. The tubes used for these samples were agitated for 2 minutes and the aqueous solution was used for Raman analysis. Raman analysis was conducted on a Renishaw inVia Raman microscope with the Renishaw WiRE 3.4 software. The source laser was 633 nm over the range of 120–4000 cm<sup>-1</sup>. Spectra were collected with 20x magnification, 100% laser power, 10s exposure time and 4 accumulations. Target peaks, at 1002 cm<sup>-1</sup> for atropine, 1000 cm<sup>-1</sup> for cocaine and 2067 cm<sup>-1</sup> for the internal standard KSCN, from the Raman spectra produced were used as the quantifying peaks.

**Results:** Atropine could easily be identified by its characteristic Raman bands despite the presence of background matrix signals arising from the textiles. The method was validated over a concentration range of 6.25–37.5 mg/cm<sup>2</sup> with a coefficient of determination R<sup>2</sup> at 0.975 for the linearity. The LOD and LOQ were estimated at 3.13 and 6.25 mg/cm<sup>2</sup>, respectively. The method was deemed precise with relative standard deviation (RSD) less than 3.53% and accurate with mean relative error (MRE) less than 3.54%. It was observed that the use of the KSCN international standard removed the need for a matrix matched calibration, allowing a calibration constructed using one type of textile to be applied to different types of textile. The Raman method was used to analyse cocaine samples prepared in the extraction medium (0.5M H<sub>2</sub>SO<sub>4</sub>) in the presence of KSCN internal standard. The quantifying peak signal intensity for cocaine was observed to be very similar to that for atropine, suggesting that a similar result would be obtained if cocaine was used in the impregnation study.

**Conclusion/Discussions:** A simple and accurate Raman spectroscopy method for the quantification of cocaine analogue impregnated in textile has been developed and validated. This proof-of-concept study has demonstrated that atropine can act as an ideal model compound to study the problem of cocaine impregnation in textile. The method has the potential to be further developed and implemented in real world forensic cases.

**Keywords:** Cocaine, Textile Impregnation, Raman Spectroscopy

## Gas Chromatography-Mass Spectrometry Determination of Heroin in Adulterated Commercial Beverage in Transport Drug Cases

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**Background/Introduction:** Drug abuse has been of significant concern to developed countries but in the recent years drug misuse has attracted attention as growing health problem in developing nations. Globally 3.6-6.9% of the adult population has used illicit drugs in the last year, drug trade expansion from Asia to Europe has driven by great demand. To avoid detection at border crossing or airport customs, drug trafficking is increasingly performed by body packing. Now, more secretive transport methods have been used as rare mode of transportation for lesser quantities, that have brought difficulties and challenges to forensic toxicologists. This year, Yuxi city public security bureau, which is located in Yunnan province, southwest border of China, cracked two transport illicit drug cases in which heroin was adulterated in commercially available beverage “JIA DUO-bao”.

**Objective:** To establish a quick method for determination of heroin in adulterated “JIA DUO-bao”. As heroin was dissolved in the beverage, it would be of crucial importance to establish an effective extraction method.

**Methods:** Two suspicious commercial beverage samples were collected, the strip experiments showed positive results. To determine the heroin in adulterated beverage, a quantifying technique of gas chromatography/mass spectrometry (GC/MS) for beverage samples was developed and heroin was extracted by solid-phase extraction (SPE).

**Results:** In present study, a simultaneous quantification method for determination of heroin and 6-acetylmorphine in beverage was developed and fully validated through added samples. Chemical decompositions of heroin to 6-acetylmorphine in beverage or during extraction were not avoided, especially under liquid-liquid extraction no matter what solvent was chosen, recovery for heroin using extraction with chloroform was 64%, however, recovery for heroin using SPE eluted with chloroform was 86%. Daily calibration for heroin (0.01-1mg/mL) and 6-acetylmorphine (0.05-1mg/mL) achieved determination coefficients of  $> 0.994$ . Limits of detection (LOD) and limit of quantification (LOQ) for heroin (LOD: 1ng; LOQ: 5ng) and 6-acetylmorphine (LOD: 5ng; LOQ: 10ng) were evaluated. Precision (heroin: 3.9%; 6-acetylmorphine: 4.1%) of this method were determined. After determination of samples, there was heroin at 3.25g and 9.13g respectively in total in two samples based on the conversion of detected 6-acetylmorphine to heroin.

**Conclusion/Discussions:** Solid-phase extraction was more effective removal of sugar and other impurities than liquid-liquid extraction avoiding decomposition of heroin. The method of solid-phase extraction combined GC/MS is suitable for the analysis of heroin in adulterated “JIA DUO-bao”.

**Keywords:** Heroin, 6-Acetylmorphine, Adulterated Commercial Beverage.

## On-site Identification of Chemical Warfare Agents by Portable Gas-chromatography Mass Spectrometry Instrument with Tri-Bed Microconcentrator and VX-G Conversion Tube

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**Background/Introduction:** In crisis and consequence management of chemical warfare/terrorism, first responders perform on-site activity for detecting dangerous materials such as chemical warfare agents (CWAs). The National Research Institute of Police Science has evaluated the detection performance of commercially available portable detection equipments such as ion mobility mass spectrometers (MS). Among those, a capillary gas chromatography (GC)/membrane-interfaced electron ionization quadrupole MS instrument exhibited valuable performance for the detection of CWAs.

**Objective:** The aim of the present work was to evaluate the detection performance of a field-portable GC-MS instrument, Hapsite ER, equipped with VX-G conversion tube and Tri-Bed concentrator, for different CWAs: choking agents, nerve agents, blister agents and lachrymators.

**Methods:** The instrument is a Hapsite ER (INFICON, East Syracuse, NY, USA) with non-evaporative getter (NEG) vacuum pump. Instrumental conditions are micro-concentration with Tri-Bed 15 mg for 3 sec or 1 min at 60°C and desorption at 180°C; carrier gas, nitrogen 2 mL/min; DB-1 column (polydimethylsiloxane fused silica; 15 m × 0.25 mm ID; 1 mm film thickness); temperature control (initial temperature of 60°C (1 min hold), ramp to 80°C at 10°C/min, then ramp to 120°C at 12°C/min, and a final ramp to 200°C (2.43 min hold) at 27°C/min; membrane, oven, probe and NEG at 80°C, 70°C, 60°C and 400°C; MS: electron ionization;  $m/z=45-300$ , 70 eV, 300 mA, 0.79 sec/scan. Hapsite ER was optionally bypassed of the micro-concentration trap and gas chromatographic separation (for analysis of gaseous CWAs), or attached with VX-G fluoridating conversion tube containing silver nitrate and potassium fluoride in front of the sampling probe (for analysis of VX or RVX). Volatile CWA's [sarin, soman, cyclohexylsarin, *O*-ethyl methylphosphonofluoridate (EtGB), *O*-isobutyl methylphosphonofluoridate (iBuGB), tabun, *O*-ethyl *N,N*-dimethylphosphoramidofluoridate (FGA), sulfur mustard, nitrogen mustard 1, 2, 3, lewisite 1] were produced in house. 2-Chloroacetophenone and chloropicrin were commercially available. Volatile CWAs were diluted with *n*-hexane, and applied to 500 mL gas sampling cylinder (GL Sciences), and vaporized by heating. Vapors of hydrogen cyanide and cyanogen chloride were generated in 10 L glass container (Kusano Science) by mixing potassium cyanide with sulfuric acid and chloramine-T solution, respectively. Vapors of phosgene and chlorine were generated by permeator (Gastec Company). The prepared vapors were applied to the Hapsite system.

**Results:** Sample vapors containing as little as 22 mg sarin, 100 mg soman, 210 mg tabun, 55 mg cyclohexylsarin, 4.8 mg sulfur mustard, 390 mg nitrogen mustard 1, 140 mg nitrogen mustard 2, 130 mg nitrogen mustard 3, 120 mg 2-chloroacetophenone and 990 mg chloropicrin per cubic meter could be confirmed after Tri-Bed micro-concentration (1 min) and automated AMDIS (explicitation needed) search within 12 min. Lewisite 1 and *o*-chlorobenzylidene malonitrile could also be confirmed, but not quantified. Vapors of phosgene, chlorine and cyanogen chloride could be confirmed by direct MS detection at concentration levels higher than 2, 140, and 10 mg/m<sup>3</sup> respectively, by bypassing the micro-concentration trap and gas chromatographic separation. Hydrogen cyanide could not be detected. No compounds were detected when the VX and RVX samples were analyzed without the conversion tube. In contrast, by attaching the GC-MS system to the VX-G conversion tube, sample vapors of VX and RVX were converted into EtGB and iBuGB, respectively, and detected in the GC-MS. From GA vapor, not only GA but also GAF were detected. Gasoline vapor interfered with detection of sarin, VX and RVX, depending on the concentrations of gasoline and the target compounds.

**Conclusion/Discussions:** The Hapsite ER system enabled the identification of volatile CWAs as low as 1 mg per cubic meter in vapor sample.

**Keywords:** Potable GC/MS, Chemical Warfare, Detection

## The In Vitro Inhibition of Opioid Metabolism by Skeletal Muscle Relaxants

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**Background/Introduction:** The opioid epidemic continues. Opioids are potent toxins that cause death by Mu-receptor-mediated respiratory depression. Our concern is blood concentrations being shifted from tolerant to toxic by drug interactions that inhibit the metabolism of the opioid. A common co-medication with opioids, particularly for treatment of back pain, is the skeletal muscle relaxants. Little is known of the potential for these drugs to inhibit cytochrome P450 (CYP)-dependent metabolism.

**Objective:** Using in vitro methods to study the potential of 9 muscle relaxants and 1 active metabolite to inhibit human CYP-dependent metabolism of buprenorphine (CYP3A4 and 2C8), methadone (CYP3A4 and 2B6) and oxycodone (CYP3A4, 2D6 and 2C18).

**Methods:** The compounds and the upper solubility-dependent concentration ( $\mu\text{M}$ ) that could be studied were: baclofen (1000), carisoprodol (200), its metabolite meprobamate (1000), chlorzoxazone (200), cyclobenzaprine (1000), metazalone (50), methocarbamol (1000), orphenadrine (1000), tizanidine (1000) and zopiclone (50). Compounds were incubated with human liver microsomes (HLM) at 3 concentrations (1, 10, 50  $\mu\text{M}$ ; 1, 30, 200  $\mu\text{M}$ ; or 10, 300, 1000  $\mu\text{M}$ ). The opioids were added along with or 15 minutes after the inhibitor and a source of NADPH (- and + pre-incubation, respectively) to test for time-dependent inhibition (TDI). Compounds were subsequently incubated with human CYPs to screen for presumptive  $\text{IC}_{50}$  concentrations. These results were then used to design  $\text{IC}_{50}$  experiments with the same CYPs. For presumptive  $\text{IC}_{50}$  concentrations below the solubility limit a full 8-concentration test was performed with the respective CYP and opioid. If the presumptive  $\text{IC}_{50}$  was close to the solubility limit, a three-concentration experiment was designed to permit potential extrapolation of an  $\text{IC}_{50}$ . For several pairings, an  $\text{IC}_{50} >$  solubility limit was set.

**Results:** Based on the screen in HLM there was no evidence of TDI. This simplified the design of subsequent experiments to the determination of  $\text{IC}_{50}$  values. At this point we have done the CYP screen, designed and started the  $\text{IC}_{50}$  experiments. The findings to date can be summarized as follows:

Skeletal Muscle Relaxant	Therapeutic Plasma Range ( $\mu\text{M}$ )	Buprenorphine to Norbuprenorphine		Methadone to EDDP * (R-/S-)		Oxycodone (OC) to Noroxycodone		OC to Oxymorphone
		CYP 3A4	CYP 2C8	CYP 3A4	CYP 2B6	CYP 3A4	CYP 2C18	CYP 2D6
		$\text{IC}_{50}$ ( $\mu\text{M}$ )						
Baclofen	1.3-2.6	1780e	>1000	>1000	>1000	942e	>1000	>1000
Carisoprodol	4.6-14	>200	330e	174e/135e	288e/398e	39.8	61.7	190e
Meprobamate	73-110	1130e	>1000	248/148	>1000	64.6	526	>1000
Chlorzoxazone	123-212	>200	>200	151e/135e	309e/309e	198e	490e	>250
Cyclobenzaprine	0.047-0.17	479	95.5	36.3/30.2	170/200	28.8	15.5	2.04
Metazalone	4.4-8.2	>50	>50	>50	>50	67.6e	9.23	>50
Methocarbamol	108-170	973	>1000	>1000	>1000	1122e	1550e	>1000
Orphenadrine	0.37-0.74	>1000	630e	87.1/79.4	224e/309e	129	178	3.52
Tizanidine	0.016-0.06	>1000	>1000	>1000	>1000	>1000	>1000	2.40
Zopiclone	0.20-0.34	>50	>50	>50	44.7e/61.7e	142e	61.7e	12.3

Note: e – 3-point extrapolation. \* - High concentration orphenadrine interfered with EDDP chromatography, 150  $\mu\text{M}$  was the upper limit that could be tested for this pathway.

**Conclusion/Discussions:** These studies demonstrate that skeletal muscle relaxants do inhibit some CYP-mediated pathways. However, when we extrapolate using 1)  $K_i = \text{IC}_{50} / (1 + S/K_m)$  and 2)  $\text{AUC}_1 / \text{AUC}_N = 1 + [I]p/K_p$ , using our current estimates for  $\text{IC}_{50}$ , only chlorzoxazone has a  $\text{AUC}_1 / \text{AUC}_N$  ratio that exceeds 2, which is does for all of the pathways studied. For CYP3A4 metabolism of oxycodone the ratio for meprobamate exceeds 1.5, suggesting caution.

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**Keywords:** Opioids, In Vitro Drug Interactions, Skeletal Muscle Relaxants

## The National Forensic Laboratory Information System (NFLIS): Expansion Feasibility Study for Drug-related Mortality and Toxicology Findings

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**Background/Introduction:** The Drug Enforcement Administration's (DEA's) National Forensic Laboratory Information System (NFLIS) is a critical resource that supports DEA's core mission of enforcing the nation's drug laws. NFLIS has become an operational information system that includes data from forensic laboratories that conduct solid-drug analyses of over 98% of the nation's approximate 1.5 million annual drug cases. NFLIS provides detailed and timely information on drugs seized by law enforcement and analyzed by these laboratories. Information from the NFLIS supports strategic and operational drug control activities at national, state, and local levels.

**Objective:** The objective of this presentation is to describe DEA's enhanced efforts to combat diversion and identify new and emerging substances by expanding NFLIS to establish two additional continuous drug surveillance programs. These programs will provide the DEA with current information on drug-related mortality and toxicology findings supplied by medical examiner and coroner offices (ME/Cs) and toxicology laboratories (TLs) to supplement the current drug seizure data from the forensic drug laboratories. This presentation will describe the expansion feasibility study conducted by NFLIS staff and present the next steps for DEA's implementation of this NFLIS enhancement.

**Methods:** Between May 2016 and October 2016, NFLIS team members traveled to nine ME/Cs and nine TLs across the United States. The purpose of the site visits was to obtain the perspectives of ME/C and TL stakeholders regarding the feasibility, logistics of, and interest in participating in a long-term national surveillance system that monitored drug-related deaths and drug-related toxicology testing. The pilot sites were strategically selected to help inform the RTI team on short- and long-term requirements for the two data collections to be developed and successfully implemented.

**Results:** Results from the feasibility study included insight and context into the data flow, data quality, reporting feasibility, infrastructure (e.g., staffing, technologies used), and additional contexts (e.g., policies and procedures, practices, participation barriers) that are important for understanding how the data from the drug-related mortality and toxicology cases may be collected, analyzed, and reported. Data from our pilot site visits resulted in 37 recommendations within the context of known DEA priorities regarding these two new data collections.

**Conclusion/Discussions:** Attendees will gain an understanding of the NFLIS enhancement, results from a feasibility study, and steps moving forward to implement this surveillance system for drug-related mortality and toxicology findings supplied by ME/Cs and TLs.

**Keywords:** National Forensic Laboratory Information System, Drug Enforcement Administration, Drug Surveillance



## Characterization of Guanidine Based Biocidal Polymers Using Liquid Chromatography and Tandem Mass Spectrometry

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**Background/Introduction:** In 2011, the Korean Center for Disease Control and Prevention, and the Ministry of Health and Welfare reported an outbreak of biocidal humidifier disinfectant agents such as PHMG and PGH induced pulmonary disease that eventually killed more than 100 people including infants and pregnant women. Subsequent toxicological studies including inhalation toxicity of PHMG and PGH from several local research groups have shown that PHMG and PGH caused severe lung inflammation and fibrosis in histopathologically studied rats, findings identical to those patients with lung injury caused by PHMG and PGH. It is surprising that the comprehensive physical and chemical characterization of guanidine based polymers are limited despite of a vast amount of researches and extensive use of these compounds as biocidal disinfectants. Here we present mass spectrometric characterization of biocidal polymers PHMG and PGH.

**Objective:** The purpose of this study was to develop an analytical method based on a liquid chromatography and tandem mass spectrometry for characterization of PHMG and PGH and further application for a routine analysis of such compounds.

**Methods:** Agilent 6545 Q-TOF LC/MS with Thermo Aquasil C18 column using MeOH based mobile phase was used for determining molecular formula as well as structural elucidation of several PHMG and PGH subunits. The separation and quantitation of PHMG and PGH were performed using AB Sciex API 6500 triple-quadrupole linear iontrap mass spectrometer with Thermo Aquasil C18 column using MeOH based mobile phase and 0.1% TFA as an additive.

**Results:** Structural analysis of PHMG revealed that PHMG has following subunits;  $[1+(141)n+16+1]$ ,  $[100+(141)n+16+1]$ ,  $[1+(141)n+58+1]$ ,  $[100+(141)n+m-1+100+16+1]$ ,  $[(141)n+1]$ ,  $[(141)n+42+1]$ , and  $[(141)n+m-1+100+1]$ Da. Similarly, PGH showed following patterns;  $[1+(173)n+16+1]$ ,  $[132+(173)n+16+1]$ ,  $[1+(173)n+58+1]$ ,  $[132+(173)n+m-1+132+16+1]$ ,  $[(173)n+1]$ ,  $[(173)n+42+1]$ , and  $[(173)n+m-1+132+1]$ Da. In both cases, the intensity of each subunit varies depending on conditions of polycondensation reaction. Multiple reaction monitoring (MRM) transitions selected from the product ion scans of PHMG and PGH were used for LC separation. The efficiency of LC was greatly improved when the reversed phased stationary phase was used with 0.1% TFA as mobile phase additive. LC analysis of PHMG and PGH produced complicated chromatographic patterns resulting from the wide distribution of polymeric subunits as well as in source fragmentation of subunits. Indirect quantitative determination of PHMG and PGH was evaluated for the first time using selective MRM transitions and acceptable linearity with the correlation coefficients higher than 0.995 was observed.

**Conclusion/Discussions:** This study investigated the use of LC-MS/MS for characterizing biocidal polymers PHMG and PGH. Precursor and product ion scans of PHMG and PGH showed characteristic mass spectra that were used for determining the molecular formula and structure. Product ion scans of PHMG and PGH subunits generated selective MRM transitions and applied for LC separation. Reversed phase stationary phase along with 0.1% TFA as mobile phase additive was enough to fully separate PHMG and PGH within 50 min. Wide distribution of polymer contents as well as in source fragmentation of the same subunits created complicated chromatographic patterns. Indirect quantitative determination of PHMG and PGH was performed for the first time and acceptable linearity was observed. Finally MRM transitions and chromatographic conditions found in this study were successfully applied for forensic investigation purpose in separating PHMG and PGH from the commercial humidifier disinfectants.

**Keywords:** Biocidal Polymers, Guanidine, LC/MS/MS

## Analysis of Fentanyl in Vitreous Humor Compared to Blood Fentanyl Levels in Postmortem Cases

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**Background/Introduction:** Fentanyl is a synthetic opioid that has about 100 times the potency of morphine. As such, it is commonly abused for its euphoric effects. Fentanyl related overdoses have increased exponentially in recent years, which may be attributed in part to heroin packages laced or cut with fentanyl. Fentanyl is also known to exhibit some postmortem redistribution. Limited information exists on fentanyl concentrations in vitreous humor, the fluid in the eyeball, and the relationship between vitreous fluid concentrations and blood concentrations, however, due to the stability of vitreous, these concentrations may insight into the fentanyl concentration at the time of death.

**Objective:** The objective was to develop and validate a method to quantify fentanyl and norfentanyl in vitreous humor from postmortem casework and subsequently compare the vitreous concentrations to corresponding whole blood concentrations.

**Methods:** A quantitative method was developed on an Agilent® 1100 Series Liquid Chromatograph coupled with an Agilent® 6430 Tandem Mass Spectrometer to determine fentanyl, norfentanyl, and select other fentanyl analogs in vitreous humor. The method was validated to document its performance characteristics. Vitreous samples (0.5mL) were prepared using solid phase extraction cartridges. Samples with paired blood and vitreous that had quantitative values for fentanyl and/or norfentanyl were compared.

**Results:** Limits of detection and quantitation were 0.6ng/mL and 1.0ng/mL with a linear range of 1-100 ng/mL for both fentanyl and norfentanyl. The bias was less than 20% at the LLOQ, low and high controls, and within and between run precision was less than 15% across the target concentrations for both fentanyl and norfentanyl. There were 13 samples with paired peripheral blood and vitreous that were positive for fentanyl, and 11 that were additionally positive for norfentanyl. Data comparing vitreous concentrations to either peripheral or central blood for both fentanyl and norfentanyl are shown in Table 1. Additional samples continue to be analyzed and will be included in the final data set.

	Vitreous to Peripheral Blood Fentanyl Concentration Ratios	Vitreous to Peripheral Blood Norfentanyl Concentration Ratios	Vitreous to Central Blood Fentanyl Concentration Ratios	Vitreous to Central Blood Norfentanyl Concentration Ratios
Mean	1.05	0.56	0.50	0.57
Standard Deviation	0.99	0.37	0.28	0.31
%CV	94.0	65.7	55.2	54.6
N	13	11	20	16

Table 1. Ratios for fentanyl and norfentanyl concentrations in vitreous to peripheral and central blood.

**Conclusion/Discussions:** The ratio of vitreous to peripheral blood was 1.05 for fentanyl indicating that the concentrations are nearly the same in the two different matrices. Comparatively, the ratio of vitreous to cardiac blood was 0.50 for fentanyl indicating the concentrations in cardiac blood are twice the concentration in vitreous. However, based on the large standard deviation and high percent coefficient of variation, definitive conclusions cannot be drawn. These results may be impacted by the small sample number. An additional limitation of the current data is that the sample set did not contain any paired peripheral and central blood to allow for those concentration ratios to be determined. Despite these limitations, the data still provides some initial pilot data into investigating the utility of vitreous humor to determine antemortem fentanyl concentrations.

**Keywords:** Vitreous, Fentanyl, Postmortem Redistribution

## **Para-Phenylenediamine Quantification in Ante-mortem and Post-mortem Matrices Using Single Step Liquid-liquid Extraction and Gas Chromatograph-mass Spectrometry: Application in Fatal p-Phenylenediamine Poisoning**

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**Background/Introduction:** *Para*-Phenylenediamine (PPD) is an important ingredient of permanent hair dye that can be found in more than 1000 oxidative hair dyes. Unfortunately, there are vast numbers of unintended and intended incidents of severe to life threatening intoxication involving this compound every year.

**Objective:** A gas chromatograph-mass spectrometry method with liquid-liquid extraction for confirmation and quantitation of p-Phenylenediamine (PPD) in blood, urine, stomach contents and liver specimens was developed and validated.

**Methods:** The PPD in postmortem specimens (2 mL of blood, urine, liver and stomach contents) was extracted by single step liquid liquid extraction with 1-chlorobutane (5 mL). The organic layer was evaporated, the residue was reconstituted with acetonitrile-pyridine (80:20, v/v) and derivatized with MTBSTFA. The oven temperature program of GC-MS was set for an initial temperature of 120 °C, increasing at 50 °C/min to 250 °C, then increasing at 10 °C/min to 320 °C and held for 0.4 min. The total run time was 10 min. In SIM mode, *m/z* 279.1, 280.1, 221.1 ions were monitored for detection of PPD and *m/z* 345, 347 ions were used to determine desalkylflurazepam (internal standard).

**Results:** The assay was linear over 0.5-50 µg/mL ( $r^2=0.99$ ). Limit of detection (LOD) and limit of quantitation (LOQ) in postmortem specimens (blood, urine, liver and stomach contents) were 0.1-0.5 µg/mL and 0.5-1.0 µg/mL, respectively. The accuracy and the precision were  $< \pm 6.3$  % of bias % and  $< 5.2$  % of CV %, which are acceptable criteria according to toxicology laboratory guidelines. Relative recoveries with 0.5, 20 and 50 µg/ml were 83.3-94.2 % (n=3), respectively.

**Conclusion/Discussions:** The developed method was applied in forensic toxicology to determine PPD in postmortem specimens in a fatal case of PPD intoxication in a 48-year-old man who was found dead on bed in a small room after vomiting on the floor. Postmortem finding showed swelling around neck, lips and tongue and died due to respiratory failure. The postmortem heart blood, peripheral blood and gastric contents were analyzed for PPD with the result of 11.3 µg/ml in heart blood, 6.2 µg/ml in peripheral blood and 45.6 µg/g in gastric contents, respectively. The concentration ratio of the heart/peripheral blood of PPD was 1.8, and the ratio of gastric contents/peripheral blood was 7.4, suggesting possible postmortem redistribution and there may be a massive amount of PPD orally ingested. This case study is the first report of lethal concentrations of PPD in postmortem specimens.

**Keywords:** p-Phenylenediamine, GC-MS, Fatal, Postmortem

## Cannabis And Fitness To Dive: About Two Cases With Fatal Outcome

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**Background/Introduction:** SCUBA diving is getting more popular across the world thanks to technological improvements in SCUBA equipment. If the number of divers is growing, so is the number of drug impaired divers. We report here two cases of accidental deaths underwater where the decedents have used cannabis before diving. The first case was a recreational SCUBA diver who fell unconscious during his ascent up to the boat. The second case was a professional diver who was diving with an umbilical cable and was found unresponsive by a colleague 24 meters down. The impairment caused by cannabis consumption is discussed in regard to fitness to dive.

**Objective:** This presentation is intended to remind the risks associated with the use of cannabis before diving.

**Methods:** Various methodologies were utilized to perform a complete toxicological screening on both cases: CO-Oximeter, immunoanalysis, headspace-gas chromatography with flame ionization detector; gas chromatography with mass spectrometry, and liquid chromatography with diode array detector or tandem mass spectrometry detector.

**Results:** All analyses were unremarkable for both cases except for the presence of cannabis. In blood, cardiac in the first case and peripheral in the second case, concentrations of THC, 11-OH-THC, and THC-COOH were respectively 1.4 and 0.8 ng/mL; 0.6 and < 0.5 ng/mL; 10.5 and 1.9 ng/mL. An autopsy was requested only in the second case and found pulmonary and cerebral edemas and marked arteriosclerosis of the coronary arteries. In the first case no autopsy was carried out but the man was overweight and was a tobacco user.

**Conclusion/Discussions:** In both cases cannabis might have played a role in the process contributing to death. SCUBA divers should be aware that the inhibitory effects of cannabis might last for hours, even if they are feeling well enough to dive. Numerous effects of cannabis are incompatible with a safe diving such as euphoria, relaxation, altered time and space perception, lack of concentration, mood change, memory loss, hallucinations. Moreover these effects are synergistic with the effects of nitrogen narcosis. The so-called Martini effect states that each 10 to 15 meters depth corresponds to a performance impairment as produced by one glass of alcohol. There are not only psychotropic effects for cannabis but also physiologic effects such as increased heart rate, hypothermia, dry mouth, nausea and dizziness. Although the scientific literature is scarce on the subject of cannabis and SCUBA diving, few articles have pointed the prevalence of cannabis use among divers and the increased risk of accident. Drug of abuse consumption by divers is mostly unknown, as well as the effects of drugs in hyperbaric conditions. Divers should be aware of the increased risk of accident when diving under the influence of cannabis and more generally under the influence of any drug of abuse.

**Keywords:** Cannabis, Diving, Accident

## Decomposition Kinetics of DDVP in Stored Human Blood Under Different Storage Conditions

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**Background/Introduction:** A number of dichlorvos (dichlorovinyl dimethyl phosphate, DDVP) poisoning cases are currently taking place in China. Dichlorvos is metabolized quickly in the body, and can be decomposed in the in vitro samples. The decomposition kinetics of DDVP in stored human blood under different storage conditions was studied. This study was supported by the Ministry of Public Security and Beijing Public Security Bureau of China (2013GABJC001), National Key Technology R & D Program (2007bBAK26B05, 2012BAK02B02-2) and 2015 Science and technology basic work special project of China poisons (SQ2015FYJ010051).

**Objective:** To observe the decomposition kinetics of dichlorvos in stored human blood under different storage conditions.

**Methods:** DDVP was added to blank pooled human cadaver blood at a concentration of 3 µg/mL. Blood was stored in four different containers (Red-top blood collection tubes, gray-top blood collection tubes, plastic tubes, plastic bags) and each group was divided into three subgroups for the common storage in China: room temperature (24°C), refrigerated (4°C), and frozen (-20°C). Specimens were analyzed at various time points to assess stability. After a liquid-liquid extraction with ethyl acetate, dichlorvos was detected by a GC/MS/MS with a multiple reaction monitoring (MRM).

**Results:** DDVP degraded more rapidly from the blood samples as the storage temperatures increased. Under each storage condition (room temp, refrigerated, and frozen), red-top blood collection tubes yielded all the most stable results, which samples start to become unstable at 3, 3, 1 hours, but the grey-top blood collection tubes yielded all the most unstable results, which samples start to become unstable at less than 1 hour. At -20°C DDVP may be detectable up to 51 days, at 4°C DDVP was undetectable after 48 hours, and at room temperature (ions) DDVP was undetectable after 17 hours.

**Conclusion/Discussions:** The decomposition kinetics of DDVP in stored human blood under different storage conditions can be useful in assisting to select the storage and temperature for medico legal cases potentially involving DDVP poisoning.

**Keywords:** Dichlorvos, Decomposition Kinetics, GC/MS/MS

## Detection of Volatile Hydrocarbons from Gasoline in Whole Blood by Solid-Phase Microextraction Gas Chromatography Mass Spectrometry (SPME-GC-MS)

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**Background/Introduction:** In November 2015, a forty-six-year-old male and his young child were found expired in the backseat of the truck with zip ties around their necks and in the early stages of putrefactive decomposition. An open water bottle with an unknown liquid was found in the cup holder along with two empty gasoline canisters located on the floor. Strangulation with the zip ties was initially suspected as the cause of death; however the autopsy findings did not correlate with the initial impression. Because gasoline inhalation can cause severe side effects including confusion, euphoria, slurred speech, impaired motor function, central nervous system depression, and death, the Laboratory proceeded with testing the postmortem specimens for the components of gasoline.

**Objective:** The objective is to present an analytical method for the detection of volatile hydrocarbons in gasoline and postmortem biological specimens, and to present two case studies where gasoline inhalation was determined as the cause of death.

**Method:** An automated solid-phase microextraction procedure (SPME) utilizing a CombiPal® autosampler was developed to isolate volatile hydrocarbons from biological specimens and gasoline submitted to the Laboratory. A 1.0mL aliquot of whole blood and 100µL of solvent headspace from the evidence were fortified with 1.0mL of 15mg/L N-propanol internal standard and transferred to the autosampler. The SPME fiber was exposed to the headspace for 5 minutes and injected on a Bruker 450 gas chromatograph (GC) coupled to a Varian 1200L mass spectrometer (MS) operating in full scan. The scan range was from  $m/z$  12 - 220. This method utilizes an 85µm Carboxen-PDMS SPME fiber and a 60m x 0.25mm x 1.4µm Restek Rtx-VMS column with a 1.0mL/minute helium flow rate in splitless mode.

**Results:** Toxicology testing of the chest fluid from the child revealed the presence of ethanol (0.063%) and 7-aminonitrazepam. In the adult, loperamide was the only drug identified in the iliac vein blood. After SPME-GC-MS analysis, the mass spectral data of the adult and the child were compared to the mass spectral data of the gasoline to determine if volatile hydrocarbons were present. The results of the analysis are in Table 1. The cause of death for both decedents was determined as inhalation of gasoline fumes/volatile hydrocarbons, and the manners of death for the child and the adult were homicide and suicide, respectively.

**Conclusion/Discussion:** In cases involving gasoline such as the two described, there are no guidelines or protocols on how to proceed with testing. Although there are reference materials available for gasoline, the makeup of the analytical standard is vague and not specific to the medical examiner case in question. Toxicologists must therefore rely upon evidence obtained at the terminal event scene as reference material and adapt to the needs of the case. Utilizing SPME-GC-MS analysis and comparing the mass spectral results of the biological specimens to those obtained of the gasoline; toxicology testing was able to aid the medical examiner in correctly determining the cause and manner of death.

Analyte	Adult	Child	Gasoline (Reference Material)
2-Methylhexane	X	X	X
3-Methylhexane	X	X	X
Acetone	X	X	
Benzene	X	X	
Ethanol	X	X	X
Hexane	X	X	X
O-Xylene	X	X	X
Octane	X	X	
P-Xylene	X	X	
Propanal	X	X	
Toluene	X	X	X
2-Hexene		X	X
Pyrrolidine	X		

**Keywords:** Solid-Phase Microextraction, Volatile Hydrocarbons, Gasoline

## A Comparison Between *Post-Mortem* Blood and Hair Samples from Victims of Violent Death in Brazil

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**Background/Introduction:** Violence is a dreadful phenomenon spread throughout the world, resulting in unfortunate events that can ultimately result in death. However, it is known that some countries play a much worrying role in this scenario than others. Brazil is one of them. The latest estimates show a worldwide homicide rate of 6.7 per 100 thousand inhabitants while in Brazil that same rate is at 29.1 per 100 thousand inhabitants. The reasons contributing to such numbers are various; within them there is the use of cocaine as the country represents a favourable geographical position when it comes to cocaine trafficking to the rest of the world. The present work has focused on identifying the use of cocaine through the detection of cocaine, benzoilecgonine and norcocaine as well as *crack* (anhydroecgonine methyl ester) and also its concomitant use with alcohol by looking at cocaethylene, in several violent *post-mortem* cases arriving at the Institute of Legal Medicine of Sao Paulo, the largest Brazilian city. Both blood and hair samples have been analysed through UPLC-MS/MS in order to distinguish between recent or chronic cocaine use.

**Objective:** The aim of this work was to evaluate the prevalence of cocaine in violent events that have ended in death, and to establish whether those individuals were under the influence of the drug and/or they were chronic cocaine users.

**Methods:** An aliquot of 100 $\mu$ L of a blood sample was pipetted into an eppendorf together with 20 $\mu$ L of the respective deuterated internal standards (cocaine-d3; benzoilecgonine-d3 and cocaethylene-d3) at a concentration of 500ng/mL. Then it was diluted with 880 $\mu$ L of a cold mixture of ACN/MeOH (80:20/v:v) and the solution was centrifuged at 9000g for 6min. After that, an aliquot of 3 $\mu$ L has been injected into the UPLC-ESI-MS/MS system. As for the hair procedure, a small piece of 50mg from the hair sample was washed with water and a mild detergent followed by the addition of 2mL of dichloromethane under 37°C for 15min. Once dried and cut into small pieces, 2mL of MeOH and 10 $\mu$ L of the respective deuterated internal standards at a concentration of 10 $\mu$ g/mL were added and left to incubate for 18h at 50°C. The next day the MeOH was transferred to a new falcon tube and dried under N<sub>2</sub> stream at 50°C. The residue was then re-suspended with 50 $\mu$ L of the mobile phase (1mM of ammonium formate with 0.1% of formic acid) and 3 $\mu$ L were also injected into the UPLC-ESI-MS/MS system. The linearity for both blood and hair analysis was of 25 - 1000ng/mL and 0.05 - 20 ng/mg, respectively. After full validation, the method has been applied to *post-mortem* blood and hair samples from 62 violent death victims.

**Results:** From the total number of samples, 21 have yielded a positive result for cocaine and some of its derivatives in blood against 32 for hair. Also, the different circumstances reported for each case have been divided into 4 categories where the highest number of deaths was homicide resulting from opposition to police intervention (31%) followed by general violent and risk behaviour (27%), drug abuse suspicion (26%) and possible suicide (16%).

**Conclusion/Discussions:** It can be seen that there is a relatively high prevalence of cocaine use involved in violent deaths either showing cocaine use at the time of death (34%) or chronic cocaine use (52%).

**Keywords:** Post-Mortem; Violence; Cocaine

## Systematic Study on the Time-Dependent Post Mortem Redistribution of Opioids

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**Background/Introduction:** Post mortem redistribution (PMR) is a well-recognized phenomenon leading to difficulties in interpretation of post mortem cases in a forensic toxicological context. Opioid drugs are widely used for their pain relieving effects but due to their highly addictive properties they are prone to misuse. The current literature that investigates the potential time-dependent post mortem redistribution of opioids focuses on blood as the matrix of choice and the cardiac-to-femoral blood ratio as a mean to assess PMR. Due to the limitation of using a single matrix, it is important to acquire more extensive data for a broad range of opioids, also incorporating alternative matrices to be able to draw further conclusions.

**Objective:** The aim of this project was to conduct a systematic study on the time-dependent PMR of opioids and their metabolites in humans. Besides blood samples, also alternative matrices such as muscle, liver, kidney, lung, spleen and adipose tissue should be evaluated for PMR in the course of this study.

**Methods:** Utilizing a computed tomography (CT)-guided biopsy tool connected to a robotic arm, 26 cases of opioid use were investigated. Post mortem biopsy samples of the femoral and heart blood (right ventricle), right lung, right kidney, liver, spleen and muscle tissue were collected from the deceased at admission to the institute (t1). At the time of autopsy, approximately 24 h later (t2), samples from the same body regions were collected, in addition to the gastric contents, urine, brain tissue and heart blood from the left ventricle. For quantitation purposes, a two-step liquid-liquid extraction was performed with subsequent LC-MS/MS (5500 QTrap) analysis using a previously validated method. Target opioid analytes for quantitation were methadone, its metabolite 2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine (EDDP), morphine, 6-monoacetylmorphine (6-MAM), codeine, fentanyl, dihydrocodeine, hydrocodone, hydromorphone, oxycodone, oxymorphone and tramadol with its metabolite O-desmethyltramadol (ODMT).

**Results:** Time-dependent concentration changes were evaluated within 10 cases of methadone use (with detection of EDDP as a metabolite), 13 cases of morphine detection and 4 cases of fentanyl use. The post mortem interval across all cases ranged from 5 to 59 hours. Methadone concentrations in femoral blood (pB) at t1 ranged from 2 to 1800 ng/mL. In most cases an increase in pB methadone concentration was observed (median +20%). In two cases, decreases in pB methadone concentrations were detected (-45 % and -66 % respectively). Adipose tissue and muscle samples, overall, showed methadone concentration decreases (median -18 % and -9 % respectively) over time. In contrast to this, EDDP concentrations in the aforementioned alternative matrices increased in the majority of cases (median +25 % and +39 % respectively) with two cases of observed decreases per matrix. The same trend of EDDP concentration increase over time was detected in pB samples. While morphine pB concentrations significantly increased over time (median 44 %), the alternative matrices showed concentration increases and decreases with no clear trend. All 4 fentanyl cases showed a significant increase in pB concentration over time with up to +117%.

**Conclusion/Discussion:** It is expected that methadone as a lipophilic base with a pKa of 8.6 and a large volume of distribution ( $V_d$ ) of 4-7 L/kg is subject to PMR by passive diffusion from surrounding tissues. With observed methadone concentration decreases in tissue samples and increases in blood sample concentrations this statement can be supported, despite observed inter-individual variations across the analyzed samples. The morphine and fentanyl concentration increases in pB are most likely to be also caused by diffusion processes e.g. from surrounding muscle tissue. Further, CT-guided biopsy sampling proved once more to be a valuable tool for investigating PMR mechanisms.

**Keywords:** Post Mortem Redistribution, Opioids, CT-Guided Biopsy



## Postmortem Fluid and Tissue Concentrations of THC, 11-OH-THC, and THC-COOH

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**Background/Objective:** Marijuana is the most commonly abused illicit drug worldwide. While marijuana is used for its euphoric and relaxing properties, it has been shown to impair memory, cognitive skills, and psychomotor function. The Federal Aviation Administration's Civil Aerospace Medical Institute conducts toxicological analysis on aviation fatalities. Due to severe trauma associated with aviation accidents, blood is not always available; therefore, the laboratory must rely on specimens other than blood for toxicological analysis in approximately 40% of cases. Unfortunately, the postmortem distribution of cannabinoids has not been well characterized. The purpose of this research is to evaluate the distribution of THC, 11-OH-THC and THC-COOH, in postmortem fluid and tissue specimens.

**Methods:** A solid phase extraction (CSTHC; UCT Inc.; Bristol, PA) following a protein precipitation with acetonitrile was utilized to isolate the desired cannabinoids. This extraction method only required 0.5 mL or 0.5 g of sample. Dried extracts were reconstituted in 100  $\mu$ L of methanol for analysis. A sensitive and robust LC/MS/MS method was developed and validated (following SWGTOX guidelines) using a Waters<sup>®</sup> Xevo TQ-S Acquity Ultra Performance Liquid Chromatography (Waters Corporation; Milford, MA) to identify and quantify THC, 11-OH-THC and THC-COOH in postmortem fluids and tissues. Eleven aviation accident fatalities (2014-2015), previously found positive for cannabinoids, were analyzed with the new procedure. Specimens evaluated, when available, included: blood, urine, vitreous humor, liver, lung, kidney, spleen, muscle, brain, heart, and bile.

**Results:** The method readily identified and quantified THC, 11-OH-THC, and THC-COOH in postmortem fluids and tissues. The calibration curve best fit model was determined by preparing and analyzing a calibration curve on 5 separate, but consecutive days (0.78–100 ng/mL). All curves best fit a linear regression model with a 1/x weighting. This method could readily determine concentrations far below the lowest calibrator (0.78 ng/mL); however, we determined this was a low enough concentration for our laboratory's needs and mission. Therefore, the LOD and LOQ were administratively determined to be 0.78 ng/mL. Carryover was evaluated for all analytes; no carryover was observed. Accuracy and precision was evaluated at 2, 20 and 80 ng/mL in blood for all analytes on 5 separate days. All controls were within 20% of the target concentration. Stability was evaluated with 2, 20, and 80 ng/mL blood controls in three ways: on-instrument stability (post-extraction stability), refrigerated stability, and freeze/thaw stability. On-instrument stability and refrigerated stability were found to be stable for 5 days (the total length of the stability study). Three freeze/thaw cycles were tested and the analytes were stable the entire time. Ion suppression/enhancement was evaluated in blood, urine, serum, liver, lung, brain, and muscle. Significant suppression was observed in tissue specimens; however, ion suppression similarly affected the deuterated internal standards, thus the quantification of these analytes in these specimens was not affected. None of the analytes suffered qualitative or quantitative interference when analyzed in the presence of commonly encountered drugs. Specimens with the highest amount of THC were the lung and kidney. While all available urine and bile specimens were positive for 11-OH-THC, only 4 of 11 blood specimens had detectable concentrations. THC-COOH was detected in all blood, urine, liver, lung, kidney, and bile specimens. The distribution of cannabinoids between blood and non-blood specimens was evaluated, but there was no consistent distribution observed between blood and any other fluids or tissues.

**Conclusion/Discussion:** A sensitive and robust LC/MS/MS method was developed and validated to detect THC, 11-OH-THC, and THC-COOH in postmortem fluids and tissues. While quantitative interpretation of non-blood postmortem fluid and tissues samples is not prudent, a majority of the non-blood specimens tested could be suitable alternative/supplemental choices for qualitative cannabinoid detection.

**Keywords:** Cannabinoids, Postmortem Distribution, LC/MS/MS

## Post-Mortem Levels and Tissue Distribution of Codeine, Codeine-6-Glucuronide, Norcodeine, Morphine and Morphine Glucuronides in a Series of Codeine-Related Deaths

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**Background/Introduction:** The analgesic effects of the widely used opiate codeine appear to be largely dependent on metabolic conversion to morphine by the polymorphic cytochrome P450 isoenzyme 2D6 (CYP2D6). Whether this also applies to the toxicity of codeine remains a matter of controversy. Although unsubstantiated by receptor affinity studies, some investigators have suggested codeine and its main metabolites codeine-6-glucuronide (C6G) and norcodeine as putative mediators of codeine toxicity. Previous studies of codeine-related deaths have reported a limited array of codeine metabolites in biological specimens, particularly in matrices other than blood and urine. Furthermore, published data regarding post-mortem changes of codeine and its metabolites are limited and inconsistent. Thus, further investigations of the concentrations and tissue distribution of codeine and its metabolites in codeine-implicated deaths are warranted.

**Objective:** To address these limitations in the literature.

**Methods:** We present levels and tissue distribution of codeine, C6G, norcodeine, morphine and the morphine metabolites morphine-3-glucuronide (M3G) and morphine-6-glucuronide (M6G) in post-mortem blood, vitreous fluid, muscle, fat and brain tissue in a series of 23 codeine-related fatalities. CYP2D6 genotype is also determined and taken into account. Quantification was performed with a validated SPE-LC-MS method.

**Results:** The series comprised 19 deaths (83%) attributed to mixed drug intoxication, 4 deaths (17%) attributed to other causes of death, and no cases of unambiguous monointoxication with codeine. The typical peripheral blood concentration pattern was C6G>>codeine>>norcodeine>morphine, and M3G>M6G>morphine. In matrices other than blood, the concentration pattern was similar, although in a less systematic fashion. Measured concentrations were generally lower in matrices other than blood, especially in brain and fat, and in particular for the glucuronides (C6G, M3G and M6G) and, to some extent, morphine. In brain tissue, the presumed active moieties morphine and M6G were <LLOQ (0.0080 mg/L and 0.058 mg/L, respectively) in a majority of cases. In general, there was a large variability in both measured concentrations and calculated blood/tissue concentration ratios. There was also a large variability in calculated ratios of morphine to codeine, C6G to codeine and norcodeine to codeine in all matrices, and CYP2D6 genotype was not a reliable predictor of these ratios. The different blood/tissue concentration ratios showed no systematic relationship with the post-mortem interval. No coherent degradation or formation patterns for codeine, morphine, M3G and M6G were observed upon reanalysis in peripheral blood after storage.

**Conclusion/Discussion:** Measured brain concentrations in this study seem to provide little support for the conception of C6G/norcodeine as mediators of codeine toxicity. The observation that the presumed active moieties morphine and M6G were <LLOQ in brain in a majority of cases is of particular interest, and warrants further investigations. CYP2D6 genotype was not a reliable predictor of codeine metabolite levels. Post-mortem formation, degradation or redistribution of the analytes was not found.

**Keywords:** Codeine, Post-Mortem, Tissue Distribution

**Update on the Department of Justice's Efforts to Curb Opioid Epidemic**

**Invited Speaker:** M.J. Menendez, JD, Department of Justice Organized Crime Drug Enforcement Task Force (OCDETF), Washington D.C

The Department of Justice Organized Crime Drug Enforcement Task Forces (DOJ/OCDETF) is tasked with, among numerous other things, tracking the rapid evolution and identification of novel fentanyl drugs at first instance in the United States, namely, international ports of entry. The unit works extensively with the Drug Enforcement agency, Center for Disease Control, and NMS Labs on these projects. A lot of time was also spent spreading the news of importance of immediate and accurate identification of these novel fentanyl analogues to policy makers, and work on medico-legal death investigation (MDI) issues for timely prosecution and cause and manner identification. This brief talk will update the attendees on these activities and the website cited below contains more information.

<https://www.justice.gov/criminal/organized-crime-drug-enforcement-task-forces>

## Evaluating the Use of Kendrick Mass Defect Analysis for Rapid Discovery of New Psychoactive Substances in Non-targeted Screening Approaches

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**Background/Introduction:** The proliferation of new psychoactive substances (NPS) has recently initiated considerable interest into the development of non-targeted screening workflows for the discovery of novel analogues without requiring certified reference materials and comprehensive mass spectral libraries. These workflows often require complex data processing software and experienced analysts to operate them and interpret results. Since NPS analogues in most classes are  $-\text{CH}_2$  homologues, Kendrick Mass Defect (KMD) analysis has been identified as a potential technique for the discovery of novel analogues that are structurally related to known analogues. However, vendor data processing software cannot currently accommodate this technique.

**Objective:** To develop and evaluate KMD analysis software for the rapid discovery of NPS analogues in authentic toxicological samples in non-targeted screening approaches.

**Methods:** Authentic toxicological samples ( $n=10$ ) confirmed to contain NPS by routine analyses utilizing a range of high-resolution mass spectrometry (HRMS) platforms (Agilent Technologies, Waters Corporation and Thermo Fisher Scientific) were selected for the KMD analysis software assessment. Averaged mass spectra containing all mass-to-charge ratios ( $m/z$ ) from selected retention time ranges were generated from MS scan data. Peak lists containing  $m/z$  values and intensity were exported as comma-separated value (.csv) files or copied to a Microsoft Office Excel worksheet and then saved as a .csv file. A Microsoft Office Excel-based KMD analysis software was developed using the Visual Basic for Applications (VBA) programming language. Briefly, the software workflow involves the importation of single or multiple .csv files, followed by the calculation of KMD values for each  $m/z$  entry normalized to  $-\text{CH}_2$ . The data can then be filtered by  $m/z$  range, intensity, mass defect and even/odd mass. KMD values which match the user-defined values (up to 8 different values can be monitored simultaneously) are highlighted and isolated for easy visualization. These  $m/z$  values can then be extracted using the corresponding native data processing software to observe the presence of distinct chromatographic peaks for the selected  $m/z$  values. In this study, a  $m/z$  range 150-500, intensity threshold  $> 100$  counts, 0.01-0.23 Da mass defect filter were applied across all sample files with only even  $m/z$  values monitored. Further strategies to reduce intrinsic  $m/z$  values were also investigated such as the application of subtraction and deconvolution algorithms prior to KMD analysis.

**Results:** Application of KMD analysis to all raw sample data files resulted in an average of 13 unique highlighted results per file, corresponding to  $>99.9\%$  reduction of the raw data. Analytes of interest (mostly synthetic cathinones) that were identified by routine analyses were correctly highlighted and mostly situated in the top 5 unique results when sorted on decreasing intensity. Extraction of remaining  $m/z$  values in the native data processing software indicated that most highlighted  $m/z$  were recurring over the selected retention time range or did not produce significant chromatographic peaks to render further interrogation.

**Conclusion/Discussions:** A rapid and intuitive Excel-based program was developed and successfully applied to MS data acquired from authentic samples resulting in the correct identification of NPS with minimal other possible candidates based on highlighted  $m/z$  values. KMD analysis can be used as a complementary technique in non-targeted screening workflows to assist in the discovery of novel compounds. There is scope to transfer the program to a conventional graphical user interface (GUI) which can import mass spectrometry data formats, enhancing its data mining capabilities.

**Keywords:** Kendrick Mass Defect, High-resolution Mass Spectrometry, New Psychoactive Substances

## Deaths Related to Abuse of 3-Methylfentanyl in the United States

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**Background/Introduction:** 3-methylfentanyl was first synthesized by the Janssen Pharmaceutical company in 1974 based on a modification to the structure of fentanyl by the addition of a methyl group on the 3 position of the piperidine ring. 3-methylfentanyl has two chiral centers resulting in two enantiomeric pairs, (±)-cis-3-methylfentanyl and (±)-trans-3-methylfentanyl. The potency of these compounds was compared to that of fentanyl, and it was demonstrated that (±)-trans-3-methylfentanyl has similar potency to fentanyl, while (±)-cis-3-methylfentanyl is approximately 8 times more potent. Further work into the relative potency of the (±)-cis-3-methylfentanyl was performed and it was determined that when separating the enantiomeric pair the (+)-cis-3-methylfentanyl was 16 times more potent than fentanyl.

**Objective:** 3-methylfentanyl was first synthesized by the Janssen Pharmaceutical company in 1974 based on a modification to the structure of fentanyl by the addition of a methyl group on the 3 position of the piperidine ring. 3-methylfentanyl has two chiral centers resulting in two enantiomeric pairs, (±)-cis-3-methylfentanyl and (±)-trans-3-methylfentanyl. The potency of these compounds was compared to that of fentanyl, and it was demonstrated that (±)-trans-3-methylfentanyl has similar potency to fentanyl, while (±)-cis-3-methylfentanyl is approximately 8 times more potent. Further work into the relative potency of the (±)-cis-3-methylfentanyl was performed and it was determined that when separating the enantiomeric pair the (+)-cis-3-methylfentanyl was 16 times more potent than fentanyl.

**Methods:** Analysis was performed using a Waters Xevo® G2-S QTOF coupled with an Acquity I-class UPLC (Waters®; Milford, MA) for the quantitation of the two enantiomeric species of 3-methylfentanyl. Chromatographic separation was achieved with an Acquity UPLC® BEH C18 (2.1 mm x 150 mm, 1.8 μm) column heated to 50°C at a flow rate of 0.4 mL/min for a total run time of 15 minutes. The mobile phases used for analysis were 5 mM ammonium formate (pH=3) and 0.1% formic acid in acetonitrile. A fit-for-purpose validation was performed with reference to the SWGTOX guidelines consisting of establishing linear dynamic range, limits of detection and quantitation, precision, accuracy and interference. Interference studies included all isomers of 3-methylfentanyl and other fentanyl analogues.

**Results:** Eight blood specimens that had screened and confirmed positive for 3-methylfentanyl without diastereomeric resolution were analyzed for (±)-cis-3-methylfentanyl and (±)-trans-3-methylfentanyl diastereomers. Average concentrations for (±)-cis-3-methylfentanyl were 0.93 (±0.44) ng/mL, with a median of 0.84 ng/mL, and for (±)-trans-3-methylfentanyl the average concentration was 0.48 (±0.22) ng/mL with a median of 0.39 ng/mL. The ratio of the (±)-cis-3-methylfentanyl to the (±)-trans-3-methylfentanyl was always greater than 1, with an average and median ratio of 1.9. The concentration of these samples was also compared to the total undifferentiated 3-methylfentanyl concentrations obtained using a method performed by a liquid chromatograph tandem mass spectrometer (LC-MS/MS), where 3-methylfentanyl was quantitated as the racemic mixture. The sum of the cis and trans enantiomers quantitated by LC-QTOF method were in agreement with the total 3-methylfentanyl result from the LC-MS/MS analysis, with a maximum deviation of less than 15%.

**Conclusion/Discussions:** The analysis of 3-methylfentanyl in forensic casework will present a challenge to analytical toxicology laboratories, as toxicologically significant concentrations are typically in the low to sub nanograms per milliliter range. Being able to distinguish between the cis and trans diastereomers of 3-methylfentanyl can assist in interpretation of case findings and drug concentrations as the (±)-cis-3-methylfentanyl is more potent than the (±)-trans-3-methylfentanyl. It is also important to ensure the analytical method has the ability to distinguish between the isomers of 3-methylfentanyl and other isobaric fentanyl analogues, such as alpha-methylfentanyl.

**Keywords:** Opiates, 3-methylfentanyl, Q-TOF

## Development of a LC-MS/MS-Based Method for the Multiplex Detection of Fentanyl Analogs in Whole Blood at Sub ng mL<sup>-1</sup> Concentrations

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**Background/Introduction:** Fentanyl, a synthetic opioid, is commonly prescribed for chronic pain management but comes with a high illicit abuse potential. A recent surge of fentanyl and fentanyl analog-related overdose deaths has risen to epidemic numbers both locally and nationally. Consequently, there is a tremendous need to detect various fentanyl analogs and their metabolites in human specimens.

**Objective:** The aim of this study was to develop and validate a detection method for 22 fentanyl analogs and metabolites in human biological fluids at sub nanogram concentrations using solid phase extraction (SPE) in conjunction with liquid chromatography triple quadrupole mass spectrometry (LC-MS/MS). All drugs meeting acceptable Scientific Working Group for Forensic Toxicology (SWGTOX) validation criteria (N = 15) were quantitated, while all others (N = 7) criteria are reported qualitatively.

**Method:** One milliliter of blood samples were combined with 100  $\mu$ L of norfentanyl-d5, fentanyl-d5 and acetylfentanyl-<sup>13</sup>C6 (10 ng mL<sup>-1</sup>). Various concentrations of calibrators (0.1 – 50 ng mL<sup>-1</sup>) and quality controls (QC-LO, QC-MED, QC-HI) were fortified then extracted using UCT Clean Screen SPE filters. Following column preparation and sample clean-up, samples were eluted using a mixture of methylene chloride/isopropanol/ammonium hydroxide (78:20:2), evaporated, and reconstituted with 100  $\mu$ L of MeOH before analysis by LC-MS/MS in a biphenyl column containing 2 mM of ammonium formate and 0.1% formic acid in water and 0.1% formic acid in acetonitrile (ACN) (total run time of 13.5 minutes).

**Results:** Validation of method parameters consisted of linearity, bias, precision, matrix effects (78 – 125%), process efficiency (59 – 104%), recovery (58 – 95%) and interferences. Despropionyl para-Fluorofentanyl and 4ANPP values were excluded from the ranges of matrix effects (57, 65%) and recovery (139, 118%) and remifentanil acid was excluded for process efficiency (40%) and recovery (32%). Interferences were evaluated with negative human blood and drugs commonly found in a toxicological setting. Large concentrations of a benzodiazepine may interfere with AH7921 and U47700, but all other analogs experienced no endogenous or exogenous interferences. Limit of detections were determined over a five-day validation period. Limits of quantitation for quantified analytes (lowest acceptable calibrator) were determined to be 0.1 – 0.25 ng mL<sup>-1</sup>. Upper limit of quantitation for fentanyl and norfentanyl was 50 ng mL<sup>-1</sup>, while all other quantified analogs had a 10 ng mL<sup>-1</sup> upper limit. Calibration curves for quantified fentanyl analogs collected from this method exhibited a consistently linear trend ( $r^2 > 0.99$ , 1/X weighted) except for fentanyl, where a quadratic fit was used. Bias (0.1 – 20%) and precision (3.9 – 17%) were determined by fortifying human blood at three different concentrations (0.35, 2.5, and 25 ng mL<sup>-1</sup>, N = 15).

**Conclusion/Discussions:** A novel LC-MS/MS-based method was developed and validated for the identification and quantification of fentanyl analogs and metabolites. This all-inclusive method has shown to be accurate, specific, and may be utilized for immediate applications in the forensic field.

**Keywords:** Method Development, Method Validation, Fentanyl Analogs

## Postmortem Detection of Despropionyl Fentanyl in Drug Related Deaths

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**Background/Introduction:** Despropionyl fentanyl (4-anilino-N-phenethyl-4-piperidine [4-ANPP, DPF]) is an immediate precursor in the illicit synthesis of fentanyl; incomplete reactivity of this final step leaves DPF as a low level contaminant which can be a specific marker of illicitly-produced fentanyl. This may be of significant use as fentanyl and its analogs are typically present at low concentrations in post-mortem specimens. The Drug Enforcement Administration (DEA) estimated 75% of fentanyl seizures contain DPF as a contaminant at levels  $\leq 2.5\%$ . Because of its use in illicit fentanyl manufacturing, the DEA designated it a Schedule II controlled substance in 2010. The Maryland Office of the Chief Medical Examiner (OCME) Toxicology Laboratory initially detected DPF in 2014 in acetyl fentanyl-positive cases, but began reporting it in postmortem toxicology specimens in March 2016 when several cases were concurrently negative for fentanyl and fentanyl analogs.

**Objective:** A retrospective analysis was conducted to characterize all DPF-positive cases from the Maryland OCME from 1 March 2016 – 28 February 2017.

**Methods:** DPF was initially identified in blood and urine specimens following an alkaline liquid-liquid extraction and analysis by gas chromatography-nitrogen/phosphorus detection and gas chromatography-mass spectrometry (GC-MS) operated in full scan mode. Confirmation was performed via solid-phase extraction and GC-MS operated in selected ion monitoring mode; DPF results are currently reported as a qualitative positive/negative. Relevant co-identified substances were characterized for DPF-positive cases.

**Results:** There were 653 DPF-positive cases (any specimen) over the 12 month period. Blood was positive for DPF in 529 cases; cocaine (142 cases, 26.8%), benzodiazepines (38, 7.2%), amphetamine/methamphetamine (11, 2.1%), and opioids (435, 82.2%) were co-identified. Blood-positive cases were additionally positive for fentanyl (142, 26.8%), acetyl fentanyl (3, 0.6%), 4-fluoroisobutyrylfentanyl (4-FIBF, 126, 23.8%), acrylfentanyl (13, 2.5%), and furanyl fentanyl (145, 27.4%). Free morphine and 6-monoacetylmorphine (6-MAM) were co-identified in 423 (80.0%) and 11 (2.1%) blood-positive specimens, respectively. Among DPF-positive blood specimens, 218 (41.2%) were neither fentanyl nor fentanyl-analog positive, 106 (20.0%) were neither free morphine nor 6-MAM positive, and 58 (11.0%) were not fentanyl, fentanyl-analog, free morphine, nor 6-MAM positive. DPF was positive in urine in 352 cases. These cases were additionally positive for fentanyl (161, 45.7%), acetyl fentanyl (26, 7.4%), 4-FIBF (80, 22.7%), acrylfentanyl (12, 3.4%), and furanyl fentanyl (156, 44.3%); 6-MAM was positive in 206 (58.5%) urine-positive specimens. Among DPF-positive urine specimens, 82 (23.3%) were neither fentanyl nor fentanyl-analog positive, 146 (41.5%) were not 6-MAM positive, and 44 (12.5%) were not fentanyl, fentanyl-analog, nor 6-MAM positive. There were 273 cases in which DPF was confirmed in *both* blood and urine. Among these, 97 (35.5%) blood specimens were neither fentanyl nor fentanyl-analog positive, 56 (20.5%) were neither free morphine nor 6-MAM positive, and 31 (11.4%) were not fentanyl, fentanyl-analog, free morphine, or 6-MAM positive. Finally, 608 cases contained DPF in *either* blood or urine; among these, 186 (30.6%) did not contain fentanyl or an analog in either blood or urine.

**Conclusion/Discussions:** The Maryland OCME has seen an increase in DPF-positive cases over the prior year. Fentanyl and its analogs were not always detectable in postmortem toxicological specimens; DPF may be a suitable marker of their use in blood and urine. It does not require a special extraction method and can be analyzed by routine GC-MS instrumentation. Initial screening of specimens for DPF, therefore, may lead to increased identification of fentanyl-positive cases and improve results interpretation.

**Keywords:** Despropionyl Fentanyl, Fentanyl, Postmortem Toxicology

## NPS Identification by Portable Raman Instrument During On-Field Drug Checking

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**Background/Introduction:** The identification of Novel Psychoactive Substances (NPS) to monitor the evolution of the narcotics market and to inform the National Early Warning System is of utmost urgency. Instruments based on Raman spectroscopy can be used for fast, accurate and inexpensive analysis of seized drugs and unknown materials, allowing simple and safe handling and reliable identification of existing and new psychoactive substances. Furthermore, portable instruments have been recently introduced into the market, making Raman spectroscopy ideal for on-site analysis.

**Objectives:** Aim of the study was to identify the drugs purchased and commonly used by partygoers and in music festivals. The efficiency of portable Raman instrumentation in the analysis of more than 300 alleged drugs, tested during 21 night events within the first formal implementation of drug checking in Italy, will be discussed. The hazard caused by the unaware intake of drugs will be also highlighted.

**Methods:** On site drug checking was performed using a Thermo Scientific TruNarc™ portable Raman analyzer equipped with a 785-nm Class IIIB laser at 250mW. Raman spectra in the interval 300-1800 cm<sup>-1</sup> were recorded. The identification of the substances was performed by comparing the spectrum of the unknown compound with those present in an on-board library containing traditional narcotics, NPS, cutting agents, and precursors. All the samples were tested during electronic dance music festivals, rave parties, GOA parties and street parades in the Italian territory, during 2016 and 2017. Whenever possible, puzzling and NPS samples were transferred to the lab for GC-MS or LC-MS/MS confirmation.

**Results:** The drug checking procedure allowed the detection of traditional drugs like MDMA (85 samples), ketamine (67 samples), amphetamine (43 samples) and cocaine (30 samples). Other findings included LSD, DMT, cutting agents, precursors and various pharmaceuticals. Furthermore, several NPS were identified, including synthetic cathinones (mephedrone, methyldone, pentylone), amphetamine-like compounds (4-FA, DOC, DOM), phenethylamines (25I-NBOMe, 25B-NBOMe, 2-CB), and tryptamines (5-MeOMiPT, 4-AcOMET). The presence of NPS in the tested samples was often in disagreement with the user's expectation.

**Conclusion/Discussions:** Drug checking by Raman spectroscopy proved effective in the identification of several NPS and the investigation of drug distribution present in various recreational settings at different times. In particular, portable Raman instrumentation has demonstrated several advantages in this context, namely the direct analysis of the sample through water, glass, and plastic bags, avoiding direct contact with the substance, with non-destructive and non-invasive testing. The on-field drug checking activity revealed the presence of several NPS in the nightlife scenario, often in replacement of the traditional illicit drugs. Since several of these substances are potentially more toxic than the usual recreational drugs, their intake poses a high overdose risk for unaware users and a life-threatening situation for them.

**Keywords:** NPS, Drug Checking, Raman



## An Efficient Screening Approach for Fentanyl Analogs Using A Single Extraction Sequential GC/MS and LC/MS/MS Analysis

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**Background/Introduction:** All suspected overdose cases admitted to the Franklin County Coroner's Office Forensic Toxicology Laboratory have required dedicated, labor intensive, replicate analysis to screen and confirm fentanyl analogs by LC/MS/MS, such as carfentanil, acrylfentanyl, furanylfentanyl, 3-methylfentanyl, fluorobutyrylfentanyl, fluoroisobutyrylfentanyl, acetylfentanyl, valerylfentanyl, etc. A duplicate extraction approach requires more biological sample to be consumed and a potential delay in case completion. Compounding this, is the ever changing array of fentanyl analogs with concentrations appearing in blood at less than 5 ng/mL making traditional GC/MS full-scan detection and immunoassay screens ineffective.

**Objective:** To describe an efficient and adaptable single extraction sequential (SES) GC/MS and LC/MS/MS protocol enabling fentanyl analog screening from routine and previously analyzed blood alkaline drug concentrates. Fentanyl analogs are typically undetectable using GC/MS full-scan detection alone or fall short of recommended forensic identification criteria without duplicate LC/MS/MS extractions. The following protocol allows for sample conservation, overall testing efficiency, and the best of both GC/MS search and LC/MS/MS detection capabilities.

**Methods:** All cases submitted for medico-legal toxicology investigation receive a routine blood basic drug solid phase extraction and GC/MS analysis on 1 mL of blood, fortified with methapyrilene as the internal standard, and reconstituted in 75  $\mu$ L acetonitrile. In February 2017, we successfully evaluated adding fentanyl-d5/norfentanyl-d5 internal standards to the blood samples at a concentration not visible to the full-scan GC/MS analysis. Fentanyl analog fortified positive and negative controls are also extracted with each batch. After the alkaline GC/MS full-scan analysis is complete, all potential overdose and SUID case extracts are separated with the positive and negative fentanyl analog controls. By directly adding 50  $\mu$ L of LC mobile phase, water with 0.1% formic acid to these extracts, fentanyl analog screening by LC/MS/MS can be performed as a sequential extension of the routine basic compound analysis. The low concentration fentanyl internal standards and analogs are easily visible by the LC/MS/MS analysis using a targeted SRM mode, down to approximately 100 pg/mL. All presumptive positive fentanyl analog cases are ordered for subsequent LC/MS/MS extraction and confirmation. SWGTOX LOD, Stability, Interference, and Suppression/Enhancement studies for qualitative screens were evaluated and demonstrated acceptable results for all analogs of interest.

**Results:** This tandem instrument protocol allows for efficient dual analysis of the same sample extract (broad scope basic drug GC/MS analysis and targeted LC/MS/MS screening) to detect low concentration fentanyl analogs, while conserving evidentiary biological samples. The LC/MS/MS analog detection method can be expanded or modified to include data dependent fragmentation, MRM, or alternate analysis of the extract by an analytical platform such as TOF-LC/MS, depending on the laboratory's available instrumentation.

**Conclusion/Discussions:** In post-mortem toxicology, ensuring a full systematic toxicological approach can be performed on a finite sample volume is a frequent challenge and limited hospital admission fluids typically exacerbate this issue. Additionally, while immunoassay detection is a common screening tool, a la cart immunoassay kit development and sensitivity for each analog may be difficult for manufacturers and not feasible for laboratory testing long term. The single extraction sequential (SES) instrument analysis can be used as an expansion of basic drug GC/MS analysis routinely performed by most forensic toxicology labs. This method is easily adaptable to any LC/MS/MS detection platform to ensure progressive detection of designer analogs in the future. This thorough testing approach enables the Franklin County Coroner's Office Forensic Toxicology Laboratory to conserve labor and resources while screening for low concentration compounds that may otherwise go undetected.

**Keywords:** Fentanyl, Analogs, Screen

## Validation of a Comprehensive UHPLC-MS/MS Method to Quantify Six Novel Fentanyl Analogues in Postmortem Specimens

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**Background/Introduction:** Since 2013, the Miami-Dade County Medical Examiner Department (MDME) has seen an increase in the number of opioid-related deaths due to the introduction of fentanyl and fentanyl analogues into the local heroin supply. From 2014 to 2015, a near 600% increase in fentanyl and fentanyl-related deaths was observed, followed by another 200% increase in 2016. In addition to fentanyl, six novel fentanyl analogues (carfentanil, furanyl fentanyl,  $\beta$ -hydroxythiofentanyl, acetyl fentanyl, butyryl fentanyl, and para-fluoroisobutyryl fentanyl) emerged during this time frame and were contributory to the cause of death in over 200 cases, with an additional 46 cases through March 2017. A comprehensive analytical method to simultaneously confirm and quantify these compounds was needed to address this increase in the number of deaths involving fentanyl and fentanyl analogues, as well as a better understanding of how the postmortem concentrations of these compounds correlate to the cause of death. Due to the nature of these drugs and their extreme potency at low concentrations, analysis by UHPLC-MS/MS was desired to ensure maximum sensitivity and selectivity.

**Objective:** The objective of this project was to develop and validate a comprehensive UHPLC-MS/MS method to quantify fentanyl and six novel fentanyl analogues in postmortem biological fluids and tissues.

**Method:** Quantitative analysis of carfentanil, furanyl fentanyl,  $\beta$ -hydroxythiofentanyl, acetyl fentanyl, fentanyl, butyryl fentanyl, and para-fluoroisobutyryl fentanyl was performed using a Shimadzu Nexera X2 Ultra High Performance Liquid Chromatograph (UHPLC) coupled to a Shimadzu 8060 Tandem Mass Spectrometer (MS/MS). Isolation of the compounds and their respective deuterated internal standards from postmortem biological matrices was performed using mixed-mode (reverse phase and ion exchange) solid phase extraction columns. Chromatographic separation was achieved within 7.25 minutes using a gradient elution (A=0.1% aq. formic acid, B=acetonitrile) with a Restek Raptor™ ARC-C18 column (50 mm x 2.1 mm x 2.7  $\mu$ m) at a flow rate of 0.65 mL/min. Mass spectrometric data was acquired using positive electrospray ionization in multiple reaction monitoring (MRM) mode. This method was validated to assess linearity, bias, precision, limit of detection (LOD), limit of quantitation (LOQ), carryover, dilution integrity, extraction recovery, matrix effects, exogenous interferences, ionization suppression/enhancement, and sample stability on the autosampler. This method will be used to analyze over 250 MDME cases in which fentanyl and/or fentanyl analogues were previously identified and/or suspected to contribute to the cause of death.

**Results:** All analytes except for carfentanil were linear from 1.0-50.0 ng/mL with an administrative LOD of 0.5 ng/mL and an LOQ of 1.0 ng/mL; carfentanil was linear from 0.2-10.0 ng/mL with an administrative LOD of 0.1 ng/mL and an LOQ of 0.2 ng/mL. Inter- and intra-day precision, bias, extraction recovery, and sample stability were evaluated for all seven compounds at low and high concentrations within their respective linear ranges. For all analytes, inter- and intra-day precision were determined to be  $\leq 5\%$ , bias was determined to be  $\leq 13\%$ , and extraction recovery was  $\geq 87\%$ . No carryover was observed in an extracted matrix blank analyzed immediately after a sample spiked at twice the concentration of the highest calibration standard. Dilution integrity was assessed at x2, x5, and x10 dilutions; percent deviation from target was determined to be  $\leq 12\%$  for all analytes. No exogenous interferences from 56 commonly identified drugs were observed. All analytes in extracted samples were determined to be stable in the refrigerated autosampler for up to 72 hours.

**Conclusion/Discussion:** A robust and sensitive UHPLC-MS/MS method to quantify fentanyl and six novel fentanyl analogues was developed and validated to analyze over 250 postmortem specimens in which these compounds were previously identified and/or suspected to contribute to the cause of death.

**Keywords:** Carfentanil, Fentanyl Analogues, UHPLC-MS/MS

## Stability of Mephedrone, Naphyrone, and MDPV in Solvents and Na<sub>2</sub>EDTA Preserved Human Whole Blood

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**Background/Introduction:** Synthetic cathinones are new psychoactive substances that are structurally derived from cathinone, the psychoactive component of *Catha edulis* (“khat”), a shrub that is indigenous to the Middle East and East Africa. These compounds are often sold in headshops and on the Internet to circumvent controlled substance legislation due to their amphetamine-like pharmacological effects. Cathinone itself degrades in the khat plant to its inactive metabolites or dimers upon contact with sunlight or heat. Current research evaluates the stability of synthetic cathinones in biological matrices, including blood preserved with the NaF/K<sub>2</sub>C<sub>2</sub>O<sub>4</sub> combination used in grey-top tubes, but does not assess their stability in the solvent-based working solutions used to make calibration curves or in blood preserved with Na<sub>2</sub>EDTA, which is used for some clinical samples.

**Objective:** To evaluate the stability of the Schedule I synthetic cathinones mephedrone, naphyrone, and 3,4-methylenedioxypropyl-alerone (MDPV) in methanol and acetonitrile-based working solutions and in human whole blood preserved with Na<sub>2</sub>EDTA over a 30-day period

**Methods:** Mephedrone, naphyrone, and MDPV samples in each matrix were spiked to a concentration of 1 mg/L on day 0 of the study and subsequently aliquoted (100 µL solvents, 1.2 mL biological matrices, n=39) for storage at three temperatures (21 °C, 4 °C, and -20 °C) (n=12 at each temperature). On day 0, deuterated internal standard corresponding to each analyte was added to three samples prior to extraction. Solvent samples were evaporated to dryness at 37 °C under compressed air, and blood samples underwent solid phase extraction using United Chemical Technologies (UCT) CLEAN SCREEN® CSDAU506 cartridges. An additional derivatization step using a 2:1 solution of pentafluoropropionic anhydride (PFPA) and ethyl acetate was required for mephedrone samples. On days 3, 7, 14, and 30 of the study, samples from each storage temperature were extracted in triplicate for GC-MS analysis. Resulting peak area ratio data were statistically evaluated for significance using single factor analysis of variance (ANOVA).

**Results:**

Drug	Matrix	Temperature (°C)	Percent loss over 30-days
Mephedrone	Methanol	21	<b>88%</b>
		4	<b>51%</b>
		-20	Stable
	Acetonitrile	21	<b>33%</b>
		4	Stable
		-20	
	Blood	21	<b>97%</b>
		4	<b>24%</b>
		-20	<b>25%</b>
Naphyrone	Methanol	21	Stable
		4	
		-20	
	Acetonitrile	21	<b>26%</b>
		4	Stable
		-20	
	Blood	21	<b>98%</b>
		4	<b>91%</b>
		-20	<b>25%</b>
MDPV	Methanol	21	<b>44%</b>
		4	<b>32%</b>
		-20	Stable
	Acetonitrile	21	Stable
		4	
		-20	
	Blood	21	<b>45%</b>
		4	Stable

**Conclusion/Discussions:** Mephedrone, naphyrone, and MDPV all exhibited instability in at least one solvent-based working solution and in human whole blood preserved with Na<sub>2</sub>EDTA at a minimum of one storage temperature examined, with freezer temperatures resulting in the highest stability overall. Methanol-based mephedrone working solutions displayed instability even when stored at 4 °C, which is a significant finding, as all reference standard manufacturers currently supply this compound in methanolic suspensions and often recommend refrigerator storage. Additionally, although past research has shown pyrrolidine-derived synthetic cathinones to be stable for up to two weeks in blood collected into grey-top tubes, it is important to be cautious in interpreting results of toxicological analysis for these compounds in blood preserved with Na<sub>2</sub>EDTA.

**Keywords:** Stability, Synthetic Cathinones, New Psychoactive Substances

## Evaluation of Artificial Drug Incorporation into Hair for the Production of Quality Control Samples

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**Background/Introduction:** The application fields of hair analysis have been growing substantially over the years and, consequently, the number of laboratories that provides this kind of service has been also raised. In this context, quality assurance for this practice has gained strength and so has the need for reference material. However, due to the lack of authentic hair samples from drug users as well as their significant high cost, this reference material is scarce. As an alternative, internal quality controls can be produced artificially by the laboratories themselves to be used routinely, generally by fortification.

**Objective:** To evaluate the phenomenon of artificial incorporation of drugs in hair to produce internal quality controls.

**Methods:** The internal quality controls were prepared according to the recommendations of the National Institute of Standards and Technology (NIST) with some adaptations. Aliquots of drug-free (brunette, blonde ?) human hair (50 mg), in triplicate, were separately incubated in a solution of water-dimethylsulfoxide (1:1) spiked with cocaine (5 µg/ml), amphetamine (3 µg/ml), MDMA (5 µg/ml), morphine (3 µg/ml) and diazepam (2 µg/ml). The incubation was performed during three, six, nine and twelve days. At the end of each period, the samples were washed with methanol and then extracted. The method of extraction consists in overnight incubation with methanol at 55 °C. An aliquot of 3 µL of the extract was directly injected in an Acquity UPLC (Waters, USA) coupled to a Quattro Premier XE mass spectrometer (Micromass, UK). The chromatographic separation was achieved on an Acquity UPLC BEH C18 column eluted with a gradient of 1 mM ammonium formate with 0.1% formic acid and acetonitrile at 400 µL/min flow rate and 45 °C. The ESI-MS parameters were set in the positive ion mode.

**Results:** The spiked concentrations of analytes were based on previous experiments by both NIST and at our laboratory. The wash residue values were insignificant, not interfering in the final quantification of all analytes.

For cocaine, the incorporation rate was progressive over the course of days, ranging from 0.15 to 0.75% with coefficients of variation (CV%) < 25 among the triplicates. Amphetamine and MDMA showed different incorporation rate (0.17 to 0.5% and 0.10 to 0.4%, respectively); however, it was notable that both have similar patterns of incorporation, showing no increase of concentration from the sixth to the ninth day (0.42% for amphetamine and 0.35% for MDMA). For diazepam, the incorporation rate was the same, during three or six days, reaching 0.57%. From the ninth to twelfth day, it was possible to observe an increase in the incorporation rate getting to 2.5 and 3.0% respectively. Low rates of morphine were attained, ranging from 0.08 to 0.25%, being the 0.25% of incorporation reached on the ninth day. As for NIST experiments, the rates for all analytes (except for diazepam) were higher possibly due to longer periods of incubation (approximately 16.5 days).

**Conclusion/Discussions:** Overall it is possible to achieve different concentrations at different time points to produce internal quality control samples, according to the laboratory interests. These data may enable laboratories to perform hair analyses and to evaluate the accuracy of their methods.

**Keywords:** Material Reference, Quality Control, Hair Analysis, Alternative Matrices

## The Invisible Threat from the Wells

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**Background/Introduction:** According to the 2011 census, Romania has a population of over 20 million inhabitants. 43.6% live in the countryside, compared to the countries in the European Union where the percentage is only 25%. Romania's poor social-economic particularities, especially in the rural area, is characterized by the existence of territories with frequent and important changes of the water purity from wells, which is used in consumption, by the nitric substances coming from the fertilizers and waste waters. These contaminants lead to the appearance of the methemoglobinemia in infants, known in literature as the "blue baby syndrome".

**Objective:** The aim of the present study is to emphasize the danger of polluted waters, particularly on children, and to inform the population about the danger from wells, and the health effects.

**Methods:** Determination of methemoglobin from blood was based on the spectrophotometric absorbtion at 620 nm with the transformation to cyanomethemoglobin.

**Results:** The methemoglobinemia may appear in cases of exposure to oxidizing substances (nitrates, nitrites, pharmaceuticals), congenital metabolism errors, and some other medical conditions. The

Pediatric Poisoning Centre at the Emergency Clinical Hospital for Children " Grigore Alexandrescu" in Bucharest reported 61 cases of acute toxic methemoglobinemia from 2010 – 2014: 51 (83.6%) consumed well water and 9 (16.4%) where exposed to pharmaceuticals. 28 cases (46%) involved children that were less than 1 year in age, while 26 cases (43%) were children between 1 to 5 years and in 7 cases (11%) the patients were over 6 years old. The methemoglobin in all patients ranged between 12.9% and 87%. Within the Legal Medicine network, of 83 methemoglobin analyses during 2012 - 2017, 22 (26.5%) were positive. The youngest subject was just 17 days old, while the oldest was 1 year and 10 months. The methemoglobin values were between 2.35% and 54%, with an average of 19.35%.

**Conclusion/Discussions:** oxic methemoglobinemia is potentially fatal, especially in babies that consume well water. The risk of consuming unchecked water is not understood by the Romanian population. There is a need to inform the public regarding the constructions and sanitary protection of wells. It has been suggested that babies be breast fed and, when this is not possible, water should be used from a trusted sources, such as when bottled

**Keywords:** Methemoglobinemia, Infants, Well Water, Nitrates

## Analysis of the Antiretroviral, Efavirenz, in Hair Samples from HIV-Infected Patients by Liquid Chromatography Tandem Mass Spectrometry: A Novel Method of Therapeutic Drug Monitoring in South Africa

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**Background/Introduction:** South Africa has the highest prevalence of HIV/AIDS compared to any other country in the world with an estimated 12.7% of the total population living with HIV. Efavirenz is a non-nucleoside reverse transcriptase inhibitor used for the treatment of HIV-1 infection. This antiretroviral (ARV) is used as a component of first-line antiretroviral therapy (ART) in South Africa. Successful response to ART largely depends on optimal adherence to treatment. Therapeutic drug monitoring (TDM) plays an important role in monitoring the response to ART by measuring ARV drug exposure. Plasma drug concentrations are generally used for TDM, however these concentrations represent more recent drug exposure and may be susceptible to 'white-coat effects', where adherence just prior to clinic visits improves. More recently, hair has been studied as an alternative matrix for TDM and concentrations of ARV's in hair have been shown to be strongly correlated with virologic outcomes and adherence. Additionally, the collection of hair samples is noninvasive and drug concentrations in hair provide information of long-term drug exposure.

**Objective:** Primarily, to develop and validate a method for the determination of efavirenz levels in small samples of hair collected from HIV-infected patients in Cape Town, South Africa. Secondly, to investigate the usefulness of using hair as a matrix to monitor ARV levels specifically within the South African context.

**Methods:** A liquid chromatography tandem mass spectrometry method for the analysis of efavirenz in hair was developed. The extraction procedure was simple and involved a simultaneous pulverization and extraction step with a 70:30 (v/v) mixture of methanol:water. The extracted efavirenz was separated on an Agilent Poroshell C18 column using an isocratic elution with a total run time of 3 min. Efavirenz was monitored in positive ionization multiple reaction monitoring mode. This method was validated from 0.625 ng/mg – 40 ng/mg using 0.2 mg hair samples. The validated method was applied to 257 hair samples, collected during three clinic visits at 16, 32 and 48 weeks, from 135 HIV-infected patients who commenced ART at a treatment center in Cape Town, South Africa.

**Results:** A liquid chromatography tandem mass spectrometry method for the analysis of efavirenz in hair was developed. The extraction procedure was simple and involved a simultaneous pulverization and extraction step with a 70:30 (v/v) mixture of methanol:water. The extracted efavirenz was separated on an Agilent Poroshell C18 column using an isocratic elution with a total run time of 3 min. Efavirenz was monitored in positive ionization multiple reaction monitoring mode. This method was validated from 0.625 ng/mg – 40 ng/mg using 0.2 mg hair samples. The validated method was applied to 257 hair samples, collected during three clinic visits at 16, 32 and 48 weeks, from 135 HIV-infected patients who commenced ART at a treatment center in Cape Town, South Africa.

**Conclusion/Discussions:** The validated method allowed for the successful monitoring of efavirenz in small amounts of hair collected from HIV-infected patients. This newly developed method, novel within the South African context, will be useful in establishing the routine analysis of efavirenz in hair to be used in the TDM context and can be further developed to include other relevant ARV's. Additionally, the results obtained from the analysis of hair samples will be used in studies investigating factors influencing adherence to ARV's.

**Keywords:** Efavirenz, Hair, LC-MS/MS

## Qualitative Identification of Fentanyl Analogues and Other Opioids in Postmortem Cases by UHPLC-Ion Trap-MS<sup>n</sup>

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**Background/Introduction:** Since 2013, the Miami-Dade County Medical Examiner Department (MDME) has seen an increase in the number of opioid-related deaths. From 2014 to 2015, a near 600 percent increase in fentanyl-related deaths was observed, followed by an additional 200 percent increase in 2016. In 2015, the first novel fentanyl analogues were identified in medical examiner cases: beta-hydroxythiofentanyl and acetyl fentanyl. In 2016, four additional fentanyl analogues emerged: para-fluoroisobutyrylfentanyl, butyryl fentanyl, furanyl fentanyl, and carfentanil, as well as U-47700. Due to the potency and subsequent low concentrations in whole blood of these compounds, a basic drug screen method by gas chromatography-mass spectrometry (GC-MS) was not sensitive enough to identify these substances in postmortem casework.

**Objective:** The objective of this work was to validate a targeted screening method for the qualitative identification of 44 opioid-related and analgesic compounds in postmortem samples, and to demonstrate the methods applicability to postmortem detection of fentanyl analogues.

**Methods:** A Thermo Scientific Dionex Ultimate 3000 Ultra High Performance Liquid Chromatograph (UHPLC) coupled to a Bruker AmaZon Speed Ion Trap mass spectrometer (Ion Trap-MS<sup>n</sup>) equipped with ToxTyper™ software, was validated for the screen of a broad range of opioid-related compounds. UHPLC separation was achieved by using a gradient elution on a Thermo Scientific Acclaim® RSLC 120 C18 column (2.1 x 100 mm, 2.2 μm) with an aqueous mobile phase of 2mM ammonium formate, 0.1% formic acid, and 1% acetonitrile in water, and an organic mobile phase of 2mM ammonium formate, 0.1% formic acid, and 1% water in acetonitrile. The ion trap was operated using electrospray ionization (ESI) with zero delay alternating polarity. Data was collected using an autoMS<sup>n</sup> mode, targeting known precursor and product-precursor ions to produce MS<sup>3</sup> spectral detail. Acquisition was achieved using a data-dependent Scheduled Precursor List (SPL) which contains 44 targeted compounds. Out of the 44 targeted compounds in the method, 27 have MS<sup>3</sup> spectral profiles. Prior to analysis, compounds were extracted from blood, brain, liver, serum, and urine using mixed-mode solid-phase extraction columns.

**Results:** Separation of all 44 compounds was achieved within the first 6 minutes of the 11 minute run time. Limits of detection (LOD) ranged from 0.1 - 5 ng/mL for all analytes. Of the 44 compounds targeted, 11 were optimized and added to the established ToxTyper™ library. Endogenous interferences from decomposed matrices and exogenous interferences from commonly detected analytes were found to be insignificant. Extracts were stable in the refrigerated autosampler for 48 hours. No significant ionization suppression/enhancement was observed.

**Conclusion/Discussion:** This method has proven to be invaluable for postmortem casework where drug paraphernalia and/or case history suggest the presence of the targeted compounds, but corresponding analysis of the case samples was negative by GC-MS. Over 500 postmortem MDME cases have been analyzed using this method since 2014. Fentanyl analogue-positive cases first appeared in 2015, with 176 identified through 2016. Of the 176 positive cases, only 24% were identified by GC-MS, with the remaining cases screening negative. A large majority of the negative cases were also negative for other drugs, resulting in an undetermined medical examiner case status. Furthermore, approximately 80% of fentanyl analogue-positive cases contained carfentanil that was initially not identified by GC-MS. Clearly, the introduction of a more sensitive screening method that provides robust spectral detail at a much lower detection limit allowed for the identification of fentanyl analogues in postmortem matrices and ultimately led to the appropriate cause of death determination.

**Keywords:** Fentanyl Analogues, Postmortem, LC-Ion Trap MS<sup>n</sup> Screening



## Broad and Comprehensive Screening of Novel Psychoactive Substances in Post-Mortem Matrices by Liquid Chromatography Tandem Mass Spectrometry (LC-MS/MS)

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**Background/Introduction:** Novel Psychoactive Substances (NPS) are newly emerging compounds, natural and synthetic, that are often sold as ‘legal’ alternatives to controlled substances. These substances can include phenethylamines, tryptamines, synthetic cathinones, dissociative drugs, and other compounds not routinely encountered in casework. These compounds may go undetected in forensic toxicology labs where suitable identification methods for NPS do not exist.

**Objective:** The aim of this study was the development of a suitable liquid chromatography tandem mass spectrometry (LC-MS/MS) method for a broad and comprehensive screening of novel psychoactive substances, allowing for their simultaneous detection in human post-mortem matrices such as whole blood and urine.

**Methods:** Extraction of target analytes was conducted using a liquid-liquid extraction scheme under basic pH conditions. The assay utilized a nominal sample volume of 1 mL. To samples, 1.0 µg/mL internal standard (Alpha PVP-D8, Amphetamine-D5, Butylone-D3, MDPV-D8, Mitragynine-D3, PCP-D5, and 2C-I-D3) and 0.5 µg/mL quality controls were added along with 0.5 mL NH<sub>4</sub>OH and 6 mL of 70:30 n-butyl chloride:ethyl acetate to 16x125 screw top tubes. Samples were placed on a gentle rocker and centrifuged for 10 minutes each. The supernatant was transferred over to 13x100 centrifuge tubes treated with 25 µL of 2% Methanol in HCl and evaporated to dryness. Samples were reconstituted with 200 µL of 90:10 water:ultrapure methanol and analyzed on an Agilent Technologies 1260 LC coupled with a 6460-triple quadrupole mass spectrometer with a Jetstream electrospray ion source operating in positive ion mode. When possible, two ion transitions were utilized for identification.

**Results:** A total of 46 NPS (and 7 deuterated internal standards) were targeted in the assay.

Phenethylamines	Cathinones	Dissociatives	Tryptamines	Opioid receptor agonists
25B-NBOMe	3-fluoromethcathinone	Ketamine	Psilocin	Mitragynine
25C-NBOMe	Alpha PVP	Norketamine	DMT	7-Hydroxymitragynine
25I-NBOMe	Alpha PBP	Deschloroketamine	5-MeO-DMT	
25I-NBOH	Alpha PPP	Methoxetamine	LSD	
2C-C	3-methoxymethcathinone	PCP		
2C-E	Methylone	3-MeO-PCP		
2C-I	Ethylone			
2-fluoroamphetamine	Butylone			
Ephedrine/Pseudoephedrine	Mephedrone			
Amphetamine	Pentedrone			
Methamphetamine	Pentylone			
Phentermine	MDPV			
MDA	N-ethylpentylone			
MDMA	Methedrone			
Methylphenidate	MDPPP			
5-IT				
DOB				
DOI				
DragonFLY				

Parameters were investigated based on SWGTOX guidelines for matrix interferences, carryover, stability, and peak resolution. All target analytes demonstrated successful results for each parameter. Initially, all compounds were detected down to a level of 6.25 ng/mL in certified negative matrix, with recovery of both transition ions within laboratory guidelines of +/- 20%. Further work demonstrated that all compounds, with the exceptions of Amphetamine, DragonFLY, and Psilocin, were detected down to a level of 2.5 ng/mL in spiked post-mortem blood and urine samples in varying states of decomposition, with transition recovery also within +/- 20%. Other drugs commonly found in the forensic toxicology lab not targeted in this method were evaluated for potential interferences including opiates, opioids, THC (Δ9 and THC-COOH), benzodiazepines, antihistamines, antidepressants, antipsychotics, and cocaine/benzoyllecgonine. During investigation, potential MS/MS breakdown products were identified. Analysis demonstrated that both 25I-NBOMe and 25I-NBOH break down to 2C-I, 25C-NBOMe breaks down to 2C-C, and DMT breaks down to Psilocin. The origins of these breakdowns are unknown, but subsequent investigations support the possibility of these occurring during instrumental analysis. Additionally, 3-methoxymethcathinone, Methedrone, and MDMA share identical precursor and transition ions. Qualifier ratios and retention time were evaluated to differentiate between the aforementioned targets.

**Conclusion/Discussion:** The data collected represents a targeted LC-MS/MS method to accurately screen for and qualitatively identify 46 NPS in whole blood and urine following a liquid-liquid extraction scheme. The robustness of this method allows for the continual addition of NPS as they appear, ensuring up-to-date simultaneous drug detection. Care must be taken when determining detection of drugs due to similar transitions or breakdowns. Future work will also include investigation into the nature of these breakdowns, as they were not the original focus of this research.

**Keywords:** Novel Psychoactive Substances, LC-MS/MS, Method Development

## Investigation of Post-Mortem Redistribution from the Bladder Using In-Vitro Models

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**Background/Introduction:** Drug concentration at the time of death can determine whether the drug was in the therapeutic or toxic range, indicating if this drug was involved in the death. However, drug concentrations from autopsy samples cannot be assumed to be the levels at time of death. During the postmortem interval (PMI) between death and autopsy there are changes occurring that can affect drug concentrations. Postmortem redistribution (PMR) is a process that involves the passive movement of drugs after death. PMR can lead to changes in postmortem drug concentrations at sampling sites. Certain organs including lungs, liver, and heart are depots for drugs to “enable” PMR. One possible depot is the bladder, with which there has been limited research on possible PMR.

**Objective:** The aim was to determine if the bladder is a PMR depot candidate and develop methodology to allow further study. We investigated the influence of temperature, pH, bladder degradation, and solution volume on diffusion from the bladder using in vitro diffusion through bladder sections, whole bladders and finally in vivo diffusion from the bladder in rat models over nine days.

**Methods:** A section of six porcine bladders was secured onto Franz cells. Acceptor chamber solutions were 20 mM pH 7.4 phosphate buffer (PBS) and 20 mM pH 5 ammonium acetate (AA). Donor solutions, dependent on experiment, contained 100 mg/L Rhodamine B or amitriptyline and nortriptyline in the respective solutions. Sampling occurred over five days. Parameters included temperature (37 °C, 20 °C, 5 °C), pH (7.4, 5), intra-variability of bladder diffusion and tissue degradation. Quantitation methods of Rhodamine B (UV, Agilent, Cary 60) and amitriptyline and nortriptyline (HPLC, Dionex Ultimate 3000) were validated according to SWGTOX guidelines. Whole bladder studies used the validated UV method for Rhodamine B. Experimental temperature was 20 °C. Full and half-filled bladders contained Rhodamine B (100 or 200 mg/L) dissolved in PBS and AA. Triplicate analysis was performed using the UV spectrophotometer at 554 nm. The in-vivo study involved catheterising a rat and inserting silver nitrate into the bladder then securing it for Computed Tomography (CT) analysis over nine days.

**Results:** Fresh and frozen bladders (pH 7.4, 5) showed the fastest diffusion at 37 °C. Degraded bladders had twice the drug diffusion through bladder tissue in PBS at 37 °C when compared to fresh bladders. The lower pH (5) took 30 hours longer than pH 7.4 for the diffusion process to begin. Different sections within the same bladder (Intra-bladder) had variation with the peak drug concentration in PBS at 2.49 mg/L and AA at 0.61 mg/L. Amitriptyline and nortriptyline had peak drug concentrations of 4.2mg/L and 12.3mg/L respectively in PBS at 37 °C. Concentration in whole bladder studies was not a significant factor affecting diffusion with peak drug concentrations for 100 mg/L at 3.47 mg/L and for 200 mg/L at 1.22 mg/L. The drug solution volume showed mixed results and was pH dependent; the highest drug concentration was in 100 mg/L Rhodamine B, with the full bladder in PBS at 3.47 mg/L and the half-filled bladder in AA at 0.67 mg/L. The rat bladder was intact for two days, and then between 2-6 days, an opening was observed with leakage of solution. However, after day 7 this solution was not observed on the CT.

**Conclusion/Discussions:** The bladder is not deemed a source of significant PMR for amitriptyline and nortriptyline for the first five days after death. These results show that the concentrations, even if diffused to the femoral vein, would not alter the associated drug concentrations to a significant degree that could alter the toxicological interpretation of a case.

**Keywords:** Postmortem Redistribution, Amitriptyline, Diffusion

## Analysis of Ethyl Sulfate in Oral Fluid by Liquid Chromatography Tandem Mass Spectrometry

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**Background/Introduction:** Oral fluid has been shown to be an acceptable alternative matrix to urine in clinical drug testing (CDT). Detection of ethanol exposure is an important component of CDT. Ethyl Sulfate (EtS) is the primary ethanol metabolite present in oral fluid. Research has demonstrated that ethyl glucuronide (EtG) concentrations are low or undetectable (<50 ng/mL) in most oral fluid samples. However, there are very few data currently available on EtS concentrations in oral fluid after ethanol exposure.

**Objective:** To develop a robust liquid chromatography tandem mass spectrometry (LC-MS/MS) method for the determination of EtS in oral fluid and to establish a practical linear range for the detection of positive patients.

**Methods:** Standard reference material obtained from Cerilliant (1 mg/mL) was spiked into drug-free oral fluid buffer to obtain calibrators in the range of 25 to 2,500 ng/mL EtS. Ethyl-d5 sulfate sodium salt (as ethyl sulfate) was utilized as internal standard (Cerilliant, 1 mg/mL). Quality control (QC) specimens included a negative (drug-free oral fluid), and 2 positives (35 and 2,000 ng/mL spiked into drug-free oral fluid). Oral fluid samples were collected using the Oral-Eze® collection device. Internal standard was added to 100 µL of the oral fluid samples before acidification with 4% phosphoric acid. The acidified EtS samples were loaded onto preconditioned weak anion exchange columns (Biotage) using a Hamilton Microlab Starlet. The columns were washed with 2% formic acid in water and methanol. Analytes were eluted with 5% ammonium hydroxide in acetonitrile. The extracts were evaporated to dryness under nitrogen at 45°C and reconstituted using 80:20 acetonitrile:methanol with 0.1% formic acid. Samples were analyzed using a Shimadzu Prominence liquid chromatograph with a Luna HILIC 3µ 100 x 4.6 mm column (Phenomenex) coupled with a Sciex 4500 Triple Quad tandem mass spectrometer operated in negative electrospray ionization mode. Separation was achieved isocratically within 4 minutes with mobile phase A of 50:50 acetonitrile:methanol with 0.2% formic acid and mobile phase B 25 mM ammonium formate in 87.5% acetonitrile. The following MRM transitions were monitored for EtS: 125-80 (quantifier), 125-97 (qualifier), and 130-98 for EtSd5. Limit of detection (LOD), within-run and between-run precision, bias, carryover, interference, and stability of the assay were tested via standard methods. Between June 21, 2016 and March 27, 2017, 4,349 patient samples were analyzed using this method.

**Results:** The calibration model was linear 25 to 2,500 ng/mL ( $r^2 > 0.998$ ), with calibrators calculated to be within  $\pm 20\%$  of target. The limit of detection was 2 ng/mL and the administrative lower reporting limit was set at the limit of quantitation of 25 ng/mL. Within-run ( $n=5$ ) and between-run precision ( $n=25$ ) had percent coefficient of variation  $< 5\%$  at 35 and 2,000 ng/mL concentrations. Bias for positive QC specimens averaged 2.3%, with a range of -4 to 10.6% ( $N=24$ ). No carryover was observed at 5,000 ng/mL concentration. Studies conducted with over 70 compounds commonly encountered in CDT (including benzodiazepines, barbiturates, opiates, synthetic stimulants, and EtG) demonstrated no interference with EtS. Extracts of QC material were stable ( $\pm 20\%$  original result) for 24 hours at room temperature or 3 days refrigerated (2°C -8°C). Of the 4,349 patient samples analyzed by this method, 169 (3.9%) were positive in the range of 25 to 2,341 ng/mL, with an average concentration of 137 ng/mL. Of the positive patients, 139 (82.2%; 3.2% of all samples) had concentrations between 25 and 200 ng/mL.

**Conclusion/Discussions:** An effective method for the analysis of EtS in oral fluid was developed for routine CDT. Based on tested patient samples, the linear range of 25 to 2,500 ng/mL is suitable for all positive patients analyzed to date.

**Keywords:** Oral Fluid, Ethyl Sulfate, LC-MS/MS

## Prevalence of *In Utero* Exposure to Drugs in a Cohort of Neonates and Mothers by an Extended Toxicological Screening in Biofluids and Hair

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**Background/Introduction:** Maternal consumption of alcohol, illicit or medicinal drugs during pregnancy exposes fetuses at severe health risk, and may result in adverse mental, physical, and psychological outcomes at birth. The identification of drugs or their biomarkers in either maternal or neonatal specimens is much more informative than interview-based methods or self reporting from mothers. Among the biological matrices that are available for analysis, neonatal hair plays an important role. Drugs or their metabolites crossing the placenta are incorporated into the growing foetal hair from the 12th week of gestation. Collection of neonatal hair at birth is useful to assess, retrospectively, the exposure to drugs during the third trimester.

**Objective:** A retrospective study is presented, aimed at analyzing the prevalence of *in utero* exposure to drugs in a cohort of neonates and mothers by means of toxicological screening in hair. The final goal was the implementation of a clinical toxicological protocol where obstetricians, neonatologists, paediatricians, toxicologists and social services collaborate to contrast the phenomenon of drug use in pregnant women.

**Methods:** Data from 80 neonate-mother dyads that underwent a clinical toxicological screening at the university hospital in the years 2015 - 2016 were made anonymously and retrospectively analysed. The recorded data for neonates included: sex, weight, head circumference, gestational age, Apgar, adverse symptoms motivating the request of a toxicological ascertainment, number and type of samples collected, type and concentration of drugs in collected samples, and clinical follow up. The recorded data for mothers included: age, medical history, pregnancy history, number and type of samples collected, type and concentration of drugs, declared/undeclared use of drugs. The toxicological data were generated by a LC-HRMS screening including more than 800 drugs and psychoactive medicines. Hair were analysed by a micropulverised method applicable to tiny amounts of hair (2-10 mg). Maternal hair were analysed in 3 cm aliquots corresponding to pregnancy trimesters.

**Results:** Hair were positive for one or more class of drugs in 65 % of neonate-mother dyads. Drugs of abuse and substitutes (cocaine and metabolites, cannabinoids, opiates, ketamine, methadone, buprenorphine), pharmaceutical drugs (benzodiazepines, barbiturates, tramadol, non steroidal analgesics, selective serotonin re-uptake inhibitors, stimulants) and nicotine were detected. Cocaine and opiates were the most prevalent in neonates, cocaine, opiate and cannabinoids in mothers. Hair drug concentrations were generally higher in mothers than in neonates. No designer drug was detected. In hair from mothers, when long enough, use was documented for the whole pregnancy period. Poor neonatal outcome and/or withdrawal symptoms were always associated with detection of *in utero* exposure to drugs. In 90% of positive cases, healthcare providers and social services had no previous documentation of use from the mothers.

**Conclusion/Discussions:** The diagnosis of foetal exposure to drugs is sensitive and specific only when the identification of xenobiotics in biological matrices is available. Hair analysis is useful for identification of xenobiotics, whereas urine is useful for follow up of child and mother. Clinicians found interest in exposure to psychopharmaceuticals during pregnancy. Health care providers, toxicologists and social services should collaborate to contrast the phenomenon of drug use in pregnant women.

**Keywords:** Drugs, Hair, Neonates

**Case Report: Unusual Urine and Oral Fluid Results After Poppy Seed Muffin Consumption**

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**Background/Introduction:** Many studies have demonstrated that consumption of poppy seeds in food can result in a positive opiate drug test. Following observed poppy seed consumption, both urine and oral fluid drug-testing literature describe positive morphine results, but codeine results are always lower than morphine or none detectable. To our knowledge there are no published reports showing codeine concentrations greater than morphine in urine or oral fluid after eating poppy-containing food products.

**Objective:** To share unusual urine and oral fluid results following consumption of concentrated poppy seed muffins.

**Methods:** In urine, 8 opiate analytes (codeine, morphine, hydrocodone, hydromorphone, norhydrocodone, oxycodone, oxymorphone, and noroxycodone) were quantitated using a validated LCMSMS method. Sample preparation consisted of hydrolysis, centrifugation, and dilution. The linear quantitative range was 25 – 15000 ng/mL, with a 50 ng/mL reporting cutoff. In oral fluid, these same 8 analytes were quantified along with several other opioids in a larger panel. Oral fluid sample preparation involved solid phase extraction. The oral fluid linear range was 1 – 250 ng/mL, with a 2.5 ng/mL reporting cutoff. Each analyte had a matched internal standard in both the urine and oral fluid analyses. Poppy seeds were purchased from Target (Archer Farms brand) and baked into lemon muffins with each muffin containing approximately 1 tablespoon of poppy seeds (4.6x the amount the recipe called for). To approximate the dose of codeine and morphine per muffin, poppy seeds were crushed and extracted in methanol for 30 min. Eleven participants consented to the study and 14 urine samples were collected. Paired oral fluid collections were obtained for 9 of the 14 urine collections. For the first participant, urine and oral fluid collections occurred at time 0, 2h, and 4h after eating 1 muffin. For the remaining participants, collections occurred only at 4h after eating the muffin.

**Results:** Each muffin contained 4.3 mg codeine and 0.45 mg morphine. For the first patient, urine and oral fluid collections immediately prior to muffin consumption were below the lower limit of quantitation. For all subsequent collections and all collections for other subjects, codeine concentrations were higher than morphine results, with 4900 ng/mL as the highest observed codeine concentration (Table 1). The mean urine codeine:morphine ratio among the 13 positive samples was 4.8 (range 3.4-7.6). In the first participant with multiple collections, urine concentrations were higher at 4h compared to 2h. In contrast, the oral fluid from this subject showed higher concentrations at 2h (220 ng/mL codeine and 4.3 ng/mL morphine). All samples were negative for 6-acetylmorphine.

Table 1. Urine (UR) and oral fluid (OF) codeine and morphine results after poppy seed muffin consumption

Subject	Urine collection time (h) after consumption	UR Codeine (ng/mL)	UR Morphine (ng/mL)	UR Codeine/ Morphine ratio	OF Codeine (ng/mL)	OF Morphine (ng/mL)	OF Codeine/ Morphine ratio
A	0	0	0		0	0	
A	2	1100	210	5.2	220	4.3	51.2
A	4	2600	340	7.6	5.6	0	
B	4	1900	530	3.6	51	4.7	10.9
C	3	1600	290	5.5			
D	4	1000	200	5	37	3.36	11
E	3	1100	240	4.6	22	0	
F	4	1000	270	3.7			
G	4	2800	820	3.4			
H	4	4900	1400	3.5			
I	4	1700	400	4.3			
J	4	820	140	5.9	75	10	7.5
K	4.5	1700	260	6.5	39	4.5	8.7
K (Day 2*)	4.25	3300	930	3.5	85	12	7.1
Mean (of positives)		1963.1	463.8	4.8	66.8	6.5	16
SD		1170.6	370	1.3	67.1	3.6	17.3
Min positive		820	140	3.4	5.6	3.4	7.1
Max positive		4900	1400	7.6	220	12	51.2
*after additional muffin on Day 2							

**Conclusion/Discussions:** We report for the first time that poppy seed muffin consumption can result in higher codeine concentrations relative to morphine, in both urine and oral fluid. These unusual results need to be verified and repeated. Limitations of this report include the small sample size, limited collections prior to eating, and the lack of further monitoring beyond 4h after consumption. Prior to this report, higher codeine concentrations relative to morphine in urine or oral fluid suggested codeine drug exposure more than poppy seed consumption. However, our findings are clinically significant in revealing that higher codeine concentrations compared to morphine in urine or oral fluid is consistent with poppy seed consumption alone.

**Keywords:** Poppy Seeds, Oral Fluid, Codeine

## Increase of Amphetamine Concentration in Cosmetically Treated Hair

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**Background/Introduction:** The retrospective study carried out on 2,954 hair samples analyzed in our laboratory for amphetamine derivatives -amphetamine (AP), methamphetamine (MA), 3,4-methylenedioxy-methamphetamine (MDMA), 3,4-methylenedioxyamphetamine (MDA), and 3,4-methylenedioxy-N-ethylamphetamine (MDEA)- revealed significant differences between the positivity rate for AP in dyed or bleached hair (31.48%) in relation to that rate in natural hair (16.17%) ( $p = 0.003$ ). Conversely, positivity rate for MDMA was lower in dyed hair than in natural hair, but these differences were non-significant ( $p = 0.828$ ). Other retrospective studies have obtained similar results, although with non-significant differences. According to several studies, the concentration of drugs in hair may decrease by bleaching or dyeing; however, at the same time, treated hair can incorporate greater quantity of drugs than intact hair under the same exposure conditions. Our finding of higher positivity rate for AP from dyed hair could suggest a greater incorporation of AP in dyed hair from sweat and sebum, favored by cuticle damage.

**Objective:** Evaluate the effect of incorporation of AP, MA and MDMA to virgin, dyed, and bleached hair from sweat or sebum, on the analytical results to those drugs.

**Methods:** Virgin, dyed, mildly as well as severely bleached locks of hair were exposed to artificial sweat or sebum containing AP, MA, and MDMA (500 ng/g), at 37°C for a time period of 8 and 24 h, respectively. After the exposure, all the hair samples were tested by GC/MS in selected-ion monitoring mode, applying an already published method, accredited to ISO/IEC 17025 standards. The differences of each drug concentration (AP, MA, MDMA) between different kind of hair were statistically compared: virgin hair vs dyed hair, virgin hair vs mildly bleached hair, virgin hair vs severely bleached hair. The differences between different drugs concentration within each kind of hair (virgin, dyed, mildly and severely bleached) were, as well, statistically compared: AP vs MA, AP vs MDMA, MA vs MDMA.

The paired samples Student's t-test was applied in cases where the concentration differences followed the normal law. In these cases the extent of the difference was calculated with a confidence interval of 95%. The paired samples Wilcoxon's t-test was applied in cases where the concentration differences did not follow the normal law.

**Results:** Regarding the incorporation of AP, MA and MDMA from artificial sweat in vitro, the analysis provides evidence that the mean concentration for each drug in dyed or bleached hair is higher than the mean concentration in virgin hair, and that the differences between means are likely to be between 0.15 and 0.57 ng/mg, depending on the drug and the compared pairs. The incorporation of different drugs in the same kind of hair shows little differences in virgin and dyed hair, but the mean concentration of MDMA in bleached hair is higher than the mean concentration of AP; the differences between means are likely to be between 0.08 and 0.16 ng/mg. Regarding the incorporation of AP, MA and MDMA from artificial sebum in vitro, the differences between mean concentrations are lower for every compared pair, in relation to the incorporation from sweat. AP is not incorporated into hair from sweat or sebum to a greater extent than MDMA.

**Conclusion/Discussions:** The effects of cosmetic hair treatments on the concentration of amphetamines, both positive and negative, should be considered when interpreting the analytical results, especially if they are close to the proposed cut-off.

**Keywords:** Hair Analysis, Amphetamines, Cosmetic Treatment



## A New Method for Human Hair Analysis of 132 Various Types of Novel Psychoactive Substances (NPS) by Tandem LC/MS

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### Background/Introduction: Introduction.

Novel Psychoactive Substances (NPS) are now frequently encountered in clinical and postmortem toxicology investigations. Sensitive, reliable and reproducible techniques are required to detect and identify these substances in a variety of different matrices. Testing for drugs in hair is well established in the field of forensic and clinical toxicology, however, limited screening procedures for NPS currently exist. Most techniques are restricted to certain classes of NPS, contain limited numbers of NPSs and require time consuming sample preparation.

**Objective:** To develop and validate a liquid chromatography / tandem mass spectrometry (LC-MS/MS) multi-analyte method to qualitatively identify (screening) 132 NPS of different classes (mainly synthetic cathinones and synthetic cannabinoids, but also amphetamine type designer drugs, piperazines and other hallucinogenic compounds) utilizing a single extraction step in hair.

**Methods:** Washed hair (20mg) was placed in 2mL BeadRuptor™ tubes containing ceramic beads and silicone O-ring. Internal standard solution (50 µL) and 1 mL of a freshly prepared mixture of methanol/concentrated hydrochloride (0.1 M hydrochloric acid in methanol) was added to each tube. The samples were pulverized on the BeadRuptor™ system, and incubated at 40 °C for 3 hours before being pulverized again under the same conditions. Mixtures were then centrifuged (16,000 rpm, 5 minutes), supernatant transferred to autosampler vials and evaporated to dryness under nitrogen at 40 °C. Extracts were reconstituted with methanol (100 µL) and aliquots of 2 µL were injected into the LC-MS/MS system (Shimadzu Nexera X2 30 AD LC and Sciex 4500 Q-Trap™ mass spectrometer) operated in MRM mode using positive electrospray ionization. Separation was performed on a Kinetex C18 column (4.6 mm×50 mm, 2.6 µm) with gradient elution from 12% to 100% eluent B (eluent A: 50 mM aqueous ammonium formate/ formic acid, pH 3.5; eluent B: acetonitrile /0.1 % formic acid). The flow rate was 1.5 mL/min, autosampler and column oven were operated at 4 °C and 30 °C respectively. The run time was 12.40 min.

**Results:** No interferences were observed among the selectivity experiments. The limit of reporting was set at 0.1 ng/mg. Matrix effects were acceptable for most NPS, both at low and high concentration with only six analytes (norephedrine, cathinone, methylcathinone, norketamine, UR-144 5-Chloro-pentyl and JWH-210) showing responses of 70-75% at low concentrations. Ion enhancement (>125 %) was observed at high concentrations for benzylpiperazine, pseudo-ephedrine, ethylone, PPP, and MDPPP. All analytes showed variation in response within 20 % of the mean. Extraction efficiencies were above 75 % for most analytes in both low and high concentrations. NPS at high concentration were extracted more efficiently than at low concentration. The lowest values in extraction efficiencies were observed for cathinones and piperazines while most synthetic cannabinoids showed excellent extraction efficiencies. Hair samples from 23 coronial cases where NPS were detected in blood or where NPS abuse was suspected were tested. Many NPS were identified including acetyl fentanyl, 25CNBOMe, MDPV, PB-22 and AB-FUBINACA, confirming hair as a useful matrix for the analysis of NPS where historical or retrospective information is sought.

**Conclusion/Discussions:** A rapid tandem LC-MS method for the targeted screening of 132 NPS in hair is described. It covers a broad range of NPS, mainly synthetic cathinones and synthetic cannabinoids, but includes also amphetamine type designer drugs, piperazines and other hallucinogenic compounds. It can be used in routine medico-legal toxicology investigations.

**Keywords:** Novel Psychoactive Substances (NPS); Hair; LC-MS

## Detection and Quantification by LC-MS/MS of Opioid Glucuronides in Hair of Opioid Users

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**Background/Introduction:** Currently most hair testing methods rely on quantification of parent drug analytes, which alone do not definitively distinguish between drug use and external contamination. One possible solution to this problem would be to confirm the presence of unique drug metabolites that cannot be present through contamination.

**Objective:** Phase II conjugated metabolites are ideal unique drug metabolites that show use because they are not products of common degradation pathways, as is the case for many Phase I metabolites, and are not commercially available drugs. The objective of this work was to determine if opioid glucuronide conjugated metabolites are present in measurable amounts in hair of opioid users.

**Methods:** Human hair (25 mg) was heated at 100 °C for one hour in the presence of M3 extraction reagent from Comedical (Trento, Italy). Samples were centrifuged and the supernatants underwent SPE prior to LC-MS/MS analysis. An LC-MS/MS method was developed and validated to quantify codeine, dihydrocodeine, codeine-6-glucuronide, dihydrocodeine-6-glucuronide, morphine, dihydromorphine, morphine-3-glucuronide, morphine-6-glucuronide, oxycodone, oxymorphone, oxymorphone-3-glucuronide, hydromorphone, hydromorphone-3-glucuronide, and 6-acetylmorphine in human hair. The calibration range was 40-1200 pg/mg for all parent compounds and 2-120 pg/mg for all glucuronide conjugates, except dihydrocodeine-6B-D-glucuronide, which had an ULOQ of 80 pg/mg.

**Results:** The method was used to analyze 46 human hair samples from known drug users, that were confirmed positive for opioids by an independent laboratory. Codeine-6-glucuronide, morphine-3-glucuronide, morphine-6-glucuronide, oxymorphone-3-glucuronide, and hydromorphone-3-glucuronide, were present in sufficient concentrations to be quantifiable in hair of opioid users. Their concentrations generally increased with increasing concentration of the corresponding parent compounds. Glucuronide concentrations in samples positive for parent analytes ranged from approximately 1 pg/mg to 25 pg/mg with a majority of samples having glucuronide concentrations in the range of approximately 1 pg/mg to 5 pg/mg. Relative to parent concentrations, average concentrations of the four detected glucuronides were: Codeine-6-glucuronide 2.33%, hydromorphone-3-glucuronide 0.94%, oxymorphone-3-glucuronide 0.77%, morphine 3-glucuronide 0.59%, and morphine-6-glucuronide 0.93%.

**Conclusion/Discussions:** This work demonstrates for the first time that codeine-6-glucuronide, morphine-3-glucuronide, morphine-6-glucuronide, oxymorphone-3-glucuronide, and hydromorphone-3-glucuronide are present at sufficient concentrations to be quantifiable in hair of opioid users.

**Keywords:** Opioid, Metabolite, Hair

## Disposition of Cannabinoids in Oral Fluid and Whole Blood after Vaporized and Smoked Cannabis

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**Background/Introduction:** Administration of cannabis products via vaporization (“vaping”) is gaining in popularity because of real and perceived benefits of vaporization technology. Vaporization of cannabis delivers drug to the user without the concomitant toxic chemicals and carcinogens produced by smoked (combusted) cannabis.

**Objective:** Profile the disposition of tetrahydrocannabinol (THC), metabolites, cannabinol (CBN), and cannabidiol (CBD) in oral fluid (OF) and blood (BL) following administration of single doses of either smoked or vaporized cannabis to infrequent users.

**Methods:** Seventeen drug-free participants completed six outpatient sessions of 8 hours duration, each separated by at least 1 week. Vaporized and smoked cannabis doses of 0, 10, and 25 mg of THC was administered to each participant using a within-subject cross-over study design. Dried cannabis containing approximately 13% total THC, 0.1% CBD, and 0.8% cannabinol (CBN) was smoked in a hand-held pipe or vaporized using a Volcano Medic tabletop vaporizer (Storz and Bickel, GmbH & Co., Oakland, CA) in each session. Participants consumed the entire dose *ad-libitum* over a period of 10 min or less. Neat OF and whole BL specimens were collected at baseline, 0.17, 0.5, 1, 1.5, 2, 3, 4, 5, 6, and 8 hr. Specimens were analyzed for cannabinoids by LC-MS-MS (Immunoanalysis Corporation, Pomona, CA). OF specimens were analyzed for THC (LOD, 1 ng/mL), THCCOOH (hydrolyzed, LOD, 0.025 ng/mL), CBD (LOD, 1 ng/mL), and CBN (LOD, 1 ng/mL). Blood specimens were analyzed (LOD, 1 ng/mL) for THC, 11-OH-THC, THCCOOH and THCCOOH-glucuronide.

**Results:** Maximum concentrations (C<sub>max</sub>) of analytes in OF and BL are listed in the table. The C<sub>max</sub> for THC in OF and BL generally occurred at the first specimen collection time point following cannabis administration. THCCOOH appearance in OF was erratic and was detected in only 7 of 17 participants in very low concentrations. Free THCCOOH in blood appeared rapidly and declined slowly whereas THCCOOH-glucuronide (THCCOOH-GL) was delayed.

Mean C<sub>max</sub> (SD, Range), ng/mL

Oral Fluid					
Route	Dose	THC	THCCOOH	CBN	CBD
Vaporized	10	85.6 (91, 5 - 383)	0.015 (0.031, 0 - 0.098)	8.2 (8, 0 - 32)	0.1 (0.2, 0 - 1)
Vaporized	25	460.7 (456, 30 - 1646)	0.017 (0.040, 0 - 0.128)	36.4 (34, 3 - 107)	0.6 (1.0, 0 - 3)
Smoked	10	169.8 (260, 11 - 1063)	0.066 (0.265, 0 - 1.095)	18.9 (27, 1 - 105)	0.3 (0.6, 0 - 2)
Smoked	25	402.1 (566, 16 - 2368)	0.016 (0.045, 0 - 0.175)	39.2 (40, 2 - 139)	0.8 (1.3, 0 - 5)
Blood					
Route	Dose	THC	11-OH-THC	THCCOOH	THCCOOH-GL
Vaporized	10	6.6 (5.7, 0 - 21.0)	1.4 (1.7, 0 - 5.0)	8.5 (7.1, 0 - 29.0)	6.1 (4.2, 0 - 14.0)
Vaporized	25	13.5 (10.7, 0 - 32.0)	2.9 (2.5, 0 - 8.0)	10.7 (7.5, 2 - 25.0)	8.9 (4.3, 2 - 17.0)
Smoked	10	3.3 (5.3, 0 - 19.0)	0.2 (0.5, 0 - 2.0)	2.6 (2.5, 0 - 8.0)	2.4 (2.6, 0 - 8.0)
Smoked	25	8.2 (10.3, 0 - 34.0)	1.1 (1.7, 0 - 6.0)	6.9 (7.1, 0 - 20.0)	7.6 (8.1, 0 - 25.0)

**Conclusions/Discussion:** Vaporized cannabis produced higher BL levels compared to smoked administration of THC, metabolites and associated cannabinoids. THCCOOH in OF was not a reliable indicator of cannabis use and was only detected in a minority of participants.

**Keywords:** Cannabis, Vaping, Smoking, THC, CBD, CBN

## Simultaneous Extraction of Propofol and its Major Metabolite Propofol Glucuronide from Hair Followed by LC-MS/MS Analyses

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**Background/Introduction:** Propofol is an intravenous short-acting anesthetic that is commonly used for induction and maintenance of general anesthesia. After administration, propofol undergoes extensive metabolism mainly by direct conjugation to propofol glucuronide. However, besides its clinical use, propofol is also misused, particularly by healthcare professionals, with a high mortality rate. Consequently, there is an urgent need for a sensitive and specific method to detect chronic propofol abuse. Confirmation of a chronic drug misuse or abstinence, respectively, can be provided by hair analysis.

**Objective:** The aim of this study was to develop a method for the simultaneous extraction of propofol and propofol glucuronide from hair samples followed by LC-MS/MS analyses. Furthermore, it should be tested if a distinction can be made between single and chronic propofol use based on detected propofol and propofol glucuronide concentrations.

**Methods:** Prior to extraction, hair samples were washed twice with H<sub>2</sub>O and methanol. After drying, hair samples were cut into 1 mm segments. After addition of methanol and internal standard (propofol-d<sub>17</sub>, propofol glucuronide-d<sub>17</sub>), samples were incubated over night in a ultrasonic bath for extraction. Subsequently, samples were filtered through syringe filters and extracts were split into two fractions. For propofol glucuronide analysis, extracts were dried on a rotary evaporator and residues were reconstituted in mobile phase. For propofol analysis, extracts were fortified with 2-fluoro-1-methylpyridinium-p-toluene-sulfonate and triethylamine and the mixtures were subsequently incubated for 10 min at room temperature to achieve direct conversion of propofol to its *N*-methylpyridinium derivative (P-FluMP). Samples were dried under a nitrogen stream and the residues were reconstituted in mobile phase. Analyses were carried out by LCESI-MS/MS in the multiple reaction monitoring (MRM) mode using two specific ion transitions for each analyte ( $m/z$  270.1 → 133.0 and 270.1 → 110.0 for propofol-FluMP,  $m/z$  353.1 → 177.0 and  $m/z$  353.1 → 113.0 for propofol glucuronide). Propofol-FluMP and propofol-d<sub>17</sub>-FluMP were detected in positive ionization mode, propofol glucuronide and propofol glucuronide<sub>17</sub> were detected in negative ionization mode.

**Results:** By using this extraction method, propofol and propofol glucuronide can be simultaneously extracted from hair samples. Subsequent LC-MS/MS analysis of propofol glucuronide in negative ionization mode allows detection of the propofol metabolite down to 7.8 pg/mg hair. After derivatization using 2-fluoro-1-methylpyridinium-p-toluene-sulfonate, propofol can be detected by LC-MS/MS in positive ionization mode with a limit of detection of 3.6 pg/mg hair. The method was successfully applied to real human hair samples.

**Conclusion/Discussions:** Simultaneous extraction of propofol and its main metabolite propofol glucuronide can be performed using the described extraction method. Due to the derivatization reaction, improvements of the ionization and fragmentation efficacy of propofol can be achieved. Both, propofol and propofol glucuronide can be detected even in small quantities.

**Keywords:** Hair Extraction, Propofol, Propofol Glucuronide

## Retrospective Demonstration of 25I-NBOMe Acute Poisoning with Dramatic Outcome, Using Hair Analysis

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**Background/Introduction:** The abuse of new psychoactive substances (NPS) has been dramatically increasing all around the world since the late 2000s and has become a serious public health problem. NPS are a challenge for the worldwide forensic community. Among NPS, a group named phenethylamine NBOMe has gained importance in the recent years. 25I-NBOMe (2-(4-iodo-2,5-dimethoxyphenyl)-N-[(2-methoxyphenyl)methyl] ethanamine) is an analog of the 2C series of psychedelic phenethylamine drugs that contain an N-methoxybenzyl (NBOMe) substituent, which significantly affects their pharmacological activities. It is a potent agonist of 5-HTA receptors and a severe hallucinogenic drug, with numerous irreversible psychedelic effects (convulsions, paranoia, coma and brain damages) which can last from 5 to 10 hours. It can be consumed most often in the form of drops or blotters by the transmucosal, sublingual or intranasal routes. The active dosage is very low, supposed to be less than 100 µg. The literature is poor in reporting cases where 25I-NBOMe was identified. Only very few clinical cases of consumers were published, suggesting a low prevalence of this compound. Blood and urine remain the matrices of choice for toxicology analysis in case of a recent exposure to a drug, whereas hair analysis can document chronic and past consumption using segmental analysis. This case is the first demonstration of NBOMe analysis in hair.

**Objective:** Retrospective demonstration of a 25I-NBOMe acute poisoning using hair analysis.

**Methods:** A 7-year-old man was supposed to have consumed some LSD during a party with friends, at end of August 2016. After some initial hallucinations, he was found unconscious a few hours later. The patient suffered from cardiorespiratory arrest, reactive bilateral mydriasis, tachycardia and seizures. Blood was sent to the toxicology laboratory for drug screening, including ethanol. No pharmaceutical, nor LSD and ethanol, were detected. 25I-NBOMe was detected and quantified in the blood sample at 6,1 ng/mL by LC-MS/MS. Six months after poisoning, the patient was still hospitalized in a functional re-education and rehabilitation service due to cognitive disorders such as infantile behaviors, disorientation in time and space, attention deficits, memory problems and difficulty thinking. Two hair strands, measuring 9.5 cm, were collected 6 months after drug consumption during a forensic clinical evaluation of brain dysfunctions for analysis of 25I-NBOMe, to establish the pattern of poisoning. A specific method to test for 25I-NBOMe in hair was developed on an ultra-high performance liquid chromatography system (Acquity class I) coupled to a Xevo TQD tandem mass spectrometer (UPLC-MS/MS) from Waters, using liquid-liquid incubation/extraction at pH 9.5 and specific ions  $m/z$  428.1 > 121.2 and 428.1 > 90.6. The limit of quantification and the limit of detection were respectively fixed at 5 pg/mg and 1 pg/mg. Hair strands were segmented to determine the historic pattern of drug use and differentiate a single exposure from a chronic exposure.

**Results:** The hair test result for 25I-NBOMe was the following: not detected (0-2 cm), not detected (2-4 cm), approx. 1 pg/mg (4-6 cm), approx. 4.9 pg/mg (6-8 cm) and not detected (8-9.5 cm).

**Conclusion/Discussions:** The result of the segment 6-8 cm coincides with the date of consumption and the low concentration detected in the segment 4-6 cm probably corresponds to the contribution of dormant hair. The toxicological significance of the measured concentrations is impossible to establish because this is the first case describing hair analysis for 25I-NBOMe. The use of hair analysis for NPS is still at the initial stages. In particular, little is known about the incorporation into the keratin matrix after intake and the correlation between their dosage, passive exposure, use frequency, and hair concentrations.

**Keywords:** 25I-NBOMe, Hair Analysis, UPLC-MS/MS

## Interpretation of Cannabis Findings in the Hair of Very Young Children: Mission Impossible

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**Background/Introduction:** Hair has been suggested since the middle of the 90's to be a suitable matrix to document repetitive exposure to cannabis. Because it is possible to detect  $\Delta^9$ -THC, cannabinal (CBN) and cannabidiol (CBD) in cannabis smoke, the identification of THC-COOH has been considered to allow the discrimination of active use.

**Objective:** Whereas the detection of drugs in a child's hair unambiguously shows drug handling in the environment of the child, it is difficult to distinguish between systemic incorporation into hair after ingestion or inhalation and external deposition into hair from smoke, dust, or contaminated surfaces. However, the interpretation is particularly important in case of children for a realistic assessment of the health risk. We present here a series of hair tests for cannabis where the interpretation was almost impossible to establish.

**Method:** Hair specimens were collected during the autopsy of the 10 children, aged 2 to 24 months, either deceased from shaken baby syndrome (SBS, n=3) or sudden infant death (SID, n=7) during 2015-2016. After decontamination, the hair specimens were tested for THC, CBN and CBD and THC-COOH. When 30 mg of hair are processed, the LOQ for THC-COOH was 0.5 pg/mg. The whole length of hair was submitted to analysis.

**Results:** The amount of hair from children can be low. This may affect the limit of quantitation of THC-COOH. Six from ten hair tests were positive for cannabis markers.

The results are presented in the Table below (all concentrations in pg/mg):

Age of child	Hair	Death type	THC	CBN	CBD	THC-COOH
23 months	brown, 9 cm	SID	1150	2300	2300	< 0.5
2 months	brown, 2 cm	SID	39	42	< 20	Not detected
2 months	brown, 2 cm	SBS	32	130	580	Not detected
2 months	blond, 1 cm	SID	190	90	760	Not tested
4 months	blond, 2 cm	SID	1890	650	1590	< 0.5
6 months	black, 2 cm	SID	1300	880	1240	Not tested

In 2 cases, it was not possible to test for THC-COOH (not enough material).

**Conclusion/Discussion:** After delivery, to avoid becoming bald, hair growing is asynchronous (variation in the anagen / catagen phases) during the first 3 to 4 months. One will observe hair loss during the first 6 months after delivery, followed by a slow growing rate during the next 6 months. After 1 year, the normal rate of hair growth (1 cm/month such as in adults) starts. The growth rate is first 0.2 mm per day, and then it increases to 0.3 – 0.5 mm per day, to finally be stabilized at about 0.35 mm per day. As a consequence, it is very difficult to put any window of detection when testing for drugs in young children. It has also been demonstrated that drugs can be incorporated during pregnancy in the hair of the foetus, which can contribute to the positive findings after delivery. Hair from children is finer and more porous in comparison with adult (risk of higher contamination by sweat and environmental smoke versus adults). The final interpretation of cannabinoid findings in the children's hair is very complicated as this can result from in utero exposure (although none of the mother admitted cannabis use during pregnancy), cannabis oral administration by the parents to obtain sedation, close contact to cannabis consumers (hands, bedding, dishes) and inhalation of side-stream smoke. Over-interpreting cannabis findings in hair can have very serious legal implication in child protection cases. Active scientists have the responsibility to inform about these limitations. They have also to resist to the pressure of lawyers and judges, who generally expect a yes or no answer.

**Keywords:** Hair, Cannabis, Children

## Estimating Time of Last Oral Ingestion of Clozapine from Whole Blood Clozapine and Demethylclozapine Concentrations

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**Background/Introduction:** The driving under the influence of medicines is increasing in China. Determining the time interval since last use of medicine is important in forensic contexts. Clozapine is an atypical antipsychotic medication which is used in clinic and involved in driving sometimes. In this study, a mathematical model was described for estimating the time of last clozapine use, which based on clozapine-to-desmethylclozapine concentration (CLZ/DMCLZ) ratio in the whole blood.

**Objective:** To explore a mathematical model for estimating time of last clozapine use.

**Methods:** After signing an informed consent, twenty-nine volunteers were recruited to join the experiment, who were 22 to 27, didn't use medicine within past two weeks, had no chronic illness. Volunteers were given an oral dose of 12.5mg clozapine. And the venous blood were collected at 0.5h, 1.5h, 3h, 5h, 8h, 12h, 24h, 36h, 51h, 82h, 130h. Clozapine and dimethyl clozapine were quantified in whole blood by solid phase extraction (SPE) and high performance liquid chromatography tandem mass spectrometry. The equation of mathematical model was derived from linear regression analysis. The relative time error of the prediction was calculated as follows:  $TE = |T - T_a| / T_a$ , TE=absolute time error, T=predicted elapsed time (hours) after clozapine use, and  $T_a$ =actual elapsed time (hours) after clozapine use.

**Results:** The mathematical model described the relationship between whole blood concentrations CLZ/DMCLZ ratios and elapsed time. Equation ( $T = 43.6R^{-1.93}$ , T=predicted elapsed time (hours) after clozapine use, R=CLZ/DMCLZ ratio) was derived by plotting the concentrations of CLZ/DMCLZ in whole blood versus the elapsed of the time after clozapine use. By the equation, the theoretical values of the time interval since last use of clozapine were predicted. The predicted accuracy by this mathematical model was within 20% in 3-51h after clozapine use, but beyond 20% in 0.5-3h and 51-130 h.

**Conclusion/Discussions:** A mathematical model for estimating time of last clozapine use after a single dose was initially established. Existing data indicate that this model may be used for the prediction of time of clozapine use by whole blood CLZ/DMCLZ concentration ratio within defined confidence limits. But we should take multiple dosing, dose dependency of clozapine metabolism, induction or inhibition of CYP enzymes on metabolism, influence of smoking on metabolism into consideration.

**Keywords:** Estimating Time of Last Oral Ingestion, Clozapine, Clozapine-to-Desmethylclozapine (CLZ/DMCLZ) Ratio

## Metabolic Fate of Alpha-Cathinones for Urine Screening Purposes Using a Comprehensive in Vitro System in Comparison to Human Liver Microsomes, Primary Human Hepatocytes, and Authentic Human Urine

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**Background/Introduction:** Alpha-pyrrolidinobutyrophenone (alpha-PBP), alpha-pyrrolidinothiophenone (alpha-PVP), alpha-pyrrolidinohexanophenone (alpha-PHP), alpha-pyrrolidinoenanthophenone (alpha-PEP), and alpha-pyrrolidinooctanophenone (alpha-POP) are synthetic derivatives of the natural alkaloid cathinone. They are expected to induce amphetamine-like effects and were reported to have appeared at least in Europe and Japan [Odoardi et al., 2016; Kudo et al., 2015]. For developing reliable toxicological analysis, it is of importance to know about their metabolic fate and their main urinary excretion products. Thus, the metabolism of these compounds has partly been studied using different systems such as pooled human liver microsomes (pHLM) [Takayama et al., 2014], primary human hepatocytes (PHH) [Swortwood et al., 2015; Swortwood et al., 2016] and human urine [Matsuta et al., 2015; Swortwood et al., 2015; Paul et al., 2015; Swortwood et al., 2016; Shima et al., 2015]. However, all used in vitro systems have characteristic drawbacks such as lack of certain reactions (pHLM) or limited availability and high variability of metabolizing enzymes (PHH).

**Objective:** This study aimed to compare published metabolism data with a recently developed and comprehensive in vitro system based on pooled human S9 fraction (pS9) [Richter et al., manuscript submitted]. This system should allow simultaneous formation of all important phase I and phase II metabolites in an easy and cost-effective way.

**Methods:** Alpha-PBP, -PVT, -PHP, -PEP and -POP were incubated with pS9 and all necessary cofactors for most important phase I and phase II reactions. Samples were collected after 1 and 8 h and analysis was performed by LC-HR-MS/MS (TF Q-Exactive Plus using a TF PhenylHexyl Accucore column).

**Results:** Except for alpha-PBP, all compounds were hydroxylated at the pyrrolidine ring. All compounds underwent lactam formation and additional oxidation was found for alpha-PVT at the thiophene ring. Further hydroxylation and ketone formation at the alkyl chain were observed for alpha-PHP, -PEP and -POP. A carboxylic acid at the alkyl chain was metabolically introduced in the case of alpha-PEP and alpha-POP. Neither glucuronides nor sulfates were found for the investigated compounds using the new vitro system.

**Conclusion/Discussions:** One study using pHLM was published for alpha-PVT [Takayama et al., 2014]. The published metabolic steps included single hydroxylations at the pyrrolidine ring, thiophene ring or the alkyl chain. Lactam formation and dihydroxylation observed with pS9 incubations were not found in pHLM. Incubations using PHH were published for alpha-PVT and alpha-PEP [Swortwood et al., 2015; Swortwood et al., 2016]. The metabolic pathways of these compounds in PHH were similar to those by the incubations with pS9. Remarkable differences were the beta-ketone reduction observed with PHH but not with pS9 and the lack of glucuronides of the dihydro and hydroxy metabolites. Hence, the total number of identified metabolites was lower with the pS9 incubations, but all main metabolic steps have been observed. Comparison of all in vitro data with authentic human urine data indicated that the total number of formed metabolites was comparable with those of PHH. Glucuronides described in human urine and PHH were not found in pS9. However, the most abundant metabolites excreted into human urine were also detected after comprehensive pS9 incubation as well. Considering time and costs needed for the preparation and incubation with pS9, this system might be a suitable alternative to PHH at least for urine screening purposes. Particularly the huge costs that are needed to perform incubations with PHH can be avoided. On the other hand, PHH provide results that are closer to the overall metabolism observed in humans, at least for the studied cathinones.

**Keywords:** Synthetic Cathinones, CYP, In Vitro Metabolism



## Analysis of the Pharmacokinetic / Pharmacodynamic (PK/PD) Relationship of 2 Smoked Doses of THC vs Placebo in Occasional (OC) and Chronic (CH) Users of Cannabis.

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**Background/Introduction:**  $\Delta(9)$ -tetrahydrocannabinol (THC) is the compound most involved in road accidents in France

**Objective:** To describe the effect of THC and its active metabolite on vigilance and driving tests in volunteers according to their level of use and for 2 doses of THC vs placebo.

**Methods:** A randomized, double-blind, cross-over (3 periods) study using a 10 and 30 mg dose of smoked THC or placebo was conducted. Two groups of 15 OC (1 or 2 joints per week) and 15 CH (1 or 2 joints per day) were included. Before each period, an oral fluid drug screening (Drugwipe 5S) was necessary negative. A blood sample for assay of THC, 11-hydroxy- $\Delta(9)$ -tetrahydrocannabinol (11-OH-THC), and 11-nor-9-carboxy- $\Delta(9)$ -tetrahydrocannabinol (THC-COOH) was performed ( $T_0$ ). Each male volunteer then consumed a joint by taking a puff of 2 seconds every 40 seconds (timed), with a total of 15 puffs. THC remaining in each cigarette was quantitated to determine the actual dose of THC consumed. A measurement of alveolar CO before and 30 minutes after the joint has been performed to characterize the extent to which the volunteer has “pulled” on the joint. A blood sample was collected 5 min after the end of the administration, then at 15 min, 30 min, 1h, 2h, 4h, 6h, 8h, 10h, 12h and 24h. At each time, Psychomotor Vigilance Tests (PVT) with measurement of the mean reaction time (mRRT, expressed in  $s^{-1}$ ) were carried out, as well as driving simulator tests (York Computer Technologies, Canada) with measurement of the Standard Deviation of Lateral Position (SDLP). A population-based PK/PD analysis was performed using NONMEM software. In the first step, a model was constructed to describe the concentrations of THC and 11-OH-THC. The covariates likely to explain the inter-individual variability of the parameters were analyzed. Then, PD models were tested to describe the PD variables (mRRT and SDLP).

**Results:** A 3-compartment model with zero-order absorption ( $C_{max}$  at the end of administration) provides the best estimate of THC concentrations. For 11-OH-THC, the concentrations are described by a 2-compartment model. The kinetic parameters are as follows: for THC,  $T_{1/2\alpha} = 5.18\text{min}$ ,  $T_{1/2\beta} = 1.06\text{h}$ ,  $T_{1/2\gamma} = 37.18\text{h}$  for a high central distribution volume of 440 L. For 11-OH-THC,  $T_{1/2\text{formation}} = 2.2\text{h}$ ,  $T_{1/2\alpha} = 20\text{min}$ ,  $T_{1/2\beta} = 4.32\text{h}$ . Among the covariates tested, only the type of consumption is significant, with a relative bioavailability (RB) multiplied by 2.41 for the CH. Moreover, the dose of 30 mg has a RB reduced by a factor of 0.677 compared with the 10 mg dose, which may evoke a possible saturation of one of the steps of the PK. The mRRT shows a delayed decrease compared to blood concentration of THC. A model with 1 effect compartment and a sigmoidal relationship allows describing mRRT. Depending on the patient group, the  $EC_{50}$  value is significantly different ( $p < 0.001$ ). CHs have an  $EC_{50}$  for the effect compartment of  $3.50\ \mu\text{mol/L}$ , vs  $1.69\ \mu\text{mol/L}$  for OC, showing that CHs need higher concentrations than OC to produce the same effect. The effect half-life (towards the effect compartment) is 1.5h, the maximum effect ( $E_{max}$ ) is  $0.68\ s^{-1}$  with a baseline value  $E_0$  of  $4.07\ s^{-1}$  (-17%). Concerning SDLP, a significant difference in the  $E_{max}$  and its duration was observed between OC and CH ( $p < 0.001$  in both cases), without dose effect. The time to reach the  $E_{max}$  is between 4h and 5h in the two groups.

**Conclusion/Discussions:** 11-OH-THC is a better blood marker than THC for recent consumption since its  $T_{1/2}$  is shorter. Cannabis use reduces alertness by increasing reaction times by about 17%, CH having a significant tolerance to this effect compared to OC, and modify the SDLP mainly in OC, the maximum effect of these effects being delayed compared to blood concentrations of cannabinoids..

**Keywords:** THC, PK/PD, Reaction Time

## Quantitative Estimation of Aluminium in Fatal Aluminium Phosphide Poisoning Cases Using Inductively Coupled Plasma-Atomic Emission Spectrophotometry

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**Background/Introduction:** Use of aluminium phosphide (AIP) as a fumigant in India is huge because of its massive use in agricultural sector. The considerable use, cheap and easy availability along with extremely high mortality enhances its misuse as a suicidal agent especially in northern part of country. Until now, the cause of death has been given on the basis of police inquest proceedings, post-mortem findings and presence of phosphine gas in viscera using a silver nitrate ( $\text{AgNO}_3$ ) test during toxicological examination. The case may be reported as false positive as putrefied viscera also give positive result and the cause of death remains inconclusive. Also it can't be commented whether it was zinc phosphide or aluminium phosphide.

**Objective:** The study was undertaken to quantitatively estimate the values of aluminium in aluminium phosphide poisoning deaths. A total of 110 poisoning cases with alleged history of phosphide poisoning were collected from Mortuary AIIMS, State Forensic Science Laboratory (FSL), Delhi & State Forensic Science Laboratory, Haryana along with 100 control cases from Mortuary, AIIMS during the three year period (2014-16).

**Methods:** Out of 110 cases, 53 cases were of aluminium phosphide poisoning and rest were of zinc phosphide poisoning. Each case consisted of three biological samples i.e. Blood, Liver and Stomach contents. After screening all the samples were then digested and quantitatively analysed using inductively coupled plasma- Atomic emission Spectrophotometry (ICP-AES).

**Results:** The values of aluminium obtained in blood, liver and stomach contents of aluminium phosphide poisoning cases (n=53) were significantly higher than the control case (n=100 cases) samples. In blood the mean value is 29.76mg/l (with range from 0 to 24 in control cases while in control case it is 4.58mg/l with range as 0 to 223.05 mg/l. Similarly the mean value of aluminium in liver and stomach contents of aluminium phosphide poisoning cases were 46.96mg/g (with range from 0 to 263.17 mg/l) and 828.66mg/l (with range from 0 to 29054.81mg/l) respectively while in control cases it is 5.95mg/g (with range from 0 to 49.88 in control cases including outlier) and 82.40 respectively. (with range from 0 to 7582.59 in control cases including outlier)

**Conclusion/Discussions:** There was almost 10 fold increase in values of aluminium in liver and stomach contents and 6 times in blood in fatal AIP poisoning cases. The values thus obtained signify that the cause of death can be opined conclusively on the basis of quantitative estimation of aluminium in aluminium phosphide poisoning along with the other screening methods.

**Keywords:** AIP, Phosphine, ICP-AES

## Forensic Toxicology: One of the Roads Towards Sustainable Development

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**Background/Introduction:** Between the 25<sup>th</sup> and the 27<sup>th</sup> of September of 2015, the Heads of State and Government and High Representatives met at the Headquarters of the United Nations to approve and launch the new 2030 Agenda, devoted to Sustainable Development, the Sustainable Development Goals (SDGs). In this new agenda, a total of seventeen goals, composed of sub-goals (targets) can be found.

**Objective:** The main goal of this presentation is to introduce the new SDGs and to highlight our individual role as forensic toxicologists and the role of our scientific societies (both TIAFT and SOFT) as part of this global movement.

**Conclusion/Discussions:** In this 2030 agenda, there are several targets of importance to us. The abuse of substances (e.g. narcotic drugs or alcohol) are still an epidemic across the world. Therefore, the UN dedicated the target 3.5 to it and we have responsibility to act upon it. But, our possibilities of action don't end here. Our contribution to projects like DRUID that actively work towards the reduction of fatalities in road crashes (Target 3.6) or to the manipulation of hazardous chemicals (e.g. pesticides), or even our duties in terms of development, improvement and training. Though, the most important and crucial goal is the seventeenth: **Partnerships**. If the future lies in the present, then nothing meaningful is achieve alone. A multi-stakeholder approach is needed. Not only between members of the society but above all between TIAFT and other institutions (e.g. UNODC or UN Global Compact Network).

In summary, this presentation pretends to discuss and highlight not only our role as stake-holders, but also the challenges ahead of us.

**Keywords:** Toxicology, Sustainability, UN

## Stability of Cannabis Compounds in Biological Fluids During Post-Analysis Custody

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**Background/Introduction:** In Forensic Toxicology, the evidences have to be maintained under custody for, at least, one year. Depending on the conditions and duration of storage, drug concentrations might have changed considerably since the first analysis.

**Objective:** To study *in vitro* stability of cannabis compounds (THC, THC-OH and THC-COOH) in blood and urine. Parameters evaluated include: time of custody, temperature, addition of preservative (blood) and pH (urine).

**Methods:** Blood samples were spiked with the three cannabis compounds to give a final concentration of 10 ng/mL. Urine samples were spiked with THC-COOH at 100 ng/mL. The prepared samples were divided into 2 groups and stored at two temperatures (4 °C and -20 °C). Each one of these groups was subsequently divided in other two groups: with and without preservative (NaF) for blood, and pH 4 and 8 in the case of urine. THC, THC-OH and THC-COOH were analyzed in blood samples by GC-MS/MS after SPE and derivatization with BSTFA. THC-COOH was analyzed in urine by GC-MS after basic hydrolysis, liquid-liquid extraction and derivatization with HFBA/HFBOH. Samples were analyzed every two weeks for a year.

**Results:** In blood samples, the worst storage conditions were at 4 °C with NaF, with recoveries, after one year, of 17.1±1.4%, 2.6±0.2% and 6.8±0.8% for THC, THC-OH and THC-COOH, respectively. On the contrary, the best conditions were at -20 °C with no influence of preservative in the case of THC-COOH (recoveries 83.2±6.6% with NaF and 86.9±0.2% without NaF) and THC-OH (recoveries of 37.0±4.5% and 42.0±0.8% with and without NaF, respectively). As far as THC is concerned, the influence of preservative is noticed, since recovery in samples with NaF is 26.1±1.4% versus 44.4±3.1% without NaF. In urine samples the best storage conditions were under freezing conditions, as well, and do not depend on urine pH; THC-COOH recoveries are 52.3±5.6% at pH 8 and 60.2±7.4% at pH 8. Recoveries after storage at 4 °C are much lower, 17.1±0.6% at pH 4 and 4.3±0.5% at pH 8.

**Conclusion/Discussions:** Previous cannabinoid stability studies have been performed in only one matrix, blood/serum or urine and have mainly focused on adsorptive losses, in blood samples, or glucuronide degradation, in urine. The parameters evaluated in this study were selected in order to replicate the normal storage conditions in a forensic toxicological laboratory, because our goal is the applicability of the results in the routine work

The study shows that cannabis compounds degrade in blood and urine samples in all the studied conditions, with the exception of THC-COOH in blood samples maintained at -20 °C. The influence of storage temperature is much higher than urine pH or the influence of adding preservative, in blood samples. From this study, we can conclude that post-analysis custody conditions play an important role on re-analysis results, and should be addressed when interpreting cannabis concentrations after one year of custody.

**Keywords:** Stability; Cannabis; Biological fluids

## Utilizing Nuclease Screening of Ligand-Aptamer Complexes to Enhance Specificity of an Aptamer-Based Cocaine Assay

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**Background/Introduction:** Cocaine is a dangerously addictive central nervous system stimulant. Detection of cocaine is of vital importance for public health and law enforcement. Screening of samples which potentially contain this illicit drug has been achieved using competitive immunoassays. Unfortunately, these immunoassays possess inherent disadvantages such as poor specificity and batch-to-batch variation. Aptamers are single-stranded oligonucleotides selected *in vitro* by Systematic Evolution of Ligands by Exponential enrichment (SELEX). Aptamers possess a unique advantage over antibodies as their specificity can be tuned during the selection process. However, it is difficult to select against all existing interferent molecules and thus unexpected cross-reactivity can be observed when performing detection in complex matrixes.

**Objective:** To improve the specificity of cocaine-aptamer sensors, we first utilized the enzyme Exonuclease III (Exo III) to selectively distinguish between cocaine-bound and interferent-bound aptamers, and then employed the enzyme Exonuclease I (Exo I) to completely digest the “Exo III marked” non-/interferent-bound aptamers into mononucleotides, generating null background signal, while leaving cocaine-bound aptamers in an intact state. The intact cocaine-bound aptamers can be readily quantified using a DNA binding dye.

**Methods:** We first utilized Exo III to achieve high specificity by selectively identifying and digesting non-bound or interferent-bound aptamers into a single-stranded state while such digestion is inhibited for the cocaine-bound aptamer. Exo I was then used to completely digest the produced single-stranded non-/interferent-bound aptamers into mononucleotides whereas this digestion is similarly inhibited for the cocaine-bound aptamers. The presence of cocaine was quantified in a label-free manner utilizing the DNA binding dye (SYBR Gold) which has high affinity for the cocaine-bound aptamer and no affinity for mononucleotides allowing for a large increase of fluorescence in the presence of cocaine.

**Results:** The synergistic digestion between Exo III and Exo I allowed for the sensitive and specific detection of cocaine in a rapid and label-free manner. Exo III intelligently identifies and digests non-/interferent-bound aptamers into a single-stranded state, whereas digestion of the cocaine-bound aptamers is inhibited, allowing for the cocaine-bound aptamers to remain in its natural double-stranded structure. Exo I which possesses high specificity for single-stranded DNA digests the remaining “Exo III marked” non-/interferent-bound aptamers into mononucleotides, while the double-stranded cocaine-bound aptamers remains resistant to such digestion. The addition of SYBR Gold produces a large fluorescence signal in the presence of the undigested cocaine-bound aptamers whereas the mononucleotides produced from non-/interferent-bound aptamers displayed zero background signal. This exonuclease assay possesses high specificity to cocaine, demonstrating limited cross-reactivity to most structurally similar cocaine metabolites as well as structurally dissimilar interferent molecules.

**Conclusion/Discussions:** The null background signal produced in the absence of cocaine allows for sensitive detection of cocaine at concentrations as low as 300 ng/mL in oral fluid within 25 min at room temperature. The use of Exo III for intelligently distinguishing between interferent-bound and cocaine-bound aptamers also allows us to improve the specificity of the existing cocaine aptamer against structurally-similar and -dissimilar compounds, preventing false-positives. The sensitivity of the exonuclease cocaine assay allows for detection of recent cocaine use in oral fluid. Future directions involve the use of target recycling strategies to reach lower detection limits. Alternatively, the isolation of higher-affinity aptamers with cross-reactivity for cocaine and its metabolites could be directly incorporated into the presented work, allowing for longer detection windows in oral fluid.

**Keywords:** Aptamer, Cocaine, Fluorescence spectroscopy

## Fast and Simple Quantitative Analysis of Phosphatidylethanol (PEth) 16:0/18:1 in Whole Blood – a Direct Biomarker Suitable for Evaluation of Long-Term Alcohol Intake

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**Background/Introduction:** The World Health Organization has estimated that 3.3 million deaths annually (i.e. 5.9 % of all deaths) are caused by alcohol consumption, thus harmful consumption of alcohol is a major health issue both in industrialized and non-industrialized parts of the world. High alcohol consumption is a risk factor for developing a range of mental as well as somatic diseases, to be involved in accidents and to commit and be subjected to criminal acts. As many subjects tend to underestimate their alcohol intake, objective measures of alcohol consumption are crucial in the diagnosis as well as the follow-up of alcohol-related conditions.

Phosphatidylethanol (PEth) is a collective term for abnormal phospholipids formed from essential fatty acids by the enzyme phospholipase D in the membranes of red blood cells when ethanol is present. In contrast to other direct biomarkers for alcohol consumption such as ethanol itself and ethyl glucuronide/ethyl sulfate, PEth has a wide detection window of about three to four weeks, depending on initial concentration and the lower limit of detection of the method. This makes PEth a suitable marker for evaluation of long-term alcohol intake.

**Objective:** A high throughput ultra-performance liquid chromatography tandem mass spectrometry (UPLC-MSMS) method for quantitative analysis of the most abundant PEth homologue, PEth 16:0/18:1, in the range of 0.05 – 4.00  $\mu\text{M}$  was validated with emphasis on simple sample preparation and short analytical runtime.

**Methods:** Patient samples were frozen ( $-80\text{ }^{\circ}\text{C}$ ) at arrival at the laboratory. PEth 16:0/18:1 and the added internal standard PEth- $d_5$  (0.55  $\mu\text{M}$ ) were extracted from whole blood (150  $\mu\text{l}$ ) by simple protein precipitation with 2-propanol (450  $\mu\text{l}$ ). Chromatography was achieved using a BEH-phenyl (2.1 x 30 mm, 1.7  $\mu\text{m}$ ) column and a gradient elution combining ammonium formate (5 mM, pH 10.1) and acetonitrile at a flow rate of 0.5 ml/min. The injection volume was 2  $\mu\text{l}$  and the method had a runtime of 2.3 minutes. The mass spectrometer was monitored in negative mode with multiple reaction monitoring (MRM). The  $m/z$  701.7 > 255.2 and 701.7 > 281.3 transitions were monitored for PEth 16:0/18:1 and the  $m/z$  706.7 > 255.3 transition was monitored for PEth- $d_5$ .

Calibration curves, lower limit of detection (LOD), lower limit of quantification (LOQ), precision, accuracy, recovery, matrix effects and pre- and post-extraction stability were included in the validation.

**Results:** The quadratic calibration curves showed  $R^2 \geq 0.999$ . LOD was 0.01  $\mu\text{M}$  and LOQ was 0.03  $\mu\text{M}$  (CV = 6.7 %, accuracy = 99.3 %). Within-assay and between-assay inaccuracies were 0.4 – 3.3 % (CV  $\leq$  7.1 %). Recoveries were 95-102 % (CV  $\leq$  4.9 %) and matrix effects after internal standard correction ranged from 107-112 %. Stability tests showed that patient samples were stable for at least five days at 30  $^{\circ}\text{C}$  and that repeated freezing ( $-80\text{ }^{\circ}\text{C}$ ) and thawing did not affect the concentration. After thawing and analysis, remaining blood from patient samples were stable at 4-8  $^{\circ}\text{C}$  for at least four weeks. Extracted samples were stable for at least five days at 10  $^{\circ}\text{C}$ .

**Conclusion/Discussions:** The method fulfills the requirements as a high throughput method with its fast and easy sample preparation and short analytical runtime. The method has been in routine use at our laboratory for over a year running approximately 70 samples per day. The robustness and reliability of the method has been proven to be good, and no specific problems have been detected.

**Keywords:** Alcohol, PEth, UPLC-MSMS

## Confirmation of Metabolites of the Neuroleptic Drug Prothipendyl Using Human Liver Microsomes, Specific CYP Enzymes and Authentic Forensic Samples – Benefit for Routine Drug Testing

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**Background/Introduction:** Prothipendyl (Dominal®) is a tricyclic azaphenothiazine derivative. It belongs to a group of low potent neuroleptic drugs that reveal low antipsychotic effects. Due to its sedating and psychomotoric damping effects, prothipendyl is used for the therapy of psychomotoric agitated patients but also for patients suffering from sleep disorder or anxiety.

**Objective:** A voluntary intake of Dominal® was conducted to confirm the formation of previously identified metabolites in vivo, to investigate their detection windows and to prove their benefit for routine drug testing.

**Methods:** In vitro investigations were performed using human liver microsomes but also specific isoforms of cytochrome P450 (CYP) enzymes (1A2, 3A4, 2C9, 2C19, 2D6). The identification of the formed metabolites was done by high performance liquid chromatography (LC)-Quadrupole-Time of Flight (QTOF)-mass spectrometry (MS). The quantification of prothipendyl concentrations and the analysis of its metabolites was achieved using LC-Triple Quadrupole (QQQ)-MS methods.

**Results:** Various metabolites, including demethylated or oxidized derivatives of prothipendyl, were detected during in vitro experiments. Metabolic reactions of prothipendyl were mainly catalyzed by CYP enzymes 1A2, 2D6, 2C19 and 3A4. *N*-demethyl-prothipendyl was predominantly formed by catalytic activity of CYP2C19 and CYP1A2, while CYP isoenzyme 3A4 was responsible for the formation of prothipendyl sulfoxide. All metabolites were proved in serum and urine samples collected after a voluntary intake of 40 mg prothipendyl. There was no extended detection for the metabolites compared to the parent compound.

**Conclusion/Discussions:** Results of the herein presented study point out the formation of various metabolites of prothipendyl in vitro and in vivo. It should be considered that some of the identified metabolites also appear as degradation / oxidation products of prothipendyl. Additional studies are required to evaluate the pharmacological / toxic activity of the identified metabolites. Moreover, genotyping of different isoforms of CYP enzymes in patient samples can be useful for a more accurate interpretation of the proven amounts of metabolites.

**Keywords:** Prothipendyl, Metabolism, CYP

## Detection of Drugs in Work Areas of Polices Stations and the Implications for Workplace Drug Testing

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**Background/Introduction:** Drug use is commonplace in the broader community and leads to accumulation of drugs on public surfaces. Daily police operations include interception and seizure of licit and illicit drugs, creating the potential for exposure of police officers. Long term storage of drug exhibits may increase the risk of exposure and the likelihood of drug residues accumulating in police facilities. While monitoring of drug residues has been carried out in external environments such as clandestine drug laboratories and cannabis growing houses, no work has been undertaken to identify residues in police stations.

**Objective:** We analysed work surfaces in 10 city and country police stations and one large scale drug storage facility in New South Wales, Australia for 22 drugs and 2 metabolites. These results were compared to a series of public sites.

**Methods:** Swabs were collected from surfaces including counters, computers, barcode readers, drug storage safes and handles. Hair and urine samples were collected from volunteer police officers working in various roles in police facilities. Swabs and hair were extracted, concentrated and analysed by LC-MS/MS for 24 drugs and metabolites. A testing cup was used to screen urine samples for amphetamine, methamphetamine, cocaine, opiates and THC, with a subset analysed by GC-MS by an external laboratory.

