Abstracts Of Platform Presentations

S-01

Urinary Markers for AB-FUBINACA Intake Determined by Human Hepatocytes Incubation and High-Resolution Mass Spectrometry

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Introduction: Synthetic cannabinoids (SC) intake increased worldwide, with purported psychotropic effects documented in drug user blogs, poison control call centers, driving under the influence of drugs, and emergency room visits. Although multiple SC classes are now DEA controlled, new cannabimimetic compounds continue to emerge. It is critically important to identify urinary SC targets to document SC intake, to link adverse effects with specific SC, and to educate the public on the dangers of designer drug use. Recently, our laboratory conducted metabolite-profiling studies of SC with human hepatocytes incubation, high resolution mass spectrometry (HRMS), and data-mining software. AB-FUBINACA, *N*-(1-amino-3-methyl-1-oxabutan-2-yl)-1-(4-fluorobenzyl)-1*H*-indazole-3-carboxamide, is a scheduled indazole derivative SC originally identified in Japanese seized herbal products with unknown human pharmacology (CB_1 Ki = 0.9 nM).

Objective: Our goal was to characterize AB-FUBINACA's metabolism with human hepatocytes incubation and HRMS to identify the best urinary targets.

Method: We incubated 10 µM AB-FUBINACA in pooled cryopreserved human hepatocytes at 37°C for 0 (control), 1, and 3 h. Reactions were quenched with cold acetonitrile. Samples were centrifuged at 15,000 g at 4°C and supernatant was stored at -80°C for further analysis. Samples were analyzed on the AB Sciex TripleTOF® 5600+ HRMS with a TOF survey scan followed by information-dependent acquisition product ion scan using ion intensity of 500 cps and mass defect filtering (MDF) as criteria, as well as dynamic background subtraction. Data were analyzed with AB Sciex MetabolitePilot™ software utilizing a number of processing algorithms including generic peak finding, MDF, neutral loss, and product ion filtering. Potential metabolites were manually evaluated and assigned a structure, if possible.

Results: Almost 80% of parent analyte was unchanged after 1 h, 45% after the 3 h incubation. Two and 7 metabolites were identified after 1 and 3 h, respectively. Two major metabolites, identified at both time points, were the products of carboxamide hydrolysis to butanoic acid (*m/z* 369.1730) and mono-hydroxylation at the butanoic acid substructure (*m/z* 385.1667). In addition, their glucuronidated forms also were identified. Other minor metabolites included mono-hydroxylation at the indazole structure (*m/z* 385.1675), carboxamide hydrolysis to butanoic acid plus mono-hydroxylation at the butyl substructure (*m/z* 386.1515), and mono-hydroxylation at the indazole substructure and internal hydrolysis (*m/z* 403.1773). Defluorination at the benzyl ring, and sulfate conjugation were not observed.

Conclusion: We present the first *in vitro* metabolic profile of AB-FUBINACA providing urinary targets for identifying drug intake. We propose targeting metabolites formed by carboxamide hydrolysis to butanoic acid or mono-hydroxylation at the butanoic acid substructure. Given the decrease of parent compound to 45% after 3 h incubation, we also suggest further investigation of metabolite half-lives and intrinsic clearance.

Keywords: AB-FUBINACA, Synthetic Cannabinoids, HRMS, Human Hepatocytes

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S-02

The Identification of Primary Metabolites of the Designer Hallucinogen 25I-NBOMe (4-Iodo-2,5-Dimethoxy-N-(2-Methoxybenzyl)-Phenylethylamine)

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Introduction: In 2010, a novel class of synthetic hallucinogens, the N-methoxybenzyl-methoxyphenylethylamine (NBOMe) derivatives, became readily available on the internet. These derivatives were first synthesized in 2000 and are potent serotonin 2A (5-HT2A) receptor agonists. The 5-HT2A receptor has been closely linked to complex behaviors including working memory and cognitive processes. It has also been implicated in the pathophysiology of affective disorders such as depression and schizophrenia. As a result, the therapeutic effects of atypical antipsychotics are due to antagonism at 5-HT2A receptors. All serotonergic hallucinogens act as 5-HT2A agonists, including the indole derivative lysergic acid diethylamide (LSD). Currently, NBOMe derivatives are sold as powders or on blotter paper with 25I-NBOMe (4-iodo-2,5-dimethoxy-N-(2-methoxybenzyl)-phenylethylamine) being the most commonly reported. Clinical presentations of severe NBOMe intoxication include tachycardia, agitation, hypertension, aggressive/violent behavior, hallucinations and continuous agitation and seizures which can persist for as long as three days.

Objective: The identification of 25I-NBOMe metabolites may serve as useful biomarkers in clinical and forensic toxicology. If these metabolites are present in blood, urine and other of specimens in greater abundance and/or have longer half-lives than 25I-NBOMe, they may be valuable biomarkers in postmortem toxicology, driving while impaired testing and all areas of clinical and forensic urine drug testing.

Method: The 25I-NBOMe reference material was obtained from Cayman Chemical Company. 25I-NBOMe was incubated in freshly prepared mouse liver microsomes. These *in vitro* samples were used to generate phase I metabolites. This animal study was approved by the Institutional Animal Care and Use Committee of Virginia Commonwealth University in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals. The 25I-NBOMe metabolites formed by the microsomes were identified using a Waters Acquity Xevo TQD LC-MS/MS system. A human serum specimen from a case of 25I-NBOMe intoxication collected approximately 11 hours post ingestion was analyzed for the potential metabolites.

Results: Mouse microsomal phase I studies yielded two O-demethylated 25I-NBOMe metabolites and several other metabolites. Mass fragmentation of O-desmethyl-25I-NBOMe indicated that demethylation occurred at either the *ortho* or the *meta* position. Chromatographic separation of O-desmethyl-25I-NBOMe indicated the presence of two demethylated metabolites occurring at either the *ortho* or *meta* position. The major metabolite appeared to be 4-iodo-2-hydroxy,5-methoxy-N-(2-methoxybenzyl)-phenylethylamine. O-desmethyl-25I-NBOMe was also identified in the serum from the case of clinical intoxication.

Conclusion: Given the efficacy and ease of synthesis of 25I-NBOMe and its many derivatives, it is likely that the abuse of these hallucinogens will become more widespread in the future. Therefore this approach to identify biomarkers will be useful in determining NBOMe derivatives in clinical and forensic specimens.

Keywords: 25I-NBOMe, 25I-NBOMe Metabolites, O-desmethyl-25I-NBOMe, Microsomes, Phase I Metabolism

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S-03

Quantification of Cocaine and Metabolites in Exhaled Breath by Liquid Chromatography High Resolution Mass Spectrometry Following Controlled Administration of Intravenous Cocaine

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Introduction: Cocaine is the most widely used illicit stimulant in the United States and Europe. There is increased interest in alternative matrices for identifying drug use in driving under the influence of drugs (DUID), workplace, clinical, and anti-doping programs. Recent studies investigating exhaled breath as an alternative matrix for detecting recent consumption of drugs of abuse, including cocaine, were primarily conducted in drug treatment patients or criminal justice programs, rather than after controlled drug administration.

Objective: To validate a liquid chromatography-high resolution mass spectrometry (LC-HRMS) method to quantify cocaine, benzoylecgonine (BE), ecgonine methyl ester (EME), and norcocaine in breath. Breath was collected with the SensAbues device following controlled intravenous (IV) cocaine administration.

Method: In this Institutional Review Board (IRB)-approved study, adult cocaine users were administered single 25 mg IV cocaine doses on Days 1, 5 and 10, after providing written informed consent. Exhaled breath specimens were collected over 3 min an hour prior to and 10 min, 0.5, 1, 1.5, 2, 3, 4, 6.5, 9.5, 12.5, and about 21 h post-administration. Filters were extracted with 9 mL 1% formic acid in water, followed by solid-phase extraction on UCT Clean Screen cartridges. Chromatographic separation was achieved with a Synergi Polar-RP column and 1 mM ammonium formate and 0.01% formic acid, and methanol mobile phases within 10.5 min. Target-MS/MS scans were acquired at 35,000 resolution with a heated electrospray source in positive mode on a QExactive. Limits of Quantification (LOQ) were 25 pg/filter, with linearity to 1,000 pg/filter. Extraction efficiencies were 83.6-126%. Ion suppression, determined by post-extraction addition, was less than -25%, except for EME and cocaine, which exhibited suppression up to -46% (n=10, CV<21%). No endogenous or exogenous interferences were observed. Analytes were stable under all tested storage conditions, except the high cocaine concentration at room temperature (-21%).

Results: Breath specimens from 8 participants were only positive for cocaine; BE, EME, and norcocaine were not detected above the LOQ. Only 6/167 specimens from 3 participants were positive for cocaine, with concentrations from 26.1-66 pg/filter (median 34.0 pg/filter). Most positive specimens were collected 1-2 h post-dosing; however, two specimens were positive at 4 and 9.5 h. Methanolic extraction of the devices, after filters were removed, yielded 32/125 positive cocaine tests (10 min to 22 h post-dosing), with concentrations from 25.2-364 pg/device (median 52.8 pg/device). BE was detected in 8/173 devices, with concentrations between 27.4-93.7 pg/device (median 43.6 pg/device).

C**onclusion:** We developed and validated a sensitive and specific method for cocaine, BE, EME, and norcocaine quantification in exhaled breath. Only 3.6% of filters were positive for cocaine from 1-9.5 h; no samples were positive for cocaine metabolites. When extracting the device itself, without the filters, 25.6% were positive for cocaine and 4.6% for BE, suggesting that the device reflects oral fluid as well as lung microparticles, while the filter, based on the design of the device, reflects only drug-contaminated microparticles. Cocaine in breath after controlled IV administration identifies recent cocaine ingestion, but its absence does not preclude recent use.

Keywords: Cocaine Metabolites, Exhaled Breath, SensAbues, LC-HR/MS

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S-04

Application of an Immunohistochemical Staining Method to Detect and Visualise the Presence of Opiates in Mice Following Peritoneal Injection of Morphine

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Introduction: Whilst it is now widely accepted that drugs can be detected in oral fluid and devices are now available to facilitate analysis of drugs in this matrix our understanding of the behaviour of drugs in the mouth and oral secretions is far from complete. It has been postulated that drugs which are administered orally either via smoke or solution have the potential to enter the mouth tissue by mechanisms other than via the circulating blood and hence providing support for a proposition that drugs can enter tissue via external contamination and may subsequently form depots in oral tissue from which they may be released over time. Previously we have shown the development of an immunohistochemical technique for the detection of cocaine and opiates in porcine tongue tissue (SOFT 2013).

Objective: The objective of research was to investigate whether morphine can be detected/visualised in oral tongue tissue and salivary glands in mice following peritoneal injection this demonstrating depot formation rather than simple passage into saliva from the blood via the acini of the saliva glands.

Method: Mice were injected with a saline solution (control), 3 mg/mL or 9mg/mL of morphine solution via peritoneal injection prior to cervical dislocation 30 minutes post injection and removal of the tongue and salivary glands. The tissue was then embedded in 10 % neutral buffered formalin for 24 hours prior to the application of an immunohistochemical staining method for opiates (SOFT 2013).The primary antibody was a polyclonal morphine-3-gluruconide antibody which was conjugated to keyhole limpet haemocyanin. The secondary antibody was a biotinylated rabbit-anti-goat antibody.

Results: Morphine administered to mice via peritoneal injections at concentrations of 3mg/mL and 9 mg/mL was shown to completely penetrate the tongue tissue with intense staining observed within the muscle bundles. As no external oral contamination occurred positive staining can only originate from diffusion from the blood into muscle tissue. Additionally morphine was shown to enter the parotid, submandibular and submaxillary gland where it was visualised within the blood vessel wall as well as the intercalated and interlobular ducts. No staining was present in the acini of the salivary gland which mainly consist of epithelial cells. Results will be presented pictorially.

Conclusion: This study demonstrated the entry of morphine into tongue and salivary gland tissues and provides support for the proposition that drugs can form depots in oral tissue from which they may subsequently be released over time. The presence of morphine in the salivary ducts strongly supports a hypothesis that drug is excreted from the salivary gland during production and excretion of saliva into the oral cavity hence contributing to the elevated drug concentrations observed in oral fluid.

Keywords: Oral Fluid, Drug Depots, Immunohistochemistry

S-05

In Vitro Reversible Inhibition of Oxycodone Cytochrome P450-Dependent Metabolism by Azole Antifungal Agents

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Introduction: The decade-long increase in mortalities associated with opioid use and abuse continues. One of the causes of these deaths may be related to co-medications, i.e. drug interactions. One commonly used opioid, oxycodone, is metabolized by cytochrome P450s (CYPs) 3A4 and 2C18 to noroxycodone and by CYP2D6 to oxymorphone. CYP3A4 and 2D6 are major sites for drug interaction. This study investigated inhibition of oxycodone CYP-dependent metabolism by ten azole antifungal agents. Six of them are used orally, which are albendazole (as its active metabolite albendazole sulfoxide), fluconazole, itraconazole, ketoconazole, metronidazole, and voriconazole. Others are applied topically (clotrimazole, econazole, miconazole and terconazole).

Objective: In vitro inhibition studies can predict potential pharmacokinetic drug interactions.

Method: Solutions of azole antifungals were made in MilliQ water with ≤ 1% of DMSO in the incubation system. These compounds have a wide range of aqueous solubility, which often limited the highest concentrations we could study. To screen for potential inhibitors, azole antifungal agents were first incubated at 3 concentrations with human liver microsome (HLM), NADPH and oxycodone. To test for time-dependent inhibition, azole antifungal agents were incubated with HLM and NADPH before adding oxycodone. A secondary screen was performed with the highest concentration of all azole antifungals with CYP2C18 or 2D6. Significant inhibitors were incubated at 6 concentrations with the respective cDNA-expressed human CYPs to determine the half maximal inhibitory concentrations (IC_{50}s). Production of noroxycodone and oxymorphone were determined by liquid chromatographic-tandem mass spectrometry.

Results: All ten azole antifungals inhibited the noroxycodone formation in HLM greater than or near 50%. Micromolar IC_{50}s for these inhibitors were determined for CYP3A4-mediated formation of noroxycodone, which ranged from 0.019 to > 500. The most potent inhibitors were clotrimazole (0.019), ketoconazole (0.042), terconazole (0.48) and econazole (1.2). For CYP2C18-mediated formation of noroxycodone, IC_{50}s ranged from 1.0 to > 500. The more potent inhibitors were econazole (1.0), ketoconazole (1.2), miconazole (5.0) and terconazole (8.0). For oxymorphone formation, only four azole antifungals: econazole (1.9), miconazole (6.5), ketoconazole (150) and fluconazole (900) inhibited above or near 50%. None of the azole antifungal agents displayed time-dependent inhibition.

Conclusion: Oxycodone and oxymorphone have opioid activity, while noroxycodone is inactive. The opioid activity of oxycodone will increase with inhibition. This is more so if oxymorphone is also increased. Therefore, among the ten azole antifungals, potent CYP3A4 inhibitors, clotrimazole, ketoconazole, terconazole and econazole are most likely to produce adverse effects under conditions of low tolerance or ingestion of higher doses of oxycodone. Especially for ketoconazole as a orally used drug, its plasma concentration can reach 9.6 μM. Although fluconazole, itraconazole and voriconazole show moderate inhibition, they could still be significant due to high concentrations from oral intake. Future studies are needed to explore *in vivo* inhibition.

Keywords: Oxycodone, Azole Antifungal Agents, Reversible Inhibition

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S-06
Complicated Toxicological Findings in a Drug-Facilitated Sexual Assault (DFSA) Case

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Introduction: Prevalence data indicates that ethanol is the number one drug identified in drug-facilitated sexual assault (DFSA) cases. Benzodiazepines and common sleeping medications are often reported as the most common secondary findings with sedative properties in DFSA casework. However, many other drugs must be considered in order to perform a thorough toxicological examination in DFSA casework.

Objective: Attendees of this presentation will learn about the pharmacokinetics and pharmacodynamics of two drugs infrequently encountered in DFSA casework, Salvia divinorium and butalbital. Additionally, attendees will be reminded of the importance of thorough screening in DFSA cases.

Method: This case involves individuals that were known to each other, and were attending a party one night. A third individual came to the party looking for his wife and found the subject and his wife (the victim) engaged in sexual activity. Specimens from both the suspect and the victim were collected within 5 hours of the incident under investigation and were submitted to the FBI Laboratory for analysis. Both the suspect and the victim reported lapses in memory during the timeframe of the assault. Additionally, witnesses described the victim as disoriented, dazed, and unable to dress herself or walk on her own in the hours following the assault. Investigatory leads indicated that Salvia was smoked by the suspect and victim, so the request of the investigators was that Salvia be identified in the biological specimens. However, laboratory protocols require that full toxicological analyses are performed on specimens from suspected DFSA victims. Specifically, specimens are screened for the following: ethanol, gamma-hydroxybutyrate, drugs of abuse including amphetamines, cannabinoids, cocaine and opioids, benzodiazepines, and other common prescription and over-the-counter medications. Preliminary findings are confirmed in a second sampling and/or in a second specimen.

Results: Ethanol was identified in the victim's blood and urine at concentrations of 0.169 ±0.023 and 0.237 ±0.031 gram percent, respectively (99.7% confidence level; k=3). Clonazepam, 7-aminoclonazepam, trazodone, citalopram and desmethylcitalopram were identified in specimens from the victim. These findings were consistent with drugs the victim admitted taking therapeutically. Salvinorin A was not identified in the victim's urine, but was identified in plant material and a pipe seized from the scene of the alleged assault. Butalbital was identified in the toxicology specimens from the victim, but was not indicated as a drug that the victim was knowingly taking at the time of the alleged assault. Ethanol was identified in the suspect's urine at a concentration of 0.086 ±0.012 gram percent. No other drugs or metabolites, including Salvinorin A, were detected in the suspect's urine.

Conclusion: A fairly significant amount of ethanol in addition to other positive toxicology findings made interpretation in this case challenging. Clonazepam, trazodone, citalopram and butalbital can all have sedating effects. Reported effects of smoking salvia are predominantly hallucinogenic in nature, but also include memory impairment. Salvinorin A, a kappa opioid agonist, and a major psychoactive component of Salvia divinorium, is very rapidly metabolized and excreted from the body. Therefore, though Salvinorin A was not detected, it is possible that Salvia was consumed during the evening. Butalbital findings were eventually linked to a Fioricet© prescription that belonged to the suspect's wife. The suspect confessed to slipping the medication to the victim, but the case was not prosecuted because the victim became uncooperative and did not want to testify in court.

Keywords: DFSA, Salvia Divinorium, Butalbital

S-07

Drug-Facilitated Crime in Pakistan: Toxicological Findings in Public Transport Passengers

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Introduction: Drug facilitated crime targeting public transport passengers is an emerging problem due to the easy availability of sedative-hypnotic drugs without prescription in Pakistan. Numerous passengers have been found drowsy or unconscious after accepting edibles offered by strangers travelling with them in the bus or train. The victims were brought to the hospital by police or rescue 111 for specific diagnosis and medical care.

Objective: To determine the frequency of criminal poisoning by identifying the sedative drugs detected among the passengers admitted in the tertiary care hospitals during September 2012 to April 2014 in Pakistan.

Method: This cross sectional study was carried out in the department of Forensic Toxicology, Armed Forces Institute of Pathology (AFIP), Rawalpindi, Pakistan. The study included a total of 208 male and female criminal drug intoxication patients aged 18 to 50 years. Patients were admitted in the tertiary care hospitals during Sep 2012 to April 2014. Patients arrived at the hospital in a drowsy or unconscious state from public transport terminals. Patients having any disease or using therapeutic medicine were excluded. Blood (5ml) in EDTA with sodium fluoride in vacutainer tubes and urine (25ml) samples were transported within 12-24 hours of the incident to the AFIP for biochemical and toxicological investigations. Blood alcohol estimation was carried out by head space-gas chromatography. Drug screening for amphetamine, barbiturates, benzodiazepines, buprenorphine, cannabinoids, cocaine metabolite, MDMA, methadone, methamphetamine, opiates, phencyclidine, and tricyclic antidepressants (TCA) were carried out on an Evidence Investigator using a biochip array kit (Randox, UK). Drug confirmations were carried out on 6010-B liquid chromatography mass spectrometer (Agilent, USA).

Results: A total of 208 adults passengers comprised of males (n=191) and females (n=17) with a mean age of 28 $\pm$ 6 years were included. Most of the victims were brought in unconscious (37%), drowsy (51%) and confused (12%) to the tertiary care hospitals of Multan (n=43), Rawalpindi (n=41), Sargodha (n=21), Lahore (n=17), Bahawalpur (n=10), Peshawar (n=2) and Hyderabad (n=2), Pakistan. Most of the patients recall traveling in the bus (48%), van (29%) and train (23%); accepting soft drinks (57%) and food items (23%) offered by strangers travelling with them. Another common feature was the loss of money, wallets and mobile phones. Blood ethanol was found in 7 victims (3%). Drug intoxication was detected in 152 (73%) of the cases and was comprised of benzodiazepines (89%), cannabinoids (6%), opiates (4%) and barbiturates (1%). These drugs were confirmed by LC-MS/MS as lorazepam (49%); diazepam (24%); oxazepam (7%); temazepam (5%); alprazolam (4%), clonazepam (3%) and nitrazepam (1%). Lorazepam in combination with diazepam and oxazepam was found in 7% of the cases. The biochip array assay has excellent correlation with LC-MS/MS for identifying drugs in biological fluid with few false positives, specificity, and fast throughput.

Conclusion: Drug facilitated crime targeting public transport passengers is an emerging forensic toxicological challenge in Pakistan. The benzodiazepines lorazepam and diazepam were most frequently used for spiking drinks or food items in the criminal poisoning among the public transport passengers. There is a serious need for forensic investigations in collaboration with law enforcement agencies to identify culprits and control the sale of sedatives/psychoactive drugs in the country. General public education and awareness programs should be organized to prevent drug facilitated crime in society.

Keywords: Drug Facilitated Crime (Robbery), Public Transport Passengers, Benzodiazepines, Biochip Array Technology, LC-MS/MS

S-08
Drug Facilitated Assault of a Living Victim of the Cleveland Strangler

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Introduction: A 39 year old woman was abducted in Cleveland, OH in April 2009 and driven to an unknown residence where she was bound and repeatedly assaulted over the course of two days by the 49 year old male driver. During the woman's captivity, the assailant forced her to drink a mixture of beer and wine and swallow an unknown tablet. The woman eventually escaped after a struggle with her assailant. The male was subsequently arrested in October 2009 as a suspect in the murders of eleven women whose remains were discovered at his Cleveland, OH duplex.

Objective: The assailant in this case, identified by media reports as the Cleveland Strangler, was known to lure his victims to his home with the promise of drugs and alcohol. The assailant preyed on women who used crack cocaine, and took it upon himself to exact punishment for their addictions. This case presents the toxicology results of a living victim of the Cleveland Strangler proximate to the time of the assault.

Method: The victim reported she voluntarily entered the assailants' vehicle after recognizing a female passenger, who asked the victim if she wanted a ride. After the passenger was dropped off, the victim was immediately assaulted by the assailant, driven to the duplex and forced inside on Wednesday at 2100 hours. The victim escaped two days later on Friday at 0930-1000 hours. The victim related that she was forcibly drugged late Thursday morning and raped four times during the 36 hour captivity. The victim was transported within one hour of her escape to an area hospital where a sexual assault kit and blood and urine specimens for toxicology were collected on Friday at 1217 hours. Routine screening of whole blood and urine for drugs of abuse and volatiles was performed using enzyme-linked immunosorbent assay (ELISA) and headspace-gas chromatography/flame ionization detection (HS-GC/FID). Subsequent confirmation of presumptive positive drug screen results was performed using gas chromatography/mass spectrometry (GC/MS).

Results: Blood screening revealed presumptive positive results for cannabinoids and cocaine/metabolites. Blood confirmation yielded the following results: 11-nor-9-carboxy-tetrahydrocannabinol (THC-COOH) 18.7 ng/ml, benzoylecgonine (BE) 19.0 ng/ml. A volatile screen and general drug screen performed on blood were negative. Urine screening revealed presumptive positive results for benzodiazepines, cannabinoids, cocaine/metabolites and opiates. Urine confirmation yielded the following results: BE positive, ecgonine methyl ester (EME) positive, THC-COOH positive, morphine positive. A general drug screen on urine revealed positive results for diphenhydramine, nordiphenhydramine, doxylamine and acetaminophen. A volatile screen on urine revealed a 0.014 g/dLethanol. The benzodiazepines did not confirm. An ELISA benzodiazepine screen using a cut off calibrator of 20 ng/mL oxazepam was used with a subsequent confirmation method with a limit of detection of 10ng/mL for the following analytes: alprazolam, α-hydroxyalprazolam, clonazepam, 7-aminoclonazepam, diazepam, nordiazepam, flunitrazepam, 7-aminoflunitrazepam, flurazepam, desalkylflurazepam, lorazepam, midazolam, oxazepam, phenazepam, temazepam and triazolam. The remains of eleven victims recovered at the duplex underwent toxicological examination: six revealed the presence of BE only, two revealed THC-COOH only, one revealed the presence of BE and fluoxetine and two were negative.

Conclusion: The assailant was charged with 85 counts of murder, rape and kidnapping and pleaded not guilty. He went on trial in June of 2011 and was convicted on July 22 on 83 counts. The jury's recommendation for the death penalty on August 10 was upheld by the judge two days later. The victim in this case did not testify during the trial; subsequently the results of her sexual assault kit and toxicology examinations were not entered as evidence.

Keywords: Drug Facilitated Assault, Serial Murder, Drugs of Abuse

S-09
Drug Facilitated Sexual Assault Survey Results

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Introduction: The Drug Facilitated Crimes (DFC) Committee designed a survey for those laboratories that analyze specimens in drug facilitated sexual assault (DFSA) cases. An email broadcast was sent out to the SOFT membership in the latter half of 2013 and to American Society of Crime Lab Directors (ASCLD) accredited labs in early 2014 asking that the labs that do this work complete the survey.

Objective: The purpose of the survey was to gain insight into the types of specimens that labs routinely analyze, the drugs that are targeted and their cut off limits in urine, the time spent in court on these types of cases, the drug(s) most commonly encountered and the assistance required to improve the labs ability to analyze these cases.

Method: The survey questions covered general demographic information. The number of DFSA cases per year, the type of specimens analyzed, how many were positive, how many went to court and what drug was involved, how the specimens were analyzed to achieve the desired sensitivity and the top 10 drugs most commonly encountered in their case work. In addition the labs were asked if there would be any interest in purchasing a proficiency test in urine modeled after a DFSA case and if the lab analyzed hair and if not, why not.

Results: Data was received from 15 labs which covered a representative cross section of the United States and one survey participate was from France. State labs comprised 41% of the respondents followed by County labs at 35%, City/Township labs at 23%, Private labs at 6% and Federal labs at 6%. The population base served was 76% from labs covering from 1,000,000 to greater than 5,000,000 with the remaining 24% covering from less than 99,999 to 999,999. The number of DFSA cases analyzed per year ranged from 5 to 250, with percent positive cases ranging from 20% to 87%, with an average of 64% and a mean of 65%. The most common specimens analyzed were blood and urine from each case. Some labs reported receiving a few cases with urine only. Labs reported testimony in court of between 0 and 10 cases per year with ethanol being the most common drug involved followed by cannabis, benzodiazepines (diazepam and alprazolam) and zolpidem. Of the top ten drugs detected ethanol was number one, followed by cannabis, then benzodiazepines, and a wide variety of drugs after that consisting of: stimulants, opiates, diphenhydramine, citalopram, quetiapine, carisoprodol, GHB, methadone, dextromethorphan, tramadol and methylone. The participates were asked to answer if their lab could meet the recommended performance limits for the drugs listed in the Recommended Maximum Detection Limits for Common DFSA Drugs and Metabolites in Urine table located on the SOFT website in the DFC Committee area. The majority of the recommended detection limits could be met by most of the labs but this question did identify some problem drugs. Drugs in which 40% or more of the labs could not meet the recommended detection limits in urine were: THC-COOH, clorazepate, phenazepam, gabapentin, cyclobenzaprine, zaleplon, zopiclone, hydroxyzine, tetrahydrozoline, scopolamine, secobarbital, butalbital, amobarbital, phenobarbital, pentobarbital, valproic acid, phenytoin, ziprasidone and clonidine. The types of assistance that the labs required to meet these recommendations were: state of the art instrumentation and additional staff. A minority of the labs required revalidation of existing methods. Labs used GC/MS, LC/MS, LC/MS/MS and low sensitivity opiate and benzodiazepine “plates” to augment traditional specimen screening and expand their menu of detected drugs. In addition reference labs were used by 33% of the participants. Regarding the DFSA proficiency offering, 67% of labs would be willing to purchase one if it was made available. Hair analysis was performed in only 13% of the labs. The reasons given for not testing hair varied but they centered on lack of protocols, instrumentation, and adequate staffing. One lab did respond that hair testing results were not accepted in their courts.

Conclusion: The survey results were not surprising in the fact that ethanol is the number one drug involved in DFSA cases and that many labs do not have the instrumentation and staff necessary to meet the recommended detection limits in urine for some drugs. The variety of drugs that was listed in the top ten drugs detected category of the survey was surprising. It was also surprising that most of the labs do not utilize hair as an additional specimen for testing. It is clear from these survey results that assistance is required to improve the majority of labs regarding their ability to more effectively perform toxicology analysis in DFSA cases.

Keywords: Drug Facilitated Sexual Assault (DFSA) Survey Results, Drug Facilitated Crimes (DFC) Committee

S-10

Post-hoc Analysis of 11-Nor-delta^9-THCV-COOH as a Marker of Cannabis Use

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Introduction: 11-Nor-delta^9-tetrahydrocannabinvarin-9-COOH (THCV-COOH) was developed as a marker for the discrimination of illicit cannabis use vs. the legitimate prescribed use of dronabinol in forensic urine specimens found positive for 11-Nor-delta^9-tetrahydrocannabinol-9-COOH (THC-COOH). A retrospective analysis of the relative concentrations of THCV-COOH and THC-COOH in these urine specimens was undertaken.

Objective: To investigate trends of cannabis use and to characterize the performance of THCV-COOH as a marker for cannabis use as opposed to the sole use of prescription dronobinol as the source of the positive drug test.

Method: The specimens analyzed were from routine workplace testing and were identified at the testing laboratory as positive for THC-COOH. The Medical Review Officers requested that testing be done to determine if Marinol was the "only" reason for the positive test. If the THCV-COOH was positive along with the THC-COOH then the person must have used marijuana, with or without Marinol. Samples were analyzed by a validated GC/MS method for both THC-COOH and THCV-COOH as their TBDMS derivatives after liquid/liquid extraction using hexane ethyl acetate (9:1). The LOQ of the method was 2 ng/mL.

Results: Based on the distribution of THC-COOH results in specimens where THCV-COOH was detected below the limit of quantitation, the estimated minimum sensitivity of the analytical methodology was calculated to be 76.4%. Overall, 79.3% of the specimens analyzed confirmed positive for cannabis use. Considering that some of the samples that were negative for THCV-COOH might be from legitimate prescription dronabinol users, and other samples with very low levels of THC-COOH to start with, the method seems to be of high reliability in detecting cannabis use as a source of the positive drug test.

Conclusion: It is concluded that testing for THCV-COOH in urine specimens provides a positive indication that the person providing the urine specimen must have ingested cannabis at some point prior to providing the specimen. Therefore, a positive THCV-COOH excludes the possibility that the positive marijuana drug test is caused solely by the ingestion of a prescription dronabinol.

Keywords: Cannabis Ingestion, Marinol, THC-COOH, THCV-COOH

S-11

Inhibition of Buprenorphine Metabolism by Proton Pump Inhibitors: Enigmatic Time-Dependent Effect of Esomeprazole

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Introduction: We are performing ongoing studies on in vitro inhibition of metabolism of opioids by different classes of potential drug interactants. One noted set of interactants is the proton pump inhibitors (PPIs). While several studies have investigated the effect of PPIs on the activities of specific cytochrome P450s (CYP450), most studies used model substrates.

Objective: Here we investigate the effect of PPIs on the N-dealkylation of buprenorphine, a CYP3A4 and 2C8 driven reaction.

Method: An initial screen was performed using three concentrations of PPI in human liver microsomes (HLM) with or without a 15-minute pre-incubation of the HLM with inhibitor and a source of NADPH. The pre-incubation tests for time-dependent inhibition (TDI). Subsequent studies with cDNA-expressed CYPs (rCYP) were conducted with 6 or more concentrations of inhibitor to determine an IC_{50}. IF TDI is suspected, tests were also conducted with a dual incubation system. From a primary incubation with 3 or more inhibitor concentrations, aliquots were then transferred (and diluted) after varyious pre-incubation times to a secondary system with buprenorphine and additional NADPH.