**Results:** Low concentrations (0.04-23 ng) of amphetamine, methamphetamine, MDMA, oxycodone and cocaine were detected on swabs taken from counters and computers in public areas at background sampling sites. Methamphetamine showed the highest concentrations of all drugs, at 23 ng per swab. In contrast, 14 drugs and one metabolite/by-product were detected in police stations. The majority of swabs taken in charge (arrest), drug storage and main reception areas tested positive for drugs at police stations. The majority of swabs collected from work surfaces and evidence balances were positive for at least one drug, but most results were less than 40 ng per swab for a 15 cm diameter sampling ring. The highest concentrations of drugs on surfaces (up to 325,000 ng) and were detected inside drug safes, on evidence bags and on balances used for weighing seized drugs. These areas correspond to the highest drug traffic areas in police stations. Higher concentrations were also detected on some surfaces in the large drug storage facility. Urine samples collected from officers working in these environments tested negative for the drugs screened. Two hair samples collected tested positive for very low concentrations of cocaine.

**Conclusion/Discussions:** While the number of drug analytes detected on work surfaces in police stations correlated with the types of drugs seized, the concentrations of drugs detected on work surfaces in city and country police stations did not differ appreciably, and did not correlate with the number of drug exhibits seized. More than 75% of swabs had drug concentrations less than 40 ng of a given drug per swab, which is similar to concentrations detected in public facilities. These results suggest that even though police stations work surfaces are routinely exposed to drug residues, the exposure risk in these areas of police stations are on par with exposure in public areas. The highest surface drug concentrations tend to be focused in high risk areas of police stations, reducing exposure risk to police officers, and allowing cleaning processes to be focussed. Both hair samples positive for cocaine were collected from police officers in high risk job roles, but since no benzoylecgonine was detected in either sample and the cocaine concentrations were so extremely low, external contamination of hair was considered a likely cause for the low-level positive cocaine results. This conclusion is also supported by negative urine tests for both officers, as well as all police officers tested during the study.

**Keywords:** Drugs, Contamination, Police



## Fast Forensic Toxicological Screening and Quantitation Under 3 Minutes with a QTOF LC-MS/MS System

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**Background/Introduction:** Mass spectrometry (MS), especially the high resolution accurate mass system such as Time-of-flight (TOF) mass spectrometry, is a great fit for forensic screening applications because the data generated from these systems provides structural information for every possible analyte. Further, QTOF is able to provide the full-scan information of the precursor ions and the product ions in high resolution. Modern QTOF systems provide the capability of fast switching between MS and MS/MS scans for obtaining sufficient structural information.

There are mainly two approaches to acquire MS/MS information for screening purpose: Information-dependent-acquisition (IDA) or Data-dependent-acquisition (DDA), and MS/MS<sup>All</sup>. For IDA-MS/MS, a survey scan is performed to collect the information on the precursor ions, followed by multiple dependent MS/MS scans for a number of most abundant precursor/candidate ions. MS/MS<sup>All</sup> with SWATH<sup>®</sup> acquisition is a novel MS/MS<sup>All</sup> technique. In every data cycle, the instrument acquires TOF-MS information first, and then it sequentially acquires MS/MS information of all precursor ions across a specified mass range in multiple pre-divided mass windows. Both IDA and SWATH are compatible with retrospective data interrogation.

LC-QTOF technology combines separation (LC) and detection (TOF-MS and TOF-MS/MS) and is widely used for screening applications. Mostly, the duration of LC runtime in these studies varies from 5 to 20 min. For high-throughput laboratories, a fast screening method is desired.

**Objective:** In this study, we aimed to develop an ultra-fast screening method under 2.5 min in a forensic toxicological setting using the SCIEX X500R QTOF system. We also aim to compare IDA-MS/MS and MS/MS<sup>All</sup> with SWATH acquisition for this fast screening method.

### Methods:

#### Sample preparation

*Dilute and shoot.* Blank urine samples were spiked with multiple drugs commonly found in forensic settings at different concentration levels. Typically, samples were diluted 10-fold in 10% methanol and centrifuged. The clear supernatants were transferred to autosampler vials and 10 mL samples were injected.

#### List of target compounds

#### LC condition

HPLC separation was performed on a 20 × 2 mm Phenomenex Hydro-RP column with cartridge holder. Mobile phase A was 10 mM ammonium formate in water and mobile phase B was 0.1% formic acid in methanol. LC runtime was 2.5 min.

#### MS and MS/MS condition (Will be provided in poster/presentation)

#### Data analysis:

Data was acquired, processed and reported in SCIEX OS software 1.2.

**Results:** Two sets of urine samples including calibrators and unknown samples were tested with two different target lists (~ 100 compounds each with some variations). The true positive rates were consistently better with SWATH<sup>®</sup> acquisition compared to IDA-MS/MS using fast LC method. This also applied to unknown urine samples due to better MS/MS coverage with SWATH. However, with longer LC method, the true positive rates between IDA-MS/MS and SWATH were nearly identical. Sensitivity of the method was evaluated for both sets of samples and will be provided in the presentation. For instance, we could detect fentanyl and norfentanyl at 1 ng/mL in urine.

**Conclusion/Discussions:** In this study, we have developed a very fast screening/quantitation method in forensic setting under 2.5 minutes using the SCIEX X500R QTOF system. Two non-targeted data acquisition methods: IDA-MS/MS and MS/MS<sup>All</sup> with SWATH acquisition were both tested and compared. The choice of IDA-MS/MS and SWATH for data acquisition is situational and should be evaluated based on the specific method requirements. In situations when high-throughput is required and a short LC method is used, the preferred data acquisition approach would be SWATH acquisition due to the complete MS/MS coverage. Otherwise, if high-throughput is not required, both IDA and SWATH acquisition can be used but the IDA-MS/MS approach is preferred because it yields cleaner MS/MS spectra for easier data interpretation and faster data processing.

**Keywords:** Screening, IDA-MS/MS, SWATH

## Frequency of Other Prescription and Illicit Drugs in Gabapentin Positive Patients

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**Background/Introduction:** Gabapentin (Neurontin/Horizant) is used to treat epilepsy, neuropathic pain, and restless leg syndrome. Gabapentin is available in 100, 300, and 400 mg capsules; 600 and 800 mg tablets; and an oral solution of 250 mg/5 mL. Generally, dosing is started low ~300 mg/day for neuropathic pain and ~900 mg/day for epilepsy. These doses can be titrated up to 1,800 mg/day for neuropathic pain and 1,200 mg/day for epilepsy. Some cases require higher doses of 2,400 to 3,600 mg/day. Gabapentin is also unique in that it is not metabolized and is excreted unchanged in the urine. While gabapentin is not one of the 100 most commonly prescribed medications it is often co-dosed with other therapeutics for the treatment of chronic pain. In this work, we present a review of gabapentin patient data together with the prevalence of other common prescriptions and illicit medications.

**Objective:** To characterize gabapentin patient data in terms of the prevalence of gabapentin prescriptions and likelihood to abuse other drugs including other pain medications and/or illicit drugs.

**Methods:** We retrospectively analyzed data from patients who were gabapentin positive from January to February 2017, and then compared the prevalence of other drugs in this population to previously published rates from January 2013 to July 2015.

**Results:** In the two months of data review, over 11,000 samples tested positive for gabapentin using a reporting MS cut-off of 0.5 µg/mL and an upper limit of linearity of 500 µg/mL. Of those, ~39% did not have a prescription listed for gabapentin. The average gabapentin concentration was 217.46 µg/mL and the median concentration was 169.0 µg/mL. These high urine concentrations re-iterate that the drug is not metabolized and is largely excreted in the urine. Females outnumbered males in this population 60% to 40% respectively. The median age was 51.8 years old and ranged from 9.2 – 99.4 years of age. These positive samples were also positive for various other prescription drugs such as opiates (33.5%), oxycodone/oxymorphone (22%), benzodiazepines (30%), barbiturates (2%), buprenorphine (20%), fentanyl (4%), and methadone (3%). These positivity rates mirror the prescription rates of the listed drugs with the exception of benzodiazepines, buprenorphine, and methadone where the prescription rates are slightly lower than the positivity rate. This could indicate a preference to abuse benzodiazepines, buprenorphine, and methadone over other prescription drugs. In terms of illicit drugs, 2% were positive for cocaine, 14% were positive for marijuana (<1% have a prescription for marijuana), 3% were positive for methamphetamine, 18 (0.16%) were positive for heroin, and 11 (0.10%) were positive for ketamine (4 have a prescription for ketamine). These patients were not positive for novel psychoactive substances (NPS) such as bath salts, or for synthetic cannabinoids. Albeit, the NPS tested may not be an exhaustive panel of all available. These statistics of potential prescription abuse and illicit use appear to be similar to the general pain population. The general pain population, with comparable demographics, demonstrated positivity rates for cocaine and marijuana of 2% and 12.2%, respectively. However, the rate of heroin positivity is lower in the gabapentin positive patients at 0.16% versus the general pain population at 1.3%.

**Conclusion/Discussions:** The relatively high percentage of gabapentin positives without a prescription indicates this may be a drug with potential for abuse. It also seems that those who are taking gabapentin are as likely as other pain patients to abuse illicit drugs, with the exception of heroin that appears to be slightly less abused in gabapentin positive patients. It is also important to test for high concentrations of this drug as it is dosed as high as 2,400 mg/day. While toxicity by gabapentin is rare, it has been reported.

**Keywords:** Gabapentin Population, Pain, Diversion, Illicits

## Quantification of Cocaine and Its Metabolites in Dried Blood Spots from a Controlled Administration Study

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**Background/Introduction:** Cocaine is one of the most common illegal drugs of abuse; yet most literature data on its deposition in the human body is available for plasma and urine. A controlled administration study carried out at King's College London aimed to look at different matrices among which were dried blood spots (DBS).

**Objective:** Here we present the application of a previously developed HPLC-MS/MS method for the quantification of cocaine (COC) and its metabolites benzoylecgonine (BE), ecgonine methyl ester (EME), norcocaine (nor-COC), cocaethylene (COC-Eth), and *meta*-hydroxybenzoylecgonine (m-OH-BE) in DBS obtained during a controlled administration study of cocaine.

**Methods:** Seven healthy male volunteers intranasally administered 100 mg cocaine hydrochloride and capillary DBS were taken before administration and at 5 min, 10 min, 30 min, 60 min, 2 h, 3 h, 6 h, 24 h, and 48 h post-administration. DBS were shipped to our laboratory and stored at -80 °C until analysis. DBS were extracted with 200 µL 2 mM ammonium acetate. The extract was precipitated with 1000 µL acetonitrile. The supernatant was evaporated and redissolved in 50 µL mobile phase A. The analytes were separated on a Phenomenex Synergi Polar-RP Column and mass spectrometric detection was carried out with a SCIEX QTrap 5500 in MRM mode with positive electrospray ionisation. The analytical method has been fully validated with LLOQs between 1.0 and 5.0 ng/mL, bias and imprecision of less than 15 % and extraction efficiencies higher than 35 %.

**Results:** COC could be detected in all participants at least for 24 h after dosing. Two participants were negative for COC after 48 h while the other five participants were still positive after 48 h. Median  $t_{\max}$  was 10 min and median  $c_{\max}$  was 404 ng/mL. As expected, BE was the predominant metabolite and was detectable in all participants after 48 h with a median  $t_{\max}$  of 120 min and a median  $c_{\max}$  of 516 ng/mL. EME was only detectable in DBS for up to 24 h after administration, peaking around the 3 h time point, with a median maximum concentration of 97 ng/mL. m-OH-BE appeared only 60 min after administration and was detectable in all patients until 6 h after administration, median  $t_{\max}$  was 3 h and median  $c_{\max}$  was 31 ng/mL. In contrast to previously published studies, we were able to quantify nor-COC in DBS after cocaine administration. However, as concentrations were fluctuating close to the LLOQ of 2.5 ng/mL, nor-COC did not prove to be a useful marker for cocaine consumption in DBS. No cocaethylene was detected in any samples, which was consistent with the participants' lack of alcohol consumption for the duration of, and prior to, the study.

**Conclusion/Discussions:** We successfully applied a method for the quantification of cocaine and its metabolites yielding data about their deposition in capillary dried blood spots following controlled intranasal administration of cocaine. Comparison of DBS concentrations with whole blood concentrations revealed only limited correlation. Bland-Altman analysis revealed large variations in the differences between DBS and whole blood concentrations and the tendency to overestimate low concentrations in DBS. *Ex vivo* degradation of COC could be excluded as the cause of the differences as the sum of COC and BE concentrations as well as the sum of COC, BE, and EME concentrations also showed little correlation. Time-dependency of correlation was also investigated but could not be confirmed. Therefore, the cause for discrepancy between DBS and whole blood concentrations of cocaine and its metabolites remains unclear and warrants further investigation.

**Keywords:** Cocaine, Dried Blood Spots, LC-MSMS

## Pharmacodynamic Comparison of Acute Cannabis Effects Following Oral, Smoked, and Vaporized Administration

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**Background/Introduction:** Cannabis is increasingly available through a burgeoning cannabis marketplace that offers a growing number of consumable products intended for use via a variety of methods of administration. Novel products and alternative routes of administration were not developed in traditional pharmaceutical pathways and there are few controlled studies that have been conducted using administration methods other than smoking.

**Objective:** The aim of the study was to directly compare the dose effects of cannabis following oral, smoked, and vaporized routes of administration.

**Methods:** The comparative pharmacodynamic effects of cannabis were assessed in healthy adults in two studies. One study was conducted to evaluate the dose effects of oral cannabis (raw plant material containing 0, 10 mg, 25 mg, and 50 mg THC doses), and another study was conducted to evaluate smoked and vaporized cannabis (raw plant material containing 0, 10 mg, and 25 mg THC doses for both routes). Both studies used a within-subjects crossover design; 17 participants completed each study, 8 of whom completed both studies. Participants had a history of cannabis use, but had not used cannabis for at least one month prior to randomization. Outpatient drug administration sessions were conducted 1 week apart until study completion. Subjective effect ratings, cardiovascular measures (heart rate, blood pressure), and cognitive performance battery (Digit Symbol Search Test [DSST], Divided Attention Test [DAT], Paced Auditory Serial Addition Test [PASAT]) were obtained at baseline and for 8 hours following drug exposure. Data from the 10 mg, and 25 mg dose conditions were compared with placebo for each route of administration to detect significant drug effects and the same doses were compared across routes of administration to detect differences in the magnitude and time course of effects.

**Results:** Significant differences from placebo were observed, and were dose dependent for subjective drug effect ratings, cardiovascular effects, and cognitive performance effects across all three routes of administration. Notable differences were observed in the time course of effects. Onset of peak drug effects was observed fifteen minutes post-exposure for smoked and vaporized cannabis followed by a gradual return to baseline over the course of the observation period. In contrast, oral cannabis drug effects did not onset until 60-90 min post-administration and remained at peak levels for a longer period of time. Further, the time course of subjective, cardiovascular and cognitive performance effects differed with cardiovascular effects occurring more immediately (peak effect at 90 min) compared with subjective drug effects (peak effect at 180 min) and cognitive performance impairment (peak effect at 300 min). Across outcomes, the magnitude of peak drug effects was comparable between the smoked and oral routes of administration, but greater following vaporized cannabis administration.

**Conclusion/Discussions:** Significant variability in the pharmacodynamic effects of cannabis was observed among increasing doses and as a function of route of administration. Oral administration was associated with a slower onset and longer duration of sustained peak effects compared with the two inhaled routes of administration. At the same doses, the magnitude of peak drug effects was comparable for both oral and smoked cannabis. This is in contrast to prior reports suggesting poor bioavailability of cannabis/THC via the oral route. Data suggest that vaporization is a more efficient route of cannabis delivery compared to smoked and oral routes of administration. This may be due to a lower rate of drug loss due to combustion and/or first pass metabolism.

**Keywords:** Cannabis, Marijuana, Pharmacodynamics

## Morphine Concentrations in Human Urine following Poppy Seed Paste Consumption

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**Background/Introduction:** Turkey is an important major legal opium poppy (*Papaver Somniferum*) producer for medicinal and scientific purposes in the world. The poppy seeds -parts of the opium poppy- are used for food and are produced about 15.000-20.000 ton/year. Using of poppy seeds to make patty, bread, cakes, paste etc. is very common in our country. Paste is a special food in Turkey; it is just made from twice grinded and burned poppy seeds for eating at breakfast traditionally. It is known that poppy seeds contain morphine and other different opiate alkaloids. The consumption of food products containing poppy seeds can lead to positive opiate urine test results. Evaluation of the results for opiate urine analysis should cover the case of drug abuse potential from natural occurrence of opiate alkaloids in poppy seed.

**Objective:** In this study, we investigated the presence of morphine in human urine after three different poppy seed paste consumption.

**Methods:** In this research; white, yellow and blue-black poppy seed pastes were analyzed. Ten selected volunteers (age between 23-25, 2 male, 8 female) were consumed pastes at breakfast (spread on bread) on three different dates, at rate of 100 grams. The illegal substance analysis was made from total 227 urine samples collected from each volunteer one hour before and after breakfast (2, 4, 6, 8, 12, 24 and 48 hours). Screening of urine samples was analyzed by enzymatic immunoassay. The confirmation of urine samples was made after hydrolysis and derivatization by Gas Chromatography-Mass Spectrometry.

**Results:** Determined alkaloids in all type of poppy seed pastes were; codeine, thebaine, morphine and papaverine.

Immunoassay results for morphine and its derivatives after creatinine normalization were found above the cut off 300 ng/ml value; for white opium poppy seed paste 63.4 % (n=45/71), for yellow 73.4 % (n=58/79), for blue-black 68.8 % (n=53/77), respectively. For white 21.13 % (n=15/71), for yellow 48.10% (n=38/79) and for blue-black poppy seeds 29.87 % were above 2000 ng/ml. In urine samples the presence of morphine was found until 48 hour in some cases (n=9). In order to evaluate consumption of opium poppy seeds, thebaine -the natural component of poppy seeds- was chosen and analyzed by GC-MS. Thebaine was positive in all urine samples after consumption of opium seed paste.

**Conclusion/Discussions:** Morphine was detected in urine samples was significantly above 2000 ng/ml, after consumption of poppy seed paste by screening test. We must remember that in workplace drug testing cut off levels is 300 ng/ml. It is reported that there is significant variations for opiate alkaloids in poppy seed products and morphine concentrations are directly linked to the morphine content of the poppy seeds. Used poppy seeds are purchased from local market in Afyon. Afyon is legal opium producing area and consuming of poppy seeds food is high in this region. Living people in Afyon said that they need more analgesic concentration for medical conditions. Attention should be paid that in Turkey traditional poppy seed paste can be eaten easily more than 100 g.

The importance of analyzing an indicator for poppy seeds such as thebaine has been shown. Because of the common use of poppy seeds food in many countries, distinguishing morphine abuse from poppy seeds consumption is very important. As a conclusion, in these circumstances, the opiate cut off levels for drug abuse screening and specific biomarkers for poppy seeds consumption should be identified in guidelines.

**Keywords:** Poppy seed, Immunoassay, GC/MS

## Chemsex Study Through 5 Cases with 2 Deaths

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**Background/Introduction:** The use of psychoactive substances in a sexual context (chemsex) and SLAM (injection of these substances) is an emerging phenomenon for several years. The abuse of these drugs is sometimes leading to emergency or intensive care units or death.

**Objective:** Investigate chemsex polydrug use by toxicological analyses and case histories through 5 cases.

**Methods:** We describe 5 men chemsex cases received between June 2016 and March 2017: 2 hospitalized and 3 forensic from which 2 postmortem cases. Clinical information and outcomes, autopsy observations and toxicological analysis results are reported. Quantitation was performed by GC/FID (ethanol, ethyl chloride), GC/MS (GHB), UPLC/DAD (sildenafil), LC/MS-MS (4-MEC, THC-COOH), identification of benzoylecgonine and 4-MEC in urine by LC/MS-MS, detection of ethyl glucuronide in urine by DRI® (COBAS® 8000).

**Results:** Case 1, 39 year-old, was admitted to Intensive Care Unit (ICU) for a coma Glasgow 10 with a reactive myosis. Outcome was favorable in 12 h. Analyses revealed sildenafil (admission blood 1.7 mg/L; urine 37.9 mg/L), 4-MEC (blood 367 µg/L; urine), GHB (blood 139 mg/L; 1656 mg/L urine) and THC-COOH (urine 78 ng/ml). 9 months before, he was admitted to ICU with the same symptomatology, favorable outcome in 24 h, same drugs identified with cocaine and MDMA, poppers use not excluded (flask in his pocket).

Case 2, 37 year-old, was admitted to emergency unit for restlessness and psychological suffering after injection of 3-MMC (reported). A few months before, he had a follow-up in the addictology department for double addiction to sex and cathinones injected during SLAM sessions for 4 years. 4-MEC was identified in 4 urine samples during therapy (one month).

Case 3, 51 year-old, is a forensic case, following his partner's death (case 4): 4-MEC identified in blood (0.29 g/L), ethyl chloride consumption reported. He has a history of acute intoxication to ethyl chloride with cerebellar syndrome (hospitalization 10 days 4 years before).

Case 4, 27 year-old, was dead by asphyxia (autopsy observation). Toxicology: 4-MEC (cardiac blood 9.83 mg/L; urine 1830 mg/L; nasal swab), ethyl chloride (cardiac blood 0.08 g/L; urine 0.005 g/L), glucuronide ethyl in urine (> 2000 ng/mL). Alprostadil in injection was reported by the partner (8 injection tracks in penis observed during autopsy) and poppers flasks were found in the flat.

Case 5, 31 year-old, was dead by asphyxia (autopsy observation). Toxicological results: Ethanol (blood 0.13 g/L; urine 0.33 g/L), GHB (peripheral blood 258 mg/L; urine 12000 mg/L), benzoylecgonine detected in urine. Poppers flasks and ethyl chloride spray were found in the flat.

**Conclusion/Discussions:** Chemsex is a recent phenomenon, concerning men who have sex with men. All our cases had taken psychoactive stimulants: 4-MEC, MDMA or cocaine. The 4-MEC blood concentration is very high in case 4 and potentially increased by postmortem redistribution. Ethyl chloride (local anesthetic) was used in 3 cases and quantified in samples for one. Blood concentrations reported as associated to death were 0.65 g/L and 1.10 g/L. The use of poppers (vasodilator) is reported in 3 cases. GHB or GBL was taken in cases 1 and 5. Concentration is high for case 5 but heart failure was not excluded, cocaine having possibly enhanced cardiac trouble. Sildenafil and alprostadil, treatment of the erectile dysfunction, are administered respectively by systemic route and local injections. Sildenafil concentrations are high in case 1, while alprostadil, endogenous compound (prostaglandin E1) was not detected by analyses (case 4). Ethanol consumption was displayed in 2 cases.

These 5 cases are showing polydrug consumption, drugs acting by different ways. Moreover, repeated administrations are leading to high blood concentrations and toxic effects, consequences of behaviors putting health in jeopardy.

**Keywords:** Chemsex, SLAM, Polydrug Use

## Phosphatylethanol (PEth) Compared with Other Direct (EtG) and Indirect (GGT/MCV, CDT) Alcohol Biomarkers to Assess the Risk Drinking Status

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**Background/Introduction:** Assessment of alcohol drinking by using laboratory investigations has important clinical and forensic applications. Apart from measuring ethanol itself in various body fluids, a number of biomarkers exist for estimating single and repeated ethanol exposures. Serum ethyl glucuronide (EtG) is a marker for single ethanol intake with extended detection time. PEth and carbohydrate deficient transferrin (CDT) are specific biomarkers of long-term alcohol exposure, while gamma-glutamyltransferase (GGT) and mean corpuscular volume of red cells (MCV) are more indirect and less specific biomarkers. Together these biomarkers constitute a panel of alcohol biomarkers. This investigation was performed in order to use samples taken from individuals with indications of risk drinking in occupational health settings.

**Objective:** The aim of this study was to study how these alcohol biomarkers compare in a population of suspected risky drinkers.

**Methods:** EDTA-blood and serum samples (n=525) were taken by venous puncture from 515 individuals (20% females) for alcohol biomarker analysis due to clinical indications of problem drinking. PEth (16:0/18:1) isomer in whole blood (cutoff 0.05  $\mu\text{mol/L}$ ) and EtG in serum (cutoff 1.0 ng/mL) were analyzed with accredited UPLC-MS/MS methods. GGT and MCV were measured with medical laboratory standard methods with reagents from Roche Diagnostics and Axonlab respectively. GGT and MCV was combined and was considered positive when both were over cutoff limits, e.i. GGT >0.75  $\mu\text{mol/L*s}$  for females, >1.05  $\mu\text{mol/L*s}$  for males and MCV >95 fL. CDT was measured as percent disialotransferrin (cutoff 2.0%) with a commercial HPLC method (Chromsystems). All samples were stored at  $-24^{\circ}\text{C}$  until analysis.

**Results:** PEth was positive in 336 samples (64%) and among these 76 were also positive for CDT, 42 also for the combined GGT/MCV and 50 were positive for all three biomarkers. Thus, out of 336 PEth positives, 168 (50%) were only positive for PEth. In the 168 cases with only a positive PEth result the mean value was 0.55  $\mu\text{mol/L}$  and median 0.32  $\mu\text{mol/L}$ . In the 50 cases that were positive in all markers the mean PEth value was 2.70  $\mu\text{mol/L}$  and median 2.42.

EtG was positive in 297 serum samples (57%) with only 16 samples being negative for PEth. From the 281 samples 228 had PEth values >0.30  $\mu\text{mol/L}$ .

Also for EtG serum concentrations there appeared to be a relation to the pattern of positive long-term markers. In the subgroup of being only positive for PEth the mean and median EtG concentrations were 299 and 38 ng/mL (n = 117), respectively. From the 168 PEth positive samples also positive for CDT, GGT/MCV or both, 164 samples were also positive for EtG. In the subgroup of 50 samples being positive for all long-term markers the corresponding EtG concentrations were 2428 and 1457 ng/mL (n = 50).

**Conclusion/Discussions:** The results support the conception that PEth is a more sensitive biomarker of alcohol exposure than CDT and GGT/MCV. The results also demonstrates that cases resulting in positive CDT and GGT/MCV is related to more heavier drinking as the PEth levels are markedly higher. In the group with only a positive PEth result the median concentration was at the limit (0.3  $\mu\text{mol/L}$ ) being used to indicate excessive drinking. The higher levels of the direct biomarkers in the subgroup being positive for all three long-term biomarkers might indicate development of tolerance leading to higher amounts of ethanol ingested.

In conclusion, PEth appears to be a biomarker of alcohol exposure with unmet sensitivity and specificity as compared with CDT, GGT/MCV. Laboratory results must always be evaluated in relation to clinical observations.

**Keywords:** Alcohol biomarker, PEth, CDT.

## Dripping meets Dabbing: Cases Using the Rebuildable Dropper Atomizer to Vape Non-traditional E-liquids and Drugs-Other-Than-Nicotine (DOTNs)

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**Background/Introduction:** In 2011, the emergence of fourth generation electronic cigarettes (e-cigs) provided a new way for users to vape non-traditional e-liquids and drugs-other-than-nicotine (DOTNs). The rebuildable dropper atomizer (RDA) is different from traditional tank e-cigs as it allows users to “drip” e-liquid directly onto the coil and wicking system in the atomizer. Users claim that this method provides a better vaping experience and doesn’t risk clogging the device when using non-traditional e-liquids. The ceramic donut cup atomizer is a device that contains no coil and wicking system, but a ceramic cup that can hold and heat the mixture deposited into the cup. Drippers and ceramic donut cups are frequently abused by e-cig users to vape illicit substances. The dripping method allows users to vape non-traditional e-liquids or DOTNs such as plant materials and drug tinctures when dissolved in a small amount of propylene glycol (PG) and/or vegetable glycerin (VG). The ceramic donut cup atomizer is capable of housing wax “dabs” or other plant material that are not capable of being dissolved in PG or VG.

**Objective:** The purpose of this presentation is to present cases where the rebuildable dropper atomizer and the ceramic donut cup atomizer were used to aerosolize non-traditional e-liquid formulations; an unidentified resin/powder (nuciferine and mitragynine) and a marijuana dab using solid phase micro-extraction gas chromatography-mass spectrometry (SPME GC-MS) and Direct Analysis in Real Time mass spectrometry (DART-MS).

**Methods:** E-liquid samples, resins, and the mouthpiece of a ceramic donut cup suspected of containing marijuana submitted to our laboratory were screened using a Jeol JMS T100LC AccuTOF DART-MS to determine if the substances had been successfully aerosolized. The helium stream was set to 350 at a flow rate of 2.0 L/min. The instrument was set to positive ion mode and orifice 1 was at 20 V. E-liquid samples and resins were aerosolized in a Plume Veil 1.5 Clone RDA by Tobacco (Peak Vapor) and a IPV mini 2 70 W battery supply (Wake and Vape) at 4.3 V for 4 seconds into a simple trapping system. The coils were hand wrapped to a total resistance of 0.6  $\Omega$  with 26 gauge Kanthal A-1 wire and organic cotton was used as the wicking material. A 7  $\mu$ m polydimethylsiloxane (PDMS) SPME fiber from Sulpelco was used to sample the aerosol for five minutes before being injected onto an Agilent GC-MS 6890/5973N Mass Selective Detector containing a HP-5MS column. The initial temperature was set to 120 °C with a ramp of 10 °C/min until 300 °C and held for 12 minutes with a total run time of 30 minutes. The mass spectrometer was set to scan mode and scanned from 40-550 m/z.

**Results:** The ceramic donut cup atomizer was determined by DART-MS to contain  $\Delta$ 9 (THC), cannabinoids, and other marijuana constituents based on library matches and comparisons to known reference standards. The presence of THC and other cannabinoid was also determined in the mouthpiece of the atomizer by DART demonstrating that THC was aerosolized. The resins/powders were determined by DART-MS to contain nuciferine and the e-liquid was determined to contain mitragynine. The RDA was successful in aerosolizing the nuciferine in the resin/powder and the mitragynine in the e-liquid.

**Conclusion/Discussions:** The ceramic donut cup atomizer and the rebuildable dripping atomizer are capable of aerosolizing non-traditional e-liquids and DOTNs. Both e-cig devices are also capable of aerosolizing resins and other materials that can be used for illicit purposes.

**Keywords:** Electronic Cigarette, RDA, SPME



## Validated Assay for Determination of Ergocristine, Ergocryptine, Ergotamine, Ergovaline, Lolitrem B, Lysergic acid, *N*-Acetyllooline, *N*-Formylloline, Peramine, and Paxilline in Horse Serum

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**Background/Introduction:** Alkaloids produced by symbiotic endophytes are suspected to be associated among other factors with ryegrass staggers, fescue toxicosis, and equine fescue oedema in horses. These can be difficult to diagnose due to unspecific symptoms, e.g. reduced food intake, hypersensitivity, inappetence, depression, and reproduction problems. Ergovalin, lolitrem B, *N*-acetyllooline, *N*-formylloline, and peramine have been discussed as causative agents. Additionally, ergotamine and paxilline may produce clinical signs similar to those of ergovaline and lolitrem B. However, analytical evidence to support the association between the above alkaloids and diseases are scarce. Previously published analytical methods were limited to single analytes (e.g. ergovaline, lolitrem B, peramine) or a specific group (e.g. ergot alkaloids).

**Objective:** Therefore, the aim of this study was to develop a validated method for sensitive multi-target determination of ergocristine, ergocryptine, ergotamine, ergovaline, lolitrem B, lysergic acid, *N*-acetyllooline, *N*-formylloline, peramine, and paxilline in horse serum using ultra high performance liquid chromatography coupled with a high resolution tandem mass-spectrometry (UHPLC-HRMS/MS) and its application to authentic samples.

**Methods:** The method was developed on a Q Exactive Focus MS coupled with a Dionex UHPLC system (Thermo Fisher Scientific, Dreieich, Germany). HRMS/MS was performed in positive heated electrospray ionization. Quantification was carried out by parallel reaction monitoring (PRM) mode using accurate fragments. Chromatographic separation was achieved by gradient elution with 5 mM ammonium formate buffer (mobile phase A) and acetonitrile (ACN) (mobile phase B) on an ACQUITY UHPLC RP18 column (100 x 2.1 mm; 1.7  $\mu$ m) (Waters GmbH, Eschborn, Germany). Serum samples (1.5 mL) were spiked with 25  $\mu$ L internal standard (IS), diluted with 4.5 mL ammonia in water (2:98 [v:v]) and loaded on Oasis HLB SPE cartridges (Waters GmbH, Eschborn, Germany) pre-conditioned with methanol and water. Elution was performed with 1 mL each of formic acid in ACN (2:98 [v:v]), pure ACN, and ammonia in ACN (2:98 [v:v]). After evaporation to dryness the samples were redissolved in a mixture of mobile phase A and B (80:20 [v:v]). The method was validated according to international guidelines with respect to selectivity, linearity, accuracy, precision, matrix effects, recovery, stability, LOQ, and LOD. In absence of analyte free horse serum, calf serum was used as surrogate matrix for quantitative parameters.

**Results:** Selectivity experiments (n= 18) showed no interferences from matrix or IS, but *N*-actylloline and *N*-formylloline were found to be ubiquitous in horse serum. Calibration ranges were analyte-dependent and in total covered concentrations from 0.1 to 50 ng/mL. Linearity was proven over the respective ranges using appropriate weighting factors. Lolitrem B and paxilline could be sensitively detected, but did not meet quantification requirements. For the other analytes, accuracy and precision were shown for at least 2 different concentrations with acceptable bias (-4.7% – 7.9%) and precision (CV 2.6% – 16.6%). Matrix effects varied from -57% to 17% and were compensated by IS. Recoveries ranged from 20% to 109% with an acceptable RSD (3.4% - 24%). Autosampler stability was given for all analytes over a period of 20 hours and freeze and thaw stability was given for at least one cycle. LOQs defined as the lowest calibrator concentrations ranged from 0.1 to 1.0 ng/mL. LOD defined as the lowest concentration, which allowed a reliable MS<sup>2</sup> identification ranged from 0.05 to 1 ng/mL. In comparison to published quantification methods the present method achieved lower LODs and LOQs with acceptable run times. Applicability was proven by analyzing authentic horse serum samples (n=32).

**Conclusion/Discussions:** The presented method allows a sensitive detection of ergocristine, ergocryptine, ergotamine, ergovaline, lolitrem B, lysergic acid, *N*-acetyllooline, *N*-formylloline, peramine, and paxilline in horse serum and reliable quantification of all but lolitrem B and paxilline.

**Keywords:** Horse serum, HRMS-MS, Alkaloids

## Targeted Human Metabolome Analysis after a Single Intake of 3,4-Methylenedioxymethamphetamine (MDMA) in Healthy, Placebo-Controlled Subjects

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**Background/Introduction:** The recreational psychoactive drug 3,4-methylenedioxymethamphetamine (MDMA; ‘ecstasy’) is widely used and its pharmacological effects are well studied. On the hormone level, administration of MDMA was shown to increase plasma levels of oxytocin, prolactin, and cortisol. However, little is known about its effect on the human metabolome in general. Metabolomics research focuses on high-throughput identification and quantification of small molecular weight molecules. Mapping the biochemical changes after drugs of abuse (DoA) exposure, can complement traditional approaches by revealing potential biomarkers of organ toxicity or discovering new metabolomic features in a time- and dose-dependent manner. From the analytical point of view, such new features attributable to DoA consumption might serve as alternative biomarkers of their consumption particularly useful in case of short detection windows or to characterize new psychoactive substances regarding similarities of action with classic substances. One of the largest challenges of metabolomics is to overcome inter-individual and intra-individual metabolite variations demanding for an experimental study design under highly controlled conditions.

**Objective:** We aimed to analyze for the first time plasma samples from a placebo-controlled study in healthy adults to explore changes in endogenous plasma metabolites in response to a single intake of MDMA.

**Methods:** Plasma samples from 15 subjects of a randomized, double-blind, placebo-controlled crossover study (ClinicalTrials.gov (NCT01771874)) taken at four different time points: 2 hours before (= tp 0) and 3 hours (= tp 1), 8 hours (= tp 2) and 24 hours (= tp 3) after the intake of 125 mg MDMA or placebo were analyzed with the commercially available AbsoluteIDQ® kit (Biocrates), respectively. Up to 188 endogenous metabolites can be simultaneously quantified by this targeted metabolomics approach. Plasma samples were prepared according to the manufacturers instructions and measured by multiple reaction monitoring (MRM) with a UHPLC system coupled to a 5500 QTrap followed by a second run employing flow injection analysis (FIA-MS/MS). Metabolite concentrations at tp 0 were set as baseline to minimize inter-day variabilities and baseline-corrected differences were calculated for tp 1, tp 2 and tp 3 respectively. Paired t-tests ( $p < 0.01$ ), principal component analysis (PCA) and partial least squares discriminant analysis (PLS-DA) of non-corrected as well as baseline-corrected data were performed. For time series analysis baseline-corrected metabolites were further tested with two-way repeated measure ANOVA ( $p < 0.05$ ).

**Results:** Many amino acids (e.g. tryptophan) revealed time-dependent changes after MDMA intake. ANOVA revealed a total of 9 metabolites which showed a significant concentration change between MDMA and placebo intake, respectively. PLS-DA showed a clear separation between MDMA and placebo intake of the uncorrected data for tp 2 and tp 3 whereas baseline-corrected data revealed no clear separation of the two groups. Paired t-tests of the single time points showed statistically significant concentration changes mainly of glycerophospholipids and the metabolic ratio of methionine-sulfoxide (Met-SO) over methionine (Met), respectively. Statistical calculations at every time point indicated that neglecting inter-day individualities results in better separations with stronger outcome, especially when applying PLS-DA.

**Conclusion/Discussions:** Changes of the metabolic ratio of Met-SO/Met may be indicative for changes in systemic oxidative stress level, whereas the increased amount of glycerophospholipids could be interpreted as an upregulation of energy production associated with changes in plasma lipoprotein, cell function and inflammation. Repeating the multivariate statistical tests with baseline-corrected metabolite concentrations showed no significant separation of the MDMA and placebo intake groups showing that a suitable ‘zero sample’ is indispensable for the outcome of statistical calculations.

In conclusion, a single intake of MDMA did not strongly alter the targeted metabolome. A repeated/frequent intake of MDMA may increase metabolomic changes thus revealing possible biomarkers. Additional information of human metabolome changes should be gained by applying an untargeted screening approach theoretically including all human plasma metabolites.

**Keywords:** Targeted Metabolomics, MDMA, Statistics

**An Attempt to Acquire the Nostradamus Effect: The First Case of Intravenous Use of the Tricyclic Antidepressant Tianeptine**

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**Background/Introduction:** Tianeptine is a tricyclic antidepressant prescribed in European countries. Currently, it is not approved for use in the United States. It is sold on websites in the United States as a nootropic or smart drug/cognitive enhancer. Tianeptine has an anxiolytic efficacy profile similar to other tricyclic antidepressants, but has a different mechanism of action. Originally thought to be a serotonin reuptake enhancer, new studies suggest that tianeptine's possible dual activation of the mu and delta opioid receptors is the initial molecular event responsible for modulation of the glutamatergic system. The activation of these receptors is believed to be responsible for causing many of the known acute and chronic effects of tianeptine, including its antidepressant/anxiety actions. The major metabolic pathway is  $\beta$ -oxidation and the principal metabolites are propanoic acid (inactive) and pentanoic acid (active) metabolites. Less than 3% of the dose is excreted unchanged in urine and the active metabolite has a half-life of 7.2 h. There are limited published case reports of tianeptine intoxication. **Case:** A 36 year-old male intentionally injected tianeptine powder dissolved in water intravenously to "help him see into the future." He became unresponsive and a bystander called emergency medical services. He was administered naloxone 1 mg IV due to suspected heroin use by medical personal and was transported to the Emergency Department (ED) around midnight. Upon arrival to the ED, it was noted the patient had excessive constriction of the pupils, a respiratory rate of six breaths per minute, and he was sedated. His toxicity was successfully reversed with two doses of naloxone 0.4 mg IV and he was placed on a naloxone infusion at 0.2 mg/hour. Approximately twelve hours post admission, the patient was alert, awake and oriented, and was discharged.

**Objective:** The objective of this study was to develop a method for the detection and quantitation of tianeptine in a urine specimen from an interesting and unusual case.

**Methods:** A high performance liquid chromatography tandem mass spectrometry (HPLC-MS/MS) method was used for the detection and quantitation of tianeptine in urine. A six-point calibration curve ranging from 1-100 ng/mL and four quality control specimens (1, 3, 30, and 75 ng/ml) were analyzed on five separate days of method validation. Sample extracts were prepared by adding 10 ng of the internal standard, protriptyline, to 200  $\mu$ L aliquots of the calibrators, controls, and urine sample followed by the addition of 200  $\mu$ L of 50:50 acetonitrile: distilled water. Samples were briefly mixed and transferred to Clean Screen FAST<sup>TM</sup> SPE columns and rapidly aspirated into auto-sampler vials. Analysis was performed using a Waters Acquity Xevo TQD HPLC-MS/MS with ESI ionization in positive ion mode.

**Results:** The patient's urine concentration was 2 ng/mL tianeptine. The linear regression correlation coefficients for each day calibration  $r^2$  were 0.9996 or greater. The limit of quantitation (LOQ) was administratively set at 1 ng/mL. Accuracy/bias of the assay was determined to be within +/- 20% of the target value for each analyte in each quality control specimen. The CV for inter-day and intra-day precision samples did not exceed 15%. Validation criteria for matrix effects, absolute recovery, carryover and specificity were acceptable.

**Conclusion/Discussions:** The developed method is robust and reliable for the detection and quantitation of tianeptine in urine. There are no other detailed reports of intravenous tianeptine abuse in the literature to date. The administration of naloxone appears to have been effective in blocking the toxic effects of tianeptine. The patient's low tianeptine urine concentration (2 ng/mL) is likely due to the extensive metabolism of tianeptine and low urine creatinine (0.11 mg/dL).

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**Keywords:** Case Report, Tianeptine, HPLC-MS/MS

## N-Acetyltaurine as a Novel Ethanol Marker in Blood and Urine

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**Background/Introduction:** To prove alcohol abstinence, various direct alcohol markers are currently analyzed in different matrices such as hair, urine, and blood. The most prominent markers are ethyl glucuronide (EtG), ethyl sulfate (EtS) and phosphatidylethanol (PEth), representing the non-oxidative pathway of ethanol metabolism. During a study with mice by Shi et al., N-Acetyltaurine (NAcT) was found to be an alcohol-related, direct marker, which is part of the oxidative pathway of ethanol metabolism. NAcT is formed when the oxidative metabolites of ethanol oxidation, acetate or acetyl-CoA, react with the non-proteinogenic amino acid taurine.

**Objective:** We investigated the NAcT concentrations in blood and urine samples obtained during a drinking study with eight subjects and compared it with the well-established marker EtG, EtS and the blood alcohol concentration (BAC).

**Methods:** Two SeQuant ZIC-HILIC (3.5  $\mu\text{m}$ , 100  $\text{\AA}$ , 150 $\times$ 2.1 mm) based LC-MS/MS methods were developed and validated to simultaneously measure creatinine (to correct for the dilution of the urine, 100 mg/dL) and NAcT in human urine (50  $\mu\text{L}$ ), and to measure EtG and NAcT in blood samples (250  $\mu\text{L}$ ). Samples were analyzed by using an acetonitrile – ammonium acetate gradient, and a 5500 QTrap instrument from Sciex. The mass spectrometer was operated in electrospray negative MRM mode and signals were corrected by their individual internal standard signal. During the drinking study, blood and urine were taken every 1.5-2 h from eight subjects who ingested 0.66 to 0.84 g/kg alcohol to reach a target blood alcohol concentration of 0.8 g/kg. The blood alcohol concentration was measured with HS-GC-FID.

**Results:** NAcT was found to be an endogenous substance which was present at a range of 1.0 to 2.3  $\mu\text{g/mL}$  in urine, and 13-31 ng/mL in the blood of alcohol-abstinent subjects. Following alcohol ingestion, NAcT concentrations increased in all subjects within 3 to 6 hours to reach a mean peak NAcT concentration of  $14\pm 2.6$   $\mu\text{g/mL}$  (range 9-17.5  $\mu\text{g/mL}$ ) in urine, and  $40\pm 10$  ng/mL (range 27-57 ng/mL) in blood. Within 24 hours, the NAcT concentrations reached endogenous levels again. Compared to the blood alcohol concentration, NAcT concentrations were found to be slightly shifted. When BAC was not detectable anymore, NAcT levels were still elevated.

**Conclusion/Discussions:** In contrast to NAcT, the two established markers EtG and EtS have the advantage of being undetectable when no alcohol was consumed, which simplifies the interpretation of the result. So far, the analysis of NAcT concentrations shows no advantage when compared to EtG or EtS analysis. Considering NAcT concentrations in blood, endogenous concentrations and alcohol induced concentrations partially overlap among different subjects. However, NAcT in urine, which shows a higher alcohol induced concentration increase, can serve as a further indication of alcohol consumption and provides insight in the oxidative pathway of ethanol metabolism.

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**Keywords:** N-acetyltaurine, Alcohol Marker, Drinking Study

## Relative Abundances of 5F-ADB and its Ester Hydrolysis Metabolite in Carboxylesterase-Containing Tissue Specimens

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**Background/Introduction:** 5F-ADB is an indazole-type synthetic cannabinoid (SC) with its first fatal report in 2015. While the blood 5F-ADB concentrations remained unreported in past fatal intoxication cases, we reported the first successful quantification in postmortem blood (0.19 ng/mL) and its human metabolism.<sup>1</sup> For indazole-type SCs with terminal amides and esters such as 5F-ADB, the major metabolic pathway appears to be the enzymatic hydrolysis by carboxylesterases (CES), which we also confirmed through urinary metabolite investigation. This extensive metabolism likely leads to the very low or even undetectable blood concentration of 5F-ADB. Since carboxylesterases are found mainly in the liver, lung, and the intestinal walls, we suspected that the abundance of 5F-ADB in its unchanged form in such organs would be very low similar to blood, and the ester hydrolysis metabolite abundance in turn would be high. Here we present the qualitative results of 5F-ADB analysis in CES-containing tissues and discuss the contribution of human carboxylesterases to the abundance of 5F-ADB and its ester hydrolysis metabolite in tissue specimens.

**Objective:** To investigate the contribution of human carboxylesterases to the abundance of 5F-ADB and its ester hydrolysis metabolite in tissue specimens.

**Methods:** 5F-ADB was purchased from Cayman Chemicals (Ann Arbor, MI). 5F-ADB ester hydrolysis metabolite was synthesized at the Aichi Prefectural Police Forensic Science Laboratory. Tissue specimens (0.5 g) were spiked with 5F-ADB at 0.5 ng/g. QuEChERS extraction method was optimized and the final extract was evaporated to dryness then reconstituted in 50  $\mu$ L of 50% methanol-10mM aqueous ammonium formate solution. Blood was prepared following our previous protocol.<sup>1</sup> Analysis was performed on a TripleTOF 5600 (AB Sciex, Framingham, MA) system coupled to a Shimadzu NexeraX2 LC system (Shimadzu Co., Kyoto, Japan) using an L-column 2 ODS (1.5 x 150 mm, 3  $\mu$ m) column (CERI, Tokyo, Japan). Other analytical parameters were as follows: 10 mM ammonium formate-5% (A) or 95% (B) methanol mobile phases; linear gradient; 0.1 mL/min flow; electrospray ionization (positive); product ion scan mode.

**Results:** Lung specimens with high fluid content posed difficulty in sufficient separation of the organic layer during QuEChERS extraction; to overcome this issue, the solvent volume was increased to 1.5 mL which ensured enough volume of the organic layer to be obtained. Optimized extraction and analytical conditions allowed successful detection of 5F-ADB at 0.5 ng/g. **Case sample:** Analysis of CES-containing tissues (liver, lung) revealed the presence of the unchanged 5F-ADB in lung but not in liver. In contrast, ester hydrolysis metabolite in blood, liver, and lung were detected at high ion intensity, confirmed through retention time and product ion spectra comparison with the synthesized reference standard, indicating high abundance of the ester hydrolysis metabolite.

**Conclusion/Discussion:** Obtained results supported our hypothesis that 5F-ADB is extensively metabolized in CES-containing tissues, suggesting that analysis of 5F-ADB in tissue specimens requires paying close attention to the presence of CES, as the unchanged drug may not be easily detectable in organs such as liver and lung where CES is known to be present. In our case sample the fact that unchanged 5F-ADB could be detected in lung but not the liver may be attributed to the route of administration, suspected to be smoking, which may have been involved in the presence of 5F-ADB at a higher abundance relative to other organs. Following the current results, validated quantitative analysis in lung and blood for the detected 5F-ADB and its ester hydrolysis metabolite is underway.

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**Keywords:** 5F-ADB, Human Carboxylesterases, Ester Hydrolysis Metabolite

## Characterization of Potency and Efficacy of a Series of New Fentanyl Analogues

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**Background/Introduction:** New psychoactive substances (NPS) are appearing on the recreational drug scene at a steady pace. Preventing the introduction of new drugs by the increase of a governmental effort to schedule dangerous compounds is not significantly reducing the rate of appearance of novel NPS. Due to the severe lack of information regarding their potency, efficacy and toxicity profiles, NPS are responsible for numerous non-fatal and fatal intoxications. Among the NPS, synthetic opioids have a significant impact on public health; the high potency and the unknown pharmacology of these compounds expose the abusers to the risk of overdosing and of incurring in various complications. Analogues of the prescription drug fentanyl are dangerously powerful synthetic opioids that have contributed to hundreds of deaths worldwide in the past years. A more accurate characterization of the pharmacology and the toxicology of these new compounds would be a useful tool to more successfully counteract the dangerous effects of fentanyl analogues.

**Objective:** The first phase of the project aimed to measure the specific activation of human  $\mu$ -receptors – known target of opioid compounds – in order to better characterize the potency and the efficacy of a series of new fentanyl analogues. The study focused on calculating  $ED_{50}$ , Hill slope and potency of the synthetic analogues, comparing them with well-known traditional opioids.

**Methods:** Receptor activation was measured in AequeoZen cell line (Perkin Elmer) expressing human  $\mu$ -receptor. In brief, phospholipase C-induced intracellular calcium level change; calcium ions bind the intracellular aequorin, which in turn oxidized coelenterazine with production of  $CO_2$  and emission of light. The luminescence was measured using a Spark 10M plate reader (TECAN).

**Results:** The ability of fentanyl, acrylfentanyl, acetylfentanyl, furanylfentanyl, 4-isobutyrylfentanyl, two metabolites of acetylfentanyl, along with morphine and buprenorphine as reference compounds, to activate the  $\mu$ -receptor was investigated. Confirming the validity of the screening system, morphine resulted as full agonist, while the lower efficacy of buprenorphine indicates a partial agonist.

Fentanyl and fentanyl analogues showed to be significantly more potent than morphine; the  $ED_{50}$  of fentanyl and of all analogues were between 0.1 ng/ml (acrylfentanyl) and 3.21 ng/ml (furanylfentanyl), with the exception of acetylfentanyl (33.4 ng/ml); the  $ED_{50}$  of morphine and buprenorphine were 200.9 ng/ml and 36.0 ng/ml respectively. The efficacy of all fentanyl analogues was comparable to morphine's, with the exception of furanylfentanyl which presented a partial agonist profile. The metabolites of acetylfentanyl induced  $\mu$ -receptor activation as well, albeit with lower potency and efficacy.

**Conclusion/Discussions:** The results, when compared to the case reports of fatal intoxications in Sweden, correlate with the empirical observations of the potency of the NPS studied: the most potent drug tested – acrylfentanyl – caused the highest number of lethal outcomes among the recorded intoxications, as well as resulting in the lowest post-mortem plasma concentrations. Moreover, the different profiles of acetylfentanyl and furanylfentanyl (respectively, high efficacy and low potency, and low efficacy and high potency) could account for the higher mortality rate of the less potent acetylfentanyl, in contrast with the lower lethal effect of the more potent furanylfentanyl. Finally, the evidence that acetylfentanyl also induces  $\mu$ -receptor activation through its metabolites – albeit weakly – highlight the need to further expand the study of NPS to include also secondary metabolites.

In this regards, the next phases of this project will study multiple classes of NPS and their metabolites, analysing their effect on various target receptors.

**Keywords:** Fentanyl-analogues, Designer Drugs,  $ED_{50}$

## Strategies for the Analysis of Synthetic Cannabinoid Metabolites Illustrated by the Separation of 24 Metabolites and Structure Analogs of AKB-48 and 5F-AKB-48

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**Background/Introduction:** With hundreds of analogs reported, synthetic cannabinoids are one of the more prevalent groups of new psychoactive substances. They are heavily metabolized, with the parent compounds rarely observed in urine, making analysis of metabolites the obvious choice for establishing drug intake. However, synthetic cannabinoid metabolism adds another level of complexity. For example, AKB-48 is mainly metabolized through hydroxylation on the adamantyl group and the pentyl chain. There are nine potential monohydroxylated metabolites and about 75 dihydroxylated metabolites. The correct identification of the exact structure is necessary to distinguish intake of different synthetic cannabinoids, i.e. to differentiate intakes of AKB-48 and 5F-AKB-48. Metabolites can sometimes be distinguished by mass spectrometry; but as the fragmentation patterns of structural analogs are often very similar, a chromatography capable of separating structurally related metabolites remains important.

**Objective:** To systematically investigate the liquid chromatography separation of 24 potential metabolites of AKB-48 and 5F-AKB-48 on five different stationary phases using four different combinations of mobile phases.

**Methods:** AKB-48, 5F-AKB-48 and four potential metabolites were obtained commercially while another 20 potential metabolites were synthesized in-house. Using ammonium acetate or formic acid with either methanol or acetonitrile, the analytes were separated on ACQUITY CSH Fluoro-Phenyl, ACQUITY CSH Phenyl-Hexyl, ACE Excel 2 C18-AR, CORTECS C18 and ACQUITY BEH C18 columns using gradients designed so that the first metabolite eluted at 1 min and the last at 10 min. Gradients were evaluated based on the worst separation of “critical pairs”, i.e., analytes with the same mass. However, adamantyl hydroxy metabolites were not considered critical pairs with pentyl hydroxy metabolites, as they can be distinguished by fragmentation patterns. The feasibility of this approach was illustrated by the development of a triple quadrupole LC-MS/MS method used to identify mono-hydroxylated metabolites in an authentic urine sample.

**Results:** The overall retention order was similar for all mobile phases on all columns, but formic acid and acetonitrile generally provided the best separation. The monohydroxylated metabolites proved to be the most difficult group, with no column achieving [complete/full] resolution. However, when the differences in fragmentation pattern were considered, the best separation was obtained on the Fluoro-Phenyl column (resolution >0.9 for all critical pairs); therefore, this column was selected for the LC-MS/MS. Four monohydroxylated metabolites were identified in the authentic urine sample with 4-eq-adamanylhydroxy AKB-48 being the most abundant.

**Conclusion/Discussions:** If all metabolite analogs cannot be separated, there is a risk of erroneously identifying urinary metabolites as identical to a co-eluting reference material. In the worst case this could lead to different laboratories reporting different major metabolites or false negatives if a laboratory used the wrong reference material. This type of study requires the custom synthesis of a large number of analogs and is therefore unfeasible in most settings. It might be possible to reduce the risk of misidentification by validating the match between reference material and authentic urine using orthogonal chromatography (multiple methods with unique separation characteristics). Unfortunately, none of the chromatographies in this study were different enough to be used this way. The reported chromatographic separations could serve as a starting point when developing assays for synthetic cannabinoid metabolites in urine, as monohydroxylated metabolites are major metabolites of most analogs and similar difficulties in separation can be envisaged.

**Keywords:** Synthesis, Synthetic Cannabinoid, Liquid Chromatography

## Phase I Metabolism of Desomorphine

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**Background/Introduction:** Desomorphine, also known as Krokodil, is a semi-synthetic opioid. It is a mu receptor agonist that is more potent than morphine but has a shorter duration of action. In a clandestine setting it is synthesized from codeine using hydroiodic acid and red phosphorus. When administered intravenously, users may experience serious dermatological sequela. Perceived by users to be a cheaper alternative to heroin, its use has been geographically limited to Russia, neighboring Soviet Republics, as well as Eastern and Central Europe. Although there have been reports of its use in the United States, none have been analytically confirmed. Until very recently, the metabolic fate of desomorphine in humans was unknown. One study was recently published (Richter, 2016) and in this report we describe additional metabolic pathways involved in its biotransformation. Bactosomes, as used in this study, have high activity compared to other *in vitro* models making them a suitable candidate for identifying minor biotransformation pathways that might otherwise be difficult to observe.

**Objective:** To evaluate the phase I metabolic pathways of desomorphine in humans using recombinant human cytochrome P450s (CYP450s) and liquid chromatography/quadrupole-time of flight-mass spectrometry (LC/Q-TOF-MS).

**Methods:** Bactosomes (Xenotech EasyCYPS) and a NADPH regenerating system (Corning Gentest) were used to generate phase I metabolites for desomorphine in a fully optimized system. Samples were analyzed using LC/Q-TOF-MS (Agilent Technologies 6530 Accurate-Mass Q-TOF LC/MS) equipped with a Poroshell 120 EC-C18 column. High resolution MS/MS spectra over a range of CID voltages were used for structural elucidation. Metabolite formation was verified by repeating individual bactosome incubations in the presence of an inhibitor. The enzymes used in the study were CYP1A2, CYP2B6, CYP2C8, CYP2C9, CYP2C18, CYP2C19, CYP2D6, and CYP3A4. The inhibitors used were ketoconazole (CYP2C9, CYP2C18, CYP2C19, and CYP3A4), fluvoxamine (CYP1A2 and CYP2D6), montelukast (CYP2C8), and ticlopidine (CYP2B6).

**Results:** Metabolic activity was observed using CYP2B6, CYP2C8, CYP2C9, CYP2C18, CYP2C19, CYP2D6, and CYP3A4. CYP3A4 is the only isozyme that has been previously reported to mediate desomorphine metabolism. This is the first report of CYP2B6, CYP2C8, CYP2C9, CYP2C18, CYP2C19 and CYP2D6 mediated pathways to date. A total of eight metabolites were identified, including nordesomorphine, desomorphine-*N*-oxide, norhydroxydesomorphine, and five hydroxydesomorphine isomers. Results were verified by the addition of an inhibitor for each isoenzyme. No metabolic activity was observed in the control bactosomes (no CYP450 gene) or blanks (CYP450 without drug). Desomorphine-D3 was used as the internal standard. Nordesomorphine was the major metabolite for all except CYP2B6, where an aliphatic hydroxydesomorphine isomer was the major metabolite.

**Conclusion/Discussion:** This study confirmed recent findings related to the phase I metabolism of desomorphine and identified new metabolic pathways involving CYP450-mediated biotransformations that have not been reported to date. As this study was conducted *in vitro*, there exists the possibility that not all of the pathways or identified metabolites will occur under *in vivo* conditions. However, the results of the study indicate that desomorphine will undergo phase I metabolism in the human body and that nordesomorphine is the major metabolite produced.

**Keywords:** Desomorphine, Cytochrome P450, LC/Q-TOF-MS



## Analysis of the Influence of 3,4-methylenedioxy-N-methamphetamine (MDMA) on the Metabolome of Zebrafish Larvae as a Simple Vertebrate Model

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**Background/Introduction:** The ever growing number of new psychoactive substances (NPS) increases the demand to assess their safety and efficacy in a fast and inexpensive manner. Zebrafish (*Danio rerio*) larvae already proved to be a complementary model for assessing the behavioral effects of new psychoactive substances. Compared to other animal models such as mice and rats, zebra fish larvae offer a cost and time saving vertebrate model with short cycles of reproduction and high throughput screening capabilities. Metabolomics offers a unique possibility to gain knowledge on the effects of NPS on biochemical pathways in vertebrates. Corresponding changes might lead to metabolome fingerprints in zebra fish larvae which are indicative for NPS use.

**Objective:** The aim of this work was the development and application of a targeted mass spectrometric method for the analysis of the metabolome in zebra fish larvae. The developed method may be applied to investigate the influence of 3,4-methylenedioxy-N-methamphetamine (MDMA) on the metabolome of zebrafish larvae after controlled drug administration.

**Methods:** MDMA toxicity was tested by exposing 8 zebrafish larvae per concentration (5, 25, 50, 100, 200  $\mu\text{M}$ ) on three different days for 24 hours, respectively. Behavior and morphological changes were assessed and a non-toxic concentration selected for metabolomics experiments. Eight zebrafish larvae were exposed to 15  $\mu\text{M}$  MDMA for 1h, 3h and 6h, respectively. Zebrafish samples were collected in 15 replicates per time point for sufficient statistical evaluation. The samples were stored at  $-80^{\circ}\text{C}$  until analysis. Zebrafish homogenization and extraction was performed using 500  $\mu\text{L}$  methanol / 0.01% phosphate buffer (85:15; v/v), stainless steel beads and the fastprep24 homogenizer (Ilkirch, France). After centrifugation the supernatant was transferred in an Eppendorf tube and freeze dried under nitrogen. The precipitate was dissolved in 50  $\mu\text{L}$  methanol / 0.01 % (v/v) phosphate buffer and 10  $\mu\text{L}$  were used for the Biocrates KIT p180 (Biocrates Inc., Washington, USA). The analysis was performed using a Thermo Fischer Ultimate 3000 UHPLC system (Thermo Fisher, San Jose, California, USA) coupled to a 5500 QTrap linear ion trap quadrupole mass spectrometer (Sciex, Darmstadt/Germany).

**Results:** MDMA showed no effects on the survival rate of zebra fish larvae at any of the chosen concentrations. Morphological changes appeared at concentrations higher than 50  $\mu\text{M}$  MDMA. All further tests were performed at an MDMA concentration of 15  $\mu\text{M}$ . The established sample preparation resulted in sufficient sensitivity to quantify up to 188 analytes from five metabolic substance classes. Standard deviations of the measured compounds were small ( $< 15\%$ ) and offered enough statistical power. The neurotransmitters serotonin and dopamine showed no concentration changes in zebra fish larvae over the chosen exposure times. Creatinine, ornithine, tyrosine and phosphatidylcholines showed statistical significant concentration changes (ANOVA-test, P-value: 0.05). Further, statistical tests for biomarker discovery need to be performed in future studies.

**Conclusion/Discussions:** A highly sensitive and reproducible sample preparation protocol was developed and applied for the measurement of up to 188 analytes in zebra fish larvae using the Biocrates KIT p180. The method was successfully applied to study the influence of MDMA on the metabolome of zebra fish larvae. Further statistical evaluation is needed for drug class biomarker evaluation in zebra fish.

**Keywords:** Zebra Fish Larvae, Metabolomics, MDMA

## Metabolic Profile Determination of U-47700 and U-49900 using Human Liver Microsomes and Authentic Human Urine Specimens

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**Background/Introduction:** U-47700, 3,4-dichloro-*N*-[2-(dimethylamino)-cyclohexyl]-*N*-methylbenzamide, a novel opioid agonist synthesized in the 1980's but first identified in forensic specimens in 2015, has proliferated in drug markets, causing overdose, death, and adverse events in many drug using subjects. The metabolites of U-47700 have been described in urine specimens taken following overdose, but rationalization of the structural identity has been speculative. U-49900, a di-ethyl analogue of U-47700, has also recently emerged in recreational drug markets and postmortem casework, but there are currently no literature reports concerning U-49900. We report the elucidation and first verification of the metabolites of these substances in human forensic toxicology casework.

**Objective:** The objective of this study was to characterize the metabolic profiles of two emerging novel opioids through incubations using pooled human liver microsomes (pHLM), with verification of identified metabolites in authentic human urine specimens. Additionally, similarities and differences in the metabolic profiles were examined for these two analogues.

**Methods:** U-47700 and U-49900 were incubated with pHLM for one hour at 37 °C. Samples were analyzed using a SCIEX (Framingham, MA) TripleTOF<sup>®</sup> 5600+ quadrupole time-of-flight mass spectrometer coupled to a Shimadzu (Kyoto, Japan) Nexera XR ultra high performance liquid chromatograph (LC-QTOF). Data files were processed using SCIEX software applications including MetabolitePilot<sup>™</sup> and PeakView<sup>®</sup>. Targeted and non-targeted data interrogation strategies were used to identify commonly encountered biotransformation products and potential metabolites. Identified metabolites were tableted for verification in authentic human urine casework specimens collected after overdose, and analytically confirmed for the presence of U-47700 (n=5) and U-49900 (n=1).

**Results:** Following pHLM incubations and specimen analysis, four metabolites were characterized and verified in authentic human urine specimens for U-47700. *N*-Desmethyl-U-47700 was identified as the most prominent metabolite. U-47700 was identified in all five urine specimens collected after overdose. The chemical composition and presence of *N*-desmethyl-U-47700 and *N,N*-didesmethyl-U-47700 were confirmed for the first time using standard reference material. Five metabolites were characterized and verified in an authentic human urine specimen for U-49900. *N*-Desethyl-U-49900 was identified as the most prominent metabolite of U-49900 during microsomal incubations, but *N,N*-didesethyl-*N*-desmethyl-U-49900 was identified as the greatest in intensity during data processing of the authentic urine specimen. U-49900 was present in the urine specimen, but at low intensity. Reference material for all U-49900 metabolites is currently unavailable, therefore proposed metabolites were based on accurate mass and fragmentation data.

**Conclusion/Discussions:** Metabolites of U-47700 and U-49900 were characterized using pHLM by LC-QTOF. U-47700 metabolites were consistent with previously proposed pathways in the literature, and structural composition was confirmed for the first time. This is the first report of metabolites associated with U-49900, an emerging novel opioid and analogue of U-47700. Based on this study, similarities and differences were noted between the metabolic profiles of U-47700 and U-49900. This may result in challenges for forensic toxicologists trying to interpret urine results of U-47700 and/or U-49900 ingestion. One metabolite of U-49900 was found to be isobaric to U-47700, with very similar fragmentation pattern and retention time. Processing software for broad-based drug screening initially identified this U-49900 metabolite as U-47700, but significant differences in fragmentation led to the conclusion that this is a chemically distinct compound. U-47700 and U-49900 were found to metabolize to a common metabolite, 3,4-dichloro-*N*-(2-amino-cyclohexyl)-*N*-methyl-benzamide, with accurate mass 301.0869 Da. These results will assist toxicologists in identifying ingestion of U-47700 and U-49900.

**Keywords:** U-47700, U-49900, Metabolism

## Toxicokinetic Modelling of JWH-210, RCS-4, and THC in Pig Serum after Pulmonary Administration

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**Background/Introduction:** Since their appearance on the drugs of abuse market, synthetic cannabinoids (SC) are gaining increasing forensic toxicological relevance and have become a tremendous public health concern. SC are sold and consumed without the knowledge of their toxicokinetic (TK) and toxicodynamic properties, because results from controlled human studies are not available. Yet, TK data are indispensable when interpreting analytical data of impaired or poisoned persons in concern of e.g. the time of intake or concentration at a particular time, especially if profound expert opinion in e.g. driving under the influence of drugs cases is required. A controlled animal TK study allowing for extrapolation to human data should therefore be helpful for better interpretation regarding these issues. In this respect, establishing a pig model suitable for cannabinoid TK studies after intravenous (i.v.) administration and allowing for prediction of human exposure provided first knowledge (Schaefer et al., *Toxicol Lett*, 2016). In consequence, these data should be supplemented with results of a TK study applying a pulmonary administration, as this is the more authentic route.

**Objective:** The first aim was to develop a TK model of the two SC 4-ethylnaphthalen-1-yl-(1-pentylindol-3-yl)methanone (JWH-210) and 2-(4-methoxyphenyl)-1-(1-pentyl-indol-3-yl)methanone (RCS-4) in comparison to  $\Delta^9$ -tetrahydrocannabinol (THC) in pigs after pulmonary administration. For evaluation of the bioavailability, the data obtained from the pulmonary administration experiments were compared to those determined in the i.v. study.

**Methods:** All experiments were approved by the governmental Committee on Animal Affairs. Six ventilated and isoflurane-anaesthetized pigs (mean body weight  $45.5 \pm 3.7$  kg) received a single pulmonary dose of 200  $\mu\text{g}/\text{kg}$  BW each of JWH-210, RCS-4, and THC via the M-neb flow<sup>+</sup> ventilation ultrasonic nebulizer MN-300/7 (Nebutech, Elsenfeld, Germany) operated in the inspiration-triggered mode. The whole dose was administered over 12 min. Blood samples were drawn before and 1, 2, 5, 10, 15, 30, 45, 60, 90, 120, 180, 240, 300, 360, 420, and 480 min after administration. The serum concentrations of the three parent compounds were determined with a validated LC-MS/MS method in positive APCI mode after SPE (Schaefer et al., *ABC*, 2015).

Non-linear mixed effects modelling approach was applied using NONMEM 7.3 (ICON development solutions, San Antonio, USA). Several structural models were tested. Model selection was based on the visual inspection of goodness-of-fit plots, precision of parameter estimates and the statistical values provided by NONMEM.

**Results:** Maximum serum concentrations ( $C_{\text{max}}$ ) were reached 10-15 min after the beginning of nebulization and amounted to  $48 \pm 10.3$  ng/mL for JWH-210,  $39 \pm 14$  ng/mL for RCS-4, and  $77 \pm 39$  ng/mL for THC. After 480 min, the parent compounds could still be quantified and had decreased to mean concentrations of  $0.88 \pm 0.54$  ng/mL for JWH-210,  $0.55 \pm 0.26$  ng/mL for RCS-4, and  $0.77 \pm 0.46$  ng/mL for THC.

The serum-concentration-time profiles of JWH-210, RCS-4, and THC were best described by three-compartment models with first order elimination processes. Absorption from the lungs to serum was modeled by first-order processes. Volumes of distributions at steady-state were estimated at 5.1, 21, and 4.2 L/KG for JWH-210, RCS-4, and THC. Systemic clearances were assessed at 0.049, 0.090, and 0.045 L/min/KG for JWH-210, RCS-4, and THC. The determination of the bioavailability after pulmonary administration revealed 24.8 %, 46.4 %, and 23.6 % for JWH-210, RCS-4, and THC, respectively.

**Conclusion/Discussions:** Three TK models were successfully developed describing the serum-concentration-time profiles of JWH-210, RCS-4, and THC after pulmonary administration to pigs. Comparing the data with the results of the i.v. study revealed similar profiles, but significantly lower  $C_{\text{max}}$  values attributable to lower bioavailability because of adhesion to the tracheal tube wall and pulmonary metabolism/accumulation. Regarding THC, the concentration-time profiles correlate with those obtained from published human smoking studies using a similar study protocol.

**Keywords:** Toxicokinetic Modelling, Cannabinoids, Pigs

## Characterization of the New Synthetic Fentanyl Derivatives 4chloroisobutyrfentanyl, 4-methoxybutyrfentanyl, Benzodioxolfentanyl, Cyclopentylfentanyl, Methoxyacetylfentanyl, and Tetrahydrofuranfentanyl and Identification of their *In Vitro* Phase I Main Metabolites

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**Background/Introduction:** In 2013 the first non-controlled fentanyl analogs emerged on the constantly growing online market of new psychoactive substances (NPS). Some of the new synthetic fentanyl derivatives have been found as cutting agent in seized heroin batches or as heroin substitute and have been associated with several intoxication cases and fatalities around the world. The potencies of designer fentanyls are expected to be in a similar range as the potency of fentanyl itself, posing an extremely high risk to human health, especially when consumed unknowingly.

**Objective:** The aim of the present study was to analytically characterize the six recently obtained fentanyl derivatives 4chloroisobutyrfentanyl (4-Cl-iBF), 4-methoxybutyrfentanyl (4-MeO-BF), benzodioxolfentanyl (Bdx-F), cyclopentylfentanyl (CycP-F), methoxyacetylfentanyl (MeO-Ac-F), and tetrahydrofuranfentanyl (THFF) and to investigate their *in vitro* phase I metabolism. The main phase I metabolites can be used as additional screening markers for designer opioids in biological samples.

**Methods:** All products were purchased as ‘research chemicals’ from online vendors in 2016. Identity and purity of the compounds were determined by gas chromatography-mass spectrometry (GC-MS), liquid chromatography-quadrupole time of flight-mass spectrometry (LC-qTOF-MS) and nuclear magnetic resonance (NMR) spectroscopy. An *in vitro* assay using pooled human liver microsomes (pHLMs) was applied for investigation of phase I metabolism. Fentanyl derivatives were incubated with pHLMs for 60 minutes at 37 °C. The samples were analyzed by LC-qTOF-MS employing full scan/bbCID (broadband Collision Induced Dissociation) and full scan/Auto-MS/MS. Data evaluation was carried out manually using the DataAnalysis software (Bruker).

**Results:** Each of the products contained the declared fentanyl derivative with purities ranging from 60 to 97%. In the GCMS analysis of Bdx-F a second signal corresponding to the amide-linked benzodioxol moiety was observed. This putative impurity probably emerged from thermal degradation in the GC injector since it could not be confirmed by NMR analysis. In general, the following metabolic phase I reactions were observed *in vitro* in the pHLM preparations: amide hydrolysis (leading to formation of 4-anilino-*N*-phenethylpiperidine (4-ANPP) or the corresponding substituted derivative (4-chloro-ANPP, not found for 4-MeO-BF)), *N*-desalkylation (leading to the corresponding nor-metabolite), as well as a couple of mono- and dihydroxylations and formation of *Noxides*. The preferred sites for hydroxylation were the piperidinyl cores, the phenethyl side chains, the corresponding amide-linked head substituent of the fentanyl core structure or the piperidinyl nitrogen for formation of the *N*-oxide. A few compounds showed more specific metabolic transformations, such as demethylation of the benzodioxol moiety of Bdx-F leading to the characteristic metabolite 3,4-dihydroxy-*N*-phenyl-*N*-[1-(2-phenylethyl)piperidin-4-yl]benzamide or formation of the demethylated metabolites of 4-MeO-BF and MeO-Ac-F (*N*-(4-hydroxyphenyl)-*N*-[1-(2-phenylethyl)piperidin-4-yl]butanamide and 2-hydroxy-*N*-phenyl-*N*-[1-(2-phenylethyl)piperidin-4-yl]acetamide, respectively).

**Conclusion/Discussions:** The analyzed fentanyl analogs show similar *in vitro* phase I metabolism patterns. The predominant metabolic reactions are *N*-desalkylation, amide hydrolysis and demethylation (for compounds comprising a methyl or methylene group). *N*-desalkylation leads to formation of compound-specific metabolites, whereas hydrolysis of the amide linker leads to a common metabolite (4-ANPP) for four of the analyzed substances (Bdx-F, CycP-F, MeO-Ac-F and THF-F). Hydroxylated metabolites were formed to different extent and abundance for the compounds investigated. The most dominant *in vitro* metabolites were used together with their parent compounds as analytical targets to update existing routine LC-MS/MS and LC-qTOF-MS screening methods for designer opioids in urine samples. The pHLM assay is a straightforward tool for prediction of main *in vivo* phase I metabolites and for identification of useful biomarkers for urine analysis, which may provide longer detection windows than the parent compound.

**Keywords:** Fentanyl Derivatives, *In Vitro* Metabolism, LC-qTOF-MS Analysis

## Identification, Characterization and Pharmacological Evaluation of New Psychoactive Substances Exemplified by the Recently Emerged Synthetic Cannabinoid CUMYL-PEGACLONE

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**Background/Introduction:** New psychoactive substances (NPSs) flood drug markets worldwide, posing persisting challenges to national authorities and forensic laboratories. Different legislations developed individual strategies to ban entire substance groups based on defined chemical structures instead of adding single substances to national narcotics laws. In response to these new laws, customized substances circumventing the respective restrictions are synthesized and marketed. The substances are added to ‘legal high’ products and offered mainly online as legal alternatives to illicit drugs. At the time of their introduction on the drug markets, often neither their chemical structure nor basic pharmacological data are available. Consequently, new substances may remain undetected by customs, police authorities and analytical laboratories. In addition, they often may pose high risks to consumers due to unforeseeable potencies and side effects.

**Objective:** The present work demonstrates a workflow for the identification of NPSs in ‘legal high’ products including structure elucidation and basic pharmacological characterization, exemplified by the recently emerged synthetic cannabinoid receptor agonist CUMYL-PEGACLONE. Furthermore, a procedure to prove consumption of the drug by urine analysis is presented.

**Methods:** Within a systematic test purchasing program, six herbal mixtures containing an unknown substance were bought from online vendors. Semi-preparative flash chromatography was used to isolate the substance in order to facilitate structure elucidation using gas chromatography-mass spectrometry (GC-MS), gas chromatography-solid state infrared spectroscopy (GC-sIR), liquid chromatography-electrospray ionization-quadrupole time of flight-mass spectrometry (LC-ESI-QToF-MS) and nuclear magnetic resonance (NMR) analysis. The binding affinity towards the cannabinoid receptors CB<sub>1</sub> and CB<sub>2</sub> and the efficacy in a cyclic adenosine monophosphate (cAMP) accumulation assay were measured. For urinary biomarker evaluation, the main *in vivo* metabolites detected after enzymatic conjugate cleavage were identified in urine samples from forensic casework using LC-MS/MS and LC-QToF-MS techniques.

**Results:** Our GC-MS analysis detected an unknown substance together with the synthetic cannabinoid receptor agonist MDMB-CHMCZCA in all six herbal mixtures. Structure elucidation confirmed the unknown substance as 5-pentyl-2-(2-phenylpropan-2-yl)-2,5-dihydro-1*H*-pyrido[4,3-*b*]indol-1-one, a hitherto unknown synthetic cannabinoid with a tricyclic  $\gamma$ -carboline core structure. As a semi-systematic name CUMYL-PEGACLONE (short form of 2-cumyl-5-pentyl- $\gamma$ -carbolin-1-one) is suggested. The substance exhibits high potency at both cannabinoid receptors as suggested by the determined binding affinities ( $K_i$  (CB<sub>1</sub>) =  $1.37 \pm 0.24$  nM;  $K_i$  (CB<sub>2</sub>) =  $2.09 \pm 0.33$  nM) and shows full agonistic activity in the cAMP accumulation assay. Two monohydroxylated metabolites were evaluated as most suitable biomarkers to prove CUMYL-PEGACLONE consumption by urinalysis.

**Conclusion/Discussions:** During a systematic online monitoring of NPSs performed within the EU-financed project SPICE Profiling, a new synthetic cannabinoid receptor agonist was identified in February 2017, shortly after the first ‘legal high’ products containing the compound were sold online in December 2016. Structure elucidation revealed that the new compound, CUMYL-PEGACLONE, bears a  $\gamma$ -carboline core structure circumventing generic definitions of NPSs in national laws like the German ‘Act to control the distribution of new psychoactive substances’ (NpSG) which took effect in November 2016. It can be assumed that the substance was exclusively designed for this purpose. In conclusion, the present workflow demonstrates a strategy to meet the ongoing challenges associated with the highly dynamic NPS market.

**Keywords:** New Psychoactive Substances, CUMYL-PEGACLONE,  $\gamma$ -carboline

## Designer Benzodiazepine Use in the Prison Population in Scotland

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**Background/Introduction:** Benzodiazepines are one of the most common classes of drugs used in Scotland as both prescribed medication and drugs of abuse. Designer benzodiazepines hit the UK recreational drug market in 2012 as a legal alternative to controlled prescription medicines. Some of these substances were novel such as flubromazepam and pyrazolam while others are used medicinally in other parts of the world such as phenazepam and etizolam. In recent years reports of adverse reactions and an increase in drug-related deaths has caused concern. The potency of some of these benzodiazepines can produce strong sedative effects at low doses which may lead to an increased risk of unintentional overdose when ingested in combination with other respiratory depressants.

**Objective:** The objective of this study was to assess the use of benzodiazepines including designer benzodiazepines within the Scottish prison population.

**Method:** Urine samples were collected from individuals undergoing admission to or liberation from 7 Scottish Prison Service (SPS) facilities during November 2015.

Urine samples were analysed using liquid-liquid extraction (LLE) followed by liquid chromatography-tandem mass spectrometry (LC-MS/MS). Twenty-two analytes were included in the method; diazepam, desmethyldiazepam, oxazepam, temazepam, chlordiazepoxide, nitrazepam, alprazolam, lorazepam, lormetazepam, delorazepam, diclazepam, phenazepam, 3-hydroxyphenazepam, etizolam, metizolam, deschloroetizolam, pyrazolam, nifoxipam, clonazolam, meclonazepam, flubromazepam and flubromazolam. LOD for all analytes - 3.75 µg/L; except nifoxipam which was 15 µg/L.

**Result:** A total of 725 urine samples were received from the SPS; 59% (n=430) of these were admission samples (AD), 40% (n=287) were liberation samples (LIB) and 1% (n=8) were unlabelled (UL) so it is unknown if they were AD or LIB. Sixteen analytes were detected in total. One or more diazepam metabolites were detected in 39% of samples (n=281; AD=237, LIB=38, UL=6). Lorazepam was present in 59 samples, (AD=54, LIB=3, UL=2) lormetazepam in 41 samples (AD=39, LIB=1, UL=1) and delorazepam in 44 samples (AD=41, LIB=2, UL=1). Etizolam was present in 22 samples (AD=20, LIB=2) and metizolam was present in 7 AD samples only. Phenazepam was present in 6 samples (AD=5, LIB=1) while 3-hydroxyphenazepam was present in 21 samples (AD=18, LIB=2, UL=1). Chlordiazepoxide and nitrazepam were present in 5 and 3 AD samples, respectively. Flubromazepam, pyrazolam and diclazepam were all positive in 1 AD sample only.

**Conclusion/Discussion:** Benzodiazepines are a large class of drugs and often metabolise, or breakdown, to other active benzodiazepines e.g. diclazepam metabolises to delorazepam, lorazepam and lormetazepam. All diclazepam metabolites are drugs in their own right and can be formed from more than one parent drug. As a result care must be taken when interpreting benzodiazepine results.

As this study was completely anonymised there was no prescription information given with each sample therefore it is unknown how many individuals were prescribed benzodiazepines. It is not possible to ascertain the extent of illicit diazepam use for this study.

Etizolam was positive for 22 samples; 7 of these samples were positive for metizolam. The source of the metizolam may have been as a result of the ingestion of both etizolam and metizolam or metizolam may be a degradation product of etizolam. Delorazepam was positive for 44 samples; 41 and 38 of these were also positive for lorazepam and lormetazepam, respectively. From these results and based on national intelligence it can be speculated that this is a result of diclazepam use. 3-hydroxyphenazepam, a known metabolite of phenazepam, as well as a designer benzodiazepine, is present in 21 samples. Phenazepam use can be confirmed for 6 of these due to the presence of the parent drug, however, the remaining 15 positives could have been the result of phenazepam or 3-hydroxyphenazepam use.

This study has provided a snapshot of benzodiazepine use amongst the prison population in Scotland.

**Keywords:** Prevalence, Benzodiazepines, Novel Psychoactive Substances (NPS)

## UHPLC-MS Impurity Profiling of Spice-Products Containing the Synthetic Cannabinoids MDMB-CHMICA Assisted by Multi-Variate Data Analysis

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**Background/Introduction:** Impurity profiling of drugs of abuse like amphetamine or heroin is an established tool to establish links between samples, providing forensically relevant information ranging from drug source, manufacturing methods and precursors, distribution networks and trafficking routes to dealer-user relationships.

**Objective:** The main aim of this work was to adapt and transfer the concept of impurity profiling from the classical drugs to the rapidly changing new psychoactive substance (NPS) market, exemplarily represented by the potent synthetic cannabinoid MDMB-CHMICA (methyl-(S)-2-(1-(cyclohexylmethyl)-1*H*-indole-3-carboxamido)-3,3-dimethylbutanoate), which was the most prevalent active substance in 'Spice'-products in Europe in 2015/16. The intention of this profiling study is to attain comprehensive information about the underlying manufacturing and distribution processes and the NPS market structure, especially links between internet shops. For that purpose more than 150 samples of 'Spice'-products from online test purchases (in 32 shops with 116 different product brands represented) and additional samples from police seizures were available for an impurity profiling study in the context of the ongoing EU-project "SPICE-profiling" (JUST/2013/ISEC/DRUGS/ AG/ISEC/4000006421).

**Method:** The herbal material was extracted twice with acetonitrile, the extracts combined and evaporated to dryness. These extracts were then submitted to flash-chromatography (Büchi X50 system using pre-packed silica gel columns with n-hexane and ethyl acetate as eluents) to isolate occurring impurities from the main component MDMB-CHMICA. All fractions containing impurities were combined to produce one solution carrying the complete impurity profile of the corresponding sample. Further measurements were carried out on an UHPLC-MS system (Dionex UHPLC coupled to a Bruker AmaZon speed ion trap mass spectrometer equipped with an ESI-source). The resulting chromatographic/mass spectrometric data sets were processed (Profile Analysis, Bruker) and evaluated by principle component analysis (PCA, Unscrambler X, Camo) to establish links between samples due to their individual impurity profiles.

**Result:** Several replicate measurements showed excellent reproducibility for the sample preparation procedure via flash-chromatography. In a separate study, more than 30 target synthesis impurities have been assigned and qualified to allow discrimination between different synthesis batches of MDMB-CHMICA. On this basis a PCA model was generated including all herbal blend samples. The resulting scores plot was evaluated by assigning the samples to external parameters like "packaging layout", "internet-shop" and "date of purchase", the latter one yielding the most striking results. When subdivided into consecutive intervals of three months, cluster formations of potentially linked samples became visible, independent of the internet shop or product brand. A plausible explanation for this finding is that the European market could mainly be supplied by successive single batches of MDMB-CHMICA from one producer, which are then distributed among major manufacturers of the herbal mixtures supplying most of the internet shops. This hypothesis is supported by the customs seizure of 40 kg pure MDMB-CHMICA at Luxemburg airport in December 2014, representing approximately 40 million single doses in a single shipment.

**Conclusion/Discussion:** Impurity profiling is not only a valuable tool for classical drugs, but can also be used to elucidate the structures of the NPS market including online shops.

**Keywords:** Synthetic Cannabinoids, Spice-Products, Impurity Profiling

## Fatalities Involving Carfentanil and Furanyl Fentanyl: Two Case Reports

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**Background/Introduction:** Carfentanil is a fentanyl analog frequently used in large animal veterinary medicine. Recently, it has been discovered in cases throughout the United States as carfentanil has been found in the heroin supply either alone or mixed with heroin and/or other fentanyl analogs. The strength of carfentanil is approximately 10,000 times greater than morphine and 100 times greater than fentanyl. In two recent cases, carfentanil was identified and ruled to be the cause of death, either alone or in combination with other drugs. Case 1 was a known heroin user. He was discovered slumped over in a running van blocking the bays of a car-wash. Two syringes, a spoon with cotton and residue, and a yellow baggie of powder were found in the van. Case 2 was living in a tent in a park with his mother who noted him to be “itching all over” and later she discovered him unresponsive with an e-cigarette and small baggie of brown powder next to him.

**Objective:** To present postmortem carfentanil and furanyl fentanyl concentrations and to demonstrate the importance of case histories, autopsy findings, and toxicological results when evaluating cases.

**Method:** Postmortem blood, vitreous and/or urine specimens were screened for volatiles by headspace gas chromatography, drugs of abuse by immunoassay and alkaline extractable drugs by full scan gas chromatography mass/spectrometry (GC/MS). Case 1 heart blood was presumptive positive for fentanyl and opiates. Case 2 heart blood was presumptive positive for benzoyllecgonine. Fentanyl confirmations were performed utilizing liquid-liquid extraction and analyzed by GC/MS in selective ion monitoring (SIM) mode. Cocaine and opiates confirmations were performed by protein precipitation and liquid chromatography tandem mass spectrometry (LC-MS/MS) and all opiate blood and urine specimens were analyzed with both hydrolyzed and un-hydrolyzed aliquots. Heart blood for both cases was sent to a reference laboratory for carfentanil and furanyl fentanyl analysis.

**Result:** The toxicology findings for both cases are presented in Table 1. The spoon was analyzed using the GC/MS screening program and the following drugs were identified by spectral library matching only: caffeine, carfentanil, diphenhydramine, fentanyl, para-fluoroisobutyryl fentanyl, furanyl fentanyl, heroin, hydromorphone, mannitol, 6-monoacetylmorphine, morphine, noscapine, and quinine. Based on pathological findings, case history, and toxicology results, the medical examiner determined the cause of death for case 1 to be intoxication by the combined effects of heroin, fentanyl, carfentanil, and furanyl fentanyl and case 2 to be intoxication by carfentanil.

**Table 1:** Drug concentrations (ng/mL) in the two presented case fatalities. Not analyzed (NA), Not detected (ND)

Case	Drug	Peripheral Blood	Heart Blood	Vitreous Humor	Urine
1	Carfentanil	NA	1.3	NA	NA
	Furanyl Fentanyl	NA	0.34	NA	NA
	Fentanyl	ND	6	NA	NA
	Morphine (total)	ND	<20	ND	Present
	Hydromorphone (total)	ND	<20	ND	Present
	6-AM	ND	ND	Present	Present
	Hydrocodone	ND	ND	Present	Present
	Hydromorphone	ND	ND	Present	Present
2	Carfentanil	NA	0.12	NA	NA
	Benzoyllecgonine	460	NA	510	NA
	Cocaine	ND	NA	40	NA

**Conclusion/Discussion:** The laboratory generally will not send out case samples for additional testing if toxicologically significant levels of drugs are found. These cases were sent to a reference laboratory for carfentanil and furanyl fentanyl analysis due to the lack of significant toxicological or autopsy findings. Additionally the scene descriptions gave a strong indication that a drug overdose occurred. Attention should be paid to the paraphernalia at the scene, injection marks, and symptoms before death. Toxicologists should be cognizant when the toxicology findings are negative or only a trace amount of drug is detected as these cases could contain carfentanil, furanyl fentanyl, or other fentanyl analogs. Targeted analysis should be performed using more sensitive instrumentation such as LC-MS/MS.

**Keywords:** Carfentanil, Furanyl Fentanyl, Postmortem



## Seven Cases of Severe Intoxication Associated with Analytically Confirmed Use of the Novel Psychoactive Substances 25I-NBOMe

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**Background/Introduction:** The novel psychoactive substance 4-iodo-2,5-dimethoxy-N-(2-methoxybenzyl) phenethylamine (25I-NBOMe) is a N-methoxybenzyl-substituted phenethylamine. The drug can be insufflated as a liquid or powder, swallowed, and taken orally. 25I-NBOMe is a very potent 5-HT<sub>2A</sub> receptor agonist with typical user doses being reported to be in the sub-to-low milligram range. Information from clinical case reports suggests that some users experience severe psychological and behavioral changes associated with 25I-NBOMe use. These include intensive auditory and visual hallucinations, severe agitation, aggression and unpredictable violent episodes. Overdose effects may include seizure and acute kidney injury. Typically, serum concentrations of severely intoxicated individuals were found to be lower than 5 ng/ml.

**Objective:** We report seven cases of analytically confirmed 25I-NBOMe toxicity, with plasma concentrations ranging from 4-81 ng/ml.

**Methods:** Plasma samples of seven patients were analyzed with LC-MS/MS using a Sciex 5600+ QqTOF mass spectrometer. Targeted and non-targeted workflows were used to detect and quantify drug compounds. Liquid-liquid-extraction was used for sample preparation.

**Results/Discussion:** On new year's eve the center of Innsbruck becomes a party destination. There are numerous events, parties and clubs. People are enjoying live music, culinary delights and colorful shows. For seven people, however, the new year's eve 2015 turned into a night of horror after consuming an unknown drug compound. The patient collective included three women and four men aged 18-35 years. Three patients represented frequent drug users that were taking part in drug maintenance treatment.

Shortly after insufflating a brown drug powder, the patients experienced visual hallucinations, confusion, agitation, and mydriasis. Furthermore, some got into a panic or became aggressive, and one patient fell into a coma. All seven patients were sent to the emergency department, where they received variable doses of lorazepam, midazolam, diazepam, and metoclopramide. Toxic effects were resolved 6-12 hours after admission. Patients were discharged without any chronic or late sequelae.

To identify the consumed drug compound, plasma samples were submitted to our laboratory for systematic toxicological analysis, which involved non-targeted and targeted LC-MS/MS analysis. In all seven plasma samples 25I-NBOMe was detected as the main active compound. The plasma concentrations ranged from 4-81 ng/ml. As far as we are aware of, these are the highest plasma levels ever reported for 25I-NBOMe. Based on available data from other intoxication cases, it is reasonable that the consumed doses would have been sufficiently high to even cause fatal intoxications. Probably, more serious consequences were prevented by interactions with other drug compounds consumed, such as morphine, methadone, MDMA or THC.

**Conclusions:** 25I-NBOMe was identified as the main active compound in seven cases of severe intoxication. Plasma concentrations turned out to be sufficiently higher than 4 ng/ml. Probable drug-drug interactions prevented fatal consequences.

**Keywords:** 25I-NBOMe Intoxication, Plasma Concentrations, Systematic Toxicological Analysis

## Comparison and Evaluation of Recombinant $\beta$ -Glucuronidases for Urine Drug Testing

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**Background/Introduction:** The prevalence and use of urine drug testing has significantly increased over the past decade. As sample volumes increase, laboratories are continually seeking ways to improve the overall efficiency of the testing process. Hydrolysis of conjugated metabolites has historically been a time consuming step in the sample preparation process, requiring incubation times of up to two hours. Recently, newer enzymes have been introduced and can achieve complete hydrolysis of most target conjugates (e.g. opiates, benzodiazepines, TCAs) in less than an hour, significantly reducing sample preparation time. Over the past 3-4 years, several manufactures have introduced these novel/second generation  $\beta$ -glucuronidases and several enzymes from different manufactures will be compared.

**Objective:** High efficiency  $\beta$ -glucuronidases have been reported to completely hydrolyze compounds of interest in a “standard” prescription/illicit drug panel in less than 60 minutes. Some manufacturers state complete hydrolysis in 30 minutes or less. Four different enzymes from three manufacturers are compared and evaluated for use in a high throughput urine drug testing laboratory. Criteria evaluated include hydrolysis efficiency, matrix interferences, method ruggedness, and cost.

**Methods:** Enzymes: Four different enzymes from three different manufacturers were evaluated: 1) IMCSzyme® (IMCS); 2) BG100® (Kura Biotec); 3) BGTurbo™ (Kura Biotec); and 4)  $\beta$ -D-Glucuronide glucuronosohydrolase (MilliporeSigma). Urine Samples: Drug free urine was fortified with glucuronide standards obtained from MilliporeSigma. Conjugated metabolites represented several drug classes of interest, including opiates (morphine, codeine, oxymorphone), benzodiazepines (lorazepam, oxazepam, temazepam), opioids (buprenorphine, norbuprenorphine, tapentadol, naltrexone), TCAs (amitriptyline), and THC (THC-COOH). Urine was spiked at concentrations from 1000 ng/mL to 20000 ng/mL. Concentrations chosen were relevant to concentrations observed in patient urine specimens. Sample preparation: Samples were hydrolyzed using each enzyme and the manufacturer’s recommended hydrolysis protocol. Hydrolysis efficiency was measured at five different time points: 15 minutes, 30 minutes, 45 minutes, 60 minutes, and 75 minutes. At each time point, the hydrolysis reaction was quenched, sample centrifuged, and an aliquot diluted for analysis by LC-MS/MS.

**Results:** All enzymes showed hydrolysis efficiency of >90% in <60 minutes for the majority of the glucuronides. Thirty to forty-five minutes was sufficient for most enzymes. Amitriptyline-, Codeine-6 $\beta$ -, dihydrocodeine-6 $\beta$ -, morphine-6 $\beta$ -, and tapentadol-glucuronide posed the greatest hydrolysis challenge, with at least one enzyme demonstrating glucuronide cleavage of <50% at 60 minutes. Even using a simple “dilute-and-shoot” sample preparation, there were no significant matrix interferences when analyzing for >50 prescription and illicit drugs/metabolites. Ruggedness studies will be determined by analyzing multiple injections and determine when/if data quality is affected. Preliminary results suggest column lifetime is around 2000 injections.

**Conclusion/Discussions:** The newer, next generation  $\beta$ -glucuronidases definitely improve testing efficiency. Hydrolysis times are reduced by >50% from our previous production method. Ruggedness was comparable or better than our current method. Cost analysis and evaluation on a variety of patient samples are compared to determine the full cost/benefit of each enzyme.

**Keywords:**  $\beta$ -glucuronidase Enzyme Hydrolysis, LC-MS/MS, Urine Drug Testing

## Toxicology Screening Approaches Take Flight: The Utilization of ESI LC/qToF Workflows in a High Throughput Laboratory Amidst the Opioid Epidemic and the Designer Drug Era

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**Background/Introduction:** The field of analytical chemistry has made great strides in technological advancements for toxicology in the last 50 years and even more so in the last 15 years with the commercial availability and affordability of a variety of LC/MS (liquid chromatography/ mass spectrometry)-based systems. There exists however the ever-present demand for fast, more accurate, more durable analytical separation and detection systems and workflows that can yield legally defensible information. This demand only grows stronger in the United States as the opioid epidemic continues and the designer drug trends define an era. The approach of using liquid chromatography and high resolution mass spectrometry in the form of ToF (time-of-flight)-based detection presents opportunity for alternative high throughput workflows that can take into account unknown compounds and foreseen challenges in linear dynamic range.

**Objective:** The goal of this project is to propose and test an alternative approach to current LC-qToF (liquid chromatography- quadrupole time-of-flight) high throughput workflows to adapt to the instrumentation limitations by simultaneous detection of compounds of both very large and very small quantities with known and unknown chemical makeup. Thus providing analytical insight to the impact of the opioid epidemic and designer drug trends within the toxicology production laboratory.

**Methods:** An LC-qToF (ESI) screen method (Lexion LC and X500R qToF) was developed for the detection of 100 + targeted compounds of interest with the focus being on natural and synthetic opioids, cathinones and cannabinoids, in addition to untargeted acquisition of data. The analytical method was tested by the analysis of a 100 + of clinical samples positive for a variety of prescription and illicit drugs/metabolites in addition to negative human urine spiked to stress the limit of detection, mass accuracy and linear dynamic range of the system.

**Results:** It was determined that the accuracy and selectivity of the system was directly correlated to the depth and robustness of the validated analytical method and corresponding analytical criteria. Specific compounds behave uniquely in the LC/qToF system and as a result revealed the strengths and weaknesses of the workflow as a high throughput screen approach. The limits of detection, spike study results and ability to retrospective analyze the sample for non-targeted knowns clearly presents the positive potential the LC/qToF in the capacity of the above workflows has in providing more informative toxicology screens.

**Conclusion/Discussions:** The ability to harness readily available LC/qToF technology to create a more comprehensive and robust clinical toxicology screen is instrumental in addressing the opioid epidemic and simultaneous designer drug infiltration. Implementing a LC/qToF screen workflow into a clinical toxicology setting that is designed to confront linear dynamic range limitations and collect data that can be analyzed retrospectively for relevance, offers the opportunity for more thorough and comprehensive treatment.

**Keywords:** LC/MS, LC/qToF, Time-of-Flight Mass Spectrometry

## Qualitative Method Validation: A First Approach Through Binary Results Applied to a Multi-Drug LC-MS/MS Method

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**Background/Introduction:** The literature describing quantitative method validation is well developed. However, information on qualitative method validation is much sparser. Qualitative methods are characterized by the binary nature of the output (presence/absence, above/below cutoff), a result fundamentally different from the continuous numerical output of quantitative methods. Although qualitative method validation protocol is covered in the SWGTOX Standard Practices, the procedures used rely on quantitative values (e.g. precision at the decision point, ionization suppression/enhancement), in what is clearly a derivative of the quantitative method validation protocol.

**Objective:** The aim of this project is to develop a method validation procedure for qualitative methods that is fully based on their binary output, and apply it to a multi-drug LC-MS/MS targeted screening method.

**Methods:** Extraction was carried out using protein precipitation. 10  $\mu\text{L}$  of deuterated internal standards solution was added to 100  $\mu\text{L}$  of blood and diluted with 100  $\mu\text{L}$  of 0.2% formic acid:methanol (50:50). Diluted samples were then precipitated with 400  $\mu\text{L}$  of acetonitrile:acetone (70:30). Following vortexing, centrifugation at 3200xg for 5 minutes was performed. A 25  $\mu\text{L}$  aliquot of the supernatant was then diluted with 180  $\mu\text{L}$  of 0.2% formic acid solution. 5  $\mu\text{L}$  of this extract was analyzed on an LC-MS/MS system (Sciex 5500 QTRAP) using a C18 column and a 13-minute gradient. Analysis of 10 samples of antemortem and postmortem blood spiked with the 40 analytes at the cutoff concentration (generally 20 ng/mL, with a few exceptions) allowed estimation of the standard deviation ( $\sigma$ ) at the cutoff concentration. The next step was the preparation of probability curves from ten blood samples spiked at  $-4\sigma$ ,  $-3\sigma$ ,  $-2\sigma$ ,  $-1\sigma$ , cutoff,  $+1\sigma$ ,  $+2\sigma$ ,  $+3\sigma$  and  $+4\sigma$ . With these results, the percentage of positive samples (i.e. samples with an area ratio above the cutoff average) was plotted as a function of the concentration.

Finally, 30 blood samples were spiked at  $-3\sigma$  and  $+3\sigma$ . Analytes were deemed to be validated if the false negative and false positive rates stemming from these results were below 10%, and if reliability, selectivity and sensitivity rates were above 90%.

**Results:** Following analysis of the 10 blood samples spiked at the cutoff, analytes were binned into three groups based on the magnitude of their standard deviation: 1.6 ng/mL (group 1), 3.3 ng/mL (group 2), and 5 ng/mL (group 3). With these groupings it was then possible to prepare samples surrounding the cutoff on the basis of the standard deviation and prepare probability curves. Theory suggests that the probability curve should be sigmoidal and this was generally observed. These results highlighted that probability curves could be used to perform a better internal standard assignment. The final experiment resulted in 31 out of 40 analytes being validated, with false positive rates oscillating between 0 and 80% and false negative rates oscillating between 0 and 10%. Reliability rate (60% - 100%), selectivity rate (90% - 100%) and sensibility rate (20% - 100%) were also calculated. Upon investigation, we determined that the deficient performance of nine analytes was attributable to a large inter-day variation of the standard variation of measured analytes, which could sometimes increase three-fold despite the use of deuterated internal standards. Work to address this issue is currently underway.

**Conclusion/Discussions:** The procedure shown here is a first attempt at a qualitative method validation procedure that respects the binary nature of the output rather than shoehorning a method validation protocol designed for quantitative methods. Because of the unforeseen problem of daily changing standard deviations, a more developed statistical approach will be necessary and is currently under development. However, the approach shown here can be applied to more stable instruments (e.g. UV-Vis spectrophotometer).

**Keywords:** Qualitative, Method Validation, Statistics

## An Activity-based Screening Method for Synthetic Cannabinoids: From Concept to Application

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**Background/Introduction:** Synthetic cannabinoids (SCs) are the largest group of compounds currently monitored in Europe by the EU Early warning system on new psychoactive substances. The number of substances, their chemical diversity and the rate at which they emerge makes this group of compounds particularly challenging in terms of detection and monitoring. Current approaches for detection of these substances (typically only present at low ng/mL concentrations in biological matrices) are based on targeted, structure-based methods, such as immunoassays or MS-based methods. However, both these approaches have important limitations (e.g. lack of cross-reactivity and prior knowledge of structure required).

**Objective:** Here, we report on the development and application of a bioassay that is based on the activity of SCs at the cannabinoid (CB) receptors. This assay may be used to perform activity profiling of new SCs and their metabolites and as a first-line screening tool to identify positive biological samples.

**Methods:** The activity-based screening method is based on the NanoBiT® technology (Promega). Activation of the CB1/CB2 cannabinoid receptor (fused to one part of luciferase) leads to the recruitment of  $\beta$ -arrestin 2 (fused to the other part of luciferase). The resulting functional complementation of luciferase can easily be monitored via luminescence. We upgraded our previously developed transient system to a stable HEK293T cell system via retroviral transductions and cell sorting. The stable system has the advantage that the workload is reduced and there is less variation between experiments. The assay was applied in a 96-well format on pure substances, as well as on extracts from urine and blood. Urine extracts were prepared from 0.5 mL, which was deconjugated and extracted with acetonitrile and ammonium formate (10M). Blood extracts were prepared from 0.5 mL, to which a carbonate buffer (pH 10) was added and extracted with n-hexane/ethylacetate (99/1). Following evaporation of the supernatant, the residue was redissolved in 100  $\mu$ L of a 50:50 mixture methanol : serum free medium. Ten  $\mu$ L of the extract (or pure compound) was used in the bioassay.

**Results:** Different SCs (XLR-11, UR-144, AB-CHMINACA and ADB-CHMINACA) and their major phase I metabolites were evaluated for their activity at CB1 and CB2, showing that several major metabolites retain their activity. Application of the bioassay on 42 + 32 genuine urine samples, 43 authentic serum samples, and 76 DUID plasma samples (with 1 SC positive sample) shows that the bioassay is capable of detecting positive urine and blood samples. From the 18 urine samples from users who consumed either UR-144 or XLR-11, 17 were scored positive (94.4%). From the 12 urine samples positive for AB-CHMINACA metabolites, only 4 were scored positive (33.3%), which was unexpected based on the activity profiling of the AB-CHMINACA metabolites which showed activity at both CB1 and CB2. Nine out of 11 (81.8%) urine samples from users who had consumed ADB-CHMINACA were scored positive. Analysis of a second batch of urine samples (n=32) resulted in an overall sensitivity of 75% and a specificity of 90.5%. For the analysis of the serum samples (containing a variety of SCs), we reached a sensitivity of 81.8% (18/22) and a specificity of 100% (21/21). In the plasma DUID samples, the single SC positive sample was identified. Here, also high THC concentrations (>20 ng/mL) yielded a slightly positive result.

**Conclusion/Discussions:** Different SCs (XLR-11, UR-144, AB-CHMINACA and ADB-CHMINACA) and their major phase I metabolites were evaluated for their activity at CB1 and CB2, showing that several major metabolites retain their activity. Application of the bioassay on 42 + 32 genuine urine samples, 43 authentic serum samples, and 76 DUID plasma samples (with 1 SC positive sample) shows that the bioassay is capable of detecting positive urine and blood samples. From the 18 urine samples from users who consumed either UR-144 or XLR-11, 17 were scored positive (94.4%). From the 12 urine samples positive for AB-CHMINACA metabolites, only 4 were scored positive (33.3%), which was unexpected based on the activity profiling of the AB-CHMINACA metabolites which showed activity at both CB1 and CB2. Nine out of 11 (81.8%) urine samples from users who had consumed ADB-CHMINACA were scored positive. Analysis of a second batch of urine samples (n=32) resulted in an overall sensitivity of 75% and a specificity of 90.5%. For the analysis of the serum samples (containing a variety of SCs), we reached a sensitivity of 81.8% (18/22) and a specificity of 100% (21/21). In the plasma DUID samples, the single SC positive sample was identified. Here, also high THC concentrations (>20 ng/mL) yielded a slightly positive result.

**Keywords:** Synthetic Cannabinoids, Bioassay, Activity-Based-Screening

## New Bioassay for Detection and Activity Profiling of Synthetic Opioids

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**Background/Introduction:** Following series of synthetic cannabinoid and synthetic cathinone derivatives, the illicit drug market shows an increased incidence of synthetic opioids, including fentanyl and its derivatives, and other chemically unrelated opioids, including U-47700. These synthetic opioids, together with the natural (e.g., morphine, codeine) and semi-synthetic opioids (e.g., hydromorphone, desomorphine) are common toxicological findings in death investigation cases.

**Objective:** Here, we report on the development and application of a bioassay that is based on the activity of (synthetic) opioids at the  $\mu$ -opioid receptors (MOR). This assay may be used for activity profiling of new synthetic opioids and as a first-line screening tool to identify opioid-positive biological samples.

**Methods:** The bioassay utilizes transiently transfected HEK293T cells, in which the NanoBiT® technology (Promega) was applied. Activation of MOR (fused to one part of luciferase) leads to recruitment of  $\beta$ -arrestin 2 (fused to the other part of luciferase). The resulting functional complementation of luciferase can be easily monitored via luminescence. The assay was applied in a 96-well format on pure substances and blood extracts. The latter were prepared by subjecting 250  $\mu$ l of blood to SPE (Waters Oasis® MCX), drying of the eluate and reconstitution in 100  $\mu$ l of serum free medium. Ten  $\mu$ l of the extract (or pure compound) was used in the bioassay.

**Results:** Several semi-synthetic opioids (hydromorphone and desomorphine) and synthetic opioids (fentanyl and derivatives such as 4-chloro-isobutyrfentanyl, 4-methoxybutyrfentanyl, acryloylfentanyl, alfentanil, benzodioxole-fentanyl, cyclopentylfentanyl, methoxyacetylfentanyl, ocfentanil, tetrahydrofuranfentanyl and other unrelated opioids (U-49900 and methene-U-47700 (U-51754)) were tested in our bioassay, showing a variety in level of MOR activation. Application of the developed bioassay on blind-coded authentic blood samples from morphine users and non-users shows that the bioassay is capable of detecting opioid activity in blood samples (sensitivity 100% (5/5), specificity 100% (7/7)).

**Conclusion/Discussions:** The developed bioassay may not only offer better insight into the potential activity of new synthetic opioids, it also offers the opportunity to serve as a first-line screening tool for opioids in biological matrices in an alternative way. Applicability of this assay will be further demonstrated using biological samples from authentic users of newly emerging synthetic opioids.

**Keywords:** Synthetic Opioids, Bioassay, Activity-Based-Screening

## A Multi-Analyte LC-MS/MS Based Screening Approach Combined with Simultaneous UV-DAD Quantification of Drugs in Blood and Urine

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**Background/Introduction:** Screening, identification and quantification of drugs in blood and urine is most relevant for routine applications in clinical and forensic toxicology. LC-MS/MS based screening procedures are powerful tools for the identification of multiple targets in a single chromatographic run. However, such screening approaches are generally less efficient regarding a simultaneous and reliable quantification of identified compounds.

**Objective:** The development of a multi-target screening method for forensic applications based on LC-MS/MS identification as well as UV-DAD quantification, combining the analytical power of both techniques.

**Methods:** Calibrations curves of multiple drugs were prepared in blood and urine and compounds were extracted with chlorobutane, pH 8. Separation was performed on a Zorbax Eclipse XDB-C18 column using a Shimadzu Prominence UHPLC including a UV-DAD detector (spectral recording from 195 – 380 nm). The buffer system (buffer A: 5 mM ammonium formiate, trifluoroacetic acid pH 2.3; buffer B: 5 mM ammonium formiate, 95% acetonitrile, trifluoroacetic acid pH 2.3,) is compatible with both UV-DAD and LC-MS analysis. Compounds were detected using an ABSciex QTrap3200 hybrid triple-quadrupole linear ion trap mass spectrometer. An initial survey MRM scan was followed by an information-dependent acquisition applying two enhanced product ion scans. Target compounds were then identified by comparing and matching MS/MS and UV spectra against our library containing the spectral data (MS/MS and UV spectra). The ABSciex Multiquant software was used to accurately quantify target compounds using the DAD signal at three selected wavelengths for each analyte.

**Results:** Here we describe the development of a multi-target approach for the simultaneous LC-MS identification and UV-DAD quantification of drugs in blood and urine. Our method allows the identification and quantification of at present over 100 drugs relevant for forensic and clinical toxicology, including common drugs of abuse, benzodiazepines, antidepressants, neuroleptics, anesthetics and barbiturates. MRM triggered fragmentation spectra as well as UV profiles were recorded for each compound to build up a combined spectral library. An MRM triggered generation of fragment spectra followed by a MS/MS library search allows the identification of target compounds, and simultaneously recorded UV-DAD signals are used for quantification. Calibration curves for each compound were previously prepared and stored; the continuous UV based calibration data provide stable signals over time. This is much in contrast to LC-MS generated data which are prone to ionization dependent fluctuations. Only one internal standard (Medazepam, 100 µg/ml) is added to the samples to compensate the extraction efficiency and match retention times. Since a baseline separation of compounds is critical for UV based quantification, the chromatographic separation is performed over 40 min, which allows interference free recording in most cases. The immediate availability of quantitative data compensates for comparably long run times. The chosen buffer system further allows the potential identification of unknown UV spectra using commercially available UV libraries (e.g. UV library of toxic compounds, Pragst).

**Conclusion/Discussions:** We present a method for simultaneous identification and quantification of multiple drugs in blood and urine. The screening approach combines LC-MS based identification power with a reliable UV-based quantification of compounds. Our method is cost efficient and can be most useful for routine applications in forensic and clinical toxicology.

**Keywords:** LC-MS/MS Screening, UV-DAD Quantification, Screening

## NIST Standard Reference Materials (SRMs) for Drug Toxicology Measurements - Past, Present, Future

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**Background/Introduction:** The SRM program provides the highest order materials to underpin measurements in a manner traceable to the SI (the International System of Units). To support the forensic toxicology measurement community, NIST provides a suite of SRMs containing ethanol-water mixtures for the calibration of alcohol breathalyzers and for blood-alcohol determinations. In the past, NIST also provided series of drugs-of-abuse SRMs in serum, hair, and urine matrices. Certified values for the parent drugs or their metabolites included benzoyl ecgonine (cocaine), THC-COOH (marijuana), morphine and morphine glucuronide, methamphetamine, phencyclidine (PCP), codeine, methadone, nordiazepam, and cotinine (nicotine). Many of these drugs-of-abuse SRMs were developed in cooperation with the College of American Pathologists. However, because of technical issues such as the unsuitability of the matrix, analyte instability, and/or poor sales, most of this series of drug SRMs have been discontinued.

**Objective:** The abuse of opioids and the advent of Novel Psychoactive Substances (NPS) including fentanyl (and its synthetic analogues) and carfentanil has reached the crisis level. NIST is ascertaining approaches to provide quality assurance materials and data to support the tremendous task of toxicological measurements in this rapidly changing landscape. NIST has several metrological tools to support quality measurements: reference materials, hosting interlaboratory measurement comparisons, providing accessible and curated data, and developing measurement technology. The objective of this discussion is to determine the needs of Toxicology community and decipher the most effective strategy(s) to address the measurement needs.

**Methods:** When the most accurate and precise measurements are required, NIST pursues the rigor of SRM production. SRM certification requires well-characterized, high purity primary standards, investigation of unit homogeneity, and statistical agreement of two independent, high accuracy measurement techniques for the measurements. This is typically a 3-year process. In the context of rapidly changing targets associated with the opioid/NPS challenge, NIST is considering less rigorous avenues. Reference Materials (RMs) require characterization of the primary standard and one reliable method to provide information values. An idea under development is to provide NIST Research Grade Materials (RGMs) which are uniform materials determined to be homogenous for a defined property. An RGM serum or urine could be 'spiked' with NPS drug metabolites to validate methods between laboratories on a common-use material. NIST also frequently hosts interlaboratory studies to assure comparability of results between laboratories, providing pre-evaluated test materials and rigorous statistical evaluation of the data.

**Results:** NIST is establishing a NIST *NPS DataHub* incorporating NMR data toward rapid and reliable identification of the primary materials. A stratified approach is being developed that would allow data addition by participating external laboratories, with defined levels of certainty based on NIST curation of the available information. We are also working on tools for the comparison of the new low resolution, tabletop NMR spectra with high field data.

**Conclusion/Discussions:** NIST is reaching-out to the forensic toxicology community to determine the most effective approaches to assure the quality of NPS measurements.

**Keywords:** Novel Psychoactive Substances, Reference Materials, Opioids



**High Throughput Protein Precipitation: Screening and Quantification of 106 Drugs and their Metabolites using LC-MS/MS.**

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**Background/Introduction:** Protein precipitation in combination with LC-MS/MS is rarely used for postmortem biological matrices analysis in forensic toxicology. However, protein precipitation (PPT) is a simple, fast and economical sample preparation process which allows faster turnaround times, especially when used in conjunction with a multi-analyte targeted screening method. Additionally, this analytical procedure reduces the need for non-specific screening (e.g. immunoassay) and general unknown screening (e.g. GC-MS).

**Objective:** The objective of this work is to develop and validate a wide-spectrum LC-MS/MS analytical method for analyte quantification and identification in antemortem and postmortem urine and blood samples, using protein precipitation as sample preparation.

**Methods:** In a 96 wellplate, 100  $\mu$ L of sample (blood or urine) was mixed with 10  $\mu$ L of internal standard solution (36 deuterated IS) before being diluted with 100  $\mu$ L of 0.2% formic acid:methanol (50:50 v:v). Diluted samples were precipitated with 400  $\mu$ L of acetonitrile:acetone (70:30 v:v). Once sealed, the wellplate was shaken for 5 minutes and centrifuged at 3200x g for 5 minutes. A 25  $\mu$ L aliquot of the supernatant was transferred into a first injection plate (plate #1) using a 96 channels pipette (Liquidator 96™) and diluted with 180  $\mu$ L of 0.2% formic acid solution. Then, another supernatant aliquot (200  $\mu$ L) was transferred in a second injection plate (plate #2) and diluted with 50  $\mu$ L of 1.5% formic acid solution. Plate #1 was used for the quantification and screening of a multi-class drug panel (106 analytes) including opioids, anti-anxiety, anti-depressant, anti-psychotics, stimulants and hallucinogens. Plate #2 was used for the analysis of cannabinoids. The plates were injected consecutively on an LC-MS/MS system (Agilent 1200 HPLC/Sciex 5500 Qtrap) with C18 columns (Agilent, Zorbax Eclipse plus C18, 2.1x50 mm or 2.1x50 mm, 3.5 $\mu$ m) using a ramp gradient of 13 and 6.5 minutes respectively. The mobile phases used are MeOH:10mM ammonium formate pH3.0 (2:98 v:v) and ACN. Scheduled MRM was used to monitor two transitions (quantifier/qualifier) for each analyte and internal standard.

**Results:** Protein precipitation solvent was optimized to provide a homogenous sample and minimize the amount of phospholipids in the extracted sample. The dilution step prior to precipitation allowed to achieve a better recovery for all analytes (>70%). Two separate injection plates were prepared in order to carry an accurate quantification of cannabinoids, which need to be diluted in a high percentage of organic solvent (>50%) to avoid adsorption on the walls of the plate wells.

This method was validated based on SWGTOX Standard Practices. Parent drugs were quantitatively validated in blood and qualitatively validated in urine. Metabolites were qualitatively validated in both matrices. Verification of the absence of conversion during the extraction was one of the additional tests performed. Calibration model was determined using statistical analysis. Between-run accuracy was 93.7%-104.2% and precision (n=3) was 6.0%-17.6% for all the analytes. The ionization suppression/enhancement measurements were between 91.9%-110.0% with a maximal 10% CV. The dilution of the sample minimized ionization suppression or enhancement. Matrix effect was evaluated at low and high (n=3) concentrations using 10 lots of blood (antemortem and postmortem). Precision, accuracy and MRM ratios met acceptance criteria for all analytes. Other evaluations such as carryover, interference and dilution integrity also met acceptance criteria. Finally, proficiency testing samples (blood and urine) were analyzed: all the substances were detected with a 70-130% accuracy.

**Conclusion/Discussions:** We successfully developed and validated a high throughput quantitative and qualitative analytical method combining protein precipitation extraction and LC-MS/MS analysis. This method is currently used for routine analysis, with more than 2000 samples analyzed at this point. With this new development, the use of immunoassay was abandoned in our lab, and the number of GC-MS requests decreased significantly ( $\approx$  50%). Since the implantation of this method, our turnaround time decreased by half.

**Keywords:** Protein Precipitation, LC-MS/MS, Cannabinoids

## Detection of Neonatal Cannabis Exposure by Umbilical Cord Analysis

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**Background/Introduction:** Cannabis remains the most prevalent drug worldwide, with 128-234 million cannabis users between 15 and 64 years old in 2014, and this prevalence is expected to increase as a growing number of states and countries are now considering legalization. According to the 2014 National Survey on Drug Use and Health, 5.3% of pregnant women smoked marijuana in the past month in the USA. Despite these data, our understanding of short and long-term effects of prenatal exposure to cannabis is poor. Objective tools to detect in utero cannabis exposure are critical but scarce. Although umbilical cord is becoming a useful alternative matrix to detect in utero drug exposure, currently there are no data about its utility to detect cannabis exposure (i.e. metabolite profile, window of detection).

**Objective:** The objective of the present work was to develop and validate an analytical method for the determination of  $\Delta^9$ -tetrahydrocannabinol (THC) and its metabolites 11-hydroxyTHC (THC-OH), 11-nor-9-carboxyTHC (THCCOOH), 8- $\beta$ -11-dihydroxyTHC (THC-diOH), THC and THCCOOH glucuronides, and cannabidiol (CBD) in umbilical cord by Ultra-High Performance Liquid Chromatography-Tandem Mass Spectrometry (UHPLC-MSMS) with dual API/ESI ionization source (DUIS) LCMS-8030 (Shimadzu, Columbia, MD). The method was applied to 15 authentic samples from cannabis-exposed newborns, which matched meconium samples had tested positive for THC, its metabolites and/or CBD.

**Methods:** Umbilical cord samples ( $0.5 \pm 0.02$  g) were homogenized in methanol using a tissue homogenizer for 5 min. After centrifugation, the supernatant was evaporated to dryness, reconstituted in 3 mL of a mixture of 0.1% formic acid in water and 0.1% formic acid in acetonitrile (70:30, v/v) and subjected to mixed mode reversed phase and cation exchange solid-phase extraction. Chromatographic separation was performed on a Kinetex F5 column, 100 x 2.1 mm, 1.7  $\mu$ m (Phenomenex, Torrance, CA) at 40 °C, with a gradient of 0.1% formic acid in water and 0.1% formic acid in acetonitrile at a flow rate of 0.5 mL/min. The total run time was 14 min. Two transitions per analyte were monitored in multiple reaction monitoring (MRM) using DUIS source in positive mode.

**Results:** Method validation included linearity ( $n=5$ ; 1-20 ng/g for THC-Glucuronide, 1-200 ng/g for THCCOOH-Glucuronide, 7-200 ng/g for THCCOOH, THC and CBD, and 10-200 ng/g for THC-OH and THC-diOH), imprecision ( $n=15$ ; 4.1-15.1%), accuracy ( $n=15$ ; 87.5-111.4%), matrix effect ( $n=5$ ; from -54.8 to -5.8%, CV from 3.4 to 35.5%), extraction efficiency ( $n=5$ ; 25-45.6%), process efficiency ( $n=5$ ; 11.6-42.2%), limits of detection and quantification ( $n=5$ ; 1-10 ng/g), interferences (no endogenous/exogenous interferences), and 24 h auto-sampler stability (no loss detected). Validation parameters were studied at 2 concentrations, low (15 ng/g, except THC-Glucuronide 5 ng/g) and high (150 ng/g, except THC-Glucuronide 15 ng/g) quality control samples. Fourteen out of the 15 authentic umbilical cord specimens tested positive for cannabis. All these samples were positive only for THCCOOH-Glucuronide, with concentrations from 1.6 to 19.1 ng/g. None of the other metabolites nor THC or CBD were detected in these samples.

**Conclusion/Discussions:** We developed and validated a specific and sensitive method for the determination of THC, its metabolites, including THC-Glucuronide and THCCOOH-Glucuronide, and CBD in umbilical cord samples by UHPLC-MSMS. The analysis of authentic samples showed a good agreement umbilical cord – meconium (93.3% match). The predominant analytes in meconium samples were THCCOOH (3.9 – 117.8 ng/g) and diOHTHC (5.4 – 887.4 ng/g), and only four samples tested positive for THC-COOH-Glucuronide (19.4 – 190.2 ng/g). In the case of umbilical cord, THCCOOH-Glucuronide was the only metabolite detected at concentrations below 20 ng/g. CBD was detected in meconium (9.5 – 335.3 ng/g) but it was not detected in umbilical cord samples.

**Keywords:** Cannabis, Umbilical Cord, Meconium

## Determination of twelve commonly found compounds in DUI cases in whole blood using fully automated supported liquid extraction and UHPLC-MS/MS

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**Background/Introduction:** The Department of Forensic Sciences annually receives approximately 6000 whole blood samples from driving under the influence (DUI) cases. In about 90% of these samples compounds known to give impairment were found, and on average three compounds were found in each sample. The most frequently found compounds, other than ethanol, were THC, amphetamine, methamphetamine, MDMA, clonazepam, diazepam, nordiazepam, oxazepam, alprazolam, nitrazepam, morphine and codeine. These drugs were confirmed and quantified by four different methods; however, several of these compounds were often found in the same sample. Thus combining the most frequently detected drugs in the same method would reduce the labour work, chemicals, instrument workload and possibly the response time.

**Objective:** To develop and validate an automated high-throughput method including the relevant compounds on 96-well plates on a Tecan robot followed by UHPLC-MS/MS.

**Methods:** To an aliquot of 100  $\mu$ L whole blood, internal standard, water, TritonX-100 and pH 9.3 buffer were added before application to the supported liquid extraction (SLE) plate. The compounds were eluted with 2x700  $\mu$ L ethyl acetate+heptane (4+1). 0.01% HNO<sub>3</sub> in MeOH was added before evaporation and reconstitution in 100  $\mu$ L MeOH+H<sub>2</sub>O (10+90). Gradient elution was performed on a C18 column (50x2.1 mm, 1.7  $\mu$ m) with MeOH and 5 mM pH 10.2 ammonium formate. The run time was 4.5 min and 1  $\mu$ l was injected on an Aquity UPLC I-Class system with a Xevo TQS tandem-quadrupole mass spectrometer (Waters). <sup>13</sup>C labelled internal standards were used for all the analytes except for alprazolam and morphine, which had deuterated analogs. Quantification was carried out with calibrators without whole blood matrix.

**Results:** The calibration curves ( $r^2 \geq 0.996$ ) covered the concentration ranges found in the DUI samples: 0.00063-0.13 mg/L THC, 0.027-4.1 mg/L amphetamine, 0.030-4.5 mg/L methamphetamine, 0.039-5.8 mg/L MDMA, 0.0013-0.51 mg/L clonazepam, 0.057-4.3 mg/L diazepam, 0.054-4.1 mg/L nordiazepam, 0.17-8.6 mg/L oxazepam, 0.0030-0.93 mg/L alprazolam, 0.014-0.84 mg/L nitrazepam, 0.086-1.7 mg/L morphine, and 0.090-1.8 mg/L codeine, respectively. The method showed satisfactory accuracy  $\leq \pm 10\%$  with the exception of nordiazepam (16.4%), nitrazepam (19%) and morphine (14%) when compared to the existing methods and external quality control samples (z-score -2.1 to 1.7, n=18 samples). The precision, estimated as the relative standard deviation of the concentration difference between results from the current method versus the quantitative results obtained with the former methods for authentic whole blood samples, was in the range 3.5 to 7.7% RSD.

**Conclusion/Discussions:** The method is used in routine forensic analysis and has shown to be robust and satisfactory for forensic analysis of DUI and postmortem whole blood samples.

**Keywords:** Driving Under the Influence of Drugs, Supported Liquid Extraction (SLE), Ultra High Performance Liquid Chromatography-Tandem Mass Spectrometry (UHPLC-MS/MS)

## Practical Application of a Screening Procedure for More than 3000 Compounds in Whole Blood by UHPLC-TOF-MS<sup>E</sup> – a 10 Months Experience

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**Background/Introduction:** LC-HR-MS as an integral part of the comprehensive screening approach is a valuable tool in forensic toxicology. The screening can be used to supplement findings from other instruments and ensure coverage of less frequently occurring targets, such as NPS and pesticides. Using data-independent acquisition allows for comparing the analytical data with large target libraries. A prerequisite for a useful LC-HR-MS forensic screening method is that the data analysis time should be suitable for high-throughput screening workflows.

**Objective:** The objective was to develop a data analysis procedure that combines a comprehensive targeted, semi-targeted and non-targeted drug screening within a reasonable data-analysis time.

**Methods:** The analytical method consisted of a protein precipitation on a fully-automated robotic TECAN platform, 3  $\mu$ L was injected onto the LC on a 15 minutes gradient elution program, and data acquisition was performed in positive mode, with data-independent acquisition mode (MS<sup>E</sup>). The instrument was controlled and data was analyzed with UNIFI 1.8. The compound library consisted of 3,000 data entries. Approximately half of the library entries had expected retention time (RT), and exact mass of one to four fragment ions. The other half of the library consisted of molecular structures, of which 100 NPS had expected fragment ions from online data sources, such as [www.highresnps.com](http://www.highresnps.com). Known endogenous compounds and contaminant masses were removed from further analysis.

The basic criteria for an identification was mass error <3 mDa, RT error <0.5 min and signal > 200 counts. The data analysis was divided into five steps. Step 1-4 were applied to all biological samples in our routine screening, and step 5 was reserved for special cases, as it required longer data analysis time and expert knowledge.

Step 1 was targeted identifications of 400 important targets in forensic toxicology, where any identification was inspected by the analyst. Around 300 of these compounds were present in three reference standard mixes running with each batch, allowing for a narrowed RT error tolerance (<0.03 min). In step 2 the remaining targeted identifications with at least one expected fragment ion were inspected. In step 3 semi-targeted identification of the 100 NPS were inspected, when at least one of the expected fragment ions were observed. In step 4 semi-targeted identifications amongst the remaining 1,400 compounds was inspected, when the observed fragmentation had been matched in-silico. Step 5 was the non-targeted screening, which was performed for unknown peaks, but narrowed down according to previously published work (Mollerup/Dalsgaard/Mardal/Linnet, 2017). Each selected unknown peak was matched against a 400,000 compounds structure library (ChemIDPlus), and positive hits were evaluated based on isotope pattern and in-silico fragment matching.

**Results:** From April 2016 to February 2017 more than 300 different compounds were identified as true positives across 2,339 whole blood samples. The total number of findings was 13,037 (average: n= 5.6 per case). Caffeine was identified in 2,145 cases (92%) and nicotine in 985 cases (42%). Most identified drugs of abuse were cocaine (38%, n= 896), amphetamine (16%, n=370) and methadone (12%, n=270). The average data analysis time per biological sample was 2 minutes, and step 1-3 can be performed by all forensic chemists taking part in this screening, while step 4 and 5 require knowledge about fragmentation rules.

**Conclusion/Discussions:** By dividing the data analysis into steps and selecting relevant identifications, the data analysis is simplified and focused. With this approach, it is possible to screen for a wide range of compounds within a reasonable timeframe.

**Keywords:** Toxicological Screening, UHPLC-TOF-MS, High Resolution Mass Spectrometry

## Implementation of an Oral Fluid Drug Testing Program in Alabama: An Evaluation of Draeger DT5000, Alere DDS2, and Randox MultiSTAT with LC/MS/MS Confirmation

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**Background/Introduction:** Oral fluid (OF) drug testing offers a rapid, non-invasive collection requiring no medical personnel. An OF specimen can be taken in close time proximity to a traffic stop and the presence of parent drugs in OF reflects recent drug use. These advantages make OF testing attractive to DUI/D prevention and roadside drug screening. With the continued evolution of OF testing and drug screening devices, it is important to evaluate this technology and these devices for fit for purpose and use prior to implementation of a statewide OF testing program.

**Objective:** The Alabama Department of Forensic Sciences (ADFS) and the Alabama Drug Recognition Expert (DRE) program conducted a proof of principle study set forth to validate the use of OF screening in the field by officers and OF confirmation testing at ADFS.

**Methods:** Approximately 100 drug users from the Clara White Mission in Jacksonville, FL volunteered for this study. The study was conducted in two cohorts: June 2016 and May 2017. The following drugs/drug classes were compared: cocaine, cannabinoids, opioids, methadone, benzodiazepines, methamphetamine, and amphetamine. Randox screens for 21 targets simultaneously, but only the aforementioned targets were assessed. We investigated the accuracy, sensitivity, specificity, positive and negative predictive values, false negative and positive rates of the following OF screening devices/instruments: Draeger DT5000, Alere DDS2, and Randox Evidence MultiSTAT. The MultiSTAT was only evaluated in the May 2017 cohort (n=50). We collected OF, blood, and urine confirmation specimens that were subsequently screened by immunoassay and quantitated by LC/MS/MS. OF samples collected in Quantisal collection devices were extracted using dispersive pipette extraction (DPX) tips and analyzed on an Agilent 6460 Triple Quad. DRE evaluations were performed to assess subject behavior and impairment.

**Results:** Time to test completion was less than 10, 5, and 17 minutes for Draeger, Alere, and Randox devices, respectively. All instruments demonstrated >90% for accuracy, sensitivity, specificity, positive and negative predictive values for cocaine, opiates, methadone, and cannabinoids with the following exceptions. The Alere DDS2 and Draeger DT5000 had sensitivities less than 90% for opioids and cocaine, respectively. The Randox MultiSTAT had inferior methadone and cannabinoid sensitivity. The devices displayed overall poor sensitivity to the benzodiazepine drug class (50-75%). The MultiSTAT was the exception demonstrating excellent sensitivity (>90%). There were only two methamphetamine positive subjects and no amphetamine positives. Approximately 45% and 40% of participants tested positive for cannabinoids and cocaine, respectively. Median oral/blood (OF:BL) drug concentration ratios were consistent with those published in the literature with low ratios (<1.0) for benzodiazepines and higher ratios (>1.0) for other targets evaluated. However, OF:BL ratios were heavily impacted by recent use and oral cavity contamination.

**Conclusion/Discussions:** This is the first study to evaluate the Randox Evidence MultiSTAT as an OF screening instrument in the field. The three OF screening instruments evaluated proved to be fit for purpose with comparable performance. However, advantages and potential areas of improvement were noted. The Randox MultiSTAT outperformed other devices in benzodiazepine sensitivity by using two antibodies tailored to this drug class. On the other hand, the MultiSTAT struggled with cannabinoid sensitivity, most likely due to using carboxy-THC as their immunoassay target. However, their software allows for lowering of the cutoff threshold which improves performance for this drug class. The Draeger DT5000 offers the advantage of testing for methadone over the Alere DDS2 (as does the Multi-stat). The Alere DDS2 received excellent officer feedback for ease of use and portability in the field. In summary, the OF drug screening instruments evaluated proved to be fit for purpose with comparable performance. Roadside OF screening combined with OF and/or blood confirmation testing offers law enforcement tools and services to improve DUI/D detection and improve highway safety. In particular, these devices are great tools to complement DRE programs by enhancing the probability of obtaining blood (or OF) search warrants in suspected DUI/D cases, especially in rural areas lacking the presence of DREs.

**Keywords:** Oral Fluid, Draeger DT500, Alere DDS2, Randox MultiSTAT

## Quantification of Pheniramine in Alternate Matrices (Hair, Nails and Saliva): Comparison with Corresponding Concentration in Conventional Matrices (Blood and Urine) in Drug Abusers

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**Background/Introduction:** Pheniramine is a commonly available over-the-counter anti-histaminic drug in Pakistan. Owing to its low cost and ease of availability, its abuse has been increasing day by day.

**Objective:** Usual matrices for pheniramine analysis are blood and urine but relative concentration in alternate matrices like hair, nails and saliva are not reported. In this manuscript, pheniramine was quantified in both usual and alternate specimens collected from ten drug abusers, ages ranged 16-50 years, on their consent.

**Methods:** Blood, urine and saliva specimens were used as such without any pre-treatment or dilution. Hair and nails specimens were washed with dichloromethane for 5 minutes. Wash extracts were also analyzed for the presence of external contaminations. Washed hair and nails specimens were dried, weighed (50mg), cut into small pieces and then incubated with 2ml of 2N sodium hydroxide solution at room temperature for 24h. Negative hair and nails specimens were also digested by same procedure. The pH of hair and nails specimens was adjusted to 9 with 6N hydrochloric acid before analysis. Gas chromatograph with inert mass spectral detection in the positive electron impact mode with DB-5MS (30m x 0.25mm x 0.25 $\mu$ m) capillary column was employed for analysis. Pheniramine was detected in all matrices during basic drug screening performed on GC-MS in full scan mode following liquid-liquid extraction. For confirmation and quantification purpose, pheniramine was extracted using Hypersep verify CX solid phase extraction (SPE) cartridges while MS detector was operated in SIM mode using the ions of m/z 169.1, 168.1, 167.1 for pheniramine while ions of m/z 260 and 455 were monitored for nalorphine (internal standard).

**Results:** Pheniramine was quantified in blood, urine, hair, nails and saliva specimens of ten drug abusers. Concentration differences between these matrices for pheniramine were evaluated with selected case histories included. No significant matrix effects were found in the concentrations of pheniramine in alternate matrices quantified with a calibration curve constructed in a blood matrix. After determining that alternate matrices can be accurately measured with blood calibrators, a comparison was made between blood, urine, hair, nails and saliva specimens for pheniramine. Moreover, pheniramine was not detected in the wash extracts of hair and nails specimens that suggested ingestion of the drug rather than external contamination.

**Conclusion/Discussions:** Analysis of drugs in alternative or unconventional matrices is of much significance due to easy and non-invasive sample collection, difficulty in adulteration and present larger detection windows than for conventional matrices (blood, urine). The comparative concentration of drugs like pheniramine in both conventional and alternate matrices is of significance for toxicological interpretation.

**Keywords:** Pheniramine, Gas Chromatography, Alternative Matrices

## Are Nails an Alternative to Air for the Assessment of Drugs of Abuse Consumption? A Correlation study

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**Background/Introduction:** Hair and nails accumulate compounds over time and allow retrospective investigation of past consumption. Owing to their long detection window (weeks/months), analysis of these matrices can provide information complementary to blood and urine analysis or can be used as standalone when compounds have already been eliminated from the body. So far, research has primarily focused on the detection of drugs of abuse (DOA) in hair, while studies in nails are scarce.

**Objective:** To assess concentrations of DOA and their metabolites in hair, finger- and toenails collected from the same individuals to evaluate differences and correlations between the matrices.

**Methods:** A total of 26 hair, 24 fingernail, and 16 toenail samples were collected from patients treated for substance use disorders at a Belgian psychiatric treatment center. Samples were analyzed by a validated liquid chromatography-tandem mass spectrometry (LC-MS/MS) method able to simultaneously detect nine DOA together with their metabolites: amphetamine, methamphetamine, 3,4-methylenedioxymethamphetamine (MDMA), 3,4-methylenedioxyethylamphetamine (MDEA), morphine, codeine, 6-monoacetylmorphine, methadone, ethylene dimethyldiphenyl pyrrolidine (EDDP), cocaine (COC), benzoylecgonine (BE), and ecgonine methyl ester (EME). The lower limit of quantification (LLOQ) of the method was 50 pg/mg for all DOA in hair and nails. Statistical evaluation of associations was performed by calculating Pearson correlation coefficients ( $r$ ).

**Results:** DOA concentrations between hair and fingernails and between hair and toenails were positively correlated, although this was not significant for all compounds. Likewise, there was a positive correlation between the DOA levels in finger- and toenails. For example, COC levels in hair and nails were significantly and positively correlated ( $p$ -value  $< 0.05$ ,  $r = 0.77$  and  $r = 0.79$ , for finger- and toenails, respectively), and there was a positive correlation between finger- and toenails ( $p$ -value  $< 0.05$ ,  $r = 0.72$ ). Concentrations of DOA observed in nails were higher compared to hair and were larger in toenails compared to fingernails. Ratios between parent compounds and their respective metabolites were assessed to evaluate differences in profiles between hair and nails. In general, these ratios were different between hair, fingernails, and toenails. For example, the ratio BE/COC in hair was on average 0.34, while in fingernails it was 1.75, and in toenails 2.36.

**Conclusion/Discussions:** Higher concentrations in nails compared to hair can be attributed to the slower growth rate of nails (0.3 cm/month for fingernails and 0.1 cm/month for toenails compared to 1 cm/month for head hair), resulting in increased accumulation of compounds in nails. As a result, cut-off values established for hair are not valid for nails. Due to the higher concentrations found in nails compared to hair, nail analysis can be valuable when low concentrations of substances are expected. Differences in parent compound to metabolite ratios can indicate important variances in accumulation of compounds between hair and nails. In summary, nails are a useful alternative to hair for monitoring long-term DOA consumption in cases where hair is not available. However, care should be taken regarding the variability in the accumulation of compounds between the matrices, and further studies are needed to acquire an in-depth knowledge of nail analysis and to define cut-off values for the analysis of DOA in nails, as they exist for hair.

**Keywords:** Hair, Nail, Drug of Abuse

## Prevalence of Synthetic Cathinones Identified by Hair Analysis in Paris and its Suburbs Over Five Years

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**Background/Introduction:** New psychoactive substances (NPS) have emerged in a threatening way in the last decades. Their identification is still a challenge because it requires highly sensitive and specific analytical methods like LC-MS/MS. The use of hair as an alternative matrix for prevalence studies is crucial, since it offers a wide detection window and allows evaluating occasional or repeated drug exposure. However, this kind of studies is still scarce. Most of the available data have been derived from self-reported use or case reports.

**Objective:** Evaluate the prevalence of NPS, primarily synthetic cathinones, consumed in Paris and its suburbs between 2012 and 2016, and identified by hair analysis using LC-MS/MS.

**Methods:** 328 hair samples were analyzed in different circumstances (addict followed-up, intoxications, other clinical or forensic investigations). 200 analytes including conventional drugs of abuse (cocaine, amphetamines, opiates) and 31 NPS were screened. 20 mg of hair was washed twice with dichloromethane, and water, dried at room temperature and powdered. It was hydrolyzed by an acidic buffer and extracted twice in alkaline condition using a mixture of hexane/ethyl acetate and chloroforme/isopropanol. 10 µL of the final residue was injected into the system. Separation was performed in a Hypersil PFP Gold column (100mmx2.1mm, 1.9µm) with the gradient of ammonium formate 2mM in formic acid and acetonitrile. The detection used a Triple Quad TSQ Vantage (ThermoFisher®) in MRM mode. The method runs 14 min and was validated according to the European Medicines Agency's guidelines.

**Results:** 96 cases were tested positive for NPS. Their prevalence was 29%, similar to that of amphetamines (25%) or opiates (33%), and was a quite lower than that of cocaine (37%). Consumers were predominantly male (72.6%). The average age was 37 years, but the highest number of cases was observed in 20-30 years-old group, with 8.3% of users being under 20 years. 4-MEC was the most frequently detected cathinone (17 cases) followed by mephedrone/3MMC (15), methylone (11) and MDPV (7). Others detected cathinones included butylone (3), methcathinone (3),  $\alpha$ -PVP (2), cathinone (1) and cathine (1). For piperazines, TFMPP (2),  $\alpha$ -PVP (2) and m-CPP (1) were found. 6-APDB was found in 2 cases, while methoxetamine, diphenidine and 4-fluoroamphetamine (4-FA) were identified in single cases. Users consumed NPS associated to cocaine, amphetamines or opiates in the same period in 64, 55, and 28 % of cases respectively. They consumed two or more NPS in 35% of cases while only one NPS was used without any conventional drugs in 9 % of cases. Ketamine (KET), dextromethorphan (DXM) and methylphenidate (MPH) still attract NPS users, and were found in 71, 17 and 5 cases respectively. Therapeutic use of KET was identified in 9 cases while a prescription of MPH was validated in one case. Cocaine was positive in 68 % of KET positive cases (a combination called CK). 71% of DXM positive cases were associated with others drugs of abuse. Identified NPS cases were increasing over time, probably related to a real increase in their consumption. Segmental hair analysis performed in 79% of cases showed a wide range of concentrations, ranging between 0.002 ng/mg and 169 ng/mg, with higher values in addict follow-up

**Conclusion/Discussions:** Prevalence of synthetic cathinones use in Paris was similar to that of conventional drug of abuse, and 4-MEC seems to be the most consumed NPS. The phenomenon of NPS use was observed particularly among young people, who were predominantly poly drugs abusers. NPS identification should be included in routine analysis, and more prevalence studies, mainly by hair analysis are needed to investigate this scourge that represents a real public health concern nowadays.

**Keywords:** New Psychoactive Substances (NPS), Synthetic Cathinones, Hair



## Determination of AB-CHMINACA and its Metabolites in Human Hair and their Deposition in Hair of Abusers

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**Background/Introduction:** Despite global efforts to control the abuse of synthetic cannabinoids, the continuous turnover from the market impedes regulation, endangering public health. N-[(1S)-1-(aminocarbonyl)-2-methylpropyl]-1-(cyclohexylmethyl)-1H-indazole-3-carboxamide (AB-CHMINACA) is the most popular synthetic cannabinoid in South Korea since its introduction in 2014. Nonetheless, few studies have been carried out on AB-CHMINACA, its metabolites, and their deposition in human hair.

**Objective:** The purpose of this study was to develop and validate an analytical method for detection of AB-CHMINACA and its six metabolites (N-[[1-(cyclohexylmethyl)-1H-indazol-3-yl]carbonyl]-L-valine (M2), 1-((4-hydroxycyclohexyl)methyl)-1H-indazole-3-carboxyl-L-valine (M3A), 1-(cyclohexylmethyl)-1H-indazole-3-carboxylic acid (M4), 1-((4-hydroxycyclohexyl)methyl)-1H-indazole-3-carboxylic acid (M5A), 4-amino-3-(1-(cyclohexylmethyl)-1H-indazole-3-carboxamido)-2-methyl-4-oxobutanoic acid (M6), 2-(1-(cyclohexyl-methyl)-1H-indazole-3-carboxamido)-3-methylsuccinic acid (M7)) in hair using a liquid chromatography tandem mass spectrometry (LC-MS/MS) system for forensic applications.

**Methods:** Hair strands (ca. 10 mg) were washed with 2 mL each of methanol, distilled water, and methanol, and cut finely into 1 - 2 mm pieces with scissors. AB-CHMINACA and its metabolites in the hair were extracted by incubating with methanol at 38 °C stirring for 20 h. JWH-018-d<sub>5</sub> and JWH-018 N-5-OH M-d<sub>5</sub> were used as internal standards. The extracts were collected and evaporated under nitrogen gas. The residues were reconstituted in mobile phases and injected to LC-MS/MS system (Agilent 1290 infinity UHPLC system-AB SCIEX QTRAP® 5500).

The chromatographic separation was performed in a Zorbax Eclipse Plus C18 (RRHD 2.1 x 100 mm, 1.8 μm, Agilent Technologies) with Zorbax Eclipse Plus C18 guard column (2.1 mm, 1.8 μm UHPLC guard column, Agilent Technologies). The mobile phase was a gradient prepared from 2 mM ammonium formate/0.2 % (v/v) formic acid in water (A) and 2 mM ammonium formate/0.2 % (v/v) formic acid in methanol (B). The initial mobile phase, 10% B, was maintained for 0.5 min and then rapidly increased to 65% B for 0.5 min. Then, the composition of B was increased gradually to 95% for 9 min and maintained for 1 min. Finally, the initial conditions were restored for 4 min. The flow rate was 300 μL/min, and the total run time was 15 min.

The multiple reaction monitoring (MRM) data were achieved with electrospray ionization in positive ion mode. Two MRM transitions were selected for each analyte.

The established method was applied to 37 authentic hair samples from suspected synthetic cannabinoid users.

**Results:** The limits of detection and quantification ranged from 0.5 to 10 pg/mg and 2 to 50 pg/mg, respectively. Good linearity was achieved within the range of 5 to 1000 pg/mg or 10 to 1000 pg/mg depending on the analyte. Intra- and inter-assay precision and accuracy values were below 15%. No significant variation was observed using different sources of hair matrices (*n*=5). AB-CHMINACA and its two hydroxylated metabolites, AB-CHMINACA M2 and AB-CHMINACA M4, were detected in 37 hair specimens among 140 suspected synthetic cannabinoids users' hairs. The mean concentration of the parent drug (299.2 pg/mg) was much higher than those of its metabolites, with mean AB-CHMINACA M2 concentrations (21.2 pg/mg) greater than mean AB-CHMINACA M4 concentrations (10.5 pg/mg) in all samples. No other metabolites were detected in the samples

**Conclusion/Discussions:** This research is the first study examining the deposition of AB-CHMINACA and its six metabolites in hair from 37 authentic hair samples collected from suspected cannabinoid abusers. A selective, accurate, and reproducible quantitative LC-MS/MS method for the simultaneous analysis of AB-CHMINACA and its metabolites in hair was developed, fully validated and successfully applied to the analysis of 37 authentic hair samples. The results suggest that AB-CHMINACA is metabolized into AB-CHMINACA M2 and M4 by amidase in hair. Therefore, the presence of AB-CHMINACA M2 and/or M4 could rule out the possibility of passive contamination for the consumption of AB-CHMINACA in hair.

**Keywords:** AB-CHMINACA, Hair Analysis, Synthetic Cannabinoids

## The Specification and Evaluation of an Oral Fluid Drug Screening Device for Use in Roads Policing in Ireland

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**Background/Introduction:** Driving under the influence of drugs (DUID) is an increasing problem for roads policing. As concluded by the ROSITA and DRUID studies there is a need for roadside drug testing and authorities need quick and reliable tools to detect those who may be driving under the influence of an intoxicant. Oral fluid is an easily accessible, non-invasive sample that is relatively quick to collect and can contain measurable amounts of a recently used drug. For this reason many point of collection drug screening devices utilize oral fluid. The Medical Bureau of Road Safety (MBRS) devised a specification to be used for evaluation of an oral fluid drug testing system with the view to choosing a device for drug screening in drivers. The Drager DrugTest 5000 was evaluated using the developed specification which considered the recommendations of ROSITA and DRUID.

**Objective:** To develop a specification necessary for an oral fluid screening system to be suitable for road traffic use and then use this specification to evaluate the suitability of the Drager DrugTest 5000 for this purpose.

**Methods:** Our specification outlined that the device must be able to identify 4 drug classes; Cannabis, Benzodiazepines, Cocaine and Opiates. Evaluation was conducted under the specifications five main headings: end user fitness for purpose (field trial), technical requirements, reader/analyser assessment, service and support and ultimate cost. Each main heading (except for cost) had a maximum achievable score. Most assessment was performed by desktop review of material submitted by the manufacturer, however technical requirements and analyser assessment were performed through laboratory based methods. On the spot oral fluid collection from donors was used for elements not related to drug testing e.g., collection volumes and times. For testing specific drug concentrations, genuine oral fluid was collected using a salivette (Sarstedt). This oral fluid was confirmed to be drug free before spiking at the appropriate concentration. Specimen collection was timed and collection volume was assessed by gravimetric analysis. The weight of 1ml of oral fluid was established for each donor collection and using this the volume of collection was estimated based on the difference between pre and post collector weight.

Sensitivity, specificity and accuracy for each of the four drug classes was performed using spiked oral fluid which was assessed based on the number of true positive and true negative results given over 15 tests at 60% and 140% of the drug cut-off. To do this human oral fluid from multiple donors was pooled and subjected to three freeze/thaw/centrifuge cycles with transfer of fluid to a fresh tube each time to avoid particulate matter. Spiked oral fluid samples were extracted by either SPE or Liquid-Liquid extraction and levels verified by validated GC-MS/MS or LC-MS/MS methods. A minimum of 80% for sensitivity, specificity and accuracy was required in order for devices to pass. A field trial and questionnaire with members of the national policing service formed part of the end user fitness for purpose assessment. The field trials objective was to evaluate the practical aspects of the systems use by the police. This field trial was not conducted in a real traffic scenario and did not involve the collection of specimens for scientific evaluation.

**Results:** The device satisfactorily detected Cannabis (THC, cut-off 10ng/ml), Benzodiazepines (Diazepam, cut-off 15ng/ml), Cocaine (Cocaine, cut-off 20ng/ml) and Opiates (Morphine, cut-off 20ng/ml). Results for sensitivity, specificity and accuracy are outlined in the table below. Specimen collection was timed during a normal oral fluid collection using the device. Average specimen collection time across 15 donations was 56sec and all donations were within the 5min limit set out in the specification. Average collection volume was 267.05µl and did not vary by greater than 20%. All volumes collected across 15 donations were less than the 500µl limit set out in the specification. The police field trial was performed using a mock checkpoint in light, dark, wet and dry conditions. The device operated in these tested conditions. Feedback from the field trial questionnaire contributed to the overall scoring for the device and incorporated questions to evaluate the end user satisfaction.

	Cannabis	Benzodiazepines	Cocaine	Opiates
Sensitivity	93%	80%	100%	93%
Specificity	100%	100%	100%	100%
Accuracy	97%	90%	100%	97%

**Conclusion/Discussion:** A specification was developed identifying the essential properties of an oral fluid drug screening device for use in roads policing in Ireland. The Drager DrugTest 5000 was evaluated using this specification and met all the criteria satisfactorily.

**Keywords:** Oral Fluid, DUID, DrugTest 5000

**LC-MS/MS determination of cannabis and metabolites in nails and hair. Analysis of real specimens from cannabis users**

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**Background/Introduction:** According to the 2016 World Drug Report, cannabis remains the most prevalent illicit drug worldwide, with an estimated 183 million people having used this drug in 2014. Keratinized matrices such as hair are widely used for workplace drug testing or in court cases mainly due to the long window of detection, allowing detection of drug use several months after any drug use. Nails also possess a long window of detection, and may be used when hair is not available. However, the use of this matrix for toxicological analysis is still at an early stage, and more studies are necessary to evaluate its usefulness and the interpretation of any results obtained.

**Objective:** The objectives of the present work were a) to develop and validate a LC-MS/MS method for the determination of  $\Delta^9$ -tetrahydrocannabinol (THC) and its metabolites 11-hydroxyTHC (THC-OH), 11-nor-9-carboxyTHC (THC-COOH), 8- $\beta$ -11-dihydroxy-THC (THC-diOH), and cannabidiol (CBD) and cannabinol (CBN) in nails and hair; b) to apply the methods to authentic specimens from cannabis users to compare cannabis incorporation into these keratinized matrices.

**Methods:** : 30 mg of nails or 50 mg of hair were washed with 5 or 3 cycles of dichloromethane for 2 min, respectively, and pulverized in a ball mill. After addition of the internal standard (IStd) mixture, samples were incubated with 1 mL NaOH 1 M for 15 min at 95°C in a bath, and subsequently extracted with Oasis MAX cartridges. For hair samples, a second extraction using Oasis MCX cartridges was necessary. Separation was performed using a Kinetex C18 column (50 x 2.1 mm, 2.6  $\mu$ m), and a gradient with 0.1% formic acid and acetonitrile at 35°C. Total run time was 10 min. Fingernails and/or toenails specimens from 4 chronic cannabis users (one of them abstinent since the month previous to sample collection), and from 3 control volunteers were analyzed. Hair specimens were available for 2 chronic users and for all the control volunteers.

**Results:** Method validation in nails and hair was performed following SWGTOX guidelines, and included selectivity (no endogenous or exogenous interferences), limits of detection and quantification (20-100 pg/mg), linearity (n=5, 20-20000 pg/mg in nails and 40-20000 pg/mg in hair for THC and CBN, and 100-20000 ng/mg for the other analytes), imprecision (n=15; from 0% to 6.4%, and from 1.1% to 6.9% in nails and hair, respectively) and accuracy (n=15; from 99.5% to 109.1%, and from 101% to 109.8%); matrix effect (from -54.1% to -23.4%, and from -74.4% to 46.1%), extraction efficiency (from 21.5% to 84.5%, and from 12.9% to 75.9%) and process efficiency (from 16.5% to 49.1%, and from 5.6% to 90%), and autosampler stability for 72 h (%loss <15%). Fingernails from the 4 chronic cannabis users were positive for THC (133.0-24569.1 pg/mg), CBD (179.7-12945.6 pg/mg) and CBN (42.7-2486.6 pg/mg), and THC-COOH was detected in one of them (<LOQ). Toenails were positive for the same analytes, but at similar or lower concentrations (THC=52.7-1009.2 pg/mg, CBD=22.4-261.3 pg/mg, and CBN=20.3-120.6 pg/mg, THC-COOH<LOQ). Hair concentrations in the 2 specimens from the chronic cannabis users were 123.9-294.6 pg/mg for THC, <LOQ-856.1 pg/mg for CBD, and <LOQ-86 pg/mg for CBN. All control samples were negative.

**Conclusion/Discussions:** THC, CBD and CBN were detected in the analyzed fingernails, toenails and hair of chronic cannabis users. However, fingernail analysis provided a greater sensitivity, as concentrations were 10 to 40-fold higher than to those observed in hair. As expected, metabolites incorporation into these matrices was very low, as only traces of THC-COOH were identified in the nails of one chronic user.

**Keywords:** Cannabis, Nail, Hair

## The Postmortem Distribution of Ranolazine in a Case of Suspected Homicidal Poisoning

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**Introduction/Background:** Ranolazine (Ranexa®), available in 500 or 1000 mg extended release tablets, is a piperazine acetamide derivative that has been in use as an antianginal agent since 2005. Reported adverse effects include: dizziness, nausea, headache, constipation, hypoglycemia, hepatotoxicity, confusion, hallucinations, dysarthria, ataxia, hypotension, seizures and cardiac arrhythmia. The specimens in this case were received from a coroner requesting identification of a large unknown peak that was detected by the coroner's toxicology laboratory in a GCMS basic drug screen.

**Objective:** The purpose of this case presentation is to report the concentration of ranolazine in blood, vitreous, kidney, brain and liver in a death attributed to a combined drug toxicity. There is limited information available regarding concentrations of ranolazine in the literature.

**Methods:** Initial identification of the unknown compound was accomplished using a basic extract of the blood and full scan LCMSMS analysis. Molecular weight and product ion spectra of the unknown large peak was identified. The method was validated using SWGTOX criteria with SPE and detection by LCMSMS. Calibration was performed in blood using six calibrators with three positive controls included with each batch. The sample size was 100 uL and the linear range for ranolazine was from 25 to 750 ng/mL, however the highest calibrator used was 500 ng/mL. Ranolazine was stable in extracted blood and urine on the LC auto-sampler for up to 72 hours. Ranolazine showed significant ion suppression in both blood and urine which was normalized by the d3 ranolazine internal standard to 3% or less. Dilution integrity was maintained over 4 different dilutions. Interference was tested with 145 common drugs and metabolites at 1000 and 5000 ng/mL and no interference was detected. Ranolazine carryover was not detected up to 2000 ng/mL. The method of standard additions was used to quantify ranolazine in non-blood samples. The tissue samples were prepared with an initial dilution of four with 0.1 M phosphate buffer pH 6.0 in order to accomplish homogenization. Dilution prior to extraction for analysis was accomplished using blank porcine blood: saline in a ratio of 1:1. Dilution amounts varied depending on the ranolazine concentration.

**Results:** Refer to tables one and two below. Additional toxicology results in ng/mL from the femoral blood included: tramadol and O-desmethyltramadol at 445 and 40, trazodone and mCPP at 317 and 39, cyclobenzaprine at 170 and duloxetine at 400. All of these drugs were also positive in the gastric contents.

	Bias	Standard Deviation	Coefficient of Variation %CV
Ranolazine LOD/LLOQ at 25 ng/mL	6%	1.68	6.3%
Precision at 51 ng/mL	- 5%	2.9	6.1%
Precision at 382 ng/mL	-13%	15	4.5%
Precision at 680 ng/mL	14%	30	4.0%
Average of all precision	-1.1%	16	5.0%

Table One – Ranolazine Validation Data in Blood, 5 replicates over 5 days

	Femoral Blood ng/mL	Liver ng/g	Brain ng/g	Vitreous ng/mL	Kidney ng/g	Gastric ng/mL	Bile ng/mL
Ranolazine	11,900	68,160	12,633	5,766	21,637	Positive	Positive

Table Two – Case Data

**Conclusion/Discussion:** Ranolazine was quantified by this method in all submitted specimens with the exception of gastric and bile. Repeat analysis of gastric and bile at various dilutions produced positive but widely variant quantitative results. The average value of three different dilutions plus standard additions was within 5 to 10% of each individual value. The blood was measured by dilution only. The average value measured in the blood was within 5% of each individual value. The coroner signed the case as acute combined drug toxicity, manner undetermined. In addition to ranolazine this method was also simultaneously validated for the quantification of tramadol, O-desmethyltramadol, trazodone, m-chlorophenylpiperazine (mCPP) and tapentadol.

**Keywords:** Ranolazine, Tissue Quantification, LCMSMS

## Postmortem Estimation of Metabolizer Type: Combining Quantification and Characterization of CYP Enzymes by Proteomics

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**Background/Introduction:** Differences in xenobiotic metabolism rate between individuals can lead to fatal consequences. For example, slow metabolizers have died, even when taking therapeutic dosages, due to their inability to clear the drug resulting in accumulation of the drug to toxic levels. Two main factors determine metabolic capacity: genetics and expression. Known genetic polymorphisms (mutations) of the key metabolizing enzyme family, cytochrome P450s (CYPs), are responsible for a range of metabolizing efficiencies. Simultaneously, differences in biological factors such as drug use, disease, age and gender lead to varying CYP expression (liver concentration) levels which also impact metabolic capacity. Current forensics practice assesses metabolic capacity only through CYP genotyping, but a poor genotype to phenotype correlation reduces the usefulness of this tool.

**Objective:** The aim of this research is to develop a more accurate postmortem estimation of metabolic capacity that encompasses both genetic assessment and quantitation of the protein expression level. This initial work focuses on CYP 2D6 and CYP 3A4.

**Methods:** A liver sample is collected during the autopsy and minced into pieces of a few mm length. The liver pieces are then mechanically blended with a 0.25 M sucrose, 150 mM NaCl, 50 mM Tris (pH 7.0) solution (1:10 mass ratio) and protease inhibitors to generate a liver homogenate. Ultracentrifugation (100 000 x g, 4°C, 1 h) is used to isolate the liver microsomal cell fraction. This microsomal pellet is resuspended in a 1.8% CHAPS, 1.2 M thiourea, 4.8 M urea solution with a Potter-Elvehjem apparatus. The purified proteins in this suspension are then denatured under the effect of the chaotropes, detergent, and heat (45°C for 20 minutes). Reduction with 4 mM dithiothreitol and alkylation with iodoacetamide allows the removal of existing disulfide bridges in the proteins. Digestion is performed with 20 µg trypsin at 37°C for 5 h. Non-MS compatible salts and detergents are removed by a simple solid phase extraction (Oasis mixed mode cartridges, Waters). The peptide extract is separated using an 18-minute step/ramp gradient (0.1% formic acid to acetonitrile) on a C18 column. Peptides are detected using MRM on an AB Sciex 5500 QTrap mass spectrometer.

**Results:** Peptides containing sites of point mutations for CYP 2D6 and CYP 3A4 are monitored in their wild-type (normal) and mutated versions. Identification of these peptides enables the genetic metabolic capacity potential for CYP 2D6\*1, \*4, \*5, \*6, \*9, \*10, \*17 and CYP 3A4 \*1, \*5, \*8, \*11, \*13 to be assessed. Simultaneously, 12 peptides not containing any point mutation site are used to quantify the abundance of CYPs. Different internal standardization schemes were tested (label-free, isotopically labeled peptide, reporter protein) for a 10-point calibration curve ranging from 0.01 to 5 pmol of CYP on column. Combining the genetic and quantitative abundance information allows calculation of an individual metabolic capacity. This procedure was applied on a set of 20 wild-type, unmutated individuals. Preliminary results highlight large biological differences between individuals of up to 800% for CYP 3A4 and 500% for CYP 2D6. Application of this methodology to individuals with a known CYP 2D6 mutation, \*4/\*4, yielded the expected result of a null metabolic capacity for CYP 2D6 and a positive metabolic capacity for CYP 3A4. Preliminary work looking into the post-mortem degradation of CYP enzymes indicates this is a relatively slow process, with CYP enzymes still present after 30 days of decomposition at room temperature.

**Conclusion/Discussions:** We have developed a more accurate method to estimate the metabolic capacity postmortem that takes into account not only genetics but, more importantly, differences in CYP expression level. This is an additional tool to help the forensic toxicologist to establish the manner of death.

**Keywords:** Pharmacogenomics, Proteomics, Metabolism

**Toxicology Laboratory Findings for the 2004 Census of Medical Examiner and Coroner Offices**

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**Background/Introduction:** In 2007, the U.S. Bureau of Justice Statistics (BJS) published a Special Report: Medical Examiners and Coroners' Offices, 2004 describing the medicolegal investigation of death in the United States. At that time, nearly one million deaths were referred to medical examiner and coroner offices (ME/C), accounting for approximately 40% of all deaths in 2004 ([www.bjs.gov](http://www.bjs.gov), June 24, 2007, NCJ 216756). However, much of the data collected on toxicology laboratory operations, casework, and practices were not included in this report. Publicly accessible BJS data were analyzed by RTI International (RTI) to report on these toxicology findings in this nationally represented sample of nearly 2,000 ME/C offices.

**Objective:** To analyze and discuss operational characteristics of forensic toxicology laboratories with medicolegal death investigation systems in the U.S., to provide insight for workload and function, budget and operations, management practices, and testing protocols.

**Methods:** The original data collection for the 2004 Census of Medical Examiner and Coroner Offices was conducted by RTI on behalf of BJS (2005-MU-MU-K011) during a 12-month period beginning in 2005. A census questionnaire was designed and coordinated with forensic expert panel review and piloting of the instrument to select ME/C offices. Mixed mode collection of data occurred by fielding the census through mail-out, email correspondence, and follow-up computer assisted telephone interviewing (CATI). Responses were received from ME/C offices by completed census questionnaires through mail, facsimile, CATI, and a web-based instrument. Data obtained from the BJS publicly accessible website was evaluated in 2017 for any remaining information that was not reported in the 2007 BJS Special Report. Questions and data providing details as to the operation of toxicology laboratories within a ME/C office or specific to toxicology testing were analyzed and will be discussed.

**Results:** Results from this study include insight and context for personnel, budgets, and workload of toxicology laboratories within these ME/C offices. When available, the data are presented by type of office and size of jurisdiction. Record keeping practices are also provided. Of the 1,998 ME/C offices responding, more than 1000 responses specific to toxicology laboratory services were examined by RTI. These data are important for understanding how the data from the drug-related mortality and toxicology cases were collected, analyzed, and reported during a one year period.

**Conclusion/Discussions:** Attendees will gain an understanding of operations, caseload and practices of U.S. forensic toxicology laboratories within ME/C systems. This is the first high response rate (85%) national data collection of this kind and the BJS anticipates funding a similar effort in 2017.

**Keywords:** Bureau of Justice Statistics, Postmortem Toxicology Practices, Medical Examiner and Coroner Systems

## The Estonian Fentanils Epidemic Revisited: 3-methylfentanyl Makes Way for Fentanyl and Furanylfentanyl

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**Background/Introduction:** According to the European Monitoring Centre for Drugs and Drug Addiction (EMCDDA), the 2014 mortality rate due to overdoses in Europe was estimated at 18.3 deaths per million for the population aged between 15–64. The highest rates were reported in Estonia (113 per million), where overdose deaths were mostly related to the abuse of fentanils. In 2005–2006, an exceptional epidemic of poisonings due to the highly potent opioid designer drug 3-methylfentanyl (TMF) was revealed among Estonian drug users with more than 50 TMF-related deaths annually, and the epidemic has continued long thereafter. Recently, many other highly potent fentanyl derivatives have entered the European illicit drug market, posing a high risk of overdose and death.

**Objective:** To profile the current fentanyl-related deaths in Estonia in terms of demographic characteristics and toxicological findings, with comparison to the situation ten years earlier.

**Methods:** The material consisted of all 54 fentanyl-related death cases from the regions of North and East Estonia during a one-year period September 2015 – August 2016. The urine samples from these cases were analysed for drugs by two complementary liquid chromatography – tandem mass spectrometric methods: LC-MS/MS and UHPLC-QTOFMS.

**Results:** The median (range) age of the deceased was 34 (18 – 68) years, and 89% were males. Blood alcohol was positive ( $\geq 0.5$  g/kg) in 26% of cases. Fentanyl was found in 51/54 (94%) of the urine samples, while in two cases fentanyl was found only in blood. Furanylfentanyl was found in six (11%) urine samples, TMF in four (7.4%) samples, acryloylfentanyl in one (1.9%) sample, and other opioids, including buprenorphine, codeine, methadone and tramadol, in 13 (24%) samples. There were 19 (35%) cases with multiple opioid findings. There were no 6-monoacetylmorphine or heroin findings. Stimulants, including amphetamine, methamphetamine and para-methoxymethamphetamine, were found in 16 (30%) urine samples. Cannabis was found in nine (17%) urine samples. Sedatives, including benzodiazepines, pregabalin, and z-drugs, were found in 31 (57%) urine samples.

**Conclusion/Discussions:** Due to its geographic position and a distinct sub-population of problem fentanyl users, Estonia has a longer history of fentanyl injection and higher levels of fentanyl-related deaths than other Northern European countries. While the total number of opioid-related deaths is still high in the country, the exceptionally long-lasting TMF epidemic has gradually subsided and TMF has been partly substituted by ordinary fentanyl and furanylfentanyl. The concomitant use of non-fentanyl opioids (24%) and sedatives (57%) is now more common than during the peak of the TMF epidemic (11% and 22%, respectively). The median age of the overdose victims has increased from 26 to 34 years at death but the proportion of males has remained high.

**Keywords:** Fentanils, Opioid-related Death, Estonia

## Analysis and Postmortem Consideration of Gabapentin and Pregabalin in North Carolina

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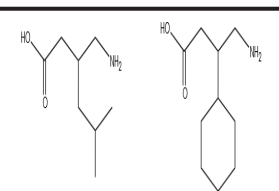
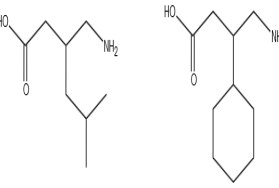
**Introduction:** Gabapentin, first marketed by Pfizer in 1993 under the brand name Neurontin, and now also under many generic formulations, is approved by the FDA for seizures in children and adults and for neuropathic pain associated with shingles. The largest market, however, is “off-label” use, with gabapentin prescribed for any number of indications, ranging from anxiety and depression to restless leg syndrome and addiction.

Following the success of gabapentin, Pfizer developed pregabalin (Lyrica), and received FDA approval for epilepsy in adults, neuropathic pain associated with shingles and diabetic peripheral neuropathy, and fibromyalgia. Like gabapentin, the bulk of prescriptions for pregabalin come as a result of “off-label” prescription, with indications like alcohol withdrawal, migraines, and social anxiety. Rampant “off-label” prescriptions for both are due largely to illegal marketing by Pfizer, which has paid \$3.7 billion in fines for doing so.

**Objective:** The purpose of this presentation is to describe the frequency of gabapentin and pregabalin in postmortem cases, screening and confirmation techniques, and considerations, such as renal disease and specimen collection sites, when interpreting cases.

**Methods:** Both gabapentin and pregabalin are screened by a multi-analyte targeted assay employing an ion-trap LC-MS. The “cut-off” for confirmation is 1.0 mg/L. Confirmation and quantitation is achieved by a validated LC-MS/MS method in blood using 0.1 mL of specimen and using gabapentin-D<sub>10</sub> and pregabalin-D<sub>6</sub> as internal standards. Positive electrospray ionization on a Thermo TSQ triple quadrupole LC-MS/MS monitors two transitions for each analyte with identification criteria based upon retention times and ion ratios. A whole blood linear calibration curve of 1.0 – 50 mg/L, as well as matrix matched controls is included with each batch of specimens.

**Results:** From 2012 through present-2017 over 2000 cases involving gabapentin or pregabalin have come through the laboratory. A summary of each is shown in the table below. Also to be presented are supporting instrumental information, case studies, and considerations for postmortem interpretation.

Postmortem Blood: 2012 - 2017		
	<b>Gabapentin (mg/L)</b>	
	Mean	15.8
	Range	1 - 250
	n =	1767
	<b>Pregabalin (mg/L)</b>	
	Mean	7.98
	Range	1 - 75
	n =	459

**Conclusion:** Gabapentin and pregabalin are prevalent in postmortem cases with wide concentration variability. Postmortem interpretation is intricate, with many factors to be considered.

**Keywords:** Gabapentin, Pregabalin, Postmortem



## Combined Drug Intoxication of Amphetamines, Cannabis and MDMB-CHMICA as a Possible Trigger in Falling from a High-Rise

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**Background/introduction:** The synthetic cannabinoid MDMB-CHMICA was first identified and reported to the EMCDDA in 2014. Since then, it has been associated with 29 fatal intoxications, suggesting a very high pharmacological and toxicological potency.

**Objective:** We present the case of a 27-year-old male who suffered a polytrauma with severe head injuries, due to a fall from a high-rise, after combined drug intoxication. Systematic toxicological analysis (STA) and NPS screenings were performed - revealing abuse of amphetamines, cannabis and MDMB-CHMICA. STA was then extended to an examination of MDMB-CHMICA organ distribution, along with its metabolites.

**Methods:** STA (immunochemical assays, HPLC-DAD, LC-ESI-MS/MS and GC-EI-MS/MS) were performed on the femoral blood, urine and gastric content (420 mL). LC-ESI-MS/MS was carried out to screen for stimulants, synthetic cannabinoids and metabolites. MDMB-CHMICA was quantitatively analyzed by standard addition (in femoral and cardiac blood, gastric content, psoas major muscle, brain, liver and kidney) and by calibration using lung and urine blanks. *In vivo* MDMB-CHMICA metabolites (amide hydrolysis product (AH), ester hydrolysis product (EH) and mono-hydroxylated (OH) MDMB-CHMICA, as well as combinations of those (EH-OH, EH-OH<sub>2</sub>) were qualitatively screened via product ion scans (PIS) and compared to *in vitro* results of a pHLM assay. Sample preparation consisted of acidic and alkaline liquid/liquid-extractions with chloroform and 1-chlorobutane, respectively.

**Results:** STA revealed abuse of amphetamine (1051 ng/mL), MDMA (275 ng/mL), MDA (22.8 ng/mL) and cannabis (9.3 ng/mL THC, THC-OH 0.9 ng/mL, 65 ng/mL THC-COOH) in femoral blood. Detected MDMB-CHMICA concentrations ranged from 0.01 ng/mL to 5.5 ng/g. Low MDMB-CHMICA concentrations were found in cardiac blood (2.1 ng/mL), femoral blood (1.7 ng/mL), psoas major muscle (1.2 ng/g) and urine (ca. 0.01 ng/mL). Higher concentrations were found in the gastric content (2.4 ng/g, 1.1 µg absolute), liver (2.6 ng/g), lung (2.6 ng/g) and kidney (3.8 ng/g), with the highest measured in brain (5.5 ng/g). Different OH-metabolites were detected in blood, urine, liver and psoas major muscle and liver. EH was found positive in liver and lung, EH-OH could only be detected in liver. PIS of brain, kidney and gastric content did not reveal any metabolite. Metabolites with a higher oxidation state (EH-OH<sub>2</sub>) were not detected *in vivo*. Nevertheless, the spectrum of detectable metabolites in urine was much smaller than expected, in contrast to previous literature descriptions of antemortem urine samples - with only OH detectable.

**Conclusion/discussion:** STA revealed acute combined drug intoxication as a possible trigger to jumping or falling from a high elevation. Synthetic cannabinoids, prone to being extensively metabolized, are usually only briefly detectable in urine shortly following consumption. The broadest metabolite spectrum was detected in liver, followed by blood and psoas major muscle. Postmortem distribution demonstrated MDMB-CHMICA was detectable in all specimens. The absence of metabolites in kidney, the small metabolite spectrum in urine and the fact that MDMB-CHMICA itself was still detectable in the urine, suggests consumption close to death. The brain concentration was approximately twice the blood concentration, likely due to the drug lipophilicity. Brain tissue is thus a particularly useful matrix for investigating synthetic cannabinoid consumption in fatal cases. Organ distribution showed MDMB-CHMICA is not highly susceptible to postmortem redistribution, but is likely slightly influencing the concentration in gastric content. The MDMB-CHMICA concentration was only somewhat higher in gastric content - and therefore in heart blood - than in femoral blood, hence not enough to conclude a high likelihood of postmortem redistribution.

The higher concentration detected in lung, liver and kidney is not an unusual finding in postmortem toxicology as those organs are highly perfused and therefore tissue accumulation of a substance is commonly observed.

**Keywords:** MDMB-CHMICA, Postmortem Distribution, Fatal Fall from a High-Rise

**Postmortem Distribution of 4-MEC, MDPV, Methoxetamine and  $\alpha$ -PVP in Deaths Arising from Poisoning**

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**Background/Introduction:** This case centres on the death of a couple (27 (A) and 28 (B) year old men), who consumed 4-methylcathinone (4-MEC), methylenedioxypropylamphetamine (MDPV), methoxetamine (MXE) and  $\alpha$  pyrrolidinopentiophenone ( $\alpha$ -PVP).

**Objective:** Postmortem samples were collected at autopsy and analysed by liquid chromatography-tandem mass spectrometry (LC-MS/MS). Two different sample preparation techniques were compared to each other – QuEChERS (QUick, Easy, CHEap, Effective, Rugged and Safe) and automated ITSP-SPE (Instrument Top Sample Preparation-solid phase extraction).

**Methods:** The detection of 4-MEC, MDPV, MXE and  $\alpha$ -PVP in kidney, liver, lungs, brain, muscle, femoral blood, and pericardial fluid (urine was not available) was performed on an LC-MS/MS system, using a biphenyl column. The QuEChERS method includes an extraction with acetonitrile and separation of interfering matrices. ITSP-SPE was carried out with mixed-mode cation exchange cartridges. Standard addition was used for quantification.

**Results:** Sample concentrations were determined for different tissues. With reference to *deceased A*, QuEChERS yielded concentrations ranging from 46  $\mu$ g/kg (liver) to 308  $\mu$ g/kg (brain) for 4-MEC; 425  $\mu$ g/L (femoral blood) to 1706  $\mu$ g/kg (liver) for MDPV and 263  $\mu$ g/L (femoral blood) to 902  $\mu$ g/kg (liver) for MXE. In contrast, ITSP-SPE revealed concentrations of 21  $\mu$ g/kg (liver) to 369  $\mu$ g/kg (brain) for 4-MEC; 137  $\mu$ g/L (pericardial fluid) to 1065  $\mu$ g/kg (muscle) for MDPV and 385  $\mu$ g/L (femoral blood) to 808  $\mu$ g/kg (kidney) for MXE. Detected concentrations of  $\alpha$ -PVP in some tissues were lower than 11  $\mu$ g/L. In results for *deceased B*, QuEChERS gave sample concentrations ranging from 8  $\mu$ g/L (heart blood) to 47  $\mu$ g/kg (brain) for 4-MEC; 3  $\mu$ g/L (heart blood) to 16  $\mu$ g/kg (liver) for MDPV and 2  $\mu$ g/L (heart blood) to 16  $\mu$ g/kg (kidney) for MXE. The ITSP-SPE concentrations for *deceased B* ranged from 8  $\mu$ g/L (heart blood) to 43  $\mu$ g/kg (lung) for 4-MEC; 1  $\mu$ g/L (heart blood) to 26  $\mu$ g/kg (kidney) for MDPV and 1  $\mu$ g/L (heart blood) to 14  $\mu$ g/kg (kidney) for MXE.

**Conclusion/Discussions:** The concentrations of 4-MEC and MDPV observed in deceased A were consistent with acute poisoning. The cause of death of deceased B could not be clearly determined. Both experimental methods showed a good bias ( $\leq \pm 14\%$ ) for fortified blood specimens. Matrix effects (ME) and recoveries (R) were determined for 4-MEC, MDPV, MXE and PVP in blood, brain, kidney and liver. With the QuEChERS approach the ME ranged between 103 and 112% and R was higher than 50% for all analytes. ITSP-SPE showed ME between 72 and 625% and R between 28 and 65%. **Conclusion:** Both preparation techniques are suitable for quantifying NPS in organ tissues and body fluids. The calculated concentrations did not fluctuate more than 35% for most matrices. The ITSP method offers advantages of automation and a lower consumption of organic solvents. QuEChERS was more time-consuming and required larger sample quantities but, for certain types of tissues and analytes, it gave higher R and no relevant ME.

**Keywords:** New Psychoactive Substances, Organ Distribution, QuEChERS

## Rectal Administration of Cocaine Resulting in Death: an Exceptional Case and Literature Review

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**Background/Introduction:** : The rectal route is a transmucosal route of administration. Rectal administration of a xenobiotic substance enables local action or direct uptake to the systemic circulation. The xenobiotic is not subjected to the effects of digestive enzymes or gastric acidity. Nevertheless, rectal administration does not circumvent the hepatic barrier as the substance is absorbed via the superior rectal veins and carried directly to the portal vein and therefore the liver. This route is especially suited to babies and infants but can be proposed for patients who are nauseous, unconscious or unable to swallow. If the use of suppositories, capsules, enemas or ointments is common practice, this is not the case for a powder inserted directly. For cocaine, this can occur in 2 situations: drug smuggling and rectal insertion for sexual purposes. In the later situation, only one paper is available in the whole literature, dating 1988. Case description: We report the case of a rich, 44-year-old, foreign financier found in the early hours of the morning roaming the streets in a state of extreme agitation. The fire brigade was called and the patient was administered a huge dose of loxapine to calm him down. The patient had a heart attack when the police arrived and could not be resuscitated. According to the call girl who was with him, the patient had gay tendencies and had inserted the cocaine powder in his rectum. Both had been drinking alcohol. The autopsy revealed moderate multi-visceral congestion, not specific to a cause of death. No abnormalities were observed on the total body x-ray. During the autopsy, samples were taken for toxicological testing.

**Objective:** Description of a fatality resulting from the use of cocaine administered rectally and discussion of the pharmacological characteristics of this route of administration.

**Methods:** HS-GC-FID was used for the detection of ethyl alcohol. HS-GC-MS was used for the detection of blood cyanide, solvents, and volatile substances. Carboxyhemoglobin concentrations in cardiac blood were determined spectrophotometrically using a CO-oximeter. LC-MS/MS (Quattro Micro) was used for the detection of common narcotic drugs and broad-spectrum blood screening. A specific method was used for cocaine and its metabolites with a UPLC-MS/MS system (Xevo TQD) after liquid-liquid extraction at pH 8.4.

**Results:** The exhaustive screening of the drugs listed in the French Pharmacopeia revealed diazepam (112 ng/ml), nordiazepam (91 ng/ml), and loxapine (2 ng/ml) in the peripheral blood, and the blood alcohol level was 0.56 g/l. All the other analyses were negative, except for cocaine and its metabolites:

Sample	Cocaine	Benzoyllecgonine	Methylecgonine	Cocaethylene
Femoral blood	681	4194	56	229
Urine	458	19550	3215	890
Vitreous humour	547	3580	980	332
Bile	458	3600	358	155
Hair	2.9	0.9	not determined	0.06

All concentrations are in ng/ml, except hair which is in ng/mg

**Conclusion/Discussions:** The thanatological and toxicological observations are concordant with death due to the use of cocaine and ethyl alcohol. The originality of this observation derives from the administration route (rectal) of the narcotic drug. The main pharmacological characteristics of this transmucosal route will be reviewed during the presentation.

**Keywords:** Cocaine, Rectal Administration, Death

## Phosphine Analysis in Postmortem Specimens: Five Cases of Fatal Aluminum Phosphide Poisoning in Children after Inhalation of Phosphine

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**Background/Introduction:** Aluminum phosphide is an effective and cheap pesticide that is commonly used worldwide, but it is also a common cause of human poisoning and carries a high mortality rate. Thousands of children are poisoned accidentally every year mainly due to their mouthing tendency. Inhalation of phosphine is one of the common routes of exposure after the action of moisture on AIP. Absorbed phosphine is rapidly metabolized into phosphite and hypophosphite.

**Objective:** To present five fatal cases of aluminum phosphide poisoning in children after inhalation of phosphine and diagnose the aluminum phosphide poisoning based on toxicological analysis.

**Methods:** The postmortem specimens were collected from rabbits as well as victims. Experiment animal models were established with rabbits exposed to phosphine generated from aluminum phosphide in contact with moisture in air. The rabbit survived for hours with the aluminum phosphide tablets placed at the top of box, and died minutes after the tablets became wet. The metabolites of phosphine were transformed into phosphine with the addition of zinc powder and sulfuric acid. The total phosphine in postmortem specimens was analyzed by headspace gas chromatography coupled with mass spectrometry.

**Results:** The metabolites of phosphine were detected in blood, urine, liver, lung, bladder and stomach ranged from 0.1 μg/mL (LOD) to 2.5 μg/mL in fatal aluminum phosphide poisoning cases. The metabolites of phosphine were also detected in tissues of rabbit, while the unconverted phosphine was detected only in the lung of one rabbit exposed to phosphine. Diagnosis of aluminum phosphide poisoning was made after postmortem toxicological analysis and confirmed by police investigation and animal experiment. Deaths of children occurred after inhalation of phosphine generated from aluminum phosphide contacting with moisture in air in all the five cases. Vomit and abdominal pain appeared in most of the children before they were sent into hospital and the pathological damage was not typical for the diagnosis of poisoning.

**Conclusion/Discussions:** The toxicological analysis of postmortem specimens provides useful information in diagnosis of aluminum phosphide poisoning in forensic science. As an important fumigation pesticide, aluminum phosphide deserves special attention, especially because of its high fatality rate.

**Keywords:** Aluminum Phosphide, Phosphine, Poisoning

## Development of a Gas Chromatography-Mass Spectrometry Method for Mexedrone in Urine

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**Background/Introduction:** New psychoactive substances continue to be problematic in the field of forensic toxicology as the sector struggles to keep up to date with substances released and those being recreationally abused. NPS use continues despite the many legal controls in place such as the UK's New Psychoactive Substance Bill (May 2016). Mexedrone (3-methoxy-2-(methylamino)-1-(4-methylphenyl)propan-1-one) is a synthetic cathinone and the alpha-methoxy derivative of mephedrone. It has similar effects to those of mephedrone although appears to be less potent. It was available online as of 2015 and continues to be recreationally used in the UK.

**Objective:** To develop and validate a quantitative GC-MS method for the detection of mexedrone in urine and to determine the stability of this analyte in urine over a 3 month period. Room temperature (23 °C), fridge (4 °C) and freezer (-20 °C) stability were assessed as well as autosampler stability and the effect of freeze thaw on concentrations detected.

**Method:** Sample preparation was carried out using CLEANSCREEN CSDAU UCT SPE cartridges. Column conditioning was done by the addition of 3 mL MeOH, 3 mL dH<sub>2</sub>O and 1 mL pH6 phosphate buffer. To samples, 3 mL of pH6 phosphate buffer was added, along with 0.5 mg/L internal standard (mephedrone-D3). Samples were then centrifuged for 10 minutes at 4500 rpm. The supernatant from each sample was transferred to SPE columns and allowed to pass through. Column washing was carried out with 3 mL dH<sub>2</sub>O, 1 mL acetic acid (1 M) and 3 mL MeOH, after which the cartridges were left to dry fully under full vacuum. Elution was carried out using 3 mL of DCM/IPA/NH<sub>3</sub> (78/20/2), followed by evaporation under a gentle stream of nitrogen and derivatization using PFPA:EtOAc (2:1) for 20 minutes at 37 °C. Samples were then evaporated to dryness, reconstituted using 50 µL of EtOAc and analyzed by GC-MS. Parameters investigated for validation included bias, linear calibration model, carryover, interferences, limit of detection, limit of quantification, precision, and stability.

**Results:** Mexedrone yielded successful results for each of the validation parameters as per SWGTOX guidelines. Samples did show significant degradation when left on the autosampler. Mexedrone levels fell 49% when reinjected over a 5 day period with 31% of the analyte degrading within the first 24 hours.

**Conclusion/Discussions:** A robust and sensitive GC-MS method was developed for the detection of mexedrone in urine. The results highlight the instability of mexedrone and that should instrumental failure occur fresh samples should be provided. Samples should be stored in the fridge (-20 °C) to prevent degradation.

**Keywords:** GC-MS, Mexedrone, NPS

## Screening and Confirmation of Fentanyl Analogues by High Resolution Mass Spectrometry using an Agilent 6545 QTOF Mass Spectrometer

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**Background/Introduction:** Fentanyl analogues and other emerging synthetic opioids have become more prevalent in recent years leading to an increase in overdose related deaths in the State of Alabama. The chemical structure of fentanyl is being modified by the addition, removal, or replacement of functional groups to circumvent legal restrictions for the exportation and sale of fentanyl. These compounds are often more potent than fentanyl and information related to their toxicity is limited. These compounds are making their way into the illicit market through the sale of the powder form of the compound, in combination with heroin, or pressed into pill form to appear as other pharmaceutical medications.

**Objective:** Develop and utilize a detection and confirmation methodology for fentanyl analogues in whole blood utilizing high resolution mass spectrometry (HRMS) screening with fragment confirmation using an Agilent 6545 QTOF Mass Spectrometer.

**Methods:** The methodology employed for this work included initial screening of suspected fentanyl analogue cases in a low energy TOF MS only scan mode and the data processed by an accurate mass library search with an in-house constructed library. The library was created by the addition of empirical formulas for currently available fentanyl analogues and other synthetic opioids for accurate mass information. Standards were acquired and their retention times and fragmentation spectra were added to the library. Confirmation of initial screening results was conducted by a second injection in high energy mode for MS/MS fragmentation data. The MS/MS data was processed by both Mass Hunter Qualitative and Quantitative Analysis software packages for the evaluation of acceptance criteria parameters for structural confirmation of the TOF screen accurate mass screening results.

**Results:** Screening and confirmation by the QTOF mass spectrometer was accomplished by conducting two experiments with extracts from a liquid-liquid extraction. The first injection was for MS only full scan data collection in TOF mode. The second injection was for MS/MS mode with varying collision energies for fragmentation of the accurate mass precursor ions. The screening algorithm within the software was optimized for appropriate sensitivity and specificity for the target analytes. General acceptance criteria for MS mode were based on a scoring algorithm of extracted ion chromatograms that included mass accuracy, retention time, isotopic spacing, and isotopic abundance. Acceptance criteria for MS/MS data were based on the type processing software utilized. Mass Hunter Qualitative Analysis software fragmentation data criteria were based on mass accuracy, fragment coelution scoring, and number of qualified fragments. Mass Hunter Quantitative Analysis software fragmentation data criteria were based on more traditional acceptance parameters of mass spectral data such as ion ratios of fragments  $\pm 20\%$ , retention time, and S/N. Utilizing this approach we were able to detect and confirm the presence of fentanyl analogues or other emerging synthetic opioid compounds present in authentic whole blood samples (see table).

Target	# of Positives
4-ANPP	9
U-47700	6
Methoxyacetyl Fentanyl	5
Acetyl Fentanyl	3
FIBF	2
Butyryl Fentanyl	2
Acryl Fentanyl	1

**Conclusion/Discussions:** Utilization of HRMS screening instrumentation has become more common for forensic applications in recent years. There is limited information available regarding methodology and acceptance criteria for screening and confirmation using TOF/QTOF instrumentation. This work demonstrates the application of an Agilent 6545 QTOF for both accurate mass screening and fragment confirmation of fentanyl analogues and emerging synthetic opioids. MS data demonstrated high sensitivity and screening capability for the target analytes. MS/MS experiments allowed for confirmation of the accurate mass MS results by traditional fragmentation data confirmation criteria. This methodology has specific advantages over traditional immunoassay and GC-MS screening procedures that are unique to HRMS instrumentation such as increased sensitivity, higher throughput, reduced data processing time, and retroactive screening capability. An additional component of this work is the presentation of acceptance criteria being utilized in our laboratory for HRMS data.

**Keywords:** TOF, QTOF, Fentanyl Analogues

**A Sensitive LC-MS/MS Assay for Quantification of Methadone and Its Metabolites in Dried Blood Spots (DBS)**

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**Background/Introduction:** Methadone, a long-acting synthetic narcotic, is widely used both in adults and children for pain control, as a replacement drug in opioid abuse, and withdrawal treatment. Methadone benefits include oral bioavailability, potent analgesic effect, prolonged duration of action and delayed development of opioid tolerance. Therapeutic drug monitoring (TDM) is recommended due to intra- and inter-individual variability of methadone pharmacokinetics that is poorly understood in neonates, infants and children.

**Objective:** To support clinical pharmacokinetics trials in pediatric population and TDM, we developed and validated a novel, automated highly sensitive liquid chromatography tandem mass spectrometry (LC-MS/MS) method for the quantification of methadone and its major metabolites 2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine (EDDP) and 2-ethyl-5-methyl-3,3-diphenylpyrrolidine (EMDP) in plasma and dried blood spots (DBS).

**Methods:** Fifty  $\mu\text{L}$  of blood was spiked with different concentrations of methadone, EDDP and EMDP and blood drops were applied to filter paper cards. DBS punches containing 20  $\mu\text{L}$  of blood were removed from the cards using a standard 6.4 mm paper hole punch and the samples were reconstituted with 100  $\mu\text{L}$  of HPLC grade water. Six hundred  $\mu\text{L}$  of protein precipitation solution (methanol/0.2M  $\text{ZnSO}_4$ , 7:3, v/v) containing the internal standard (D9-methadone) at a concentration of 1 ng/mL was added. Samples were vortexed for 2.5 min and centrifuged (4°C, 13,000 g, 8 min). After centrifugation, supernatants were transferred into autosampler vials. The extracts were analyzed using LC-electrospray-MS/MS in combination with online extraction. One hundred  $\mu\text{L}$  of the sample supernatant was injected onto the extraction column (4.6x12.5 mm, 5  $\mu\text{m}$  particle size, C8) and was washed with a flow of 4 mL/min. The analytes were back-flushed onto the analytical column (4.6x50 mm, 3.5  $\mu\text{m}$  particle size, C8). Mobile phases were 0.1% formic acid and methanol. The LC system was interfaced with an AB Sciex API4000 MS/MS via a turbo spray source. The mass spectrometer operated in the positive multiple reaction monitoring (MRM) mode. The ion transitions monitored were for methadone  $m/z= 310.2/265.1$ , for EMDP  $m/z= 264.2/235.0$  and for EDDP  $m/z= 278.2/234.2$ . For the internal standard, D9-methadone, ion transitions were  $m/z= 319.2/268.1$ . Total run time was 3.2 minutes.

**Results:** The method was validated as considered fit for a clinical assay. For the DBS, lower limit of quantifications were 0.1 ng/mL and linear ranges were 0.1-100 ng/mL ( $r^2 > 0.99$ ) for methadone, EDDP, and EMDP. Inter-batch mean analytical bias was 91.2-99.8% and mean imprecision was 7.0-14.2% CV (n=18). Recovery from plasma ranged between 86.9%-102.6% and in DBS between 39.1% and 68.8%. Post column infusion showed no matrix effects for methadone, EDDP and EMDP (n=10 different individuals) in either matrix. No carry-over was detected.

**Conclusion/Discussions:** This assay enables the sensitive and reliable measurements of methadone, EDDP and EMDP in a DBS following a simple finger-, arm or heel- stick without the need for an IV blood draw, making it suitable for application in pediatric population. The method could be utilized to analyze specimens collected during pharmacokinetics studies and therapeutic drug monitoring in both pediatric and adult population and DBS can easily be collected at home and then mailed.

**Keywords:** Methadone, DBS, TDM

## Development and Validation of an ICP-MS Method to Determine Levels of Arsenic, Mercury and Lead in Whole Blood and Urine Samples

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**Background/Introduction:** The heavy metals lead (Pb), mercury (Hg), and arsenic (As) are ubiquitous environmental pollutants with high neurotoxic and nephrotoxic potential. Acute or chronic exposure can cause severe health problems including GI illness, pulmonary edema, anemia, renal tubular necrosis, and neurological damage. Thus, reliable laboratory testing is needed to measure heavy metals in a patient's blood and urine. Atomic absorption spectrometry has been a traditional method for laboratory assessment of heavy metal levels. However, this method has a low throughput and requires a relatively large quantity of sample. Inductively coupled plasma mass spectrometry (ICP-MS), which has superior detection capability and throughput, is gradually replacing atomic absorption spectrometry in metal and trace element testing in clinical laboratories. However, few publications describing an ICP-MS method of heavy metals in human biological samples are available.

**Objective:** Develop and validate a sensitive, accurate, and high-throughput ICP-MS method to simultaneously determine arsenic, mercury, and lead levels in whole blood and urine samples.

**Methods:** Whole blood and urine samples were diluted 1:400 in 0.5% nitric acid with 200 ppb gold, 5 ppb gallium, and 1 ppb thulium. The 6-point calibration curve for As (2.5-80 ppb), Pb (10 – 320 ppb), and Hg (4 – 128 ppb), as well as 2 levels of urine controls and 2 levels of blood controls, were included with each batch of patient samples. Two milliliters of diluted sample were aspirated through an autodiluter (Elemental Scientific SC-4Q) and injected into ICP-MS (Thermo scientific, iCAP Q with collision cell technology (CCT) gas module). Kinetic energy discrimination (KED) mode was employed in data acquisition.

To validate this method, the following characteristics were determined: lower limit of detection (LOD), lower limit of quantitation (LOQ), linearity, within-day and between-day precision, accuracy, carry-over, and patient comparison with another ICP-MS method (Perkin Elmer Elan 6000 model). Precision was assessed using 2 levels of urine controls and 2 levels of blood controls, with each sample running 5 times for 5 consecutive days. Accuracy was assessed using 4 CAP and UK proficiency testing samples. Forty samples of each analyte were compared between 2 ICP-MS instruments.

**Results:** This method allows simultaneous measurement of As, Pb, and Hg. It only requires 35 microliters of sample, and it takes approximately 100 minutes to run a batch of 80 patient samples allowing high-throughput analysis. The LOQ were 2.5 ppb for As, 10 ppb for Pb, and 4 ppb for Hg. Calibration curves of each analyte exhibited consistent linearity and reproducibility in the range of 2.5 to 80 ppb for As, 10 to 320 ppb for Pb, and 4 to 128 ppb for Hg, with regression coefficients higher than 0.995 for all analytes. Within-day and between-day precisions of each analyte are shown in Table 1. Quantitation of CAP and UK proficiency testing samples (n = 4, each 3 times) were within the acceptable ranges. No carryover was observed for any of the analytes. Comparison study showed an agreement of As, Pb, Hg levels between with the two ICP-MS methods, with correlation coefficient higher than 0.98 for all analytes.

Table 1. Within-day and between-day CV in urine and blood sample for arsenic, mercury and lead.

		Urine, low level	Urine, high level	Blood, low level	Blood, high level
As	Within-day CV, %	1.1	0.75	2.2	2.3
	Between-day CV, %	1.3	1.1	3.9	2.2
Hg	Within-day CV, %	3.3	2.0	5.1	2.7
	Between-day CV, %	5.9	6.0	8.5	3.8
Pb	Within-day CV, %	1.4	0.9	0.7	1.1
	Between-day CV, %	4.1	1.3	3.2	1.5

**Conclusion/Discussions:** An accurate and sensitive ICP-MS method was developed and validated to quantitatively measure As, Hg, and Pb in blood and urine.

**Keywords:** ICP-MS, Heavy Metal, Arsenic



## Fast and Simple Screening of 40 Drugs in Diluted Blood and Urine Samples by UHPLC-MS/MS: Application in Emergency Toxicology

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**Background/Introduction:** We developed A simple approach to overcome challenges in emergency toxicological analysis, using UHPLC-MS/MS for the extraction and detection of analytes in blood and urine from the following drug classes: analgesics, benzodiazepines, antidepressants, anticonvulsants, drugs of abuse and pesticides. These substances are relevant in the context of emergency toxicology in Brazil.

**Objective:** The purpose of this work was the development and application of a fast and simple multi-analyte procedure for the identification of 40 relevant drugs to clinical and forensic toxicology using a liquid chromatography-mass spectrometry method.

**Methods:** *Urine sample extraction:* An aliquot of 100  $\mu\text{L}$  from urine samples with 20  $\mu\text{L}$  of the internal standards (IS; 0.5  $\mu\text{g}/\text{mL}$ ) was mixed with 75  $\mu\text{L}$  of ammonium acetate buffer and 5  $\mu\text{L}$  of beta-glucuronidase (500U) and incubated at 55° C for one hour. The sample was diluted with 800  $\mu\text{L}$  of methanol and water (60:40, v/v). The sample was centrifuged at 9000 g for six minutes. An aliquot of 15  $\mu\text{L}$  was injected in the UHPLC-MS/MS system. *Blood sample extraction:* 800  $\mu\text{L}$  of an acetonitrile/methanol mixture (80:20, v/v) was added to the blood samples spiked with internal standards (IS; 0.5  $\mu\text{g}/\text{mL}$ ), and the mixture was shaken for 30 seconds. After centrifugation for 6 min at 9,000 $\times$ g, an aliquot of 3  $\mu\text{L}$  was injected. The LC system consisted of a Nexera X2 UHPLC coupled to a LC-MS 8050 mass spectrometer (Shimadzu, Japan). The chromatographic separation was achieved on a Raptor Biphenyl column (50 mmx 3 mm, 2.7  $\mu\text{m}$ ; Restek, USA) eluted with flow rate of 600  $\mu\text{L}/\text{min}$  and 45 °C. Based on electrospray ionization (ESI) mode of the substance, two chromatographic methods were development in parallel. Positive ESI method: The mobile phase consisted of 1 mM ammonium formate with 0.1% formic acid and acetonitrile. The total run time was 6.0 min including re-equilibration at the initial conditions. Negative ESI method: The mobile phase

**Results:** The procedure showed to be very simple, rapid and only a small volume of blood and urine sample was necessary. The newly developed method has been validated for limits, intra-day and inter-day precision, accuracy, selectivity and matrix effects. For all other analytes, linear weighted regression models were used. The lower limits of quantification (LLOQs) were encountered in the range of 0.005 $\mu\text{g}/\text{mL}$  and 0.02  $\mu\text{g}/\text{mL}$ . The developed method was successfully applied to 201 real samples collected at the Poison Control Center of São Paulo (PCC-SP) between November 2014 and August 2016. In most of the analyzed samples (blood and urine), at least one analyte was detected. In some cases, multiple drugs were detected in the same sample. The predominant drug classes were: drugs of abuse (37 cases), followed by benzodiazepines (27 cases), anticonvulsants (22 cases), antidepressants (18 cases) and acetaminophen (5 cases). From a total of 201 patients with suspected poisoning, 53.7% have gone to PCC-SP due to substance abuse and 28.8% as attempted suicide. From the latter, at least one analyte of cocaine has been detected in 23% of the samples.

**Conclusion/Discussions:** In this study, a multi-analyte method for determination of drugs and pesticides in blood and urine was applied and it was possible to conclude that the sample preparation technique is relatively cheap, easy and fast to perform. This confirms its applicability and importance to emergency toxicological analysis and it could be also very useful in both fields of clinical and forensic toxicology.

**Keywords:** Emergency Toxicology, LC-MS/MS, Multi-Analyte

## Investigation of Potential Hydrolysis-Related Interferences in Amphetamine Positive Samples

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**Background/Introduction:** Beta glucuronidase is commonly used as an enzymatic catalyst when total drug concentration must be determined in urine samples. Glucuronide conjugation affects numerous drug classes, but robust hydrolysis methods are lacking, especially for opiate compounds which have shown increasing trends of abuse in the United States. There are many published studies that characterize optimal hydrolysis conditions, but very few study how these parameters impact the stability of other unconjugated compounds in the specimen. Specifically, we are concerned about potential compounded interferences with amphetamine-like substances in the presence of an enzyme source. Our work here compares several methods and demonstrates how these interferences can be eliminated by applying adequate extraction techniques prior to analysis.

**Objective:** Investigate the concern that various beta glucuronidase preparations contribute to an interference that could alter the detection of amphetamine-like substances in urine samples and develop a sample preparation methodology to remove interferences and restore stability.

**Methods:** Instrumental analysis was performed via a Shimadzu 8050 Liquid Chromatographer Mass Spectrometer. Separation of all analytes was achieved using a UCT Selectra® DA Biphenyl HPLC column. Ten blank urine lots were then fortified at either 100 or 1000 ng/mL of a working stock solution containing morphine, codeine, 6-acetyl morphine, amphetamine, methamphetamine and MDMA. An appropriate amount of enzyme and internal standard working solution was also added to each sample. Four different enzymes, three from abalone and one from helix pomatia, were obtained. Of the three abalone enzymes, two were purified formulations. Each urine sample contained a final concentration of 5,000 Fishman units/mL of said enzyme in question. Following an incubation period of 60 minutes at 65°C, samples were prepared for instrumental analysis one of three ways: one-fold dilution and centrifugation, one-fold dilution and clean-up via a UCT Clean Screen FAST® filter column or liquid/liquid extraction. Three patient samples that had previously screened positive for both amphetamines and opiates were also analyzed via the above method. In order to elucidate the origin of any interference seen, each urine sample was also screened independent of spiking standards and enzymes.

## Results:

Sample Name	Presence of Amphetamine Interference Following Enzyme Addition and Sample Clean-Up											
	Purified Abalone A			Purified Abalone B			Crude Helix Pomatia			Crude Abalone		
	D+S	L/L	FAST	D+S	L/L	FAST	D+S	L/L	FAST	D+S	L/L	FAST
Urine 1	X	None	None	X	None	None	X	X	X	X	X	X
Urine 2	X	None	None	X	None	None	X	X	None	X	None	None
Urine 3	NONE											
Urine 4	None			None			X	X	None	None		
Urine 5	None			None			X	X	None	None		
Urine 6	X	None	None	X	None	None	X	X	None	X	X	None
Urine 7	None			None			X	X	None	None		
Urine 8	None			None			X	X	None	None		
Urine 9	None			None			X	None	None	None		
Urine 10	NONE											
Patient 1												
Patient 2												
Patient 3												

Note: Liquid/liquid and/or FAST clean-up was not carried out if interference was not seen following dilute and shoot

**Conclusion/Discussions:** We observed a large contribution of unwanted matrix components in hydrolyzed urine samples when using the “dilute and shoot” or liquid extraction methods. The use of Clean Screen FAST® columns allowed for the removal of unpurified enzyme components in the majority of urine samples affected by the use of enzyme C. If an unpurified beta glucuronidase source is utilized for enzymatic hydrolysis, there is an increased probability that an artifact could present itself that may interfere with the analysis of amphetamine-like substances. Depending on the length and resolving power of a laboratory’s chromatographic method, the interference in question may co-elute with amphetamine leading to inaccuracies in data interpretation and analysis.

**Keywords:** Amphetamine, Hydrolysis, Interference

## Evaluation of a New Extraction Procedure for the Preparation of Cannabis-based Olive Oil for Therapeutic Use: Determination of $\Delta^9$ -tetrahydrocannabinol and Cannabidiol Concentrations

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**Background/Introduction:** Since 2013 Cannabis-based preparations, containing the two main cannabinoids of interest,  $\Delta^9$ -tetrahydrocannabinol (THC), and cannabidiol (CBD), can be used for therapeutic purposes, such as palliative care, neurodegenerative disorder treatment and other therapies. The administration route is strictly ruled by the Italian government, that allows the pharmacists to import cannabis preparations from other countries and provide drugs to patients, only after medical prescription. The preparations may consist of a drug partition in sachets, capsules or through the extraction in certified olive oil. The sachets and capsules do not require any specific analysis, because the THC and CBD concentrations are already certified by the supplier. On the contrary, whenever cannabis is extracted/administered through olive oil, the THC and CBD levels in this alternative matrix must be measured and certified through sensitive and specific analytical procedures.

**Objective:** the aims of the study were: a) to develop and validate a new liquid chromatographic-tandem mass spectrometric (LC-MS/MS) method for the identification and quantification of THC and CBD in olive oil; b) to evaluate the extraction efficiency and reproducibility of a new commercial extractor on the market.

**Methods:** the olive oil was simply diluted three consecutive times, using organic solvents with increasing polarity index (n-hexane → isopropanol → methanol). The sample was then direct injected into LC-MS/MS system, operating in Multiple Reaction Monitoring Mode, in positive polarization. The method was then fully validated.

**Results:** The method assessed to be linear over the range 0.1-10 ng/ $\mu$ L for both THC and CBD. Imprecision and accuracy were within 12.2% and 16.9% respectively; matrix effects proved to be negligible; THC concentration in oil is stable up to two months at room temperature, whenever kept in the dark. CBD provided a degradation of 30% within ten weeks. The method was then applied to olive oil after sample preparation, in order to evaluate the efficiency of extraction of a new generation instrument. Temperature of extraction is the most relevant factor to be optimized. Indeed, a difference of 2 °C (from 94.5°C to 96.5°C, the highest temperature reached in the experiments) of the heating phase, increases the percentage of extraction from 54.2% to 64.0% for THC and from 58.2% to 67.0% for CBD. The amount of THC acid and CBD acid that are decarboxylated during the procedure must be check out in the future.

**Conclusion/Discussions:** The developed method was simple and fast. The extraction procedure proved to be highly reproducible and applicable routinely to cannabis preparations.

**Keywords:**  $\Delta^9$ -tetrahydrocannabinol, Cannabidiol, Olive Oil

## Differentiation of Regioisomeric Analogues of Amphetamine and Methamphetamine by Ultra-high Performance Supercritical Fluid Chromatography

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**Background/Introduction:** Drastic increase of new psychoactive substances (NPS) is one of the biggest problems for controlling drug abuse in the world. For forensic chemists, it is essentially important to establish powerful analytical techniques which are applicable to NPS, in particular to structurally similar compounds such as ring-substituted regioisomers.

**Objective:** In this study, we developed a method to achieve chromatographic differentiation of ring-substituted regioisomers of amphetamine (AP) and methamphetamine (MA) as the example of NPS. These types of regioisomers are one of the most difficult compounds to differentiate by commonly used chromatography.

**Methods:** Differentiation was performed by ultra-high performance supercritical fluid chromatography (UHPSFC) with ultraviolet absorption (UV) spectroscopy and mass spectrometry (MS). Optimized chromatographic conditions were investigated by systematically changing the experimental conditions including not only stationary phase but also chromatographic parameters such as temperature and co-solvent proportion.

**Results:** We have found that the retention of UHPSFC showed unique behavior in comparison to liquid chromatography. For example, the retention time did not show monotonic behavior to the change of column temperature. By using these properties, optimization of chromatographic conditions was attained. As a result, analogues of AP and MA with the substituents of methyl, methoxy, fluoro, chloro and bromo groups on either of 2-, 3-, or 4- positions on the benzene ring were separated by UHPSFC with the co-solvent of methanol containing ammonia as the additive. Except for methyl-MAs (about 10minutes), all regioisomers were separated within 6 minutes by the method developed. UV spectrum and mass spectrum of each compound were obtained to estimate the ability to identify the type and the position of substituents. The limit of detection (LOD) was evaluated by MS. The LOD obtained was about 1-10 pg on column except for methyl-APs (about 50 pg).

**Conclusion/Discussions:** Although mass spectra provided the information on the types of substituents, it was difficult to estimate their positions on the benzene ring. Meanwhile, UV spectra sensitively reflected the position of the substituents. This meant that the combination of UV spectroscopy and MS was very useful to identify the regioisomer. Therefore, it was concluded that UHPSFC with UV spectroscopy and MS was expected to be a powerful analytical technique of structurally similar NPS with splendid identification capability.

**Keywords:** Ultra-high Performance Supercritical Fluid Chromatography, New Psychoactive Substances, Regioisomer

## New Psychoactive Substances Produce Reactive Metabolites – A Possible Mechanism of Toxicity?

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**Background/Introduction:** Most xenobiotics, when metabolized, form non-toxic products; however, some chemical structures have the potential to generate highly reactive metabolites. If not detoxified, reactive metabolites can covalently bind to endogenous structures such as proteins or DNA and subsequently affect their function. Research, mostly performed retrospectively on failed pharmaceuticals, has shown that reactive metabolites can cause various toxicities, such as hepatotoxicity, drug-induced immune reactions, necrosis and idiosyncratic toxicities. Assessment of reactive metabolites has therefore become a standard task during pre-clinical evaluation of new drug candidates. In contrast, new psychoactive substances (NPS) do not undergo any toxicological testing before being marketed. On closer inspection, a worrying number of NPS contain structural features that are known to form reactive metabolites. In fact, several metabolism studies have identified so-called ‘smoking gun metabolites’, i.e. metabolites which are likely generated from reactive metabolites and are therefore good indicators for their short-lived existence. Finally, disregarding psychoactive effects, many case reports describe severe adverse reactions, which might be related to reactive metabolites and subsequent organ toxicity, e.g. liver damage, abdominal pain, dermatitis, rhabdomyolysis and kidney failure.

**Objective:** In this pilot study, we tested a selected set of eleven NPS for their ability to produce reactive metabolites.

**Methods:** To identify potential reactive metabolites, we performed HLM incubations with three established trapping agents glutathione (GSH, traps soft electrophiles), potassium cyanide (KCN, traps iminium ions) and methoxylamine (MXA, traps hard electrophiles such as aldehydes). In this experiment, reactive metabolites are trapped as adducts. Merging findings from our own or published metabolism studies and case reports with results from theoretical structure evaluations, we compiled a list of NPS candidates suspected to form reactive metabolites. Eleven compounds were included for the initial screening: JWH-018, JWH-200, JWH-210, XLR-11, 5F-PB-22, MDPV, MDPPP, 5-MeO-DALT, methiopropamine, glaucine and MT-45. Compounds were incubated at 10  $\mu$ M for 1h with HLM in the presence of NADPH-regenerating system and either no additional trapping agent or 10 mM GSH, 20 mM KCN and 10 mM MXA, respectively. Clozapine, rimonabant and prazosine served as positive controls. All samples were analyzed via liquid chromatography high-resolution mass spectrometry on an Agilent QTOF G6550 coupled to an Agilent 1290 Infinity UH-PLC system with an Acquity HSS T3 column. Mass spectrometric data were acquired in data-dependent acquisition mode collecting MS/MS spectra for abundant candidates.

**Results:** For each tested compound, we found between one and eleven different adducts for a single combination of NPS and trapping agent, showing that NPS can form reactive metabolites, in some cases to a surprisingly high extent (XLR-11, 5-MeO-DALT). We identified adducts for all three trapping agents suggesting the existence of different reactive metabolites, such as aldehydes, iminium ions and epoxides. GSH adducts were detected for 5-MeO-DALT and MT-45; cyanide adducts for JWH-200, MDPV, glaucine and MT-45; MXA adducts for all compounds except glaucine and MT-45.

**Conclusion/Discussions:** Many NPS show atypical and severe toxicities, which, to date, are not understood well. One possible mechanism for the observed toxicities are the reactive metabolites identified in this study. Important to realize, the sheer existence of adducts in the trapping assays is not enough to link reactive metabolite formation to toxicity. The extent of the covalent binding to biomolecules, the dose of the drug and frequency of consumption also play a pivotal role. Therefore, this preliminary data are currently expanded in a more comprehensive study to answer the question if reactive metabolites contribute to the toxicity of NPS.

**Keywords:** New Psychoactive Substances, Reactive Metabolites, High-resolution Mass Spectrometry

**The ETORA-project: Evaluation of Toxicity of New Drugs of Abuse Based on Analysis.**

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**Background/Introduction:** A new challenge for the clinical health services is the dramatic increase in the number of substances available on the drug market during the last decade (1). A primary source of purchase is internet shops, enabling global distribution and recruitment of other user groups than traditional drug users. Synthetic cannabinoids and synthetic cathinones have been the most common groups, but designer benzodiazepines, LSD-analogues and designer opioids have become increasingly important, all representing potent drugs with potentially harmful, and for some compounds even lethal, effects. An evaluation of substances sold from internet shops revealed that the content can be variable, both regarding purity and the substances in the sample (2). Users might therefore not know which compound they consume, or how to estimate the dose. This increases the risk for drug overdoses. Due to this inherent lack of knowledge, analysis of biological samples is the only way to correlate clinical effects with substance abused. Most hospital clinics will, however, not have the analytical methods available, or resources to keep a repertoire and reference materials updated. In addition, very limited, or no, data exist on pharmacology, toxicity, and behavioral effects of these drugs.

**Objective:** The aim of the project is to uncover which NPS used in Norway lead to hospitalization, and study their geographic distribution, effects, and toxicity as well as to further develop methods for NPS detection in biological samples.

**Methods:** Patients were recruited by the Norwegian Poison Information Centre, targeting patients admitted to hospital due to suspected exposure to new psychoactive substances. This inclusion criterion is not specific, and patients with self-admitted use of NPS, intake of centrally stimulating and/or hallucinogenic compounds, use of cannabis or derivatives, intake of plants or mushrooms for intoxication, or hospitalization after use of weight loss products, will be eligible for inclusion. De-identified samples were sent to the Department for Forensic Sciences at Oslo University Hospital for analysis. Synthetic cannabinoids, THC and GHB are screened by UHPLC-MS/MS, alcohol by ADH and NPS and other common drugs of abuse by LC-QTOF. De-identified medical records are being collected from the hospitals, and will be compared to analytical findings of new psychoactive substances as well as alcohol and classical drugs of abuse, to relate clinical symptoms, intoxication and harm potential for these new substances. The severity of poisoning will be graded based on the clinical symptoms observed using the standardized Poisoning Severity Score (3) based on available medical records and notes at the Poisons Information Centre.

**Results:** Up to now 57 cases from 25 different hospitals have been included in the study, and 35 samples have been analyzed. The majority of samples only had alcohol or common drugs of abuse such as MDMA, amphetamines, THC, GHB and LSD. Some NPS such as 3-fluorophenmetrazine, pyrazolam, mitragynine, 2C-B and bk-MDMA have been found.

**Conclusion/Discussions:** As the Norwegian Ethics Committee only allowed for surplus material to be used in the project the sending of material before destruction has been a problem, and only about 50% of the samples have been sent for analysis. Most cases of intoxication are due to multi-drug intake, making conclusions about intake of a substance and symptoms of intoxication difficult to make. Although the active agent was usually suggested in the dialogue with the Poison Information Centre the suggested agent was not always found; for three cases with assumed intake of 2C-B, one was positive for 2C-B, one for LSD, while one only had positive alcohol and clonazepam results. A summary of results and examples of cases will be given.

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**Keywords:** New Psychoactive Substances, Poison Information Centre, Drug Analysis

## A Qualitative/Quantitative LC-MS/MS Workflow for Comprehensive Drug Detection in Oral Fluid Samples

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**Background/Introduction:** Oral fluids have gained a lot of interest in clinical, workplace drug testing, and suspected drug-driving offences to verify recent drug consume. Compared to other biological samples such as blood and urine, oral fluid offers sample collection advantages, which include a less invasive collection protocol and no need for special collection facilities and a same sex collector. Furthermore, the parent drug is frequently prominent in oral fluid and may reflect free plasma concentrations, providing a better correlation with pharmacodynamic effects, such as impaired performance. Oral fluid, as the test matrix, has certain limitations as well. Drugs present in oral fluid are usually at lower concentrations than that found in urine; together with the small volume of oral fluid available for collection, drug detection can become a significant challenge. Therefore, systematic toxicological analysis of oral fluids typically involves LC-MS/MS multianalyte assays. These approaches enable the detection, confirmation and quantification of a selected number of targets with low limits of detection. The strength of targeted assays to be capable of specifically detecting targets is their weakness: the assays cannot provide analytical information on compounds that are not included in the target list. To overcome this limitation, non-targeted LC-MS/MS techniques have been developed. These assays are commonly used for the analysis of diverse biological samples. So far, however, the concept has hardly been realized for oral fluid samples.

**Objective:** We demonstrate the applicability of a qualitative/quantitative workflow involving non-targeted LC-MS/MS for comprehensive and sensitive drug detection in oral fluid.

**Methods:** Oral fluid was collected with the GBO saliva collection system. The portion of oral fluid in the mixture was determined spectrophotometrically using standards and controls included in the package. Solid phase extraction with the Phenomenex Strata-X 33  $\mu\text{m}$  polymeric reversed phase cartridge was used to prepare oral fluid samples for LC-MS/MS. 500  $\mu\text{L}$  of saliva were processed to yield 50  $\mu\text{L}$  of extract which was submitted to non-targeted LC-MS/MS on a Sciex TripleTOF 5600+ and to targeted analysis on a Sciex QTRAP 4000 instrument. Targets of the multianalyte assay included cocaine, amphetamine, methamphetamine, MDMA, opiates, opioids, and metabolites thereof.

**Results/Discussion:** We have developed a qualitative/quantitative LC-MS/MS workflow for oral fluid analysis. The workflow involves sample collection with the GBO saliva collection system, sample processing by solid-phase extraction, as well as non-targeted and targeted LC-MS/MS analyses. The workflow was validated according to published guidelines. Validation of the non-targeted assay involved check of selectivity, detection capability and reliability of identification. As part of the validation of the quantitative multianalyte assay the parameters selectivity, linearity of calibration, limits of detection, accuracy, precision, matrix effects and recovery were tested. Both assays were found to be fit for the intended purpose. For the targeted assay, limits of quantification below 0.5 ng/mL were reached. With the non-targeted approach, compounds were detectable at concentrations as low as 1-5 ng/mL. To prove the suitability of the developed workflow for the analysis of authentic samples, 60 patient samples were screened. Obtained results correlated well with those reported by a reference lab employing multi-target LC-MS/MS for 65 drugs only. The clear advantage of the newly developed workflow was its ability to detect compounds not included in a preselected target list. With the two workflows 514 drugs and metabolites were identified. 37% of compounds were detected with both workflows, 48% with the qualitative/quantitative LC-MS/MS workflow and 15% with the reference assay only. Out of the 75 missing compounds, however, only 27 compounds had concentrations larger than 5 ng/mL; clearly, the majority of missing compounds were hardly detectable by the non-targeted LC-MS/MS technique.

**Conclusion:** The developed qualitative/quantitative LC-MS/MS workflow using oral fluid as sample was demonstrated to be fit for forensic drug screening applications.

**Keywords:** Oral Fluid, Drug Screening, Non-Targeted LC-MS/MS

## U-47700 Threat to Personal and Public Health

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**Background/Introduction:** U-47700, 3,4-dichloro-N-[2-(dimethylamino)cyclohexyl]-N-methyl-benzamide, is a selective **agonist** of the  **$\mu$ -opioid receptor**, developed in the 1970s as a synthetic alternative to morphine. It acts at the  **$\mu$  receptor** with a  $K_d$  value of 5.3 nM compared to 910 nM for the  **$\kappa$ -opioid receptor** and is 7 times more potent than morphine in animal models. U-47700 became the lead compound of different selective kappa-opioid receptor ligands that are used in research. Recently, a huge production of these opioids has invaded USA and Europe. More than twenty overdoses and several deaths in the USA had initially been associated with U-47700 in early 2016. As of November 2016, the DEA confirmed at least 46 fatalities associated with U-47700. Considering the threat to public health and safety, the U.S. Drug Enforcement Administration and several European countries has placed U-47700 into Schedule I of their Controlled Substances Acts. The effects of U-47700 are similar to other opioids and its toxicities should be similar to the opiate toxidrome.

**Objective:** We report the case of young man, 28 years old, presenting severe intoxication after insufflation of U-47700, with subsequent confirmation of this substance in blood, urine and hair samples, and seized material.

**Methods:** A 28-year-old man was found irresponsive with cyanosis and agonal respiration in his apartment. Glasgow coma scale was 3. He was intubated and taken to the local emergency department where he recovered after supportive care. Urine, blood and a white powder found at his home were collected and analysed using GC-MS and LC-HRMS. Later, his pubic hair was collected. A standard comprehensive toxicology screening was performed. A quantification of the parent molecule U-47000 was accomplished. Using liquid chromatography–high resolution mass spectrometry (LC-HRMS) and liquid chromatography–high resolution multiple mass spectrometry (LC-HRMS/MS) on an Orbitrap, the presence of U-47000 and its phase I and phase II metabolites in blood, urine and hair was proposed.

**Results:** U-47700 was identified in the seized white powder and all biological samples. No other opioid nor designer drug could be detected in blood and urine. The findings are shown in the table below, where a + or a ++ sign indicates the relatively higher abundance of metabolites with respect to the parent molecule.

<i>Specimen</i>	Blood on admittance	Blood 24 hs later	Urine on admittance	Urine 24 hs later
Parent drug (ng/mL)	94	3	5.2	5.4
N-desmethyl metabolite	+	++	++	++
N-desmethyl hydroxy metabolite	+	+	++	++
N-di-desmethyl metabolite	+	++	++	++
N-di-desmethyl hydroxyl metabolite	+	+	+	+

In pubic hair, properly washed before extraction, U-47700 was determined at 3 ng/mg, together with its four metabolites at relatively lower concentrations. Cocaine, Benzoylcegonine, Cocaethylene, Nor-cocaine, Mephedrone, Ketamine, Nor-Ketamine, MDMA, and Tetrahydrocannabinol, were also detected, revealing a history of poly-drug use. Once the guy recovered, he told physicians that he was using U-47700 for the first time. The presence of the molecule and its metabolites in pubic hair collected only three days after the acute intoxication must be ascribed to abundant sweating during the event, rather than to incorporation from blood into the growing hair matrix.

**Conclusion/Discussions:** U-47700 is a relatively recent illicit drug that is easy to obtain over the web notwithstanding bans in almost all occidental Countries. It has the potential to cause significant morbidity and mortality and methods are needed to rapidly identify it in biological matrices. In the first reported death involving U-47700, it was detected at 1,460 ng/mL in post-mortem femoral blood. In other post-mortem cases, the mean and median concentrations (n = 16) in blood were 253 ng/mL ( $\pm 150$ ) and 247 ng/mL within a range of 17–490 ng/mL. The blood concentration observed in the present case falls within the reported range; the rapid intervention of supportive cares and the absence of concomitant drug use were probably crucial in saving life.

**Keywords:** Designer Drugs, Respiratory Failure, U-47700



## Novel Scheduling of U-47700 in the State of Ohio

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**Background/Introduction:** Abuse trends for novel compounds often appear at rates that outpace the ability of State and Federal policy makers to respond. Seeking to resolve this issue, state and local agencies in Ohio have engaged more significantly in data sharing practices. Such was recently the case in the scheduling of 3,4-dichloro-N-[2-(dimethylamino)cyclohexyl]-N-methyl-benzamide, also known as U-47700. U-47700 is structurally categorized as an opioid and is an isomer of AH-7921. U-47700 was produced by Upjohn in 1978 as a synthetic alternative to morphine. U-47700 exerts its pharmacologic effects as a mu-opioid receptor agonist and has approximately 7.5 times the potency of morphine. Opioid novel psychoactive substance (NPS) drugs are popular recreational substitutes for heroin, fentanyl or morphine. NPS drugs can be taken orally, smoked, snorted, intravenously or rectally. They are sold on the internet as 'legal highs' and often mixed with heroin or other psychoactive substances. There have been more than 60 deaths in the US and EU countries involving U-47700. The effects of U-47700 are similar to other opioids and include analgesia, sedation and mild euphoria. U-47700 toxicities should be similar to the opiate toxidrome and include respiratory depression, altered mental states, pulmonary edema, coma, bradycardia, hypotension, hypothermia, nausea, and vomiting. As of November 14, 2016, DEA confirmed at least 46 fatalities associated with U-47700.

**Objective/Method:** The State of Ohio Board of Pharmacy has authority to add to Ohio's list of controlled substances, while crime laboratories are frequently the first entities capable of identifying abuse trends. The objective is to use Ohio's novel scheduling of the synthetic opioid U-47700 to underscore the impact of interagency cooperation in mitigating the trafficking and abuse of novel compounds.

**Results:** Confirmed cases of fatal overdoses in Ohio and Pennsylvania initiated drug scheduling research to be conducted by the Ohio Attorney General's Bureau of Criminal Investigation (BCI). After BCI's research was provided to the State of Ohio Board of Pharmacy, the Board cast a swift and unanimous vote which classified U-47700 as a schedule I opium derivative under rule 4729-11-02 of the Ohio Administrative Code. The next day, Ohio Governor John Kasich signed an executive order authorizing the Board to take emergency action and subjecting U-47700 to criminal drug penalties as of May 4, 2016. U-47700 is also scheduled in the states of Wyoming, Georgia and the countries of Finland and Sweden. On November 14, 2016, the U.S. Drug Enforcement Administration placed U-47700 into Schedule I of the Controlled Substances Act.

**Conclusion/Discussions:** Due to cooperation between crime laboratories and agencies with authority to schedule novel compounds, U-47700 was scheduled in Ohio less than two weeks after it was identified as a substance of concern for the state.

**Keywords:** Drug Scheduling, U-47700, Novel Psychoactive Substances

## Simple and Rapid Screening Procedure for 66 Synthetic Cannabinoids by Liquid Chromatography-tandem Mass Spectrometry and its Application for Forensic Toxicology Purposes

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**Background/Introduction:** In recent years, many synthetic cannabinoids (SC) have appeared on the drug market. These substances sold as 'herbal highs' or 'research chemicals' belong to different chemical classes. According to reports of the European Monitoring Centre for Drugs and Drug Addiction (EMCDDA), more than 160 SC were introduced to the European market up to 2015 and 256 SC are currently listed by the United Nations Office on Drugs and Crime (UNODC). Despite the increasing number of SC, there are few comprehensive screening methods for their detection in biological specimens. The variety of SC, their low active doses, low concentrations in biosamples, as well as rapid and numerous metabolic changes create great analytical problems. The analytical methods should be sensitive and selective, and moreover, screening procedures should be open, i.e. ensuring the possibility of continuous inclusion of new compounds.

**Objective:** In abovementioned context, the purpose of this study was to develop a fast and simple liquid chromatography-tandem mass spectrometry (LC-MS/MS) screening procedure for detection and identification of 66 SC in whole blood.

**Methods:** Blood samples (0.2 mL) were precipitated with acetonitrile (0.6 mL). Analyses were performed on an Agilent Technologies 1200 series liquid chromatograph connected to a 6460 Triple Quad mass spectrometer. The separation was achieved a Kinetex C18 2.6u 100Å (100×4.6 mm) column (Phenomenex). The mobile phase consisted of a mixture of 0.1% formic acid in acetonitrile (v/v) and 0.1% formic acid in water (v/v) was delivered under the following flow rate conditions: 0 min – 0.5 mL/min, 1 min – 0.5 mL/min, 3.5 min – 0.8 mL/min, 10 min – 0.8 mL/min, 10.5 min – 0.5 mL/min, 16 min – 0.5 mL/min, and the following mobile phase gradient conditions (shown in relation to acetonitrile content): 0 min – 40%, 1 min – 40%, 3.5 min – 60%, 4.5 min – 90%, 10 min – 90%, 10.5 min – 40%, 16 min – 40%. Dynamic multiple reaction monitoring (dMRM) with positive ion detection was applied (retention time window was set at 1 min). The total number of transitions monitored was 199, and the total analytical run time was 16 min.

**Results:** Despite differences in chemical structures, the method allowed the simultaneous detection and identification of 66 SC from different groups, among others, naphthoylindoles, phenylacetylindoles, naphthylmethylindoles, cyclopropylindoles, adamantoylindoles. The application of the gradient flow rate and gradient mobile phase conditions made that all of the compounds were well differentiated by their retention times and/or transitions. The retention times of compounds were from 2.53 to 9.15 min. Prepared blood calibration curves (number of replicates for each level, n = 3) were linear in the range of from 0.1-5 to 100 ng/mL with  $r^2$  in the range of 0.9958-0.9993. The limits of detection (LODs) established for 49 compounds (for the signal-to-noise ratio equalling 3 (S/N=3) for the transition with the lowest intensity) were in the range 0.001-0.48 ng/mL making this assay suitable for the analysis of biological material.

**Conclusion/Discussions:** We developed a sensitive LC-MS/MS method for simultaneous identification of 66 SC in blood. The developed procedure allows performing rapid screening analysis and requires only 0.2 mL of blood. The procedure can be easily expanded for more substances. The procedure was successfully applied to the analysis of forensic blood samples in routine casework. Such methods are needed for forensic and clinical laboratories due to the ever-increasing spectrum of new SC.

**Keywords:** Synthetic Cannabinoids, Blood Screening Analysis, LC-MS/MS

## Screening and Quantitative Analyses of New Psychoactive Substances in 11 Emergency/Fatal Cases Using UHPLC-Q-TOFMS Coupled with Ion Mobility Separation

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**Background/Introduction:** In recent years, numerous analogues of new psychoactive substances (NPS) have been widely distributed globally and unexpected effects associated with the intake of mixtures of these substances are causing concern. Ion mobility is an additional dimension of separation, based on molecular size and shape. Combining this ion mobility separation (IMS) technique with an ultra-high performance liquid chromatography-quadrupole time-of-flight mass spectrometry (UHPLC-Q-TOFMS) should provide analytical benefits, including the highly selective detection of various analogues in samples with a high matrix burden.

**Objective:** For more sensitive and selective analyses, a screening method for 500 NPS and their metabolites in human biological samples was developed using UHPLC-IMS-Q-TOFMS. Moreover, this method was applied to examine these substances in whole blood, serum and urine samples obtained from 11 emergency/fatal cases, and the quantitative analyses were also carried out for the substances detected in the samples.

**Methods:** The screening analyses for 500 NPS and their metabolites were made by an UHPLC-IMS-Q-TOFMS (Synapt G2-Si, Waters) in a HDMS<sup>E</sup> mode (sequential acquisition of mass spectra at low energy and high collision energy). In addition to retention times and accurate masses provided by the HDMS<sup>E</sup> in the presence of the protonated molecule and fragment ions, collision cross section (CCS) values were measured and all data were stored in a mass spectra database. Chromatographic separation was performed in a gradient mode (0.1% formic acid and 0.1% formic acid/acetonitrile) using a CORETECS C18 column (2.7  $\mu\text{m}$ , 2.1 mm x 150 mm, Waters). Human whole blood, serum and urine samples were obtained from 11 emergency/fatal cases (total 18 samples) caused by the consumption of liquid or herbal products adulterated with NPS (from 2013 to 2016). The whole blood and serum samples were analyzed after removing proteins and lipids using a filtration plate (Captiva ND<sup>Lipid</sup>, Agilent Technologies), and the urine samples were extracted with *t*-butyl methyl ether after enzymatic hydrolysis. For the quantitative analyses, a UHPLC-tandem mass spectrometry (MS/MS) in a multiple reaction monitoring (MRM) mode was used.

**Results:** The screening method was established based on four index parameters, such as the accurate masses of the protonated molecule and fragment ions, the retention times (1.0-24.4 min) and the CCS values (120.2-220.8  $\text{\AA}^2$ ) for the 500 substances. The detection limits of the control substances added to the control biological materials ranged from 0.5 to 5 ng/mL. Using the analysis in combination with the IMS, the background interferences on the mass chromatograms were reduced and the detection sensitivities of each substance were increased. These results led to the analysis narrowing down by less than half of the candidate substances by **library searching** the mass spectral database. As a result of the screening analyses for the 18 biological samples, 17 kinds of NPS (10 cathinones, 4 synthetic cannabinoids such as 5F-QUPIC, 5F-AB-PINACA, 5F-AMB and AB-CHMINACA, diphenidine, 5-APDB and AH-7921), 15 metabolites of synthetic cannabinoids, 2 metabolites of cocaine and 18 medicines were detected. In particular, 6 cathinone derivatives and AH-7921 were simultaneously detected in one fatal case. By the quantitative analysis of the serum sample from this case, MDPPP (587.4 $\pm$ 30.1 ng/mL), AH-7921 (235.5 $\pm$ 10.0 ng/mL), MPHP (114.2 $\pm$ 5.9 ng/mL),  $\alpha$ -PHPP (92.9 $\pm$ 4.7 ng/mL), 4F- $\alpha$ -PVP (30.1 $\pm$ 0.6 ng/mL),  $\alpha$ -PBP (3.67 $\pm$ 0.09 ng/mL) and 4-MeO- $\alpha$ -PVP (0.60 $\pm$ 0.01 ng/mL) were detected.

**Conclusion/Discussions:** Acquiring the UHPLC-Q-TOFMS analytical data combined with the added specificity from the ion mobility made it possible to discount chromatographically co-eluting compounds and background interferences. The UHPLC-IMS-Q-TOFMS is useful for screening analyses of various analogues of NPS and their metabolites with low concentrations in biological materials. In the last 3 years, the number of NPS distributed in Japan dramatically decreased, while types of newly-emerged substances tended to diversify. To prevent health risks caused by these substances, continuous and dedicated monitoring is essential.

**Keywords:** New Psychoactive Substances, Biological Samples, UHPLC-IMS-Q-TOF MS

## The Elephant (Tranquilizer) in the Room: Carfentanil

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**Background/Introduction:** 4-carbomethoxyfentanyl, also known as carfentanil, is a derivative of the pharmaceutical medication fentanyl and was first developed by Janssen Pharmaceutica in 1974. Carfentanil is a mu opioid receptor agonist and is estimated to be 5,000-10,000 times more potent than morphine in animal (non-human) models. Carfentanil, a Schedule II controlled substance in the USA, is marketed as Wildnil® as its only approved medical use in the USA is as an immobilizer or tranquilizer of large exotic animals, such as bears, bison, elephants, elk, and rhinoceros. In 2016 news media began to report the appearance of carfentanil as an additive to street heroin in the USA. During this same time various agencies in the USA reported an increased number of life-threatening hospital admissions and fatalities.

**Objective:** Attendees of this presentation will learn about the pharmacology/toxicology of carfentanil via literature review, discussion of the methods of analysis for carfentanil in blood specimens, survey of overall toxicology results in our laboratory from September 1, 2016 to March 31, 2017, and presentation of three case reports in which carfentanil was attributed as cause or contributing cause of death by the pathologist.

**Methods:** Routine toxicological screening in blood was completed for opiates/oxycodone and cannabinoids via an enzyme linked immunosorbent assay (ELISA), volatiles by headspace gas chromatography with flame ionization detection (GC-FID), and a comprehensive drug screen by liquid chromatography with tandem mass spectrometry (LC-MS/MS). Carfentanil analysis was performed via a protein precipitation extraction with acetonitrile and LC-MS/MS on a Waters Acquity UPLC™ coupled to a Waters Quattro Premier XE™ tandem quadrupole mass spectrometer. Scope of analysis for fentanyl analogs included 8 other fentanyl analogs and opioid research chemicals. Method validation was completed according to in-house method validation standard operating procedures for quantitative analysis of novel psychoactive substances. Attributes assessed during validation included linearity, accuracy and imprecision, carryover, exogenous drug interferences, and ion suppression/matrix selectivity.

**Results:** From September 1, 2016 – March 31, 2017, 984 blood samples were analyzed for the presence of carfentanil. Carfentanil was detected in 341 of the cases resulting in a 34.6% positivity rate. Blood concentrations ranged from 10.2-2,000 pg/mL with a mean blood concentration equal to 175 pg/mL and a median blood concentration equal to 89.0 pg/mL. Positive specimens originated from 9 states – Florida, Illinois, Indiana, Kentucky, Michigan, Ohio, West Virginia, Tennessee, and Wisconsin. Three representative cases will be presented in detail to include case circumstances/history, autopsy findings, and toxicology.

**Conclusion/Discussions:** Carfentanil emerged as a drug of abuse/contaminant in street heroin in 2016 in the USA. The validated method proved to be accurate and reliable for the detection and quantitative analysis of carfentanil in blood specimens with 34.6% of all specimens analyzed for this drug showing positive results. Carfentanil mean blood concentration was very low (175 pg/mL). In each of the three cases discussed, carfentanil was certified as the cause or contributing cause of death by the pathologist.

**Keywords:** Carfentanil, LC-MS/MS, Fatalities

## A Study of the Illicit Drugs Situation in Hong Kong by Examination of Local Drug Seizures

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**Background/Introduction:** The planning and implementation of prevention and treatment programmes of a country/region associated with illicit drug use requires more specific information on its patterns of drugs use situation. While cannabis, amphetamines and opioids/opiates were among the three most commonly used drugs at the global level, usage of other illicit drugs may vary from one country/region to another. In particular, ketamine has becoming a major drug of abuse in Hong Kong, and that the emergence of new psychotropic substances (NPS) has also brought about concerns in recent years because of their vast diversity of chemical structures with little knowledge available about their harmful effects.

**Objective:** To study the analytical data obtained from drug seizures between 2011 and 2015 submitted by law enforcement agencies to reveal and discuss the illicit drugs situation in Hong Kong.

**Methods:** As the only designated laboratory responsible for illicit drug analysis in Hong Kong, all analytical results obtained from cases submitted by law enforcement agencies in 2011-2015 were included in the study. Identification and quantitation of drugs were performed using validated methods. If the presence of illicit drug(s) was confirmed, quantitative analysis would normally be performed. For NPS, as the purpose of examination was mainly for disposal, only qualitative analysis was performed.

**Results:** An average of 5334 cases with 20926 items per year was examined in 2011-2015. Ketamine was the most commonly encountered drug, contributed to 34% of the cases analyzed followed by methamphetamine (MA) (20%), heroin (14%), cocaine (13%), cannabis (7%), zopiclone (4%), midazolam (4%), NPS (2%) and nimetazepam (1%). While ketamine was the most frequently encountered illicit drug in the five-year period, a general decline in cases that found to contain ketamine. Also, a decreasing trend is also noted for heroin while increasing number of cases was found to contain MA and cannabis. The purity of MA remained high and rather constant with an average purity ranged from 96-97%. On the contrary, general decreases in purities for ketamine and cocaine, together with a slight increase in purity for heroin are observed.

Although there were only an average of less than 2% cases related to NPS examined in the 5-year period, about 10-fold increase in cases examined was observed from 2011 to 2015, and with a wide range of NPS encountered: 16 synthetic cathinones, 2 plant-based substances, 10 synthetic cannabinoids, 7 phenethylamines, 1 piperazine, 2 tryptamines and 1 ketamine analog. It is noted that substances related to cathinones (i.e. synthetic cathinones and plant-based substances containing cathinone/cathine) contributed to the majority of NPS cases, and that ethylone and methylone were the two most commonly detected NPS. Some NPS were transient in nature i.e. they appeared in a short period and subsequently disappeared in the case submissions. For instance, the synthetic cannabinoid, JWH-073, and the phenethylamine, 2C-E, were detected in 2011 but no case was found with these drugs since 2012 and 2013 respectively.

**Conclusion/Discussions:** The illicit drugs situation through examination of illicit drug seizures in Hong Kong revealed that ketamine, MA, heroin, cocaine and cannabis were commonly encountered. While purity of MA remained high, a general decline in the purity of ketamine and cocaine was noted. Although the proportion of NPS case submissions seemed to be relatively low, the NPS evolved constantly with successive structural modifications; that posed analytical challenge to devise and develop methodologies suitable for determination of the newly encountered NPS. In view of the large number of NPS as reported to UNODC (i.e. over 644 by Dec. 2015), it is envisaged that more new NPS with diverse or even unknown chemical structures would be encountered.

**Keywords:** Illicit Drugs, New Psychotropic Substances

## Methodical Approach for the Identification of Novel Psychoactive Substances (NPS)

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**Background/Introduction:** The number and volume of novel psychoactive substances (NPS) encountered by the Jefferson County Coroner and Medical Examiner's Office (JCCMEO) are increasing. Identifying these compounds is challenging and often requires more than the analytical resources housed within the laboratory.

**Objective:** In an effort to manage the evolving NPSs issue, our laboratory identified a key resource, a collaborative service offered by Cayman Chemical, to be used in conjunction with other tools including: combined in-house analytical chemistry skills and case information, professional networking, and monitoring of social networking and illicit drug user platforms.

**Methods:** The approach for identifying NPSs started with combining data collected from routine toxicological analyses with case information. General laboratory methods employed by our laboratory are immunoassay and gas chromatography mass spectrometry. Collaborations were with scientists at Cayman Chemical. Professional networking involved colleagues from other laboratories and members of SOFT. Social networking sites and illicit drug user platforms monitored included: Twitter®, Instagram®, Facebook®, Reddit.com, erowid.org, bluelight.org, tripsit.me, and blogspot.com to name a few.

**Results:** The methodical approach employed by the JCCMEO forensic toxicology laboratory started with combining known information (i.e. suspect/decedent history, investigator notes, medical examiner body diagram notes, etc.) with the analytical data (i.e. positive immunoassay screens, spectrum, molecular ion, retention time, etc.) obtained during drug screening. This combined information was used to narrow the potential unknown into a class of drugs. This information and data was then shared with colleagues around the country and ultimately submitted to scientists at Cayman Chemical. Through the collaborative efforts with Cayman Chemical, several structures were identified as possible matches. From mass spectral analyses by the toxicologists, the structures were narrowed down and a custom standard was ordered. Upon confirmation of retention time and spectral characteristics, the method was validated and the unknown was identified and quantified. For interpretation purposes, a search was performed in literature, on social networking internet sites, and on illicit drug user platforms. Information obtained from these sources varied but often consisted of obtained form (crystalline, powder, etc.), routes of administration, estimated dosing and duration of action, and a list of both desired and adverse physiological and psychological effects.

**Conclusion/Discussions:** Identification and interpretation of NPS abuse is challenging, albeit necessary, for forensic agencies; however, with the ever-changing scope, little scientific literature is available. To ensure proper identification and provide pharmacologically relevant information, toxicologists must be willing to use all available resources including but not limited to monitoring social networking and illicit user platforms, industry data sharing/networking, continued case study publications, and collaborations with reference material manufacturers.

**Keywords:** Novel Psychoactive Substance, Forensic Toxicology Resources, Drug Identification

## HighResNPS.com – an Internet Database for Liquid Chromatography - High Resolution Mass Spectrometry Screening for New Psychoactive Substances

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**Background/Introduction:** The number of new psychoactive substances (NPS) is constantly increasing which makes it challenging to keep the screening libraries updated with the relevant analytical targets. Liquid chromatography coupled High Resolution Mass Spectrometry (LC-HRMS) screening methods frequently utilize accurate mass of fragment ions for identification, in addition to retention time and accurate mass of precursor ions. The fragment ion information is obtained with data independent acquisition or data dependent acquisition. Both tend to generate similar fragment ions, when acquired with similar conditions.

**Objective:** We set out to build a crowd-sourced, free-of-charge mass spectral database for NPS that could be used by HRMS users, independent of instrument and software system.

**Methods:** The contributing laboratories defined their user profile with experimental setup and location. NPS data entries consisted of exact mass of protonated molecular ions with exact mass of the three highest intensity fragment ions; preferably corresponding molecular formulas, retention time on the defined system, International Chemical Identifier (InChI) key, and systematic chemical name (IUPAC). Furthermore, upload of acquired mass spectra and molecular structure was encouraged. The uploaded data and spectra were not reviewed, but users were encouraged to report any inconsistencies. The database could be viewed directly on the homepage, or exported as an Excel spreadsheet, making it compatible with most screening platforms after minor formatting.

**Results:** Currently, 11 users from 9 laboratories in 7 counties have contributed with 318 entries to the database with experimental data containing at least one fragment ion. 66% of the uploaded data were based on reference standards. Synthetic cannabinoids and their metabolites constitute more than 60% of the database and opioids and their metabolites account for around 15% of the entries. 74% of the entries in HighResNPS are present in the European Database on New Drugs (EDND) governed by the European Monitoring Centre for Drugs and Drug Addiction (EMCDDA) and 12% of the entries were metabolites. In total, the database contained 264 unique compounds with at least one fragment ion, covering 29% of EDND. Fifty compounds were submitted by at least two laboratories, where 98% (n=49) had at least one fragment ion in common, and 60% (n=30) had two or three fragment ions in common. The database contained 29 isomer groups; 70 compounds in total. Twenty isomer groups were distinguishable by at least one fragment ion. The majority of the non-distinguishable isomers were position isomers, such as ortho-, meta-, and para-methyl-amphetamine.

**Conclusion/Discussions:** . The overlapping entries of the database verify that similar fragment ions can be observed from identical compounds across different LC-HRMS systems. The inclusion of fragment ions from other labs can reduce false positive identifications, when no reference standard is available in-house. HighResNPS can serve as a useful add-on in LC-HRMS semi-targeted and non-targeted screening workflows in clinical or forensic toxicology cases.

**Keywords:** Toxicological Screening, New Psychoactive Substances, High Resolution Mass Spectrometry, Internet Database

## Deaths Caused by New Psychoactive Substances (NPS) in the Expert's Opinions for Medico-Legal Purposes

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**Background/Introduction:** The phenomenon of abusing new psychoactive substances (NPS) has resulted in numerous new problems emerging in various investigative fields. On the one hand, such issues are represented by analytical problems associated with newly emerging compounds; their currently available list is extremely extensive and according to the data presented by the European Monitoring Centre for Drugs and Drug Addiction (EMCDDA), it contains approximately 600 substances. On the other hand, there is a problem of understanding and gaining knowledge on the ways the xenobiotics affect the human organism and estimating the resulting social dangers. Among the emerging investigative fields, there is the analysis of fatalities in medico-legal expert opinion formulating. Although the fatalities do not pose a quantitative problem when compared to other poisonous substances (e.g. alcohol), yet, the gravity of the phenomenon in its social aspect must attract the attention of clinicians, opinion-issuing experts, toxicologists or lawyers.

**Objective:** Assessment of the effect of taking new psychoactive substances (NPS) in 16 selected cases from medico-legal practice.

**Methods:** The toxicological analysis aiming at quantitative evaluation in postmortem blood was performed employing chromatographic methods coupled to tandem mass spectrometry (GC-MS/MS, LC-MS/MS) following the isolation of SPE (solid phase extraction) from the biological matrix. The results provided the basis of determining the cause of death.

**Results:** In the course of the toxicological analysis, the authors demonstrated the presence of 13 psychoactive compounds belonging to various chemical groups, namely 4-methylethcathinone (4-MEC), bk-MBDB, methedrone, methcathinone, 3-methylmethcathinone (3-MMC), 4-chloromethcathinone (4-CMC), pentedrone, 5-MAPB,  $\alpha$ -pyrrolidinovalerophenone ( $\alpha$ -PVP), UR-144, XLR-11, AB-CHMINACA and U-47700.

The analyzed fatalities pointed to two categories of deaths:

- 12 cases of accidental deaths – the demonstrated NPS was a direct cause of death with the underlying mechanism of circulatory and respiratory failure;
- 4 cases of suicidal deaths – the demonstrated NPS was an indirect cause of deaths that included 2 cases of hanging, 1 case of jumping from a high altitude and 1 multiorgan trauma.

**Conclusion/Discussions:** The vast majority of deaths were noted in males aged 16-41 year-old and with a history of taking drugs. They were predominantly of an accidental character, while the suicide evoked by psychoactive substances was only one. The types of NPS determined in the presented fatalities confirm the dynamics of the designer drugs market, reflecting the current offers. The character of accidental deaths following NPS taking indicates the unpredictability of their effect on the human organism. In case of NPS, the cause-effect relations that include the relation between a toxic substance and its effect are complex and poorly understood. An easy access of young people to NPS leads to social damages that are difficult to estimate, such as destruction of personality, mental diseases, destruction of social bonds, entering the social margin, the ability to ruining health and finally bringing death.

**Keywords:** New Psychoactive Substances (NPS), Fatal Poisonings, Suicidal Deaths



## Untargeted Determination of Low-Dosed NPS in Blood Plasma by LC-high Resolution Mass Spectrometry

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**Background/Introduction:** The World Health Organization (WHO) continues to report abuse of new psychoactive substances (NPS) with a rapidly increasing number of highly potent analogues of traditional drugs such as LSD and heroin (World Drug Report, 2016). The high potency of these compounds results in very low blood concentrations being observed usually in the range of 0.1-10 ng/mL. Furthermore, NPS are being continuously modified to avoid legal consequences and to circumvent analytical detection. Therefore, highly sensitive methods need to be developed, which can easily be adopted to the latest NPS appearing on the market.

**Objective:** The aim of this work was to design a simple, robust, and fast method for simultaneous determination of 21 highly potent NPS from different classes such as 2Cs, NBOMes, LSD derivatives, and fentanyl derivatives in human blood plasma using universal sample preparation and an untargeted liquid chromatography-high resolution mass spectrometry (LC-HRMS) method. The method should also be validated in accordance to EMA guidelines for quantitative procedures.

**Methods:** Different extraction methods were tested for sample preparation. Finally, an established liquid-liquid extraction for blood plasma [1] was used. After adding methanolic internal standard solution (trimipramine-d3, 1 mg/L final concentration) and saturated aqueous sodium sulfate solution, the extraction was carried out with a mixture of diethyl ether and ethyl acetate (50:50, v/v), followed by evaporation, and reconstitution with methanol/2 mM aqueous ammonium formate containing 0.1 % formic acid (50:50, v/v, pH 3.4). A 5- $\mu$ L aliquot was injected onto the LC-HRMS system (Dionex Ultimate 3000, TF Q-Exactive Plus), running in alternating full scan (FS) and all ion fragmentation (AIF) mode. Identification used two specific AIF fragment ions in addition to the protonated molecule in FS. Quantification was carried out via FS data using an eight point calibration model (0.1-40 ng/mL).

**Results:** The extraction method was shown to be suitable for all tested NPS and is expected to allow also extraction of newly appearing compounds. The MS method allowed identification down to 0.1 ng/mL and quantification down to 0.25 ng/mL. The validation criteria were fulfilled for most of the tested compounds except for the NBOMe derivatives, one 2C-derivative and butyryl fentanyl, which failed at accuracy and/or precision or at the acceptance criteria for matrix effect, respectively.

**Conclusion/Discussions:** The developed method allowed, although designed as open and untargeted procedure, the reliable identification down to 0.1 ng/mL of 21 compounds, which should be sufficient to detect all analytes after recreational use in human blood plasma. Further analytes can easily be added at least for their identification. Additionally, the short turn-around time of the method allows the application within clinical routine.

**Keywords:** New Psychoactive Substances, Analytical Toxicology, Plasma Quantification

## In Vivo and in Vitro Metabolism and Detectability Studies on the New Psychoactive Substance 4-EA-NBOMe by Means of GC-MS, LC-MS<sup>n</sup>, and LC-HR-MS/MS

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**Background/Introduction:** *N*-(ortho-methoxybenzyl)-4-ethylamphetamine (4-EA-NBOMe) is a new member of the emerging group of NBOMe derivatives. The first NBOMes, such as 25B-, 25C-, and 25I-NBOMe, were based on a phenethylamine core structure (also known as 2Cs), while 4-EA-NBOMe is the *N*-methoxybenzyl derivative of 4-ethylamphetamine. So far, there are no studies on the metabolites of this compound. It could be assumed that 4-EA-NBOMe may lead to stimulating and hallucinogenic effects.

**Objective:** The aims of the presented work were to study the *in vivo* and *in vitro* phase I and II metabolism of 4-EA-NBOMe using rat urine and pooled human S9 fraction (pS9) incubations and its detectability in standard urine screening approaches (SUSAs) using GC-MS, LC-MS<sup>n</sup>, and LC-HR-MS/MS.

**Methods:** After application of 4-EA-NBOMe to a male Wistar rat for toxicological diagnostic reasons (10, and 1 as well as 0.1 mg/kg for metabolism and toxicological detection studies, respectively), urine was collected over 24h. The urine was treated with acetonitrile, evaporated, reconstituted (2-fold concentration) and analyzed for phase I and II metabolites by LC-HR-MS/MS (TF Q-Exactive Plus) according to Michely et al. [1]. For the detectability studies, authors' SUSAs by GC-MS (TF ISQ), LC-MS<sup>n</sup> (TF LXQ), and LC-HR-MS/MS (TF Q-Exactive) were applied to the two low dosed rat urine samples. Due to the lack of authentic human urine, additional incubations with pS9 (n = 2) were performed according to Richter et al. [2] to compare rat and human metabolism. Finally, initial CYP activity screenings with the described LC-HR-MS/MS method were performed to identify CYP isozymes involved in the initial steps.

**Results:** 4-EA-NBOMe was mainly metabolized by oxidation of the ethyl group to acetophenone, to benzoic acid, or to phenylacetic acid, by hydroxylation, and all combined with *O*-demethylation as well as by glucuronidation of the main phase I metabolites in rats. With exception of the oxidation to benzoic acid, all main metabolic reactions could be confirmed in the incubations with pS9. Intake of 4-EA-NBOMe was detectable only via its metabolites by all SUSAs after both low dose administrations in rats. Initial CYP activity screenings revealed the involvement of CYP1A2, CYP2C19, and CYP3A4 in hydroxylation and further oxidation, CYP1A2 and CYP2B6 in *O*-demethylation, and CYP2B6 and CYP3A4 in *N*-demethoxybenzylation.

**Conclusion/Discussions:** The study shows that 4-EA-NBOMe was extensively metabolized and that an intake could be detected by both GC-MS and LC-MS-based screening approaches. The main targets for screening should be the phenylacetic acid metabolite with and without additional hydroxylation and/or *O*-demethylation. Since several CYPs were involved in initial metabolic steps, interactions might not be expected.

1. Michely JA, Helfer AG, Brandt SD, Meyer MR, Maurer HH (2015) *Anal Bioanal Chem* 407:7831-7842
2. Richter LHR, Flockerzi V, Maurer HH, Meyer MR (2017) *J Pharm Biomed Anal* 143:32-42

**Keywords:** 4-EA-NBOMe, Metabolism, SUSAs

## In Vivo and in Vitro Metabolism and Detectability Studies on the New Psychoactive Substance 4-EA-NBOMe by Means of GC-MS, LC-MS<sup>n</sup>, and LC-HR-MS/MS

Caspar A.T.<sup>1</sup>, Westphal F.<sup>2</sup>, Meyer M.R.<sup>1</sup>, and Maurer H.H.<sup>1</sup>, <sup>1</sup>Department of Experimental and Clinical Toxicology, Saarland University, Homburg (Saar), Germany, <sup>2</sup>State Bureau of Criminal Investigation Schleswig-Holstein, Section Narcotics/Toxicology, Kiel, Germany

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**Conclusion/Discussions:** The study shows that 4-EA-NBOMe was extensively metabolized and that an intake could be detected by both GC-MS and LC-MS-based screening approaches. The main targets for screening should be the phenylacetic acid metabolite with and without additional hydroxylation and/or *O*-demethylation. Since several CYPs were involved in initial metabolic steps, interactions might not be expected.

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2. Richter LHR, Flockerzi V, Maurer HH, Meyer MR (2017) *J Pharm Biomed Anal* 143:32-42

**Keywords:** 4-EA-NBOMe, Metabolism, SUSA

## Two Hospitalizations and One Death After Exposure to Ortho-Fluorofentanyl

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**Background/Introduction:** Designer fentanyl analogues have caused concern in recent years due to their increasing availability and high potency, resulting in a high risk of accidental overdose and death. Fluorinated fentanyl derivatives are frequently seized, but reports on their clinical or postmortem toxicology are unavailable.

**Objective:** We describe two hospitalizations and one death after the ingestion of ortho-fluorofentanyl.

**Methods:** *Patient histories:* Two males in their early twenties were admitted to hospital after snorting a white powder. They both lost consciousness and respiratory function, but responded to cardiopulmonary rescue therapy and naloxone administration, and were discharged the following day in unremarkable condition. From one of the patients, a serum sample was drawn for drug analysis, and the white powder was also submitted to the laboratory. A few days later, one of the two males was found dead in his home. A snorting straw with crystalline deposits found at the scene, as well as femoral blood and urine samples collected at autopsy, were submitted for toxicological analyses.

*Analysis:* A drug screening by LC-QTOF-MS showed that the white powder as well as the crystals from the snorting straw contained fluorofentanyl. A specific search identified the same substance in the biological samples from the two men. To differentiate between ortho-, meta-, and para-fluorofentanyl (2-, 3- and 4-fluorofentanyl), the three isoforms were purchased and a LC-MSMS method employing a chiral column was developed that allowed the separation and quantitation of all three fluorofentanyl isomers.

**Results:** Specific analyses revealed that the white powder contained ortho-fluorofentanyl. The serum sample from one of the hospitalized males contained ortho-fluorofentanyl in a concentration of 2.5 ng/mL (6.9 nmol/L), whereas the whole blood and urine samples obtained from the other male at autopsy contained 2.4 and 3.9 ng/mL (6.8 and 11.0 nmol/L), respectively. Several other drugs were present in biological samples from both men, although not in toxic concentrations.

**Conclusion/Discussions:** In the absence of previous data, the evaluation of concentrations is difficult, but may possibly be interpreted in light of known toxic concentrations of the parent compound fentanyl. Due to the lack of other causes of death and the assumed high potency and possibility of lethal respiratory depression of fentanyl derivatives, we conclude that the death was caused by ortho-fluorofentanyl. This case illustrates that combining analyses of drug seizures or paraphernalia with analyses of biological samples is sometimes crucial to identify the culprit drug.

**Keywords:** Ortho-fluorofentanyl, LC-QTOF-MS, Postmortem

## Characterization of 8 Post-Mortem Cases Positive for U-47700

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**Background/Introduction:** In Finland, buprenorphine has long been the major cause of opioid overdose deaths, and synthetic cathinones have dominated the designer drug scene. However, in recent years some fatalities caused by synthetic opioids other than buprenorphine have been witnessed. U-47700, or 3,4-dichloro-N-[2-(dimethylamino)cyclohexyl]-N-methylbenzamide, belongs to a group of synthetic  $\mu$ -opioid receptor agonists originally developed by pharma industry in the 1970's. Although never used in medical treatment, the drug has recently gained publicity as a dangerous abused research chemical.

**Objective:** In this study we aimed to characterize death cases positive for U-47700 in terms of blood and urine concentrations, other toxicological findings, and demographic information.

**Methods:** In 2016, comprehensive post-mortem toxicology was performed in 6380 death cases representing about 13% of all fatalities in Finland in that time. In all relevant cases, screening for both traditional and new psychoactive substances in urine was performed by ultra-high performance liquid chromatography coupled with high-resolution time-of-flight mass spectrometry. After a positive screening result, U-47700 was quantified by gas chromatography-mass spectrometry.

In this register-based study, all cases positive for U-47700 were examined in terms of toxicological data, background information, and the circumstances of the death.

**Results:** There were 8 cases positive for U-47700 in 2016. All of the deceased were male and their mean (range) age was 26 (23-29) years. One of the deceased had no other psychoactive substance, besides U-47700, in his blood. In all other cases, other drugs were detected, such as amphetamine, cocaine, benzodiazepines and buprenorphine. The median (range) blood concentration of U-47700 was 0.25 (0.15-2.0) mg/L. In the mono-intoxication case, the U-47700 blood concentration was 0.27 mg/L.

**Conclusion/Discussions:** U-47700 has recently emerged on the drug market. It is readily available online and constitutes thus a serious health risk worldwide. In Finland, a series of 8 death cases positive for U-47700 in a short period of time is quite exceptional. Keeping analytical methods up-to-date with the ever-expanding variety of novel psychoactive substances is a growing challenge for all forensic laboratories. Co-operation between authorities and international communication enables the timely incorporation of relevant substances into the screening programs in clinical and forensic toxicology.

**Keywords:** Post-Mortem Toxicology, NPS, Synthetic Opioids

## Metabolism and Biliary Excretion of the Synthetic Cannabinoid 5F-PY-PICA by Human and Rat Hepatocytes, and Sandwich-Cultured Rat Hepatocytes

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**Background/Introduction:** Analytical strategies for detecting drugs in biological samples rely on information on metabolism and excretion. Such parameters are usually studied for therapeutic drugs, whereas data are sparse or absent for new psychoactive substances (NPS). The NPS 5F-PY-PICA was first reported as a synthetic cannabinoid on the European drug market in 2015, and is unique in having a pyrrolidine ring attached to the carbonyl linker. Incubation with suspension primary hepatocytes can identify analytical targets for toxicological screening, but does not provide information on the route of elimination and thus in which biological specimens to screen for these targets. Sandwich-cultured hepatocytes form functional bile canaliculi, making them suitable for *in-vitro* differentiation between basolateral and biliary efflux of analytical targets

**Objective:** The objectives of the study were to identify metabolites of the synthetic cannabinoid 5F-PY-PICA in pooled human liver microsomes (pHLM), pooled human hepatocytes (pHH), freshly isolated rat hepatocytes (fiRH), and sandwich-cultured rat hepatocytes (SCRH), and identify which of the analytical targets are eliminated into the bile. Furthermore, the metabolic pathways observed in the two species were compared.

**Methods:** Metabolites were identified after incubation of 5F-PY-PICA with pHLM, pHH, suspended fiRH, or SCRH after 3 days of culturing. Rat hepatocytes were harvested following a two-step perfusion protocol. The SCRH were prepared with fiRH between layers of rat-tail collagen. The cell culture medium was changed daily, and the presence of functional bile canaliculi was visualized by fluorescence imaging with 5(6)-carboxy-2',7'-dichlorofluorescein. After 3 days of culturing, 10  $\mu$ M 5F-PY-PICA was loaded onto the SCRH in culture medium for 3 or 24 h. Efflux of 5F-PY-PICA and the produced metabolites was initiated with the addition of either Hanks' balanced salt solution (HBSS) or HBSS without divalent cations. Removing divalent cations from the buffer opens the tight junctions between the hepatocytes, and releases the bile content into the buffer. Internal standard (IS)-corrected areas of 5F-PY-PICA and the main metabolites in the different HBSSs were used to differentiate between basolateral and canalicular efflux from the hepatocytes, and then to calculate the biliary efflux index. A two-sided t-test ( $\alpha=0.05$ ) comparing the IS-corrected areas in the different buffers from time points 5 and 15 minutes of efflux served to identify statistical significance in biliary efflux of the analytical target. The metabolites were identified and quantified using liquid chromatography – high resolution – mass spectrometry / mass spectrometry (LC-HR-MS/MS)

**Results:** The proposed metabolic pathways consisted of degradation of the pyrrolidine ring through hydroxylation, oxidation, and *N*-dealkylation. Also, hydroxylation of the indole moiety and alkyl side chain were observed, as well as oxidative defluorination with subsequent oxidation at the  $\omega$ -position, and combinations thereof. Phase II metabolites consisted of glucuronides and one glutathione conjugates. A total of 24 metabolites were identified across the investigated metabolic systems. The main metabolite in pHH was the defluoro-COOH- $\omega$ -5F-PY-PICA (M1), in pHLM it was the defluoro-HO- $\omega$ -5F-PY-PICA, whereas the main metabolite in fiRH and the SCRH was the COOH-pyrrolidine-5F-PY-PICA (M2).

The IS- corrected area of 5F-PY-PICA and its metabolites M1 and HO-indol-glucuronide-5F-PY-PICA (M3) 5F-PY-PICA, were significantly higher ( $p<0.050$ ) in the HBSS without divalent cations than in the HBSS

**Conclusion/Discussions:** The metabolic pathway of 5F-PY-PICA is consistent with reports on the metabolism of other pyrrolidinophenone-type psychoactive substances and synthetic cannabinoids. 5F-PY-PICA, M1, and M3 are proposed as analytical targets for bile analysis in forensic screening protocols. Significant biliary excretion of these targets indicates that biliary elimination can be important in the disposition of the synthetic cannabinoid 5F-PY-PICA. The metabolite M2 should be one of the main urinary targets of 5F-PY-PICA

**Keywords:** Hepatobiliary Elimination, Synthetic Cannabinoids, Sandwich-Cultured Hepatocytes

## Does “Bath Salts” Consumption Increase the Risk of Danger? Reporting Four Cases with Forensic Implications in Spain

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**Background/Introduction:** The use of recreational drugs, also called legal highs, has been increasing, particularly due to easy access via the Internet. In 2009 and 2010, a significant rise in the abuse of a new group of synthetic cathinones also known as “bath salts” was reported in Western Europe.

**Objective:** We present here four documented cases of exposure in Spain with a complete identification of substances in biological samples.

**Methods and Results:** All drugs and metabolites were detected using gas chromatography-mass spectrometry (GC-MS) and liquid chromatography-mass spectrometry (LC-MS-MS). **Case 1:** A 19-year old Caucasian male was arrested for assaulting his father during a family argument. He had gone to a night party and, according to him, did not ingest anything but half a bottle of rum and some beers. In a discotheque, he took a random drink a glass from a shelf and drank it. He described a feeling of elation, irritability and several episodes of amnesia which explained why he did not remember the argument with his father. He thought there was something poured in the drink because of the effects he experienced. According to his testimony he began cannabis use at the age of 17 with a daily consumption of 1 gram smoked and occasionally consumes what he called “MD”. 11-nor- $\Delta^9$ -tetrahydrocannabinol-9-carboxylic acid, methylone, and its metabolites were detected in urine. **Case 2:** A Caucasian, 33-year-old male was found hanged in his bathroom. There was no data of substance abuse or suicidal ideation. The medical examiner reported the cause of death as hanging and the manner of death was determined to be suicide. Alpha-pyrrolidinopentiophenone (alpha-PVP) and 4-methylcathinone (4-MEC) were found in blood and urine. Cocaine and its metabolites were detected only in urine. **Case 3:** A 53-year-old male went to the hospital explaining that he had inhaled a “white substance”. He remembered that he had taken money with the credit card but was unsure of the circumstances. At hospital arrival, he had palpitations, thoracic pain, and was agitated. He was treated with intravenous clorazepate. The case was defined as drug facilitating crime. The toxicological results showed alpha-PVP in blood (0.009 mg/L) and urine. Other toxicological findings were nordiazepam and lorazepam and related metabolites. **Case 4:** A 39-year-old Colombian male was restrained by the police due to the fact that he showed agitation and apparently persecutory psychosis and irrational behavior. The emergency services found him in asystole in the street. He was being treated with anti-retrovirals. Preliminary immunoassay analysis in urine made by the emergency service (Samur) yielded positive findings for amphetamines and benzodiazepines. The toxicological results from autopsy samples showed alpha-PVP in blood (1.20 mg/L) and urine along with cocaine, amphetamine, MDMA, lormetazepam, and alprazolam.

**Conclusion/Discussions:** The four cases described here bring to the attention the severe toxicity including behavioral abnormalities and self-injurious symptoms induced by easily accessible products such as synthetic cathinones. The analytical detection is a challenge faced by toxicologists due to the increasing number of compounds that appear constantly and the necessity of having modern laboratories with very sensitive and highly specific analytical instrumental techniques. The synthetic cathinones can be missed with a routine drug screening. Therefore, a thorough history obtained from the patients and relatives is important. Regarding the forensic implications, from these toxicologists’ point of view, there is obviously an increased risk of danger while consuming “Bath Salts”. This fact should be taken into consideration by authorities in order to adjust legislation with celerity to real life circumstances.

**Keywords:** Methylone, 4-MEC, Alpha-PVP

## Whole Blood and Urine Cannabinoid Levels After Smoking High-CBD Marijuana

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**Background/Introduction:** Cannabidiol (CBD) is a major constituent of marijuana that lacks the psychoactive effects of  $\Delta^9$ -tetrahydrocannabinol (THC), but possesses pharmacological activity which is explored for therapeutic applications. In Switzerland, only plant material with a THC content of 1% or higher is controlled by the narcotics legislation. High-CBD/low-THC marijuana products can be legally sold and their consumption has recently become increasingly popular in Switzerland. Due to the trace amounts of THC, the consumption of high-CBD marijuana might cause positive urine drug screen results and detectable THC blood levels potentially rendering the consumer non-compliant with abstinence requirements or unable to drive from a legal point of view.

**Objective:** Qualitative and quantitative analysis of cannabinoids in whole blood and urine after smoking high-CBD marijuana.

**Methods:** Two healthy subjects (one female, one male, regular tobacco smokers) participated in two experimental sessions, separated by three weeks, in which they either smoked one cigarette (ad libitum for up to 15 min) or four cigarettes (within 1 h) containing a 1:1 mixture of high-CBD marijuana (8.53% CBD, 0.43% THC, 0.01% CBN) and tobacco. Blood samples were collected up to 1.1 h and urine samples up to 27.8 h after the start of smoking. Urine samples were qualitatively analyzed for cannabinoids by enzyme immunoassay (EIA) screening on a Beckman Coulter AU480 analyzer using the Immunalysis HEIA Cannabinoids kit, with the P.I.A.<sup>2</sup> THC lateral flow immunoassay (LFA) test from Protzek and with the Drug-Screen-Multi 12Z test from Nal von Minden. Determination of CBD, THC and its metabolites 11-OH-THC and THC-COOH (quantitative) as well as CBD-acid A (qualitative) in whole blood and urine (200  $\mu$ L) was achieved by LC-MS/MS analysis following protein precipitation with acetonitrile and liquid-liquid extraction with butyl acetate, respectively. Urine samples were enzymatically hydrolyzed prior to extraction. Linear ranges were 0.5 – 20  $\mu$ g/L for CBD, THC and 11-OH-THC; and 2.5 – 100  $\mu$ g/L for THC-COOH.

**Results:** All urine samples tested negative by the three qualitative immunoassays (each using a 50  $\mu$ g/L cut-off for THC-COOH). Urinary CBD, THC, 11-OH-THC and THC-COOH concentrations were in the range of < 0.5 – 166.0  $\mu$ g/L, 0 – 1.5  $\mu$ g/L, 0 – 5.5  $\mu$ g/L and 0 – 12.9  $\mu$ g/L, respectively, as determined by LC-MS/MS. 11-OH-THC and THC-COOH were not detected in blood after smoking a single CBD-marijuana cigarette, whereas THC and CBD blood levels reached a maximum of 1.4 and 23.4  $\mu$ g/L, respectively. After smoking four cigarettes within 1 h, THC, THC-COOH and CBD blood levels reached a maximum of 2.1  $\mu$ g/L, < 2.5  $\mu$ g/L and 38.8  $\mu$ g/L, respectively, whereas 11-OH-THC was not detected. CBD-acid A, the biogenetic precursor of CBD, was detected in all blood samples as well as in urine within 30 min after smoking four cigarettes.

**Conclusion/Discussions:** The amounts of high-CBD marijuana smoked in this study did not cause any positive urine drug screen results for cannabinoids employing the common 50  $\mu$ g/L cut-off for THC-COOH. The highest observed THC blood concentration (2.1  $\mu$ g/L) was below the Swiss legal THC limit for driving which is set at 2.2  $\mu$ g/L (Swiss analytical cut-off of 1.5  $\mu$ g/L plus a confidence interval of 30%). In other countries enforcing lower legal THC limits, however, the THC concentrations observed in this study would deem proof of the consumer's acute impairment and inability to drive.

The cannabinoid blood and urine levels found after smoking high-CBD marijuana are currently being investigated further and more systematically.

**Keywords:** Marijuana, Cannabidiol, Drug of Abuse Testing



## The Application of Central Composite Design to Liquid-liquid Extraction Followed by LC-MS/MS Analysis for the Simultaneous Quantification of 14 Anti-hypertensive Drugs and Metabolites in Human Urine

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**Background/Introduction:** Arterial hypertension is a chronic disease with high prevalence worldwide (25%). According to the 2014 Annual Report of the American Association of Poison Control Centers, cardiovascular drugs are ranked in the top 25 of drug categories mostly involved in fatalities in the United States based on their National Poison Data System. The detection of anti-hypertensive drugs in biological matrices also plays an important role in compliance testing and in doping control.

**Objective:** To develop and validate liquid-liquid extraction (LLE) methods using central composite design (CCD) followed by LC-MS/MS to quantify 14 anti-hypertensive drugs and their metabolites (amlodipine, atenolol, bendroflumethiazide, bisoprolol, canrenone, doxazosin, furosemide, losartan, losartan-COOH, norverapamil, ramipril, ramiprilat, spironolactone, verapamil) in human urine.

**Methods:** LC-MS/MS: Agilent HPLC 1260 Infinity Series coupled to a G6420 triple quadrupole mass spectrometer utilizing both positive and negative electrospray ionization. Following optimization, reversed-phase chromatography was achieved on a Phenomenex Gemini C18 (150x2.0mm, 5 $\mu$ m) column, held at 40°C, using gradient elution with deionized water and methanol for mobile phases A and B (both supplemented with 0.1% formic acid). The total run-time was 20min. Dynamic multiple reaction monitoring (dMRM) mode was used to detect two transitions per analyte (quantifier and qualifier) and one transition for each internal standard.

LLE optimization using CCD: As the method included multi-class drugs with acidic, basic or amphoteric behavior, different pKa and different polarities, two LLE methods were optimized, one acidic and one basic. Firstly, pH from 1.5-4.0 and 10.0-13.0 was tested using formic, phosphoric and hydrochloric acids, and NH<sub>4</sub>OH and NaOH, respectively. The time of extraction (3-17min) and volume of solvent (0.35-1.00mL) were optimized using a two-level factorial, full factorial CCD. To a 2mL microcentrifuge tube the following was added: 100 $\mu$ L of urine, 100 $\mu$ L of a methanolic solution containing all drugs and internal standards (100ng/mL), 200 $\mu$ L of acid/base and 0.35-1.00mL of methyl-tert-butyl ether (MTBE). Tubes were vortex-mixed (3-17min, 2500rpm) and then centrifuged (5min, 13000rpm). The organic layer was transferred to a vial and dried under nitrogen at ambient temperature. The extract was reconstituted with 500 $\mu$ L of mobile phase A and 10 $\mu$ L was injected onto the LC-MS/MS.

**Results:** Acidic extraction: furosemide, losartan, losartan-COOH, ramipril and ramiprilat were extracted by acidic LLE (1.25M formic acid, pH2). The surface plots from CCD resulted in an optimum volume of solvent of 0.8mL and a mixing-time of 3min. Acceptable linearity (1/x weighting, R<sup>2</sup>>0.99, n=5) was obtained for all analytes using 7 calibrators between 10-2000ng/mL (50-2000ng/mL for furosemide) with an LOD=1ng/mL (losartan, ramipril) or 10ng/mL (furosemide, losartan-COOH, ramiprilat). Using quality control samples (QC1=30ng/mL, 150ng/mL for furosemide; QC2=800ng/mL; QC3=1600ng/mL), the following validation parameters were assessed: accuracy=89-105% (n=25), intraday precision (%CV)<15% (n=5) and inter-day precision <7% (n=5). Using QC1 and QC3, recoveries=96-108% for furosemide, losartan and losartan-COOH and 47% for ramipril and 13% for ramiprilat (n=10) and matrix effects ca.  $\pm$ 10% (n=10) were determined.

Basic extraction: the remaining drugs were extracted from basic urine (2.5M NH<sub>4</sub>OH, pH 13) with 1.3mL of MTBE and a 3min mixing-time. Acceptable linearity (1/x weighting, R<sup>2</sup>>0.99, n=5) using 7 calibrators (10-2000ng/mL) was obtained with LODs of 0.1ng/mL (bisoprolol, canrenone, norverapamil, verapamil), 1ng/mL (amlodipine) or 10ng/mL (remaining drugs). Using QC1-3, the accuracy was within 86-108% (n=25), and intra- and inter-day precision <15% (n=5) and <8% (n=5). Using QC1 and QC3 the average recovery (n=10) ranged between 82-116% (except atenolol, 16-19%) and matrix effects of -17-9% (n=10).

**Conclusion/Discussions:** The methods were successfully optimized using CCD and validated according to SWGTOX guidelines. The proposed method for determining urinary anti-hypertensive drugs and their metabolites showed adequate performance for assessment of clinical or forensic toxicological data.

**Keywords:** Liquid-liquid Extraction, Hollow-fiber Liquid-phase Microextraction, Central Composite Design

## Exploitation of Cancer Patient with Mystical Herbal Preparations: A Case Report

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**Background/Introduction:** Today, there is a growing interest in alternative and supplementary treatments as well as herbal products. Recently, undesirable and unexpected intoxications and adverse drug interactions of the products in question have been reported. These cases indicate that there is a need for strict regulations, particularly regarding the production, licensing, marketing and supervision of herbal products introduced to the market under the name of alternative and supporting plants. While some products are frequently advertised on web sites, others may even abuse societies' moral and spiritual values. Especially in a fatal disease such as cancer, products exploit people's hope by promising "mystic" recoveries. The prices of prepared herbal formulas are sometimes even more expensive than pharmaceuticals, which creates fraudulent circumstances.

**Case History:** A 40 years old female patient was admitted in 2016 to the General Internal Medicine Service in Istanbul due to high fever, fatigue and somnolence. She reported that she was diagnosed with myometrial sarcoma and had an operation in May 2016. Continuous neutropenia condition without taking any chemotherapeutic drug made the case suspect. After repeated interviews, the patient finally confessed that she had started using an herbal medicine that she saw on the internet during the radiotherapy session. Despite all the treatments she was given, her condition did not change, presumably, because of the formulation she used. After long explanations regarding to adverse effect, she finally gave her consent to give the herbal sample for a toxicological analysis by the Forensic Toxicology laboratory.

**Method:** Firstly liquid-liquid extraction (LLE) was carried out with ethyl acetate and the preparative thin layer chromatography was applied to the sample; then purified substance was analyzed by Gas Chromatography-Mass Spectrometry.

**Results/Discussion:** One of the major compounds found in chromatography was Inuviscolide which indicates the presence of *Inula viscosa* L. compatible with patient's statement. In herbal medicine, *Inula viscosa* L. has many properties such as anticancerous, antibacterial, cytotoxic and anti-inflammatory. This plant is recently used as "cancer herb" in Turkey individually without considering the type of cancer thoroughly. It is known that several compounds of *Inula viscosa* (such as alkaloids and sesquiterpene lactones, sesquiterpene acids, triterpenes, sterols, alantolactones, isoalantolactones, and flavonoids) have apoptosis inducing effects. Regular use of this plant extract possibly gave rise to myelosuppression and, apoptosis of neutrophils. Although any chemotherapeutic agents were administered to the patient, absolute neutrophil count found below 500, unexpectedly. As a conclusion, the use of *Inula viscosa* L. caused a severe medical conditions as well as a delay in the antitumor therapy, which unfortunately caused tumor growth.

**Keywords:** *Inula viscosa* L, Herbal Preparations, Intoxications, Fraud

## Toxicological and Clinical Findings from Presentations Involving Drug Intoxication at a UK Emergency Department

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**Background/Introduction:** The rise in the number and availability of novel psychoactive substances (NPS) compounded by increasing purity of some classical drugs of abuse and the abuse of prescription medication has resulted in complex and challenging clinical presentations. Within a UK emergency department (ED), current point of care tests do not accurately identify specific substances causing drug intoxications in patients.

**Objective:** To determine the range of substances present in patients attending a large tertiary ED within the UK and their associated clinical findings.

**Methods:** Patients presenting at the Glasgow Royal Infirmary ED between May-November 2015 were sampled. Patients included in the study were selected based on clinical suspicion or stated ingestion of NPS/drugs of abuse. Medical staff recorded age, gender and clinical symptoms such as the Glasgow Coma Scale (GCS).

Medical staff attempted to collect venous whole blood and/or urine. Biological samples were sent to Forensic Medicine and Science (FMS) at the University of Glasgow for toxicological analysis. A range of analytical techniques were employed for the identification of NPS, classical drugs of abuse (including ethanol) and prescription/over-the-counter medications.

**Results:** Between May-November 2015, 98 cases were received from patients. The age range of patients was 14-55 years (median 24, n=92) and the majority of the patients were male (85%). The clinicians recorded that 47% of cases may have involved poly-drug use. Synthetic cannabinoid receptor agonists (SCRAs) were the most commonly reported/suspected substance used (24%) followed by an "unknown" (23%) and MDMA (21%).

Following toxicological analysis at least one substance was detected in 95 presentations and only in 3 presentations were no substances detected. Poly-drug use was detected in 71% of presentations. The most commonly detected substances were ethanol (43%), diazepam and metabolites (27%), cannabis metabolites (21%), SCRAs (19%), MDMA and metabolites (17%) and cocaine and/or metabolites (16%). The designer benzodiazepines detected included etizolam (5%), phenazepam (2%) and diclazepam and metabolites (1%). In 3 individual presentations 25B- and 25C-NBOMe (n=1), methoxphenidine (n=1) and alpha-PVP (n=1) were detected.

The clinical findings of all presentations were reviewed. A Poisoning Severity Score (PSS) was calculated for the majority of patients (n=93). A PSS of 3 (graded clinically as severe) for central nervous system was determined in 20% of presentations with 70% of these involving poly-drug use. A PSS of 3 for cardiovascular system was determined in 2% of presentations. Both of these presentations involved SCRAs (5F-AKB48 and 5F-PB22 (n=1) and MDMB-CHMICA, 5F-AKB48 and 5F-PB22 (n=1)). A PSS of 3 for metabolic balance was determined in 3 presentations. In the first presentation no substances were detected, in the second 5F-AKB48 and 5F-PB22 were detected and in the third MDMB-CHMICA, 5F-AKB48 and 5F-PB22 were detected.

**Conclusion/Discussions:** This data provides insight into the range of substances responsible for acute intoxication in the Glasgow area. The study detected a range of substances including NPS, classical drugs of abuse and prescription/over-the-counter medication. As many of the presentations involved poly-drug intoxication (71%) it is difficult to match individual clinical symptoms with specific substances, however this study can be used as guidance for the type of substances which may be present and the clinical symptoms presented through poly-drug use and single drug use where the data allows.

**Keywords:** Clinical Toxicology, NPS, Drug Intoxications

## Lead Toxicity after Consumption of Weight Loss Supplements

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**Background/Introduction:** This clinical case report documents lead toxicity in a 41 year old female who traveled to India and bought tablets and powder at a local pharmacy for weight loss. She was instructed to take 1 tablet twice per day and 1 teaspoon (tsp) of powder at night. She took the treatment for 12 days with the onset of symptoms occurring approximately 1 week after the treatment regimen ended. The patient's symptoms included insomnia, abdominal pain, anorexia, lower limb myalgias and paresthesias with difficulty walking which subsequently progressed to upper extremity paresthesias. A whole blood lead level was requested. Analyses of the powder and tablets were also requested.

**Methods:** Blood (250 µL) was diluted 1 in 20 with an internal standard/nitric acid diluent prior to analysis. The powder (11.4 mg) was dissolved in concentrated nitric acid. Further dilutions of this solution were performed. These samples were diluted with deionized water with subsequent addition of an internal standard/nitric acid diluent. The tablet was weighed and crushed with 10.7 mg used for analysis. Both nitric acid and sonication were used to dissolve the crushed tablet. Further dilutions of this solution were performed. These samples were then diluted with deionized water with subsequent addition of an internal standard/nitric acid diluent. All samples were analyzed using a matrix matched calibration curve (whole blood or water as appropriate) on a Perkin Elmer NexION 300D Inductively Coupled Plasma – Mass Spectrometer (ICP-MS) in standard mode.

**Results:** Whole blood lead concentration was determined to be 5.10 µmol/L (106 µg/dL). The powder contained 0.7 mg/g of lead. By extrapolation, if the patient consumed 1 tsp of powder a day, 2.7 mg of lead was consumed daily, which translates to a weekly consumption of 18.9 mg, with a total lead consumption of 32.9 mg over the course of 12 days. Assuming the patient dissolved the 1 tsp of powder in 250 mL of water for consumption purposes, the water would have contained 11.0 mg/L of lead. The concentration of lead in the tablets was below the limit of detection of the method (1.0 µg/L) and is not included in this report. The patient was managed with chelating agents [intramuscular dimercaprol and intravenous calcium disodium ethylenediaminetetraacetic acid (EDTA)] over the course of 19 days. Approximately 5 months after the initial presentation the blood lead level remains elevated at 0.85 µmol/L (17.6 µg/dL). The patient's current main complaint is soreness at the dimercaprol intramuscular injection site.

**Conclusion/Discussions:** The lead concentration in the whole blood sample is substantially higher than the established normal range of <0.5 µmol/L (10 µg/dL). Even 5 months after the initial presentation and management with chelating agents the patient's lead level is still above normal. At one time the Joint Food and Agricultural Organization of the United Nations/World Health Organization Expert Committee on Food Additives (JECFA) published a provisional tolerable weekly intake of lead to be 25 µg/kg body weight. This value was later withdrawn and JECFA concluded it was not possible to establish a new threshold that would be considered safe. The patient described in this case report would have consumed more than 9 times that provisional level. According to the Canadian Guidelines for Drinking Water Quality the maximum allowable limit for lead in drinking water is 0.010 mg/L. For comparison purposes, assuming the patient dissolved the 1 tsp of powder in 250 mL of water for consumption purposes, the water consumed would have contained more than 1100 times the maximum lead allowed in drinking water. The literature contains other reports of elevated lead from consumption of herbal and Ayurvedic medicines. For example, Gunturu et. al. (2011) reported elevated blood lead levels in an adult female after consuming an Ayurvedic medicine and in 2016 the U.S. Food and Drug Administration recalled specific dietary supplements because of elevated lead levels. The case reported here adds to the literature of patients naively consuming uncontrolled dietary supplements and experiencing adverse effects.

**Keywords:** Lead, Toxicity, ICP-MS

## Urinary 11-nor-9-carboxy-9-tetrahydrocannabinol (THC-COOH) Concentrations in US Military Specimens Analyzed at the Forensic Toxicology Drug Testing Laboratory, Oahu, Hawaii (2012 – 2016).

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**Background/Introduction:** Marijuana is a Schedule I drug under the U.S. Controlled Substances Act. Marijuana use is prohibited for Federal employees and Army, Air Force, Navy, Marine Corps, and Coast Guard service members (SM), among other drugs of abuse. There are five Department of Defense (DOD) drug testing laboratories; one is located at the Tripler Army Medical Center (TAMC), Oahu, Hawaii. The TAMC forensic toxicology drug testing laboratory (FTDTL) primarily tests urine specimens collected from SMs stationed in the Midwestern and Western US including states and/or US territories in the Pacific (HI, AK, Guam) and countries in Asia and Middle East.

**Objective:** The study objectives are to compare overall marijuana positivity rates from 2012 to 2016, determine if the positivity rate is higher in states where cannabis has been legalized for medicinal or recreational use than states without approved recreational or medical marijuana use, and discuss whether reported urinary concentrations have increased over this time period.

**Methods:** Marijuana use is determined by confirming for 11-nor-9-carboxy-9-tetrahydrocannabinol (THC-COOH), a metabolite of delta-9-tetrahydrocannabinol (THC), by gas chromatography mass spectrometry. The DOD THC-COOH confirmation cutoff is 15µg/L. Positive THC-COOH results reported between 2012- 2016 were extracted from the TAMC FTDTL laboratory information management system. Blind proficiency specimens were excluded from the analysis. Data and statistical analyses were performed in MS Excel, GraphPad v.6.0, and MiniTab v.17.3.

**Results:** Between 2012 and 2016, TAMC FTDTL reported 13427 (0.30%) out of 4,481,462 specimens positive for THC-COOH with concentrations ranging from 15µg/L to 24,198µg/L. About 53.4% of the positive specimens were reported between 15 and 100µg/L, while 0.1% reported at ≥6001µg/L THC-COOH. The five-year period median concentrations (µg/L) were 89 (2012), 74 (2013), 83 (2014), 102 (2015), and 100 (2016) with positivity rates of 0.33%, 0.23%, 0.28%, 0.31%, and 0.37%, respectively. Median [range] THC-COOH concentration in specimens (n = 4998) collected from AK, WA, and CO was 94µg/L (15 – 24,198) compared to 79.5µg/L (15 – 6835µg/L) in specimens (n = 2084) collected from AZ, HI, CA. Median [range] THC-COOH concentration for specimens (n = 4921) collected in TX and OK was 87.0µg/L (15 – 19,207). Five year overall positivity rates among these three groups were: 0.5%, 0.3%, 0.7%, respectively. However, THC positivity rate was highest in specimens collected from TX and OK at 1.3% in 2016.

**Conclusion/Discussions:** Marijuana use in SMs stationed in states with medical marijuana is lower than those stationed in states with recreational marijuana. Although the overall THC positivity rate remained relatively low at 0.3%, the median urinary THC-COOH concentration has steadily increased with the highest THC-COOH concentration reported at 24,198µg/L. Increased THC-COOH concentrations could be attributed to marijuana cultivars bred with higher THC concentrations and SMs potentially eating edibles or smoking THC oil via e-cigarettes. We present here useful information that could assist clinical and forensic toxicologists in data interpretation. Higher urinary THC-COOH concentrations also prolong the window of detection, which is critical to the DOD drug-testing and deterrence program.

*Disclaimer: The views expressed in this abstract are those of the authors and do not reflect the official policy or position of the Department of the Army, Department of Defense, or the US Government.*

**Keywords:** Marijuana, U.S. Military, 11-nor-9-carboxy-9-tetrahydrocannabinol

## A Comparison of ELISA and Rapid LC-MS/MS Drug Screening Techniques for Urine Specimens

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**Background/Introduction:** Toxicology laboratories commonly employ immunoassay methodologies to screen urine specimens. These results are then used to direct further testing. Perceived advantages of immunoassays include reduced sample preparation times and lower costs when compared to confirmatory techniques. However, there are limitations when using this methodology that can affect the detection of a class of drugs in a sample. In addition, the number of immunoassay panels needed to encompass a broad drug screen may be cost and time prohibitive for routine screening. Due to these limitations, mass spectrometry screening approaches have gained favor in the toxicology field as a screening technique. When utilizing liquid chromatography tandem mass spectrometry (LC-MS/MS), minimized sample preparation can be achieved since extraction and derivatization steps are not needed. Broad drug panels can be created utilizing this methodology, which can make LC-MS/MS more cost and time effective for screening urine specimens when compared to immunoassays.

**Objective:** The objective of this study was to compare ELISA and LC-MS/MS screening techniques in terms of sensitivity, selectivity, and cost for urine human-performance cases that were submitted to the University of Miami Toxicology Laboratory.

**Methods:** A Dynex™ DS2® Automated ELISA System was used with Neogen® ready-to-use ELISA kits to screen the urine samples. Each sample was screened using up to 10 validated ELISA assays. The LC-MS/MS method uses 50 µL of a urine sample fortified with an internal standard (IS) mix that is then diluted with 50 µL of ammonium acetate buffer and 20 µL of deionized water. Enzymatic hydrolysis was performed for 15 min at 50° C with KURA BIOTEC® BGTurbo® β-glucuronidase solution. The sample was then transferred to a vial and injected on an Agilent 1260 Infinity HPLC coupled to a 6460 Triple Quad MS in positive electrospray ionization mode. Chromatographic separation was performed using a Poroshell 120 EC-C18 (3.0 x 50 mm, 2.7 µm) column with a gradient elution (Run time 12.2 minutes). Dynamic multiple reaction monitoring (MRM) was used to target 49 compounds.

The LC-MS/MS results of 54 authentic urine samples were compared to previously obtained ELISA results. In addition to the analytical results, a cost analysis was performed to further compare these two screening methods.

**Results:** The limit of detection for drugs in the LC-MS/MS method was between 2-20 ng/mL for more than 90% of the target compounds. In the 54 samples that were analyzed, 8 cases had low concentrations of drugs and/or metabolites that were not detected by the comparable ELISA assay, and an additional 11 drugs and/or metabolites were detected using the LC-MS/MS screen that would not have been detected by typical ELISA assay panel. However, in nine cases THCA was not detected by the LC-MS/MS method but was detected using ELISA. The cost to analyze one sample for one ELISA assay costs approximately \$1; to analyze one sample on a typical 10-panel immunoassay would cost approximately \$10. The cost to run one sample on the LC-MS/MS screen for all 49 compounds is approximately \$2.

**Conclusion/Discussions:** After comparing the cutoff concentrations of the screening methods, the LC-MS/MS has better sensitivity and can detect a larger array of drugs at lower concentrations when compared to ELISA. In addition, the LC-MS/MS panel encompasses more compounds; to achieve a similar drug panel for immunoassay, more than 20 different ELISA kits would be needed and this would drive the screening cost up to approximately \$20 per sample when the LC-MS/MS is approximately \$2. While the LC-MS/MS has a more expensive start-up cost when compared to the ELISA, the larger detection capability, sensitivity, and lower per sample cost can be useful for toxicology laboratories.

**Keywords:** ELISA, LC-MS/MS, Drug Screening

## Chiral Separation and Quantification of R/S-Amphetamine in Urine by Ultra-High Performance Supercritical Fluid Chromatography Tandem Mass Spectrometry

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**Background/Introduction:** Amphetamine is a central nervous system (CNS) stimulant that is abused worldwide. It is also used in the treatment of medical conditions such as attention deficit hyperactivity disorder (ADHD) and narcolepsy. The amphetamine molecule contains a chiral center and its enantiomers exhibit differences in pharmacological effects, with the S-enantiomer mediating a greater CNS stimulant activity than the R-enantiomer. In Norway, the majority of prescribed amphetamine consists of the pure S-enantiomer, but therapeutic formulations containing the R-enantiomer in various proportions are also available. Illegal amphetamine remains available mainly as a racemic mixture of the R- and S-enantiomers. To distinguish between legal and illegal consumption of amphetamine a reliable method for chiral separation is thus required. In this regard, supercritical fluid chromatography (SFC) has several potential advantages over UPLC, including rapid separation of enantiomers due to low viscosity and high diffusivity of supercritical CO<sub>2</sub>, the main component in the SFC mobile phase.

**Objective:** To develop a robust and specific ultra-high performance supercritical fluid chromatography tandem mass spectrometry (UHPSFC-MS/MS) method for enantioselective separation and quantification of R/S-amphetamine in urine, suitable for routine purposes.

**Methods:** Urinary samples (0.5 mL) mixed with 25 µL internal standard R/S-amphetamine-d<sub>3</sub> were diluted with 0.5 mL 0.05 M ammonium acetate pH 6 and extracted using a CX express 96-well plate (Biotage). The column was washed with 0.05 M ammonium acetate (1 mL) and methanol (1 mL). The analytes were eluted with 0.5 mL methanol/NH<sub>4</sub>OH (95:5, v/v). The eluates were added 10 µL 0.3 M HCl, evaporated to dryness under O<sub>2</sub> (Ultravap 40 °C) and dissolved in 100 µL 2-propanol. The analysis was performed on a Waters Acquity Ultra Performance Convergence Chromatography (UPC<sup>2</sup>) Xevo TQS. Separation was obtained using a Chiralpak AD-3 column (Chiralpak Technologies) with a mobile phase consisting of CO<sub>2</sub> (A) and 0.2% cyclohexylamine in 2-propanol (B). The gradient profile was 5-15% B in 0-0.2 min, 15-30% B in 0.2-4 min, 30% B in 4-5 min, 30-5% B in 5-5.5 min and 5% B in 5.5-6 min. The mobile phase rate was 1.3 mL/min, the column temperature was 30 °C and the injection volume was 2 µL. The make-up solvent (0.1% NH<sub>4</sub>OH in 2-propanol) flow rate was 0.3 mL/min. MS/MS detection was performed with positive electrospray ionization and two multiple reaction monitoring transitions.

**Results:** The additive cyclohexylamine was essential for the separation of R/S-amphetamine, but caused extensive ion suppression of the analytical signal. A sample concentration was therefore required and an extraction method was thus developed and validated. With the applied extraction method satisfactory signal at the lowest calibration level was obtained. In addition, the signal was also improved by lowering the desolvation temperature in the ion source from 650 °C to 250 °C. The calibration range was 50-5000 ng/mL and the limit of detection was 25 ng/mL. The between-assay relative standard deviations were in the range of 2.9%-7.6%. Recovery was 90% and matrix effects ranged from 100 to 104%. The method has been successfully implemented in routine use in our laboratory the past year for both separation and quantification of R/S-amphetamine in more than 500 urine samples.

**Conclusion/Discussions:** The UHPSFC-MS/MS method has proven to be a robust and specific tool for the separation and quantification of R/S-amphetamine in urine.

**Keywords:** R/S-Amphetamine, Urine, UHPSFC-MS/MS

**Drug and Substance Abuse in Refractory Epilepsy**

Raafat Abdeldayem\*, Maha Hazem

**Background/Introduction:** Seizures often occur in substance abusers.**Objective:** is to study the etiology of non-response to antiepileptic drugs by estimating their serum levels and screening of drugs and substance abuse in patients with resistant epilepsy.**Methods:** this study was conducted in epilepsy outpatient clinic, neurology department, Mansoura University Hospital. After exclusion those with organic brain lesion and who were not compliant to antiepileptic treatment, 924 patients with intractable epilepsy were included. They were subjected to:

- Toxicology screen for detection of drug and substances abuse by analysis of urine and blood samples.
- Measurements of the level of antiepileptic drugs in the blood (carbamazepine, valproic acid, phenytoin).

All assays run on the system use of homogenous immunoassay technique EMIT (Enzyme Multiplied Immunoassay Test) and confirmed by GC/MS (gas Chromatography/Mass Spectrum).

**Results:** Confirmed Positive results for drugs and substances abuse were detected in 246 of 924 patients (26.62%) by GC/MS. Cannabis was the first abused drug (29.27%), opiates was the second drug abused by patients (21.95%) followed by alcohol (17.88%), benzodiazepine (16.26%) tricyclic antidepressants (8.54%) and finally barbiturate constituted (6.1%). Only 17 patients show serum level of antiepileptic drugs (carbamazepine, valproate and phenytoin) within therapeutic range, but 169 patients' levels were below it and 60 patients with levels above it.**Conclusion/Discussions:** Substances abuse may be the cause of resistant epilepsy as they are epileptogenic by themselves or due to drug-drug interaction with the antiepileptic.*Recommendations*

- A screening test for drug and substances abuse is performed if drug abuse or withdrawal is suspected in patients with resistant epilepsy even if patients deny the use of them.
- To confirm the results of EMIT, further study is needed by using GCMS (gas chromatography mass spectrum) as it is more sensitive and more specific than EMIT system.

**Keywords:** Urine Samples, Blood Samples, EMIT, GC / MS



**Preliminary study for driving under the influence of Alprazolam, Clonazepam and Diazepam in Istanbul Title**

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**Background/Introduction:** Benzodiazepines are frequently prescribed drugs due to their effects on the central nervous system in the treatment of sleep disorders, anxiety and muscle pain. Moreover, addiction potential of these drugs makes them the most abused prescription drug group across the globe. The effects of benzodiazepines on human body impair road safety as well.

There are only a few studies on driving under the influence of drugs (DUID) and traffic accidents involving benzodiazepines in Turkey.

**Objective:** The aim of this study was to determine the prevalence of some benzodiazepines' usage among drivers in traffic accidents and its effects on driving ability. Blood alcohol concentrations of drivers were also investigated in addition to benzodiazepines.

**Methods:** Blood and urine samples were collected from injured drivers involved in traffic accidents at The Emergency Clinic of Haseki Training and Research Hospital, which is one of a pilot hospital in Istanbul. Sociodemographic form and case information of drivers such as age, gender, time of accident and admission to hospital etc. were recorded for the interpretation of results. For blood alcohol analysis, 2 mL whole blood was collected into tubes containing EDTA. Urine samples of drivers were also collected into sterile plastic containers. Whole blood samples were analyzed using Head Space/Gas Chromatography-Mass Spectrometry (HS/GC-MS), urine samples were stored at -80°C until analysis. Extraction method was also optimized for urine samples of drivers. Samples were hydrolyzed with 50 µL enzyme β-glucuronidase/arylsulfatase within pH at 5.5 (adjusted with acetate buffer 0.1M) at 55°C for 60 minutes prior to solid phase extraction (SPE) column (OASIS HLB 3CC, 60MG). Then, they were also derivatized with 50 µL BSTFA+1%TMCS in 40 µL of ethyl acetate/acetonitrile (1:1) for clonazepam determination.

**Results:** A GC/MS method was optimized for determination of clonazepam, diazepam and alprazolam in urine samples. Docosane was used as internal standard. Seven-points calibration curves were ranged from 2.5 to 60 µg/mL ( $R^2= 0.998$ ) for clonazepam-TMS, 1.25-30 µg/mL ( $R^2= 0.999$ ) for diazepam and 5-120 µg/mL ( $R^2= 0.999$ ) for alprazolam. Five of 29 blood samples were determined as positive for alcohol up to now. Besides, urine samples are being analyzed using the optimized extraction and GC/MS method, and results will be presented in the final presentation. At the end of the study, it is intended to reach 100 injured drivers for the research.

**Conclusion/Discussions:** Unfortunately, these drugs can be obtained easier than illicit drugs by means of multiple sources like doctors, pharmacists, family, friends and other illegal ways. Impaired driving involved prescription drugs are a public health problem in the World. With this study, it is aimed to create awareness about the potential risks of nonmedical use and abuse of benzodiazepines for drivers in Turkey.

**Keywords:** Benzodiazepine, DUID, Alcohol

**Finding a Needle in a Haystack: Detection of Designer Opioids and Benzodiazepines in DUID Casework**

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Due to their sedative properties and abilities to enhance effects of alcohol, opiates and benzodiazepines are the most frequently encountered drugs in DUID casework after cannabinoids. While commonly abused drugs such as morphine as a heroin metabolite, alprazolam, and clonazepam remain highly prevalent, designer counterparts (“designer opioids” and “designer benzodiazepines”) gained popularity in 2016.

The aim of this project was to assess which designer opioids and benzodiazepines have been reported in driving cases from the past 6 months. Additionally, this study addresses the importance of complete toxicology and step-wise approach.

From November 2016 through mid-April 2017, 7,156 blood cases were submitted to NMS Labs for the routine immunoassay screen (Immunoanalysis Corporation Direct ELISA kits) for DUID investigation. Of these, 11.4% (n=819) were also screened by LC/TOF-MS (an Agilent 1290 Infinity HPLC coupled with an Agilent 6230 TOF LC/MS) for expanded scope. Designer opioids and benzodiazepines were separately quantified using LC-MS/MS (a Waters TQD Tandem Mass Spectrometer with a Waters Acquity Ultra Performance LC System).

Of 7,156 cases examined, designer opioids were identified as out of scope findings from LC/TOF-MS screen and confirmed in 30 cases (0.42%). Furanyl fentanyl was the most identified compound (n=20), followed by carfentanil (n=4), 3-methyl fentanyl (n=4), butyryl fentanyl/isobutyryl fentanyl (n=3), para-fluorobutyryl fentanyl/FIBF (n=2), U-47700 (n=2), and acetyl fentanyl (n=1). Five cases had more than two compounds present. Although these cases were initially suspected for heroin intoxication based on scene investigation, case history, and observations, 77% (n=23) were negative for morphine; morphine was detected in seven cases (23%) with only two cases positive for 6-monoacetyl morphine. Evaluating additional findings, fentanyl was detected in eleven cases (37%), while five cases (17%) had no other finding. Cannabinoids (n=10, 33%), benzodiazepines (n=7, 23%), and cocaine and metabolite (n=4, 13%) were also found in combination with designer opioids. Blood concentrations (mean, median, and range [ng/mL]) for three most prevalent compounds were examined: furanyl fentanyl (37, 1.55, 0.11-710); carfentanil (0.94, 0.96, 0.41-1.4); and 3-methyl fentanyl (0.48, 0.25, 0.2-1.2).

The compiled data also revealed eleven cases (0.15%) with at least one designer benzodiazepine present. These cases were identified either by out of scope findings from LC/TOF-MS screen or mismatch between positive ELISA screen and negative LC-MS/MS confirmation for the routine benzodiazepine panel. Etizolam was reported in 45 % of these cases (n=5) with mean and median blood concentrations of 60 and 70 ng/mL (range; 19-100 ng/mL). Delorazepam and clonazepam were reported in three cases, followed by diclazepam (n=2) and flubromazolam (n=1). Of these, nine had additional illicit drugs on board at low concentrations or prescription drugs at the concentrations consistent with therapeutic use, whereas designer benzodiazepine was the only finding in two cases by both screen methodologies (ELISA and LC/TOF-MS). Reviewing OD/OD<sub>0</sub> (normalized Optical Density) of benzodiazepine ELISA screen data, all eleven cases were flagged as presumptive positive. This supports our in-house studies which demonstrated that etizolam, delorazepam, diclazepam, clonazepam and flubromazolam were all cross-reactive on the benzodiazepines ELISA kit at a concentration of at least 20 ng/mL. With a better understanding of designer benzodiazepine cross-reactivity on the routine benzodiazepine ELISA kit, two samples screened positive that were not confirmed and had no finding on the cases resulted in etizolam at 53 and 51 ng/mL.

Although detection of designer opioids and benzodiazepines in driving cases is not common, reporting of these designer compounds is significant especially for cases with no other findings. LC/TOF-MS and ELISA have the potential to identify the presence of designer compounds. Lastly, when routine toxicology testing is negative or does not support case history and observations, we must consider designer counterparts of the drugs of the same class and conduct additional specialized toxicology testing.

**Keywords:** Designer benzodiazepines, Designer opioids, DUID casework

## The Impact of the Illicit Fentanyl and Heroin Crisis on DUID Investigation Cases

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Often we hear that the current opioid epidemic is the biggest drug crisis in the United States. The numbers of heroin users tripled between 2007 and 2014. Concurrently, availability of illicit fentanyl became widespread due to low production cost and its high potency. White powder packaged and sold as heroin are commonly mixed with or replaced by illicit fentanyl. The illicit fentanyl and heroin abuse epidemic has reached the DUID population, resulting in a significant increase in fentanyl positive cases.

The goal of this project was to evaluate the fentanyl trend in DUID casework since 2011.

Between January 2011 and March 2017, a total of 11,968 DUID cases were analyzed at NMS Labs for the expanded drug toxicology screen. Blood samples were screened by ELISA and GCMS (an Agilent 6890N GC coupled with an Agilent 5975 MSD) or LC/TOF-MS (an Agilent 1290 Infinity HPLC coupled with an Agilent 6230 TOF LC/MS). Of the submitted cases, 486 (4.1%) cases were quantitatively confirmed for fentanyl and its metabolite, norfentanyl by LC-MS/MS (a Waters TQD Tandem Mass Spectrometer with a Waters Acquity Ultra Performance LC System).

Data analysis of confirmed fentanyl positive cases clearly demonstrated a dramatic fentanyl trend over a six year period. In 2011, fentanyl was reported as a top 25 drug detected in the expanded drug analysis of approximately 130 compounds with 1.39% positivity (n=18 of 1,296). Steady increase in fentanyl positivity rates were documented from 2012 (1.52%; n=38 of 2,501), 2013 (2.20%; n=43 of 1,954) through 2013 (2.55%; n=51 of 2,003). By 2014, fentanyl positive cases tripled to 4.32% (n=87 of 2,012) and fentanyl became the third most commonly reported compound after buprenorphine (5.25%) and desmethylertraline (4.69%). We observed another huge jump in 2016, where fentanyl was reported in more than 10% of the analyzed cases (n=177 of 1,753). Month-to-month changes in fentanyl, morphine and 6-monoacetylmorphine (6-MAM) confirmation prevalence since January 2015 to March 2017 were also examined. It revealed that morphine positivity peaked at 15.7% in July 2015, but has decreased to less than 10% in the last three months (6.54-9.59%). On the other hand, fentanyl is on a steep raise averaging 16% positivity in the first three months of 2017. Positivity for 6-MAM ranged from 1.85 to 4.59% with no apparent patterns or trends.

Of 336 confirmed fentanyl positive cases in the same last 27-months period, 71% (n=239) were found without morphine and 6-MAM (**A**), 22% (n=75) with morphine but no 6-MAM (**B**) and only 6.5% (n=22) with both morphine and 6-MAM (**C**). Mean and median fentanyl concentrations are the highest for **A**, followed by **B** and **C**. Blood fentanyl concentrations (mean, median and range [ng/mL]) were as follows: **A** (5.6, 4.4, 0.14-26); **B** (4.8, 3.8, 0.51-22); and **C** (4.3, 2.8, 1.1-11). These fentanyl positive cases were most frequently accompanied by cannabinoids (25% in **A**; 37% in **B**; and 50% in **C**), followed by benzodiazepines (18% in **A**; 29% in **B**; and 32% in **C**) and cocaine (6.7% in **A**; 32% in **B**; and 27% in **C**). Designer opioids (acetyl fentanyl, furanyl fentanyl, carfentanil, U-47700, butyryl fentanyl/isobutyryl fentanyl, para-fluorobutyryl fentanyl/FIBF and 3-methyl fentanyl) were reported in addition to fentanyl in seventeen cases. Lastly, methadone and buprenorphine, two of the most commonly prescribed medications for treatment of opioid addiction, were reported in 12 (3.6%) and 3 (0.89%) cases, respectively.

The high prevalence of fentanyl in drivers continues in 2017. Heroin users experiencing more sudden rush and intense high from fentanyl in tainted heroin is a major public health concern. DUID investigators also need to be more aware of fentanyl as an abused drug for suspected narcotic intoxication and ensure it is included in toxicology analysis.

**Keywords:** Illicit Fentanyl, Opioid Epidemic, DUID

## Changing Drug Prevalence in Drivers Killed in Motor Vehicle Accidents

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**Background/Introduction:** The prevalence of drugs in drivers has steadily increased over the last decade in Victoria, Australia. The pattern of drug use has also changed over time with new classes of drugs now emerging as potentially impairing substances for road users. While Victoria Police has been proactive in the enforcement of illegal drugs in driving for several years using both an impairment model and random roadside testing of oral fluid, there are a number of other drugs used by drivers involved in fatal collisions. These include analgesics (including opioids), anti-depressants and benzodiazepines.

**Objective:** The aim of this study was to review drug driver fatality data over a 7-year period from 2010 – 2016 for drivers killed in Victoria.

**Methods:** Blood was collected from deceased drivers and submitted for a full toxicological investigation irrespective of type or cause. In this study only cases that were on-road motor vehicle crashes were included. Crashes that occurred off-road, or those that were classified as natural or suicide were excluded. When a death occurred in hospital following a road accident, the relevant ante-mortem specimens were analysed. Toxicological analysis consisted of screening and quantification for alcohol (GC-FID) and a comprehensive range of prescription and illicit drugs (tandem LC/MS/MS) in femoral or ante-mortem blood. The legal limit for driving in Australia is 0.05 g/100mL (%).

**Results:** In total, 997 deceased drivers were submitted for full routine toxicology from 2010 – 2016. The prevalence of drivers found to be positive for alcohol or any drug was 50.7%. The number of alcohol positive drivers (blood alcohol concentration - BAC > 0.05%) decreased from 21.5% in 2010 to 19.5% in 2016. The lowest prevalence of alcohol ever recorded in drivers was 17.4% in 2015. The rate of stimulant positive drivers however increased from 5.9% to 19.5% over the same period. The number of drivers with benzodiazepines halved from 10% to 5.2%, while opioids and antidepressants remain unchanged over the 7-year period at around 8% and 12%, respectively. The number of drivers killed in Victoria with cannabis (D9-THC) over this period also remained relatively unchanged with 1 in 6 drivers positive for this drug (>2 ng/mL).

**Conclusion/Discussions:** On average, there are approximately 120-170 drivers killed in Victoria each year. Half of these drivers have been shown to be positive for alcohol or drugs. The prevalence of alcohol continues to decline while the number of drivers positive for stimulants and cannabis is of concern; especially the rate of increase for stimulants despite the introduction of random roadside testing in Victoria since 2004 for illicit drugs. The prevalence of stimulants in the driving population also reflects the increased consumption of stimulants (methylamphetamine) in Australia more broadly. Additional road safety strategies will need to be introduced to deter drivers from driving with illicit drugs. The proportion of drivers who lost their lives in Victoria with a BAC greater than 0.05% has declined from 38% in 1987 to 17.4% in 2015; this figure has been achieved through extensive random preliminary breath testing at the roadside and a community education program targeting alcohol consumption and driving. Systematic approaches to toxicology demonstrate that additional measures need to be adopted for the increase in stimulant positive drivers.

**Keywords:** Drugs, Blood, Postmortem

## Cannabinol and Cannabidiol Prevalence in Driver and Post-Mortem Populations in 2016

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**Background/Introduction:** In 2015, the Orange County Crime Lab added Cannabinol (CBN) and Cannabidiol (CBD) to their testing panel to include other common cannabinoids besides THC, Hydroxy-THC and Carboxy-THC. The presence of CBN in blood is associated with use of Marijuana plant products. CBD concentration can vary among THC products, but tend to be of higher concentration in products meant for medicinal use, especially for epilepsy treatment. Some studies have recognized the potential use of CBN and CBD as markers of recent use as they do not persist as long as THC in the blood.

To evaluate the prevalence of CBN and CBD within Orange County, CA, ante-mortem driver and post-mortem blood samples submitted from January 1, 2016 to December 31, 2016 were evaluated.

**Objective:** The objective was to determine how often Cannabinol and Cannabidiol were detected in driver and post-mortem blood samples tested for Cannabinoids by LC/MS/MS.

**Method:** A Tecan Freedom EVO 200 was used to pipet all samples, standards, and reagents. Prior to extraction the urine samples were hydrolyzed in thirty minutes using ICMS®  $\beta$ -glucuronidase. In a 96-well pretreatment plate, 150  $\mu$ L of blood or urine sample was added with 275  $\mu$ L of 0.1% formic acid and 25  $\mu$ L of deuterated internal standard for all five cannabinoids. A vacuum manifold applied sufficient vacuum to pull sample onto the sorbent where it sat for 5 min. The drugs were then eluted with 1.8 mL of hexane:ethyl acetate. After the elution solvent was evaporated, samples were reconstituted in mobile phase and injected on a Waters Acquity UPLC with HSS T3 column. The aqueous and organic mobile phases used were 100% water and acetonitrile each with 0.1% formic acid. The LC method consists of a 5 min gradient that elutes all drugs with baseline separation. A Waters Xevo-TQS collected MRM data for quantitation.

**Result:** A total of 3,183 Cannabinoid analyses were completed on samples submitted between January 1, 2016 and December 31, 2016. Of these exams, 2,204 were conducted on ante-mortem drivers and 410 on post-mortem blood samples. Among exams on driver samples, CBN was detected 25 times in concentrations ranging from 1.02 to 3.25 ng/mL, and CBD was detected 30 times in concentrations ranging from 1 to 79.5 ng/mL. CBN was detected 12 times in post-mortem blood exams in concentrations from 1.03 to 11.1 ng/mL and CBD was detected 5 times and ranged from 1.15 to 2.32 ng/mL. Table 1 summarizes the results.

Table 1. Prevalence and concentration ranges of Cannabinol and Cannabidiol in ante-mortem driver and post-mortem blood samples.

	Cannabinol		Cannabidiol	
	Drivers	Post-mortem	Drivers	Post-mortem
Prevalence	25	12	30	5
% Prevalence	1.13 %	2.93%	1.36%	1.22%
Concentration Range	1.02 to 3.25 ng/mL	1.03 to 11.1 ng/mL	1 to 79.5 ng/mL	1.15 to 2.32 ng/mL

**Conclusion/Discussion:** During the evaluation period, submitted samples had low occurrence of CBN and CBD in samples tested for Cannabinoids. CBD had higher concentrations in drivers than in post-mortem samples.

In some cases CBD was found in higher concentration than THC in the same sample while CBN was consistently found at concentrations below that of THC.

**Keywords:** Cannabinol, Cannabidiol, Cannabinoid Prevalence

## The Influences of Drug Abuse on Driving Ability—A Pilot Study of Simulated Driving

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**Background/Introduction:** : The issue of driving under the influence of drugs (DUID) has gained considerable international attention over the last several years. Epidemiological studies have produced varied results concerning the effects of drugs on crash. However, drug effects on driving remain the subject of considerable debate.

**Objective:** To evaluate the influences of drug abuse on driving ability by simulated driving parameters.

**Methods:** Forty volunteers were divided into two groups (each group has twenty). Group 1 was voluntary detoxification person who used methamphetamine just 3 to 5 hours before went to drug rehabilitation center; Group 2 was normal control. Each volunteers has a driver's license. To access driving performance, participants conducted simulated drives. The test road is a two-way four-lane, with non-motorized lanes, sidewalks and non-separated facilities. It contains straight line (3min driving time, 3km long), road area with traffic light control (500m at the intersection, 2 in the intersection), pedestrian crossing facilities (to test the driver to let the line) , Road central construction area (detection of driver avoidance behavior). The total length of the experiment is about 10min. Volunteers' blood and saliva samples were collected for laboratory analysis

**Results:** All the samples of group1 were positive for methamphetamine. The concentration of methamphetamine in the blood of group 1 was 1.8~276.2ng/mL, while The concentration of methamphetamine in the saliva samples was 0.9~154.8ng/mL. There was a significant difference between the two groups in the following aspects: Maximum speed, maximum speed; standard deviation of vehicle speed; standard deviation of lane offset; standard deviation of steering wheel angle; maximum throttle opening; maximum brake pedal strength; standard deviation / maximum longitudinal acceleration; standard deviation / maximum lateral acceleration.

**Conclusion/Discussions:** There is a significant difference between the drug addicts and the normal drivers in the event of a measure of driving stability and safety, and the driving behavior of drug addicts is generally radical.

**Keywords:** DUID, Methamphetamine, Simulated Driving, Driving Ability

## Whole Blood THC Concentration Decrease in One Hour Period Based on 27 Real Case Analyses

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**Background/Introduction:** Assessment of the influence of drugs on a driver's psychomotor performance is problematic in cases of drugs that undergo rapid distribution to the body's tissues, extensive metabolism, or rapid decomposition. Tetrahydrocannabinol (THC) is one of these problematic drugs. Blood from drivers is very often collected more than one hour from the time that the impaired driver is pulled over by the police. Unfortunately, blood collection sometimes takes more than three hours. In the case of accidents, the elapsed time may be even longer, sometimes reaching five or six hours. The elapsed time has a significant influence on THC concentration in blood due to distribution and metabolism. Based on case files and blood collection reports delivered to toxicology laboratories from region of south Poland, the mean time of blood collection in routine investigations was 2 hours and 40 minutes (median 2 hours and 20 minutes) and in road accidents was 3 hours (median 2 hours and 50 minutes). In this study, we investigate the effect that these delays have on blood THC concentrations.

**Objective:** We measure the decrease in blood THC in a group of 27 drivers from whom blood samples were collected initially ( $t=0$ ) and again an hour later ( $t=1h$ ). In 5 drivers, a third blood sample was collected 2 hours after the initial ( $t=2h$ ). Collection of multiplied blood samples in one hour period is allowed by polish law regarding certain type of road crimes.

**Methods:** We measure the decrease in blood THC in a group of 27 drivers from whom blood samples were collected initially ( $t=0$ ) and again an hour later ( $t=1h$ ). In 5 drivers, a third blood sample was collected 2 hours after the initial ( $t=2h$ ). Collection of multiplied blood samples in one hour period is allowed by polish law regarding certain type of road crimes.

**Results:** The decrease in THC concentrations at the various time points was recorded for THC concentrations greater than 2 ng/mL, at which the decreases exceeded the imprecision of the method. In two cases, THC concentration did not decrease. For THC concentration in the range of 2-3 ng/mL, the decrease in  $t=1h$  samples was in the range of 0.2-0.8 ng/mL, and for the range 3-5 ng/mL it was in the range of 0.2-1.0 ng/mL. In one case the concentration increased by 0.12 ng/mL. In all cases, THC-COOH concentration was in the expected range (1.1-58.6 ng/mL). The lowest decrease of THC concentration after 2 hours of initial sample was 0.48 ng/mL.

**Conclusion/Discussions:** The performed study urged us to comment on interpretation of THC level near the cut-off values. In our opinion, the retrospective calculations could be done only for whole blood concentrations higher than 2 ng/mL and the estimation should be limited to one hour period. Proposed calculations could be used for assessing whether one or two hours earlier (at incident time) THC concentration exceeded legal cut-off.

Based on the results, we conclude that for THC concentrations ranged from 2 to 5 ng/mL, the observed decrease is usually between 0.2 and 1.0 ng/mL.