Results: For five PPIs, the following apparent IC_{50}s (μM) were determined in HLM without (-) or with (+) 15 minute pre-incubation: omeprazole, (-) 20.9, (+) 2.5; esomeprazole, (-) 9.8, (+) < 1.0; lansoprazole, (-) > 100, (+) > 100; pantoprazole, (-) 120, (+) 16.2; and rabeprazole (-) 79.4, (+) 60.3. For all but lansoprazole, pre-incubation had an impact. Respective IC_{50}s (μM) determined with rCYP3A4 and 2C8 were: omeprazole, 220 and 65; esomeprazole, 90 and 65; lansoprazole, > 100 and 90; pantoprazole, > 200 and 200; and rabeprazole, 150 and 70. The reversible inhibition (no pre-incubation) was generally stronger in HLM than in CYPs. Dual-incubation studies were then conducted for omeprazole, esomeprazole, lansoprazole, pantoprazole and rabeprazole with rCYP3A4, and omeprazole and esomeprazole with rCYP2C8. Only rabeprazole produced pre-incubation time- and inhibitor concentration-dependent decreases in CYP3A4 activity. When we performed the dual incubation using esomeprazole and HLM. Inhibition was pre-incubation time- and inhibitor concentration-dependent.

Conclusion: While TDI has previously been observed with CYP2C19 and PPIs, the TDI with rabeprazole is the first such observation for CYP3A4. An enigma arose between HLM and rCYP results for TDI with most PPIs; while TDI was observable in HLM, it was not with CYP3A4 or 2C8. We hypothesize that metabolism of esomeprazole (and likely other PPIs) at another CYP produces a potent inhibitor of CYP3A4 and/or CYP2C8 metabolism of buprenorphine.

Keywords: Buprenorphine, Drug Interaction, Time-Dependent Inhibition, Proton Pump Inhibitors

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S-12

Morphine and Codeine Concentrations in Human Urine Following Controlled Poppy Seeds Administration of Known Opiate Content

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Introduction: Opiates are an important component of drug testing programs due to their high abuse potential. Poppy seed ingestion can confound interpretation of urine opiate tests; other investigators report urine morphine concentrations of 7.5-18,000 µg/L. Detailed opiate elimination studies after controlled administration of poppy seeds with known morphine and codeine content are not available.

Objective: To investigate urine opiate pharmacokinetics after controlled oral administration of poppy seeds with known morphine and codeine content.

Method: Healthy adults were administered two 45g uncooked poppy seed oral doses 8h apart, each containing 15.7mg morphine and 3mg codeine (commercially available seeds with the highest morphine concentration). Urine was collected *ad libitum* up to 32h after the first dose. Specimens were analyzed with the Roche Opiates II immunoassay, at 2,000 and 300µg/L cutoff concentrations, and the ThermoFisher CEDIA® Heroin Metabolite (6-acetylmorphine, 6AM) and Lin-Zhi 6AM immunoassays, with 10µg/L cutoffs, to determine if poppy seed ingestion could produce positive results in these heroin marker assays. In addition, all specimens were quantified for morphine and codeine by GC/MS (LLOQ=300 µg/L).

Results: Participants (N=22) provided 391 urine specimens over 32h following dosing; 26.6% and 83.4% were positive for morphine at 2,000 and 300µg/L GC/MS cutoffs, respectively. For the 19 subjects who completed the study, morphine concentrations ranged from < 300 to 7,522µg/L, with a median peak concentration of 5,239µg/L. The median first morphine-positive urine sample at 2,000µg/L cutoff concentration occurred at 6.6h (1.2-12.1), with the last positive from 2.6 to 18h after the second dose. No specimens were positive for codeine at 2,000µg/L, but 20.2% exceeded 300µg/L, with peak concentration of 658µg/L (284-1540). The Roche Opiates II immunoassay had efficiencies greater than 96% for both cutoffs. The CEDIA 6AM immunoassay had a specificity of 91% (immunoassay positive specimens contained no 6AM by GCMS). The Lin-Zhi assay had no false positive results.

Conclusion: Following ingestion of poppy seeds with a high content of morphine and codeine, morphine was detected in urine above 2000µg/L as early as 1.2h and for up to 18h after the last dose. No urine codeine concentration exceeded 2000µg/L. All immunoassays had high efficiencies. These results provide valuable information for interpreting urine opiate results.

Keywords: Poppy Seeds, Urine, Morphine, Codeine, Controlled Dose

S-13

Plasma and Oral Fluid Morphine and Codeine Concentrations after Controlled Poppy Seed Administration of Known Opiate Content

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Introduction: Opiates are included in drug testing programs because of their high abuse potential. To correctly interpret positive opiate results, it is necessary to rule out poppy seed ingestion, although few studies in the literature address plasma and oral fluid (OF) opiate concentrations after controlled poppy seed consumption. Moreover, most did not evaluate administered poppy seeds' morphine and codeine content.

Objective: To determine plasma and oral fluid morphine and codeine concentrations after ingesting poppy seeds containing 31.4 mg morphine and 6.2 mg codeine in two divided doses.

Method: Participants provided written informed consent to this NIDA Institutional Review Board-approved study. Participants received two 45 g doses of raw, uncooked poppy seeds. Doses were administered at 0900 and 1700 h on Day 1. Plasma was collected via an indwelling venous catheter and OF with the Quantisal™ device; specimens were collected before and up to 32 h after the first dose. Specimens were analyzed by a fully validated LC-MS/MS method. Linear ranges for morphine and codeine in both matrices were 1-500 µg/L. Inter and intra-day precision and accuracy were ≤ 9.0% CV and 92.4-104%, respectively. Extraction efficiencies and matrix effects were 82.1-103% and -72.2-4.3%, respectively. OF and plasma concentrations were evaluated at multiple cutoffs and OF/plasma ratios and correlations were determined.

Results: Maximum OF morphine and codeine concentrations (C_{max} median 17.5 [range 3.6-76.5] and 6.4 [2.1-23.8] µg/L, respectively) were significantly greater than maximum plasma concentrations (5.4 [2.8-8.4] and 1.5 [1.2-2.0] µg/L, respectively). OF and plasma concentrations were significantly correlated, but large inter- and intra-subject variabilities in OF/plasma ratios were observed. Based on the proposed Substance Abuse and Mental Health Services Administration (SAMHSA) 40 µg/L and Driving Under the Influence of Drugs and Medicines (DRUID) 20 µg/L cutoffs, 41.2% and 70.6% of participants had at least1 morphine-positive OF specimen, respectively, with all OF negative by 1 and 2.5 h post-dose, respectively.

Conclusion: Positive OF morphine and codeine results are possible after ingestion of large doses of uncooked poppy seeds, indicating OF 6-acetylmorphine monitoring is the best means of distinguishing licit poppy seed consumption from heroin consumption. Morphine last detection times after poppy seed intake were not significantly different between plasma and OF. Plasma opiate concentrations cannot be estimated based on OF results due to large OF/plasma ratio variability.

Keywords: Oral Fluid, Plasma, Poppy Seeds

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S-14

A Case Study of the Possible Bacterial Reduction of Nitrobenzodiazepines in Urine

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Introduction: Nitrobenzodiazepines, which are classified as a group of benzodiazepines containing a nitro group at the 7-position of the aromatic ring, namely, nimetazepam, nitrazepam and flunitazepam, are prescription drugs used for the treatment of sleep disorder, anxiety or epilepsy. In Singapore, abuse of benzodiazepines, primarily nimetazepam, has become prevalent since the year 2000. Currently, only nimetazepam and flunitazepam are Class C controlled drugs under Singapore's First Schedule of the Misuse of Drugs Act (CAP. 185). A stability issue of nitrobenzodiazepines has been observed in urine where conversion of nitrobenzodiazepines to their corresponding 7-amino-benzodiazepines can occur. This may pose a concern to the detection of these drugs in urine if the testing is delayed. In spite of literature sources reporting a notable decrease of nitrobenzodiazepines in urine, there are few extensive studies that identify the species of bacteria which could be the cause of the degradation.

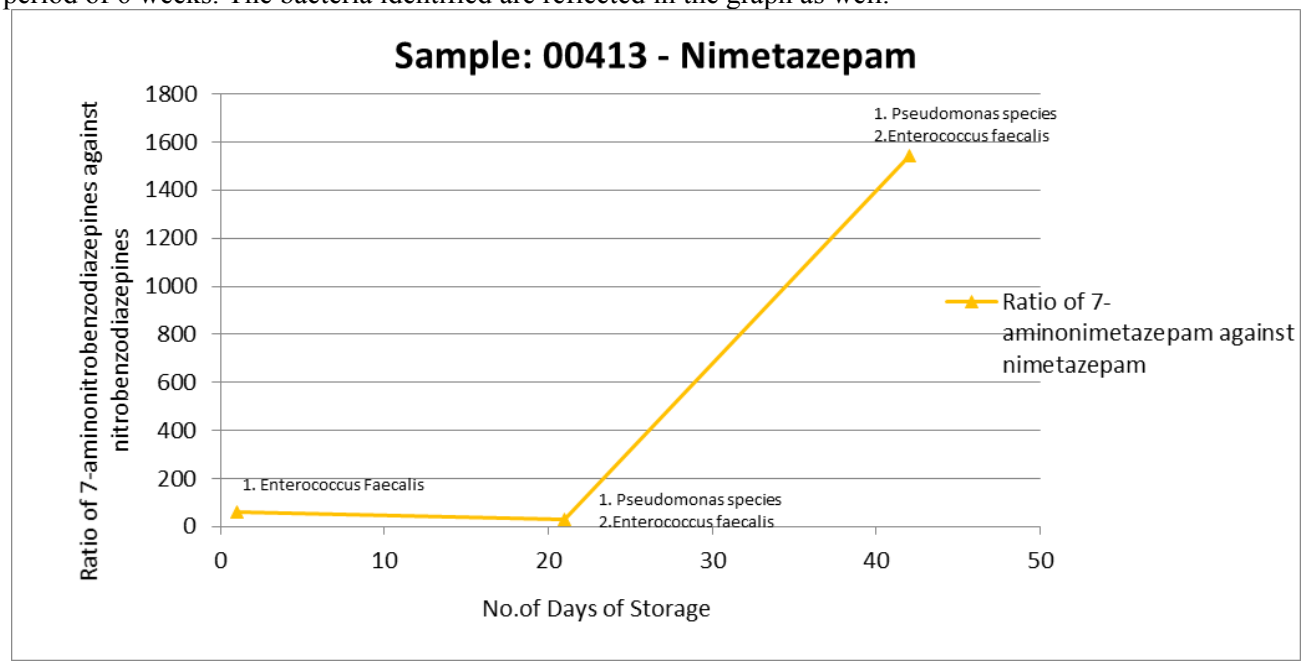
Objective: A comprehensive study of 77 urine specimens was carried out to examine the stability of these nitrobenzodiazepine analytes, namely nimetazepam and/or nitrazepam in urine.

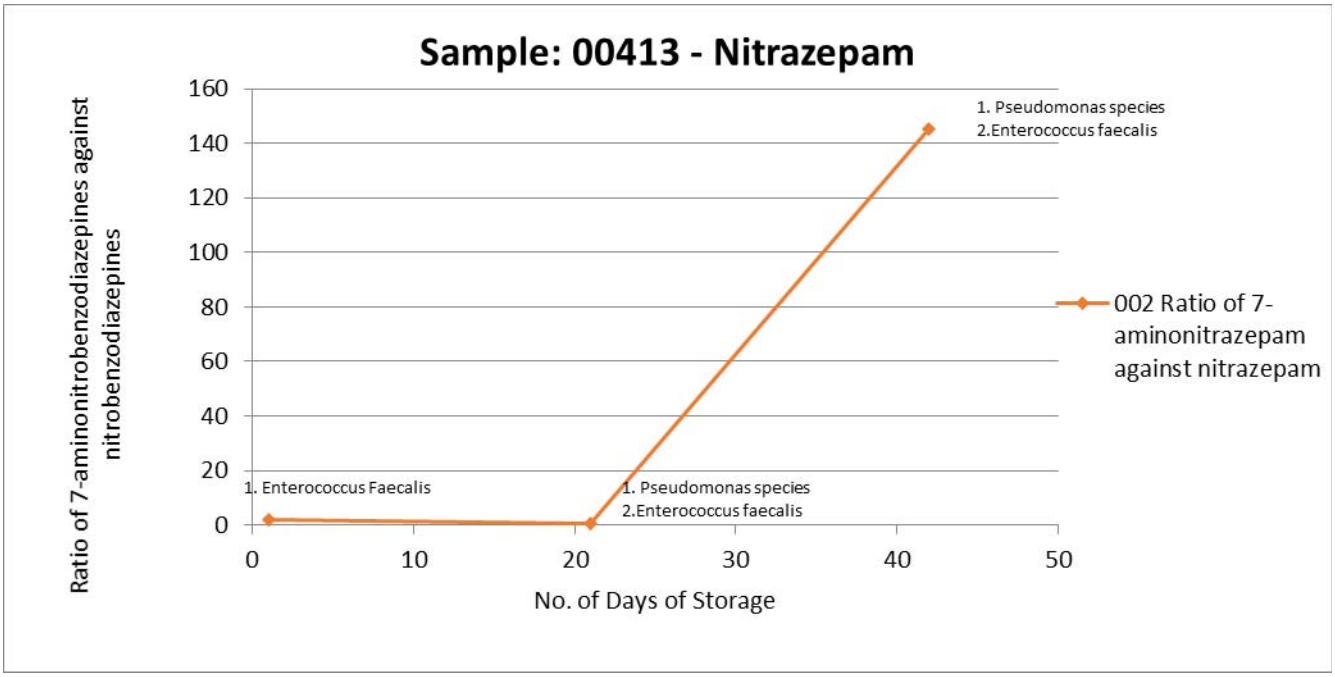
Method: The stability of nimetazepam and/or nitrazepam in urine was studied at storage condition of 4°C over a period of 6 weeks or more. Duplicate urine specimens were extracted and analyzed using liquid chromatography-tandem mass spectrometry (LC-MS/MS). Response ratio of the drug analytes to their corresponding 7amino-benzodiazepines was monitored for each of the specimens over the period. The identity of the bacteria in the urine specimens was also confirmed using microbiology testing.

Results: From the study, it was observed that over a period of a few weeks, certain urine specimens exhibited significant loss of nitrobenzodiazepines while others showed little or no degradation. The increase of 7-amino forms in urine specimens corresponded with observed degradation of the nitrobenzodiazepines. *Enterococcus species*, a genus of bacteria which causes urinary tract infection, were found to be present in most of the urine specimens with notable decrease of the monitored drugs. Other species identified were *Proteus mirabilis* and *Enterococcus Faecalis*. All these bacteria contain oxygen sensitive enzymes, which are known to reduce the nitroaromatic drugs to their corresponding amino forms.

<u>Urine specimen with significant degradation</u>

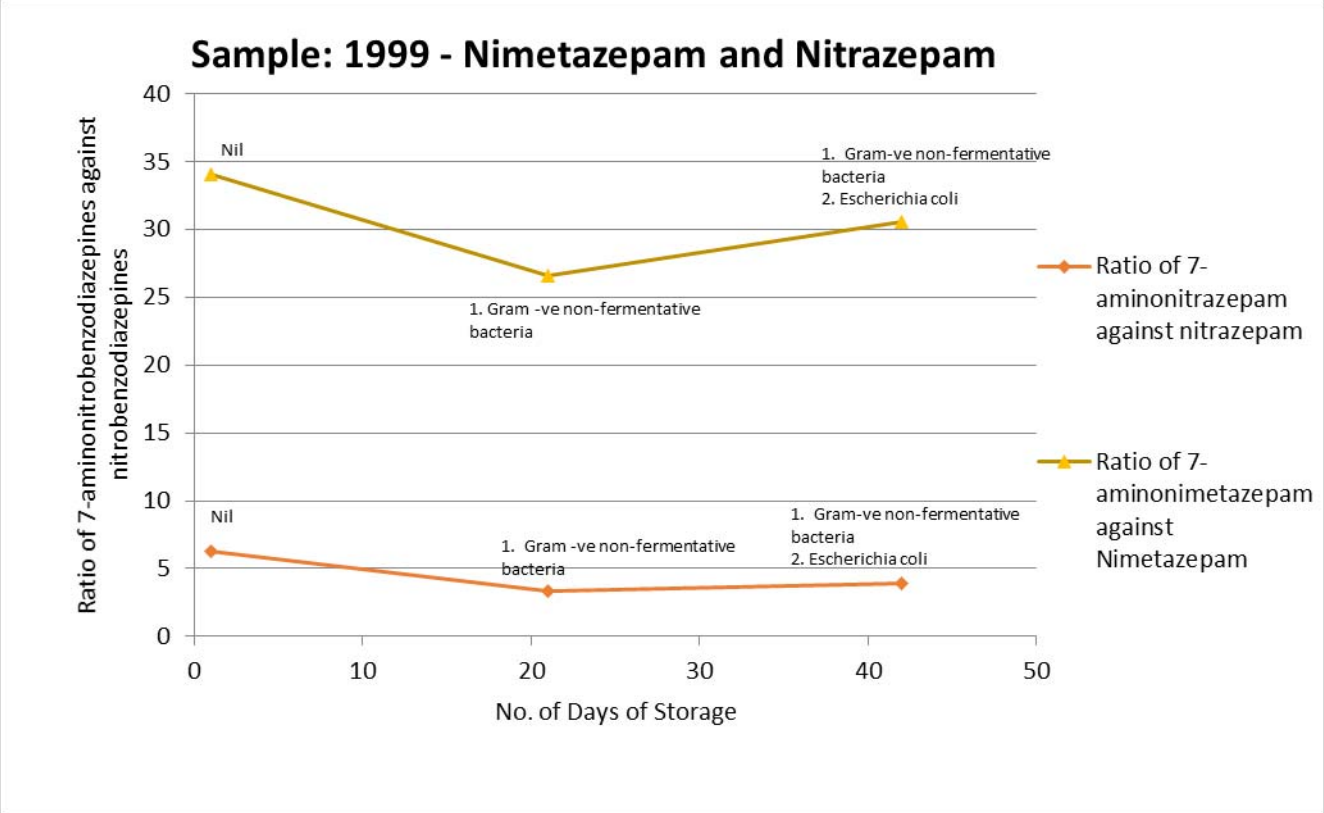
This particular sample exhibited a degradation of nitrobenzodiazepines (both nimetazepam and nitrazepam) over a period of 6 weeks. The bacteria identified are reflected in the graph as well.





Urine specimen with no degradation

The ratios of 7-aminonitrobenzodiazepines against the nitrobenzodiazepines are relatively constant throughout the storage period of 6 weeks.



Conclusion: This case study thus provides evidence that the presence of certain bacteria in urine may cause conversion of nitrobenzodiazepines to their respective 7-amino-benzodiazepines. The contamination of these urine specimens by endogenous or exogenous infectious bacteria and the propagation of these bacteria even at the laboratory's storage condition could cause the degradation of nitrobenzodiazepines in urine. Hence, analysis of nitrobenzodiazepines should be done promptly to prevent possible degradation that could affect the analytical results.

Keywords: Nitrobenzodiazepines, Urine, Degradation, Bacteria

S-15

Urinary Methamphetamine and Amphetamine Isomer Concentrations Following Controlled Vicks VapoInhaler Administration

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Introduction: Legitimate use of legal intranasal decongestants containing l-methamphetamine may complicate interpretation of urine drug tests positive for amphetamines.

Objective: To determine if Vicks VapoInhaler administration according to the manufacturer's recommendations produces amphetamine-positive immunoassay screening tests and/or false-positive d-amphetamine or d-methamphetamine confirmation tests in urine.

Method: Twenty-two healthy adults were each administered one dose (two inhalations in each nostril) of a Vicks VapoInhaler every 2 h (manufacturer's recommendation) for 10 h on Day 1 (6 doses), followed by a single dose on Day 2. Every individual urine void was collected for 32 h after the first dose and assayed by three commercially available amphetamines immunoassays and for d- and l-amphetamine-specific isomers by GC-MS with > 99% purity R-(-)-α-methoxy-α-(trifluoromethyl)phenylacetyl (MTPA) derivatives and 10 µg/L lower limits of quantification. Twenty-four blind proficiency samples were included for assay control and to provide d-methamphetamine and d-amphetamine positive samples for immunoassay evaluation.

Results: Seventeen participants were administered all doses. No d-methamphetamine or d-amphetamine was detected in any of the 391 urine specimens by GC-MS. The median l-methamphetamine maximum concentration was 62.8 µg/L (range 11.0-1440). Eleven participants (64.7%) had detectable (> LLOQ) l-methamphetamine 11 h after the last dose. Only two subjects had detectable l-amphetamine, with maximum concentrations (62.3 and 22.8 µg/L) coinciding with l-methamphetamine peak levels (1440.0 and 989.0 µg/L, respectively), and always ≤ 4.3% of the parent's maximum. Three amphetamines immunoassays, EMIT® II Plus, KIMS® II, and DRI®, had excellent characteristics (see table below), showing little cross-reactivity with the R(-)/l-isomers.

	EMIT® II Plus	KIMS® II	DRI®
Sensitivity	100%	100%	100%
Specificity	97.8%	99.6%	100%
Efficiency	97.8%	99.6%	100%

Conclusion: Following the manufacturer's recommended doses of Vicks VapoInhaler, no d-amphetamine or d-methamphetamine was detected in urine by GC-MS above 10 µg/L. Only 2 participants had l-methamphetamine concentrations ≥ 250 µg/L, the total methamphetamine cutoff concentration for federally regulated drug testing programs. However, neither participant exceeded the additional amphetamine cutoff concentration of ≥ 100 µg/L required in those same programs. These high immunoassay specificities indicate that drug testing laboratories screening with these methods will have few specimens requiring confirmation due to the presence of l-methamphetamine in urine at or above the methamphetamine cutoff.

Keywords: Vicks VapoInhaler, l-Methamphetamine, Urine

Supported by the National Institutes of Health, Intramural Research Program, NIDA and SAMHSA.

S-16

Benzofuran Analogues of Amphetamine and Methamphetamine: Metabolism and Detectability of 5-APB, 6-APB, 5-MAPB, and 6-MAPB in Urine Using GC-MS and LC-(HR)-MS^n Techniques

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Introduction: 5-(2-Aminopropyl)benzofuran (5-APB) and *N*-methyl-5-(2-aminopropyl)benzofuran (5-MAPB), and their isomers 6-APB and 6-MAPB are novel psychoactive substances (NPS). 5- and 6-APB were described as inhibitors of the norepinephrine, dopamine, and serotonin reuptake transporters (Iversen et al., EJP, 2013). This fits with the described euphoric and entactogenic effects as well as with the side effects hyperthermia, insomnia, tachycardia, and anxiety. Some of these compounds were scheduled in some European countries and unscheduled in the US; nevertheless, they were still available over the internet.

Objective: The aims of this work were to identify metabolites of the benzofuran analogs in rat urine and human liver microsomal incubations (HLM) and to investigate the CYP enzymes involved in the main metabolic reactions. In addition, their detectability within our standard urine screening approaches (SUSA) by GC-MS or LC-MS^n (Welter et al., ABC, 2013) was investigated. Furthermore, the differentiation of the isomers should be elucidated.

Method: The compounds were synthesized by either the Department of Pharmacology and Therapeutics, St. James's Hospital, Dublin or the School of Pharmacy & Biomolecular Sciences, John Moores University, Liverpool. They were checked for identity and purity by GC-MS and LC-HR-MS^n. Urine samples were collected over 24 h from male Wistar rats after administration of either 10-20 or 1-3 mg/kg BW of the drugs for toxicological diagnostic reasons. For the metabolism studies, the high dose urine samples were prepared by protein precipitation, by solid-phase extractions (SPE, C18), or by enzymatic hydrolysis, SPE (HCX), and acetylation. The samples were analyzed by GC-MS (Agilent GC-MSD) or LC-HR-MS^n (ThermoFisher Orbitrap Velos) according to Welter et al., ABC, 2013. Incubation conditions for CYPs and HLM were chosen according to Welter et al., ABC, 2013. For SUSA, the low dose urine samples, with concentrations that correspond to a described users' low single dose of the respective drug (30 mg 5-APB/6-APB or 10 mg 5-MAPB/6-MAPB), were prepared by acid hydrolysis, extraction, and acetylation for GC-MS or protein precipitation for LC-MS^n (ThermoFisher LC-LXQ). For the differentiation studies, the low dose urine samples were analyzed by GC-MS after enzymatic hydrolysis, SPE (HCX), and heptafluorobutyrylation according to Peters et al., JMS, 2003.

Results: All drugs were mainly hydroxylated, most probably at the furan part, leading to lactones followed by their hydrolysis and glucuronidation of the resulting hydroxy or carboxy moiety. The MAPBs were additionally *N*-demethylated. In the HLM incubations, only the *N*-demethyl MAPBs could be detected. For the APBs, no metabolites could be observed possibly due to low formation rates. CYP1A2, CYP2B6, CYPC19, and CYP2D6 were capable of catalyzing the *N*-demethylation of 5-MAPB, whereas CYP1A2 and CYP2D6 that of 6-MAPB. After low dose application, APBs alone or MAPBs and the *N*-demethyl metabolites were detectable by both SUSAs. Both SUSAs did not allow distinguishing between the corresponding 5- or 6- isomers, but differentiation was possible by GC-MS after heptafluorobutyrylation.

Conclusions: APBs and MAPBs were metabolized only to a low extent and thus, the parent compounds were the main targets for urinalysis. Assuming similar kinetics in humans, the authors' SUSA should be suitable to prove an intake of APBs or MAPBs in human urine by detection of the corresponding parent drugs.

Keywords: Benzofuran Analogs of Amphetamine and Methamphetamine, Urine, GC/MS, LC/MS/MS

S-17

Multi-Drug and Metabolite Quantification in Postmortem Blood by Liquid Chromatography-High Resolution Mass Spectrometry: Comparison with Nominal Mass Technology

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Introduction: Drug and metabolite findings in medical examiner casework often require quantitative analyses to provide interpretive assistance in determination of cause and manner of death. The proliferation of pharmaceutical and designer agents has added to both the volume and diversity of quantitative work in postmortem toxicology laboratories. Multi-analyte methods have been employed to improve quantitative workflow in postmortem toxicology, and selective reaction monitoring employing nominal mass spectrometry technology has been the predominant analytical approach. Currently, high resolution mass spectrometry is used primarily in postmortem drug screening as an alternative to nominal mass methods. Additional evaluation of this advancing mass spectroscopy technology is needed for quantitative casework.

Objective: The objective of this study was to develop and validate a liquid chromatographic-high resolution mass spectrometry method for quantification of frequently quantified analytes in postmortem cases and to compare casework performance with nominal mass analysis.

Method: Simultaneous quantitative analysis of cocaine, benzoylecgonine, cocaethylene, morphine, codeine, hydrocodone, hydromorphone, dihydrocodeine, oxycodone, oxymorphone, methadone, EDDP, meperidine, dextromethorphan, citalopram and diphenhydramine was performed on postmortem blood. Analysis was performed by Waters Acquity ultra-performance-liquid-chromatography (UPLC) interfaced by positive electrospray ionization with a Waters Xevo G2 QTof hybrid quadrapole time-of-flight mass spectrometry. Following mixed mode solid phase extraction of blood, UPLC-MS^E/TOF analysis was performed by MS^E acquisition in single-stage full-spectrum (m/z 50-1000) mode where precursor and fragment ions at low (6eV) and ramped (10-40 eV) collision energies are acquired, respectively. Quantification was performed with precursor ion data using analyte-matched deuterated internal standardization and a post-acquisition mass extraction window (MEW) of $\pm$5 ppm.

Results: Optimized solid phase extraction conditions were developed, resulting in greater than 60 percent recovery and less than 20 percent matrix effect for all analytes and internal standards. The MEW of $\pm$5 ppm was determined as optimal for analyte ion recovery and reduction of endogenous ion interference. Validation of defined lower limits of detection (10 ng/mL) and quantification (25 ng/mL) was demonstrated for all analytes, along with quantification across a dynamic linear range of 25-3000 ng/mL (R^2 >0.99). Fragment and isotope ions, as well as precursor ion abundance at low and high collision energies, were evaluated as additional parameters of analyte identification. For several analytes, common fragment ions were identified for analyte and internal standard acquisitions, limiting their use in ion ratio evaluation. Quantitative comparison of UPLC-MS^E/TOF with a previously validated UPLC-MS/MS method performed with an Acquity UPLC/TQD tandem MS system showed comparable precision and accuracy along with concordance for 253 positive ($y = 1.002x + 1.523$; $R^2 = 0.993$) and 2269 negative analyte findings in 159 postmortem cases.

Conclusions: Previous casework experience has shown that multi-analyte quantification improves work flow in the forensic toxicology laboratory. The current study demonstrates multi-analyte quantification by UPLC-MS^E/TOF with full spectrum data acquisition and without requirement of analyte-specific optimization of ion acquisition, as needed when using UPLC-MS/MS. Analytical performance and correlation studies demonstrate accurate quantification by UPLC-MS^E/TOF and therefore extended the potential application of high resolution mass spectrometry technology in postmortem toxicology casework.

Keywords: Postmortem Blood, Drug Quantification, High Resolution Mass Spectrometry, Ultraperformance Liquid Chromatography, ToF Mass Spectrometry, Nominal Mass Spectrometry, Medical Examiner Casework

S-18

A Direct Analysis in Real Time AccuTOF™ Mass Spectrometry (DART-MS) Method for the Analysis of 25I-NBOMe and Other Dimethoxyphenyl-N-[(2-methoxyphenyl)methyl]ethanamine Derivatives

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Introduction: A new class of "2C" serotonin 5-HT2A receptor agonist designer drugs, dimethoxyphenyl-N-[(2-methoxyphenyl) methyl]ethanamine (NBOMe) derivatives, are easily obtained over the internet with resultant abuse in the United States, Europe and Asia. Stimulation of 5-HT2A receptors is responsible for the hallucinogenic effects of recreational drugs such as lysergic acid diethylamide (LSD). The most commonly abused NBOMe derivative is 2-(4-iodo-2,5-dimethoxyphenyl)-N-[(2-methoxyphenyl) methyl]ethanamine (25I-NBOMe) which is commonly called "Bomb" or "Smiles". At the present time there are fifteen different NBOMe derivatives that are known to have been or are available as a designer drug. These designer drugs quickly evolve and rapid analysis is necessary, which is achieved by direct analysis in real time AccuTOF™ mass spectrometry (DART-MS).

Objective: To present a direct analysis in real time AccuTOF™ mass spectrometry (DART-MS) method for the analysis of NBOMe derivatives and to present the application of this method to blotter papers obtained via the internet that were advertised to contain NBOMe derivatives.

Methods: The analysis was performed using a direct analysis in real time ion source coupled to a JEOL JMS T100LC AccuTOF™ mass spectrometry (DART-MS) operated in positive-ion mode and controlled by Mass Center software version 1.3.4 m (JEOL Inc. Tokyo, Japan). Twelve different NBOMe derivatives were analyzed to determine a limit of detection (LOD), inter- and intra-day specificity and fragmentation patterns of the NBOMe derivatives. The studies were conducted with the DART-MS orifice 1 set at 20, 60 and 90V. At 20V, the DART-MS produced a protonated mass signal and the 60 and 90V settings produced fragmentation of the compound. Precision and accuracy of the data was evaluated by a mass difference of +/- 5 mmu and mass accuracy of +/-24 ppm. Blotter papers were analyzed directly and as methanol extracts, and the results were confirmed by high pressure liquid chromatography triple quadrupole mass spectrometry (HPLC/MS/MS).

Results: The LOD was determined to be 10 µg/mL, and the fragmentation of the NBOMe derivatives increased with the voltage settings. The DART-MS was reproducible within and between days, and differentiation of several NBOMe derivatives within a single mixture was possible. Equivalent data was obtained from the blotter papers and extract solutions. The blotter papers were determined to have a major component of either 25I-NBOMe, 25C-NBOMe or 25B-NBOMe and minute amounts of NBOMe derivative impurities including 25H-NBOMe, 25D-NBOMe, 25C-NBOMe, and/or 25I-NBOMe.

Conclusion: The present study validated the use of direct analysis in real time AccuTOF™ mass spectrometry as a screening tool for the rapid and accurate detection of NBOMe derivatives on blotter paper or in methanol with no sample preparation.

Funding: This work was supported by the National Institutes of Health [P30DA033934].