**Keywords:** THC, Retrospective Study

## Low-Doses of Alcohol in Light Drinkers Who Drive a Simulated Motorcycle

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**Background/Introduction:** Alcohol use continues to be a prominent factor in motorcycle crashes. International statistics reveal a high incidence of low blood alcohol concentration (BACs  $\leq 0.05\%$ ) in motorcyclists involved in traffic crashes. Previous experimental studies showed that alcohol negatively affects selected cognitive and motor skills. However, the current understanding of BAC effects on motorcycle performance is insufficient. The present pilot study used a riding simulator to investigate the effects of low alcohol dosages on driving ability of light drinkers.

**Method:** Double blind, cross-over design. 24 light drinkers (13 females/11 males) aged between 24-42 years, all with valid driving licenses for at least 3 years, were recruited. The study was carried out through the Honda Riding Trainer (HRT) simulator and BAC were monitored by the Drager Alcotest 6510. Moderate amounts of alcohol were administered to participants – based on Hume-Weyers formula - during 2 sessions (with an interval of a week in between) of moped riding simulation. In both sessions, participants had to face some of the most frequent risky scenarios in on-road riding.

The HRT is a validated training instrument (Vidotto et al. 2011) providing the rider both good awareness of traffic situations and learning of safe behaviours: trained people are shown to increase some skills such as visuospatial attention and hazard avoidance which lead to crashes reduction.

Study was preceded by a single session of familiarization, almost a week before treatments.

Half of participants underwent the alcohol condition in the first session and the placebo condition in the second one (1<sup>st</sup> Arm); while the other half had the opposite sequence (2<sup>nd</sup> Arm). Statistical analysis was carried out on the basis of the HRT scores (A - D scale: with “A” corresponding to the best behavior of safety driving and “D” the worst: crash). The dependent variables were the differences in the number of specific HRT scores (delta) observed in the two sessions (GLM analysis). Considered results were: the number of crashes and the number of safely faced situations. Positive values indicated an increased frequency for that specific score.

**Result:** BAC levels were always well below (mean value: 0.3 g/L) the limit allowed by Italian traffic law (0.5 g/L). Mean values are reported below:

1 <sup>st</sup> Arm-ALCOHOL GROUP IN			2 <sup>nd</sup> Arm-ALCOHOL GROUP IN		
FIRST SESSION			SECOND SESSION		
FIRST SESSION (ALCOHOL)			FIRST SESSION (NON ALCOHOL)		
A	B+C	D	A	B+C	D
9.1	11.1	3.7	10.6	9.3	4
SECOND SESSION (NON ALCOHOL)			SECOND SESSION (ALCOHOL)		
A	B+C	D	A	B+C	D
9.9	10.6	2.5	7.9	12.3	2.8

Regarding driving behavior, the low BAC caused significant differences, with a reduction of safe behavior (“A” score) and an increase of risky behaviors (“B” and “C” scores) compared to non-alcohol session.

The number of crashes (“D” score) did not significantly differ between alcohol and non-alcohol group. Crashes decreased in the second sessions in both the Arms of the study.

The intermediate scores (B and C, indicating a situation faced in an unsafe way although without accidents) are greatly increased in the second session for the alcohol group [ $F(1,21) = 6.358$ ;  $p < .05$ ]. In other words: the delta in the number of safely faced scenes is significantly different between the two Arms [ $F(1,21) = 6.109$ ;  $p < .05$ ], showing an increase in the first Arm, while a reduction is observed for the other Arm.

**Conclusion/Discussion:** Low doses of alcohol in light drinkers produce measurable performance impairment in motorcyclists. The small number of participants, however, may be responsible of some not statistically significant data. Nevertheless, although the BAC reached was low (mean 0.3 g/L), an increased number of risky behaviors was recorded. The driving ability became significantly more dangerous, in particular in the group drinking during the second session of the experiment (2<sup>nd</sup> Arm), when subjects become more confident with the instrument, felt safer to operate them but they were influenced by alcohol. It happened despite the expected improvement in



driving skills derived by the growing confidence with the instrument: the HRT is validated and used to evaluate the learning ability to drive (training) and subjects were tested in a phase corresponding to the rising part of the learning curve. It can be assessed that alcohol counteracted and prevailed on the beneficial effects of training.

**Keywords:** Alcohol, Riding Trainer Simulator, BAC (Blood Alcohol Concentration)

**Recommendations for Toxicological Investigation of Drug-Impaired Driving and Motor Vehicle Fatalities – 2017 Update**

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**Background/Introduction:** Beginning in 2004, the National Safety Council (NSC) started investigating laboratory practices in driving under the influence of drugs (DUID) cases by looking at screening and confirmation scope as well as cutoffs in blood and urine matrices.<sup>1</sup> Based on the results of this investigation and a survey distributed to toxicology laboratories, recommendations were published in 2007 and updated in 2013. This report describes the latest round of updates to these recommendations.

**Objective:** The purpose of this update was to provide toxicology laboratories with a recommended standardized comprehensive testing scope for the most commonly encountered drugs in DUID and traffic fatality investigations, and provide appropriate screening and confirmation thresholds.

**Method:** These updates were based on data collected from a survey completed by 70 laboratories routinely performing DUID or traffic fatality toxicology, and administered under the auspices of the National Safety Council’s Alcohol, Drugs and Impairment Division (NSC-ADID). Laboratories were surveyed about their analytical practices, specifically with respect to matrices tested, scope, and cutoff concentrations for screening and confirmation. Subsequently, a consensus meeting with representatives from 24 laboratories with diversity with respect to agency type, testing volume and geographic location convened, reviewed and updated the recommendations using a modified Delphi process.

**Result:** As a result of the above process, screening and confirmatory thresholds were established or updated for blood, urine and oral fluid specimens for 35 drugs in the categories of cannabis, CNS stimulants, CNS depressants, and narcotic analgesics. The principal changes in this round of recommendations included moving butalbital, phenobarbital, and phencyclidine from Tier I (mandatory) to Tier II (optional), due to changes in prevalence. In addition, buprenorphine, fentanyl, tramadol, and their metabolites were moved from Tier II to Tier I due to increased prevalence and their known potential for causing impairment. Other additions, mostly novel psychoactive substances (NPS) were made to the list of Tier II compounds.

**Conclusion/Discussion:** Updates to the 2013 cutoffs and recommended test menu will be distributed by the NSC-ADID in 2017. Compliance with the guidelines will be continually monitored and updated regularly.

**References:**

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**Keywords:** Driving Under the Influence of drugs, Cutoffs, Guidelines

## An LC-MS/MS Method for the Analysis of Carfentanil, 3-Methylfentanyl, Furanyl Fentanyl, Acetyl Fentanyl, Fentanyl, and Norfentanyl in Postmortem and Impaired-Driving Cases

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**Background/Introduction:** In July of 2016, carfentanil (CF) emerged in Northeast Ohio resulting in 25 deaths within a 30-day period. All were related to the presence of CF either alone or in combinations with heroin and fentanyl analogs. Some fentanyl analogs like CF and 3-methylfentanyl (3-MF) do not cross-react with the Immunalysis ELISA fentanyl assay. According to the Centers for Disease Control and Prevention (CDC), a cause of death (COD) may be attributed to any amount of CF present in urine in association with an appropriate investigation history. Since then, a handful of driving under the influence of drugs (DUID) antemortem blood specimens have also tested positive for CF by reference laboratories. This contradicts the CDC guidance and prompted us to conclude that a quantitative confirmation of CF would be useful for distinguishing lethal and non-lethal concentrations.

**Objective:** Develop an LC-MS/MS method to identify and quantify fentanyl, the metabolite norfentanyl (NF), acetyl fentanyl (AF), furanyl fentanyl (Fu-F), 3-MF, and CF in 2 mL of sheep blood.

**Method:** Analysis was performed by LC-MS/MS using a Thermo Scientific™ UltiMate™ 3000 Rapid Separation LC instrument coupled with TSQ Vantage tandem mass spectrometer (Thermo Fisher Scientific, San Jose, CA). The chromatographic separation was achieved by using a core-shell technology Kinetex® F5 column (50 x 2.1 mm I.D., particle size 1.7 µm; Phenomenex, Torrance, CA) fitted with a 1.7 µm SecurityGuard ULTRA Cartridge UHPLC F5® (3 x 2.1 mm I.D., Phenomenex). The following mixtures were used as mobile phases: 0.1% formic acid in water (Solvent A) and 0.1% formic acid in acetonitrile (Solvent B). The optimized flow rate was 0.4 mL/min using a gradient elution with initial conditions of 95:5 (Solvent A:Solvent B) for 1 min, adjusting to 5:95 over 2 min, with constant flow for 0.25 min and returning to 95:5 at 3.4 min with a total run time of 5 min. The column oven was maintained at 40°C, and the autosampler was maintained at 5°C. The injection volume was 10 µL, and the autosampler needle was purged with acetonitrile/water (50:50) between injections to minimize carryover. Detection was achieved using a Vantage mass spectrometer equipped with a heated electrospray ionization source (HESI). Source conditions were the following: spray voltage 3 kV, vaporizer temperature 311°C, capillary temperature 150°C, S-Lens RF amplitude 54, sheath and auxiliary gas pressures 10. Argon was used as the collision gas. The ion transitions were monitored with a 0.4 *m/z* window in positive mode using multiple reaction monitoring (MRM). TraceFinder™ 3.1 software was used for both data acquisition and analysis.

**Result:** The limit of detection (LOD) for NF, AF, Fu-F, 3-MF and CF is 0.050 ng/mL and fentanyl at 0.50 ng/mL. Linear calibration curves with 1/x weighting in sheep blood were established between 0.10 – 4.0 ng/mL for AF, 3-MF, and CF, 0.20 – 4.0 ng/mL for NF and Fu-F, and 1.0 – 40 ng/mL for fentanyl. Low and high control accuracy data for blood indicates that all analytes have a bias < 20%. Intraday and interday reproducibility of < 20% (except AF, fentanyl and 3-MF were < 25%). Low and high control accuracy data for vitreous humor indicates that all analytes have a bias < 20%, intraday and interday reproducibility of < 20% (except low control AF < 23% interday).

**Conclusion/Discussion:** The sensitive method has been validated for blood and cross-validated for vitreous humor against a blood calibration curve according to the Scientific Working Group for Forensic Toxicology (SWGTOX) standard practices. A qualitative confirmation of these analytes has also been validated for urine and bile.

**Keywords:** Carfentanil, 3-Methylfentanyl, DUID

## Prevalence of New Psychoactive Substances in Belgian Drivers

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**Background/Introduction:** Driving under the influence of drugs (DUID) is a major worldwide problem, and as a result, has increasingly become a focus for law enforcement worldwide. In cases where roadside controls are performed, action against DUID often starts with an on-site immunological screening test focusing on classic illicit drugs. The Belgian DUID procedure is based on an on-site oral fluid (OF) test (Drugwipe<sup>®</sup>5S, Securetec) which focuses on cannabis, cocaine, amphetamines and heroin use. Thus, on-site detection of new psychoactive substances (NPS) and licit drugs is not yet established.

**Objective:** In a previous study presented at TIAFT Brisbane by our research group, the positivity rate of NPS (7%) was only established in blood samples of positively screened drivers. The major drawback was the lack of screening drivers with signs of recent drug use, but with a negative Drugwipe<sup>®</sup>5S (and thus maybe abusing other compounds such as NPS). The current study focuses on the prevalence of NPS in suspected drivers with a negative on-site test by analyzing Drugwipe<sup>®</sup>5S pads containing OF for (il)licit drugs and NPS.

### Methods:

Negative Drugwipe<sup>®</sup>5S tests (n=199, actually collected and analyzed) were conserved when signs of recent drug use were detected by police officers. Two of the three pads of these Drugwipe devices were soaked in 300 µL of methanol during 1 hour at room temperature. After centrifugation, 50 µL of IS solution (IS: methyl-clonazepam and β-OH-ethyltheophyllin) were added to 100 µL of the obtained supernatant, then subsequently evaporated and reconstituted with 100 µL of a mixture (80:20, v:v) of methanol/formic acid 0.1 % and ammonium formate buffer 5mM at pH 3. This solution was analyzed for NPS detection with 2 different chromatographic systems using 10 µL for method 1 (M1) and 15 µL for method 2 (M2). M1 is a LC-HRMS non targeted screening method (UPLC-Xevo G2-XS QTOF) and M2 is a LC-MS/MS MRM targeted method (UPLC-Xevo TQS).

### Results:

Twenty one out of the 199 (10.6%) Drugwipe<sup>®</sup>5S screening tests that were obtained from negatively screened drivers were positive for a NPS. Seventeen various NPS were detected (n) : mephedrone (6), ketamine (2), mCPP (1), 3-FPM (1), 5MeO-DALT (1), MDPV (2), 4-MEC (4), 4-FA (1), NEB (1), JWH020 (2), FUB-JWH018 (1), ethylphenidate (2), AM-694 (1), MXE (1), 5F-AKB48 (1), MDMB Chmica (1) and HU210 (1). Moreover, 21% of the samples contained a classical drug, 5% an analgesic drug, 0.5% a benzodiazepine/hypnotic, 2% an antidepressant, 3% an antipsychotic, 2% an antiepileptic drug and 1% methylphenidate.

### Conclusion/Discussion:

A high level of poly-drug use including combinations with NPS, licit and classical drugs was observed. The presence of NPS in the Belgian DUID population is proven with NPS positivity rates of 7% in blood and 10.6% in OF. More future research concerning the effects of combined drug use on driving ability and the physical/psychological signs after NPS use should be done to establish a better on-site detection of drivers under the influence of NPS by police officers.

**Keywords:** NPS, Oral Fluid, DUID

## Driving Under the Influence of....You Must Be Kidding!

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**Background/introduction:** The role of drugs in driving is a constant interpretative challenge in toxicology, not least due to the potential variety of drugs that could be involved, including poly-drug use and the issues of new psychoactive substances (NPS). A wide range of screening is imperative to encompass many possible drugs and knowledge of the latest abused drugs is an essential part of the investigative process.

**Objective:** The purpose of this presentation is to highlight specific instances of unexpected toxicological findings in road traffic deaths to highlight the potential involvement of new drugs, plant toxins and older drugs of abuse. Analytical and stability information regarding new hallucinogens will also be presented.

**Methods:** Analysis of post-mortem biological fluid was performed as part of routine casework using a combination of immunoassay (drugs of abuse), HPLC-DAD (basic, neutral and acidic drugs, general screening), LC-MS/MS (non-targeted and targeted MRM) and GC-MS/MS (cannabinoids).

**Results:** The vast majority of driving cases (both non-fatal and fatal) investigated by the laboratory involve alcohol, cannabis and/or cocaine or other stimulant drugs of abuse. However, in some selected instances of road traffic fatalities, unusual findings were detected in post-mortem samples. Case 1: driver veered into on-coming traffic; taxine alkaloids (yew tree), medical treatment drugs (ephedrine, atracurium, ketamine) present. Case 2: rider of scooter hit by another vehicle; heroin (morphine 512 ng/mL, M3G 2285 ng/mL, M6G 317 ng/mL, low codeine, noscapine, papaverine, 6-MAM, paracetamol), diazepam (0.99 mg/L), methadone (0.25 mg/L), pregabalin (2.4 mg/L), alcohol (urine only 25 mg/dL) found. Case 3: motorcyclist fell off on approach to roundabout; MDMA (~0.7 mg/L), THC-COOH (1.2 ng/mL), 4-MeO-PCP (1.5 mg/L), therapeutic codeine (<0.1 mg/L), paracetamol (<20 mg/L), ibuprofen, alcohol (urine only 28 mg/dL) found. Case 4: driver thought to have caused a multi-vehicle collision; THC (5.4 ng/mL) and apparent 1P-LSD detected. 1P-LSD could not be measured as it had degraded following screening and prior to quantitative analysis. Studies found significant instability of the drug.

**Conclusion/Discussion:** The presence of a plant toxin (taxine alkaloids) in Case 1 was completely unexpected but provided a likely explanation for the incident and potential contribution to death. In Case 2, the presence and concentrations of morphine, diazepam and methadone in particular could constitute potential fatal toxicity, yet the individual was involved in an unrelated collision with another vehicle and the drugs were not the cause of death. Case 3 and 4 involve relatively recent NPS; specifically, PCP and LSD hallucinogenic derivatives (4-MeO-PCP and 1P-LSD) which present a likelihood of impairment. The findings show that unexpected compounds may be involved in road traffic incidents with no initial evidence or suggestion of their use. Consequently, an analytical protocol for wider testing should be considered as well as the potential for ever-evolving NPS with a consideration of their stability. Even where use may be expected, the findings in relation to an alternative cause of death are somewhat surprising and toxicologists need to continue to be aware of those concentrations where drugs may not have directly caused fatal toxicity.

**Keywords:** Road Traffic, NPS, DUID

## Targeted Analysis of Sports Doping Compounds Using GC-MS/MS Technology

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**Background/Introduction:** The detection of use of performance enhancing drugs in sports competitions has been a challenge for the analytical chemist since the first reports for horse doping control in the beginning of the 19th century. Most modern methods involve chromatography- gas chromatography (GC) or liquid chromatography (LC) coupled to a mass spectrometer (MS). The GC-MS has been an important technique for doping control since the 1960s. The World Anti-doping Agency (WADA) regularly publishes a list of prohibited substances that need to be detected at a minimum concentration in urine; this is the Minimum Required Performance Limits (MRPL). The single quadrupole GC/MS has been workhorse of the doping control lab and has been used extensively for screening and confirming the presence of doping agents. While SIM analysis by GC-MS is sensitive enough for most doping agents it suffers from poor selectivity. Selectivity is especially important when analyzing compounds from urine matrix where many endogenous compounds can be present which are chemically and structurally similar and there is a high chance of similar fragments. Triple quadrupole GC/MS increases the selectivity of the analysis by eliminating the interfering matrix ions when run in Selected Reaction Monitoring (SRM) mode.

**Objective:** The objective of this presentation is to show sensitivity and selectivity advantages of the GC-MS/MS over the single quadrupole GC-MS. We also discuss the utility of software tools like AutoSRM and Timed-SRM in enabling easier method development for the user.

**Methods:** A simple sample preparation was processed in four steps: an enzymatic hydrolysis, liquid-liquid extraction, evaporation and trimethylsilylation. The instrumentation used was Thermo Scientific's TRACE 1310 Gas Chromatograph coupled to TSQ 8000 Evo GC-MS/MS system.

**Results:** We show that we are able to meet WADA's MRPL criteria for over 100 banned substances with lower limits of detection (LLOD) well below half of MRPL for majority of the compounds. We show the utility of GC-MS/MS in eliminating the noise from the interfering matrix ions, thus improving selectivity and reducing the chances of false positive identification. We also highlight the ease of obtaining optimum transitions using AutoSRM software. One of the most cumbersome parts of the SRM method development is assigning the dwell times for each of the transitions. Timed-SRM mode feature in TSQ 8000 Evo automatically optimizes the dwell times for the compounds such that each compound has the highest dwell time possible.

**Conclusion/Discussions:** We show that GC-MS/MS is a highly selective and sensitive technique for doping analysis and along with the use of software tools like AutoSRM and timed-SRM is a capable workhorse of the modern doping control laboratory.

**Keywords:** GC-MS/MS, Doping Analysis, Anabolic Androgenic Steroids (AAS), Triple Quadrupole

## Application of Q-TOF-based Metabonomics Techniques to Analyze the Changes on Rats' Plasma Metabolic Profile Following Death Due to Acute Intoxication of Phorate

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**Background/Introduction:** Organophosphorus pesticides (OPs) were widely used in the world and OPs poisoning is a major public health issue, especially for suicide by ingesting organophosphorus chemical in developing countries. Because its high effectiveness and low cost, phorate has been indiscriminately used in many areas of China, especially in the northern part of China. Identification of deaths caused by acute phorate intoxication is a major task for forensic scientists in China. The most common way of assessing exposure to organophosphorus pesticides is to detect their dialkylphosphate (DAP) metabolites in the urine or hair. The methods to analyze OPs and their metabolites in different tissues have been established. The toxicity of phorate has also been extensively studied, but the changes in metabolic profile after acute exposure to phorate have not been completely elucidated in the forensic literature. Therefore, there is need to identify novel biomarkers that can be applied to interpretation of the toxicological results.

**Objective:** To investigate the metabolic profile of acute phorate poisoning in rats in order to identify some biomarkers in plasma. Moreover, the study also investigated the dissimilar biomarkers induced by phorate at different doses. The novel biomarkers in rat's plasma after acute phorate intoxication were found on the basis of multivariable analysis. The results would contribute to new useful method of forensic toxicological investigation of phorate intoxication.

**Method:** The metabonomics of rat plasma at different doses of acute phorate intoxication was analyzed based on Q-TOF analysis. The original raw data were converted to a format of mzXML data by Waters xevo G2 Q-TOF MarkerLynx and preprocessed using XCMS. XCMS was employed for peak finding, peak filtering, peak alignment, and for reporting the mass to charge (m/z), retention time, and peak area in each sample. Using two dimension matrixes that includes the peak number (RT-m/z pair), sample name and ion intensity. Powerful multivariate analysis performs in principal components analysis (PCA) and partial least squares-discriminant analysis (PLS-DA) Using Simca-P 11.0. PLS-DA is used to reveal the net treatment effect on the subjects to detect the ions that have the greatest effect on the variance. And then differential variable was screened by S-Plot, Variable Importance on Projection (VIP) of PLA-DA variable model and loading plot. The identification of potential biomarkers was achieved by comparison with free online databases, such as the Human Metabolome Database (HMDB) (<http://www.hmdb.ca>), Metlin (<http://metlin.scripps.edu>), using exact mass and MS/MS spectra.

**Result:** A total of 11 metabolites were significantly changed in groups exposed to phorate at LD<sub>50</sub> and 3 LD<sub>50</sub> level compared with the control group. Plasma metabonomics analysis showed that DETP can be a distinctive biomarker of acute phorate intoxication. Uric acid, acetylcarnitine, succinate, gluconic acid and phosphatidylcholine (PC) (36:2) levels increased and pyruvate decreased in all groups exposed to phorate. ceramides (Cer) (d 18:0/16:0), palmitic acid and lysophosphatidylcholine (lysoPC) (18:1) levels were changed after 3 LD<sub>50</sub> dosage.

**Conclusion/Discussion:** DETP can be considered as a distinctive biomarker of acute phorate intoxication. It was confirmed that the levels of various metabolites were changed in phorate acute intoxication groups compared with control group in plasma analysis using LC-Q-TOF/MS. The changes of metabolites indicated that it may cause energy metabolism disorder especially TCA cycle after acute exposure phorate, as well as liver, kidney and nervous system functions. In addition, our study suggested that metabonomics is capable of representing the differences between the various dose of phorate intoxication. The dose-dependence relationship between apoptosis, fatty acid metabolism disorder and toxicity were more obvious. These findings will hopefully lead to the discovery of novel biomarkers that allow the identification of before death or after death invade body and interpretation of false positive results.

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**Keywords:** Biomarker, Metabonomics, Acute Intoxication

## Evaluation of Dried Blood Spot Sampling and UHPLC-HRMS for Large-Scale Screening of Drugs and Pharmaceuticals

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**Background/Introduction:** Dried blood spot (DBS) is an attractive solution with respect to invasive classical venepuncture by reducing cost and facilitating storage and shipment. In addition to the reduction of pathogenic risks, DBS also provides an efficient support for sample preparation prior to MS-based analysis. Nevertheless, working with low volumes of blood requires highly sensitive analytical strategies. The implementation of high resolution mass spectrometer (HRMS) hyphenated with ultra high performance liquid chromatography (UHPLC) could allow the development of highly sensitive large-scale methods of screening. Those kinds of methods hyphenated with DBS could be an asset for both clinical and forensic toxicology screenings.

**Objective:** The goal of this study was to develop a toxicology screening procedure using DBS and untargeted UHPLC-HRMS approach. A sensitive UHPLC-HRMS method has been developed and cross-validated with another MS-based approach used in our laboratory routine.

**Methods:** A UHPLC-HRMS method was developed for DBS analysis on a HRMS instrument (Q Exactive Plus). This method was developed using two 10  $\mu$ L DBS spiked with a representative panel of drugs and pharmaceuticals. A double organic solvent extraction procedure followed by a reconstitution in water was established in order to keep a single injection strategy. The samples were then analyzed in a full-scan data dependent acquisition (DDA) in positive polarity using an inclusion list currently containing around 1000 substances. A short switching to a targeted selected ion monitoring (Tsim) data dependent acquisition in negative polarity was performed for the specific detection of 11-nor-9- carboxy-delta-9-tetrahydrocannabinol (THC-COOH). The large-scale method developed with 142 substances is allowing the successful detection of wide classes of substances including new psychoactive substances (NPS). Forensic and clinical toxicology cases were then analyzed with this method and cross-validated with a published LC-MS based targeted screening procedures.

**Results:** Method evaluation was performed on a subset of 32 selected substances covering all classes of interest. Limits of detection (LOD) and identification (LOI) were evaluated as well as matrix effect, extraction recoveries and overall process efficiency showing good results. LOD was under 5 ng/ml for almost every substance tested and the extraction process showed good efficiency. Comparison of forensic and clinical toxicology cases analyzed with this method and a published targeted method used for routine analysis was performed. Overall there was a good correlation between the substances detected and identified allowing to cross-validate this method. Identification is performed using a combination of 4 criteria based on the exact mass ( $m/z$ ), the retention time, the isotopic pattern, and a library search for fragmentation spectra. The identification of any substance requires the combination of at least 3 of those 4 criteria. This large-scale method can be easily adapted to implement new potential substances released in the market.

**Conclusion/Discussion:** The recent development of highly sensitive HRMS allows the use of DBS sampling for large-scale toxicological screening, including the possibility of analyzing THC-COOH and NPS from few microliters of blood.

**Keywords:** DBS, Large-Scale Screening of Drugs, HRMS



## General Unknown Screening – Peak Extraction and Hit Optimization Using a Design of Experiment Approach

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**Background/Introduction:** General unknown screening (GUS) in biological matrices becomes more and more crucial in forensic toxicology with an ever growing number of new psychoactive substances (NPS) entering the drug market. Liquid-chromatography coupled to high resolution quadrupole time-of-flight mass spectrometry (LC-HR-QToF) provides a suitable analytical platform to meet this challenge. While data-dependent acquisition (DDA) approaches are still widely used, the greater possibilities provided by data independent acquisition (DIA) approaches are more promising for GUS. The disadvantage of DIA approaches is the huge amount of data produced with sometimes endless lists of possible hits. Consequently, software parameters (e.g. XIC widths, mass tolerance, threshold values etc.) helping in generating actual hits and minimizing useless data must be carefully optimized, to really take advantage of the infinite possibilities of DIA.

**Objective:** The aim was to optimize the algorithm parameters of the non-targeted peak finding option in PeakView® software using a design of experiment (DOE) approach. The factors with the largest effects should be identified and optimized in the fewest possible number of experimental runs.

**Method:** Blank whole blood samples were spiked with low or high concentration mixes of 22 DUID core substances covering authentic blood concentration ranges, respectively. Screening was performed on a Sciex TripleTOF® 6600 in SWATH mode coupled to a Thermo UltiMate 3000 HPLC (Synergi 2.5u Polar-RP 100A 100 x 2.0 mm). Cycle time was 1.6 sec including survey scan (100 to 1000 m/z) and 27 SWATH windows (Width: 25 Da; 140-800 m/z). Measuring was in positive ion mode and total run time was 25 minutes. Data analysis was performed with PeakView® 2.2. Optimization of parameters for the peak finding process was made using the DOE software MODDE Go (MKS Umetrics, Umea, Sweden).

**Result:** Before optimization, 15 out of 22 high concentrated compounds (HCC) and 10 out of 22 low concentrated compounds (LCC) were found in the very large number of possible peaks provided by the non-targeted peak finding option in PeakView®. After a simultaneous optimization of parameters (e.g. peak detection sensitivity, mass tolerance, signal width etc.) with MODDE, 19 out of 22 HCC and 11 out of 22 LCC were found. (22 of 22 with given chemical formula) with 10 times less peaks to check for.

**Conclusion/Discussion:** Compared to usual approaches in the laboratory, where typically only 1 parameter is changed at the very same time and consequences are noted, MODDE allowed to vary all relevant factors simultaneously. Thus, identification and optimization of factors with the largest effects in the fewest possible number of experimental runs was possible. It could be shown that the optimization provides a 30 percent better efficiency with more than 10 times less workload, thus helping in efficient evaluation of the DIA data. The DOE approach can be recommended for a lot of typical optimization tasks in the forensic laboratory.

**Keywords:** General Unknown Screening, High Resolution Mass Spectrometry, Design of Experiment Approach

## Ultra-Rapid Targeted Screening and Semi-Quantitation of 327 Basic and Neutral Drugs and Poisons in Post-Mortem Whole Blood by Scheduled MRM LC/MS-MS

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**Background/Introduction:** The number and diversity of medicinal and illicit substances used by the Australian public has continued to rise. This poses a challenge for toxicology laboratories involved in death investigations to cover as many drugs as possible in their systematic toxicological analysis. Likewise, the number of samples submitted to the toxicology laboratory has also increased, creating pressure on analysis turn-around times. While general unknown screening via high-resolution mass spectrometry may be one solution to identify a broad range of analytes in forensic casework, equipment cost or time-consuming data interrogation is prohibitive in high-throughput laboratories. In order to maintain a timely high quality toxicology service, an improved rapid technique for drug analysis was required.

**Objective:** The aim of this study was to develop a semi-quantitative analysis technique utilizing small sample volume, simple extraction procedure and single point calibration for semi-quantitation with automated data processing to yield rapid analysis turnaround times.

**Method:** In total, 327 of the most commonly used medicinal drugs or other drugs of abuse drugs in Australia were monitored for screening and semi-quantitation, including classes of amphetamines, benzodiazepines, betablockers, anaesthetics, analgesics, stimulants, opioids, anti-depressants, anti-psychotics, anticonvulsants, cannabinoids and multiple classes of novel psychoactive substances such as synthetic opioids, benzodiazepines, stimulants, hallucinogens and cannabinoids. Analytes were paired with 11 deuterated internal standards (morphine-d3, ephedrine-d3, methamphetamine-d9, MDMA-d5, zopiclone-d4, cocaine-d3, fentanyl-d5, haloperidol-d4, trimipramine-d3, diazepam-d5 and THC-d3). For extraction, 100uL of post-mortem whole blood was added to a 2 mL polypropylene tube, along with a standard mixture (either A or B, each containing one-half of the analytes included in the method). Samples were then mixed with 0.2 mL of 2M trizma buffer (pH 9.2) and extracted using 1mL chlorobutane:isopropanol (9:1) for 5 min using a multi-tube vortexer. Samples were centrifuged and the solvent layer transferred to autosampler vials and evaporated to dryness using nitrogen gas. Extracts were reconstituted in 200 uL mobile phase mixture and 20 uL was injected into the LC-MS/MS and analysed over 5 minutes (1min re-equilibration). Samples were separated using a Shimadzu Prominence HPLC system with a C18 column (Kinetex C18, 4.6×50mm, 2.6µm) using gradient elution with a mobile phase of 50 mM ammonium formate buffer (pH 3.5)/acetonitrile. The drugs were detected using a Sciex™ 3200 QTRAP™ mass spectrometer (ESI+, two MRMs per analyte).

### Result:

The method was fully validated in accordance with international guidelines. Matrix effects and extraction efficiencies were acceptable with most analytes showing >80% response and low variation (within 25% SD). Cannabinoids were most affected by matrix and yielded poorest recovery values. Linearity was acceptable ( $r^2 > 0.99$ ) for the majority of compounds. Precision, accuracy and repeatability calculations were performed using a one-point calibration, and found to be satisfactory for 78 analytes at all levels and parameters while 170 analytes were acceptable for all parameters at both medium (therapeutic) and high (toxic) concentration. Most analytes outside the acceptance criteria showed positive bias at low concentration (sub-therapeutic levels). Quality Assurance Program samples were re-analysed and compared to the program's reported results as well as results from the laboratory's existing validated method. All previously reported analytes were detected including additional drugs and metabolites that were not included in the laboratory's previous method such as, oxymorphone, asenapine, amlodipine, tapentadol, furanyl fentanyl and acetyl fentanyl. The method described has been successfully implemented for use in the laboratory's routine overnight analysis service for the analysis over 5000 cases per year.

**Conclusion/Discussion:** The fast and reliable extraction technique combined with rapid LC-MS/MS analysis of a broad range of medicinal and drugs of abuse using automated data processing allows for the opportunity of greater throughput, and decreased turnaround times with overnight, or the possibility of same-day, analysis results.

**Keywords:** Drugs, Blood, Postmortem

## Retention Time and Ion Mobility Collision Cross Section Prediction with Artificial Neural Networks for UHPLC-HRMS and UHPLC-IMS-HRMS Drug Screening

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**Background/Introduction:** Liquid-chromatography coupled to high-resolution mass spectrometry (LC-HRMS) is widely used in forensic laboratories, with targeted screening methods relying on reference standard materials for retention time (RT), and exact masses of precursor and fragment ions. Additionally, instruments with ion mobility enable the use of collision cross section (CCS) as an identification parameter. Keeping these targeted screening methods updated with the continuous addition of new psychoactive substances can be challenging, therefore approaches using shared or predicted reference values have been utilized with semi-targeted and non-targeted screening. Artificial neural networks (ANNs) allows for modelling of complex relationships between molecular descriptors (such as molecular properties, constitutional descriptors, and functional group counts) and reference values (RT/CCS).

**Objective:** The aim was to generate prediction models for RT and CCS based on ANNs. The objectives were to evaluate a previously developed ANN model for RT prediction on new data gathered under different LC conditions in a different laboratory; to develop and train ANNs for prediction of CCS; and evaluate if both RT and CCS could be predicted by a single model. Predicted reference values are expected to ease semi-targeted and non-targeted workflows, by adding additional parameters for selection and identification.

**Method:** Two datasets were used. Dataset I: RT reference values for 827 compounds acquired by LC-HRMS with gradient elution and a total runtime of 15 min. Dataset II: A 357 compound subset of dataset I, with CCS values for the proton adduct measured on an LC-Travelling wave ion mobility-HRMS system. 20% and 10% of dataset I and II, respectively, were retained for external validation of prediction accuracy. In total, four ANNs were trained and optimized: Model 1 on dataset I and model 2 on dataset II predicting RT; model 3 on dataset II predicting CCS; and model 4 on dataset II predicting both RT and CCS. During the ANN optimization, the dataset was split into training, verification and test set (70:15:15). Each ANN was optimized in four steps, based on the best performing network. Model 1 and 2 were based on previously selected descriptors; while feature selection was used to select relevant descriptors for model 3 and 4. Additionally, one variable least-squared regression was tested for prediction of CCS based on molecular weight (MW) and molecular refractivity (MR).

**Result:** All models were four-layered multilayer perceptron networks. Total correlation of predicted versus measured RT for the external test sets were:  $R^2 = 0.87, 0.81, \text{ and } 0.82$ ; with 61%, 56% and 53% with sub-minute errors, and 91%, 84%, and 84% within 2 minutes for model 1 (n=166), model 2 (n=506) and model 4 (n=506), respectively.

The total correlation of predicted versus measured CCS for the external test set were:  $R^2 = 0.96, 0.96, 0.94, \text{ and } 0.95$ ; with 61%, 61%, 53%, and 58% with less than 2% error, and 100%, 94%, 89%, and 89% with less than 5% error for model 3 (n=36), model 4 (n=36), MW (n=36), and MR (n=36), respectively. The most influential molecular descriptor in the prediction of RT was LogD (pH=3), and descriptors used for ANN prediction of CCS were based on MW, MR, and number of atoms.

**Conclusion/Discussion:** ANN improved overall prediction accuracy compared to the one-variable correlation of CCS values. Prediction accuracy of RTs was unsurprisingly best for model 1, likely due to larger training set. However, RT comparison of model 2 and 4, and CCS comparison of model 3 and 4, showed it was possible to predict both RT and CCS with a single ANN. While predicted reference values are no substitute for measured reference values, it can prove to be an effective filter, when a large number of false positives are observed in semi-targeted screening.

**Keywords:** *In-Silico* Prediction, Semi-Targeted Screening, Collision Cross Section

## Dried Blood Spots (DBS) Increase the Stability of Synthetic Cathinones

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**Background/Introduction:** Cathinones are beta-ketone amphetamine analogues that occur naturally in the *Catha edulis* (Khat) plant. Its effects include tachycardia and hypertension as well as psychoactive effects such as euphoria and increased alertness. Cathinone derivatives have been known and investigated since the late 1920s for their medical use (e.g. bupropion). Since the mid-2000s, synthetic cathinones have emerged as drugs of abuse worldwide, showing several intoxication and fatal cases as reported in literature on a regular basis. The poor stability of synthetic cathinones in biological samples represents a challenge to forensic toxicology laboratories.

The use of dried blood spots (DBS) has gained popularity as an alternative sampling procedure in both clinical research and forensic toxicology.

**Objective:** The aim of this work was to investigate the stability of synthetic cathinones (benzedrone, butylone, mephedrone and pentylone) in DBS, under different periods of storage and temperatures. Prior to the stability study, an LC-MS/MS method was developed and fully validated according to SWGTOX guidelines.

**Methods:** DBS sample preparation: analyte fortified whole blood was spotted onto a 6 mm diameter pre-punched Whatman 903 DBS paper cards disc. The blood spots were allowed to dry at room temperature for 3 hours before extraction. For stability study, low (75 ng/mL) and high (750 ng/mL) QC samples were prepared, stored in plastic bags with desiccants at 3 temperatures (room, 4 °C and -20 °C), for 0, 4, 7, 18, 25 and 45 days.

A DBS punch was placed in a 2 mL polypropylene tube. The extraction was performed with 300 µL of internal standard solution (MD-MA-d<sub>5</sub> 10 ng/mL in methanol). The tubes were vortexed for 10 min and centrifuged at 10,500 g x 5 min. The organic phase (200 µL) was transferred to autosampler vials, and 2 µL of injection onto LC-MS/MS system.

### Instrumentation

A liquid chromatography-tandem mass spectrometry system (Shimadzu LCMS8040) equipped with an electrospray ionization (ESI) source was used. The chromatographic separation was performed with a C18 column (Kinetex XB-C18, 50 x 4.6 mm, 2.6 µm, Phenomenex®), maintained at 40°C. The mobile phase consisted of ultra-pure water (A) and methanol (B), both of containing formic acid (0.1%, v/v). The mobile phase flow rate of 0.35 mL/min. Two MRM transitions were selected for each analyte.

**Result:** The method's limits of detection (LOD) and quantitation (LOQ) were 10 and 25 ng/mL, respectively. Linearity was obtained from 25 to 1,000 ng/mL for the four cathinones ( $r > 0.99$ ,  $1/x^2$  weighted linear regression). The precision (% RSD) between-run and within-run weren't higher than 6.6% and 5.8%, respectively, for all the QC, and the accuracy (bias) weren't greater than 5.8%. The matrix effect varied between 88.1-108.9% for low QC (75 ng/mL) and 83.4-94.3% for high QC (750 ng/mL). No carryover or interference of common pharmaceuticals and drugs of abuse were observed.

Pentylone, mephedrone and benzedrone weren't stable in DBS (average concentration fell outside of the ±20% range) at room temperature after 4 days of storage. For butylone, the concentration decrease to values more than 20% of the initial 7 days after DBS preparation. When storage at 4 °C, benzedrone's high QC wasn't stable after 45 days. All analytes were stable within 45 days of study at -20 °C.

### Conclusion/Discussion:

According to the literature, cathinones are reported as instable in whole blood, in some cases the analyte concentration drop dramatically in few days. Considering this scenario, dried blood spots are an excellent alternative, since it improves considerably the stability of these analytes, especially when stored at -20 °C.

**Keywords:** Dried Blood Spots, Synthetic Cathinones, New Psychoactive Substances

## A Simple Online Extraction LC/LC Atmospheric Pressure Ionization MS/MS Assay for the Analysis of 11 Cannabinoids and Metabolites in Human Plasma and Urine

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**Background/Introduction:** Cannabis is by far the most widely cultivated, trafficked, and abused illicit drug. Cannabis consumption is generally monitored by the detection of  $\Delta^9$ -tetrahydrocannabinol (THC) and its metabolites, 11-hydroxy-THC (11-OH-THC) and 11-nor-9-carboxy-THC (THCCOOH), in plasma or urine. Recently, the surge of marijuana legalization and the use of other cannabinoids such as cannabidiol (CBD) in preclinical, clinical and observational trials increased the need for sensitive and specific analytical assays to establish dose-effect relationships of THC and other cannabinoids.

**Objective:** We developed and validated an online extraction high-performance liquid chromatography coupled to tandem mass spectrometry (LC/LC-MS/MS) method for simultaneous quantification of 11 cannabinoids and metabolites including THC, 11-OH-THC, THCCOOH, THCCOOC glucuronide (THCCOOH-gluc), cannabinol (CBN), CBD, cannabigerol (CBG), cannabidivarin (CBDV),  $\Delta^9$ -tetrahydrocannabivarin (THCV) and 11-nor-9-carboxy-THCV (THCV-COOH) in human plasma and urine.

**Methods:** Human plasma or urine were spiked with the appropriated concentrations of cannabinoids and 200  $\mu$ L was extracted using a simple one-step procedure. Eight hundred  $\mu$ L of protein precipitation solution (methanol/0.2M ZnSO<sub>4</sub>, 7:3, v/v) containing internal standard mix at a concentration of 5 ng/mL was added. Samples were vortexed for 10 min and centrifuged (4°C, 26,000 g, 10 min). After centrifugation, supernatants were transferred into HPLC vials. The extracts were analyzed using LC-APCI-MS/MS in combination with online extraction. Two-hundred and fifty  $\mu$ L of the sample supernatant was injected onto the extraction column (4.6x12.5 mm, 5  $\mu$ m particle size, C8) and was washed with a gradient flow of 0.5-1.5 mL/min over 1 minute. The analytes were back-flushed onto the analytical column (4.6x50 mm, 2.7  $\mu$ m particle size, C18). Mobile phases were 0.1% formic acid and a mixture of acetonitrile:methanol:isopropanol (60:20:20). The HPLC system was interfaced with an API5000 MS/MS via a turbo V ion source operated in positive atmospheric pressure chemical ionization (APCI). The mass spectrometer run in the multiple reaction monitoring (MRM) mode.

**Result:** The method was validated according to FDA guidelines. Linear ranges were 0.39-400 ng/mL for THC, THCCOOH and CBD; 0.78-400 ng/mL for CBG, THCV, THCVCOOH, CBC and CBDV; 1.56-400 ng/mL for 11-OH-THC and CBN and 3.91-2000 ng/mL for THCCOOH-glu. Acceptance criteria for intra- and inter-batch accuracy (85-115%) and precision (<15%) were met for all compounds in all matrices except for THCV and CBDV in plasma for which the assay is considered semi-quantitative. Mean extraction efficiency ranged 60-103% in plasma and 94-101% in urine. Mean matrix effect ranged -17-7.9% in plasma and -4.6-11.7% in urine. No carry over was detected in both plasma and urine. To date, 352 plasma and 93 urine samples collected during clinical and observational studies in marijuana product users were analyzed with the presented methods.

**Conclusion/Discussion:** We present a validated, high-throughput, sensitive and specific method for quantification of eleven cannabinoids that can be used for clinical monitoring and research studies.

**Keywords:** Cannabinoids, Online extraction, Plasma

## The Usual Suspects: Quantifying Eight Common Drugs in Miami-Dade by LC-MS/MS

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**Background/Introduction:** The Miami-Dade County Medical Examiner Department (MDME) Toxicology Laboratory processes approximately 2500 cases per year, with many requiring multiple quantitative tests. A review of postmortem casework data determined that the most common drugs requiring quantitative analysis included morphine, codeine, 6-acetylmorphine (6-AM), benzoylecgonine (BEC), cocaine, cocaethylene, fentanyl, and alprazolam. In the past cases requiring quantitation of these drugs were assigned up to four different analytical methods, most requiring derivatization for analysis by gas chromatography tandem mass spectrometry (GC-MS/MS). With an increasing caseload due to the opioid epidemic in South Florida and the constant high volume of cases requiring quantitative analysis of cocaine and alprazolam, a new analytical approach was needed to reduce the needed sample volume, reduce the number of tests required, and decrease the turnaround time for these cases.

**Objective:** The objective of this work was to develop and validate a quantitative method for eight commonly identified drugs (morphine, codeine, 6-AM, BEC, cocaine, cocaethylene, fentanyl, and alprazolam) at the MDME for the purpose of reducing analysis time, sample and solvent volumes required for analysis, and overall case turnaround time.

**Method:** A Shimadzu Nexera X2 Ultra High Performance Liquid Chromatograph (UHPLC) coupled to a Shimadzu 8060 Tandem Mass Spectrometer (MS/MS) was used to develop and validate a method to quantify morphine, codeine, 6-AM, BEC, cocaine, cocaethylene, fentanyl, and alprazolam in postmortem biological specimens. The analytes and their deuterated internal standards were isolated from the biological specimens by solid phase extraction using mixed-mode columns. HPLC separation was achieved using a Restek Raptor biphenyl column at 50°C with a gradient elution at a flow rate of 0.650 mL/min. Positive mode electrospray ionization MS/MS analysis was performed using multiple-reaction monitoring. The total runtime of the method was 3.25 minutes with selective quantitation of each analyte within this time. The method was validated in accordance with published SWGTOX method validation guidelines.

**Results:** Bias, inter-day precision, and intra-day precision were evaluated for all analytes at low and high concentrations within their linear ranges and were determined to be  $\leq 5\%$  for all analytes. Extraction recovery from whole blood was also evaluated for all analytes at low and high concentrations within their linear ranges and was calculated to be  $\geq 85\%$  for all analytes. All extracted samples were determined to be stable on the autosampler for up to 72 hours. The table below details the limit of detection (LOD), limit of quantitation (LOQ), and linear range of the eight analytes as determined by the method validation.

Analytes	LOD	LOQ	Linear Range
6-AM	0.5 ng/mL	1.0 ng/mL	1-50 ng/mL
Morphine	5.0 ng/mL	10.0 ng/mL	10.0-500.0 ng/mL
Codeine	5.0 ng/mL	10.0 ng/mL	10.0-500.0 ng/mL
Fentanyl	0.5 ng/mL	1.0 ng/mL	1-50 ng/mL
Cocaine	5.0 ng/mL	10.0 ng/mL	10.0-500.0 ng/mL
BEC	20.0 ng/mL	40.0 ng/mL	40.0-2000 ng/mL
Cocaethylene	5.0 ng/mL	10.0 ng/mL	10.0-500.0 ng/mL
Alprazolam	5.0 ng/mL	10.0 ng/mL	10.0-500.0 ng/mL

**Conclusion/Discussion:** This quantitative method was able to combine four separate quantitative analyses into one comprehensive quantitative method. The required analysis time for these eight compounds decreased from a total of 1 hour 56 minutes to just 6.5 minutes, per case sample analyzed in duplicate. The required sample volume necessary for quantitative analysis of postmortem casework, performed in duplicate for each analysis, decreased from 8 mL to 1 mL per case. This method also lowered the LOD for these compounds compared to their previous methods, optimized their linear ranges, and eliminated the need for derivatizing reagents for analysis by GC. This method also helped in lowering the overall case turnaround time within the MDME Toxicology Laboratory at a time when it would have been expected to rise due to the opioid epidemic.

**Keywords:** LC-MS/MS, Method Development, Method Validation

## Liquid Chromatography Ion Mobility Quadrupole Time of Flight Mass Spectrometry (LC-QTOF-IM-MS) as an Alternative to Immunoassay and Gas Chromatography Mass Spectrometry (GC-MS) for Urine Drug Screening

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**Background/Introduction:** Comprehensive urine drug-screening (UDS) requires laborious immunoassay (IA) and GC-MS and/or LC-MS/MS analyses. IA screens are prone to false positives (FP) and false negatives (FN). A single GC-MS analysis can seldom encompass all drug classes.

**Objective:** Develop a LC-QTOF-IM-MS method as an alternative to IA and GC-MS for confirmatory UDS and assess its feasibility to concomitantly perform semi-quantitative confirmatory UDS.

### Method:

(a) Sample Preparation for LC-QTOF-IMS-MS: To 250  $\mu$ L of urine sample 250  $\mu$ L buffer (ESI+: 2M ammonium acetate, 0.02 M NaOH, ESI-: 2M acetic acid) and 500  $\mu$ L acetonitrile internal standard solution (nordiazepam-d5, morphine-d3, amphetamine-d8,  $\Delta$ 9-THC-COOH-d9) was added and vortex mixed for 1 min at room temperature. The sample was centrifuged for 1 min and upper layer was separated and dried under a stream of  $N_2$  at 45-50  $^{\circ}$ C. The residue was reconstituted in 100  $\mu$ L acetonitrile/water (10:90) and 10  $\mu$ L extract separated on ACQUITY<sup>®</sup> UPLC BEH C18 1.7  $\mu$ m, 2.1x50 mm column over 8.5 min using acetonitrile/0.1% formic acid and water/5mM ammonium formate/pH=3 gradient and analyzed on Waters<sup>®</sup> Vion IMS QTOF system on a ESI+ mode. Barbiturates and THC metabolites were detected on ESI- over 7.5 min on an ACQUITY<sup>®</sup> UPLC HSS C18 1.8  $\mu$ m, 2.1x150 mm column.

(b) Retention time (RT, min), drift time (DT, ms) and collisional cross section (CCS,  $\text{\AA}^2$ ) were determined for 140 prescription drugs, metabolites and illicit drugs.

(c) N=40 urine samples with clinically ordered comprehensive UDS (IA (Roche Integra)+GCMS) were analyzed by LC-QTOF-IM-MS. GC-MS method used an extraction buffer (pH=9.5) and ~1-5 mL urine sample.

(d) Serial dilutions of multi-drug control material from UTAK<sup>®</sup> (Part name: PM100, BenzoPLUS, DAU High Cut-off) were used as calibrators and Liquicheck<sup>®</sup> solutions from Biorad<sup>®</sup> (Part Name: 461-S1, 462-S2, 463-S3, 466-S1 low opiate) were used as QC samples to assess feasibility to concomitantly perform semi-quantitative confirmed UDS. Analytical measurement range (AMR) and (inter, intra assay) imprecision and accuracy were determined.

### Result:

(a) LC-QTOF-IMS-MS (~5-6 min/sample prep, 8.5 min (ESI+) + 7.5 min (ESI-)) than for GC-MS (~15-20 min/sample, 17.5 min). A chromatographic run time of 15 min and a longer LC column offered no advantage on LC-QTOF-IM-MS ESI+ method. GC-MS needed manual interpretation whereas LC-QTOF-IMS-MS generated an automated report that can be potentially interfaced. Isobars including morphine and hydromorphone ( $M+H^+$ : 286.1346) and codeine and hydrocodone ( $M+H^+$ : 300.1593) were separated on LC.

(b) Variability in drift times and CCS were  $\pm 2\%$  across multiple injections for most compounds studied.

(c) LC-QTOF-IMS-MS detected more true positives compared to IA+GC-MS. LC-QTOF-IMS-MS detected 60 more compounds (example: fentanyl, hydrocodone, designer amines, benzodiazepines, glucuronides) that were missed by IA+GCMS. THC use could be confirmed in all THC-COOH positive samples by IA (cut off: 50 ng/mL) by LC-QTOF-IM-MS method. GCMS screen could not detect THC-COOH due to a basic extraction pH mandating a separate THC-COOH-specific analysis for cannabinoid exposure confirmation.

(d) LC-QTOF-IMS-MS semi-quantitation was acceptable for benzodiazepines, opioids and benzoylecognine. Accuracy (%recovery): 85-120%; AMR: nordiazepam = 37.5-1000 ng/mL, morphine = 37.5-2500 ng/mL, benzoylecognine = 37.5-750 ng/mL and amphetamine = 500-2000 ng/mL. Amphetamine and methamphetamine sensitivities were difficult to improve due to in-source fragmentation. %CV for intra and inter assay imprecision were acceptable: nordiazepam: 4-14%; Morphine: 8-18%, benzoylecognine: 7.5-14%.

**Conclusion/Discussion:** LC-QTOF-IMS-MS detected several clinically relevant drugs and metabolites that IA+GC-MS missed in clinical samples. LC-QTOF-IMS-MS is more sensitive and specific for all clinically relevant drug classes than IA+GCMS and can perform semi-quantitative urine drug confirmation in a single step

**Keywords:** Ion-mobility-mass-spectrometry, Urine Drug Screen, LC-QTOF-IM-MS

## Ionic Liquid-Based Dispersive Liquid-Liquid Micro-Extraction Combined with LC-MS/MS Analysis for the Quantification of Benzodiazepines in Whole Blood Forensic Cases

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**Background/Introduction:** To date, thorough clean-up of complex biological samples remains an essential part of the analytical process. The solid phase extraction technique is the well-known standard, however, its main weaknesses are the labor-intensive and time-consuming protocols. In this respect, dispersive liquid-liquid micro-extractions seem to offer less complex and more efficient extraction procedures. Furthermore, ionic liquids – liquid salts – are emerging as new promising extraction solvents, thanks to their non-flammable nature, negligible vapor pressure and easily adaptable physiochemical properties.

**Objective:** In this study, we investigated whether ionic liquids can be used as an extraction solvent in a dispersive liquid-liquid micro-extraction procedure for the extraction of a broad range of benzodiazepines and benzodiazepine-like hypnotics in whole blood samples.

**Method:** One mL whole blood was transferred into a conical glass tube. Subsequently, 1.0 mL aqueous buffer pH 8.0 and 60 µL ionic liquid (1-butyl-3-methylimidazolium hexafluorophosphate) were added to the blood sample. The glass tube was mixed for 5 min at 50 rpm. Phase separation was induced by centrifugation for 6 min at 3500 rpm. 10 µL of the lower ionic liquid layer was collected and diluted 1:10 in MeOH. The final extract was analyzed using LC-ESI(+)-MS/MS analysis in scheduled MRM scan mode.

**Result:** The optimized analytical method was successfully validated for 7-aminoflunitrazepam, alprazolam, bromazepam, clobazam, clonazepam, clotiazepam, diazepam, estazolam, ethyl loflazepate, etizolam, flurazepam, lormetazepam, midazolam, oxazepam, prazepam, temazepam, triazolam, zolpidem and zopiclone. The method showed good selectivity for endogenous interferences based on 12 sources of blank whole blood. No benzodiazepine interferences were observed, except for clorazepate and nordiazepam, which were excluded from the quantitative method. Matrix-matched calibration curves were constructed covering the whole therapeutic range, including low toxic plasma concentrations. Accuracy and precision results met the proposed acceptance criteria (bias, repeatability and intermediate precision < 15 % or < 20 % at LOQ) for the vast majority of compounds, except for brotizolam, chlordiazepoxide, cloxazolam, flunitrazepam, lopraxolam, lorazepam and nitrazepam, which can only be determined in a semi-quantitative way. Recoveries were within the range of 24.7 % - 127.2 % and matrix effects were within 20.0 % - 92.6 %. Both parameters were tested using 5 sources of whole blood and coefficients of variance were below 20 %. Finally, the method was evaluated for the analysis of a certified quality control and real-life forensic samples.

### Conclusion/Discussion:

Overall, the applicability of ionic liquids as promising solvents for the extraction of benzodiazepines in whole blood samples has been proven. Moreover, a fast and easy ionic liquid-based dispersive liquid-liquid micro-extraction procedure was developed for the quantification of 19 benzodiazepines and benzodiazepine-like hypnotics.

**Keywords:** Ionic Liquid, Benzodiazepines, Dispersive Liquid-Liquid Micro-Extraction



**Validation of a Rapid Quantitative LCMSMS Method for Opioids, Cocaine, and Cocaine Metabolites in Biological Matrices**

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**Background/Introduction:** The proliferation of liquid chromatography tandem mass spectrometry (LCMSMS) in forensic toxicology makes the development and validation of methods using generally accepted guidelines essential. The Toxicology Section of the Virginia Department of Forensic Science (VADFS) receives DUI/DUID, death investigation, and other police cases for analysis. The prevalence of polydrug use extends to all case types and hinders laboratory efficiency. Traditionally, analyses are completed by individual drug class which subsequently requires a case to be analyzed using multiple analytical methods. To assist in increasing laboratory efficiency, a combined quantitative protein precipitation extraction of opioids, cocaine, and cocaine metabolites using liquid chromatography tandem mass spectrometry (LCMSMS) has been developed and validated. The validated method not only uses a decreased sample volume, but also combines four analytical techniques into one which significantly increases laboratory productivity.

**Objectives:** To validate a quantitative LCMSMS method for the analysis of opioids, cocaine, and cocaine metabolites including: morphine, oxycodone, hydromorphone, codeine, oxycodone, 6-monoacetylmorphine, hydrocodone, fentanyl, acetyl fentanyl, tramadol, meperidine, methadone, benzoylecgonine, cocaine, and cocaethylene in whole blood and other postmortem matrices.

**Methods:** A thorough validation was conducted for each target compound. The validation was completed in accordance with the Scientific Working Group for Forensic Toxicology (SWGTOX) method validation guidelines and VADFS validation requirements. Aspects evaluated were accuracy and precision, sensitivity, calibration model, ion suppression/enhancement, recovery, carryover, interferences, dilution integrity, and post-extraction stability. An acetonitrile protein precipitation extraction was validated using 1.0 mL of sample and 2.0 mL of acetonitrile. Samples were vortexed for approximately 15-30 seconds and centrifuged for 15 minutes at approximately 2800 rpm. After centrifugation, samples were frozen at approximately -20 °C for thirty minutes. The supernatant was transferred and dried down between 50-60°C with nitrogen. Samples were reconstituted in 5mM ammonium formate and 0.01% formic acid prior to positive mode dynamic MRM LCMSMS analysis.

**Results:** All compounds passed the comprehensive validation. Pooled accuracy and precision for all compounds was within the predetermined acceptance criteria  $\pm 20\%$  at three concentration levels. The best fit calibration model was established using statistical analysis, residual plots, and coefficient of determination. The best fit calibration model for all compounds was weighted (1/x) quadratic with the exception of morphine, tramadol, and cocaine which were weighted (1/x) linear. Three dynamic ranges were utilized within the validation based on therapeutic as well as lethal concentrations for each compound. The limit of detection (LOD) was determined using replicate analysis of serial dilutions. The lowest LOD was demonstrated with fentanyl and was determined to be 0.0625 ng/mL. All other LODs were less than 1.0 ng/mL. The limit of quantitation was established as the lowest calibrator concentration since extrapolation beyond the dynamic range will not be performed. Ion suppression/enhancement and recovery was evaluated in blank blood, postmortem blood, liver, and urine samples. Although suppression and decreased recovery were noted during the validation, the factors did not negatively impact the overall accuracy and precision of the method. Further, all compounds were deemed stable for seven days post-extraction.

**Conclusion/Discussions:** The validation of a combined quantitative method for the analysis of opioids, cocaine, and cocaine metabolites using LCMSMS was successful. The method requires less sample volume and combines four analytical methods into one efficient robust method. The rapid protein precipitation in conjunction with an 11 minute analysis time enables increased laboratory efficiency. Therefore, use of the fully validated method can aid in streamlining forensic toxicology analyses.

**Keywords:** Quantitative Method Validation, Tandem Mass Spectrometry, Opioids, Cocaine

## Marijuana and Driving in Alabama: A 10 Year Retrospective Study

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**Background/Introduction:** Marijuana continues to be one of the most commonly found drugs in impaired driving cases. Although classified by the DEA as a Schedule I drug, medical and recreational marijuana have been legalized in 28 and eight states as well as the District of Columbia, respectively. As a result of the push towards decriminalization, more focus has been placed on testing for marijuana use and the effects of use on driving. Many states have looked to adopt per se statutes for delta-9-tetrahydrocannabinol (THC), the active ingredient in marijuana. These statutes would be similar to the 0.08% per se for ethanol.

**Objective:** To investigate the prevalence of THC in driving under the influence and traffic crash cases analyzed by the Alabama Department of Forensic Sciences over a 10 year period, the concentrations of THC and its metabolites, the time between the incident and collection, and the demographics of these subjects.

**Methods:** Driving under the influence and traffic accident cases were evaluated over a 10 year period (2006-2016). All cases were screened by enzyme immunoassay using either a Tecan Freedom Evo75 with Immunalysis reagents or a Randox Evidence Analyzer. Confirmation and quantification of THC as well as its two metabolites, 11-nor-9-carboxy-delta-9-THC and 11-hydroxy-delta-9-THC, were performed by liquid-liquid extraction followed by analysis using LC/MS/MS. For cases with a quantitative result for delta-9-THC and 11-nor-9-carboxy-delta-9-THC, the estimated time since last use was calculated using the models created by Huestis et al.

**Results:** Between 2006 and 2016, 764 blood samples from DUI and traffic crash cases in Alabama were positive for THC. Demographic information was available for 715 of these cases. In 79% of these cases, the subject was male between 16 and 64 years of age and in 21% of cases the subject was female between 17 and 57 years of age. For males, the median THC concentration was 2.8 ng/mL with a range of 1.0-28 ng/mL compared to 2.7 ng/mL for females with a range of 1.0-15 ng/mL. 499 subjects were Caucasian (70%), 203 were African American (28%), nine were Hispanic (1%), two were Indian (< 1%), and two were unspecified (<1%). In cases where the time of the incident and the time of collection were noted, the median time difference was 112 minutes with a range from 8 minutes to 16.2 hours. The median time since last use was calculated with a lower and upper limit of 40 minutes and 7.5 hours, respectively. The median THC concentration for samples collected after a DUI stop, traffic crash, and traffic death were 2.9 ng/mL, 2.7 ng/mL, and 4.8 ng/mL, respectively. The percentage of cases at or above different cutoff concentrations was also calculated.

# of Cases	At or above Amount	% from Total Cases
726	≥ 1 ng/mL	95
521	≥ 2 ng/mL	68
339	≥ 3 ng/mL	44
214	≥ 4 ng/mL	28
154	≥ 5 ng/mL	20
118	≥ 6 ng/mL	15
84	≥ 7 ng/mL	11
70	≥ 8 ng/mL	9
51	≥ 9 ng/mL	7
39	≥ 10 ng/mL	5

**Conclusion/Discussions:** In Alabama, the average THC concentration found in blood samples from drivers has increased over the last ten years. On average, the concentration of THC found in drivers was less than 5 ng/mL (the proposed per se in many states), with 80% being below this cutoff. The time difference between the incident and collection varied widely and law enforcement should be encouraged to collect blood as close to the incident as possible.

**Keywords:** Marijuana, DUI, THC

## Can Serum EtG Help the Interpretation of BAC in Road Traffic Accidents in Case of Delayed Blood Collection?

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**Background/Introduction:** Drunk-driving is an important risk factor of road accidents. Since the driving ability impairment is correlated with the blood alcohol concentration (BAC), the traffic laws of the most of the countries established BAC legal limits to drive. The Italian traffic law fixed three different values: 0.5, 0.8 and 1.5 g/L, respectively, with different penalties. The presence of legal limits to drive and the rapid elimination curve of ethanol in blood require that the blood collection is performed immediately after the crash.

However, according to the literature and also in our experience the blood collection in traffic accidents is performed approximately 1.5–4 h after the event, thus complicating the interpretation of the role of alcohol intoxication in the accident [1].

Ethylglucuronide (EtG) is a minor metabolite of alcohol with a longer detection window than ethanol. EtG reaches the maximum blood concentration after a median of 3 h (range 2–4.5) after  $C_{\max}$  of ethanol and can be determined in serum up to 8 h after complete ethanol elimination [2]. The delayed kinetic of EtG in comparison to that of ethanol can provide useful information on the portion of the BAC curve in which the subject was at the moment of blood collection.

**Objective:** The aim of the present work was to determine both BAC and serum EtG (sEtG) in a group of 500 drivers involved in non-fatal traffic accidents in order to study the possibility of using sEtG to evaluate BAC results in case of delayed sample collection.

**Methods:** Ethanol was measured by HS-GC-FID (LOQ: 0.1 g/L) while sEtG concentration was determined, after serum precipitation with methanol, by LC-QQQ MS by MRM analysis monitoring the following transition: 221 m/z → 75, 85 m/z for EtG; 226 m/z → 75, 85 m/z for EtG-D5 (I.S.). The method was linear in the range 0.1–20 µg/mL, the LLOQ being 0.1 µg/mL.

**Results and Discussion:** About 60% of subjects showed both BAC and sEtG negative. About 18% of the subjects showed BAC positive and sEtG positive, while about 22% showed BAC negative and EtG positive. No cases with positive BAC and negative sEtG were found.

In the positive cases the concentration range of ethanol was 0.16–3.8 g/L (mean  $1.42 \pm 0.91$ ) and that of sEtG was 0.17–9.1 µg/L (mean  $2.59 \pm 2.18$ ).

Plotting the BAC data versus sEtG data a significant correlation was obtained ( $R = 0.788$ ) even if a dispersion of data was observed. Evaluating the outliers, many subjects with similar BAC and different sEtG were identified. A specific study was performed on these subjects by evaluating BAC, sEtG and time of blood collection in order to identify the portion of the ethanol curve at the time of sampling. These cases will be presented and discussed in detail focusing the attention on that cases in which sEtG evaluation allowed for a better comprehension of the case.

### References:

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**Keywords:** Serum EtG, Traffic Accidents, BAC

## Furanyl Fentanyl, Butyryl Fentanyl, and U-47700 in Blood and Urine of Drivers Arrested for Driving Under the Influence of Drugs (DUID)

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**Background/Introduction:** Synthetic opioids such as fentanyl and its designer analogs are  $\mu$ -opioid agonists often several fold more potent than morphine. They have become increasingly common in the last few years, occasionally appearing on their own and frequently in heroin preparations. Heroin users may be unaware that a batch contains these compounds. When detected, they are often attributed postmortem (along with any other opioids or central nervous system depressants which would act synergistically) as the primary cause of death. Obtaining toxicological concentrations of fentanyl analogs from non-fatal cases and evaluating their effects is challenging due to their potency (making them relatively rare to detect in living individuals) and users' propensity toward polypharmacy. Interpretation of both DUID and postmortem casework involving these compounds would be improved with increased reference concentrations from non-fatal cases to compare with those obtained postmortem, and by evaluating observations of driver behavior with these compounds.

**Objective:** To identify and evaluate drugged driving cases with designer opioids furanyl fentanyl, butyryl fentanyl, and U-47700.

**Methods:** The laboratory performs comprehensive routine drug analysis on blood and/or urine for drugged driving cases, including volatiles, cannabinoids, opioids, stimulants, benzodiazepines, sedatives, antipsychotics, antidepressants, "z-drugs", and antihistamines. The laboratory database was searched for drugged driving cases submitted where designer opioids furanyl fentanyl, butyryl fentanyl, or U-47700 were confirmed.

**Results:** Eleven drivers (8 M, 3 F, ages 24-52 [age not available for 3 cases]) from 2016-January 2017 were identified with at least one of the target compounds. Reasons for stop included failure to stay in single lane, "driving erratically", "odd behavior", and a motor vehicle accident. Specimens submitted to the laboratory for toxicological analysis included blood (5 cases) and urine (7 cases):

Case	Specimen	Furanyl Fentanyl	Butyryl Fentanyl	U-47700	Fentanyl	Norfentanyl	Morphine	6-MAM	Codeine
A	Blood			8.6	3.6	23.8	12		
B	Blood	1.3		3.4	5.1	3.3	<5		
C	Blood	1.2					<5		
D	Blood	1.4					<5		
E	Blood	<0.5							
E	Urine	3.1				0.8			
F	Urine	30.9			75.6	Pos	Pos	Pos	Pos
G	Urine	<0.5			65.8	84.4	Pos	Pos	Pos
H	Urine	3.1			122	261	Pos	Pos	Pos
I	Urine	17.3			799	Pos	Pos	Pos	Pos
J	Urine	1.6	101		2.0	8.4	Pos	Pos	Pos
K	Urine		15.7		83.6	518	Pos		Pos

Concentrations in  $\mu\text{g/L}$ . Where provided, urine concentrations are semiquantitative.

Other substances detected in blood included alprazolam (cases A, C), cannabinoids (C, E), cocaine/metabolites (B, C, D, K), difluoroethane (E), duloxetine (D), and quetiapine (A). Other substances in urine included amphetamine/methamphetamine (H), cannabinoids (E, F, G, H, K), clonazepam/7-aminoclonazepam (I, K), cocaine/metabolites (E, F, H, I, J, K), cyclobenzaprine (K), difluoroethane (E), gabapentin (K), hydrocodone/hydromorphone (E, J, H, K), oxycodone/oxymorphone (E, J, H, K), quetiapine (I), tramadol/O-desmethyltramadol (H), and venlafaxine/desvenlafaxine (F). All cases were negative for alcohol.

**Conclusion/Discussions:** Although blood is the preferred matrix for driving impairment cases, urine is easier to collect and was the only specimen in 6/11 of these cases. This highlights the need to educate law enforcement officers on the importance of blood in DUID cases, and illustrates the necessity of officers' observational reports in addition to toxicological findings. Although the fentanyl analogs have increased in prevalence, they remained relatively rare in our DUID casework. Concentrations of furanyl fentanyl and U-47700 in non-fatal DUID case blood are presented, adding to the currently limited body of data. Furanyl fentanyl was only detected in these drivers' blood at consistently low concentrations, but this does not preclude potentially higher concentrations in living drivers. More data are needed to establish a more complete picture. Because these compounds are often used to cut heroin and in conjunction with other drugs, polypharmacy challenges correlation of impairing effects with concentrations.

**Keywords:** Furanyl Fentanyl, Butyryl Fentanyl, U-47700, Opioids, Driving

## Alcohol in Combination with Other Drugs Among Fatally Injured Victims in Sao Paulo, Brazil

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**Background/Introduction:** Protocols in medical research need constant revision and improvement, especially regarding forensic investigation procedures that are essential in guiding strategies for relevant health issues such as injuries associated with substance use. This is particularly important for the Latin American region, where budget constraints limit the ability to implement large-scale drug testing routine procedures and the scarce data available on alcohol- and drug-related injuries hampers the ability of evaluating public health outcomes in the long-term.

**Objective:** To present a new protocol created for investigating alcohol and other drug use among fatally injured subjects taking into account the injury context for both intentional and non-intentional injuries. The city of São Paulo was used as a model to generate a probability a representative sample of fatal injuries that could reduce costs attributed to blood toxicological analyses necessary for injury surveillance and generate reliable data for public health monitoring systems.

**Methods:** Post-mortem blood specimens were obtained from a probability sample of all fatally injured victims autopsied (N=365) during a 19-month period (2014-15). Blood alcohol concentration was measured using headspace gas chromatography equipped with flame-ionization detection (HS-GC-FID). Other drugs, including amphetamines, barbiturates, benzodiazepines, cannabis, cocaine, opioids (fentanyl, methadone, morphine and oxycodone), and phencyclidine were screened by enzyme-linked immunosorbent assay (ELISA) and confirmed/quantified as necessary by gas chromatography-mass spectrometry (GC-MS) or liquid chromatography tandem mass spectrometry (LC-MS-MS). Toxicology information was interpreted in combination with injury context data retrieved from police records about cause, time and place of injury, including criminal history from the victims studied.

**Results:** More than half of the victims (55.3%) were confirmed positive for at least one substance. Alcohol was the most consumed substance among victims (30.1%), followed by cocaine (21.9%), cannabis (14%), and benzodiazepines (11.5%). Traffic-related casualties presented the highest proportion of alcohol-positive victims (42.9%) and of those consuming alcohol simultaneously with any other drug use (21.4%), while suicide cases presented the lowest proportion of victims positive for alcohol (13.6%), but a high prevalence for other drug use (25%). Homicides had the highest proportion of positivity for any substance (59.6%) and also for any other drug use (45.2%). Those who used drugs, in combination or not with alcohol, were more likely to have been injured in a public setting than a private place ( $p<0.05$ ). Victims who had at least one previous criminal conviction were significantly more likely ( $p<0.05$ ) to have used illicit drugs (cannabis and cocaine) before the event compared to those who did not have a criminal background.

**Conclusion/Discussions:** We estimated that one in every two fatal injuries in the city of Sao Paulo is associated with substance use. The health burden attributed to alcohol and other drug use has reached significantly higher levels in Latin American countries such as Brazil compared to the rest of the world; therefore, data-based monitoring systems with reliable and systematic information on all drugs, and not only alcohol, are urged to be implemented in order to guide evidence-based public health strategies aiming to reduce substance-related injuries in developing countries.

**Keywords:** Alcohol, Drugs, Injuries

**Characteristics of Alcohol-Impaired Drivers in the City of Houston (January 2014 - February 2017): Did Hosting Super Bowl LI cause a Spike in BAC?**

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**Background/Introduction:** Driving under the influence of ethanol poses a great public safety risk. The National Highway Traffic Safety Administration reported 418 traffic fatalities in 2014 in Harris County, which encompasses the city of Houston. Of these fatalities, 49% had blood alcohol concentrations (BACs) above 0.08 g/dL. As the fastest growing US city above 1 million residents, Houston gains new drivers each day. Houston welcomed additional drivers in February 2017 when it hosted Super Bowl LI.

**Objective:** This study aimed to examine case data associated with traffic accident and impaired driving arrests in Houston over multiple years. The number of cases, distribution of BACs, and demographics were examined. The potential for a sharp rise in the incidence of impaired driving while hosting the Super Bowl was considered, along with annual events and holidays with which alcohol is often associated. This analysis was intended to provide a better understanding of the impaired driver population observed in a large city, and potentially identify high-risk times of the year for impaired driving.

**Methods:** The driving while intoxicated or driving under influence of drug cases, fatal and non-fatal, analyzed by Houston Forensic Science Center for alcohols in blood samples with offense dates falling between 01/01/2014 and 02/28/2017 were included. The samples were collected by Houston Police Department from drivers or suspects as indicated in the laboratory information management system. BAC (overall and divided into 8 groups from <0.050 g/dL to >0.300 g/dL) and demographics including age (<21, 21-44, 45-65, and >65 years), sex, and race/ethnicity were evaluated. Cases were analyzed for ethanol by headspace gas chromatography interfaced with dual flame ionization detectors. The LOQ was 0.010 g/dL and range of linearity was 0.010-0.500 g/dL for ethanol (with the exception of 2014 when the LOQ (linear range) was 0.02 g/100 dL (0.020-0.400 g/dL)).

**Results:** Over the 38-month period examined, 5974 items were analyzed by HFSC, with a mean (median) BAC of 0.173 g/dL (0.179 g/dL) and age of 36 (34) years. The majority of the drivers (80%) were male (19% female); there was no significant difference in mean BAC between the two groups (ANOVA, P=0.8). Overall, the highest proportions of cases were from 21-44 year olds (76%), Whites (70%), and had BACs falling between 0.150-0.199 g/dL (32%). While the Harris County population grew by approximately 5% from 2014 to 2016, the number of alcohol cases received increased by 32%; the yearly mean BAC, however, decreased from 0.181 g/dL in 2014 to 0.166 g/dL in 2016. Thirteen percent more cases were analyzed in February 2017 than in February 2016; however, no significant difference between mean BACs were observed (t-test, P=0.5). The proportion of cases submitted for testing with BAC none detected increased each year (<1% in 2014 and 2015 to 7% in 2016). The distribution of drugs in those samples was examined; the five most prevalent drugs detected were cannabinoids, alprazolam/metabolite, cocaine/metabolites, carisoprodol/meprobamate, and phencyclidine.

**Conclusion/Discussions:** Alcohol is often part of the experience of major sporting events, holidays, and social events. Australian researchers observed significant increases in the presentations of acutely intoxicated patients on the day leading up to public holidays and major sporting events (Lloyd et al. *Addiction*, 2012). Of randomly selected attendees exiting professional football and baseball games in Minnesota, 40% were found to have a positive BAC (Erickson et al. *Alcohol Clin Exp Res* 2011). The 13% increase in submissions from February 2016 to February 2017 may be attributed to Houston hosting Super Bowl LI on February 5, 2017. However, the impact of ride sharing, public transit, and police patrols for impaired driving should be considered to better understand the impact of these data.

**Keywords:** Ethanol, Impaired Driving, Houston

## A Suspected Mobbing Case with Doxylamine

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**Background/Introduction:** Doxylamine is a common temporary nighttime sleep support for insomnia. It is also an active ingredient used for the relief of cold and allergy symptoms. The minimum necessary dose for sleeping can be as low as 6.25 mg, while the active dose is 25 mg in commercial tablet form. The half-life of doxylamine is approximately 6 hours and the lethal dose is 25–250 mg/kg body weight. Doxylamine is regularly involved in intentional and unintentional intoxication cases. For example, intoxication with doxylamine in combination with alcohol and morphine (1), a case resulted in sinus tachycardia (2), and two overdose cases (3) have also been reported in the literature. In this study, a possible mobbing (physical or psychological harassment in the workplace) case with drugging is presented.

**Case history:** A 39-year-old woman submitted an open cup of beverage claiming it was a black teabag infusion. She requested toxicological testing due to her complaints of tremor, weakness and state of sleep after she drank the beverage. According to the woman's statement, the previous day a cup of tea was served to her by a workmate in the workplace, and she felt sleepy, weak and dizzy after drinking it. The next day a beverage was again served by the same person, and after taking a few sips of that drink, she again felt sleepy and weak. It was then that she suspected the beverage, and decided to have a toxicological test carried out.

**Methods:** The administered infusion was extracted by liquid–liquid extraction (LLE) in basic conditions (pH 10): 2 mL of case liquid was taken and the pH was adjusted to 10 with 0.01 M NaOH. LLE was performed with ethyl acetate: *n*-heptane (1:1) and docosane was used as internal standard (IS) (30 µg/mL). The extract was analyzed by gas chromatography-mass spectrometry (GC-MS) using a newly developed method. Spiking a tea bag sample with two different concentrations of doxylamine (50 and 175 µg/mL) and caffeine (20 and 75 µg/mL) was carried out for the quality control studies.

**Results:** The results showed that the infusion contained doxylamine and caffeine. The retention times of caffeine, doxylamine and docosane (IS) were 6.63, 7.02 and 8.02 min, respectively. The MS ions were 67, 82, 109 and 194 *m/z* for caffeine and 58, 71, 167, 180 *m/z* for doxylamine. Five-point calibration curves were generated by incremental amounts of doxylamine and caffeine using the following ranges respectively; 25-250 µg/mL and 5-100 µg/mL. The correlation coefficient for doxylamine was found 0.999 and 0.998 for caffeine. The quantitative results were found to be 69.3 µg/mL for doxylamine and 24.8 µg/mL for caffeine. Assuming the regular volume of a tea cup is 200 mL, a 13.86-mg/per cup doxylamine dose was adequate to reveal the described symptoms. Additionally, the calculated 4.96-mg/per cup caffeine was considered a normal quantity for commercial teabag brands.

**Conclusion/Discussions:** If her claim is true, then it is indisputable that the case in question is a mobbing, regardless of the intention. However, considering the opposite situation, the complainant may have added the drug to the beverage afterwards to accuse her workmate of mobbing. The controversial part is that, even if the biological samples from the complainant had been analyzed, it would not be enough to clear up the case certainly, due to the possibility that the complainant could have added the drug to the drink and consumed it. This shows that the analytical proof of mobbing is considerably hard and not always enough by itself. Doxylamine has often previously been reported in the literature in drug-facilitated crimes, however, there is no case reported related to mobbing in which doxylamine was included.

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**Keywords:** Doxylamine, Mobbing, Doxylamine-drugging



## High Variation in BAC Back-extrapolation even During Controlled Dosing

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**Background/Introduction:** It is accepted that a toxicologist may report an extrapolated ethanol concentration in a blood sample collected from a living person. The context is usually traffic accidents or sexual assaults where the blood sample is obtained several hours after the incident. A pre-requisite is that all ethanol has been absorbed and that the person is in the elimination phase. Ethanol elimination rates vary between individuals and have been categorized as slow (0.08-0.12 ‰), moderate (0.12-0.15 ‰), rapid (0.15-0.25 ‰), and ultra-rapid (0.25-0.35 ‰). A common approach for back extrapolation of ethanol concentrations is to use either a moderate elimination rate of 0.15 ‰/hour or to use ranges from slow to rapid, not necessarily using the ultra-rapid rates.

**Objective:** Therefore, we aimed to investigate how close to the true value these estimation were in an experimental setting with a real-life drinking scenario.

**Methods:** Fifteen subjects (20-66 yo, 11 males and 4 women) administered two doses of ethanol, first 0.51 g/kg of beer (approximately 1 L) and two hours later either 0.25, 0.51 or 0.85 g/kg of whiskey (N=5 in each group). A total of 17 blood samples were drawn in vacutainer tubes, with potassium oxalate and sodium fluoride, over seven hours. Samples were analyzed with headspace gas chromatography and flame ionization detection. The individual elimination rate was determined from 4-7 blood concentrations in the elimination phase. Values 3 hours post dose (t1) were estimated using the 6 hours post dose (t2). The accuracy of back-extrapolation was calculated by comparing the difference between the true value and the calculated using either the individual elimination rate or three evidence based rates of 0.10, 0.15, and 0.25 ‰/hour.

**Results:** The individual elimination rates varied between 0.10 and 0.21 ‰/hour with 9 rapid, 4 moderate, and 2 slow eliminators. Using the individual elimination rates the estimated BAC at t1 deviated from the true value by -8 to +27%. Using the low elimination rate of 0.10 ‰/hour the estimated BAC at t1 deviated from the true value by -42 to +26%. Using the moderate elimination rate of 0.15 ‰/hour the estimated BAC at t1 deviated from the true value by -25 to +74%. A rate of 0.25 ‰/hour gave only overestimations with deviation ranging from +6 to + 171 %.

**Conclusion/Discussions:** The elimination of ethanol has been studied extensively and the scientific base for extrapolation is valid as long as there are evidence that the subject is in the elimination phase. However, using population data to calculate an individual BAC is merely a rough estimation as shown by our data. Both underestimations and overestimation are equally important to recognize depending on the type of case. Therefore, the toxicologist must not only appreciate these short-comings but also communicate them to the court. We also suggest, that in some cases, a second blood sample is drawn to calculate the individual elimination rate at the time of the event. We conclude that depending on the elimination rate used in back-extrapolation the true value can be severely flawed and care must be taken to avoid misinterpretation in courts.

**Keywords:** Ethanol, Back-extrapolation, DUI

## Toxicology Findings in 1000 Alleged Drug-Facilitated Sexual Assault (DFSA) Cases Over a Two-Year Period (2105-106) in the United States

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**Background/Introduction:** DFSA involves the act of slipping a drug into a beverage designated to incapacitate without consent and subsequent practice of sexual assaults. In 2015, 90,185 rapes were reported to law enforcement, according to the FBI Uniform Crime Reports. The most common substances used in DFSA cases include: ethanol, GHB, benzodiazepines, opioids, Z-drugs, antihistamines, barbiturates and traditional drugs of abuse such as cocaine, cannabinoids and amphetamines.

**Objective:** To investigate the number and variety of substances encountered in DFSA cases in the United States and provide sociological and toxicological information in order to contribute to a better understanding about the factors involved in DFSA.

**Method:** The data from 1000 DFSA cases submitted to NMS Labs in 2015 and 2016 were reviewed. Screening analysis were performed by immunoassay (ELISA (blood), EMIT (urine)), GC-MS screen for basic drugs, GC-MS for GHB, GC-FID for alcohol, LC-MS for benzodiazepines and LC-MS for flunitrazepam. All positive results from screening were verified by LC-MS/MS.

**Result:** In cases in which gender was available, 562 (71.68 %) were female with an average of age of 26.8 (median 23.5, range 1 – 64 years), and 51 (6.51 %) were male, with an average of age of 26.5 (median 25, range 3 – 55 years). Not given gender represents 21.81 % (n=171) of this population. Blood and urine were collected from the alleged victims. Urine was submitted in 83.7 % of the cases, and blood was submitted in 73.3 % of the cases. Both matrices were collected in 15.2 % of the cases. Overall, 66 substances were found positive in blood and 97 substances were found positive in urine. The most prevalent substances identified overall, in blood and in urine samples from the cases reviewed are listed in **Table 1**. The drugs most frequently found in combination with alcohol were: Ethanol + THC/metabolites (6.9 %); Ethanol + AMPH/METH (3.6 %); Ethanol + COC/met (3.4 %); Ethanol + Diphenhydramine (2.4 %); Ethanol + Citalopram/Escitalopram/met (2.0 %); Ethanol + Clonazepam/met (2.0 %); Ethanol + Lorazepam (1.9 %); Ethanol + Alprazolam/met (1.5%); Ethanol + Diazepam/met (1.3 %); Ethanol + GHB (1.2 %); and Ethanol + Fluoxetine/met (1.2 %). Benzodiazepines and GHB are frequently reported as being associated with DFSA. In this series, benzodiazepines were found in a total of 210 cases (21 %): Clonazepam/7-amino Clonazepam (76 cases, 36.1 %); Alprazolam/ $\alpha$ -Hydroxyalprazolam (72 cases, 34.2 %); Lorazepam (66 cases, 31.4 %); Diazepam/met (37 cases, 17.6 %); Oxazepam (13 cases, 6.1 %); Temazepam (9 cases, 4.2 %); Midazolam/1-Hydroxymidazolam (6 cases, 2.8 %) and Chlordiazepoxide (1 case, 0.4 %). GHB was found in 59 cases (56 urine samples and 3 blood samples), mean concentrations of 11.0 mg/L (median 3.1, range 2 – 270 mg/L). Cut-off concentrations of 5 or 10 mg/L have been proposed to distinguish endogenous from exogenous GHB. 5 cases (8.4 %) had concentrations of greater than 10 mg/L.

**Conclusion/Discussion:** As expected, ethanol, THC/met and benzodiazepines were found at high prevalence in both matrices. It was surprising to find so many cases of stimulants as AMPH/METH and COC/met, despite the fact of being common drugs of abuse, stimulants are usually less reported in DFSA cases. GHB findings were representative, although just a few number of cases (5) can be considered exogenous. The absence of alcohol and drugs and some cases may represent time delay to collect samples. There is no information about the voluntary or involuntary ingestion of these compounds but, whether voluntary or not, drug use may predispose subjects to greater risk of sexual assault.

**Table 1.** Most common substances in DFSA cases.

Overall		Blood		Urine	
	%		%		%
Ethanol	30.7	Ethanol	17.0	THC/THCCOOH/11-OH-THC	30.3
THC/THCCOOH/11-OH-THC	28.8	THC/THCCOOH/11-OH-THC	9.6	Ethanol	30.1
AMPH/METH	16.5	AMPH/METH	6.5	AMPH/METH	16.2
COC/BE/CE/MEC/EEC	10.4	Clonazepam/met	3.9	COC/BE/CE/MEC/EEC	11.1
Clonazepam/met	7.6	Alprazolam/met	2.7	Clonazepam/met	7.8

**Keywords:** Drug-Facilitated Sexual Assault, Forensic Toxicology, United States

## Detection of Carfentanil in Driving Under the Influence Cases in Palm Beach County, FL, USA

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**Background/Introduction:** Palm Beach County, Florida, has been labeled "the recovery capital of America" by several media outlets due to the proliferation of drug treatment centers. In South Florida there are more than 320 licensed drug treatment centers of which a significant number are located within Palm Beach County (1). In recent years, a large number of sober homes for recovering addicts have also opened. Media reports anecdotally state that many sober homes are unregulated, unaccredited and that corruption by the owners have contributed to increased opioid related overdoses (2-3). As recently as 2010 Florida was home to 90 of the top 100 oxycodone prescribing physicians in the nation. Through legislative efforts that included a prescription drug monitoring database as well as law enforcement efforts, by 2013 there were no Florida doctors on the top 100 list. As a result in the reduction in the availability of pharmaceutical opioids there has been a marked increase in the use of heroin and synthetic opioids (4). Synthetic opioids like fentanyl, carfentanil, furanyl fentanyl and other fentanyl related compounds are frequently identified in suspected heroin. It is also common to see complex mixtures of heroin, fentanyl, and other synthetic opioids, rather than a single source compound. Concomitantly this has led to an increased incidence of the identification of these compounds in forensic toxicology cases.

**Method:** A volatile analysis was performed on all blood specimens. A case management protocol was used to limit the number of drug screens that were performed. The case management protocol specified that a drug screen was only conducted when the case involved a fatality (to an individual other than the driver, as postmortem testing is not conducted by this laboratory) or if the blood ethanol concentration (BAC) was less than 0.15 g/dl for a routine driving under the influence (DUI) case. From January 1 of 2017 to June 4 of 2017, 108 blood specimens were received as part of DUI investigations and 69 (64%) were analyzed for drugs. Blood specimens were screened using a basic extraction with scan GC-MS and an 11 panel ELISA for amphetamines, barbiturates, benzodiazepines, buprenorphine, carisoprodol, cocaine/ benzoylecgonine, fentanyl, methamphetamines, opiates, oxycodone/oxymorphone, and cannabinoids. The cutoff levels used for the blood ELISA analysis as compared to the 2013 recommendations are listed in Table I (5). All positive results were confirmed with GC-MS and/or LC-MSMS. Targeted analysis for select fentanyl analogs was performed by LC-MSMS after liquid-liquid extraction of 200 ul of blood with methyl tert-butyl ether. This qualitative procedure was validated using the SWGTOX guidelines (6) and included evaluation of selectivity/specificity, ionization suppression/enhancement, sensitivity, precision, reportable range, carryover, extract stability, ruggedness/robustness, and a case sample comparison. All parameters were determined to be suitable for the qualitative identification of the target compounds including the validated limit of detection (LOD) for each compound. The target compounds and LODs were acetyl fentanyl (0.2 ng/ml), beta-hydroxythiofentanyl (0.1 ng/ml), butyryl fentanyl (0.1 ng/ml), carfentanil (0.02 ng/ml), fentanyl (0.2 ng/ml), furanyl fentanyl (0.1 ng/ml), kavain (5 ng/ml), mitragynine (2 ng/ml), MT-45 (5 ng/ml), and U-47700 (5 ng/ml). Targeted analysis was performed based on the case history provided by the investigating officers. Targeted analysis was conducted if there was a history of heroin use, naloxone was administered, or the toxicology results obtained through routine analysis did not explain the severe CNS depression that was observed (e.g. low levels of fentanyl were detected, but the patient required administration of naloxone to regain consciousness).

**Result:** From January 1 to June 6 of 2017 a significant decrease in the number of cases that were positive for ethanol (> 0.2 g/dl) and that were above the statutory limit of 0.08 g/dl were observed when compared to the same time period from 2016. As a result the total number of drug screens that were performed had a similar increase since a case management protocol was employed as can be observed in Table II. In the first 5 months of 2017 carfentanil was the most frequently identified drug in driving under the influence of drugs (DUID) cases in Palm Beach County, FL. Carfentanil was identified in one-third of the 69 cases in which a drug test was conducted. The top five drugs that were identified in the 69 cases are listed in Table III. Case drug identification results from the first five months of 2016 are compared to the 69 cases from the same time period in 2017 with the exception of carfentanil, which was not included in the scope of analysis in 2016.

**Conclusion/Discussion:** Although only qualitative analysis was performed, many of the cases involving carfentanil had analytical results near the LOD of 0.02 ng/ml. The high incidence of carfentanil observed in these case studies demonstrates the importance of incorporating appropriately sensitive analysis of fentanyl analogs that are encountered in the jurisdiction of the laboratory. Through the analysis of drug seizures in Palm Beach County, carfentanil has been and continues to be the most commonly identified fentanyl analog with 146 exhibits identified from 1/1/17 to 6/4/17 followed by furanyl fentanyl with 87 exhibits identified. Carfentanil was rarely identified alone (only 1 case since August of 2016) and was most commonly identified with fentanyl (57% of cases), morphine (43%), and alprazolam (30%). Additionally, in over half of the cases a benzodiazepine was also identified (alprazolam, diazepam, or lorazepam), usually at low therapeutic levels. Little has been reported on the involvement of carfentanil in DUID cases. Many of the cases reported herein involve an individual that was passed out behind the wheel of a vehicle that was not moving; as might be expected due to the potency of the drug. However, at least 14/29 (48%) of the DUID cases involved some type of crash. Since the majority of individuals in these cases were found unresponsive (at least 55%), it is likely they were driving a motor vehicle when they passed out. Common opioid overdose symptoms such as agonal breathing, constricted pupils, and blue or purple skin discoloration were observed in those individuals that were found unresponsive. Other symptoms included slurred speech, droopy eyelids, lethargy, difficulty with balance, and slow response to questions. Synthetic opioids such as carfentanil pose a significant risk to traffic safety and at least in this jurisdiction are very commonly identified in DUID cases. The high incidence of carfentanil observed may

be in some part due to the high concentration of drug recovery programs also present in this region of the United States.

Table I. ELISA cutoff levels for blood analysis compared to 2013 recommendations (5)

Assay	Blood Cutoff (ng/ml)	Recommended (ng/ml)
Amphetamines	50	20
Barbiturates	100	300
Benzodiazepines	12.5	50
Buprenorphine	2	Not included
Carisoprodol	1000	500
Cocaine/Benzoylcegonine	50	50
Fentanyl	1	Not included
Methamphetamine/MDMA	50	20
Opiates	25	10
Oxycodone	12.5	10
THC	10	10

Table II. Blood DUI Casework summary from January 1 to June 6

Analyte	# of Cases	2017 <i>n</i> = 69		2016 <i>n</i> = 48	
		% of Cases	# of Cases	% of Cases	
Carfentanil	23	33%			
Alprazolam	22	32%	10	21%	
Fentanyl	21	30%	11	23%	
Morphine	20	29%	14	29%	
Delta-9-THC	17	25%	17	35%	

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**Keywords:** Carfentanil, DUID, Synthetic Opioids

## A Deaths Related to the Synthetic Opioid U-47700: Blood/Liver Concentrations Distribution

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**Background/Introduction:** Following series of synthetic cannabinoid and synthetic cathinone derivatives, the illicit drug market has begun to see increased incidence of synthetic opioids including fentanyl and its derivatives, and other chemically unrelated opioid agonists including AH-7921 and MT-45. Between August 2016 and January 2017 we reported four deaths of men aged 16-41 year, related to U-47700 (*trans*-3,4-dichloro-*N*-(2-(dimethylamino)cyclohexyl)-*N*-methyl-benzamide) at the Department of Forensic Medicine in Kraków. This opioid has been reported to be present in the heroin supply and to be gaining popularity among recreational opioid users, but were initially developed by Upjohn - pharmaceutical companies in the 1970's as candidates for development as potential analgesic therapeutic agents.

Deaths caused by new psychoactive substances generate problems that create ever-expanding research areas, including analytical, clinical and medico-legal issues, thus leading to development of systemic databases. The problem is illustrated in the present paper by fatal cases representing accidental poisonings involving U-47700, which were the subject of complex and detailed investigations in the analytical, pathological, toxicological and medico-legal aspect.

**Objective:** Complex assessment of the toxic effects of taking new psychoactive substance - U-47700 in the selected cases from medico-legal practice.

**Methods:** A SPE/LC-ESI-MS-MS method was developed and validated for the analysis of U-47700 in blood and liver specimens. The liver samples before extraction were enzymatic digested with Subtilopeptidase A. A total of 4 postmortem cases, initially believed to be new psychoactive substances overdoses, were submitted for quantitative analysis.

**Results:** The analytical ranges for U-47700 were 10-1500 ng/ml and 100-10000 ng/g for blood and liver, respectively. Within the scope of the method, U-47700 was the only confirmed drug in 4 of the cases. Additionally, identification study of blood and liver samples carried out of the means LC-tandem-MS in the full scan option (*m/z*: 50-650 amu) confirmed desmethyl-U-47700 metabolite. In the case 1 concentration of U-47700 was 1140 ng/ml for both blood specimen taken during resuscitation and autopsy blood. In the same case, the concentration of U-47700 in the liver sample was 2900 ng/g. In the cases 2, 3 and 4 concentrations of U-47700 were 360; 1140 and 300 ng/ml for autopsy blood and 800; 5000 and 1400 ng/g and liver specimens, respectively.

The autopsy of the four cases demonstrated minor skin abrasions and bruising, pulmonary edema and congestion of the internal organs. In the case 4 autopsy showed spot marks after injections.

Histopathology of the collected sections demonstrated cardiac muscle congestion, disseminated perivascular fibrosis, parenchymal degeneration of single muscle fibers, interstitial edema; pulmonary congestion, edema and unequal aeration; hepatic congestion, small droplet steatosis of disseminated hepatocytes; nephrotic congestion, hyalinization and fibrosis of single glomeruli; cerebral congestion. In the case 1 and 2 histopathology showed elements of foreign substance in the bronchi and vesicles, suggesting an aspiration. In the case 2 confirmed purulent pneumonia.

**Conclusion/Discussions:** U-47700 is one of the most toxic opioids showing euphoric effects and thus a substance characterized by a high lethal potential.

In all the presented cases, the postmortem findings were very similar. Both macroscopically and microscopically, the authors noted profound congestion of the internal organs with blood extravasation and pulmonary edema. Such observations are seen in acute cardiorespiratory failure and although they are not typical for a single, tangible cause of death, yet they fully correlate with the toxic mechanism of U-47700 effect exerted on a human body.

**Keywords:** Accidental Fatal Poisonings, U-47700, LC-ESI-MS-MS

## Post-mortem Beta-hydroxybutyrate and Ketoacidosis in Forensic Toxicology Casework

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**Background/Introduction:** Ketoacidosis is a metabolic disturbance that might be caused by uncontrolled diabetes, chronic alcoholism, intoxication or conditions such as hypothermia or infection. In suspected diabetes related deaths glucose should be measured in vitreous. In addition, the analysis of beta-hydroxybutyrate (BHB) can be used to diagnose alcoholic ketoacidosis (AKA) and diabetic ketoacidosis (DKA). Furthermore, it is helpful to analyze for BHB and glucose in diagnosing hyperosmolar hyperglycemic state (HHS). In the literature, it has been suggested that the analysis of acetone can be used as a screening biomarker for acidosis. However, the presence of acetone does not necessarily mean that other ketone bodies, such as BHB, are elevated.

**Objective:** Our aim was to investigate how BHB and glucose concentrations were interpreted in autopsy cases where the immediate cause of death was related to acidosis or diabetes.

**Methods:** In Sweden all forensic pathology departments send e.g. blood and vitreous humour, when available, to one toxicology laboratory for analyses. Information from the forensic autopsy and the results from the forensic toxicological analyses are stored in two databases. In these databases we identified all cases during 2016 with the ICD9 codes 250 (diabetes mellitus) or 276 (acidosis) as the immediate cause of death. For these cases all results from laboratory analyses were retrieved. The threshold for a pathological finding of BHB was considered 250 µg/g blood and for glucose it was 6.9 mmol/L vitreous humour.

**Results/Discussion:** In total 93 cases were identified during the study period. The immediate cause of death was DKA in 68 cases of which BHB and glucose were analyzed in 20 and 61 cases, respectively. In 19 of these cases the BHB concentrations was above 250 µg/g (range 470 to >1000 µg/g blood) and 54 had a glucose concentration above 6.9 mmol/L in vitreous humour. In the DKA group more than two thirds of the cases were diagnosed without the support of a BHB analysis and 14 also without the support of a high glucose.

The immediate cause of death was AKA in 22 cases. BHB and glucose were analyzed in 19 and 18 cases, respectively. Eighteen cases had BHB concentrations close to or above 250 µg/g (range 220 to >1000 µg/g blood) and the glucose concentrations were below 6.9 mmol/L in all of the 18 cases. In four of the cases where BHB was above 250 µg/g, acetone in blood was negative (<0.1 promille). In the AKA group all but four were supported by toxicological analyses.

In three cases HHS was stated as the immediate cause of death, in none of which BHB was analyzed.

**Conclusion:** We conclude that DKA often is stated as immediate cause of death without the support of toxicological analyses whereas AKA almost never is. An explanation for this could be that cases of suspected DKA often have other supporting evidence. To increase the scientific basis for diagnosis we recommend that BHB is analyzed in cases where diabetes mellitus or chronic alcoholism is suspected regardless if glucose is high or normal or if acetone is negative.

**Keywords:**

## Comparative Study of Post-mortem Concentrations of Antidepressants in Different Matrices

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**Background/Introduction:** Peripheral blood is considered to be the golden standard for measuring post-mortem drug concentrations. In several cases peripheral blood is however not available, but information regarding drug findings might be crucial to determine the cause of death. Antidepressants are often detected in samples from forensic toxicology cases. When peripheral blood samples are not available, other matrices might be an alternative, but the literature investigating if concentrations in other matrices collected post-mortem can be used to evaluate concentrations of antidepressants is limited.

**Objective:** The aim of the study was to provide knowledge of post-mortem drug concentrations in different matrices for a large number of drugs and medicinal products and to investigate if drug concentrations in peripheral blood, cardiac blood, pericardial fluid, vitreous humour, and two different muscles (vastus lateralis and psoas) were comparable. Results for antidepressants will be presented.

**Methods:** Samples were collected from medicolegal autopsies in the period June 2013 – June 2016, from cases where the forensic pathologists expected positive toxicological results due to the circumstances. All six matrices were collected on the same day. After screening in peripheral blood, all matrices from the cases positive for the antidepressants amitriptyline, nortriptyline, trimipramine, mianserin, mirtazapine, citalopram, paroxetine, sertraline, and venlafaxine were analysed using a validated UHPLC-MS/MS method [1]. Care was taken to analyse all the six matrices from a single case together in the same analytical series to minimise differences in concentrations due to experimental factors. Concentrations above the limit of quantification (LOQ) were compared. LOQ was 0.006 mg/L for amitriptyline and venlafaxine, 0.003 mg/L for citalopram, mianserin, nortriptyline, and trimipramine, 0.001 mg/mL for mirtazapine, 0.002 mg/L for paroxetine, and 0.008 mg/L for sertraline.

**Results:** In the collected material of 173 cases, one or more of the nine different antidepressants were detected in peripheral blood in concentrations above LOQ in a total of 44 cases. Variations in concentrations were large as could be expected. Findings were done in all matrices, except for some cases with amitriptyline, nortriptyline, and sertraline where levels in vitreous humor were below LOQ. Ratios between the other matrices and peripheral blood, except vitreous humor, varied between 0.4 and 3 for the majority of the cases. For amitriptyline and nortriptyline higher ratios were found for several cases, especially for the muscles. Concentrations in vitreous humor were generally lower than for the other matrices. For citalopram, mirtazapine, mianserin, and venlafaxine the ratios between vitreous humor and peripheral blood were between 0.15 and 1, whereas for amitriptyline, nortriptyline, paroxetine, sertraline, and trimipramine the majority of the ratios were between 0.01 and 0.3. Concentrations in pericardial fluid were quite comparable to peripheral blood for most of the antidepressants (ratios between 0.5 and 2), but amitriptyline and venlafaxine had a tendency towards higher concentrations in pericardial fluid.

**Conclusion/Discussions:** All the investigated matrices, except vitreous humor, are suitable for analyzing post-mortem concentrations of the nine different antidepressants. For citalopram, mirtazapine, venlafaxine, and mianserin vitreous humor could also be used, bearing in mind that the concentrations in general are lower than in peripheral blood. Pericardial fluid can be available in some cases where peripheral blood is difficult to sample due to e.g. large bleedings, and seems to be a good alternative matrix which can be handled in the same manner as blood. The concentration levels in the different matrices were comparable to the concentrations in peripheral blood in the majority of the cases, showing that alternative matrices as investigated here are likely to provide important knowledge in autopsy cases where peripheral blood is not available. Analyses of more than one matrix are recommended, as the concentration ratios can vary between the matrices and this might give a more consistent result. Concentrations from any post-mortem samples must however, always be interpreted with caution, as changes as i.e. degradation, formation, post-mortem redistribution, and putrefaction are likely to take place. More studies are warranted to investigate cases where the corps has been exposed to burns, injuries, severe putrefactions etc.

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Quantitative determination of fifteen basic pharmaceuticals in ante- and post-mortem whole blood by high pH mobile phase reversed phase ultra high performance liquid chromatography–tandem mass spectrometry. *Journal of Chromatography B* 2013; Volum 927, s. 112-123.

**Keywords:** Post-mortem Fluids and Tissues, Antidepressants, Concentration Ratios

## Interpretation of Percent Carboxy-Heme from Nonstandard Postmortem Specimens Employing Carbon Monoxide-Iron Ratio

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**Background/Introduction:** Carbon monoxide (CO) poisoning is one of the leading causes of both accidental and intentional poisoning deaths in the United States. The preferred matrix to confirm CO poisoning is the testing of blood. However, there are specific circumstances when normal postmortem blood specimens are unavailable; these cases include those that are decomposed, embalmed, and fire-dried. When this occurs, toxicology testing relies on the use of alternate specimens such as spleen, kidney, and dried blood to determine carbon monoxide concentrations. These alternate specimens require specialized testing for CO, a technique reported previously in *Middleberg et al 1993*. In order to provide a more accurate estimation of the percent carboxy-heme saturation in nonstandard postmortem specimens, iron (Fe) content is also determined, and then a ratio is calculated between the two results.

**Objective:** Interpretation of percent CO saturation (%CO) in blood samples is well studied, with >10% capable of causing significant adverse effects; however, interpretation of %CO in tissue samples is less clear. This current study seeks to examine the utility of using spleen, muscle, brain, liver and dried blood specimens for carbon monoxide testing.

**Methods:** For this study, data were collected from the analyses of 307 samples tested from 2013-2016 for CO from nonstandard postmortem specimens. CO was quantified by headspace gas chromatography/mass spectrometry (GCMS), with subsequent testing of the same tissue sample for Fe by digesting the sample in acid and analysis by inductively coupled plasma – optical emission spectroscopy (ICP-OES). Once CO and Fe results are obtained from their respective analyses, the values were substituted into an equation which assumes 100%CO saturation of Fe associated heme. The ratio of CO to Fe is 1:1 molar or 1:2 by weight (CO 28, Fe 56). Using the assumption that all Fe binds to CO, the %CO is estimated. A known limitation of the method is that the calculated result underestimates the actual %CO as it assumes that all Fe present in the sample is associated with heme capable of binding CO.

**Results:** Of the 307 cases analyzed for Co/Fe ratio, 5 cases included both tissue and blood. These samples had an average difference between the blood and the tissue values of 30% (range, 12.5-43.8%). The %CO results varied within the same case when multiple nonstandard specimens were tested. However, Fe content is not standard in all tissue types, so that change affects the %CO. Ninety-three (93) cases of the cases reviewed were suspected CO deaths either by poisoning or by fire related circumstances. Of these 93, 4 were none detected and the %CO for positive cases ranged from 0.32 – 68% with a median of 11%; 34 of these cases had a result >10%. From the 307 cases, 69 spleen samples were analyzed. Thirteen (13) of these spleen samples were from suspected CO related deaths. The %CO ranged from 1.1-61%, with 1 case >30%, 5 cases >10% but <30%, and the remainder <10%. In comparison to unknown or non-official CO related deaths, there were 56 cases and the %CO ranged from 0.55-58%; 17 cases were >30%. Similar evaluations of case related findings were performed on other specimen types.

**Conclusion/Discussions:** Overall, cases with no history were a limiting aspect to this study. The utility of %CO in nonstandard specimens is difficult to assess when case history is lacking. Many of these cases had results >10%CO and >30%CO. There were also many instances of >10 %CO with suspicion of CO poisoning, and these results may lead to a different assessment of cause of death. Ultimately, carbon monoxide testing in nonstandard postmortem specimens has been shown to provide useful information in some cases when traditional blood samples were not readily available.

**Keywords:** CO Poisoning, Postmortem, Tissues



## Post-mortem Redistribution (PMR) of Delta-9-tetrahydrocannabinol (THC) Demonstrated by an Investigation into Blood THC Concentration Discrepancies Between Ante-mortem and Post-mortem Specimens

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### Background/Introduction

Given its physicochemical properties, delta-9-tetrahydrocannabinol (THC) is a drug that is expected to be susceptible to the phenomenon of post-mortem redistribution (PMR). It is therefore somewhat surprising that predictable and definitive trends have not been established in regards to the PMR of THC. At the Victorian Institute of Forensic Medicine (VIFM) a femoral blood specimen is collected upon the admission of a body to the mortuary, thereby providing a toxicological specimen that minimizes the effects of PMR. We have previously reported discrepancies in THC concentrations between paired admission specimens and those taken at autopsy (sometimes up to days later). In this study, we analyzed blood from individuals who had presented to hospital and subsequently died whilst receiving medical care. Both ante-mortem bloods taken prior to death and post-mortem admission bloods were analyzed for the quantitation of THC and compared.

### Objective

To determine if blood THC concentrations in paired specimens change from the period prior to death to the time of collection at post-mortem admission.

### Methods

Twenty cases were analyzed over a 4 year period where **both** ante-mortem and post-mortem specimens were available from individuals who had died in hospital. In all cases, the deceased was in intensive care from hospital admission until death and therefore had no possible exposure to cannabis during this period. Cases were identified where THC concentrations in the post-mortem admission blood specimens were higher than corresponding ante-mortem specimens requested from the treating hospital, and specifically where only the post-mortem concentrations would have been reportable by laboratory guidelines. Police and hospital records were examined to determine the period between presentation to hospital and death, in addition to the nature of the incident resulting in the initial hospitalization. All deaths occurred in metropolitan Melbourne, resulting in the body being admitted promptly to the VIFM. THC was analyzed using a fully validated LC/MS-MS method for blood specimens.

**Results:** The mean post-mortem blood THC concentration was 10.9 ng/mL (median 7.0 ng/mL, range 1.5 – 59.8 ng/mL, n=20), while the mean ante-mortem blood concentration was 0.6 ng/mL (median 0.4 ng/mL, range 0 – 2.5 ng/mL, n=20). Duration of hospitalization ranged from 20 min to 26 days (median 14 hours). Causes of death (determined by the pathologist) consisted of motor vehicle incident drivers/passengers/pedestrians, suicide, drug overdose, natural causes and ‘unknown’ findings; (mainly suspected heroin overdoses, and one case where novel psychoactive drug-use was confirmed).

**Conclusion/Discussion:** This study revealed a surprising number of cases where post-mortem blood THC concentrations were higher than corresponding ante-mortem hospital specimens, despite the deceased individuals having no possible access to cannabis during their hospitalization. Furthermore, the relative magnitude of these concentration discrepancies demonstrated that significant post-mortem changes can occur with THC in blood, where some cases showed no measureable THC in the ante-mortem specimens yet produced substantial post-mortem concentrations. While the exact mechanisms behind these post-mortem changes are unknown, these findings indicate that the forensic community continues to be faced with substantial challenges in interpreting post-mortem blood THC concentrations. The limit of reporting for toxicology cases with a positive blood THC at VIFM is 2 ng/mL; hence, in all these cases THC would not have been reported if only the ante-mortem specimen had been analyzed. Conversely, in the absence of ante-mortem specimens and hospital information, THC would have been reported only in the post-mortem blood of all 20 cases; notably, this could have had particular implications in regards to the interpretation of recent cannabis use prior to death in the motor vehicle accident victims in this cohort. Further caution in the interpretation of post-mortem blood THC concentrations is required given the degree to which THC can redistribute after death.

**Keywords:** THC, Redistribution, Post-mortem

## Determination of Fentanyl, Norfentanyl and New Fentanyl Derivatives in Plasma by GC Triple Quadrupole with Application in Clinical Toxicology Analysis

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**Background/Introduction:** Fentanyl (a piperidine derivative) is a powerful synthetic opioid analgesic and anaesthetic, 50-100 times stronger than morphine. These powerful effects are also observed in the fentanyl derivatives, such as thiofentanyl, acrylfentanyl and valerylfentanyl. Due to their mechanism of action, acting predominantly at the  $\mu$ -opiate receptor, they produce psychoactive effects leading people to abuse of these substances causing many cases of acute intoxication and deaths worldwide. In Brazil, these substances were found in the streets in the form of blotters, tablets and colored powder. Overdose results in respiratory depression which is reversible with naloxone if diagnosed early. Blood concentrations of approximately 7 ng/mL or greater have been associated with fatalities, then analysis of fentanyl and fentanyl derivatives requires highly selective and sensitive methodologies.

**Objective:** The aim of this work was the development of a method based on liquid-liquid extraction followed by acetylation to quantify fentanyl, norfentanyl, thiofentanyl, acrylfentanyl and valerylfentanyl in plasma by gas chromatography-triple quadrupole mass spectrometry (GC-MS/MS). The method was successfully applied in cases of emergency analysis.

**Methods:** An aliquot of 300  $\mu$ L of plasma were extracted with 1.2 mL of 2-chlorobutane at basic pH after the addition of internal standard (cocaine-d<sub>3</sub> 100 ng/mL). After vortexing for 3 minutes, sample was centrifuged at 12.500 rpm for 5 mins, the supernatant was collected, evaporated to dryness and then acetylated (50  $\mu$ L of pyridine and 50  $\mu$ L of acetic anhydride + 5 mins for 400 W microwave). The mixture was evaporated to dryness and reconstituted with 50 $\mu$ L of methanol and transferred to vial insert. Two microliters were injected onto the GC-MS/MS. The analyses were conducted using a Shimadzu GCMS-TQ8040 triple quadrupole with ultra-inert capillary column (5% phenyl stationary phase, with dimensions of 15 meter x 0.25 mm I.D. x 0.25  $\mu$ m film thickness) operated in the multiple reaction monitoring (MRM) mode. Optimized retention time, MRM transitions and collision energies were obtained from empirical determination using the MRM Optimization Tool<sup>®</sup>.

**Results:** The respective transitions and retention time optimized were: fentanyl (245.0 > 146.0; 245.0 > 158.0; 11.657 min), acetyl-norfentanyl (231.0 > 158.1; 217.0 > 158.1; 9.787min), thiofentanyl (245.0 > 146.0; 245.0 > 158.0; 11.721 min), acrylfentanyl (243.0 > 200.0; 243.0 > 158.0; 11.737 min), valerylfentanyl (273.0 > 146.0; 273.0 > 189.0; 12.441 min), cocaine-d<sub>3</sub> (185.0 > 85.0; 185.0 > 91.0; 8.809 min). Total run 15.3 min. The method was linear from 0.5 to 50 ng/mL for all analytes ( $r > 0.99$ ), accuracy > 85%, precision CV < 9.7% for all intra and inter-day assay. **Case 1:** A 31-y-old man had been admitted into the intensive care service unconscious after college party with severe respiratory depression, hypotension and a green powder in nasal mucosa. Toxicology analysis detected: 1.3 g/L of ethanol in blood, cannabinoids in urine and 6.1 ng/mL of fentanyl and 0.6 ng/mL of norfentanyl in plasma. Fentanyl was also detected in green powder collected from nasal mucosa. **Case 2:** Nurse contacted the poison control center requesting an antidote for LSD, ecstasy and alcohol in emergency case of young male who arrived unconscious, with respiratory depression and vomiting. His friends reported they drank vodka, and ingested LSD blotter and ecstasy pills. Toxicology analysis detected: 0.8 g/L of ethanol in blood, MDMA in urine, LSD not detected in blood and urine, and 10.5 ng/mL of fentanyl, and not detected norfentanyl (LOD: 0.5 ng/mL) in plasma.

**Conclusion/Discussions:** Fentanyl, norfentanyl and fentanyl derivatives in plasma can be rapidly assessed with the present method by GC-MS/MS which when compared to LC-MS equipment becomes a cheaper alternative for forensic and clinical toxicology laboratories. The method was successfully applied in clinical toxicology to diagnose acute intoxication by these opioids.

**Keywords:** Fentanyl Derivatives, GC-MS/MS, Clinical Toxicology Analysis

## Changes and Trends in the Novel Illicit Opioids use in 2016 and 2017 from a Large Postmortem Population

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**Background/Introduction:** Novel illicit opioids have emerged as significant novel psychoactive substances (NPS) in the past two years. The drugs that achieve popularity at any given time are changing rapidly. In our casework, as the novel substances initially detected (e.g. Carfentanil, Furanyl fentanyl, U-47700) become scheduled or fall out of favor with recreational users, new substances appear to replace them (e.g. Acryl fentanyl, Butyryl fentanyl). This constant change poses a significant challenge to agencies that monitor for changing patterns in emerging drug use, especially toxicology laboratories that first have to develop testing for these substances in biological specimens.

**Objective:** Toxicological data identifying novel illicit opioid use in postmortem and human performance toxicology casework, in addition to drug seizure data, provides intelligence to law enforcement and public health agencies such as the Drug Enforcement Agency (DEA) and Centers for Disease Control (CDC). We describe how tracking the change in popularity of six novel illicit opioids over time, and geographically linking toxicology results aids in the understanding of the true scope and dynamic change of the opiate epidemic at a regional level.

**Methods:** Through routine testing at the National Medical Services (NMS) Labs, multiple incidental findings outside the scope of routine analysis were documented regarding the detection of novel illicit opioids using Liquid Chromatography/Time of Flight Mass Spectrometry (LC-TOF/MS) screening. A panel was developed using Liquid Chromatography-Tandem Mass Spectrometry (LC-MSMS) to confirm and quantify 19 designer opioid compounds. Between November 2016 to January 2017, data was compiled to investigate positivity rates as well as demographic information for six of the most common novel illicit opioids detected.

**Results:** A total of 844 novel opioid positive results were obtained during this time period, 655 of which were positive for the following: Carfentanil (242; 36.9%), PFBF/FIBF (113; 17.2%), Furanyl Fentanyl (372; 56.7%), Butyryl Fentanyl/Isobutyryl Fentanyl (8; 1.2%), Acryl Fentanyl (128; 19.5%), and U-47700 (140; 21.3%) either on its own or in some combination with another novel illicit opioid. A total of 218 cases were positive in some combination of novel opioids, of which PFBF/FIBF and Furanyl Fentanyl (85; 38.9%), Furanyl Fentanyl and U-47700 (72; 33.0%), and Carfentanil and Furanyl Fentanyl (64; 29.3%) were found to be the most common. Carfentanil, Furanyl Fentanyl, and U-47700 also were positive in combination in 20 cases (9.17%). The change in positivity for each analyte month to month was also evaluated. From a demographic standpoint, most the positive cases were found in males (440) versus females (136), with most cases (428) involving individuals between 20 and 49 years old. Statewide distribution of novel illicit opioids detected in 33 different states and Canada shows the heaviest regions with novel illicit opioids from NMS Labs casework were the Northeast [NJ (57), NY (21), & PA (77)], the Midwest [IL (53), MI (178), & OH (25)], and Florida (94).

**Conclusion/Discussions:** Due to the limitation of only having results for cases sent to NMS Labs, the data produced may not accurately produce a comprehensive distribution of these novel substances nationwide. However, it does provide drug intelligence for regions that need to be aware of not just traditional opioids, but their designer counterparts as well. Further investigation is being conducted into April 2017, as the growth in popularity of a second generation of novel illicit opioids has been observed. Overall, this will expand the evaluated timeline of the presence of these compounds in the market to gain a better understanding of the shifts that occur in recreational use and regional locations.

**Keywords:** LC-MS/MS, NPS, Opioids

## Distribution and Stability of Acetyl fentanyl and Acetyl norfentanyl in a Heroin Related Death

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**Background/Introduction:** Deaths resulting from the use of heroin either mixed or substituted with fentanyl or similar opioid analogues have risen dramatically over the past several years. These compounds have no approved medicinal purpose though numerous emergency room visits have been reported in conjunction with their use. Many of these overdoses and/or deaths may be associated with a criminal case, so reanalysis could be required at a future date. As a result, establishing data on the effects of long term storage is important to the interpretation of toxicology findings. In this work, we report a death where heroin and acetyl fentanyl were discovered through comprehensive toxicology screens. In order to examine the postmortem distribution and stability of these drugs, we assayed a variety of blood sources and tissues proximal to autopsy collection and then repeated the measurements after a year in storage.

**Objective:** To present the distribution of acetyl fentanyl and metabolite concentrations in various matrices and investigate the stability of acetyl fentanyl and acetyl norfentanyl in these specimens when stored frozen after a 1 year period.

**Method:** 6-acetylmorphine, acetyl fentanyl, and several metabolites were isolated from a variety of matrices using an alkaline solid phase extraction. Samples were analyzed using liquid chromatography tandem mass spectrometry

**Results:** A 22 year old male was found unresponsive in his secure barracks room on post. There was no evidence of foul play at the scene, nor any notes expressing suicidal ideations. Investigation of the scene revealed evidence of recent intravenous drug use – a belt tourniquet, syringe with needle, several small empty packets labeled “Crazy Samurai”, and a clear plastic bag containing a powdery residue. A field test indicated that the latter was an opioid. At autopsy, there were approximately three needle punctures measuring less than 1 mm with small surrounding contusions on the right antecubital fossa. Furthermore, the lungs displayed moderate pulmonary congestion and edema (combined lung weight 1500 grams). In the toxicology examination, routine immunoassays signaled the presence of opiates, 6-acetylmorphine, and fentanyl. More extensive mass spectrometry screens determined the fentanyl to be a variant known as acetyl fentanyl. Acetyl fentanyl and metabolite concentrations for both analyses are displayed in the table below:

		(ng/mL)			(ng/g)					
		Heart blood	IVC blood	Vitreous	Liver	Heart	Kidney	Spleen	Lung	Brain
After Autopsy	Acetyl fentanyl	18	20	52	77	27	53	36	88	26
	Acetyl norfentanyl	6.2	6.8	4.1	40	7.6	19	6.5	12	6.8
After 1 year	Acetyl fentanyl	15	18	50	73	32	40	41	77	23
	Acetyl norfentanyl	7.3	9.7	6.6	62	13	28	9.4	18	---
Additional Autopsy Findings	6-Acetylmorphine	< LOQ	< LOQ	31	--	16	--	103	3.0	28
	Morphine	411	381	78	1096	674	1061	1131	1360	358
	Codeine	11	11	4.9	22	23	40	55	40	30

IVC, Inferior Vena Cava; LOQ, Limit of Quantitation; --, None Detected.

**Conclusion/Discussions:** Acetyl fentanyl and acetyl norfentanyl were quantitated in a variety of matrices at the time of autopsy and again after a one year time interval. A comparison of the concentrations from both analyses support a profile of good stability under these conditions since the values remained largely unchanged. This postmortem case provides a relevant example of how novel opioids can still be analyzed after a long period in storage. These results also provide a reference for tissue concentrations when interpreting toxicology in a case where blood specimens might not be available for analysis.

**Keywords:** Acetyl Fentanyl, Distribution, Stability

## Development of a Screening Method for Fentanyl Analogues Using UPLC-QTOF-MS with Data Independent Acquisition (DIA) in MS<sup>E</sup> Mode and its Application in a Retrospective Analysis of 2339 Authentic Blood Samples

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**Background/Introduction:** Since the recent appearance of new fentanyl analogues and the increment of related overdose deaths worldwide, the development of sensitive screening methods to determine fentanyl analogues in biological samples and seizures should be a continuous improvement task in forensic laboratories.

**Objectives:** The aims of the present study were the development and implementation of a targeted screening method for fentanyl analogues in authentic whole blood samples using liquid chromatography with high resolution mass spectrometry in elevated collision energy mode (MS<sup>E</sup>) and its application for a retrospective analysis of authentic whole blood samples from 2016.

**Method:** The method was developed for the detection of 52 4-anilidopiperidine related fentanyl analogues using ultrahigh performance liquid chromatography quadrupole time-of-flight mass spectrometry (LC-QTOF-MS). Targeted identification of fentanyl analogues was based on exact mass of the precursor ion and predicted product ions, and retention time (RT) when available. Prediction of the product ions was performed under the assumption that all 4-anilidopiperidine related fentanyl analogues follow common fentanyl-specific cleavages. Sample preparation was performed by protein precipitation on a fully automated robotic setup and the separation was performed by a 15 min gradient. Thirteen fentanyl analogues were selected for validation of the method, and matrix effect (ME), extraction recovery (RE), process efficiency (PE), limit of detection (LOD) and of identification (LOI) were evaluated. The retrospective analysis was applied for the analysis of 2339 authentic blood samples. Additionally, to enhance the confidence for the identification and diminish false positives, an *in silico* method was created to predict the RT for those fentanyl analogues with unknown RT using ACD/ChromGenius software, based on structural similarities (similarity coefficient *Dice*) between each compound from the database and the targeted structure, and on the experimental liquid chromatography setup used in the developed screening method.

**Result:** The proposed hypothesis was supported by the analysis of fentanyl analogues with available reference standards in the laboratory (n = 16) and available published sources, online mass spectral databases and published literature. It was observed that these compounds with a 4-anilidopiperidine skeleton are fragmented at the C-N bond between the piperidine ring and the amide group; in 15 out of 16 of the cases the formed carbocation was the most abundant fragment ion in the collision cell. Furthermore, the formed carbocation is characteristic of the fentanyl analogues group structure.

Evaluation of ME showed a small ion enhancement effect (110 - 123 %) for all compounds; RE ranged from 67 to 81 % and PE from 81 to 98 %. LOD was 0.0005 mg/kg, except for the least retained compounds in the chromatographic system, acetylnorfentanyl and norfentanyl, with a LOD of 0.001 mg/kg and a LOI of 0.005 mg/kg, while for the rest of the compounds LOIs were 0.001 mg/kg or 0.002 mg/kg. In the retrospective analysis of 2339 blood samples the major finding was fentanyl (56 cases) and alfentanyl (5) (confirmation of other fentanyl analogues is ongoing). Norfentanyl was also detected in 6 cases but it was most likely a metabolic product. A preliminary *in silico* study to calculate RT for fentanyl analogues showed good prediction accuracies (92-116 %), except for those compounds with RT < 4 min; and calculated *versus* experimental RT correlation showed a  $r^2$  of 0.96.

**Conclusion/Discussion:** A targeted screening method for 52 fentanyl analogues was successfully developed, validated and implemented for the analysis of authentic blood samples, where the identification of targeted fentanyl analogues can be achieved tentatively without the use of reference standards.

**Keywords:** Time-of-Flight Mass Spectrometry, Screening, Fentanyl Analogues

## The Impact of Buprenorphine/Naloxone Ratio on Apparent Adherence to Suboxone® Therapy

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**Background/Introduction:** Suboxone® is a substance abuse product formulated as a 4-to-1 buprenorphine-to-naloxone ratio. Urine drug monitoring is employed to inform clinicians about patients' adherence with their therapy. Consequently, the drugs and metabolites monitored are a crucial part of providing adequate information about patients' consistency with their prescribed regimen. Currently, simple buprenorphine and/or norbuprenorphine positivity are used as the primary indicator(s) of adherence with Suboxone® therapy. Naloxone detection was previously deemed as less reliable; however, hydrolysis has since revealed significant improvements in naloxone detection.

**Objective:** The results of liquid chromatography/tandem mass spectrometry (LC/MS/MS) analysis of a large population of Suboxone® therapy patients indicates that the presence of both co-dosed drugs and their ratios can provide additional insight about consistency with Suboxone® therapy.

**Methods:** Data was acquired from patients who were prescribed Suboxone® and submitted urine samples for both naloxone and buprenorphine LC/MS/MS analysis. Samples were diluted 2X with IS and enzyme buffer solution followed by 30 minute incubation at 60°C. Extracted samples were reconstituted in 50% methanol: 50% water and run on a 3 minute gradient using a Restek Raptor™ Biphenyl column. Analytical ranges for buprenorphine and norbuprenorphine were 2 ng/mL to 1,000 ng/mL and 10 ng/mL to 2,500 ng/mL for naloxone. The results of 33,670 patient samples were used to determine the impact of hydrolysis on naloxone testing along with the value of not only testing for both analytes but also the impact of the ratio of the two analytes as a method of determining apparent adherence to Suboxone® therapy.

**Results:** Buprenorphine alone, when used to monitor Suboxone® usage in patients yields an apparent adherence rate of approximately 97.6%. Furthermore, 90% of patients tested were positive for naloxone and 97.3% were positive for norbuprenorphine while 88.4% were positive for all three analytes. Previous reports have suggested that naloxone is negative more frequently than buprenorphine making it an unreliable indicator of consistency with Suboxone® therapy. However, results of this study suggest that positive naloxone results are to be expected and can be used to monitor Suboxone® therapy. Hydrolysis has a significant impact on naloxone testing with results increasing from 34% naloxone positivity without hydrolysis to 100% positivity with hydrolysis. Most importantly, the ratio of buprenorphine-to-naloxone in patient samples can present great value in understanding abuse or misuse potentials in patients on Suboxone® therapy. The median and mean total buprenorphine-to-naloxone ratios were found to be 1.72 and 3.57 respectively. It is noteworthy that the formulated ratio of buprenorphine-to-naloxone in Suboxone® is 4.00. Total buprenorphine is equivalent to the summation of buprenorphine and adjusted norbuprenorphine. Thus, the ratio of total buprenorphine-to-naloxone is a close approximation of the initial formulation. Further examination of these results can provide insight into patients who are likely misusing or abusing their medications or may have genetic deficiencies.

**Conclusions/Discussions:** An in-depth understanding of the drug-to-metabolite and drug-to-drug ratios can present an innovative method for clinicians to interpret patient results. Unlike normal opiates, monitoring for simple positivity of buprenorphine and norbuprenorphine cannot rule out abuse or non-adherence to treatment. Hence using the ratios proposed herein affords a more accurate estimate of apparent adherence. Total buprenorphine-to-naloxone ratios that are significantly larger than the mean of 3.57 may indicate sample adulteration or abuse while lower ratios may indicate infrequent medication use. Furthermore, higher total buprenorphine-to-naloxone ratios can indicate oral or improper sublingual administration while lower ratios can indicate intravenous use. By hydrolyzing urine samples, more accurate levels of naloxone can be determined. Together with buprenorphine and norbuprenorphine, these hydrolyzed naloxone levels can provide more accurate estimates of apparent adherence than simply monitoring the individual drugs alone.

**Keywords:** Suboxone® Therapy, Buprenorphine, Naloxone

## New Psychoactive Substances (NPS)-Related Deaths in Taiwan During the 2012–2016 Period

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**Introduction:** The 2014 World Drug Report by the UNODC (United Nations Office on Drugs and Crime) reported an increased number of detected new psychoactive substances (NPS) from 251 in July 2012 to 348 in December 2013, while the number of psychoactive substances controlled on an international level was 234. In recent years, NPSs have for example been found in instant coffee or milk tea packets in nightclub and within the party scenes in Taiwan.

**Objective:** UNODC's 2016 World Drug Report emphasized that "understanding the NPS problem in order to address it more effectively requires efforts ... to improve ... forensic capacity related to NPS detection and ... monitoring systems". This study reports the analytical methodologies, trends and toxicological findings of the 204 known NPS-related fatalities in Taiwan during the 2012–2016 period.

**Methods:** Postmortem blood samples were routinely screened using liquid-liquid extraction (Toxi-tubes® A), followed by GC/MS, LC/ion-trap/MS, and LC-QTOF/MS with automated library search protocols. NPSs were quantified by LC-MS/MS or GC/MS using deuterated analog internal standards. The LOQ established for the analysis of these NPSs was 0.010 µg/mL.

**Results:** Among the 204 cases reviewed, the mean age of the NPS-related fatalities was 29.0, ranging from 15 to 54, while 162 (79.4%) of these deaths were men and 42 (20.6%) were women. Top ten NPSs detected were: ketamine (n = 149; 29.2%), PMA/PMMA (n = 117; 22.9%), ethylone (n = 62; 12.2%), methylone (n = 52; 10.2%), 4-chloroamphetamine (n = 41; 8.0%), 4-fluoroamphetamine (n = 31; 6.1%), 5-MeO-MiPT (n = 26; 5.1%), mephedrone (n = 14; 2.7%), butylone (n = 13; 2.5%), and 25B-NBOMe (n = 5; 1.0%). There was evidence of poly-drug use in 95% of these NPS-positive cases, including 1 to 5 other drugs. Average NPSs detected per case increased from 1.9 in 2012 to 4.1 in 2016. Furthermore, these NPSs were ruled as the cause of death in 156 cases (76%). Mean concentrations of ketamine (n = 140), PMA (n = 113), PMMA (n = 61), ethylone (n = 60), 4-chloroamphetamine (n = 39), methylone (n = 36), 4-fluoroamphetamine (n = 30), mephedrone (n = 11), and butylone (n = 11) were 1.03, 3.24, 2.28, 2.66, 0.59, 5.28, 0.37, 1.84, and 12.17 mg/mL, respectively. Fifty (25%) fatalities occurred during or shortly after activities at a club, party, or rave.

**Conclusion:** Out of the total numbers (2927, 3003, 3513, 3654, and 3557 in 2012, 2013, 2014, 2015, and 2016 respectively) of forensic postmortem toxicological cases received and analyzed in our laboratory, NPS-related deaths increased from 0.34% in 2012 to about 2% in 2016. Growing use of NPSs has become a critical public health concern — given the lack of understanding on these substances' toxicity, addiction potential, and withdrawal symptoms. "Greater efforts [are] needed to enhance forensic capacity and monitoring systems for new psychoactive substances" (UNODC's 2016 World Drug Report).

**Keywords:** New Psychoactive Substances, Postmortem toxicology, Fatalities

## Separation of Ortho, Meta and Para Isomers of Methylmethcathinone (MMC) and Methylethcathinone (MEC) Using LC-ESI-MS/MS: Application to Forensic Serum Samples

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**Background/Introduction:** Separation and identification of constitutional isomers is an important issue in forensic toxicology. Despite the structural similarity, positional isomers often show different pharmacological properties and thus can exhibit differences with respect to their toxicity. Additionally, besides these pharmacological and toxicological effects, the legal status is also of great importance.

**Objective:** The aim of this study was the development of a validated LC-MS/MS method for the separation of the ortho, meta and para isomers of methylmethcathinone (MMC) and methylethcathinone (MEC). Retrospective measurements of samples with suspicion of a recent MMC or MEC consumption collected in the period from June 2014 to August 2016 were also performed.

**Methods:** For sample preparation, 200 µl of the serum sample was fortified with 10 µl butylone<sub>3</sub> [1 µg/ml] and a subsequent protein precipitation was done using 200 µl methanol. After vortexing and centrifugation, 50 µl of the supernatant was diluted with 150 µl water. Chromatographic separation of the isomers was achieved using a Restek® Raptor Biphenyl column (100 mm x 2.1 mm, 2.7 µm particle size). The mobile phase consisted of (A) 0.1% formic acid in water/methanol (95:5, v/v) and (B) 0.1% formic acid in methanol. Mass spectrometric measurements were performed in the multiple reaction monitoring (MRM) mode using the following specific ion transitions:  $m/z$  178.1 → 145.1 and 178.1 → 160.0 for MMC isomers,  $m/z$  192.1 → 174.0 and 192.1 → 144.0 for MEC isomers.

**Results:** Reliability of the method was confirmed under consideration of the validation parameters selectivity, linearity, accuracy and precision, analytical limits, processed sample stability, matrix effects and recovery. Application of the method to ten real serum samples revealed the proof of a recent MMC or MEC consumption, respectively, in eight cases. Isomers of MMC could be detected in three of these eight cases, of which two were positive for 3MMC and one was positive for 2-MMC. The other samples were tested positive for 3MEC. In none of the samples 4MMC, 2MEC or 4MEC could be detected.

**Conclusion/Discussions:** A selective LC-MS/MS method for the separation and clear identification of the ortho, meta, and para isomers of MMC and MEC was developed and validated. Only substances that were not governmentally controlled at that point of time could be detected, reflecting the rapid response of the recreational drug market to newly enacting drug laws.

**Keywords:** Methylmethcathinone (MMC), Methylethcathinone (MEC), Positional Isomers



## Prevalence of Synthetic Cannabinoid Receptor Agonists in Sub-populations in Scotland

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**Background/Introduction:** Synthetic Cannabinoid Receptor Agonists (SCRAs) have been a concern for forensic toxicologists since their appearance on the market in the mid-late 2000s. The rapid emergence and turnover of hundreds of different compounds has created a challenging environment in which to determine the extent of their use. More recently reports of adverse reactions and death, as well as the determination of extreme potency in some compounds, has highlighted the need for sensitive, accurate and reliable methods of detection for this group of drugs.

Prevalence data is becoming available for certain geographical areas and subsections of populations but there is a perception that SCRA use is widespread and problematic, certainly within the prison population in the United Kingdom. Unfortunately data is currently sparse as to whether this perception is scientifically justified.

**Objective:** The objective of this study was to assess the use of SCRAs within different sections of the Scottish population

**Methods:** Urine samples were collected from individuals undergoing admission to or liberation from 7 Scottish Prison Service (SPS) facilities; individuals under supervision of the Scottish Drugs Court (SDC); and individuals receiving treatment from the National Health Service Greater Glasgow and Clyde Forensic Directorate (NHS GGC FD) psychiatric services.

Aliquots (0.5 mL) were subjected to a simple liquid-liquid extraction (LLE) protocol and analysed by Liquid Chromatography – tandem Mass Spectrometry (LC-MSMS) for the following analytes:

AB-CHMINACA, AB-CHMINACA metabolite 1A, AB-CHMINACA metabolite 2, AB-FUBINACA, AB-FUBINACA metabolite 2B, AB-FUBINACA valine metabolite, AB-PINACA, 5F-AB-PINACA, AKB48, AKB48 N-(5-hydroxy pentyl), AKB48 N-pentanoic acid, 5F-AKB48, 5F-AKB48 N-(4-hydroxy pentyl), AM-2201, AM-2201 N-(4-hydroxy pentyl), AM-2201 N-(5-hydroxy indole), APICA, APICA N-(4-hydroxy pentyl), BB-22, BB-22 3-carboxy indole, MDMB-CHMICA, MDMB-CHMICA O-desmethyl acid metabolite, MDMB-CHMINACA, 5F-MDMB-PINACA, 5F-NPB-22, PB-22, PB-22 N-(5-hydroxy pentyl), PB-22 N-pentanoic acid, PB-22 N-pentanoic acid-3-carboxy indole, 5F-PB-22, 5F-PB-22 3-carboxy indole, STS-135, and STS-135 N-(4-hydroxy pentyl) These analytes were deemed to be the most commonly available in the UK and thus most likely to be encountered. The data was collated and indications of the prevalence of SCRAs in these subpopulations were drawn.

**Results:** A total of 725 urine samples were received from the SPS cohort. One or more SCRA compound was detected in 2.9% (n=21) samples. All positive samples were admission samples, with the exception of 1 which was labelled neither admission nor liberation. A total of 73 urine samples were received from the SDC cohort, with only 1 sample (1.4%) being found positive for any of the SCRAs tested. A total of 95 urine samples were received from the NHS GGC FD cohort. All samples were negative for SCRAs in this group.

**Conclusion/Discussions:** Whilst these studies cannot be judged as true prevalence studies as consent was required by ethical review and not all SCRAs were included in the panel, invaluable information was obtained as to the use of SCRAs in different sub-populations in Scotland. It is acknowledged that sample sizes were relatively small, however, data was collected on the specific SCRA compounds in use and the demographics in which SCRA use might be more common. The number of positive results indicates that SCRA use is relatively rare within the populations included, which is contrary to the opinion expressed by many media outlets reporting on the subject. Even so, reports of the effects of SCRAs have shown they can be highly potent compounds with significant risk of harm to health, so an awareness of their prevalence is essential. This information can inform toxicologists, medical professionals, prison staff and lawmakers and allow improvements in the way SCRA use is managed in society. How many and which SCRA's were targeted in method? Address whether this panel was expected to as comprehensive as it would need to be to get a true prevalence rate.

**Keywords:** Synthetic Cannabinoid Receptor Agonists, Prevalence, Biological Fluids

## Simultaneous Detection of New Psychoactive Substances from a Single Sample of Human Urine by Incorporating New Tests Alongside Already Existing Test Sites on a Biochip Array

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**Background/Introduction:** New Psychoactive Substances (NPS) have been defined as those that are not listed under the United Nations 1961 or 1971 conventions, but may pose a serious threat to public health. Such 'legal highs' have been around since the 1980s, but the market for these drugs is rapidly evolving and expanding. Over the last 4 years NPSs like AB-CHMINACA, AB-PINACA, alpha PVP and UR-144/XLR11 have become popular in the legal high market. In this context the availability of screening tools adaptable to the new trends in the NPS drug market is of value. Biochip array technology not only enables the screening of multiple NPS from a single sample, but also has the capacity to adapt the biochip arrays to meet the testing needs, incorporating new tests alongside already existing tests

**Objective:** The aim of this study was to update a current biochip array, which enables the simultaneous screening of benzylpiperazines, JWH-018, mephedrone, MDPV, mescaline, phenylpiperazines and salvinorin A, by adding new test sites on the biochip surface for the simultaneous detection of the new NPS: AB-CHMINACA, AB-PINACA, alpha PVP and UR-144/XLR11.

**Methods:** Simultaneous-competitive chemiluminescent immunoassays, defining eleven discrete test sites on the biochip surface, were employed. Multiple antibodies and conjugates were produced, screened and selected to ensure compatibility of the new tests with the original tests already present on the biochip array. The assays can be applied to the semi-automated Evidence Investigator and fully-automated Evidence analyzers. The results are semi-quantitative. Chosen cut-offs for detection of specific analytes were validated through determination of percentage recovery to spiked concentration at -50% cut-off concentration and +50% of the cut-off concentration in negative human urine. Each sample was ran in triplicate across 5 separate runs to determine both the percentage recovery and inter-assay precision. The sensitivity of individual assays were determined through analysis of twenty different negative urine samples to assess the respective 'Limit of Detection' (the mean concentration detected plus three standard deviations).

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**Results.:** Drugs detected at respective validated cut-offs include: AB-CHMINACA (2ng/mL), AB-PINACA (5ng/mL), alpha-PVP (1ng/mL), benzylpiperazines (10ng/mL), JWH-018 (10ng/mL), mephedrone (5ng/mL), mescaline (7.5ng/mL), phenylpiperazines (7.5ng/mL), salvinorin A (0.5ng/mL) and UR-144/XLR-11 (5ng/mL). Recovery (%) of the -50 cut-off and +50% of the cut-off concentrations was achieved within 70-130% range of the spiked concentration apart from alpha-PVP -50% cut-off sample on Evidence Investigator (131%) and mescaline -50% cut-off sample on Evidence (138%). All samples spiked below the cut-off reported negative and all samples spiked +50% cut-off reported positive. The Limit of Detection for each analyte was less than 50% of the cut-off concentration. Inter-assay precision values (n=15), expressed as CV(%) were  $\leq 20\%$  for all assays at three different concentration levels

**Conclusion/Discussions:** The results indicate applicability and optimal analytical performance of this biochip array for the simultaneous detection of the NPS AB-CHMINACA, AB-PINACA, alpha-PVP and UR-144/XLR-11 as well as benzylpiperazines, JWH-018, mephedrone, mescaline, phenylpiperazines and salvinorin A from a single human urine sample. By incorporating new tests alongside current tests on a biochip surface, biochip array technology shows test flexibility and adaptability in combating the ever changing market of NPS use. Accurate and sensitive screening of a broad range of NPS facilitates the drug testing process especially when facing the rapidly evolving and expanding market of 'legal highs'.

**Keywords:** New psychoactive substances, Biochip Array, Drug screening

## A Fatal Poisoning Involving 2-fluorofentanyl

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**Background/introduction:** A fatal intoxication involving 2-fluorofentanyl, a potent synthetic opioid analgesic, is reported. This is the first serious case associated with 2-fluorofentanyl that to our knowledge have been reported. 2-fluorofentanyl is the common name for N-(2-fluorophenyl)-N-[1-(2-phenylethyl)-4-piperidinyl]propanamide (InChIKey: BKUWDIVZCJNXRA-UHFFFAOY-SA-N). Structurally it is an ortho-fluoro derivative of fentanyl. It is a new illegal substance on the drug scene, as the first reports on this drug in Europe appeared in 2016; First in Denmark in March, then in Ireland and Sweden in April [European information system and database on new drugs, EDND]. In the present case from Denmark, a 23-year-old male was found dead in his apartment on March 16th 2016, approximately 3 days after being seen alive. A small plastic bag with approx. 1 g of white powder was found in the waist of his pants.

**Objective:** To determine the cause of death by analyzing postmortem samples from the deceased and to identify the seizure found at the scene of death.

**Methods:** Medico-legal autopsy was performed on the deceased, during which time peripheral whole blood, bile, liver tissue, muscle tissue and gastric content samples were submitted for toxicological examination; all samples were screened by ultra-performance liquid chromatography high-resolution time-of-flight mass spectrometry (UPLC-HR-TOFMS) and quantified by specific UPLC-MS/MS methods. Powder recovered from the scene of death was identified using gas chromatography–mass spectrometry (GC-MS), UPLC-HR-TOFMS and Nuclear Magnetic Resonance (NMR) (<sup>1</sup>H, <sup>19</sup>F and <sup>13</sup>C).

**Results:** The powder was unambiguously identified to be 2-fluorofentanyl. 2-fluorofentanyl was detected by UPLC-HR-TOFMS in all matrices collected from the autopsy. Post-mortem femoral blood concentrations found were: 2-fluorofentanyl 0.012 mg/kg (UPLC-MS/MS); alcohol < 0.2 ‰ (w/w) (GC-FID) likely post-mortem production as the body was moderate decomposed; buprenorphine 0.0004 mg/kg (UPLC-MS/MS) prescribed; quetiapine 0.088 mg/kg (UPLC-MS/MS) prescribed; venlafaxine 0.089 mg/kg (UPLC-MS/MS) prescribed; o-desmethylvenlafaxine 0.31 mg/kg (LC-MS/MS), metabolite of prescribed drug. The autopsy findings were consistent with acute poisoning. The decedent had a history of drug abuse. The route of administration of 2-fluorofentanyl was unknown. However, we presume the drug was snorted as no syringe was found at the scene of death.

**Conclusion/discussions:** Based on the toxicological findings, the cause of death was determined to be a fatal overdose with 2-fluorofentanyl. 2-fluorofentanyl is a very potent and dangerous drug. The purity of the powder found at the scene of death was high, thus making it easy to cause a fatal intoxication. Further, a fatal blood concentration of this potent drug is difficult to detect even when using highly sensitive methods like UPLC-HR-TOFMS. To our knowledge, the present abstract reports the first quantification of 2-fluorofentanyl in biological specimens in a fatal intoxication.

**Keywords:** 2-fluorofentanyl, Fatal Intoxication, UPLC-HR-TOFMS

## Analysis of Selected Designer Benzodiazepines by Ultra High Performance Liquid Chromatography with High-Resolution Time-of-Flight Mass Spectrometry

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**Background/Introduction:** In the last decade, increasing number of intoxication by new designer drugs has occurred. Two large classes of new designer drugs have been described recently, namely synthetic cathinones and synthetic cannabinoids. During the last five years, new psychoactive substances – designer benzodiazepines (DBZDs) - have been detected and seized on illegal drug scene. DBZDs have related structures as therapeutically used benzodiazepines (BZDs), but they are not used such the medical purposes. Pyrazolam was among the first non-therapeutic benzodiazepines abused. The potential risk of DBZDs abusing is not only in addiction, but also in serious health complications and consequences, because the pharmacology and toxicology of DBZDs are not deeply described unlike in case of BZDs used for the legal therapy.

**Objective:** The aim of the work was developed a new UHPLC-ESI-TOF-MS method for selective and sensitive separation, identification and determination of selected DBZDs (namely pyrazolam, phenazepam, etizolam, flurbomazepam, diclazepam, deschloroetizolam, bentazepam, meclonazepam, nimetazepam and flubromazolam) in human serum.

**Methods:** The blood samples obtained from 100 healthy volunteers were collected and centrifuged at 2,500 rpm for 5 min. The obtained sera were collected and pooled. The pooled serum was analyzed to ensure negative results for the studied analytes and used as a matrix for method validation and spiked sample analyses. Liquid-liquid extraction (LLE) with butyl acetate was used for the samples preparation. All LC-MS analyses were carried out using a UHPLC UltiMate 3000 RSLC System (Dionex, Sunnyvale, CA, USA) connected with a UHR-TOF Maxis Impact HD (Bruker Daltonics, Billerica, USA). Chromatographic separations were performed at 40 °C on a reverse phase analytical column Acclaim RS 120, C18 2.2 µm, 2.1 x 100 mm (Thermo Fisher Scientific, Waltham, USA). The UHPLC UltiMate 3000 RSLC System contained binary rapid separation pumps, solvent rack with two degasser channels, an analytical in-line split loop, a thermostated autosampler and a thermostated column. The chromatographic conditions used were as follows: injection volume 5 µL; flow rate 0.5 mL/min; mobile-phase solvents used (A) water/ACN 99/1 (v/v) with 2 mM ammonium formate and 0.1 % formic acid (v/v) and (B) ACN/water 99/1 (v/v) with 2 mM ammonium formate and 0.1 % formic acid (v/v); gradient protocol: 1% B (0 min, 1-min hold), 70% B (5 min, 0.2-min hold) and 1% B (6.3 min, 0.7-min hold); the total run time was 7 min.

**Results:** The method was validated in terms of linearity, LOD, LOQ, matrix effects, specificity, precision, accuracy, recovery and sample stability. The LOD values were in the range from 0.10 to 0.15 ng/mL. The recoveries were higher than 84 %. The application of the developed UHPLC-ESI-TOF-MS method was demonstrated on the analyses of spiked serum samples.

**Conclusion/Discussions:** A new method of UHPLC-ESI-TOF-MS simultaneous separation, identification and quantification of ten DBZDs was developed and validated. The method is selective and sensitive for potential application as a method for STA in case of DBZDs abuse.

**Keywords:** Designer Benzodiazepines, Liquid Chromatography, Mass Spectrometry

## How Generic Narcotics Laws (Do Not) Influence the Legal High Market

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**Background/Introduction:** New Psychoactive Substances (NPS) have become a substantial part of the drug market for many years now. They are mainly distributed via online shops in the form of products like herbal blends, bath salts and e-liquids, or as research chemicals. Based on the models from other European countries, the German legislative put into force a law based on a generic definition of new psychoactive substances (NpSG) on November 26<sup>th</sup>, 2016. Two substance groups of NPS were banned by definition of chemical structures: substances derived from 2-phenethylamine and cannabimimetics/synthetic cannabinoids.

**Objective:** The aim of the present work is to provide an overview of how the new legislation affected the product range of legal-highs available in online shops. For this purpose, the online market of NPS was systematically monitored in the frame of the EU-financed project 'SPICE Profiling'.

**Methods:** Herbal blends, bath salts, e-liquids, and research chemicals were bought via the internet on a regular basis. The total monitoring period encompasses one year – starting from September 2016 until the beginning of the TIAFT 2017. Till March 2017 162 products were bought within the monitoring program. The analysis consisted of a solvent extraction followed by GC-EI-MS analysis. The spectra were compared to in-house libraries, the Cayman Spectral Library and the SWGDRG Mass Spectral Library. Unknown substances were structurally characterized by NMR after isolation with flash chromatography.

**Results:** From September 2016 until the end of November 2016 68 products were bought in 15 different shops. They mainly contained NPS (66%) or narcotics (24%). After the entry into force of the NpSG, 90 products were ordered in 18 different internet shops between December 2016 and March 2017. 20% contained NPS which are covered by the NpSG and compounds not covered by the NpSG were detected in 24% of the cases. The percentage of products containing narcotics increased to 47%. Already in December 2016, six herbal blends have been acquired containing an unknown substance which was identified as Cumyl-PEGACLONE. The core structure of this compound is a  $\gamma$ Carboline not covered in the NpSG yet. Until end of March 2017, Cumyl-PEGACLONE was detected in 21 herbal blends.

**Conclusion/Discussions:** During the regular monitoring of online shops the percentage of narcotics in the ordered products significantly increased. This effect was seen right after the NpSG came into force. Based on banning most of the NPS by means of the NpSG the shop owners obviously moved to selling narcotics. In particular, the synthetic cannabinoids MAM-2201, UR-144 and JWH-210 were detected, although they had been disappeared from the market a couple of years ago after being banned. Furthermore, the percentage of NPS currently not covered by the NpSG strongly increased. It is very likely that the synthetic cannabinoid Cumyl-PEGACLONE was specifically designed for the German market to circumvent the NpSG.

**Keywords:** New Psychoactive Substances, Monitoring, Narcotics

## Using Direct MS and Microextraction for Drug Screening from Biological Matrices

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**Background/Introduction:** The field of illicit drug testing is a constantly changing environment with rapid development of unregulated designer and synthetic compounds. The difficulty for forensic testing facilities is the fact that these compounds are not detected under normal ELISA testing methods; therefore, more specific MS based approaches are necessary. The extraction mechanism for Biocompatible Solid Phase Micro Extraction (BioSPME) combined with DART-MS was used to rapidly screen for selected drugs of abuse analogs at ng/mL levels from biological matrices. By employing a fast and accurate screening method, the amount of samples that require confirmatory analysis can be reduced and laboratories can increase throughput and decrease costs associated with their drug screening programs..

**Objective:** By eliminating steps that are necessary in some of the more extensive SPE procedures (i.e. elution and evaporation), the BioSPME method obtained reproducible and accurate results in less time. Advantages over current methodologies with respect to the time of preparation, solvent usage and pre-concentration of the analytes to achieve screening detection limits will be demonstrated.

**Methods:** A model set of drugs were fortified in plasma, saliva, and urine to demonstrate the BioSPME sampling technique with direct MS detection. Samples are aliquotted into 96 well plates and extracted by immersion of the BioSPME fibers. The BioSPME fibers are then directly analyzed with DART-MS system.

**Results:** The method demonstrated reproducible extraction efficiencies from human saliva with accuracies ranging from 81-120% for all analytes and relative standard deviations (% RSD) ranging from 1.9-7.0%. Table 1 shows the average accuracies and % RSD's.

Table 1. Average measurement accuracies with %RSD from saliva samples

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**Conclusion/Discussions:** Using BioSPME fiber tips to extract the samples reduced the sample preparation steps as compared to SPE techniques, while still providing matrix removal. Incorporation of direct MS analysis with the DART-QDa system enabled analytical results in seconds, and with no solvent usage as compared to HPLC analyses. Combining the simple BioSPME extraction procedure with DART-MS produced a fast, reproducible screening method for high throughput analysis of biological samples.

**Keywords:** Microextraction, DART-MS, Drug Screening

## Differentiation of *o*-, *m*-, *p*-fluorine Positional Isomers of Synthetic Cannabinoid 'AB-FUBINACA' and Synthetic Cathinone 'Fluoromethcathinone' Using Triple Quadrupole Energy-Resolved Mass Spectrometry

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**Background/Introduction:** Mass spectrometric differentiation of positional isomers is crucial for the analysis of new psychoactive substances (NPS) in order to determine whether they fall under legal restrictions or not, as for some controlled substances, legal restrictions may not include their isomers. However, it is difficult to differentiate *o*-, *m*-, and *p*-halogen positional isomers on the phenyl ring of synthetic cannabinoids and synthetic cathinones, because they exhibit similar retention properties and mass spectral patterns. Legal and analytical difficulties prompted us to obtain reliable evidence for differentiating such positional isomers. In this study, we utilized gas chromatography–electron ionization–triple quadrupole mass spectrometry in product ion scan mode and investigated the product ion spectra as a function of collision energy (energy-resolved mass spectrometry; ERMS) of *o*-, *m*-, and *p*-fluorine positional isomers of both 'AB-FUBINACA' and 'fluoromethcathinone'.

**Objective:** To differentiate *o*-, *m*-, and *p*-fluorine positional isomers on the phenyl ring of AB-FUBINACA and fluoromethcathinone using ERMS.

**Methods:** The *o*-, *m*-, and *p*-fluorine positional isomers of AB-FUBINACA and fluoromethcathinone were purchased from Cayman Chemical (Ann Arbor, MI). Product ion spectra were obtained by GCMS-TQ8040 (Shimadzu Corporation, Kyoto, Japan) with fused-silica capillary columns SH-Rxi-5SilMS (30 m × 0.25 mm i.d.; 0.25 μm film thickness) operated in electron ionization mode at 70 eV. 1-μL of the working standard methanol solutions (20 μg/mL) were injected into the mass spectrometer. Quantum chemical calculations were performed with Gaussian 09 Rev.D. The geometries were optimized, and the vibrational frequencies and the zero-point energies were calculated with the density functional theory (DFT) method at the B3LYP/6-31G(d,p) level [1].

**Results:** ERMS analyses of the *o*-, *m*-, and *p*-fluorine positional isomers of AB-FUBINACA showed while the detected product ions were identical, slight differences were observed in the relative abundances of the product ions at *m/z* 109 and *m/z* 253. In order to make the differences clearer, the logarithmic values of the abundance ratio of the ion at *m/z* 109 to *m/z* 253 ( $\ln(A_{109}/A_{253})$ ) were plotted against collision energy. The values showed *meta* < *ortho* < *para* relationship at each collision energy, suggesting that the three isomers required different energies for cleaving the indazole moiety from the fluorobenzyl group [2]. The differences in the  $\ln(A_{109}/A_{253})$  values of the three isomers were statistically significant according to Tukey's test. Additionally, the relationships between  $\ln(A_{109}/A_{253})$  and collision energy were linear with high correlation coefficients, decisively separating the three approximation lines. The relative standard deviations (%RSD) determined by the intra-day and inter-day variabilities of the abundances of the two ions at *m/z* 109 and 253 were < 7.2% and < 9.2%, respectively. DFT calculation indicated that the dissociation reactivity for cleaving between the indazole moiety and the fluorobenzyl group was in the order of *meta* < *ortho* < *para*, which confirmed the relationship of the three AB-FUBINACA isomers obtained by ERMS. The present method was applicable to real case samples. Using the same methodology, the *o*-, *m*-, and *p*-positional isomers of fluoromethcathinone were significantly differentiated by comparing the  $\ln(A_{95}/A_{123})$  values, whose results were also confirmed by DFT calculation.

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- 1. Conclusion/Discussions:** We developed a differentiation method for *o*-, *m*-, and *p*-fluorine positional isomers of both AB-FUBINACA and fluoromethcathinone based on the product ion abundances using ERMS strategy. Our methodology would be applicable to the determination of the fluorine substituted positional isomers of other similarly structured NPS.

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**Keywords:** NPS, Positional Isomer Differentiation, Energy-Resolved Mass Spectrometry

## Development of a New Biochip Array for the Detection of Multiple Illicit Fentanyl Compounds and Derivatives from One Urine Sample

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**Background/Introduction:** Opioid analgesics are commonly used in health care, yet they have substantial abuse potential. This danger is emphasized by the fact that opioids are responsible for more than 20,000 overdose deaths per year in the United States. More recently, a new wave of illicit synthetic opioids including fentanyl derivatives has emerged. Fentanyl compounds such as acetylfentanyl, butrylfentanyl, furanylfentanyl, acrylfentanyl and carfentanil have all been observed in casework in 2016. The danger of these compounds is highlighted by carfentanil, which is 10,000 and 100 times more potent than morphine and fentanyl respectively. It is therefore important to detect these compounds in the screening step of drug testing. Evidence biochip array technology provides a platform for the simultaneous determination of multiple drugs of abuse from a single sample using matrix dedicated kits. The miniaturization of the immunoassays reduces the volume of sample and reagent per test and increases the results output.

**Objective:** The aim of this study was to develop a new biochip array to maximize the detection of illicit fentanyl compounds and derivatives from human urine samples. Multiplexing becomes more complex when the target antibodies are structurally similar; standardizing antigens can be detected on more than one target, similarly, optimal conjugates can also operate more than one assay.

**Methods:** . In-house made polyclonal antibodies and conjugates were used. The antibodies to fentanyl, norfentanyl, ocfentanyl, furanylfentanyl, remifentanil and sufentanil were immobilised and stabilised on the biochip surface in discrete test regions (DTRs). Six simultaneous competitive chemiluminescent biochip-based immunoassays, applied to the Evidence Investigator biochip analyzer, were employed. The signal output is inversely proportional to the concentration of drug in the sample. Each DTR was assessed with the six optimal conjugates and the six standardizing antigens to determine how the assays could be multiplexed. Specificity with over 20 different fentanyl compounds and limit of detection (LOD) with negative human urine samples (n=18) were determined. The sample volume required was 25µL. The results are semi-quantitative.

**Results:** Four of the six antibodies responded well to ocfentanyl antigen therefore this was selected as the standardizing compound for these assays with an assay range of 0-20ng/mL. Remifentanil and sufentanil are more specific antibodies so both antigens were required in the multi-analyte calibrators, 0-2ng/mL and 0-5ng/mL respectively. Three antigen-HRP conjugates were used for the multiplex array. Fentanyl conjugate was specific for the fentanyl assay, ocfentanyl bound to both norfentanyl and ocfentanyl antibodies. Sufentanil conjugate bound to both the remifentanil and sufentanil antibodies. 25 fentanyl compounds were screened across the six assays. On review of the furanylfentanyl specificity profile, no additional compounds were detected with this assay; both furanylfentanyl and its metabolite norfuranylfentanyl were detected on the norfentanyl assay, 106% and 22% respectively. Based on this, the furanylfentanyl assay was removed. The remaining five assays were required to achieve a full cross-reactivity profile with the 25 fentanyl derivatives. There was high detection of the key compounds; acetylfentanyl and acrylfentanyl were detected on the fentanyl assay 95% and 100% respectively. Butrylfentanyl and furanylfentanyl were detected on the borfentanyl assay 150% and 106% respectively. Carfentanil was detected 121% on the remifentanil assay. Resulting LODs were  $\leq 0.5\text{ng/mL}$  for all five assays. Remifentanil LOD was 0.16ng/mL.

**Conclusion/Discussions:** The results indicate that this new biochip array is applicable to the simultaneous detection of multiple illicit fentanyls from a single human urine sample. Biochip array technology is suitable for combating the ever changing market of designer opioid use as it presents test flexibility and adaptability. A reliable multiplex screening of a broad range of opioids from one sample, facilitates the drug testing process especially when facing the rapidly evolving and expanding market of 'designer drugs'.

**Keywords:** Biochip Array, Fentanyl, Screening



## Phase I Metabolism of the New Synthetic Cannabinoid Cumyl-4CN-BINACA and Detection in Human Urine Samples

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**Background/Introduction:** Synthetic cannabinoids (SCs) are a class of new psychoactive substances (NPS) commonly sold as ‘legal highs’ via online shops. The SC Cumyl-4CN-BINACA (1-(4-cyanobutyl)-*N*-(1-methyl-1-phenylethyl)-1*H*-indazole-3-carboxamide) was originally described in a patent application of Bowden and Williamson from 2014 (compound SGT-78). The compound emerged in January 2016 on the European drug market and was first identified by the Hungarian police in a herbal mixture. Other cumyl derivatives covered in the patent, like Cumyl-PINACA or Cumyl-5F-PINACA, have been detected on the drug market before. In contrast to other prevalent SCs, Cumyl-4CN-BINACA has an aliphatic nitrile function which is a rarely observed feature within this class of drugs. In the Institute of Forensic Medicine in Freiburg (Germany), the substance was detected in 19 ‘legal high’ products purchased in 2017 (total number of purchased products 109). This sharp increase in prevalence necessitated the development of analytical methods for the detection of this drug. Since urine samples are usually the preferred matrix for drug abstinence testing and SCs are known to be extensively metabolized prior to renal excretion, the main *in vivo* metabolites have to be identified for a reliable detection in urine.

**Objective:** Aim of this study was the identification of reliable Cumyl-4CN-BINACA consumption markers for urine analysis and the application to authentic case samples.

**Methods:** A collective of 16 authentic human urine samples was investigated using an LC-ESI-MS/MS system. Findings were confirmed by LC-ESI-qToF-MS. Sample preparation included conjugate cleavage using *beta*-glucuronidase and salting out assisted liquid-liquid extraction (SALLE) with acetonitrile/ammonium formate. Due to the lack of commercially available reference standards, a pooled human liver microsome (pHLM) assay was conducted to generate *in vitro* reference spectra of Cumyl-4CN-BINACA phase I metabolites. The detected metabolic profile obtained by the pHLM assay was compared to the *in vivo* metabolic profiles in human urine. The two most abundant *in vivo* phase I metabolites were included in an LC-MS/MS-based routine screening method for SC metabolites.

**Results:** In total, 28 phase I metabolites of Cumyl-4CN-BINACA were detected *in vivo*. The observed metabolic pathway included *N*-desalkylation, hydroxylation, formation of dihydrodiols, formation of the 4-hydroxybutyl metabolite and further oxidation to the butanoic acid metabolite, as well as combinations of these reactions. The butanoic acid metabolite and a metabolite mono-hydroxylated at the cumyl moiety were the most abundant phase I metabolites in the urine samples analyzed. Screening of 1,611 authentic urine samples between January and March 2017 resulted in a total of 204 samples positive for SC metabolites (13%). Among these Cumyl-4CN-BINACA uptake could be confirmed in 50 samples (3%).

**Conclusion/Discussions:** The nitrile function of the compound is subject to extensive metabolism (formation of the 4-hydroxybutyl metabolite and further oxidation) leading to metabolites highly abundant in human urine samples. This might also apply to other substances with a terminal nitrile function. It seems likely that this reaction is comparable to the hydrolytic defluorination of compounds with a terminal fluorine atom at the *N*-alkyl side chain. An alternative mechanism might be the hydrolysis of the nitrile function with subsequent decarboxylation combined with hydroxylation in position 4 of the *N*-alkyl chain. The butanoic acid metabolite was the most abundant phase I metabolite in human urine among the investigated sample collective and should be targeted for reaching maximum sensitivity (as required for drug abstinence testing). The most abundant *in vivo* phase I metabolite with intact nitrile function, and therefore a highly specific Cumyl-4CN-BINACA marker, was a metabolite mono-hydroxylated at the cumyl moiety. Among the samples tested positive for SC in 2017, 25% were positive for metabolites of Cumyl-4CN-BINACA indicating a rapidly growing distribution of this compound. For this reason it is strongly recommended to update screening methods for SCs, facilitating the detection of this compound and its main metabolites.

**Keywords:** LC-MS/MS, New Psychoactive Drugs, SGT-78

**Opioid Toxidrome after Cocaine Sniffing: First Case in Europe of Non-Fatal Overdose by Carfentanil Laced Cocaine**

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**Background/Introduction:** An increasing number of opioid overdoses and deaths have been associated with the use of fentanyl and its synthetic analogues, particularly in north America. These “ultra-potent” opioids are often associated with heroin or cocaine regarding their low cost of producing; most of illicit fentanyl analogues are not mentioned on the convention on narcotic drugs (1961).

**Objective:** In 2017, a white powder, presented as being cocaine, was given to a 41-year-old man who sniffed it during a private party. Within a few minutes, he was comatose and presented a miosis and dyspnea. His respiration was quickly medically assisted by the emergency unit. The patient was then hospitalized in an intensive care unit. Unfortunately, since he was thought to have sniffed cocaine only, he was not treated by naloxone. His recovery was complete without any sequelae and came back home after a few days.

**Methods:** Samples of plasma at hours-31 and urine at hours-1 were available. A first screening by GC/MS was performed in urine in full scan mode. A targeted analysis on 9 derivatives of fentanyl was performed in plasma and urine by LC/MS-MS. 200 µL of sample were added to 200 µL of precipitant reagent (sulfosalicylic acid 500 mg/mL) with deuterated internal standard (fentanyl-D5). After centrifugation, supernatant was injected in a 2D-chromatographic system (oasis HLB/X Select Waters) coupled with API4000 Sciex. MRM transitions were m/z 395.2/335.3 or 113.1 or 246.2 for carfentanil; m/z 291.2/231.0 or 142.0 or 113.0 for norcarfentanil and 342.3/188.2 for fentanyl-D5. Cocaine and metabolites were also quantified by LC/MSMS.

**Results:** In urine, the screening by GC/MS revealed the presence of cocaine and its metabolites benzoylecgonine (BE), ecgonine-methylester (EME), anhydroecgonine-methylester as well as the presence of levamisole as adulterant and drugs that have been administered during the hospitalization (lidocaine and midazolam). The analysis by LC/MS-MS revealed the presence of carfentanil and its metabolite norcarfentanil (concentration < 0.05 µg/L in plasma and 8.8 µg/L in urine). In plasma, only residues of BE were present after 31 hours. In urine, BE and EME were present at the following concentrations: 570, 1230 and 367 µg/L.

**Conclusion/Discussion:** The results demonstrated the exposure to carfentanil were consistent with the clinical opioid toxidrome of the patient. No other opioids or fentanyl analogues have been identified by the screenings.

Carfentanil is 10,000 more potent than morphine on opioid receptor which might be responsible of overdose cases and thus represent a major health problem.

**Keywords:** Carfentanil, Fentanyl, Opioid Overdose

## Jefferson County Fentalogues: A 6 Month Review

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**Background/Introduction:** Fentanyl analogues (i.e. fentalogues), are a nationwide problem; Jefferson County Alabama is no exception. These Novel Psychoactive Substances (NPS) have grown in popularity due to their ability to create the desired effects experienced by abuse of other opioids without violating current laws. This quest for "legal highs" has resulted in the availability of hundreds of fentalogues.

**Objective:** Fentalogues are increasingly problematic for the Jefferson County Coroner and Medical Examiner's Office (JCCMEO). In the last six months, a total of 18 deaths, representing 2% of all toxicology requests, have been related to fentalogue use. The resulting data has been trended by following metadata: age, race, sex, drug history, and analyte concentrations.

**Methods:** From August 2016 to January 2017, five separate fentalogues were identified during routine postmortem toxicological analyses. Drug screening for fentanyl and its analogues was performed using a fentanyl immunoassay kit obtained from Immulysis®. Positive screens were confirmed by basic liquid-liquid extraction followed by gas chromatography mass spectrometry (GC/MS) analysis using mepivacaine as the internal standard. Fentalogue spectra are included in both the Scientific Working Group for Drug (SWGDRUG) and the Cayman Chemical reference libraries. Upon positive identification of a fentalogues, certified reference materials were obtained to confirm and quantify results.

**Results:** Fentalogues encountered by JCCMEO included: carfentanil, acryl fentanyl, despropionyl fentanyl (4-ANPP), fluoro fentanyl, and methoxy acetyl fentanyl. There were 28 incidences of these fentalogues over 18 cases. Of the 18 decedents, the mean age was 36 with a median of 31 years of age. There were 14 Caucasians and 4 African Americans. Males accounted for 61 % (n=11); females accounted for 39% (n=7). A history of heroin abuse or heroin metabolites was identified in 72% cases (n=13). A history of cocaine abuse is associated with 4 of the 18 cases (22%). Methoxy acetyl fentanyl accounted for 36% (n=10) of the fentalogues reported with a mean concentration of 0.110 mg/L and a median of 0.014 mg/L. The fentalogue precursor, despropionyl fentanyl (4-ANPP), was encountered in 25% (n=7) of the cases with a mean concentration of 0.0081 mg/L and a median of 0.0059 mg/L. Acryl fentanyl was also present in 25 % (n=7) of the cases. Carfentanil occurred in 11% or 3 cases with a mean 0.012 mg/L and a median of 0.011 mg/L. Fluoro fentanyl was present in 3% (n=1) of the cases. An accurate mean and median could not be determined for acryl fentanyl and fluoro fentanyl due to the differences between the Limit-Of-Detection and Lower Limit-Of-Quantification.

**Conclusion/Discussions:** Results indicate the fentalogue problem in Jefferson County Alabama is most prevalent in Caucasian males in their early 30's with a history of illicit drug use, primarily heroin. Furthermore, the median and mean concentrations of these fentalogues align with those related to fentanyl toxicity (3.0 – 28 ng/mL, Baselt 10<sup>th</sup> Ed.). Although not obvious, this observation is true for methoxy acetyl fentanyl. When we considered the time frame of these cases, methoxy acetyl fentanyl was initially determined at concentrations ranging from 0.132 mg/L to 0.449 mg/L (n=4). After two weeks, the concentrations dropped significantly ranging from 0.005 mg/mL to 0.011 mg/L (n=6). Based on these concentration differences, it is proposed that this new fentalogue was spiked into the heroin supply at high doses to generate demand. Once the reputation, and thereby demand, for methoxy acetyl fentanyl was established, dosing was reduced. In short, fentalogues have become problematic in Birmingham, AL. Furthermore, observed demographic trends and toxic concentration for these specific fentalogues are consistent with those published.

**Keywords:** Fentanyl Analogs, Methoxy Acetyl Fentanyl, Fluoro Fentanyl, Acryl Fentanyl, 4-ANPP, Carfentanil, Forensic Toxicology

## Evaluation of New Developed Antibodies to AH-7921, Carfentanil, MT-45, and U-47700 for Application to ELISA Based Screening of Designer Opioid Use

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**Background/Introduction:** In recent years, designer opioid abuse have continuously emerged as a major issue for law enforcement, health-care professionals and wider society. Synthetic opioid deaths increased by 79% during 2013–2014 across 27 US states. However, the fatality incidence is likely underestimated as designer opioids are often not detected by drug tests commonly employed by forensic professionals. It is therefore important to detect these compounds in the screening step of the drug testing process.

**Objective:** The aim of this study was to evaluate the feasibility of antibodies raised to AH-7921, carfentanil, MT-45, and U-47700 for ELISA based screening of designer opioid use. These antibodies will be intended to cross-react with metabolites and related compounds of the target drug.

**Methods:** Immunogens were developed and administered to adult sheep to initiate polyclonal antiserum production. The antisera generated were employed in the development of ELISAs. The selected Ig fraction derived from each antiserum was immobilized and stabilized on a 96-well micro-titre plate. The analyte, if present in the sample, competed with horseradish peroxidase labelled conjugate for antibody binding sites on the microtitre plate. Absorbances were read at 450nm. The feasibility of a cut-off for a specific ELISA was confirmed by determining the recovery of negative urine and whole blood samples spiked with the target drug at the cut-off and 50% above and below the cut-off. Specificity was determine by calculating the percentage difference in half maximal inhibitory concentration ( $IC_{50}$ ) between the standardising compound and cross-reactant of interest.

**Results:** The analytical evaluation of the new developed antibodies in ELISA showed: for the antibody to AH-7921, the assay range was 0-2.5ng/mL in urine and 0-10ng/mL in whole blood (1:4 dilution); the cut-off was 1ng/mL in urine and 2ng/mL in whole blood (1:4 dilution) and cross-reactivity of 92.4% was found with the main urinary metabolite nor-AH-7921. For the antibody to carfentanil, the assay range was 0-2ng/ml in urine and 0-16ng/mL in whole blood (1:8 dilution); the cut-off was 1ng/ml in urine and 2ng/ml in whole blood (1:8 dilution). 25 fentanyl group compounds were screened and the assay was standardised to remifentanyl; cross-reactivity of 162.6% was found for carfentanil, 90.9% for remifentanyl acid, 65.5% for norcarfentanil, 30.2% for alfentanil and 12.8% for sufentanil. For the antibody to MT-45, the assay range was 0-50ng/mL in urine (1:10 dilution) and 50ng/mL in whole blood (1:10 dilution); the cut-off was 5ng/mL in urine (1:10 dilution) and 5ng/mL in whole blood (1:10 dilution) and cross-reactivity of 139.3% was found for hydroxyl MT-45. For the antibody to U-47700, the assay range was 0-600ng/mL in urine (1:10 dilution) and 0-1200ng/mL in whole blood (1:20 dilution); the cut-off was 10ng/ml in urine (1:10 dilution) and 25ng/ml in whole blood (1:20 dilution) and cross-reactivity of 32.3% was found for bi-desmethyl-U47700 and 19.8% for N-desmethyl U-47700.

**Conclusion/Discussions:** The results indicate applicability of the newly produced antibodies in the use of ELISA screening of the synthetic opioids AH-7921, carfentanil, MT-45, and U-47700 in urine and blood. These ELISAs are highly sensitive and show detection of appropriate urinary metabolites. ELISAs generated with these antibodies will provide a useful analytical tool in detecting very low concentrations of designer opioids in urine and blood samples. This will assist criminal justice and forensic professionals in detecting and combating the ever rising tide of designer opioid use within society.

**Keywords:** Designer Opioid, ELISA, Screening

**RTI-111 – New Research Chemical or Old Acquaintance?**

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**Background/Introduction:** The phenyltropane RTI111 (methyl (1*R*,5*S*)-3-(3,4-dichlorophenyl)-8-methyl-8-azabicyclo[3.2.1]octane-2-carboxylate), also called dichloropane or O-401, is a stimulant drug showing high resemblance to cocaine regarding chemical structure as well as psychotropic effects. In the early 1990s this substance and other substituted phenyltropane derivatives were synthesized to investigate the binding affinity of cocaine-like substances at monoamine transporters. Recently, this substance emerged on the research chemical market being marketed as a legal substitute for cocaine. The first seizure of this substance was reported to the European Monitoring Centre for Drugs and Drug Addiction (EMCDDA) in late 2016 by the Swedish police, while another sample of RTI-111 was collected by the Slovenian National Forensic Laboratory.

**Objective:** In the last months (December 2016 - March 2017) two cases of an assumed RTI-111 uptake were submitted to our laboratory; samples were stored at -20°C until analysis. Case 1 comprised a serum sample and case 2 a femoral blood and a urine sample. For investigation of these samples our screening method for designer stimulants had to be updated with RTI-111 and its main metabolites.

**Methods:** RTI111 was purchased online as a research chemical and tested for identity and purity by means of GC-MS and NMR analysis. Enhanced product ion scans were performed and the three most abundant ion transitions were included into an existing LC-MS/MS screening method for designer stimulants. Additionally, three putative ion transitions covering two anticipated metabolites were included. As plausible metabolites and in analogy to the metabolism of cocaine, the ester hydrolysis metabolite and the product of *N*-demethylation were chosen. LC-ESI-MS/MS parameters of RTI-111 were optimized to gain maximum sensitivity. Sample preparation of urine and serum specimens consisted of an automated solid phase extraction (Aspec<sup>®</sup> GX274, Gilson) using Chromabond<sup>®</sup> Drug cartridges (Macherey-Nagel). LC-ESI-MS/MS analysis was performed on a Shimadzu HPLC system coupled to a SCIEX QTRAP<sup>®</sup> 4000 mass spectrometer operated in positive ionization mode (serum: LOD = 0.2 ng/mL, LOQ = 0.5 ng/mL; urine: LOD = 0.3 ng/mL, LOQ = 1.0 ng/mL). As no reference material was available for LC-MS/MS analysis of the assumed metabolites, supplemental LC-QToF-MS experiments were conducted on a Bruker impact II<sup>TM</sup> system.

**Results:** All three samples tested positive for RTI-111. In case 1 (serum) a concentration of 0.6 ng/mL RTI-111 was detected, while case 2 showed 1.0 ng/mL RTI-111 in femoral blood and 4.5 ng/mL RTI-111 in urine.

While in case 1 (serum) only the ester hydrolysis metabolite was detected, both assumed metabolites were detected in case 2 (femoral blood and urine). The signals for the ester hydrolysis metabolite showed the highest abundance in all three samples, while the *N*-demethylation product was only found in lower (femoral blood) or equal intensities (urine) compared to RTI-111. The findings were qualitatively verified by LC-QToF-MS analysis.

**Conclusion/Discussions:** Unchanged RTI-111 seems to be a suitable consumption marker in human serum and urine samples. However, considering the rapid metabolic breakdown of cocaine and other ester compounds, it seems advisable to additionally monitor metabolites of this substance like the *N*-demethylated compound and in particular the ester hydrolysis product. The ester hydrolysis product showed relatively high signal intensities both in urine and serum (RTI-111/RTI-111 ester hydrolysis product: 0.84% for case 1 (serum), 6.2% for case 2 (femoral blood), and 16% for case 2 (urine)). Therefore, this metabolite likely offers the broadest window of detection. Further studies on pharmacodynamics and pharmacokinetics of RTI-111 are needed for conclusive interpretation of RTI-111 (and metabolite) findings.

**Keywords:** RTI-111, LC-MS/MS, Metabolites

## Prevalence of Novel Psychoactive Substances and Classic Drugs of Abuse in Pain Management and Mental Health Populations

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**Background/Introduction:** Novel psychoactive substances (NPS) are abused due to their ability to produce more intense psychostimulant effects than common illicit drugs and their easy accessibility. Novel psychoactive substances are classified according to stimulant or hallucinogenic effects or according to their chemical family. Synthetic cathinones (bath salts) are commonly used as cheaper substitutes for methamphetamine and cocaine while mitragynine (Kratom) have opiate-like effects in high doses and have been reportedly used as opioid substitutes. This study explores the prevalence of alpha-PVP (a synthetic cathinone) and mitragynine, two frequently tested NPS, along with instances of use of other synthetic cathinones, synthetic cannabinoids and common drugs of abuse including —opiates, opioids, benzodiazepines, barbiturates, amphetamines, cocaine, heroin, ketamine, and marijuana.

**Objective:** An existing LC/MS/MS method for Bath Salt analysis was updated to include alpha-PVP and mitragynine which have been reported by NMS to be amongst the most frequently abused NPS. The prevalence in the tested population for these two designer drug substances was compared with that of other designer drugs as well as common drugs of abuse.

**Methods:** Data analysis was conducted on 79,598 discrete patient urine specimens submitted for alpha-PVP confirmation test, of which 8,864 also requested a mitragynine confirmation test, along with 2,067 patient specimens submitted for mitragynine confirmation testing only. LC/MS/MS analysis was conducted on samples diluted 5X with internal standard solution and analyzed on a Waters TQD LC/MS/MS platform. Details of the validation of the bath salts method which includes alpha-PVP and mitragynine can be found in earlier reports.

**Results:** Of the 79,598 submitted alpha-PVP requests, 6 patient specimens (0.008%) were reported positive. Of the subset of these patients submitted for testing of other drug categories, non-medical use was confirmed for opiates at 5.0%, benzodiazepines at 6.6%, amphetamine at 2.9% while illicit use was confirmed for methamphetamine at 2.4%, cocaine at 3.3%, and THCA at 10.3%. Furthermore, of the 10,926 patient specimens tested for mitragynine, 13 (0.12%), were found positive. Of the subset of these patients submitted for testing of other drug categories non-medical use was confirmed for opiates at 5.9%, benzodiazepine at 6.3%, amphetamine at 4.2% while illicit use was confirmed for methamphetamine at 3.0%, cocaine at 4.6%, and THCA at 21.0%. None of these patients tested positive for synthetic cannabinoids, another popular class of NPS.

**Conclusion/Discussions:** NPS are perceived to be more “popular” due to their ease of access and enhanced psychological effects. However, this did not correlate with the results of our tested pain management and mental health patient populations. The positivity rate for cocaine in both alpha-PVP and mitragynine populations, 3.3% and 4.6% respectively, are notably higher than the ~2% reported for the general population. Furthermore, 21.0% of the mitragynine population tested positive for marijuana which is nearly double the ~12.2% prevalence reported for the general population. Though there were seemingly trifling instances of alpha-PVP and mitragynine use, the high occurrence of cocaine and marijuana use in this population justifies the need for clinicians to monitor all potential drugs of abuse. Results suggest that these patients are a “high risk” and likely to abuse drugs not prescribed.

**Keywords:** Novel Psychoactive Substances, Alpha-PVP, Mitragynine

## Analysis of Fentanyl and Designer Fentanyl Derivatives in Urine using SPE and HPLC-MS/MS

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**Background/Introduction:** Fentanyl abuse is drastically on the rise in the United States. In addition to traditional fentanyl, modified versions of the drug are also being abused (i.e. fentanyl analogs). With the list of derivatives on the rise forensic toxicologists need the ability to rapidly identify not only fentanyl, but its modified forms as well. Generally, these compounds have varying levels of potency when compared to fentanyl, correlating to a wide range of encountered concentrations in biological fluids. While immunoassays are typically employed as a reliable, first-step screening tool, they have proven to be inaccurate when applied to the initial detection of these novel, synthetic drugs/metabolites. On account of this, a universal extraction approach for both current compounds in addition to emerging analytes is therefore vital to both screen and confirm their presence. Presented is a rapid, three step SPE procedure, that includes a concentration step, for the identification and quantification of fentanyl and its major urinary metabolite norfentanyl, in addition to seven “designer” opioid compounds: U-47700, desmethyl U47700, W-18, W-15, U58800, acryl fentanyl and furanyl fentanyl.

**Objective:** To develop a high throughput method to extract designer opioids from urine using SPE prior to LC-MS/MS analysis.

**Methods:** Urine samples were prepared by adding 1 mL of 100 mM phosphate buffer (pH 6.0) and internal standards to 1mL of urine. Samples were then applied directly to the SPE cartridge without any conditioning. Columns were washed with 3 mL of deionized water followed by 3 mL of 100 mM acetic acid. After drying the cartridges for 10 minutes under full positive pressure, analytes were eluted with 3 mL of methanol containing 2% ammonium hydroxide. Samples were evaporated to dryness and reconstituted with 100 uL of mobile phase.

Instrumentation	
HPLC System	Shimadzu Nexera X2 LC-30AD
MS System	Shimadzu LCMS-8050
HPLC Column	UCT Selectra® PFPP, 100 x 2.1 mm, 3 µm
Guard Column	UCT Selectra® PFPP, 10 x 2.1 mm, 3 µm
Column Temperature	40°C
Flow Rate	300 µL/min
Injection Volume	5 µL

Mobile Phase Gradient		
Time (min)	%Mobile Phase A Water + 0.1% Formic Acid	%Mobile Phase B Acetonitrile + 0.1% Formic Acid
0.0	90	10
5.0	0	100
8.0	0	100
8.1	90	10
12	90	10

**Results:** Recoveries were evaluated by fortifying samples at three varying concentrations. Data was calculated by dividing the chromatographic peak areas of urine samples spiked prior to extraction by those that were spiked post-extraction. Excellent recoveries were achieved for the range of analytes included in this study. On average, the recovery for samples spiked at 5 ng/mL (n=5) was 93%, for samples spiked at 20 ng/mL (n=5) it was 87% and for samples spiked at 50 ng/mL (n=5) it was 99%. The responses for the representative compounds were linear with R<sup>2</sup> values ranging from 0.96 to 0.99 over a concentration range of 10-200 ng/mL.

**Conclusion/Discussions:** Due to the large variation in fentanyl-like drugs mentioned in recent case reports, a panel of seven new derivatives were selected along with fentanyl and its major urinary metabolite norfentanyl for extraction and analysis. The universal SPE methodology was designed keeping in mind the continuously evolving target of designer opiate-like drugs and other novel analgesics. A reduction in wash steps and elimination of column conditioning allows for an overall savings on both analyst time and solvent usage.

**Keywords:** Fentanyl, SPE, Designer Drugs

**Stability and Validation of 26 Synthetic Cannabinoids in Urine at Various storage Conditions by LC-MS/MS**

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**Background/Introduction:** The abuse of novel psychoactive substances (NPS) has increased over the past decade, with synthetic cannabinoids (JWH, K2 or Spice) being a significant contribution to NPS pervasiveness. Synthetic cannabinoids refer to a growing number of mind-altering chemicals that can be either smoked from sprayed on dried plant material or vaporized and inhaled from e-cigarettes liquids. These drugs of interest target the CB1 and CB2 receptors of the brain, much like marijuana, but with toxic side effects. Recently, the DEA proposed placing the chemical compounds AB-CHMINACA, AB-PINACA and THJ-2001 under Schedule I classification of the U.S. Controlled Substances Act. A challenge to detection of this class of drugs is the composition of synthetic cannabinoid products is constantly changing to avoid legislation. This method was designed to quantify a large list of relevant synthetic cannabinoids (26) and determine their stability at various storage conditions.

**Objective:** To develop a sensitive, reliable and reproducible method for quantification of synthetic cannabinoid metabolites in urine and determine stability in urine at different storage temperatures.

**Methods:** Negative urine samples were fortified with known concentrations of standards and left stored under four conditions: room temperature (72°C), refrigerated (8°C) and freezer (-20°C) with both continuously frozen and freeze/thaw samples. The samples were stored for 3 months and tested at weekly intervals. Samples were extracted with organic solvent under acidic conditions, evaporated, reconstituted and analyzed on Agilent 1260 Infinity series LC system coupled with 6460 QQQ with JetStream Technology and ESI source. Chromatographic separation was achieved on a C-18 column under isocratic conditions. The method was validated using SWGTOX guidelines including: limit of detection (LOD), limit of quantitation (LOQ), accuracy, precision, matrix effects and interferences.

**Results:** Validation results included LOD (0.25 ng/mL), LOQ (0.5 ng/mL), acceptable accuracy ( $\pm 20\%$ ), precision (%RSD $<15\%$ ), no significant matrix effects ( $<\pm 20\%$ ) and no detected interferences. Stability of the 26 synthetic cannabinoids in urine was evaluated under four conditions with frozen storage conditions producing the most stable results over a 3 month period.

**Conclusion/Discussions:** The extraction and LC-MS/MS method developed for analysis of urine for 26 synthetic cannabinoids is precise, sensitive and reproducible at forensically relevant concentrations.

**Keywords:** Synthetic Cannabinoids, THJ-2201, Stability



## Phase I Metabolism of the Synthetic Cannabinoid CUMYL-PEGACLONE

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**Background/Introduction:** Synthetic cannabinoid receptor agonists (SCRAs) are a structurally diverse class of new psychoactive substances (NPSs). Most SCRAs that gained popularity as drugs of abuse are based on indole or indazole core structures. CUMYL-PEGACLONE (5-pentyl-2-(2-phenylpropan-2-yl)-2,5-dihydro-1*H*-pyrido[4,3-*b*]indol-1-one) is a SCRA with a novel structural feature that emerged on the German drug market in December 2016. In contrast to previous SCRAs, the linker group, connecting a bulky substituent to the core structure, is included into a tricyclic  $\gamma$ -carboline core. To date, this substance has been detected in our laboratory in 24 ‘legal high’ products. To prove consumption of the drug by analysis of urine samples, an analytical method looking for the main *in vivo* phase I metabolites had to be developed as the parent compounds of SCRAs are usually not detectable in urine due to their extensive metabolism.

**Objective:** The aim of this study was to investigate the *in vitro* and *in vivo* phase I metabolism of CUMYL-PEGACLONE in order to characterize its metabolic pathways, to identify reliable consumption biomarkers of the drug in urine and to demonstrate their applicability to authentic forensic cases.

**Methods:** As reference standards of CUMYL-PEGACLONE and its metabolites were not commercially available, the compound was purified from a ‘herbal mixture’ using semi-preparative flash chromatography. Pooled human liver microsomes (pHLM) were incubated with the isolated substance in order to generate *in vitro* phase I metabolites, and to record reference spectra by LC-MS/MS and LC-qTOF-MS. The ion transitions of the tentative *in vitro* main metabolites were integrated into an existing LC-MS/MS screening method and a total of 30 authentic urine samples were subsequently screened and found positive for CUMYL-PEGACLONE. These samples were used to identify the main metabolites in human urine samples after enzymatic cleavage of conjugates as reliable biomarkers for drug uptake.

**Results:** In total, 21 *in vivo* phase I metabolites were detected in the authentic urine samples after conjugate cleavage using  $\beta$ -glucuronidase. The metabolic reactions included mono- and di-hydroxylation, *N*-desalkylation, and the formation of a carbonyl function at the pentyl moiety in combination with mono-hydroxylation. Six *N*-desalkylated metabolites mono-hydroxylated at the core ring system could only be detected *in vivo*. Within the analyzed set of urine samples, mono-hydroxylated metabolites showed the highest abundances. By comparing the product ion spectrum of the parent compound with the characteristic product ions of these metabolites, the oxidative biotransformation was found to most likely occur at the  $\gamma$ -carboline core and the pentyl moiety, respectively.

**Conclusion/Discussions:** These *in vitro* and *in vivo* phase I metabolism studies revealed that CUMYL-PEGACLONE is an extensively metabolized SCRA. The parent compound was not detected in any of the urine samples. The metabolic reactions occurred mainly at the pentyl moiety and the  $\gamma$ -carboline core. The mono-hydroxylated metabolites are suitable targets for LC-MS/MS urine analysis of the substance. These metabolites facilitate detection of CUMYL-PEGACLONE consumption in human urine as proven by the analysis of 30 authentic specimens. The diversity of substances on the highly dynamic drug market limits the availability of reference standards for most NPSs and their relevant main metabolites. *In vitro* experiments using pHLMs offer a suitable approach to identify metabolites and generate reference spectra of phase I metabolites to circumvent this obstacle. Although SCRAs are expected to be extensively conjugated (e.g. by glucuronidation) as part of phase II metabolism, the detected phase I metabolites are reliable biomarkers in urine analysis as conjugate cleavage is commonly used prior to analysis in routine practice and enhances the overall sensitivity.

**Keywords:** CUMYL-PEGACLONE, Synthetic Cannabinoid Receptor Agonists, Phase I Metabolism

**Fentanyl and Fentanyl Related Substances Reported in NFLIS, 2014–2016**

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**Background/Introduction:** The National Forensic Laboratory Information System (NFLIS) is a program of the Drug Enforcement Administration (DEA), Diversion Control Division, which systematically collects drug identification results and associated information from drug cases submitted to and analyzed by Federal, State, and local forensic laboratories. These laboratories analyze controlled and noncontrolled substances secured in law enforcement operations across the country. NFLIS represents an important resource in monitoring illicit drug abuse and trafficking, including the diversion of legally manufactured pharmaceuticals into illegal markets. NFLIS data are used to support drug scheduling decisions and to inform drug policy and drug enforcement initiatives nationally and in local communities around the country.

**Objective:** The objective of this presentation is to present updated findings on fentanyl and fentanyl related substances submitted to State and local laboratories from January 1, 2014 through December 31, 2016, and analyzed within three months of each calendar year reporting period.

**Methods:** National annual estimates, regional trends, and reports by Federal laboratories for fentanyl and selected fentanyl related substances are presented. This presentation highlights reports of specific substances such as fentanyl, acetyl fentanyl, furanyl fentanyl or carfentanil by State between 2014 and 2016.

**Results:** In 2015, fentanyl was the 9<sup>th</sup> most frequently identified drug by State and local laboratories in the United States. Of the 14,440 fentanyl reports identified in 2015, more than three-quarters were identified by laboratories in the Northeast (5,896 reports) and Midwest (5,253 reports). About one-fifth of fentanyl reports were identified by laboratories in the South (3,013 reports). Few fentanyl reports were identified by laboratories in the West (278 reports). In 2015, fentanyl (4<sup>th</sup>), acetyl fentanyl (10<sup>th</sup>), and butyryl fentanyl (12<sup>th</sup>) were among commonly reported narcotic analgesics.

**Conclusion/Discussions:** Attendees will gain an understanding of the status of fentanyl and fentanyl related substances submitted to and analyzed by crime laboratories. NFLIS publicly shares aggregated and analyzed data through various reports throughout the year including highlights of current drugs of concern such as those presented here. These publications can benefit crime laboratory managers by increasing their awareness of emerging substances and other drug trends.

**Keywords:** National Forensic Laboratory Information System, Drug Enforcement Administration, Fentanyl Related Substances

## Development of Solution Based Certified Reference Materials of Carfentanil and Related Compounds

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**Background/Introduction:** Carfentanil is an extremely potent analog of fentanyl, used as a large animal anesthetic, and marketed under the trade name Wildnil. It is 100 times more potent than fentanyl, making it 10,000 times more potent than morphine and lethal on the microgram scale. Heroin and other street drugs are sometimes laced with carfentanil and other fentanyl analogs, causing an epidemic of overdose deaths in the United States and Canada. This also represents as a hazard for law enforcement personnel who come in contact with these compounds as they are readily absorbed through the skin. The presence of carfentanil in the illicit drug market has led to a need for high purity native and stable-labeled certified reference materials (CRM) of the drug and its metabolites for accurate quantitation.

**Objective:** High purity reference standards are vital in identification and quantification of carfentanil and its metabolites. Synthesis and formulation of these compounds presented challenges due to its acute toxicity. The target compounds were synthesized, certified, and then formulated into reference standards.

**Methods:** Carfentanil oxalate, carfentanil-D<sub>5</sub> oxalate, and norcarfentanil oxalate were synthesized using the Ugi Reaction followed by methanolysis to yield the desired compounds. In the case of Norcarfentanil oxalate, further hydrogenation was required to get to the final target. Due to difficulty in handling of the free base of carfentanil, all targets were converted into the oxalate salt adducts. Identity was established through <sup>1</sup>H-NMR, 2D-NMR, and mass spectrometry. Purity was established through HPLC/UV, Karl Fisher, GC/FID headspace, and inorganic microash analysis. Isotopic purity of carfentanil-D<sub>5</sub> oxalate was established through LC/MS-SIM analysis. Development studies for solution stability of the certified reference materials were executed in methanol and acetonitrile.

**Results:** Carfentanil oxalate, carfentanil-D<sub>5</sub> oxalate and norcarfentanil oxalate were successfully synthesized in high purity. Rigorous handling procedures were developed to ensure the safety of employees when working with these substances. Solution stability was established in methanol for formulation of the certified reference materials.

**Conclusion/Discussions:** Certified solution standards of carfentanil oxalate, carfentanil-D<sub>5</sub> oxalate and norcarfentanil oxalate were manufactured for use in clinical toxicology, forensic analysis, and research applications. They were manufactured and packaged taking into account their acute toxicity through oral, inhalation, and skin contact.

**Keywords:** Reference Materials, Carfentanil

**“Death Grip” Intoxication - Identification of 5-Fluoro-ADB in Human Performance and Postmortem Case Samples**

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**Background/Introduction:** The use of synthetic cannabinoids has become widely distributed throughout the United States and Europe. Synthetic cannabinoids are a structurally diverse group of compounds that are used to imitate the effects of traditional cannabinoids and have been found to interact with the CB<sub>1</sub> and CB<sub>2</sub> receptors. The main pharmacologically active component in these synthetic cannabinoids mixtures is constantly evolving with multiple generations of compounds being reported. 5-Fluoro-ADB (aka 5F-MDMB-PINACA), a potent agonist of the CB<sub>1</sub> receptor, was recently confirmed in both a human performance and postmortem case. There is currently limited information regarding the effects of this drug on human performance and post-mortem samples.

**Objective:** The objective is to present two cases, one human performance and one postmortem case, where a validated method was used to confirm the presence of 5-fluoro ADB. Plant like material in a bag labeled “Death Grip” was seized in both cases.

**Methods:** Blood samples were subjected to a solid phase extraction method that utilized Agilent Bond Elute PCX SPE cartridges. Briefly an acetonitrile crash was performed on the sample, centrifuged and supernatant decanted. 2mL of ammonium acetate buffer was added and then the mix was extracted on the SPE cartridges, which were then washed with buffer and DI water: methanol (50:50). The columns were eluted with acetonitrile: ammonia hydroxide (98:2) and then evaporated and reconstituted in mobile phase. The samples were then analyzed on an Agilent 1200 Series High-Performance Liquid Chromatograph (HPLC) coupled to an Agilent 6460 tandem mass spectrometer. A gradient method that employed an Agilent Poroshell 120 PFP (3.0 × 50 mm, 4 μm) analytical column was used. The mass spectrometer was operated in positive electrospray ionization (ESI) with a dynamic Multiple Reaction Monitoring (dMRM) acquisition method that targeted 30 new synthetic cannabinoid compounds. The method was validated using SWGTOX guidelines for a qualitative confirmation. The plant material from one case was extracted in methanol and analyzed using the same analytical methodology. The other material was analyzed using full scan Gas-Chromatography-Mass Spectrometry (GC-MS) in full scan mode with comparison to an analytical standard.

POSTER

**Results:** Human performance case: A white male, aged between 20–40 yr. was observed driving at high speed in the incorrect lane of travel and had a head-on collision with another vehicle. The subject was observed by police officers to discard a packet of “Death Grip” from the vehicle. The subject’s family had reported the person as missing for the past 48 hrs. Routine toxicology testing resulted in no drugs or alcohol being present. The blood sample was analyzed with the new method, and 5-fluoro-ADB was confirmed in two blood samples, one containing EDTA as an anti-coagulant, the other with sodium citrate. The seized material “Death Grip” also was analyzed and confirmed positive for 5-fluoro-ADB using the same analytical methodology LC-MS/MS. Postmortem case: A black male, aged between 40–60 yr. was found unconscious along the side of a road. Witnesses advised that the decedent had consumed several beers and smoked “tobacco that was synthetic marijuana”. Shortly after, the descendant began pacing the roadway, and collapsed. The decedent was known to heavily consume alcoholic beverages and smoke tobacco. Scene investigation revealed a black, plastic bag nearby labeled “Death Grip” incense. Toxicology testing revealed 0.303% and 0.309% of ethanol in iliac vein blood and ocular fluid, respectively. Urine was positive for 11-nor-9-carboxy-Δ<sup>9</sup>-THC; no other drugs were identified in the decedent’s blood or urine. Both 5-Fluoro-ADB and AM-2201 were confirmed in the blood sample. Analysis of the “Death Grip” incense material by GC full scan MS identified the presence of 5-fluoro-ADB.

**Conclusion/Discussions:** A validated LC-MS/MS method was used to confirm the presence of 5-fluoro-ADB in both a human performance and a postmortem case, which was otherwise not identified in routine testing. These two cases illustrate the necessity of forensic laboratories to continuously expand their scope of testing to keep up with current drug trends. These cases also illustrate the importance of drug paraphernalia submissions, which proved invaluable in directing the laboratory to include this compound in a targeted synthetic cannabinoids method.

**Keywords:** Synthetic Cannabinoids, 5-fluoro-ADB, LC-MS/MS

## Metabolomics of N-ethyl Pentylone: Study Involving a Designer Cathinone Analogue using Human Liver Microsomes

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**Background/Introduction:** In the past few years, the emergence of novel psychoactive substances (NPS), including cathinone analogues, into the recreational drug market has introduced various challenges in forensic analytical toxicology regarding adequate and timely detection. N-ethyl Pentylone, as well as other “-lone” synthetic simulants, is a cathinone derivative that presents psychostimulant effects. While its physiological and toxicological actions have not been characterized yet, information regarding ingestion from online forums and toxicology laboratory is apparent. To date, there are few *in vitro* studies involving the metabolomics of cathinone derivatives, and there are no studies characterizing the metabolomics of N-ethyl Pentylone.

**Objective:** The aim of this study was to identify major and minor Phase I metabolites of N-ethyl Pentylone in order to generate a predicted human metabolic pathway, as well as to determine if commonalities are present with other synthetic cathinones.

**Methods:** N-ethyl Pentylone was *in vitro* incubated with pooled human liver microsomes, using a protocol previously developed and verified against diazepam. The resulting metabolic mixture was further prepared to remove unwanted cellular material and debris. Analysis was performed using a SCIEX TripleTOF® 5600+ quadrupole time-of-flight mass spectrometer coupled to a Shimadzu Nexera XR ultra high performance liquid chromatograph (LC-QTOF). Data processing was performed using MetabolitePilot™ to identify the major and minor metabolites present through the use of commonly encountered biotransformations. Metabolite identity was determined based on fragmentation pattern, and accurate fragment masses, as well as relative retention time.

**Results:** - During this study, *in vitro* incubation of N-ethyl Pentylone resulted in the identification of six metabolites. The two major metabolites, those highest in intensity, were associated with N-deethylation (M1) and demethylenation (M2) biotransformations (Figure 1). The remaining metabolites, those lower in intensity, were associated with demethylation, hydroxylation, N-deethylation and demethylenation, and ketone reduction biotransformations. Structural composition was determined dependent on analytical capabilities. Standard reference material is currently unavailable for all metabolites, therefore absolute structural certainty was not achieved.

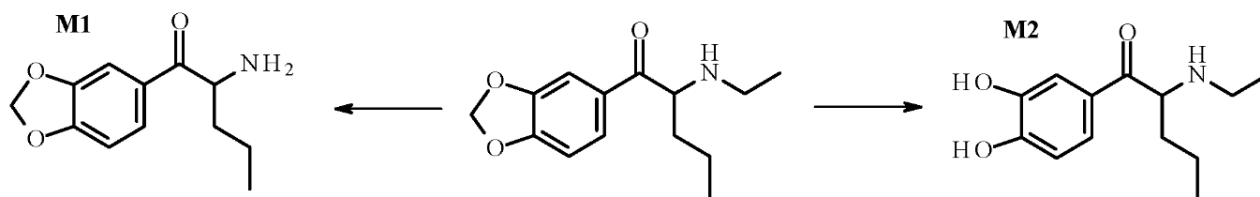


Figure 1: Metabolism of N-ethyl Pentylone (center)

**Conclusion/Discussions:** As a result of this study, major and minor metabolites of N-ethyl Pentylone have been characterized, and could serve as important biomarkers in identifying the ingestion of N-ethyl Pentylone. Interestingly, several similarities were noticed between the metabolism of N-ethyl Pentylone and other “-lone” synthetic cathinones. N-desethyl-N-ethyl Pentylone shares a common metabolite with Pentylone (N-desmethyl-Pentylone). Synthetic cathinones are often found in conjunction with other synthetic cathinones, so the presence of this metabolite could complicate the determination of drug ingested. N-desethyl-N-ethyl Pentylone is isobaric to Ethylone, Butylone, and Dimethylone. This could further complicate the identification of these compounds during broad-based drug screening, potentially resulting in positive screen but negative confirmation results. Desmethyl-N-ethyl-Pentylone was found to be isobaric to Pentylone, but retention time and mass spectral library data shows a chemical difference between these two compounds. The results from this study should be considered when evaluating the ingestion of N-ethyl Pentylone following cases of intoxication or death.

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**Keywords:** N-ethyl Pentylone, Human Liver Microsomes, LC-QTOF

**Screening method for NBOMes in Whole Blood Using LC-MS/MS Precursor Ion Scan and Library Search**

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**Background/Introduction:** Consumption of novel psychoactive substances (NPS), analogues of illegal drugs of abuse produced in order to circumvent the law, has grown worldwide and concerns government agencies related to drug enforcement and public health. NBOMes are highly potent NPS that causes intense hallucination, tachycardia, hypertension, agitation, and aggressiveness at low doses.

Previous studies usually analyze NBOMes in biological fluids using target methods based on liquid chromatography-tandem mass spectrometry (LC-MS/MS) in multiple reactions monitoring (MRM) mode, which provides great selectivity and sensitivity. There are no screening methods to date, such as immunoassay, for NBOMes in biological fluids. Precursor ion scan (PIS) is a very popular technique to study classes of compounds, which have a diagnostic fragment ion, such as NBOME. Data-dependent acquisition mode in mass spectrometry is a data collection in which a fixed number of precursor ions are subjected to a second type of mass selection, product ion scan analysis.

**Objective:** The aim of this work was to develop a screening method for NBOME compounds in whole blood, using liquid chromatography-tandem mass spectrometry. Precursor ion survey scans were followed by information-dependent acquisition-enhanced product ion scan experiments on an ABSciex 5500 QTRAP mass spectrometer.

**Methods:** For sample preparation, 300 µL of whole blood was transferred to a 2 mL polypropylene tube. The extraction was performed with 300 µL of a saturated solution of sodium tetraborate and 1.2 mL of methyl tert-butyl ether. The tube was vortexed for 5 min at 2,000 rpm, centrifuged at 8,000 rpm for 5 min, and 900 µL of organic phase was transferred to a clean tube. The solvent was evaporated at 40 °C under nitrogen stream, resuspended with 500 µL of methanol, transferred to an autosampler vial and 20 µL were injected into the LC-MS/MS system.

The analyses were performed with an Agilent 1260 Infinity LC system coupled to an ABSciex 5500 QTRAP mass spectrometer. The chromatographic separation was performed with a C18 column (Atlantis T3, 150x3 mm, 3 µm, Waters®), maintained at 40°C. The mobile phase consisted of ultra-pure water (A) and acetonitrile (B), both of containing formic acid (0.1%, v/v), gradient elution and flow rate of 0.50 mL/min. The mass spectrometer was set to operate with an electrospray source, in positive ion mode and a data-dependent acquisition, using precursor ion scan as survey scan – compounds which presented the NBOME class specific fragment,  $m/z$  121, with intensity greater than 10,000 cps activates an enhanced product ion scan (PIS) experiment, collecting the substance spectrum (collision ion spread, 35 eV ± 15 eV). The compounds were identified by comparison with an in house MS/MS spectrum library.

**Results:** The developed method was able to detect several NBOMes, e.g. 25H-, 25D-, 25G-, 25E-, 25C-, 25I- and 25B-NBOME in concentrations lower than 100 pg/mL. The method was also able to provide a semi quantitative estimation of NBOMes concentration. The method presented great selectivity, without false positive against common drugs of abuse and pharmaceuticals.

**Conclusion/Discussions:** Data-dependent precursor ion survey scans, followed by information-dependent acquisition-enhanced product ion scan experiments was a feasible tool for selective screening analysis of NBOME in whole blood. Therefore, data-dependent PIS mode using the specific NBOME class compound fragment,  $m/z$  121, in tandem with enhanced product ion scan mode is a robust approach for rapid untargeted NBOME screening, which leads to identification of NBOME structure.

**Keywords:** NBOMes, LC-MS/MS Data-Dependent Acquisition Mode, Precursor Ion Scan

## Prevalence of Recreational Drugs and New Psychoactive Substances Based upon Geographical Areas in the United States

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**Background/Introduction:** In recent years, the emerging trends and use of new psychoactive substances (NPS) has been a major health concern within the United States. Additionally, the abuse of opioids and other drugs considered “recreational” has been on the rise in the United States. This rise in NPS and recreational drugs has created a national crisis, resulting in challenges to forensic toxicology laboratories to detect these substances.

**Objective:** The objective of this study was to assess drug patterns from various parts of the United States with suspected recreational drug and/or NPS use.

**Methods:** LC-MS/MS was used to drug screen 1402 urine specimens collected from four sites throughout the United States during 2015 and 2016. The four sites consisted of two from the Mid-Atlantic region, one from the Midwest, and one from the Pacific island region. The specimens were from populations consisting of adult parolees and probationers, prison inmates, and emergency rooms. Drug screening was performed on an LC-MS/MS operated in positive-ion electrospray ionization mode. The LC-MS/MS testing protocol used four screening methods/panels -- a Synthetic Cannabinoid Panel (LOD 0.2 ng/mL), a Designer Drug Panel (LOD 5.0 ng/mL), a THC/Barbiturates/Buprenorphine/LSD Panel (LOD 1.0 – 25 ng/mL), and a “General” Drug Panel (LOD 4.0 – 25 ng/mL) -- to identify the presence of 134 analytes in urine. The criteria for the identification of substances were based upon chromatography and multipoint calibration.

**Results:** Of the 1402 urine specimens screened, 886 specimens (63.2%) contained at least one drug or drug metabolite. The most commonly detected single drug class for all sites was cannabinoids (THCCOOH), which were detected in 202 (22.8%) of the positive specimens. Every site had a different prevalent drug. The most prevalent drugs detected in specimens from the four sites were: Mid-Atlantic site #1: THCCOOH (25.6% out of 270 total specimens); Mid-Atlantic site #2: Morphine (46.1% out of 490 total specimens), Midwest: THCCOOH (11% out of 304 total specimens), and Pacific island region: Methamphetamine/Amphetamine (22% out of 338 total specimens). Thirty-one specimens (11.4%) from Mid-Atlantic site #1 contained at least one synthetic cannabinoid, in which AB-PINACA-N-COOH was the most prevalent. Mid-Atlantic site #2 had 25 specimens (5.1%) that contained at least one synthetic cannabinoid and/or metabolite, in which UR-144-N-COOH was the most prevalent.

**Conclusion/Discussion:** When the data is analyzed as a whole, it becomes evident that sites which are geographically very close together, such as Mid-Atlantic site #1 and the Mid-Atlantic site #2, still demonstrated a clear difference in prevalent drug use. It is important to note that for all four sites marijuana was either the most prevalent drug or the second most prevalent drug detected; thus, demonstrating that marijuana is still a popular drug for abuse. Additionally, Mid-Atlantic site #1 includes an area where marijuana is legal. This study demonstrates the usefulness of broad-based drug screening to determine the actual drug trends throughout the United States.

**Keywords:** Prevalence Study, New Psychoactive Substances, LC-MS/MS Drug Screening

## Ecstasy, Molly, and MDMA: The Novel Stimulants Infiltrating the Designer Drug Market

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**Background/Introduction:** “Ecstasy” and “Molly” (short for “molecule”) are common slang drug terms used among populations associated with club and rave cultures. These terms are used to describe tablet, powder, crystal, and capsule preparations, believed to contain 3,4-methylenedioxymethamphetamine (MDMA). Historically, “Ecstasy” was used synonymously with MDMA, but as “Ecstasy” was diluted, cut, and mixed with other components, the term “Molly” became more prevalent to denote pure MDMA. In the current culture, “Ecstasy” is usually used to describe pressed tablet formulations, while “Molly” is used to describe powders or crystals, often prepared in capsules. The term novel stimulant was used in this study to denote emerging stimulants, excluding MDMA which was categorized separately.

**Objective:** Our hypothesis was that drug users use the terms “Ecstasy,” “Molly,” and MDMA non-specifically and interchangeably, and are generally unaware of the identity of the substance or substances ingested. To evaluate this hypothesis, self-reported drug use was compared against toxicological findings in specimens provided by survey respondents.

**Methods:** Blood, urine, and/or oral fluid specimens were collected from participants attending a large multi-day electronic dance music festival in Miami, Florida. The participants completed a structured survey involving age, gender, and recreational drug use, including use of “Ecstasy,” “Molly,” and MDMA. This study was approved by the Institutional Review Board at Arcadia University. Collected specimens were screened for therapeutic drugs, common drugs of abuse, and novel psychoactive substances (NPS), including novel stimulants, using liquid chromatography quadrupole time-of-flight mass spectrometry. Positive screen results were confirmed by validated liquid chromatography tandem mass spectrometry and gas chromatography mass spectrometry methods.

**Results:** During this three-year study, 72 participants indicated in their survey responses recent use of “Ecstasy,” “Molly,” and/or MDMA, and provided at least one biological specimen for analytical confirmation. Of these subjects, 65 (90.2%) indicated only one drug term, answering “Ecstasy,” “Molly,” or MDMA; while 7 (9.8%) participants indicated a combination of multiple terms. Of the 65 participants designating only one drug term, 10 (15.4%) indicated “Ecstasy” use, 37 (56.9%) indicated “Molly” use, and 18 (27.7%) indicated MDMA use. One participant indicated the use of methylenedioxyamphetamine (MDA), a metabolite of MDMA but also a drug sometimes ingested in parent form. This participant tested positive for MDA and MDMA. Correlation of specimens results by drug term response are listed in Table 1.

Table 1: “Ecstasy,” “Molly,” and MDMA Responses by Drug Positivity

	“Ecstasy” (n=10)	“Molly” (n=37)	MDMA (n=18)	Multiple Terms (n=7)
MDMA Only	1 (10.0%)	12 (34.4%)	2 (11.1%)	1 (14.3%)
Novel Stimulant Only	1 (10.0%)	12 (34.4%)	9 (50.0%)	5 (71.4%)
MDMA and Novel Stimulant	2 (20.0%)	6 (16.2%)	5 (27.8%)	1 (14.8%)
Negative	6 (60.0%)	7 (18.9%)	2 (11.1%)	0 (0.00%)

Novel stimulants identified in specimens provided with responses of “Ecstasy” use included alpha-PVP and ethylone; responses of “Molly” use included alpha-PVP, 4-fluoroamphetamine, methylone, dimethylone, ethylone, and butylone; responses of MDMA use included alpha-PVP, 4-fluoroamphetamine, methylone, dimethylone, ethylone, butylone, and dibutylone; responses of multiple terms included alpha-PVP, methylone, ethylone, and butylone.

**Conclusion/Discussions:** The results from this study indicate that there are inconsistencies between admission to drug use and toxicological findings in this population. Novel stimulants were analytically confirmed in biological specimens regardless of respondent drug terminology. Of the 72 participants who indicated use of “Ecstasy,” “Molly,” or MDMA, MDMA without a novel stimulant was only confirmed in 16 (22.2%) participants, while 41 (56.9%) were positive for at least one novel stimulant. While several factors could account for this finding, it is interesting to note the majority (60.0%) of specimens linked to “Ecstasy”-only survey responses were negative for MDMA and other novel stimulants.

**Keywords:** Ecstasy, MDMA, Novel Psychoactive Substances



**Case Report: A Sudden Death Following the Consumption of ADB-FUBINACA**

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**Background/Introduction:** In Europe 637 new psychoactive substances have been reported to the EMCDDA since 1997 and the largest number, 172 substances belongs to the group of synthetic cannabinoids (SC). SC appeared in Hungary around 2011 and became very popular within a short time. Unlike THC which is a partial receptor agonist, SC are full receptor agonists which can be the cause of the large number of reported health problems related to ADB-FUBINACA in Hungary.

**Objective:** To investigate on the death of a 37 year old man, died suddenly after the consumption of a herbal smoke toxicological analysis of post mortem blood, urine and tissues (liver, kidney, heart, spleen, lungs, stomach, small and large intestine) were undertaken. The urine and blood samples were tested for drugs of abuse (DoA), new psychoactive substances (NPS) and medicines. The tissues were analysed for SC and ADB-FUBINACA was quantified in all samples.

**Methods:** Isolute HXC cartridges for solid phase extraction were used for the preparation of 1 mL blood and urine. From the acidic and alkaline fractions 10 mL was injected in a Shimadzu UHPLC-MS/MS instrument. There were three different methods, one for DoA and NPS (273 monitored substances) used only for screening, one for barbiturates and one for other sedatives and tranquillizers. All UHPLC-MS/MS analysis were performed on Kinetex C18 column using formate buffer and acetonitrile (containing 0.1% formic acid) as eluents. The samples prepared this way were analysed with GC/MS and HPLC-DAD techniques as well for medicines. For the confirmatory measurements of SC 1 mL blood and 0.5 mL urine samples were extracted by supported liquid extraction with ethyl-acetate. For urine samples enzymatic hydrolyses (*E.coli*  $\beta$ -D-glucuronidase) was applied before extraction. As to the tissues, 2 g aliquots were analysed for ADB-FUBINACA *via* standard addition technique. The analyses were done on the same instrument (UHPLC-MS/MS) and under similar conditions but with different gradient and by using 3 MRM transitions for every SC compound.

**Results:** For five aliquots of the blood sample the following compounds were detected with the following average concentrations: 17.24 ng/mL ADB-FUBINACA, 17.8 ng/mL mirtazapine, 81.4 ng/mL carbamazepine, 3.6 ng/mL clonazepam and 119.0 ng/mL 7-amino-clonazepam. From the two aliquots of the urine sample the following average concentrations were measured: 0.73 ng/mL ADB-FUBINACA, 25.5 ng/mL AB-FUBINACA carboxylic acid, 62.5 ng/mL mirtazapine, 29.0 ng/mL carbamazepine, 13.5 ng/mL clonazepam and 1334 ng/mL 7-amino-clonazepam. The quantitative analysis of ADB-FUBINACA from the tissues gave the following results: 117 ng/g in liver, 43 ng/g in kidney, 32 ng/g in lungs, 18 ng/g in spleen, 12 ng/g in heart, 1.7 ng/g in stomach and <1 ng/g in small and large intestines.

**Conclusion/Discussions:** According to the circumstances, death occurred within a short time after smoking a herbal cigarette. None of the medicinal drugs found in blood was in a toxic range. The common metabolite of AB-FUBINACA and FUB-AMB were detected only in the urine without the presence of any parent compound which may indicate a former use or a smaller concentration in the cigarette. In the literature, ADB-FUBINACA blood concentrations were reported in two cases. One reported about supraventricular tachycardia and severe confusion with 15.6 ng/mL ADB-FUBINACA and 5.6 ng/mL AB-FUBINACA, in blood. The other case was about a death associated with the use of ADB-FUBINACA, where 7.3 ng/mL was measured in blood (together with 1.1 ng/mL THC and 4.7 ng/mL THC-COOH). In 70 blood samples from alive SC users measured in our institution the average blood concentration of ADB-FUBINACA was 10.5 ng/mL (0.09-125 ng/mL). As blood concentrations of ADB-FUBINACA seem to lack straight correlation with toxicity, and no other naturally occurring disease or circumstance had been unfolded at the autoptic scene, the official cause of death was the consumption of synthetic cannabinoids.

**Keywords:** LC-MS/MS, ADB-FUBINACA, Case Report, Death

## Metabolism of Butyrylfentanyl and Beta-Hydroxythiofentanyl in Human Hepatocytes

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**Background/Introduction:** Last years have seen the (re)emergence of synthetic opioids sold as new psychoactive substances (NPS), in particular fentanyl analogs. Two compounds belonging to this family of drugs are butyrylfentanyl (or butyrfentanyl; 1/8 of the potency of fentanyl) and beta-hydroxythiofentanyl (potency unknown) - both have been detected in Europe and the US, where they have caused numerous fatalities. In butyrylfentanyl, fentanyl's propionyl chain is replaced by a butanoyl chain; in beta-hydroxythiofentanyl, fentanyl's ethylphenyl is replaced by a hydroxyethyl thiophene substructure. Both compounds were temporarily placed into Schedule 1 of Controlled Substances in the US. One of the first steps after the encounter of a new compound on the market is the investigation of its metabolism as this will help analytical detection, assessment of drug interactions and interpretation of cases. Data on the metabolism of butyrylfentanyl and beta-hydroxythiofentanyl is limited or not existent. Butyrylfentanyl metabolism was investigated in human liver microsomes and one authentic case and hydroxylation and N-dealkylation to form the nor metabolite were found to be the main metabolic steps in HLM, while hydroxylation and carboxylation of the butanoyl chain produced the major metabolite *in vivo*. No metabolism studies have been performed for beta-hydroxythiofentanyl yet.

**Objective:** To characterize the metabolism of butyrylfentanyl and beta-hydroxythiofentanyl in cryopreserved human hepatocytes.

**Methods:** Butyrylfentanyl or beta-hydroxythiofentanyl were incubated at 10  $\mu\text{mol/L}$  with human hepatocytes in a 96-well plate at 37 °C for up to 5 h. After quenching the reaction with 125  $\mu\text{L}$  ice-cold acetonitrile and centrifugation, the supernatant was injected undiluted onto the liquid chromatography-quadrupole time-of-flight mass spectrometer (LC-QTOF). Chromatographic separation was performed on an Agilent 1290 Infinity UHPLC system with an Acquity HSS T3 column at 60 °C with 10 mM ammonium formate in 0.05% formic acid (A) and 0.05% formic acid in acetonitrile (B) run in gradient over 19 min. Mass spectrometric data was obtained with an Agilent 6550 QTOF mass spectrometer with an electrospray ionization source and auto MS/MS acquisition.

**Results:** For butyrylfentanyl, we identified eleven metabolites generated by N-dealkylation, hydroxylation, amide hydrolysis, N-oxidation, and combinations of these reactions. The most intense metabolite was the nor metabolite, followed by the desbutyryl and several hydroxy metabolites at the ethyl linker and butyryl chain.

For beta-hydroxythiofentanyl, we identified ten metabolites, which were generated by N-dealkylation, dehydrogenation (probably hydroxylation followed by loss of water), piperidine opening and reduction of the formed aldehyde, hydroxylation, thiophene oxidation, and direct glucuronidation.

**Conclusion/Discussions:** For each NPS emerging on the market, forensic toxicologists are challenged to determine suitable analytic targets, often metabolites. Similar to fentanyl, N-dealkylation at the piperidine nitrogen is usually suggested to be the major biotransformation for new fentanyl analogs. For butyrylfentanyl, the results of the *in vitro* hepatocyte experiment show that, indeed, the nor metabolite is formed in abundance and that hydroxylation and amide hydrolysis form further minor metabolites. Steuer et al. found the carboxy metabolite to be major in one authentic case, but similar to their *in vitro* HLM experiment we did not find significant signals for the carboxy metabolite in our experiment. However, two metabolites were hydroxylated at the butyryl chain, one likely to be the precursor for the carboxy metabolite. Ultimately, samples from several authentic cases will be needed to reliably determine which metabolites are suitable analytical targets. For beta-hydroxythiofentanyl, the metabolic profile in hepatocytes suggests, again, that the nor metabolite, which is shared with fentanyl, is the major metabolite. It is recommended to also target more specific metabolites that still contain all relevant substructures, particularly the thiophene ring (e.g. the dehydrogenated metabolite), in order to distinguish beta-hydroxythiofentanyl from fentanyl consumption.

**Keywords:** Fentanyl Analog, Metabolite Identification, LC-HRMS

## Development of an LC-MS/MS Method for the Simultaneous Determination of 40 Synthetic Cathinones in Urine with Multi-Mode Reversed Phase Column Using pH Gradient Elution and Stability of the Synthetic Cathinones in Urine

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**Background/Introduction:** Synthetic cathinones are a sub-category of new psychoactive substances which have emerged all over the world causing significant social issue. New analogs with varying effects and potencies are constantly introduced into the illegal drug market, so the detection of a broad-spectrum of synthetic cathinones in biological samples represents a challenge for forensic toxicologists.

**Objective:** The focus of this research was to develop and validate a method for the simultaneous quantification of 40 cathinone derivatives including 13 metabolites in urine by liquid chromatography-tandem mass spectrometry(LC-MS/MS), as well as evaluate their stability in urine samples stored up to 4 weeks at room temperature, 4 and -20 .

**Methods:** Analytes were recovered via liquid-liquid extraction and quantified using D<sub>8</sub>-a-PVP as the internal standard(IS). In order to overcome limitations of standard reversed-phase column to separate isobaric synthetic cathinones, we adopted a multi-mode reversed-phase column, Scherzo SS-C18, which was composed of strong ionic ligands and C18 ligands, and used pH gradient elution. Mass analysis was performed on a QTRAP mass spectrometer in positive electrospray ionization (ESI) mode with multiple reaction monitoring (MRM).

**Results:** The essential validation parameters including selectivity, LOD, LLOQ, linearity, intra- and inter-assay precision and accuracy, recovery, and the matrix effect were satisfactory. The limit of detection and quantitation were 0.5-5 ng/mL and 2.5-10 ng/mL, respectively. In stability tests, total losses were observed in urine samples stored at room temperature for 4 weeks for some analytes. At 4 , losses up to -46% occurred however all compounds were stable if stored at -20 . The developed method was successfully applied to 42 authentic urine samples from drug suspects. As a result we confirmed a-PVT, a-PHPP, a-PVP and methcathinone in 5 urine samples.

**Conclusion/Discussions:** The differences in stability of synthetic cathinones occurred depending on the storage temperature and the chemical structure of the cathinone. These results showed that the storage temperature should be taken into account to improve the stability of synthetic cathinones in urine. The analytical separation using multi-mode reversed phase column employing pH gradient was a useful technique to separate low molecular amines which that exhibit some limitation to analysis with normal reversed-phase column. This developed LC-MS/MS method will be very useful for monitoring inappropriate use of synthetic cathinones in forensic or clinical toxicology laboratories.

**Keywords:** Synthetic Cathinones, LC-MS/MS, pH Gradient Elution, Stability

## Characterization and *In Vitro* Phase I Microsomal Metabolism of Designer Benzodiazepines – an Update Comprising Flunitrazolam, Norflurazepam and 4'-Chlorodiazepam

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**Background/Introduction:** The number of newly appearing benzodiazepine derivatives on the new psychoactive substances (NPS) drug market has increased over the last couple of years, totaling in 17 'designer benzodiazepines' monitored at the end of 2016 by the European Monitoring Centre for Drugs and Drug Addiction (EMCDDA). While other classes of NPS such as synthetic cannabinoid receptors agonists or synthetic cathinones are mainly consumed for hedonistic reasons, designer benzodiazepines may also be applied as 'self-medication' by persons with anxiety disorders or by users of stimulant and hallucinogenic drugs ('stand-by medication' to counteract unpleasant overstimulation). Additionally, designer benzodiazepines such as flubromazolam have been detected in fake alprazolam tablets, and according to the EMCDDA these counterfeit medicines have become an important part of the illicit drug market in some European countries. Offer of metabolites (e.g. norflunitrazepam, 3-hydroxyphenazepam) or designer benzodiazepines being metabolized to licensed benzodiazepines (e.g. diclazepam, cloniprazepam) pose a particular challenge with regard to correct interpretation of analytical findings.

**Objective:** The aim of the present study was to characterize the three benzodiazepines flunitrazolam, norflurazepam and 4'-chlorodiazepam (Ro5-4864) which are offered as 'research chemicals' on the Internet and to identify their main *in vitro* phase I microsomal metabolites. The information obtained can be used to update analytical methods for the detection and identification of benzodiazepines in biological samples.

**Methods:** All products were obtained as research chemicals via Internet shops in 2016 in Germany; norflurazepam was additionally obtained in the form of tablets. For identification and characterization of the compounds, nuclear magnetic resonance (NMR) spectroscopy, gas chromatography–electron ionization mass spectrometry (GC–EI-MS), liquid chromatography–tandem mass spectrometry (LC–MS/MS), and liquid chromatography–quadrupole time-of-flight-mass spectrometry (LC–QToF-MS) were applied. The main *in vitro* phase I metabolites were investigated by incubation with pooled human liver microsomes (pHLMs). The obtained solution and blank pHLM samples were analyzed using enhanced product ion (EPI) scan experiments on a LC-MS/MS system. Additionally, precursor ion scans based on the fragmentation pattern of the parent compounds as well as Q-ToF analysis (positive ESI; fullscan/bbCID & fullscan/AutoMS/MS) were conducted.

**Results:** For all compounds the structural formula declared by the vendor was confirmed by GC-MS, LC MS/MS, LC-Q-ToF-MS analysis and NMR spectroscopy. The purity of the compounds was 95% or higher in all cases. The observed metabolic steps for flunitrazolam were monohydroxylation, dihydroxylation and reduction of the nitro function. The detected *in vitro* phase I metabolites for norflurazepam were hydroxynorflurazepam and dihydroxynorflurazepam. 4'-chlorodiazepam biotransformation consisted of *N*-dealkylation and hydroxylation.

**Conclusion/Discussions:** The three benzodiazepines flunitrazolam, norflurazepam, and 4'-chlorodiazepam were structurally characterized, and their respective *in vitro* main phase I microsomal metabolites tentatively identified applying a combination of LC-MS/MS and HRMS techniques. Certainly, all described metabolites are prone to undergo further phase II metabolic transformations *in vivo*, such as *O*- and *N*-glucuronidation, and acetylation of the amino moiety of the respective metabolites of flunitrazolam. It has to be noted that 4'-chlorodiazepam and its metabolites show an almost identical LC-MS fragmentation pattern to diclazepam and its metabolites (delorazepam, lormetazepam and lorazepam), making a sufficient chromatographic separation inevitable for unambiguous identification. The sale of norflurazepam, a metabolite of the prescribed benzodiazepines flurazepam and fludiazepam, presents a particular risk for incorrect interpretation of analytical findings.

**Keywords:** Designer Benzodiazepines, *In Vitro* Metabolism, Ro5-4864

**Non-Pharmaceutical Fentanyl Encountered by the Alabama Department of Forensic Sciences (ADFS)**

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**Background/Introduction:** In recent years, fentanyl and non-pharmaceutical fentanyl (NPFs) overdoses are on a rise in the United States. CDC indicated fentanyl encounters increased 160% from 2014 to 2015. It is difficult to measure the true extent of NPF use as these compounds are often excluded from routine toxicology screens. Moreover, when a new molecule is identified and scheduled, manufacturers are often quick in introducing a new analogue to the market, forcing agencies into an endless chase. At ADFS, unconfirmed presumptive positive fentanyl cases are analyzed by QTOF LC/MS to investigate the presence or absence of NPFs.

**Objective:** To highlight and investigate NPF cases received by the Alabama Department of Forensic Sciences.

**Methods:** Drug screenings were performed by enzyme-linked immunoassay using a Randox Evidence Analyzer and LC/MS using an Agilent 6545 QTOF. Confirmation or quantification of presumptive positive cases were performed in-house via liquid-liquid extraction followed by GC/MS analysis or by National Medical Services Labs via LC/MS/MS analysis.

**Results:** The results for 16 NPFs related cases received by ADFS from September 30, 2016 to April 12, 2017 were 4-ANPP (n=10), U-47700 (n=7), Methoxyacetyl Fentanyl (n=5), FIBF (n=2), Furanyl Fentanyl (n=1), and Acryl Fentanyl (n=1). *Case 1:* A 30 year old male was found dead at home by his children. His wife was found unresponsive in the bedroom but was later recovered at a hospital. The grandmother stated that the decedent's wife made a stop to "obtain drugs" after work. She also stated that, "This was their first time. They do not have any needle marks." Toxicology results reported: 22 ng/mL of morphine, 0.18 ng/mL of Furanyl Fentanyl, 0.20 ng/mL of 4-Anilino-N-Phenethylpiperidine (4-ANPP), 0.91 ng/mL of Acryl Fentanyl, and 0.52 ng/mL of U-47700. *Case 2:* A 30 year old female was found unresponsive and not breathing by her sister at 0100. She became responsive after her sister slapped her, threw water, and performed CPR on her. 5 hours later, she became unresponsive again. The decedent was transported to a hospital and was pronounced dead. There were visible needle track marks on her right arm. It was stated that she had been using heroin laced with fentanyl. Toxicology results reported: 4-ANPP present, U-47700 present, 60 ng/mL of methamphetamine, and 24 ng/mL of amphetamine. COD: Multiple drug intoxication MOD: Accident. *Case 3:* A 29 year old male and a female friend received an unmarked black box at the post office. Upon arrival at a friend's residence, they stayed outside. The female friend returned to the residence and began having a breathing episode. Narcan (naloxone) was administered and she recovered. The male decedent was found unresponsive on the roadway with fresh needle track marks on his arms. He was known to have a history of substance abuse. Toxicology results reported: 4-ANPP present, 55 ng/mL of methamphetamine, 35 ng/mL of amphetamine, and 69 ng/mL of alprazolam. COD: Multiple drug overdose MOD: Accident

**Conclusion/Discussions:** In ADFS, there was a 54% increase in fentanyl overdoses from 2014 to 2015. Additionally, we identified 16 NPFs positive cases with the first appearing on September 30, 2016. The most encountered NPFs in ADFS were 4-ANPP, methoxyacetyl fentanyl, FIBF, furanyl fentanyl, and acryl fentanyl along with U-47700. U-47700, commonly known as PINK, is a novel synthetic opioid with approximately 7.5x more potency than morphine. Law enforcement should be informed of the rapid rise of NPFs use among substance abusers. Fentanyl-related overdoses are similar to overdoses induced by other opioids, which are characterized by respiratory depression, stupor and miosis. Toxicology laboratories should enhance their screening methodology to include analysis for NPFs.

**Keywords:** Fentanyl Analogues, 4-ANPP, U-47700

## Designing A Molecular Imprinted Polymer Based QCM Biosensor for Simple Synthetic Cannabinoid Testing in Urine

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**Background/Introduction:** Synthetic cannabinoids (SCs), molecules that mimic the effects of the active ingredient of marijuana, have gained popularity over the last decade. The analysis of synthetic cannabinoids in human matrices is of particular importance in the fields of forensic and clinical toxicology. Although there are countless different structures of synthetic cannabinoids available and these numbers are rising, the two most well-known are aminoalkylindole synthetic cannabinoids (*JWH-018* and *JWH-073*). The diversity of these structures presents a challenge in detection of SCs. Although typically mass spectrophotometry is used for chemical identification of the compounds Quartz Crystal Microbalance (QCM) biosensors, a member of mass-sensitive chemical sensors, have been getting researchers' attention because of their properties such as high selectivity, low cost, portability, easy-to-use, stability and simplicity. In order to create sensitive QCM biosensor surface, although several methods can be applied, the most promising approach is molecular imprinting technique. The methodology mainly depends on the molecular recognition, is a type of polymerization which occurs around the interested molecules called as a template and creates specific cavities in the highly cross-linked polymeric matrices.

**Objective:** The objective of the present study was to engineer a robust, stable, fieldable and selective molecularly imprinted QCM biosensors for the detection of common use SCs *JWH-018*, *JWH-073* and their major metabolites *JWH-018 pentanoic acid*, *JWH-073 butanoic acid* respectively in artificial urine in real time.

**Methods:** *JWH-018*, *JWH-073* and their major metabolites *JWH-018 pentanoic acid*, *JWH-073 butanoic acid* imprinted nanoparticles (NPs) were prepared and attached to the surface of QCM chip and prepared as a biosensor. Prepared molecularly imprinted (MIP) NPs were characterized by Fourier transform infrared spectroscopy (FTIR), scanning electron microscope (SEM), Zeta-size, and transmission electron microscope (TEM). The synthetic cannabinoids imprinted and non-imprinted. QCM chips were characterized with ellipsometry, atomic force microscopy (AFM) and contact angle measurement. Specificity and selectivity of imprinted and non-imprinted QCM biosensor chips were determined and kinetics and isotherm parameters were calculated by applying association kinetics analysis. Reproducibility of the imprinted QCM biosensors was tested in the final step. Real-time and fast measurement, high sensitivity and specificity, no need of labeled reagents are the unique properties of QCM biosensors.

**Results:** The prepared MIP based QCM biosensors, characterized with the procedures mentioned in the "methods" section, were sensitive enough to yield signals from SCs *JWH-018*, *JWH-073* and their major metabolites *JWH-018 pentanoic acid* and *JWH-073 butanoic acid* in spiked artificial urine in ppm and ppb levels (0.5 ppb-5 ppm). Further developments are in order to enhance the sensitivity of the biosensor to allow the detection of the above-mentioned compounds in the ppt range.

**Conclusion/Discussions:** MIP-based QCM nanosensors possess the potential to become precise, reliable and economic approaches for the detection of synthetic drugs in biological samples, due to their novel and innovative technique of detection. They innovatively combine identification and detection capabilities in one portable system. Given the remarkable advantages of a MIP-based QCM sensory system for detection of illicit drugs over conventional detection methods, further studies to improve the practicality and in-field usability of such nanotechnology-based biosensors are well warranted.

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**Keywords:** Forensic Toxicology, Biosensor, Synthetic Cannabinoids

## Metabolic Studies of Synthetic Cannabinoid APINAC

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**Background/Introduction:** APINAC (AKB-57, ACBL(N)-018, adamantane-1-yl-1-pentyl-1H-indazole-3-carboxylate) was first detected in smocking mixtures in the black market in Australia and South Korea. In September 2016 a powdery substance (m=1.97 g) containing APINAC was confiscated in the Saratov region (Russian Federation) in the course of a police investigation. At the time of writing this report, APINAC has not been included into the list of narcotic drugs, psychotropic substances and their precursors subject to control in the Russian Federation.

**Objective:** The aim of the present study was to investigate the metabolism of APINAC in rats by using liquid chromatography-mass-spectrometry with tandem quadrupole-time-of-flight mass spectrometry (LC-QTOF). Metabolites detected by LC-QTOF were characterized by gas chromatography-mass spectrometry (GC-MS) for their routine identification in human urine samples.

**Methods:** Rats were administrated intravenously with 15 mg/kg of APINAC dissolved in dimethylsulfoxide (0.5 mL/kg). Rat urine samples were analyzed by using GC-MS and high-resolution mass spectrometry (QTOF). Data were acquired via TOF scan, followed by Auto MS and triggered product ion scans. The LC-QTOF experiments were performed on a system consisting of a model 1290 Infinity HPLC System and a model 6540 quadrupole time-of-flight mass spectrometer (both from Agilent, Santa Clara, CA, USA). Chromatographic separation of the analytes was achieved on a ZORBAX Eclipse Plus C18 column (100 × 2.1 mm i.d., particle size 1.8 μm; Agilent). The column oven was set to 40 °C; the autosampler was set to 4 °C. The GC-MS analyses were performed in full-scan mode (50-750 amu) on the system comprising a 7890B series gas chromatograph coupled to 5977 series mass selective detector (Agilent Technologies) with electron ionization (EI) at 70 eV. An HP-5MS column (30 m × 0.25 mm × 0.25 μm, Agilent)

**Results:** The predominant metabolic pathway for APINAC was ester hydrolysis, which was similar to PB-22, yielding a wide variety of N-pentylindazole-3-carboxylic acid metabolites. Ten metabolites for APINAC were identified, with the majority generated by hydroxylation, carbonylation and carboxylation with or without glucuronidation. As expected, APINAC was extensively hydrolyzed. APINAC metabolites can be divided into two major groups: N-pentylindazole-3-carboxylic acid metabolites (M1–M5) and 1-adamantanol metabolites (M6–M9). In both groups hydroxylation was the most common biotransformation. Interestingly, APINAC metabolites without ester hydrolysis were not detected.

**Conclusion/Discussions:** In the present study, we have investigated the metabolism of APINAC, one of the newest synthetic cannabinoids, by using an in vivo rat model. To our knowledge, this is the first trial to investigate the APINAC metabolism. The predominant metabolite was N-pentylindazole-3-carboxylic acid. The identification of glucuronidated 1-adamantanol or glucuronidated dihydroxy-adamantane is crucial to conclude that the parent compound is APINAC. The N-pentylindazole-3-carboxylic acid was subject to various transformations giving ten metabolites in total. The identification of the metabolites was made by both LC-QTOF-MS(/MS) and GC-MS to enable others to identify APINAC by either method. The question whether the profile of authentic human urinary APINAC metabolites meets in full or in part that of the rat in vivo model awaits further studies.

**Keywords:** APINAC, Synthetic Cannabinoids, Structural Elucidation, LC-QTOF, GC-MS

**Determination and Validation of 5f-NPB-22 and SF-ADBICA in Whole Blood and Urine**

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**Background/Introduction:** Synthetic cannabinoids (SCs) are the most rapidly growing class of recreational designer drugs. The rapid growth in popularity of SCs use among teens and adults is of serious in Turkey as all of the world. SCs mimic effects of  $\Delta^9$ -tetrahydrocannabinol. These products are saturated to various plants in order to make herbal appearance. They are labeled “not for human consumption” and also their chemical structures are rapidly evolving. They are known as *Bonsai* in Turkey. 5F-ADBICA and 5F-NPB-22 are detected in *Bonsai* plants which are recently captured by police forces.

**Objective:** The aim of this study was to determine and validate of 5F-ADBICA and 5F-NPB-22 in whole blood and urine by LC/MSMS. Urine metabolites of these products are not identified in the literature due to them being new generation SCs.

**Methods:** Briefly, samples were prepared by extracting 250  $\mu$ l of blood with 2 ml acetonitrile: ethylacetate (75:25). The tubes were capped and rotated for 15 min. The samples were then centrifuged for 10 min at 3500 rpm. The organic layer was transferred to another tube and evaporated to dryness at 45°C under a stream of nitrogen. The dried extract was reconstituted with “volume” methanol. 0.5 ml of urine samples, 50  $\mu$ l  $\beta$ -glucuronidase and 0.5 ml phosphate buffer (pH 6) were incubated at room temperature. Afterwards, 1.5 ml ice-cold acetonitrile and 0.5 ml of a 10 M ammonium formate solution were added. The mixture was shaken, centrifuged, and 1 ml of the organic layer was transferred into a separate vial and evaporated to dryness under a stream of nitrogen. The chromatographic separation was performed with an pentafluorophenylpropyl (PFPP) column (Allure 50x2.1x50 mm i.d., 5  $\mu$ m, Restek, Bellefonte, PA, USA) using a gradient binary with 10 mM ammonium formate (A) in ultrapure water and methanol (B). Positive ionization electrospray was used with nitrogen as the cone. The detector and interface voltage was 1.88 kV and 4.5 kV. Heat block temperature was 400°C, desolvation temperature was 250°C. Run time is 20 min.

**Results:** The method was validated in terms of limit of detection and quantification, stability, extraction recovery (85-89%), carry over, matrix effect, linearity (0.05-10 ng/ml) intra-assay and inter-assay precision (CV < 20%).

**Conclusion/Discussions:** The analytical method developed allowed the analysis of new generation synthetic cannabinoids in Turkey. A simple, cost effective and accurate LC/MSMS method is for simultaneous quantification of 5F-ADBICA and 5F-NPB-22 in whole blood and urine.

**Keywords:** 5F-ADBICA, 5F-NPB-22, Biological Samples



## Synthetic Cannabinoids 5F-ADB and ADB-FUBINACA in Postmortem Samples of Inmates in Alabama

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**Background/Introduction:** The US Department of Justice estimates that 70% of inmates have used drugs regularly or have committed a drug-related offense. Of particular concern are novel psychoactive substances that may escape routine jail or prison drug monitoring. As more of these compounds have been placed on the Schedule I list, newer compounds have been appearing in case samples, such as 5F-ADB and ADB-FUBINACA. 5F-ADB and ADB-FUBINACA are members of the newest generation of synthetic cannabinoids.

**Objective:** To investigate postmortem and overdose data pertaining to inmate deaths in Alabama. We present three cases involving synthetic cannabinoids 5F-ADB and ADB-FUBINACA detected in postmortem samples of individuals who died while incarcerated.

**Methods:** Full toxicological analysis is conducted on inmate deaths. This includes ethanol by HS/GC/MS, common drugs of abuse using either the Randox or Tecan immunoassay systems, liquid-liquid extraction for basic and weak acid/neutral drugs followed by GC/MS, and quantification as needed by either liquid-liquid or solid phase extraction followed by either GC/MS or LC/MS/MS. If the case history indicated possible synthetic cannabinoid use, that analysis was performed but it is not part of routine testing.

**Results:** Prevalence of the most common drugs found in inmate cases was determined. Interestingly, a rash of synthetic cannabinoid overdoses was identified in December, 2016 that coincided with the arrest of a prison guard for drug distribution. Synthetic cannabinoids were among the drugs found in the guard's possession. We highlight three cases below:

Case # 1: A 51 year old white male was found dead sitting in a chair in the shower room of the prison, covered in bruises. After interviewing other inmates, it was determined that the decedent had fallen and hit the back of his head before becoming unresponsive. He was transported to the hospital where x-rays determined he had three skull fractures, and he was placed on life support. Five days later, he was declared brain dead and life support was removed. According to medical records, he had a history of falls. Analysis for synthetic cannabinoids was requested as the decedent had a history of drug use. Toxicological findings were positive for ADB-FUBINACA and 5F-ADB. Cause of death was blunt force trauma and the manner of death was accident.

Case # 2: A 46 year old black male was found unresponsive in his cell, lying on the bed. It was stated that he may have been smoking synthetic cannabinoids. The only toxicological finding was the presence of ADB-FUBINACA. The cause of death was listed as synthetic cannabinoid toxicity and the manner of death as accident.

Case # 3: A 27 year old white male was observed to be jumping on his bed at approximately 1am. At 2am, he was observed by another inmate to come out of his cell, stop and look up at a fan, then collapse face first onto the floor. Other inmates stated that the decedent was smoking FLAKKA that night. Toxicological findings were only positive for 5F-ADB. The cause of death is listed as synthetic cannabinoid toxicity and the manner of death as accident.

**Conclusion/Discussions:** In addition to maintaining communication between investigators, medical examiners, and toxicologists, it may be useful to include synthetic cannabinoids as part of routine screening for the inmate population. As these cases demonstrate, unusual behavior cannot necessarily be attributed to deaths associated from synthetic cannabinoid use. In such situations, toxicologists can play a key role in assisting law enforcement to identify potential drug distribution rings within the jail and prison systems.

**Keywords:** 5F-ADB, ADB-FUBINACA, Inmates

## Furanyl-Fentanyl Sold Illicitly in Toronto, Canada as Fentanyl Pills – A Clinical Case Investigation Through Urine Drug Screening

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**Background/Introduction:** A 28 year-old male receiving methadone maintenance therapy in an outpatient program self-reports to his physician in January 2017 that he purchases illicit pills from the street for personal consumption. He describes the pills packaged similarly to prescription oxycodone. He consumes 5-10 tablets/day, feels euphoric after use and experiences symptoms consistent with opioid withdrawal when he does not take them. The pill supplier claims the product contains fentanyl. Fentanyl is increasingly found in illicit drugs produced by clandestine laboratories and sold in Toronto, Canada. A point-of-care testing (POCT) urine drug screen is performed in the physician's office and is positive for fentanyl. This specimen is then sent to a laboratory but screens negative for fentanyl/nor-fentanyl by qualitative liquid chromatography tandem mass spectrometry (LC-MS/MS). Over the next 3 months,  $N=4$  additional urine specimens tested positive for fentanyl by the POCT immunoassay but negative by qualitative LC-MS/MS.

**Objective:** Investigate potential fentanyl analogue(s) as the cause of the discrepant fentanyl POCT and LC-MS/MS urine drug screening results.

**Methods:** Immunoassay-based (IA) testing for fentanyl was performed by: lateral flow POCT tests from (a) BTNX Inc. (10 ng/mL fentanyl/20 ng/mL nor-fentanyl cut-off) and (b) Innovacon (SureStep Multi-Drug One Step Test Panel (20 ng/mL nor-fentanyl cut-off)); and (c) a laboratory-based DRI fentanyl screen (ThermoScientific; 2 ng/mL fentanyl cut-off). Alternative mass spectrometry testing was performed by: (a) qualitative LC-MS/MS screening using Waters Acquity LC system paired with Waters Xevo TQD triple-quadrupole mass spectrometer, ESI+ operated (fentanyl/nor-fentanyl 25 ng/mL cut-off); (b) quantitative LC-MS/MS confirmation testing for fentanyl/nor-fentanyl, sufentanil/nor-sufentanil (1 ng/mL cut-off) using Shimadzu LC system coupled to AB Sciex 4000 triple-quadrupole mass spectrometer, ESI+ operated; and (c) qualitative high resolution mass spectrometry (LC-HRMS/MS) screening using ThermoFisher LC Accela system coupled to Q-Exactive<sup>TM</sup> quadrupole-orbitrap mass spectrometer (HESI; ion polarity switching; 3-step energy ion fragmentation); a full scan (non-threshold)/MS<sup>2</sup> program identifies compounds based on monoisotopic (exact) mass, retention time (RT) and MS<sup>2</sup> spectra.

**Results:** All ( $N=5$ ) urine specimens screened positive for fentanyl when tested by the respective lateral flow POCT devices and the lab-based IA test. All mass spectrometry tests were negative for fentanyl/nor-fentanyl. All quantitative LC-MS/MS testing did not show any evidence of sufentanil use. Testing by LC-HRMS/MS did not identify acetyl-fentanyl and carfentanil nor their metabolites in the MS<sup>2</sup> drug library searches of any specimen. LC-HRMS/MS screening with the exact masses of several additional fentanyl analogues was then initiated in one of the specimens. Sufentanil was not found. The strongest peak was extracted at 10.99 min RT and corresponded to the exact mass of furanyl-fentanyl, M+H 375.2067 (<1 ppm). At the indicated RT, the significant mass fragments identified corresponding to: (a) the M+H molecular ion ( $m/z$  375); and (b) mass fragments specific to the N-phenethyl piperidine portion of the fentanyl molecule ( $m/z$  79, 105 and 188). This fragmentation pattern was confirmed using mzCloud Advanced Mass Spectral Database software (HighChemLLC). The presence of furanyl-fentanyl was additionally supported by mass spectral findings consistent with its metabolism by dealkylation (removal of phenethyl) to nor-furanyl-fentanyl or deacylation (removal of furancarboxy) to 4-anillino-N-phenethyl-piperidine. All specimens showed the same LC-HRMS/MS derived findings.

**Conclusion/Discussion:** This case represents one of the first reports of urine drug screening identifying the illicit presence of furanyl-fentanyl use in our area. Although there are relatively limited cross-reactivity data available from the IA vendors, the presence of furanyl-fentanyl and related analytes may be responsible for the positive IA screening results. The ability of conventional LC-MS/MS-based urine testing to identify fentanyl analogues may be constrained by the specific analytes targeted by the method, but overcome with high resolution mass spectrometry procedures. This finding is of particular interest for forensic drug use investigations.

**Keywords:** Furanyl-Fentanyl, False Positive Fentanyl Immunoassay Screens, High Resolution Mass Spectrometry Screening (UP-LC-HRMS/MS)

## Novel Automated Approach for the Analysis of $\Delta^9$ -tetrahydrocannabinol and its Two Main Metabolites in Blood Using Tip-On-Tip Filtration and DPX Extraction

Kaylee R. Mastrianni\*, William E. Brewer, DPX Technologies, LLC Columbia SC

**Background/Introduction:** Marijuana is the most widely abused drug in the US. As marijuana continues to be decriminalized and legalized, the need for easy, fast, accurate, and sensitive quantitative methods of  $\Delta^9$ -tetrahydrocannabinol, the active ingredient, and associated metabolites increases. Blood is the traditional matrix for determining impairment for suspected DUID cases. The complex nature of blood can make drug determinations with LC-MS/MS difficult due to matrix effects which manifest as ion suppression/enhancement. Therefore, sample preparation is a necessity. Previously published work generally requires large sample volumes and complex sample preparation in order to achieve essential sensitivity. We propose a fully automated approach that uses novel automated Tip-On-Tip filtration followed by a quick DPX clean-up for easy, high throughput analysis of THC and its metabolites in blood.

**Objective:** Our aim was to develop a method that minimizes sample volume and automates a fast and easy filtration and clean-up procedure to obtain a sensitive quantitation of  $\Delta^9$ -tetrahydrocannabinol (THC), 11-hydroxy- $\Delta^9$ -tetrahydrocannabinol (11-OH-THC) and 11-nor-9-carboxy- $\Delta^9$ -tetrahydrocannabinol (THC-COOH) in whole blood using LC-MS/MS with method validation according to the SWGTOX guidelines.

**Methods:** Aliquots of 100  $\mu$ L of each sample (calibrator, control, blank, case sample) were transferred into a well plate. The plate was added to the Hamilton NIMBUS96 system with an acetonitrile reservoir, 0.1 M formic acid reservoir, filter tips, and DPX WAX-S tips. Acetonitrile (300  $\mu$ L) was added to the blood and the solution was aspirated and dispensed multiple times to facilitate thorough protein precipitation. The total volume was aspirated and then the filter tips were attached through an automated movement of the robotic head. The total solution was then dispensed through the filter tip into a clean well plate. The Tip-On-Tip was then discarded, 50  $\mu$ L of the 0.1 M formic acid was added to the filtrate and DPX WAX-S (10 mg WAX resin and 10 mg salt) tips were picked up. The DPX WAX-S tips are then used to aspirate and dispense the sample solution three times. This allows for extraction of matrix and subsequent partitioning of the acetonitrile and the aqueous phase. The CO-RE tips are then used to transfer 100  $\mu$ L of the supernatant (acetonitrile layer) to a clean well plate, which is then transferred to the LC-MS/MS for injection.

**Results and Discussion:** The method was very accurate and precise. The bias of the assay did not exceed 6% for any analyte over each concentration. The precision (coefficient of variation, %) did not exceed 11% for any analyte over each concentration for both within and between run variations. The calibration models were linear, each correlation coefficient was greater than 0.99 with a 1/x weighting. The method was free from carryover at 50 ng/mL, the highest calibrator. Matrix effects manifested as ion suppression did not exceed 15%. The extraction efficiency of the automated SPE was 91% for 11-OH-THC, 98% for THC, and 92% for THC-COOH. Limits of detection were calculated to be 0.45 ng/mL for 11-OH-THC, 0.6 ng/mL for THC, and 2.5 ng/mL for THC-COOH.

**Conclusion:** The method presented herein provides an easy, fully automated approach to analyzing THC, THC-COOH, and 11-OH-THC in whole blood using LC-MS/MS. Protein precipitation and the extraction are fully automated on a Hamilton NIMBUS96 platform. The method is robust, with high precision and accuracy. The extraction time, after loading of the plates and solvents on the system deck, takes less than 5 minutes to perform with up to 96 samples simultaneously.

**Keywords:** Blood, THC, Automation

## Rapid Automated Protein Crash Method Using Novel Tip-On-Tip Filtration

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**Background/Introduction:** The bottleneck of blood analysis is often the vortex/centrifugation process required for protein precipitation. We propose a novel automated method (patent pending) as an alternative using wide bore tip mixing followed by in-tip filtration. The wide bore tip thoroughly mixes the sample solution with a precipitant to provide efficient protein precipitation. Previous studies performed in our laboratory have shown that wide bore mixing of acetonitrile with blood provides consistent recoveries of drugs when compared to 30 seconds of rigorous vortex mixing. Subsequently, the wide bore tip containing the precipitated sample solution is transferred and fitted into a secondary filter tip. The sample is then dispensed through the filter tip into a clean well plate for analysis (or further sample preparation). This provides an automated filtration step without complex accessories such as a positive pressure manifold or centrifuge. For increased sample clean-up during the filtration process, different solid phase extraction sorbents can be added. We present three filtration options: filter only, addition of C18 sorbent, and the addition of phospholipid removal sorbent.

**Objective:** The objective of the study was to define the utility of Tip-On-Tip filtration as a viable automated protein precipitation technique for comprehensive and rapid drug analysis in whole blood.

**Methods:** The Hamilton NIMBUS system was loaded with a 96 well plate of blank or spiked whole blood in 100  $\mu$ L aliquots, a reservoir of acetonitrile (5% acetic acid for HybridSPE® sorbent), a blank well plate, filter tips, and wide bore tips. The NIMBUS system picks up the wide bore tips, then aspirates 300  $\mu$ L of acetonitrile and dispenses it into the well plate containing blood. The wide bore tips mix the blood and acetonitrile vigorously through multiple aspirations and dispenses. The crashed solution is then aspirated and held in the tip as the system moves to the filter tip position. Once at the filter tip position, the NIMBUS 96-head moves downward and easily fits into the filter tips and picks them up. This Tip-On-Tip then moves to a clean well plate where the crashed solution is dispensed through the filter tip. The total automated extraction time (after loading of the system deck) takes approximately 3 minutes. The filtrate was solvent evaporated and reconstituted in 100  $\mu$ L of 10% methanol in water for injection onto the LC/MS. Filter tips contained no sorbent, 25 mg HybridSPE® sorbent (Supelco), or 10 mg C18. An alternative method included the use of anhydrous  $MgSO_4$  to remove water and consequently some matrix components.

**Results:** The total ion chromatogram of a LC/MS scan of the acetonitrile-crashed whole blood was compared with the filter tips without sorbent, and containing C18 or Supelco HybridSPE® sorbent. Matrix effects and recoveries for 40 common drugs of abuse were monitored. This method provided acceptable recoveries (>70%) for most of the analytes studied. The addition of the sorbents to the filter tip showed a reduction in the background in the full MS scan of the blank blood sample matrix. This reduction in matrix not surprisingly showed a corresponding reduction in matrix effects of many of the drugs being monitored. Higher responses and lower ion suppression effects were obtained with HybridSPE® sorbent compared to C18. The use of C18 in this “Tip-On-Tip” method may serve as a means of protecting the LC/MS system by preventing the introduction of matrix components that could build up in the LC column and ionization source, essentially acting as a disposable guard cartridge. With this product, we believe LC/MS maintenance issues can be greatly reduced. The use of  $MgSO_4$  was shown to both reduce the background in the LC/MS chromatograms and the time for solvent evaporation.

**Conclusion/Discussion:** A rapid and readily automated method for protein precipitation for sample preparation of whole blood was presented. The method provides good recoveries of various drugs, and addition of some sorbents can be incorporated to facilitate clean-up and provide better sensitivity.

**Keywords:** Blood, Automation, Protein Precipitation

## Analysis of Commercially Available Beta-Glucuronidase Enzymes and Optimum Hydrolysis Conditions in Urine for Licit and Illicit Drugs Using In-Well hHydrolysis

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**Background/Introduction:** Most drugs, both licit and illicit, are excreted in urine as glucuronide conjugates. Hydrolysis using beta-glucuronidase converts the glucuronidated metabolites back to their “free,” or non-conjugated form, increasing sensitivity for LC/MS-MS analysis. Hydrolysis using red abalone (BG100™, Kura Biotech, Los Angeles, CA), abalone (Campbell Science, Rockford, IL), and two recombinant (IMCSzyme®, IMCS, Irmo, SC and BGTurbo, Kura Biotech, Los Angeles, CA) beta-glucuronidase enzymes were evaluated in-well to determine which provided the most efficient hydrolysis of glucuronide metabolites without effecting the overall recovery of non-conjugated compounds. A glucuronide control was used to determine the extent of hydrolysis that occurred. A non-conjugated control was also evaluated to determine if signal suppression occurred as a result of hydrolysis.

**Objective:** An in-well hydrolysis solid phase extraction (SPE) plate was compared to a conventional SPE plate (hydrolysis was performed separately prior to extraction in the conventional plate). Various enzymes and incubation parameters were evaluated to determine differences in performance between the plates, optimal hydrolysis conditions, and variations in enzyme efficiency and analyte recovery.

**Methods:** A glucuronide control was prepared at 100 ng/mL using eight glucuronide compounds: 4 opiates (morphine-3-b-D-glucuronide, codeine-6-b-D-glucuronide, hydromorphone-3-b-D-glucuronide, oxymorphone-3-b-D-glucuronide), 2 benzodiazepines (oxazepam, lorazepam), norbuprenorphine, and 11-nor-9-carboxy THC (THC-COOH) (Cerilliant, Round Rock, TX). A second control was made using 56 non-conjugated drugs and metabolites. Enzyme was added. Samples were extracted via SPE using EVOLUTE® EXPRESS CX 10 mg 96-well or EVOLUTE® HYDRO CX 10 mg plates (Biotage, Charlotte, NC). Samples were hydrolyzed at room temperature for 30 minutes or 55°C for 10 or 30 minutes, extracted by SPE, and analyzed using a Shimadzu UPLC (Shimadzu, Long Beach, CA) coupled to a 5500 triple quadrupole mass spectrometer (Sciex, Framingham, MA).

**Results:** Norbuprenorphine was fully hydrolyzed (at least 80% hydrolysis) by all enzymes except for the Campbell enzyme when using a heated incubation. When using a room temperature incubation, hydrolysis efficiency was lower. THC-COOH was better hydrolyzed at lower temperatures and incubation times. Lorazepam and oxazepam were fully hydrolyzed by all enzymes under all conditions. The opiates were much more difficult to hydrolyze. None of the enzymes were able to fully hydrolyze all of the opiates under any conditions. However, the BGTurbo enzyme proved to be the most successful when using a 30 minute 55°C incubation. Hydromorphone and oxymorphone were fully hydrolyzed; morphine had 70% hydrolysis efficiency, and codeine had 45% hydrolysis efficiency. The Campbell enzyme was the least effective in hydrolyzing the opiate compounds and did not fully hydrolyze any of the opiates under any conditions. Recovery of the 56 compounds in the panel was also investigated to determine if any of the enzymes caused suppression of other analytes of interest. For the most part, the recoveries were within 10% for all compounds when using all enzymes. There were a few exceptions to this. The zolpidem metabolite (zolpidem-phenyl-4-COOH) had some recovery differences between enzymes. However, recoveries were all still above 50%. When comparing an in-well hydrolysis plate with an SPE plate, hydrolysis efficiencies and recoveries were similar between plates.

**Conclusion/Discussions:** When looking at the data, none of the enzymes were able to fully hydrolyze the opiate compounds. The THC-COOH may have had better hydrolysis efficiency at lower times and temperatures due to some of the non-conjugated THC-COOH converting after the glucuronide was cleaved. Overall, the BGTurbo enzyme yielded the highest hydrolysis efficiencies for the most glucuronide compounds. The Campbell enzyme yielded the lowest hydrolysis efficiencies, particularly for the opiate compounds. When using an in-well hydrolysis plate, time is saved as a sample transfer step is eliminated. The risk for cross-contamination is also reduced.

**Keywords:** Hydrolysis, Solid-Phase Extraction, Beta-Glucuronidase Enzymes

## Evaluation of Solid Phase Extraction (SPE) Compared to Supported Liquid Extraction (SLE+) as Sample Preparation Techniques for LC-MS/MS Detection of 49 Drugs and Metabolites in Umbilical Cord Tissue

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**Background/Introduction:** Prenatal drug exposure has adverse effects on fetal development and child outcome. Early detection is necessary for effective management of newborns at risk for acute complications including neonatal abstinence syndrome (NAS). Umbilical cord drug testing provides an accurate identification of *in utero* exposure and alternative to meconium as it offers various advantages i.e., ease of collection, specimen availability, and fast turnaround times. We previously published LC-MS/MS screening methods for assessment of in utero drug exposure using umbilical cord testing [1, 2]. Based on the complex nature of tissue samples, an efficient and robust sample clean-up procedure is needed to minimize matrix interference. In this study, we evaluate mixed-mode cation exchange SPE compared to SLE+ for extraction of a broad range of drugs and metabolites from umbilical cord tissue prior to analysis by LC-MS/MS.

**Objective:** Develop an extraction procedure for 49 drugs and metabolites from umbilical cord tissue using SPE for LC-MS/MS detection.

**Methods:** Previously screened drug-free umbilical cord specimens were used to prepare calibrator and controls. De-identified residual patient specimens, calibrator (cutoff) and controls (negative, 50% cutoff, and 150% cutoff) were weighed (1.50g±0.01), 3.00 mL of water and stainless steel UFO beads were added. Samples were placed in -80°C for 15 minutes prior to homogenization on the Bead Ruptor 24 followed by centrifuged at 0°C for 10 minutes. Congealed lipids were removed and deuterated internal standard (IS) mixture was added to 1.00 mL of supernatant from each sample. 0.900 to 1.00 mL supernatant was loaded on ISOLUTE® SLE+ 1 mL columns (Biotage, NC) and various combinations of water-immiscible solvents (DCM, IPA, ACN, ethyl acetate) were evaluated for extraction. Supernatants for SPE were diluted 1:1 with 0.100 M phosphate pH 6.0 and loaded on EVOLUTE® EXPRESS CX 60 mg columns preconditioned with methanol and buffer. Columns were washed with buffer, 0.1% acetic acid (optimized) and dried at 30 psi for 6 minutes. 1mL hexane was added to the columns to remove residual water then dried for another 6 minutes. 50:50 hexanes: ethyl acetate was used for elution of acidic and neutral drugs/metabolites then a second elution of basic drugs/metabolites was performed with 78:20:2 DCM:IPA:NH<sub>4</sub>OH after an acidic methanol wash. All eluates were dried under nitrogen (40°C) using a TurboVap® (Biotage) and reconstituted in 90:10 water:methanol. LC-MS/MS analysis was performed using our previously published method with some modifications [described in the poster].

**Results:** 95:5 ethyl acetate: IPA was the optimal elution solvent for SLE+ however, after sample reconstitution the extracts were dirty and the recoveries obtained varied greatly (<70 to >200%). Cleaner extracts were obtained with 98:2 DCM:ACN / 95:5 ethyl acetate:IPA elution solvent but the recoveries of a majority of drugs/metabolites were compromised. Two-step elution using mixed-mode cation exchange SPE provided the optimal recoveries for most drugs /metabolites and resulted in good quality extracts for direct LC-MS/MS analysis. Both SLE+ and SPE methods were unable to extract buprenorphine, buprenorphine glucuronide and m-hydroxybenzoylcgonine from cord tissue. A single point calibration standard (cutoff) with regression passing through zero was used to evaluate the SPE method performance including accuracy, intra and inter-assay imprecision, carry over and matrix effect. Authentic patient samples were used for qualitative method comparison.

**Conclusion/Discussions:** Mixed-mode cation exchange SPE offers superior selectivity for a wide range of drug classes and metabolites in umbilical cord tissue providing optimal extract cleanliness ideal for LC-MS/MS analysis. SLE+ was limited due to co-extraction of lipid interference and resulted in highly variable recoveries. The SPE method described is robust, effective and has acceptable performance for routine extraction of 49 drugs of abuse and metabolites from umbilical tissue for assessment of in utero drug exposure.

**Keywords:** Umbilical Cord, Sample Preparation (SPE), LC-MS/MS

## The Application of UPLC/MS/MS to the Study of Amygdalin Stability, Metabolism Prediction and Toxicity

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**Background/Introduction:** Amygdalin is the subject of scientific interest from 19<sup>th</sup> century. However, amygdalin is a potential toxic substance. It is a poisonous cyanogenic glycoside in many plants. The source of amygdalin are notably seeds of apricot, peach, apple and other fruits, especially from Rosaceae family. It is also found in other plants like lima beans, clover, sprouting seeds, in cherries, orange, plums, papaya, almonds, nectarines and in particular, bitter almonds. It is found in heavy concentration in the stone fruit kernels. So called vitamin B17 is also found in grains like millet and buckwheat, as well as macadamia nuts, bamboo shoots, mung beans, butter beans, and some garden peas. Since the early 1950s amygdalin was promoted as alternative of cancer prevention and treatment. The studies have found amygdalin to be ineffective of the treatment of cancer as well as toxic or lethal when taken by mouth. Moreover as a vitamin B17 is easy available on nutritional supplement on common food market.

**Objective:** Food supplements containing amygdalin are further widely used for cancer prevention and treatment. Injection form of this remedy is applied intravenously. There are many controversies of the matter of the application of amygdalin. Our main scope of interest is focused on the better understanding of the role of amygdalin in nutrition, its physico-chemical properties and also its behaviour under different analytical conditions, mainly amygdalin solubility and stability in solvents with different degree of polarity and pH reaction.

**Methods:** The UPLC ( UltiMate 3000 Binary RSLC) separation was performed with 0.1% formic acid (FA) in water(A)/ methanol : acetonitril, 50:50 (B) gradient ( from 5% to 95 % B during 4.5 minutes) on Hypersil GOLD 50 x 2.1 mm, 1.9 mm .The total time of the analysis was 5.1 minutes. The mass spectrometer LTQ XL ( Thermo Fisher Scientific ) was operated in negative H- ESI mode. The spray voltage was set at 3.8 kV. The capillary temperature was 275 °C, sheath gas was set at 45 units, auxiliary gas was set at 15 units, capillary voltage was -46 V, tube lens was -135 V, heater gas was adjusted on 330 °C .

**Results:** We have studied chemical addition products of amygdalin in various solvents under negative H-ESI conditions. The optimization of H-ESI probe parameters, mainly set up temperatures on the capillary, and heater gas to minimize the abundance of ion 323 m/z (the glucopyranosyl ring cleavage) played a key role. The 502 m/z was observed in pure water , water +FA (0.1%) and methanol with 0.1% FA as the adduct of formiate resulting in product negative ion M+45. The m/z 492/494, 3/1 (Cl) was dominant in 2M HCl solution resulting in M + 35/M +37 negative chlorine A+2 ion. The H<sub>2</sub>O adduct of amygdalin (M + 19) was formed in alkaline solution with sodium hydroxide (1M solution).

**Conclusion/Discussions:** We report the effect of various solvents on the precursor ion of amygdalin in negative H-ESI mode using linear ion trap system. This conclusion was important as one of key way out for quantitative determination of this glycoside in kernel and other herbal extracts.

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**Keywords:** Amygdalin, B17, Cyanogenic Glycoside, UPLC/MS/MS, Negative H-ESI

**Enzyme Activity Determined with Phenolphthalein-Glucuronide Correlates Weakly with Hydrolysis Efficiency**

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**Background/Introduction:** Since Fishman *et al.* introduced phenolphthalein-glucuronide in the 1940s as a viable chromogenic substrate for testing  $\beta$ -glucuronidase activity, it has been widely used as a rapid and simple way to determine activity. The activities of commercially available  $\beta$ -glucuronidase are advertised with a value of units per milliliter, a measure of how many micrograms of phenolphthalein will be liberated per hour. It is typically perceived that the higher the activity, the higher the rate of hydrolysis of drug glucuronides found in various biological fluids. However, this work presents data that contradict this common belief.

**Objective:** Demonstrate that  $\beta$ -glucuronidase activity as determined with a chromogenic assay using phenolphthalein-glucuronide does not necessarily correlate with hydrolysis efficiency of common drug metabolites. Monitor enzyme activity and test hydrolysis efficiency of five different  $\beta$ -glucuronidases.

**Methods:** Five  $\beta$ -glucuronidases from various sources were assayed with phenolphthalein-glucuronide using their respective buffers at temperatures of 25°C and 37°C. For the hydrolysis efficiency test, drug-free urine was spiked with 500 ppb of common drug metabolites, including opiates, benzodiazepines, and tricyclic antidepressants. Five master mixes were prepared for each glucuronidase containing the enzyme, its respective buffer, water, and internal standards. The spiked urine was incubated with each master mix in triplicate at 58°C for time periods of 0, 15, 30, 45, and 60 minutes. Samples were then extracted and eluted using DPX WAX/RP tips and analyzed on a Thermo Scientific™ Vanquish™ UHPLC system coupled with a Thermo Scientific™ Endura™ Triple Quadrupole Mass Spectrometer.

**Results:** Three  $\beta$ -glucuronidases with varied protein concentrations and advertised as having activities between the ranges of 50,000 to 150,000 U/mL had nearly identical measured activities as well as hydrolysis efficiencies. Two  $\beta$ -glucuronidases which are advertised at >100,000 U/mL did not hydrolyze most compounds as efficiently as a  $\beta$ -glucuronidase that is advertised as >50,000 U/mL. One  $\beta$ -glucuronidase whose activity determined by the phenolphthalein-glucuronide assay as being up to 14-fold greater than that of the other  $\beta$ -glucuronidases had the lowest hydrolysis efficiency.

**Conclusion/Discussions:** While  $\beta$ -glucuronidases with a higher advertised activity are expected to have higher hydrolysis efficiency, this is not necessarily the case. Enzyme activity determined with phenolphthalein-glucuronide does not directly correlate with the enzyme's ability to hydrolyze certain drug metabolites. Consumers should be aware of this discrepancy before purchasing a  $\beta$ -glucuronidase based on enzyme activity.

**Keywords:** Phenolphthalein-glucuronide,  $\beta$ -glucuronidase, Hydrolysis Efficiency



## LC/MS/MS Analysis with On-line Cartridge for Removal of Phospholipids from Protein Precipitation Biological Fluid Samples

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**Background/Introduction:** Phospholipids are abundantly (at the mg/mL level) present in biological fluids such as blood, plasma, serum, cerebrospinal fluids, among others. They are often co-extracted with a broad range of analytes of interest during sample preparation. The phospholipids present in a sample are notorious in producing various issues in liquid chromatography/mass spectrometry (LC/MS) based bioanalysis. They may cause ion suppression or, in rarer cases, ion enhancement, in MS detection. They also tend to buildup on a reversed-phase (e.g. C18 and C8) column, fouling the chromatographic separation and ultimately shortening the column lifetime. Consequently, the accuracy, reproducibility, and sensitivity of the LC/MS bioanalysis may be greatly compromised if the phospholipids are not removed. We have developed a HybridSPE®-Phospholipid technology for selective and rapid depletion of phospholipids from biological samples prior to LC/MS analysis of small molecules. The technology utilizes the affinity of zirconia particles for selective binding and removal of phospholipids. The technology was introduced a few years ago in two product formats: 96-well filter plates for high throughput sample preparation and cartridges for low sample volume, respectively.

**Objective:** To introduce a new product format, in-line cartridge, as an alternative option of phospholipid removal and sample preparation. The setup of the in-line cartridges with an LC/MS column is devised and their efficiency in phospholipid removal from protein precipitated plasma samples is evaluated. Their applicability was demonstrated with two sets of compounds of different chemical properties.

**Methods:** In-line cartridges (2 cm x 4.0 mm I.D.) were made by packing about 100 mg of zirconia-coated silica material into each cartridge. These cartridges act much like guard columns and were configured with LC/MS for the removal of phospholipids in matrices while allowing the analytes to pass through. Rat plasma spiked with analytes was firstly protein precipitated by vortex mixing the rat plasma with the precipitation solvent (e.g. acetonitrile with 1% formic acid) at 1:3 ratio. Then the mixture was centrifuged at 10000 rpm x 3 min and the resulting supernatant was collected for the LC/MS analysis.

**Results:** An in-line cartridge packed with zirconia-coated silica particles was set up with a LC/MS for in-line phospholipid removal during LC/MS analysis of biological samples. Performance testing shows the in-line cartridges are capable of removing >95% of phospholipids from 1  $\mu$ L of plasma samples even after 300 consecutive injections. Compounds of varied properties including basic (risperidone, clomipramine, tamoxifen), hydrophilic (digoxin and digitoxin) and hydrophobic (25-OH Vitamin D3, 3-epi-25-OH-Vitamin D3, 25-OH Vitamin D2 and 3-epi-25-OH Vitamin D2) were tested. For all of the tested analytes, a recovery of 94%-102% was achieved with a reproducibility (RSD) of 1%-5%. Additionally, narrow and symmetric peaks were observed with a peak width at half height <6 s and tailing factors 0.9-1.3, respectively.

**Conclusion/Discussions:** An in-line cartridge packed with zirconia-coated silica particles has been successfully developed for in-line phospholipid removal during LC/MS analysis of biological samples. While the phospholipids in the matrix samples are effectively removed by the on-line cartridge, the analytes are not retained and well recovered. The narrow and symmetric peak shapes indicate there is minimum or no negative effect on the LC/MS/MS analysis from the on-line cartridges.

**Keywords:** LC/MS/MS, On-line Cartridge, Phospholipid removal, Biological Fluids

**Urine Clean-up for EtG/EtS Analysis on LCMSMS and Automation on Robotic Platforms**

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**Background/Introduction:** DPX tips provide a fast, accurate, and simple clean-up method for analyzing alcohol metabolites (ethyl glucuronide / ethyl sulfate (EtG/EtS) in urine. This method is fast and efficient and highly amenable to automation on any number of robotic platforms. Analysis of EtG/EtS is a common test run in many toxicology laboratories. Sample prep is usually very minimal due to their water solubility, highly polarity and inability to bind to common solid phase extraction (SPE) resins. Methods that do incorporate SPE usually require harsh solvents and strong acid. We have developed a quick and easy cleanup method utilizing a mixture of ionic exchange resins that requires no organic solvents.

**Objective:** To provide a fast, efficient, and environmentally friendly sample preparation method for the analysis of EtS/EtG in urine for liquid chromatography tandem mass spectrometry (LCMSMS) analysis.

**Methods:** To a well plate containing 50ul of unhydrolyzed urine is added 200ul of an aqueous ammonium formate/formic acid buffer, pH approx. 5. The mixed mode CX/WAX (10mg) tips are used to aspirate and dispense the sample five times. The extraction was performed on an Integra Viaflo 96 semi-automated robotic platform. Aspirate and dispense speeds can be set from 1 – 10 with 1 being the slowest. For aspiration, the speed was set to 2 and for dispense the speed was set to 4. Dwell time between aspirate and dispense steps was 10 seconds. Another well plate containing 200ul of the same buffer as above is aspirated in the tip and dispensed into the original well plate containing the sample. The sample is now ready for injection. Analysis was performed on a Thermo TSQ Vantage triple quadrupole instrument with an Agilent 1260 HPLC using an Agilent Poroshell EC-C18 column (3.0 x 50mm, 2.7um) with a 10 uL injection.

**Results:** Correlation coefficients were greater than 0.99 for the range of 100 - 10000 ng/mL. Relative standard deviation was calculated using 5 replicate extractions at 1000 ng/mL. RSDs for both EtS and EtG were lower than 5. Yields for EtS and EtG were 42% and 90% respectively. The lower yield for EtS was due to binding to the WAX resin. Due to the ionization response of EtS in the MSMS a cut-off of 100 ng/ml was easily achieved. EtG does not bind effectively to the resin resulting in higher recoveries and a cut-off of 250 ng/ml was also easily achieved as a result. S/N at the cut-offs were 90 and 136 for EtS and EtG respectively.

**Conclusion/Discussions:** By using a cation exchange resin with an anion exchange resin to remove a broad range of acidic and basic contaminants we have produced a reliable sample preparation method that provides the necessary recoveries, sensitivity, and reproducibility in a high throughput setting. The ability to automate the sample preparation method makes the analysis non-tedious as well as rapid.

**Keywords:** DPX, EtS/EtG, Extraction

**Esterase Activity in Commercially Available Crude  $\beta$ -Glucuronidases Correlates to Loss of Heroin and Cocaine Metabolites**

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**Background/Introduction:** Small molecule drugs are cleared from the body by being glucuronidated or sulfated in the liver. These phase II conjugated metabolites then travel through the circulatory system and are excreted in biological fluids, including saliva, blood, and urine. Clinical laboratories utilize a hydrolysis process to liberate these molecules from their glucuronide or sulfate conjugates to facilitate their detection by a range of analytical techniques including liquid chromatography tandem mass spectrometry (LC-MS/MS). Glucuronide hydrolysis could be achieved with strong acid, but this can lead to analyte degradation and conversion.  $\beta$ -glucuronidase is a cleaner option to hydrolyze glucuronides without a non-specific compound degradation. However, we demonstrate that esterase contamination is present in many of the commercially available  $\beta$ -glucuronidases. This contamination correlates with conversion of acetylated opiates and other esterified drugs. It is important to note this contaminant when using  $\beta$ -glucuronidase or sulfatase to monitor the pharmacokinetics of esterified drugs and steroids.

**Objective:** To demonstrate esterase contamination in various commercial crude  $\beta$ -glucuronidases and to show that esterase-contaminated enzymes convert 6-monoacetylmorphine (6-MAM), a specific metabolite of heroin, to morphine, skewing the readout to false negative for heroin usage. Similarly, contaminated enzymes also convert benzyloecgonine (BZE), a specific metabolite of cocaine, to ecgonine (ECG), skewing the readout to false negative for cocaine usage.

**Methods:** Various commercial  $\beta$ -glucuronidase enzymes (abalone, limpet, snail, and recombinant) were tested. A fluorogenic assay was employed to rapidly detect esterase activity. LC-MS/MS analysis was carried out to quantitate 6-MAM to morphine conversion and BZE to ECG conversion. Drug-free urine was fortified with 500 ng/mL of either 6-MAM or BZE. Samples were hydrolyzed for 0, 1, and 2 hours. At each time point, the hydrolyzed samples were immediately extracted using CX or WAX/RP tips (DPX Technologies). The LC-MS/MS analysis was performed on a Thermo Scientific™ Vanquish™ UHPLC system coupled with a Thermo Scientific™ Endura™ Triple Quadrupole Mass Spectrometer.

**Results:** Esterase contamination was observed in various commercial  $\beta$ -glucuronidase enzymes when using a fluorogenic substrate. Contaminated enzymes show the conversion of 6-MAM to morphine, and BZE to ECG. This potentially skews the readout to false negative for the usage of heroin and cocaine, respectively. The level of esterase contamination correlates with the amount of converted analytes. These conversions are not observed when using  $\beta$ -glucuronidases with undetectable esterase activity

**Conclusion/Discussions:** The esterase contamination correlates with conversion of acetylated opiates and other esterified drugs. It is important to note this contaminant when using  $\beta$ -glucuronidase or sulfatase enzymes to monitor the pharmacokinetics of esterified drugs and steroids.

**Keywords:**  $\beta$ -glucuronidase, 6-MAM Conversion, Benzyloecgonine Conversion

## Targeted Forensic Screening and Semi-Quantitation of Drugs in Plasma Using High Resolution Accurate Masses Detection and On-Line Sample Preparation

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**Background/Introduction:** High Resolution Accurate Masses detection coupled to liquid chromatography has proved to be a powerful tool for forensic screening purposes. The Orbitrap systems have also showed excellent results for quantitation analysis in numerous targeted studies.

**Objective:** The aim of this work was therefore to generate a large screening panel in a short chromatographic run, and then combine the screening capabilities of a Q Exactive Focus mass spectrometer to the quantitation of 41 drugs in plasma matrix. In both screening and quantitation methods the results can be strongly affected by the sample preparation, therefore the method was developed with on-line sample preparation based on TurboFlow technology.

**Methods:** For the generation of the spectral library and compound database, the 1530 standard solution were injected with the HPLC method to obtain retention times and MS/MS spectra. From these standards, 41 compounds were selected to run quantitative work in plasma matrix. Protein precipitation was performed to the samples and they were extracted the on-line using two TurboFlow columns (Cyclone P 50x0.5mm and Phenyl 50x0.5mm) connected in series. The analytes were then transferred to an Accucore Phenyl Hexyl 100x2.2mm 2.6 $\mu$ m column for separation. The total run time for extraction and separation was 16.75 minutes.

The screening method was established for 1433 compounds that were retained by the extraction columns. The screening method was then established in these conditions with identification based on the accurate mass of the parent compound, retention time of the compound, its isotopic [pattern matching score, the presence of at least two fragment ions, and its library matching score based on mzVault library search algorithm. The method was then partially validated. To this end, 41 compounds were selected from the panel, covering different compound classes, retention times and polarities. Limits of detection (LOD), quantification (LOQ) and identification (LOI) were obtained for these 41 compounds. The LOD was defined as the lowest concentration for which a peak is observed for the analyte for three different plasma matrices. The LOQ was defined as the lowest concentration for which a quantitation has an accuracy with a bias and a %RSD both inferior to 20%. The LOI was defined as the lowest concentration for which a compound can be identified based on the following conditions: m/z of the parent (< 5 ppm), isotopic pattern match, fragment ion presence, and MS<sup>2</sup> spectra matching. The 41 compounds were divided in two groups for the generation of calibration curves, one group tested from 0.1ng/mL to 250ng/mL and one group from 10ng/mL up to 5000ng/mL. The concentration ranges were selected according to the concentration generally assessed for the compounds in each group.

**Results/Discussion:** The method presented here was suitable for a quantitative approach considering that the calibration curves were linear from LOQ up to 250ng/mL for the first group of compounds, and from LOQ up to 5000ng/mL for the second group. A compound database and a spectral library for the screening of 1433 compounds were implemented on a Transcend II TLX-1 system coupled to a Q Exactive Focus high resolution accurate mass spectrometer. The panel includes both positively and negatively ionized compounds such as drugs of abuse and metabolites, antidepressants, beta-blockers, antibiotics, pesticides and other classes, and its duration is of 16.75 minutes. A partial validation was performed in plasma. The compounds can be used as a basis for the method validation since they cover different drug classes, retention times and polarities.

**Keywords:** Forensic Screening, Orbitrap Technology, TurboFlow Technology

## Extraction Optimization for LC-MS/MS Quantitation of Cannabinoids and Metabolites in Umbilical Cord Tissue and Meconium

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**Background/Introduction:** Little is known about the long term effects on neonates after *in utero* exposure to cannabis. Being the most abused drugs amongst pregnant women, a better understanding of cannabis metabolite patterns/concentrations is essential.

**Objective:** To develop a sample preparation method for umbilical cord tissue and meconium to extract tetrahydrocannabinol (THC), 11-nor-9-carboxy-THC (THCA), cannabinol (CBN), cannabidiol (CBD), and 11-hydroxy-THC (11-OH-THC).

**Methods:** Mixed mode cation (CX) and anion (AX) exchange columns were evaluated for extraction efficiency, matrix effect, reproducibility, and cleanliness of final extracts. Biotage Evolute AX and CX SPE columns (60mg/3mL, Biotage, Charlotte, NC) were used under the same extraction procedure to examine both base hydrolysis and enzymatic hydrolysis. Ultimately base hydrolysis was used for both sample matrices. The extraction method selected for routine use is shown in Table 1. THCA-glucuronide was added to batch controls to monitor hydrolysis completion. Extracts were analyzed on an AB SCIEX 5500 mass spectrometer interfaced with CTC PAL HTC-xt DLW autosampler and Agilent 1260 infinity series HPLC pump. Two transitions for each analyte and corresponding internal standards were monitored in negative mode electrospray ionization using scheduled MRM. A Agilent Poroshell 120 EC-C18 column, 3.0x50mm, 2.7 $\mu$ m was used to develop a 4.6 minute HPLC profile with mobile phase A: 5mM ammonium bicarbonate pH 9.5 in water and mobile phase B: methanol.

**Results:** Overall, more than 40 combinations of extractions methods were examined in triplicate to determine the method associated with the highest recoveries and lowest imprecision. Reproducibility was drastically affected by pH change on CX SPE columns versus AX SPE column. CX SPE columns showed the highest overall recovery and the cleanest final samples but had consistent reproducibility complications. AX SPE columns gave sufficient recoveries, reproducible results, and visibly clean samples. Recoveries for THC, THCA, 11-OH-THC, and CBN in umbilical cord and meconium ranged from 71-99% and 73-83%, respectively. Extraction of CBD was unsuccessful in umbilical cord while 27% was recovered in meconium. The analytical measurement range for umbilical cord (0.20-5.0 ng/g) and meconium (5.0-1000 ng/g) varied considerably due to the differences in metabolite concentration seen in patient samples. Ultimately, two different sample clean-ups were needed to get reproducible results, clean samples, and acceptable recoveries.

**Table 1:** Extraction details

<u>Extraction procedure AX SPE column</u>		
<u>Description</u>	<u>Meconium</u>	<u>Umbilical Cord Tissue</u>
<b>Sample volume</b>	0.25±0.01g	1.0±0.10g
<b>Homogenization liquid</b>	Methanol	
<b>Centrifuge</b>	10 minutes at 0°C and 14,000rpm	
<b>Base hydrolysis</b>	Heat samples at 70 °C for 15 min in 0.5M NaOH	
<b>SPE wash 1</b>	1mL 85:15:1 H <sub>2</sub> O:CAN:NH <sub>4</sub> OH	1mL 1% ammonium hydroxide
<b>SPE wash 2</b>	1mL 50:50 Hexane:EtOH	1mL methanol
<b>Elution</b>	2% acetic acid in 90:10 Hexane:EtOH	2% acetic acid in methanol
<b>Reconstitution</b>	200 $\mu$ L 60:40 methanol:water	

**Conclusion/Discussions:** The described AX extraction and LC-MS/MS method was robust, sensitive, reproducible, and accurate for monitoring cannabinoids and metabolites in umbilical cord tissue and meconium. With the increasing prevalence of cannabis usage in pregnant women this method supports studies designed to understand the patterns of THC and metabolites and the long term effects on neonates.

**Keywords:** Umbilical Cord Tissue/Meconium, Cannabinoids, LC-MS/MS

## Anodyne by Design; Detecting Designer Opioids in Pain Management

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**Background/Introduction:** In an effort to combat opiate abuse, tighter restrictions have been implemented regarding prescribing opiates and opioids and their formulations. While these changes have undoubtedly reduced the prevalence of prescription opiate abuse, they have also left a subset of individuals searching for alternatives to satiate addictions or to assuage personal opioid-seeking motivations, and thus potentially contribute to a notable increase in heroin use as well as a rapid proliferation of designer opioids. Multiple classes of designer opioids exist stemming from academic research and drug development projects, and each compound has the potential for clandestinely applied structural permutations. Each of the esoteric designer opioids evaluated herein possesses its own unique pharmacology and toxicology and all have at least some reported use by opiate-seeking individuals. Almost all designer opioids are invisible to standard opioid testing methodologies, which could further motivate an individual to seek out designer opioids and thereby conceal their opioid use and / or opioid supplementation.

**Objective:** While there have been multiple high profile reports of designer opioid use, it is difficult to assess the true prevalence of these drugs. We hypothesized that individuals abusing opioids while within a pain management setting are likely to be early adopters of designer opioids. We set out to survey this population to estimate the prevalence of designer opioids.

**Methods:** Pain management samples containing evidence of heroin use through the presence of 6-AM were deemed to have been donated from opiate abusers; these same samples were then selected for subsequent designer opioid testing. Independent liquid chromatography tandem mass spectrometry (LC-MS/MS) screening and confirmation methods were developed targeting parents and metabolites of designer opioids. Metabolites without available certified reference standards were monitored as qualitative supporting evidence. The assays monitored for the parent drugs and metabolites of the following designer opioids:

Targeted Analytes		
Fentanyl	Valeryl fentanyl	IC-26
Acetyl fentanyl	Ocfentanil	AH-8533
Acetylfentanyl-4-methylphenethyl	<i>para</i> -Fluorobutyryl fentanyl	AH-8529
Furanyl fentanyl	AH-7921	Carfentanyl as Norcarfentanil
Butyryl fentanyl	MT-45	W-18
3-Methylfentanyl	U-47700	W-15
<i>para</i> -Fluorofentanyl	4-Methoxy butyrylfentanyl	W-19
$\beta$ -hydroxy-thiofentanyl	Acrylfentanyl	

A 90-second LC-MS/MS screening procedure was fully validated targeting a single MRM transition for each compound. A secondary conditional LC-MS/MS confirmation procedure was also developed and fully validated utilizing independent chromatographic parameters, two transitions per analyte, and additional targeted metabolites and internal standards. The lower limit of quantitation for targeted parent drugs ranged from 100 to 400 pg/mL with non-quantitative limits set for additional metabolites.

**Results:** 775 samples from suspected heroin users enrolled in chronic pain clinics were surveyed for designer opioids. Unsurprisingly, 43% contained either fentanyl or norfentanyl. This may result from an actual prescription for fentanyl or from the fact that heroin is frequently cut with fentanyl. The next most prevalent designer opioid was furanyl fentanyl, where parent drug was present in 12% of samples and accompanied by its primarily excreted dihydroxy metabolite. Parent acetyl fentanyl and *para*-fluorobutyrylfentanyl along with their respective metabolites were each detected in approximately 8% of the samples. Kratom, and U-47700 in combination with their respective metabolites were detected in 1% or less of the heroin positive samples.

**Conclusion/Discussions:** Results indicate designer opioid use in pain management patients exists, but their use is not widespread. Use of fentanyl and fentanyl analogs dominate within this population, whereas use of the more esoteric designer opioids occurs in this population, but at a very low frequency.

**Keywords:** Designer Drugs, Opiates, Pain Management

## Cross-Reactivity of Novel Opioids, Fentanyl Analogs, Designer Benzodiazepines and Mitragynine with Various Immunoassay Kits

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**Background/Introduction:** Novel psychoactive substances (NPS) are modified variants of either controlled substances or research chemicals abused for their psychoactive effects. The continuous structural modification trend, which began with synthetic cannabinoids, recently shifted to opioid compounds such as acetylfentanyl and U-47700. In addition to the recent emergence of novel opioids, designer benzodiazepine use has been suspected in casework involving these substances. Many of these esoteric compounds are not very well understood due to the little information available regarding the pharmacological and toxicological effects, which has created challenges in forensic interpretation and analyses. During initial screenings with immunoassays, NPS may or may not cross-react with specific kits; this can lead to potentially missing toxicologically relevant compounds or discrepancies between immunoassay screening results and confirmatory analysis data.

**Objective:** The objective of this study was to assess the cross-reactivity of novel opioids, fentanyl analogs, designer benzodiazepines, and mitragynine against multiple commonly used enzyme-linked immunosorbent assay (ELISA) kits.

**Methods:** 1.0 mL stock spiking solutions were prepared at various concentrations of 0.01, 0.1, 1.0, and 5.0 ng/ $\mu$ L using aqueous solutions containing the analytes of interest. Stock solutions containing novel opioids U-47700, U-50488, MT-45, W-15, W-18, and AH-7921 were spiked in duplicate into 400  $\mu$ L of PBS buffer (pH 7.0) samples for final concentrations of 100, 500, and 1000 ng/mL. Stock solutions containing fentanyl analogs furanyl fentanyl, butyryl fentanyl, para-fluorofentanyl (PFF), and 4-ANPP were spiked in triplicate into 400  $\mu$ L of PBS buffer (pH 7.0) samples for final concentrations ranging from 0.1 to 100 ng/mL. Selected designer benzodiazepines bromazepam, clonazepam, diclazepam, etizolam, flubromazepam, flubromazolam, phenazepam, and pyrazolam were spiked in triplicate into 200  $\mu$ L of PBS buffer (pH 7.0) and human whole blood samples for final concentrations ranging from 5.0 to 50 ng/mL. Mitragynine was spiked in triplicate into 400  $\mu$ L of PBS buffer for final concentrations of 100, 500, and 1000 ng/mL. Using Immulysis<sup>®</sup> ELISA opiate, oxycodone, fentanyl, and benzodiazepine kits, the immunoassay analyses were carried out following the manufacturer's protocol with a TECAN Freedom Evo 150. The plates were washed using an ELX405TS Plate Washer prior to the addition of TMB substrate and left sitting for 25 minutes. Acid stop solution was added and optical densities were measured using TECAN Sunrise.

**Results:** Mitragynine and the selected novel opioid compounds U-47700, U-50488, MT-45, AH-7921, W-15, and W-18 were determined to exhibit no cross-reactivity with ELISA opiate and oxycodone kits at the studied concentrations. Fentanyl analog compounds 4-ANPP, butyryl fentanyl, PFF, and furanyl fentanyl were determined to have 0.44%, 88%, 61%, and 74% cross-reactivity with the ELISA fentanyl immunoassay, respectively. At the concentrations assessed with the ELISA benzodiazepine immunoassay, designer benzodiazepines flubromazepam, bromazepam, clonazepam, diclazepam, etizolam, flubromazolam, phenazepam, and pyrazolam showed 81.9, 182, 125, 94.5, 158, 104, 101, and 247% cross-reactivity in PBS buffer and 81.7, 172, 108, 80.2, 143, 102, 92.3, and 246% in human whole blood, respectively.

**Conclusion/Discussion:** As expected, NPS that lacked structural similarities to traditionally abused drugs, such as novel opioids, exhibited no cross-reactivity. In cases where no cross-reactivity is observed, substances could be missed and may require additional screening techniques to detect them in specimens. Substances that are structurally similar to traditional drugs, such as fentanyl analogs and designer benzodiazepines, exhibited varying degrees of cross-reactivity in comparison to the analyte standard. Samples that initially screened positive, but fail to confirm, should be subjected to further testing and investigated for emerging NPS.

**Keywords:** Novel Psychoactive Substances (NPS), Screening, Enzyme-Linked Immunosorbent Assay (ELISA)

**Ritalinic Acid in Urine: Modelling Data to Assist with Determination of Patient Adherence**

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**Background/Introduction:** Methylphenidate is a central nervous system stimulant that has been primarily used for treatment of attention deficit hyperactivity disorder (ADHD). 80% of a single oral dose of methylphenidate is excreted in urine as the primary metabolite, ritalinic acid. Methylphenidate shares similar psychotropic effects and evokes pharmacological pathways as found with amphetamines and cocaine. Recent reports estimate diversion rates of stimulant drugs > 30%. Therefore, it is important to monitor prescribed methylphenidate usage to help identify abuse and diversion.

**Objective:** The objective of this study was to develop a transformed and normalized mathematic model of patient ritalinic acid values to provide a Gaussian distribution that can assist in determining whether patients are consistent with the concentration range of a large patient population previously tested for this medication.

**Methods:** Data was acquired from patients who were prescribed oral methylphenidate (Ritalin, Focalin, Methylphenidate tablets, etc.) and submitted urine samples for ritalinic acid LC/MS/MS analysis. Samples were diluted 6X with internal standard (IS), ritalinic acid-D10, and enzyme buffer solution followed by 30 minute incubation at 60°C. Extracted samples were reconstituted in 50% methanol: 50% water and run on a 4.5 minute gradient using a Phenomenex Kinetex 2.6µm Phenyl-Hexyl 100Å, 50 x 4.6mm column. The analytical range for ritalinic acid was 500 ng/mL to 100,000 ng/mL. The results from 7305 patient samples were used to develop the transformed and normalized model using “R”, a language for statistical computing and graphics.

**Results:** Transforming and normalizing these historical data results in a near Gaussian distribution. A separate analysis of 636 randomly selected patients, not included in the patient population used to develop the model, was used to assess the validity and robustness of the model. This assessment found that 68.4% of patients within  $\pm 1$  standard deviation (SD), 94.3% of patients within  $\pm 2$  SD, and 5.7% of patients out of  $\pm 2$  SD, where a true Gaussian distribution has 68.2% of data within  $\pm 1$  SD, 95.4% of data within  $\pm 2$  SD, and 4.6% out of  $\pm 2$  SD.

**Conclusion/Discussions:** In the present study, using about 2 years of urine ritalinic acid data generated by LC/MSMS, we have developed a mathematic model that provides a historical distribution of expected patient ritalinic acid values from patients prescribed methylphenidate. The resulting near Gaussian historical distribution model provides a tool for the physician to quickly assess whether their patient is consistent with this historical population of patients prescribed the drug and who tested positive for ritalinic acid. While this comparison alone would not give a definitive judgement for adherence with a treatment, combined with patient interviews, patient history, and other clinical criteria, it adds another piece of information in the medical professionals' arsenal against abuse, misuse, and diversion of methylphenidate.

**Keywords:** Ritalinic Acid, LC/MSMS, Mathematic Model



## Evaluation of Liquid Chromatography Time-of-Flight Mass Spectrometry (LC-TOF-MS) as a Replacement Screening Tool in a Full-Service State Forensic Toxicology Laboratory

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**Background/Introduction:** The Washington State Patrol Toxicology Laboratory provides full-service testing for ~15,000 deaths and driving under the influence of drugs (DUID) case submissions annually. The Laboratory frequently performs between two to four separate drug screens to aid in identifying which drugs are present in each case submission. These methods include an immunoassay screen, a basic drug screen, an acidic-neutral drug screen, and separate screens for benzodiazepines and other prescription medications not detected using the preceding general screens.

**Objective:** The objective of this research was to develop and validate a broad ranging screening method using liquid chromatography time-of-flight-mass spectrometry (LC-TOF-MS) and to compare and evaluate the efficiency, accuracy, sensitivity and breadth of the new LC-TOF-MS screening method with the Laboratory's current screening approach.

**Methods:** Blood samples (0.2 mL) are precipitated with a measured amount of acetonitrile containing the internal standards methamphetamine-d14, diazepam-d5, morphine-d6, and hexobarbital. Extracts were analyzed using an Agilent Technologies 1200 Series LC System with an Agilent 6230 TOF-MS. The LC is equipped with 5 mM ammonium formate in water (Solvent A) and methanol as the organic mobile phase (Solvent B). The MassHunter software uses an algorithm to determine a score of the compound based on retention time, accuracy, isotope abundance, and spacing. An in-house database was created using certified reference materials.

**Results/Discussion:** A drug screening procedure was developed for the detection of over 100 compounds in biological samples by LC-TOF-MS by blood precipitation in positive and negative modes. The experimentally determined limit of detection (LOD) of the compounds was determined at 5ng/mL for benzodiazepines, opiates, amines and other drugs of abuse and basic drugs in positive mode. However, the LOD evaluated in method validation was set at 10 ng/mL, a level comparable to the lower limit of quantitation for the laboratory's confirmation test methods. In negative mode, the LOD was determined at 0.2mg/L for barbiturates, valproic acid, gabapentin, and antiepileptic medication. Results for within-run and between-run precision and accuracy at the LOD will be presented as will the analysis of carryover, matrix selectivity (including liver homogenate, urine and serum), sample storage stability, and an assessment of ion suppression/enhancement. Following preliminary case comparisons, the developed LC-TOF-MS method was able to detect several relevant compounds that were not detected using the current immunoassay and basic drug screen using gas chromatography-mass spectrometry, including buprenorphine, gabapentin, and 6-acetylmorphine.

**Conclusion/Discussions:** A broad screening method utilizing LC-TOF-MS was developed. However, it was determined that a separate method is needed for the detection of 11-nor-9-carboxy- $\Delta^9$ -tetrahydrocannabinol (THCA) and  $\Delta^9$ -tetrahydrocannabinol (THC). These compounds will be screened using an LC-MS-MS after a blood precipitation with acetonitrile for sample preparation. THC cannot be detected with the TOF and the LOD for the metabolite is 250ng/mL. It was also determined that the mobile phase is stable for a maximum of 7 days. After 7 days, certain compounds such as the opiates will have a shift in retention times.

This research was funded through a NIJ grant (NIJ-2014-3732).

**Keywords:** LC-TOF-MS, Drug Screen, Biological Samples

## Development Of Homogeneous Enzyme Immunoassay For The Detection Of Bath Salts I ( $\alpha$ -pyrrolidinophenones) in Human Urine

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**Background/Introduction:** Cathinone, a  $\beta$ -keto phenylethylamine, is a monoamine alkaloid found in the *shrub Catha Edulis* (khat). The psychostimulatory effects of cathinone are considered to be the same as amphetamine. Synthetic variants of cathinone, known as “bath salts”, can be much stronger than the natural product. Synthetic cathinones are potent central nervous system (CNS) stimulants known to inhibit the reuptake of dopamine, norepinephrine and serotonin. Some of the typical effects include tachycardia, increase in blood pressure, insomnia, anorexia, general malaise, irritability and migraine.  $\alpha$ -pyrrolidinophenones are the second-generation synthetic cathinones with substituted pyrrolidino ring. The abuse of  $\alpha$ -pyrrolidinophenone drugs (MDPV,  $\alpha$ -PVP,  $\alpha$ -PHP, TH-PVP, 4-Methoxy PV8,  $\alpha$ -PHP, PV9, etc) has increased worldwide recently and currently there is no automated immunoassay available to detect  $\alpha$ -pyrrolidinophenone drugs, and there exists a need to develop a new homogeneous enzyme immunoassay.

**Objective:** To develop a new homogeneous enzyme immunoassay that can detect bath salts I, ( $\alpha$ -pyrrolidinophenones) in urine using DRI® Technology.

**Methods:** The assay is based on competition between drug labeled with glucose-6-phosphate dehydrogenase (G6PDH) and free drug in the urine sample for a fixed amount of antibody binding sites. In the absence of free drug from the sample, the specific antibody binds the enzyme labeled drug causing a decrease in enzyme activity. This phenomenon creates a direct relationship between the drug concentration in urine and enzyme activity. The enzyme activity is determined spectrophotometrically at 340 nm by measuring its ability to convert NAD to NADH. The DRI Bath Salts-I Assay uses a 100 ng/mL cutoff. The reagents and calibrators are liquid ready-to-use, with calibrators ranging from 0 ng/mL to 1000 ng/mL. The performance of the assay was evaluated on the Beckman Coulter AU680 analyzer. Patient samples were analyzed by LC-MS/MS to detect the following compounds:  $\alpha$ -pyrrolidinophenone drugs including  $\alpha$ -PVP, MDPV, MDPPP, MPHP,  $\alpha$ -PBP,  $\alpha$ -PVT, Naphyrone, and other synthetic cathinones including Mephedrone, Flephedrone, Methcathinone, Methedrone, Pentedrone, Pentylone, Methylone, Butylone, Bazedrone, 3,4-Dimethylcathinone, 4-Ethylmethcathinone, 4-Methylethcathinone. Precision, method comparison, cross-reactivity and interference studies were performed to determine the overall performance of the assay.

**Results:** The assay uses a 100 ng/mL  $\alpha$ -PVP as a cut-off calibrator with controls at  $\pm$  50% of the cut-off, and the assay shows high cross-reactivity to bath salts I ( $\alpha$ -pyrrolidinophenone drugs): 168% cross-reactivity to Methylenedioxypropylone (MDPV), 148% cross-reactivity to TH-PVP, 147% cross-reactivity to 4-methoxy PV8, 120% cross-reactivity to  $\alpha$ -PHP, 100% cross-reactivity to  $\alpha$ -PVP, 67% cross-reactivity to PV9, and 53% cross-reactivity to Methylenedioxy-alpha-pyrrolidinobutyrophenone (MDPBP). The assay has minimal cross-reactivity to opiates, and has no significant cross-reactivity to structurally unrelated and concomitantly used compounds. The assay demonstrated excellent precision in both qualitative (<1.0 % CV) and semi-quantitative (<10.0 % CV) modes. Method comparison of 647 authentic urine samples showed > 90% agreement between immunoassay and LC-MS/MS.

**Conclusion/Discussion:** The preliminary data on the DRI Bath Salts-I assay indicates excellent specificity and sensitivity to  $\alpha$ -Pyrrolidinophenone drugs, without any significant cross-reactivity to other commonly abused compounds.

**NOTE:** The assay is currently in development and is not approved by FDA.

**Keywords:**  $\alpha$ -pyrrolidinophenone, Synthetic Cathinones, Immunoassay (DRI)

## Development of a New Homogeneous Enzyme Immunoassay for the Detection of Zolpidem and its Metabolite Zolpidem-Phenyl-4-COOH in Human Urine

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**Background/Introduction:** Zolpidem, sold under trade names such as Ambien and Stilnoct, is a Schedule IV drug under the Controlled Substances Act in the U.S. and is used to treat sleep disorders. It is an ideal insomnia drug because it has a quick onset with minimal residual daytime effects. It is a short-acting non-benzodiazepine compound of the imidazopyridine class that increases the activity of GABA, an inhibitory neurotransmitter, by binding to GABA<sub>A</sub> receptors at the same location as benzodiazepines<sup>1</sup>. Zolpidem is metabolized rapidly into zolpidem phenyl-4-COOH and zolpidem 6-COOH, with only 1% of parent drug excreted in the urine. Zolpidem phenyl-4-COOH accounts for > 50% of all metabolites excreted in the urine, while zolpidem 6-COOH comprises 11% of all metabolites. Most commercially available immunoassays detect only zolpidem, and have low cross-reactivity to its metabolites, which reduces the window of detection.

**Objective:** The objective of this study was to develop a new homogeneous enzyme immunoassay that can detect zolpidem and its metabolite(s) in urine using the CEDIA® Technology.

**Methods:** CEDIA Technology is based on the bacterial enzyme  $\beta$ -Galactosidase which has been genetically engineered into two inactive fragments, Enzyme Acceptor (EA) and Enzyme Donor (ED). These fragments spontaneously re-associate to form an active enzyme. In the absence of analyte from the sample, the specific antibody binds the ED-drug conjugate causing a decrease in enzyme activity. The free drug in the sample will compete for the limited number of antibody binding sites, making the ED-drug conjugate available for complementation to form an active enzyme. This phenomenon creates a direct relationship between the drug concentration in urine and enzyme activity. The enzyme activity is then determined spectrophotometrically at 570 nm. The performance of the assay was evaluated on the Beckman Coulter AU680 analyzer. The zolpidem assay uses a 20 ng/mL cutoff calibrator with controls at  $\pm$  50% of the cutoff. The reagents are lyophilized and the calibrators and controls are liquid ready-to-use. Urine samples were obtained from pain management laboratories. The samples were analyzed by LC-MS/MS to determine the levels of zolpidem (LOD: 0.1 ng/mL, LOQ: 0.2 ng/mL), zolpidem Phenyl-4-COOH (LOD: 1 ng/mL, LOQ: 1.7 ng/mL) and zolpidem 6-COOH (LOD: 0.5 ng/mL, LOQ: 1.5 ng/mL). Method comparison and cross-reactivity studies were performed to determine the overall performance of the assay.

**Results:** Using a 20 ng/mL cut-off calibrator, the selected monoclonal antibody is specific to zolpidem, with approximately 50% cross-reactivity to its major metabolite zolpidem-phenyl-4-COOH. It does not cross-react with zolpidem 6-COOH, zaleplon or zoplicone. The antibody has no significant cross-reactivity to other imidazopyridine compounds, such as XLR-11, PB-22 pentanoic acid, JWH-018-N-hydroxypentyl metabolite, AB-PINACA, AB-FUBINACA, AB-CHIMINACA, or structurally unrelated compounds, such as opioids, tricyclic antidepressants, phenobarbital, chlorpromazine, efavirenz, diphenhydramine and synthetic cathinones. Method comparison study using 475 urine samples showed > 95% agreement between immunoassay and LC-MS/MS.

**Conclusion/Discussion:** The preliminary data on the CEDIA® Zolpidem Assay indicates excellent specificity and sensitivity to zolpidem and its major metabolite zolpidem Phenyl-4-COOH, without any significant cross-reactivity to other commonly abused drugs.

### Reference

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**NOTE: The assay is currently in development and is not approved by FDA.**

**Keywords:** Zolpidem, Metabolites, Immunoassay (CEDIA)

## Development of a Homogeneous Enzyme Immunoassay for the Detection of AB-PINACA, its Metabolites and Related Compounds in Human Urine

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**Background/Introduction:** Synthetic cannabinoids (SCs) are a class of rapidly growing designer drugs which are being used as recreational drugs. In the past few years, forensic researchers increasingly encountered SCs, AB-PINACA, MAB-CHMINACA and AB-FUBINACA, a new class of SCs which include the indazole-carboxamide (INACA) compounds. These INACA SCs are cannabimimetic *in vivo* and act as high potency agonists of the Type 1 and Type 2 Cannabinoid Receptors (CB1 and CB2). Intoxication incidences significantly increased in the past two years, indicating that these INACA SCs pose an emerging threat to the public health in the US and Europe. In responding to the need for an automated immunoassay, Thermo Fisher Scientific developed an enzyme immunoassay to detect multiple INACA SCs in human urines.

**Objective:** To develop an immunoassay for the detection of AB-PINACA parent compound and its metabolites using CEDIA® Technology.

**Methods:** The CEDIA Technology is based on the bacterial enzyme  $\beta$ -Galactosidase which has been genetically engineered into two inactive fragments, Enzyme Acceptor (EA) and Enzyme Donor (ED). These fragments spontaneously re-associate to form an active enzyme. In the absence of analyte from the sample, the specific antibody binds the ED-drug conjugate causing a decrease in enzyme activity. The free drug in the sample will compete for the limited number of antibody binding sites, making the ED-drug conjugate available for complementation to form an active enzyme. This phenomenon creates a direct relationship between the drug concentration in urine and enzyme activity. The enzyme activity is determined spectrophotometrically at 570 nm. The performance of the assay was evaluated on the Beckman Coulter AU680 analyzer. The CEDIA AB-PINACA assay uses a 20 ng/mL cutoff calibrator with controls at  $\pm 50\%$  of the cutoff. The assay calibration range is 0 to 100 ng/mL. The reagents are lyophilized and the calibrators and controls are liquid ready-to-use. Urine samples were obtained from several reference or academic laboratories and were analyzed by LC-MS/MS for parent and metabolites of AB-PINACA and its related compounds.

**Results:** The assay demonstrates  $> 80\%$  cross-reactivity to least 9 compounds: AB-CHMINACA M1A, 5F-AB-PINACA, AB-CHMINACA M1B, AB-PINACA 5-hydroxypentyl, AB-PINACA 4-hydroxypentyl, AB-PINACA N-4-fluoropentyl, AB-PINACA pentanoic acid, AB-FUBINACA, 5F-AB-PINACA-N-4-hydroxypentyl. The assay can also detect 24 other synthetic cannabinoids and/or their metabolites. Method comparison study using 90 human urine samples showed  $> 80\%$  agreement between immunoassay and LC-MS/MS.

**Conclusion/Discussion:** The CEDIA AB-PINACA immunoassay demonstrates excellent precision and specificity to several AB-PINACA compounds without significant cross-reactivity to other structurally unrelated compounds and other concomitantly used drugs.

**NOTE:** The Assay is for Criminal Justice and Forensic Use only.

**Keywords:** AB-PINACA, Metabolites, Immunoassay (CEDIA)

**Method Validation for Simultaneous Quantification of Fentanyl, Nor-fentanyl and Acetyl Fentanyl in Blood and Identification in Urine using LC-MS/MS**

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**Background/Introduction:** entanyl is a potent synthetic opioid acting as a strong  $\mu$ -receptor agonist with a potency 100 times that of morphine. As a result of its availability as a prescribed drug and its potency, it has been abused for decades. Additionally, clandestine laboratories have begun manufacturing fentanyl and its analog, acetyl fentanyl, leading to greater availability and misuse, often leading to overdose. A low therapeutic range makes it difficult to detect using conventional GC/MS methods, so a method was developed and validated using liquid chromatography tandem mass spectrometry (LC-MS/MS) to improve sensitivity.

**Objective:** The LC-MS/MS method will allow for the qualitative identification and quantitation of acetyl fentanyl, fentanyl, and fentanyl metabolite nor-fentanyl. Qualitative analysis is performed in urine. The drugs are extracted using CEREX Clin II solid phase extraction (SPE) columns and subsequently analyzed using LC-MS/MS dynamic multiple reaction monitoring. Isotopically labelled internal standards are used in the quantitative analysis of fentanyl, nor-fentanyl and acetyl fentanyl. The SWGTOX guidelines were followed for validations.

**Method:** Isotopically labelled internal standards were added to samples followed by 5 mL of pH 4.5 sodium acetate buffer. Samples were sonicated, centrifuged and applied to Cerex Clin II SPE columns preconditioned with ethyl acetate and methanol. The columns were washed with potassium carbonate buffer pH 9 and deionized water, before elution with ethyl acetate and ammonium hydroxide at 98:2 ratio. Samples were reconstituted with mobile phase (90% water/0.1% formic acid, 10% LC/MS grade methanol) and transferred to auto sampler vials before injecting onto an Agilent 1200 series LC coupled to an Agilent 6410 Triple Quadrupole Mass Spectrometer. HPLC separation was attained by using water with 0.1% formic acid and a gradient of methanol over an Agilent Zorbax C18 HPLC column (1.8  $\mu$ M X 2.1 x 50 mm) at 50° C and analyzed using dynamic multiple reaction monitoring. Validation was performed using SWGTOX guidelines for calibration model fits, accuracy and precision, sensitivity measured by the limit of detection (LOD) and limit of quantitation (LOQ), recovery and efficiency, carryover, interference, ion suppression/enhancement, dilution integrity and stability.

**Results:** All analytes were determined to fit to a linear 1/x weighted curve with an  $R^2 \geq 0.999$  with an LOQ of 1 ng/mL and an upper limit of quantitation of 100 ng/mL. The LOD of Fentanyl, Nor-Fentanyl and Acetyl Fentanyl in blood is 0.12ng/ml, 0.41ng/ml and 0.25 ng/ml, respectively. The LOD for urine is 1ng/ml.

Percent accuracy at three concentrations ranged from 0.44-4.35%, between run bias %CV of 1.52-3.18% and within run bias %CV of 2.67-5.65%. All of the numbers are within acceptable CV values of  $\pm 20$  %. No significant carryover, interference from matrix effects or drugs of abuse, or ion suppression/enhancement effects were noted. Furthermore, samples proved stable over 5 days and were successfully quantitated at dilutions of 1:2, 1:4 and 1:10.

**Conclusion/Discussions:** A method for the quantitation of fentanyl, nor-fentanyl and acetyl fentanyl in postmortem and ante mortem blood, and confirmation in urine was successfully developed and validated using SPE and LC/MS-MS. The sensitivity of this method allowed us to successfully confirm and quantitate fentanyl, nor-fentanyl and acetyl fentanyl that were routinely not seen by GC/MS in our laboratory. Additionally, this method was developed using a methanol mobile phase instead of acetonitrile which has a large cost advantage.

**Keywords:** Fentanyl, Analogues, LC/MS/MS

## The Optimization of Extraction and Derivatization for Quantitative Analysis of Common and New Amphetamine- and Cathinone-Derivatives in Serum and Urine Using GC/MS

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**Background/Introduction:** Amphetamine, methamphetamine, MDMA and certain cathinones (e.g. N-ethyl-hexedrone, 4-chloro-meth-cathinone, 4-methyl-N-ethyl-norpentedrone, 4-chloro-ethylcathinone and N-ethyl-pentylone) are popular drugs in Hungary. These compounds are controlled partly due to generic list of new psychoactive substances. We need to input new target compounds and to reoptimize our method to be able to give up-to-date results in the growing number of forensic cases.

**Objective:** Our goal was to reoptimize our previous method for the analysis of amphetamine and cathinone type designer drugs in serum and urine. This comprises the optimizing of the conditions of extraction and derivatization. The second aim of this study was to validate the GC-MS method for serum determining selectivity for all analytes, and linearity and accuracy for main amphetamines, and furthermore to find an easy way to adjust the retention times for all analytes.

**Methods:** The sample preparation was done by addition of 200 ml buffer solutions to 1.3 ml sample, followed by liquid-liquid extraction with 250 ml toluene. The derivatization with N-methyl-bis-trifluoroacetamide (MBTFA) was performed in two steps: parallel with the extraction (extractive acylation) and after phase separation (off line acylation). We used D5-analogues of 5 amphetamines as internal standards. The extracts were analyzed using a Shimadzu GC/MS-QP2010 Ultra GC-MS. Capillary column: HP-5MS (length: 25 m, diameter: 0.2 mm, film: 0.33 mm), splitless time: 0.5 min. SIM was scheduled in 11 ion sets.

**Results:** We used urine samples for the method optimization. The optimal buffer composition for the parallel determination of primary, secondary and tertiary amines was 3:5 (v/v) ratio of cc.  $\text{NaHCO}_3$  and 3 M KOH (pH ~9.5). The optimal parameters for derivatization are as follow: addition of 30 ml MBTFA (extractive acylation) and then 30 ml MBTFA to the separated organic phase for off line derivatization (80 °C, 30 min). With this method we are able to monitor 195 target compounds (amphetamines, synthetic cathinones and other basic drugs) with one measurement. The results of selectivity were appropriate except for some positional isomers (e.g. 3/4-fluoromethamphetamine, 3/4-fluoromethcathinone, 3/4-MMC). There were no co-eluting matrix compounds at the retention time of amphetamine (AM), methamphetamine (MA), MDA, MDMA and MDEA. Calibration samples were spiked with this 5 compounds at the following concentrations: 10, 20, 40, 80, 120, 160, and 200 ng/ml. The  $r^2$  of the linearity was  $>0.997$  for these analytes. The within assay precision (RSD) at the concentration of 20, 80 and 120 ng/ml lies between 5.0–7.2 %, 3.8–7.9 % and 2.4–4.2 %, respectively. Bias values at given concentrations are 94–108 %, 98–105 % and 95–101 %, respectively. Concerning sensitivity, the minimal S/N values (RMS) for serum samples spiked at 10 ng/ml found to be 144, 49, 124, 33 and 62 for AM, MA, MDA, MDMA and MDEA. For the identification, Kovats-type retention indices are registered by use of Retention index standard for GC (Sigma), containing normal alkanes of C8-C32. If the capillary column is degraded or the GC method is slightly modified, retention time values can be predicted and adjusted easily by use of the registered retention indices of analytes and the Automatic Adjustment of Retention Time Function (AART) of the GCMSSolution software with an accuracy of  $<0.4\%$ . Reinjection of analytes can be omitted by this function during method update.

**Conclusion/Discussions:** We are presenting a simple and selective GC-MS method for the identification of potentially more than 200 amphetamine and cathinone type designer drugs in serum and urine after liquid-liquid extraction and two-step acylation. The assay ensures complete derivatization of the hindered amino groups and the quantitative determination of analytes with satisfying validation parameters.

**Keywords:** GC-MS, Amphetamine-Derivatives, Cathinone-Derivatives, MBTFA

**Development and Validation of a New and Highly Sensitive rFab-based SEFRIA™ Immunoassay for the Detection of Phencyclidine in Oral Fluid**

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**Background/Introduction:** Phencyclidine (PCP) is an illegal hallucinogenic street drug that was formerly developed as an intravenous anesthetic agent in 1957 and was discontinued due to its side effect that resulted in lethargy, disorientation, hallucinations and loss of coordination. The hallucination and “out of body” experience led to its abuse. As a drug of abuse, it can be administered by nasal insufflation or intravenous injection, but it is typically sprayed on plant material and smoked. Oral Fluid is a great viable matrix option for testing drugs of abuse and offers many advantages over other biological matrices (like urine). One of the main benefits is the noninvasiveness and simplicity of sample collection. Therefore it can be performed almost anywhere under direct supervision with a minimum risk of alteration. SEFRIA™ is a competitive homogenous enzyme immunoassay and an optimal platform for immunoassays that require a low detection limit. This big advantage over traditional G6PDH –based homogeneous enzyme assays (HEIA) stems from its unique methodology, which employs the re-association of two inactive enzyme fragments to form an active enzyme (tetramer). One of the enzyme fragments is attached to the analyte of interest and competes with the analyte in the sample for the antibody binding site. The enzyme activity is directly proportional to the drug concentration in the testing specimens.