Keywords: DART-MS, 25I-NBOMe, Blotter Paper

S-19
Analytical Method Comparison for Broad Based Screening of Designer Drugs

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Background: Certain social groups have displayed willingness to experiment with drugs. In order to sample what new compounds are on the market, a population of electronic dance music festival attendees was surveyed. This group of individuals was targeted with the assumption that the prevalence of novel designer drugs would be significantly higher than the normal population. Beginning in 2009, "legal high" products containing designer drugs gained popularity in the United States presenting unique challenges to toxicology laboratories across the country. As Federal and State governments rushed to restrict the legality of these products and schedule their illicit components, manufacturers simply modified the compounds to retain their legality. Toxicology laboratories were left scrambling to identify and incorporate testing methods and procedures for all the new designer drugs being encountered. A multifaceted approach for the detection and identification of drugs in urine samples collected from 104 concert attendees was investigated. The samples underwent screening via three separate techniques these methods were compared for ease of use, breadth of analyte detection, sensitivity and ability to detect unknown compounds.

Objective: The objective of this presentation is to identify the technique(s) best suited for a broad based screen for designer drug compounds. Urine samples with a presumed high positive rate of novel psychoactive substances were analyzed using multiple testing platforms to aid in this determination.

Methods: An IRB approval was attained for the sample collection process of this study. Urine samples (n=104) underwent analysis using the Hitachi P-Modular immunoassay screen for MDMA, Cocaine, Opiates, Oxycodone, 6-AM, Amphetamines, Barbiturates, THC, K2 (JWH018 N-5-COOH), Methamphetamine, Benzodiazepines, PCP and LSD. Each specimen underwent two solid phase extractions for alkaline drugs. One aliquot underwent a full scan GC-MS analysis while the other was subjected to analysis using a high resolution mass spectrometer for comparison to a database of over 1100 compounds. The full battery testing approach was utilized to evaluate the effectiveness of each technique against one another.

Results: The tested specimens were positive for a variety of stimulant type compounds, including novel psychoactive substances. Overall, only six of the 104 urine samples screened completely negative via all three technologies. The immunoassay testing identified 65 positive samples that would require further confirmatory testing. The GC-MS analysis identified 85 positive samples, 24 of which were only positive for Nicotine/Cotinine and the UPLC-QTOF identified 97 positive samples, 22 of which were positive for only Nicotine/Cotinine. The immunoassay range of detectable compounds encompassed both acidic and basic analytes; but wasn't able to identify or elucidate unknowns, or detect a variety of the novel designer drugs. The GC-MS provided more analytical information for specific compounds present in the urine specimens; however this assay is limited to basic compounds that are thermally stable and volatile. The UPLC-QTOF provided the most information regarding the presence of novel designer drugs; but this assay too is limited to basic compounds and data interpretation is more time intensive.

Conclusion: Overall, the UPLC-QTOF appeared to elucidate the most compounds present in the urine specimens tested. Complementary techniques are recommended, however, to ensure that common analytes encountered (like tetrahydrocannabinol metabolites) are not excluded from the reported results.

Keywords: Designer Drugs, LC-QTOF, GCMS, Novel Psychoactive Substances

S-20

Validation of Calibration Models: Development and Testing of a Practical Procedure

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Introduction: In 2013, the Scientific Working Group in Forensic Toxicology published its *Standard Practices for Method Validation in Forensic Toxicology*. It recommended choosing the simplest calibration model that fit the concentration-response relationship based on evaluation of the standardized residuals plot. This non-quantitative strategy is adequate for identifying poor models but is open to inter-analyst variations and does not provide a simple definitive result. Other methods such as ANOVA lack-of-fit for linear unweighted models and significance of the second order term in quadratic models are suggested but not developed nor evaluated.

Objectives: To replace evaluation of the residuals plot with a stepwise model selection process (weight and order) and to develop a confirmatory test for the adequacy of fit using LC-MS/MS data.

Methods: Five replicate calibrations, each with nine calibrants (5-1000 ng/mL), were obtained using an LC-MS/MS method for 50 analytes including benzodiazepines, amphetamines, opioids and cocaine. For each analyte an F-test ($\sigma^2_{ULQ}/\sigma^2_{LLQ}$) determined if the data was heteroscedastic. In these cases, a linear relationship between variance and concentration indicated required 1/x weighting, whereas a x^2 relationship indicated required $1/x^2$ weighting. A quadratic term was included in the model when a partial F-test found that the increase of the regression sum of squares was significant (α=0.05). Both ANOVA lack-of-fit and residuals normality testing (Cramer-von Mises) were tested as model validation tools.

Results: The data for all analytes was heteroscedastic, with variances increasing proportionally to x^2 so $1/x^2$ weighting was used. The partial F-test concluded that ten analytes were satisfactorily calibrated with a linear calibration function, while the remaining required a quadratic function. The ANOVA lack-of-fit test found that none of the chosen models could be validated (all $p \leq 9.9 \times 10^{-3}$) whereas this situation arose with only 7 analytes using Cramer-von Mises normality testing.

Conclusion / Discussion: The use of a simple F-test for heteroscedasticity and selection of an appropriate weighting is required for adequate least-squares fitting. Inclusion of a quadratic term is always tempting but only justified when testing shows the model's explained variance is significantly improved. Care must be exercised as tests involving residuals, or sum of squares, need adjustment to take into account the weighting factor. These modifications were applied to the partial F-test, ANOVA lack-of-fit and Cramer-von Mises normality testing. ANOVA lack-of-fit testing was expected to be useful but systematically declared the model as having an improper fit due to its sensitivity to experimental design (too many replicates in combination with too few calibrants). Instead, Cramer-von Mises normality testing for the weighted residuals was more robust and therefore recommended for validation. Analytes with an inadequate fit should not be validated and should be further investigated and modified (e.g. reduction of the dynamic range, extraction modification) before complete validation.

Examination of bias results confirmed the adequacy of the model chosen through heteroscedasticity and partial F tests. It also illustrated how an overfit can result from a choice of calibration model based on these values alone. The process used here is systematic, unbiased and allows simpler approach of the calibration model selection and validation.

Keywords: Calibration, SWGTOX, Validation, Linearity, ANOVA Lack-of-Fit, Weight, Quadratic, Heteroscedasticity, F-test, Residuals, Normality Testing

S-21

Application of Data-Mining to Forensic Casework in Identifying Methoxetamine, NBOME's and Pyrrolidinophenones

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Background: LC-TOF/MS provides a sensitive and specific technique for drug screening using high resolution mass and retention time data to identify analytes, by comparing them to a defined scope incorporated into a molecular weight and retention time database. LC-TOF/MS collects all scans during the analysis of a sample which can subsequently be data-mined to obtain analytical information about compounds that may not have been included in a targeted scope. Thus, if a new drug becomes a concern at a point in time based on new information about its use, data acquired prior to that knowledge can be retrospectively reprocessed to determine if it was present in historical samples.

Objective: This presentation describes the use of a data-mining technique to re-evaluate historical LC-TOF/MS data to retrospectively assess older data from samples that may have been discarded, for the presence of novel analytes.

Methods: In routine LC-TOF/MS, the identification of an unknown analyte relies on it being included in the scope of the TOF database, which can pose a challenge to forensic laboratories in having to continuously update the corresponding database. Each analyte in a TOF method needs to be calibrated and controlled for, to ensure proper identification due to retention time drift. To take advantage of the inclusive scanning capabilities of LC-TOF/MS without continuously adding costly reference standards to calibrator and control solutions, a data-mining database was developed by identifying retention times and accurate mass measurements for analytes of interest that were not considered of high enough prevalence to be included in routine testing. Processing run scans with the data-mining database or an alternate target drug compound list provides an opportunity to identify other emerging analytes of forensic interest.

Results: An example is presented where a data-mining database was successfully used to identify methoxetamine in an impaired driving case. The blood sample was submitted for routine analysis using an Agilent 6230 LC-TOF/MS with Mass Hunter software to screen for 232 target compounds. While the initial analysis was negative, based on case history and the presence of a non-target peak in the sample, the data file was reprocessed against an alternate database containing out-of-scope designer drugs of interest, and got a positive match to methoxetamine in addition to MDPV, based on accurate mass and retention time. The sample was forwarded for LCMSMS confirmation with a final result of 300 ng/mL methoxetamine. Methoxetamine is a novel psychoactive substance that is structurally related to ketamine, and is considered to have similar impairing effects. Other examples leading to the detection of NBOME compounds, and pyrrolidinophenones will also be presented.

Conclusions: The inclusive nature of LC-TOF/MS scans provides an opportunity to identify analytes of forensic interest that are not included in routine drug tests, but requires careful processing of data.

Keywords: LC-TOF, Data-Mining, Methoxetamine

S-22

The Role of the Forensic Toxicologist in a Pharmaceutical Drug Diversion Program

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Introduction: Pharmaceutical quality control and diversion are relevant issues in the forensic community, but these issues are not adequately addressed. Health care workers have a substance abuse incidence similar to the general public, but with a higher incidence of abuse of opioids and benzodiazepines. The forensic toxicologist may be involved from initial testing of pharmaceutical diversion samples, to pre-employment, and random or for cause urine drug testing of the health care workers. This drug testing may carry over into compliance testing so that the health care worker may maintain their licensure or accreditation. Our laboratory has been involved in a pharmaceutical quality assurance/diversion detection program for over 20 years. The program verifies the identity and concentration of pharmaceuticals prepared by the in-house pharmacies, post-use commercial pharmaceutical products, and pharmaceuticals obtained/discovered in non-controlled environments or from patients that have unexpected reactions. In addition to in-house samples, those from surrounding institutions are also analyzed.

Objective: After review of over 150,000 specimens over a 10 year period, we present 5 cases that represent the various aspects of the program. While these cases represent a small percentage of the total number of samples analyzed, they encompass the aberrant cases seen.

Method: Pharmaceutical preparations are submitted to the laboratory each month for analysis. These samples come from various pharmacies throughout the health system and area medical centers; they represent post-use (2/3) and compounded (1/3) samples. Over 120 samples per month are routinely analyzed by a previously published method for various anesthetic, analgesic and therapeutic agents, including fentanyl (1/3), opiates (1/3), midazolam (1/6) and miscellaneous drug(s) (1/6). “For cause” samples are also analyzed for electrolytes and other compounds, to verify that the samples have not been substituted with readily available solutions.

Results: The number of samples with suspected errors analyzed each month is low (< 5%), and fortunately, the number of samples with actual errors is very low (< 0.1%). The cases to be presented involve drug diversion, pharmacy preparation errors and non-pharmacy preparation errors, which are issues a forensic toxicologist can play a role in their outcome. The majority of suspected samples involve potential drug diversion of anesthetics, fentanyl, from high use areas such as operating rooms and maternity; or analgesics, morphine and hydromorphone from patient controlled analgesia (PCA).

Conclusion: The role of the forensic toxicologist in a pharmaceutical drug diversion program is important because there are many legal issues and implications that can arise from diversion of pharmaceuticals. Our program has shown the necessity to routinely analyze pharmaceutical preparations to identify potential diversion and compounding issues, in order to diminish or prevent issues that may have long standing legal implications for the medical center and health care workers.

Keywords: Prescription Drug Diversion, Prescription Drug Abuse, Pharmacy Preparation Error

S-23

Trends in Driving Under the Influence of Drugs in the Commonwealth of Virginia; Second Sample Drug Testing Program, 1990-2013

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Introduction: The Commonwealth of Virginia has unique statutes specifically addressing second sample independent testing (SSIT) of blood collected for driving under the influence of drugs (DUID). Statutes for SSIT have changed over the past 20 years. In SSIT, two vials of whole blood are collected from the suspect charged with DUID, the evidentiary vial is sent to and analyzed by the Commonwealth's Division of Forensic Sciences laboratory (DFS); the second vial is sent to and analyzed by the SSIT laboratory chosen by the defendant. Prior to July 2000, DFS performed testing on the evidentiary vial and notified the SSIT laboratory which drugs were detected. The SSIT lab analyzed the second vial for those drugs, whenever possible. Both the evidentiary and secondary vial results were mailed by the separate entities to the appropriate jurisdiction. For the period of July 2001 to July 2003, the statutes were changed to provide true independent testing. The SSIT laboratories screened and quantified DUID specimens under Virginia statutes. Beginning July 2003, the SSIT laws were revised to their present form. Now, both vials are sent to DFS and the defendant has 90 days to file a judicial motion for independent analysis by an SSIT laboratory of their choosing.

Objective: The forensic toxicology laboratory at the Virginia Commonwealth University (VCU) Health Systems present a survey of DUID drug findings in the Commonwealth of Virginia SSIT program from 1990 thru 2013, in order to identify any potential drug trends in impaired drivers in Virginia.

Method: We performed a comprehensive review of drug findings from the Virginia SSIT program DUID specimens. The review involved 3,291 specimens submitted from 1990 thru 2013. Toxicology findings were grouped in accordance with the time periods of the three different SSIT statutes.

Results: During the three time periods evaluated, marijuana was detected in 38%, 40% and 50% of SSIT specimens. The prevalence of cocaine and phencyclidine (PCP) was consistent at approximately 8% and 5%, respectively; prevalence of diazepam/nordiazepam declined from 11% to 6%, while alprazolam increased from 7% to 15% over the 23 years of this survey. Oxycodone findings increased from 0% prior to 1999 to 8% in 2013, while zolpidem increased from 0.1% to 15% over the same time period. Since 2000, 35% of the drug findings involved two or more drugs.

Discussion: After alcohol, marijuana remained the most commonly detected drug in DUID cases, which is similar to the latest published data on substance abuse in Virginia. The prevalence of other illicit drugs such as cocaine and PCP has remained constant. The greatest increase in specific drugs detected, was oxycodone and alprazolam which reflects their increased use in pain management. The most dramatic increase in drug prevalence was the sleep-aid, zolpidem which has become a major problem in impaired driving. Changes in drug prevalence corresponds more to the popularity or availability of a particular drug, than changes in the DUID statutes. Changes in the 'driving under the influence' statutes appear to have little effect on DUID findings.

Keywords: Driving Under the Influence (DUID), Drug Prevalence Data, Virginia

S-24

Evaluation of the Effectiveness of a Wash Procedure for the Removal of Methadone Spiked Drug-Free Hair Samples

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Background: The Society of Hair Testing (SoHT) recommends laboratories should investigate to what extent their wash procedure removes surface contamination, however no recognised protocol exists on how to achieve this. Contaminating hair with known quantities of drug is the first step in the process but with so many potential routes of external contamination: smoking, sweat, sebum, urine or through hand to hair contact, replicating these scenarios *in vivo* is a significant challenge. Many studies have been published that attempt to address this by exposing hair to smoke, soaking in solution or by rubbing drugs directly onto the surface of hair. The process of soaking drug-free hair in solution was chosen as an ideal model for evaluating the effectiveness of the in-house wash procedure as a known amount of drug is added and the soaking process is most likely to result in a homogenous contaminated sample.

Objective: To evaluate the effectiveness of the in-house wash procedure to remove methadone spiked drug-free hair samples.

Method: Nine drug-free hair samples were incubated in an aqueous solution of methadone equivalent to a concentration of 10 ng/mg for a period of 30 or 60 minutes. The hair samples were removed from solution and allowed to dry before washing two times with deionised water and then two times with dichloromethane. All four washes were analysed along with the hair sample utilising liquid chromatography tandem mass spectrometry following solid-phase extraction. The percentage of methadone recovered was calculated for each wash and the hair extract.

Results and Discussion: The increase in the incubation period did not result in an increase in the amount of drug contaminating the hair however there was significant variation in the total percentage of methadone recovered from the nine different hair samples ranging from 11.1 to 88.3%. The first dichloromethane wash was the most effective of the washes at removing methadone in eight of the nine hair samples whilst in four samples the amount of methadone recovered from the hair extract was much higher than that recovered from the individual washes. In contrast, in five samples the amount in a single wash (first dichloromethane) was higher than in the hair extract while in one sample the first water wash was more effective than the rest of the washes. The sample that had the highest amounts of methadone measured in the hair had been bleached while the sample with the least amount of methadone in the hair had not been treated.

Conclusion: Variations in hair porosity and hair treatments are the most likely explanation for the difference in the amount of drug contaminating the hair samples. The variation in recoveries indicates the likelihood of methadone being actively incorporated into the hair rather than simply contaminating the surface of the hair samples.

Keywords: External Contamination, Wash Protocol, Drug Incorporation

S-25

Psychomotor Effects in Occasional and Frequent Smokers Following Controlled Smoked Cannabis

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Introduction: Δ^9-Tetrahydrocannabinol (THC), the main psychoactive ingredient in cannabis, was the most prevalent illicit drug detected in injured drivers in Victoria, Australia (9.8%), and cannabinoids were found in 8.6% of nighttime drivers' blood and/or oral fluid in the 2007 US Roadside Survey. Cannabis impairs psychomotor performance, cognition, and driving ability in driving simulators and on-the-road driving tests, and is thus a concern for public safety.

Objective: To evaluate smoked cannabis' neuromotor effects in occasional and frequent smokers.

Method: Fourteen frequent (≥ 4x/week) and 11 occasional (< 2x/week) adult cannabis smokers (18 Males, 7 Females) provided written informed consent for this IRB-approved study. Participants entered the secure research unit approximately 19h prior to participation in the smoking session to preclude intoxication at the time of initial cannabis dosing. Participants smoked one 6.8% THC (54mg) cannabis cigarette. The "critical tracking task" (CTT) measured participant's ability to control a displayed error signal in a first-order compensatory tracking task. The point at which participants can no longer compensate for the error is the main performance measure (lambda-c). The "divided attention task" (DAT) measured participants' ability to divide attention between the CTT (set at 50% of their optimal performance) and monitoring the four screen corners for the number "2" among asynchronously changing numbers. Control losses, mean absolute tracking error (in mm), number of correct "2" detections (hits), false alarms (FA), and reaction time (RT) are the primary performance measures. Participants were trained before the study session to achieve stable task performance and minimize practice effects. The tasks were performed at baseline (1.75 h prior to dosing), and at 1.5, 3.5, 5.5, and 22.5h after smoking began. General Linear Model (GLM) repeated measures ANOVA was utilized to compare scores. Simple contrasts evaluated differences between each post-smoking time point and baseline.

Results: Occasional smokers had significantly more difficulty compensating for CTT tracking error compared to frequent smokers 1.5h after smoking. The DAT revealed that, compared to baseline, hits were significantly decreased (fewer "2" detections) and RT was significantly increased at 3.5h, independent of group; there was a trend towards increased RT at 1.5 and 5.5h. Time×group effects (indicating group differences) were present on DAT for tracking error at 1.5h (with frequent smokers having less and occasional smokers having more errors than their baselines), hits at 3.5h (with occasional smokers having fewer hits than frequent smokers), FA at 1.5h (with occasional smokers having more and frequent smokers having less than their baselines), and increased RT (with occasional smokers having delayed RT at 1.5 and 3.5h). Trends ($p<0.01$) were noted for decreased DAT hits in occasional smokers at 1.5 and 5.5h. No residual impairment was noted after 22.5h, as FA significantly decreased and control losses tended to be lower in occasional smokers at this time.

Conclusion: Cannabis impairs psychomotor function following controlled cannabis smoking, especially in occasional smokers, suggesting some tolerance to psychomotor impairment in frequent users. These data have implications for DUID cases, as drivers' ability to appropriately control a car can be impaired following cannabis smoking for up to 3.5h, as demonstrated in these psychomotor tasks.

Keywords: Cannabinoids, Psychomotor, Impairment, DUID

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S-26
The Changing Landscape of Marijuana Legislation and DUID Testing in Colorado

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Introduction: The state of Colorado is currently under scrutiny as the subject of what is effectively an ongoing experiment in progressive marijuana policy. This policy has progressed slowly, encompassing the better part of the last 15 years of recent history. In 2000 the use of medical marijuana was legalized in Colorado. On November 6, 2012, *Amendment 64* legalized the private consumption of cannabis. On May 28, 2013, a "permissible inference" for a whole blood THC concentration of 5ng/mL was passed. Most recently on January 1, 2014, recreational marijuana retail stores were opened to the public.

Objective: From January 2011 through February 2014, the results of testing whole blood samples collected from Colorado drivers suspected of DUID involving cannabis were analyzed for trends and changes in rates of testing and positivity for THC, along with limited demographics.

Method: All law enforcement cases (n=12,082) where whole blood samples were submitted for testing were examined and considered for this report. Submissions to the laboratory consist primarily of DUI/DUID cases, where all law enforcement samples are routinely analyzed for the presence of ethyl alcohol. Additional testing for other drugs is performed only upon request by the submitting agency. Of the cases where drug screens were performed (n=4,235), samples yielding a positive cannabinoid screen (n=2,621) were subjected to a quantitative THC confirmation, and the following demographic factors were determined: age and gender of subject, and time elapsed between time of stop and time of blood draw/collection. THC was confirmed in 1,848 cases. Due to the laboratory's decrease in its analytical limit of detection (LOD) for THC (from 2ng/mL to 1ng/mL), all samples confirmed at < 2ng/mL THC were not considered for this report. Following this adjustment, there were 1,598 samples confirmed for THC at ≥ 2 (LOD). The change in LOD was due to a change in instrumentation from gas chromatography-mass spectrometry (GC/MS) to liquid chromatography-tandem mass spectrometry (LC-MS/MS) in April 2013. The limit of quantitation (LOQ) also changed at the same time from 5ng/mL to 1ng/mL for THC. Samples which were confirmed as positive with a LOD of 2ng/mL but less than 5ng/mL (LOQ prior to April 2013) were included in the report (n=231) but were not included in any calculations.

Results: The overall positivity rate of cannabinoid screening, for all tested samples, remained consistent at ~ 64%. The percentage of cases with both positive cannabinoid screening and THC confirmation rose significantly from 2011 through 2013. Due to the limited data available for 2014, only data from the months of January and February were included along with the full annual data from years 2011 through 2013.

Total Number of Samples by Category and Year With Percentage of Samples Confirmed Positive for Δ9-tetrahydrocannabinol (THC) in Whole Blood Samples.

Time Period	LE Requests	CS	Positive Screens	Confirmed THC	% CS with Confirmed Positive THC
2011	1595	453	283	79	28%
2012	1691	485	324	190	59%
2013	7420	2774	1659	1076	65%
2014*	1376	523	355	253	71%
2011-2014 Total	**12082**	**4235**	**2621**	**1598**	**61%**
J & F 2011	223	56	35	12	34%
J & F 2012	220	57	37	22	59%
J & F 2013	379	135	85	61	72%
J & F 2014	1376	523	355	253	71%
J & F 2011-2014 Total	**2198**	**771**	**512**	**348**	**68%**

*2014 only January and February data included.

†† Abbreviations used: CS, Cannabinoid Screens; LE, Law Enforcement; Conf., Confirmation; J & F, January and February.

Total Number of Samples by Category and Year With Percentage of Samples Confirmed Positive for Δ9-tetrahydrocannabinol (THC) in Whole Blood Samples.

Time Period	LE Requests	CS	Positive Screens	Confirmed THC	% CS with Confirmed Positive THC
2011	192	108	64	25	39%
2012	204	87	58	40	69%
2013	238	117	86	68	79%
2014*	27	11	9	9	100%
2011-2014 Total	**661**	**323**	**217**	**142**	**65%**

*2014 only January and February data included

†† Abbreviations used: CS, Cannabinoid Screens; LE, Law Enforcement; Conf., Confirmation; J & F, January and February.

Conclusion: The mean and median THC concentrations for these cases were 8.1ng/mL and 6.3ng/mL, respectively (range of 2ng/mL to 192ng/mL, n=1367). Approximately 40% of all cases confirmed at ≥ 2ng/mL THC were also below the 5ng/mL current "permissible inference" THC level in Colorado. Of all subjects included in these cases, 87% were male, with a mean age for both male and female subjects of 22 years (range: 14-77 years). The median elapsed time between the stop and blood collection was 1.05 hours. The significant increase in DUID THC confirmations by a Colorado laboratory over the last 3+ years documented in this report is undoubtedly a finding of interest. Sufficient information is currently lacking to effectively interpret the specific cause of the significant increase seen in case submissions with positive THC confirmations. Data collected on DUID cannabis/THC cases in Colorado, offers valuable insight into the possible impact of changes in marijuana legislation on public and traffic safety.

Keywords: THC, DUID, Driving, Marijuana

S-27

Stability of Free and Glucuronidated Cannabinoids in Blood and Plasma Collected in Plastic Gray-Top Sodium Fluoride Tubes and Stored in Polypropylene Tubes Following Controlled Smoked Cannabis

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Introduction: Blood and plasma cannabinoid stability is important for test interpretation, and is best studied in authentic rather than fortified samples. No stability studies with authentic specimens after controlled cannabis smoking exist for gray-top sodium fluoride tubes, commonly employed in forensic drug testing.

Objective: Our objective was to assess cannabinoids stability in authentic blood and plasma specimens collected on ice in polyethylene gray-top sodium fluoride tubes, centrifuged (plasma) and stored in polypropylene tubes after controlled smoked cannabis.

Method: Low and high blood and plasma pools were created for each of three participants after smoking a cannabis cigarette within 2 h of collection and stored in polypropylene cryotubes. The stabilities of Δ9-Tetrahydrocannabinol (THC), 11-hydroxy-THC (11-OH-THC), 11-nor-9-carboxy-THC (THCCOOH), cannabidiol (CBD), cannabinol (CBN), THC-glucuronide, and THCCOOH-glucuronide were determined after 1 week at room temperature (RT), 1, 4, 12 and 26±2 weeks at 4˚C and 1, 4, 12, 26±2 and 52±4 weeks at -20˚C. Concentration changes > ±20% were considered unstable. Specimens were analyzed via our validated, published LC-MS/MS method with limits of quantification (LOQ) in blood and plasma of: 1, 1, 1, 1, 1, 0.5 and 5 µg/L for THC, 11-OH-THC, THCCOOH, CBD, CBN, THC-glucuronide and THCCOOH-glucuronide, respectively.

Results: Stability results are summarized in the table below.

Duration of acceptable storage stability for cannabinoids in authentic blood and plasma samples stored in gray-top tubes.

Analyte	Temperature	Blood	Plasma
THC	RT	1 week	1 week
	4˚C	12 weeks	12 weeks
	-20˚C	26 weeks	52 weeks
11-OH-THC	RT	1 week	1 week
	4˚C	26 weeks	26 weeks
	-20˚C	52 weeks	52 weeks
THCCOOH	RT	1 week	< 1 week
	4˚C	26 weeks	4 weeks
	-20˚C	52 weeks	52 weeks
THCCOOH-glucuronide	RT	< 1 week	< 1 week
	4˚C	1 week	1 week
	-20˚C	12 weeks	12 weeks

Stability could not be determined for THC-glucuronide, cannabidiol and cannabinol since initial concentrations exceeded LOQ in ≤ two participants.

Conclusion: Blood and plasma specimens should be stored at -20˚C for no more than 12 weeks to assure accurate THC, 11-OH-THC, THCCOOH and THCCOOH-glucuronide quantitative results. THCCOOH-glucuronide hydrolysis can confound analysis of unconjugated THCCOOH in blood and plasma if specimens are stored at 4 or -20˚C for more than 4 or 52 weeks, respectively. Laboratories quantifying THC in blood specimens should store specimens at -20˚C and analyze within 26 weeks; refrigerated storage requires analysis within 12 weeks for assuring accurate THC blood concentrations.

Keywords: Cannabis, THC, Glucuronides, Plasma, Whole Blood, Stability

This research was supported by the Intramural Research Program of the National Institute on Drug Abuse, National Institutes of Health.

S-28

Evaluation of 2-(4-iodo-2,5-dimethoxyphenyl)-N-[(2-methoxyphenyl)methyl]ethanamine (25I-NBOMe) in the Mouse Using a Battery of Behavioral Paradigms

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Introduction: Recently, the abuse of a new class of designer hallucinogenic drugs, dimethoxyphenyl-N-[(2-methoxyphenyl) methyl]ethanamine) (NBOMe) derivatives that are widely available via the internet have become a concern to public health and safety. In vivo testing has demonstrated that NBOMe derivatives are potent serotonin 5-HT2A receptor agonists. The 5-HT2A receptor has been closely linked to complex behaviors including working memory and cognitive processes. It is also implicated in the pathophysiology of affective disorders such as depression and schizophrenia. Stimulation of 5-HT2A receptors is responsible for the hallucinogenic effects of recreational drugs such as lysergic acid diethylamide (LSD). There is little to no data concerning the pharmacological, toxicological and behavioral effects of NBOMe derivatives. NBOMe derivatives are not suitable to administer to humans. However, data may be readily obtained in a battery of behavioral paradigms after NBOMe derivative administration to mice.

Objective: To evaluate the effects of 2 mg/kg (n=12) of 2-(4-iodo-2,5-dimethoxyphenyl)-N-[(2-methoxyphenyl)methyl]ethanamine (25I-NBOMe), the most popular abused NBOMe derivative, or vehicle (n=11) in subcutaneously injected mice using a battery of behavioral paradigms.

Method: The behaviors assessed included head-twitch response (HTR)**,** number of rears, body temperature, hyperreflexia, number of convulsions, time freezing and distance traveled. The HTR is a reliable way to distinguish hallucinogenic and non-hallucinogenic 5-HT2A agonist properties of drugs in rodents. Body temperature, hyperreflexia, and convulsions were evaluated due to clinical case reports indicating that NBOMe derivatives induce hyperthermia, agitation, bizarre behaviors and convulsions in human patients. To further characterize the behavioral effects of 25I-NBOMe, time freezing and distance traveled were measured. Videotapes from time of injection to 30 min post injection were scored for HTRs, rears and convulsions. Hyperreflexia and change in body temperature were evaluated 35 min post injection. Time freezing and distance traveled were determined in a 10 min test 40 min post injection. Experiments were approved by the Institutional Animal Care and Use Committee at Virginia Commonwealth University.

Results: 25I-NBOMe induced HTRs which peaked within the first 15 min of testing and decreased after this time point. Significant rearing and convulsions were found in all 25I-NBOMe treated mice. 25I-NBOMe also produced a slight increase in body temperature at 35 min but had no effect on distance traveled or time freezing.

Conclusion: The effects of 25I-NBOMe can be measured and evaluated using this battery of behavioral tests. Such tests can and have provided quantified data as to the relative potency and efficacy of classic hallucinogens. Additionally, somatic responses such as convulsions may indicate particular dangers of the abuse of NBOMe derivatives.

Keywords: 25I-NBOMe, Designer Hallucinogens, Behavioral Paradigms, Mice

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S-29
Etizolam: There's a New Benzodiazepine in Town

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Introduction: Etizolam is a benzodiazepine analog that has been used as a sedative-hypnotic drug in Asian and European countries since 1983. It is prescribed in 0.5-1 mg tablets that are to be taken orally, with total daily doses ranging from 0.5 to 3 mg. Currently, this drug is not scheduled by the DEA and is only available online in the United States. In the past year, Etizolam was detected in two cases in Los Angeles County, which the authors believe are the first postmortem cases involving this drug in the United States.

Objective: To introduce viewers to a new "legal" benzodiazepine and present what the authors believe are the first two postmortem cases in the United States where Etizolam was detected.

Method: Etizolam was recovered by a basic Toluene liquid/liquid extraction, followed by a solid phase extraction using United Chemical Technologies (UCT) Clean-Up® Silica columns and detection by GC/ECD. A method validation was performed using a standard obtained from Cerilliant® and SWGTOX guidelines, in which Etizolam was integrated into a currently existing method that the laboratory uses for analyzing other halogenated benzodiazepines.

Results: Etizolam was successfully validated and quantitated in two postmortem cases that Los Angeles County encountered in the past year. In regards to cross-reactivity, 2.5 ng/ml Etizolam was equivalent to a 5.0 ng/ml Oxazepam standard utilizing an Immunalysis Benzodiazepine kit. In an LC/MS/MS analysis, the 343 precursor ion was shared with Triazolam, as were product ions 239, 308, and 315. However, the two drugs were able to be distinguished by the retention time. Co-elution with Triazolam was also evident utilizing a GC/ECD, but they could not be separated. The first postmortem case encountered by the laboratory involved an accidental multiple drug intoxication including Etizolam, and had heart blood, femoral blood, and vitreous levels of 549, 351, and 60 ng/ml, respectively. The second case was also an accidental multiple drug intoxication, with heart blood and femoral blood Etizolam levels of 30 and 16 ng/ml, respectively.

Conclusion: Etizolam is a benzodiazepine analog that has been added to the ever-changing list of legal highs that can be purchased online in the United States. Benzodiazepines alone do not often cause death due to their high therapeutic indices; however, they can have a contributory role in the presence of other drugs, such as the two cases experienced by Los Angeles County. Viewers of this presentation will gain awareness that Etizolam has arrived in the United States and be able to apply this information to their own laboratories.