**Objective:** The objective of this project was to develop and validate a new highly sensitive SEFRIA™ immunoassay for the rapid detection of PCP in human saliva.

**Methods:** An anti-PCP recombinant fragment antibody (rFab)-based SEFRIA™ immunoassay was developed. The assay was designed to detect PCP in oral fluid.

**Results:** The immunoassay is a semi-quantitative and qualitative method with a semi-quantitative reportable range of 5 to 40ng/mL with control levels at ±25% of cutoff. The cutoff of the assay will be 10ng/mL. The qualitative precision of the assay is less than 2% CV. The SEFRIA™ immunoassay was validated with a total of 71 oral fluid samples collected in-house. Some of these samples were spiked with varying concentrations of PCP below and above the cutoff value.

		Confirmation (10ng/mL)	
		N	P
SEFRIA™ (10ng/mL)	N	44	0
	P	0	27

The sensitivity, specificity and accuracy of the assay were found to be 100%, 100% and 100%, respectively.

**Conclusion/Discussions:** An anti-PCP recombinant fragment antibody (rFab) has been successfully developed in house with a very high specificity and it also has been demonstrated to be suitable for SEFRIA platform to achieve 10ng/mL as a cutoff. We believe it is the first report regarding a rFab-based PCP immunoassay.

**Keywords:** Enzyme Immunoassay, Phencyclidine, Oral Fluid

## Development of a Homogeneous Enzyme Immunoassay for the Detection of UR-144/XLR-11 and Structurally Related Synthetic Cannabinoids in Human Urine

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**Background/Introduction:** Synthetic cannabinoids are a class of synthetic compounds chemically similar to  $\Delta^9$ -tetrahydrocannabinol (THC), the psychoactive ingredient in marijuana. Synthetic cannabinoids interact with the same receptors as THC, causing similar physiological effects, but often with higher potency and higher health risks to the users. Synthetic cannabinoids are typically sprayed on a mixture of shredded plant material and marketed as incense that can be smoked. Short term effects of synthetic cannabinoids include loss of control, lack of pain response, increased agitation, pale skin, seizures, vomiting, profuse sweating, uncontrolled / spastic body movements, elevated blood pressure, heart rate and palpitations. In addition to physical signs of use, users may experience dysphoria, severe paranoia, delusions, hallucinations and increased agitation. Many synthetic cannabinoids, including UR-144 and XLR-11, have been placed into Schedule I of the Controlled Substance Act CSA. Thermo Fisher Scientific developed a CEDIA® UR-144/XLR-11 assay to meet the urgent need for high throughput screening of these compounds.

**Objective:** To develop an immunoassay for the detection of synthetic cannabinoids structurally related to UR-144 and their metabolites in human urine.

**Methods:** CEDIA® technology is based on the bacterial enzyme b-Galactosidase which has been genetically engineered into two inactive fragments, Enzyme Acceptor (EA) and Enzyme Donor (ED). These fragments spontaneously re-associate to form an active enzyme. In the absence of analyte from the sample, the specific antibody binds the ED-drug conjugate causing a decrease in enzyme activity. The free drug in the sample will compete for the limited number of antibody binding sites, making the ED-drug conjugate available for complementation to form an active enzyme. This phenomenon creates a direct relationship between the drug concentration in urine and enzyme activity. The enzyme activity is then determined spectrophotometrically at 570 nm. The performance of the assay was evaluated on the Beckman Coulter AU680 analyzer.

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The CEDIA® UR-144/XLR-11 assay uses a 10 ng/mL cutoff calibrator with controls at  $\pm 50\%$  of the cutoff. Calibration range of the assay is 0 to 60 ng/mL. The reagents are lyophilized and the calibrators and controls are liquid ready-to-use. Patient samples were obtained from reference laboratories. The samples were analyzed by LC-MS/MS to determine the levels of UR-144 major metabolites, UR-144 N-pentanoic acid and UR-144 N-5-hydroxypentyl. Method comparison and cross-reactivity studies were performed to determine the overall performance of the assay.

**Results:** The selected monoclonal antibody detects UR-144, its major metabolites and structurally related derivatives (including XLR-11, XLR-12, and other analogs). The assay exhibits low cross-reactivity with a number of other synthetic cannabinoids which may be detected at higher concentrations. The antibody has no significant cross-reactivity to opioids and other un-related compounds tested. Method comparison study using 83 urine samples showed  $> 95\%$  agreement between immunoassay and LC-MS/MS.

**Conclusions/Discussion:** The CEDIA® UR-144/XLR-11 Assay demonstrates reliable detection of UR-144-related synthetic cannabinoids, without significant cross-reactivity to other commonly abused or prescribed drugs.

**NOTE:** The assay is for Criminal Justice and Forensics use only.

**Keywords:** Synthetic Cannabinoid, UR-144, XLR-11, Metabolites, Immunoassay (CEDIA)



## Development of a Highly Sensitive Oral Fluid ELISA for PINACA and Related Synthetic Cannabinoids

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**Background/Introduction:** We have previously presented results for a sensitive ELISA for AB-PINACA/ADB-PINACA and their metabolites, as well as structurally similar synthetic cannabinoids in human urine. These compounds mainly consist of a core indazole or indole carboxamide ring structure, with a pentyl side chain. Several of these compounds are also DEA schedule I substances. Saliva testing for drugs of abuse offers certain advantages over urinalysis, such as ease of specimen collection and difficulty of adulteration, but has not been utilized to its full potential, due to lack of sensitivity of detection methods. In this study, oral fluid specimens were collected using the Quantisal™ collection device, that has a volume indicator showing when 1 mL of oral fluid sample is collected, which is then diluted with 3 mL of a stabilization/transportation buffer.

**Objective:** To use oral fluid as an alternate screening matrix for the parent synthetic cannabinoids, AB-PINACA, ADB-PINACA, 5F-AB-PINACA, 5F-ADB-PINACA and other analogous designer drugs, using a highly sensitive ELISA.

**Method:** A broad-cross reactivity polyclonal antibody to AB-PINACA and structurally similar compounds was developed and immobilized on a microtiter plate. AB-PINACA calibrators (25 µL) at neat concentrations of 0.1, 0.25, 0.5 and 1 ng/mL that were pre-diluted 1:4 in synthetic negative oral fluid and oral fluid specimens (25µL) collected with the Quantisal™ device, were pipetted on a microtiter plate and allowed to pre-incubate for 30 minutes. This was then followed by addition of PINACA-HRP enzyme conjugate (100µL) and then incubated for 60 minutes at ambient temperature. The plate was washed 6 times with DI water and then incubated with enzyme substrate for 30 minutes at ambient temperature. The reaction was stopped with 1N hydrochloric acid and read at dual wavelengths of 450 and 650 nm, using a Tecan microplate reader.

**Result:** The ELISA is capable of a detection cutoff of 0.25 ng/mL neat oral fluid. The assay shows a high cross-reactivity for AB-PINACA (100%), ADB-PINACA (120%), 5F-AB-PINACA/5F-ADB-PINACA (200%). It further cross-reacts with AB-FUBINACA (80%), AB-CHMINACA (60%), MAB-CHMINACA (80%), and 5F-ADBICA (50%). The assay was validated with 52 oral fluid specimens, some of which were obtained from a clinical laboratory.

ELISA		LC-MS/MS	
		+	-
	+	10	0
	-	1	41

10 specimens were true positives by ELISA and LC-MS/MS, containing AB-FUBINACA, ranging from 8-27 ng/mL, while 41 specimens were true negatives. However, one specimen containing 17.6 ng/mL of AB-FUBINACA could only be read as borderline positive by ELISA, possibly due to some degradation over time. The assay showed intra and inter-day imprecision less than 10% CV, with a detection limit (LOD) of 0.1 ng/mL neat oral fluid.

**Conclusion/Discussion:** A highly sensitive ELISA with a cutoff of 0.25 ng/mL has been developed, to allow oral fluid to be used as an alternate screening matrix to urine, for detection of PINACA compounds. Using this assay, a broad range of indazole/indole carboxamide synthetic cannabinoids can be detected.

**Keywords:** AB-PINACA, Synthetic Cannabinoids, Oral Fluid

**Development and Validation of a Polyclonal Antibody for Detection of the Crack Cocaine Biomarker AEME in Oral Fluid**

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**Background/Introduction:** Cocaine is a stimulant that is used recreationally, but presents a severe addiction problem worldwide. The effect of its use is the release of dopamine in the brain, leading to a feeling of euphoria. Typically to achieve a high, cocaine hydrochloride powder is snorted and hence the parent drug is detectable in oral fluid, which then undergoes hydrolysis in the liver to its main metabolite benzoylecgonine, excreted in the urine. Crack cocaine is a purer crystallized form of cocaine free base, which when smoked, due to absorption directly into the bloodstream, reaches the brain in a few seconds, thereby making it the most addictive form of cocaine. Due to the pyrolysis of crack cocaine at around 90°C, it is rapidly converted to anhydroecgonine methyl ester (AEME) or methylecgonidine. AEME has been found to be more toxic to the body organs like the heart and lungs, than other by-products of cocaine. It gets reabsorbed in oral fluid, thereby making it an appropriate and direct biomarker for crack cocaine use. Saliva testing for drugs of abuse also presents certain advantages over urinalysis, such as ease of specimen collection and difficulty of adulteration.

**Objective:** To develop a sensitive and specific antibody for the detection of AEME in oral fluid, as a biomarker for crack cocaine use.

**Method:** An AEME specific polyclonal antibody was developed by immunization of sheep with a suitable AEME antigen. The purified immunoglobulin G fraction was immobilized on an ELISA microtiter plate. AEME calibrators (25 µL) at concentrations of 1, 2.5, 5, 7.5, 10, 25 and 50 ng/mL in synthetic negative oral fluid were pipetted on the microtiter plate, followed by addition of AEME-HRP enzyme conjugate (100µL) and allowed to incubate for 60 minutes at ambient temperature. The plate was washed 6 times with DI water and then incubated with enzyme substrate for 30 minutes at ambient temperature. The reaction was stopped with 1N hydrochloric acid and read at dual wavelengths of 450 and 650 nm, using a Tecan microplate reader.

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**Result:** The newly developed antibody is capable of an AEME limit of detection (LOD) of 1 ng/mL. It does not show any specificity for anhydroecgonine, cocaine or benzoylecgonine up to 100,000 ng/mL, as well as other structurally non-similar compounds up to 100,000 ng/mL. The antibody was challenged with 70 oral fluid specimens previously confirmed by GC-MS for AEME, with concentrations ranging from about 5-150 ng/mL, all of which were found to be positive by the ELISA screen. The assay was also challenged with 30 oral fluid specimens collected from cocaine free individuals and all were found to be negative by ELISA.

**Conclusion/Discussion:** A new and highly specific antibody has been developed for the detection of AEME in oral fluid, as a convenient and direct biomarker for crack cocaine use. The antibody is very sensitive and capable of a limit of detection of 1 ng/mL AEME.

**Keywords:** AEME, Crack Cocaine, Oral Fluid

## Drugs of Abuse Extraction from Whole Blood using Supported Liquid Extraction (SLE) and Extrahera Automation Prior to UPLC-MS/MS Analysis.

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**Background/Introduction:** Whole blood continues to be a valuable tool in forensic toxicology for the immediate and near-term detection of illicit drugs, and in cases where no other sample is available. Screening drugs of abuse can be complicated due to the wide variation of functional groups associated with different analyte classes. Most extraction techniques cannot extract all analytes using a single procedure without using non-optimal extraction protocols resulting in compromised extract cleanliness. Supported liquid extraction allows for the simultaneous analysis of cross functional analytes in a single extraction protocol without forfeiting extract cleanliness. This poster discusses the potential for a single extraction protocol for various drugs of abuse classes prior to UPLC-MS/MS analysis.

**Objective:** The objective was to develop an automatable, common extraction procedure for multiple drug panels from whole blood matrix using supported liquid extraction (SLE) prior to UPLC-MS/MS analysis. The drug suites included amphetamines, benzodiazepines, cocaine, opiates, ketamine, buprenorphine, Z-drugs and carboxy-THC.

**Methods:** Blank whole blood was spiked with drugs from various panels at 13 ng/mL for the analytes and appropriate deuterated internal standards. Whole blood extraction was performed using ISOLUTE SLE+ 1 mL capacity columns evaluating pH control and optimum extraction solvent combinations. The resultant extracts were evaporated to dryness and reconstituted in mobile phase for subsequent UPLC-MS/MS analysis. Samples were analyzed using a Waters ACQUITY UPLC coupled to a Quattro Premier XE triple quadrupole mass spectrometer. Positive ions were acquired using electrospray ionisation in the MRM (multiple reaction monitoring) mode.

**Results:** Initial investigations focused on sample treatment. Extraction conditions were evaluated using spiked whole blood pre-treated 1:1 (v/v) with various ammonium hydroxide (aq) concentrations from 0.1% up to 2%. 0.1% NH<sub>4</sub>OH (aq) was selected as the pre-treatment as many of the drugs are basic in nature, however an environment at this pH value also allowed concomitant extraction of THC-COOH.

Loading 1 mL of pre-treated whole blood led to the extraction of matrix components in the form of red blood cells. Evaluation of various loading volumes from 500 to 900 µL resulted in selection of 750 µL (1:1, v:v) as the optimal loading volume for extract cleanliness. DCM, DCM/IPA and MTBE combinations with various modifiers were evaluated for extraction efficiency. BZE could only be extracted efficiently when using DCM as the extraction solvent. The optimum extraction protocol provided recoveries greater than 70% for nearly all drugs spiked into whole blood. BZE and Buprenorphine recovered 47% and 52% respectively. RSD values for all drugs were under 10%. Lower recoveries for BZE and Buprenorphine did not prevent quantitation at sub-ng/mL concentrations. Evaporative effects were eliminated using 0.05 M HCl in methanol prior to evaporation.

Calibration curves constructed from 1-500 ng/mL demonstrated excellent linearity and coefficients of determination,  $r^2 > 0.99$  for all analytes. LLOQs were determined to be sub-ng/mL for each analyte when extracting 375 µL of whole blood, with the exception of THC-COOH (5 ng/mL).

MRM analysis for common phospholipid molecules demonstrated the optimum method yields extremely clean extracts. This method was transferred to the Extrahera sample automation platform for repeat investigations into recovery, reproducibility and linearity and in nearly all analytes, the automation approach improves upon the manual approach for acquired data while also saving operator time.

**Conclusion/Discussions:** This poster describes the suitability of ISOLUTE SLE+ for the rapid and reliable extraction of multiple analyte panels from whole blood in a single, fully-automatable assay on the Extrahera platform, prior to UPLC-MS/MS analysis.

**Keywords:** SLE (Supported Liquid Extraction), Whole Blood, Drugs of Abuse

## Validation of Mitragynine Immunoassay on Tecan Freedom Evo 75 with Randox ELISA Kit

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**Background/Introduction:** Mitragynine is the main active ingredient found in the *Mitragyna speciosa*, medicinal plants native to South East Asia. The leaves are often referred to as kratom. Mitragynine acts on the  $\mu$  and  $\delta$  opioid receptors and at high doses can produce opioid-like analgesic effects. At low doses, mitragynine can produce stimulant-like effects. There has been an increase of cases reporting Mitragynine, indicating there is a need to develop a robust screening method. The Tecan Evo 75 using the Randox ELISA kit described herein provides a sensitive and quick immunoassay screening option in whole blood for both human performance (DUI) and postmortem applications.

**Objective:** To validate the Randox Mitragynine ELISA kit in 96-well format in whole blood using a semi-automated Tecan Freedom Evo 75 instrument.

**Methods:** 96-well plates were coated with Mitragynine antibody by Randox. Whole blood samples were diluted 1:4 with diluent (150  $\mu$ L sample + 450  $\mu$ L diluent). 50  $\mu$ L of diluted sample was added to each well followed by 75  $\mu$ L of horseradish peroxidase labeled antigen (conjugate) to allow for competitive binding. Incubation time with the antibody, enzyme substrate (TMB), and stop solution (HCl) were 30, 20, and 5 minutes, respectively. These times were monitored carefully to enhance reproducibility. Optical density was measured at 450 nm by UV/VIS spectrophotometry. The cutoff for mitragynine was set to 2 ng/mL. Precision at 50% below the decision point (low – 1 ng/mL), at the decision point (cutoff – 2 ng/mL), 50% above the decision point (1.5X – 3 ng/mL), and 100% above the decision point (high – 4 ng/mL) was monitored in triplicate over 5 days for mitragynine. 26 previously analyzed samples were evaluated to determine false positive/negative rates and to assess the assay's ability to reliably detect compounds at concentrations commonly observed in routine casework. Interference was evaluated in ante- and post-mortem blood specimens. As a component of their in-house validation, Randox studied the following parameters: intra-assay precision, limit of detection, interference, and cross-reactivity. Demographics, autopsy findings, and other commonalities were evaluated in 24 mitragynine positive cases between 2014-2017.

**Results:** Intra-day precision (CVs) was 1.1% – 17.9 % for mitragynine. Between-day precision is shown below. All concentration points had CVs less than 20%.

Target	Between-day Precision (CV%)			
	Low (1 ng/mL)	Cutoff (2 ng/mL)	1.5X (3 ng/mL)	High (4 ng/mL)
Mitragynine	14.9%	14.2%	11.3%	14.1%

There were no false negatives or false positives in the twenty-six previously analyzed. The cases were previously analyzed using GC/MS or LC/MS/MS. There was no interference in negative blood specimens or with commonly encountered drugs of abuse and therapeutic drugs. All subjects were Caucasian and 63% were male. The median age of males and females was 33 and 35, respectively. Both antemortem and postmortem cases were tested; however, postmortem cases accounted for 71% of the positives. Evaluation of autopsy reports revealed pulmonary edema as a common finding.

**Conclusion/Discussion:** We were one of the first forensic toxicology laboratories to validate the Randox Mitragynine ELISA assay. Sufficient precision around the cutoff and the lack of false positives/negatives indicate that the assay has the ability to presumptively identify or eliminate mitragynine. Since these data suggest this assay is a highly specific preliminary test for mitragynine, it is an additional tool for laboratories to increase their scope of analysis.

**Keywords:** ELISA, Mitragynine, Immunoassay

## Simultaneous Determination of Amphetamine Derivatives and New Psychoactive Substances in Urine by Gas Chromatography-Mass Spectrometry

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**Background/Introduction:** Despite efforts to prevent the spread of new psychoactive substances (NPS) such as synthetic amphetamine derivatives, many have appeared recently on the market. Due to high potential for their abuse, reliable analytical methods were required to detect and identify these substances in biological samples.

**Objective:** The objective of the study was to develop and validate the gas chromatography-mass spectrometry (GC-MS) method for the simultaneous determination of thirteen amphetamine derivatives and NPS (amphetamine; AP, 4-fluoroamphetamine; 4FA, methamphetamine; MA, 4-fluoromethamphetamine; 4FMA, 4-chloroamphetamine; 4CA, para-methoxyamphetamine; PMA, 4-chloromethamphetamine; 4CMA, 6-(2-aminopropyl)benzofuran; 6-APB, 4-methylenedioxyamphetamine; MDA, para-methoxymethamphetamine; PMMA, 6-(2-methylaminopropyl)benzofuran; 6-MAPB, 3,4-methylenedioxymethamphetamine; MDMA, 5,6-methylenedioxy-2-aminoindane; MDAI) in human urine, as substrate for the detection of residues of illicitly administered substances.

**Methods:** The internal standard solution (50  $\mu$ L, 0.5 ng/mL) was added to 1 mL of urine, followed by 1 mL of 0.02 M phosphate buffer (pH 7.4), and 50  $\mu$ L of concentrated ammonium hydroxide. The urine samples were extracted with 1.5 mL of ethyl acetate. The upper layers were evaporated to dryness, derivatized using trifluoroacetic anhydride (TFAA) at 70 °C for 15 min, and analyzed by GC-MS.

**Results:** The validation parameters included selectivity, linearity and limits of quantification (LOQ), intra- and inter day precision and accuracy, recovery and stability. The linear ranges were 2-100 ng/mL for AP, 4FA, 4FMA, 4CA, PMA, 6-APB, MDA, and MDAI, 10-250 ng/mL for 4CMA, PMMA, and 6-MAPB, 25-1000 ng/mL for MA and MDMA, with good correlation coefficients ( $r^2 > 0.9963$ ). The intra-day and inter-day precisions were within 11.9% and 12.5%, while the intra-day and inter-day accuracies were ranged from -10.6 to 13.0% and -11.0 to 6.8% for the nominal concentration at all studied levels, respectively. The LOQs for each analyte were 2 - 25 ng/mL. The extraction recoveries ranged from 69.3 to 96.4%. Stability was considered acceptable for all analytes in the studied matrices. The applicability of the developed method was examined by analyzing urine samples from drug abusers.

**Conclusion/Discussions:** This study describes a reliable GC-MS method for the simultaneous determination of thirteen amphetamine derivatives and NPS in human urine. The method introduces high speed centrifugation at 20000 g to produce cleaner supernatants with the reduced interference from the chemical background noise. The method has been useful to detect multiple drug use.

**Keywords:** New Psychoactive Substances, Urine Analysis, GC-MS

**Recommendations for the determination of matrix suppression in biological samples by UPLC-ESI-MS/MS: Extending Quality Measures in Forensic Toxicology**

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**Background/Introduction:** Ion suppression or enhancement of analytes due to incomplete removal of matrix components can negatively impact analytical methods. Based on the Scientific Working Group for Forensic Toxicology Standard Practices for Method Validation in Forensic Toxicology, suppression/enhancement can be evaluated using two approaches: post-column infusion or post-extraction addition. By using one of these two approaches, laboratories can assess extraction efficacy to gauge impact on crucial validation parameters. Additional options for quantitative determination of matrix interferences will be presented.

**Objective:** Development of a comprehensive strategy to determine the effect of both mass selective and non-selective endogenous interferences on the analysis of drugs of abuse extracted from biological fluids.

**Methods:** De-identified drug-free human blood and urine samples (n=12) were extracted at Boston University. Extracts were shipped to PerkinElmer (Downers Grove, IL) for analysis. The analytes morphine, BZE, THC-COOH, Fentanyl, 6MAM, and hydrocodone (Cerilliant Corporation, Round Rock, TX) were selected to capture the effect of matrix suppression on analytes that elute at different time points during the chromatographic run. Different sample preparation techniques were compared. Protein precipitation (PPT) was completed using cold acetonitrile and 6% formic acid. A solid phase extraction (SPE) method was employed with UCT DAU Clean-Screen 130mg/3mL cartridges (UCT, Inc. Bristol, PA). Samples were diluted 1:10 with phosphate buffer for a final volume of 1 mL. Before loading samples, SPE cartridges were conditioned with methanol and 100 mM phosphate buffer. Cartridges were washed with DI H<sub>2</sub>O, 0.1N HCl, and methanol. Analytes were eluted with methylene chloride: isopropyl alcohol: ammonium hydroxide (77:20:3) and dried for 5 min. A PerkinElmer (Shelton, CT) QSight™ 220 MS/MS equipped with a coaxial electrospray ionization (ESI) source operated in positive mode was used. The operational mode was MRM. The MS/MS instrument was equipped with a StayClean™ interface to manage dirty samples without instrument downtime. The UPLC was a PerkinElmer Altus A30 liquid handling system. Compound parameters were optimized at PerkinElmer (Bolton, ON). The column was a Brownlee Phenyl Hexyl (100 x 2.1 mm, 2.7 µm). Mobile Phase A was 0.1% formic acid (aq). Mobile Phase B: 50:50 methanol/acetonitrile with 0.1% formic acid. Injection volume was 10 µL. Analytical run time was 7.5 min at a gradient flow rate of 0.5 mL/min. Post-column infusion studies were completed using a syringe pump connected to a T-union. The syringe pumped delivered 50 µg/mL standard solution at a rate of 50 µL/min. Matrix variables were loaded onto the autosampler at varying levels to assess the ability of dilution to minimize the effect of chemical noise.

**Results:** The post-column infusion approach was effective in mapping the suppression events versus retention time to help predict future issues with quantitation for specific analytes. It was suspected that early eluting analytes would be most affected by salts. It was determined that later eluting analytes were most affected by phospholipids via MRM monitoring of lipid and phospholipid transitions. The data collected from these experiments also guided optimization of dilution levels, and SPE optimization of wash volumes and chemistry. The detection of phospholipids after SPE inspired an orthogonal cleanup attempt pairing SPE with PPT and phospholipid depletion strategies. Although adding an additional step, this improved sample cleanliness and overall reduction in chemical noise.

**Conclusion/Discussions:** Pairing the qualitative information obtained from the post-column infusion experiments, a quantitative description of matrix suppression across the linear dynamic range of the method was evaluated by comparing the slopes of a calibration curve of solution standards versus extracted matrix. This value was also compared against a matrix suppression values obtained as a single point calibration. The sum of this multi-layer approach gives the most realistic depiction of the effect of matrix suppression versus any one technique alone.

**Keywords:** UPLC-MS/MS, Matrix Interferences, Biological Samples, Sample Preparation, Method Development

## Wash and Elution Analysis of both pH Insensitive and Responsive Analytes in Urine for Large Drugs of Abuse Panels using Polymeric Mixed-Mode Cation Exchange

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**Background/Introduction:** The majority of pain management drugs are usually restrained to classes of opioids, benzodiazepines, cannabinoids, and stimulants; although their subtle dissimilar intermolecular traits can offer remarkably different analgesic, anxiolytic or other off-label effects, their similarities often provide an opportunity for their isolation via pH modulation through common functional groups such as amines (opioids and stimulants) or imines (benzodiazepines). Herein, we demonstrate that a large urine panel, comprised of 44 DOA's, from multiple drug classes, can be simultaneously screened by mix-mode cation exchange despite their disparate intermolecular traits, by selecting appropriate organic wash and elution conditions that enable excellent sample recovery and detection.

**Objective:** In an effort to build a comprehensive panel and include pH insensitive analytes, carisoprodol and meprobamate, we set out to examine the retentive and recovery effects of varying concentrations of organic wash solvent. Additionally, we examined the pH sensitive analytes, pregabalin and gabapentin, upon altering organic elution composition under polar-protic and polar-aprotic conditions. We therefore investigated the effects of varying concentrations of organic wash and elution solvents upon carisoprodol, meprobamate, with pregabalin and gabapentin, respectively.

**Methods:** Interfering matrix components were removed using EVOLUTE® EXPRESS CX solid phase extraction (SPE) plate under the auspice of the Extrahera™ automation sample prep workstation. Each sample was analyzed with 44 compounds representing four major DOA classes at 50 ng/mL in 150 uL of user-supplied drug-free urine. Enzymatic hydrolysis was performed IMCSzyme beta-glucuronidase and acidified with 200 uL of 4% phosphoric acid. Samples were loaded into the Extrahera™ sample prep workstation and extracted using a 30 mg EVOLUTE® EXPRESS CX 96-well SPE plate. Each well was pre-treated with methanol and water followed by sample loading and an acidified aqueous wash. The second wash varied among samples from 0 to 100% MeOH (aq) in 10% increments. Analytes were eluted with two 0.5 mL aliquots of DCM/IPA [78:20] or varying concentrations of MeOH/ACN with 2% NH<sub>4</sub>OH. The elution solvent was evaporated and reconstituted with 150 uL of 10% methanol, and immediately analyzed via LC/MS-MS. Extracted analytes were chromatographically resolved over 8.6 minutes on a Phenomenex Kinetex Phenyl-Hexyl 2.6 mm, 50 x 4.6 mm column prior to injection and analyzed using a Sciex 5500 triple quadrupole mass spectrometer (Sciex, Foster City, CA.).

**Results:** Carisoprodol and Meprobamate peak areas declined rapidly when washing with 60-100% methanol with an accompanying decrease in signal to noise, whereas the majority of analytes possessed recoveries >90%. A recovery study using 10% methanol wash demonstrated strong affinity for the CX sorbent with carisoprodol and meprobamate at 25, 50, and 100 ng/mL resulting in 95-110% and 97-112%, respectively. Conversely, a 50% methanol wash yielded 16-28% and 1.8-2.8% recoveries for both carisoprodol and meprobamate, respectively, over the same analytical range. When substituting methanol and acetonitrile for DCM/IPA, both gabapentin and pregabalin were recovered at substantially higher yields. Increasing the ratio of methanol in the elution volume from 0% to 20, 30, or 40% resulted in enhanced recovery of both gabapentin (> 100%) and pregabalin (> 85%), whereas the same increase with acetonitrile was deleterious.

**Conclusion/Discussions:** Carisoprodol and meprobamate can be simultaneously isolated using mixed-mode cation exchange when carefully considering the organic composition under SPE washing conditions. Although these DOA's possess at least one carbamate functional group, this "ester-amide" hybrid does not behave as an acid or base within the recognized pH range of 1-14. Consequently, they remain pH insensitive and must rely upon the hydrophobic retention mechanism of the sorbent's reverse phase characteristics. Lastly, both gabapentin and pregabalin require at least a 20% polar-protic solvent for enhanced recovery and are insensitive to organic washes under a cation-exchange mechanism.

**Keywords:** Solid Phase Extraction, Pain Management Drugs

**Hydrolysis Efficiency Comparison of Two Beta-Glucuronidases: BG100 and BGTurbo**

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**Background/Introduction:** At the time of drug testing in urine or similar matrix, drugs must be de-conjugated in order to unify the readings and achieve accurate analyte quantification. To perform this de-conjugation, efficient chemical or enzymatic hydrolysis is needed. The efficiency of a hydrolysis method is normally related to analyte hydrolysis, incubation time, analyte artifacts and downstream effects. When an enzymatic hydrolysis is performed, the results must be reliable. For this, a previous optimization step is very important to perform the biocatalysis under optimized conditions. Kura Biotec, an enzyme-producing company focused to toxicological analysis, has developed two beta-glucuronidases: BG100® and BGTurbo®, which promote the release of the compound most commonly conjugated to drugs in the body: glucuronic acid. Both enzymes have showed to be excellent alternatives to be applied in drug testing. But for this to have been possible, hydrolysis conditions were previously optimized.

**Objective:** The aim of this work is to show optimized hydrolysis conditions of two beta-glucuronidases produced by Kura Biotec: BG100 (First generation enzyme) and BGTurbo (Second generation enzyme) to be applied in drugs testing on urine samples in order to improve its performance.

**Methods:** Two different beta-glucuronidases were purified through salt precipitation and chromatographic techniques. In order to find optimal catalytic conditions of genetically enhanced BGTurbo, pH was evaluated between 33 and 300 mM (final) preparing Tris-HCl buffer (pH 7.0-7.5), sodium phosphate buffer (pH 6.5-7.2) and ammonium bicarbonate buffer (pH 7.0-8.0). Hydrolysis temperature was evaluated between 25 and 65 celsius degrees. For BG100  $\beta$ -glucuronidase, pH was assessed between 3.0 and 6.5 preparing sodium acetate and ammonium acetate buffers varying molarity between 23 and 460 mM (final). BG100 activity was evaluated between 20 and 80 celsius degrees. For BGTurbo and BG100, activity was measured by the release of phenolphthalein from phenolphthalein glucuronide (PPG) per hour at pH 5.0 and 6.8 respectively, incubating at 37°C. Also, hydrolysis efficiency for both enzymes was evaluated on different % urine (v/v) samples of spontaneous urination from volunteers not exposed to drugs. By last, we show how source of different beta-glucuronidases impacts on % analyte recovery, incubation time and necessity of post-hydrolysis clean-up when the enzymes hydrolyze various types of glucuronide conjugates.

**Results:** BG100 beta-glucuronidase showed to be more active at acidic pH than BGTurbo enzyme which was a neutral enzyme. BG100 was more thermophilic than BGTurbo due to its optimum hydrolysis temperature was 70°C at 20 minutes of incubation time. BGTurbo has an optimum temperature of 50°C but hydrolysis time was only 10 minutes being up to 12 times more efficient than other  $\beta$ -glucuronidases tested, obtaining above 85% analyte recovery when was incubated with different glucuronide conjugates. When different percentages of urine on total hydrolysis mix volume were tested, BG100 prefers diluted urine (up to 4%) while BGTurbo did not show significant activity differences when was incubated with diluted urine between 10 and 40 % (v/v).

**Conclusion/Discussions:** The choice of which beta-glucuronidase to use in drugs testing will depend on what users want to get. Partially purified BG100 beta-glucuronidase, deconjugate glucuronides (and sulfate esters in low proportions) by catalyzing hydrolysis on body fluids as urine, under optimized acidic and high temperature conditions. This enzyme would be more efficient when urine samples with very acidic pH values (4.0-5.0) are tested. Whereas that BGTurbo, a highly chromatography purified enzyme, allows to catalyze a broader-spectrum of conjugated glucuronides, even those slowest to hydrolyze like codeine-6-glucuronide, under optimized neutral and high temperature conditions. BGTurbo would act more efficiently on neutral or slightly alkaline urine samples within a few minutes of hydrolysis (<15 minutes).

**Keywords:** Drugs Testing, Second Generation, Beta-Glucuronidases



**Development of an Analytical Method for the Detection of Non-declared Anabolic Steroids in Dietary Supplements in Brazil**

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**Background/Introduction:** Dietary supplements are used to increase muscle mass, aerobic capacity and physical performance, to reduce body fat, lose weight, delay the aging process and to attract the attention of many amateur and professional athletes. Around the world, billions of dollars were profited from the market of supplements. In Brazil, their trade is significantly high and the national laws classify them as foods and the supervision of their production and marketing does not follow the same rigor as that of medicines, promoting the practice of adulteration and contamination of these products by pharmacologically active substances. One of the classes of substances most commonly found as adulterants are the anabolic steroids. The presence of non-declared anabolic steroids in these formulations consists not only of a public health problem, but also an issue for professional athletes, sports federations and anti-doping laboratories.

**Objective:** The aim of the present study was to develop a new analytical method based on a liquid chromatography-tandem mass spectrometry (LC-MS/MS) to investigate 11 possible anabolic steroids (testosterone, methyltestosterone, testosterone propionate, testosterone decanoate, nandolone decanoate, stanozolol, trembolone, dehydroepiandrosterone, androstenedione, methasterone, oxandrolone) as adulterants in dietary supplements used in Brazil.

**Method:** The chromatographic method was optimized using a LC-MS/MS and a C18 column Kinetex (100 mm x 3 mm, 2.6  $\mu$ m). The mobile phase consisted of water: methanol, 50:50 (v v<sup>-1</sup>). A gradient elution, at constant flow of 0.550 mL min<sup>-1</sup> and temperature of 45°C, was optimized for appropriate chromatographic separation of all 11 analytes. For the sample treatment, 500 mg of each matrix (protein, carbohydrate and aminoacid supplements) was dissolved in 5 mL of methanol. The mix was then shaken for 1 min, sonicated for 30 min and then centrifuged for 5 min at 3320xg at 4°C. . An aliquot of the supernatant was diluted 10000-fold with water and an aliquot of 30  $\mu$ L was finally injected into the instrument. The internal standard used was 17-OH-progesterona-d $\delta$ . The atmospheric pressure chemical ionization (APCI) was the chosen ionization form.

**Results and Discussion:** Limits of detection (LOD) and quantitation (LOQ), selectivity, linear range, matrix effect (ME), heteroscedasticity and precision (intra and inter-day) were performed for all of 11 target analytes. The range of concentrations considered for this study was based on the therapeutic concentrations (TC) of each steroid. The LOD and LOQ were above 10% of the TC for all substances showing the applicability of the proposed method for the identification of both adulteration and contamination levels in supplement samples. No ME was found in the 10000-fold dilution. Analytical curves exhibited linearity between 10-4000 ng/dg and the r<sup>2</sup> was higher than 0.99 for all substances. The method showed to be selective, precise and accurate (RSD%<15%). The total time of separation chromatography was lower than 13 minutes.

**Conclusion:** The results obtained allowed the applicability of the analytical method based on LC-MS/MS for detection of contaminants as well as the intentional addition of active substances in different supplement samples. Besides using an accessible technic, the method proposes a simple, cheap and practical sample treatment followed by the analysis by LC-MS/MS.

**Keywords:** Dietary Supplements, Adulteration, Anabolic Steroids

**LC-MS/MS Analysis of Phytocannabinoids and their Metabolites in Urine, Oral Fluid and Blood****Rory M Doyle\***, Thermo Fisher Scientific

**Background/Introduction:** Phytocannabinoids are compounds that occur naturally in the cannabis plant. These cannabinoids are abundant in the viscous resin that is produced by the trichrome glandular structures in the cannabis plant. The phytocannabinoids are water insoluble but are soluble in alcohol and fat and other non-polar organic solvents. Of over 480 different compounds present in the cannabis plant, only around 66 have been identified as cannabinoids and about 18 have been shown to be relevant. The main psychoactive cannabinoid is the delta-9-tetrahydrocannabinol ( $\Delta^9$ -THC). The cannabinoids are separated into various subclasses depending on whether they are psychoactive or not and their affinity for the CB1 and CB2 receptors as agonists or antagonists. Phytocannabinoids can reduce pain perception in the brain, are neuroprotective and can relieve convulsions or seizures, anxiety, nausea and inflammatory changes.

**Objective:** Liquid chromatography triple quadrupole (QQQ) mass spectrometry (LC-MS/MS) is suited for rapid analysis of multiple analytes. A highly sensitive and specific LC-MS/MS analytical method has been developed for the quantitation of Phytocannabinoids and their metabolites that include- cannabidiol (CBD), cannabidiolic acid (CBDA), cannabinol (CBN), cannabinolic acid (CBNA), cannabidivarin (CBDV), cannabidivarinic acid (CBDVA), cannabigerol (CBG), cannabigerolic acid (CBGA), cannabichromene (CBC), tetrahydrocannabinol (THC), tetrahydrocannabinolic acid (THCA-A), 11-hydroxy-delta9-THC (OH-THC), 11-nor-9-carboxy-delta9-THC (COOH-THC), tetrahydrocannabivarin (THCBV), cannabicylol(CBL), cannabicitran (CBT) cannabinol monomethyl ester (CBME) and cannabiodiol (CBND) in urine, oral fluid and blood. Various sample preparation techniques were developed that included dilute and shoot in urine and oral fluid, protein crash in blood and liquid-liquid extraction for all matrices. One dimensional (1D) chromatographic configurations achieved the required sensitivity and is capable of quantitating the Phytocannabinoids and their metabolites over their relevant dynamic range. Therefore, a simple and accurate quantitative analytical method was developed for the quantitative measurement of Phytocannabinoids and their metabolites in urine, oral fluid and blood.

**Methods:** A Thermo Fisher Endura in positive and negative electrospray mode and a Vanquish Horizon HPLC system were utilized for this analysis. 100  $\mu$ L of urine, oral fluid and blood were used for the analysis of the Phytocannabinoids and their metabolites. Various columns were evaluated and an Accucore C18 100 x 2.1mm, 2.6  $\mu$ m was used with a water:acetonitrile mixture containing 0.1% Formic Acid gradient that achieved baseline chromatographic separation in an approximately 6 minute run time. Quantitative analysis was performed using scheduled reaction monitoring (SRM) transition pairs for each analyte and internal standard in positive and negative mode and accuracy of the method was verified using serum reference materials from UTAK, pooled controls and human samples.

**Results:** Good linearity and reproducibility were obtained across the dynamic range of the Phytocannabinoids and their metabolites with a coefficient of determination  $R^2 > 0.95$  for all compounds in the various matrices. The lower limits of detection (LLOD) and lower limit of quantitation (LLOQ) were determined to range from 0.25 to 2.5 ng/ml and excellent reproducibility was observed for all compounds (CV < 15%) in all matrices.

**Conclusion/Discussions:** A sensitive, simple, specific and accurate liquid chromatography QQQ mass spectrometry method was developed and verified for the simultaneous measurement of Phytocannabinoids and their metabolites in urine, oral fluid and blood.

**Keywords:** Phytocannabinoids, Mass Spectrometry, Biological fluid

## A Comparison of Screening and Quantitative LC-MS/MS Analysis of Drugs and their Metabolites in Urine, Oral Fluid and Blood on Triple Quadrupole and Quadrupole Orbitrap Mass Spectrometers for Forensic Use

Rory M Doyle\*, Thermo Fisher Scientific

**Background/Introduction:** Forensic toxicological analysis requires the identification and characterization of numerous drugs within biological samples that must be screened and then confirmed before the final result can be released to the court. Mass spectrometry has become a significant tool for toxicological determination and the different platform types that are now available can allow for the easy drug screening and confirming of numerous drug classes in biological fluids. The various mass spectrometer platforms available have different specificities and sensitivities based on their resolution potentials and thus can be positioned within the forensic lab to achieve the best results.

**Objective:** Liquid chromatography triple quadrupole (QQQ) and quadrupole Orbitrap (QE) mass spectrometry (LC-MS/MS) are suited for rapid analysis of multiple analytes for screening and quantitative purposes. Sensitive and specific LC-MS/MS analytical methods have been developed for the screening and quantitation of over 400 drugs of the following drug classes: stimulants, benzodiazepines, antidepressants, opioids, muscle relaxants, hallucinogens, etc. on both the QQQ and the Q Exactive instrument platforms. Simple sample preparation techniques such as dilute and shoot for urine and oral fluid, and protein crash for blood, and one dimensional (1D) chromatographic configurations achieved the required sensitivity and are capable of screening and quantitating the drug analytes over their relevant dynamic range.

**Methods:** A Thermo Scientific™ TSQ Endura™ and Q Exactive™ Focus mass spectrometers in positive and negative eElectrospray mode and a Thermo Scientific™ Vanquish™ HPLC system were utilized for this analysis. 100 uL of human urine, oral fluid and blood were used for the analysis of the various drug classes. Various columns were evaluated and initially a Thermo Scientific™ Accucore™ C18 100 x 2.1 mm, 2.6 um column with water:methanol mixture containing 0.1% formic acid and 5 mM ammonium formate gradient achieved baseline chromatographic separation in an approximately 8 minute run time for all matrices. Screening and quantitative analysis was performed using full scan data dependent MS2 analysis with inclusion list and using selective reaction monitoring (SRM) transition pairs respectively for each analyte and internal standard in positive and negative mode. The accuracy of the method was verified using reference materials from UTAK and human samples.

**Results:** Good linearity and reproducibility were obtained across the dynamic range of the drugs with a coefficient of determination  $R^2 > 0.95$  for all drugs in the various matrices on both instrument platforms. The lower limits of detection (LLOD) and lower limit of quantitation (LLOQ) were determined and excellent reproducibility was observed for all compounds ( $CV < 15\%$ ) in all matrices. The TSQ Endura instrument gave more sensitive quantitation results while the Q Exactive was not as sensitive but definitely a useful tool for screening and quantitation where the cut-offs are better than with immunoassay techniques.

**Conclusion/Discussions:** SA sensitive, simple, specific and accurate liquid chromatography QQQ and QE mass spectrometry methods were developed and verified for the simultaneous screening and quantitative measurement of over 400 drugs and their metabolites in urine, oral fluids and blood.

**Keywords:** Triple Quadrupole, Orbitrap, Mass Spectrometry

## Detection of Morphine Glucuronides of High Heroin Positives in Oral Fluid

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**Background/Introduction:** Testing for the presence of drugs of abuse in oral fluids has gained popularity due to various advantages that oral fluid offers over other matrices such as blood and urine. The noninvasive and observed collection procedure of oral fluid sampling makes adulteration almost impossible. Specifically in the case of heroin testing, oral fluids has proven to give better evidence regarding the impairment and the origin of the metabolites. Heroin is a widely abused drug in the United States. Heroin overdose causes suppression of breathing, coma, permanent brain damage and death. Heroin consumption is generally determined by the presence of its major metabolites: 6-monoacetylmorphine and morphine in oral fluid. Morphine further metabolizes into morphine-3-glucuronide and morphine-6-glucuronide. One of the arguments posed against oral fluid drug testing is the contamination of the collection pad with residual drugs in the mouth. The detection of the drug metabolites in oral fluid provides defense against this argument. However, morphine glucuronides have not been reported in oral fluid due to their low abundance and hydrophilic nature. Commonly used reverse phase liquid chromatography mass spectrometry methods yield very low sensitivity to these metabolites. In this research work, we developed a liquid chromatography-tandem mass spectrometry (LC-MS-MS) method to detect morphine glucuronide metabolites and other heroin metabolites in oral fluid.

**Objective:** To develop and validate LC-MS-MS method that can accurately quantify morphine-3-glucuronide, morphine-6-glucuronide, heroin, 6-monoacetylmorphine and morphine in high heroin positive samples.

**Methods:** Oral fluid samples were collected with a Quantisal™ collection device. The collection device is made of a pad and buffer system. The pad is placed in the mouth, then placed into the buffer solution and sealed. The collected oral fluid samples were purified with solid phase extraction (SPE) prior to LC-MS-MS analysis. SPE was performed in a vacuum manifold using an Agilent PCX cartridge. SPE process is as follows: 200 µL sample and 3% phosphoric acid in water were mixed in a rotary shaker. The wells were preconditioned with methanol and water and the samples were loaded into the wells. The wells were washed with 3% phosphoric acid in water and methanol. The compounds were eluted with 5% NH<sub>3</sub> in methanol. The eluent was dried under nitrogen and reconstituted with aqueous mobile phase. Chromatographic separation was performed on an Acquity UHPLC system (Waters) equipped with Zorbax Eclipse C8 RRHD (100X2.1 mm, 1.8 µm) analytical column (Agilent Technologies). Electrospray ionization mass spectrometry was performed on a TQD instrument (Waters). Analysis was performed in positive ionization (ESI+) and multiple reaction monitoring (MRM) mode.

**Results:** The developed method was validated according to Scientific Working Group for Forensic Toxicology (SWGTOX) guidelines. The linear dynamic range for morphine-3-glucuronide and morphine-6-glucuronide was 0.5-80 ng/mL. The linear dynamic range for heroin was 500-20,000 ng/mL. The linear dynamic range for 6-acetylmorphine and morphine was 1000-40,000 ng/mL. The acceptance criterion for the accuracy and precision was that % relative error and %CV should be ≤20% for QC samples. The results of the accuracy and the precision values were within the acceptance criteria for all the analytes. In addition, selectivity, matrix effect and recovery were calculated for the LC-MS-MS method. Four hundred and thirteen samples were analyzed using the assay. Range of morphine-3-glucuronide was 0.8-205 ng/mL and range of morphine-6-glucuronide was 0.5-121 ng/mL. On average, concentration of morphine-3-glucuronide in a heroin positive sample was five times higher than that of morphine-6-glucuronide.

**Conclusion/Discussions:** The validation data indicate that the method is accurate, precise and robust and is suited for toxicology confirmation applications in a production setting. Morphine-3-glucuronide and morphine-6-glucuronide were reported in oral fluid for the first time.

**Keywords:** Heroin, Oral Fluid and Morphine Glucuronides

## Opioid Quantitation in Blood using Dispersive Pipette Extraction on the Integra Viaflo 96 and LC-MS/MS

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**Background/Introduction:** According to the Centers for Disease Control and Prevention, overdose deaths involving prescription opioids have quadrupled since 1999, and so have sales of these prescription drugs. From 1999 to 2015, more than 183,000 people have died in the U.S. from overdoses related to prescription opioids. With the high number of cases needing confirmatory testing, an efficient process is key. Automation improves accuracy and precision and brings uniformity to operations that are prone to human error. Dispersive Pipette Extraction (DPX) technology is a non-traditional SPE technique in which loose sorbent is contained between two porous barriers within a pipette tip. The sorbent is mixed with solution by simply aspirating and dispensing. With seamless integration onto robotic liquid handlers, like the Integra Viaflo 96. DPX WAX tips contain weak anion exchange sorbent on a styrene divinyl benzene backbone for removal of non-polar and anionic sources of ion suppression.

**Objective:** To validate a semi-automated dispersive pipette extraction (DPX) for the analysis of opioids using LC-MS/MS in an effort to replace our existing Solid Phase Extraction (SPE) and Gas Chromatography/Mass Spectrometry (GC/MS).

**Methods:** Drug standards were purchased from Cerilliant Corporation and Lipomed. DPX WAX tips were purchased from DPX Labs, LLC (Columbia, SC). Extraction was performed using an Integra semi-automated pipetting unit with DPX WAX tips. Analyses were performed using an Agilent 1290 LC coupled to a 6430 Triple Quad equipped with Thermo Scientific's Accucore RP-MS 2.1 X 100 2.7  $\mu\text{m}$  column. The following analytes were validated: codeine, morphine, 6-monoacetylmorphine (6-MAM), hydrocodone, hydromorphone, oxycodone, oxymorphone, fentanyl, tapentadol, tramadol, methadone, and meperidine. Mobile phases consisted of 5 mM Ammonium Acetate with 0.1% formic acid in water (A) and 0.1% formic acid in acetonitrile (B). Injections of 10  $\mu\text{L}$  were introduced into a gradient elution from 98% A to 98% B over 11 minutes.

The method was validated following SWGTOX guidelines for accuracy and precision, limits of detection, linearity, calibration model, matrix and analyte interference, carryover, matrix effects/ion suppression, dilution integrity and stability. Previously analyzed cases were evaluated to assess fit for purpose.

**Results:** Between-run mean accuracy ranged from 83.4% - 115%. Between-run precision had coefficient of variations (CVs) less than 9% for all compounds (range 2.6% - 8.9%). The lower limit of quantitation was determined to be 0.5 ng/mL for Oxymorphone, Fentanyl, and Hydromorphone; 1 ng/mL for 6-MAM, 5 ng/mL for Morphine, Meperidine, and Tapentadol, and 10 ng/mL for Codeine, Oxycodone, Hydrocodone, Tramadol, and Methadone. The upper limit of quantitation was 1000 ng/mL for codeine, morphine, hydrocodone, oxycodone, oxymorphone, tapentadol, tramadol, methadone, and meperidine, and 100 ng/mL for 6-MAM, oxymorphone, hydromorphone, and fentanyl. The calibration curves were a linear with 1/x weighing for all compounds with the exception of morphine and fentanyl which were quadratic 1/x weighted. No significant interference from matrix effects or common drugs of abuse was observed. Carryover was evaluated and not present up to 1000 ng/mL. Results from previously analyzed samples were consistent with original results.

**Conclusion/Discussions:** A quantitation of opioids was successfully developed and validated using semi-automation, DPX and LC/MS/MS techniques. The extraction minimizes sample volume, use of organic solvent, allows for additional targets, a wider linearity range, and eliminates the need for separate analyses for confirmation. Of our monthly caseload, 50% produces presumptive positives within the aforementioned analytes. The new method will cut down extraction/analysis time by more than 80%. Forensic laboratories will benefit from incorporating methods that are easily automated.

**Keywords:** Semi-Automation, Dispersive Pipette Extraction, Opioid

## Analyte-Dependent Stability of Synthetic Cathinones

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**Background/Introduction:** Synthetic cathinones are a class of designer drugs that have been increasing in popularity since the late 2000s according to the National Forensic Laboratory Information System (NFLIS). Numerous case reports have identified synthetic cathinones in both ante-mortem and post-mortem toxicology investigations. Several studies to date have documented the instability of certain cathinone species in biological samples. In this report we describe a systematic approach to identify analyte dependent differences in stability between cathinone species to facilitate the interpretation of toxicological results.

**Objective:** To determine how the structural characteristics of the synthetic cathinones influence stability in various biological matrices.

**Methods:** Twenty-two synthetic cathinones were selected to include secondary and tertiary amine (pyrrolidinyl) cathinones, with and without ring substituents. Blood and urine, fortified with all twenty-two analytes, was monitored over a period of six months at 32°C, 20°C, 4°C and -20°C. Specimens were analyzed in duplicate, using appropriate sampling intervals (hours, days, weeks, and months) using a validated liquid chromatography quadrupole/time of flight mass spectrometry (LC-Q/TOF-MS) assay. Half-lives were determined for each of the cathinones in blood and urine at each temperature. Substituents on the aromatic ring (unsubstituted, substituted or methylenedioxy substituted) and the nitrogen (secondary or tertiary amine) were evaluated in terms of stability (Table 1). When significant drug loss was evident (>20%), analyte-dependent differences in stability were evaluated statistically using analysis of variance (ANOVA).

Secondary Amine			Tertiary Amine	
Unsubstituted	Substituted	Methylenedioxy	Non-Methylenedioxy	Methylenedioxy
Buphedrone	3,4-DMMC	Butylone	MPBP	MDPBP
Ethcathinone	3-FMC	Ethylone	Naphyrone	MDPV
Methcathinone	4-EMC	Etylone	Pyrovalerone	
Pentedrone	4-FMC	Methylone	$\alpha$ -PVP	
	4-MEC	Pentylone		
	Methedrone			
	Mephedrone			

Table 1

**Results:** Instability (>20% loss of target concentration) was observed for several synthetic cathinones stored at elevated or ambient temperature within hours of storage. One-way ANOVA was used to determine if there was a statistical significance in degradation *within* and *between* the synthetic cathinone subgroups. Pyrrolidine-type cathinones (tertiary amines) were significantly more stable than their secondary amine counterparts. Among the secondary amines, no significant differences were observed between unsubstituted and ring substituted cathinones. However, addition of the methylenedioxy group had a significant stabilizing effect on both secondary and tertiary analogs. As a result, cathinones containing both a pyrrolidine and a methylenedioxy group exhibited the greatest overall stability, while fluorinated cathinones, notably 3-FMC, was the least stable.

Half-lives derived from first order rate constants also highlighted analyte dependent differences in stability. At ambient temperatures in blood, half-lives ranged from 1-6 days for the least stable secondary amines, to approximately 3 months for the methylenedioxy substituted tertiary amines. Similar analyte dependent differences were observed in blood and urine over a range of temperatures.

**Conclusion/Discussions:** The stability of synthetic cathinones is highly dependent on the chemical structure of the specific analogue. The presence of the pyrrolidinyl group on the tertiary amine makes them significantly more stable than their secondary amine counterparts. The methylenedioxy group also contributes a significant stabilizing effect. When considering cathinone stability, substitutions at the aromatic ring,  $\alpha$ -carbon and nitrogen play an important role. Analyte dependent differences in cathinone stability should be carefully considered in toxicological investigations.

**Keywords:** Synthetic Cathinones, Stability, Blood, Urine

## Quantification of Suvorexant in Blood Using LC-Q/TOF-MS

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**Background/Introduction:** Suvorexant is a novel drug for the treatment of insomnia that was approved for use by the FDA in 2014. In 2015, suvorexant became commercially available under the trade name Belsomra®, and was placed under Schedule IV of the Controlled Substances Act by the DEA. Unlike other common treatments for insomnia, such as benzodiazepines and non-benzodiazepines that act on GABA receptors, suvorexant is a dual orexin receptor antagonist that is believed to have a lower abuse potential compared to other sedative hypnotics. Suvorexant is highly lipophilic and has a long half-life (~12 hours). Although these properties are not favorable from the standpoint of impaired driving, case reports have yet to be reported. To date there have been limited reports that describe the analysis of suvorexant in biological samples. Here we describe an analytical procedure for the quantitative determination of suvorexant in whole blood.

**Objective:** To validate a method for the quantification of suvorexant in blood using quadrupole time-of-flight liquid chromatography/mass spectrometry (LC-Q/TOF-MS).

**Methods:** A simple acidic/neutral liquid-liquid extraction was performed for the isolation of suvorexant from 0.5 mL whole blood. Estazolam-D5 was used as the internal standard. An Agilent Technologies 6530 Accurate-Mass LC-Q/TOF-MS equipped with electrospray ionization (ESI) technology was used for the detection of suvorexant. Mobile phase A and B consisted of 0.1% formic acid in deionized water and 0.1% formic acid in acetonitrile, respectively. Gradient elution was performed for the chromatographic separation of the compounds using a Poroshell 120 EC-C18 column. The column temperature was maintained at 35°C with a flow rate of 0.4 mL/min. The gradient elution profile consisted of a 40% B to 80% B ramp between 0-3 minutes, a hold of 80% B for 1 minute, and then decrease to 40% B until 5 minutes. This assay was validated according to the Scientific Working Group for Toxicology (SWGTOX) Standard Practices for Method Validation in Forensic Toxicology guidelines. The extraction efficiencies of various solvents in blood were also evaluated in addition to limit of detection, limit of quantitation, precision, accuracy, matrix effects, interferences, carryover, and stability using this assay.

**Results:** The recovery of suvorexant was evaluated using four different extraction solvents (*N*-butyl chloride, ether/toluene (1:1), hexane/ethyl acetate (9:1), and *tert*-butyl methyl ether). Due to the high lipophilicity of the drug, no significant differences in analytical recovery were observed ( $p=0.12$ ). However, *N*-butyl chloride demonstrated improved reproducibility. A weighted quadratic calibration model was selected (0-250 ng/mL). Limits of detection and quantification in whole blood were 0.5 and 1 ng/mL, respectively.

**Conclusion/Discussions:** Sedative hypnotic drugs feature prominently in forensic toxicology investigations. We describe a new analytical procedure for the quantification of suvorexant in whole blood using LC-Q/TOF-MS that will aid in the identification of this new drug in forensic toxicology casework.

**Keywords:** Suvorexant, Blood, LC-Q/TOF-MS

## Analysis of Dextromethorphan and Metabolites in Skeletal Remains by UPLC-qToF-MS: Effects of the Dose-Death Interval on Drug and Metabolite Levels

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**Background/Introduction:** This work formed part of a research program investigating drug and metabolite disposition in skeletal remains following various drug exposure conditions and postmortem environments.

**Objective:** This work reports the analysis of dextromethorphan (DXM) and its metabolites dextrorphan (DXT), N-desmethyldextromethorphan (DMDXM) and N-desmethyldextrorphan (DMDXT) in decomposed skeletal remains of rats (n=14) acutely exposed to DXM (75 mg/kg i.p.) by UPLC-qTOF-MS, and the influence of dose-death interval (DDI) on relative analyte distribution.

**Methods:** Following dosing, rats were separated into three groups, with groups ( $n_i=4$ ) of animals euthanized 45, 90 or 135 min post-dose, and one group (n=2) acting as drug-free controls. Rats decomposed to skeleton outdoors in secure caging. Bones were recovered, sorted by skeletal element, washed with phosphate buffer (PBS, 0.1 M, pH6), methanol and acetone, then dried under ambient conditions and pulverized to a powder. Pulverized bone samples (0.1g) underwent methanolic extraction under vigorous agitation using a TissueLyser<sup>®</sup> for 45 minutes. Calibrants were prepared at concentrations ranging from 1-1000 ng/mL in drug-free bone tissue extract (BTE). A 200 $\mu$ L aliquot of each calibrant or extract was diluted with 800 $\mu$ L of 1:1 acetonitrile:water. Internal standards were added to each sample (50 ng D3-DXM and D3-DXT). Samples were filtered using Clean Screen FASt<sup>®</sup> 96 well plates (100 mg, United Chemical Technologies, Bristol, PA). Extracts were analyzed using an Acquity<sup>®</sup> UPLC equipped with a G2-XS qToF MS (Waters, Milford, MA) in MSE mode. The UPLC-TOF MS method was validated by assessment of 8 standard curves ( $n_i=3$  per calibrant) on each of 8 different days. Analyte identification was by retention time ( $\pm 0.01$  min), parent mass  $\pm 5$  mDa, and isotope pattern. Acceptable precision and bias (< 20%) were achieved for all analytes using quadratic curve fitting. Matrix effects were less than 25% and recovery was greater 80% for all analytes. LOD and LOQ were administratively set to 1 ng/mL.

**Results:** Bone analyte levels were expressed as mass-normalized response ratios (RR/m). Kruskal-Wallis (KW) analysis tested whether skeletal element was a main effect for analyte level and analyte level ratio for each DDI. Skeletal element was a main effect for DXM and DXT level in DDI1-DDI3, for DMDXM level in DDI2 and for DMDXT level in DDI2, DDI3 ( $p<0.05$ ). Skeletal element was a main effect for analyte level ratios (DXT/DXM, DMDXM/DXM, DMDXT/DXM, DMDXM/DXT, DMDXT/DXT, DMDXM/DMDXT) in 11/15 scenarios, most consistently for DMDXM/DXM and DMDXT/DXM (DDI1-DDI3) and least consistently for DMDXT/DXT (DDI3 only). DDI was a main effect for DXM and DMDXM level for all skeletal elements, for DXT level in scapula and skull, for DMDXT in rib, tibia and scapula. DDI was a main effect for analyte level ratios in 15/35 scenarios, most consistently for DMDXT/DXM and DXT/DXM (4/7 and 3/7 skeletal elements, respectively) and least consistently for DMDXT/DXT (scapula only).

**Conclusion/Discussions:** In conclusion, UPLC qToF-MS provided a simple and reliable means for analysis of DXM and metabolites in bone. The relatively short DDIs were best discriminated by DXM and DMDXM levels. When using analyte ratios to offset challenges in measurement

**Keywords:** UPLC-qToF-MS, Bone, Dextromethorphan



**Forensic Drugs of Abuse Analysis Using an Automated, Simultaneous In-Tip Extraction Technique**

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**Background/Introduction:** In the forensic toxicology laboratory, proper sample preparation can be very time consuming and can represent a considerable bottleneck to throughput and analysis. Matrix effects are a major concern to downstream LC-MS/MS analysis. In-tip Dispersive Pipette eXtraction (DPX) is a new solid phase extraction (SPE) technology that minimizes sample processing steps and decreases reagent use compared to typical SPE plate-based techniques. Pipette tips containing DPX chemistry allow for a high-throughput extraction method that can be fully automated. This in-tip chemistry extraction method is highly reproducible and provides the necessary sensitivity for target analyte quantification and analysis typically required in forensic laboratories.

**Objective:** The objective of this work is to demonstrate an automated in-tip extraction method for the processing of forensic samples for 36 drugs of abuse analysis within a 96-well platform.

**Methods:** Microplates containing hydrolyzed urine were loaded on to a robotic platform. The automated method starts by filling a 96-well microplate with 200  $\mu$ L of water (for the wash step) and 150  $\mu$ L of 1% FA (formic acid) in methanol (for the elution step). The DPX tips (mixed mode with RP/WAX) were then conditioned by aspirating 30% methanol from a solvent reservoir. After conditioning, the sample solutions, containing 150  $\mu$ L urine + 100  $\mu$ L mixture of buffer, enzyme and internal standards, were aspirated and dispensed three times in order to bind the drugs of abuse targets to the sorbent. Water was then aspirated and dispensed to remove sample matrix components such as salts, urea, and creatinine. The analytes of interest were eluted by aspirating and dispensing 1% FA in methanol three times. The eluent was diluted until an appropriate percentage of methanol was reached for injection. In this case, 1,050  $\mu$ L of water was added (to make the final solution 12.5% methanol). Analysis was performed on a Thermo TSQ Vantage triple quadrupole instrument with an Agilent 1260 HPLC using an Agilent Poroshell EC-C18 column (3.0 x 50 mm, 2.7  $\mu$ m) with a 10  $\mu$ L injection.

**Results:** From start to finish, this fully automated dispersive SPE method takes approximately 15 minutes in order to prepare two 96-well microplates of samples. Results from this method are linear, accurate, and reproducible. All correlation coefficients were greater than 0.99 for the range of at least 12.5-400 ng/mL, with most analytes being linear from 6.25-800 ng/mL. Relative standard deviation was calculated using 7 replicate extractions at 400 ng/mL and ranged from 1.6 to 8.0%. Limits of detection (LOD) were calculated as  $3.3 \cdot \sigma / m$ , where  $\sigma$  is the standard deviation of the lowest non-zero calibrator and  $m$  is the slope of the calibration line. Limit of quantitation (LOQ) was calculated as  $10 \cdot \sigma / m$ . LOD ranged from 0.50 to 18 ng/mL and LOQ ranged from 1.5 to 54 ng/mL. While it should be emphasized that confirmatory tests and cutoff levels are highly dependent on LC-MS/MS instrumentation and procedures, for improved sensitivity and lower LODs and LOQs, solvent evaporation can be employed to concentrate the extracts. Elution volume can also be increased to 500  $\mu$ L to increase recovery as well.

**Conclusion/Discussion:** The method described herein demonstrates adequate recoveries, sensitivity, and reproducibility for reliable sample preparation in a high-throughput forensic laboratory setting. Complete automation of the sample preparation method provides a less tedious and faster process compared to manual processing for analysis of drugs of abuse; two 96-well microplates are processed in 15 minutes.

**Keywords:** Drugs of Abuse, Urinalysis, Automation

## A Modified QuEChERS Approach for the Extraction of Common Prescription and Illicit Drugs from Liver Prior to LC/MS-MS Analysis

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**Background/Introduction:** The high demands placed on toxicology laboratories to produce accurate results in a concise period of time has fueled a mass transition towards universal sample preparation techniques. These broad approaches are fast and efficient as they are able to encompass more analytes and drug classes within a given panel; however, since many of these methods are not as selective, analysts may be compromising on analyte recovery and sample cleanliness in order to increase throughput. One possible solution toxicology laboratories are beginning to explore that provides an optimum balance between producing reliable results and saving time and cost on analysis is the QuEChERS (Quick, Easy, Cheap, Effective, Rugged and Safe) approach. Crossing over from the food safety industry, this technique was originally developed in 2003 for multi-residue pesticide analysis in fruits and vegetables. Since its conception, this approach has been utilized for sample clean up on a wide variety of matrices to include animal-based products (meat, fish, kidney, chicken, milk, honey), cereals and grains, and other food produce market sectors (wines, juices, fruit and vegetables). The QuEChERS method has not only proven to be simple to perform, but also is rugged enough to withstand any necessary modifications that make it amenable to complex matrices such as those commonly encountered in a forensic toxicological setting. The study explores a modified QuEChERS approach for sample clean-up for one of toxicology's more difficult specimens to work with, post-mortem liver.

**Objective:** To evaluate an alternative method for the extraction of common drugs from postmortem liver samples prior to analytical analysis by LC-MS/MS.

**Methods:** Blank bovine liver samples were homogenized using a Robot-Coupe Blixer® at a 1:4 ratio by weight with deionized water. Blank liver homogenate (2 mL) was fortified with appropriate amounts of working standards prior to being added to 15 mL centrifuge tubes containing QuEChERS salts (800 mg magnesium sulfate (MgSO<sub>4</sub>) and 200 mg sodium chloride (NaCl)) and 2 mL of acetonitrile containing 5% ammonium hydroxide. Samples were briefly vortexed to break up any salt agglomerates prior to shaking for 5 minutes at a rate of 1000 strokes/minute using a SPEX Geno/Grinder®. After shaking, samples were placed into a centrifuge and spun for 10 minutes at a speed of 3000 rcf. Further sample cleanup was performed by adding 1 mL of the centrifuged supernatant to 2 mL micro-centrifuges tubes containing 150 mg of MgSO<sub>4</sub> and 50 mg endcapped, silica based C18 sorbent. Samples were vortexed at a rate of 100 strokes/minute for 1 minute, then placed into a centrifuge and spun for 5 minutes at a rate of 3000 rcf. A 500 µL aliquot of the final extract was then dried to completion for concentration purposes and reconstituted in 100 µl of the appropriate mobile phase for analysis by LCMS. Recoveries were calculated by dividing the chromatographic peak area of samples spiked prior to extraction by the peak area produced by samples that were spiked into a pre-extracted blank matrix. Excellent recoveries were achieved for the range of analytes included in this study. Recoveries were evaluated by fortifying samples at two varying concentrations.

**Results:** On average, the recovery for samples spiked at 75 ng/g was 81 % and for samples spiked at 300 ng/g it was 83 %. The responses for the representative compounds were linear with R<sup>2</sup> values ranging from 0.93 to 0.99 over a 6-point concentration range of 0-500 ng/g, with the lowest spiked calibrator being 25 ng/g for all analytes.

**Conclusion/Discussions:** Preliminary studies have shown that this modified QuEChERS-based method for the extraction of prescription and illicit drugs from postmortem liver should be considered as a reliable alternative when extracting multiple drug classes from challenging biological matrices.

**Keywords:** QuEChERS, Postmortem Toxicology, LC-MS

## Enantioselective Analysis of Citalopram and Demethylcitalopram in Human Whole Blood by Chiral LC-MS/MS and Application in Forensic Cases

Sys Stybe Johansen\*

**Background/Introduction:** Citalopram is one of the most frequently used antidepressants in Denmark. It is marketed as a racemic mixture (50:50) of S- and R-enantiomers as well as of the S-enantiomer alone, which is the active enantiomer named escitalopram that processes the inhibitory effects.

**Objective:** In this study, a chiral liquid chromatography-tandem mass spectrometry (LC-MS/MS) method is developed for the measurement of citalopram and demethylcitalopram enantiomers in whole blood and applied to forensic cases.

**Methods:** Whole blood samples (0.10 g) were extracted with butyl acetate after adjusting the pH with 2 M NaOH. The analytes were separated on a 250 x 4.6 mm Chirobiotic V, 5 µm column by isocratic elution with methanol:ammonia:acetic acid (1000:1:1) using an ultra-high-pressure liquid chromatography (UHPLC) system. Quantification was performed by tandem mass spectrometry (MS/MS) using multiple reaction monitoring in the positive mode with two transitions per analyte.

**Results:** The analysis was validated according to Peters et al. (FSI 2007, 165, 216) and EN ISO/IEC 17025:2005 issued by the Danish Accreditation and Metrology Fund (DANAK) with some modifications. The limit of detection (LOD) and quantification (LOQ) were 0.001 and 0.005 mg/kg of both enantiomers for the two analytes, respectively. Linear behaviour was obtained for all enantiomers from LOQ to 0.50 mg/kg blood with absolute recoveries from 71 to 80%. The method showed an acceptable precision and accuracy as the obtained coefficient of variation and bias values were  $\leq 16\%$  for all enantiomers. A correlation with the racemic method, where no suppression was determined for citalopram and its main metabolite (REF), was assessed and found to be good (BIAS  $< 7.2\%$ ) demonstrating that matrix effects were minimal. The total chromatographic run time was 20 min.

**Conclusion/Discussions:** After the successful validation of the method, the analysis was successfully applied to authentic blood samples from forensic investigations. In about 30% of 200 citalopram positive cases only S-citalopram was responsible for the positive screening. This concurs with the number of defined daily doses (DDD) sold of escitalopram respectively citalopram within the three years investigation period.

**Keywords:** Escitalopram, Chiral LC-MS/MS, Post-Mortem Blood

**Simultaneous Quantitation of Criminal Drugs of Abuse in Whole Blood by QuEChERS and LC-MS/MS**

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**Background/Introduction:** Analysis of biological samples such as blood for toxicological purposes conventionally requires pretreatment of the sample by protein precipitation (PP), liquid-liquid extraction (LLE) or solid-phase extraction (SPE). There is currently no definitive method of pretreatment for all analytes of toxicological significance with methods entailing issues such as the inability to remove interferences effectively, complex handling procedures and targeted selectivity. QuEChERS, an acronym for Quick, Easy, Cheap, Effective, Rugged, and Safe, is a sample preparation procedure that is widely used for multi-residue pesticide analysis in food and agricultural products. Originally, QuEChERS extraction involves two steps: (i) extraction-partitioning followed by (ii) dispersive solid phase extraction (dSPE) purification. A simplified QuEChERS method applying only salting-out extraction without the use of the interference scavengers was investigated with the purpose of recovering all common drugs of abuse of diverse physicochemical properties from whole blood.

**Objective:** The aim of this study was to develop and validate a lean, sensitive and cost-effective multi-target UPLC-MS/MS method for the simultaneous determination of 43 psychoactive drugs of abuse in whole blood based on QuEChERS extraction. The selected analytes represent the most frequently detected drugs of abuse and their metabolites in the forensic samples received for toxicological analysis at ESR, New Zealand – opiates/opioids (13), amphetamines (7), cocaine (2), benzodiazepines (12), synthetic stimulants (3) and miscellaneous therapeutic drugs with abuse potential (6). The method was validated in accordance with recommendations by SWGTOX Standard Practices for Method Validation in Forensic Toxicology with respect to selectivity, matrix effects (ME), extraction efficiency (EE), accuracy, precision, limits of detection (LOD), limits of quantitation (LOQ), extract stability and dilution integrity.

**Methods:** To 100 $\mu$ L of fortified blood, 200 $\mu$ L cold ACN and (0.045  $\pm$  0.001g) QuEChERS salts were added sequentially, vortexed, centrifuged and 50 $\mu$ L of the supernatant reconstituted in mobile phase for analysis; 20 $\mu$ L of the final extract was injected. Separation was performed by gradient reverse phase liquid chromatography on a Kinetex™ Biphenyl (2.1mm x 50mm, 2.6 $\mu$ m particle size) column at a flow rate of 0.5mL/min with mobile phases consisting of H<sub>2</sub>O with 0.1% formic acid (eluent A) and MeOH with 0.1% formic acid (eluent B). The analytes were detected using a Sciex® 5500 Q-Trap™ quadrupole mass spectrometer operated in positive electrospray ionisation (ESI+) and scheduled multiple reaction monitoring (MRM) mode monitoring three transitions per analyte and one transition per internal standard.

**Results:** Chromatographic separation with baseline resolution of the 43 compounds was obtained in less than 6min, with an additional 2 min re-equilibration time. Insignificant ME (-13.4 to 9.9%) and reproducible EE (%RSD: 0.9 to 6.7%) was observed. Calibration was established ( $r^2 \geq 0.99$ ) from 0.002-0.01 mg/L to 0.2-1 mg/L, depending on analyte. Within-day and between day imprecision were within acceptance limits of  $\leq 20\%$ . Accuracy data were also within the acceptance interval of  $\pm 20\%$  of expected concentrations.

**Conclusion/Discussions:** An efficient sample clean-up method using QuEChERS salting out extraction has been validated for selective, sensitive, accurate and precise quantitation of 43 common drugs of abuse in whole blood. This multi-target method does not require the use of dedicated equipment, needs only minimal amounts of solvent and in conjunction with automated data processing has high throughput capability that affords a significant increase in productivity and reduction in cost. This robust assay has been successfully applied to more than 300 human performance and postmortem cases from December 2016, providing quick turnaround forensic results as well as information on epidemiological prevalence.

**Keywords:** QuEChERS, Drugs of Abuse, Multi-Target

## Evaluation of Three Different Extraction Procedures for the Forensic Analysis of Drug-Facilitated Crimes by Liquid Chromatography Tandem Mass Spectrometry (LC-MS/MS)

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**Background/Introduction:** Lately, drug-facilitated crime (DFC) has become an increasing problem. DFC is the general term that includes criminal offenses in which the victims were under the influence of psychotropic substances. Amongst the DFCs criminal offenses, rape and/or other sexual assaults are the most common, being called Drug-Facilitated Sexual Assault (DFSA). The real number of DFCs cases around the world are not known precisely and the number of unreported cases is estimated to be high. In order to produce evidences in these cases, the forensic laboratories must develop selective and sensitive methods for the widest number of drugs that can be used on DFCs.

**Objective:** The objective of the present study was to compare three sample treatment procedures and to apply the best one for the development of a semi-quantitative method for screening of DFCs in urine and whole blood samples by liquid chromatography tandem mass spectrometry (LC-MS/MS).

**Methods:** The extraction procedures described below were evaluated for the forensic analysis on DFCs cases.

- (A) Solid Phase Extraction (SPE) – This procedure was realized using a mixed mode cartridge (Bond-Elute Certify - Agilent Technologies) aiming the extraction of acidic and basic analytes using two different solvent mixtures for elution. Half milliliter of samples was used.
- (B) Protein precipitation with acetonitrile – Acetonitrile kept in -20° C was used to protein precipitation and further supernatant collection. Two hundred microliters of samples were used.
- (C) Liquid-liquid extraction (LLE) – The LLE procedure was carried out using 1-chlorobutane in basic pH. One milliliter of samples was used.

Residues were evaporated under a gentle nitrogen stream and reconstituted with 0.1 mL of aqueous formic acid (0.1%) in every procedures. The internal standards were the same for these three procedures: GHB D6 (5 µg/mL) and 7-amino flunitrazepam D7, benzylecgonine D3, clonazepam D4, cocaine D3, imipramine D3, MDMA D5, morphine D3, THC-COOH D3 (25 ng/mL). The procedures were tested to extract the following classes of drugs and metabolites: GHB, benzodiazepines, Z-drugs, antihistamines, antidepressants, analgesics/narcotics, cannabinoids, cocaine, amphetamines, LSD and others. The drugs and metabolites were selected according to the UNODC guidelines for DFCs analysis and their availability in our laboratory. The cut-offs values used in this work were also those recommended by the UNODC guideline.

Liquid-chromatography analysis was performed in gradient mode on a Zorbax Eclipse plus C18 (2.1 x 100 mm, 1.8 µm) on an Agilent 1290 Infinity. The mobile phase consisted of A: aqueous formic acid (0.1%) and B: acetonitrile (with 0.1% of formic acid). The flow rate was set at 0.4 mL/min and the column was kept at 45° C. Tandem mass spectrometry was performed in positive/negative polarity, using an electrospray ionization (ESI) source on an Agilent 6430.

**Results:** Every procedures showed similar performance and were also capable to extract most of analytes at their recommended cut-offs values. However, the procedure C failed to extract GHB, zolpidem, zopiclone, haloperidol, morphine and THC-COOH and the procedure A failed to extract GHB, while the procedure B could successfully extract all analytes. The limit of detection (LOD) was determined by analyzing decreasing concentrations of each analyte in the three extraction procedures. The linearity was assessed (procedure B only) by means of the coefficient of determination ( $r^2$ ). Despite the method of analysis have been developed for semi-quantitative purposes, according the cut-off values of each analyte, the procedure B also presented good linear responses for all analytes ( $r^2 > 0.99$ ), at the range of 1 (or 5) to 50 ng/mL.

**Conclusion/Discussions:** The procedure B was elected as the best extraction method, being effective to extract every analytes at their recommended cut-offs, for also consuming low amounts of samples and solvents and for being a very simple and quick extraction method. Moreover, the procedure B could provide good linearities for all analytes.

**Keywords:** Sexual Assault, Sample Treatment, LC/MS-MS

## Method Validation of 39 Pain Management Drugs in Six Common Toxicological Matrices using DPX-WAXS Tips and LCMSMS

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**Background/Introduction:** When working in an antemortem and postmortem toxicology laboratory it is most efficient to develop methods that can quantitate many commonly detected drugs in multiple assays simultaneously. The wide range of concentrations of drugs and metabolites observed in blood, urine and tissues make it challenging to create multidrug assays. Opioids have recently taken over as the most commonly seen drug class at the Orange County Lab. Other drugs that are commonly ingested with opiates, such as muscle relaxants and tricyclics would be beneficial to quantitate on the same assay to reduce the number of samplings and extractions for cases.

**Objective:** To develop an LCMSMS method that uses a single sample preparation for the quantitation of 6-monoacetylmorphine (6-MAM), acetaminophen, acetylfentanyl, amitriptyline, baclofen, benzoylcegonine, buprenorphine, carisoprodol, cocaethylene, cocaine, codeine, cyclobenzaprine, dihydrocodeine, EDDP (methadone metabolite), fentanyl, gabapentin, hydrocodone, hydromorphone, ketamine, meperidine, meprobamate, metaxalone, methadone, methocarbamol, methorphan, mitragynine, morphine, N-desmethyltramadol, norbuprenorphine, normeperidine, norpropoxyphene, nortriptyline, O-desmethyltramadol, oxycodone, oxymorphone, pregabalin, propoxyphene, tapentadol and tramadol in blood, urine, brain, liver, stomach contents, and other aqueous samples.

**Method:** A thorough validation was conducted for each compound listed above. The validation was completed in accordance with the SWGTOX method validation guidelines and the Orange County Crime Lab validation requirements. Aspects evaluated within the validation were calibration curve, bias and precision, ion suppression and enhancement, sensitivity, specificity, carry-over, dilution integrity, method deviations and stability. The final check of the method was to run 30 previously analyzed samples to ensure the results from new method are within 20% of the results from the previously used method.

**Results:** An acetonitrile protein precipitation extraction was validated using, 50  $\mu$ L of deuterated internal standard, 0.5 mL of sample (blood, urine, liver homogenate, brain homogenate, or stomach content homogenate), and 1.5 mL of acetonitrile. After centrifugation, the supernatant was then rapidly "cleaned-up" using DPX-weak anion exchange with salt (WAX-S) tips that selectively remove matrix interferences from the samples by dispersive SPE. The samples were processed by aspirating and mixing the sample solutions with the WAX-S sorbent using a pneumatic extractor, which allowed up to 48 samples to be processed simultaneously in just a few minutes. The samples were then diluted in mobile phase and injected onto a Waters Acquity UPLC coupled to a Waters TQ-S triple quadrupole mass spectrometer utilizing positive electrospray ionization in MRM mode. A Phenomenex Kinetex® 1.7  $\mu$ m biphenyl column (2.1 x 100 mm) held at 40°C with a gradient mobile phase using 0.1% formic acid in water and 0.1% formic acid in acetonitrile at 0.4 mL/min was used for chromatographic separation. A quadratic calibration model with a weight of 1/x and no forcing through the origin was used for all drugs. Limit of quantitation (LOQ) was administratively decided for each drug based on previous casework and literature references. Instrumental limit of detection and LOQ were also determined, but not used in routine casework. No significant ion suppression or enhancement was seen during the validation. None of the over 100 drugs or 30 matrices, including decomposed blood and tissue, tested caused any interference for any drug or internal standard.

**Conclusion/Discussion:** This method was shown to have adequate accuracy and precision for the quantitation of the all 39 drugs in the six matrices. Due to results from the bias and precision experiments, propoxyphene and mitragynine cannot be quantitated in brain, 6-MAM cannot be quantitated in liver, N-desmethyltramadol cannot be quantitated in brain and urine, and norbuprenorphine can only be quantitated in blood. The method allows for accurate quantitation of targeted drugs in complex matrices while significantly reducing sample volume and sample preparation and analysis time.

**Keywords:** Method Validation, LCMSMS, Pain Management Drugs

## Application of a Fully Automated Biochip Analyzer to the Simultaneous Detection of 20 Drugs of Abuse and Creatinine in Less than 20 Minutes from One Urine Sample

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**Background/Introduction:** Rapid drug screening is of vital importance in workplace, hospital and criminal drug testing in the case of overdose, criminal acts and where safety is critical. Urine is the testing sample of choice for many drug tests. However, because it is easy to dilute it is important to check for adulteration by examining the amount of creatinine present.

**Objective:** This study presents the application of the rapid screening system Evidence MultiSTAT, which is based on biochip array technology, to high density immunoassay based simultaneous detection of 20 drugs of abuse and creatinine from a single urine sample in less than 20 minutes whilst maintaining sensitivity and accuracy.

**Methods:** Simultaneous competitive chemiluminescent immunoassays on a biochip surface applied to the Evidence MultiSTAT analyzer were employed. This fully automated system processes a self-contained cartridge containing all the components required for the immunoassay reactions and has the capacity to assess two biochips in under 20 minutes. Sampling 25µl of urine against a cut-off sample, the results obtained are qualitative.

Precision was determined by preparing and assessing samples at +50% of the cut-off and -50% of the cut-off against a cut-off sample. The samples were analyzed twice per day for 10 days on 2 different analyzers giving a total of 40 replicates. The qualitative results were determined and presented as percentage agreement.

Accuracy was determined as follows: 100 negative urine samples were used: 40 negative samples and 60 spiked samples (spanning the cut-off). Each sample was run against a cut-off sample, a qualitative result was determined for each of the 20 drug classes and creatinine and the results presented as percentage agreement.

In addition, 30 authentic urine samples were collected and assessed. A qualitative result was determined for each of the drug classes and the results presented as percentage agreement to LC/MS.

**Results:** The following drug classes were detected with the associated cut-off values: AB-Pinaca (2.5ng/mL), alpha-PVP (5ng/mL), amphetamine (200ng/mL), barbiturates (200ng/ml), benzodiazepines (150ng/mL), benzoylecgonine/cocaine (150ng/mL), buprenorphine (1ng/mL), ETG (750ng/mL), fentanyl (2ng/mL), JWH-018 (20ng/mL), 6-MAM (5ng/mL), methadone (300ng/mL), methamphetamine (200ng/ml), opiate (200ng/mL), oxycodone (50ng/mL), THC (20ng/mL), tramadol (5ng/mL), UR-144 (10ng/mL) and creatinine (20mg/dL).

The evaluation of the repeatability (40 replicates +50% and -50% analyzed across 2 analyzers) and accuracy (40 negative and 60 spiked samples) showed percentage agreement of >90% for all assays. The multi-analytical screening of 30 authentic samples showed positive for one or more of the analytes present on the array, with the highest positivity rate seen for THC and benzoylecgonine / cocaine. The percentage agreement with LC/MS was as follows: 100% for 6-MAM and oxycodone, 97% for benzodiazepines, methadone and opiate, 93% for amphetamine, buprenorphine, methamphetamine, and THC and 80% for benzoylecgonine/cocaine. All samples screened positive for the presence of creatinine at greater than 20mg/dL indicating that no sample adulteration had occurred.

**Conclusion/Discussions:** The data indicates that 20 drug classes and creatinine can be screened in less than 20 minutes from a single urine sample by using the fully automated Evidence MutiSTAT system. The cut-offs achieved are extremely sensitive and applicable for a urine matrix with reproducible and accurate results. This reported new application, utilizing the biochip array technology, is an effective, reliable, quick and user friendly solution for urine drug testing.

**Keywords:** Biochip Array, Rapid Drug Screening, Urine

## Validation of an Automated Multi Component Method Using Protein Precipitation LC-MS-MS for the Analysis of Whole Blood Samples

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**Background/Introduction:** Analytical chemical methods used in forensic toxicology normally cover classes of drugs with the same kind of chemical structure and properties. Multi component methods covering a wide range of chemical structures and properties have been described, but have been used for the analysis of oral fluid or plasma. To our knowledge, only a few multi-methods exist for the extraction of drugs from whole blood, however, solid phase extraction (SPE) is often used for sample preparation in these methods, but is a very time-consuming and labour-intensive method. Protein precipitation is a much simpler and faster sample pre-treatment method than SPE, and protein precipitation also has the ability to cover a wider range of components. However, pipetting of whole blood, especially post-mortem blood, can be challenging, because the samples are differing in regard to viscosity, sedimentation and coagulation.

**Objective:** The aim was to develop a robust automated analytical method for whole blood samples based on protein precipitation. The setup should improve the speed, robustness, and reliability of ante- and post-mortem whole blood sample preparation for toxicological analysis; from the primary sample tube to a 96-deepwell plate ready for injection on the liquid chromatography mass spectrometry (LC-MS/MS).

**Methods:** The automated method was based on a previously published manual method (Sørensen et al., 2013). This method is a multi-component method for 47 analytes using protein precipitation by addition of methanol and acetonitrile. Deuterium-labelled internal standards were added to the samples prior to extraction. The extract was centrifuged, filtered, evaporated, resolved in eluent and analysed directly with LC-MS/MS. High and low quality control samples (QCs), consisting of blank blood samples spiked with the analytes, were included in each run. As a method comparison experiment the samples and controls were analysed by both the manual and automated sample preparation methods on the same day, and by the same laboratory technician. The automated procedure used a Freedom EVO 200 platform from Tecan AG, Switzerland. The sample extracts were injected on the same LC-MS/MS instrument using the same batch of eluent. The analysis was repeated with new samples, controls and laboratory technicians on 8 occasions.

**Results:** Linearity plots between the manual and the automated procedure for samples of ante- and post-mortem blood showed linear tendencies with a correlation factor of 0.9897, slope of 1.0005, and intercept of 0.0008. The samples showed good correlation in analyte concentrations. Comparison of QC's between the automated and manual procedures showed the same repeatability ( $RSD_R$ ) for both low and high controls, with some deviations. When using an automated procedure, the  $RSD_R$  was lower for 30 out of 47 components, and the bias was closer to zero for 43 out of 47 components for the low QC. For the high QC the  $RSD_R$  was lower for the automated procedure for 27 of the analytes, and the bias was closer to zero for 13 out of 47 components. Compared to the original validation parameters, most of the components had a better RSD, when using the automated procedure.

**Conclusion/Discussion:** The overall analytical performance of the method was improved for most of the analytes when using automated sample preparation. The risk of sample mix up was minimized, resulting in better traceability of the samples and repetitive work was avoided. In conclusion, an automated protein precipitation sample preparation method has been successfully developed and implemented for whole blood samples.

**Keywords:** Automated Protein Precipitation, Whole Blood, LC/MS/MS.



## LC-MS-MS Method Development and Analysis of Stimulants, Opiates, Synthetic Opiates, PCP, and Benzodiazepines in Wastewater. Preponderance of these Drugs During Football Games

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**Background/Introduction:** The analysis of municipal wastewater to study real time drug usage and trends was first proposed in 2001 by Daughton, using sewage-based epidemiology (SBE). Four years later, Zuccato *et al.* were the first to put SBE in practice, in Italy. Since 2005, SBE has been used in cities in Europe, North America, Asia, and Australia. Recently, efforts have turned toward monitoring changes in drug usage during special events. Sporting events draw a large number of attendees to one location, so it is important to determine whether these events are resulting in higher drug usage in the venue and community. Even though Gerrity *et al.* (2011) studied the effect of a major televised sporting event, it was done at a different location from the event. However, no prior studies have analyzed the direct wastewaters from the sporting venue and the surrounding areas. Various stimulants, opiates, benzodiazepines, and miscellaneous drugs were evaluated in the City of Oxford, MS (Oxford) and at the University of Mississippi (Ole Miss) by analyzing wastewater samples collected from both places during football games and on normal days. For LC-MS/MS analysis, these drugs were split up based on their drug class into a total of 4 panels. Stimulant drugs were Panel 1, opiates were Panel 2, synthetic opiates and PCP were Panel 3, and benzodiazepines were classified into Panel 4.

**Objective:** A method was developed for the analysis of stimulant drugs, opiates, synthetic opiates, PCP, and benzodiazepines in wastewater samples using liquid chromatography coupled with tandem mass spectrometry (LC-MS-MS). The LC-MS-MS system consisted of a Shimadzu Prominence HPLC with a dual pump, a vacuum solvent microdegasser, a controlled-temperature autosampler, and an MS-MS detector (Applied Biosystems/MSD Sciex Qtrap 3200 with a turbo-ion ESI source operating the positive-ion multiple reaction monitoring, or MRM, mode). Separation was achieved on a Synergi Hydro-RP column (150 x 3.00 mm; 4  $\mu$ m; 80 Å) from Phenomenex (Torrance, CA, USA).

**Methods:** For the sample preparation, thirty-five (Panel 1), thirty-six (Panel 2), thirty-four (Panel 3), and thirty (Panel 4) wastewater samples were extracted and analyzed using the validated procedures. Although the validated methods used 1 mL aliquots of negative wastewater, 15 mL (Panels 1-3) and 30 mL (Panel 4) of wastewater was extracted from each sample; this pre-concentration step was added due to the low concentrations found within the wastewater samples. When calculating the concentrations of the samples, this additional volume was taken into consideration.

**Results:** The LOD and LOQ ranged from 0.075 – 2.5 ng/mL for these methods, varying for different compounds and metabolites. The within-batch %CV for the 10 ng/mL control ranged from 0.9-14.7, and the batch-to-batch %CV ranged from 1.9-11.5. The within-batch %CV for the 50 ng/mL control ranged from 0.8-11.9, and the batch-to-batch %CV ranged from 1.7-12.3. The collected samples were analyzed using a validated method and found to contain Amp, Meth, MDMA, MDA, Coc, BE, codeine, hydrocodone, hydromorphone, morphine, norhydrocodone, oxycodone, oxymorphone, tramadol, EDDP, meperidine, normeperidine, methadone, alprazolam,  $\alpha$ -OH-alprazolam, nordiazepam, oxazepam, and temazepam. None of the samples contained MDEA, 6-MAM, fentanyl, norfentanyl, PCP, chlordiazepoxide, flurazepam, 2-OH-ethylflurazepam, 7-NH<sub>2</sub>-flunitrazepam, and  $\alpha$ -OH-triazolam.

**Conclusion/Discussions:** Four LC-MS-MS methods were successfully validated for the analysis of stimulants, opiates, synthetic opiates, PCP, and benzodiazepines in wastewater samples. The methods were reproducible for all the drugs and were applied to analyze wastewater samples collected from the University of Mississippi and City of Oxford around football game times. These methods can be helpful for law enforcement agencies (such as the DEA and the local police department) to know the drug flow at any community.

**Keywords:** Wastewater, Drugs of Abuse, LC-MS/MS

**Validation of a Screening Method in Visceral Blood And Bile By GC/MS as Alternative Matrices to Peripheral Blood**

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**Background/Introduction:** In forensic toxicology, the screening for non-targeted drugs in post-mortem samples is a great challenge. Although, liquid chromatography-mass spectrometry (LC-MS) has become increasingly important in recent years, LC-MS is expensive and not available to all laboratories. Gas chromatography-mass spectrometry is still useful in comprehensive drug screening, especially for the possibility to use large reference libraries.

Although urine is often the preferred matrix it is not always found in an autopsy, so we replaced it with bile and blood obtained from tissue (visceral blood). As in urine the concentration of drugs and metabolites also tends to be high in bile and visceral blood.

**Objective:** The validation plan for bile and visceral blood was established according to the concept delineated by SWGTOX, based on Scope of the Method for Qualitative confirmation/identification. Carryover, interference, detection limit and stability were the parameters validated.

**Methods:** Seventy-eight reference substances (barbiturates, antidepressants, benzodiazepines, amphetamines, cocaine, opioids etc.) were kindly provided by pharmaceutical companies and The United Nations and were split into thirteen (13) groups. Different sources of the blank matrix of bile and visceral blood were fortified at a concentration of 1 mcg/ml and 5 mcg/ml using prazepam as an internal standard at a concentration of 5 mcg/ml with each group of drugs and it was considered the substance detected when the signal-to-noise ratio was more than 3:1.

HCL 6N was added to 1 ml of bile which was then centrifugated and the sample was adjusted to pH 8-9 using buffer Tris, a double liquid-liquid extraction was made using dichloromethane and afterwards dichloromethane: isopropanol (4:1).

1.5 ml of buffer Tris was added to 3ml of visceral blood. Extrelut columns were used for extraction with chloroform: acetone (80:20).

Organics phase were evaporated to dryness and re-dissolved in methanol then injected into the CG-MS in full scan screening condition.

Selectivity was evaluated using several blank matrices of bile and visceral blood. Stability to two freeze/thaw cycle was estimated. Several concentrations were used to determine carryover.

**Results:** It was possible to detect most of the seventy-eight drugs at a concentration of 1 mcg/ml in our assay conditions, no interferences were detected. At concentrations of 20mcg/ml, some drugs have carryover.

**Conclusion/Discussions:** Our laboratory has a high number of samples in our routine work, we analyze both matrices visceral blood and bile (when they are available) for general unknown screening. This CG-MS method is quick and relatively low cost. The applicability in authentic postmortem samples was successful and has improved the number of positive samples in relation with peripheral blood and tissue.

**Keywords:** Bile, Postmortem Samples, Visceral Blood

## Reliable Determination of Cyanide, Thiocyanate, and Azide in Whole Blood by GC-MS and its Application in NAGINATA-GC-MS Screening

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**Background/Introduction:** Cyanide and azide, which are inorganic toxic anions, are important target compounds to be analyzed in forensic toxicology because they are sometimes used in crime. Cyanide and its metabolite, thiocyanate, and azide in human biological fluids are commonly analyzed by GC-MS after derivatization with pentafluorobenzyl bromide (PFBBR) using extractive alkylation technique. However, the published method has some drawbacks, such as the narrow linearity range of the calibration curve and rapid damage to the GC-MS column.

**Objective:** In this study, we examined each step of the representative methods<sup>1-2)</sup> and tried to establish a more reliable method to determine the above compounds in human whole blood. Further, we examined the applicability of the established method to NAGINATA-GC-MS screening that can quickly identify the anions and give approximate concentrations without using toxic anions as standards.

**Methods:** The following steps were examined: 1) The deproteinization method; we examined various kinds of acids and solvents to find the most suitable deproteinization agent. 2) Internal standard (IS); benzyl derivatives of cyanide, thiocyanate, and azide and isotopes of potassium cyanide ( $K^{13}C^{15}N$ ) were examined as candidate IS compounds, and the linearity range of the calibration curve was compared with those methods using tribromobenzene (TBB). 3) Cause of the column damage; we searched for a causative agent of the column damage including a derivatization reagent and the phase transfer catalyst, tetradecyldimethylbenzylammonium chloride (TDMBA). 4) Conditions of GC-MS; the conditions of GC-MS that can separate the azide derivative from the derivatization reagent, PFBBR, were determined as follows:

Apparatus, Agilent 7890B GC-5977B MS; Column, Agilent HP-5MS UI (30 m × 0.25 mm i.d., 0.25 μm film thickness); Temperature: Column, 40°C (2 min)–10°C/min–100°C–20°C/min–300°C (5 min); Injection port, 250°C; Interface, 280°C; Carrier gas, Helium; Ionization, EI-full scan; Constant pressure mode (RTL compound diazepam-d<sub>3</sub>, RT = 17.919 min).

5) Mass spectrum, retention time, and peak area ratio of qualifier ion to target ion of PFB derivative of each compound and IS (TBB) were registered to the database of NAGINATA software (Nishikawa). Further, the information on each calibration curve was registered.

**Results:** The analysis of cyanide in whole blood was possible only when the blood was deproteinized with trichloroacetic acid. We decided to analyze thiocyanate and azide without the deproteinization step because the recovery rate of these compounds significantly decreased with any kind of acids and solvents.  $K^{13}C^{15}N$  was selected as the IS for cyanide determination to give a good linearity of the calibration curve. The calibration curves of azide and thiocyanate were linear using the respective benzyl derivative as well as TBB as the IS. The damage to the column was found to be caused by the phase transfer catalyst (TDMBA) moving into organic layer and remaining in the column. The catalyst was successfully eliminated by passing through an ethyl benzoic sulfonic silica gel column (Agilent Bond Elut-SCX, 50 mg). The calibration curve of each compound was linear in the concentration range from 0.02 to 1.0 μmol/ml, and the precision and accuracy data were satisfactory. The three compounds were quickly identified using NAGINATA software, and the approximate concentration of each compound in whole blood was obtained at the same time.

**Conclusion/Discussions:** A simpler and more reliable method of determining cyanide, thiocyanate, and azide in human whole blood was developed. Because NAGINATA-GC-MS screening can quickly identify these poisons without using toxic compounds as standards, it should be useful in forensic department as well as the laboratories of emergency hospitals.

**Keywords:** Cyanide, Thiocyanate, Azide

## Quantitative Determination of Ethyl Glucuronide and Ethyl Sulfate in Postmortem and Antemortem Whole Blood Using Phospholipid Removal 96-Well Plates and UHPLC-MS/MS

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**Background/Introduction:** Postmortem ethanol formation is a well-known problem in forensic toxicology. The hip-flask defence, i.e. claiming ethanol intake after an incident, is difficult to refute by only ethanol analysis. The non-oxidative metabolites of ethanol, ethyl glucuronide (EtG) and ethyl sulphate (EtS) are direct ethanol metabolites and can be used to distinguish antemortem ethanol intake from postmortem formation of ethanol and in addition can be a helpful tool in assessment of the hip-flask defense.

**Objective:** To develop and validate a high-throughput method for determination of EtG and EtS in antemortem and postmortem whole blood on 96-well phospholipid removal plates followed by UHPLC-MS/MS.

**Methods:** To an aliquot of 100  $\mu$ L whole blood, internal standard and water was added before protein precipitation with 400  $\mu$ L ice-cold acetonitrile (ACN). The supernatants were filtered through a 96-well phospholipid removal plate from Phenomenex (Torrance, CA, USA). The filtered samples were evaporated to dryness and reconstituted in 150  $\mu$ L water/ACN/formic acid (99/0.1/0.1, v/v). Identification of compounds was performed using multiple reaction monitoring (MRM) in the negative mode. EtG and EtS deuterium labelled-internal standards were used. Gradient elution was performed on a C18 column (100x2.1 mm, 1.8  $\mu$ m) with MeOH and 0.1% formic acid. The run time was 4 min and 0.5  $\mu$ l was injected on an Aquity UPLC I-Class system with a Xevo TQS tandem-quadrupole mass spectrometer (Waters).

**Results:** Satisfactory linearity was achieved ( $R^2 \geq 0.999$ ) for EtG in the range 0.09-22.2 mg/L (0.4-100  $\mu$ mol/L) and EtS 0.025-6.3 mg/L (0.2-50  $\mu$ mol/L). The limit of detection (LOD) and limit of quantification (LOQ) were 0.009 and 0.067 mg/L (0.04 and 0.3  $\mu$ mol/L) for EtG and 0.0025 and 0.019 mg/L (0.02 and 0.15  $\mu$ mol/L) for EtS, respectively. Accuracy (bias) and precision (relative standard deviation) were studied at four concentration levels of spiked whole blood samples, and were  $\leq \pm 10\%$ . The use of deuterium labelled-internal standard appeared to be appropriate for the EtG and EtS assay and the matrix effects were found to be negligible in spiked whole blood samples. Method comparison was carried out with a previously used UHPLC-MS/MS method and satisfactory agreement was achieved.

**Conclusion/Discussions:** A sensitive and specific method for quantitative determination of EtG and EtS in antemortem and postmortem whole blood has been developed. The method has been running on a routine basis for about 1 ½ years, and has proven to be robust and reliable with satisfactory long-term precision and bias, and with z-score  $< \pm 1$  for external quality control samples.

**Keywords:** Ethyl Glucuronide (EtG) and Ethyl Sulphate (EtS), Phospholipid Removal Plates, Ultra high Performance Liquid Chromatography-Tandem Mass Spectrometry (UHPLC-MS/MS)

**Highly Sensitive Determination of Alendronate in Human Plasma and Dialysate Using HPLC-MS/MS**

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**Background/Introduction:** Alendronate is widely used for prevention and treatment of osteoporosis. However, the drug is very difficult to detect due to its strongly hydrophilic properties.

**Objective:** The simple and sensitive determination of alendronate in human samples is required for the therapeutic drug monitoring (TDM) of effective treatment. We established a simple and sensitive method for the analysis of alendronate in human plasma and dialysate using a MonoSpin SAX<sup>®</sup> extraction, and HPLC-MS/MS following methylation with trimethylsilyldiazomethane.

**Methods:** Plasma or dialysate samples (500  $\mu$ l) spiked with the alendronate and alendronate-*d*<sub>6</sub> (IS) were diluted with 500  $\mu$ l of distilled water. The MonoSpin SAX<sup>®</sup> column was pre-activated with 750 mM NaF and distilled water. The sample mixture was loaded onto the activated column and then centrifuged at 15,000 *g* for 1 min. It was then washed with distilled water, followed by 2% TFA aqueous solution to elute the drugs from the column by centrifuge at 8,000 *g* for 1 min. The eluate was evaporated with the evaporator, reconstituted in 20  $\mu$ l of distilled water, and methylated using 200  $\mu$ l each of methanol and trimethylsilyldiazomethane. After the incubation at room temperature for 40 min, the solution was evaporated to dryness under a stream of nitrogen. The residue was dissolved in 100  $\mu$ l 10 mM ammonium acetate and a 10- $\mu$ l aliquot was injected into the HPLC-MS/MS.

**Results:** The chromatographic separation of the derivatives for alendronate and alendronate-*d*<sub>6</sub> was achieved on a *L-column2* ODS metal-free column (50 mm x 2 mm i.d., particle size 3  $\mu$ m) with a linear gradient elution system composed of 10 mM ammonium acetate (pH 6.8) and acetonitrile at a flow rate of 0.4 ml/min. Quantification was performed by multiple reaction monitoring (MRM) with positive-ion electrospray ionization (ESI). Distinct peaks appeared for alendronate and the internal standard on each channel within 1 min. The regression equations showed good linearity from 2.0–100 ng/0.5 ml for the plasma and 1.0–100 ng/0.5 ml for the dialysate, with the limits of detection at 1.0 ng/0.5 ml plasma and 0.5 ng/0.5 ml for dialysate, respectively. Extraction efficiencies of alendronate for plasma and dialysate were 41.1–51.2 % and 63.6–73.4 %, respectively. The coefficient of variation (CV) was less than 5.5%. The method was successfully applied to actual analyses of real plasma and dialysate samples, derived after intravenous administration of alendronate, which followed the present method.

**Conclusion/Discussions:** The present method will be useful for simple and sensitive determinations for alendronate in clinical analyses. We are currently conducting trials with this technique for the detection of other classes of strongly hydrophilic properties in human body fluid samples.

**Keywords:** Alendronate, Plasma and Dialysate, Tandem Mass Spectrometry (MS/MS)

**Determination of Valproate in Human Samples by Modified QuEChERS Extraction and GC-MS/MS**

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**Background/Introduction:** Valproate is widely accepted as a drug of first choice for patients with epilepsy. It is also associated with misuse or abuse.

**Objective:** The rapid and sensitive determination of valproate in human samples is required for diagnosis and effective treatment of intoxication and for forensic purposes. We established a high sensitivity QuEChERS (Quick, Easy, Cheap, Effective, Rugged, and Safe) extraction and GC-MS/MS method for the analysis of valproate in human samples.

**Methods:** For QuEChERS extraction, 200  $\mu$ l of plasma samples spiked with valproate and secobarbital-*d*<sub>5</sub> (IS) were diluted with 1,300  $\mu$ l of distilled water. Acetonitrile (1 ml) was added followed by 0.4 g MgSO<sub>4</sub> and 0.1 g NaOAc. After a centrifugation step (2,000 g  $\times$  10 min), 1 ml of the supernatant was transferred to a dSPE tube containing 150 mg MgSO<sub>4</sub> and 50 mg C<sub>18</sub>. This mixture was vortexed and centrifuged at 3,000 g for 5 min, and then the upper layer was evaporated to dryness under a stream of nitrogen. The residue was dissolved in 40  $\mu$ l ethyl acetate and a 1- $\mu$ l aliquot was injected into the GC-MS/MS.

**Results:** The chromatographic separation of the compounds was achieved on a fused-silica capillary column Rxi-5Sil MS (30 m  $\times$  0.25 mm i.d.; 0.25  $\mu$ m film thickness) and detected with electron impact ion source (EI) operating MS/MS mode. The regression equations showed good linearity ( $r > 0.9998$ ) from 50–5000 ng/ml for the plasma, with the limits of detection at 10 ng/ml plasma. Extraction efficiencies of valproate for plasma were 71.2–103.5 %. The coefficient of variation (CV) was less than 8.3%. The method was successfully applied to actual analyses of an autopsy sample, due to the valproate intoxication.

**Conclusion/Discussions:** The present method will be useful for high sensitive determinations for valproate in clinical and toxicological analyses. We are currently conducting trials with this technique for the detection of other drugs in human body fluid samples.

**Keywords:** Valproate, QuEChERS Extraction, GC-MS/MS

## **Ion Mobility Derived Collision Cross Section as an Additional Measure to Support the Rapid Analysis of Drugs of Abuse and Toxic Compounds Using Electrospray Ion Mobility Time-Of-Flight Mass Spectrometry**

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**Background/Introduction:** Increases in cases involving drugs of abuse leads to a heavy case load for law enforcement agencies, intensifying the need for a rapid screening technique. Additionally, traditional methods take a long time to get the chromatographic results, which is a burden for crime investigation. Consequently, high-throughput screening methods that can quickly finish identification are desirable.

**Objective:** The aim of the present study is to demonstrate the use of ion mobility as a tool to support the rapid analysis of drugs of abuse and toxic compounds. Ion mobility spectrometry, which could be used together with tandem mass spectrometry, allows for the measurement of the collision cross section (CCS) and provides information about the shape of an ionic molecule in the gas phase. This technique enables instantaneous determination of drugs and toxic compounds in samples with confidence.

**Methods:** In this current study, the hyphenation of ion mobility spectrometry (IMS) with high-resolution quadrupole time-of flight mass spectrometry (QTOFMS) was used to measure the CCSs of 124 common drugs of abuse and toxic compounds in positive ionization mode. In order to insure the empirical CCS values were stable and thus may add more confidence in the identification process, two internal standards were involved for the mobility calibration and accuracy estimation in the CCS measurements. Then we systematically studied the reproducibility of the CCS measurements whether in vitro or in vivo on this commercial instrument.

**Results:** In this study, we generated a CCS database of the 124 drugs of abuse and toxic compounds, studied the reproducibility of the CCS measurements whether in vitro or in vivo on this commercial instrument systematically. This work provides the proof of concept for the analysis of drugs of abuse and toxic compounds based on accurate collision cross section and mass measurements without the need for derivatization and pre-fractionation protocols, thus significantly reducing the cost and analysis time. The CCS measurements showed high intra- and inter-day precision, and they were not affected by complex sample matrices such as extracted blood and urine. The method was applied to real samples, which were collected from forensic cases.

**Conclusion/Discussions:** Eventually, when applying the proposed procedure to several real samples from a forensic laboratory, the availability of IM-MS instrument made it feasible to perform the identification and confirmation of the detected compounds. Therefore, it is considered that ion mobility-derived collision cross section could be used as an additional measure to support the rapid analysis of drugs of abuse and toxic compounds.

**Keywords:** Abuse Drugs and Toxic Compound, Ion Mobility, Collision Cross Section

## Ultrasound can be Applied in the Derivatization of Drugs of Abuse? A Pilot Study with Silylation of Amphetamine, MDA and MDMA using GC/EI-MS and Univariate Experimental Optimization

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**Background/Introduction:** In the analysis of polar and/or less volatile organic compounds by gas chromatography the derivatization reactions are essential. However, this step requires additional energy consumption due to necessity of high temperatures and long reaction times. In this sense, new methods have been proposed as alternatives to the traditional protocols. The ultrasound radiation is one of the emerging methods that have been successfully applied in organic reactions and has potential for derivatization of compounds of forensic interest as drugs of abuse.

**Objective:** Evaluation of the ultrasound use in silylation derivatization of amphetamine, MDMA and MDA.

**Methods:** 10  $\mu\text{L}$  of a 10  $\mu\text{g}/\text{mL}$  methanolic solution of amphetamine, MDMA, MDA and deuterated internal standards (amphetamine- $\text{d}_{11}$  and MDMA- $\text{d}_3$ ) were dried, diluted in ethyl acetate to a final volume of 80  $\mu\text{L}$  and derivatized with N-Methyl-N-(trimethylsilyl) trifluoroacetamide (MSTFA). The reactions were performed in three conditions: (1) following a conventional derivatization protocol by heating at 85°C for 20 minutes, (2) at room temperature, without temperature control and (3) in ultrasonic bath, at a frequency of 40 kHz and potency of 100 W. To select the optimal conditions for ultrasound assisted derivatization, univariate experimental optimization was applied and three variables, temperature, time and volume of MSTFA, were tested, at three levels each, setting constant two variables and studying the effects in the third one. The levels of each tested parameter were: for time, 5, 10 and 20 min; for volume of MSTFA, 10, 20 and 30  $\mu\text{L}$ ; and for temperature, 24, 30 and 40°C. The analyses were performed in GC-MS with electron impact (EI) detector. The univariate analysis was performed using one-way ANOVA followed by Tukey-Kramer test for multiple comparisons, when significant differences were detected.

**Results:** Comparing the results of the different derivatizations, no statistically significant differences were observed, for confidence level of 95%, for amphetamine and MDA. For MDMA, conventional derivatization was significantly different from the ultrasound assisted derivatization ( $p = 0.018$  and  $F\text{-value} = 5,682$ ), resulting in a higher analytical response. In the optimization of ultrasonic derivatization conditions, no statistically significant differences were observed among the selected range of values for time and ultrasound bath temperature. However, better precision (with coefficient of variation less than 15%) was obtained for reactions conducted for 10 min and at 24°C. In the study of MSTFA volume influence, no statistically significant differences were observed but the volume of 10  $\mu\text{L}$  was not sufficient to derivatize all samples and the volume of 30  $\mu\text{L}$  of MSTFA was the best condition based in the precision of the analytical responses observed for amphetamine, MDA and MDMA.

**Conclusion/Discussions:** Although ultrasound assisted derivatization does not promote an increase in the analytical response relative to conventional derivatization, the statistical analysis shows that both conditions are comparable. According to the analysis of the results, based on the ANOVA and precision criteria, the best conditions were 30  $\mu\text{L}$  of MSTFA, 10 min of reaction and an ultrasonic bath temperature of 24°C. The time of 10 min allows a large analytical gain over the conventional protocols. In addition, it is known that increasing the temperature of the medium reduces the rate of a reaction under ultrasound radiation and the selection of the lowest tested temperature goes according to this physico-chemical principle of sonochemical reactions. In summary, our results are promising since it showed that the ultrasound can be successfully applied in silylation reactions of amphetamine, MDA and MDMA, suggesting its potential for derivatization of other drugs and/or to other types of derivatization reactions.

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**Keywords:** Sonochemistry, Derivatization, Gas Chromatography



**A New Perspective for an Old Method to Quantify Drugs in Different Matrices by LC-MS/MS.**

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**Background/Introduction:** Over the past few years, requirements for the validation of quantitative methods have become more exhaustive. This is linked in part to adherence of toxicology laboratories to accreditation entities such as ISO 17025 and ASCLD/LAB. Although this generates a more rigorous testing that ensure the adequacy of analytical methods, such validations are time consuming and sometimes at odds with the speed requirements of coroner cases. Here, we present an application of the standard addition technique to perform a fast quantification of a drug which is not part of an accredited method of the toxicology laboratory. This approach is simple, fast, and cheap, requires minimal validation and uses less than 1 mL of biological matrix. It was used to respond quickly to a coroner's demand to analyze quantitatively vortioxetine (Trintellix®) in a suicide case. Vortioxetine is a prescription drug used to treat major depressive disorder (MDD) in adults. Like other antidepressants, it may increase the risk of suicidal thoughts. Since vortioxetine is not detected at low concentrations by the GC-MS general unknown screening, nor part of the LC-MS/MS targeted screening method used in our laboratory, another approach was necessary to provide a quick and reliable result to the coroner.

**Objective:** The goal of this project is to use standard addition to rapidly quantify vortioxetine in post-mortem whole blood by LC-MS/MS, without going through a full method validation process.

**Methods:** 100 µL of the case's post-mortem whole blood was added to 6 different wells of a 96-well plate. 2 µL of a methanol vortioxetine solution of varying concentrations was then added to each well, generating doped concentrations between 1.0 to 80.0 ng/mL. Fortified samples were then diluted with 100 µL of methanol:water (50:50) and vortexed. Protein precipitation was performed using a solution of acetone:acetonitrile (30:70) followed by centrifugation at 3200xg and 20°C for 5 minutes. 25 µL of supernatant was transferred into an injection plate and diluted with 180 µL of 0.2% aqueous formic acid, the final sample composition matching the mobile phase in organic solvent proportion. Gradient separation of 5 µL was performed with 10 mM ammonium formate in water with 5% methanol (mobile phase A) and acetonitrile (mobile phase B) at a 0.6 mL/min flow on a Eclipse Plus C18 3.5µm, 2.1 X 100 mm using a Agilent HPLC 1260. Two MRM transitions were monitored using positive electrospray ionization on an AB Sciex 5500 QTrap instrument. The concentration in post-mortem whole blood was estimated by calculating the value of the X intercept of the linear curve plotting the area (y axis) as a function of doped concentration (x axis).

**Results:** Accuracy (89 to 109%) and precision (intra-batch 5.2%) of this standard addition method was confirmed to be adequate through blind analysis of quality controls. The presence of vortioxetine was confirmed in femoral blood, cardiac blood and urine following protein precipitation extraction of the matrices (see Methods) and comparing the retention time and MRM ratio to a spiked standard. The concentration of vortioxetine in cardiac blood and femoral blood were found to be 31 ng/mL and 3.8 ng/mL, respectively. No interference was observed.

**Conclusion/Discussions:** We presented here a simple, fast and low cost alternative to quantify drugs not present in analytical methods under the toxicology laboratory's validation scope. This method uses a small volume of biological matrix and yields reliable results rapidly, without requiring a full validation. This standard addition technique is an interesting approach to use for either new drugs, or drugs that are rarely present in case work and therefore are not part of a fully validated method.

**Keywords:** Protein Precipitation, Standard Addition, LC-MS/MS

## Evaluation of the Ark™ Pregabalin Urine Assay: Application in Postmortem Toxicology Investigations

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**Background/Introduction:** In Australia, pregabalin (Lyrica®) is a drug approved for the treatment of partial seizures and neuropathic pain. Recently, reports have indicated an increase in the misuse and abuse of pregabalin, as demonstrated by an increase in the number of prescriptions and fatalities involving this drug [1]University of Hertfordshire, School of Life and Medical Sciences, College Lane Campus, Hatfield, AL10 9AB, UK, f.schifano@herts.ac.uk.</auth-address><titles><title>Misuse and abuse of pregabalin and gabapentin: cause for concern?</title><secondary-title>CNS Drugs</secondary-title><alt-title>CNS drugs</alt-title></titles><periodical><full-title>CNS Drugs</full-title><abbr-1>CNS drugs</abbr-1></periodical><alt-periodical><full-title>CNS Drugs</full-title><abbr-1>CNS drugs</abbr-1></alt-periodical><pages>491-6</pages><volume>28</volume><number>6</number><keywords><keyword>Amines/administration & dosage/\*adverse effects/therapeutic use</keyword><keyword>Cyclohexanecarboxylic Acids/administration & dosage/\*adverse effects/therapeutic</keyword><keyword>use</keyword><keyword>Humans</keyword><keyword>Pregabalin</keyword><keyword>\*Prescription Drug Misuse/statistics & numerical data</keyword><keyword>Substance-Related Disorders/\*epidemiology/etiology</keyword><keyword>gamma-Aminobutyric Acid/administration & dosage/adverse effects/\*analogs &</keyword><keyword>derivatives/therapeutic use</keyword></keywords><dates><year>2014</year><pub-dates><date>Jun</date></pub-dates></dates><isbn>1179-1934 (Electronic. Immunoassays are ideal as a preliminary screen for large numbers of samples as these assays can typically generate a result within a few hours. However, any positive results require confirmatory testing to rule out potential cross reactivity as specificity can be an issue.

**Objective:** The aim of this project is to assess the suitability of the ARK™ Pregabalin Urine Assay for the detection of pregabalin in post mortem urine specimens. Screening for the presence of pregabalin using immunoassays in death investigations can enable the rapid detection of this drug in medico-legal casework.

**Methods:** Twenty urine samples from coronial cases previously confirmed to contain pregabalin in blood by a validated liquid chromatography tandem mass spectrometry (LCMS/MS) method were analysed [2]. Additionally, 81 randomly selected post mortem urine samples were analysed for the presence of pregabalin. Cross-reactivity was investigated using another gabapentanoid (gabapentin) at spiked concentrations of 0.4, 0.5 and 2 mg/L. Qualitative immunoanalysis of all urine samples using the ARK™ Pregabalin Urine Assay was performed on the Beckman Coulter AU480 Chemistry Analyser. Briefly, the immunoassay kit employs a cut-off concentration of 0.5 mg/L. Spiked urine specimens (Low @ 0.25 mg/L and high @ 0.75 mg/L) were run as negative and positive controls, respectively. Confirmatory testing of post mortem urine samples involved sample preparation by a liquid-liquid extraction (LLE) and analysis performed using LC-MS/MS (0.1-10 mg/L).

**Results:** No cross-reactivity was observed at the spiked concentrations of gabapentin investigated (0.4, 0.5 and 2 mg/L). All 20 urine samples selected from coronial cases with confirmed pregabalin levels in post mortem blood, were shown to be positive for pregabalin by immunoanalysis. The performance of the immunoassay based on QC data was within the assay limits (low level 0.3 mg/L, with an accuracy of 13.2%, 5.5% precision and 4.7% repeatability and high level 0.6 mg/L, with an accuracy of 4.3 %, 1.7% precision and 1.3% repeatability). The urine concentration of pregabalin for all 20 cases tested by LC-MS/MS were found to be greater than 10 mg/L (the upper limit of quantitation) and not re-analysed diluted (results confirmed as above the 0.5 mg/L immunoassay cut-off). LC MS/MS acceptance criteria included a curve correlation coefficient  $r^2$  value > 0.99 and quality control accuracy of 30%. The performance of the LC-MS/MS analysis at low concentration (0.3 mg/L) was acceptable with percentage residual standard deviations (%RSD) of -6.88% for accuracy and 19.78% and 10.09% intra- and inter-assay responses (n = 6), respectively. At high concentration (0.9 mg/L) performance was also acceptable with -11.11 RSD% for accuracy and 15.74% and 8.79% intra- and inter-assay responses (n = 6), respectively. Immunoanalysis determined 10 positive results from the 81 (11%) post mortem urine samples screened. The lowest quantified concentration of pregabalin in urine by LC-MS/MS was 2.4 mg/L, far exceeding the 0.5 mg/L cut-off concentration. A single false-positive result (rate of 1.4%) was found when comparing the immunoassay findings to the results obtained from LC-MS/MS analysis. This urine was sampled from a decomposed body and the sample failed our in-house suitability test for interfering substances of a cloned-enzyme donor immunoassay.

**Conclusion/Discussions:** Analysing post mortem urine samples for pregabalin by immunoassay enables effective/accurate high throughput screening for the detection of pregabalin in cases where its presence may not have been suspected. Pregabalin is excreted mostly unchanged in urine and therefore cases positive for pregabalin in blood were expected to be positive for pregabalin in urine [1]University of Hertfordshire, School of Life and Medical Sciences, College Lane Campus, Hatfield, AL10 9AB, UK, f.schifano@herts.ac.uk. </auth-address><titles><title>Misuse and abuse of pregabalin and gabapentin: cause for concern?</title><secondary-title>CNS Drugs</secondary-title><alt-title>CNS drugs</alt-title></titles><periodical><full-title>CNS Drugs</full-title><abbr-1>CNS drugs</abbr-1></periodical><alt-periodical><full-title>CNS Drugs</full-title><abbr-1>CNS drugs</abbr-1></alt-periodical><pages>491-6</pages><volume>28</volume><number>6</number><keywords><keyword>Amines/administration & dosage/\*adverse effects/therapeutic use</keyword><keyword>Cyclohexanecarboxylic Acids/administration & dosage/\*adverse effects/therapeutic</keyword><keyword>use</keyword><keyword>Humans</keyword><keyword>Pregabalin</keyword><keyword>\*Prescription Drug Misuse/statistics & numerical data</keyword><keyword>Substance-Related Disorders/\*epidemiology/etiology</keyword><keyword>gamma-Aminobutyric Acid/administration & dosage/adverse effects/\*analogs &</keyword><keyword>derivatives/therapeutic use</keyword></keywords><dates><year>2014</year><pub-dates><date>Jun</date></pub-dates></dates><isbn>1179-1934 (Electronic. Even though this relationship cannot be assumed, the results show that the ARK™ Pregabalin Urine Assay is a reliable test to show the presence of pregabalin in urine when pregabalin has been administered. The screening for pregabalin in urine using the ARK™ Pregabalin Urine Assay and subsequent confirmatory analysis of positive cases for the pregabalin concentrations in blood may serve as a useful method to determine the toxicological significance of pregabalin in coronial death investigation.

1. Schifano, F., *Misuse and abuse of pregabalin and gabapentin: cause for concern?* CNS Drugs, 2014. **28**(6): p. 491-6.
2. Di Rago, M., et al., *Fast targeted analysis of 132 acidic and neutral drugs and poisons in whole blood using LC-MS/MS.* Forensic Sci Int, 2014. **243**: p. 35-43.

**Keywords:** Pregabalin, Urine, Immunoassay

**Rapid detection of illegal drugs in urine by MEPS™-DART-TOF-MS analysis**

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**Background/Introduction:** In Japan, both use and possession of stimulant drugs is prohibited; nevertheless, stimulant offenses (mainly related to the use and possession of methamphetamine) account for over 80% of drug crimes. Our laboratory receives a large number of high-urgency requests for analysis of urinary methamphetamine; thus, a rapid analytical technique is desirable. A method based on solid-phase extraction (SPE) pretreatment and Liquid Chromatography or Gas Chromatography analysis is typically used for the determination of illegal drugs in urine. However, SPE requires long sample processing time, and the chromatographic examination is lengthy and time-consuming. Direct Analysis in Real Time (DART)-TOF-MS has been developed in recent years, in which the samples are introduced directly into the ion source and illegal components are rapidly detected. However, because DART-TOF-MS causes ionization of the entire sample, the sensitivity to target species may be affected by the matrix effect. In this study, micro extraction by packed sorbent (MEPS™), in which the cartridge is integrated into the syringe needle, was used as a pretreatment for simultaneous sample purification and concentration, and DART-TOF-MS was applied to the rapid urinalysis for illicit drugs.

**Objective:** The objective of this study is to develop a rapid qualitative analytical technique based on MEPS™-DART-TOF-MS for urinary methamphetamine, amphetamine, MDMA, and MDA. Limit of detection (LOD) and limit of quantification (LOQ) values of the developed method were calculated, and the analytical utility was assessed.

**Methods:** The optimum temperature of the DART ion source and MEPS™ conditions (load frequency, choice of eluate, volume of eluate, and selection of internal standard) were examined by the following method.

Sample solutions were prepared by adding 0.29 mL of a buffer solution (pH 12) and 10 µL of a 10 µg/mL aqueous solution of internal standard to 0.3 mL of urine to which methamphetamine, amphetamine, MDMA, or MDA was added. C18 was chosen as the solid-phase support for MEPS™, and was conditioned with methanol and buffer solution (pH 12). The sample solution (100 µL) was loaded on the MEPS™, and the solid phase was washed with 100 µL of buffer solution (pH 12) and 100 µL of 5% methanol aqueous solution. Next, the elution was carried out by passing each eluate through the MEPS™. After elution, a drop of eluate was deposited on the needle tip and introduced into the DART ion source for measurement.

**Results/Conclusion:** A constant detection sensitivity was achieved at a DART ion source temperature of 350 °C, and the optimum frequency of loading sample was five times. The best recovery rate was obtained using a 0.1% formic acid methanol solution as the eluate. In addition, the recovery rate was slightly improved when buffer solution (pH 12) was used as the washing solution instead of pure water. As for the amount of eluate, a volume of 50 µL gave the best recovery. Peak area ratio reproducibility for methamphetamine was evaluated using 2-fluoromethamphetamine, 4-fluoroamphetamine, methoxyphenamine, 4-fluoromethcathinone, 4-ethylmethcathinone, and methiopropamine as internal standards, and 2-fluoromethamphetamine gave the best result. The LOD and LOQ of the developed method under the optimized conditions for methamphetamine, amphetamine, MDMA, and MDA were measured to be 5 ng/mL and 16 ng/mL, 8 ng/mL and 20 ng/mL, 7 ng/mL and 20 ng/mL, and 7 ng/mL and 20 ng/mL, respectively. Moreover, a calibration curve was prepared in the range of 20 to 2000 ng/mL, and good linearity was obtained for all analytes. These results suggest that the DART-TOF-MS analysis combined with MEPS™ provides a useful tool for the rapid determination of methamphetamine, amphetamine, MDMA, and MDA in urine samples.

**Keywords:** MEPS, DART, Urine

## Extraction and Quantification of Methadone and its Metabolites in Skeletal Tissue of Chronical Dosed Rats

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**Background/Introduction:** In forensic sciences, the most common ante- and postmortem collected specimens for toxicological analysis include blood, urine, saliva but also gastric contents, vitreous humor and bile. However, when extended time has elapsed before discovery of the body these specimens are often degraded or not available at all due to decomposition. In this case, bone may be the only tissue left. Nevertheless, very limited research can be found on the drug disposition in bone, making interpretation very difficult. Aside to that, methadone is the most common prescribed substitution therapy in opioid addiction and linked to almost 50% of the fatalities due to drugs abuse in Belgium. Therefore, this study investigates the disposition of methadone and its metabolites in bone after chronical dosage.

**Objective:** In this study, we investigate whether skeletal tissue can be used as an alternative matrix for the detection and interpretation of chronic methadone usage. An extraction and quantification method is developed and the distribution pattern of methadone and its metabolites in skeletal tissue of chronically dosed rats is investigated.

**Methods:** For this study, an animal model is used. Male Wistar rats (n = 12) weighing 320-380 g and aged between 80-87 days are administered an intraperitoneal daily dose of 0 (n=6) or 3 mg/kg methadone (n=6) for 139 days. The daily doses are derived using allometric calculations and the Maximal Recommended Tolerated Dose (MRTD) for humans as recommended by the FDA. All drug preparations are diluted using saline in order to create an injection volume of 0.6-0.8 ml. 24 hours after the last injection, animals are euthanized using CO<sub>2</sub>. This experiment was approved by the Ethical Committee Animal Experimentation of the University of Leuven (P 113/2011). Instantly, blood is collected in tubes containing sodium fluoride. Femora, tibia, ulna, radius, humerus and scapula are removed by dissection. These bones are cleaned by scraping the soft tissue off with a scalpel. After this cleaning step, single full bones or bone parts are extracted with methanol at room temperature for 72h. The final extract is analyzed using LC-ESI(+)-MS/MS in scheduled MRM mode for methadone, EDDP, EMDP and methadol. Deuterated internal standards are used to quantify methadone and its metabolites. Matrix-matched calibration curves were constructed and linear ( $R^2 > 0.99$ ) for all analytes. For methadone, The range was set from 0.50-20 ng/mg and for the metabolites from 0.05-2 ng/mg using 7 concentration levels. LOQ was set on 0.5 ng/mg for methadone and 0.05ng/mg for both metabolites with sufficient precision (< 20%).

**Results:** Methadone and its metabolites are proven detectable and quantifiable in bone tissue of chronically dosed rats using a fast and easy methanol extraction. The concentration of methadone and its metabolites found in blood and bone are shown to be very variable among different rats although the daily dosage (in mg/kg) was the same. Moreover, methadone concentrations are shown to vary within one bone of the same rat. The highest concentrations are found near the heads of the joints. The lowest concentrations are detected near the middle part of the bone. This can be explained by the difference in vascularization of the different bone parts. Furthermore, the concentration ratios of metabolite (EDDP or EMDP)/methadone are estimated around 0.05 in full bone tissue.

**Conclusion/Discussions:** Overall, the applicability of bones as an alternative matrix for detection of chronic methadone use is proven. Moreover, an easy extraction procedure is developed and the distribution pattern of methadone and its metabolites in skeletal tissue is described. However, no correlation between dosage and bone concentration is found. Therefore, it can be expected that forensic interpretation regarding usage of methadone based on bone concentrations is impossible.

**Keywords:** Methadone, Skeletal Tissue, Chronical Dosage

**Automated Desorption, SPE Extraction, and LC/MS/MS Analysis of Dried Blood Spots**

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**Background/Introduction:** The extraction of dried blood spots (DBS) typically involves manual intervention. First, a small disc is punched out of the center of a dried blood spot card. Following solvent extraction of the sample, it is also common to include further cleanup using solid phase extraction to improve detection limits or exchange solvents for compatibility with subsequent chromatographic separations. Modern analytical labs are looking to automation to help reduce solvent usage and increase sample throughput while ensuring the high quality of the resulting data.

**Objective:** In this report, the complete automation of dried blood spot analyses by the robotic autosampler is discussed. Examination of a new, automated DBS analyzer that allows samples to be rapidly and effectively desorbed, extracted, and injected into a LC/MS/MS system, is described.

**Methods:** A single robotic X-Y-Z coordinate autosampler commonly used for sample introduction in GC or HPLC can be used to perform a wide variety of sample preparation techniques using a single instrument and controlling software. This sampler can also be configured as part of the LC/MS/MS system. The automated DBS analyzer can also be configured with a fraction collector to provide DBS extracts for subsequent GC/MS analyses.

**Results:** Automated DBS extraction methods for a variety of analytes from different matrices were examined and resulting precision and accuracy data are provided. The DBS-SPEXos-LC/MS/MS method proved to be accurate and precise. For example, for dried rat blood spots, accuracy data averaged 91.7% (range: 82.6% - 104%) and precision data averaged 5.75%CV (range: 2.47% -11.5%) for all compounds analyzed. For dried bovine blood spots, accuracy data averaged 108% (range: 98.6% - 119%) and precision data averaged 5.31%CV (range: 0.288% -10.2%) for all compounds analyzed.

**Conclusion/Discussions:** The development of automated dried blood spot extraction methods was achieved using the GERSTEL DBSA and SPEXos Options with MAESTRO software. Drug compounds of forensic interest, including ketamine, amitriptyline, and warfarin, for example, were successfully extracted from dried blood spot samples using an automated DBS-SPEXos procedure coupled to LC/MS/MS analysis using an Agilent 6460 Triple Quadrupole Mass Spectrometer.

**Keywords:** Forensics, Sample Preparation, Dried Blood Spots

## Headspace Solid-Phase Microextraction and Gas Chromatography-Mass Spectrometry for Determination of Cannabinoids in Human Breast Milk

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**Background/Introduction:** Human milk is a highly complex biological fluid, and it is considered to be the ideal food for infants during their first months of life. On the other hand, licit and illicit substances consumed by the breastfeeding woman can pass into the milk and may cause harmful effects to the infant. Drug extraction from this matrix can be an analytical challenge due to its high protein and lipid content.

**Objective:** Since marijuana is the most commonly used illicit drug at the global level, the aim of the present study was to develop a headspace solid-phase microextraction (HS-SPME) method for the determination of  $\Delta^9$ -tetrahydrocannabinol (THC), cannabidiol (CBD) and cannabinol (CBN) in human breast milk using gas chromatography-mass spectrometry.

**Methods:** 0.05 mL of breast milk was transferred into a 4 mL screw vial, followed by the addition of 7.5  $\mu$ L of the deuterated internal standards (THC- $d_3$ , CBD- $d_3$ , and CBN- $d_3$ ) at a concentration of 1  $\mu$ g/mL. The pH of the solution was adjusted to 10 by using 10  $\mu$ L of 1 M NaOH followed by addition of 940  $\mu$ L of bicarbonate buffer (pH 10). The salting out effect was achieved by adding 0.25 mg of NaCl. The vial was sealed with a Mininert valve and the sample solution was heated at 70 °C with magnetic stirring. For extraction of cannabinoids, the needle of the SPME device was inserted through the septum of the Mininert valve, and the 100  $\mu$ m polydimethylsiloxane fiber (PDMS) was exposed to the headspace for 40 min. After the exposure time, the SPME device was directly inserted into the GC injector port at 250 °C for thermal desorption

**Results:** The limits of detection and quantification were 10 and 20 ng/mL, respectively, for all analytes. The calibration curve was linear over a concentration range of 20–200 ng/mL ( $r^2 = 0.99$ ). Precision and accuracy were performed by analyzing breast milk samples containing the analytes at three quality control (QC) levels: low (40 ng/mL), medium (90 ng/mL) and high (160 ng/mL) at six replicates each. Intraday and inter-day precision was not greater than 13.3% (relative standard deviation), and accuracy bias ranged from 5.5 to 17.9%. One hundred and nine human milk samples were randomly collected and analyzed. Nineteen of these breast milk samples were collected from women who had declared prior drug use and one of them could be confirmed THC-positive at 20 ng/mL, even though CBD and CBN were not detected. In addition, another sample, without previous report of drug use during life or pregnancy was confirmed THC-positive at 31 ng/mL. CBD was also detected in this sample although it could not be quantified as its concentration was lower than the LOQ.

**Conclusion/Discussions:** The present method has shown to be reliable for the determination of THC, CBD and CBN in human breast milk in a very low sample volume (0.05 mL). However, its high LOQ may represent a significant limitation as some biological samples may be at lower concentrations. The HS-SPME procedure was demonstrated to be a rather promising low cost and environmental-friendly technique. In addition, the SPME is very suitable for automated extraction procedures.

**Keywords:** Breast Milk, Headspace Solid-Phase Microextraction, Marijuana

**Qualitative Analysis for Multiple Drug Classes and Metabolites in Serum/Plasma by LC-MS/MS**

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**Background/Introduction:** Each year, millions of Americans present to the emergency department due to an adverse drug reaction. A multi-analyte drug screen panel can be utilized clinically to identify commonly prescribed drugs, over-the-counter medications, and illicit drugs. The identification of commonly used drugs can assist clinicians in providing supportive care for patients with adverse drug reactions [1]. This work presents a developed and validated method to extract 175 drugs and their respective metabolites out of serum/plasma with LC-MS/MS detection.

**Objective:** To expand the quantity of detectable analytes for testing of drugs and drug metabolites in overdose cases.

**Methods:** PLD+ columns (Biotage, Charlotte, NC) were used for the extraction process. The columns were placed on a 96-deep well plate. A crash solvent, consisting of acetonitrile and a mix of internal standards (50-400 ng/mL) was aliquoted into each PLD+ column at a volume of 750  $\mu$ L [2,3,4]. 250  $\mu$ L of control, calibrators, or patient sample was added to their appropriately labeled column. The supernatant was then pushed through the column (no preconditioning necessary) at  $4.1 \times 10^4$ - $8.3 \times 10^4$  pascal (or 1 drop per second) and collected. The samples were dried at 35 °C under nitrogen for approximately 30 minutes, or until visibly dry, at  $1.4 \times 10^5$ - $2.1 \times 10^5$  pascal. Each well was reconstituted in 100  $\mu$ L of a 95:5 solution of clinical laboratory reagent water: HPLC grade methanol. The 96-deep well plate was covered and analyzed by an ABSciex Triple Quad 5500. This assay is an improvement to the previous method used which involved acid/base extraction and analysis by GC-MS.

**Results:** The semi-quantitative or qualitative assay in which the presence of an analyte above or below the respective analyte cutoff concentration would signify reported interpretations as “present” or “not detected”. The cutoffs in this assay are analyte specific and range from 1 to 375 ng/mL. Samples spiked at 50% (n=4 over five days, n=20 total) of the cutoff screened negative; samples spiked at 150% (n=20) of the cutoff screened positive. Any carryover observed was less than the cut-off concentrations. Accuracy and selectivity of calibrators, QC, and spiked samples were within  $\pm 20\%$  of expected values for all 175 analytes. An average recovery range of 68-132% was achieved for all 175 compounds. This is notable due to the varying physical properties of compounds amongst the 19 different classes of compounds (antiarrhythmic, anticoagulant, anticonvulsant, antidepressant, antihistamine, anti-inflammatory, antipsychotic, barbiturates, benzodiazepines, cannabinoids, decongestant, hallucinogens, hypoglycemic, muscle relaxants, nicotine, opioids, sedative-hypnotics, stimulants, and tricyclic antidepressants). The new extraction method greatly cleaned up samples eliminating the need for several blank runs between samples. The new method afforded a 42.9% increase in the detection of compounds in comparison to testing by GC-MS. Consequently, the LC-MS/MS method had a 57.1% agreement rate with the old extraction and GC-MS method.

**Conclusion/Discussions:** This new assay allows for a single extraction method and LC-MS/MS detection method for 175 compounds in one panel that covers 19 different drug classes, allowing for a more dynamic range of drug analysis.

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**Keywords:** LC-MS/MS, Serum/Plasma, Drug Screen



**Evaluation of the Stability of Dried Blood Spots as Quality Controls for Drugs of Abuse**

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**Background/Introduction:** Due to many advantages such as minimally invasive sampling, storage, transport and small volume, dried blood spot (DBS) sampling gained more interest in forensic and clinical toxicology during the last decade. Thus, many methods have been published for screening or drug quantification. However, only few attention has been paid for DBS as quality control (QC) materials for drugs of abuse.

**Objective:** The aim of this study was to evaluate the stability of DBS QC materials for drugs of abuse purpose.

**Methods:** Blank blood sample and 2 blood samples containing NaN<sub>3</sub> and NaF as preservative and spiked with different concentration (level 1 and level 2) of 13 drugs of abuse (amphetamine [24 and 70 µg/L], methamphetamine [24 and 71 µg/L], MDMA [24 and 70 µg/L], MDE [24 and 70 µg/L], MDA [23 and 70 µg/L], cocaine [15 and 71 µg/L], benzoylecgonine [29 and 117 µg/L], ethylcocaine [13 and 66 µg/L], 6MAM [10 and 38 µg/L], free morphine [9.7 and 70 µg/L], free codeine [9.4 and 66 µg/L], oxycodone [21 and 72 µg/L], dihydrocodeine [24 and 68 µg/L]) were used. 10 µl DBS were obtained using HemaXis microfluidic device (DBS system SA, Switzerland). 10 devices (each containing 4 spots) were used for the evaluation of two storage conditions (room atmosphere and under vacuum) at the worst temperature for blood samples storage, namely room temperature. Analyses of 10 µl DBS were performed at D1 (first day of analyses), D1+30 days (D2 – day 2 of analyses) and at D1+90 days (D3 – day 3 of analyses), by LC-MS/MS methods after organic extraction procedure.

**Results:** No drug was detected in blank DBS. All relative standard deviations (N=10 DBS) were comprised for the different drugs between 2.8% and 14% for level 1 (median: 7.8%), and between 1.9% and 12% for level 2 (median: 6.7%). For amphetamine, 6MAM, free morphine and dihydrocodeine, no significant difference (P>0.05) was observed between the two conditions of storage (room atmosphere and under vacuum). For methamphetamine, MDMA, MDE, MDA, cocaine, benzoylecgonine, ethylcocaine, and free codeine, at least one level on one day showed significant difference (P<0.05) between the two conditions of storage, showing a decrease of the concentration without vacuum. For oxycodone, all levels on all days showed highly significant differences (P<0.001) between the two conditions of storage, showing a decrease of the concentration without vacuum. No significant difference (P>0.05) was observed for morphine concentration at both levels between D1 and D3. For MDMA, MDE, cocaine, benzoylecgonine, ethylcocaine, free codeine, and dihydrocodeine, no significant difference (P>0.05) was observed at level 1 between D1 and D3, but significant differences (P<0.05) were observed at level 2 between D1 and D3, showing a decrease of the concentrations over the time. For amphetamine, methamphetamine, MDA, and oxycodone, significant differences (P<0.05) were observed at both levels between D1 and D3, showing a decrease of the concentrations over the time.

**Conclusion/Discussions:** Generally, when kept at room temperature, DBS as QC materials for drugs of abuse have to be stored under vacuum condition. Moreover, the stability of DBS QC at room temperature depends on the substances and the delay of conservation. Storage at lower temperature such as -20°C should improve stability, and must be investigated.

**Keywords:** Dried Blood Spot (DBS), Drugs of Abuse (DOA), Quality Controls (QC)

**Simultaneous Quantitative and Qualitative Determination of 80+ Forensic Toxicology Drug Candidates and Metabolites using Triggered Dynamic Multi Reaction Monitoring with LC/Tandem MS.**

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**Background/Introduction:** The ability to detect an ever increasing number of toxicological drug classes and their metabolites over wide ranges of sample concentration with analytical sensitivity, accuracy and confidence can be challenging for any single analysis. To address these requirements a method has been developed to analyze for a typical list of 80+ common toxicology analytes in under ten minutes using a unique Triggered Dynamic Multi Reaction Monitoring (tDMRM) utilizing low dwell time MS/MS capability of down to 0.5ms. Qualitative spectral library matching scores together with quantitative accuracy and precision over a typical concentration range will be illustrated herein.

**Objective:** To develop a fast & inclusive targeted confirmation method for 80+ analytes with additional qualitative spectral library matching capability.

**Methods:** Eighty commonly analyzed toxicology drugs and metabolites were chosen at random to demonstrate method proof of principle. Two primary DMRM transitions were utilized for each analyte and internal standard in the method, with a further three transitions chosen and waiting only to be triggered if thresholding criteria are met. UTAK negative matrix urine samples and calibrators were spiked with analytes and ISTDs, extracted using Bond Elut PCX SPE cartridges and reconstituted into mobile phase. Analytes were separated using an Agilent UHPLC system with Poroshell 120 EC C18 column (100 x 3mm) over a ten minute gradient using 5mM ammonium formate/0.01% formic acid in water and 0.01% formic acid in methanol as mobile phases. The Tandem Mass Spectrometer used was an Agilent 6470. Blood and plasma matrices will be evaluated in future studies.

**Results:** The tDMRM MS functionality only monitors for potential analyte targets during their elution window, thus facilitating more efficient dwell time allowances for such large analyte numbers. Calibrators across the range of 1-500ng/ml showed excellent linearity  $R^2 > 0.998$ , precision (n=5) typically  $< 5\%$  RSD and excellent spectral library match scores  $> 90\%$  across the concentration range for each analyte.

**Conclusion/Discussions:** The tDMRM method functionality greatly increases the number of toxicological analytes that are able to be analyzed in a set amount of time. Herein we demonstrate analytical sensitivity, linear range, good precision for confirmations with accurate and sensitive spectral library match integrity.

**Keywords:** Triggered Dynamic Multi Reaction Monitoring, tDMRM

## Quantitative Analysis of Gastric Content: A Standardized Preparation Method Using Protein Precipitation and LC-MS/MS Analysis

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**Background/Introduction:** The toxicological analysis of gastric content is routinely done in various cases such as suicide, intoxication and poisoning. While preparing for an upcoming validation under SWGTOX and ISO 17025 guidelines of an LC-MS/MS targeted screening method, we realized that the literature does not address specifically the issues associated to preparation and analysis of gastric content. This matrix is unique due to the almost infinite possibilities in term of physical (viscosity, volume, density, homogeneity) and chemical (concentration, analytes, pH) properties. Due to the distinctive nature of this biological matrix, there is a need for extensive optimization and validation of a preparation method.

**Objective:** The aim of this project is to develop a simple, efficient and standardized methodology for the preparation of gastric contents, their extraction by protein precipitation and their LC-MS/MS analysis.

**Methods:** Samples of gastric contents of various viscosities and homogeneity levels were blended and analyzed with our LC-MS/MS targeted unknown screening methods to ensure the absence of detectable drugs and metabolites. Whole pills were added to blank gastric contents and blended again until a smooth texture was obtained. For this first foray into the preparation of gastric contents by protein precipitation, acetaminophen, naproxen and ibuprofen were tested due to their typical large doses. 1 and 10% of the sample of the gastric content was then taken with a volumetric pipette or by weight if the high viscosity prohibited the volumetric approach. The sample was then diluted with a volume of methanol:water (1:1). The dilution volume was selected to ensure that the final concentration after extraction was within the dynamic range of the LC-MS/MS method for the targeted analyte. Protein precipitation was performed using a solution of acetone:acetonitrile (30:70) followed by centrifugation at 3200xg and 20°C for 5 minutes. 20 µL of supernatant was transferred into an injection plate and diluted with 200 µL of MeOH:0.2% aqueous formic acid (1:9). Gradient separation was performed with 10 mM ammonium formate adjust to pH 3,5 with formic acid in water with 5% methanol (mobile phase A) and methanol (mobile phase B) on a C18 column, and analysis on an AB Sciex 5500 QTrap instrument. The diluted gastric content sample is quantified using a calibration curve freshly prepared in whole blood.

**Results:** Aliquot sizes 1 and 10% of the initial volume or mass of the gastric contents were tested to achieve representative sampling of the initial matrix. A 10% sample of the initial volume or mass of the gastric content proved to be efficient, yielding accurate quantification results. Methanol:water (1:1) solution was used to dilute the gastric content in order to achieve a balance of the solubility of hydrophobic and hydrophilic molecules. An array of different drugs from different families (for example amphetamines, opioids, NSAIDS) were confirmed to be stable in this dilution solution for 72 hours at 4°C. Addition of small (2-3) and large (15-20) quantities of pills to the gastric contents were tested. The initial blended mixture proved to be homogeneous for the analytes tested, with less than 10% CV between samples (i.e. good precision). Accuracy was also found to be adequate, varying between 80 and 120%.

**Conclusion/Discussions:** We have developed a standardized methodology for preparing gastric contents and extract them using protein precipitation. Targeted screening can then be carried using a standard LC-MS/MS analysis. This method is quick, efficient and has been shown to have adequate accuracy and precision for a certain number of test analytes. Future work will look into the performance of this method for the analysis of other drugs such as benzodiazepines, amphetamines and cannabinoids in gastric contents.

**Keywords:** Gastric Content, Protein Precipitation, LC-MS/MS

## Quantification of High Therapeutic Concentration Compounds: A Piece of a Novel High Throughput Protein Precipitation Extraction and LC-MS/MS Analysis Workflow

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**Background/Introduction:** Recently, our laboratory eliminated the use of immunoassay screening by developing a rapid, specific and cheap workflow to screen and quantitate the compounds constituting 95% of our findings in cases. A protein precipitation extraction combined with three different LC-MS/MS analysis methods were created to quantify or detect more than 100 compounds in a total combined run time of 25 minutes. The LC-MS/MS method presented here has been developed to analyze “over the counter” drugs (acetaminophen, ibuprofen, salicylic acid), anticonvulsivants (gabapentin, pregabalin, levetiracetam, valproic acid, phenytoin, topiramate), barbiturates (barbital, butalbital, phenobarbital), GHB, BHB and other analytes with a high therapeutic blood concentration (celecoxib and naproxen). It is necessary to analyze these compounds in a separate method because of their  $\mu\text{g/mL}$  concentration range, much higher than most other xenobiotics which are typically in the  $\text{ng/mL}$  range.

**Objective:** The aim of this project is to develop and validate a novel quantification method for 16 xenobiotics with a high therapeutic blood concentration.

**Methods:** This method is designed for the analysis of antemortem and postmortem blood and urine. Quantification is performed using a set of 7 ascending standards, containing the 16 targeted drugs over two orders of magnitude. The specific concentration ranges of the calibration curves are selected so that the vast majority of our cases will fall between the LOQ and ULQ. Each calibration curve is freshly doped in whole blood. The extraction is carried out using protein precipitation. A 100  $\mu\text{L}$  sample aliquot is first doped with 10  $\mu\text{L}$  internal standard solution. It is then diluted with 100  $\mu\text{L}$  of methanol:0.2% formic acid solution (1:1 v:v) and precipitated with 400  $\mu\text{L}$  of an acetone:acetonitrile (3:7 v:v) organic solvent mixture, followed by centrifugation at 3200xg and 20°C for 5 minutes. 20  $\mu\text{L}$  of supernatant is aliquoted and diluted with 200  $\mu\text{L}$  of methanol:0.2% formic acid (1:9 v:v). 5  $\mu\text{L}$  of this diluted sample is then separated using a 8.7 minutes gradient chromatography on a Agilent Eclipse Plus C18 2,1x100mm, 3,5  $\mu\text{m}$ . The Sciex 5500 QTRAP mass spectrometer parameters are optimized to allow acquisition in the positive and negative polarities in the same run and to compensate the very broad response differences between the analytes.

**Results:** During method development, special care was taken to reduce ionization effects that could occur due to the high concentration of the analytes involved. The chromatography was designed to increase as much as possible the resolution between GHB and BHB, two highly hydrophilic isomers. This method is currently undergoing validation according to the SWGTOX Standard Practices. As of now, accuracy (80% to 120%) and precision (<20%) were deemed to be adequate for all analytes. Calibration models were selected and validated using a statistical approach. Limits of quantification were estimated and varied between 0.05 and 3  $\mu\text{g/mL}$ , depending on the analyte. Carryover and interferences were either absent or below an acceptable threshold (2% of LOQ). Dilution integrity was assessed for a dilution factor of 2, 20 and 50<sup>th</sup> fold. Stability was evaluated for analytes in blood for 6 and 24hrs at room temp and 4°C, 7 days, 1 month and 2 month at 4°C. Finally, thanks to the use of deuterated analogues as internal standards, matrix effects were mostly compensated both in antemortem and postmortem blood (70% to 130%).

**Conclusion/Discussions:** A novel quantification method for 16 xenobiotics with a high therapeutic blood concentration has been developed and validated based on SWGTOX Standard Practices. It uses protein precipitation as an extraction technique and LC-MS/MS for sample analysis. Although further optimization could still be carried to reduce the number of internal standards used, this is a specific, fast and cheap analysis method for high therapeutic blood concentration xenobiotics. Together with the two other LC-MS/MS analysis methods, it provides a comprehensive screening and quantification workflow that allows the elimination of the immunoassay.

**Keywords:** High Therapeutic Concentration Drugs, Protein Precipitation, LC-MS/MS

**Strategies for Multiple-Target Screening using LC-MS/MS with Merged Spectrum Database for Forensic Toxicology**

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**Background/Introduction:** In Forensic Toxicology, LC/MS/MS has become a preferred method for the routine quantitative and qualitative analysis of drugs of abuse. It allows for the simultaneous analysis of multiple compounds in a single run, thus enabling a fast and high throughput analysis. An ideal screening method should cover a large number of drugs and provide spectral information for confirmation– all in a single analysis.

**Objective:** In this study, we developed Multiple-Target Screening (MTS) method for forensic toxicology to reduce false positive and negative using MS/MS spectral library database. MTS method consists of multiple reaction monitoring (MRM) and product ion scans at three collision energies to confirm the compound identification based on mass spectral library searching. The mass spectral library was created using certified reference materials from over 1,200 compounds including illicit drugs, psychotropics, hypnotics, pesticides and other substances for forensic toxicology.

**Methods:** The MTS parameters were set to a single MRM per compound with threshold triggered MS/MS at 3 collision energies (15, 30, 45V) enabling confirmation of fragmentation widely. Library searching was performed on all CE spectral data in addition to a merged-CE spectrum.

Biological sample preparation was carried out by the modified QuEChERS extraction method. Treated samples were measured using a Nexera UHPLC system and tandem mass spectrometer (Shimadzu Corporation, Japan). Samples were separated on a Phenomenex Kinetex XB-C18 (100x2mm, 2.6µm) at a column temperature of 40 °C for 15 min. A flow rate of 0.3 mL/min was used together with a binary gradient system. 10mM ammonium formate with 0.1% Formic acid in water and 10mM ammonium formate with 0.1% Formic acid in methanol were used for mobile phases.

**Results:** We evaluated MTS method with modified QuEChERS using 50 standard drugs spiked into human whole blood. The peak area was calculated for each compound and the percentage recovery and matrix effect were confirmed. Recovery was over 90 % and matrix effect was small.

Next, some different matrices (blood, serum, urine, etc.) were prepared using modified QuEChERS and 50 drugs (1ng/mL and 10 ng/mL) were confirmed with MTS method. Most compounds can be identified as the first hit in a spectral based library matching with merged-CE spectrum.

**Conclusion/Discussions:** A MRM triggered product ion spectra method with merged-CE spectrum matching to identify compounds in biological samples is effective for forensic toxicology.

**Keywords:** Ultra-high speed LC/MS/MS, Multiple-Target Screening, Merged Spectrum Library

**Analysis of Fifteen Rodenticides in Blood and Urine Samples by On-Line Solid Phase Extraction and Liquid Chromatography-Mass Spectrometer Triple Quad**

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**Background/Introduction:** With the prohibition of acutely toxic rodenticides, chronic rodenticides have been widely used in rat killing because of their efficiency and low toxicity. In recent years, due to improper use and management of rodenticides, people and livestock poisoning has frequently happened. These events endangered have raised health concerns for the population.

**Objective:** To develop a new and sensitive analytical method for the simultaneous determination of 15 rodenticides in blood and urine samples.

**Methods:** The blood and urine samples were treated with acetonitrile, followed by dilution, centrifugation, and filtration. The resulting solution was purified by on-line, polymer based, hydrophilic-lipophilic balance (HLB) column. The samples were eluted on a ZORBAX Eclipse Plus C18 column with a methanol and 0.01 mol/L ammonium acetate aqueous solution. The analytes were detected in dynamic multiple reaction monitoring (DMRM) mode under positive and negative electrospray ionization (ESI<sup>+</sup>/ESI<sup>-</sup>) mode and quantified by external standard method.

**Results:** The analytical process developed is 10.1 minutes long. Fifteen rodenticides were separated well with high sensitivity. The correlation coefficients within the investigated mass concentration ranges of the 15 rodenticides were  $\geq 0.9978$  in blood and  $\geq 0.9965$  in urine by quadratic equation fitting. The LODs varied from 0.10 ng/mL to 5.00 ng/mL, and the LOQs varied from 0.50 ng/mL to 10.0 ng/mL. The recoveries at three spiked levels in blood and urine ranged from 81.8% to 109.6%, whereas RSDs ranged from 0.3% to 3.6% ( $n=6$ ).

**Conclusion/Discussions:** A new and sensitive analytical method for the simultaneous determination of 15 rodenticides in blood and urine samples was developed. The blood and urine samples were treated with acetonitrile, diluted, centrifuged, and filtrated, and then purified by on-line column. After that, the samples were directly detected by QQQ. QQQ is sensitive and selective, while on-line SPE is quick, easy, reusable and automated. This method is effective and reliable for the rapid detection and accurate quantification of the fifteen anticoagulant rodenticides in whole blood and urine samples.

**Keywords:** On-Line Solid Phase Extraction (on-line SPE), Liquid Chromatography-Mass Spectrometer Triple Quad (LC/MS QQQ), Rodenticides

**Discrimination of Human Urine from Animal Urine Using <sup>1</sup>H-NMR**

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**Background/Introduction:** Among the biological samples, urine has been the traditionally used matrix in drug testing. Some drug abusers try to cheat drug testing by using animal urine. To combat such practice, we have developed urinary analysis to discriminate human urine from animal urine. The application of quantitative <sup>1</sup>H-NMR spectroscopy is well suited for quick and accurate analysis of complex mixture. In this study, <sup>1</sup>H-NMR analyses were carried out for all samples (Human 359, Rat 370, Pig 12, Horse 10, Cat 8, Dog 13, Cow 10, Monkey 10). Acquired spectral data of the whole set were submitted to pattern recognition methods such as unsupervised principal component analysis (PCA) and supervised orthogonal partial least squares discriminant analysis (OPLS-DA). Then, targeted metabolite profiling method was applied for acquired spectra.

**Objective:** The purpose of this study was to compare and differentiate human from animal urine samples through the use of quantitative <sup>1</sup>H-NMR and to find biomarkers that can be used for the discrimination of human urine from animal urine.

**Methods:** All urine samples were centrifuged at 13,000 rpm for 5 min. The 630 µL of urine supernatant was mixed with 70 µL of D<sub>2</sub>O containing 20 mM TSP-d<sub>4</sub> and then transferred into 5 mm NMR tube. The NMR experiments were carried out on a 600 MHz spectrometer (Agilent Technologies, USA) operating at 600.17 MHz and equipped with 7600-AS autosampler at 299.1 K. The spectra were acquired with a presaturation pulse sequence to suppress the water peak. Acquisition time was 1.998 sec and relaxation time was 1.52 sec. All spectra were acquired with 128 scans. Each <sup>1</sup>H-NMR spectrum between 0.5 ppm and 10 ppm was binned by 0.01 ppm intervals and water peak area (4.6 ppm ~ 4.9 ppm) was excluded. Multivariate analysis was performed using SIMCA-P + 12.0 software (Umetrics, Sweden). Chemomx NMR suite 7.1 software (Chemomx Inc., Canada) was used for qualitative and quantitative analysis of urine components

**Results:** In pattern recognition method, OPLS-DA score plot showed that human urine groups have different patterns from animal urine groups. In targeted metabolite profiling method, we found major metabolites that distinguish human and animal urine; Rat (2-oxoglutarate, succinate, acetate, N,N-dimethylglycine), Cat (1-Methylnicotinamide), Dog (acetate, lactate, succinate, 1-Methylnicotinamide, Kynurenate), Cow (acetate, pyruvate, glycine, creatine, benzoate), Horse ( acetate, benzoate), Monkey (benzoate)

**Conclusion/Discussions:** Human and animal urine were distinguished by NMR spectra. Several characteristic metabolites were found and could be used as biomarkers for distinguishing human and animal urine.

**Keywords:** Quantitative <sup>1</sup>H-NMR, Biomarker, Pattern Recognition

**Observation of Alkaloids Condition in Buxus Sempervirens by the Method of TLC**

D.A. Zulfikariyeva , Z.A. Yuldashev, N.R. Alakbarova

**Background/Introduction:** Buxus sempervirens is widely used in decorative gardening. In homeopathic medicine, drugs made from leaves buxus sempervirens are used as sudorific, antifebrile, and diuretics. In addition, the extracts are used in treatment of rheumatic disease. However, it is necessary to point out that all parts of the plant, especially the leaves, are toxic. The Buxus contains about 70% steroidal alkaloids, the most poisonous of which are buxine and cyclobuxine. Initial symptoms poisoning by buxus sempervirens are vomiting, diarrhea, and cramps. During the 12-24 hours following ingestion, death can occur due to respiratory depression. On autopsy can be observed, hyperemia and swelling of stomach and intestinal mucosa. Until now, methods for detecting and determining buxus alkaloids in biological samples had not been developed. In this work, we report the development of a method to analyze cyclobuxine and buxine extracted from plant material by thin layer chromatography (TLC).