Keywords: Etizolam, Benzodiazepine Analog, Postmortem Toxicology

S-30
Examination of a Rare Suicidal Overdose Involving Milnacipran

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Introduction: Milnacipran is a serotonin/norepinephrine reuptake inhibitor (SNRI) indicated for the treatment of depression or fibromyalgia. The therapeutic range of milnacipran in blood is judged to be about 0.1 to 0.5 mg/L. Unlike its counterparts, venlafaxine and duloxetine, it is considered to be more potent and effective since it possesses nearly equal inhibitory activity at serotonin and norepinephrine transporters. It appears to be inactive at dopamine transporters, which is consistent with its low abuse potential. This presentation addresses a rare occurrence of intentional overdose by the use of milnacipran. Only one other report discusses a direct fatality involving milnacipran, with a blood concentration of 21 mg/L. Other articles have described acute toxicity from deliberate overdoses, but the patients recovered without requiring aggressive medical treatment or developing subsequent damage. We report our experience with a fatal intoxication from milnacipran, at a blood concentration of 40 mg/L, which is twice that of the other report and ten times higher than expected steady-state therapeutic concentrations.

Objective: To alert the forensic toxicology community about an intentional overdose case involving milnacipran at a higher blood concentration than has previously been reported.

Method: Alkaline-extracted GC/MS analysis on heart blood revealed an unknown peak characterized by a 246 m/z parent mass with a dominant 204 m/z base peak. Further studies using liquid chromatography time-of-flight mass spectrometry (LC-TOF/MS) showed the protonated accurate mass to be 247.1878 m/z, while liquid chromatography tandem mass spectrometry (LC-MS/MS) transition ions of 247 m/z to 230 m/z and 247 m/z to 100 m/z aided in the confirmation of milnacipran.

Results: In this report, we discuss the case of a 33 year-old female who exhibited a history of suicidal and accidental overdoses from prescription medications. Family members discovered her body upon their return from a shopping trip. Pathological examinations were unremarkable besides obesity, but the usually associated cardiac complications were not observed. Traditional toxicology assays revealed low therapeutic levels of ibuprofen, metaxalone, and pregabalin. After identifying milnacipran by mass spectrometry screens, tandem mass spectrometry was used to confirm the relative concentrations in blood and gastric contents, which were consistent with suicide:

Femoral blood	40 mg/L
Gastric contents	379 mg/L

Conclusion: Milnacipran is considered safer than tricyclic antidepressants (TCAs) and the newer selective serotonin reuptake inhibitors (SSRIs) as it is well tolerated, its metabolites are pharmacologically inactive, and does not undergo significant metabolism by the cytochrome P450 system. Our finding is particularly interesting since milnacipran has few adverse effects reported during therapeutic administration. It is believed that the mechanism of milnacipran toxicity is based on the development of neuroleptic malignant serotonin syndrome, which may include agitation, hallucinations, and coma, as well as cardiovascular disturbances such as tachycardia, hypertension, and hyperthermia. While reports show that others have survived toxic levels of milnacipran, this case highlights a rare fatality whereas scant information exists on this subject. As SNRIs gain more popularity and widespread use, these events may become more prominent. The lessons learned from this presentation will raise awareness about the methods to identify and measure milnacipran, including interpretive support for postmortem toxicology consultations.

Keywords: Milnacipran, Suicide, Overdose, Postmortem

S-31
Loperamide Related Deaths in North Carolina

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Introduction: Loperamide (Imodium®) has been accepted as a safe and effective over-the-counter anti-diarrheal drug with low potential for abuse. It is a potent mu-opioid receptor agonist however, when taken as advised, the drug has little CNS activity with most of the drug not reaching systemic circulation due to its poor bioavailability. The recommended dosing should not exceed 8 mg per day, although users seeking an opioid-like high from this drug reportedly take it in excess of 200 mg per dose. Since late 2012, the North Carolina Office of the Chief Medical Examiner has seen several cases involving loperamide.

Objective: In eight cases involving the abuse of loperamide, pathologists recognized the drug as being primary or additive to the cause of death. These data, along with informative case studies and two cases with concentrations consistent with natural causes, will provide the toxicology community with a better understanding of loperamide found in postmortem cases.

Method: When taken as prescribed, loperamide will typically not be detected in the routine GC/MS-NPD organic base screen performed in our laboratory. When taken in excess, loperamide and its metabolite will be detected late in the chromatogram after trazodone and is then confirmed using LC/MS/MS.

Results:

Manner	Case #	Central Blood (mg/L)	Peripheral Blood (mg/L)	Liver (mg/kg)
Natural (n=2)	1	0.066	0.034	0.55
	2	0.019	0.004	0.15
***Drug Overdose (n=8)**	3	0.72	0.54	20
	4	0.33	0.41	0.83
	5	0.8	0.5	12
	6	0.34	0.17	4.6
	7	1.8	n/a	34
	8	0.39	0.42	8.3
	9	n/a	0.89	2.5
	10	n/a	n/a	6.5

n/a= not available; *Single or multidrug toxicity

Conclusion: The recreational use of loperamide is on the rise in North Carolina. As a result, our laboratory has developed an LC/MS/MS method for quantitation whenever loperamide is detected on the routine basic organic screen. Since loperamide only produces an opioid-like high in extremely large doses, the presence of numerous empty bottles at the scene or a significant quantity of pills in the decedent's stomach does not necessarily indicate that the manner of death is suicide. As a result, caution should be exercised when investigating and interpreting deaths involving loperamide.

Keywords: Loperamide, Postmortem, LC/MS/MS

S-32

Determination of Methylone, Butylone, alpha-PVP, MDPV, and Naphyrone in Blood by LC-MS/MS

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Introduction: The Khat plant from which cathinone is derived has been a staple in East Africa and southern Arabia and is used by the indigenous people on a daily basis. Cathinone in the United States however was branded a Schedule I drug because of its mind altering effects and has steadily become a mainstream drug of abuse. The Miami-Dade Medical Examiner Department (MDME) has seen a rise in cases involving synthetic cathinones requiring the need to develop a validated comprehensive quantitative procedure in blood. The method developed includes the cathinones most commonly observed in our cases: methylone, butylone, alpha-PVP, MDPV, and naphyrone.

Objective: The purpose of this research was to develop and validate a quantitative method for five commonly seen cathinones using LC-MS/MS and apply this method to the analysis of approximately 80 positive cases in blood from 2011 to 2014. During this time period, the Medical Examiner Department has seen a shift from exclusively methylone cases to the current trend of alpha-PVP as drug scheduling laws have evolved.

Method: Using SWGTOX guidelines a method was developed and validated for the quantitation of five synthetic cathinones. Prior to this method, all positive cathinone cases received by the laboratory were analyzed qualitatively. Blood samples were collected either during hospital admission or at the time of autopsy and stored in tubes containing both a preservative and anti-coagulant. Biological specimens were extracted by mixed-mode solid phase extraction using a positive pressure manifold followed by analysis using a Varian model 320 TQ LC/MS. The mobile phase, 2 mM ammonium formate in water and acetonitrile (80:20), at a 0.5 uL/min flow rate employed a gradient to separate on a Zorbax C18 column. Positive mode electrospray ionization-MS analysis was performed using multiple-reaction monitoring (MRM) mode. One principal MRM transition was used for quantitation and two additional transitions to serve as a qualifier for each analyte.

Results: The linear range for each analyte was determined to be 25-1000 ng/mL with regression models weighted by a factor of 1/x. All R^2 values were a minimum of 0.990. The LLOQ is 25 ng/mL and the LOD is 10 ng/mL. The bias, within-run precision and between-run precision for each analyte met the criteria for validation (+/-20%) in terms of accuracy, repeatability, and precision as calculated at three different control concentration levels (40 ng/mL, 200 ng/mL, and 750 ng/mL). Matrix effects, extraction recovery, and process efficiency were evaluated for all drugs and were deemed acceptable by SWGTOX guidelines. Endogenous interferences from different blood sources and interferences from commonly encountered analytes were assessed and found to be insignificant. The extraction was successfully applied to case blood samples with high recovery and minimal matrix effects.

Conclusion: A quantitative method was developed for the analysis of methylone, butylone, alpha-PVP, MDPV, and naphyrone in blood. A linear range of 25-1000 ng/mL was used to evaluate blood levels in cases that ranged in concentration from less than 25 ng/mL to greater than 1000 ng/mL.

Keywords: Methylone, Butylone, alpha-PVP, MDPV, Naphyrone, LC-MS/MS

S-33

Ethyl Glucuronide and Phosphatidylethanol as Brain Biomarkers for Determining Chronic Alcohol Use

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Introduction: The direct measurement of ethanol is the most commonly used measure for alcohol use but the short detection window limits its ability to distinguish between acute and chronic use. Additionally, in the post mortem setting, objective information concerning the decedent's alcohol consumption history may be limited. The detection of the long-term direct alcohol biomarkers, EtG and PEth, in brain tissue may provide objective post-mortem data for alcohol consumption classification.

Objective: The objective of this study was to determine if human brain was a suitable specimen type for the detection of the objective long-term alcohol biomarkers EtG and PEth in a postmortem setting.

Method: Homogenates of brain tissue (1g) in 3mL of acetone were prepared using a BulletBlender® – Homogenizer Cell Disruptor. The homogenates were subjected to SPE and the eluates were evaporated under a stream of nitrogen. The extracts were reconstituted in 0.5mL of mobile phase and separation was achieved with an Agilent Zorbax Eclipse Plus (50mm x 2.1mm, 1.8mm particle size) C-8 column and a Phenomenex Synergi Polar RP (50mm x 2.0mm, 2.5mm particle size) C-18 column for PEth and EtG, respectively. The PEth MSMS method assayed a single isoform of PEth (palmitoyl/oleoyl) by monitoring the *m/z* 702.0 > 281.3 and *m/z* 702.0 > 255.3 transitions for PEth and the *m/z* 733.0 > 281.4 transition for its internal standard PEth -d_{31}. EtG was monitored using the *m/z* 221.1 > 75.0 and *m/z* 221.1 > 85.0 and the *m/z* 226.1 > 75.0 and *m/z* 226.1 > 85.0 for its internal standard EtG -d_5. The study comprised of 36 samples of brain tissue from the Brain Endowment Bank™ (Miami, FL) where next-of-kin provided informed consent. Of the subjects selected, 12 subjects were reported to be social drinkers and 24 chronic alcoholics. The social drinkers were selected based on negative BAC at autopsy, next of kin interviews, medical records, and negative liver pathology. The chronic alcoholics were selected based on documented history and alcoholism as a contributing factor to death. Of the 24 chronic alcoholics, 12 cases had measureable BAC at autopsy.

Results: The limits of detection for PEth and EtG were 2ng/g and 1ng/g and the limits of quantitation were 4ng/g and 3ng/g, respectively. The intra- and inter-assay imprecision and bias were < 14.7%. Both assays were linear up to 200ng/g. The mean measured concentrations of EtG and PEth were significantly different between the social drinkers (12.91 ± 4.76 ng/g and 301.9 ± 127.6 ng/g, respectively) and the alcoholics (318.6 ± 87.6 ng/g and 3013.4 ± 683.7 ng/g, respectively).

Conclusion: This study demonstrated that brain tissue is a suitable specimen type for detection of the long-term direct alcohol biomarkers EtG and PEth. Additionally, the results presented here suggest that the detection of EtG and PEth in post mortem brain tissue provides objective data concerning the previous drinking history of the decedent especially in cases where the BAC are negative.

Keywords: Ethyl Glucuronide; EtG; Phosphatidylethanol; PEth; Brain Tissue; Chronic Alcohol Use

S-34

Citalopram / Escitalopram: A Cautionary Tale of Enantiomers in Postmortem Forensic Toxicology

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Introduction: The complete investigation of the cause or causes of sudden death is an important civic responsibility. Establishing the cause of death rests with the medical examiner, coroner, or pathologist, but success or failure in arriving at the correct conclusion frequently is dependent upon the combined efforts of the pathologist and toxicologist. Case history and initial autopsy findings: **t**he decedent was a 92 year-old white male five feet-four inches in height and weighing 189 pounds and was under hospice/nursing home care. In addition to necessary physical care, the visiting nurse also administered his prescribed medication. The decedent was found dead in bed at his home in the morning by his visiting nurse. He had a medical history of, but not limited to; hypertension, coronary artery disease, osteomyelitis, hyperlipidemia, chronic renal failure and dementia. An autopsy was performed on the decedent approximately five days after his death. The autopsy disclosed the following significant pathology: 100% occlusion of the left anterior descending artery, severe aortic atherosclerosis, myocardial fibrosis, dilated cardiomyopathy and aspiration pneumonia. The initial cause of death was determined to be "atherosclerotic cardiovascular disease" and the manner of death ruled "natural causes".

Objective: To make toxicologists aware of the potential dire consequences of the failure to understand, consider or communicate the significance of stereoisomers in postmortem toxicology. A racemic mixture is not a single compound, but two different compounds, stereoisomers/enantiomers. Enantiomers have the same physical and chemical properties; but, due to structural difference in space, usually exhibit different physiological or pharmacological effects or intensity of effects.

Method: The postmortem toxicology findings from iliac blood collected at autopsy were as follows: total morphine, 0.43 mg/L and citalopram, 0.87 mg/L. The laboratory applied a gas chromatographic -NPD method using an HP 5MS 25 m x 0.25 mm x 0.25 μm achiral column that did not resolve citalopram from escitalopram. No additional prescription drugs or drugs of abuse were detected. Vitreous humor was negative for alcohols.

Results: The decedent had been prescribed among other drugs, morphine for pain and escitalopram (tradename, Lexapro) for depression. Morphine was determined not to contribute to his death. However, citalopram was deemed to be present at a lethal concentration. Based upon these findings the cause of death was amended to "citalopram poisoning" even though the concentration was within the generally accepted therapeutic range. It was noted that the decedent was prescribed only escitalopram (Lexapro) and not citalopram (tradename, Celexa). It was concluded he was administered a lethal dose of a drug which he was not prescribed. Therefore, the manner of death changed from "natural causes" to "homicide". The decedents' son was prescribed citalopram daily. Following an extensive police investigation the son was suspected of murdering his father. While a homicide indictment was in preparation, the author was contacted and resolved the issue. The Death Certificate was again amended to its original status and no charges were filed.

Conclusion: The Medical Examiner was unaware that the toxicology laboratory applied testing methods that could not separate citalopram and escitalopram. The toxicology report did not asterisk the citalopram or even report "citalopram/escitalopram" as one line item knowing they could not differentiate them.

Keywords: Enantiomers, Citalopram, Escitalopram, Postmortem Interpretation

S-35

Deadly Spring Cleaning: An Investigative Approach to Identifying Inhalants in Postmortem Specimens

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Introduction: Volatile organic compounds (VOCs) are commonly used in compressed air duster products, as well as in solvents and other commercial and household products. Identified as a cheap and legal alternative to illicit drug use, adolescents and young adults are abusing these VOCs in the form of inhalants, and as a result may experience severe toxicity or death. The postmortem investigation of cases involving huffing of VOCs and inhalants is a multifaceted approach requiring open communication between law enforcement, first responders, death investigators, pathologists, and toxicologists; without this approach, the cause and manner of death in huffing cases may be incorrectly determined.

Objective: The objective of this presentation is to offer background information as it relates to huffing VOCs and inhalants and to present case studies illustrating the information necessary for assisting in the determination of cause and manner of death. The analytical difficulties in identifying and confirming VOCs and inhalants by automated SPME-GC-MS, as well as demographic trends of deaths involving huffing in the State of Florida will be discussed.

Method: Routine toxicological analyses of postmortem biological fluids and tissues are unable to detect many abused VOCs; specialized extraction methods and instrumentation are necessary for specific detection. An automated SPME-GC-MS method utilizing a Bruker 450 gas chromatograph coupled with a CombiPAL autosampler and Varian 1200 mass spectrometer was developed to qualitatively detect over sixty low to moderate molecular weight VOCs. This method was optimized and validated to qualitatively detect commonly abused VOCs such as toluene, 1,1-difluoroethane (DFE) and 1,1,1,2-tetrafluoroethane (TFE) with a limit of detection of 5 mg/L. This full scan method utilizes an 85µm Carboxen-PDMS SPME fiber and a 60m x 0.25mm x 1.4µm Restek Rtx-VMS column with a 1.0 mL/min helium flow rate in the splitless mode. Case study information and demographic trends that will be presented were obtained from the Miami-Dade Medical Examiner Department and the Florida Department of Law Enforcement's annual statistical report, *Drugs in Deceased Persons*.

Results: Since 2006, the Miami-Dade Medical Examiner Department has had eighteen deaths involving VOCs and inhalants, most of which included DFE, nitrous oxide, methyl ethyl ketone (MEK), and helium. Throughout the State of Florida, a near 400% increase in the number of deaths attributed to huffing has been observed in the previous seven years. Inhalant abuse trends show that white, adolescent and young adult males abuse VOCs. In many cases, circumstances surrounding these inhalant-related deaths are similar, such as social and medical history of the deceased, autopsy findings, and the presence of huffing agents at the scene. In many instances, it was the initial postmortem investigation and on-going communication between agencies that led the laboratory to test for and ultimately identify the VOCs.

	Analyte	Detected	COD	Age
Case 1	DFE	X	X	14
Case 2	DFE	X	X	25
Case 3	DFE	X	X	34
Case 4	Multiple VOCs	X	X	27
Case 5	Nitrous Oxide		X	31
Case 6	DFE	X	X	37
Case 7	Helium		X	23
Case 8	Hydrogen Sulfide		X	50
Case 9	DFE	X	X	26
Case 10	Nitrous Oxide		X	35

	Analyte	Detected	COD	Age
Case 11	DFE	X	X	57
	MEK	X	X	
Case 12	DFE		X	27
Case 13	Nitrous Oxide		X	28
	MEK	X		
Case 14	DFE	X	X	26
Case 15	DFE	X	X	31
Case 16	Helium		X	56
Case 17	Helium		X	20
Case 18	Ethyl Chloride	X	X	39

Conclusion: A specialized SPME-GC-MS screening method was developed and validated to qualitatively detect commonly abused VOCs and inhalants in eighteen cases involving huffing at the Miami-Dade Medical Examiner Department. Despite the difficulties faced when testing for abused VOCs and inability to detect other abused inhalants, the open communication and sharing of information between all agencies involved has led to a proper determination with regard to cause and manner of death.

Keywords: Volatile Organic Compounds, Inhalants, Huffing, Postmortem Investigation of Death

S-36
Case Reports: Fatalities Associated with the Synthetic Cannabinoid, AB-PINACA

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Introduction: In 2014, a new synthetic cannabinoid, AB-PINACA, emerged in our toxicology casework. This cannabinoid is quite different structurally from previous generations of synthetic cannabinoids. Currently, the chemical is not controlled by the United States Federal Government though a closely related structural derivative, ADB-PINACA, is now a Schedule I compound. In January-April 2014, our laboratory quantitatively identified AB-PINACA in blood specimens in two non-related postmortem cases. No published literature exists on the detection of AB-PINACA in postmortem toxicology.

Objective: We present an analytical method for the quantitative detection of AB-PINACA in postmortem blood specimens and describe two postmortem cases, to include pertinent autopsy findings and decedent histories, together with the quantitative results for AB-PINACA determined in postmortem blood.

Method: Routine toxicological analyses for postmortem blood were completed for opiates/oxycodone by enzyme linked immunosorbent assay (ELISA), volatiles by headspace gas chromatography with flame ionization detection (GC-FID), and a comprehensive drug screen by liquid chromatography time of flight mass spectrometry (LC/ToF). Synthetic cannabinoid analysis was performed via liquid chromatography electrospray ionization tandem mass spectrometry (LC/MS/MS). For the synthetic cannabinoid analysis, specimens were extracted via a liquid-liquid extraction at pH 10.2 into hexane:ethyl acetate. The analytical method was validated according to in-house validation standard operating procedures, including accuracy and precision, carryover, and exogenous drug interferences. In addition to AB-PINACA, the directed synthetic cannabinoids assay scope consisted of the following analytes; 5F-AB-PINACA, 5F-ADB-PINACA, 5F-PB-22, AB-PINACA, ADB-PINACA, AM-2201, BB-22, Cl-2201, JWH-015, JWH-018, JWH-019, JWH-073, JWH-081, JWH-122, JWH-210, JWH-250, PB-22, UR-144 and XLR11.

Results: Case 1: A 19 year old male had been smoking a synthetic cannabinoid product for several hours. Observed behavior throughout the day included poor coordination, instability, slowed/deliberate movements, sedation, balance issues and occasional loss of consciousness. All of these cognitive and psychomotor effects have been observed in synthetic cannabinoid-related casework. None of the typically reported agitation, irritability, paranoia, or hallucinations was observed by the witnesses. In the evening and after a couple of hits from a mixed cannabis and synthetic cannabinoid product, he lost consciousness again and started snoring. Friends tried to wake him, but they were unsuccessful. Approximately 2.5 hours later, he was found unresponsive and not breathing. Autopsy blood toxicology findings were AB-PINACA (32.8 ng/mL), THC (1.1 ng/mL), THC-COOH (14.5 ng/mL), and ethanol (0.147%).
Case 2: A 37 year old male collapsed at a mobile home that law enforcement described as a "K2 house". Upon review of the scene, there were packages of synthetic cannabinoid related products strewn all over the floor. Packages included "Bizarro", "Black Diamond", "Brainfreeze", and "No Mames". Iliac blood toxicology findings were AB-PINACA (12.2 ng/mL) and ethanol (0.171%). Vitreous electrolytes and chemistries were unremarkable. AB-PINACA, along with other synthetic cannabinoids, was detected in four of the five product brands tested. The cause of death for both cases was ruled as synthetic cannabinoid-related.

Conclusion: AB-PINACA was an emerging federally uncontrolled novel synthetic cannabinoid in early 2014. This validated LC/MS/MS method proved to be accurate and reliable for the detection and quantitation of AB-PINACA in whole blood specimens. We detected AB-PINACA in two unrelated postmortem cases. With the scarcity of available published literature specifically on AB-PINACA, we believe these case reports will be a good addition to existing literature surrounding synthetic cannabinoids in postmortem toxicology.

Keywords: Synthetic Cannabinoids, Fatality, AB-PINACA, LC/MS/MS

S-37

Studies on the Metabolism and Detectability in Urine of Five Tryptamine-Derived Novel Psychoactive Substances (NPS) Using GC-MS, LC-MS^n, and LC-HR-MS/MS Techniques

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Introduction: Among the novel psychoactive substances (NPS), tryptamine derivatives are becoming more and more popular as hallucinogens. In contrast to some older drugs (e.g. 5-methoxy-*N*,*N*-dialkyltryptamine), only few data currently exist on the metabolic fate and detectability of the newer derivatives.

Objective: The aim of this study was to investigate the metabolic fate in rat urine and human liver microsomes (HLM) and detectability of five typical representatives of the new generation of tryptamine-derived drugs namely *N*,*N*-diallyltryptamine (DALT), 5-fluoro-DALT, 7-methyl-DALT, 5-methoxy-DALT, and 5,6-methylendioxy-DALT in urine, which either have been encountered on the street or are in anticipation to become drugs of abuse.

Method: Test substances were synthesized and provided by School of Pharmacy and Biomolecular Sciences, Liverpool John Moores University. Identity and purity were checked by GC-MS and LC-HR-MS/MS. Rat urine samples were collected over 24 h after administration of either 20 or 0.1 mg/kg BW each. For metabolism studies after high dose, urine samples were prepared either by protein precipitation or liquid-liquid extraction, with or without previous enzymatic conjugates cleavage. The underivatized and/or acetylated extracts were then analyzed by GC-MS (Agilent GC-MSD) or LC-HR-MS/MS (ThermoFisher Q-Exactive). For standard urine screening approach (SUSA) after low dose, representing anticipated common users doses, urine samples were prepared by acid hydrolysis, extraction, and acetylation for GC-MS or protein precipitation for LC-MS^n (ThermoFisher LC-LXQ) and LC-HR-MS/MS according to Wink et al., DTA, 2014. Incubation conditions for HLM were chosen according to Welter et al., ABC, 2013.

Results: According to the identified metabolites, *N*-dealkylation, aromatic and aliphatic multiple hydroxylation followed by glucuronidation and sulfation could be proposed as general metabolic pathways of the DALTs in rats. The main metabolic reactions were confirmed using HLM incubations. Because of the common, relative low doses, the tested NPS could not be detected by the GC-MS and LC-MS^n SUSAs, but in overdose cases, the above-mentioned metabolites should be detectable by both assays assuming similar metabolism and kinetics. However, the much more sensitive LC-HR-MS/MS allowed the detection of all tested NPS and/or their metabolites even after low doses.

Conclusions: The five DALT-derived tryptamines were extensively metabolized and thus the metabolites should be the targets for urinalysis. The authors' SUSAs using GC-MS and LC-MS^n did not allow proving the intake of commonly used low doses in contrast to the more sensitive LC-HR-MS/MS.

Keywords: Metabolism, Designer Drugs, GC-MS, LC-(HR)-MS, DALT Derivatives

S-38

Linear Pharmacokinetics of 3,4-Methylenedioxypyrovalerone (MDPV) and its Metabolites in the Rat: Relationship to Pharmacodynamic Effects

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Introduction: 3,4-Methylenedioxypyrovalerone (MDPV) is a commonly abused synthetic cathinone in the United States and is associated with dangerous side effects. MDPV is a dopamine transporter blocker that is 10-fold more potent than cocaine as a locomotor stimulant in rats. Previous *in vitro* and *in vivo* metabolism studies identified 3,4-dihydroxypyrovalerone (3,4-catechol-PV) and 4-hydroxy-3-methoxypyrovalerone (4-OH-3-MeO-PV) as the two primary MDPV metabolites. Knowledge of MDPV and its metabolites pharmacokinetics could provide clues to the drug's mechanism of action. Additionally, pharmacokinetic data with MDPV in animal models can aid forensic toxicologists in interpreting findings from urine, blood, oral fluid and hair specimens from human subjects exposed to MDPV.

Objective: Determine for the first time, MDPV and its metabolites pharmacokinetic profiles in rat and its relationship to pharmacodynamic effects.

Method: Groups of 7 rats received 0.5, 1 or 2 mg/kg subcutaneous (MDPV. Blood was collected by an indwelling jugular catheter before dosing and at 10, 20, 30, 60, 120, 240 and 480 min thereafter. Plasma specimens were analyzed by liquid chromatography coupled to high-resolution tandem mass spectrometry (Q-Exactive, Thermo Scientific, Fremont, CA). Plasma pharmacokinetic data were further analyzed with Phoenix/WinNonlin (version 6.3; Pharsight, Mountain View, CA, USA) to determine noncompartmental pharmacokinetic constants including maximum concentration (C_{max}), time of maximum concentration (T_{max}), area-under-the-curve (AUC), last measured concentration (C_{last}) and elimination half-life ($t_{1/2}$). Horizontal locomotor activity (HLA) and stereotypy were calculated over multiple 20-min time frames.

Results: C_{max} and AUC for MDPV and two metabolites increased proportionally with administered dose, showing linear pharmacokinetics. MDPV exhibited the highest C_{max} at all doses (74.2-271.3 µg/L) and 4-OH-3-MeOH-PV the highest AUC (11,366-47,724 min·µg/L), being the predominant metabolite. MDPV time to C_{max} (T_{max}) was 12.9 -18.6 min, while 3,4-catechol-PV and 4-OH-3-MeO-PV peaked later with T_{max} 188.6 - 240 min after subcutaneous dosing. HLA correlated positively with plasma MDPV concentrations ($R > 0.6$, $P < 0.025$), while HLA and stereotypy correlated negatively with MDPV metabolites.

Conclusion: MDPV showed linear pharmacokinetics in rats, and 4-OH-3-MeOH-PV was the predominant metabolite. Our results suggest that the parent compound mediates motor stimulation after systemic MDPV administration, but additionally, metabolites may be inhibitory, may not be active, or may not pass the blood brain barrier.

Keywords: MDPV, Synthetic Cathinone, Stereotypy, Locomotion, Metabolites

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S-39

Controlled / Unapproved Drugs in Dietary Supplements and Labeling Subterfuge—Statistics and a Call for Better Regulation

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Background: Under the Dietary Supplement Health and Education Act (DSHEA), products sold as dietary supplements are ineffectually regulated by the FDA. Consumers are forced to rely on label information and self-education. This largely unregulated market has generated a rampant problem of product adulteration, misuse of prescription pharmaceuticals, analogues in "natural" remedies, and the practice of incomplete and/or misleading labeling, such as using a lesser-known chemical alias in lieu of well-known names with the intention of deceiving the consumer. A comprehensive database of commercial dietary supplements was used to compile an overview of recent abuses by the supplement industry to increase awareness of these practices and to demonstrate the need for better regulation.

Objective: Provide data on recent abuses of the unregulated supplement industry by determining the number of frequently used banned substances and prescription medications found in commercially available dietary supplements and to provide statistics on the use of chemical aliases with the possible intent to deceive the consumer.

Method: A comprehensive dietary supplement database (The Aegis Shield®) was analyzed for the incidence of banned substances in a large population of commercial dietary supplements. Additionally, the incidence of prescription drug adulteration, including diuretics, anorexiants, erectile dysfunction medications (and analogues) was evaluated in this supplement population. Recent FDA recalls and consumer alerts were included in this compilation of data.

Results: Of 63,606 products in our database, 179 were found to contain hemp or hemp derivatives, 69 contain peptide hormones banned by various sporting agencies, 44 potentially contain IGF-1, 285 contain synephrine, 108 contain methylhexaneamine (DMAA), and 9 contain designer amphetamine-like compounds. Additionally, 68 products contain sibutramine, 91 contain prescription erectile dysfunction medications or their chemical analogues (e.g., sildenafil, tadalafil, and sulfoaildenafil), and 1 contains a prescription diuretic (triamterene). Chemical aliases are also routinely used to mislead consumers. For example, 82 chemical aliases can be used to describe DMAA, a powerful stimulant linked to a number of deaths in recent years, and 147 can be used to describe synephrine, a banned stimulant and anorexiant. Furthermore, "deer antler" is a common alias for IGF-1, "bitter orange" is a common alias for synephrine, and "geranium extract" is a common alias for DMAA. Other manufacturers may hide banned substances behind phrases such as "proprietary matrix," "proprietary substance," or trademarked names. In the Aegis Shield® database, there are 1,547 proprietary and unknown ingredients, accounting for 2.4% of the total ingredients in the database. It should be noted that the aforementioned ingredients were classified as unknown substances even after attempts to contact the manufacturers.

Conclusion: Data from the Aegis Shield® database on banned substances and commonly used chemical and trade aliases are provided with the intent of increasing awareness of the misleading practices discovered in the dietary supplement industry. This information is of use to the public and regulatory agencies working toward improving oversight of this industry.

Keywords: Dietary Supplements, Hemp, Diuretics, ED Drugs, Peptide Hormones, Sibutramine, DMAA

S-40

Incidence of Designer Steroids and Prohormones in Commercially Available Dietary Supplements

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Introduction: Anabolic androgenic steroids (AAS) were added to the U.S. Federal Schedule of Controlled Substances in 1990. However, with the passing of The Dietary Supplement Health and Education Act (DSHEA) in 1994, the sale of steroid precursors as dietary supplements became unregulated and supplement manufacturers continue to take advantage of this loophole. The U.S. government attempted to establish more rigorous regulations with approval of the Anabolic Steroid Control Act of 2004 (ASCA). The intent of ASCA was to limit availability and discourage use of AAS, but it inadvertently encouraged the clandestine synthesis of designer steroids and prohormones. These compounds are generally believed to be produced to avoid regulations and evade detection and identification by laboratories. Additionally, they are added to dietary supplements without efficacy, safety, or toxicity assessments. Athletes may inadvertently test positive for banned substances and there have been toxic reactions and deaths attributable to supplement use. Recently, the Designer Anabolic Steroid Control Act of 2014 (DASCA) was introduced to the U.S. Congress with the aim of closing the loophole in ASCA and giving authorities more power to regulate such products.

Objective: Determine the number of designer steroids and prohormones listed on the labels of commercially available dietary supplements for the purposes of increasing awareness of this practice and assisting regulatory agencies in identifying these manufacturers.

Method: Data was extracted from a database of approximately 63,600 dietary supplements and 2,813 ingredients (37,095 aliases) available for purchase in retail stores and on the internet. Each ingredient was evaluated and designer steroids/prohormones were included in the count. Pharmaceutical and over-the-counter medications were eliminated from the results.

Results: Of the 2,813 ingredients listed in the database, 165 (5.86%) were identified as AAS, designer steroids, prohormones, or structurally similar compounds; 79 are currently associated with commercial products. Glucocorticoids, progesterones, estrogens, phytosteroids, and ecdysteroids were not included, obviating the possibility of an even larger threat to athletes and supplement users. These 79 steroid/prohormone compounds were identified 581 times in 388 products, which accounts for 0.61% of the 63,606 products in the database. Many products were found to contain multiple compounds (up to 8 were found in some supplements). When atypical dietary supplement products (e.g., tea, flavored water, snack food) are eliminated, this accounts for 0.92% of the dietary supplements in the database (42,382).