**Objective:** Developing a technique for the analysis of cyclobuxine and buxine alkaloids using TLC methods.

**Methods:** Raw materials were the dried above ground part of a plant. The materials were crushed to the size of the particles passing through a sieve with openings with a diameter of 1 mm. About 10 g of crushed raw materials were placed in a flask with a capacity of 250 ml, flowed 150 ml of ethyl oxide, ammonia solution till it reaches to PH=9 and shook up the mixture during 1 h. The extract was merged from the raw materials. The ether extract was quickly filtered through cotton wool into a 200 ml flask. 5 ml of water was added to the extract, vigorously shaken and left until the ether layer turned into clear appearance, after Extracted liquid was transferred to a tap funnel with a capacity of 200 ml. From the ether extract, the alkaloids were extracted again as much as possible with a 1% solution of hydrochloric acid in portions of 20, 15, 10 ml. The re-extracted acid was neutralized by concentrated solution of ammonia before forming alkaline reaction (pH=9) which was checked by phenolphthalein. The alkaloids were extracted with chloroform sequentially in portions of 20, 15, 10 ml. Chloroform extracts were filtered and was distilled off at room temperature. The dry residual content was dissolved in 1 ml of ethanol. The extracted alkaloids were examined by thin layer chromatography.

**Results:** As an optimal mobile phase, a mixture of ethyl alcohol and diethyl ether was chosen in a ratio of 8:2. In order to display a zone of of buxine and cyclobuxine alkaloids localization whith reagent of Dragandorf modified with Munye was used (an orange-red spot is formed), secondly the reaction diazotixation was applied with a solution of sodium nitrite, 10% hydrochloric acid and an alkaline solution of  $\beta$ -naphthol (a red color is formed.) Chromatographic plates were examined under UV rays at a wavelength of 254 nm, with alkaloids appearing as dark brown spots, and Rf values of the test substances reached Rf = 0.72 in alcohol extraction.

**Conclusion/Discussions:** Buxus sempervirens alkaloids buxine and cyclobuxine were extracted from plant material and detected using a TLC method. In Buxus sempervirens the research was conducted by the TLC method, which is considered as the most effective method. The content of alkaloids in extract composition was proven by colorful reaction during the research.

**Keywords:** Buxus Sempervirens, Detection, TLC



**Development of a Quantitative LC-MS/MS Assay for Codeine, Morphine, 6-Acetylmorphine, Hydrocodone, Hydromorphone, Oxycodone, and Oxymorphone in Neat Oral Fluid**

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**Background/Introduction:** In 2015 the U.S. Department of Health and Human Services (HHS) proposed revisions to the mandatory guidelines for federal workplace drug testing programs that would allow oral fluid testing as an alternative specimen (to urine), and allow testing for oxycodone, oxymorphone, hydrocodone, and hydromorphone in addition to the current opiate analytes: 6-acetylmorphine, morphine, and codeine.

**Objective:** The development and validation of an analytical method for the analysis of codeine, morphine, 6-acetylmorphine (6-AM), hydrocodone, hydromorphone, oxycodone, and oxymorphone in neat oral fluid by LC-MS/MS. The assay was developed in accordance with the revised mandatory guidelines for opioid confirmation in regulated workplace drug testing at dilution factors consistent with those encountered when using collection devices, while being independent of any specific collection device.

**Method:** Drug-free human neat oral fluid was collected, diluted 1 to 2 with a proprietary synthetic oral fluid developed at RTI and fortified with opioids. Samples were prepared using solid-phase extraction and quantified using an Agilent Technologies (Santa Clara, CA) 1290 liquid chromatograph interfaced to an Agilent 6490 triple quadrupole mass spectrometer operating in positive electrospray ionization (ESI) mode. Quantification was validated by evaluating the calibration model, precision, accuracy, carryover, dilution integrity, limit of detection (LOD) and limit of quantitation (LOQ), matrix effect, interference and stability.

**Results:** LODs ranged from 0.01 to 0.04 ng/mL with hydrocodone and hydromorphone having the lowest LODs. The LOQ was 0.4 ng/mL (20% of the cutoff) for 6-AM and 1.5 ng/mL (10% of the cutoff) for all other analytes. Excellent accuracy and precision results were obtained and were within acceptable limits for all analytes. Within-run and between-run precision were < 5% for all analytes except for hydrocodone, which had 6.2 %CV between runs. Diluting samples by factors of 1:10 and 1:25 did not negatively impact the accuracy and precision of the assay. Analysis of blank oral fluid matrix did not produce interferences for any of the target analytes or ISTDs. The potentially interfering compounds evaluated were dihydrocodeine, norhydrocodone, noroxycodone, noroxymorphone, norcodeine, normorphine and heroin. Noroxymorphone, norcodeine, and normorphine, did not interfere with any of the analytes or ISTDs. Noroxycodone appeared to suppress 6-AM-d<sub>3</sub> slightly, resulting in higher than acceptable 6-AM accuracy (121%). Substituting 6-AM-d<sub>6</sub> as the internal standard resolved the interference. Dihydrocodeine (302 m/z) interfered with codeine-d<sub>3</sub> (303→165 m/z, and 303→152 m/z) as these compounds have similar molecular weights and retention times. Substituting codeine-d<sub>6</sub> as the internal standard resolved the interference. Matrix effects, while evident, could be controlled using matrix-matched controls and calibrators with deuterated internal standards. All analytes were deemed stable at room temperature for up to 72 hours in the extracted sample solutions.

**Conclusion/Discussion:** This work presented a validated method for the analysis of opioids in a neat oral fluid matrix using LC-MS/MS.

**Keywords:** Neat Oral Fluid, Opioids, LC-MS/MS

**Advanced Workflow for Quantification of Glucuronide Metabolites in Biological Matrices by LC-MS.****Know More in Less Time**

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**Background/Introduction:** Urine drug testing is an important tool in monitoring compliance to pain management regimens as well for detection of illicit drugs. Current workflows for detection of glucuronidated drug metabolites in biological matrices via LC-MS are challenged with multiple obstacles. Among those are sample traceability, ease and duration of sample processing, enzyme stability as well as limited LC column life-time, which is directly related to the cleanliness of the sample applied onto LC-MS.

**Objective:** We aim to develop an advanced 96-well ultrafiltration plate-based workflow that is reliable, reproducible and faster by combining the hydrolysis and cleanup into one step. Such a workflow allows for shorter hands-on time, lower sample-to-sample variability and overall lowers workflow costs.

**Methods:** The optimized workflow is based on combining enzymatic hydrolysis and filtration into one step by depositing beta-glucuronidase enzyme on a 96-well ultrafiltration plate. As a first test, a number of recombinant beta-glucuronidases were screened for assay suitability assessing both enzymatic activity and thermostability. Urine samples containing metabolites of interest can be directly applied onto the filter plate and enzymatic reaction will take place in a plate followed by a manifold-assisted ultrafiltration step. The ultrafiltration step allows for hydrolyzed drug metabolite to pass through, while retaining high MW species. SDS-PAGE analysis was implemented on the flow through to ensure successful filtration (retention) of proteins by the filter membrane. Preservation of enzymatic activity was verified by a colorimetric assay using phenolphthalein-glucuronide as a substrate. Obtained flow through from the experimental set up with known varying amounts of drug-glucuronides was subjected to LC-MS analysis to demonstrate that analyte detection is robust and reproducible.

**Results:** Generated data indicates that proposed workflow does not interfere with qualitative metabolite detection. Concentrations as low as 20 ng/mL of pre-hydrolysis parent metabolite were detectable by LC-MS. Enzymatic activity of plate-deposited beta-glucuronidase enzyme remained unchanged after 2 days incubated at RT as judged by read-outs from the colorimetric assay using phenolphthalein-glucuronide as substrate. The ultrafiltration step was shown to be capable of removing proteinaceous species as judged by quantification of SDS-PAGE gel band intensity of the flow through fraction in comparison to pre-filtration. Very low non-specific binding to the filter membrane was observed for codeine as confirmed by 100% recovery via LC-MS. Recoveries of other deconjugated drugs varying in polarity are being evaluated at the moment. Minimal influences on plate performance in regards to filtration times, enzymatic activity, and drug recovery were observed by using synthetic urine samples in comparison to phosphate buffer facilitated reaction.

**Conclusion/Discussions:** The 96-well ultrafiltration plate facilitated workflow proposed here opens doors to a shorter, automated, robust, and reproducible method for the qualitative detection of glucuronide metabolites in biological matrices by LC-MS. The thermostable beta-glucuronidase allows analysts to keep the filter plate with deposited enzyme on the deck of automated liquid handling system for as long as 48 hrs, eliminating the need to provide fresh aliquots of enzyme every two hours as well as minimizing differences in hydrolysis efficiencies due to enzyme instability. Inclusion of a filtration-based cleanup step allows for removal of LC-column damaging impurities, thus increasing column lifetimes. Work is currently underway to determine the quantitative range for proposed workflow.

**Keywords:** Beta-Glucuronidase, Drugs of Abuse, Workflow

## The Analysis of Common Antiepileptic Drugs in Human Urine by LC-MS/MS

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**Background/Introduction:** The use of liquid chromatography coupled with mass spectrometry (LC-MS/MS) in therapeutic drug monitoring and toxicology labs has increased significantly over the years. LC-MS provides sensitivity, speed, and the ability to simplify sample preparation. The Raptor™ Biphenyl column was developed to complement high-throughput LC-MS/MS analyses by combining the increased efficiency of superficially porous particles (SPP) with the resolution of Ultra Selective Liquid Chromatography™ (USLC™) technology. In this example, a simple dilute and shoot method was developed for 14 common antiepileptic drugs in urine using a Raptor™ Biphenyl column.

**Objective:** The intent of this study was to develop a simple dilute-and-shoot method for the analysis of 14 antiepileptic drugs in human urine with a rapid run time of 5.5 minutes.

**Methods:** Human urine samples were diluted in 0.1% formic acid in water and injected into a Shimadzu Nexera UHPLC equipped with an AB SCIEX API 4500™ MS/MS. Detection was performed using electrospray ionization in positive ion mode with scheduled multiple reaction monitoring (MRM). The separation was performed using water and methanol mobile phases modified with 0.1% formic acid under gradient conditions on a Restek Raptor™ Biphenyl 2.7µm, 100 x 2.1mm column.

**Results:** Linearity, precision, and accuracy experiments were performed during method development. Purchased human urine was fortified with 14 drug analytes and their deuterated internal standards. The calibration range for most analytes was from 10 to 1000 ng/mL; R values were all greater than 0.990. Accuracy and precision were determined by fortifying human urine at a concentration of 800 ng/mL prior to dilution. Mean values at this level ranged from 88% to 110% of nominal concentrations for all analytes. Coefficient of variation (CV) was calculated for the determination of precision and ranged from 6.2% to 10.5%.

**Conclusion/Discussions:** The Raptor™ Biphenyl column was excellent for the simultaneous analysis of 14 antiepileptic drugs in human urine. The accurate and reproducible analysis can be achieved in less than 5.5 minutes of chromatographic run time and is thus applicable for low-cost and high through-put analysis in therapeutic drug monitoring and toxicology labs.

**Keywords:** Antiepileptic Drugs, LC-MS/MS, Raptor Biphenyl

**The Analysis of Common Drugs of Abuse in Human Urine by LC-MS/MS**

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**Background/Introduction:** The use of liquid chromatography coupled with mass spectrometry (LC-MS/MS) in forensic toxicology labs has increased significantly over the years. LC-MS provides sensitivity, speed, and the ability to simplify sample preparation. The Raptor™ Biphenyl column was developed to complement high-throughput LC-MS/MS analyses by combining the increased efficiency of superficially porous particles (SPP) with the resolution of Ultra Selective Liquid Chromatography™ (USLC™) technology. In this example a simple dilute and shoot method was developed for 10 common drugs of abuse and their metabolites in urine using a Raptor™ Biphenyl 5µm column. The ultra-low back pressure of the 5µm particle column allows even conventional 400 bar LC systems to take advantage of this high speed separation with a total analysis time of 5 minutes.

**Objective:** Provide a universally fast and easy method for the quantitative analysis of drugs of abuse in diluted urine.

**Methods:** Human urine samples were diluted 5X in mobile phase A and injected into a Shimadzu Prominence UFLCXR HPLC equipped with an AB SCIEX API 4000™ MS/MS. Detection was performed using electrospray ionization in positive ion mode using scheduled multiple reaction monitoring (MRM). The separation was performed using water and methanol mobile phases modified with 0.1% formic acid under gradient conditions on a Restek Raptor™ Biphenyl 5µm, 50 x 2.1mm column.

**Results:** Preliminary linearity, precision and accuracy, and matrix effects experiments were performed during method development. Purchased human urine was fortified with 10 drug analytes and their deuterated internal standards. The calibration range for codeine and 6-MAM is from 5.00 to 750 ng/mL. The calibration range for benzoylecgonine is from 1.00 to 500 ng/mL. The calibration range for the remaining 7 analytes is from 1.00 to 750 ng/mL. Accuracy and precision were determined by fortifying human urine at a concentration of 50.0 ng/mL prior to dilution. Mean values at this level ranged from 91.9% to 103% of nominal concentrations for all analytes. Coefficient of variation (CV) was calculated for the determination of precision and ranged from 2.90% to 6.54%. Matrix effects were assessed by infusing a solvent standard (post column) prepared at a concentration of 50 ng/mL while injecting a blank matrix sample diluted 5x in mobile phase A. Areas of ion suppression would result in dips in the response of the solvent standard. There was no evidence of ion suppression during the time period of analyte elution.

**Conclusion/Discussion:** Innovations in SPP column technology allow for faster and more sensitive LC-MS/MS assays. The Raptor™ Biphenyl 5µm column provides highly retentive, selective, and rugged reversed-phase separations with ultra-low back pressure. Now even conventional LC systems can benefit from the speed and efficiency of the Raptor™ Biphenyl.

**Keywords:** Drugs of Abuse, LC-MS/MS, Quantitative, Human Urine, Raptor™ Biphenyl

## The Analysis of Emerging Drugs of Abuse: Updating an Existing Method with New Compounds

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**Background/Introduction:** The determination of psychoactive drugs and their metabolites has become routine in many forensic toxicology laboratories. The optimization of analysis time, resolution between metabolites, method robustness, and the ability to add emerging compounds is of ultimate importance when developing an efficient method for validation. The Raptor™ Biphenyl column combines the speed of superficially porous particles (SPP) with the resolution of highly selective USLC® technology to give the analyst the ability to produce fast dilute and shoot methods while staying current with the ever changing landscape of illegal drugs.

**Objective:** Provide an expanded method for the fast and easy analysis of 22 synthetic cannabinoids, 12 metabolites, and salvinorin A resulting in complete resolution of isobars and separation from matrix interferences in diluted human urine.

**Methods:** The method investigations were performed on a Waters Acquity I-class equipped with a Xevo TQ-S and a Shimadzu Nexera equipped with a SCIEX 4500. Both systems utilized electrospray ionization in positive ion mode. Standards were prepared in human urine and were diluted 3x in a 0.2 µm PVDF Thomson SINGLE StEP® Filter Vial with 50:50 water:methanol prior to analysis. Data was collected with MRM windows of approximately ± 30 seconds. Chromatographic optimization resulted in complete resolution of isobars and separation from major matrix interferences of a representative pooled urine sample. Water and acetonitrile mobile phases modified with 0.1% formic acid were used under gradient conditions on a Restek Raptor™ Biphenyl 2.7µm, 50 x 3.0mm column equipped with a Raptor™ Biphenyl EXP 2.7µm, 5 x 3.0mm guard.

**Results:** Chromatographic separation is essential for analyzing synthetic cannabinoids JWH-018 and JWH-073 and their metabolites due to the presence of multiple positional isomers among the mono-hydroxylated metabolites. Since these positional isomers have identical molecular weights and very similar fragmentation patterns, they are indistinguishable by MS/MS detectors and chromatographic resolution is required for positive identification. Previously a method was presented for the comprehensive screen of 17 synthetic cannabinoids, 12 metabolites and 5 internal standards with a cycle time of 5 minutes. All positional isomers were resolved on the Raptor™ Biphenyl column making it possible for the most abundant metabolites from a given parent compound to be identified in authentic samples. Today, laboratories are faced with the difficult task of keeping up with the ever-growing list of synthetic cannabinoids illicit drug makers produce to avoid legal classification and detection. In an effort to determine the ability of the original method to keep pace with the rapidly changing list, 5 emerging synthetic cannabinoids (i.e. AB-PINACA, AB-FUBINACA, PINACA, 5F-PB-22, and PB-22) and salvinorin A were prepared in human urine and analyzed using the same methodology.

**Conclusion/Discussion:** The analysis of synthetic cannabinoids and their metabolites can be a difficult and challenging task. The Raptor™ Biphenyl provides solutions to the chromatographic and validation issues surrounding this analysis. It has the ability to provide highly retentive, selective, and rugged reversed-phase separations, allowing for the simultaneous analysis of 22 synthetic cannabinoids, 12 metabolites, and salvinorin A. It has been demonstrated that analyte lists can easily be expanded as new synthetic cannabinoids are introduced. The speed of SPP allows analysis times to become shorter. The unique selectivity of the biphenyl phase allows isomer separation to be easily achieved.

**Keywords:** Synthetic Cannabinoids, Salvinorin A, LC-MS/MS

**A Novel Solution for EtG/EtS Analysis in Human Urine by LC-MS/MS**

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**Background/Introduction:** Ethyl Glucuronide (EtG) and Ethyl Sulfate (EtS) are unique biomarkers of alcohol use. The analysis of EtG and EtS offers many advantages for abstinence monitoring including the detection window (~3 days), stability in stored specimens (non-volatile), and specificity. EtG and EtS are both polar which makes them difficult to retain via reversed-phase chromatography. Both compounds are also very sensitive to matrix interferences which can result in being unable to achieve low limits of detection. Isobaric interferences can also make quantitation impossible. In this study, a simple dilute and shoot method was developed and validated for the analysis of EtG and EtS in human urine by LC-MS/MS.

**Objective:** Validate a method that provides a simple, fast, and sensitive measurement of EtG and EtS in human urine.

**Methods:** Pooled human urine (pre-screened to confirm absence of EtG/EtS) was fortified with EtG and EtS from 50-5,000 ng/mL for both analytes. Urine calibration samples, QC samples, and twenty selectivity lots were diluted 20-fold in the working internal standard (25 ng/mL for EtS-d5 and 100 ng/mL for EtG-d5 in 0.1% formic acid in water). The samples were vortexed and centrifuged prior to injection on a Raptor EtG/EtS column (100x2.1mm, 2.7  $\mu$ m). The mobile phases used were 0.1% formic acid in water (aqueous phase) and 0.1% formic acid in acetonitrile (organic phase) and the chromatographic separation was achieved with a gradient elution of 5-35% organic phase in two minutes. Primary method validation was performed on a Shimadzu XR coupled with a SCIEX API 4000<sup>TM</sup> mass spectrometer using electrospray ionization in negative ion mode. In order to evaluate method ruggedness, precision and accuracy sets were also performed on a Shimadzu Nexera UHPLC coupled with a SCIEX Triple Quad<sup>TM</sup> 4500 mass spectrometer and a Waters Acquity I-Class UPLC<sup>®</sup> system coupled with a Xevo<sup>®</sup> TQ-S mass spectrometer using multiple column lots.

**Results:** EtG and EtS were successfully resolved from matrix interference. The selectivity lots did not show additional interferences that would impact quantitation. The calibration linearity was acceptable for both analytes with  $R^2$  values  $\geq 0.999$  and % deviation of less than 10.0%. Three levels of QC samples were analyzed for accuracy and precision across multiple days, instrument platforms, and column lots. Mean accuracy values ranged from 90%-101% of the nominal concentration for QC low, mid, and high samples and 89-105% for the QC LLOQ for both analytes. The %RSD did not exceed 10% for any set of QC samples throughout the study.

**Conclusion/Discussion:** An easy dilute and shoot method was developed and validated for the quantitative measurement of EtG and EtS in human urine. The analytical method was demonstrated to be fast, reproducible, and rugged.

**Keywords:** EtG/EtS, Alcohol Metabolites, Dilute and Shoot

**Comparison of the Concentrations of Clonazepam (CLO)/7-Aminoclonazepam (7-ACLO) in the Blood of Drugs-Driving (OUI) and Drug Facilitated Crimes (DFC) cases**

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**Background/Introduction:** As members of the benzodiazepine class of drugs, the analysis of CLO/7-ACLO in antemortem (OUI) taken in drugs-driving cases and drug facilitated crime (DFC) blood samples are routinely performed by forensic toxicology laboratories. In this present study, samples of whole blood from both OUI and DFC cases are presented having been analyzed by the same extraction method (solid phase extraction (SPE) and liquid chromatography-tandem mass spectrometry (LC-MS/MS).

**Objective:** This study was initiated with the idea of comparing the concentrations of CLO/7ACLO in DFC blood samples with those found in OUI cases when extracted and analyzed using LC-MS/MS. The data will assist those analysts involved in the interpretation of CLO and metabolite in both OUI and DFC types of cases.

**Methods:** Samples of blood (1 mL (Calibrators, controls, and test containing deuterated internal standards were diluted with 4 mL of phosphate buffer (pH 6), after which the samples were extracted on mixed mode SPE columns. The SPE columns having being pre-conditioned with methanol, deionized (DI H<sub>2</sub>O) water and 0.1 M phosphate buffer prior to sample loading. The SPE cartridges were washed with DI H<sub>2</sub>O and 0.1 M phosphate buffer/acetonitrile and dried. The SPE cartridges were then washed with hexane. and dried. Each SPE column was eluted with a solution of ethyl acetate containing 2% ammonium hydroxide. The samples were evaporated to dryness under nitrogen at 40 °C, and the residues dissolved in 100 µL of mobile phase (0.1 % aqueous formic acid/acetonitrile containing 0.1 % formic acid (95:5)) for LC-MS/MS analysis performed in positive multiple reaction monitoring (MRM) mode (details presented).

**Results:** The limits of detection/quantification for this method were determined to be 1.0 ng/mL for both analytes (CLO/7-ACLO). The method was found to be linear from 1.0 ng/mg to 500 ng/mL ( $r^2 > 0.99$ ). Recoveries were found to be greater than 93 %. Interday and Intraday analysis of the compounds were found to < 8 % and < 10 %, respectively. Matrix effects were < 7%. The results of DFC cases involving 48 males (mean age = 26 yrs): the median values (ng/mL) were: 9 (CLO): (range (0 to 26.0)), 11 (7-ACLO): (range (1.2 to 39.0)) respectively; for DFC cases involving 92 females (mean age = 22 yrs): the mean values (ng/ mL) were: 7 (CLO): (range (0 to 19.0)), 10 (7-ACLO): (range 1.0 to 23.0)), respectively. With OUI cases: For 211 male cases (mean age= 32 yrs): the mean values (ng/ mL) were: 29 (CLO): (range 1.1 to 96.0)), 36 (7-ACLO) : (range 1.8 to 119.0)), respectively; in 115 females (mean age = 27yrs): the mean values (ng/mL) were: 22 (CLO): (range 1.0 to 76.0)), 27 (7-ACLO): (range 2.5 to 78.0), respectively.

**Conclusion/Discussions:** This study shows that using a single extraction method and LC-MS/MS for the analyses, CLO and its metabolite from OUI and DFC cases can be evaluated. The interpretation of concentrations of CLO and 7-ACLO in DFC samples relative to those obtained from OUI subjects should be evaluated based not only on toxicology but case and medical history too. The increased concentrations of the metabolite may be due to *in situ* degradation as well as metabolism, this should also be taken into consideration.

**Keywords:** Clonazepam, SPE, Blood

**Definitive (UPLC-MS/MS) Drug Screening in Urine Confirmed by UPLC-MS<sup>E</sup>/QTOF Analysis in Forensic Casework**

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**Background/Introduction:** Presumptive immunoassay screening for drugs and their metabolites (analytes) in urine has been replaced in our court-related casework by definitive screening employing nominal mass UPLC-MS/MS analysis with threshold accurate calibration (TAC) as previously reported (*Journal of Analytical Toxicology* 2016; 40: 628-638). TAC technique utilizes a novel approach for normalization of matrix effects by dual analysis of each donor specimen i.e. with and without addition of reference-analytes. The assay is calibrated using a single calibrator sample prepared at threshold interpretive cut-off concentration; comparison of normalized TAC ratio enables qualitative identification of threshold-positive analytes. The transition to definitive screening by the TAC technique has extended selectivity and sensitivity for detection of large analyte panels in the opiate, opioid, opioid antagonist, benzodiazepine, sympathomimetic, cathinone, cocaine, hallucinogen, gabapentinoid, sedative and designer drug classes. In forensic casework the expansion in definitive drug detection requires availability of a second test based upon a different analytical method, with at least equivalent sensitivity and selectivity, for confirmation of all tentative positives.

**Objective:** The objective of this analytical work is to develop a complementary confirmatory method for all screened agents. The method uses an alternate chromatographic separation coupled with high resolution time-of-flight (TOF) mass spectrometry and is also based on the TAC principle of matrix effect normalization. An additional aim was to validate routine use of the confirmation method for forensic defensibility in the definitive screening program.

**Method:** Dual aliquots (50  $\mu$ L) of threshold calibrator, controls and tentative-positive case specimens are aliquoted into adjacent "neat" and "spiked" wells of a 96-well plate with addition to the spiked well of a reference analyte mixture containing all analytes at three times the positive-threshold concentration. Methapyrilene was added to neat and spiked wells to assess UPLC sample volume precision. Further addition of purified  $\beta$ -glucuronidase to all wells was followed by hydrolysis incubation for one hour at 55°C. After final addition of 0.5 mL of starting mobile phase to all wells, 5  $\mu$ L of well contents was analyzed with use of a Waters ACQUITY I-Class UPLC system interfaced in positive electrospray ionization mode with a G2-XS QTOF in MS<sup>E</sup> mode, all under UNIFI<sup>®</sup> software control. Chromatographic gradient separation in 15 min was performed with an ACQUITY UPLC HSS C18 analytical column and analyte identification criteria included: retention time ( $\pm 0.4$  min of reference analyte), precursor mass ( $\pm 5$ ppm) and at least one fragment ion within  $\pm 5$ ppm of exact mass. Supplementary data, to support identification, included additional fragment ions and isotope ratios. A UNIFI calculation of a TAC ion response ratio (neat/(spiked – neat) was used to identify analytes that reached, or exceeded, the positive threshold concentration. Analyte-specific threshold concentrations range from 10-500 ng/mL.

**Results:** Performance data for ten analytical runs was used to assess threshold precision and accuracy of the method. Quality control testing was performed at 40%, 75%, 125% and 500% of threshold concentration along with analysis external proficiency testing specimens. Cross-over testing of de-identified case samples showed 100% concordance with positive and negative analyte findings in the definitive screening method in over 150 donor urine specimens.

**Conclusion/Discussion:** A UPLC-MS<sup>E</sup>/QTOF method employing the TAC technique has been developed for confirmation of large panels of drugs and drug metabolites screened by a previously reported definitive method using nominal mass UPLC-MS/MS analysis. Accuracy of confirmation testing has been validated with quality control, proficiency and case sample cross-over studies. The confirmatory method is validated for routine use in forensic casework and offers the potential for unrestricted identification of additional analytes due to the full spectrum data capture associated with the TOF-MS<sup>E</sup> technology.

**Keywords:** Urine Drug Confirmation Testing, UPLC-MS<sup>E</sup>/QTOF Analysis, Threshold Accurate Calibration (TAC)



## UHPLC-QTOF-MS for Dynamic Targeted Screening and Quantification of Synthetic Cannabinoids in Urine

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**Background/Introduction:** High resolution mass spectrometry techniques such as liquid chromatography quadrupole time of flight mass spectrometry (UHPLC-QTOF-MS) have been proven sensitive and accurate for drug screening purposes and methods can relatively easily be updated.

**Objective:** The aim of this study was to develop a sensitive quantitative screening method in urine covering metabolites of the most frequently seized synthetic cannabinoids in Norway at the given time (AB-Fubinaca, AB-Pinaca, AB-Chminaca, AM-2201, AKB48, 5F-AKB48, BB-22, JWH-018, JWH-073, JWH-081, JWH-122, JWH-203, JWH-250, PB-22, 5F-PB-22, RCS-4, THJ-2201 and UR-144) using UHPLC-QTOF-MS. As synthetic cannabinoids undergo extensive metabolism parent drugs were not included in the method.

**Methods:** Sample preparation and clean up based on solid phase extraction in a 96 well-plate format was chosen to maintain the quality of the analytical column and for preconcentration purposes. The samples were treated with  $\beta$ -glucuronidase prior to extraction and liquid handling was partially automated using a robot and a positive pressure unit. The UHPLC-QTOF-MS instrument consisted of a 1290 Infinity UHPLC system and a 6550 QTOF-MS system both from Agilent. Chromatographic separation was achieved using a Zorbax Eclipse Plus C18 Rapid Resolution HD (2.1x100 mm, 1.8 mm) and a gradient with 0.1 % formic acid in water and 0.1 % formic acid in acetonitrile. The sample was first injected with the QTOF operating in positive ionization and MS-only mode at a scan rate of 2 Hz. The identification of the metabolites was based on MS-data and retention time conformity. Further confirmation of positive findings was done after a second injection in targeted MS/MS mode acquiring MS/MS-data and using a library for collision induced dissociation spectra comparison.

**Results:** The limit of quantification varied between 0.05 and 10 ng/ml for the different metabolites. The matrix effect was mainly between 80 and 120 %. Extraction recovery was above 75 % except for the metabolites of JWH-210. Intra-sequential precision was better than 10 % except for RCS-4-N-pentanoic acid and inter-sequential precision was better than 15 % except for the metabolites of JWH-210. The accuracy was, with some exceptions, between 85 and 115 %. The applicability of the method is currently tested by analyzing urine samples ( $n \approx 1000$ ) from subjects in drug withdrawal programs where synthetic cannabinoid screening had been requested. The rate of findings and the concentration of the drugs detected will be presented. The preliminary results on 700 screened samples are showing a rate of positive findings at 2.8 %. Metabolites of AB-Fubinaca, AKB48, 5F-PB-22, BB-22, JWH-018 and JWH-073 are detected so far. A strategy to add new compounds to the screening method, ensuring that it is always updated, will be presented.

**Conclusion/Discussions:** A method for identification and quantification of synthetic cannabinoids in urine using UHPLC-QTOF-MS was validated. The method was proven to be sensitive, selective and robust for routine use.

**Keywords:** Synthetic Cannabinoids, Urine Screening, High Resolution Mass Spectrometry

## Application of a New Random Access Fully Automated Biochip Analyzer, Evidence Evolution, to the Simultaneous High Throughput Screening of Twenty Drugs of Abuse in Urine

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**Background/Introduction:** Drug impaired driving is becoming a major problem in the US and Worldwide. In 2013, recommendations for the toxicological investigation of drug-impaired driving and motor vehicle fatalities were reported. By using biochip array technology, multiple analytes can be detected from a single sample leading to test consolidation and an increase in the screening capacity in testing settings. The biochip array DOA ULTRA/DUID has 20 simultaneous immunoassays for drugs of abuse relevant in testing driving under the influence of drugs (DUID) casework.

**Objective:** The aim of this study was to evaluate the applicability of the new random access, fully automated biochip analyzer Evidence Evolution to the simultaneous high throughput screening of 20 drugs of abuse in urine employing the biochip array DOA ULTRA/DUID. With this system, the time to first result is 36 minutes; 60 samples can be loaded per hour and 20 different biochip arrays can be on-board at any one time.

**Methods:** The biochip array DOA ULTRA/DUID kit applied to the Evidence Evolution biochip analyser was used for the simultaneous detection of amphetamine, methamphetamine, barbiturates, benzodiazepine 1 and 2, buprenorphine, cannabinoids (THC), cocaine/benzoylcegonine, dextromethorphan, fentanyl, ketamine, meprobamate, methadone, opiates, oxycodone 1 and 2, phencyclidine, tramadol, tricyclic antidepressants and zolpidem. Simultaneous competitive chemiluminescent biochip-based immunoassays were employed. Ligands were immobilized and stabilized to the biochip surface defining an array of twenty discrete test sites. The signal output is inversely proportional to the concentration of drug in the sample. The assays are semi-quantitative. The system has dedicated software to process, report and archive the data produced. The sample volume required is 10  $\mu$ L of neat urine.

**Results:** Recommended urine cut offs were achieved for all assays on the Evidence Evolution analyzer: amphetamine 200ng/mL, methamphetamine 200ng/mL, barbiturate 200ng/mL, benzodiazepine 1 and 2 100ng/mL, buprenorphine 5ng/mL, cannabinoids 50ng/mL, cocaine/benzoylcegonine 150ng/mL, dextromethorphan 20ng/mL, fentanyl 2ng/mL, ketamine 750ng/mL, meprobamate 500ng/mL, methadone 300ng/mL, opiates 200ng/mL, oxycodone 1 and 2 100ng/mL, phencyclidine 25ng/mL, tramadol 5ng/mL, tricyclic antidepressants 100ng/mL and zolpidem 10ng/mL. The limit of detection was determined by running 20 negative urine samples, the resultant mean concentrations plus three standard deviations were less than 50% of the cut-offs required. The cut-off values were further validated by assessing inter-assay precision. Urine samples were spiked with the appropriate drug compound 50% below, at the cut-off, and 50% above the recommended cut-off. Three replicates were assessed over 5 separate runs and the inter-assay precision, expressed as CV(%), was <20% for all levels across all assays. Intra-assay precision was also assessed with 20 replicates of a low, mid and high concentration sample within one run; resultant intra-assay precision was <20% for all levels across all assays.

**Conclusion/Discussions:** The results indicate applicability of the DOA ULTRA/DUID biochip array kit the simultaneous testing of drugs of abuse from a single sample of urine when applied to the new random access fully automated analyzer Evidence Evolution. The twenty immunoassays arrayed on each biochip surface presented both the desired sensitivity and reproducibility required to achieve screening at the recommended cut-offs. With a time to first result of 36 minutes and with the capacity to assess 60 samples per hour, this system represents a new valuable multi-analytical screening tool for test consolidation and increased screening capacity in test settings.

**Keywords:** Biochip Array, Drugs of Abuse Screening, Biochip Analyzer

**High-Throughput Determination of Carbapenem Drugs in Human Plasma by HILIC-MS/MS**

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**Background/Introduction:** Carbapenems are  $\beta$ -lactam antimicrobial agents and then time-dependent antibiotics. The regimen should be based on pharmacokinetic-pharmacodynamic (PK-PD) theory and be useful in clinical practice including against anaerobic infections.

**Objective:** The rapid and sensitive determinations for these drugs in human samples are required for the therapeutic drug monitoring (TDM) of effective treatment, and diagnosis of intoxication for forensic purposes. We established a new high-throughput HILIC-MS/MS for the analysis of 5 carbapenems (biapenem, panipenem, imipenem, doripenem and meropenem) in human plasma.

**Methods:** Plasma samples (20  $\mu$ l) spiked with the 5 carbapenems and piperacillin (IS) were diluted with 80  $\mu$ l of 10 mM ammonium acetate followed by a simple protein precipitation with 400  $\mu$ l of acetonitrile. After centrifugation, the clear supernatant extract (10  $\mu$ l) was directly injected into the HILIC-MS/MS, without any solvent evaporation and reconstitution steps. The chromatographic separation of the carbapenems was achieved on a Unison UK-Amino HILIC column (50 mm x 3 mm i.d., particle size 3  $\mu$ m) with a linear gradient elution system composed of 10 mM ammonium acetate (pH 6.8) and acetonitrile at a flow rate of 0.5 mL/min.

**Results:** MRM obtained by this method provided high specificity for determinations of the 5 carbapenems in plasma samples. Distinct peaks appeared for each drug and the IS on each channel within 3.5 min. All drugs spiked into plasma showed recoveries of 24–85%. The regression equation for the 5 drugs showed excellent linearity from 50 to 2000 ng/0.02 ml of plasma, and the limits of detection ranged from 25 ng/0.02 ml. The intra- and interday coefficient of variations for all drugs in plasma were less than 6.3%. The present method was successfully applied to actual plasma determinations of doripenem or meropenem.

**Conclusion/Discussions:** The present method will be useful for high-throughput determination of carbapenems in clinical and toxicological analyses. We are currently conducting trials with this technique for the detection of other classes of drugs in human body fluid samples.

**Keywords:** Carbapenem Drugs, Interaction Liquid Chromatography (HILIC), Tandem Mass Spectrometry (MS/MS)

**Validation of i-STAT®1 for the Quantification of Electrolytes and Glucose in Postmortem Vitreous Humor**

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**Background/Introduction:** The analytical value of vitreous humor as a specimen in postmortem forensic toxicology has been known for some time. Most medical examiner laboratories outsource the analysis of this important specimen for electrolyte and glucose measurements. This can be both time-consuming and costly. The utility of the i-STAT®1 medical device to measure electrolytes and glucose in whole blood samples has been demonstrated for over two decades in a clinical setting through single-use disposable cartridges that introduce samples to the i-STAT®1. Different cartridge types allow for the analysis of various analytes including sodium, potassium, chloride, creatinine, urea nitrogen, and glucose. With only 100 µl of sample, results are obtained in under four minutes.

**Objective:** To validate the use of i-STAT®1 to quantitate sodium, potassium, chloride, creatinine, urea nitrogen, and glucose in postmortem vitreous humor.

**Methods:** Validation studies were performed as suggested by the Scientific Working Group for Forensic Toxicology and the Organization of Scientific Area Committees.

**Results:** All analytes of interest demonstrated a percent error less than  $\pm 10\%$  for both accuracy and precision studies. Drug interference and stability studies were performed with many of the analytes demonstrating a percent error less than  $\pm 20\%$ . Throughout drug interference and stability studies, results from all analytes of interest were acceptable except for potassium, which gave inconclusive results. However, creatinine and chloride must be evaluated carefully as there was significant interference with some commonly encountered drugs. Vitreous samples with high concentrations of ethanol revealed interferences for all analytes and was considered a limitation for this method of analysis.

**Conclusion/Discussions:** Overall, the i-STAT®1 was accurate and precise for its intended use. Parallel studies with other techniques and further investigation in the extent of drug interferences are required before employing this technique. As vitreous humor has been used in forensic medicine to aid in diagnostic interpretation, the i-STAT®1 has great potential to give accurate results in a timely and cost-effective manner.

**Keywords:** Vitreous, Electrolytes, Postmortem

**Elevated Blood Lead Levels in Children 2009-2016**

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**Background/Introduction:** Childhood lead toxicity is a preventable environmental disease with long-lasting adverse health and behavioral effects. Public health services and healthcare professionals throughout the United States have dedicated more than four decades of effort to screen children, especially those at high risk, for lead exposure and to identify and limit primary sources of lead. Environmental policies together with laboratory testing and case management have been instrumental in reductions of blood lead levels (BLLs) in the United States.

According to the 2007-2010 National Health and Nutrition Examination Survey (NHANES), the mean BLL estimate was 1.3 µg/d - a 90% decrease compared to the 12.8 µg/dL estimate from the NHANES II 1976-1980. The Centers for Disease Control and Prevention (CDC) changed BLLs deemed safe from 25 µg/dL to 10 µg/dL (1991), and then to “no safe BLL” (2012). “No safe BLL” is based upon absence of BLLs without effects, and that low BLLs are associated with intellectual deficits, attention deficit behaviors, and poor academic achievement. These effects of lead poisoning are irreversible and emphasize the shift in public health care from preventing exposure after detecting lead exposure to primary prevention.

Current CDC recommendations use the NHANES 97.5<sup>th</sup> BLL percentile (5.0 µg/dL) as the upper reference interval threshold to identify children with elevated BLLs. Based upon the 5.0 µg/dL threshold, the CDC estimates 450,000 children in the United States have elevated BLLs. The NHANES analysis includes demographic categories with long-standing disparities of risk such as poverty and housing age. A limitation of the NHANES is the low number of children with elevated BLLs (9 children in 2007-2008; 6 children in 2009-2010), which makes BLL population estimates difficult to interpret.

This Quest Diagnostics Health Trends™ study extends our original six-year retrospective study of a large national clinical laboratory database and now has more than 6 million BLL results. Originally, we analyzed BLLs three years before and three years after the May 2012 CDC changes. The data now includes BLLs from tests performed May 2015-September 2016.

**Objective:** To evaluate trends in blood lead levels (BLLs) in children <6 years old. This Quest Diagnostics Health Trends™ report builds upon previously reported National Health and Nutrition Examination Survey (NHANES) data with a much larger national group and adds more granularity and novel assessments.

**Methods:** This study (May 2009-September 2016) is based on analysis of >6 million BLL results (including >4.6 million venous results) from children <6 years old living in all 50 states and the District of Columbia. We evaluated yearly changes and examined demographic categories including gender, pre-1950s housing construction, PIR, Medicaid enrollment status, and geographic regions. The Cochran-Armitage test was used to analyze trends in proportions of children with blood lead levels  $\geq 5.0$  µg/dL.

**Results:** Of children <6 years old, 2.9% exhibited BLLs  $\geq 5$  µg/dL (high BLL). Generally, BLLs declined over time for all groups. There were significant differences in high BLLs based on gender, pre-1950s housing construction quintiles, PIR<1.25 and PIR>5 (all  $p<0.01$ ) as well as Health and Human Services Regions.

**Conclusion/Discussions:** Examination of >4.6 million BLL venous results in children <6 years old provides a robust, detailed analysis of BLL group results by geography and other criteria that are not available using the NHANES. Progress in reducing the burden of lead toxicity is a public health success story that is incomplete, with some identified factors posing larger, ongoing challenges.

**Keywords:** Blood Lead Levels, Children, Regions, Pre-1950s Housing Construction, Poverty Income Ratios

## A Validated Analytical Method for Synthetic Cannabinoids and Metabolites in Urine Using Liquid Chromatography Tandem Mass Spectrometry

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**Background/Introduction:** Synthetic cannabinoids are a group of new psychoactive substances (NPS) acting as agonists at the cannabinoid receptors and mimic the effects of  $\Delta^9$ -tetrahydrocannabinol. Due to the increasingly emerging of synthetic cannabinoids into the illicit drug market, many have been controlled under our Misuse of Drug Act since 2014. Hence, there is a need for our laboratory to continuously develop analytical method for the new synthetic cannabinoids under the list of controlled drugs.

**Objective:** To validate the addition of nine new synthetic cannabinoids/metabolites, namely 5-fluoro-AKB48 N-(4-hydroxypentyl) metabolite, 5-fluoro-UR-144, 5-fluoro-AKB48, MAM-2201 N-(4-hydroxypentyl) metabolite, THJ-018, THJ-2201, BB-22, PB-22 and 5-fluoro-PB-22 to our existing analytical method for 15 synthetic cannabinoids in urine using liquid chromatography tandem mass spectrometry (LC-MS/MS) according to the SWGTOX guidelines.

**Methods:** A urine sample of 0.5 ml was mixed with internal standard (AM2201 N-(4-hydroxypentyl) metabolite -d<sub>3</sub>) and buffered with pH 5 ammonium acetate. The mixture was incubated at 60 °C for 1 hour with 500 Fisherman units of a mixture of  $\beta$ -glucuronidase/sulfatase from abalone (*Haliotis rufescens*). The sample was basified with alkaline buffer before loading onto a Biotage Isolute SLE+ cartridge, and extracted with ethyl acetate. The eluate was evaporated to dryness and reconstituted with 500  $\mu$ l of water and acetonitrile mixture (1:1, v/v) for LC-MS/MS analysis. Chromatographic separation was achieved on a CORTECS UPLC C18 column, (2.1 x 100 mm, 1.6  $\mu$ m) using gradient elution comprising of solvent A (10 mM ammonium formate in water) and solvent B (acetonitrile with 0.1% formic acid), at a flow rate of 0.4 mL/min. Mass spectrometric data was acquired with 3 transitions per analyte in multiple reaction monitoring (MRM) mode using positive electrospray ionization.

**Results:** The 24 analytes were eluted within 12 minutes, and the detection of the existing 15 synthetic cannabinoids was not affected by the addition of 9 new synthetic cannabinoids. No significant interferences from the matrix, structurally-similar analytes and other common drugs of abuse were observed. The limits of detection were found to range from 0.1 – 0.5 ng/ml based on the requirement of 3 MRM transitions. Extraction recoveries using the SLE method were found to be in the range of 58% to 89% for all 9 analytes at concentrations of 2, 10 and 20 ng/ml. Ion suppression/enhancement was evaluated at concentrations of 2 and 10 ng/ml, and was found to be between -19.4% to 7.5% (n=10) with internal standard correction. The analytes in the urine extracts were found to be stable for at least a week when kept at 4°C except for 5-fluoro-AKB48 (4 days) and THJ-2201 (2 days).

**Conclusion/Discussions:** Nine new synthetic cannabinoids/metabolites were successfully added to an existing LC-MS/MS analytical method for 15 synthetic cannabinoids in urine. The method was successfully validated according to the SWGTOX guidelines for qualitative methods.

**Keywords:** Synthetic Cannabinoids, NPS, LC-MS/MS

**Validation of Generic Sample Extraction Workflow for Analysis of 62 Drugs in Urine by LDTD-MS/MS (Screening) and LC-MS/MS (Confirmation)**

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**Background/Introduction:** To improve analysis by lowering operating costs and increasing throughput all the while maintaining quality data reporting, a generic extraction tool combined with LDTD-MSMS (Laser Diode Thermal Desorption) and LC-MSMS analysis was validated for screening and confirmation analysis, respectively. Validation parameters based on screening method described in SAMHSA (Substance Abuse and Mental Health Services Administration) guideline were used for the LDTD-MS/MS method and quantitative analysis for LC-MS/MS method.

**Objective:** A generic extraction method for all drug polarities is used for presumptive and definitive validation method. 62 drugs are analyzed in urine from different classes (opiates, benzodiazepines, amphetamines, barbiturates, etc.). Precision at the decision point, interference study and stability tests are evaluated for LDTD-MS/MS screening method. All quantitative analysis parameters for the LC-MS/MS confirmation methods are tested.

**Methods:** The following drugs are spiked in urine at a 50%, 100% and 150% of the required concentration for the screening validation. For the confirmation analysis, calibration curve from 1 to 1000 ng/mL in urine is prepared. Urine samples are first hydrolyzed. 50  $\mu$ L of urine sample is mixed with 100  $\mu$ L of internal standard/Buffer/b-glucuronide (IMCSzyme, 20000 IU/ml) solution, added to the SPE-LDX-1 cartridge (SPEWare) and hydrolyzed at 55°C for 30 minutes. The sample is pushed through the packing and then washed (500  $\mu$ L HCl (0.1N) and 800  $\mu$ L Hexane:Ethyl acetate (98:2)). Barbiturate group, THCC (11-Nor-9-Carboxy-THC), Meprobamate and Carisoprodole are eluted with ethyl acetate then all other drugs are eluted using basic elution solution. 4  $\mu$ L of each elution solution are spotted on LazWell plate (96 well plate, Phytronix) for LDTD-MS/MS (LDTD: W-960, Phytronix and MS/MS: TQ-S, Waters) screening. Elution 1 and elution 2 were mixed and injected on LC-MS/MS (LC: UHPLC Nexera, Shimadzu and MSMS: LCMS-8060, Shimadzu) system using generic gradient on C18 column in ESI ionization mode.

**Results:** LDTD-MS/MS operated in MRM mode allows rapid measurement of all drugs desorbed simultaneously. Specific transitions are monitored for each drug. Certain drugs are analyzed using positive ion mode: Benzodiazepine, opiate, amphetamine, antidepressant and more. Others are screened using negative ion mode: barbiturate group and THCC. Precision at the decision point, interference study and stability are reported for the screening approach. All compounds give good responses around decision point with no overlap and good stability. Two transitions (quantifier and qualifier) are used for LC-MS/MS confirmation method. Within-run and between-run precision lower than 20% is reached and bias within  $\pm 20\%$ .

**Conclusion/Discussion:** A generic sample preparation of urine samples combined with LDTD-MS/MS and LC-MS/MS system is validated. All parameters followed criteria of regulatory guideline. This analytical procedure is now ready for real sample analysis.

**Keywords:** Drug, Validation, Screening/Confirmation

**Treatment of Alcohol Dependence in France: Analytical and Toxicological Aspects**

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**Background/Introduction:** There has been in France a recent increase of baclofen prescription, centrally acting gamma-aminobutyric acid agonist, since introduction for the treatment of alcohol addiction management at high daily doses exceeding 250 or 300 mg/day (compassionate use program for baclofen in France). So, intoxication with baclofen seems likely to increase in France, as growing interest has made it a “French craze”. Most recently, nalmefene, a selective opioid receptor antagonist was further licensed in 2014 in Europe for the reduction of alcohol consumption in adults with a high drinking risk level.

Many methods are available for the analysis of these two molecules and in particular for baclofen. Due to the amphoteric nature of baclofen, it is difficult to extract it efficiently from biological specimens but many published methods describe simple sample precipitation procedures with determination in liquid chromatography with UV detection, tandem mass spectrometry (Kintz et al, *Tox Ann Clin* 2015; Labat et al, *Tox Ann Clin* 2016) or high resolution spectrometry (Labat et al, *Biomed Chromatogr* 2017). One of the main interests of this last method is the identification of two baclofen metabolites in plasma samples of patients treated with baclofen but we don't know at that time what new information could bring quantification of these no active metabolites in the clinical settings or toxicological activity.

**Methods:** Many published LCMSMS methods described quantification used baclofen-d4 as internal standard with linear range in general between 10 to 2000 ng/mL for all biological matrices (Kintz et al, *Tox Ann Clin* 2015; Labat et al, *Tox Ann Clin* 2016). These methods validate specific, simple method of determination baclofen in plasma, urine, whole blood or alternative samples. Intra- and inter-day precisions were below 10% and accuracies were between 90 and 110% for baclofen. No matrix effect was observed.

**Results:** In our laboratory, a method in LCMSMS with one step sample precipitation in acidic conditions was validated according to the European Medicines Agency (EMA) guidelines and was successfully applied to two group of patients, first patients with baclofen monitoring (plasma concentrations between 12 and 1399 ng/mL with daily doses from 30 to 240 mg, n = 36 plasma from 36 patients treated) (Labat, *Biomedical Chromatography* 2017) and to patients with baclofen intoxications ( maximum plasma concentrations between 222 to 14600 ng/mL in plasma for doses described between 250 to 3500 mg, n = 15). In 9 intoxication cases, half-life were observed between 4.8 and 21 h and in these cases, it has been interesting to realize two samplings a day to monitoring baclofen elimination.

In 2016, the French Society SFTA (Société Française de Toxicologie Analytique) has developed a new external quality control (EEQ) for the baclofen plasma determination and added the molecule in the screening EEQ in urine, plasma and whole blood. For the first one, 62% (20/32) determinations in LCMSMS were kept in the statistical treatments with a mean of 359 ng/mL (between 172 to 546 ng/mL). No satisfactory results were achieved from laboratories with GC-MS or LC-DAD methods. EEQ proposed as screening in urine/ plasma or urine/whole blood described less successful results in GC-MS, LC-DAD or LC-MSMS method screening with only 20% (4/24 and 7/28) positive detection of the molecule for LC methods.

For nalmefene detection, it is not still systematically included in general targeted screenings but fewer analytical problems were observed with this molecule in targeted opiate screenings.

**Conclusion/Discussions:** Baclofen, a small molecule with amphoteric nature was difficult to detect in classical screening conditions at low concentrations. It seems that a specific method to determine baclofen is necessary not to miss out its detection. These initial results show the interest of the EEQ in France and ask of the interest to add nalmefene in quality control system.

**Keywords:** Alcohol Dependence, Baclofen, Treatment



## Evaluation of KIMS Immunoassays on a Cobas c 501 Analyzer for Drugs of Abuse and Ethyl Glucuronide in Urine for Abstinence Control and for Drugs of Abuse in Serum Using Typical Clinical Cut-Offs

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**Background/Introduction:** Reliable urine screenings for drugs of abuse and alcohol consumption are essential for the implementation of abstinence control programs. For the medico-psychological assessment (MPA) during driving license re-granting a high sensitivity of the applied screening methods is highly important, as very low drug concentrations have to be detected. On the other hand, in clinical settings, drug intoxications have to be detected rapidly by analysis of blood/serum. Immunoassays are typically used for drug screenings and have to be adapted to the range of expected concentrations, where appropriate.

**Objective:** Evaluation of modified kinetic interaction of microparticles in a solution (KIMS) drugs of abuse and ethyl glucuronide immunoassays on a Roche cobas c 501 analyzer for abstinence control in urine and analysis of serum samples.

**Methods:** Urine: the following parameters (and target concentrations in ng/ml) were evaluated: cannabinoids (10), opiates (25), cocaine (metabolite) (30), amphetamines (50), methadone (metabolite) (50), benzodiazepines (50), and ethyl glucuronide (100). A semi quantitative application covering five to six calibration points was used and precision, accuracy, onboard calibration stability, , sensitivity, specificity, and cross reactivity for a variety of drugs and their metabolites and new psychoactive substances (NPS) were evaluated using quality control and at least 150 authentic urine samples per parameter. Authentic positive samples with concentrations of 60 % to 200 % of the target concentrations were applied. The concentrations of the quality controls were approx. +/-25 %, and 3 to 10 times the respective target concentrations.

Serum: the following parameters (and target concentrations in ng/ml) were evaluated: cannabinoids (50), cocaine (metabolite) (300), amphetamines (300), methadone (metabolite) (300), benzodiazepines (200), and barbiturates (200). A qualitative application with a one-point calibration was used to evaluate the assay performance at the respective target concentration using at least 140 authentic and spiked positive and negative samples per parameter.

**Results:** Urine: precisions (intraday and interday relative standard deviation (RSD %)) and accuracies (bias) at three concentrations were 12% or lower for all parameters. The calibrations remained stable (deviations < 25%) for at least 28 days for all assays except amphetamines (21 days), therefore the instrument needs to be calibrated only every three to four weeks. Satisfactory cross reactivity was determined for the relevant analytes and also for several new psychoactive substances (NPS). The sensitivity at the chosen cut-off was 100% for all parameters except methadone metabolite EDDP (92%) and fully met the sensitivity criteria for MPA urine testing.

Serum: Most of the immunoassays were able to discriminate well between positive and negative samples in the respective range of the target concentration. Unexpectedly, the cannabinoids immunoassay showed positive results in samples having free THC-COOH concentrations well below the target concentration of 50 ng/ml; this might be due to high cross reactivity of other metabolites.

**Conclusion/Discussions:** The modified KIMS immunoassays on a cobas c 501 can be applied to reliably and sensitively detect drug or alcohol consumption in abstinence control programs and drugs of abuse in clinical intoxication cases.

**Keywords:** Urine Screening, Medico-Psychological Assessment, Sensitivity

**Cannabinoids Detection in Bronchoalveolar Lavages of Heavy Cannabis Smokers with Lung Disease**

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**Background/Introduction:** Cannabis is one of the most widely abused substances throughout the world and the second most smoked psychotropic substance after tobacco. It is difficult to accurately assess the specific effects of cannabis smoking and to distinguish them from the ones of tobacco. Nevertheless, it is known that Cannabis smoke affects the lungs similarly to tobacco smoke, causing symptoms such as increased cough, sputum, hyperinflation and chronic bronchitis. Furthermore, the chronic use can also cause serious lung diseases and airway obstruction. Finally, cannabis can weaken the immune system, leading to eventual lung infections such as pneumonia.

The relationship between cannabis smoking and pulmonary functions or respiratory complications is poorly understood and the objective assessment of causal effects of cannabis in the above reported diseases have been scarcely demonstrated.

**Objective:** To develop and validate a method for the identification and quantification of delta-9- tetrahydrocannabinol (THC) and its metabolites 11-hydroxy-THC (11-OH-THC) and 11-nor-9-carboxy-THC (THC-COOH) and non psychoactive cannabinoids, cannabinol and cannabidiol in bronchoalveolar lavages (BALs) collected from hospitalized patients with lung diseases and a long history of heavy cannabis consumption. The interval of time between BAL collection and last cannabis smoking was in the range 2 – 35 days.

**Methods:** For the extraction of cannabinoids from BALs, 1 ml sample was added with 300 uL of 0.1 N NaOH and 3mL of hexane/ethyl acetate (9:1). The solvent was then evaporated to dryness. Trimethylsilyl derivatives were prepared and then analyzed by a Agilent HP 7890A gas chromatograph coupled with an Agilent MSD 5975C. The used capillary column was an HP-5MS (15 m × 0.25 mm I.D coated with a 0.25 µm film). The GC conditions were as follows: the column temperature was hold for 3.5 min at 70°C; increased to 200°C at 40°C/min and then increased to 290°C at 10°C; the injection port temperature was 250°C. The mass analyzer operated by electron impact (70 eV) in selected ion monitoring mode (SIM). Quantitative analysis was carried out recording ions *m/z* 303, 371, 386 for THC and *m/z* 306, 374, 389, *m/z* cannabinol, *m/z* 390, 337, 301 cannabidiol, *m/z* 367, 382 310 11-hydroxy-THC (11-OH-THC) and *m/z* 488, 473, 371 for 11-nor-9-carboxy-THC (THC-COOH) and for THC-*d*3 internal standard. The method was fully validated according to international guidelines.

**Results:** The obtained results showed that 6 cases out of 15 were positive for THC, cannabinol and cannabidiol (THC concentration range: 0.5 -25.4 ng/ml; cannabinol 0.4-25.6 ng/ml; cannabidiol 0.6-39.8 ng/ml). In two samples the presence of 11-OH-THC was also measured (1.5 ng/ml and 3.3 ng/ml) while THC-COOH was not detected in BALs. In the 6 positive cases the last cannabis smoking was between the previous 2-14 days, whereas in the other 9 negative cases the last cannabis smoking was between the previous 16-35 days. Surprisingly, only in two of the 6 positive cases urine was found positive for THC-COOH.

Surprisingly, 12 patients declared themselves as non- cigarette smokers stating that they smoked the few tobacco mixed with cannabis to prepare the joint. Unfortunately, at the time of the study nicotine and its metabolite cotinine were not measured in BAL.

**Conclusion/Discussions:** A method for the detection and quantification of cannabinoids in BAL has been developed and validated. The application of this method to 16 cases allowed the identification of 6 positive cases, supporting that BAL collected from patients with lung disease is a valid alternative matrix to detect cannabinoids with a broad detection window up to 14 days, although it is necessary to take into account that these are preliminary data with a limited number of cases, therefore it needs confirmation with a wider sample. This is the first time that cannabinoids are detected in BAL, demonstrating the presence of a drug with its metabolites in a target organ of consumers who present a lung disease. This occurrence let hypothesize a role of cannabinoids in the development of the disease and prompted an investigation on possible associations between cannabis smoking and clinical outcomes in these patients with lung disease with an eventual cytotoxic effect of cannabinoids themselves.

**Keywords:** Cannabinoids, Bronchoalveolar Lavages, Lung Disease

**Injuries in People Convicted for Driving Under the Influence of Alcohol: The Role of Non-Illicit Psychoactive Drugs**

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**Background/Introduction:** One of the main risks for people convicted of Driving Under the Influence of Alcohol (DUIA) is the increased occurrence and re-occurrence of Motor Vehicle Collision (MVC) and other injuries (i.e. domestic, occupational and sportive). The use of medications is a widespread event in the DUIA population and many drugs are known to impair cognitive functions and consequently driving abilities. The effects of these substances is often additive/synergistic when combined with alcohol and this is the reason why non-illicit drugs could be an important but underrated factor for accident causation. The purpose of this study is to find out elements which could be accounted for as risk factors for accident causation in a population convicted for DUIA, with the intent of carrying on repressive and preventive measures which could be accomplished through the delay or denial of driving licence regranting. These preventive actions are meant to avoid the circulation of those dangerous subjects on the roads.

**Objective:** Evaluate the role of medications and other variables such as age, gender and laboratory findings as risk factors for MVC and generic (domestic, occupational and sportive) injuries.

**Methods:** 5003 DUIA people seeking restoration of their driving licenses were examined between April 2005 to December 2015 at the Legal Medicine Section of the Università Politecnica delle Marche, Ancona, Italy. About 2000 of these were involved in MCV and other kinds of accidents. Information about their gender, age, marital status, education, laboratory findings and medications was collected.

**Results:** Of the entire population studied, 32 % were involved in one or more MVC, 5 % had at least one work injury and 10 % were involved in other kind of accidents (domestic, occupational and sportive). About 8% were consuming non-illicit drugs: anxiolytics, antidepressants, hypnotics, antiepileptics, neuroleptics and antihistamines were the main drug classes found. Considering the population of people who had accidents, the majority were male (1743) but female gender was associated with a higher risk of accidents (M vs F, OR = 0.659 CI: 0.507 to 0.858). They were mainly aged 30-49, with a higher incidence of injuries in people older than 50 (OR = 1.014 CI: 1.006 to 1.023). The majority had an average education (high school degree) but a low level of education was accounted for as a risk factor (higher education OR = 0.588 CI: 0.423 to 0.817). Other important elements related were the use of medications, in particular antiepileptics (OR = 2.740 CI: 1.241 to 6.052) and anxiolytics (OR = 1.976 CI: 1.296 to 3.011) and drink-driving relapses (OR = 1.726 CI: 1.447 to 2.059). Single status seemed to be a protective factor (OR = 0.708 CI: 0.591 to 0.848).

**Conclusion/Discussions:** Accident-proneness in people seeking restoration of their driving licenses after being stopped for driving under the influence of alcohol was analyzed. Some elements contributed to risk factors: antiepileptics and anxiolytics use, drink-driving relapses and age. Contrarily, being single or male and or having a high education appear to be protective factors. General Practitioners should prescribe the appropriate medications or choose the safest alternatives. Furthermore, while examining a patient seeking restoration of his driving license, many variables should be taken into account, i.e. the number of drink-driving relapses, age, gender, etc. The investigation of these risk factors could be the first important step to reduce the incidence of both MVC and generic accident causation in this particular population.

**Keywords:** Driving Under the Influence of Alcohol (DUIA), Motor Vehicle Collision (MVC), Medications

**LC-MS/MS Method Development and Validation for the Quantitation of AB-PINACA Pentanoic Acid Metabolite and UR-144 Pentanoic Acid Metabolite in Urine**

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**Background/Introduction:** Synthetic cannabinoids (SCs), originally developed as research tools, are now highly abused novel psychoactive substances that are illegal in many European countries and in the United States. Due to the use of these illegal drugs, it is important for drug testing laboratories to have a sensitive and specific way of monitoring these compounds. The parent compounds of SCs are not excreted in urine; however, their common metabolites are, and thus they can be detected in urine. Immunoassays have different cross-reactivities for different SCs classes and it is challenging to keep pace with changing analyte targets. Robust and precise analytical methods are needed to quantitate these compounds in biological matrices for forensic purposes.

**Objective:** To develop and validate a sensitive, specific and robust quantitative method for the AB-PINACA pentanoic acid metabolite and UR-144 pentanoic acid metabolite.

**Methods:** A quantitative method using Thermo Scientific™ TurboFlow™ technology was developed for the quantitation of AB-PINACA pentanoic acid metabolite and UR-144 pentanoic acid metabolite in the urine matrix. Chromatographic analysis was performed using Thermo Scientific Transcend™ Aria LC system and a Thermo Scientific Hypersil™ GOLD 100x3 mm, 5 μ column. MS analysis was carried out on a Thermo Scientific TSQ Vantage triple stage quadrupole mass spectrometer equipped with a heated electrospray ionization (HESI-II) probe. Standard curves were prepared by spiking blank urine matrix with increasing concentrations of both the metabolites (0 to 150 ng/mL). Quality control (QC) specimens were prepared in a similar manner at concentrations corresponding to the low, middle and high end of the calibration range. Intra- and inter-run precision and robustness were determined by analyzing five replicates of each QC level with a calibration curve on five different days. Other validation studies included limit of quantitation, method comparison, and dilution linearity. Bench top, freeze/thaw, and autosampler stability studies were also included in the validation.

**Results:** A TurboFlow LC-MS/MS method was developed and validated for the quantitation of AB-PINACA pentanoic acid metabolite and UR-144 pentanoic acid metabolite in urine matrix. The LC-MS/MS method resolves both analytes with total analysis time of less than 7 minutes. The calibration curves were linear from 0.5 to 150 ng/mL, with  $R^2$  values greater than 0.999. The limit of quantitation was 0.5 ng/mL. The intra-run precision and accuracy (relative to gravimetrically-based target) values obtained were 0.2% to 4.7% CV and -0.3% to 0.8% bias, respectively, for AB-PINACA pentanoic acid metabolite and 0.5% to 5.0% CV and -0.5% to 0.3% bias, respectively, for UR-144 pentanoic acid metabolite. The inter-run precision and accuracy obtained for AB-PINACA pentanoic acid metabolite were equally acceptable and ranged from 2.3% to 2.8% CV and -0.02% to 0.3% bias, respectively. The results for UR-144 pentanoic acid metabolite were 1.8% to 3.0% CV and -0.3% to -0.01% bias, respectively, indicating that the method is accurate and reproducible. In addition, both metabolites were stable under storage conditions of 4°C and room temperature for 5 hrs, and after three freeze-thaw cycles. The dilution linearity regression equation and correlation for both metabolites were linear with  $R^2$  of 0.999 and above. Minimal carryover was observed over the range. The method comparison data (n = 28) excellent correlation between an immunoassay in development versus LC-MS/MS values with a slope of 1.0053, intercept of 1.0432 and  $R^2$  of 0.988 for AB-PINACA pentanoic acid metabolite and a slope of 1.1011, intercept of 0.431 and  $R^2$  of 0.9988 for UR-144 pentanoic acid metabolite.

**Conclusion/Discussions:** The developed TurboFlow LC-MS/MS method for the quantitation of AB-PINACA pentanoic acid metabolite and UR-144 pentanoic acid metabolite in urine matrix is demonstrably sensitive, specific, robust, accurate and reproducible and could be successfully used in the field forensic toxicology.

**Keywords:** B PINACA Pentanoic Acid Metabolite, UR-144 Pentanoic Acid Metabolite, LC-MS/MS Method

**Detection of Noscapine and Papaverine as Heroin Biomarkers in Pain Management Compliance Testing**

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**Background/Introduction:** Opioid usage in the United States (USA) has increased over the past few years with prescriptions increasing from 76 million in 1991 to 207 million in 2013. Illicit heroin use has also increased with overdose deaths quadrupling between 2000 and 2015. In many laboratories, 6-acetylmorphine (6-AM) is used as the heroin biomarker and is reported to be detectable in urine 2 – 8 hours after heroin use. Due to 6-AM's short detection window, alternative biomarkers, noscapine and papaverine, have been proposed for heroin screening. Noscapine and papaverine have been reported to have longer detection windows than 6-AM, approximately 24 hours and 24-48 hours respectively. Papaverine has been found to have higher urine concentrations than 6-AM. Paterson et al and Trathen et al reported that by screening for noscapine and papaverine metabolites together, the specificity for heroin detection approached 100%. Heroin is synthesized from the morphine present in opium. Opium and poppy seeds contain morphine and other alkaloids, including codeine, noscapine and papaverine, which may be detected in illicit heroin in varying concentrations depending on the method used for heroin synthesis and the opium's geographical origin. Detection of noscapine and papaverine can be used to differentiate between prescription (diamorphine) and illicit heroin use. In many countries, noscapine is an approved drug in over-the-counter cough medications and as an anti-tumor agent. However, neither drug is approved for use in the USA.

**Objective:** To evaluate noscapine and papaverine as heroin biomarkers for use in pain management compliance testing.

**Method:** Over 600 urine specimens routinely analyzed for opiates were also analyzed for noscapine and papaverine, using 6-AM-D6 as the internal standard, by Ultra Performance Liquid Chromatography tandem mass spectrometry (UPLC-MS/MS). Sample preparation included enzyme hydrolysis followed by solid phase micro-extraction. Linearity was performed in duplicate over 3 days, using calibrators prepared by fortifying blank urine at 0.5, 1.0, 2.5, 5.0, 10, 50, 100 ng/ml. The cutoff (decision point) calibrator (2 ng/ml) and controls ( $\pm$  50% of the decision-point calibrator) were analyzed in triplicate over 5 days. Selectivity and matrix effect were evaluated according to SWGTOX guidelines. Two commercially available poppy seed products were analyzed for their morphine, codeine, noscapine and papaverine content.

**Results:** In the 600+ urine specimens analyzed, morphine  $\geq$  1000ng/ml was detected in 82 samples (13%) and in combination with codeine, 32 samples (5%). 6-AM (LOD 5 ng/mL) was detected in 29 samples (4.8%); in combination with morphine and codeine, 16 (2.5%) and morphine only, 13 (2.1%). Noscapine was detected in 8 samples (1.3 %), all except one was positive for 6-AM and morphine. Papaverine was detected in 5 samples (0.8%). Both noscapine and papaverine were detected in 3 samples, which were also positive for morphine, codeine and 6-AM. One sample was also positive for 6-acetyl codeine. No endogenous compounds were found to interfere with the assay. Matrix effect, at the decision point, was noscapine (107%) and papaverine (178%). In the poppy seed products, morphine content was <0.01% and 0.02%; codeine and noscapine were present (<0.005%), and, papaverine was not detected in both products.

**Conclusion/Discussions:** Noscapine and papaverine were detected in specimens with other heroin biomarkers. Noscapine and papaverine, with longer detection times than 6-AM would presumably be more prevalent than 6-AM in urine but they were found in fewer specimens and at lower concentrations than 6-AM. Detection of noscapine and papaverine was evidence of illicit heroin use. 6-AM appears to be the better biomarker of heroin use in this study of the chronic pain patient population. An evaluation of the noscapine and papaverine metabolites may be a worthy study for identifying longer detection time heroin biomarkers.

**Keywords:** Heroin, Heroin Biomarkers, Noscapine, Papaverine

**Carboxyhemoglobin Levels in Transit Police Officers of Arequipa-Perú.**

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**Background/Introduction:** Carbon monoxide (CO) is one of the most important toxic related to human activity. The exchange of carbon monoxide from the air we breathe and the human body is controlled by physical and chemical processes. The mechanisms of induction of toxic effects by forming carboxyhemoglobin are fully known, mostly due to the induction of a hypoxic state in many tissues of various organs. Smaller amounts of COHb lead to oxygen deprivation of the body causing tiredness, dizziness, and unconsciousness.

**Objective:** Determine the levels of carboxyhemoglobin (COHb) in relation to the personal characteristics of the personnel of the Traffic Police Officers of Arequipa City.

**Methods:** The study consisted of 30 traffic policemen officers to which blood samples were taken to determine the levels of carboxyhemoglobin by the spectrophotometric method.

**Results:** We determined that the levels of carboxyhemoglobin (COHb) of the Transit Police in the city of Arequipa vary, in relation to the personal characteristics such as the age group and the characteristics of work that they present. Regarding the age, it was observed that in the police agents exposed 20% have low levels of carboxyhemoglobin and 6.67% high, but about the unexposed ones, their totality have low levels of carboxyhemoglobin. Carbon monoxide poisoning is based on the assessment of symptomatology, source of exposure and COHb plasma concentration. Regarding sex, 18 males were observed, of whom the exposed ones presented a 13.33% low levels of carboxyhemoglobin and 53.33% high, and the ones unexposed presented low levels. The years of service is significant, 53.33% have 1-4 years of service of which the total has low levels of carboxyhemoglobin, medium-exposed police where 6.67% have low levels and 40.00% high. The exposure time is an average of 7 to 9 hours with 66.67% of agents that work this amount of time, of which the exposed ones 20.00% have high levels of COHb and 13.33% low, about the unexposed ones all have low levels of carboxyhemoglobin

**Conclusion/Discussions:** We found that levels of carboxyhemoglobin (COHb) of the members of the Transit Police officers in Arequipa vary in relation to personal characteristics such as age group and working characteristics presented.

**Keywords:** Carboxihemoglobin, Carbon Monoxide, Toxic

**Toxicological Findings in a Case of Oral Exposure to the Herbicide Fluroxypyr 1-methylheptyl Ester**

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**Background/Introduction:** To our knowledge, toxicological findings in herbicide poisoning are rare. We present a case of fluroxypyr 1-methylheptyl ester poisoning caused by intentional contamination of the water reservoir of a coffee-maker. A 50 year old male made coffee and after drinking, the victim experienced drowsiness, dizziness and some gastrointestinal distress, for which he was treated with pantoprazole. He was kept in the hospital for one day, no further medical treatment was required.

**Objective:** To present toxicological findings of oral exposure to fluroxypyr 1-methylheptyl ester in human matrices.

**Methods:** Blood and urine of the victim were collected during hospitalization. After drinking two sips of coffee, the victim felt ill and was hospitalized. 4 Hours after drinking the coffee blood samples were taken. Water from the water reservoir and the cup of coffee (containing coffee) was secured by the police. Ethanol and GHB analysis, a targeted screening (by UPLC-tandem MS and UPLC-ToF-MS), a general-unknown screening (by HPLC-UV and GC-MS) and volatile compounds analysis (by HSGC-MS) were performed in blood and/or urine. The water and coffee were diluted 1,000 to 10,000 times with methanol and analyzed by a general-unknown screening (HPLC-UV and GC-MS) and for the presence of volatile compounds by HSGC-MS. Screening results showed the presence of fluroxypyr 1-methylheptyl ester and/or fluroxypyr in the different matrices. These results were confirmed and the compounds semi-quantified in different matrices.

**Results:** Analysis of the blood and urine demonstrated the presence of ethanol, venlafaxine and metabolites as well as ibuprofen. Toxicological general unknown screening (HPLC-UV and GC-MS) indicated the presence of fluroxypyr in blood and urine. Targeted analysis showed the presence of fluroxypyr in blood (~0.02 mg/l) and in urine (~1.5 mg/l). No fluroxypyr 1-methylheptyl ester was found in these matrices. Fluroxypyr 1-methylheptyl ester was, however, found in the water (~600 mg/l) and coffee (~45 mg/l). The water and coffee also contained small amounts of fluroxypyr (< 10 mg/l). C3 and C4 alkylbenzenes were also present in the water and coffee. The blood sample contained low levels of toluene, ethylbenzene and xylenes. No volatile compounds were identified in the urine.

**Conclusion/Discussions:** Herbicide poisoning by ingestion is rare. Our case presents the first oral exposure in human to the herbicide fluroxypyr 1-methylheptyl ester. This case suggests that fluroxypyr 1-methylheptyl ester in man is rapidly (within 4 hours) hydrolyzed to fluroxypyr. The estimated ingested dose of ~5 mg fluroxypyr 1-methylheptyl ester did not seem to result in a life-threatening situation.

**Keywords:** Fluroxypyr, Herbicide, Human

**Neurometabolic Therapy of Toxic Encephalopathy in Patients with Acute Alcohol Poisoning**

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**Background/Introduction:** One of the most dangerous complications of ethanol intoxication is toxic and hypoxic encephalopathy (TSE). Therefore, intensive care TSEs should be aimed at reducing toxic load, and the timely correction of universal mechanisms of lesions.

**Objective:** evaluation of the effectiveness of rational neuroprotection in complex intensive therapy toxic-hypoxic encephalopathy in patients with acute alcohol poisoning.

**Methods:** This study included 46 men aged 27-55 years. Patients were divided into 2 groups: I group - 22 patients treated with neuroprotective complex includes 1. Infusion of «Reosorbilakt» («Arterium», Ukraine), 400 ml 2 times a day from the date of receipt within 3 days. 2. Infusion or «Suktinasol» («Remedi», Uzbekistan) 400 ml 2 times a day for 3 days from receipt. 3. Injections of 20% solution of «Tiotsetam» («Arterium», Ukraine), a dose of 10 ml per day after recovery of consciousness, ranging from 2 days before the patient is discharged from hospital. Group II - 24 patients received conventional therapy, which includes fluid resuscitation with crystalloid and colloid solutions, sedation neuroleptics (droperidol) and benzodiazepines (sibazon, dormicum), the B and C groups of vitamins, the maximum therapeutic doses. Comparability of groups ensures the absence of significant differences in age, severity of condition (patients 30-55 years). All patients underwent a comprehensive examination by laboratory methods. Blood alcohol concentration was determined in Clinical Analyser at admission and in dynamics at 6, 12, 24 hours. The Glasgow scale was used to evaluate the conscious level of subjects. During the period of recovery of consciousness assessed the severity of intellectual disability using a scale Mini-Mental State Examination (MMSE) of 10 positions on the 2nd, 5th day.

**Results:** Blood ethanol level in both groups at admission was  $3,1 \pm 1,4$  g / l, and the level of consciousness on a scale of Glasgow was  $7,2 \pm 1,4$  points. The therapeutic effect of neuroprotective complex was estimated at a comparative perspective on the regression of clinical manifestations of the disease, and detoxification - on the degree of reduction of ethanol in the blood. Against the background of the therapy is already on the 2nd day in 17 patients in the test group was restored to the level of consciousness of  $14 \pm 1,2$  points in 3 - to  $11 \pm 1,2$  points, in 2-consciousness was 8 points that due to the late delivery of patients to the clinic. Reduction of blood ethanol concentration was already observed after 6 hours on average to  $1,6 \pm 0,4$  g / l, and after 12 hours the concentration decreased to nearly normal -  $0,7 \pm 0,2$  g / l. In the comparison group, the restoration of the level of consciousness on the 2nd day in patients averaged  $11 \pm 1,0$  points which is 1.3 times longer than in the main, the ethanol concentration in the blood after 6 hours was  $2,6 \pm 0,5$  g / l, and after 12 hours  $1,3 \pm 0,3$  g / l, which is higher than the main group 1.6 and 1.8 times, respectively. Study of cognitive function in patients in group I showed a significant improvement in the level of intelligence, study of intelligence on a scale MMSE showed that patients in group I on the 2nd day there is a slight cognitive impairment -  $22 \pm 1,4$  points, and on the 5th cognitive deficit was almost stopped and the total score was  $28 \pm 1,6$  points. The patients in the control group, the performance of intelligence on the 2nd and 5th day were 1.4 and 1.5 lower than in the main group.

**Conclusion/Discussions:** The developed rational neurometabolic therapy improves treatment results due to a significant reduction in the period of recovery of consciousness and cognitive functions.

**Keywords:** Alcohol, Poisoning, Toxic encephalopathy



**Anhydrous Caffeine: Overdose in Tablespoons**

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**Background/Introduction:** Caffeine is the most widely consumed psychoactive drug and is a potent stimulus of the central nervous system. Included in methylxanthine class, it is a crystalline purine alkaloid that is found in vegetation. Its antagonistic effect on adenosine receptors contributes to caffeine's effect on drowsiness and wakefulness. The FDA categorizes caffeine as "generally recognized as safe"; allowing for exemption from many additive requirements and thus is largely unregulated; leading to abuse of the stimulant. Caffeine intoxication, as defined by the DSM-IV, occurs after ingestion of caffeine in amounts greater than typically found in caffeinated beverage or tablets (average 400-500 mg). Toxic overdose occurs with consumption of greater than 10 grams per day. The introduction of anhydrous caffeine has allowed for possible lethal dosage of caffeine in tablespoon-sized amounts.

**Method/Results:** This case presents the rapid effects of anhydrous caffeine on the adult population. A 22 year old male presented to the emergency department with nausea, vomiting and palpitations. The patient has no significant past medical history. He reported taking 3 tablespoons of anhydrous caffeine to increase his energy for work. Laboratory studies revealed elevated creatine kinase 37029 U/mL (reference range 40-230 U/mL) and elevated liver function tests; findings consistent with rhabdomyolysis. A comprehensive drug analysis and a caffeine level was performed and the caffeine level was 119.6 mcg/L (reference range 10-20 mcg/L). The patient was managed with aggressive hydration and potassium supplementation.

**Discussions:** This case shows the importance of raising awareness of the adverse effects of caffeine, specifically anhydrous caffeine.

**Keywords:** Caffeine, Overdose, Case Study

## Ultra-high Performance Liquid Chromatography-tandem Mass Spectrometric Method for the Determination of Fluoxetine and Norfluoxetine in Dried Blood Spots

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**Background/Introduction:** Fluoxetine (FLU) is the most widely prescribed antidepressant drug in the world, being present in many clinical and forensic toxicology cases. FLU is biotransformed by CYP2D6 to the active metabolite norfluoxetine (NFLU). The therapeutic range for trough plasma concentrations is 150-500 ng/mL for the sum of FLU+NFLU. Therapeutic drug monitoring of FLU is recommended for dose titration and for special indications or clinical problem solving. Dried blood spots (DBS) obtained from finger pricks can be used as an alternative for phlebotomy for obtaining clinical samples for FLU and NFLU measurements.

**Objective:** The objective of this study was to develop and validate a method for determination of FLU and NFLU in DBS using ultra-high performance liquid chromatography-tandem mass spectrometry.

**Methods:** One DBS disk with 8 mm of diameter (Whatman 903) was used for FLU and NFLU extraction with 400  $\mu$ L methanol:acetonitrile (3:1, v/v), containing the internal standard fluoxetine-D6 (FLU-D6), at 45 °C. The extraction solvent was evaporated, recovered with initial mobile phase and injected into the Ultimate 3000 LC system (Thermo Scientific). The chromatographic separation was performed in an Accucore C18 column (100 x 2.1 mm, p.d. 2.6  $\mu$ m) maintained at 40 °C and eluted at a mobile phase flow rate of 0.4 mL/min. The mobile phase consisted of formic acid 0.1% (eluent A) and acetonitrile containing 0.1% formic acid (eluent B). Initial eluent composition was 80% A, maintained for 1 min, followed by 5-minute gradient to 50%, which was held for 1 min. Total run time, including re-equilibration, was 12 min. Samples were analysed using a TSQ Quantum Access triple quadrupole mass spectrometer (Thermo Scientific) in the positive electrospray mode. The MS conditions were as follows: capillary voltage of 4.5 kV; sheath gas nitrogen at a flow rate of 20 arbitrary units; auxiliary gas nitrogen at flow rate of 15 arbitrary units; collision gas argon; vaporizer temperature of 380 °C; and ion transfer capillary temperature of 210 °C. The following transitions were used for MRM acquisition: FLU m/z 310-44.5 (quantitation) and m/z 310-42.6 and 310-117.4 (qualification); NFLU m/z 296-134.3 (quantitation) and m/z 296-30.5 and 296-105.3 (qualification); IS (FLU-D6) m/z 316.2-44.5 (quantitation) and m/z 316.2-42.7 and 316.2-187.8 (qualification). Linearity was evaluated in the range of 10-750 ng/mL. Precision and accuracy were evaluated at 40 ng/mL, 200 ng/mL and 600 ng/mL. Within-assay precision and between-day precision were calculated by one-way ANOVA. The acceptance criteria for accuracy were mean values within 15% of the theoretical value and for precision a maximum CV of 15% was accepted. The assay was applied to 18 paired samples of plasma and DBS, collected from patients prescribed FLU.

**Results:** Retention times of FLU, FLU-D6, and NFLU were 4.72, 4.68 and 4.4 min, respectively. The assay was linear in the range of 10 to 750 ng mL<sup>-1</sup>. Considering all QC levels, accuracy was 99.6-107.8% for FLU 99.5-107.2% for NFLU. Precision was 3.5-9.22% for FLU and 2.43-10.42% for NFLU. Concentrations of FLU and NFLU in DBS and plasma samples (n=18) were highly correlated, with *r* of 0.966 and 0.977, respectively. On average, DBS concentrations of FLU were 1.71 times higher than plasma levels and DBS concentrations of NFLU were 1.57 times higher than plasma levels, indicating significant binding to erythrocytes.

**Conclusion/Discussions:** FLU and NFLU can be measured with adequate accuracy and precision in DBS samples within the clinical relevant range of concentrations. DBS levels of FLU and NFLU from a small cohort of patients were highly correlated with plasma concentration. The developed assay can be used to estimate plasma concentrations of FLU and NFLU from capillary DBS samples.

**Keywords:** Fluoxetine, Dried Blood Spots, LC-MS/MS

**Insistent Problem of Turkey: Mass Methanol Poisoning in Cukurova Region**

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**Background/Introduction:** : Illegal alcohol consumption and related methanol intoxication with fatal results is still a problem in Turkey, especially in Cukurova Region which is located at southern part of the country.

**Objective:** To emphasize the importance of blood methanol analysis in forensic toxicology laboratory for recognition and diagnosis of the methanol intoxicated patients.

**Methods:** In this study, methanol intoxicated 48 patients, who admitted to Cukurova University Faculty of Medicine Balcali Hospital between September 2016-February 2017, were presented with their clinical features and with our forensic laboratory results.

**Results:** All data were evaluated by SPSS programme; the mean age was 55 years old, mean methanol concentration was  $92.8 \pm 87.6$  mg/dL (range 9.1-35+0 mg/dL), mean pH was  $7.1 \pm 0.23$  (6.6-7.4), and  $p\text{CO}_2$  was  $32.54 \pm 11.93$  mmHg (15.3-58.2), and  $\text{pHCO}_3$  was  $11.7 \pm 6.81$  mmol/L (4.2-27.8). The most common symptoms and signs on admission were visual disturbances (33%) and rests were headache, nausea, vomiting, dyspnea. Conventional hemodialysis was given to 34 patients (71%), antidotal treatment applied for 14 patients (ethanol for 79% and fomepizole for 21% of patients). Eleven of 48 patients were died.

**Conclusion/Discussions:** Methanol poisoning is still fatal issue due to delayed admission to hospital and/or late diagnosis. Improvement of rapid and effective forensic toxicological analysis of methanol in blood is essential for urgent and optimal care of intoxicated patients. Besides, due to high taxes of alcoholic beverages, uncontrolled and illegal products are put on market time to time and Turkey faces to mass methanol intoxication continually. New government policy about price of alcoholic beverages should be determined and implemented immediately.

**Keywords:** Methanol Poisoning, Forensic Toxicology, Symptoms, Cukurova Region,

## Driving Under the Influence of Drugs in North Moravia in the Czech Republic

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**Background/Introduction:** Over the past period, there has been a significant increase in requests from law enforcement authorities in criminal proceedings (especially the Police of the Czech Republic), for the examination of drug use by vehicle drivers. In 2005, 11 tests were required in total while in 2015 we have reported a massive increase to a record of 866 tests for addictive substances among drivers. In 2014, the Government issued a Decree no.458/2013 Collection of Laws of the Czech Republic, which sets the limit values of addictive substances from which the driver is considered to be affected by such addictive substance. The aim of this retrospective study was to create a summary of toxicological tests of all blood samples examined for the presence of drugs in the period of 2005-2016, particularly among passenger car drivers. Examinations of blood samples were carried out in the Laboratory of Toxicology, Institute of Forensic Medicine of the University Hospital Ostrava.

**Objective:** On the basis of the driver's suspicious behavior, police conducts an on-site traffic control test for addictive substances in the driver's saliva. In the case of positive result, a sampling of biological material for toxicological analysis is carried out with the consent of the driver. Police determines the requirements for individual examinations of addictive substances in the blood from the results of preliminary tests, driver's behaviour and information gathered on-site. In the event of a fatal accident biological material for toxicological analysis is obtained in an autopsy. Determination of the substance in the blood sample is carried out mainly by gas chromatography – mass spectrometry. The limit value established for methamphetamine in the Czech Republic is 25 ng/ml for delta-9-tetrahydrocannabinol (THC) 2 ng/ml.

**Methods:** The data used in the retrospective study were obtained from the laboratory tests performed. We then processed and evaluated the data on the basis of scientific research methods and their combinations (analysis, synthesis, research and comparison).

**Results:** During the assessed period addictive substances were determined in the blood samples of 3879 drivers. Out of this, 94% of examinations were performed in men, 6% in women and a total of 18 drivers died in an accident due to intoxication. The age range varied from 15 to 59 years old. 87% of positive tests for addictive substances were proven to drivers aged 19 to 35 years. The drivers captured were most frequently under the influence of methamphetamine (45%), followed by THC (32%) and a combination of THC and MA (21%). In rare cases, drivers were influenced by other addictive substances such as cocaine and opiates. The limit concentration value was exceeded in 94% of examinations in the case of methamphetamine and 72% of examinations in the case of THC.

**Conclusion/Discussions:** There has been a significant increase in requests for the examination of drugs in the blood of drivers during the monitored period of 2005-2016. This is not only due to the increase of drug users, but also better quality assessment by the police when checking drivers. Driving under the influence of drugs is most commonly observed in male drivers within the age group of 19-35 years. Methamphetamine and THC were the most often proven addictive substance.

**Keywords:** Metamphetamine, THC, Driver

**Detection of Illicit Drugs in Oral Fluid from Truck Drivers in the State of Sao Paulo, Brazil**

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**Background/Introduction:** The use of psychoactive drugs can alter the driver's perception, increasing the risk for the occurrence of traffic accidents. In Brazil, the use of stimulants such as amphetamine and cocaine occurs among truck drivers due to their extensive daily work journey with little rest. Moreover, many of them have depression due to loneliness on the roads, which could contribute to the use of illicit drugs. In 2014, truck drivers were involved in 30% of all traffic accidents in federal highways in Brazil, and the use of illicit drugs could be responsible for many of them.

**Objective:** The objective of this study was to investigate the use of cocaine, amphetamine and cannabis by truck drivers in the State of Sao Paulo, Brazil.

**Methods:** Truck drivers included in this study were participating in health preventive actions carried out by the Federal Highway Police, entitled "Health Commands on the Roads". Drivers provided oral fluid samples, collected with the Quantisal™ device, and socio-demographic and occupational data were recorded using a structured questionnaire in order to study any association with the toxicological results. The samples were screened using an ELISA test and those that tested positive were confirmed by UPLC-MS/MS. The samples were collected during the years of 2014 and 2015.

**Results:** 762 oral fluid samples were included in this study. Only two of the drivers refused to participate. Of all samples 5.2% (n=40) were positive for psychoactive substances. Cocaine or benzoylecgonine were found in 2.1% (n=16). Amphetamine was found in 1.4% (n=11) and THC in 0.5% (n=4). In nine OF, more than one substance was found. Cocaine and amphetamine were present in one sample, another three presented cocaine and tetrahydrocannabinol (THC), amphetamine and THC were detected in one. In addition, meprobamate was found in three samples, in one was found cocaine and in another two, amphetamine. In one sample, alprazolam was found with amphetamine. The drivers who presented positive samples for any drugs were all men with an average age of 36.9 years. The majority was married (52.5%), and studied less than 8 years. 52.5% of the participants were self-employed and had experience of 13.2 years as truck drivers. This group of truck drivers drove an average of more than 1,100 km for 10.5h a day. Drivers who consumed amphetamines had the longest average of distance (1,700 km) of all participants.

**Conclusion/Discussions:** The use of psychoactive substances by truck drivers was fairly common and cocaine was the most frequently detected drug among truck drivers.

**Keywords:** Drugs, Truck Drivers, Oral Fluid

**Determination of Glufosinate in Whole Blood Samples from Emergency Room Patients Using LC-MS/MS**

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**Background/Introduction:** Glufosinate is non-selective phosphorus-containing amino acid-type herbicide developed in the 1980s, widely used in herbicide market. Glufosinate poisoning is characterized by various neurological symptoms such as disturbances of consciousness, convulsions and apnea that appear several hours after ingestion. Ever since paraquat was banned from use in Korea, the number of intoxicated glufosinate patients has increased in emergency departments. However due to its ionic and water soluble characteristics and amino-phosphonate structure, it is very difficult to establish the detection method of glufosinate, especially in biological specimens by GC/MS and LC/MS. There have been many attempts to establish the analytical methods of glufosinate including the use of derivatives or hydrophilic interaction chromatography (HILIC) columns.

**Objective:** In order to establish the method for the analysis of glufosinate in whole blood by LC-MS/MS, whole blood samples from emergency room patients were examined by using HILIC columns, without derivatization after the sample preparation procedure.

**Methods:** Whole blood samples were collected from seven patients of Chungnam national university hospital. The mean age of the patients with glufosinate poisoning was 61.4 years. Samples were extracted with solid-phase extraction (SPE) after deproteinization and then were analyzed by LC-MS/MS using HILIC column. Mobile phases used in this study were 0.3% formic acid in acetonitrile and 0.3% formic acid water. Negative electrospray ionization modes and multiple reaction monitoring (MRM) were used. The method was developed without derivatization for rapid analysis in whole blood. For method validation, linearity, limit of detection (LOD), limit of quantitation (LOQ), intra- and inter-day precision, and accuracy were studied. The established method was applied to analysis sample of actual poisoning patients.

**Results:** Glufosinate was detected in 7 whole blood samples using HILIC columns, without derivatization by LC/MS/MS. The concentration range of glufosinate is 2.0 ~ 70.0 µg/ml in the patients. Validation data showed that the correlation coefficient was over 0.99, showing a good linearity. The LOD and LOQ are below 0.1 µg/ml and 0.5 µg/ml, respectively. Accuracy and precision in intra- and inter-day were respectively between 80 ~ 120 % and below 15%. The difference in glufosinate concentration between gender and age was not observed.

**Conclusion/Discussions:** The method for the analysis of glufosinate in whole blood by LC-MS/MS was established in whole blood samples by using HILIC columns, without derivatization by LC/MS/MS. This method was applied to determine glufosinate in emergency patient cases and proved to be applicable to actual cases.

**Keywords:** Non-Selective Herbicide, Hydrophilic Interaction Chromatography Columns, Actual Poisoning Patients, Method Validation

**Development of a Rapid Multi-Target Screening Analysis Suitable to Emergency Toxicology in Urine by GC-MS and LC-MS/MS**

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**Background/Introduction:** It is important to identify toxicants within the golden hour to treat intoxicated patients in the emergency room. In order to establish the rapid method for simultaneous screening of multiple toxicants, GC-MS and LC-MS/MS were used to determine targeted and unknown toxicants in urine.

**Objective :** The purpose of this study is to develop a rapid simultaneous screening method using GC-MS and LC-MS/MS for the determination of multiple toxicants in urine samples collected from intoxicated patients in emergency room

**Method:** 265 urine samples from February 2015 to March 2017 were collected from Chungnam University Hospital emergency room and analyzed within one day. Urine samples are cleaned up by using Waters Ostro (pass-through type) and examined by GC-MS and LC-MS/MS. After analysis by GC-MS, the library search for unknowns was conducted by in-house Mass spectral databases with the Automated Mass spectral Deconvolution and Identification System (AMDIS). In addition, Chemstation software was mobilized to identify toxicants. For LC-MS/MS analysis, the 3200 Qtrap LC-MS/MS and Cliquid software (AB scx) was used for a simultaneous multi-targeted screening.

**Results/Discussion:** A rapid multi-target screening method by GC-MS and LC-MS/MS was developed to determine toxic substances in urine. The result by urine analysis was compared with the medical report by family to check the reliability of information. By using Ostro extraction and in-house data base, it was possible to screen urines for toxic substances within three hours. With this method, 265 urine samples were examined and it was noted that zolpidem, acetaminophen, and escitalopram, were detected in 49, 29 and 16 cases respectively and the most frequently encountered drugs in emergency room patients. The targeted and unknown toxicants were well searched by in-house mass spectral databases and commercial ones in all specimen studied. AMDIS & Chemstation software were used for GC-MS analysis and Cliquid 2.0 software was used for LC-MS/MS analysis.

**Conclusion:** The rapid multi-target screening methods by GC-MS and LC-MS/MS developed in the study proved to be applicable to the actual hospital poisoning samples.. This method will efficiently use to detect toxic substances within 3 hour in emergency cases.

**Keywords:** Multiple Toxicants, Targeted and Unknown Toxicants, Most Frequently Encountered Drugs, AMDIS, Pass Through

## LC-MS/MS Analysis of Large Drug Panels in Urine Using Three Sample Preparation Techniques

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**Background/Introduction:** Many laboratories desire a single liquid chromatography tandem mass spectrometry (LC-MS/MS) method to quantitate 50 or more drug analytes in urine for increased throughput and efficiency. Challenges in sample preparation result as acidic, basic and neutral compounds from multiple drug classes must be extracted from specimens with adequate recoveries using a single method. Here, we compare extraction of a large panel of drugs from hydrolyzed urine using three sample preparation techniques.

**Objective:** To evaluate three different sample preparation strategies for large drug panels in urine.

**Methods:** Samples were analyzed using a 5500 triple-quadrupole mass spectrometer (Sciex, Framingham, MA) in electrospray ionization mode, and a Nexera X2 UPLC system (Shimadzu, Columbia, MD). Standards came from Cerilliant (Round Rock, TX). LC separation was achieved by gradient separation using water and methanol acidified with 0.1% formic acid and a 50x3mm Raptor biphenyl column (Restek, Bellefonte, PA). Three sample preparation techniques were evaluated: supported liquid extraction (SLE, ISOLUTE SLE+), reverse phase solid phase extraction (SPE, EVOLUTE EXPRESS ABN), and mixed-mode reverse phase cation exchange SPE (EVOLUTE EXPRESS CX), all Biotage, Charlotte NC. Drugs evaluated were: codeine, morphine, hydrocodone, hydromorphone, oxycodone, oxymorphone, 6-monoacetyl morphine (6-AM), methadone, buprenorphine, fentanyl, meperidine, tramadol, tapentadol, naloxone, alprazolam, chlordiazepoxide, clonazepam, diazepam, lorazepam, midazolam, zolpidem, butalbital, phenobarbital, secobarbital, amphetamine, methamphetamine, ritalinic acid, 3,4-methylenedioxymethamphetamine (MDMA), cocaine, benzoylecgonine (BE), 9-carboxy-tetrahydrocannabinol, phencyclidine (PCP), ketamine, norketamine, amitriptyline, nortriptyline, pregabalin, gabapentin, meprobamate, carisoprodol and major metabolites. Drug free urine was fortified at 25, 50 and 100ng/mL. Samples were analyzed in triplicate. 200 $\mu$ L of pH 4 acetate buffer and 13 $\mu$ L of beta-glucuronidase enzyme (Campbell Science, Rockford, IL) were added to 200 $\mu$ L of urine and incubated at 65°C for 30 minutes. Samples were pretreated with 400 $\mu$ L of 4% phosphoric acid (H<sub>3</sub>PO<sub>4</sub>) for mixed mode SPE and 0.1% ammonium hydroxide (NH<sub>4</sub>OH) for reverse phase SPE and SLE. 400 $\mu$ L of treated sample were loaded onto ISOLUTE SLE+ plates and eluted with 90:10 dichloromethane:2-propanol. 550 $\mu$ L of treated sample were loaded onto the SPE columns. The ABN columns were washed with 0.1% NH<sub>4</sub>OH and 10% methanol, and eluted with 90:10 dichloromethane:2-propanol. The CX columns were washed with 4% H<sub>3</sub>PO<sub>4</sub> and 50% methanol, and eluted with 78:20:2 dichloromethane:2-propanol:NH<sub>4</sub>OH or 78:20:2 dichloromethane:methanol:NH<sub>4</sub>OH. All samples were dried under nitrogen at 40°C and reconstituted in 150 $\mu$ L of 90:10 mobile phase. Recovery was determined by comparing area counts of samples fortified before and after extraction.

**Results:** Average values (n=9) are reported. SLE worked well (< 90% recovery) for most drugs except 9-carboxy-tetrahydrocannabinol, 7-aminoclonazepam, norfentanyl, n-desmethyltapentadol, hydromorphone (80-85%), norhydrodocone and morphine (70-75%), amphetamine and zolpidem-phenyl-4-COOH (50%). Less than 50% recovery was seen for ritalinic acid, pregabalin and gabapentin. Reverse phase SPE using ABN achieved 80 to >90% recovery for semi-synthetic opioids, most benzodiazepines, barbiturates, cocaine, BE, carisoprodol, meprobamate, ketamine, norketamine, and PCP. Recovery of 9-carboxy- tetrahydrocannabinol, amitriptyline and nortriptyline was about 70%. Lower recoveries (<10 to 50%) were observed for codeine, morphine, hydrocodone, oxycodone and their metabolites, amphetamine, methamphetamine, ritalinic acid, pregabalin, gabapentin, and naloxone. Mixed mode SPE yielded 80 to >90% recovery for most compounds except poor recovery (<30%) was seen for barbiturates, ritalinic acid, pregabalin, gabapentin, meprobamate, and carisoprodol using 78:20:2 dichloromethane:2-propanol:NH<sub>4</sub>OH as the elution solvent. Recoveries for these analytes (except barbiturates) improved when 2-propanol in the elution solvent was replaced with methanol.

**Conclusion/Discussions:** Sample preparation for methods with 50 or more drugs and metabolites in urine is challenging. SLE and mixed-mode reverse-phase cation-exchange SPE yielded the best results for most compounds of interest. The choice of sample preparation technique should be selected based on the drugs of interest and desired sensitivity. Compromises in recovery for some analytes must be considered for methods that include acidic, basic and neutral compounds with different chemical properties.

**Keywords:** Urine Drug Testing, Sample Preparation, LC-MS/MS



## Urine and Hair Drug Testing by ELISA/ LCMSMS Comprehensive Method Reveals Multiple Drug Chronic Exposure of an Infant.

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**Background/Introduction:** A 15 month old female child was admitted to the Emergency Room of a Hospital in the Houston Area suffering from serious pharmacologic symptoms. Upon testing a urine sample collected in the ER Methamphetamine was detected, and confirmed at 187,196 ng/mL and Amphetamine at 6,832 ng/mL by LCMSMS.

**Objective:** Additional testing was performed to determine the possibility of a chronic drug exposure of the child. This was accomplished by performed on a hair sample of 1.5" length with an approximate time frame of growth of 3 months. We were requested by the attending physician to also test an unwashed hair sample to verify external drug exposure.

**Method:** The hair samples both pre- washed with buffer, and not- washed, were screened by ELISA on a Tecan Freedom Evo analyzer (Immunalysis, Ca).

Positive screening indications were then confirmed in a LCMSMS Agilent 6460 system utilizing Mass Hunter software in the D-MRM mode for data analysis.

### Result:

Washed sample		Unwashed sample	
DRUG	CONC (pg/mg)	DRUG	CONC (pg/mg)
Delta9-THC	133	Delta9-THC	18
6 Acetyl Morphine	4623	6 Acetyl Morphine	1152
Morphine	168	Morphine	79
Methamphetamine	27,424	Methamphetamine	99,884
Amphetamine	754	Amphetamine	2,622
Cocaine	167	Cocaine	333
Benzoylgonine	21	Benzoylgonine	43
Diphenhydramine	144	Diphenhydramine	1,111
Carisoprodol	303	Carisoprodol	409
Meprobamate	19	Meprobamate	44
Dextromethorphan	17		

**Conclusion/Discussion:** It is noted that the washing method yielded higher positive Delta9-THC results and higher 6-AM, Morphine, and Dextromethorphan LCMSMS concentrations. The only reason for such a discrepancy maybe attributed to cosmetic interference (soap residues). In the other hand the unwashed sample, diminished the presence of THC while it revealed higher concentrations of Methamphetamine and Amphetamine. In the case of the latter the increased concentrations of the Methamphetamine/ Amphetamine maybe the result of more recent external contamination. The presence of drug metabolites i.e. Amphetamine, Morphine, and Meprobamate are disturbing and signify possible ingestion of the parent drugs of Methamphetamine, Heroin and Carisoprodol by this infant, at least over a three month period. The high concentrations in the hair sample of some of the drug analytes and the corresponding metabolites, may support the opinion that the drug ingestion/exposure occurred chronically.

**Keywords:** ELISA, LCMSMS, Hair Testing

## Lamotrigine Poisoning as Evidence of Munchausen Syndrome by Proxy: A Paediatric Case Report Documented Using Hair Analysis

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**Background/Introduction:** Munchausen syndrome by proxy is a form of abuse where a caregiver deliberately produces or feigns illness in people under their care, frequently a child, so that they receive medical attention that gratifies the caregiver.

Case report. The case involves a 16 month old child who has been reported to have gone to accident and emergency 32 times since birth, of which 13 visits were followed by the child being hospitalised for several days in various different healthcare structures. Besides the digestive and ENT troubles, the reasons for hospitalisation were essentially neurological signs such as trembling or abnormal movements, always indicated by the mother. None of the explorations carried out (notably neurophysiological) allowed an unequivocal explanation to be found for the neurological symptoms of the child as reported by the mother. During the periods of hospitalisation, no malaise episode was detected. About four months before the actual diagnosis was made, following an admission into hospital for the umpteenth time and once again following the indications given by the mother, the existence of the beginnings of an epileptic syndrome was noted justifying putting in place an anti-epileptic treatment using levetiracetam. Further episodes of tonic-clonic seizures, while under treatment, were observed, this time by healthcare professionals; these attacks were sufficiently suspect that finally 2 months later, the hypotheses of Munchausen syndrome by proxy was mentioned. The first toxicological analyses show the presence of lamotrigine at a toxic concentration (74 mg/L) in the blood of the child, which may explain the symptoms observed several months earlier. Analysis of the child's hair was required to confirm if the child has been exposed to lamotrigine - the mother's antiepileptic treatment - since birth.

**Objective:** A case of Munchausen syndrome by proxy is described in a child aged 16 months following toxicology hair analysis.

**Method:** Hair lock (18cm) was decontaminated twice and then segmented (4 segments of 4 cms) to cover the period in question. Each segment was cut into small pieces (< 1mm) followed by a liquid-liquid extraction process and analysed by GC-MS and LC-MS/MS.

**Result:** The results confirmed regular lamotrigine use during the previous fifteen months (102, 96, 63 and 45 ng/mg of hair starting from the most proximal segment). Levetiracetam and 7-aminoclonazepam were identified in the segments of hair corresponding to the periods of treatment.

**Conclusion/Discussion:** Without ruling out from the results obtained the likelihood of *in utero* exposure (the child was not breast fed), hair analysis has confirmed that the child was exposed to lamotrigine since birth. Evidence of repeated overdoses of this drug will have caused the onset of the observed clinical neurological signs (a paradoxical effect of antiepileptic medication) that led to the diagnosis of true epilepsy and the implementation of an antiepileptic treatment. The mother was identified as having Munchausen syndrome by proxy and the child was placed in care. No further recurrence of a convulsion has been observed since the epileptic treatment has been stopped. This case illustrates the difficulty in diagnosing this serious and potentially very dangerous psychological illness, where the objective of the sufferer is always to attract attention via the victim.

**Keywords:** Munchausen Syndrome by Proxy, Lamotrigine, Hair Analysis

## Evaluation of a New Fully Automated Rapid Screening System for the Simultaneous Detection of Drugs of Abuse from a Single Oral Fluid Sample Collected with the NeoSal Device

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**Background/Introduction:** Rapid drug screening can play a vital role in the workplace, reduced time to first result could speed up the confirmation of an individual's impairment. Oral fluid provides an ideal matrix for testing in this environment with a quick, simple and non-invasive collection method. The application of a new fully automated rapid screening system based on biochip array technology, the Evidence MultiSTAT, for the simultaneous detection of 20 drug classes in under 20 minutes from a single sample of oral fluid collected using the Intercept I2 collection device was previously reported.

**Objective:** To investigate further the applicability of the system to the multi-drug screening of oral fluid samples collected with other collection devices, this study reports the evaluation of the Evidence MultiSTAT for the rapid detection of 20 drug classes from a single sample of oral fluid collected with the NeoSal collection device.

**Method:** Simultaneous competitive chemiluminescent immunoassays, defining discrete test sites on a biochip surface, were applied to the fully automated Evidence MultiSTAT analyzer. This system processes a self-contained cartridge containing all the components required for the immunoassay reactions and has the capacity to assess two biochips in under 20 minutes. The NeoSal collection device was used for sample collection. Assay accuracy was determined by assessing 100 samples (40 negative samples and 60 spiked samples spanning the cut-off). Each sample was ran against a cut-off sample, a qualitative result was determined for each of the 20 drug classes and the results presented as percentage agreement to the spiked value. Repeatability was determined by assessing samples at +50% of the cut off and -50% of the cut-off against a cut-off sample (one replicate of each sample was analyzed twice per day for 10 days on 2 different analyzers giving a total of 40 replicates). The qualitative results were determined and presented as percentage agreement. Authentic positive samples (n=48) externally sourced and collected with the same collection device, were screened using this system and results reported while waiting for confirmation.

**Result:** The following drug classes were detected with the associated cut-off values: buprenorphine, LSD and fentanyl (1.5ng/mL), alpha-PVP (2.5ng/mL), 6-MAM (3ng/ml), methadone, THC and tramadol (5ng/mL), PCP (7ng/mL), oxycodone (10ng/mL), benzodiazepines and opiate (15ng/mL), JWH-018 (20ng/mL), UR-144 (25ng/mL), benzoylecgonine (30ng/mL), barbiturate and amphetamine (60ng/mL), ketamine (65ng/mL), and methamphetamine (70ng/mL). The evaluation of the accuracy (40 negative and 60 spiked samples) showed percentage agreement of 100% for all except 5 assays (benzodiazepine 2 and 6-MAM 99%, THC 98%, ketamine 95%, and JWH018 90%). The evaluation of repeatability (+50% and -50% cut-off samples analyzed across 2 analyzers) showed a percentage agreement of 100% for all assays with the exception of PCP, alpha-PVP and buprenorphine, which was 97.5%. The multi-analytical screening of 48 positive authentic samples showed the following positive results: 1 (alpha-PVP, fentanyl, 6-MAM and methamphetamine), 2 (amphetamine, JWH-018), 4 (methadone), 7 (opiate), 21 (THC), 22 (benzoylecgonine), these screening results are currently being confirmed.

**Conclusion/Discussion:** The data indicate suitability of the Evidence MultiSTAT system to detect in under 20 minutes, 20 drug classes from a single sample of oral fluid collected with a Neosal collection device. The results showed accuracy and reproducibility, with cut-offs extremely sensitive. Optimal analytical performance was previously reported when this system was applied to oral fluid samples collected with the Intercept I2 device with favorable comparison with a confirmatory method. This rapid multi-screening device has the potential to be used with samples obtained with different collection devices presenting different laboratory equipment demands and thus facilitating oral fluid testing to laboratories with different equipment accessibility.

**Keywords:** Biochip Array, Oral Fluid Collection, Rapid Drug Screening

## Evidence by Hair Analysis of 3-MMC Consumption by Two Polydrug Abusers

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**Introduction/Objective:** During the last years, new psychoactive substances (NPS), particularly synthetic cathinones, have invaded the street drug market. Several fatal intoxications have been reported in Europe involving NPS. In the last 24-month period, 11 cases of poisoning with a synthetic cathinone have been reported by the Bordeaux Addictovigilance Centre. Blood and urine concentrations have been established for the majority of these cases but very few were documented using hair tests. We present here the results for two young men, consumers of NPS, particularly of 3-methylmethcathinone (3-MMC) with a focus on hair period

Case reports: Subject 1, 33 years old, was found wandering the street after killing his wife and 2 year old daughter in an outburst of violence 24 hours earlier. A blood specimen was immediately collected. Subject 2, 38 years old, was found deceased in his home. There was no trace of violence or traumatic lesions on the body. Both subjects were known as polydrug abusers. In the home of the 2 subjects, cannabis, crack pipes and bags with the inscriptions “Go gaine chemical powder” or “3-MMC” were found. A blood sample and chest hairs were collected for subject 1. An autopsy was performed on subject 2 and various specimens including hair were collected. Powders contained in bags seized at the home were also submitted for analysis.

**Method:** Identification of seized materials was performed with gas chromatography-mass spectrometry (GC-MS) using commercial and free specialized mass spectra libraries. Blood toxicological analyses were performed by gas chromatography with flame ionization detection (GC-FID), high performance liquid chromatography-diode array detection (HPLC-DAD), gas chromatography-mass spectrometry (GC-MS) and liquid chromatography-mass spectrometry (LC-MS/MS, XevoTQD) with validated methods. Chest and head hair segments were analyzed by LC-MS/MS with a specific method after pH 9.5 alkaline incubation.

**Result:** Blood sample for subject 1 was tested 48 h after the crimes and revealed the use of cannabis (THC: 0,5 ng/mL) and 3-MMC (3 ng/mL) Chest hair analysis revealed a regular consumption of cannabis, occasional consumption of cocaine, pholcodine, codeine and various psychoactive drugs (diazepam, hydroxyzine, zopiclone, levomepromazine, alimemazine). Analysis of the blood sample obtained during autopsy for the subject 2 revealed GHB (154 mg/L), amphetamine (938 ng/mL), mephedrone (604 ng/mL) and 3-MMC (613 ng/mL).

Results for hair analysis are presented in the table below for both subjects :

NPS identified	Subject 1 (chest hair)	Subject 2(head hair)
3 MMC	14 pg/mg	17.1 ng/mg
4 MEC	<10 pg/mg	-
Mephedrone	-	19.6 ng/mg
Methoxetamine	260 pg/mg	-
Ethylphenidate	41 pg/mg	-

**Conclusion/Discussion:** Our results are in concordance with the few previously published reports on hair analysis for 3-MMC. These reports illustrated the use of synthetic cathinones easily accessible on the Internet and provide useful information regarding the toxicological data of cases involving designer stimulants, highlighting the need to consider the potential involvement of such drugs when presented with suggestive incident circumstances.

**Keywords:** Hair, Synthetic Cathinone, 3-MMC

## Is 5-Hydroxytryptophol (5-HTOL) Glucuronide in Hair a Potential Alcohol Misuse Indicator? A Comparison with Ethyl Glucuronide

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**Background/Introduction:** Ethyl glucuronide (EtG) is often measured in urine and considered as recent alcohol consumption indicator. Moreover, EtG can accumulate into hair to document alcohol use over months (abstinence, repeated consumptions or misuse). Alternatively to EtG, 5-hydroxytryptophol (5-HTOL) glucuronide, often measured in urine and also considered as recent alcohol consumption indicator, may also accumulate in hair, offering new possibilities as alcohol misuse indicator. A aim of this study is to determine the possibility to consider 5-HTOL glucuronide in hair as alcohol consumption marker as EtG. Another aim of this study is to investigate the production of 5-HTOL glucuronide consecutive to 5-HTOL increase following ethanol consumption, and its accumulation into hair as ethyl glucuronide accumulates directly from ethanol.

**Method:** EtG and 5-HTOL glucuronide were measured in hair of 19 patients involved in abstinence program. These molecules were extracted from washed (water, then acetone) hair by milling and solid phase purification - Biotage Evolute Express AX columns (150 mg, 6mL). A volume of 5  $\mu$ L of internal standard (EtG-d5) at a concentration of 1  $\mu$ g/mL and 1 mL of distilled water were added to the homogenized hair powder during the milling. Finally, hair samples were reconstituted in 50  $\mu$ L of water with formic acid 0.1 % and analysed (10  $\mu$ L) by liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS). Calibrations points were set at 0.2, 0.5, 1.0, 2.0, 5.0 and 10 ng prepared on 30 mg of blank hair: 6.7, 17, 33, 67, 170 and 330 pg/mg.

**Result:** The results obtained with 19 individuals (4 women aged from 22 to 52 y.o., 15 men aged from 21 to 72 y.o.) have shown that 5-HTOL glucuronide concentrations in hair were not correlated to EtG concentrations (Pearson's correlation coefficient of 0.08), whatever EtG concentration. Several hypotheses can be formulated to explain this absence of correlation such as diet composition and patients' medication that are known to influence 5-HTOL glucuronide concentration. Moreover, in urine, 5-HTOL must be normalised by 5-HIAA to take into account the other generating sources of 5-HTOL. As 5-HIAA is not extensively glucuronised, it seems too random to monitor 5-HIAA glucuronide in hair to normalise 5-HTOL glucuronide hair concentration.

**Conclusion/Discussion:** If 5-HTOL / 5-HIAA ratio can be used simultaneously to EtG to detect recent alcohol consumption in urine, 5-HTOL glucuronide seems to present a bad efficiency and correlation to EtG in hair. However, this assumption should be confirmed on a more important pool of hair samples and with a simultaneous survey of diet and drink.

**Keywords:** Hair Analysis, Ethyl Glucuronide, 5-Hydroxytryptophol Glucuronide, Alcohol Misuse

**Oral Fluid vs. Urine Analysis to Monitor Synthetic Cannabinoids and Classic Drugs Recent Exposure**

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**Background/Introduction:** Urine is a common biological sample to monitor recent drug exposure; however, its supervised collection intrudes on donor's privacy and there is risk of adulteration. Oral fluid (OF) is an alternative matrix of increasing interest in forensic toxicology, easy to collect and with low biohazard and adulteration risk. Although urine has a wider window of detection than OF, the overlap between detection times in OF and urine can be improved by using sensitive methods, and if a repeated ingestion of high doses of drugs happens. Limited data are available about OF vs. urine drug disposition, especially for synthetic cannabinoids.

**Objective:** The objective of the present study was to compare urine and OF as biological matrices to monitor recent exposure to synthetic cannabinoids and classic drugs among HIV-infected homeless individuals in New York City (NYC).

**Method:** Thirteen participants were recruited at the harm reduction and AIDS service Boom! Health Inc., in the South Bronx, NYC. After the participant provided informed consent to participate in the study, the biological samples were collected once per week for 12 weeks during the Summer of 2016. Participants were also interviewed after each sample collection about their health behaviors, including recent drugs consumption. Urine samples were collected in plastic cups and OF samples were collected in Quantisal® **OF collection devices. Delta-9-tetrahydrocannabinol (THC), amphetamines, benzodiazepines, cocaine and opiates were analyzed in urine samples by the enzyme multiplied immunoassay technique Viva-Jr (EMIT, Siemens), and in OF samples by solid phase extraction and liquid chromatography tandem mass spectrometry (LC-MSMS, LCMS-8030, Shimadzu) with a limit of quantification (LOQ) 1-5 ng/mL.** Eleven synthetic cannabinoids and 13 metabolites were analyzed in urine and the 11 parent compounds in OF by supported liquid extraction and LC-MSMS with a LOQ 0.5-1 ng/mL.

**Result:** A total of 70 matched urine and OF samples were collected. For synthetic cannabinoids, 5 OF samples (samples 1, 3, 4, 6 and 7) were positive for AB-FUBINACA (0.8-38.9 ng/mL). For urine, 4 samples tested positive for synthetic cannabinoids; sample 2 for PB-22 and 5-Fluoro-PB-22 (1.4 ng/mL), sample 4 for AB-FUBINACA (3 ng/mL), sample 5 for UR-144 5-pentanoic acid (8.9ng/mL) and UR-144 4-hydroxypentyl (1.7 ng/mL), and sample 8 for UR-144 5-pentanoic acid (3 ng/mL). In only one case, OF and urine results matched for the synthetic cannabinoid AB-FUBINACA (sample 4). For cannabis, 40 samples tested positive in urine and 30 in OF, showing an 85.7% match. In the case of cocaine, 37 urine samples and 52 OF samples were positive (75.7% match). Twenty-four urine samples screened positive for opiates, and 25 OF samples were positive for morphine, codeine and/or 6-monoacetylmorphine (81.4% match). For benzodiazepines, 23 samples were positive in urine and 25 in OF, showing an 85.7% match.

**Conclusion/Discussion:** These results offer new information about synthetic cannabinoids and classic drugs disposition between urine and OF. OF is a good alternative matrix to urine for monitoring cannabis, cocaine, opiates and benzodiazepines recent use; however, synthetic cannabinoids showed mixed results. Although uncommon for synthetic cannabinoids, parent compounds AB-FUBINACA, PB-22 and 5-Fluoro-PB-22 were detected in urine at low concentrations (1.4-3 ng/mL). AB-FUBINACA was more frequently detected in OF (5 cases) than in urine (one case), and urine tested positive for PB-22, 5-Fluoro-PB-22 and for UR-144 metabolites while OF samples were negative for those compounds. No metabolites were analyzed in the OF method, and metabolites for AB-FUBINACA, MAM2201, PB-22 and 5-Fluoro-PB-22 could not be included in the urine method due to the lack of reference material. Monitoring additional metabolites in urine and OF may improve the agreement between both matrices for synthetic cannabinoids.

**Keywords:** OF, Urine, Synthetic Cannabinoids

## Development and Validation of a New SEFRIA™ Immunoassay for the Detection of Oxycodone in Oral Fluid

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**Background/Introduction:** The epidemic of opiate/opioid addiction and misuse continue to plague the world. Currently, the number of people killed by opioid overdose in the United States has been quadrupling since 2000. Oxycodone is a semi-synthetic opiate, which may be synthesized from the opioid alkaloid thebaine. It is generally prescribed for the relief of moderate to severe pain. Saliva is an alternative matrix to test drugs of abuse with many advantages over other biological matrices. Oral fluid samples are considered to give a good indication of current drug use, similar to blood samples, which can be especially useful for driving under the influence cases. SEFRIA™ is a competitive homogenous enzyme immunoassay, which depends on the re-association of two inactive enzyme fragments to form an active enzyme (tetramer). One of the enzyme fragments is attached to the analyte of interest and competes with the analyte in the sample for the antibody binding site. The enzyme activity is directly proportional to the drug concentration in the testing specimens.

The SEFRIA™ methodical advantage over the traditional G6PDH –based homogeneous enzyme assay (HEIA) platform is that it has much lower detection limits. Therefore, the SEFRIA™ immunoassay is an ideal platform for oral fluid drug testing when immunoassays with very low cutoffs are required.

**Objective:** The objective of this project was to develop and validate a new highly sensitive SEFRIA™ immunoassay for the rapid detection of oxycodone in human saliva.

**Method:** An anti-Oxycodone recombinant fragment antibody-based SEFRIA™ immunoassay was developed and validated with LCMS confirmed saliva specimens. The assay was designed to detect oxycodone in oral fluid.

**Result:** The immunoassay is a qualitative and semi-quantitative method with a semi-quantitative reportable range of 15 to 100 ng/mL. The cutoff of the assay is 30 ng/mL. The qualitative precision of the assay is less than 2% CV. The SEFRIA™ immunoassay was validated with a total of 96 urine samples previously analyzed by LCMS. The sensitivity, specificity and accuracy of the assay were found to be 100%, 95% and 98%, respectively.

		Confirmation (30ng/mL)	
		N	P
SEFRIA™ (30 ng/mL)	N	40	2*
	P	0	54

\*borderline samples: SEFRIA 28.2 ng/mL and 25.7 ng/mL, Mass Spec. 41 ng/mL and 33 ng/mL, respectively.

**Conclusion/Discussion:** A highly sensitive SEFRIA™ immunoassay has been developed for the broad detection of oxycodone in human saliva. When applied to authentic specimens the assay correlated well with LCMS results.

**Keywords:** Enzyme Immunoassay, Oxycodone, Oral Fluid

**Quantitative Analysis of Cocaine, its Metabolites and Adulterants in Oral Fluid**

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**Background/Introduction:** There has been a significant upward trend in the outbreak of cocaine adulteration. The major concern is the intensified effect of the drug in presence of the additives, which shows relatively more adverse effects than its unadulterated counterpart. Common adulterants of cocaine include, but are not limited to; lidocaine, procaine, benzocaine, caffeine and levamisole that extend their own risk and fatality. The analysis of these compounds can provide wealth of information in the medicolegal death investigation and can serve as an extensive data base for future reference. While different forms of biological matrices (whole blood, urine) are available for analysis, the preference for an oral fluid specimen becomes almost obvious in cases like driving under influence of drugs (DUID). This form of analysis is time sensitive and demands on-site (roadside or a traffic stop) specimen collection for accurate drug quantification. Drug testing from oral fluid is more desirable because it is friendly, non-invasive and assures least chance of adulteration. Numerous oral fluid collection (OFC) devices are available in the market that provide a preservative solution to secure the stability of the sample. These preservative solutions contain varying degree of excipients that needs to be removed efficiently from the sample to prevent loss of mass spec (MS) instrument sensitivity and downtime. In this work, we present an efficient sample clean up procedure that suffice good recovery and reproducibility of the compound of interest.

**Objective:** The intent of this communication is to develop a comprehensive method for quantitation of cocaine, its metabolites and adulterants for forensic drug testing, employing a solid phase extraction (SPE) in conjunction with LC/MS/MS.

**Method:** Our analyte panel consists of nine compounds including the parent, cocaine. Cocaethylene, benzoylecgonine and norcocaine are among the metabolites, and the rest comprises the most abundant adulterants such as lidocaine, procaine, caffeine, benzocaine and levamisole. A polymeric, strong cation exchange sorbent, Strata-X-C in 96-well plate format is utilized for sample extraction. We employed Intercept i2®, a commercially available oral fluid collection (OFC) device (from Orasure Technologies, Pennsylvania) for sample collection and transport. A strong wash comprising of 50% acetone followed by an elution solvent composing of ethyl acetate, Isopropanol and ammonium hydroxide was chosen for optimum clean up and analyte recovery respectively. A Kinetex 2.6u, Polar C18, 50x2.1 dimension column was employed for LC/MS/MS analysis using a SCIEX API 5000 instrument under ESI in positive mode. Mobile phase consisting of 0.1% formic acid and methanol was utilized for a 3 minutes gradient.

**Result:** A 50% acetone wash substantially reduced the most of the excipients present in the oral fluid transport buffer. The majority of the analytes showed absolute recovery ranging from 65 to 95% except caffeine. The neutral and very polar caffeine recovered 40%, losing the rest in the aggressive (50% acetone) wash applied for optimum cleanup of the samples. Calibration curves were constructed from the spiked saliva ranged from 0.25 ng/mL to as high as 300 ng/mL with a quadratic fit of 1/x weighting factor. Three levels (low, mid and high) of QC samples were analyzed producing a precision below 15% and accuracy ranging between 85-115% for 6 replicate samples.

**Conclusion/Discussion:** The recovery for all analytes tested, resulted in high yield except caffeine. The extraction procedure significantly eliminated the quantity of excipients present in the transport buffer resulting in ultrapure and clean extract.

**Keywords:** Oral fluid testing, Adulterants, Sample Prep



## Getting Hair Done: Analyzing Drugs of Abuse in a Complex Matrix

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**Background/Introduction:** Drug testing in hair provides the ability to detect chronic drug use. This is in comparison with other biological matrices such as urine, blood, or oral fluid which are only suitable for monitoring more recent drug use. Hair is a very complex matrix and presents significant challenges for analysis by LC-MS/MS. Typical hair extracts display a high amount of background and matrix interferences. These matrix components can also cause ion suppression and make detecting low analyte concentrations difficult.

**Objective:** Using a combination of sample preparation and chromatographic separation, we aim to reduce the impact of matrix effects from hair samples during LC-MS/MS analysis.

**Methods:** Donor hair samples were first washed several times with methanol and water and allowed to dry completely. For the solid-liquid extraction procedure, the hair was clipped into very small segments (~1mm) and 20 mg was weighed out. Samples were soaked overnight (~14 hours) in extraction solvent with internal standard at room temperature. The supernatant was then decanted and the vial was rinsed with an additional 1 mL extraction solvent. The rinse was combined with the supernatant and evaporated to dryness at 50°C under a gentle nitrogen stream (~4-5min). Samples were re-suspended prior to undergoing a sample cleanup procedure. Liquid-liquid and solid phase extraction sample clean-up techniques were attempted and results compared. Multiple hair matrices including black, red, light brown, and salt-and-pepper hair were investigated. Solid-liquid extraction solvents composed of methanol, acetonitrile, and acetone were compared. Acidic, basic, and neutral extracts were qualitatively tested for relative cleanliness and analyte recovery. Alkyl, phenyl, and combination stationary phase columns were screened under acidic reversed-phase conditions for best resolution of matrix interferences. A variety of acidic, basic, and neutral probes from a typical drug panel were tested. Analytes included amphetamine, methamphetamine, morphine, 6-MAM, codeine, THC, THC-COOH, cocaine, benzoylecgonine, cocaethylene, and diethylpropion. LC-MS/MS data were collected on a Sciex Triple Quad 4500 with ESI source operating in positive and negative (for THC). A Kinetex 2.6µm Phenyl-Hexyl, 50x3mm column was chosen as the final candidate. A combination of aqueous 10mM ammonium formate and 0.1% formic acid in methanol provided the most appropriate solution. A total run time of 7.5 min was achieved on an Agilent 1260 LC system.

**Results:** Samples extracted with acetone displayed the lowest background, especially for THC and THC-COOH. Acidic and basic extractions resulted in the loss of 6-MAM and cocaethylene. Neutral extractions using both liquid-liquid and solid phase extraction techniques, respectively, were unable to completely remove interference peaks in the THC-COOH transition 345 --> 326.8. Consequently, these interferences needed to be chromatographically resolved during the HPLC method. This was achieved by increasing the column length and gradient runtime

**Conclusion/Discussions:** Through effective use of sample preparation and chromatographic techniques, we were able to resolve matrix interferences present in hair samples for LC-MS/MS analysis.

**Keywords:** Hair Testing, Pain Panel, LC-MS/MS

**Development of an Alternative Method for the Determination of 11-nor-9-carboxy-tetrahydrocannabinol in Hair Using LC-MS/MS and its Application to Drug Abusers Hair**

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**Background/Introduction:** Cannabis is the second most-commonly abused illicit drug after methamphetamine in South Korea. In order to prove cannabis consumption, 11-nor-9-carboxy-tetrahydrocannabinol (THC-COOH), the metabolite of tetrahydrocannabinol (THC), is screened for in hair analysis. In Korean forensic laboratories, the cut-off for THC-COOH is set at 0.05 pg/mg when using gas chromatography-tandem mass spectrometry (GC-MS/MS). The analytical procedures by GC-MS/MS are somewhat complicated because derivatization is required prior to application onto the system. Currently, a liquid chromatography-tandem mass spectrometry (LC-MS/MS) is commonly available in many toxicology lab in Korea

**Objective:** In this study we developed an alternative method for the determination of THC-COOH in hair using LC-MS/MS, and examined whether this method could be applied to the authentic samples by examining the distribution of THC-COOH in the head hair of Korean drug abusers in 2016.

**Methods:** Possible contaminants on the surface of hair samples were eliminated by washing twice with 2 mL methanol and distilled water, respectively. Hair samples (about 20 mg) were then digested with 1M NaOH, and extracted with mixed organic solvents (n-hexane : ethyl acetate) or with mixed anion exchange cartridges (Oasis® MAX, Waters) and applied to a LC-MS/MS system (Agilent 1290 infinity UHPLC coupled to an AB Sciex Qtrap® 5500 MS/MS). Identification and quantification of THC-COOH and THC-COOH-D<sub>3</sub> (internal standard) were made using the multiple reaction monitoring (MRM) mode at m/z 245, 191 and m/z 248, respectively (quantifier ions are underlined). The following validation parameters were evaluated: selectivity, linearity, limits of detection (LODs), limits of quantification (LOQs), precision, accuracy, matrix effect and recovery

**Results:** LODs and LOQs of the method was 0.1 pg/mg. Good linearity was achieved for the analyte of interest in the range from LOQ to 50 pg/mg. The method showed an acceptable precision and accuracy, which were less than 15%. The result of matrix effect and recovery studies were satisfactory. The concentrations of THC-COOH in head hair (no of cases=524, no of segments=681) ranged from 0.10 to 78.37 pg/mg (average=2.45 pg/mg, median=1.13 pg/mg). By statistic evaluation of drug concentration data, the ranges proposed were: lower (minimum to the 25<sup>th</sup> percentile); middle (25<sup>th</sup> to 75<sup>th</sup> percentile); and upper (above the 75<sup>th</sup> percentile), i.e., 0.10-0.51 pg/mg, 0.51-2.85 pg/mg and 2.85-78.37 pg/mg, respectively.

**Conclusion/Discussions:** This method was successfully validated and was successfully applied in the analysis of authentic human hair samples. Although the cut-off of this method (0.1 pg/mg) was higher than that of other Korean forensic lab, the presented method was convenient as it did not include the derivatization step needed in GC-MS/MS. However, further work is necessary on our LC-MS/MS method in order to meet the 0.05 pg/mg cut-off.

**Keywords:** THC-COOH, Hair Analysis, LC-MS/MS

**JWH Synthetic Cannabinoid Isomers: Characterization and Localization Using MALDI-MS<sup>n</sup> by Hair Imaging**

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**Background/Introduction:** Hair strands analysis documents punctual or regular drugs of abuse (DOA) consumption. Current conventional and destructive methods (GC- or LC-MS/MS) usually require a great amount of hair to ensure the detection of various DOA families, which is not compatible with low hair availability case. At the beginning of the 2000s, the hair analysis was associated with Matrix-Assisted Laser Desorption Ionization mass spectrometry (MALDI) and new opportunities appear, such as high-throughput profiling or drug monitoring. Mass spectrometry imaging offers the unique possibility to map with high spatial resolution several tens of species into only one intact hair, such as synthetic cannabinoids (SCs). The SCs have multiple positional isomers but minor structural modifications lead to significant influence on SCs pharmacokinetic and their affinity for the cannabinoid CB1 and CB2 receptors, and thus on their potency *in vivo*. Combined with imaging, MALDI-MS<sup>n</sup> has the technical capacity to monitor concomitant complex drugs consumption in unique hair and to determine exact composition of drugs mixture.

**Objectives:** This work demonstrates the high efficiency of the MALDI-MS<sup>n</sup> and imaging combination to decipher along human hair strands, three JWH synthetic cannabinoid isomers with a methyl group positional change on the SCs skeleton.

**Methods:** Drug-free hair samples were soaked overnight in a mixed solution of JWH-007, JWH-019 and JWH-122, to simulate concomitant drug consumption. Two intact hair samples were fixed onto an indium tin oxide glass blade using double sided tape and sprayed with  $\alpha$ -cyano-4-hydroxycinnamic (CHCA) matrix. Standard solutions of JWH and hair soaked were analyzed and mapped using MALDI QIT-TOF (Nitrogen laser, 337 nm wavelength, 20 Hz acquisition speed) and MALDI-7090<sup>TM</sup> TOF-TOF (Nd:Yag laser, 355 nm, 500 Hz) mass spectrometers, in a mass range 50-500 Da. Fragments ions were produced through high energy fragmentation (TOF-TOF) and low energy fragmentation (QIT) process. For imaging experiments, data were acquired at a raster size of 80  $\mu$ m and retreated using the BioMap software.

**Results:** MALDI-MS/MS spectra of JWH standard solutions (precursor ion at  $m/z$  356.2) were obtained. MS/MS fragmentation of the JWH-122 (main peaks at  $m/z$  169.1 and 214.1) clearly discriminate this product from the two other isomers, while JWH-007 and JWH-019 (main peaks at  $m/z$  155 and 228) produced the same MS/MS spectra. MS<sup>3</sup> spectra of JWH-007 and JWH-019 were then obtained with a precursor ion at  $m/z$  228. Thereafter, MALDI QIT-TOF-MS<sup>3</sup> on JWH-007 and JWH-019 isomers produced characteristic fragment ions that enabled the differentiation between them. While MS<sup>3</sup> fragmentation of  $m/z$  356 >  $m/z$  228 allows to measure a single ion at  $m/z$  158.1 for the JWH-007, four ions at  $m/z$  116.1, 130.1, 144.1 (main peak) and 158.1 were obtained for JWH-019. To obtain chronological information of SCs consumption along intact hair samples, MALDI-MSI was combined with efficient MS fragmentation. Since MS<sup>3</sup> fragmentation ( $m/z$  356 >  $m/z$  228) has led to formation of an ion at  $m/z$  158.1, common to JWH-007 and JWH-019, the question then arose of the capacity to pinpoint a concomitant consumption of several SCs. The SCs monitoring using MALDI-MS<sup>3</sup> imaging demonstrated several specific areas along hair where one cannabinoid prevails over the others, confirming presence of JWH-007 and JWH-019.

**Conclusion/Discussion:** The MALDI QIT-TOF provides on one hand identification of JWH isomers using MS<sup>3</sup> and in the other hand, imaging in single human hair samples. The combination of MALDI-MS<sup>n</sup> and imaging is a real improvement: without an extensive hair samples preparation, a high mapping resolution (laser pitch  $\sim$ 100  $\mu$ m) for a precise chronological consumption history and a MS<sup>3</sup> fragmentation to determine exact composition of complex mixture, open new ways for precise elucidation of complex drugs in forensic issues using the potency of the QIT-TOF/MS<sup>n</sup> fragmentation.

**Keywords:** Hair Analysis, Drugs Monitoring, Imaging, Isomers, MALDI

## The Brazilian Regulation to Obtain a Professional/Commercial Driver's License Requires Hair Drug Testing: One Year of Results from an Expensive and Doubtful Effectiveness of this Regulation

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**Background/Introduction:** On March 2, 2015, the Law 13.103, which altered the Brazilian Traffic Code, was enacted, making it mandatory to perform “*wide detection window*” toxicological analyses using scalp, body hair or nails to assess the use of psychoactive substances (cannabis; cocaine, including crack and cocaine paste; opiates, including codeine, morphine and heroin; amphetamines; methamphetamines; ecstasy; amphetamine; propylhexedrine; mazindol) to obtain or renew a professional driver's license. After intense debate and confrontation from public and scientific associations against this proposal, because it was not based upon any scientific evidence to support its effectiveness, testing of “hair” became mandatory on March 2nd, 2016. A positive result leads to a temporary suspension of the right to drive for 90 days, and the driver is obliged to undertake a new exam, as well as a full medical checkup, having to pay for those procedures once again. The purpose of testing commercial drivers for drug use in real time is to deter prospective employees and current drivers from using drugs that are known to impair drivers' abilities; however, it is far more important to know if a commercial driver is using specified drugs or metabolites in real time, something that hair drug testing cannot assess.

**Objective:** Discuss the effectiveness of the law regarding hair drug testing for professional drivers in Brazil.

**Methods:** Data provided by the Ministry of Cities from March 2016 until January 2017 were analyzed regarding official toxicological results. The analyses were performed by eight laboratories accredited by the Brazilian government. The methodology involved screening by immunoassays and confirmation by mass spectrometry. Reports from professional drivers who had undertaken the mandatory toxicological analysis were also reviewed.

**Results:** From 978,651 samples collected in the various Brazilian territories, 12,984 (1.32%) were positive for at least one substance. It was not possible to get information about which substances were detected, neither sociodemographic characteristics from drivers tested. Many drivers reported: a) not being able to pay for the tests (which cost around US \$100); b) no counterproof is offered when the result is positive; c) many of them were not able to drive due to a delay in test results (the exam is valid for 60 days); d) presumptive positive results caused embarrassment for the driver.

**Conclusion/Discussion:** In summary, we believe that the results obtained from the hair drug tests performed among Brazilian drivers show that the current law is an extremely doubtful measure from a public safety perspective, since it identified less than 2% of drivers as potential users, and certainly does not reflect driving under the influence of any substance. Furthermore, the testing is far costlier for the driver when compared to urine drug testing. Additionally, there was no evidence that this law was effective in reducing crashes and the considerable economic loss to drivers awaiting test results or with false-positive results should not be overlooked. Alcohol is still the major substance associated with traffic crashes and investing on enforcement directed to alcohol-impaired driving could produce more benefits rather than focusing on hair drug testing that has dubious effectiveness. It is clear that different measures such as enhanced enforcement and current random or post-accident urine drug testing are responsible for a remarkable reduction of traffic injuries and deaths in other countries. These measures can be summarized as: effective training of traffic enforcement officials specialized in identifying drivers under the influence of alcohol and/or drugs; evaluation of recent drug use in biological samples, such as blood, urine and/or oral fluid; and public education campaigns. There is no report in scientific literature that nails are a good biological matrix to detect drug use by drivers.

**Keywords:** Hair, Drugs, Brazil

**Positivity rates of Drugs in Patients treated with Buprenorphine Combinations Used to Treat Opioid Dependence: A Comparison of Oral Fluid and Urine Using Paired Collections and LC-MS/MS.**

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**Background/Introduction:** Drug testing provides objective information regarding recent drug use and is considered one of several important tools in the diagnosis and treatment of substance use disorders (SUD) such as opioid dependence. While urine drug testing is well understood and has traditionally been used for this purpose, oral fluid testing has a number of attributes that may make it an attractive alternative, including ease of collection, resistance to adulteration, and enhanced sensitivity for certain medications and illicit drugs. However, there are relatively few studies directly comparing oral fluid to urine for the evaluation of this patient population.

**Method:** A retrospective review of deidentified oral fluid (OF) and urine (UR) drug test results from Millennium Health's laboratory database was performed for patients prescribed buprenorphine or buprenorphine/naloxone combination products (Suboxone®, Subutex®, Zubsolv® or Bunavail®) that are typically used in the treatment of opioid dependence. Buprenorphine dosage forms typically used to treat pain, such as Butrans® and Belbuca™, were excluded from the analysis. Between December 1, 2014 and October 1, 2016, the laboratory received 5061 paired oral and urine collections from 2746 patients prescribed buprenorphine products used to treat opioid dependence. Specimens were tested using a high throughput quantitative LC-MS/MS method for 35 medications, metabolites and illicit drugs. Positivity rates for each drug were determined by totaling positive results for each drug and metabolite combination. Results were tabulated and compared, revealing important differences in the detection rates between these matrices.

**Results:** In this study, higher detection rates were observed in OF vs. UR for cocaine (15.7% vs. 7.9%), opiates (13.4% vs. 10.0%), oxycodone (8.6% vs. 3.7%), amphetamine (15.6% vs. 13.3%) and methamphetamine (5.8% vs. 3.7%). Comparable rates were observed for fentanyl and tramadol. Lower detection rates were observed for benzodiazepines. Combinations of oxazepam, nordiazepam, temazepam and diazepam were detected in OF vs. UR at a rate of 6.5% vs. 8.7%. Detection rates for other benzodiazepines in OF vs UR were alprazolam (10.7% vs. 11.8%), clonazepam (7.5% vs. 16.2%) and lorazepam (1.2% vs. 2.2%). Consistent with previous literature, lower detection rates were also observed in OF vs. UR for hydromorphone (0.8% vs. 4.5%), oxymorphone (1.7% vs. 3.0%), buprenorphine (80.1% vs. 92.9%), and cannabinoids (15.5% vs. 19.5%).

**Conclusion/Discussion:** Clinicians may find OF advantageous for the detection of specific drugs and medications in certain clinical situations, for example, when detection of cocaine, heroin or oxycodone is considered critical for monitoring a patient in treatment for substance use disorders. There may also be a potential advantage to using UR in combination with OF by carefully selecting tests best suited for detection in their respective matrix. To our knowledge, this is the largest inter-matrix patient comparison study using paired collections and direct to definitive testing.

**Keywords:** Oral Fluid, Buprenorphine, Substance Use Disorders

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## Selected Opioid Levels in Hair Samples

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**Background/Introduction:** Hydrocodone and oxycodone are frequently prescribed opioid pain medications that are also subject to abuse. The commonly measured metabolic products of these opioids are hydromorphone and oxymorphone; however, these compounds can be prescribed independently as medications. In addition, hydrocodone has been reported as a product of codeine metabolism.

**Objective:** Herein, we document our findings of samples positive for the opioids hydrocodone, hydromorphone, oxycodone and oxymorphone using head and body hair samples analyzed by the Psychemedics laboratory. We include the range of head and body hair concentrations, as well as ratios of oxycodone to oxymorphone and hydrocodone to hydromorphone.

**Methods:** Samples were identified as presumptive positive by FDA cleared immunoassay screens (K111926, K123799). The confirmation process consisted of an extended aqueous wash followed by an AB SciEx API 3200 for LC/MS/MS quantitation. Cutoffs for the screening and confirmation were set at 2 ng/10 mg hair, with the limit of detection (LOD) established administratively at 0.50 ng/10 mg for this data set.

**Results:** The results are summarized in the tables below. Oxymorphone and hydromorphone were considered as metabolites when either drug was present at  $\geq 0.50$  ng/10 mg hair. Codeine was considered as a potential source of hydrocodone when present at  $\geq 0.50$  ng/10 mg hair.

OXYCODONE AND OXYMORPHONE RESULTS

All Oxycodone (OXYC) Samples $\geq 2.00$ ng/10 mg								
		Head, ng/10 mg			Body, ng/10 mg			
Drug	n	Range	Median		n	Range	Median	
OXYC	11904	2.00 to 709.0	7.61		4017	2.00 to 620.0	7.6	
OXYC $\geq 2.00$ , Oxymorphone (OXYM) $\geq 0.50$ ng/10 mg								
Drug	n	Range	Median	Avg Ratio OXYC to OXYM $\pm$ S.D.	n	Range	Median	Avg Ratio OXYC to OXYM $\pm$ S.D.
OXYC	2312	2.01 to 709.0	50.25	49.36 $\pm$ 34.23	650	2.04 to 569.0	50.66	49.77 $\pm$ 30.76
OXYM		0.50 to 92.8	1.09			0.50 to 94.2	1.11	

HYDROCODONE AND HYDROMORPHONE RESULTS

All Hydrocodone (HYC) Samples $\geq 2.00$ , no Codeine (COD) Excluded $\geq 0.50$ ng/10 mg								
		Head, ng/10 mg			Body, ng/10 mg			
Drug	n	Range	Median		n	Range	Median	
HYC	12279	2.00 to 438.0	9.6		5063	2.00 to 665.0	10.3	
HYC $\geq 2.00$ , COD $< 0.50$ ng/10 mg								
Drug	n	Range	Median		n	Range	Median	
HYC	9361	2.00 to 335.0	10.3		3882	2.00 to 665.0	11.0	
HYC $\geq 2.00$ , Hydromorphone (HYM) $\geq 0.50$ , no COD Excluded $\geq 0.50$ ng/10mg								
Drug	n	Range	Median	Avg Ratio HYC to HYM $\pm$ S.D.	n	Range	Median	Avg Ratio HYC to HYM $\pm$ S.D.
HYC	1903	2.08 to 438	59.6	66.30 $\pm$ 38.02	866	2.15 to 665	66.0	70.63 $\pm$ 32.93
HYM		0.50 to 36.4	0.91			0.50 to 24.9	0.94	
HYC $\geq 2.00$ , HYM $\geq 0.50$ , COD $< 0.50$ ng/10 mg								
Drug	n	Range	Median	Avg Ratio HYC to HYM $\pm$ S.D.	n	Range	Median	Avg Ratio HYC to HYM $\pm$ S.D.
HYC	1460	2.02 to 438	62.6	69.33 $\pm$ 36.03	678	2.15 to 665	66.1	71.20 $\pm$ 32.42
HYM		0.50 to 36.4	0.87			0.50 to 17.9	0.94	

**Conclusion/Discussions:** Similar range, median and ratio of parent drug to metabolite for each opioid were found in head and body hair. With codeine removed as a potential source of hydrocodone, the number of samples positive for hydrocodone decreased, while the median and average ratios remained similar for hydrocodone positive samples as well as hydrocodone positive samples with hydromorphone metabolite.

**Keywords:** Opioids, Hair, LC/MS/MS

POSTER

**Surface Enhanced Raman Spectroscopy as a Method for Toxicological Drug Screening of Cannabinoids in Saliva**

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**Background/Introduction:** The use and abuse of synthetic cannabinoids has become a global issue due to their easy access and growing popularity in young adults. As more of these drugs become illegal, new synthetic legal versions of these drugs are being made, which presents problems for the forensic scientist as standard methods may not detect the target drug. The most common method of screening for drugs of abuse in biological samples is the immunoassay. However, this method presents some disadvantages, particularly for newly synthesized compounds which may not respond to the test. Other problems include cross-reactivity between different synthetic cannabinoids, hook effects, and high cut-off values for determining if the drug is present. More advanced methods have also been used, such as GC-MS, however these procedures involve complex sample preparation and long run times. A potential solution to this issue is surface enhanced Raman spectroscopy. Traditional Raman spectroscopy is an under-utilized technique for the detection and identification of drugs due to its inherent low sensitivity. However, when Raman spectroscopy is performed in the presence of metallic nanoparticles, the signal can be enhanced by several orders of magnitude due to localized plasmonic effects. This process is known as surface enhanced Raman spectroscopy (SERS). The addition of aggregating agents, generally inorganic salts, further increase this signal due to the creation of hot-spots resulting from nanoparticle interactions. This method has recently been confirmed to work for the toxicological detection of benzodiazepines with limits of detection ranging from 1- 200 ng/mL.

**Objective:** The objective of this project is to develop an alternative screening method that is more rapid and selective, than immunoassays, to detect synthetic cannabinoids in saliva samples.

**Methods:** Gold nanoparticles were prepared using a sodium citrate reduction. The concentration, absorbance, size, and zeta potential of the nanoparticles was analyzed before and after the addition of mono ( $K^+$  and  $Na^+$ ), di ( $Ca^{2+}$  and  $Mg^{2+}$ ), and trivalent ( $Al^{3+}$ ) chloride, sulfate, and nitrate aggregating agents to assess the effect on the SERS enhancement.  $MgCl_2$  was used as an aggregating agent at varying concentrations. This approach has been previously demonstrated, by our research group, to detect and discriminate between a variety of synthetic cannabinoids including JWH-018, JWH-030, JWH-073, JWH-081, JWH-122, JWH-175, AM-2201, AM-694, MAM-2201 and some of their metabolites in standard solutions. Blank saliva was submitted to SERS for the identification of the matrix effects. Spiked saliva samples were prepared at toxicologically relevant concentrations and were submitted to SERS analysis after desalting and centrifugation.

**Results:** The nanoparticle analysis shows a decrease in absorption peak intensity with an increase in salt concentrations. For divalent and trivalent salts, the zeta potential decreases with an increased salt concentration demonstrating nanoparticle destabilization. However, monovalent cations show an increase in zeta potential with an increase in salt concentration. From synthetic cannabinoid analysis, 0.015M of  $MgCl_2$  was determined to be the optimal aggregating agent. Using this SERS method, synthetic cannabinoids could be detected at concentrations as low as 18 ng/mL. The Raman spectra of each synthetic cannabinoid could be differentiated from each other.

**Conclusion/Discussions:** Previous studies determined that the SERS enhancement was due to the anion interaction with the nanoparticle. However, these results show that the cations can have an effect on SERS enhancement via the cations interaction with the surface of the nanoparticle to cause aggregation. Analysis of the nine synthetic cannabinoids demonstrate that SERS can be a useful and more comprehensive alternative to immunoassays in the detection of synthetic cannabinoids in saliva.

**Keywords:** SERS, Synthetic Cannabinoids, Toxicology



**Drugs of Abuse Extraction from Oral Fluid using Supported Liquid Extraction (SLE) Following Collection with NeoSal, Prior to GC/MS Analysis**

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**Background/Introduction:** Drug screening using oral fluid has gained popularity over recent years due to its simple, non-invasive collection means. Screening drugs of abuse can be complicated due to the wide variation of functional groups associated with different analyte classes. Most extraction techniques cannot extract all analytes using a single procedure without using non-optimal extraction protocols resulting in compromised extract cleanliness. Supported liquid extraction allows for the simultaneous analysis of cross functional analytes in a single extraction protocol without forfeiting extract cleanliness.

**Objective:** The objective was to develop common extraction protocols for the determination of a range of drugs of abuse from oral fluid using supported liquid extraction (SLE) and GC/MS, following the collection of specimens from the NeoSal collection device. The drug suites included amphetamines and synthetic cathinones, barbiturates, benzodiazepines, cocaine, opiates, cannabinoids and synthetic cannabinoids

**Methods:** Negative oral fluid samples for method development purposes were obtained using the NeoSal collection device. To ensure maximum oral fluid extraction samples were pH modified using concentrated NH<sub>4</sub>OH prior to loading onto SLE+. The target for elevated pH was between 8-8.5 to provide a balance for the extraction of the basic drugs but also to avoid any potential hydrolysis of 6-MAM to morphine. Final pH control used 18 µL of neat NH<sub>4</sub>OH resulting in a loading pH of 8.0. Extraction evaluation was performed loading 1 mL on ISOLUTE SLE+ 1 mL capacity columns. The water-immiscible solvents evaluated for each separate panel included MTBE, DCM, 95/5 DCM/IPA, and 95/5 hexane/EtOAc. Various derivatization protocols were used, depending on the drug panel and will be shown in the final poster.

GC/MS analysis was performed with an Agilent 7890 GC and a 5975 MSD, following sample injection of 2 µL in splitless mode. Chromatography was performed on an Agilent J&W DB-5 capillary column; 30 m x 0.25 mm ID x 0.25 µm using 1.2 mL/min helium. Positive ions were acquired using electron ionization operated in SIM mode

**Results:** The optimum extraction for the majority of drugs took place at pH 8.0. No degradation of 6-MAM was observed in this pH environment. Recoveries were greater than 70% and corresponding RSDs were lower than 10% for all analytes. Calibration curves were constructed from 5-250 ng/mL and good linearity was universally achieved, demonstrating coefficients of determination > 0.99 for all analytes. Lower limits of quantitation will be shown in the final poster. When utilizing optimum pH control and extraction solvents, it was possible to extract a wide range of drugs of abuse with varying logP and pKa values, demonstrating the possibility of minimal extraction procedures. For amphetamines, synthetic cathinones, barbiturates, benzodiazepines, cocaine, opiates and cannabinoids, a common sample preparation protocol was possible. This involved a pH modification to 8.0 with 18 µL concentrated NH<sub>4</sub>OH and 2 elution volumes of 2.5 mL 95/5 DCM/IPA (v/v). The derivatization option is then selected according to the functionality of the drug under analysis. Optimum synthetic cannabinoid extraction took place at the lower, unchanged pH environment with an elution protocol of 2x 2.5 mL 95/5 hexane/ethyl acetate (v/v).

**Conclusion/Discussions:** This poster describes the suitability of ISOLUTE SLE+ for the rapid and reliable extraction of a range of drugs of abuse from oral fluid prior to GC/MS analysis, with minimal preparation steps. The drug suites included amphetamines and synthetic cathinones, barbiturates, benzodiazepines, cocaine, opiates, cannabinoids and synthetic cannabinoids.

**Keywords:** SLE (Supported Liquid Extraction), Oral Fluid, Drugs of Abuse

**New Product in Turkey Market: Hemp Extract Cold Beverages**

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**Background/Introduction:** Turkey is important tea market in the world and ice tea is one of the most popular products in the recent years. “Cannabis Ice Tea” has been sold in our country. This product has an orange can, “Swiss” (Switzerland) and “C” (Cannabis) are written beside the “Hemp” image which is on the bottom of the ice. The “C” symbol on the box and the “+” symbol from the Swiss flag give a positive impression of cannabis. Ice Tea and same beverage design energy drinks are marketed to the world by an Austrian company with different packaging slogans and designs such as “Original Hemp Ice Tea, Cannabis Ice Tea and Cannabis Energy”. It has been determined that cannabis is the most commonly used illegal substance in Turkey. In researches it was determined, the cannabis use rate at least once in life time as 0,7% and the average age of the first time use of cannabis was  $20,89 \pm 3,99$ . Cannabis ( $\Delta^9$ - tetrahydrocannabinol and its derivatives) is a prohibited substance to be procured, sold and consumed under the Turkish Criminal Code.

**Objective:** In this study, we aimed to evaluate ingredients of the substances written on the can of “Cannabis Ice Tea” which is presented as psychoactive substance cannabis positive.

**Methods:** The drink was analyzed by immunoassay and chromatographic methods whether it contains prohibited substance under the relevant legal regulations. In the immunoassay studies;  $\Delta^9$ -THC, cocaine, amphetamines, synthetic cannabinoids, opiates and benzodiazepine groups were analyzed in our laboratory. Over 2000 parameters including  $\Delta^9$ -THC, cannabidiol, cannabitol, amphetamine and its derivatives, analgesics / nonsteroidal anti-inflammatory drugs (NSAID), antidepressants, antihypertensives, hypnotics / sedatives, neuroleptics, opiates / opioids and other substances and caffeine were analyzed by Gas Chromatography-Mass Spectrometry in MVZ Labor Dessau GmbH Laboratory, Germany.

**Results:** “Cannabis Ice Tea” was analyzed by immunoassay and parameter results were determined to be negative according to cut off values. The obtained analysis results by chromatographic method;  $\Delta^9$ -THC, cannabidiol, cannabitol, amphetamine and its derivatives, analgesics / nonsteroidal anti-inflammatory drugs (NSAID), antidepressants, antihypertensives, hypnotics / sedatives, neuroleptics, opiates / opioids substances were negative, while caffeine was positive for “Cannabis Ice Tea” (62  $\mu\text{g/mL}$ ).

**Conclusion/Discussions:** The sales policy for “Cannabis Ice Tea” is followed by exaggerated taste and sense descriptions. According to the analytical results; caffeine-containing beverages were not containing any cannabis or another prohibited psychoactive substance, can create an incentive effect that suggests the use of cannabis in the subconscious mind with a hemp logo on ice. According to the Turkish Food Codex, the expression “caffeine contains” in products with a caffeine amount of more than 1.0  $\mu\text{g/mL}$  is clearly indicated in the distinctive and remarkable color and size of the product under the name of the product on the same surface as the brand. Products with a low caffeine amount of 1.0  $\mu\text{g/mL}$  should be written “caffeine free”. Cannabis which is shown as a product compatible with natural life, and thus the image of cannabis can be normalized and directed especially in young people. In general hemp plant, known to contain psychoactive substances, is required to increase awareness of the use of visuals in sales and necessary legal arrangements, studies and inspections.

**Keywords:** Ice Tea, Cannabis, THC, Caffeine

**Identification of “Kratom” (*Mitragyna speciosa*) Alkaloids in Commercially Available Products**

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**Background/Introduction:** “Kratom” is the common name for the botanical *mitragyna speciosa*. It is a tree native to Southeast Asia in which leaves contain the psychoactive alkaloids mitragynine and 7-hydroxymitragynine. Kratom is often ingested as teas, chewed, or smoked. Both alkaloids are acute mu-opioid receptor (MOR) activators which cause respiratory depression, constipation, sedation, nausea, and itching. High doses can cause euphoria which make it a major drug of abuse. Overdose of MOR activators is the leading cause of accidental death in the United States. Recently the Drug Enforcement Administration (DEA) placed Kratom on Schedule 1, but, due to public outcry (White House demonstration, phone calls to Congress, and a 100,000 signature petition sent to the White House), it was almost immediately removed.

**Objective:** The purpose of this study was to qualitatively analyze commercially available products for “Kratom” (*Mitragyna speciosa*) alkaloids by JMS-T100LC AccuTOF™ Direct Analysis in Real Time Mass Spectrometry (DART™-MS) and Gas Chromatography-Mass Spectrometry (GC-MS).

**Method:** Eleven kratom based products were obtained from various tobacco shops, “headshops” and via the internet including: Choice brand capsule and powder, Krave brand capsule, Lucky brand powder, King Kratom and Purple Haze e-liquids with 0 mg nicotine and 12 mg nicotine, Mojo brand capsule, O.P.M.S Liquid Kratom concentrate, and a K. Kratom chocolate bar. These products and methanol extracted samples were analyzed for psychoactive alkaloids and other components. Screening was performed using DART™-MS that was calibrated with PEG 600, a helium stream temperature of 350°C, and run in positive ion mode at 20V. Data was processed by T.S.S Pro version 3.0. Confirmation and quantitation was performed using an Agilent 6890N/5973 GC-MS instrument with an HP5MS column (30 m x 0.25 mm x 0.25 µm). The oven temperature program was set at 275 °C then ramped to 300 °C at 10 °C/min, then held for 12 mins for a full run time of 30 mins.

**Results:** A robust method was formulated and identified the psychoactive alkaloids mitragynine and 7-hydroxymitragynine along with the four other most abundant alkaloids, corynantheidine, corynoxine, paynantheine, and speciofoline, in all 11 unregulated commercial products.

**Conclusion/Discussions:** Kratom is a major drug of concern and is available to the public in many forms that are inexpensive, and easy to purchase. Despite the public’s frustration with the DEA, it needs to be understood that Kratom is highly prone to abuse and unregulated.

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**Keywords:** Kratom, Mitragynine, Electronic Cigarettes

## Drug Analysis Quality Assurance Program for Russian Forensic Toxicology Laboratories. One Step Forward to Assure and Improve Quality

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**Background/Introduction:** It is known that forensic toxicology laboratories participate in quality assurance programs in order to monitor and demonstrate the quality and reproducibility of their analyses. Participation in proficiency tests can report many advantages to forensic toxicology laboratories. It represents an important tool to assess and monitor the quality performance of the laboratory for a specific test and it provides a means of comparison with other laboratories in the same field. All the organizations that carry out proficiency testing for drug analytes mainly use spiked biological samples. We have developed the program of external quality assurance using urine and hair samples obtained from drug consumers.

**Objective:** According to Russia's Law the use of drug QC standards in laboratories is prohibited. The aim of the present study was to create a Russian program of external quality assurance using real urine and hair samples obtained from drug consumers. The main goal was development of methods with minimum false positive and negative results, harmonization of GC-MS and HPLC-MS/MS methods in Russian laboratories and generation of MS libraries with permanent updates.

**Methods:** For proficiency testing of laboratories we used authentic samples: 1) Real multicomponent urine samples containing common drugs, NPS and their metabolites. 2) Real hair samples from NPS consumers.

The samples for Russian program of external quality assurance before starting and after finishing of round test were analyzed by GC-MS, UPLC-MS/MS, UPLC-IT-TOF for confirmation of structures and quality of long term storage.

**Results:** The number of laboratories which participated in the Russian program for external quality assurance was 23 laboratories in 2015 and 49 in 2016. In the biannual tests, during 2015 to 2016 the laboratories that participated in the program obtained the following accuracy results: for example, the substances which were determined in urine and hair control samples 8, 9, 11 (2015):

**Urine\_PT\_8:** Naproxen, carbamazepine, metabolites of PB-22F, AB-CHMINACA, XLR11, THC-COOH, amphetamine, nicotine, acetaminophen, cotinine, metabolites of  $\alpha$ -PVP, theobromine, desomorphine, morphine, tropicamide.

**Urine\_PT\_9:** Metabolites of PB-22F, AB-CHMINACA, XLR11, MDMB (N)-Bz-F, ADB-FUBINACA, AB PINACA,  $\alpha$ -PVP and its metabolites, MDPV, nicotine, cotinine, paracetamol, theobromine.