Conclusion: The aim of this work is to provide more information about the widespread practice of designer steroid and prohormone production by supplement manufacturers. The Aegis Shield® database is one of the most comprehensive collections of consumer dietary supplement products, providing expert evaluation of the supplement label in layman's terms. Examination of this database has shown a significant number of products are commercially available containing steroidal compounds. It should be noted this analysis was limited to disclosed ingredients, and that inadvertent contamination due to poor manufacturing practices and deliberate adulteration with banned substances are rampant problems in the supplement industry. This information is of importance to regulatory agencies attempting to discourage and punish this practice.

Keywords: Dietary Supplements, Designer Steroids, Prohormones

S-41

LC-MS Screening for Illicit and Therapeutic Drugs in Equine Urine Using Solid Phase Extraction

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Introduction: To ensure therapeutic and illicit drug-free equine racing, regulatory authorities apply drug testing to equine samples. To ensure uniformity across jurisdictions, several regulatory agencies have implemented screening limit thresholds that must be met by testing laboratories. The use of enzyme-linked immunosorbent assays (ELISA) for screening of equine samples had been a staple of qualitative initial testing. ELISA can be effective since it can screen for different classes of compounds, but the constant release of new drugs and their metabolites can make its use laborious and expensive with the addition of extra plates. The limits-of-detection (LOD) can be too high for screening limit thresholds. The use of liquid chromatography tandem mass spectrometry triple quadrupole (LC-MS QQQ) allow laboratories to screen for many different classes of drugs using either one or two extractions at concentrations significantly lower than ELISA.

Objectives: The use of mixed mode solid phase extraction (SPE) columns from United Chemical Technologies (UCT) followed by an Agilent 6400 series LC-MS QQQ analysis to screen for over 180 compounds from such classes as opioids, bronchodilators, corticosteroids, non-steroidal anti-inflammatories, and anti-tussives was investigated.

Methods: Two milliliter aliquots of drug-free urine were fortified with the compounds of interest. Sample preparation included an overnight enzyme hydrolysis at 37°C. The pH of the samples was then adjusted to 6. The solid phase columns were conditioned with methanol and 100 mM phosphate buffer (pH 6). The sample was added, followed by a wash with 100 mM phosphate buffer (pH 6). The pH of the columns was lowered by adding 1 mL of 1 M acetic acid. Acidic drug elution was performed with 1 mL of ethyl acetate. Basic drug elution was performed using 1 mL of 78:20:2 of dichloromethane: isopropyl alcohol: ammonium hydroxide. The elutions were dried down in a water bath at 40°C under a stream of nitrogen. The samples were reconstituted in 100 µL of 1:1 0.2% formic acid in water: methanol. 25µL was injected for analysis by the LC-MS QQQ operating in positive electrospray ionization under dynamic multi-reaction monitoring (MRM) mode.

Results: The use of the UCT mixed mode column allowed successful extraction recovery of all the compounds of interest. The use of two elutions provided greater extraction efficiency of compounds with differing pKas. Extraction recoveries for the compounds of interest ranged from 5% to 98%. Matrix effect was also investigated and found to vary from -80% to 105%. LODs were investigated and ranged from 140 pg/mL to 25000 pg/mL for all compounds.

Conclusions: The use of UCT columns for qualitative screening for substances of abuse in equine racing was found to be successful. The LODs for the compounds of interest were found to be of sufficient and allowed for effective screening for the drugs of interest at typical therapeutic ranges.

K**eywords:** SPE, LC-MS-MS, Equine Urine

S-42

Utility of a Cocaine Extended Wash Kinetics Calculation on Extensively Washed Drug Chemists Hair

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Background / Introduction: Current practices of hair decontamination are diverse and not a mandatory part of cocaine hair testing guidelines. Although many researchers have discussed the potential utility of washes, few have adopted extensive washing practices and no consensus standards exist. Several studies have examined how effective various washing protocols are at removing cocaine. Since these studies deposited cocaine onto the exterior surface of cocaine-free hair, it is unclear if these cocaine contamination studies realistically mimic external exposure.

Objective: Examine the hair from drug chemists actively working with cocaine to realistically evaluate external exposure to cocaine. Evaluate the utility of a cocaine extended wash kinetics calculation by comparing the analysis of hair from drug chemists to a cocaine user (antemortem).

Methods: Hair samples from twenty-seven drug chemists and a cocaine user were weighed and washed with n-propanol followed by a series of five phosphate buffer washes. The last wash sample was saved for analysis. Hair samples were digested overnight. The washes and hair samples were extracted via solid phase extraction, quantitatively analyzed for cocaine (COC), benzoyl-ecgonine (BE), cocaethylene (CE), and norcocaine (NC), and qualitatively analyzed for aryl hydroxy cocaine metabolites (para, meta, and ortho). The cocaine extended wash kinetics calculation takes the amount of cocaine in the final wash, multiply that value by 5 and subtract this value from the amount of cocaine measured in the hair sample. The final COC in hair after this calculation is annotated as COC_{final}.

Results: Forty percent of washes from Drug chemists contained COC and 15% contained BE but no other cocaine metabolites. Eight of the hair samples from Drug chemists contained COC (four $\geq$ 500 pg/mg), BE $\geq$ 100 pg/mg, and a BE/COC ratio from 0.24 to 0.73. None of the Drug chemists' hair contained NC, CE, or any of the hydroxycocaine metabolites. The four Drug chemists COCfinal were $\leq$ 500 pg/mg after the application of the cocaine extended wash kinetics calculation.
Hair washes from a cocaine user contained only COC and BE. The first three hair segments contained COC ranging from 1300 to 4270 pg/mg, had a COC_{final} ranging from 1040 to 3730 pg/mg, and were positive for BE, CE, NC, para hydroxycocaine, and meta hydroxycocaine.

Conclusion / Discussion: Without thoroughly washing hair samples, implementing a cocaine extended wash kinetics calculation, and analyzing the hair for cocaine metabolites other than BE, it is nearly impossible to differentiate passive exposure to cocaine from active use of cocaine. The lack of implementing these procedures may inadvertently cause a drug chemist to be mislabeled as either a cocaine user or someone who is chronically exposed to cocaine. However, drug chemists and others who have legitimate contact with cocaine in the course of their profession will not be identified as cocaine positive when a cocaine extended wash kinetics calculation and additional guidelines are applied.

Keywords: Cocaine, Hair, Wash Criteria, Liquid Chromatography-Tandem Mass Spectrometry

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The Major Significance of Minor Cocaine Metabolites (Aryl Hydroxycocaines) in Cocaine Users' Hair

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Background / Introduction: External contamination studies have suggested that cocaine positive hair results could be obtained from contaminated hair samples. It is unclear if passive exposure to cocaine would mimic these studies. If the detection of minor cocaine metabolites could be used to differentiate passive exposure to cocaine from active cocaine use, the results may have considerable forensic value.

Objective: To determine if cocaine user's hair could be differentiated from those exposed to cocaine in the course of their daily work.

Methods: Hair samples from nineteen drug users (postmortem; 15 known cocaine users), one antemortem cocaine user, and twenty-seven drug chemists were weighed and washed with n-propanol followed by a series of five phosphate buffer washes. The last wash sample was saved for analysis. Hair samples were digested overnight. The washes and hair samples were extracted via solid phase extraction. Quantitative analysis for COC, benzoylecgonine (BE), cocaethylene (CE), and norcocaine (NC) was by LC/MS/MS using a C8 column. Qualitatively analysis for aryl hydroxycocaine metabolites was by LC/MS/MS using a C6 phenyl column; the isomers separated chomatographically (para then meta then ortho). The cocaine extended wash kinetics calculation takes the amount of cocaine in the final wash, multiply that value by 5 and subtract this value from the amount of cocaine measured in the hair sample. The final COC in hair after this calculation is annotated as COC_{final}.

Results: No hydroxycocaine metabolites were detected in any of the hair washes (n=47). Hydroxycocaine data from postmortem cocaine user's hair were as follows: thirteen were positive for para, fourteen were positive for meta, and six were positive for ortho. The antemortem cocaine user's hair contained para and meta hydroxycocaine in the three most proximal 2-cm segments. None of the Drug Chemists' hair contained any of the hydroxycocaine metabolites.

Conclusion / Discussion: Since benzoylecgonine, cocaethylene, and norcocaine may be present in street cocaine samples, their detection cannot be used to ultimately distinguish cocaine use from cocaine exposure. Although meta hydroxycocaine has been identified as a cocaine impurity, on the order of 0.01%, para and ortho hydroxycocaine have not. When para hydroxycocaine was identified, samples contained uncorrected COC > 1250 pg/mg and either COC_{final} < 500 pg/mg (gross external contamination) or > 1000 pg/mg. Meta hydroxycocaine was identified in all of the cases that contained para hydroxycocaine. When ortho hydroxycocaine was identified, samples contained uncorrected COC > 4750 pg/mg and either COC_{final} < 500 pg/mg (gross external contamination) or > 3750 pg/mg. Since only chronic cocaine users' hair contained aryl hydroxycocaine metabolites, their detection can be used to differentiate cocaine use from cocaine exposure.

Keywords: Cocaine, Hair, Aryl-Hydroxycocaines, Liquid Chromatography Tandem Mass Spectrometry

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Draeger® DrugTest 5000 On-Site Oral Fluid Cannabinoid Screening Performance After Cannabis Vaporization

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Background: Oral fluid (OF) is an advantageous sampling matrix for drug screening due to ease of collection, non-invasiveness, and facility for on-site testing. On-site screening tests for cannabis exposure are increasingly common, but limited data exist for on-site OF devices following vaporized cannabis inhalation, despite its growing popularity as a smoking alternative.

Objective: To evaluate the Draeger® DrugTest 5000 (DDT-5000, Lübeck, Germany) on-site OF screening device for cannabis detection following inhalation via vaporizer.

Methods: Current occasional (≥1x/last 3 months, ≤3 days/week) cannabis smokers provided written informed consent and OF specimens for this IRB-approved controlled cannabis administration study. Participants received placebo or low-dose alcohol (approximately 0.067% peak BAC) and orally inhaled 500 mg placebo, low (2.9%)-Δ^9-tetrahydrocannabinol [THC], or high (6.7%)-THC cannabis (Volcano® Medic vaporizer, Storz & Bickel, Tuttlingen, Germany). OF specimens were collected prior to and 0.17, 1.43, 2.33, 3.33, 4.33, 5.33, 6.33, 7.33, and 8.33 h post-dose, and analyzed with the DDT-5000 at 5 µg/L THC screening cutoff. Paired confirmatory OF specimens were obtained with the Quantisal™ collection device (Immunalysis, Pomona, CA) and quantified for THC, metabolite 11-nor-9-carboxy-THC (THCCOOH), cannabidiol and cannabinol (respective limits of quantification 0.5 µg/L, 15 ng/L, 1 and 1 µg/L) by a published validated method. DDT-5000 diagnostic sensitivity (100*true positives [TP]/(TP+false negatives [FN])), specificity (100*true negatives [TN]/(TN+false positives [FP])), and efficiency (100*(TN+TP)/(TN+TP+FN+FP)) were calculated for different confirmation cutoffs. Low- vs. high-THC times of last detection (t_{last}) were compared for different screening/confirmation cutoffs via Mann-Whitney *U* Test. Fisher's Exact Test was utilized to compare diagnostic Draeger performance in the presence and absence of alcohol (measured in breath), at baseline and up to 4.3 h post-inhalation.

Results: Overall, 1657 OF screening-quantification specimen pairs from 43 smokers (26 M, 17 F, ages 21-42) were compared. DDT-5000 diagnostic performance was as follows for various confirmation cutoffs:

Quantitative Confirmation Cutoffs µg/L (THC) ng/L (THCCOOH)	Sensitivity %	Specificity %	Efficiency %	Median [Range] t_{last} (h) Low[a], High[b]	*p*-value (Low vs. High)
THC ≥5	61.4	98.1	82.5	3.33[a,b] [0.17-≥8.33]	ns
THC ≥2	48	99.6	70.4	3.33[a,b] [0.17-≥8.33]	ns
THC ≥1	41.4	99.8	61.4	3.33[a,b] [0.17-≥8.33]	ns
THC ≥2+THCCOOH ≥20	49.5	81.4	72.4	3.33[a] [0.17-≥8.33] 3.83[b] [1.43-≥8.33]	ns
THC ≥1+THCCOOH ≥20	45.6	80.8	70.1	3.33[a] [0.17-≥8.33] 3.83[b] [1.43-≥8.33]	ns

The presence of alcohol did not affect Draeger performance for cannabinoid detection.

Conclusions: The DDT-5000 demonstrated good specificity and efficiency for OF obtained after cannabis vaporization, but sensitivity was lower than after smoking a cannabis cigarette with the same THC potency (sensitivity 90.7% at THC≥2, Desrosiers *Clin Chem* 2012). Volatilization by hot air is a different heating mechanism than combustion, altering the properties of inhaled vapor versus smoke. Draeger collection involves moving the device throughout the entire mouth, whereas Quantisal devices are held sublingually. These and other factors may contribute to the observed sensitivity differences relative to smoking. Median t_{last} was between 3-4 h for evaluated cutoffs, but for all cutoffs some specimens were positive for at least 8.33 h.

Keywords: Vaporizer, Oral Fluid, Draeger, On-Site, Efficiency

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Comparison of Laboratory Based Oral Fluid Results with Field Based Screening Results Using the Alere DDS2 Mobile System and Dräger DrugTest 5000

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Background / Introduction: Oral fluid is gaining popularity as a specimen in drug impaired driving investigations. Ease of collection and minimal potential for adulteration make it an ideal specimen for point-of-contact drug testing. Options for on-site screening devices have rapidly proliferated, with several devices available to law enforcement, often without validation or effectiveness data provided.

Objective: The objective of this study was to compare the performance of two instrumented point-of-contact oral fluid devices and determine the extent to which their findings could be confirmed using laboratory-based liquid chromatography/tandem mass spectrometry (LCMSMS) and gas chromatography mass spectrometry (GCMS) methods.

Methods: Four sites in California (Fullerton, Los Angeles, Bakersfield and Sacramento) were selected for this assessment and assigned by the California Office of Traffic Safety (OTS) one of two oral fluid drug screening devices, the Dräger DrugTest 5000® and the Alere DDS® 2 Mobile System. Both devices target common drugs of abuse including amphetamine, methamphetamine, cocaine, benzodiazepines, opiates, THC, and methadone (Dräger device only). The Dräger device positive cutoff concentrations ranged from 5 ng/mL for THC to 50 ng/mL for amphetamine. In comparison, the Alere DDS® 2 Mobile System ranged between 25 ng/mL for THC and 50 ng/mL for amphetamine and methamphetamine. For the confirmatory method, all cutoffs were ≤10 ng/mL, with the exception of chlordiazepoxide and dextromethorphan. Samples were collected according manufacturer guidelines from individuals pulled over on suspicion of impaired driving, or who had displayed signs of impairment and were recruited as subjects for a Drug Recognition Expert (DRE) certification class. An additional sample was collected with a Quantisal collection device, which was submitted to the laboratory (NMS Labs) for confirmatory analysis.

Results: Between the two devices, four counties, and 108 subjects, a total of 734 test results were generated resulting in an overall 94% sensitivity, 99% specificity, and 98% accuracy when compared to the laboratory results. Further, the total number of false negatives (11) and false positives (4) represent less than three percent of the data. In Sacramento and Fullerton, both of which were using the DDS® 2 Mobile System, the overall sensitivity was 96% and accuracy was 98%. In these two locations, there were no false positive results and only two false negative results (two incidences of amphetamines being detected in the lab which had not been detected in the field). For the two locations, Bakersfield and Los Angeles, using the DrugTest 5000®, the overall sensitivity was 93% with 97% accuracy. The DrugTest 5000®generated a total of 9 false negatives and 4 false positive results between the two locations. False positive results for amphetamine (2), benzodiazepines (1), and THC (1) were observed. False negative results were produced for amphetamine (6), THC, cocaine, and benzodiazepines (1 each).

Conclusions: Using oral fluid point-of-contact devices in conjunction with a collected oral fluid specimen for confirmatory testing provides an effective and forensically defensible means for collecting legally admissible evidence of drug consumption by impaired drivers. In this assessment, both devices yielded a high number of confirmable positive results, while minimizing the number of false positive or false negative results.

Keywords: DUID, Oral Fluid, Drug Testing, Roadside Screening

S-46

Differentiation of Amphetamine Source Based on Oral Fluid Chiral Analysis: A Preliminary Investigation

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Background: Both methamphetamine (METH) and amphetamine (AMP) are central nervous system stimulants currently classified as a Schedule II because of their limited medicinal properties. Both continue to be widely abused in the USA however there are medications which are not considered to be illicit. METH metabolizes to AMP, which may be the cause of a positive result, and AMP is also used as a specific drug. While a chiral separation of the *l*-isomers from *d*-isomers of these drugs will allow differentiation between legal and illegal forms of METH, there are other prescription medications which are formulated as the *d*-isomers. Adderall® is a mixture of neutral salts of *d*-amphetamine sulfate, amphetamine sulfate, *d*-isomer of amphetamine saccharate, and *d, l*-amphetamine aspartate. For each tablet, the combination of salts and isomers results in a 3:1 ratio of *d:l* amphetamine. Oral fluid is becoming increasingly popular as a specimen for the detection of drugs in many areas including driving under the influence of drugs, and workplace testing. Basic drugs incorporate well into oral fluid; therefore it is an excellent matrix for the measurement of amphetamines.

Objective: To determine the source of AMP detected in oral fluid specimens from different research projects based on the extent of isomer presence.

Methods: Oral fluid specimens from three different research populations were subjected to extraction, chiral derivatization and LC-MS/MS analysis.

1. Volunteer currently prescribed Adderall® (10 mg; 2-3 times/day)
2. Volunteer drivers stopped randomly in roadside surveys
3. Volunteer nightclub attendees on entrance and exit from dance events

Results:

1. Specimens from the volunteer taking prescription Adderall® showed mean *d*-AMP and *l*-AMP concentrations of 133ng/mL and 44ng/mL respectively (median 125ng/mL; 44ng/mL) with an average ratio of 2.9:1 (median 2.8:1). Even though the mean elimination half-life ($t_{1/2}$) for *d*-AMP is shorter than the $t_{1/2}$ of the *l*-isomer (9.7 - 11 hours vs. 11.5 - 13.8 hours), the measured ratio held throughout the sample collection times (over 31 hours).
2. Of the specimens taken from drivers (n = 22), 10 specimens were 100% *d*-AMP with no *l*-AMP present; the other 12 specimens had an average *d:l* amphetamine ratio of 2.8: 1 (range 1.5 – 9.8). Vyvanse® is a pro-drug for *d*-AMP, therefore may be an explanation for the presence of *d*-AMP only.
3. Specimens taken from 5 individuals at entrance and exit of dance events showed *d*-METH and *d*-AMP but no *l*-METH or *l*-AMP in either specimen (n=3). One subject had approximately equivalent amounts of *d*-AMP and *l*-AMP in both entrance and exit specimens; the final volunteer had equivalent amounts of *d*-METH (417ng/mL) and *l*-METH (397ng/mL) in entrance oral fluid (*d*-METH = 417ng/mL; *l*-METH = 397ng/mL), but not on exit (*d*-METH = 39ng/mL; *l*-METH not detected).

Conclusion: Our preliminary findings show that an approximate 3:1 ratio of *d:l*-AMP in oral fluid specimens, with no methamphetamine present, is potentially indicative of Adderall® use. Chiral analysis may be used for differentiation of prescription AMP from illicit intake, although more research is needed.

Keywords: Oral Fluid, Chiral Amphetamine Analysis, Interpretation

S-47

Comparison of Methods for Extraction of Drugs from Umbilical Cord Tissue

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Introduction: Umbilical cord tissue has gained interest as a specimen for detection of *in utero* drug exposure (1-4). The tissue must be homogenized and the drugs extracted prior to analysis by immunoassay or mass spectrometry. Tissue preparation in the laboratory requires complete homogenization of the tissue for extraction of drugs and metabolites, the elimination of carryover or contamination, and the ability to take the homogenization extract for further sample preparation and clean up prior to analysis. High throughput methods may be required to maintain time to result and overall turn-around time.

Objective: To compare homogenization and extraction methods for the detection of drugs in umbilical cord tissue.

Methods: Four types of tissue homogenizers: a Tissue-miser (Thermo-Fisher, Walthem, MA), Ball Mill (Fritsch, Idar-Oberstein, Germany), Geno-grinder (SPEX Sample Prep, Metuchen, NJ) and a Cryo-prep (Covaris, Woburn, MA) were evaluated for homogenization of cord tissue. The tissue miser blends tissue with a stainless steel probe. The Ball Mill and Geno-Grinder use oscillation and stainless steel balls for homogenization. The Cryo-prep freezes the tissue and pulverizes it. Tissue was homogenized in methanol (except for Cryo-prep) and dried by centrifugal vacuum evaporation (CVE). The amount of tissue (1.0-3.0 g) and volume of methanol (1-3 mL) varied depending upon the method evaluated. The dried residues were re-constituted (conditions varied depending upon method evaluated) and extracted using Strata XB, Strata X (Phenomenex, Torrance, CA) and XCEL (UCT, Bristol, PA) solid phase extraction columns, Biotage SLE+ supported liquid extraction columns (Biotage, Uppsala, Sweden), QuEChERS (Agilent Technologies, Santa Clara, CA) and Toxi-tubes (Agilent Technologies). Samples were analyzed for 68 drugs and metabolites using a previously published liquid chromatography time-of-flight mass spectrometry method (5). Results were evaluated based on TOF criteria, including retention time, mass accuracy and match score (5).

Results : All instruments provided adequate homogenization of the tissue, but the Covaris Cryo-prep was selected for ease of use, cleanliness, degree of homogenization and increased throughput. Extraction methods had good recoveries for most drugs of interest:

E**xtraction Results Summary**

Drug Class	n	Cutoff (ng/g)	Strata X		Strata X Drug B		Xcel		Toxi-tubes		QuEChERS		Biotage SLE+	
			n	Average Score	n	Average Score	n	Average Score	n	Average Score	n	Average Score	n	Average Score
Benzodiazepine	12	3.0	12	74	12	78	11	83	12	61	12	79	12	82
Benzodiazepine metabolite	8	3.0	8	82	8	82	2	90	7	74	7	87	8	87
Z Drugs	2	3.0	2	45	2	82	0		2	92	2	79	2	93
Opioid	13	0.6-1.3	11	52	13	71	3	34	10	63	10	78	13	70
Opioid Antagonist	2	2.5	2	45	2	87	0		2	95	2	82	2	78
Opioid metabolite	11	0.6-1.3	10	54	10	72	2	27	7	69	10	77	11	73
Stimulant	12	2.5	10	65	11	77	2	45	11	58	12	88	12	62
Hallucinogen	2	1.3-7.5	2	67	2	67	1	42	0		2	84	1	69

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The Biotage SLE+ was selected for further method development based on cleanliness of the sample, quality of the TOF data and ease of use.

Discussion and Conclusions: Several different methods of homogenization and extraction can be used to prepare umbilical cord tissue for analysis for drugs of abuse, however throughput, sample cleanliness and ease-of-use makes some methods a better, more elegant solution for sample preparation.

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Keywords: In Utero Drug Exposure, Umbilical Cord Tissue, Sample Preparation

S-48

Optimized Umbilical Cord Homogenization for the Detection of In Utero Drug Exposure by Liquid Chromatography-Time-of-Flight Mass Spectrometry

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Introduction: Meconium has become the specimen of choice in many laboratories for the detection of in utero drug exposure. Recent publications (1-4) from our laboratory and others have shown the utility of umbilical cord as an alternate specimen type for the assessment of in utero drug exposure. Umbilical cord is believed to have a detection window comparable to meconium; however, the tissue itself presents a unique challenge to the clinical laboratory in regards to sensitivity and sample preparation protocols.

Objective: This study was conducted to determine if an existing, cryogenic umbilical cord preparation could be replaced with an efficient and inexpensive method using stainless steel beads.

Method: 55 drug and metabolite compounds were measured in umbilical cord tissue to compare an existing cryogenic tissue preparation against a new preparation using stainless steel bead homogenization. To prepare the sample, 1gram of umbilical cord was added to a 5mL Eppendorf tube. Next, 2mL of a 5ug/mL, DNase and water solution was added. Lastly 5 UFO (5.6mm) stainless steel beads were added to each tube. Samples were then homogenized for 5 minutes using a Storm model bullet blender from Next Advance. Homogenized samples were then extracted using a previously validated supported liquid extraction method (4), followed by analysis using liquid chromatography time of flight mass spectrometry with an Agilent 1260 chromatographic system and Agilent 6230 time-of-flight mass spectrometer . Raw instrument area counts were used to determine analytical recovery as a function of ion suppression and extraction efficiency of the new procedure. Ion suppression was determined by comparing neat standards to those in matrix.

Results: Increased drug recovery and reduced ion suppression were achieved for the majority of the compounds. Several barbiturate and opioid compounds had decreased total recoveries. Cocaine and its metabolites demonstrated enhanced sensitivity. Many of the benzodiazepine compounds showed decreased extraction efficiency combined with reduced ion suppression, resulting in a total overall increase in recovery. Compounds in the stimulant class showed reduced ion suppression, increased extraction efficiency and overall increased total recovery. These results indicate equivalent or better overall recovery when compared to the current cryogenic homogenization protocol. In addition, the new preparation reduced the inter-assay imprecision for the majority of the analytes.

Conclusion: The stainless steel bead homogenization protocol reduced the procedure time by 90 minutes. Recovery using the bead homogenization procedure proved to be comparable or superior to the current procedure for the majority of analytes. Elimination of the cryogenic tissue tube, liquid nitrogen, and a reduction in technician time provided a significant cost savings to the laboratory.

Keywords: Umbilical Cord, Drug Exposure, Cryogenic, LC/QTOF/MS

S-49

Development and Validation of a Method for Analysis of NBOMe Drugs in Blood, Plasma and Urine by LC-MS/MS

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Introduction: Reports of toxicity associated with the abuse of designer stimulants have seen a significant increase in recent years. These compounds are often advertised as safe and legal alternatives to their banned counterparts and are available for purchase at head shops or via the internet. A large proportion of designer drug substances are substituted phenethylamines, including members of the 2C class. Since the 2C compounds have become regulated, various substitutions to the central 2C structure have resulted in altered hallucinogen and stimulant activity, creating a new class of designer stimulants. N-benzylmethoxy (NBOMe) derivatives of the 2C compounds are variants specifically substituted at the 4-position on the phenethylamine structure. Numerous articles reporting both fatal and non-fatal intoxication with NBOMe drugs, with or without analytical confirmation are increasing in prevalence worldwide.

Objective: The objective of this study was to develop and validate a quantitative method for the simultaneous analysis of twelve NBOMe drugs from blood, plasma and urine using liquid chromatography tandem mass spectrometry (LC-MS/MS).

Methodology: Mescaline-NBOMe, 25C-NBOMe, 25H-NBOMe, 25P-NBOMe, 25D-NBOMe, 25B-NBOMe, 25T2-NBOMe, 25I-NBOMe, 25E-NBOMe, 25T7-NBOMe, 25T4-NBOMe, and 25N-NBOMe, along with 25I-NBOMe-D_3 as the internal standard were extracted from biological specimens (1.0mL) made basic with the addition of pH 9 borate buffer and extracted into hexane and ethyl acetate (90:10). The organic phase was removed and evaporated to dryness, reconstituted in mobile phase, then analyzed by LC-MS/MS. The separation was accomplished on an Acquity BEH C18 column, on a Waters Quattro Micro LC-MS/MS instrument. Assay performance was characterized consistent with the protocol for method validation recommended by the Scientific Working Group for Forensic Toxicology (SWGTOX) with regards to accuracy, bias and precision, limit of detection and quantitation, calibration model, selectivity/specificity, carryover, matrix effect, dilution integrity and stability.

Results: Linearity was verified from 0.1 to 100 ng/mL for each analyte using a 1/x weighting. Accuracy/bias and precision for the assay were determined not to exceed CV values of ±15% over the linear range of the assay. No significant interference from matrix effects or other common drugs of abuse were observed. Recovery was >70% for all compounds with ion suppression <25% and no carryover following a 100ng/mL standard.

Conclusion: A method was successfully developed and validated for the detection and quantitation of twelve NBOMe drugs in blood, plasma and urine by LC-MS/MS. This provides a stable and robust platform for the analysis of these dangerous designer drugs in forensic casework.

K**eywords:** NBOMe, LC-MS/MS, Validation, Blood, Plasma, Urine

S-50

Stability of 14 Benzodiazepines and Three Sedative Hypnotics at Different Storage Conditions in Five Toxicological Matrices Over Eight Months

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Introduction: Benzodiazepines and sedative hypnotics are some of the more frequently abused drugs in both driving under the influence of drugs cases and coroner cases. In most cases, blood or urine is analyzed but liver, stomach contents and brain are also commonly investigated matrices for these drugs. Stability in sample matrices is an important factor in accurately determining concentration in casework samples as some samples may wait weeks or months to be analyzed.

Objective: The objective was to evaluate the stability of 17 drugs spiked in blood, liver, brain, urine, and stomach contents over an eight month period. Spiked samples were stored in the refrigerator or freezer and spiked at high and low concentrations of alprazolam, chlordiazepoxide, clonazepam, diazepam, estazolam, flunitrazepam, flurazepam, lorazepam, midazolam, nordiazepam, oxazepam, phenazepam, temazepam, triazolam, zaleplon, zopiclone, and zolpidem.

Method: Initially, 50 µL of internal standard containing 11 deuterated forms of the drugs was added to 0.25 mL of blood or urine or 0.25 g of liver homogenate (1:3), brain homogenate (1:2), or stomach content homogenate (1:100). The protein was precipitated out of the sample with 0.75 mL acetonitrile. Samples were then "cleaned-up" using DPX-WAX tips that selectively remove matrix interferences from the samples by dispersive solid phase extraction. *The final eluant was diluted with mobile phase and injected onto a Waters Acquity UPLC coupled to a Waters TQ-S triple quadrupole mass spectrometer utilizing positive electrospray ionization in multiple reaction monitoring mode. A Waters BEH C18 1.7* µm *column (2.1 x 100 mm) held at 40°C with a gradient mobile phase of 0.1% formic acid in water and 0.1% formic acid in acetonitrile at 0.4 mL/min was used for chromatographic separation. The samples were extracted in triplicate every two weeks for the first six weeks. They were then extracted in triplicate on a monthly basis for eight months.*

Results: The concentrations were calculated using a calibration curve created from seven extracted standards. The calibration model used for all drugs was a quadratic curve, weighted $1/x^2$ with no forcing through zero. Decreases greater than 10% were seen in zopiclone and the nitrate based benzodiazepines (clonazepam, flunitrazepam, nitrazepam, and flurazepam) within the first two weeks in all matrices and increased to greater than 20% within the first month. After four months, zopiclone could not be detected in any matrix, at either concentration, in both storage conditions. For the other drugs, they appeared more stable in freezer conditions rather than refrigeration.

Conclusion: In the more aqueous based matrices, such as urine and stomach contents, many of the drugs significantly decreased in concentration within the first two months as expected since the majority of the drugs are unstable in water regardless of storage conditions. After six months, all drugs decreased by at least 30% in all matrices and in all storage conditions.

Keywords: Benzodiazepines, Storage Conditions, Stability, Blood, Tissues

S-51

Impact of Novel Accurate Mass MS/MS^{ALL} Acquisition and Processing Techniques on Forensic Toxicological Screening

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Introduction: (For research use only, not for use in diagnostic procedures). Rapid forensic toxicology screening by high resolution mass spectrometry is a powerful technique. However, some compounds cannot be unambiguously identified with high resolution MS1 measurements alone. MS/MS fragmentation yields confident identifications of these compounds, but how to ensure quality MS/MS of these compounds? Data dependent techniques, although very powerful, cannot guarantee the measurement of all possible MS/MS candidates. Targeted MS/MS ensures acquisition of the target compounds, but limits the number of compounds. Data-independent techniques, such as SWATH™ acquisition (the MS/MS of all possible candidates), improve identifications significantly and enable retrospective analysis of the data.

Objectives: To evaluate the impact of improvements to SWATH acquisition, including variable precursor window sizes, overlapping windows and deconvolution of MS/MS from multiple precursors.

Methods: Urine was spiked with over 120 drugs and compounds often found in forensics screening panels. The data was collected on a Triple TOF® 5600 system using one of the following methods 1) using a TOF-MS survey scan with IDA-triggering of up to 20 product ion scans or 2) SWATH acquisition. For SWATH acquisition, the precursor isolation window width was varied for each MS/MS experiment, or the windows were overlapped between each cycle. Data was processed in PeakView® software 2.0, using a research prototype of MasterView™ software.

Results: Astemizole and Amilodipine both demonstrated the advantage of having narrower SWATH isolation windows. The overlap 20 Da SWATH window acquisition (after demultiplexing was performed) resulted in MS/MS that were significantly reduced in interferences. The library match purity scores were improved from 2.2 % to 97.5% and 38.8 % to 92.7% respectively. Having narrower isolation windows improves the specificity of MS/MS data, but at a cost. Either accumulation times must be decreased (which would make signal to noise worse) or cycle times will get longer (reducing the number of points across a peak). We show with the example of Berberine that using overlap SWATH acquisition that the demultiplexed MS/MS can approach the quality of a true 10 Da SWATH acquisition MS/MS, while having an improved cycle time. Deconvolution of SWATH MS/MS was also shown to improve library match purity scores. Unprocessed SWATH MS/MS had significantly lower purity scores for many compounds. Simple background subtraction resulted in MS/MS of much better quality. Two other deconvolution techniques were tried. Method A was similar to techniques used for deconvolving GC-MS signals, and was implemented to run on an NVIDIA 660 graphics card. Method B is novel technique making use of principal components variable grouping (PCVG) to obtain a SWATH MS/MS. When the techniques were combined, results were equivalent to those achieved using unit resolution IDA. For a few compounds, IDA was not triggered, resulting in no identification. While the SWATH acquisition was able to confidently identify these compounds with good purity scores.

Conclusion: SWATH acquisition methods acquire MS/MS for all compounds, at every time point, achieve identification results comparable to unit resolution IDA methods and overlap SWATH acquisition can improve cycle times and improve identification results.

Keywords: Comprehensive Accurate Mass Acquisition, Unknown Screening, Data Independent Acquisition

S-52

A Comprehensive Qualitative Screen for 50 Designer Drugs in Postmortem Blood Using LC-Ion Trap MS/MS

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Background / Introduction: Over the past three years, the evolution of designer drugs in Miami has shifted dramatically from methamphetamine and MDMA, to methylone and more recently, alpha-PVP and other synthetic cathinones. Due to the frequency with which these compounds are detected in postmortem cases, the development of a targeted blood screen was essential. The current blood drug screen method used in the toxicology lab at the Miami-Dade County Medical Examiner Department (MDME) utilizes Gas Chromatography-Thermionic Specific Detector (GC-TSD) with confirmation by Gas Chromatography-Mass Spectrometry (GC-MS). Although a number of designer drugs are detected with this current screening protocol, it is possible that some compounds are undetectable due to low concentration or sensitivity.

Objective: The objective of this work was to develop and validate a comprehensive qualitative screening method for 50 designer drugs in postmortem blood using Liquid Chromatography-Ion Trap Mass Spectrometry in accordance with current SWGTOX guidelines.

Methods: A Thermo Scientific Dionex UltiMate 3000 Ultra High Performance Liquid Chromatograph (UHPLC), coupled to a Bruker AmaZon SL Ion Trap MS, was validated to screen for a broad range of designer drugs and stimulants, including but not limited to synthetic cathinones, tryptamines, piperazines and amphetamines. HPLC separation was achieved using gradient elution on a Phenomenex Kinetex Biphenyl column (100mm x 3mm, 2.6um) at 40^{o}C using 0.1% formic acid, water, and methanol (90:10) mobile phase. Positive mode electrospray ionization MS analysis was performed using enhanced resolution scan mode between 50-500 m/z. A scheduled precursor list, identifying targeted analytes, was embedded in the MS method. This precursor list allows the MS method to trigger MS/MS analysis on the targeted analytes, based on specified retention times and molecular ions. Prior to submission to the instrument, compounds were extracted from blood using a mixed-mode solid-phase extraction procedure using a positive pressure manifold.

Results: Separation of an array of designer drugs was achieved within 5 minutes. The majority of the target analytes were determined to have a limit of detection between 10-25 ng/mL. Endogenous interferences from different blood sources and analytes were assessed and found to be insignificant. Based on neat injections of certified reference standards, an in-house library was created, which identified the 50 targeted compounds based on retention time, parent ion, and daughter ion spectra.

Conclusion: This targeted assay is the first time UHPLC-Ion Trap MS/MS has been utilized for qualitative drug screening in the toxicology laboratory at the MDME. Using this method, the presence of methylone, alpha-PVP, butylone, ethylone and other compounds has been confirmed in MDME cases received between 2011 and 2014. In addition to confirming the presence of designer drugs in cases, future use of this method will allow for the detection of specific compounds in unknown samples when case history suggests potential abuse. The sensitivity of the AmaZon SL ion trap allows the laboratory to detect those drugs not amenable to GC/MS analysis and those not detected in the routine basic drug screen.

Keywords: Designer Drugs, Postmortem, LC-Ion Trap MS/MS Screening

S-53

High Resolution Mass Spectrometry: The Comprehensive Toxicology Screen of the Future?

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Background: The traditional approach to drug testing in urine has been an initial panel of immunoassays followed by specific confirmatory tests, but alternative testing workflows using mass spectrometry have been proposed. Liquid chromatography tandem mass spectrometry (LC-MS/MS) techniques are ideal for identifying a targeted set of compounds, but targeted data collection has limitations that impact its use as a screening tool. High resolution mass spectrometry (HRMS), which allows exact determination of molecular weight, has been proposed as an alternate screening technique. Liquid chromatography-HRMS has several advantages over LC-MS/MS, including the ability collect untargeted data and to review data retrospectively. Data can be analyzed using targeted, suspect and untargeted techniques. Analyzing HRMS data with a combination of these three techniques could produce a more complete accounting of the contents of a sample.

Objective: Our objective was to determine how to best use each high resolution data analysis tool to increase the compounds covered by our urine drug screen while minimizing data analysis time.

Methods: Urine samples were diluted 1:5 before injection. HRMS data was acquired with an ABSciex TripleTOF®5600 operating in positive mode, collecting full scan data with IDA triggered acquisition of product ion spectra. Data was analyzed with PeakView® and MasterView® software. HRMS product ion spectral library was built in house and LC-MS/MS meta library was supplied by ABSciex.

Results: Our method was validated for a total of 220 drugs of various classes with LLODs ranging from 5-100 ng/mL. We empirically determined the optimum parameters for targeted, suspect, and untargeted data analyses. Method performance was evaluated using patient urine samples where the analyst was blinded to the contents. Targeted screening results were compared with prescription information, immunoassay results and LC-MS/MS data to determine positivity. Suspect and untargeted results were compared to the results of the targeted analysis. Additional drugs not included in the targeted analysis were detected by both suspect (n=5) and untargeted (n=4) analyses, but the yield of drugs per unit of analyst time was much higher for suspect than for untargeted analysis.

	Targeted	Suspect	Untargeted
Sensitivity	0.97	0.54	0.35
Specificity	0.99	0.99	0.99

Table 1. Performance characteristics of data analysis techniques. Targeted analysis was the most sensitive, followed by suspect, followed by untargeted. Specificity was comparable across techniques.

Conclusions: Targeted searching yields high confidence results but requires detailed information about each compound that can only be obtained by purchasing a standard. Suspect searching returns results with lower confidence but requires less information about each compound. Untargeted searching produces results with lowest confidence and is time consuming but requires no a priori information about compounds. Using suspect and untargeted analysis to supplement targeted analysis can increase the number of compounds in a screen without increasing costs to the laboratory, especially if the instrument has a large library.

Keywords: High Resolution Mass Spectrometry, Urine Testing, Data Analysis

S-54
Qualitative Analysis of Methadone in Untreated Urine by DART™ MS

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Background: Workplace drug testing, pain management clinics, and toxicology laboratories test for the presence of methadone using urine matrix. The presence of methadone in urine can confirm compliance with treatment as well as indicate potential abuse. Current testing protocols utilize a variety of expensive and time consuming methodologies ranging from immunoassays to liquid/gas chromatography mass spectrometry screening.

Objective: This study focuses on the development and evaluation of two Direct Analysis in Real Time, DART™, enabled mass spectrometers for the qualitative analysis of methadone in untreated urine in an effort to streamline the analysis process. The two instruments selected for this study are DART™ TOF MS and DART™ QTRAP™ MS. The DART™ MS does not rely on chromatographic columns for analyte separation but rather, the mass spectrometer, allowing for data acquisition in real time, 1-2 minutes per sample. When coupling this technology with a procedure that uses minimal sample preparation, analysis time and supply costs are both decreased.

Methods: The experimental procedure employed by this study requires 500 µL of untreated urine fortified with deuterated methadone as an internal standard. Instrumental analysis is conducted with the ionization source at a 45º angle from the sampling platform and a sample volume of 10 µL analyzed in duplicate. The sample is analyzed as a wet spot on a glass microscope cover slide by both DART™ TOF MS and DART™ QTRAP™ MS. The DART™ TOF MS provides a putative identification of the analyte while the DART™ QTRAP™ MS provides fragmentation spectrum for confirmation. Total time for analysis on each instrument is less than 4 minutes resulting in a confirmed positive finding in less than 10 minutes.

Results: Studies were performed to establish the effectiveness and reliability of these analytical methods. These studies included: limit of detection, selectivity/specificity, stability, and robustness. Both instruments established a LOD of 250 ng/mL and positively identified 86% of the samples at this concentration. During selectivity/specificity experiments, 64 commonly encountered analytes and metabolites, including EDDP, were analyzed with samples consisting of both fortified specimens and previous case specimens. Both instruments detected methadone without interference within this study. The stability of spiked samples stored at 4ºC was evaluated over a 5-day period with similar results obtained between day 1 and day 5. Studies to determine the robustness of the method were performed by two scientists and the data were consistent.

Conclusion: These studies determined that DART™ MS technology provides a selective/specific, economical, and time efficient platform for the analysis of methadone in untreated urine. This is the first study where DART™ MS technology has been used in both the screening and confirmation process and the data suggests further investigation into the analysis of biological specimens by DART™ MS could be advantageous.

Keywords: Bioanalysis, DART™ TOF MS, Methadone, DART™ QTRAP™ MS

Abstracts Of Poster Presentations

P-01

Comprehensive and Confident Identification of Narcotics, Steroids and Pharmaceuticals in Urine

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Introduction: Monitoring of patients in psychiatric hospitals and clinics has traditionally relied on targeted methods of analysis and are often limited by compound lists. These screening methods are not comprehensive and result in incomplete pictures of patients activities. Gas chromatography – mass spectrometry (GC-MS) provides a fast and convenient method for the analysis of urine samples. Urine based measurements exhibit advantages over other biological fluids such as blood or plasma. Samples are easily collected and contain relatively high concentrations of pharmaceuticals, illicit drugs and steroids. In addition, compounds are typically detectable over extended periods of time.

Objective: The objective of this study was to develop a comprehensive fast and accurate method for qualitative analysis of illicit drugs and their metabolites in urine.

Materials and Methods: Samples were obtained from a European psychiatric clinic and were first screened for drugs of abuse using immunological tests (e.g., CEDIA from Thermo Scientific) and then analyzed by nominal mass GC-MS for confirmation. Simple positive or negative drug detection results in urine samples was all that was required by facility physicians. Quantitative work, if necessary, was performed using blood samples. In this study, instrumental analyses were conducted using gas chromatography-high resolution time-of-flight mass spectrometry (LECO Corporation Pegasus GC-HRT). The GC-HRT produced high resolution (R = 25,000 at m/z = 218.98508), accurate mass data (< 1ppm) which was used for robust formula determinations (molecular, fragment and adduct ions) and spectral comparisons to large, well-established databases. HRT data acquisition involved collection and storage of all high resolution ions. This is in contrast to many traditional GC-MS and LC-MS/MS systems that are typically run at lower resolution and generate data that is difficult to compare across different instrument platforms due to different acquisition parameters (e.g., collision energies). These nominal mass spectrometers do not have the proper resolution to reduce background and/or isobaric interferences.

Results: The methodology utilized in this study resulted in conclusive identification of compounds through the utilization of complementary EI and CI high resolution mass spectrometry. The workflow involved quick sample preparation (10 minutes), followed by 12 minutes of comprehensive data collection. In general, patient urine samples were found to contain nicotine, prescription drugs, opiates and their metabolites. These compounds were identified through spectral similarity (SS,perfect score=1000) comparisons to commercially available libraries and accurate mass formula determinations (Mass Accuracy, MA in ppm). For example, a representative set of compounds found in one patient's sample included: Nicotine (SS=936,MA =-0.55), cotinine (SS=911,MA =0.00), 3-hydroxycotinine (SS=921,MA=0.05), acetaminophen (SS=908,MA=0.23), codeine (SS=871,MA=0.52), morphine (SS=917,MA=0.15), and quetiapine (SS=880,MA=-1.4). Another patient's urine sample contained androst-16-en-ol (SS=866,MA=-1.01), aetiocholanolone (SS=926,MA=0.01), 3-methoxyphenothiazine (SS=883,MA=-0.08) and levopromazine (SS=837,MA=0.19), as well as, metabolites that were used to confirm the presence of steroids, narcotics and pharmaceutical compounds detected in the sample.

Conclusion: GC-HRT facilitated the rapid, robust and confident identification of drugs in urine. The analysis workflow provided a reliable mechanism for qualitative screening of samples. The combined utility of CI with EI ionization resulted in comprehensive detection and characterization of drugs, pharmaceuticals and their metabolites.

Keywords: Gas Chromatography, High Resolution Time-of-Flight Mass Spectrometry, Qualitative Urine Analysis

P-02

A Workflow for Comprehensive Analysis of Forensic Samples Using Electron and Chemical Ionization High Resolution Time-of-Flight Mass Spectrometry

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Introduction and Objectives: The production of unregulated recreational drugs has been increasing at an alarming rate since the mid-2000s. Relatively simple organic transformations produce novel and licit psychotics that can elude detection by standard analytical methods. Systematic screening for drugs is typically based on targeted GC/MS methods relying on comparisons to reference data (e.g., spectral similarity, retention times, etc). Detection and characterization of novel synthetic drugs through these routine methods is challenging due of lack of standards and inappropriate targeted instrumental techniques. These synthetic drugs are chemically diverse and often found in varying concentrations in complex plant materials. In this study, a LECO Corporation Pegasus® GC-HRT (Gas Chromatography - High Resolution Time-of-flight mass spectrometry) operating in high resolution mode was used for the rapid and confident identification of drugs in complex matrices.

Materials and Methods: Botanical, powder and drug residue samples were obtained from a collaborating forensic laboratory. Different sample preparation methods were used and evaluated for extraction efficiency. For example, samples (0.05 g) were extracted with organic solvent (1 – 2 mL of either chloroform or methanol), filtered and analyzed by GC-HRT. Alternatively, samples were mixed with either water or aqueous base and extracted by solid phase micro extraction (SPME, 2cm-50/30 um DVB/Carboxen/PDMS Stable Flex from Supelco) or extracted with organic solvent, dried, and derivatized with MSTFA prior to analysis by GC-HRT. Instrumental analyses included a combination of high resolution electron and chemical ionization mass spectrometry (EI & CI-MS).

Results: A robust workflow was tested and found to be effective for comprehensive analysis of complex forensic samples. The workflow included proper sample preparation, data acquisition, EI/CI-HRT data comparison and database searching. For example, a seized dry botanical sample was analyzed using this methodology and found to contain a wide variety of components including terpenes, terpenoids, aldehydes, acids, esters and heterocyclic compounds. Spectral similarity values for a representative set of 27 nitrogen-containing compounds detected in was determined to be a Khat sample, ranged from 710 to 975 out of a possible score of 1000. These compounds included phenylethylamine, nicotine, cathinone and cathine, along with various substituted alkaloids. The analysis of another confiscated botanical sample (Pokeweed) was found to contain two synthetic cannabinoids that were identified as XLR-11 and one of its structural isomers. EI-HRT mass accuracy values for these cannabinoids were -0.36 ($M^{+\bullet}$, m/z = 329.21482) and -0.91 ppm ($M^{+\bullet}$, m/z = 329.21465) respectively. The formula, $C_{21}H_{28}FNO$, for these isomeric compounds were confirmed using CI-HRT data which displayed the corresponding protonated molecular $[MH]^+$ at m/z = 330.22298 (mass accuracy = 0.65 ppm) and m/z = 330.22265 (mass accuracy = -0.35 ppm).

Conclusion: The combination of EI and CI-HRT data facilitated conclusive identification of compounds in complex forensic samples. The instrument's high resolving power minimized background interferences, while mass accuracy values below 1 ppm allowed for robust elemental formula determinations. In addition, complete and high quality spectral data facilitated library searches against accurate and nominal mass libraries (e.g., NIST, Wiley).

Keywords: Drug Analysis, GC-MS, High Resolution Mass Spectrometry

P-03

Development of an Ultra-Rapid, High-Throughput Analysis Method of Synthetic Phenethylamines (25I-NBOMe and 25B-NBOMe) in Urine, Saliva and Serum by Laser Diode Thermal Desorption-Mass Spectrometry

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Background / Introduction: Emerging drugs of abuse have been introduced on the illegal drug market and pose a challenge for the analytical forensic toxicologist. These drugs have increased in numbers and vary across different chemical classes making them difficult to detect by current analytical methods. In November 2013, the United States Drug Enforcement Administration issued a final order to temporarily schedule three synthetic phenethylamines into the Controlled Substances Act: 25I-NBOMe, 25C-NBOMe, and 25B-NBOMe.

Objective: A comprehensive detection and quantification method for the analysis of emerging drugs of abuse in biological matrices is needed. We developed an ultra-rapid, high-throughput and cost effective analytical method for the detection of 25I-NBOMe and 25B-NBOMe using the LDTD coupled to an API-5500 MS/MS system. Method development and validation were performed in urine, saliva and serum matrices and the analysis time needed was 9 seconds per sample.

Methods: Urine, serum and Orasure saliva extraction buffer were spiked with 25I-NBOMe and 25B-NBOMe at concentrations ranging from 0.1 to 100 ng/mL in order to build calibration curves and evaluate the extraction method. The extraction procedure consisted of using a 50 µL sample aliquot to which were added 20 µL of the internal standard (25I-NBOMe-d3) in methanol, 200 µL of sodium carbonate (0.5M at pH 10) and 200 µL of hexane:ethyl acetate (75:25, v/v). Samples were mixed and centrifuged (2 minutes/4000 rpm). A 6 µL aliquot of the organic phase was transferred into a 96-well plate (LazWell) and evaporated to dryness. Samples were desorbed using a laser pattern consisting of a 6 second ramp to 45% laser power, held for 2 seconds and shut off in 0.1 seconds. The LDTD-MS/MS was operated in MRM mode and simultaneously measured all the compounds in the extracts. The transitions used for the quantitation of 25I-NBOMe and 25B-NBOMe were m/z 428→121 and m/z 380→121, respectively. The ionization was performed in APCI positive mode.
The following validation parameters were evaluated: Linearity, intra-run and inter-run accuracy/precision, extracted sample stability and drugs cross reactivity.

Results: Calibration curves had excellent linearity with R^2 values between 0.998 and 0.9998 in urine, Orasure saliva extraction buffer and serum. The intra-run accuracy and precision across the calibration curves were between 91 to 112% and 0.3 to 6.9% for both compounds, respectively. Following the extraction procedure, all samples were stored at 4°C to evaluate the drugs temporal stability in wet state. After a waiting period, all samples were re-spotted and analyzed. A wet stability greater than 12h was obtained with a accuracy and precision between 93 to 109% and 0.6 to 11.2% for concentrations equivalent to the LOD, respectively. 32 different (Amphetamines, Benzodiazepines, Opiates, Cannabinoids and Hallucinogens) drugs were tested at a final concentration of 1µg/ml for the cross reactivity assay. No interference was obtained for all drugs.

Conclusion / Discussion: The LDTD technology provides a selective, sensitive and ultra-rapid analysis method, i.e. 9 seconds per sample, for the detection and quantification of 25I-NBOMe and 25B-NBOMe drugs in urine, saliva and serum samples at concentrations between 0.1 to 100 ng/mL.

Keywords: High Throughput, LDTD-MS/MS, NBOMe Drug, Saliva, Serum, Urine

P-04

High-Throughput Analysis of Opiate in Hair Samples Using Laser Diode Thermal Desorption (LDTD) Coupled to Mass Spectrometry

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Background / Introduction: Since the hair root is vascularized during its growth, illicit drugs present in the blood stream may enter the hair shaft via the root where they will be sequestered. Therefore, the use of illicit drugs can be revealed by analyzing a small hair sample. To increase the analysis throughput of hair samples, the laser diode thermal desorption (LDTD) coupled to tandem mass spectrometry (MS/MS) was used for the identification and quantification of opiates.

Objective: The detection and quantification of drugs in hair samples is traditionally performed by LC-MS/MS or GC-MS analysis that require several minutes due to separation time. The LDTD-MS/MS instrument significantly reduces analysis time and thus increases the sample throughput with runtimes of 9 seconds sample-to-sample. In this study, our goal was to validate a quantitative method for the following opioid drugs in hair: 6-acetylmorphine, codeine, morphine, hydrocodone, hydromorphone, oxycodone and oxymorphone, using the LDTD coupled to MS/MS.

Methods: Negative hair matrix samples were obtained from drug-free individuals and analyzed prior to preparing matrix matched calibration standards (50, 100, 200, 500 and 1000 pg/mg hair). A 10 mg hair sample was pre-washed with 2 ml of DCM and 2 ml of ethanol. Samples were then digested with a mixture of MeOH:TFA (90:10) according to a previously reported method from the literature. Following digestion, Methoxamine (MOX) derivatization and purification on SPE (CleanDrug, 1cc/10mg) was performed. After extraction, samples were evaporated and reconstituted with 100 µL of MeOH:water (75:25, v/v). 4 µL aliquot was transferred into a 96-well plate and evaporated to dryness. Samples were desorbed from the LazWell plate using a laser pattern (6 second ramp to a laser power of 45%, held for 2 seconds). The carrier gas (compressed air) was set at a flow rate of 3 L/min. The MS/MS quantification and confirmation transitions where used for each drug in APCI positive ionization mode. The LDTD-MS/MS method was cross validated against an LC-MS/MS method using positive ESI mode.

Results: Calibration curves had excellent linearity with R^2 values between 0.9967 and 0.9997. The intra-run accuracy and precision across the calibration curves for all compounds was between 81 to 110% and 0.7 to 12.0%, respectively. Ten different negative hair samples were evaluated to verify the selectivity of the analytical method. The LDTD-MS/MS technique developed was tested on 30 real samples and successfully cross validated by an LC-MS/MS method.

Conclusion / Discussion: The LDTD technology provides an ultra-rapid analysis method, i.e. 9 seconds per sample, for the detection and quantification of opioid drugs. Hair digestion followed by MOX derivatization and an SPE extraction method combined with the LDTD-MS/MS allowed for accurate, precise and stable results for the analysis of opiate drugs in hair samples.

Keywords: LDTD-MS/MS, Opiate, Hair, High-Throughput

P-05

Ultra-Fast Screening Method of Drugs in Urine Using Rapid and Simple Sample Preparation Combined to Laser Diode Thermal Desorption Mass Spectrometry (LDTD-MS/MS)

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Background / Introduction: Toxicology laboratories generally use screening methods to obtain a semi-quantitative response for drug samples. Some screening techniques are fast but less specific and generate by far too many false positive results. Confirmation of those additional false positive samples is both time and cost consuming.

Objective: Laser Diode Thermal Desorption Mass Spectrometry (LDTD-MS/MS) offers specificity combined with an ultra-fast analysis for an unrivaled screening method. To develop this application, we focused on performing fast and simple extraction method using urine sample evaporation followed by organic/aqueous dilution. 31 drugs of abuse from different classes (opiates, benzodiazepines, amphetamines, etc.) are analyzed simultaneously, with quantitative screening results obtained in less than 9 seconds per sample.

Methods: The following drugs were spiked in urine at 50, 100, 500 and 1000 ng/mL: Methamphetamine, Amphetamine, PCP, Imipramine, MDA, MDMA, MDEA, Benzoylecgonine (BZE), 6-AM, Codeine, Morphine, Hydrocodone, Hydromorphone, Oxycodone, Oxymorphone, 2-OH-ethylflurazepam, 7-aminoclonazepam, Alprazolam, Diazepam, Estazolam, OH-alprazolam, OH-triazolam, Lorazepam, Nordiazepam, Oxazepam, Temazepam, OH-midazolam, 7-aminoflunitrazepam, Chlordiazepoxide, Clonazepam and Flunitrazepam. The sample treatment include mix of 25 µL urine sample, 15 µL internal standard in methanol and 200 µL 0.1%TFA in acetonitrile. Samples are vortex and evaporated to dryness. 400 µL of a mixture of Hexane and Ethyl Acetate (1:1) is added to the vial and vortexes for sample reconstitution. After mixing, 4 µL are spotted onto the individual wells of the analyzing plate. Uses of buffer and B-glucuronidase addition are also evaluated with the same treatment.

Results: LDTD-MS/MS operated in MRM mode allows rapid measurement of all drugs desorbed simultaneously. Specific transitions are monitored for each drug to quantitate calibrator level. Ionization is performed in positive mode. Analysis includes spiked drugs in urine, potentially interfering drug and 38 real samples. All compounds give linear response from 50 or 100 ng/mL to 1000 ng/mL. Potential cross reactivity is evaluated by monitoring all transitions while desorbing individual drug spiked at 1000 ng/mL. Cross reactivity between Codeine/Hydrocodone and Morphine/Hydromorphone are observed as they have same elemental composition and fragmentation pattern. For those isobaric compounds, confirmation method has to include separation. Drug concentrations in real sample were also evaluated in LC-MS/MS with a long gradient to separate each drug class. For each drug in real samples, correlation of the data generated by LDTD-MS/MS and LC-MS/MS, false positive and false negative results are evaluated. No false negative result was obtained for the drug analysis; however the benzodiazepine group requires B-glucuronide enzyme treatment for drug detection. This treatment generates chemical noise leading to false positives ranging from 0% to 37%. No false negatives observed.

Conclusion / Discussion: The LDTD technology allows robust drug screening in urine samples with quantitation from 50-100ng/mL to 1000ng/mL. Sample-to-sample run time of 9 seconds is achieved with the capability to simultaneously analyze 31 drugs.

Keywords: Ultra High Throughput, LDTD-MS/MS, Drug Screening

P-06

Pain Management Compliance Testing for Antidepressants Targeted for Neuropathic Pain by Ultra-Fast HPLC Triple Quadruple Mass Spectrometry

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Introduction: Pain management compliance testing (PMCT) is becoming an important subfield in the drug-testing arena. Antidepressants and anticonvulsants are increasingly being prescribed to patients suffering from neuropathic pain, which can be caused by and/or coincide with diabetes, stroke and depression. PMCT is performed to determine patient compliance, establishing therapeutic drug concentrations, effectiveness of treatment, and prevent drug divergence. Currently, there are a select number of tricyclic antidepressants (TCAs), serotonin reuptake inhibitors (SRIs) and anticonvulsants approved for the treatment neuropathic pain. The low therapeutic index and high toxicity of some of these drugs, specifically the TCAs, result in the necessity for reliable toxicological methods for these drugs in pain management patients.

Objective: To develop a sensitive, specific and rapid ultra-fast high-pressure liquid chromatography/mass spectrometry/mass spectrometry urine method for the detection and quantifications of the four TCAs: amitriptyline, nortriptyline, desipramine and imipramine; four SRIs: citalopram, duloxetine, venlafaxine and o-desmethylvenlafaxine and two anticonvulsants, pregabalin and gabapentin, that the Food and Drug Administration has approved for use in pain management.

Methods: 500 µL aliquots of the calibrators, controls and samples were diluted with 1500 µL of diluent containing (5:3:2) ethanol: water: buffer solution (pH 6). The samples were then vortexed, centrifuged and extracted into auto-sampler vials using FASt^{TM} columns with a United Chemical Technologies Positive Pressure Manifold. Analysis was performed on a Shimadzu Scientific (Columbia, Maryland) LCMS-8030 Triple Quadrupole Liquid Chromatograph Mass Spectrometer (LC/MS/MS), equipped with a Restek Corporation Ultra BiPhenyl Liquid Chromatography Column (3 µm x 50 mm x 3.0 mm) and Ultra Guard Cartridge (5 µm x 10 mm x 2.1 mm). Mobile phase A contained water with 0.1% formic acid and mobile phase B contained methanol with 0.1% formic acid. Flow rate was set at 0.8 ml/min. The detection was accomplished by multiple-reaction monitoring via an electrospray ionization source operating in the positive ionization mode.

Results: Qualifying and quantifying transition ions, optimal collision energies for each drug and their corresponding deuterated internal standards were determined. The calibration curves were linear over the investigated concentration range, 50-2,000 ng/mL for each analyte. Selectivity, accuracy, precision and three stability studies were performed for the described method. In addition, known patient samples were analyzed to test the validity and reliability of this method.

Conclusion: A sensitive, rapid and accurate urine method was developed and optimized for the antidepressants and anticonvulsants approved for use in treating neuropathic pain. FASt^{TM} sample extraction was performed in seconds directly into autosampler vials. This method was developed for pain management compliance samples in high through-put urine drug testing laboratories; however, research and other types of forensic laboratories could readily adapt this efficient, cost-effective method.

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Keywords: PMCT, TCAs, SSRIs, Anticonvulsants, Urine Drug Testing, HPLC/MS/MS, Neuropathic Pain

P-07

A Comprehensive 'All Ions' Screening Method with Simultaneous Fragment Confirmation of Opiates, Opioids, Benzodiazepines, Amphetamines and Illicit Drugs by LCMS

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Introduction: Recently many forensic laboratories have become interested in the employment of a comprehensive high resolution, accurate mass screen for compounds of forensic interest with a simultaneous confirmation of fragment ions in a single MS run as an alternative to the traditional screen and confirm model. High resolution, accurate mass LCMS techniques have become increasing popular and accepted in the forensic community due to the ease of use, quality of data and results, along with efficiency. Employment of several figures of merit, including accurate mass, retention time, theoretical isotope modelling, in addition to a personal compound database library (PCDL) match, are key qualifying parameters to confirming the identification of an analyte.

Objective: In this study we developed a method for the detection and further fragment confirmation for a wide variety of forensic and toxicological compounds in whole blood and urine during a single LCMS run employing Agilent's 'All Ions' technique. Data is acquired in MS only mode, and fragment ions are obtained in source by varying the fragmentor voltage parameter on the mass spectrometer to obtain the corresponding fragment ions for further confirmation in data analysis.

Methods and Materials: Experiments were conducted using an Agilent 1290 HPLC coupled to a 6230 TOF. Both urine and blood samples were prepared using an acetonitrile protein crash and subsequently diluted in 50:50 methanol:water. Standards for all compounds were also prepared in the same diluent to confirm accurate chromatography and retention time. A custom PCDL containing 106 compounds of interest was composed from Agilent's comprehensive Forensic Toxicology Personal Compound Database Library and used to analyze the samples in data analysis.

Results: Positive results for all analytes in standard solutions were confirmed in qualitative data analysis using the 'all ions' workflow and fragment confirmation via PCDL matching and retention time coelution. Unknown whole blood and urine samples were also screened and confirmed with positive hits for compounds meeting the method criteria with fragment confirmation. The sensitivity and specificity was assessed for all compounds ranging in concentrations from 500 ng/mL – 1.0 ng/mL with an instrument resolution if 30,000. Chromatography resolved any interferences from multiple compounds of the same drug class present in the samples.

Conclusions: We present a LCMS method to screen and confirm at the same time, a large panel of common forensic compounds using Agilent's 'all ions' technique for samples in whole blood and urine.

Keywords: Mass Spectrometry, QTOF, Accurate Mass, Forensic Toxicology, LCMS

P-08

Development and Validation of a LC/QToF Mass Spectrometry Comprehensive Toxicology Screen in Urine

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Introduction and Objective: The screening of samples for large numbers of unknown compounds is a significant challenge for laboratories. The list of therapeutic drugs, pain management drugs, drugs of abuse, and other compounds being manufactured, sold, used and abused continues to grow. Our objective was to develop a screening method using state-of-the-art technology that would be faster, have a more versatile library, and improved data interpretation than our existing GCMS method. A UPLC/Time of Flight Mass Spectrometry method using the Waters Acquity UPLC Xevo G2 QToF was developed and validated. Time-of-flight mass spectrometry (ToF) measures the time it takes ions of different masses to move from the ion source to the detector via a flight tube. The ion's flight time is proportional to its mass. High mass accuracy (5 ppm), fast scan rate (10 full scans per second) and high resolution along with fragmentation information from the quadrupole (Q) provide specific data used for analyte identification.