**Urine\_PT\_11(blank):** urine caffeine, theobromine.

**Hair\_PT\_5v:** MDPV, XLR11 (thermal isomer).

**Conclusion/Discussions:** The first Russian program for the external quality assurance of drug analysis in urine and hair using authentic samples was organized. Qualitative performance was good in the majority of laboratories, with a low incidence of false results. The main problem was the reporting of false positive results in BLANK PT sample. The false positive results were obtained from the nine out of the 49 participating laboratories and 23 out of the 49 participating laboratories had a problem with a correct identification of target analyses, and only 17 laboratories achieved satisfactory results for all compounds. Overall 40 out of the 49 participating laboratories found not less than 70-80% of target substances. The results demonstrated that the performance was fair and further improvement is needed. Nevertheless, the program has provided a good external mechanism for participants to evaluate their analytical capability and to identify any testing inadequacy in urine and hair analysis for drugs.

**Keywords:** Drug Analysis, Proficiency Testing, Urine, Hair

## Evaluation of the NeoSal™ Oral Fluid Collection Device: A Side-by-Side Study in Drugs of Abuse Populations

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**Background/Introduction:** Oral Fluid (OF) is an ideal matrix for drug testing because collection is non-invasive and can be easily observed. There are a variety of OF collection devices on the market that have a wide range of performance characteristics, differing in collection (stimulated vs. non-stimulated) and volume of stabilizing buffer. As collection device continue to improve and new ones become available, it is important to evaluate these devices to determine ease-of-use and performance characteristics in authentic collection settings. As well as determine if they are comparable with other collection devices, to ensure accurate drug testing and flexibility when choosing an OF collection device. The NeoSal™ is a newly available OF collection device from Neogen®. It consists of a collection pad to collect unstimulated OF, a volume indicator, and a collection tube containing a stabilizing buffer. For ease of use in laboratory processing, the collection pad can be discarded and only the buffer is needed for analysis.

**Objective:** The goal of this study was to determine the ease-of-use and reliability of test results of the NeoSal™ OF collection device compared to another common collection device, the Quantisal™ from Immunalysis®.

**Methods:** Fifty NeoSal™ and Quantisal™ devices were provided on a volunteer basis to drugs abuse clients of Forensic Fluids Laboratories in Kalamazoo, MI. Manufacturer instructions were provided to sample collectors and followed for both devices. Oral fluid samples were collected in tandem, first with the Quantisal™ device followed by the NeoSal™ device. Samples were sealed and sent at ambient temperature to Forensic Fluids Laboratories and analyzed on the day they were received. Samples were prepared by solid phase extraction using Agilent PCX Plexa 96-well plate followed by LC/MS/MS analysis by Waters Acquity UPLC I-Class LC system paired with Waters Xevo TQ-S mass spectrometer. All samples were tested for the presence of over 100 analytes, including drugs of abuse and therapeutic medications.

**Results:** Overall, the NeoSal™ performed well compared to the Quantisal. Of the 48 samples returned to the laboratory, 46 were positive for at least one analyte. Drugs found included opioids (9 samples), THC (5), cocaine (3), benzodiazepines (3), and a variety of medications including venlafaxine (4), gabapentin (5), dextromethorphan (3), and naltrexone (3). In over 90% of the paired samples, drugs were found positive in both devices, with the exceptions of the NeoSal™ sample being positive for cotinine (2 samples) and THC (1 sample), and the Quantisal® being positive for the cocaine metabolites BZE and EME in one sample. Heroin was found in 2 samples with concentrations of 6-acetylmorphine and morphine ranging from 1.2-3.6 ng/mL for both devices. Two samples were positive for cocaine, BZE and EME at concentrations between 0.8 and 17.2 ng/mL. THC concentrations were higher in the NeoSal™ (1.6-622 ng/mL) than the Quantisal® (0-311.2 ng/mL), potentially resulting from tandem collection. Cotinine, an alkaloid found in tobacco and a metabolite of nicotine, was the most prevalent analyte found in 92% of positive samples. Neat OF cotinine concentrations ranged from 4-764 and 15.2-772 ng/mL in the NeoSal™ and Quantisal®, respectively, with an average concentration difference of  $\pm 18\%$  in paired samples.

**Conclusion/Discussions:** This preliminary study demonstrates that the NeoSal™ OF collection device performs comparably with the Quantisal® device. Tandem OF sample collection was used in this study, which could contribute to some of the variability in performance. To determine performance characteristics for a wider range of drugs, more studies are needed in controlled populations with a higher prevalence of specific drug classes.

**Keywords:** Oral Fluid, Collection Device, Drug testing

**Detectability of Hydromorphone and Codeine in Oral Fluid Samples from Patients in Substitution Therapy**

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**Background/Introduction:** Hydromorphone (H) is an abuse-relevant opioid which is increasingly included in drug screening methods for opiates and opioids. On the other hand H is a minor metabolite of morphine and can be frequently found in urine or oral fluid (OF) samples after heroin abuse or morphine (M) consumption. In Germany opiate addicts can now be substituted with Substitol™ (morphine sulfate, Mundipharma) as an alternative to Methadone/Polamidon™ or buprenorphine. Interpretation of drug screening results of these patients is additionally complicated by the fact, that Substitol™ contains up to 0.1% w/w codeine (C; pers. communication Mundipharma) which can be detected in urine and oral fluid samples. C, however, is also detectable after street heroin or C consumption.

**Objective:** The aim of this study was to collect data on the prevalence and concentration ranges of H and C in oral fluid samples from patients in Substitol™ therapy and from other substitution therapies after heroin abuse.

**Methods:** Of samples were collected using the Greiner-Bio-One SCS device. SRM multi-target drug screening in OF was performed after salting-out assisted liquid/liquid extraction with acetonitrile and 10 M ammonium acetate. Separation was conducted on an UPLC/MS-MS (Waters Acquity/Xevo TQ-S) with a BEH Phenyl 1.7 µm, 2.1 mm x 150 mm column within 6 minutes. Three transitions were monitored for 65 substances including H, 6-acetylmorphine (6-AM), M, C, 6-acetylcodeine (6-AC). OF/buffer was fortified at 0.5 ng/mL with 60 corresponding deuterated standards prior sample preparation. The cutoff in neat saliva was set at 0.1 ng/mL for H and at 1 ng/mL for all other opiates. Calibration range was from 0.025 to 20 ng/mL (16 points) for each opiate. OF samples (n = 663) from 133 patients in Substitol™ therapy (99 m, 34 f) were analysed for H, C, M, 6-AC and 6-AM. Dosing range was 30 to 2400 mg/d morphine sulphate with 60% of the patients receiving a dose between 800 and 1400 mg/d. In addition, 1827 OF samples from 1106 patients (847 m, 259 f) in Methadone/Polamidon™ or buprenorphine therapy positive for 6-AM were evaluated for H and C.

**Results:** H was detected in 96.8% (n = 642) of the OF samples from the patients in Substitol™ therapy from which 220 samples were in the concentration range 0.1 to 0.99 ng/mL and 418 ranged from 1.0 to 20.0 ng/mL. Only 4 samples were >20 ng/mL. C was detected in 40.6% of the OF samples (n = 269). However, 115 of these were also positive for 6-AM, indicating that the C prevalence in this patient group due to contaminated Substitol™ would be lower. The C concentration ranged from 1.0 to 18.9 ng/mL for 145 OF samples while the corresponding M concentration was always >20 ng/mL. Eight samples revealed M and C concentrations >20 ng/mL which was most possibly due to oral contamination. One C positive sample was excluded due to obvious non-compliance (M = 7.4 ng/mL). H was detected in only 37.7% (n = 688) of the OF samples from patients in Methadone/Polamidon™ or buprenorphine therapy from which 597 were in the concentration range 0.1 to 0.99 ng/mL and 91 ranged from 1.0 to 16.9 ng/mL. C, however, was detected in 75% of the samples with 47.7% having C concentrations >20 ng/mL.

**Conclusion/Discussions:** Detection of H in OF samples of patients in Substitol™ maintenance therapy can be regarded as “normal case”. H concentrations >20ng/mL may indicate H consumption. C detection in this pat. group is less frequent and concentrations are <20 ng/mL. After heroin abuse C can be detected more often and at higher concentrations while H detection is less frequent in the Methadone/Polamidon™ and buprenorphine patient group.

**Keywords:** Hydromorphone, Codeine, Oral Fluid

## Evaluation of Voltage, Resistance, and Glycol Ratio on Glycol and Nicotine Size Distribution Within an Aerosol Produced by an Electronic Cigarette

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**Background/Introduction:** Electronic cigarettes (e-cigs) vaporize a glycol formulation. These e-liquids, comprised of propylene glycol (PG), vegetable glycerin (VG), flavorants, and/or active ingredients, such as nicotine, produce condensation aerosols. The particle size of the aerosolized droplets plays a major role in drug deposition in the respiratory system. In general, larger particles will deposit in the upper respiratory tract, while smaller particles are more likely to penetrate deeper into the lung, increasing their probability of being absorbed into the blood. These devices can be easily modified to change the aerosol “cloud” produced. These modifications include coil type, battery voltage, as well as glycol composition. These modifications may affect the particle size of the aerosol produced, potentially changing the bioavailability of any inhaled active ingredient.

**Objective:** To determine the effect of various e-cigarette modifications, such as coil resistance, battery voltage, and glycol composition, on the particle size of the condensation aerosol.

**Method:** An electronic cigarette, KangerTech AeroTank, preassembled atomizer, and an eGo-V2 variable voltage battery was used to generate aerosols. The aerosol was fractionated by particle size using a 10-stage Micro Orifice Uniform Deposit Impactor (MOUDI) Model 100 at a flow rate of 30 mL/min. Each stage of the MOUDI represented a different particle size range, from 0.05 to 18  $\mu\text{m}$ . This sampling procedure was carried out with three preassembled atomizers, each with different coil resistances of 1.5, 1.8, and 2.2  $\Omega$ . Three e-liquid formulations composed of 100% PG, 100% VG, and 50:50 PG:VG were prepared at 12 mg/mL nicotine. Each e-liquid was also used to generate samples at 3.9, 4.3, and 4.7 V. Glycol deposition was determined by gravimetric analysis, while nicotine deposition was quantitated using LC-MS/MS. The concentration of nicotine on each stage of the MOUDI was determined using a Quattro Micro MS with a Shimadzu SCL HPLC system. Chromatographic separation was performed on an Agilent Polaris 5-Si A 50 x 3.0 mm HILIC column. The injection volume was 10  $\mu\text{L}$  with a flow rate of 0.4 mL/min. The total run time for this method is 4.5 minutes and the instrument was operated in multiple reaction monitoring mode (MRM) monitoring the following *m/z* transitions: nicotine, 163>130 and 163>117; and nicotine-d4, 167>134.

**Results:** A direct correlation between glycol and nicotine depositions was observed, with the majority of particles centered between 0.3-0.5  $\mu\text{m}$  in diameter, representing pulmonary deposition. No significant statistical differences were found between the particle size distribution of different coil resistances or battery voltages. The 100% PG e-liquid formulation produced slightly larger particles with a MMAD between 0.54-1  $\mu\text{m}$ , while the 100% VG and 50:50 PG:VG formulations produced particles with a MMAD between 0.31-0.54  $\mu\text{m}$ .

**Conclusion/Discussions:** The particle size of aerosols produced by e-cigarettes appears to be largely influenced by the glycol composition of the e-liquid being aerosolized. However, compared to traditional cigarette smoke, the aerosols produced by e-cigs have similar particle size production, predominantly around 0.3  $\mu\text{m}$ . Regardless of the coil resistance, battery voltage, or e-liquid, this model of e-cigarette is capable of producing small enough particle of an active drug, nicotine, to be deposited in the lung for absorption into the blood.

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**Keywords:** E-cigarette, Particle Size, Aerosol

## Establishing a Baseline for Drug use in California Nightclubs: A Retrospective Analysis of 2015 and 2016

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**Background/Introduction:** Oral fluid was collected from night club patrons, young working adults over the legal drinking age (21) in San Francisco, who agreed to participate in a study on club safety. Following the success of a California resolution to approve legalization of marijuana for recreational use in November 2016, we retrospectively established a baseline of drugs intake in these clubs.

**Objective:** To determine any effect of marijuana legalization in California by conducting a retrospective study of oral fluid samples analyzed in 2015 and 2016 and establish a baseline against which future surveys (2017 and 2018) can be compared.

**Methods:** Participants were recruited as they entered the club and were again intercepted as they left; oral fluid specimens (Quantisal®) were collected as they exited. Nightclubs were chosen in San Francisco based upon size (a minimum of 200 patrons on typical weekend evening) and to reflect diversity of patrons (e.g., racial/ethnic diversity, sexual preference). Specimens were sent to Immunalysis for drug analysis; the test panel included marijuana (THC); synthetic cannabinoids; cocaine/benzoylcegonine; amphetamine, methamphetamine, MDMA, MDA; codeine, morphine, 6-acetylmorphine, hydrocodone, hydromorphone, oxycodone, oxymorphone; methadone; PCP and ketamine.

**Results:** In 2015, 662 specimens were collected of which 228 were positive for one or more drugs (34.4%). The vast majority of positives were THC (63.1%), followed by cocaine/BZE (35.5%) and MDMA (15.7%). No PCP, morphine, codeine, 6-AM or methadone was detected but ketamine was present in 2 samples (0.8%).

In 2016, 407 specimens were collected. 102 were positive for one or more drugs (25%). THC accounted for 73.5% of the positives, cocaine/ BZE 49% and MDMA 21.5%. No PCP, morphine, codeine, 6-AM or methadone was detected but ketamine positives rose to 4.9% and hydrocodone rose from 0.4% in 2015 to 5.8% in 2016. In 2016 no methamphetamine was detected in any sample, while amphetamine alone was present in 4 samples (3.9%). It is likely this was because of medication use (e.g. Adderall®) rather than amphetamine abuse, particularly because the mean and median were close and the concentrations were not high. The average and median drug concentrations are shown in Table 1.

**Table 1. Drug concentrations (ng/mL)**

Mean	THC	COC/BZE	AMP/METH	MDMA	HYC	OXYC	Ketamine
2015	121	COC: 2072 BZE: 479	AMP: 272 METH: 1263	4930	22	11047	1752
2016	197	COC: 895 BZE: 553	AMP: 223	1328	191	0	2245
Median							
2015	38	COC: 95 BZE: 147	AMP: 55 METH: 218	661	22	2990	1752
2016	36	COC: 245 BZE: 174	AMP: 183	427	79	0	425

**Conclusion/Discussions:** While the mean THC concentration in oral fluid rose from 121ng/mL to 197ng/mL, the median value remained approximately the same. This value in future surveys following increasing potency of cannabis products coupled with the recent legalization of cannabis will be an interesting indication of California drug use among young adults. The mean and median concentrations for MDMA dropped between 2015 and 2016 even though MDMA accounted for a higher percentage of positive results. For oxycodone, there were no positives in 2016, but hydrocodone use increased from one positive in 2015 to six positives in 2016. The number of ketamine positives increased from 2 in 2015 to 5 in 2016; however, the number of positive samples in these drug classes is too low to deduce any trends. Among young adults who patronize nightclubs, marijuana continues to be the overwhelming drug of choice, followed by cocaine and MDMA. This data serves as a baseline with which to compare future data following marijuana legalization in the state of California.

**Keywords:** Oral fluid, Cannabis, Nightclubs



## Hair Analysis as a Forensic Tool in the Detection of Drug Use in Juvenile Detainees in Thailand: Two Year Experience

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**Background/Introduction:** Juvenile crimes in Thailand are apparently increasing these days. One of the most common characteristics of juvenile offenders is substance abuse. The treatment and re-habilitation program of these juvenile offenders generally involved urine testing to monitor drug use. However, testing urine for drugs is limited by the narrow window of time in which substances and their metabolites can be detected, which makes especially intermittent drug use difficult to detect. Hair analysis for drugs of abuse provides long-term information on drug use ranging from a week to several months. The window of detection is limited only by the length of the hair, then the drug user is not able to hide the fact that drugs have been used. Therefore, the testing of hair specimens to supplement urine testing can be an effective tool for developing a baseline measure and monitoring drug use in a detainee who was at high risk for substance use.

**Objective:** The aim of this study was to use hair analysis as an effective tool for monitoring of drug use in juvenile detainees in Thailand.

**Methods:** Hair samples were obtained from 304 detained juveniles from the Department of Juvenile Observation and Protection, whose urine samples were tested negative, during January 2015 to August 2016. In case of temporally release, hair specimens of detainee were cut to a length prior to testing in order to represent an approximate period of release; otherwise the hair was investigated in full length. Samples were analyzed for 23 drugs and metabolites including classes of amphetamines, benzodiazepines, opiates, anti-depressants and other classes of drugs by a fully validated LC-ESI-MS/MS method. The cut-off value was 0.2 ng/mg of hair for amphetamines, opiates and methadone and 0.5 ng/mg of hair for cocaine.

**Results:** Thirty one hair samples (10%) of juvenile offenders were tested positive for drugs. The drug most frequently detected was ketamine (n=18; 55%), followed by methamphetamine (n=12; 36%) and MDMA (n=3; 9%). Most of cases are single drug use (n=22) whereas poly-drug use, 2 or more drugs in hair was verified in 3 samples. None of sample can be detected for cannabis. The reason for this phenomenon is that marijuana is not as easily detected in hair by the method used in this study.

**Conclusion/Discussions:** This study show that hair analysis proved to be an efficient tool for monitor drug use of juvenile detainees, complement to urine analysis, in which hair testing provide the fact that drug has been used and prevent the hidden intermittent drug use. This is the first time to establish the new effective tool to monitor drug use of Thai juvenile offenders.

**Keywords:** Drug of abuse, Hair Analysis, Juvenile

## Ethanol in E-liquids: Concentration in 35 Formulations by Headspace Gas Chromatography with Flame Ionization Detector (HS-GC-FID) and the Impact on the Temperature of the E-cigarette Coil

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**Background/Introduction:** Personal battery powered vaporizers or electronic cigarettes were developed as an alternative nicotine delivery system to traditional cigarettes. Electronic cigarettes and their e-cigarettes liquid formulations were unregulated until May 2016 when the FDA swept them under their regulatory authority. These formulations are typically composed of propylene glycol and/or vegetable glycerin, flavoring components, and an active drug, such as nicotine. The regulations will require that all ingredients in the e-liquids are disclosed to the consumer on the label.

**Objective:** The aim of this study was to identify the concentration of ethanol in e-liquids and characterize the effect of ethanol on the generation of heat of the coil when activated by measuring the maximum temperature output during aerosolization.

**Methods:** A method routinely employed for the quantification of ethanol in the analysis of forensic and clinical specimens, headspace gas chromatography with flame ionization detector (HS-GC-FID), was used to analyze thirty-five commercially available e-liquids purchased from various sources containing a variety of flavors and active ingredients. The chromatographic separation was performed on a Restek BAC-2 column. A linear calibration was generated for ethanol with limits of detection and quantification (LOD/LOQ) of 50 mg/L. A rebuildable atomizer modeling the Kanger Aerotank Clearomizer was used and hand-built coils were made with 30, 32, and 34 gauge Nichrome wire. The wires were wrapped in both a contact, and non-contact fashion, tested at 3.5 V to 4.7 V and the resistance was measured at 1.8  $\Omega$ . The e-liquids were made with a 50:50 (w:w) ratio of propylene glycol and vegetable glycerin containing 0% ethanol or 10% ethanol by volume. A dual IR laser temperature sensor was used to measure the maximum temperature of the e-cig coils (Micro-Epsilon, Raleigh, NC). The detection range covered 100 °C to 600 °C and was recorded by Compact Connect version 1.9.8.6.

**Results:** The study identified that ethanol is a common component of e-liquids and was found in 33 out of 35 e-liquids. Ethanol concentrations ranged from none detected to 21 mg/mL. The mean amount of ethanol detected was 25 mg/mL and the median detected was 0.63 mg/mL. The only e-liquid listed to contain ethanol was measured at 56 mg/mL. Ethanol causes more rapid volatilization and effects the aerosolization process. Temperatures for e-liquids containing 0% ethanol showed a significantly lower temperature than for 10% ethanol. The range of temperatures observed for 0% ethanol were between 170 °C and 240 °C and between 180 °C and 260 °C with 10% ethanol.

**Conclusion/Discussions:** The ethanol in these products may have been used as a flavorant or as a solvent; the reason cannot be fully ascertained. Additionally, the implications of vaping ethanol, effects on bioavailability, and how the pyrolytic products affect users' health are also unknown. The effects of temperature of the coil due to the ethanol content is not fully understood, but may lead the user to modify their vaping practice from product to product to improve their experience. As a result, unintentional consequences may occur, especially if drugs other than nicotine are in e-liquid.

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**Keywords:** Ethanol, E-Cigarettes, E-Liquids, Headspace-GC-FID, Temperature

**Analysis of Mixed Fruit Chews Medibles Containing THC**

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**Background/Introduction:** The legalization of marijuana in the United States for both medicinal and recreational use has increased in the past few years. Currently 28 states have legalized marijuana for medicinal use and eight states and the District of Columbia have legalized marijuana for recreational use. The US Drug Enforcement Administration (DEA) has classified marijuana as a Schedule 1 substance. The Food and Drug Administration (FDA) does not regulate formulations or products that contain marijuana/or marijuana extracts in states that have “legalized” marijuana. Tetrahydrocannabinol (THC) and Cannabidiol (CBD) are the two most common cannabinoids found in these formulation or products. Marijuana edibles or “medibles” are typically candies and bake goods labeled with THC and/or CBD and the cannabinoids come from marijuana or marijuana extracts. THC is the major psychoactive compound of marijuana. CBD found in marijuana is reported to have medical properties including analgesic, anticonvulsant, and anti-psychotic activity. Presented is the high performance liquid chromatography/tandem mass spectrometry (HPLC-MS/MS) analysis of indica and sativa 10 mg “Spot Mixed Fruit Chews” in Strawberry and Orange Cream flavor.

**Methods:** The Samples were analyzed using a validated high sugar medible procedure. In brief, a seven point calibration curve was prepared in a “gummy matrix” (0.8 – 80 µg/g for THC and CBD). The Limit of Detection (LOD) and Limit of Quantitation (LOQ) were administratively set at 0.8 µg/g. Drug free controls with and without the internal standard (ISTD) were also analyzed with each batch. Calibrators, controls and fruit chews were prepared by dissolving the samples in approximately 0.25 mg/mL deionized water. Samples were then heated to 58°C for 5 min and then allowed to cool to room temperature. Fruit chews were then diluted to within the linear range of the assay. The ISTD, 8.0 µg/g THC-d3, and 500 µL of acetonitrile was then added. Samples were then extracted in to auto-sampler vials using a UCT Clean Screen FAST<sup>TM</sup> column and placed on the HPLC-MS/MS for analysis. Chromatographic separation was performed on a reverse phase C18 Rapid Resolution 4.6x75 mm column. The mobile phase was 20 mM ammonium formate in water (A) and 20 mM ammonium formate in methanol (B) isocratic at 10:90 for 6 min followed by a gradient to 0:100 over 1 min. The column flow rate was 0.5 mL/min. The mass spectrometer was operated in positive ionization mode with the acquisition mode in multiple reaction monitoring (MRM). The following transition ions (m/z) were monitored in MRM mode with their corresponding collision energies (eV) in parentheses: CBD: 315>43 (35) and 315>93 (23) THC: 315>43 (35) and 315>122 (29); and THC-d3: 318>93 (25) and 318>123 (35). The total run time for the analytical method was 8.0 min.

**Results:** The indica 10 mg “Spot Mixed Fruit Chews” Strawberry (n=3) and Orange Cream (n=3) flavors were found to contain 8.6±2.3 and 10.9±3.4 mg of THC. The sativa 10 mg “Spot Mixed Fruit Chews” Strawberry (n=3) and Orange Cream (n=3) flavors were found to contain 8.3±0.5 and 9.9±0.9 mg of THC. All samples were found to contain <0.10 mg of CBD.

**Conclusion/Discussions:** Limited information is available as to the accuracy of labeled contents of medible products in the United States as an ever-increasing number of states allow for some type of local legalization. The analytical method used for high sugar containing medibles was effective in the analysis of the fruit chews. The concentrations found in these fruit chews were similar to the labeled concentration of 10 mg THC.

This work was supported by the National Institute of Drug Abuse (NIDA) of the National Institutes of Health (NIH) grants: P30DA033934.

**Keywords:** Tetrahydrocannabinol, Medible, HPLC-MS/MS

## Stability of Laboratory Prepared Quality Control Materials and the Analysis of Commercially Available Brownies

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**Background/Introduction:** In just over a decade, the legalization of marijuana in the United States by state governments for both medicinal and recreational use has increased to 28 states. Federally, the US Drug Enforcement Administration (DEA) has classified marijuana as a Schedule 1 substance, and the only legal formulation of delta-9-tetrahydrocannabinol (THC) the US Food and Drug Administration (FDA) regulates is Marinol<sup>®</sup>. The FDA does not regulate any formulations or packages of marijuana that are currently marketed in states with legalized marijuana. Marijuana edibles or “medibles” are typically, packages of candies and baked goods consumed for medicinal as well as recreational marijuana use. These products contain the major psychoactive drug in marijuana (THC) and/or cannabidiol (CBD), which has reputed medical properties. In previously presented work with laboratory baked edible quality control (QC) materials, THC and CBD were found to be stable at room temperature for at least 3 month. This work did not analyze any commercially available baked goods. Also, to routinely use these laboratory prepared QC materials for analysis, extended stability information would be useful.

**Objective:** To evaluate the stability of baked brownie edible materials over a one year time period for use as QC material in the analysis of commercially available edible baked good products using a previously validated ultra-high pressure liquid chromatography tandem mass spectrometer method (UPLC-MS/MS).

**Methods:** Per previously presented work, laboratory baked QC materials were prepared at 5 mg and 10 mg THC and CBD equivalent brownie bite serving and stored at room temperature in resealable plastic bags. Two commercially prepared brownies labeled to contain 10 mg THC, were also analyzed. In summary, 10 ng of THC-D<sub>3</sub> was added to 25 mg of calibrator, QC or sample, then 2mL of methanol was added. The sample was vortexed for 1min, stood for 2min, centrifuged at 10,000g for 5min., and an aliquot was transferred to the autosampler. The UPLC-MS/MS was a Waters Acquity UPLC with TQD mass spectrometer (Waters Corporation, Milford, MA) with a Zorbax Eclipse XDB-C18, 3.5um, 4.6x75mm column. (Agilent Technologies, PA). The mobile phase was 20mM ammonium formate in water (A) and 20mM ammonium formate in methanol (B), isocratically 10:90 for 6min followed by a gradient to 0:100 over 1min. Prepared QC and samples were analyzed using a seven-point calibration curve (0.8–80µg/g) THC and CBD. QC and samples were analyzed in triplicate each analytical run.

**Results:** Laboratory prepared 5 mg and 10 mg THC and CBD QC stability is presented in the table below. The two commercially available brownies, labeled Cannabis Sativa and Cannabis Indica, were found to contain 10.9±1.2 of THC and 9.2±0.6 mg of THC, respectively. Both brownies were found to contain < 0.5 mg of CBD.

Baked Controls (n=3) Mean±SD	5 mg		10 mg	
	THC	CBD	THC	CBD
24 h	4.8±0.6	4.6±0.2	9.0±1.3	9.5±1.2
72 h	5.2±0.5	5.1±0.1	11.9±0.5	11.9±0.5
1 week	5.2±0.3	5.6±0.2	11.4±0.9	12±0.9
1 Month Stability	5.6±0.5	5.7±0.7	10.3±0.9	10.9±1.1
2 Month Stability	5.6±0.7	6.0±0.3	10.3±0.9	10.8±0.3
3 Month Stability	5.9±0.5	5.8±0.2	12.0±1.0	11.9±1.1
4 Month Stability	4.9±0.3	6.0±0.2	9.8±0.3	9.3±0.3
5 Month Stability	4.3±0.3	4.2±0.6	7.6±0.3	7.4±0.5
6 Month Stability	4.7±0.1	4.4±0.1	7.9±0.7	7.8±0.5
9 Month Stability	4.7±0.9	5.6±0.6	9.4±1.6	11.0±1.2
12 Month Stability	3.9±0.2	4.9±0.7	9.3±0.3	11.0±0.3

**Conclusion/Discussions:** Laboratory prepared brownie QC materials containing THC and CBD, which were stored at room temperature, were stable for a minimum of 9 – 12 months. The concentrations of THC found in the two commercially available brownies were consistent with the labeled THC concentration on the packages, but CBD would have been expected to be present in the Cannabis Indica labeled package. This is to be expected with the limited availability of accuracy and enforcement of labeling on edible products.

**Acknowledgements:** This project was supported by the National Institute of Health (NIH) Center for Drug Abuse Grant P30DA033934.

**Keywords:** Medibles, Cannabinoids, QC Material

## The Use of Fentanyl with Illicit Drugs has increased in Ontario, Canada

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**Background/Introduction:** Fentanyl is increasingly found in illicit drugs produced by clandestine laboratories and sold in Ontario, Canada. Comprehensive and specific information regarding trends in the prevalence of fentanyl and illicit drug use in this region is not widely available. Urine drug screening positivity rates for a defined population within a specific geographical area has recently been used to obtain this information, but no such studies specifically examining the prevalence of fentanyl and illicit drug use in a cohort of Canadian subjects has been published.

**Objective:** Identify multi-year trends in the use of fentanyl and illicit drugs by examining qualitative liquid chromatography tandem mass spectrometry (LC-MS/MS) based urine drug screening positivity rates for subjects tested within a healthcare setting in Ontario, Canada.

**Methods:** All LC-MS/MS urine drug screening results from 2014 ( $N=136,864$ ), 2015 ( $N=153,329$ ) and 2016 ( $N=106,687$ ) were retrospectively reviewed. All urine specimens underwent  $\beta$ -glucuronidase enzymatic hydrolysis and protein precipitation prior to testing. Screening was performed using a Waters Acquity liquid chromatography system paired with a Waters Xevo TQD triple quadrupole mass spectrometer operated in positive-ion electrospray ionization mode (+ESI). This LC-MS/MS testing protocol screens for a total of  $N=63$  different compounds using their respective positive/negative cut-off concentrations. The positivity rates for: fentanyl (25 ng/mL cut-off); norfentanyl (25 ng/mL cut-off); amphetamine (250 ng/mL cut-off); benzoylecgonine (100 ng/mL cut-off); cocaethylene (100 ng/mL cut-off); levamisole (10 ng/mL cut-off); 6-acetylmorphine (10 ng/mL cut-off); MDMA (250 ng/mL cut-off); methamphetamine (250 ng/mL cut-off); and THCA (40 ng/mL cut-off) were tabulated. Urine specimens were considered positive for fentanyl if they screened positive for fentanyl and/or norfentanyl. To identify the prevalence of fentanyl and illicit drug use/co-ingestion, the relative positivity rates of each illicit drug within all fentanyl positives was also determined.

**Results:** The positivity rates for: cocaethylene (0.4% to 0.6%); levamisole (8.2% to 8.0%); MDMA (0.1% to 0.1%); and THCA (29.6% to 29.5%) did not change significantly ( $p \leq 0.05$ ) from 2014 to 2016. Fentanyl (6.0% to 6.6%); amphetamine (3.4% to 5.8%); benzoylecgonine (9.6% to 11.5%); 6-acetylmorphine (0.6% to 1.1%); and methamphetamine (2.9% to 4.9%) positivity rates all significantly increased from 2014 to 2016. The positivity rates of several illicit drugs identified in specimens that tested positive for fentanyl significantly increased from 2014 to 2016: amphetamine (8.2% to 18.4%); benzoylecgonine (35.4% to 48.6%); cocaethylene (1.1% to 2.6%); levamisole (28.5% to 38.2%); 6-acetylmorphine (2.9% to 15.8%); and methamphetamine (9.1% to 20.1%). The positivity rates for: MDMA (0.1% to 0.3%) and THCA (72.5 to 71.6%) within fentanyl positive specimens did not significantly change from 2014 to 2016

**Conclusion/Discussions:** A review of LC-MS/MS urine toxicology screening results identified clear trends in both fentanyl and illicit drug use. From 2014 to 2016 the use/co-ingestion for fentanyl and several illicit drugs (cocaine; heroin; and methamphetamine) significantly increased within the studied cohort. The increased prevalence of illicit fentanyl and/or its inclusion in illicit drugs within Ontario, Canada may be contributing to these trends. Fentanyl use is also frequently associated with the use of cannabinoids. Tabulating and communicating trends in urine drug screening positivity rates would serve to educate the forensic community, physicians and the broader public about drug use within their communities.

**Keywords:** Fentanyl, Illicit Drug Use, Co-Ingestion of Fentanyl and Illicit Drugs

**Optimized Digestion and Extraction of Endogenous Gamma Hydroxybutyrate (GHB) in Human Hair**

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**Background/Introduction:** Gamma Hydroxybutyrate (GHB) is an endogenous compound that is present throughout the body. It has also been associated with drug facilitated sexual assault, due to its rapid onset and short detection window in blood and urine. Due to delays in reporting and specimen collection, hair is sometimes collected, due to its extended window of detection. However, the endogenous nature of GHB and the means by which endogenous ranges are determined in the matrix are complex.

**Objective:** The purpose of this research was to evaluate the efficiency and performance of digestion and extraction methods capable of identifying endogenous concentrations of GHB in human head hair.

**Methods:** Using an Omni BeadRuptor12, pulverized hair (~25 mg) was agitated for 60 seconds in 2 mL tubes filled with 2.4 mm metal beads and methanol. Following agitation, samples were centrifuged at 3500 RPM, transferred to a conical tube, and evaporated under air at 40 °C. Samples were reconstituted in deionized water, acidified and isolated using liquid-liquid extraction. Samples were injected onto an Agilent 1290 Infinity Liquid Chromatograph system equipped with an Agilent 6470 Triple Quadrupole Mass Spectrometer. The mobile phase used was 0.1% formic acid in water and methanol (96:4, v/v) with isocratic flow rate of 0.4 mL/min. Hair samples from non-GHB users were utilized as part of an IRB approved study. A comparison of previously published hair digestion methods including enzymatic digestions (pronase E and proteinase K), digestion under alkaline conditions, and the optimized solvent agitation was conducted to examine the impact digestion technique have on results.

**Results:** Linearity was observed between 0.1 and 50 ng/mg ( $R^2=0.999$ ). Bias and precision were evaluated at three concentrations (2.0, 10.0, and 25.0 ng/mg) in triplicate over five days. Bias was 5%, 8%, and 1% for 2.0, 10.0, and 25.0 ng/mg respectively. Inter-assay precision was 7%, 7%, and 8% for 2.0, 10.0, and 25.0 ng/mg, respectively. Intra-assay precision ranged from 3-10%, 5-12%, and 3-7%, respectively. No carryover was detected following injection of the highest calibrator. The limits of detection and quantitation were 0.1 ng/mg and 0.2 ng/mg, respectively. Matrix ion suppression or enhancement was evaluated by equipping a T-connector to the ion source housing to allow post column infusion of the analyte (equivalent to 5 ng/mg). Due to absence of true negative matrix, five samples of low concentrations of GHB were evaluated. From the five extracts, ion suppression or enhancement was not present at the retention time of GHB. One-way ANOVA ( $\alpha=0.05$ ) and two-tailed T-test were used to determine significance between the different digestions techniques evaluated.

**Conclusion/Discussion:** For the described assay, parameters such as linearity, bias, precision, detection and quantitation limits were within acceptable ranges. Endogenous GHB concentrations were low using solvent-based agitation. Significant differences in endogenous GHB concentrations were observed between chemical, enzymatic, and solvent-based agitation. These results highlight how differences in digestion efficiency and extraction can influence thresholds of endogenous GHB in hair.

**Keywords:** GHB, Hair, LC/MS/MS

## Analysis of Clonazepam in Hair: Segmental Analysis in Drug Facilitated Sexual Assault Cases

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**Background/Introduction:** In drug facilitated sexual assault (DFSA) cases, hair analysis is an important tool for retrospective monitoring of the ingestion or administration of drugs. In recent years clonazepam is more general in DFSA cases in China. This study results of different cases of 6 victims who had intoxicated with clonazepam. The aim of this study was to show the value of segmental hair analysis in DFSA cases.

**Methods:** Hair were collected from the vertex region of the scalp of 6 females, who claimed to have been sexually assaulted after drinking a soft drink spiked with drug. The hair was cut into segments of 2 cm from the root and analyzed following our previously published work: washing, drying, milling under liquid nitrogen conditions and soaking in extraction solvent in cool ultrasonic bath, then measured by ultra-high pressure liquid chromatography-tandem mass spectrometry (UPLC-MS/MS).

**Results/Discussion:** Clonazepam and its metabolite 7-aminoclonazepam were detected in the hair section of the 6 female (Table.1). Based on a higher concentration in hair and can prove that drug has been used in the body (excluding the possibility of contamination), metabolite is a better marker than clonazepam itself to provide information of drug exposure history in DFSA cases.

Table.1. Distribution of clonazepam and its metabolites in the hair of 6 female

Case Num	Analytes	Hair section						
		0-2cm	2-4cm	4-6cm	6-8cm	8-10cm	10-12cm	12-end
1	Clonazepam	20.7	17.9	-	-	-	-	-
	7-aminoclonazepam	51.6	47.2					
2	Clonazepam	-	-	4.1	20.2	8.1	-	-
	7-aminoclonazepam	-	-	-	+	-	-	-
3	Clonazepam	-	5.3	7.7	34.2	16.6	-	-
	7-aminoclonazepam	-	13.2	29.6	192.1	11.7		
4	Clonazepam	18.7	5.1	-	-	-	-	-
	7-aminoclonazepam	83.3	7.5					
5	Clonazepam	4.2	-	-	-	-	-	-
	7-aminoclonazepam	11.2	-	-	-	-	-	-
6	Clonazepam	-	-	4.5	32.1	+	-	-
	7-aminoclonazepam	-	-	47.1	176.5	6.3	-	-

Maximal concentration is observed in single hair segment in each case. The concentration of clonazepam and 7-aminoclonazepam were 4.1-34.2 pg/mg and 3.5-192.1 pg/mg respectively. In series published paper, the presence of clonazepam in hair of patients who take medical treatment for a long time at concentrations of about 1156 to 10400 pg/mg. By contrast, the clonazepam in hair of those who just take drugs single time were lower than 100 pg/mg. In the DFSA cases involved benzodiazepines and other related hypnotics, the concentration of the analytes is an indication of whether the complainant was take drugs more than a single dosage. Moreover, the growth rate of head hair is known to vary from about 0.5 to 1.5 cm per month, analysis can provide an approximate window of exposure. The maximal concentration were presented in first segment (0-2 cm) of hair in case 1, case 4 and case 5, while victims in these three cases claimed they have been offend one or two month ago. In case 2, case 3 and case 6, the maximal concentration of analytes were presented in fourth segment (6-8 cm), while complainant involved in these cases claimed they have been offend six, seven and nine month ago respectively. As average growth rate of hair is 1cm/ months and relative security, the "peak" of

the target compound in hair segments is closely related to the actual time of ingestion (assault). The time interval from be assaulted to the hair collected is inextricably linked with the length of the hair that finds the maximal concentration in these cases (the value of “month” approximately equal to “cm”).

**Conclusion:** Clonazepam and 7-aminoclonazepam were detected at pg/mg level in hair collected from victims who claimed to have been offended after a single dosage of spiked drink. The detection of metabolites in the positive segments indicated the fact that the drug is been administered. Furthermore, based on the numerical equivalence relation between the “month” and “cm”, the hair can provide objective evidence of the incident time. Positive segments explained the drug-taken facts and approximate time of the assault. These useful information were valuable in forensic investigation, especially in the DFSA cases.

**Keywords:** Clonazepam, Hair, Sexual Assault



**Correlation between Lead and Iron Blood level and Some Biochemical Parameters in Gas Stations Workers****Hatem Abdul Moniem Ahmed\***

**Background/Introduction:** Toxic metals sometimes imitate the action of an essential element in the body, interfering with the metabolic process to cause illness. Many metals, particularly heavy metals are toxic, but some heavy metals are essential. Heavy metals contamination is a serious problem and the unit's tendency of these compounds to gather and accumulate in living organisms has been accompanied by the great technological development wasteful in the use of these metals has reached a high level of risk, leaving a huge burden on the environment.

**Objective:** The main goal of this study is determine the lead, iron levels and liver (ALT, AST) and kidney (creatinine) functions in the blood samples of gas station worker in Riyadh city.

**Method:** This study is carried out on 25 male's blood samples as control and 34 males of gas station workers as the patient. The level of lead and iron were measured by Atomic Absorption Spectrometer 240FS AA, from Agilent Technologies with (Graphite Furnace) GTA 120) æ PSD 120 Programmable Sample Dispenser, and carrier gas was Argon, complete blood count (CBC) by Mindary BC-3000 plus auto hematology analyzer while liver and kidney enzymes by spectrophotometer UVmini-1240.

**Result:** Toxic metals sometimes imitate the action of an essential element in the body, interfering with the metabolic process to cause illness. Many metals, particularly heavy metals are toxic, but some heavy metals are essential. Heavy metals contamination is a serious problem and the unit's tendency of these compounds to gather and accumulate in living organisms has been accompanied by the great technological development wasteful in the use of these metals has reached a high level of risk, leaving a huge burden on the environment. This study showed that the number of white cells in the samples under test were significantly elevated with the decrease is relatively small for MCV and slightly higher in the MCHC with no increase in concentration of lead in blood samples under test, while the concentration of iron companion decrease with high concentration of lead in some samples under test, as well as increase in liver enzymes (ALT, AST) compared to the control sample and also there is an increase in creatinine in workers at stations compared to the control samples.

**Keywords:** Iron, Lead, CBC, ALT, AST, Creatinine, Atomic Absorption Spectrophotometry

**Drugs of Abuse and the Manner of Death in an Urban Area of Brazil**

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**Background/Introduction:** The association between psychoactive substance use and violent death is well known. In homicides, the association can be due to direct psychopharmacological effects (e.g. cocaine), from the violence from attempts to obtain money for drugs and the violence that results from the dynamics of drug markets.

In Brazil, the latest UNODC World Drug Report (2012) estimated that 0.5% of the population aged 15-64 uses opiates; 0.7% uses cocaine; and 0.2% ecstasy. However, the consumption of drugs is rising, especially the use of crack, which is the cause for several government programs to try to decrease the number of drug users, and the toxicological results of violent death victims is essential in order to consider the right conducts to face this crescent problem.

**Objective:** The aim of this work is to determine the incidence of abused drugs and the manners of death in a urban area in Brazil.

**Method:** The studied population consisted of violent deaths victims (250 individuals) admitted in the Departamento Médico Legal (DML) in Vitória, Espírito Santo, in the period of 2011 and 2012. All the demographic data (age, gender and manner of death) were obtained from records kept in the DML. Vitreous humor samples were collected by puncture of the eyes of the deceased and storage at -20° until analysis.

Ethanol was measured in vitreous humor by Gas chromatography - flame ionization detector (GC-FID) and Headspace sampling. The method was fully validated using 100 µL of the sample and 500 µL of n-butanol as internal standard.

All vitreous humor samples were extracted by solid phase extraction technique and analyzed into Gas chromatography - mass spectrometry (GC-MS) to quantify drugs of abuse. The method was able to quantify amphetamines and derivatives (amphetamine, methamphetamine, MDA, MDMA, and MDEA), cocaine and metabolites (cocaethylene, benzoylecgonine, anhydroecgonine methyl ester), and opiates (morphine, codeine, dihydrocodeine, 6-monoacetylmorphine, methadone). The LOQ for all drugs was 10 ng/mL, precision and accuracy were evaluated at three concentrations and were less than 15% for all compounds under investigation.

**Result:** Most of the victims were male (85.4%), the age ranged from 14 to 84 years old (mean: 32.82 ± 14.41). Homicide was the most frequent type of death (46.2%) which 89.5% were killed from a gunshot wound, traffic accidents (44.1%), suicide (2.4%) and the others (drowning, fall from height, airplane crash and undetermined) totaled 7.2%. Psychoactive substances (amphetamines, cocaine, opiates and alcohol) tested positive in 60.4% of the cases. Cocaine was the most prevalent drug with 23.2% of the cases, in a third of them the crack usage was identified by the detection of anhydroecgonine methyl ester. Alcohol alone was present in 19.2% of the samples, and alcohol and cocaine in 12.8% of the cases. Other drugs included amphetamines (13 cases) and codeine (1 case).

In the homicides victims the prevalence of psychoactive substances is higher, 70.2% of the cases positive for drugs or alcohol. The most prevalent drug is cocaine (36.0%), cocaine and alcohol was identified in 16.7% and only alcohol in 12.3% of the cases.

Alcohol was the substance most detected in traffic accidents (24.8%), cocaine was present in 11.0% of the samples, the association between cocaine and alcohol was also present in 11.0% of the victims samples.

**Conclusion/Discussion:** The high rates of positive results for psychoactive substances in these investigated deaths strongly suggests the association between drug and alcohol and violent deaths. Cocaine is the most prevalent drug in cases of homicide and could be an explanation of the high incidence of homicides in the cities studied, almost one thousand for year. Alcohol continues to be a problem in traffic accidents, although Brazil adopts a zero tolerance for drinking and drive.

**Keywords:** Drugs of Abuse, Manner of Death, Urban Area of Brazil

## Investigation in Stability of Eight Synthetic Piperazines in Human Whole Blood Under Various Storage Conditions Over Time

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**Background/Introduction:** Synthetic piperazines have been associated with multiple fatalities and were one of the top 25 reported drugs in 2011 nationwide. While circumventing legislative controls, piperazine derivatives are encountered in forensic casework as “legal” alternatives to ecstasy. These compounds share similar psychological effects with ecstasy which in turn has led to their popularity as “party pills”. The long-lasting duration of synthetic piperazines, especially when 1-benzylpiperazine (BZP) is mixed with 1-(3-trifluoromethylphenyl)-piperazine (TFMPP), has also made them desirable to users to receive enhanced hallucinogenic effects. Unforeseeable challenges may arise with the analysis and interpretation of such drugs, such as postmortem redistribution, enzymatic reactions, matrix interferences, and the lack of reference materials.

**Objective:** The purpose of this research was to investigate the stability of synthetic piperazines in human whole blood under various storage conditions and time ranges. A total of eight synthetic piperazines were assessed on their degrees of degradation using a Shimadzu Ultra-Fast Liquid Chromatograph with SCIEX 4000 Q-Trap Electrospray Ionization Tandem Mass Spectrometer (UFLC-ESI-MS/MS) in positive ionization mode. These analytes included: BZP, 1-(4-fluorobenzyl)-piperazine (FBZP), 1-(4-methylbenzyl)-piperazine (MBZP), 1-(4-methoxyphenyl)-piperazine (MeOPP), 1-(para-fluorophenyl)-piperazine (pFPP), 1-(3-chlorophenyl)-piperazine (mCPP), 2,3-dichlorophenylpiperazine (DCPP), and TFMPP.

**Method:** All samples were prepared by spiking certified reference standards (Cayman Chemical, Ann Arbor, MI, U.S.A.) of each synthetic piperazine into certified drug-free human whole blood (UTAK Laboratories, Inc., Valencia, CA, U.S.A.) independently at a final concentration of 1000 ng/mL. Mixed samples consisting of the eight piperazines were also stored at room temperature (20°C), 4°C and -20°C for one, three, six, nine and twelve months in dark sealed containers. Solid phase extraction (SPE) was performed with Drug of Abuse (DAU) mixed-mode copolymeric columns (Clean Screen®, UCT Inc., Levittown, PA, U.S.A.) followed by evaporating to dryness. All samples were then reconstituted with 250 µL of 50:50 mixture of methanol and 2mM ammonium formate buffer with 0.2% formic acid (Fisher Scientific, Waltham, MA, U.S.A.). Analysis was performed in triplicate. Separation was achieved using a reversed-phase column (Kinetex® F5, Phenomenex®, Torrance, CA, U.S.A.) with a binary gradient of a 2mM ammonium formate buffer with 0.2% formic acid and methanol with 0.1% formic acid. The total run time was 11.5 minutes and the flow rate was 0.4 mL/min. BZP-d7, mCPP-d8 and TFMPP-d4 (Cerilliant, Round Rock, TX, U.S.A) were used as internal standards.

**Result:** Benzyl piperazines were more stable than phenyl piperazines over time under all storage conditions. As an example, MBZP when refrigerated had more than 70% remaining after 12 months while MeOPP was not detected at room or refrigerated temperature after six months. There was a smaller degree of degradation when samples were kept frozen or refrigerated, with data suggesting that case samples with synthetic piperazines should be kept frozen or refrigerated even when analysis will occur in  $\leq 30$  days. Matrix interference was present due to slight coagulation observed in some blood samples, where MBZP on Day 270 was quantified at an exceptionally high concentration of 1420 ng/mL. Drug-drug interaction was also observed but the exact stability pattern of phenyl piperazines when mixed together could not be determined from this data set alone due to analyte concentration inconsistency found between Day 91 and 270.

**Conclusion/Discussion:** This research project demonstrated a robust method to examine synthetic piperazines degradation patterns in blood. Storing samples at room temperature should be avoided due to detrimental impacts on stability of piperazine compounds. Nonetheless, phenyl piperazines stored for more than six months will show analyte degradation regardless of storage conditions.

**Keywords:** Synthetic Piperazines, Stability, UFLC-MS/MS

## Evaluation of Toxicological Risk Due to the Presence of Bisphenol A in Thermal Papers

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**Background/Introduction:** Bisphenol A (BPA) is a chemical present in thermal papers, which are widely used in commercial applications. In 2015, the European Food Safety Authority (EFSA) performed a risk assessment study, based on recent toxicity data, and established a Tolerable Daily Intake (TDI) of 4 µg/kg body weight/day for BPA. Objective: To evaluate the presence of BPA in thermal paper samples obtained in Brazil, establishing its potential toxicological risk in individuals highly exposed to BPA, as workers in toll stations, which manually deliver thermal paper receipts to all drivers. This particular group of individuals have an estimated manipulation of 1,100 thermal paper receipts per day.

**Method:** The concentrations of BPA were determined in 20 thermal paper samples. A 30 mg paper sample was placed in a screw glass tube and mixed with 2 mL of methanol, followed by 2 cycles of 30 seconds homogenization in vortex and 10 minutes sonication. After, 100 µL of the solution was transferred to a new tube and diluted with 5 mL of methanol. A 10 µL aliquot was injected into a HPLC with fluorescence detection system with a Zorbax C8 column (150x4.6 mm, 5 µm) at 30 °C. Mobile phase was 1 % of acetic acid in water, acetonitrile and methanol (60:35:5, v/v/v), at 0.9 mL/min. BPA calibration curves were in the range of 0.5-10 µg/mL. The estimated daily intake (EDI) was calculated as follows:  $EDI = k \times C \times HF \times HT \times AF \times 10^6$  (ng/day), where: k is the paper-to-skin transfer coefficient (21522.4 ng/s); C is the concentration in thermal paper in µg/g; HF is the handling frequency; HT is the handling time (5 s); AF is the absorption factor of BPA by skin (27 %) (Liao and Kannan, 2011). A body weight of 70 kg was used as default for the adult population

**Result:** The assay was linear in the range of 0.5 to 10 µg/mL. Considering all QC levels, accuracy was 102.7-109.9 % and precision was 1.88-1.96 %. The limit of detection was 0.15 µg mL<sup>-1</sup>, corresponding to a concentration of 0.05 mg/100 mg BPA in a 30 mg sample. The frequency of detection was 65 %, with concentrations ranging from 16.43 to 20.2 mg BPA/g paper. Considering the high frequency of thermal paper handling (1,100 times/day), the exposure values ranged from 5.77 to 9.20 µg/kg body weight/day. The lowest value found exceeded the value of TDI 4 µg/kg body weight/day.

**Conclusion/Discussion:** This was the first study to evaluate such a heavy manipulation of thermal paper with respect to BPA exposure. Previous studies assumed frequency rates of handling of 150 times/day for occupationally exposed population (Liao and Kannan, 2011; Rocha et al., 2015). Literature overview of BPA concentrations in thermal paper varied from non-detected (Lassen et al., 2011) to 42.8 mg BPA/g paper (Rocha et al., 2015). The frequency for BPA detection, in previous studies, ranged from 44 % (Environmental Working Group, 2010) to 100 % (Östberg and Noaksson, 2010). In the evaluated group, manipulation of thermal paper is a significant contributor to the overall BPA exposure, particularly when added to the other sources of from which individuals may be habitually exposed. BPA risk evaluation is especially important for workers with extreme exposures, requiring careful monitoring and exposure mitigation measures, such as the use of gloves and other protective measures.

Liao C, Kannan K. Widespread occurrence of bisphenol A in paper and paper products: implications for human exposure. *Environ Sci Technol* 2011; 45:9372-9.

**Keywords:** Bisphenol A, Thermal Paper, Risk Assessment

## A Routine Toxicology Case Under Scrutiny

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**Background/Introduction:** In June 2009, a routine post-mortem case involving a single drug (promethazine) was reported to the Coroner in New Zealand. The blood level of promethazine was 0.7 milligrams per litre (mg/L) and was reported with some reference levels for comparison but no conclusion. Subsequently, a person was charged with and convicted of murder in a highly-publicized trial in late 2013. In 2014, a New Zealand magazine article on the case prompted three experts (two American and one British) to volunteer their services *pro bono*, and the appeals process began. In addition, two Australian experts were also involved. These individuals were all qualified and had presented evidence in their own jurisdictions. The appeals, which were concluded at the Supreme Court of New Zealand were based around the interpretation of the toxicology result. This was despite the presence of a significant volume of other evidence in the case (the toxicology was only 0.2% of the original murder trial transcript).

**Objective:** To discuss the immense scrutiny an apparently routine toxicology case was subject to from international experts and to demonstrate why we have to treat all cases as potentially high profile.

**Method:** This presentation describes the reporting and expert witness process for a toxicology case subjected to repeated appeals, involving conflicting expert opinions from toxicologists on three different continents.

**Result:** The input from multiple experts meant multiple opinions and statements. Interestingly in this case, the usual suspects (the technique, result or chain of custody) were not challenged. The challenges were around the references in the report, the choice of analyses and samples, and the range of possible conclusions that could be drawn from the promethazine level. It was not obvious until late 2015 that opinions were being formed on either the toxicology casefile (which was disclosed in full to the defence) or the original trial testimony.

**Conclusion/Discussion:** Experts in New Zealand are required to assist the court, remain impartial and not act for either adversarial party. This can be facilitated by co-operation between multiple expert witnesses to eliminate any inadvertent errors and discuss areas of agreement before the trial, sometimes called 'hot tubbing'. In recent years, this approach has grown in popularity, and in my experience has been very useful. In this particular case, only one of the other experts (an Australian) co-operated in this way. The others preferred to adopt the combative adversarial approach, more commonly reflected in their home jurisdictions than in New Zealand today. In 2017, global news means anyone anywhere may get involved in a high-profile case. For expert witnesses serving a jurisdiction where their duty is to the court, we should be prepared for input from international experts working to different norms. This was an apparently routine toxicology case that could have been signed by any qualified toxicologist, it would not have been flagged as 'high profile'. In the future I would be proactive via the court and in my affidavits to get some consultation with the experts rather than just reacting to each expert each time.

**Keywords:** Testimony, Post-Mortem Toxicology, Expert Witness

**Quantification of Haloperidol and Its Metabolites in Postmortem Blood and Urine by UHPLC-MS/MS**

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**Background/Introduction:** Haloperidol (H) is a typical butyrophenone type antipsychotic drug (first generation), commonly used for the treatment of schizophrenia and the control of Tourette's disorder in children and adults. It mainly acts as a blocker of dopamine D<sub>2</sub> receptors. In animals and humans, H is known to be metabolized to reduced haloperidol (RH) by reduction and 4-(4-chlorophenyl)-4-hydroxypiperidine (CPHP) by oxidative *N*-dealkylation.

**Objective:** According to *AGNP Consensus Guidelines for Therapeutic Drug Monitoring in Psychiatry: Update 2011*, therapeutic reference ranges/recommended drug concentrations of haloperidol (in blood) are between 1 and 10 ng/mL. Some literature has indicated that intoxication might occur when blood concentrations exceed 50 ng/mL. The purpose of this study is to develop a simple and effective UHPLC-MS/MS based methodology for accurate quantification of H, RH, and CPHP in postmortem blood and urine at low levels.

**Method:** Blood or urine samples (1 mL) were mixed with sodium carbonate/bicarbonate buffer (pH = 9.5) and extracted with dichloromethane:*n*-hexane:ethyl acetate:dichloroethane (1:1:1:1 in volume). Extracts were evaporated and reconstituted in the initial gradient-composition of the mobile phase for injection onto the UHPLC-MS/MS system. Haloperidol-d<sub>4</sub> (for H and RH) and zolpidem-d<sub>6</sub> (for CPHP) were used as internal standards. Chromatographic separation was achieved using an Agilent Zorbax SB-Aq (100 mm×2.1 mm i.d., 1.8 μm particle) analytical column at 50°C. The mobile phase was consisted of 0.1% formic acid (v/v) in water and methanol, with gradient, 0.32 mL/min flow rate, and a 9-minute total run time. Mass spectrometric analysis was performed by Agilent Jet Stream Technology electrospray ionization in positive-ion multiple reaction monitoring (MRM) mode, using optimized collision energy for each of the selected precursor ions (for analytes and internal standards) to monitor two transitions.

**Result:** Method validation was performed using drug-free blood and urine that was fortified with 5–100 ng/mL of H and 10–200 ng/mL of RH and CPHP. Analytical parameters obtained included: (a) average extraction recovery, derived from 4 different sources of blood and urine, was higher than 70%, except for CPHP (40%); (b) matrix effect (ion suppression) was observed, but adequately compensated for by the adopted internal standards; (c) intra-/inter-day precision and accuracy ranges (in %CV) for blood were 1.26–11.24%/1.58–12.45% and 89–107.3%/94–105%, while the corresponding ranges for urine were 1.66–6.12%/1.63–20.78% and 81.1–125.8%/87–107.9%; (d) calibration linearity (r<sup>2</sup>) for all (H, RH, CPHP) analytes were >0.991 with the limits of detection for H, RH, CPHP at 0.05, 0.1, 1 ng/mL for blood and 0.05, 0.1, 2 ng/mL for urine, while the limits of quantification for H, RH, CPHP at 0.25, 0.1, 1 ng/mL for blood and 0.1, 0.2, 2 ng/mL for urine.

**Conclusion/Discussion:** This relatively simple protocol was effective and reliable for the routine identification and quantification of H, RH, and CPHP in blood and urine. This method was successfully applied to the analysis of postmortem and antemortem specimens from 32 forensic cases in 2016. Concentration (ng/mL) ranges of R, RH, and CPHP found in these specimens were 1.56–71.3, 0.81–172, and 1.28–67.7 for blood and 1.32–130, 1.31–78.6, and 3.64–256 for urine, respectively.

**Keywords:** Haloperidol, Postmortem specimens, UHPLC-MS/MS

**A Death Involving Mitragynine (Kratom)**

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**Background/Introduction:** Mitragynine is the principal psychoactive alkaloid of the kratom plant (*Mitragyna speciosa*). Kratom is widely used as a recreational drug in Southeast Asia where the leaves are often chewed for their stimulant and analgesic effects. It is now appearing in the western world in various forms, including powder, plant, capsules, tablets, liquids, gum/resin, and drug patch. According to a Forbe's article, the American Kratom Association estimates that there are 3-5 million people using kratom in the U.S. The DEA attempted to place mitragynine and 7-hydroxymitragynine on Schedule I of the Controlled Substances Act. However, due to a letter signed by several lawmakers, the DEA has delayed the ban on kratom. Some adverse effects of kratom use/abuse are agitation, irritability, tachycardia, nausea, drowsiness, hypertension, hepatotoxicity, psychosis, seizure, insomnia, hallucinations and death.

**Objective:** There is limited literature/data regarding postmortem mitragynine blood levels, toxic levels and toxic effects. This poster presents the findings of a suicide by hanging involving one of the highest postmortem mitragynine level reported.

**Method:** : Samples submitted for testing included femoral blood, subclavian blood, and vitreous. Subclavian blood was tested for volatiles by headspace GC, and subclavian blood was used for drug screening by Enzyme Multiplied Immunoassay Technique (EMIT) and liquid-liquid alkaline extraction for GC/MS analysis. Vitreous was also used to confirm volatiles by headspace GC. Mitragynine confirmation was performed on femoral blood by an external laboratory by LC-MS/MS.

**Result:** The decedent, a 29 year old white male, was found in a semi-seated position while partially suspended by a ligature between the fence and shed of his backyard. Per the detective on scene, the decedent and his significant other had broken up and were arguing via text messaging. The subject sent a text stating his intent to commit suicide. The decedent's brother had committed suicide months earlier, and the decedent stated that he was going to see his brother. The significant other responded immediately and went to the residence with a friend to check on him. The decedent's phone was found in the trash and the decedent was partially suspended from the shed. 911 was called but no medical intervention was attempted. According to the decedent's aunt, the decedent was a recovering alcoholic and drug addict. Rigor mortis was absent, but livor was present. An external examination was performed. A package filled with kratom pills was found in his jeans pocket. No autopsy was performed. The initial drug screen was positive for mitragynine, acetaminophen, nicotine, cotinine, and caffeine. Subclavian blood was positive for ethanol at 83 mg/dL and vitreous tested positive at 98 mg/dL. Mitragynine was confirmed at 980 ng/mL.

**Conclusion/Discussion:** The highest postmortem blood mitragynine level documented is 600 ng/mL (Baselt 10<sup>th</sup> ed.). The femoral blood mitragynine level in this case is 980 ng/mL. The cause of death was asphyxia due to hanging; the manner of death was suicide. Severe symptoms of kratom abusers have included psychosis and hallucinations, which may influence reasoning and cognitive function. However, there is limited literature data regarding mitragynine toxicity levels and toxic effects. The high mitragynine level observed in this case may be useful for establishing the compound's effect on behavior, and its potential contributory role in subsequent death investigations involving mitragynine.

**Keywords:** Mitragynine, Kratom, Death Investigation

**Death Following Accidental Injection of Atracurium: Results of Toxicological Investigations**

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**Background/Introduction:** Atracurium is a nondepolarizing skeletal muscle relaxant. A literature search for papers on poisoning by atracurium cited in Medline revealed very few cases and most of them concern suicide cases. We report here the case of a fatal accidental injection of 5 mL of atracurium, caused by a confusion with trimebutine which have the same drug packaging. After completion of a colonoscopy in an hospital, a patient complained of abdominal pain. After a doctor prescription, a nurse was charged to IV administrate 5 mL of trimebutine. Returning to the room a few minutes later, the nurse noted the death of the patient. All injection material was immediately thrown away in a trash.

**Objective:** To confirm that atracurium had been injected instead of trimebutine and to determine laudanosine (a recognized by-product of atracurium) concentrations in peripheral blood, vitreous humor and bile.

**Method:** Laudanosine was identified in the post-mortem samples by LC-MS-MS on a Waters Xevo TQD system and quantification was performed using high-performance liquid chromatography coupled to diode-array detection. In addition to laudanosine determination, a comprehensive toxicological screening was performed on post-mortem blood using LC-HR/MS on a Waters Xevo-G2 XS QTOF system

**Result:** Trimebutine was not detected in the blood, confirming the error of the administered product. Very high levels of laudanosine were present in peripheral blood (2400 ng/mL), vitreous humor (183 ng/mL) and bile (188 ng/mL). The peripheral blood/vitreous humor ratio was 13.1. Morphine (6.2 ng/mL), oxazepam (125 ng/mL) and pholcodine (299 ng/mL) were also present in blood.

**Conclusion/Discussion:** The presence of morphine, oxazepam and pholcodine in blood at nontoxic concentrations is in relation with the treatment of the patient. The injection of atracurium instead of trimebutine was demonstrated by the results of toxicological investigations. To our knowledge, only two authors have reported such accidental injection of atracurium: one concerned an intramuscular injection of atracurium in seven newborns, and the other case reported the death of 15 children after being vaccinated against measles. Unfortunately, in these two cases, toxicological investigations were not performed. In cases of suicide with atracurium, some authors have reported cardiac blood values ranging from 280 to 917 ng/mL for laudanosine. The very high peripheral blood value observed in our case may be explained by the intravenous injection of a bolus of 50 mg of atracurium. This concentration is however within the therapeutic ranges that are reported in anaesthesiology, but only when patients are mechanically ventilated.

**Keywords:** Laudanosine, Atracurium, Intoxication



## Ethanol and Ethyl Glucuronide Concentrations in Post Mortem Femoral Blood (FB), Urine and Vitreous Humor (VH)

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**Background/Introduction:** Alcohol is an important parameter in post-mortem investigations aimed to explain the cause of death. The interpretation of post mortem alcohol concentrations in blood or urine can, however, be challenging because of post mortem alcohol formation during the putrefaction process. VH is known as a matrix, which is less prone to decomposition and can be used in toxicology analysis, even in an advanced state of decay of the body. The correlation between VH and blood is known to be strong for ethanol, but is unknown for its phase-II metabolite ethyl glucuronide (EtG). Similar to ethanol, EtG-concentration in blood and urine can also be influenced by bacterial synthesis or degradation.

**Objective:** The aim of this study is to increase knowledge about EtG in VH as an additional marker for differential diagnosis of ante-mortem alcohol consumption.

**Method:** VH, urine samples and FB was collected from 117 consecutive autopsy cases. All samples were stored at – 24°C until analysis and were analysed for ethanol and EtG and urine samples additionally for creatinine. Urinary EtG values were adjusted for creatinine. EtG analysis in FB and VH (cutoff 1 ng/mL) and urine (cutoff 100 ng/mL) was performed with a validated UPLC-MS/MS method. Ethanol was measured with GC-FID-Headspace.

**Result:** EtG was positive in VH, FB and urine in 62 cases and ethanol in 38 cases. EtG was negative in all three matrices in 37 cases and ethanol was negative in 58 cases. The ethanol-concentrations in FB and VH (slope:1.2,  $r^2=0.96$ ) and in FB and urine (slope:1.3,  $r^2=0.94$ ) were in good agreement. When EtG-concentration in VH versus EtG-concentration in FB was plotted, five separate groups could be identified:

Group 1 (n=11): Mean/median EtG-concentration [ng/mL] was >4500 in VH and FB and >650000 in urine, while mean/median ethanol-concentration [g/L] was >1.5 in all three matrices. Mean/median EtG-concentration ratio for: FB/VH: 0.96/0.62; FB/urine: 0.13/0.007

Group 2 (n=12): Mean/median EtG-concentration: VH: 999/960; FB: 7096/6560; urine: 412220/288411 while ethanol-concentrations was >1.5 in all three matrices. Mean/median EtG-concentration ratio for: FB/VH: 27.1/7.97; FB/urine: 0.03/0.024.

Group 3 (n=9): Mean/median EtG-concentration: VH: 1447/1283; FB: 803/683; urine: 330289/336181; ethanol-concentration: VH: 1.28/0.70; FB: 1.05/0.87; urine: 1.72/1.74. Mean/median EtG-concentration ratio for: FB/VH: 0.59/0.58; FB/urine: 0.005/0.003.

Group 4 (n=6): Mean/median EtG-concentration: VH: 346/343; FB: 627/628; urine: 785725/77010; ethanol-concentration: VH: 1.75/1.79; FB: 1.49/1.58; urine: 1.92/2.02. Mean/median EtG-concentration ratio for FB/VH: 2.18/1.89; FB/urine: 0.053/0.009.

Group 5 (n=79): Mean/median EtG-concentration: VH: <50; FB: <50; urine: <6000 while mean/median ethanol-concentration was < 0.05 in all three matrices. In eight cases EtG could be detected only in VH (two pos. for ethanol in FB), in five cases in VH and urine, in two cases only in VH and FB and in two cases only in urine.

**Conclusion/Discussion:** The strong correlation between ethanol concentrations in FB and urine with VH was not observed for EtG. This might reflect different rate of formation of EtG between individuals and/or different kinetics in VH compared to FB and urine. A possible explanation for the observed groups could be the state of ethanol and EtG metabolism at time of death. According to this suggestion the groups represent the following ethanol and EtG metabolic states:

Group 1: VH and FB nearly in equilibrium state for both analytes (high concentrations).

Group 2: EtG maximum concentration in VH has not yet been reached

Group 3: EtG and ethanol in blood in elimination phase, EtG VH values higher due to previous consumption of alcohol

Group 4: single ethanol abuse, no EtG accumulation from previous alcohol consumption

Group 5: not defined

The data supports that there is a time delay for equilibrium between blood and VH EtG-concentrations.

**Keywords:** Vitreous Humor, Ethyl Glucuronide, Ethanol

## Estimating and Evaluating Metabolic Capacity of CYP3A4 in a Intoxication Case Using a Proteomics and Mechanistic Pharmacokinetic Simulation Approach

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**Background/introduction:** Toxic or fatal drug concentration can be achieved, despite the fact that the patient has taken the medication as prescribed. Such an intoxication could be ascribed to reduced drug elimination (metabolism and excretion) capacity. The major elimination pathway for a number of drugs is by metabolism through the cytochrome P450 (CYP) enzyme system present in the liver. The CYP isoforms CYP1A2, CYP2C19, CYP2D6, CYP2C9, and CYP3A4 are major contributors to the elimination of a large number of drugs. There is a great interindividual variability in the CYP enzyme activity due to differences in the amount of the CYP protein expressed in liver tissue and polymorphisms in CYP genes encoding enzymes with variable activities. Using genotyping it is possible to estimate the activity of CYP2C9, CYP2C19, and CYP2D6. Genotyping is much less predictive for activity estimation of CYP1A2 and CYP3A4. After death CYP-proteins quickly lose their activity which makes it impossible to estimate hepatic CYP activity by standard methods e.g. in vitro assays using probe drugs and a human liver microsomal (HLM) preparation. Targeted mass spectrometric methods can be used to quantify human CYP HLM-levels. For HLM there has been shown a clear relation between CYP activity and measured CYP-levels by mass spectrometric methods.

**Objective:** For a fatal intoxication case regarding a poor metabolizer of both CYP2C19 and CYP2D6 it was investigated if reduced activity of CYP3A4 could be the cause for higher than expected venlafaxine (VEN) and quetiapine (QUE) concentrations.

**Method:** The intoxication case CYP3A4 HLM-level was estimated using an in-house developed LC-MS-MS method. Absorption, distribution, metabolism and excretion (ADME) of extended release (ER) VEN and immediate release (IR) QUE were simulated using the population based mechanistic pharmacokinetic simulator Simcyp ver. 16 (Certara Inc., USA). A minimal physiological based pharmacokinetic model of distribution was assumed. Simulations were performed using 100 virtual individuals having same weight and height as the deceased. The simulations were performed for enough days to ensure steady state conditions.

**Result:** The CYP3A4 level for the intoxication case was 8.6 pmol/mg HLM, or 15% of an average HLM level. The deceased took 150 mg VEN ER formulation twice daily and 100 mg QUE IR formulation twice daily. VEN and QUE post mortem whole blood levels were 4.5 mg/kg and 0.36 mg/kg. Pharmacokinetic simulations of VEN and QUE were performed at CYP3A4 levels normally found in a population and at the for this case estimated CYP3A4 level. For normal CYP3A4 levels the simulations predicted average (range) concentrations of VEN and QUE to be 1.0 (0.44–2.1) mg/kg and 0.071 (0.013–0.18) mg/kg. Using the case-specific estimated CYP3A4 level, the simulations predicted average (range) concentrations of VEN and QUE to be 1.4 (0.54–2.7) mg/kg and 0.37 (0.11–0.74) mg/kg.

**Conclusion/discussion:** The evaluation of a measured CYP-level for a given case is non-trivial since a number of factors will influence the pharmacokinetics of a given drug e.g. other metabolizing CYP-enzymes, CYP enzyme kinetics, renal excretion, hepatic blood flow, and microsomal protein pr. gram of liver. Population based pharmacokinetic simulations were performed at normal population CYP3A4 levels and at the CYP3A4 level found in this case, but with variation in other factors important for drug pharmacokinetics. The measured QUE concentration were in line with simulation results using case estimated CYP level, further substantiating the assumption of reduced CYP3A4 activity for this poisoning case. VEN simulations using a case estimated CYP3A4 level gave results slight higher than average values, this result points towards additional reasons for the high VEN concentration. The presented results are preliminary since post mortem stability of CYP enzymes has yet to be established and the predictability of the simulations has not been investigated with this kind of approach.

**Keywords:** Proteomics, Pharmacokinetics, CYP3A4

**Utility of Forensic Radiology and Determination of Intracardiac Gases Composition for Diving Fatalities Diagnosis**

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**Background/Introduction:** The determination of the cause and circumstances of death during diving is always complex. Indeed, postmortem gas occurrence could derive from barotrauma, decompression sickness, resuscitation procedure, postmortem off-gassing and decomposition. Today, the forensic imaging tools such as Multidimensional Computed Tomography (MDCT-Scanner) and Magnetic Resonance Imaging (MRI) allow to orientate the diagnosis with the gas bubbles distribution according to the diving profile and postmortem delay. These techniques are very informative and allow a preliminary diagnosis without body dissection. However, these approaches should be validated by the composition of the gas bubbles to avoid misinterpretations, especially caused by decomposition. Intracardiac gases sampling protocol which has been initially developed for taphonomy documentation may become a precious tool in diving fatalities interpretation.

**Method:** Six cases of scuba diving fatalities occurred in Swiss lakes during the last 5 years were studied. Bodies were CT Scanned, examined and five were autopsied. Intracardiac gases were sampled following gas sampling protocol under laser guidance and analyzed by gas-chromatography coupled to thermal conductivity detection (GC-TCD). The different gaseous compositions were compared with specific emphasis on cardiac carbon dioxide (CO<sub>2</sub>) and related to the diving profiles, postmortem delays, resuscitation procedures and finally confronted to autopsy results and medico legal conclusions.

**Result:** Intracardiac gases compositions were very useful to differentiate decomposition gases (hydrogen H<sub>2</sub>, hydrogen sulfide H<sub>2</sub>S and methane CH<sub>4</sub>) to other gases. This avoids a false interpretation by forensic radiologist, because decomposition can be generated rapidly after death. Radiological interpretation of intracardiac gases distribution in bodies showing this type of gases should therefore be excluded. The intracardiac gaseous CO<sub>2</sub> concentration was found really informative when interpreted simultaneously with the diving profile. This concentration is clearly positively linked to the depth in case of postmortem off-gassing after drowning underwater and in case of barotrauma with body recovery at surface.

**Conclusion/Discussion:** Intracardiac gases sampling and analysis is become an useful step accompanying the forensic radiological diagnosis. It is also helpful to complete the medico legal conclusion about circumstances and cause of death. Additional cases are needed to statistically strengthen these observations.

**Keywords:** Intracardiac Gases, Diving Fatality, Forensic Imaging

**Detection of Cannabinoids in Forensic Serum Samples by Immunoassay: HEIA™ Versus GC-MS**

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**Background/Introduction:** Based on the high prevalence of cannabis use in most countries, a useful routine screening for cannabis in human serum specimens is a frequent request in forensic laboratories. The reliable detection of cannabinoids –  $\Delta^9$ -THC, 11-OH-THC and 11-COOH-THC – in human blood and serum specimens is, therefore, a central part of the basic forensic screening panel.

**Objective:** Only few immunoassays are suitable for the measurement of these substances in native human serum and plasma. In this study, the performance of the Immunalysis HEIA™ assays for Cannabinoids (both urine (U) and oral fluid (OF) reagents) were tested for routine use with forensic serum specimens. Results were compared against the respective chromatographic results obtained by GC-MS.

**Method:** The studies comparing the two immunoassays (HEIA™ U, HEIA™ OF) were performed in the forensic toxicology laboratory in Salzburg, Austria, and a laboratory in Passau, Germany. A total of 408 serum samples were analyzed. Adjusted calibration curves for the immunoassay screening were prepared by spiking the respective concentrations of 11-COOH-THC into a drug free human serum pool matrix. For comparison testing, all samples were quantified with a forensically validated chromatographic routine method (GC-MS (ISO 17025)), using an 0.5 ng/mL cut-off for each of the three analytes ( $\Delta^9$ -THC, 11-OH-THC and 11-COOH-THC – free substances only, w/o prior hydrolysis). Cut-off levels at 10 ng/mL were used for the immunoassay determinations. Finally, sensitivity, specificity and overall accuracy were calculated against the chromatographic results.

**Result:** All initial performance checks over the calibration range resulted within expectations. 7 of the 408 forensic serum samples were excluded from the reporting because of strong hemolysis. Comparing the results (401 each) of the two immunoassays – HEIA™ U reagents and the HEIA™ OF reagents for the detection of cannabinoids, all 401 serum specimens gave concordant results. The overall NPV was calculated as 1.0000. When comparing the results against confirmation, an overall accuracy of 99.8% and an NPV of 1.0000 were determined for both the HEIA™ U and the HEIA™ OF immunoassay.

**Conclusion/Discussion:** Our data demonstrate that both the HEIA™ U and the HEIA™ OF immunoassay for cannabinoids are viable options for the screening of native human serum specimens in a forensic setting. Both reagents show an excellent performance when compared to the GC-MS confirmation method, with a total accuracy of 99.8% each. Looking at the overall results, we can clearly recommend the HEIA™ U and OF reagent for the detection of cannabinoids when running a screening of forensic serum specimens with immunoassays.

**Keywords:** Immunoassay, HEIA™, THC, Cannabinoids

**Detection of Opiates, Cocaine and its Metabolites in Forensic Serum Samples by Immunoassay: HEIA™ Versus GC-MS**

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**Background/Introduction:** The reliable detection of cocaine (COC) and its metabolites benzoylecgonine (BE) and ecgonine methyl ester (EME) as well as the detection of opiates – morphine (MOR), codeine (COD), 6-acetylmorphine (6-AM) and dihydrocodeine (DHC) – in human blood specimens is a central part of the basic forensic screening panel.

**Objective:** Only few immunoassays are suitable for the measurement of these substances in native human serum and plasma. In this study, the performance of the Immunalysis HEIA™ assays for benzoylecgonine, the main cocaine metabolite (both urine (U) and oral fluid (OF) reagents) and the Immunalysis HEIA™ assays for opiates (both urine (U) and oral fluid (OF) reagents) were tested for routine use with forensic serum specimens. Results were compared against the respective chromatographic results obtained by GC-MS.

**Method:** The studies comparing the immunoassays (HEIA™ U, HEIA™ OF) were performed in the forensic toxicology laboratory in Salzburg, Austria, and a laboratory in Passau, Germany. A total of 224 forensic serum samples were analyzed. Adjusted calibration curves for the immunoassay screening were prepared by spiking respective concentrations of BE and MOR into a drug free human serum pool matrix. For comparison testing, all samples were quantified with a forensically validated chromatographic routine method (GC-MS (ISO 17025)), using a 3.0 ng/mL cut-off for each of the three “cocaine” analytes (COC, BE & EME) and a 3.0 ng/mL cut-off for each of the four “opiate” analytes (MOR, COD, 6-AM & DHC). Cut-off levels at 10 ng/mL were used for the immunoassay determinations. Sensitivity, specificity and overall accuracy against the chromatographic results were calculated.

**Result:** All initial performance checks resulted in precisions below 12 % CV over the calibration range. 6 of the 224 forensic serum samples were excluded from the reporting because of strong hemolysis. Comparing the results (218 each) of the two immunoassays – HEIA™ U reagents and the HEIA™ OF reagents for cocaine and its metabolites, 214 serum specimens gave concordant results, while 4 serum specimens showed discordant results (3 positive only in HEIA™ U and 1 positive only in HEIA™ OF). The overall NPV was calculated as 0.9806. When comparing the results against confirmation, an overall accuracy of 99.1% and an NPV of 0.9935 were determined for the HEIA™ U and an overall accuracy of 99.1% and an NPV of 0.9871 for the HEIA™ OF immunoassay, respectively.

Comparing the results of the two immunoassays for opiates – HEIA™ U reagents and the HEIA™ OF reagents, all 218 serum specimens gave concordant results. Therefore, the overall NPV was calculated as 1.000. When comparing the results against GC-MS, an overall accuracy of 100% was determined for both the HEIA™ U and the HEIA™ OF immunoassay.

**Conclusion/Discussion:** Our data demonstrate that both the HEIA™ U and the HEIA™ OF immunoassays for cocaine metabolite and opiates are viable options for the screening of native human serum specimens in a forensic setting. All reagents tested showed an excellent performance when compared to the GC-MS confirmation method, with total accuracies of 99.1% for cocaine and 100% for opiates. Looking at the overall results, we can clearly recommend the HEIA™ U and OF reagents to be used for the detection of opiates as well as for cocaine and its metabolites when running a screening of forensic serum specimens with immunoassays.

**Keywords:** Immunoassay, HEIA™, Cocaine, Opiates

**Implementation of an Automated Sample Integrity Check for the Immunological Screening of Forensic Serum Specimens**

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**Background/Introduction:** Sample integrity plays an important role in the screening for drugs of abuse with immunoassays. In forensic screening routines, prevention of false negative screening results is of particular interest. Especially when testing alternative matrices like human serum or blood, saliva and hair extract, the reliable detection of matrix interferences or other disturbing influences on the enzyme activity and immunoassay performance is a useful analytical tool.

**Objective:** Increasingly, immunoassay screening for drugs of abuse, including alternative matrices, is consolidated on automated clinical chemistry analyser platforms. In order to minimize human errors and streamline the daily routine process in the laboratory, an automated check for sample integrity and viability is invaluable. During this study, we developed and implemented two fully automated sample integrity checks for routine use with ante mortem forensic serum specimens – one for use with the Immunalysis HEIA™ Urine (U) and one for the HEIA™ Oral Fluid (OF) assays.

**Method:** The studies implementing the sample integrity check for the two immunoassay systems (HEIA™ U & HEIA™ OF) were performed in the forensic toxicology laboratory in Salzburg, Austria and a laboratory in Passau, Germany. Adjusted parameter settings for the Immunalysis HEIA™ U and OF PCP reagents were referenced against a drug free human serum pool matrix (100 % enzyme activity). The HEIA™ PCP reagents were used, since the prevalence of PCP in the Austrian market equals zero. A total of 511 serum samples were analyzed. To evaluate and prove the benefit of the two sample integrity checks, the concentrations for cocaine and its metabolites as well as opiates and cannabinoids were determined using HEIA™ U and OF immunoassays.

**Result:** The acceptance range was set from 93% up to 105% enzyme activity. Comparing the results of the 511 forensic ante mortem serum specimens, 467 samples gave concordant integrity results within the specified acceptance range, while 14 samples gave a concordant integrity result outside the acceptance range. The remaining 30 samples gave discordant results (14 outside the range only in HEIA™ U and 13 outside the range only in HEIA™ OF). With one exception, all conspicuous samples showed reduced enzyme activity (9 samples even showed a sample integrity check of zero) and, therefore, a tendency towards false low or even false negative results in the immunoassay screening.

**Conclusion/Discussion:** Our data demonstrate that the newly implemented sample integrity checks for the Immunalysis HEIA™ U and HEIA™ OF immunoassays provide a useful additional analytical tool for the detection of matrix influences and may help to reduce false negative screening results primarily caused by enzyme suppression or other unspecific interferences.

**Keywords:** Immunoassay, HEIA™, Sample Integrity, Sample Validity

## The Pigeon Poppy Seed Defence – Analysis of Opiates as Markers of Doping Use in Racing Pigeons

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**Background/Introduction:** Subjects caught driving under the influence of morphine often claim the use of poppy seeds in their defence. These seeds from the *Papaver somniferum* plant naturally contain a number of opiates including morphine and codeine. Though highly known in human forensic cases this defence recently surfaced in the Belgian pigeon racing. As with any international sport adulteration is likely to occur, all the more when large amounts of money are involved (and price winning pigeons can easily be sold for several \$ 100 000). Over the course of the last year the appearance of morphine, of which the performance-enhancing effects are still under debate, has been noted. The sudden use of a drug that has been on the list of forbidden substances for years, as well as the large number of pigeons having tested positive in the same period, raised the suspicion of another source of contamination, with the consumption of poppy seeds being a plausible explanation.

**Objective:** Our current research aimed to develop a gas chromatography-tandem mass spectrometry (GCMS/MS) method for the identification and quantification of the 5 major opiates found in poppy seeds: morphine, codeine, thebaine, papaverine, and noscapine. Different administration conditions (controlled administration, free choice with limited access to food, free choice with food at libitum) were applied to investigate the pharmacokinetics of these opiates in pigeons.

**Method:** Six pigeons were subjected to each of the above mentioned conditions. Faeces samples were collected every 3 hours for 2 days following administration, and twice daily thereafter. Samples were stored at -20 °C until analysis. Samples were spiked with labelled internal standards prior to ultrasonication-assisted extraction in methanol and enzymatic deconjugation in sodium acetate buffer. pH was adjusted to 6 by addition of phosphite buffer. Solid-phase extraction with a non-polar C8 – cation exchange mixed-mode cartridge and dichloromethane/isopropanol containing ammonia as eluting agent was used in sample clean-up. The resulting extracts were evaporated and reconstituted in ethyl acetate prior to BSTFA-TMCS derivatisation and GCMS/MS analysis.

**Result:** Following the administration of about 172.5 mg poppy seeds (containing approximately 20 µg of morphine), concentrations of up to 2 500 ng morphine per g faeces were detected in the samples collected. Morphine concentrations reached peak levels 6 hours after administration, returning below the detection limit within 2 days. Codeine and thebaine, a marker for opiates from natural sources rather than pharmaceuticals, could be detected as well. Our current set-up was unable to detect papaverine or noscapine in the samples, as their concentrations present in the poppy seeds were below our quantification limit of 0.2 ng/g.

**Conclusion/Discussion:** The method successfully detected and quantified the opiates present in the pigeon faeces samples. Our experiments showed a transient increase in the opiate concentrations following administration of poppy seeds, followed by a rapid decrease. Enterohepatic recirculation has been noted as is also known to occur in humans. However, due to the small size of the seeds, it seems unlikely that the birds would eat them voluntarily.

**Keywords:** Doping, Poppy Seed Defence, Pigeons

## Development and Validation of a UHPLC-HRMS/MS Quantification Method for Hypoglycin A in Horse Serum in Cases of Atypical Myopathy in Horses.

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**Background/Introduction:** Atypical myopathy (AM) is a fatal disease in horses. The etiopathology is marked by sudden onset of muscle weakness and stiffness up to lateral recumbency. Other symptoms are sweating, trembling, postural and respiratory muscle degeneration, rhabdomyolysis, and myoglobinuria, leading to dark-brown colored urine. The mortality rate is rather high with 85% and the majority of the affected horses die within 72 hours after occurrence of clinical signs. Hypoglycin A (HGA) is naturally present in e.g. maple seed and has been discussed as a cause of AM. HGA underlies a rapid metabolism to the toxic methylene-cyclopropyl-acetic-acid (MCPA); which inhibits acyl-CoA dehydrogenase and reacts with some essential cofactors (coenzyme A and carnitines) of the  $\beta$ -oxidation pathway. In 2016 outbreaks of AM were reported in Belgium, France, UK, Switzerland, Netherlands, Ireland, and Germany.

**Objective:** Aim of this study was the development and validation of a rapid and simple quantification method for HGA which also enables identification of metabolites in authentic horse serum using ultra high performance liquid chromatography coupled with a high resolution tandem mass-spectrometry (UHPLC-HRMS/MS) and the application to authentic samples.

**Method:** The method was developed on a Q Exactive Focus MS coupled with a Dionex UHPLC system (Thermo Fisher Scientific, Dreieich, Germany). HRMS/MS was performed in positive heated electrospray ionization mode. Quantification was carried out by parallel reaction monitoring (PRM) mode using accurate fragments. While metabolite identification was achieved by accurate full-scan (FS) MS and data dependent (dd) MS<sup>2</sup>. Chromatographic separation was performed by isocratic elution with 50 mM ammonium formate buffer (mobile phase A) and acetonitrile (mobile phase B) (15:85 [v:v]) on a Synchronis HILIC column (100 x 2.1 mm) (Thermo Fisher Scientific, Dreieich, Germany).

Serum samples (250  $\mu$ L) were worked up by protein precipitation using 1 mL of acetonitrile. The solutions were kept for 2 hours in the freezer. After centrifugation, 900  $\mu$ L of supernatants were evaporated to dryness and redissolved in 30  $\mu$ L of a mixture of mobile phase A and B (80:20 [v:v]). The calibration range was 100 - 2000 ng/mL according to published serum concentrations in affected horses. The method was validated according to international guidelines with respect to selectivity, linearity, accuracy, precision, matrix effects, and recovery and applied to authentic samples.

**Result:** Selectivity experiments (n = 20) showed no interferences. Linearity was proven over the whole calibration range with 1/ $x^2$  weighting. The following parameters relate to low and high concentration levels, respectively. HGA showed acceptable accuracy and precision data (bias 0 % - 11 %; RSD 3.3% – 5.4%). LOQ was defined as lowest calibrator (100 ng/mL), and was well below the lowest published serum concentration of affected horses. Matrix effects were strong (-79% and -84 %), but reproducible (RSD 1.4 % and 2.9 %). Recovery was 57 % and 60 % with low RSD (15 % and 3.8 %). In comparison to published methods the present method does not require extensive sample preparation or derivatization for quantification of HGA in concentration ranges reported in cases of AM. Applicability of the method was shown by analysis of horse serum specimens from a total of 10 authentic AM cases. In all of these specimens, relevant amounts of HGA were found (560 – 2000 ng/mL). Additionally, in all serum samples MCPA carnitine was preliminarily identified as metabolite based FS and ddMS<sup>2</sup> data.

**Conclusion/Discussion:** The developed assay allows reliable quantification of HGA as well as identification of possible metabolites in serum samples and was successfully applied to authentic samples of AM affected horses. The method will be helpful to further study the association of HGA in AM in horses.

**Keywords:** Atypical Myopathy, Hypoglycin A, UHPLC-HRMS/MS



## Postmortem Distribution of N-ethylpentylone

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**Background/Introduction:** N-ethylpentylone (NEP) is a novel synthetic cathinone with properties similar to cocaine, MDMA and other common amphetamines. The OCME of Maryland initially detected NEP in the summer of 2016. During a period of 5 months from August 2016 through December 2016, 19 cases related to NEP were investigated by the office.

**Objective:** Analysis of various biological specimens for NEP allows for the study of its postmortem distribution. Due to the limited case studies and information available, this data will impact the forensic science community by providing medical examiners and toxicologists with an analytical method and postmortem concentrations for NEP.

**Method:** NEP was first identified in blood and urine specimens following an alkaline liquid-liquid extraction and analysis by gas-chromatography mass spectrometry (GC-MS) and gas chromatography nitrogen/phosphorous detection (GC-NPD). NEP elutes shortly after caffeine and its spectrum has a base peak of  $m/z$  100. Quantitation was performed via a newly developed and validated solid phase extraction followed by GC-NPD analysis. The new method allowed for quantitation of NEP in a variety of biological specimens including blood, urine, bile, liver, kidney and vitreous humor. A specimen volume of two mL was extracted except for urine (1:2), bile (1:8), liver/ kidney homogenates (1:5), which were extracted at the specified dilutions. A six point calibration curve (10 to 500 ng/mL) and two controls (30 and 300 ng/mL) were used for quantitation.

**Result:** Case specimen concentrations of NEP (ng/mL or ng/g) are summarized below.

Case	Cause of Death	Heart Blood	Peripheral Blood	Urine	Bile	Liver	Kidney	Vitreous Humor	Other Notable Toxicology (Blood)
1	Homicide	N.A.	>500 (C)	N.A.	>500	>500	>500	>500	
2	Homicide	N.A.	50 (C)	>500	120	290	190	80	
3	Homicide	N.A.	26 (C)	N.A.	N.D.	100	N.D.	30	
4	Homicide	>500	N.A.	>500	>500	>500	>500	>500	
5	Homicide	>500	160 (C)	>500	>500	>500	>500	470	
6	Suicide	N.A.	180 (C)	>500	>500	350	300	110	
7	Suicide	75	N.A.	230	N.A.	N.A.	N.A.	150	Benzoylcegonine
8	Suicide	16	N.D.	60	N.A.	N.A.	N.A.	22	Butylone, Dibutylone, Methamphetamine
9	Suicide	350	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	Diphenhydramine
10	Natural	130	130 (S)	470	150	>500	110	37	
11	Drowning	>500*	N.A.	N.A.	N.A.	N.A.	N.A.		
12	Pentylone intoxication	500	N.A.	N.A.	N.A.	N.A.	N.A.	>500	Pentylone
13	NEP intoxication	190	N.A.	N.A.	N.A.	N.A.	N.A.	200	
14	NEP intoxication	350	N.A.	N.A.	N.A.	N.A.	N.A.	>500	
15	Complications of NEP use	170	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	
16	Mixed drug intoxication	390	300 (F)	N.A.	N.A.	430	>500	>500	Fentanyl and Methadone
17	Complications of NEP intoxication	N.A.	21 (F)	180	>500	210	110	60	
18	Mixed drug intoxication	50	40 (S)	N.A.	N.D.	180	N.A.	N.A.	Morphine, Furanyl fentanyl, 4-FIBF, DPF
19	NEP intoxication	16	13 (F)	90	N.D.	N.D.	N.D.	60	

\*Decomposition fluid was extracted.

Abbreviations: *4-FIBF* 4-fluoroisobutyrylfentanyl, *DPF* despropionylfentanyl, N.A. not available, N.D. none detected, C cavity, S subclavian, F femoral

**Conclusion/Discussion:** The OCME of Maryland has seen a remarkable increase in NEP positive cases since its first detection in August 2016. It is easily identified and quantified in various biological matrices and can be analyzed by routine GC-NPD instrumentation. There does not appear to be a significant difference between central and peripheral blood concentrations in this limited data set. NEP liver concentrations were generally higher than blood concentrations, suggesting that NEP may be sequestered in the liver. These data also suggest that vitreous humor is an appropriate specimen for the detection of NEP use, but it is of limited interpretative value. All cases were autopsied within 24 hours of receipt.

**Key Words:** N-ethylpentylone, Postmortem Toxicology, Synthetic Cathinones

**High Blood Concentrations: Cause of Death or Caused by Death?**

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**Background/Introduction:** The concept of post-mortem redistribution (PMR) has been known for more than 40 years and still has a major impact on the interpretation of xenobiotics level in casework. PMR might result in artificially elevated concentrations of drugs caused by various changes that occur after death. Consequently, PMR must be considered when interpreting toxicological results in order to determine the specific role of the xenobiotic in the cause and circumstances of death.

**Objective:** Two interesting fatal cases where PMR could have led to wrong interpretation of quetiapine and flurazepam post-mortem concentrations are presented.

**Method:** Our autopsy practices involve sampling of the heart blood, femoral blood, ocular fluid, urine, liver and gastric content, when available. Routine analysis include a solid phase extraction sample preparation followed by a GC-MS/NP and LC-MS/MS analysis. It also includes ethanol and other volatiles analysis by headspace GC-FID. All positive results must be confirmed to be included in the final toxicological report. When high blood concentrations are found, gastric content is assayed to provide information on the route of absorption and on the circumstances of death.

**Result:** Case 1: A 49-year-old male died of a self-inflicted craniocerebral trauma caused by a 9-mm handgun with a 1 day post-mortem interval. The toxicological screening of the cardiac blood revealed 20 µg/mL of quetiapine, suggesting a toxic/lethal concentration. The gastric content analysis revealed 600 mg of quetiapine, however it was undetected in femoral blood. The absence of quetiapine in femoral blood suggests diffusion from gastric content to cardiac blood. Based on these results, quetiapine was only reported in gastric content in the final toxicological report.

Case 2: A 53-year-old male was found in a state of putrefaction at home, the post-mortem interval being more than 7 days. His death was attributable to coronary artery disease (atherosclerotic). In cardiac blood, flurazepam was found at 220 ng/mL, suggesting a toxic concentration. The gastric content revealed the presence of flurazepam at 7 mg while the femoral blood concentration was found to be 7 ng/mL. In contrast to flurazepam, its active metabolite (desalkyl) concentration was comparable in cardiac (27 ng/mL) and femoral blood (25 ng/mL). However, the metabolite was undetected in gastric content; therefore suggesting diffusion of the parent drug to the cardiac blood. Based on femoral blood results, flurazepam was interpreted at therapeutic concentration in the final toxicological report.

**Conclusion/Discussion:** Even using quality autopsy samples, rigorous analytical processes and/or sophisticated and sensitive techniques, the scientific expertise of a forensic toxicologist is irreplaceable. Indeed, the choice of blood source is crucial, as seen in these two cases. This reiterates the importance of the use of peripheral blood to evaluate the role of a xenobiotic in the cause and circumstances of death.

**Keywords:** Post-mortem Redistribution, Quetiapine, Flurazepam, Post-Mortem Analysis

## A Case of Devious Poisoning with Sodiumazide

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**Background/Introduction:** A 60 years old woman suffered from mobility disorder since a few months before death and was diagnosed with Guillain Barré syndrome with progressive polyneuropathy. The woman was found in need of resuscitation and died 12 hours after hospitalization. The first screening in the hospital pointed at an ethylene glycol intoxication. Autopsy on the body identified no anatomical cause of death. The police investigation showed that both the victim and her sister had become acutely ill after drinking a spirit called Beerenburg. Later investigations showed that the suspect ordered sodium azide at two different occasions. In the blood samples of the deceased, collected during both hospitalization and postmortem, azide concentrations were demonstrated between 1 and 6.1 mg/l.

**Objective:** A case report of fatal poisoning with sodiumazide.

**Method:** Toxicological examination was conducted on six blood samples which were collected during hospitalization at various time points while the victim was still alive, on body material obtained during obduction and on Beerenburg spirit. The azide was identified and quantified after derivatization with PFBBr by using GC-MS. The stability of azide in the spirit was studied during 2 weeks. Hair analysis was performed on 4 segments of 2 cm each.

**Result:** Symptoms/diseases displayed in the medical record were pulmonary edema, reduced mobility, M. Guillan- Barré, nausea and emesis, dizziness, headache, coma, hemodynamic instability, polyneuropathy, lactic acidosis, numbness feet to knees and hands, Sjögren, panic attacks, hyperventilation and foot elevator palsy.

Azide was quantitated in all the blood samples that were collected during hospitalization in concentrations between 1.6-6.1 mg/l, depending on the time of collection. The calculated half-life of azide was 2, 5 hours.

In postmortem bodymaterial, azide was only found in femoral blood and vitreous humor (respectively 1 and 2 mg/l). No azide was identified in stomach contents, contents of small intestine, heart blood, liver tissue, bile, brain tissue or urine. Furthermore, some pharmaceuticals were found that may have been administered during hospitalization. Cyanide (a metabolite of azide) was not found in postmortem blood.

The spirit contained an azide-concentration of 1.7 g/l. Azide was stable for at least two weeks (at room temperature). In none of the hair segments azide was demonstrated (limit of detection < 0.1 ng/mg).

**Conclusion/Discussion:** The measured blood concentrations of azide in the hospital samples are high concentrations, corresponding with concentrations that have been measured in persons who died after deliberate ingestion of sodium azide. Interestingly, most postmortem materials were negative for azide, except for vitreous humor and femoral blood. This stresses the importance of investigating multiple tissue samples and types and, when available, antemortem collected material. This case also shows that azide has a relative short half-life time. According to the verdict, the fatal dose of azide must have been administered with the manipulated spirit. Earlier administrations or chronic poisoning, which could explain the Guillain Barré symptoms and the progressive polyneuropathy of the victim, could not be excluded nor proven by solely the toxicological investigations. The negative hair analysis does not exclude exposure to azide. The cause of death was certified as a fatal intoxication with sodium azide and the suspect was found guilty of murder/manslaughter by the high court.

**Keywords:** Azide, Intoxication, Human

**Toxicological findings in two victims of fatal poisoning with fentanyl**

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**Background/Introduction:** Fentanyl is a synthetic narcotic opiate with strong analgesic properties, being about 100 times more potent than morphine. It is used for the treatment of chronic pain and it is administered by diverse routes mostly using transdermal patches. Because of its high potency, fentanyl is also misused and abused.

Here we present a case of fatal fentanyl poisoning in two victims. The man and woman were found together dead at home. At first, there was suspicion for a carbon monoxide poisoning and the two victims were transported outside the house and reanimated. A screening test in urine indicated the presence of amphetamines, morphine and benzodiazepines. To determine the cause of death, forensic autopsies were ordered and toxicology was performed.

**Objective:** To present and interpret toxicological findings of fentanyl in different biological matrices.

**Method:** A complete forensic autopsy was performed according to standard protocols. Toxicology consisted of alcohol and GHB analysis, a quantitative analyses for common drugs and a targeted screening for drugs, pharmaceuticals and pesticides by LC-TOF. Positive screening results were confirmed and quantified by LC-MS-MS.

**Result:** Forensic autopsies showed a light weighted 22 year old man of 56 kg, 1.75 m length and a 31 year old woman of 55 kg, 1.65 m length. The preliminary autopsy reports revealed macroscopically no indication for morbid abnormalities and no cause of death could be given.

Toxicological analyses demonstrated the presence of fentanyl among other drugs. In the man, non-toxic concentrations of morphine, MDMA and noroxycodone in femoral blood were measured and in the woman, non-toxic concentrations of MDMA and MDA were measured. The concentrations of other detected drugs were low.

Targeted analysis of fentanyl showed the following results for the man: 0.17 mg/l in femoral blood, 0.31 mg/l in heart blood, 0.25 mg/l in vitreous humor, 1.8 mg/kg in liver and positive in urine. For the woman the following results were obtained: 0.34 mg/l in femoral blood, 0.64 mg/l in heart blood, 0.43 mg/l in vitreous humor, 3.3 mg/kg in liver and positive in urine.

**Conclusion/Discussion:** In postmortem studies, there is considerable overlap in fentanyl concentrations found in fatal cases, cases of fentanyl related deaths and cases of natural deaths overlap. Measured concentrations vary widely from 0.003 up to 0.46 mg/l in postmortem blood. The variation in concentrations between and within groups may be explained by postmortem redistribution, reanimation and tolerance. Because of possible postmortem redistribution, we measured concentrations of fentanyl in other matrices in addition to blood.

In both victims very high concentrations of fentanyl were measured in all analyzed matrices. The distribution of fentanyl in these matrices were comparable between the victims, however the concentrations in the woman were twice as high. The heart blood/femoral blood ratio of fentanyl was around 2 and is comparable with other literature data. The concentrations measured in vitreous humor were very high and were in between the femoral blood and heart blood fentanyl concentration, indicating at least some time delay between intake and death. The liver and vitreous humor fentanyl concentrations were extremely high, to our knowledge the highest liver and vitreous humor concentrations published so far were 0.61 mg/kg and 0.13 mg/l. In both victims, the death could be explained by the very high concentrations of fentanyl found in the bodies.

**Keywords:** Fentanyl, Fatal Poisoning, Postmortem Redistribution

## A Lethal Combination of Drugs During a Prolonged Chemsex Session: Increased Risk with GHB, Methamphetamine, Alprazolam, Anabolic Steroids and Antiretroviral Drugs.

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**Background/Introduction:** Chemsex is a specific form of recreational drug use involving specific drugs (e.g., GHB/GBL, ketamine, methamphetamine, mephedrone) alone or in combination to enhance or prolong sexual sessions. Chemsex mainly occurs amongst men who have sex with men (MSM). It is not a new phenomenon but the mainstream media are becoming increasingly aware of the issue and drug treatment centers in Europe, particularly in the UK, are overwhelmed by the number of men seeking help. GHB is the drug most linked to acute harm out of those used in chemsex.

**Objective:** We present the first known chemsex related death in our region. A 36 years old HIV positive man died during prolonged and close sexual sessions, as visible on videos made available. The concentrations of GHB measured in urine, peripheral and cardiac blood, vitreous, various tissues and hair were found to be exogenous and compatible with other reported deaths with GHB or GHB associated with other substances. Antiretroviral drugs, anabolic steroids, cocaine metabolites, amphetamines and benzodiazepines were also present in body fluids and tissues.

**Method:** GC-MS, LC-MS/MS and LC-QTOF-MS were used for screening purposes. GHB determination in biological liquids and tissues was performed by GC-MS after liquid/liquid extraction (LLE) and trimethylsilyl (TMS) derivatization. Cocaine, amphetamines and benzodiazepines were determined by LC-MS/MS after LLE or SPE extractions. Anabolic steroids were determined by enzymatic hydrolysis and LLE followed by GC-MS/MS (after TMS derivatization) and LC-MS/MS analyses. GHB quantitation in hair was performed by LC-MS/MS; briefly, hair samples were washed twice with dichloromethane, pulverized and extracted in methanol under agitation for 1 hour.

**Result:** Cocaine metabolites, amphetamine, methamphetamine, alprazolam, OH-alprazolam, diazepam, nordiazepam, GHB, sildenafil, testosterone, boldenone and metabolites, methandienone and metabolites, nandrolone metabolites, 1-androstenedione and metabolites, androsta-1,4,6-triene-3,17-dione (ATD) and metabolites as well as antiretroviral drugs were detected in urine. Methamphetamine (25 µg/l), alprazolam (40 µg/l) and anabolic steroids concentrations determined in peripheral blood were compatible with therapeutic levels. Amphetamine, diazepam and nordiazepam were compatible with sub-therapeutic/non-toxic levels. No cocaine was detected in blood or urine. GHB was determined in biological liquids and tissues: urine (500 mg/l), peripheral blood (180 mg/l), cardiac blood (180 mg/l), vitreous (140 mg/l), bile (170 mg/l), lungs (190 mg/kg), liver (92 mg/kg), kidneys (190 mg/kg), heart (120 mg/kg), brain (100 mg/kg), spleen (130 mg/kg) and gastric content (630 mg/l). No ethanol or volatiles were detected in blood. Screening hair analysis revealed the presence of: cocaine and metabolites, amphetamine, methamphetamine, MDMA, MDA, ketamine, sildenafil, alprazolam, GHB. GHB quantitation in hair: segment 1 (first centimeter; 240 ng/mg) and following segment 2 (cm 2 to 4; 18 ng/mg).

The detected GHB concentrations were above the normally accepted post-mortem cut-off values for endogenous GHB: 30 mg/l for urine/femoral/peripheral blood and 50 mg/l for cardiac blood/vitreous. A clear endogenous cut-off level for postmortem hair GHB is not defined yet but endogenous values of 4.4 ng/mg were reported in the literature.

During autopsy we found massive pulmonary edema and presence of foreign material in the upper airways (of gastric origin), in absence of any traumatic injury.

**Conclusion/Discussion:** In the presented case, the risk of death was likely risen by the simultaneous presence, in the body of: GHB, methamphetamine, alprazolam, anabolic steroids and antiretroviral drugs. The death was likely due to respiratory depression.

**Keywords:** Chemsex, HIV, GHB, Anabolic Steroids, Alprazolam, Methamphetamine Postmortem, Hair Tissues, Case Report

## Retrospective Analysis of Fentanyl Concentrations in 80 Post Mortem Overdoses

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**Background/Introduction:** The Nassau County Medical Examiner's office saw an increase in fentanyl overdoses in 2015, then tripling in 2016. The large majority of these 80 post mortem cases were involving individuals with a history of drug abuse. Due to this, many cases involve mixed drug intoxication, primarily with heroin, and cocaine.

**Objective:** The objective of this research was to analyze and compare data from 80 fentanyl overdoses spanning 2015-16. The compared data groups were cases consisting solely of fentanyl vs. cases where fentanyl was detected along with 6MAM, morphine, cocaine and benzoyllecgonine.

**Method:** A retrospective review of all cases in which fentanyl was detected from 2015 and 2016 was conducted. This consisted of a review of all post mortem case data including investigation notes, medical history, demographics, and toxicology results. Cases were screened using GC/MS and LC/MSMS with quantitation being performed by GC/MS.

**Result:** A total of 80 postmortem overdose cases were determined to be positive for fentanyl over a two-year period spanning 2015 and 2016. Only 2 of these were determined suicide by the pathologist due to inappropriate use of transdermal patches. The other 78 having a cause of death recorded as accidental overdose.

The average age of the accidental overdose victim was 36, with a range of 18-62. White males made up 67.5% of the data group followed by 17.5% white female and 10% Hispanic male. 13.7% (n=11) of the 80 fentanyl overdoses were only positive for fentanyl while 86.3% (n=69) were positive for fentanyl along with additional compounds. The mean blood concentration for fentanyl were calculated when independent of any other compounds vs. when present in conjunction with other substances.

Fentanyl only (n=11)	Total Mixed Drug Intoxication (n=69)	Fentanyl + Heroin (n=24)	Fentanyl + Cocaine (n=14)	Fentanyl + Heroin + Cocaine (n=9)
13.3 ng/mL	20.3 ng/mL	22.8 ng/mL	12.8 ng/mL	26.5 ng/mL

**Conclusion/Discussion:** As indicated by the data, care must be taken when interpreting fentanyl concentrations as pertaining to overdose due to a large portion of instances involving mixed drug intoxications. The measured concentration of fentanyl in cases where heroin was also detected was determined to be almost double the concentration found in cases only containing fentanyl. As a note, post mortem redistribution of fentanyl was observed with heart blood levels being consistently 1.4x the value of femoral blood, which was consistent with previous studies (Anderson and Muto, 2000).

**Keywords:** Fentanyl, Heroin, Cocaine

### Postmortem Analysis of Duloxetine (Cymbalta) in North Carolina

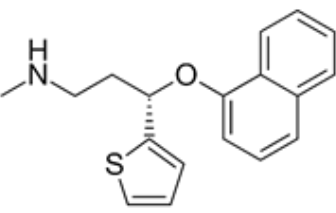
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**Background/Introduction:** In 2004 the FDA approved duloxetine (Cymbalta) in the United States for the treatment of depression. Since, duloxetine has been approved for diabetic neuropathic pain, generalized anxiety disorder, major depressive disorder, fibromyalgia, and chronic musculoskeletal pain. In addition to these indications, physicians can prescribe duloxetine for “off-label” use. While the primary anti-depressant mechanism of action is as a serotonin-norepinephrine reuptake inhibitor (SNRI), duloxetine also displays central analgesic properties via serotonergic and noradrenergic pathways. Due to its wide scope of indications and dual mechanism of action, duloxetine is often encountered in post-mortem cases.

**Objective:** The purpose of this study was to evaluate the magnitude and role of duloxetine in North Carolina Office of the Chief Medical cases, as well as determine key characteristics such as postmortem redistribution.

**Method:** Duloxetine is identified by a routine organic bases screen employing a liquid-liquid extraction and detection by GC-MS. Confirmation and quantitation are achieved by a validated LC-MS/MS method in blood and liver using 0.1 mL (g) of specimen and duloxetine-D<sub>3</sub> as internal standard. Positive electrospray ionization on a Thermo TSQ triple quadrupole LC-MS/MS monitors two transitions each for duloxetine (298 → 154, 45) and duloxetine-D<sub>3</sub> (301 → 157, 48), with identification criteria based upon retention time and ion ratios. A whole blood linear calibration curve of 0.04 – 10 mg/L, as well as matrix matched controls is included with each batch of specimens.

**Result:** From 2014 through 2016, 119 cases involving duloxetine were identified and confirmed by the laboratory. Data is summarized in the table below. Also to be presented are supporting analytical information, case studies, and postmortem interpretation.

	Duloxetine					
		Blood (mg/L)		Liver (mg/kg)	C/P	L/P
		Central	Peripheral			
Mean	0.51	0.49	9.0	1.63	18.9	
Range	0.01 - 2.4	0.05 - 2.8	0.66 - 63	0.01 - 7.4	2.5 - 67	
n =	76	118	116	75	115	

**Conclusion/Discussion:** Duloxetine is not infrequent in post-mortem cases, and is detected in 1.4% of our postmortem cases. Based upon central to peripheral blood (C/P) and liver to peripheral blood (L/P) ratios, duloxetine is expected to exhibit postmortem redistribution. As with SSRIs, triptans, and other drugs, duloxetine can contribute to serotonin syndrome. Duloxetine’s postmortem behavior and pharmacology must be carefully considered when evaluating its role in drug related deaths.

**Keywords:** Duloxetine, Cymbalta, Postmortem, LC-MS/MS



## Vitreous Humor Chemistry Analyses: An Important Tool in Postmortem Case Evaluations

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**Background/Introduction:** Vitreous humor is a routine sample in postmortem evidence collection. It is a clear, acellular fluid in the eye isolated from bacterial contamination and biochemical changes for up to 120 h after death. This makes it an excellent sample, not only for alcohol and drug analysis, but also for analysis of electrolytes (sodium, potassium, and chloride), glucose, creatinine, and urea nitrogen to assist the pathologist in determining the cause and manner of death. Despite widespread use, expected values associated with particular pathologies are scarce.

**Objective:** To characterize postmortem vitreous chemistry results in cases involving diabetes, renal failure, dehydration, water intoxication, sudden infant death syndrome (SIDS), and acute ethanol intoxication.

**Method:** Vitreous samples, collected at autopsy are received by the laboratory and aliquoted in 0.5 – 1.0 mL capped test tubes and refrigerated until scheduled testing (within 7 days). On the day of analysis, samples are sonicated and analyzed by ion specific electrodes for electrolytes, enzymatic photometry for glucose and urea nitrogen, and enzymatic/colorimetric spectrophotometry for creatinine using a Carolina Biolis 24i automatic tabletop chemistry analyzer. Vitreous chemistry results, and cause and manner of death were extracted from 2013-2016 previous analyzed postmortem cases. Cause of death was searched using the key words: *diabetes, renal failure, dehydration, water intoxication, sudden infant death syndrome (SIDS), acute ethanol intoxication, and ethylene glycol*. Vitreous results in the various subgroups were analyzed for mean, standard deviation, and maximum values and compared to the normal vitreous chemistry patterns described by Rose and Collins, "Vitreous Postmortem Chemical Analysis", CAP NewsPath.

**Result:** 5468 cases from 2013-2016.

Analyte (units)	# of cases	Sodium (mEq/L)	Potassium (mEq/L)	Chloride (mEq/L)	Glucose (mg/dL)	Urea Nitrogen (mg/dL)	Creatinine (mg/dL)	Drug concentration range
Normal Values**		135 - 150	< 15	105 - 135	< 200	8 - 20	0.6 - 1.3	
Diabetes	538	138 ± 21 (177)	12.9 ± 11.6 (49.2)	104 ± 18 (182)	15 ± 379 (1326)	43 ± 80 (288)	1.6 ± 4.0 (18.6)	
Renal Failure	84	140 ± 17 (160)	13.0 ± 10.4 (36.4)	106 ± 16 (138)	77 ± 246 (552)	80 ± 97 (252)	4.7 ± 6.3 (20.1)	
Dehydration	35	156 ± 32 (206)	14.0 ± 10.2 (36.4)	120 ± 27 (143)	159 ± 518 (958)	98 ± 128 (276)	2.1 ± 3.7 (8.3)	
Water Intoxication	1	120	4.7	72	36	35	0.8	
SIDS	79	139 ± 11 (155)	15.4 ± 6.3 (24.8)	108 ± 9 (130)	45 ± 57 (138)	11 ± 7 (26)	0.2 ± 0.1 (0.5)	
Acute Ethanol Intoxication	82	139 ± 19 (153)	13.7 ± 9.9 (29.6)	106 ± 13 (114)	41 ± 104 (248)	14 ± 17 (43)	0.5 ± 0.5 (1.8)	0.137 - 0.626 g/100 mL
Ethylene Glycol	5	144 ± 16 (159)	16.0 ± 8.8 (18.5)	110 ± 12 (120)	135 ± 291 (352)	22 ± 14 (31)	1.1 ± 0.5 (1.4)	<55.5 – >1113 mg/L

\*Results are displayed as mean ± 2SD, (max observed)

\*\*Rose, K.L., Collins, K.A. **Vitreous postmortem chemical analysis**. NewsPath. College of American Pathologists,- June2015([http://www.cap.org/apps/docs/newspath/0812/vitreous\\_postmortem\\_chemical\\_analysis.pdf](http://www.cap.org/apps/docs/newspath/0812/vitreous_postmortem_chemical_analysis.pdf))

**Conclusion/Discussion:** Renal failure (elevated creatinine and urea nitrogen) and water intoxication (sodium <135, chloride <105, potassium <15) cases showed predictable vitreous chemistry patterns. Diabetes cases showed an elevated glucose with wide variability, possibly complicated by glucose instability in unpreserved collection vials. Glucose is stable stored with preservatives such as sodium fluoride, but preservative would render the sample useless for sodium testing. Diabetes cases also show elevated creatinine and urea nitrogen as diabetes is a systemic disease affecting kidney function. Dehydration cases show elevated sodium, urea nitrogen, and creatinine supporting the expected pattern; however, the chloride average was in the normal range. Results in the SIDS cases were normal except for low creatinine levels. In acute ethanol intoxication cases, no significant pattern was detected. The ethylene glycol

cases showed a slight increase in urea and creatinine averages reflecting altered kidney function. We conclude there is justification in establishing in-house expected ranges for postmortem vitreous chemistries in particular diseases. Factors such as decomposition and methodology should be considered in further studies.

**Keywords:** Vitreous Chemistries, Electrolytes

**Study on the Postmortem Distribution of Lidocaine and its Metabolite MEGX and GX in Anesthesia Accident Death Dogs**

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**Background/Introduction:** The anesthesia accident death of Lidocaine is currently taking place in China. The postmortem distribution of lidocaine in anesthesia accident death dogs were studied. This study was supported by the NSFC (81172906), National Key Technology R & D Program of China (2007BAK26B05), and National Science and Technology special project work (SQ-2015FYJ010051).

**Objective:** To observe postmortem distribution of lidocaine and its metabolite MEGX and GX in different tissues and body fluids of anesthesia accident death dogs.

**Method:** 18 male dogs were randomly divided into A, B and C groups. The dogs were given lidocaine hydrochloride respectively with a dose of 10.665mg/kg through subarachnoid administration or epidural and femoral vein. The cardiac blood, peripheral blood, liver, spinal cord, spleen, kidney, bile, brain of the dog cadavers were collected to detect the content of lidocaine, MEGX and GX by the high performance liquid chromatography Mass Spectrometer(LC-LIT-MS ) with a linear range of 0.01-160ng/g(ng/mL) , 2-160ng/g(ng/mL) or 0.04-160ng/g(ng/mL) for lidocaine, MEGX or GX respectively, and a LOQ of 0.004ng/g(ng/mL), 1ng/g(ng/mL) or 0.01ng/g(ng/mL) for lidocaine, MEGX or GX.

**Result:** The highest content of MEGX and GX respectively was detected in the lung(3638.0±1799 ng/g and 428.9±304 ng/g and 227.2±210 ng/g) and kidney (944.97±884 ng/g and ) after dog death by subarachnoid administration, the least content of MEGX and GX respectively was in the bile; the highest content of MEGX and GX respectively was in the lung (3938.67±47.5 ng/g and 1403.93±228.26 ng/g) after dogs death by epidural administration, the least content of MEGX and GX respectively was in the bile (80.48± 64.25ng/mL and 50.32±0.7ng/mL); The highest content of MEGX and GX respectively was in the spleen (5419.9±1692ng/g )and kidney (2301.5±117ng/g) after dogs death by femoral vein administration, the least content of MEGX and GX was in the bile(372.2±58.ng/mL and 92.5±17ng/mL).

**Conclusion/Discussion:** Lidocaine and its metabolites MEGX, GX could be detected in anesthesia accident death dogs by different ways. The postmortem distribution of MEGX or GX was similar between subarachnoid and epidural injection death dogs, but different between subarachnoid and intravenous or epidural and intravenous injections; The lung could be as the first choice for examination when MEGX and GX need to be detected in the forensic identification of lidocaine poisoning death cases.

**Keywords:** Lidocaine, MEGX, GX, Postmortem Distribution

**Study on the Decomposition Kinetics of Carbofuran in Preserved Blood and Liver of Carbofuran Exposed Dogs**

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**Background/Introduction:** In our first study reported at TIAFT in (year), we discussed that the decomposition of carbofuran in sodium citrate preserved blood occurred faster than in blood containing no anticoagulant/preservative. Benzofuranol is the metabolites and decomposers. In this study, we further research the decomposition kinetics of carbofuran and benzofuranol in preservative containing blood. This research was supported by funds of China (2007BAK26B05, 2012BAK02B02) and projects of Shanxi Province (20130313020-2, 2014-032).

**Objective:** To observe the decomposition kinetics of carbofuran in the preserved blood and liver of dogs exposed to carbofuran.

**Method:** Three dogs were given an intragastric administration of carbofuran with a dose of  $4LD_{50}$ . Blood and liver specimens were sampled and divided into four portions and separately stored at 20°, 4°, -20°, 1% sodium fluoride, at 20° (blood), or 4% formaldehyde at 20° (liver). Carbofuran and benzofuranol were detected qualitatively and quantitatively by GC-MS/MS with a linear range of 0.1-20 µg/g (µg/mL) and a LOQ of 0.010 µg/mL for carbofuran and 0.010ug/ml for Benzofuranol at time points - 0h, 1<sup>st</sup>d, 5<sup>th</sup>d, 20<sup>th</sup>d, and 30<sup>th</sup>d.

**Result:** After storage for 30 d, no carbofuran and benzofuranol was detected in all dog blood specimens stored at 20°C, 4°C, -20°C and 20 °C (1% NaF). The carbofuran concentration detected in preserved blood and liver at -20° decreased slower than the blood concentrations of samples stored at 20° and 4° (P<0.05). The concentration of carbofuran detected in blood at 20° and 20° (1%NaF) decreased to 85% and 81% of initial concentration after 1 day storage. On the 4<sup>th</sup> d, it was 79% and 75% of initial concentration. The concentration of carbofuran detected in liver at 20° and 20°( 4% formaldehyde) on the 2<sup>nd</sup> decreased to 42% and 50% of initial concentration. On the 5<sup>th</sup> d, it was 0% and 5% of initial concentration. Benzofuranol could be detected at 0h in preserved cadaver blood and the blood concentrations showed a time-dependent rise trend. After storage for 30 d, no carbofuran was detected in liver stored at 20°C, 4°C and 20°C (4% formaldehyde), while carbofuran was detected in liver stored at -20°C. Benzofuranol could be detected in all preserved cadaver liver samples.

**Conclusion/Discussion:** Decomposition of carbofuran and its metabolite occurred faster in the sodium citrate preserved blood than it occurred in specimens containing heparin or no preservative. . The observed decomposition kinetics of carbofuran in preserved blood and liver of dogs exposed to carbofuran can be useful in assisting interpretation of postmortem carbofuran blood concentrations.

**Keywords:** Carbofuran, Decomposition Kinetics, GC/MS/MS

**Comparison of Serum and Whole Blood Concentrations in Acute Poisoning from Prescribed Drugs**

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**Background/Introduction:** In a forensic autopsy, toxicological analysis is typically performed using whole blood, as drug concentrations in femoral venous blood are considered to be unaffected by postmortem change. The results are compared with reference data and a diagnosis is reached. However, these reference data mostly involve clinical serum or plasma concentrations of compounds. It is not conducive to use data on clinical concentrations for comparison because significant changes in blood drug concentrations may occur after death. Routine analyses of whole blood are rarely performed in clinical toxicology, even though whole blood is the primary sample in postmortem cases. Unfortunately, only a few basic data have been found for comparison of serum drug concentrations to whole blood drug concentrations. Therefore, we have compiled serum and whole blood concentrations for each compound in acute toxicological cases.

**Objective:** This study aimed to compare serum and whole blood concentrations of blood-poisoning compounds in acute poisoning cases.

**Method:** Authentic whole blood and respective serum samples were routinely collected from patients at the Department of Emergency and Critical Care in our University Hospital, who were diagnosed with blood poisoning our university hospital, at Department of Emergency and Critical Care. Serum samples were stored in freezer (-30°C). Whole blood was collected as EDTA blood samples. As for a case that some compound was detected in serum, whole blood was performed same screening. 48 cases serum and whole blood paired samples were screened for pharmaceutical drugs using LC-MSMS. Subsequently, blood was continuously collected from patients with unexpectedly high concentrations whether ratio of serum and blood concentration change occur in accordance with decreased blood concentrations. Samples were analyzed within 2 days of arrival to the laboratory.

**Result:** Drugs detected most frequently in routine blood screening were benzodiazepines, tricyclic antidepressants, and antipsychotics. The result were characterized as follows: (1) concentrations of many compounds were less than two times higher in serum than whole blood, and the ratio remained constant regardless of concentration; (2) the ratio was >1 regardless of concentration; (3) the serum and whole blood concentration ratio of quetiapine was reversed with a decrease in concentration.

**Conclusion/Discussion:** Most cases of intoxication resulted in suicide attempt. We consider these results useful in the diagnosis in suspected fatal poisonings. Further studies should focus on patients with consistent drug concentrations in whole blood and serum concentrations, including combination effects from polysubstance use compared with those of a single drug.

**Keywords:** Acute Poisoning, Therapeutic Drug Concentrations, Serum/Whole Blood Ratios

**Mass Spectrometric Analysis of Drugs Consumed in Drug Consumption Rooms in the City of Frankfurt**

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**Background/Introduction:** The first drug consumption room in Frankfurt am Main was established in 1995 in an attempt to deal with the precarious situation in Germany's largest open drug scene near Frankfurt central station with about 200 deaths in public spaces at that time. These rooms intend to help relocate drug consumption from public areas to a controlled, hygienic and safe environment. These rooms are also seen as an important element to minimize drug-related health problems (e.g. infection risk) and promote contact of drug users with employees of drug help programs. Since 2000, the 3<sup>rd</sup> Amendment of the German Narcotics Law serves as a legal foundation for drug consumption rooms, legalizing already existing institutions and enabling the start of new drug help projects.

**Objective:** The German Narcotics Law explicitly prohibits the analysis of drugs from users ("Drug Checking"). However, the responsible authorities agreed on analysis of the consumed drugs and a scientific evaluation. The main objective of the project is to gather information on the type and quality of the drugs used by these clients with a special focus on the prevalence of New Psychoactive Substances (NPS) in street drugs.

**Methods:** Drug packing materials and used syringe filters were collected anonymously by employees of the consumption rooms and sent to our lab for analysis. In addition, information on the consumed drug if stated by the user was noted. Materials were analyzed qualitatively after solvation with methanol. If weighable amounts of powder could be found in the packings, a semi-quantitative analysis was performed. LC-MS<sup>n</sup> screening was accomplished using Bruker's Toxtyper 2.0. The automatic data evaluation was also used for quantitative evaluation of detected compounds using the peak area of the respective molecular ion in MS<sup>1</sup>, the peak area of an assigned internal standard, and the data of a previously analyzed calibrator (one-point-calibration).

**Results:** The results of drug samples analyzed up to now from this pilot study show, that heroin and cocaine are still the drugs predominantly used in this group of heavy drug users. Caffeine, phenacetin, lidocaine, and levamisole were the main cutting agents found in the filters and drug packages. In some of the materials stated as heroin by the users, cocaine was found in addition to heroin and opium alkaloids.

**Conclusion/Discussions:** The used LC-MS<sup>n</sup> approach allows automated identification and semi-quantitative determination of the active ingredients and cutting agents of drug preparations with active ingredient contents detectable down to 1% by weight. If lower levels are expected and quantification is of interest, the dilution step during sample preparation can easily be adjusted to match the linear calibration range of the calibration. LODs are typically in the range of 0.05 % or lower which is of particular interest for detecting highly potent opioids like fentanyl derivatives potentially added to heroin preparations.

**Keywords:** LC-MS<sup>n</sup>, Harm Reduction, Drug Consumption Rooms

**Development of a Method to Distinguish Amphetamine and Methamphetamine from the Other Amphetamine-like Amines by LC/MS**

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**Background/Introduction:** In recent years,  $\beta$ -methylphenylethylamine (BMPEA) and phenpromethamine are reported to be detected in some kinds of sporting supplements made in the USA. BMPEA and phenpromethamine are isomers of amphetamine (AP) and methamphetamine (MA), respectively. They are classified as stimulants by the World Anti-Doping Agency, although they are not classified as controlled substances. It is also reported that using electrospray ionization tandem mass spectrometry techniques combined with liquid chromatography (LC) systems, BMPEA and phenpromethamine produce almost identical spectra to AP and MA, respectively. Various AP and MA isomers or analogs have the potential to be abused or marketed. It is important for forensic chemists to distinguish these amphetamine-like amines from AP and MA to avoid misidentification. 17 amphetamine-like amines were selected by their structural features and attempted to distinguish them from AP and MA by LC/MS. Three brands of LC/MS instruments were used and compared.

**Objective:** The aim of this study was to establish a method to distinguish AP and MA from 17 amphetamine-like amines by LC/MS. Parameters of the mass spectrometer were optimized. Additionally, two brands of sporting supplements made in the USA were analyzed to detect amphetamine-like amines.

**Methods:** 19 amines including AP, MA, BMPEA and phenpromethamine were analyzed with three brands of LC/MS instruments. Authentic amines were dissolved or diluted with methanol to concentration of 10  $\mu\text{g/mL}$  (powder) or 10-100  $\text{nL/mL}$  (liquid). MS/MS product ion spectra were acquired with LC-Q-TOF-MS (Inst. A) and source-induced dissociation (SID) spectra were acquired with both LC-Q-TOF-MS (Inst. B) and LC-TOF-MS (Inst. C). The conditions of the mass spectrometer were evaluated by the difference of spectra by varying the orifice voltage (i.e. fragmentor voltage, cone voltage etc.) or collision energy (CE). The mobile phase consisted of mixture of component A: 5 mM ammonium formate, adjusted to pH 3 using formic acid and component B: 0.1 % formic acid in acetonitrile (95:5). An isocratic elution was employed. Two brands of supplement (PHENADRINE and VANISH) were purchased over the internet and analyzed with Inst. A.

**Results:** Adopted LC conditions separated the 19 amines well. 13 of the 17 amphetamine-like amines were distinguishable from AP and MA by SID spectra acquired using Inst. B and C. Four amines, 1-phenylpropan-1-amine, 3-phenylpropan-1-amine, BMPEA and phenpromethamine were not distinguishable. Two AP isomers, 1-phenylpropan-1-amine and 3-phenylpropan-1-amine were distinguished from AP by MS/MS product ion spectra acquired using various CE with Inst. A. None of the three instruments could distinguish BMPEA and phenpromethamine from AP and MA. Two supplements were analyzed and four amphetamine-like amines, *N*-methyl-2-phenylethan-1-amine, phenpromethamine, *N,N*-dimethyl-2-phenylethan-1-amine and phenethylamine were detected from one of them (PHENADRINE).

**Conclusion/Discussions:** SID spectra and MS/MS product ion spectra obtained with three instruments showed almost same set of ions and their base peaks were identical. This study shows that fragment information obtained by SID with single quadrupole mass spectrometer is as useful as MS/MS product ion spectra with tandem MS. However, with some amphetamine-like amines, various CE of tandem mass spectrometer was necessary to distinguish them from AP.

**Keywords:** Source-induced Dissociation (SID), Sporting Supplements, Amphetamines

**Multidisciplinary Strategy for NPS Detection in Seized Material and Study of 5-MAPB and bk-2C-B Activities on hCAs**

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**Background/Introduction:** New psychoactive substances (NPS) include a large variety of molecules bearing a multitude of chemical moieties. They are usually designed or conceived on known chemical scaffolds of either “classical” drugs of abuse or agents usually used for pharmaceutical purposes.

**Objective:** In consideration of the large amounts and raising variety of chemical structures currently present within the illegal market, it is stringent the need from all both Law Enforcement Agencies and Forensic Laboratories to potentiate their available tools in order to speed both the detection and identification of unknown substances.

NPS exert their activities by means of interaction with a large variety of biological receptors. In this context the Carbonic Anhydrases (CAs; EC 4.2.1.1) represent a high valuable target since they reversibly catalyze the hydration of carbon dioxide to afford bicarbonate and proton. This reaction is fundamental in human physio/pathology and is also deeply involved in modulation of the central-nervous-system (CNS) activities by means of interference with various biochemical pathways. The in vitro screening of NPS on CAs indeed represents a valid contribution in deeply understanding the biochemical mechanisms underpinning the physiological responses generated.

**Methods:** Herein we report a multidisciplinary approach comprising liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS), gas chromatography hyphenated to mass spectrometry (GC-MS) and solution nuclear magnetic resonance (NMR) techniques applied to seized materials. The samples above described were identified as 1-(benzofuran-5-yl)-*N*-methylpropan-2-amine (5-MAPB) as the major constituent in the white powder, 2-amino-1-(4-bromo-2,5-dimethoxyphenyl)ethan-1-one (bk-2C-B) as the major constituent in the yellow powder, whereas the grey-red powder contained the above substances in mixture with 3-(2-aminopropyl) indole ( $\alpha$ -methyltryptamine, AMT).

5-MAPB and bk-2C-B were tested in vitro on the most abundant isoforms among the 15 reported in humans (i.e. I and II) by means of the carbon dioxide hydration assay conducted with a stopped-flow technique.

**Results:** By means of the multidisciplinary approach here reported we identified NPS from seized samples without the use of commercially available standards. The kinetic data obtained as triplicate, showed that 5-MAPB and bk-2C-B were both activators of the hCA I isoform with  $K_A$ s of 29.7 and 4.0 fold stronger when compared to the reference compound histamine (HST), whereas they showed weak inhibitory properties when compared to the standard CA inhibitor acetazolamide (AZM). Such a remarkable differences in kinetic activities on the CA isoforms clearly resulted from different allocation of the ligands within the enzymatic clefts. For the CA I it is reasonable to assume that 5-MAPB and bk-2C-B, in analogy to the standard CA activator HST, are able to establish multiple hydrogen bond connections, thus allowing the protons produced from the CO<sub>2</sub> hydration reaction to be easily extruded out of the enzyme. This also represents the rate-limiting step in catalysis of this reaction. On the contrary the weak inhibition data for the CA II isoform don't allow any particular consideration at this step.

**Conclusion/Discussions:** The multidisciplinary approach here reported proved highly efficient, reliable and fast in identifying NPS without the use of commercially available standards. The in vitro kinetic investigation of 5-MAPB and bk-2C-B on the most abundant and physiological relevant CAs revealed a remarkable enhancement of activity for the isoform I and very weak inhibition for the isoform II. Since all CA isoforms vary for kinetic properties, cellular and tissue localization, such preliminary results clearly indicate that NPS modulate CAs activities with various intensities and divergent directions. Further exploration of NPS on all kinetically active CAs will contribute to better understand their role on NPS-triggered biological mechanism at the CNS level.

**Keywords:** New Psychoactive Substances (NPS), Seized Material, Carbonic Anhydrases (hCAs)



## Fatal Strychnine Poisoning

Christina Fields-Zinna\*, Amanda Cooke, Georgia Bureau of Investigation

**Background/Introduction:** Strychnine is a potent central nervous system (CNS) stimulant and convulsant, typically causing death by asphyxia in between clonic or tonic convulsions. Though very rare, poisonings occur from ingestion of rodenticides, as strychnine has no common therapeutic applications. Its use is banned in various parts of the world, and restricted in many Western countries.

**Objective:** A postmortem case involving a fatal ingestion of strychnine is presented. Decedent is a 59-year-old white male found by a relative after expressing suicidal intentions. He appeared to be experiencing a seizure and was unresponsive. Resuscitation attempts failed and he was pronounced dead. Cause of death for decedent is determined to be acute strychnine poisoning per toxicological testing of blood and liver.

**Methods:** Quantitative analysis employs a precipitation procedure and liquid chromatography-tandem mass spectrometry LC/MS/MS. Specifically, 200  $\mu$ L of specimen is treated with 2.5 mL of acetone, then the supernatant is transferred and dried. The residue is reconstituted with 1 mL of a methanol/formate buffer (50:50) solution. The sample was injected on an Applied Biosystems QTRAP with Electrospray ionization in positive mode.

**Results:** Strychnine levels in the blood and liver is 26 mg/L and 120 mg/kg, respectively. Diphenhydramine is also present in the blood at less than 0.125 mg/L and in the liver at 1.1 mg/kg.

**Conclusion/Discussions:** Investigational reports indicate this as successful suicide attempt, and the medical examiner's report designates cause of death as acute strychnine poisoning as levels are consistent with lethal levels. Even though no strychnine was found on the property, the decedent did own a large farm with reasonable access to rat poison. This is the second case of strychnine poisoning in a 14 year span as determined by the Georgia Bureau of Investigation Division of Forensic Sciences.

**Keywords:** Strychnine, Poisoning, LC/MS/MS

**Development of an In-Vitro Drug Delivery Efficiency Test for a Pulmonary Toxicokinetic Study in Pigs**

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**Background/Introduction:** Gaining profound knowledge of the toxicological profile of synthetic cannabinoids (SC) requires the determination of their toxicokinetic (TK) and toxicodynamic properties. As data from controlled human studies are usually not available, animal models suitable for cannabinoid TK studies have to be developed. In a former pilot study, a pig model has been proven to be appropriate for the elucidation of the TK properties of the two SC 4-ethylnaphthalen-1-yl-(1-pentylindol-3-yl)methanone (JWH-210) and 2-(4-methoxyphenyl)-1-(1-pentyl-indol-3-yl)methanone (RCS-4) as well as  $\Delta^9$ -tetrahydrocannabinol (THC) after intravenous administration. However, since SCs are preferably smoked, a model reflecting authentic user habits should be established.

**Objective:** Performing a pulmonary TK pig study using the above-mentioned cannabinoids required the development of an in-vitro efficiency test that allows for reliable determination of the quantity and reproducibility of which the drug dose is intratracheally delivered to ventilated pigs by nebulization.

**Methods:** JWH-210, RCS-4, and THC (dose 1 mg in 2 mL ethanol each) were nebulized (for 12 min) under ventilation using the M-neb flow+ ventilation ultrasonic nebulizer MN-300/7 (Nebutech, Elsenfeld, Germany) operated in the inspiration-triggered mode (< 0.2 mL/min). The aerosol was delivered via the inspiratory limb and the tracheal tube through a glass fiber filter (1.6  $\mu$ M pore size, Sartorius AG, Goettingen, Germany). Ventilation was identical to the set-up of future TK studies: mixture of oxygen and air (1:2 vol/vol; FiO<sub>2</sub> of 0.30; Respirator ABV-U; F. Stephan, Gackebach, Germany), 500 mL tidal volume, 2 % isoflurane. The experiments were repeated six times. Afterwards, the drugs were extracted from the glass fibre filters using 10 mL ethanol and ultrasonication for 10 min. The supernatants were then diluted twice (1:20 with ethanol, 1:10 with 0.1% aqueous formic acid). After adding 100  $\mu$ L of internal standard solution (5  $\mu$ g/mL JWH-210-d9, RCS-4-d9, THC-d3), the extracts were analyzed by LC-MS/MS. For the assessment of the extraction efficiency, a reference solution of JWH-210, RCS-4, and THC (same concentrations) was added to glass fibre filters (n = 4) and the drugs were extracted applying the same procedure. The ratios (peak area of drug target ion/peak area of internal standard target ion) were compared to the corresponding ratios of an analyzed reference solution (same concentrations). For the assessment of the delivery efficiency, the respective ratios of the extracted nebulized drugs were compared to those of the extracted spiked drugs.

**Results:** Spiking the glass fiber filters with the reference drug solution revealed extraction efficiencies of  $101.5 \pm 2.2$  % for JWH-210,  $100 \pm 1.3$  for RCS-4, and  $101.8 \pm 1.9$  for THC. In comparison, nebulization of the drugs through the tracheal tube and the glass fiber filter yielded delivery efficiencies of  $78.8 \pm 5.0$  % for JWH-210,  $70.5 \pm 6.9$  % for RCS-4, and  $70.8 \pm 7.9$  % for THC.

**Conclusion/Discussions:** As prerequisite of a TK study in pigs with pulmonary administration of JWH-210, RCS-4, and THC, an in-vitro test system had to be developed in order to determine the delivery efficiency of the drug aerosol. The loss of about 20-30 % of the administered dose found in these preliminary experiments might be attributable to adhesion of the lipophilic drug aerosol particles e.g. to the tracheal tube wall. Nevertheless, the minor standard deviations indicate an acceptable reproducibility, leading to the conclusion that this administration system is suitable for application in the TK study.

**Keywords:** Drug Delivery, Cannabinoids, Pigs

**Postmortem Endogenous Ethanol Production Detected by Court-ordered Transdermal Alcohol Monitoring**

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**Background/Introduction:** A 49 year old male was pronounced deceased inside his apartment during a well-being check. In mid 2016, the subject allegedly hit and critically injured a young girl with his motorcycle and was arrested on suspicion of driving under the influence of alcohol. He was arraigned and released on condition of wearing a transdermal alcohol sensor (TAS) bracelet. A month later, a well-being check of the deceased was conducted following complaints about a foul odor emanating from the subject's apartment. Upon entry, the decedent's decomposing remains were found lying supine on a bed. Medications secured from the scene included a bottle of hydrocodone tablets prescribed seven days prior, which showed evidence of abuse based on pill count. There was no evidence of alcohol consumption. The cellular phone of the decedent showed outgoing text messages, with the last sent five days prior to discovery of the body.

**Objective:** To demonstrate if transdermal alcohol is released following putrefaction and the microbial production of artifact ethanol.

**Methods:** Interrogation of the TAS by the manufacturer to obtain transdermal alcohol concentration (TAC) values during the decedent's antemortem, perimortem and postmortem stages. In addition, conventional blood alcohol concentration (BAC) analysis by HS-GC-FID was performed on the peripheral femoral blood.

**Results:** At autopsy, significant decomposition changes were present. The TAS affixed to the ankle of the decedent continuously recorded a TAC of 0.000 mg/dL for 3 days after the last sighting of the deceased. A positive reading (0.005 mg/dL) was then recorded. The TAC subsequently increased gradually, and measured 0.009 to 0.011 mg/dL over the following 24 hours and remained stable for the next 24 hours prior to declaration of death. The Office of the Chief Medical Examiner was contacted within the hour of discovery, in which the body was being handled from this period on, registering a spike in ethanol up to 0.047 mg/dL at this time. Following transport to the morgue and placement in the refrigerator, the TAC measurement dropped to 0.019 mg/dL two hours later and to 0.000 mg/dL by 6 hours thereafter. In comparison, the BAC of the cardiac/central specimen collected at autopsy was 0.10 mg/dL.

**Conclusion/Discussions:** Endogenous ethanol production occurs in a subset of decomposing bodies due to putrefaction by microorganisms. This is the first reported case in which likely postmortem alcohol production is documented prospectively by a TAS. The transient increase in ethanol upon movement of the body suggests expedited release and exposure of the ethanol from dermal tissue by mechanical manipulation.

**Keywords:** Transdermal Alcohol Sensor, Decomposition, Ethanol

**Quantitation of Aerosolized Methamphetamine from Electronic Cigarettes by Gas Chromatography/Mass Spectrometry (GC/MS): Does Increasing the Voltage Increase the Dose?**

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**Background/Introduction:** The use of electronic cigarettes (e-cigs) has expanded from a nicotine delivery system to an illicit drug delivery system. The internet is rife with websites, blogs and forums informing users how to modify e-cigs to deliver illicit drugs while maintaining optimal drug delivery of their device. With the exception of the flavoring agents, when e-liquids are “vaped,” an odorless vapor is produced. Therefore, when e-liquids are formulated to contain illicit drugs, users perceive they can “vape” illicit drugs in public without detection.

**Objective:** The goal of this study was to assess the effect of voltage on the concentration of aerosolized methamphetamine produced by an e-cig using Gas Chromatography Mass Spectrometry (GC/MS).

**Methods:** Three e-liquid formulations were prepared containing 30, 60 and 120 mg/mL methamphetamine in 50:50 (v:v) propylene glycol (PG):vegetable glycerin (VG). The e-liquids were “vaped” using a KangerTech AeroTank, 1.8  $\Omega$  preassembled atomizer, and an eGo-V2 variable voltage battery for 4 sec at 2.3 mL/min at either 3.9, 4.3, and 4.7 V. Five replicates were analyzed for each e-liquid concentration at each voltage (n=45). The aerosolized product was captured using a water trap. A 1 mL aliquot of the water was extracted using ammonium hydroxide and 1-chlorobutane. Analysis was performed using an Agilent GC/MS 6890N/5973 Mass Selective Detector instrument with an Agilent HP-5MS column (0.25 mm x 30 m x 250  $\mu$ m). Each sample was analyzed in split mode of 6:1. The initial oven temperature was 120 °C, with a ramp to 200 °C at 10 °C/min, followed by 30 °C/min to 280°C, for a total run time of 10.67 min. The samples were initially analyzed in scanning mode from 40-550 m/z. Kruskal-Wallis test was performed in JMP Pro 12.2.0 in order to assess the statistical difference between the concentrations of aerosolized methamphetamine at each voltage. The measured concentrations of the quality controls were within the  $\pm$  20% limit of their theoretical concentrations. Interday and intraday precision was assessed by the percent coefficient of variation and was determined to be within  $\pm$  20% of the other calculated concentrations. Interday and intraday bias was assessed by percent difference and was determined to be within  $\pm$  20% of the theoretical concentrations. No carryover was observed and percent recovery ranged from 102 to 108%.

**Results:** The aerosol contained methamphetamine, PG, and VG. The concentration of aerosolized methamphetamine was determined to be  $800 \pm 600$  ng/mL,  $800 \pm 600$  ng/mL, and  $1000 \pm 800$  ng/mL at 3.9, 4.3 and 4.7 V, respectively. The Kruskal-Wallis test was used to determine that the medians at each voltage were not different (p=0.6482).

**Conclusion/Discussions:** Methamphetamine was successfully aerosolized using the KangerTech e-cig device. The characterization of the vapors produced from e-liquids containing methamphetamine provides an understanding of the dose delivery dynamics of e-cigarettes. The concentration of aerosolized methamphetamine was not statistically different when methamphetamine e-liquids were vaped at three different voltages.

**Funding:**

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**Keywords:** Methamphetamine, Electronic Cigarettes, E-liquids

**Post-Mortem-Drug-Screening in Dental Hard Tissue Samples by LC-QToF MS**

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**Background/Introduction:** When dealing with burnt, severely putrefied or skeletonized bodies, traditional sample materials for toxicological analysis (e.g., blood, tissue, urine) are often unavailable. In these cases dental hard tissue is one of the remaining materials applicable for post-mortem toxicology. Full scan based screening methods using liquid chromatography-quadrupole time-of-flight mass spectrometry (LC-QToF-MS) are a valuable tool for forensic analysis of these materials due to the possibility of combined qualitative and quantitative analysis as well as retrospective data evaluation.

**Objective:** In this study the applicability of a screening workflow – initially developed for the detection and identification of xenobiotics in human serum and urine samples<sup>[1]</sup> to dental hard tissue as an alternative matrix for post-mortem toxicology was evaluated.

**Methods:** In cases with a known history of drug intake one whole tooth was obtained during autopsy. The pulp was removed, the teeth separated into enamel, crown and root dentin, and if present carious dentin, and powdered using a diamond burr. The powdered dental materials were extracted with methanol (3 x 0.5 mL) under ultrasonication (3 x 60 min). The extracts were dried, reconstituted in eluent, and analyzed on an LC-QToF-MS-system (impact II, Bruker Daltonik, Bremen, Germany). The MS was operated in positive electrospray ionization (ESI) full scan and broadband CID (bbCID) mode, respectively. The screening was performed using a database containing 2054 drugs, drugs of abuse, metabolites, and other exogenous and endogenous compounds. The applied assay is routinely used in our lab for toxicological screening of urine, serum and hair samples. To estimate the assay's detection limits in authentic samples, all compounds detected were subsequently quantified by liquid chromatography tandem-mass spectrometry (LC-MS/MS) in multiple reaction monitoring (MRM) mode<sup>[2,3]</sup>. Additionally all compounds detected in routine post-mortem toxicology were also added to the confirmation method. For all samples complete post-mortem toxicology results (QToF and/or LC-MS/MS) were available, so the screening results could be compared to those of femoral blood, cardiac blood, urine, stomach contents, and hair to evaluate the performance of the QToF-screening.

**Results:** Drugs and drugs of abuse found in body fluids, tissue samples, and hair could mostly be detected in dental materials with the applied high resolution mass spectrometry (HRMS) assay. In dental materials, several opioids (8), benzoylecgonine, and ecgonine methyl ester, numerous benzodiazepines (7), anti-psychotic drugs, antidepressants, and – in one case – the synthetic cannabinoid MDMB-CHMICA were detected. The screenings findings and those from routine toxicology were in good agreement. The performance seems comparable to targeted MRM methods since the assay was able to detect compounds at concentrations between LOD and LOQ determined for the LC-MS/MS method used<sup>[2,3]</sup> (Morphine for example was identified by the assay at a concentration of 1.6 pg/mg). In contrast the QToF-screening also allows for retrospective data analysis.

**Conclusion/Discussions:** The analysis of dental hard tissue may represent a useful alternative matrix for post mortem toxicology, especially if there is no other material available. Based on a study conducted earlier, the incorporation of medical and illicit drugs into dental hard tissue depends on the compound's physico-chemical properties and seems to occur mainly via the blood stream. The preliminary results of this study are promising so far. However, further investigations especially regarding possible quantitation but also regarding matrix effects will be needed to completely implement the assay in routine post-mortem analysis.

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**Keywords:** Post-mortem Toxicology, LC-QToF-MS, Dental Hard Tissue

**Detection of Dimethylether in a Blood Sample of a Deceased Person after 3 Days Storage at Room Temperature**

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**Background/Introduction:** A 32 year old woman with a history of a volatile substance abuse was found dead in her bed in her apartment. Beside the body 16 emptied hair spray bottles were found. Police investigations revealed a history of several failed detoxification therapies. While the external examination was conducted, a characteristic hair spray odor was apparent.

**Objective:** Detection of volatile ingredient of hair spray in blood and examination of the detection time window of dimethylether

**Methods:** The case was subject to a full medicolegal autopsy and subsequent toxicological examination. This involved an immunoassay screen in urine and blood for opiates, methadone, buprenorphine, benzodiazepines, amphetamines, methamphetamines, cannabinoids, and cocaine metabolites. Extracts of urine, blood, and gastric content were screened by GC-MS in scan mode. Blood was additionally analysed by LC-MS/MS for more than 100 active pharmaceuticals in a multi target method and by another method for synthetic cannabinoids. Gastric content was proofed for insecticides. Additional analyses using headspace GC-FID for volatile substances in blood were performed. An aliquot of the blood sample was stored at room temperature in an open tube, another one at 4 degree centigrade in a sealed tube and a third one frozen at -20 degree centigrade. Aliquots of the first and second blood sample were taken daily for 10 days and analyzed afterwards using headspace GC-FID.

**Results:** Internal legomedical examination revealed sticky, yellow foamed fluids in the upper and lower airways. Additionally non-specific findings like general congestion, pulmonary hemorrhagic edema and brain edema were found. Toxicological analysis of blood revealed the presence of traces of doxepine, nordoxepine, nordazepam and the volatile substance dimethylether. Afterwards examination of the time window regarding the detection of dimethylether in the blood sample over days was performed where dimethylether was detectable in open stored blood samples at room temperature for even 3 days and in sealed blood samples stored at 4 degree centigrade for at least 10 days.

**Conclusion/Discussions:** Detection of the volatile substance dimethylether, a widely used gas propellant in hair spray can be possible in blood even 3 days after a sample was stored at room temperature in an open tube. At 4 degree centigrade in a sealed tube dimethylether was detectable for at least 10 days.

**Keywords:** Dimethylether, Hair Spray, Headspace

**Distribution of Furanyl Fentanyl in an Accidental Acute Death**

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**Background/Introduction:** Fatalities from synthetic opioids have continued to reach new epidemic proportions over recent years. Due to the sparsity of research in new opioid analogues and their toxicity, commonly observed lethal concentrations of these drugs have yet to be determined. However, fentanyl analogues have been detected in multiple cases of overdose-related fatalities throughout the world in recent years. The prevalence of furanyl fentanyl in postmortem casework contributes to the opioid related deaths that are amongst half of drug-induced deaths in the United States. In this case study, a 23-year-old man in San Francisco who was using blue pills labeled as oxycodone was found dead. Initial toxicology screening showed no presence of oxycodone in blood. However, a positive fentanyl immunoassay result was obtained and analysis of pills collected at the scene were undertaken.

**Objective:** To demonstrate furanyl fentanyl concentrations in acute drug toxicity casework and the distribution of the drug in postmortem specimens.

**Methods:** ELISA immunoassay was used for initial screening of therapeutic and drugs of abuse, including fentanyl. A GC/MS screen in full scan mode showed the presence of furanyl fentanyl and its precursor and metabolite, 4-anilino-N-phenethyl-piperidine (4-ANPP) in the pills. Further quantitation of fentanyl analogues by a reference laboratory was then performed.

**Results:** Analysis of postmortem samples revealed concentrations of furanyl fentanyl at 1.9 ng/mL in peripheral blood, 2.8 ng/mL in cardiac blood, and ~55,000 ng in gastric contents. The metabolite 4-ANPP was also detected at 4.3 ng/mL in peripheral blood and 5.8 ng/mL in cardiac blood. Concentrations of both analytes were found in the vitreous humour < 0.20 ng/mL. Liver 4-ANPP concentrations of > 40 ng/g were also detected however urine was negative for both analytes.

**Conclusion/Discussions:** This case study of acute furanyl fentanyl overdose in a young male thought to be using oxycodone highlights illicit drug users are often subject unknown drug entities. The toxicological analysis provides preliminary information of the distribution of furanyl fentanyl and its metabolite in a range of postmortem specimens and collection sites. Future case series and retrospective studies on this fentanyl analogue in postmortem cases is warranted to demonstrate toxic levels.

**Keywords:** Furanyl Fentanyl, Postmortem, Acute Drug Overdose

## Postmortem Cocaine and Metabolites in Several Tissues. Eight Cases Reported and Interpretation of Finding

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**Introduction/Objective:** Stability problems and the possibility of redistribution are a challenge when interpreting post mortem (PM) tissue concentrations. For cocaine (CO), stability problems are well known while the PM redistribution is believed to be a minor problem. However, the CO stability and its related compound along large periods were not sufficiently studied. We present eight cases with post mortem data for cocaine in different PM periods.

**Methods:** Eight young male who died by CO overdose were studied.

Post mortem whole blood from femoral vein, liver, brain, kidney and urine were appropriately taken and kept to -20°C. The quantification of CO and its metabolite benzoylecgonine (BE) Ecgonine methyl ester (EME) was performed using UPLC-DAD and GC-MS. IS: C-d3, BE-d3

**Results:** The table shows the results in each eight cases. Post mortem concentrations of cocaine and EME were between 1.5 -38 µg/g tissue.

Case	PM-t (days)	Liver	Kidney	Brain	Blood	Urine
1	1	----	----	CO	-	BE
2	15	CO, EME	----	CO	----	----
3	120	CO, EME	CO, EME	CO, EME	-	----
4	15	CO	CO	CO	----	----
5	32	----	----	CO	CO	CO
6	90	----	----	CO	-	----
7	18	CO, EME, CC	CO, EME, CC	CO, EME,CC	-	-
8	18	CO, CE	CO, CE	CO, CE	EME	----

PM-t: postmortem time, CO: cocaine, EME: methylecgonine, CE: coca ethylene, BE: Benzoylecgonine, CC: cinammylcocaine, - : negative --- sample no received

**Conclusion/Discussions:** Based on the review of case and autopsy report, the cause of death was assumed be related to toxicity of cocaine. However, this interpretation was complicated by presence of other compounds and that cocaine related compound have a particular toxicity such CC (cardiotoxic) and CE. Note that intact CO was found in brain tissue in all cases independently of post-mortem time. BE was no found, except in urine of case 1. EME was detected in tissue after 4 months. We conclude that the post mortem concentration of CO in brain is very important for the interpretation of forensic cases.

**Keywords:** Cocaine, Metabolites Postmortem, Tissue, Interpretation



**Determination of Biomarkers in Vitreous Humor for the Estimation of Postmortem Interval by LC/MS/MS**

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**Background/Introduction:** Many studies have been conducted to estimate the post mortem interval (PMI) for very long time, but few studies in Korea. In this study, in order to estimate the postmortem interval, firstly appropriate biomarkers were researched and the analysis of biomarkers by LC/MS/MS was established to measure the concentration of biomarkers. As biomarkers in vitreous humor, Hypoxanthine (Hx), L-lactic acid and uric acid were selected, because Hypoxanthine (Hx) which is known to be highly correlated to PM and there have been reported that L-lactic acid revealed the correlation of its concentration and time after death. In addition, the presence of uric acid can be a marker to indicate the blood contamination in vitreous humor.

**Objective:** In order to estimate the postmortem interval, the biomarkers selected in Vitreous Humor were analyzed by LC/MS/MS and their concentration was compared with time after death to calculate the PMI.

**Methods:** A vitreous humor was collected from cadaver with a known time of death at the National Forensic Service (NFS) in Korea. 16 Vitreous Humors samples (12 male and 4 female) with a mean age of 56.1 years (range, 27-71 years) were studied. Vitreous Humors were extracted by a solid-phase extraction with OASIS MAX cartridges. Agilent 1260 infinity HPLC system and Sciex 3200 Q-trap mass spectrometer were used for the quantification of Hx, uric acid and L-lactic acid in vitreous humor. Chromatographic separation was performed by using 0.1% formic acid in water and methanol as mobile phase. The multiple reaction monitoring (MRM) of ion transitions monitored was m/z 137.0 > 110.0, 119.0 for Hx, 166.9>124.1 for uric acid and 5-(p-methylphenyl)-5-phenylhydantoin 267.2>163.3 as an Internal Standard (IS). Lactic acid was separated into L-lactic acid and D-lactic acid. Multiple Reaction Monitoring (MRM) of ion transitions monitored was m/z 308.0>89.0 for L-, D-lactic acid while 308.1>92.1 L-lactate-3,3,3,-d3 as IS for lactic acid.

**Results:** Hx and uric acid as biomarkers in vitreous humor were well separated and measured by LC/MS/ MS. L-lactic acid and D-lactic acid were well isolated by (+)-O,O'-diacetyl-L-tartaric anhydride ( $\geq 97\%$ ) (DATAN) as a derivatizing reagent in LC/MS/ MS. As a result, Hx and L-lactic acid were detected in all 16 samples, whereas uric acid was detected in 14 samples indicating two specimens contaminated with blood. The concentration of Hx ranged from 100  $\mu\text{M}$  to 400  $\mu\text{M}$  in all 16 cases. The concentration of Hx was 100 mM in vitreous humor which was collected within 24h after death, 200 mM in 48 h, 300 mM in 72 h and 400 mM in 100 h. It was very interesting finding that the longer the Time after death the higher the concentration of Hx was observed in Vitreous Humor. The difference in HX concentration between gender and age was not observed.

**Conclusion/Discussions:** Hx and l-lactic acid in Vitreous humor were selected as biomarkers for PMI and well analyzed by LC/MS/ MS. The presence of uric acid was a good indicator for the blood concentration. Hx was proved to be a good marker for PMI showing the longer the Time after death the higher the concentration of Hx in Vitreous Humor. It is the first study to select the biomarkers for PMI in Korea.

**Keywords:** Time after Death, Hypoxanthine (Hx), L-lactic acid

## Evaluation of Tip-Washing Technology for Forensic Applications

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**Background/Introduction:** Disposable pipette tips are a large expense for analytical toxicology laboratories, so implementation of a pipette tip washing device could reduce the consumption of plastic tips by allowing for reuse after washing. The Grenova TipNovus Mini is designed to wash and dry 6-8 tip racks per hour using a single consumable item, a cleaning solution. The use of a tip washing system could help to cut down both laboratory costs as well as environmental waste.

**Objective:** To determine if the TipNovus Mini could be useful in a clinical or forensic toxicology laboratory to help cut down on tip waste.

**Method:** Precision and carryover of washed tips versus unwashed tips were assessed to determine if the TipNovus Mini could be used in the Oklahoma State University Forensic Toxicology and Trace Laboratory. Tips were used to process 50 microliters (50 ul) of urine that was fortified with high concentrations (10 ug/mL) of analytes that might be present in forensic and clinical specimens, including opiates (hydrocodone, hydromorphone, norhydrocodone, noroxycodone, oxycodone, oxymorphone), benzodiazepines (nordiazepam, oxazepam, temazepam) and cannabinoids (THC-COOH), and other analytes (amphetamine, gabapentin, tramadol).

Tips were used in an automated platform (Hamilton Microlab® NIMBUS) and also in a manual format. Specimens were processed with dilute and shoot methodology, followed by injection and analysis via liquid chromatography- tandem mass spectrometry (LC-MS/MS) using a Shimadzu 8040 system. Statistical analyses for each analyte, consisting of One Way ANOVA with Tukey's Multiple Comparisons Test, were performed in GraphPad Prism®.

**Result:** No significant differences ( $p < 0.05$ ) were detected in results obtained with washed tips versus unwashed tips, demonstrating similar values and precisions for both types of tips. There were no data trends indicating carryover of any of the classes of analytes.

**Conclusion/Discussion:** The TipNovus Mini demonstrated potential utility in reducing lab waste and cost, without degrading performance.

**Keywords:** Tip Washing, Validation, Urine