Material and Methods: A retention time marker, benzocaine, 50 uL was added to 500 uL of urine sample. The samples were diluted to final volume of 2 mL with 5 mM ammonium formate and then filtered using UCT Clean Screen FASt columns and positive pressure manifold. Waters Acquity UPLC in combination with the Xevo G2 QToF with MS^e mode allowing for continual scanning across the entire acquisition range of the assay was used to analyze each sample. Compounds were identified by accurate mass, retention time and fragmentation information with set criteria matched with a validated targeted database using ChromaLynx software.

Results: To create and validate an in-house database, we investigated 168 of commonly encountered pharmaceutical compounds including prescription, over –the-counter and illicit drugs using the known standards and compared them with Waters' database which consists of 972 compounds by ChromaLynx software. One hundred and fifteen patient samples were compared in parallel by this method and the current GCMS full scan method. Thirty six (31%) samples were in concordance by both methods. More compounds were detected in sixty eight (59%) of samples by LC-QToF method. The additional findings were verified by the medication list in each patient's medical history. Eleven (10%) patients had compounds that were not found by the LC-QToF method. They are primarily barbiturates and NSAID's. The precision of retention time for retention time marker is 0.84%. LOD for each of the 168 compounds was evaluated and most of them were below 1000 ng/mL.

Conclusion: We demonstrated that this LC accurate mass time-of-flight mass spectrometry method is accurate, stable and robust for use as routine qualitative toxicology screening of urine.

Keywords: LC/MS, TOF, Urine, Database

P-09

Applications of a New Headspace GC-MS Validated Method for Volatile Organic Compounds in Different Forensic Matrices

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Introduction: Volatile organic compounds (VOCs) are of great interest in forensic toxicology as they come from a variety of products commonly used in daily routines, both domestic and workplace. Because these products are extensively used, available and are an inexpensive/legal alternative to traditional street drugs, VOCs have a significant potential for abuse with adverse effects on the central nervous system (CNS). Furthermore, a multitude of VOCs can also evolve in the early stages of human decomposition as well as in fire related deaths. In our laboratory, a headspace GC-FID method is used routinely for ethanol, isopropanol, acetone, methanol and n-propanol quantitation. A complementary method with an extensive list of VOCs is of great importance to expand our testing panel in impaired driving, sexual assault and death investigation cases.

Objective: A sensitive and specific qualitative method is required when an unidentified suspected VOC is detected using the routine headspace GC-FID method or when VOC use/presence is suspected in casework. This method has simplified sample preparation to minimize the loss of VOCs.

Method: This 30 minute method allows for the qualitative analysis of 65 VOCs, with tert-butanol as the internal standard, using an Agilent 6890/5973 GC-MS system with a DB-1MS column. Forensic biological (blood, urine, gastric content and brain) and non-biological matrices can be analyzed. A 1 mL or 1 g sample with 0.15 g of sodium chloride in a 5 mL headspace vial is prepared. The headspace is manually sampled and analyzed after a 20 minute incubation on a dry block at 60°C. An extensive list of VOCs contained in different products is covered by this method, such as aerosol propellants, adhesives, paints, removers, cleaning products, anesthetics, gas (ex: Freon®), nitrites (poppers), industrial solvents and fuel.

Results: This method was successfully validated according to SWG-TOX recommendations which include specificity, sensitivity, carryover, matrix effects and identification of gaseous VOCs. Examples of forensic case work includes presence of isoflurane above the limit of detection of 25 ng/mL and identification of 1,1-difluoroethane in impaired driving cases. Chloroform, toluene, heptane and diethyl ether, have also been found in suspicious death and homicide cases above the limit of detection of 25, 50, 75 and 250 ng/mL, respectively. Validation results will be presented for all of the VOCs tested. In addition, different VOCs produced during the early stages of human decomposition are also identified as well as typical hydrocarbon profiles in fire related deaths.

Conclusion: This easy, fast and comprehensive qualitative validated method enhances our toxicological expertise in terms of volatiles analysis and expands our contribution to the police, coroners and pathologists' investigations. This extended volatiles qualitative method should be used in any forensic case where there is a suspicion of VOC use or preliminary detection in the routine headspace GC-FID method.

Keywords: Volatile Organic Compounds, Biological Matrices, Headspace-GC-MS

P-10
Evaluation of a Precision Drug Screening Method by Perkin Elmer AxION DSA-TOF for Blood

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Background / Introduction: The abuse of illicit and prescription drugs has always been an important sociological issue. As more drugs are scheduled under federal and state regulation, the Orange County Crime Laboratory (OCCL) has seen an increase of requests for a large variety of drugs. Currently, the OCCL uses ELISA to screen biological samples for the presence of 7 drug classes using 100 µL of sample. This screening analysis requires specialized reagents and is time consuming. In order to determine specific compounds, several different extractions are performed, using much of a limited supply of biological evidence. A new way to quickly screen for a variety of unknowns is needed.

Objective: The Perkin Elmer AxION Time of Flight (TOF) was investigated as a new screening instrument due to its limited reagent use and quick analysis time.

Method: The TOF was calibrated using a syringe pump and Agilent APCI/APPI tuning solution. Four specific ions within the tuning mix were used as internal standards. A 100 µL aliquot of sample was placed in a test tube and vortexed with 100 µL of cold 0.2 M $ZnSO_4$ and 100 µL of cold acetonitrile. The solution was centrifuged for 5 mins at 1500 rpm. Finally, 10 µL of clear supernatant was placed on the Direct Sample Analysis (DSA) screen and analyzed for about 20 sec at a temperature of 250°C and nitrogen gas flow of 3.0 L/min. AxIOn Solo software was used to analyze the data.

Results: Aqueous and porcine blood samples were spiked at toxicological limits of detection (LOD) taken from OCCL's toxicology confirmation methods and literature concentrations. Less than 65% of the aqueous drug standards were identified at the tested concentrations. Less than 50% of the spiked porcine blood standards were identified at the tested concentrations. When comparisons were made between the OCCL's analyses using confirmation methods and the TOF's screening results, it was determined that over 85% of drugs found in antemortem samples by OCCL methods were not identified by the DSA-TOF. In postmortem samples, over 90% of drugs found by OCCL methods were not identified by the DSA-TOF. It was determined that the DSA-TOF was able to identify compounds that are not currently screened for at the OCCL in over 25 of the samples tested. It is estimated that ~35% of OCCL's cases may have additional reportable compounds not detected by the current screening method.

Conclusion / Discussion: Upon completion of this study, it was determined that further testing on the DSA-TOF is required prior to implementation. Sample preparation time and instrument analysis time of screening improved significantly. There were consistent issues with the instrument's ability to detect acidic compounds, contributing to the high number of false negative results. Several compounds, such as synthetic compounds and cannabinol, not currently tested for by OCCL were detected in several of the samples tested using this method. Further investigation needs to be made as to the source of the high number of false negative results with regards to blood samples before DSA-TOF can be considered for precision drug screen.

Keywords: Time of Flight, DSA, Method Development, Drug Screening

P-11

Development and Validation of a Robust LC-MS/MS Method for the Fast Quantitation of Phosphatidylethanol, a Direct Alcohol Biomarker, in Dried Human Blood Spots Using a Small Footprint LC-MS/MS System

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Background: Direct Alcohol Biomarkers are used by clinicians and researchers as a tool to monitor ethanol ingestion. Ethyl Glucuronide has a detection window of 1-5 days; Phosphatidylethanol (PEth) an abnormal phospholipid formed in the blood after excessive alcohol consumption may be detected up to 1 month following excessive consumption. Traditionally, PEth has been measured in whole blood collected via venipuncture and shipped to the laboratory. The introduction of PEth dried blood spot (DBS) testing eliminates the need for venipuncture and the specimens may be shipped with minimal biohazard concerns. The instrumentation for this high thru-put assay required a large footprint, multiplexed Liquid Chromatography Tandem Mass Spectrometry system (LC-MS/MS).

Objective: The objective of this study was to develop a robust high throughput LC-MS/MS method for the detection of PEth in DBS using a smaller footprint system.

Method: The assay was validated using the proposed Scientific Working Group for Forensic Toxicology, (SWGTOX) guidelines. PEth Negative blood was pooled and fortified with PEth 16:0,18:1. Controls were prepared across the linear range of the assay. The blood was spotted on Whatman 903 paper and 3x 3-mm punches were taken from each DBS. After the addition of deuterated PEth internal standard (PEth-d_{31}) and 1mL of Methanol the DBS samples were incubated at room temperature for 1 hour. The Methanol was evaporated under Nitrogen at 40º C and the residue was reconstituted in 100 µL of the A mobile phase (50% 2mM Ammonium Acetate/25% Acetonitrile/25% Isopropanol). The analysis was performed using an ABSciex 5500 tandem mass spectrometer equipped with an Eksigent Micro 200 LC system. A HALO C8 column (2.7-µm particle size, 0.5 × 50 mm) was heated to a constant 30°C in the column heater. The Eksigent LC flow was 0.050 mL/min with a gradient of 2% B mobile phase (60% Acetonitrile/40% Isopropanol) increasing to 98% over 2.5 minutes with 0.5 minutes of re-equilibration time. The instrument operated with electrospray ionization in the negative mode. The monitored transitions were *m/z* 701→281 and *m/z* 701 →255 for PEth and *m/z* 732→281 and *m/z* 732→286 for PEth-d_{31}.

Results: The limit of detection was 4.0 ng/mL and the method was linear from 8 to 400 ng/mL ($r^2 > 0.998$). Recovery of PEth was found to be greater than 80%. Inter-day and intra-day imprecision and bias of PEth were <10%**.** This method was been applied to authentic specimens for three months with minimal daily maintenance and no instrument down time. The Micro 200 LC occupies 9 sq. ft. of bench top space, uses 3x 110 volt power outlets, and uses less mobile phase than the more complex multiplexed system which occupies 27 sq. ft. of bench top space and uses 12x 110 volt power outlets.

Conclusion: The method and analytical platform presented here is simpler, robust, and rapid in the detection of PEth in DBS while maintaining the required sensitivity, precision and accuracy. With no change to the extraction method, sensitivity, precision and accuracy of the method we have decreased the footprint of the instrumentation by 60%, decreased solvent consumption by 94%, decreased electrical power needs by 75% and increased thru put by 3 fold.

Keywords: Phosphatidylethanol, PEth, LC-MS/MS, Mirco LC, Dried Blood Spot

P-12

A Comprehensive Screening of Illicit and Pain Management Drugs from Whole Blood Matrix Using SPE and LC/MS/MS

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Background: Drug analysis from whole blood is gaining popularity due to a more complete measurement of analytes in the biological system. Historically, most drug screens have been performed using immunoassay and similar technologies. These techniques often lack specificity and sensitivity necessary for today's complex legal requirements. In this poster, we demonstrate a fast, robust technique for the screening of a broad range of drugs from whole blood utilizing SPE and tandem mass spectrometry. We attempted analysis of forty one compounds, which include opiates (both natural and synthetic), amphetamines, benzodiazepines, phenylpiperidines, a muscle relaxant, and some illicit drugs (PCP and cocaine metabolite)

Objective: Utilizing advanced sample preparation techniques we simplify a complicated matrix to allow for a fast and successful multi-component analysis by LC/MS/MS.

Method: Six pretreatment options for whole blood were investigated. The volume of whole blood used was 0.5 mL. A 5% zinc sulfate and acetonitrile combination worked for most classes. Benzodiazepines had a better overall response with 10% MeOH in ACN. However, there was not a single pretreatment procedure that worked well for all classes of compounds. The supernatant from the previous step was loaded onto the Strata-X Drug B 30mg/3mL cartridges. Salts and most endogenous components were removed using a two-step, 0.1% formic acid and 30% methanol, wash. Analytes were eluted using 2x500 uL of ammoniated IPA/ethyl acetate solution. Samples were then acidified and evaporated to dryness at 40-45°C under a gentle nitrogen stream. The dry residue was re-suspended in mobile phase and injected onto the column.
The chromatographic separation was performed on a Kinetex 2.6um Biphenyl 50x3.0mm column in five minutes. The best choice of mobile phase was 0.1% formic acid in water and 0.1% formic acid in methanol. The detection was achieved on an AB Sciex Triple Quad 4500 and/or a 4000 QTrap LC/MS/MS system. All analytes were detected under positive polarity and MRM scan function using two mass transitions. A MRM-IDA-EPI combination scan was used on a small sample set to verify the peak purity by comparing the MS/MS spectrum against neat standard.

Results: A detection limit of 10ng/mL was achieved for most tested analytes using this method, with tramadol, propoxyphene, fentanyl, carisoprodol and meprobamate produced strong signals yielding a possible lower detection limit. The absolute lowest detection levels for these analytes have not yet been determined. Although a full calibration method was not vigorously tested, the upper end of the calibration range was deemed to be 1000ng/mL. A quadratic fit with 1/x weighting factor was employed for calibration purposes. Early eluting analytes were well separated from the ion suppression zone by a retention factor of two (k'), or by three times the system dead volume (t_0). Isomeric/isobaric compounds were completely resolved by the biphenyl LC column by at least a factor of 2. A resolution value of 3.2 was obtained for morphine and hydromorphone.

Conclusion: The described procedure provides a simple and reliable solution to screen a wide range of illicit and pain control compounds from a complicated matrix.

Keywords: LC/MS/MS, Whole Blood, Illicit Drugs, SPE, Reversed-Phase Chromatography

P-13
LC-TOF-MS Analysis of 100 False Positive Amphetamine Immunoassay Urine Specimens

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Introduction and Objective: Urine drug screens are frequently performed by immunoassay (IA) due to ease of use, rapid result and ability to detect specific drugs or drug classes. However, immunoassays are limited by their sensitivity and specificity when compared to complex methods such as chromatography coupled to mass spectrometry. IA drug screens are prone to interferences and can produce false-negative and false-positive results, due largely to the cross-reactivity profiles of the capture antibodies. The purpose of this retrospective study was to evaluate the performance of an in-house urine amphetamine (AMP) and MDMA (Ecstasy or Molly) EMIT drug screen which reflexes positive results, when the cut-off is >300 ng/mL (AMP) and >500 ng/mL (MDMA), to confirmation by LC-MS/MS. We evaluated 21,189 specimens that were screened by EMIT. 4.0% were positive by EMIT and confirmed positive by LC-MS/MS, 94.4% screened negative, and 1.6% samples were false positive (positive by EMIT and confirmed negative by LC-MS/MS). We evaluated 100 false positive specimens by LC-TOF-MS using a targeted database to identify compounds that may have caused the false positive IA results. We were unable to detect false negative amphetamine specimens.

Methods: 100 false positive IA specimens for AMP/MDMA were analyzed using an1260 liquid chromatograph coupled to a 6230 TOF (Agilent Technologies, Santa Clara, CA) using a method previously published (1). Full scan data from 100-1000 m/z were collected. Data were analyzed using MassHunter software and the Personal Compound Database Library (Agilent). Specimens were diluted five-fold with Nanopure water prior to analysis. Criteria for a positive identification were retention time ±0.15 minutes, mass error ±10ppm, and match score >60. Compounds were identified using a targeted database of compounds likely to cause a false positive IA response gathered from the literature and other sources. Cutoffs ranged from 20-100 ng/mL.

Results: Eleven compounds suspected of causing a false positive screen result were detected in 61 of the 100 specimens. 35 specimens had only one compound detected, 12 had 2 compounds, 9 had 3 compounds, and 5 specimens had 4 compounds detected. There were no false positive amphetamine results.

Compound	n	Average Score	Average Mass Error (ppm)	Average RT Diff
Bupropion	15	81	0.1	0.014
Cathine	5	81	4.5	0.040
Fluoxetine	18	95	-0.5	-0.044
m-Chlorophenylpiperazine (mCPP)	16	76	1.1	-0.062
Methylenedioxypyrovalerone (MDPV)	1	76	4.6	-0.022
Phentermine	10	94	-1.8	0.046
Pyrovalerone	3	93	-3.3	0.044
Ranitidine	4	90	0.7	0.033
Salbutamol	2	64	-1.1	-0.005
Trazodone	27	94	-0.5	0.017
Propranolol	5	94	-1.2	0.007

Discussion and Conclusions: We performed a targeted screen by LC-TOF-MS to identify compounds that were structurally similar to AMP/MDMA and compounds that are known to cause false positives by IA. Full scan high resolution exact mass data and retention time enabled sensitive identification of drugs that may not have been detected by a traditional targeted MRM LC-MS/MS approach. Samples which had a false positive IA result but no identification by LC-TOF-MS are unknown compounds or bath salts or their analogs for which no standard to verify identification is available. **References;** Marin SJ, et al, *J Anal Tox.* Sep;36(7):477-86 (2012)

Keywords: Amphetamine, False Positive Immunoassay, LC-TOF-MS

P-14
Ultrafast Detection of Drugs of Abuse in Urine

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Introduction: Forensic toxicology laboratories routinely profile urine for drugs of abuse to detect potentially positive individuals. These presumed positive individual samples are then further analyzed by a GC/MS/MS or LC/MS/MS to confirm presence of analytes of interest. The sensitivity and specificity achieved during the initial detection method is critical for both accuracy of the investigation and efficiency of processing. Mass spectrometry provides such sensitivity and specificity but often lacks in efficiency due to its speed of analysis that is typically minutes per sample.

Objective: In the present study, we sought to create an ultrafast method for qualitatively measuring drugs of abuse in urine with using a novel SPE/MS/MS technology.

Methods: Calibration standards were created by spiking drug free urine with 1000 ng/mL of each analyte. Serial dilutions were used to achieve remaining concentrations. Standards and human samples were prepared by glucuronidase treatment followed by a 1 to 50 dilution with water. An automated online SPE system in combination with a triple quadrupole mass spectrometer was used to analyze the samples. Each sample was evaluated by two drug panels. Analysis time for each panel was 14 s with a 9 s blank injection between samples to prevent any carryover contamination. A single transition was used per analyte with a class-specific internal standard transition used to normalize the data. More than a dozen commonly abused drug analytes were evaluated including amphetamines, PCP, benzoylecgonine, benzodiazepines, and buprenorphine. The LLOQs for these analytes were the following: amphetamines (50 ng/ml), PCP (20 ng/ml), benzoylecgonine (50 ng/ml), benzodiazepines (100ng/ml), and buprenorphine (50 ng/ml). To further investigate the integrity of this method, the results were compared to a quantitative LC/MS/MS analysis using the same triple quadrupole mass spectrometer. The LC/MS/MS method utilized a 7.5 minute methodology and included both quantifying and qualifying ions for each analyte of interest.

Results: More than 50 human urine samples were analyzed. There was excellent correlation between human samples testing positive with the fast SPE/MS/MS analysis to the LC/MS/MS evaluation. No samples tested negative by SPE/MS/MS but positive by LC/MS/MS for a false negative. The false positive rate was less than 5% for most analytes. Benzoylecgonine for example had one false positive with all other samples correctly identified.

Conclusions: This ultrafast SPE/MS/MS methodology provides a means of profiling human urine samples for common drugs of abuse. It provides the sensitivity and specificity of mass spectrometry without compromising on speed of analysis.

Keywords: Drugs of Abuse, SPE/MS/MS, Screening, Urine, RapidFire

P-15

Development of a Fast Direct Injection TOF-MS Screen for Common Prescription and Non-Prescription Drugs in Urine

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Background / Introduction: It is important to know all drugs (prescription and non-prescription, licit and illicit) that a patient may be taking to understand possible side effects and drug interactions. Therefore, a method to simultaneously screen for commonly prescribed and over-the-counter medications in urine that includes acetaminophen, cetirizine, metoprolol, nicotine, cotinine, phentermine, and zolpidem, just to name a few, was developed using a novel direct injection-TOF technology. Screening by this technique provides a rapid, comprehensive, and selective mode of analysis of these Common Medicines.

Objectives: This work was developed to show that TOF accurate mass and associated criteria (i.e., isotopic distribution) alone can be used to screen for compounds in urine without the need for full chromatographic separation. Eliminating full chromatographic separation also increases the throughput of the method allowing for increased test numbers per instrument per day.

Methods: Reference standards for thirty commonly prescribed and over-the-counter drugs and/or their metabolites along with an internal standard were acquired from Cerilliant (Round Rock, Texas) or Sigma Aldrich (St. Louis, Missouri). Method development and sample analysis were completed on an Agilent (Santa Clara, California) 6530 Quadrupole-Time-of-Flight and an Agilent 1290 UPLC system with a microwell plate autosampler. A Restek biphenyl guard column was used for the updated screen with "no chromatographic separation" in place of a Phenomenex Kinetex phenyl-hexyl LC column (50 x 2.1 mm, 2.6 um) that was used in the original confirmation method with chromatographic separation. Urine samples were diluted two-fold and extracted with an SLE column (Biotage, Charlotte, North Carolina) using an automated system (ALD III System, SPEware, Baldwin Park, California) prior to the screen. The method performance for the updated "no chromatographic separation" method (~1 min cycle time) and the original chromatographic separation method (6 min cycle time) were assessed in terms of peak shape, mass accuracy, and overall analytical scores including isotopic patterns.

Results: Screening cut-offs were set at 50 ng/mL for all compounds except acetaminophen, caffeine, cetirizine, oxcarbazepine/carbamazepine epoxide, and topiramate, which were set at 100 ng/mL. Data was analyzed in a semi-quantitative manner using a single-point calibration, compound match score, and signal-to-noise ratio for each compound. If the compound match score was ≥ 70, the signal-to-noise ratio was ≥ 5, and the concentration was above the cut-off control, then the sample was deemed positive for that indicated analyte. Using these criteria, authentic patient positive urine samples were run in parallel with an existing UPLC-QTOF method with good correlation of positivity results.

Conclusion / Discussion: Screening for 30 commonly prescribed and over-the-counter medications in urine, along with select metabolites was successful with TOF accurate mass and associated criteria alone, eliminating the need for complete chromatographic separation. For compounds that were known to have isobaric interferences, the samples were run on the original method for confirmation. The method compared well with an existing UPLC-QTOF method.

Keywords: Direct Injection, TOF, Screen

P-16
Using UPLC®/MS/MS for Workplace Drug Testing in the European Union

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Introduction*:* In recent years workplace drug testing (WPDT) laws have been implemented in certain geographies for workers employed in specific industry sectors particularly those in safety-critical job roles. Random drug testing in the workplace is aimed not only at reducing costs in terms of lost productivity and absenteeism, but also at ensuring safety for the individual and the wider community.

Objective: To develop a semi-quantitative UPLC/MS/MS method for the following 21 substances which are commonly screened for in Italian workplace drug testing schemes: normorphine, morphine, norcodeine, dihydrocodeine, 6-MAM, codeine, ephedrine, amphetamine, methamphetamine, MDA, MDMA, MDEA, MBDB, benzoylecgonine, cocaine, methadone, EDDP, ketamine, cTHC, norbuprenorphine, buprenorphine, norbuprenorphine-glucuronide, buprenorphine-glucuronide and cTHC-glucuronide as well as the following class specific internal standards morphine-d6, amphetamine-d11, benzoylecgonine-d8, buprenorphine-d4, methadone-d9 and carboxy-THC-d3 . The method must be sensitive enough to detect the analytes at concentrations lower than the currently applied cut-offs and ideally, not require lengthy sample hydrolysis.

Methods: Un-hydrolysed urine samples (200µL) were prepared by simple 5-fold dilution with 5% acetonitrile. Diluted samples (8µL)were separated on a Waters ACQUITY UPLC I-class system with flow through needle using an ACQUITY UPLC BEH C18 column (100 x 2.1mm, 1.7µm), with 0.1% formic acid and acetonitrile as the mobile phases and a total chromatographic run-time of 7.0 minutes. Substances were analysed using a XEVO TQD with polarity switching in electrospray ionisation for detection in either positive or negative mode. Authentic urine samples were analysed utilising this method and the results were compared to those obtained using both immunoassay and GC/MS techniques.

Results: The developed method was assessed for accuracy, precision and linearity both intraday and over a 5 day period. Over the 5 day study the r^2 values for linearity, over a concentration range from zero to greater than 125% of the cut-off values, were all above 0.995. The %RSD for calibrator & QC replicates over the 5 day period at each concentration was <10% and the achieved values were within 10% of target. Matrix effects were investigated in 6 separate urine samples at 3 concentrations and ranged from -33% (normorphine) to +40% (benzoylecgonine). Limit of detection and lower limit of quantitation for each analyte were determined and shown to be below currently applied cut-off levels. Carryover was shown to be both less than 50% of the limit of detection following injections at 5000ng/mL for each analyte. Each analyte was shown to be free from interference from a panel of commonly encountered interferants including metabolites and glucuronides of the selected analytes. More than 100 authentic samples were analysed and the method correctly identified those analytes assigned as putative positive by the immunoassay techniques. Those analytes that screened positive by UPLC/MS/MS gave excellent correlation when compared to the results from the GC/MS method.

Conclusions: This work presents a fast, sensitive and reliable method for screening a panel of analytes in a workplace drug testing laboratory. It demonstrates excellent correlation with alternative immunoassay and GC/MS methods. Moreover, where GC/MS values were available, the semi-quantitative values obtained by this method showed very good agreement.

Keywords: Workplace Drug Testing Urine, UPLC®/MS/MS

P-17

Analytical Evaluation of an Enzyme-Linked Immunosorbent Assay for the Specific Detection of Norketamine in Human Urine

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Introduction: The drug ketamine is a dissociative anaesthetic with some hallucinogenic effects, used in human and veterinary medicine. It is a substance of abuse due to its ability to produce dissociative sensations and hallucinations. It has also been used to facilitate sexual assault. This drug can alter numerous functions in the brain and can produce psychological addiction. Chronic ketamine abuse can produce toxicity to the gastrointestinal and urinary tract. It is a controlled substance in several countries. Ketamine is rapidly absorbed when administered through the intramuscular intranasal and oral routes. Biotransformation of this drug primarily takes place in the liver. The most important pathway is N-demethylation to norketamine. The predominant route of elimination is by liver metabolism. The major metabolite norketamine is a biotransformation product, which reduces the likelihood of it as a contaminant when detected in urine samples. This is relevant in forensic applications.

Objective: The aim of this study was to evaluate an enzyme-linked immunosorbent assay (ELISA) for the specific measurement of norketamine in human urine. This ELISA represents an effective screening tool for monitoring ketamine intake with applications in clinical, toxicology, therapeutic, forensic and regulatory fields.

Methods: A competitive colorimetric immunoassay was employed. The capture antibodies were immobilised and stabilised on the 96-well microtitre plate surface. The analyte, if present in the sample, competes with the horseradish peroxidase labelled conjugate for antibody binding sites on the microtitre plate. Absorbances were read at 450 nm. The signal is inversely proportional to the concentration of drug in the sample. The sample can be either diluted after a rapid centrifugation (1 minute) prior application to the microtitre plate or for enhanced sensitivity can undergo extraction procedure. This procedure involves addition of solvent to urine followed by elution of the upper organinc layer and evaporation to dryness. Following resuspension of the dried extract the sample is ready for application to the microtitre plate.

Results: The ELISA was standardised to norketamine, the % cross-reactivity for ketamine was 2.89% and for dehydronorketamine 4.47%; phenylcyclidine (PCP) elicited a negative response at a concentration of 2000 ng/ml. The limits of detection (LODs) in human urine were 10.8 ng/ml (rapid dilution method) and 0.69 ng/ml (extraction procedure). The intra-assay precision (n=12), expressed as %CV, was $\leq$ 10% and the %recovery ranged from 71 to 109%, for both sample preparation methods. A collection of 15 urine samples with ketamine and norketamine values assigned by GC/MS were assessed with this ELISA and an agreement of 100% was obtained.

Conclusions: The results indicate that this ELISA is specific for norketamine, the major metabolite of ketamine. The LOD of the assay for the rapid sample preparation method of simple dilution is 10.8 ng/ml but is reduced to 0.69 ng/ml when the sample extraction procedure is applied, which meets guidelines set out by the Society of Forensic Toxicologists (SOFT, *http://soft-tox.org/sites/default/files/SOFT%20DFC%20Rec%20Det%20Limits%201-2014.pdf*), in urine (recommended cut-off 10 ng/ml for ketamine/norketamine). This ELISA represents an effective screening tool for monitoring ketamine use or misuse.

Keywords: Norketamine, ELISA, Screening, Urine

P-18
Cross-Platform Comparison of Rapid Benzodiazepine Analyses

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Objective: The assessment of the analysis of benzodiazepines in urine compared across three innovative fast (less than 20 seconds per sample) sample introduction platforms is presented.

Background: Benzodiazepines are commonly used drugs that replaced barbiturates in the sedative-hypnotic market in the 1960s and 1970s as a safer alternative. However, by the 1980s their potential for abuse and dependence became clear, though this did not result in a drastic decrease in the number of prescriptions written. Because of their widespread use and abuse, as well as their regulation by the U.S. Drug Enforcement Agency, therapeutic drug monitoring is used to measure patient compliance or divergence. The extensive use of benzodiazepines translates into a large number of samples to be analyzed daily, leading to faster methods being more desirable. Traditional analyses involving chromatographic separations are often time-consuming; they are limited by flow rates, the time needed to physically separate analytes on-column before injection into the tandem mass spectrometer, and the need to re-equilibrate columns following a gradient elution. Even with multiplexing, it is difficult to run an analysis in under one minute per sample with traditional HPLC/MS/MS methods, demonstrating a distinct need for faster sample introduction and analysis to handle the large testing volume.

Methods: This cross-platform comparison was performed with 50 randomly selected positive authentic urine samples for 7-aminoclonazepam, alpha-hydroxyalprazolam, alprazolam, lorazepam, nordiazepam, oxazepam, and/or temazepam and compared to results from obtained from a traditional HPLC/MS/MS method that had been previously validated on a Thermo TSQ Quantum Access Max system. All samples were hydrolyzed with β-glucuronidase at 60°C for 30 minutes, then diluted further and spun down for analysis, which was performed concurrently across the three sample introduction platforms tested. The instruments studied included the Thermo Transcend (fully multiplexed turboflow) system coupled to a QExactive mass spectrometer, a Phytronix LDTD (laser diode thermal desorption) front end coupled to a QExactive mass spectrometer, and a RapidFire (online SPE) system coupled to an Agilent 6550 mass spectrometer.

Results: Each of the three platforms examined offered significantly faster analysis times (less than 20 seconds per sample) when compared to a traditional HPLC/MS/MS method. While all platforms required sample hydrolysis, two of the three needed additional preparation after hydrolysis. Transcend was able to utilize the hydrolyzed samples immediately, while the RapidFire required an additional dilution step before analysis, and the LDTD needed further preparation and dilution prior to sample injection. Despite these additional steps, total sample analysis time remained comparable across the novel platforms, and all demonstrated greatly reduced time requirements compared to the HPLC method.

Conclusion: The three rapid analysis platforms examined all displayed comparable results to each other and to those found by traditional HPLC/MS/MS; however, all three novel platforms demonstrated at least a 5x decrease in analysis time and a lower cost per sample than the traditional method.

Keywords: Benzodiazepines, Urine, Rapid Analysis

P19

Sensitive Detection of Tramadol and Its Major Active Metabolite in Urine and Blood with an Enzyme-Linked Immunosorbent Assay

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Introduction: Tramadol is a synthetic opioid-receptor agonist that has been used clinically as a narcotic analgesic since 1977. Tramadol inhibits reuptake of certain monoamines (norepinephrine, serotonin), which contributes to its analgesic effect. Tramadol has two primary metabolites, N-desmethyl and the active metabolite O-desmethyl tramadol, the latter presenting greater affinity for μ-opioid receptors and twice the analgesic potency of the parent compound. Elimination of tramadol and its metabolites is predominantly via the kidneys. Impairing side effects from the usage of tramadol include dizziness, confusion, light-headedness or fainting spells, drowsiness, seizures and respiratory depression. Tramadol misuse is becoming more common. In addition the sale of tramadol on the black market is a major public health issue both in the United States and internationally.

Objective: The aim of this study was to evaluate the analytical performance of an enzyme-linked immunosorbent assay (ELISA) for the sensitive detection of tramadol and its major active metabolite O-desmethyl tramadol in urine and blood requiring simple sample preparation. This represents a useful screening tool for applications in toxicology.

Methods: A competitive colorimetric immunoassay was employed. The capture antibodies were immobilised and stabilised on the 96-well microtitre plate surface. The analyte, if present in the sample, competes with the horseradish peroxidase labelled conjugate for antibody binding sites on the microtitre plate. Absorbances were read at 450 nm. The signal is inversely proportional to the concentration of drug in the sample. The urine samples were diluted after a centrifugation step of 1 minute prior application to the microtitre plate. Whole blood samples, collected and prepared in accordance with the collection tube manufacturer's recommendations, were diluted prior application to the microtitre plate. For the percentage recovery a stock solution of tramadol (1μg/ml) was prepared and assessed via HPLC; this was used to spike urine and blood samples at concentrations spanning the assay range (3.2 ng/ml, 12 ng/ml, 60 ng/ml).

Results: The assay was standardised to tramadol and the metabolite O-desmethyl tramadol was also detected with a cross-reactivity of 57%. The limit of detection values were 1.40 ng/ml (urine) and 0.86 ng/ml (blood) for an assay range 0-100 ng/ml. The intra-assay precision (n=12**),** expressed as CV, was <7.0% for different concentration levels. The percentage recovery ranged from 84% to 117% (urine) and from 88% to 109% (blood).

Conclusions: Data indicate that tramadol and its major metabolite O-desmethyl tramadol can be detected with this ELISA. The limit of detection value in urine (1.40 ng/ml) is below the minimum performance limit (10 ng/ml) recommended by the Society of Forensic Toxicologists (SOFT,*http://softtox.org/sites/default/files/SOFT%20DFC%20Rec%20Det%20Limits%201-2014.pdf*), the LOD was lower in blood (0.86 ng/ml). The sample preparation for both matrices is simple, making this ELISA a useful screening tool for applications in toxicology.

Keywords: Tramadol, Tramadol Metabolite, ELISA, Screening

P-20

Development of the First Generic and Highly Sensitive Polyclonal Antibody for the Detection of the Hallucinogenic Family "25-NBOMe" Designer Drugs

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Introduction: The new class of the hallucinogens called 25-NBOME or NBOMe compounds are N-methyoxybenzyl derivatives of the 2C-X series (substituted 2, 5-dimethoxyphenethylamines). They act as potent serotonin agonists, particularly at the 5-HT2A receptors. 25-NBOMe compounds are described as highly powerful hallucinogens, showing psychoactive effects similar to LSD. They are active at very low dose (micrograms) and are generally administered on paper placed under the tongue. It may also be vaporized and inhaled. The onset of effects is rapid and the duration is 2-4 hours, but can sometimes last much longer, depending on the dose. Side effects may last up to 7 days. Users experience visual hallucinations, euphoria, dilated pupils and changes in perception of time. Undesired side effects include confusion, scrambled communication, paranoia, panic and seizures. Death has been reported in several cases. These substances are now a scheduled I. 25-NBOMe compounds include: 25B-NB2OMe, 25C-NB2OMe, 25D-NB2OMe, 25E-NB2OMe, 25H-NB2OMe, 25I-NB2OMe, 25N-NB2OMe, 25P-NB2OMe, 25T2-NB2OMe, 25T4-NB2OMe, 25T7-NB2OMe, Mescaline-NB2OMe**.**

Objective: The aim of this study was to develop the first generic and highly sensitive polyclonal antibody to the hallucinogenic compounds 25-NBOMe designer drugs. This antibody could be used in the development of efficient immunoassays for the broad determination of these compounds in different matrices (i.e. urine, blood, oral fluid and hair samples).

Methods: The 25-NBOMe hapten was synthetized in multi-steps and conjugated to carrier protein (BTG) for the preparation of the immunogen using standard methods of conjugation. The immunogen was administered to adult sheep on the monthly basis to provide target-specific polyclonal antiserum. IgG was extracted from the antiserum and evaluated via competitive immunoassay (ELISA). The specificity (expressed as % cross-reactivity) was determined by generation of calibration curves for 25I-NB2OMe and each of the cross-reactants tested. Cross-reactivity was calculated: %cross-reactivity=[IC50(25I-NB2OMe)/IC50(cross-reactant)]x100. The IC50 for each analyte was calculated by taking 50% of the optical density (OD) from the zero calibrator and reading this OD value from the x-axis (concentration in ng/ml) of the respective calibration curve. This concentration corresponded to the inhibitory concentration that produced 50% inhibition.

Results: The initial evaluation of the polyclonal antibody showed broad specificity profile to 25-NBOMe compounds and to O-Desmethyl metabolites (Table 1), no cross-reactivity was observed towards 2C-X series and DO-X series. The sensitivity, expressed as ~~a~~ half maximal inhibitory concentration (IC_{50}) ranged from 0.096 ng/ml (25H-NB2OMe) to 0.887 ng/ml (25N-NB2OH) (Table 1).

Conclusions: The polyclonal antibody developed for the detection of 25-NBOMe family presents broad specificity profile and is highly sensitive. This antibody is suitable for the development of generic immunoassays for the screening of these hallucinogenic compounds in human biological fluids (i.e. urine, blood, oral fluid and hair) for forensic and toxicology applications.

Table 1. Polyclonal Antibody to 25-NBOMe Designer Drugs: Specificity and Sensitivity

Compound	IC50 (ng/ml)	% Cross-reactivity
25I-NB2OMe	0.164	100
25P-NB2OMe	0.147	111.6
Mescaline-NB2OMe	0.1	164
25B-NB2OMe	0.138	118.8
25D-NB2OMe	0.104	157.7
25E-NB2OMe	0.13	126.2
25H-NB2OMe	0.096	170.8
25(NH2)-NB2OH	0.615	26.7
25N-NB2OH	0.887	18.5

Keywords: 25-NBOMe, Hallucinogens, Designer Drugs, Antibodies, Immunoassays

P-21

Development of the First Highly Sensitive Polyclonal Antibodies for the Detection of the New Synthetic Cannabinoid "APINACA"

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Introduction: The new wave of synthetic cannabinoid compounds include N-(1-adamantyl)-1-pentyl-1H-indazole-3-carboxamide referred to as APINACA or AKB48. This compound is a pentyl indazole compound with strong affinity for the CB2 receptor. First identified in Japan 2012, APINACA has since been banned in some countries and placed under temporary schedule I in the Unites States. This compound cannot be detected by existing screening assays for synthetic cannabinoids therefore development of specific tests for the detection of this compound and metabolites is of critical importance for forensic testing in the continuing battle to detect the expanding menu of designer synthetic cannabinoid drugs.

Objective: The aim of this study was to develop the first highly sensitive polyclonal antibodies towards N-(1-adamantyl)-1-pentyl-1H-indazole-3-carboxamide referred to as APINACA or AKB48. Two antibodies were developed to ensure the detection of the parent compound (AKB48) and related compounds. These antibodies are of value in the development of immunoassays for the detection and the quantification of these compounds in biological matrices.

Methods: The N-(1-adamantyl)-1-pentyl-1H-indazole-3-carboxamide haptens were synthetized in multi-steps and conjugated to carrier protein (KLH) for the preparation of the immunogens using standard methods of conjugation. The immunogens were administered to adult sheep on a monthly basis to provide target-specific polyclonal antiserum (polyclonal antibodies A and B). IgG was extracted from the corresponding antiserum and evaluated via competitive immunoassay (ELISA). The specificity (expressed as % cross-reactivity) was determined by generation of calibration curves for the analyte used for assay standardisation and each of the cross-reactants tested. Cross-reactivity was calculated: %cross-reactivity=[IC_{50}(analyte)/IC_{50}(cross-reactant)]x100. The IC_{50} (half maximal inhibitory concentration) was calculated by taking 50% of the optical density (OD) from the zero calibrator and reading this OD value from the x-axis (concentration in ng/ml) of the respective calibration curve. This IC_{50} concentration corresponded to the concentration that produced 50% inhibition.

Results: The initial evaluation of polyclonal antibody A**,** standardised to AKB48 N–Pentanoic acid metabolite, showed excellent sensitivity IC_{50}: 0.2ng/ml. Polyclonal antibody B, standardised to parent compound AKB48**,** exhibited an IC_{50} of 2ng/ml and showed a broader specificity profile, with detection of 3'-OH AKB48 (%cross-reactivity: 1163.5%), N-(5-fluoropentyl) AKB48 analogue (5F-AKB48, 5F-APINACA) (%cross-reactivity: 98.9%), 3'-OH SDB001 (%cross-reactivity: 47.3%) and STS-135 (%cross-reactivity: 8.4%).

Conclusions: The results indicate that the developed polyclonal antibodies are highly sensitive and enable the determination of APINACA/AKB48 and related compounds. These antibodies are suitable for the development of generic immunoassays for the detection and the quantification of these compounds in human biological fluids (urine, blood, oral fluid and hair) for forensic and toxicology applications.

Keywords: Synthetic Cannabinoids, AKB48, APINACA, Antibodies, Immunoassays

P-22

Newly Developed Enzyme-Linked Immunosorbent Assay for the Sensitive Detection of Buprenorphine and Metabolites in Urine and Blood

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Introduction: The opioid buprenorphine, a partial agonist of the μ-opioid receptor, is used for treating pain and opiate addition. It is a beneficial medication when used as prescribed, it has however abuse liability. Buprenorphine is a Schedule III narcotic under the Controlled Substances Act. Buprenorphine is extensively metabolized in humans, the primary route is N-dealkylation to the active metabolite norbuprenorphine. Both compounds undergo glucuronidation. The availability of immunoassays with low detection limits for the screening of the parent compound and metabolites are advantageous to monitor buprenorphine maintenance and detection of nonprescribed use.

Objective: The aim of this study was to evaluate the analytical performance of a newly developed enzyme-linked immunosorbent assay (ELISA) for the sensitive detection of buprenorphine and metabolites in urine and blood. This represents a useful screening tool for monitoring the use or misuse of this compound in clinical, toxicological and forensic settings.

Methods: A competitive colorimetric immunoassay was employed. The capture antibodies were immobilised and stabilised on the 96-well microtitre plate surface. The analyte, if present in the sample, competes with the horseradish peroxidase labelled conjugate for antibody binding sites on the microtitre plate. Absorbances were read at 450 nm. The signal is inversely proportional to the concentration of drug in the sample. Urine samples were centrifuged for 1 minute. Whole blood sample preparation was carried out in accordance with the collection tube manufacturer's recommendations. Both urine and whole blood were diluted 1 in 4 and only 50μl of sample were required.

Results: The assay was standardised to buprenorphine and the metabolites norbuprenorphine and norbuprenorphine-3β-D-glucuronide were detected with % cross-reactivity of 499% and 139% respectively. The limit of detection values were 0.75 ng/ml (urine) and 0.57 ng/ml (blood). The intra-assay precision (n=12**),** expressed as mean %CV, was <3.0% for different concentration levels. The percentage recovery from fortified urine and blood samples with an HPLC assigned stock, ranged from 111% to 128% (urine) and from 95% to 117% (blood). A collection of blood samples with buprenorphine values assigned by GC/MS were assessed with this ELISA and an agreement >90% was obtained.

Conclusions: The results indicate that with this sensitive ELISA buprenorphine and the metabolites norbuprenorphine and norbuprenorphine-3β-D-glucuronide can be detected. The limit of detection value in urine (0.75 ng/ml) is below the minimum performance limit (1 ng/ml) recommended by the Society of Forensic Toxicologists (SOFT, *http://soft-tox.org/sites/default/files/SOFT%20DFC%20Rec%20Det%20Limits%201-2014.pdf*), the LOD was lower in blood (0.57 ng/ml). The sample preparation for both matrices is simple, which makes this ELISA a useful screening tool for applications in toxicology.

Keywords: Buprenorphine, Buprenorphine Metabolites, ELISA, Opioid, Screening

P-23

Development of the First Generic Polyclonal Antibodies for the Detection of the DO-X and 2C-X Series of Compounds

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Introduction: Phenethylamines are a group of synthetic compounds which include many designer drugs such as dimethoxyphenylpropanamine (DO-X) and the dimethoxyphenylethanamine (2C-X) series of compounds. These drugs were primarily developed by subtly changing the chemical structure of existing compounds to maintain the ecstasy-like effects of these illegal drugs, whilst creating products which are currently not covered by existing laws. Their use has increased dramatically in the last 5 years, with several fatalities occurring recently.

Objective: The aim of this study was to develop the first generic polyclonal antibodies against the DO-X and 2C-X families of synthetic compounds. These antibodies could be used for the development of generic immunoassays for the detection of these synthetic phenethylamines in human samples.

Methods: The two antibodies were produced via immunogens comprising DO-X hapten and 2C-X hapten conjugated to carrier molecules and administered to adult sheep on a monthly basis to provide target-specific polyclonal antiserum against each immunogen. IgG was extracted from the antiserum and evaluated via competitive immunoassay (Enzyme-linked Immunosorbent Assay) for each of the targets.

Results: Polyclonal antibody for the detection of DO-X compounds: the assay was standardised to 2,5-dimethoxy-4-bromo-amphetamine (DOB) and presented a broad specificity profile for other DO-X compounds (Table 1). Amphetamine, metamphetamine, MDMA, MDEA, MBDB methcathinone, phentermine, mephedrone, tryptamine, tyramine, cathinone, pseudoephedrine and putrescine caused no interference with the assay at a concentration 100 ng/ml. The Limit of Detection (LOD, calculated as mean concentration of 20 negative samples, plus 3 standard deviations) was 0.8ng/ml in whole blood and 1.1ng/ml in urine (assay range 0-40ng/ml for both matrices). Intra-assay precision, expressed as CV, was <5%. -Polyclonal antibody for the detection of 2C-X compounds: results from the initial evaluation stages are presented. The assay was standardised to 2C-B and presented a broad specificity profile for other 2C-X compounds (Table 1). The sensitivity was assessed using a buffer based matrix and was expressed as half maximal inhibitory concentration (IC_{50}), the value obtained was 0.743ng/ml (assay range 0-20ng/ml). Intra-assay precision, expressed as CV, was <7%.

Conclusions: The two polyclonal antibodies developed are suitable for the development of generic competitive immunoassays for the detection and the quantification of the designer drugs DO-X and 2C-X series in human biological fluids with important forensic and toxicological applications.

Polyclonal antibody to DO-X compounds Specificity		**Polyclonal antibody to 2C-X compounds Specificity**	
Compound	**% Cross-**	**Compound**	**% Cross-**
DOB	100	2C-B	100
Bromo-DragonFLY (HCL)	96	2C-B fly	137.3
DOI HCL	73	2C-T7	110.1
DON	57	2C-T2	81.1
DOET	50	2C-C	63.4
DOM	49	2C-I	60.3
DOC	47	2C-D	59.3
2,4,5-Trimethoxyamphetamine	5	2C-P	39.3
		2C-E	34
		2C-N	29.9
		3C-B fly	<3.7

Keywords: DO-X Compounds, 2C-X Compounds, Polyclonal Antibodies, Generic Immunoassays, Screening

P-24
Evaluation and Validation of a Rapid Multiplex Immunoassay from Randox

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Introduction: The immunoassay is an integral part of the general screening in toxicology. Our current immunoassay platform (AxSYM from Abbott) is presently being discontinued. Following a survey of the different technologies available, the Randox biochip array platform was selected and evaluated. Its high throughput, suitability for ante and post-mortem samples, as well as its broad range of analytes made it the method of choice for our laboratory.

Objective: Our first objective was to evaluate 2 different biochip arrays DoA I+ and DoA IV for blood and urine sample on the Evidence Investigator. Our second objective was the validation of these 2 arrays for blood samples on the Evidence. A urine custom chip has been ordered and will also be validated on the Evidence.

Methods: The Randox protocol for sample analysis was followed: blood centrifugation at 4 000 rpm for 20 min followed by a ¼ dilution in kit diluent. The evaluation consisted mainly in a comparison of results obtained on previously analyzed casework on the AxSYM analyzer as well as confirmatory analysis by GC/MS and LC/MS/MS methods. Cut-off levels were selected to minimize false-positives and false-negatives in ante and post-mortem samples. The validation follows the SWG-TOX recommendations and evaluates the limit of detection, cut-off level precision and accuracy, carry-over, stability of samples on-board, specificity and comparison of a second set of previously analyzed cases. The assays included in the validation for the blood samples were: amphetamine, barbiturates, benzodiazepines, buprenorphine, cannabinoids, cocaine metabolite, methamphetamine, methadone, MDMA, opiates, phencyclidine, tricyclic antidepressants on DoA I+, and ibuprofen, salicylate and acetaminophen for the DoA IV. The urine custom chip additionally contains fentanyl, generic opioids and ketamine assays.

Results: Our evaluation of the technology shows that the assays of the biochip array, other than the amphetamine families, have a % agreement between 89-100% compared to 66-97% agreement for the AxSYM analyzer. False-positives are obtained for putrefied samples on the amphetamine and methamphetamine assays. The limits of detection increased from ante-mortem, femoral, cardiac to post-mortem blood. Amphetamine, methamphetamine, MDMA and salicylate had LODs above the cut-off levels for cardiac and putrefied blood only. No carry-over was observed. The cut-off levels are acceptable with %CV below 20% and accuracies of 70-130% for most analytes. Stability has not yet been evaluated.

Conclusion: The Randox biochip array technology uses a single aliquot for the simultaneous detection of multiple analytes. This cuts down on the preparation and analysis time compared to other immunoassays. Based on the evaluation, the comparison study and validation results, this method was selected to replace our previous immunoassay for routine analysis.

Keywords: Immunoassay, Biochip, Randox, Validation, Evidence

P-25

Detection of Fentanyl and Metabolites Alongside the Designer Drug Analogue Acetylfentanyl in Urine and Blood with an Enzyme-Linked Immunosorbent Assay

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Introduction: Fentanyl is a synthetic opioid analgesic, eighty times more potent than morphine. It is used in medicine as an intravenous anaesthetic/analgesic and in pain management as transdermal patches and lozenges. In high doses fentanyl causes euphoria, marked muscular rigidity and respiratory depression. It is a Schedule II drug under the Controlled Substances Act. Fentanyl is extensively metabolized in humans, the primary route is N-dealkylation to norfentanyl. It has been shown that the concentration of norfentanyl in urine after transdermal application of fentanyl was greater than fentanyl and that it persisted for longer. Other metabolites of fentanyl have been identified. In addition the use of a designer drug analogue, acetyl fentanyl, has increased in use since 2013 with confirmed fatalities and non-fatal overdoses. Immunoassays enabling the detection of all these compounds facilitate the screening of samples in the drug testing process.

Objective: This study reports the analytical performance of an enzyme-linked immunosorbent assay (ELISA) for the detection of fentanyl, metabolites and the designer drug analogue acetyl fentanyl in urine and blood. This represents a useful screening tool in clinical, toxicological and forensic settings.

Methods: A competitive colorimetric immunoassay was employed. The capture antibodies were immobilised and stabilised on the 96-well microtitre plate surface. The analyte, if present in the sample, competes with the horseradish peroxidase-labelled conjugate for antibody binding sites on the microtitre plate. Absorbances were read at 450 nm. The signal is inversely proportional to the concentration of drug in the sample.

Results: The assay was standardised to norfentanyl, the parent compound was detected with cross-reactivity of 790% and benzylfentanyl, thienylfentanyl, acetyl fentanyl, ω-hydroxyfentanyl, (±) cis-3-methylfentanyl and α-methylfentanyl were also detected with cross-reactivity ranging from 134% to 20%. Heroin, morphine, 6-monoacetylmorphine elicited negative response at 250 ng/ml. The limit of detection values were 0.51 ng/ml (urine) and 0.67 ng/ml (blood). The intra-assay precision (n=12**),** expressed as CV, was <5.0% for different concentration level. Assessment of positive samples (20 urine and 20 whole blood) with this ELISA and gas chromatography mass spectrometry (GC/MS) showed 100% agreement (cut-off 1 ng/ml).

Conclusions: The results indicate that with this ELISA norfentanyl, fentanyl, acetyl fentanyl, benzylfentanyl, thienylfentanyl, ω-hydroxyfentanyl, (±) cis-3-methylfentanyl and α-methylfentanyl can be detected. The limit of detection value in urine (0.51 ng/ml) is below the minimum performance limit (1 ng/ml) recommended by the Society of Forensic Toxicologists (SOFT, *http://soft-tox.org/sites/default/files/SOFT%20DFC%20Rec%20Det%20Limits%201-2014.pdf*). The ELISA also showed favourable agreement with GC/MS. This ELISA represents a useful screening tool in the drug testing process.

Keywords: Fentanyl, Fentanyl Metabolites, Acetyl Fentanyl, Screening, ELISA

P-26

Development of a Sensitive ELISA for the Alcohol Biomarker Ethyl Glucuronide in Urine

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Background: In order to establish sobriety and/or abstinence for driver license reinstatement, it has become necessary to monitor alcohol biomarkers in body matrices at a much lower concentration than currently available immunoassays can detect. Following alcohol consumption, UDP glucuronosyl transferase enzymes facilitate conjugation of a small amount of ethanol to glucuronic acid to form ethyl glucuronide (EtG). EtG is one of three phase II ethanol metabolites, along with ethyl sulfate (EtS) and fatty acid ethyl esters (FAEE). The detection window for EtG in urine lasts up to 4 days, compared to about 24 hours for FAEE and falls between the short and long-term markers, making it a meaningful biomarker for alcohol use. Currently, due to severe matrix effects and to avoid false positives, most commercially available immunoassays are designed to detect EtG in urine at a 500 or 1000 ng/mL cutoff. Hence, there is an urgent need to develop a highly specific and sensitive immunoassay to meet the 100 ng/mL cutoff guideline established by the German Society of Toxicological and Forensic Chemistry, for zero alcohol tolerance programs.

Objectives: To develop the first commercially available semi-quantitative ELISA capable of:

a) Detecting EtG in urine at a 100 ng/mL cutoff, with fewer false positives/negatives than current immunoassays.
b) Using rFab technology to minimize undesired matrix effects.
c) Low EtG cutoff for potential application to other forensic matrices (e.g. hair and blood), that are not well-suited to homogeneous or EMIT assays.

Method: A highly specific EtG rFab antibody was immobilized on a microtiter plate. Calibrators, controls and urine specimens were diluted 1:20 in 0.1M phosphate buffered saline, pH 7.0. 10□L of each were pipetted in duplicate directly onto the microtiter plate, followed by the addition of HRP enzyme labeled EtG conjugate (100µL) and then incubated for 60 minutes. The plate was washed with DI water, then incubated with TMB substrate (30 min). The reaction was then stopped with 1N HCl and read at 450 and 650 nm using a microplate reader.

Results: The assay has a detection limit of 50 ng/mL (neat urine) and a screening cutoff of 100 ng/mL. The dose-response curve was linear from 50 to 1000 ng/mL, with no observed cross-reactivity to EtS, common alcohols or other glucuronides. The intra and inter-day imprecisions of the assay were <10%. The assay was validated with 157 LC-MS confirmed urines from clinical laboratories, containing a wide range of specimens from negatives, to close to cutoff, to >200,000 ng/mL. The ELISA detected 34 specimens as true positives, 116 true negatives, with 7 false positives (4%), for an accuracy of 96%.

Conclusion: We have described a sensitive semi-quantitative ELISA for detection of ethyl glucuronide in urine at a 100 ng/mL cutoff. This method should prove very useful to laboratories performing zero tolerance alcohol testing in driver license reinstatement programs.

Keywords: Ethyl Glucuronide, EtG, ELISA, Urine

P-27

Validation and One-Year Review of the Randox Evidence Analyzer at the Alabama Department of Forensic Sciences

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Background/Introduction: In 2013, the National Safety Council's Alcohol, Drug and Impairment Division and other experts convened to make recommendations for matrix, scope of analysis, and cutoffs for drug screening in the toxicological investigation of drug-impaired driving and motor vehicle fatalities. The Randox Evidence Analyzer offers a solution with its biochip array technology for competitive immunoassay testing. The Analyzer is a fully automated instrument with the ability to streamline drug screening by saving analyst time and sample volume.

Objective: To validate a Randox Evidence Analyzer Custom Biochip in whole blood for DUI/D and postmortem testing and review its performance over one-year in a high throughput forensic toxicology state crime laboratory. The 17 target custom biochip included the following assays: amphetamine, methamphetamine, barbiturates, benzodiazepines 1 and 2, cannabinoids, cocaine metabolite, opiate, fentanyl, tramadol, opioids, carisoprodol, methadone, zolpidem, tricyclic antidepressants, dextromethorphan, and buprenorphine.

Methods: Using guidelines from SWGTOX Standard Practices for Method Validation in Forensic Toxicology, we evaluated the following parameters: precision, bias, interference, limit of detection, and false positive/negative rates. Between-day precision and bias at the decision point (cutoff) was assessed in triplicate over five different runs at three different concentration pools: 50% below the decision point (low), at the decision point (cutoff), and 50% above the decision point (high). 18 blank antemortem and 18 postmortem blood samples were analyzed to determine the limit of detection and investigate endogenous interferences. Limit of detection (LOD) was determined as mean x 3SD. 127 previously analyzed samples were investigated to determine false (+) & (-) rates. We also reviewed over 2500 cases from 2013-2014 to further document false positive/negative rates, instrument issues, and control trends. Lastly, we compared over 400 cases simultaneously screened by the Analyzer and the Tecan Freedom Evo using Immunalysis reagents.

Results: Cutoffs were chosen in light of the above-mentioned report recommendations and instrument capability. Only 4 targets displayed false negatives during validation (all <3%). False positive rates were satisfactory with all targets (all <12%). Between run precisions (CV%) were less than 20% at the cutoff for all targets (range: 8.1% - 19.1%). Five targets had biases greater than R=1.30. 12 out of the 17 targets did not display overlap between the low control and the cutoff. Per SWGTOX guidelines it is desired for the mean ($\pm$ 2 SDs) to be between 50% below the decision point concentration and the decision point concentration. It is important this range not overlap the decision point. However, the low false negative and positive rates indicate that the assay has sufficient precision around the cutoff to correctly identify a sample as positive or negative. LODs were fit for purpose and no endogenous interferences were observed. We utilized intra- and total-assay precision, cross-reactivity, interference from commonly encountered analytes from Randox in-house studies.

Conclusion / Discussion: The Randox Evidence Analyzer has proven to be a useful tool for drug screening in a high throughput laboratory as demonstrated by the validation and one-year review of casework. Our routine drug screen includes 88% of the drugs listed in tier I of the "Recommendations for Toxicological Investigation of Drug-Impaired Driving and Motor Vehicle Fatalities" published in JAT (plus five from tier II). For the compounds in tier I, 61% of the cutoffs met or exceeded the same recommendations. Our validated method screens for all recommended drugs with the exception of PCP. Laboratories should strive to enhance their scope of analysis at adequate cutoffs for DUI/D and postmortem testing.

Keywords: Immunoassay, Randox, Method Validation, DUID

P-28

Development and Validation of a Highly Sensitive Homogeneous Immunoassay for the Detection of Ketamine and Its Metabolites in Urine

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Introduction and Objective: Ketamine is an anesthetic agent used in the United States since 1972 for veterinary and pediatric medicine. However, in the past decades, it has gained popularity as a street drug used at clubs and raves (1). Ketamine metabolizes by N-demethylation to norketamine and further dehydrogenates to dehydronorketamine. After 72 hours of a single dose, 2.3% of ketamine is unchanged, 1.6% is norketamine, 16.2% is dehydronorketamine, and 80% consists of hydroxylated derivatives of ketamine (2). There is currently no commercially available homogeneous immunoassay that targets ketamine and its metabolites. The objective of this project was to develop and validate a new high throughput homogeneous enzyme immunoassay (HEIA) for the rapid detection of ketamine and its urinary metabolites at much lower cutoff concentration than any current commercially available immunoassay based products.

Methods: An anti-ketamine polyclonal-based homogeneous immunoassay was developed and validated with authentic urine specimens previously confirmed by LC-MS/MS. The assay was designed to detect ketamine and its metabolites in urine at 100ng/ml as cutoff concentration.

Results: The reportable range of the assay was 50 to 500ng/mL with the cutoff concentration of ketamine set at 100ng/mL. Norketamine metabolite sample was spiked at 400ng/mL into urine shows a 25% cross reactivity while dehydronorketamine samples spiked to urine at concentration of 100,000 ng/mL did not cross react. There was no interference with commonly used therapeutics and drug of abuse with the ketamine HEIA. The intra-day and inter-day coefficient of variation (% CV) for the qualitative assay was less than 1.0%. The HEIA was validated with a total of 91 urine samples previously analyzed by LC-MS/MS. The LC-MS/MS concentration of ketamine and norketamine were 82 to 45,000ng/mL and 120 to 26,000ng/mL, respectively. The negative specimens used for the screening were suspected to be true negatives. The sensitivity, specificity and accuracy of the assay were found to be 100%, 100% and 100%, respectively when LC-MS/MS cutoff concentration was set as 100ng/ml for ketamine or norketamine.

		Confirmation (100ng/mL)	
		Negative	**Positive**
HEIA (100ng/mL)	**Negative**	40	0
	Positive	0	51

Conclusion: This is a first report on a high throughput homogeneous enzyme immunoassay for the detection of ketamine and its metabolites in human urine. When applied to authentic specimens the assay correlated very well with LC-MS/MS results.

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Keywords: Immunoassay, Ketamine, Norketamine, Urine Screen

P-29

Development and Validation of a Highly Sensitive Homogeneous Immunoassay for the Specific Detection of Methadone Metabolite (EDDP) in Urine

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Introduction and Objective: Opiates addiction is a major public health issue in the United States and the methadone maintenance program, although controversial, is an effective treatment of opiate addiction. Methadone is a synthetic opiate agonist that metabolizes by mono and di-N-methylation to form EDDP and EMDP. Methadone possesses similar pharmacologic properties to some opiates, like heroin and morphine, but does not cause euphoria or intoxication with stable dosing. The measurement of EDDP for compliance is a preference in the methadone treatment program due to individuals occasionally spiking their own urine with methadone as a diversion and not ingesting it. Therefore, the commercially available methadone immunoassay may test positive for methadone, but negative for EDDP. The objective of this project was to develop and validate a high throughput homogeneous enzyme immunoassay (HEIA) for the specific detection of EDDP in urine at 100ng/mL cutoff.

Methods: An anti-EDDP rFab was obtained from Alere San Diego and the homogeneous immunoassay was developed at Immunalysis Corp and validated with authentic urine specimens previously confirmed by LC-MS/MS. The assay was designed to detect EDDP in urine at 100ng/mL as cutoff concentration.

Results: The reportable range of the assay was 50 to 500ng/mL while the cutoff concentration of EDDP was set at 100ng/mL The intra-day and inter-day coefficient of variation (% CV) for the qualitative assay was less than 1.0%. The HEIA was validated with a total of 107 urine samples previously analyzed by LC-MS/MS while using 100ng/ml as cutoff concentration for both methods. The sensitivity, specificity and accuracy of the assay were found to be 97%, 99% and 98%, respectively. The calculation of sensitivity, specificity and accuracy are as follows: Sensitivity = number of true positives/ (number of true positives + number of false negatives), Specificity = number of true negatives/ (number of true negative + number of false positives), Accuracy = number of true positive + number of true negative/total samples. Most importantly, methadone sample spiked to urine at 500,000ng/ml was screened negative with this assay.

		Confirmation (100ng/mL)	
		Negative	**Positive**
HEIA (100ng/mL)	**Negative**	68	1*
	Positive	1*	37

*The concentrations of EDDP in those two specimens are around cutoff range.

Conclusion: When applied to authentic specimens the homogeneous enzyme immunoassay for the detection of EDDP in urine correlated very well with LC-MS/MS results.

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Keywords: Immunoassay, EDDP, Methadone, Methadone Metabolite

P-30

The Analysis of Plasma Cannabinoids by Gas Chromatography –Tandem Mass Spectrometry

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Objectives: Two important pharmacologically active compounds in marihuana are delta-9-tetrahydrocannabinol (THC) and cannabidiol (CBD). A method utilizing gas chromatography-tandem mass spectrometry (GC-MS/MS) was developed to test for CBD, THC, and two prominent THC metabolites, hydroxy-THC (OH-THC) and carboxy-THC (COOH-THC) in human plasma.

Methods: Calibrators and controls were prepared in blank human plasma. The calibrator ranges were: THC and OH-THC, 0.1 to 100 ng/mL; CBD, 0.25 to 100 ng/mL; COOH-THC, 0.5 to 500 ng/mL. Controls for THC, OH-THC, and CBD were 0.3, 5, and 80 ng/mL, for COOH-THC, 1.5, 25, and 400 ng/mL. A 1 mL volume of each calibrator, control, blank, or sample was added to separate 16x100 mm silanized glass culture tubes. Each tube was fortified with internal standards (25 µL of 0.1 ng/µL THC-d_3,OH-THC-d_3, CBD-d_3 /0.5 ng/µL COOH-THC-d_3). Analysis preparation involved acetonitrile precipitation, liquid-liquid extraction with hexane:ethyl acetate (9:1), and MSTFA derivatization. The GC-MS/MS analysis used a 7890A GC interfaced with a 7000 MS Triple Quadrupole and operated by Masshunter© B05 00.412 software (Agilent Technologies, Santa Clara, CA). A DB-1ms (30 M x 25 mm, 0.1 µm film) column (Agilent) was employed. The GC conditions used splitless injection, 260˚C injection temperature; 280˚C transfer line temperature and GC oven temperature program (100˚C, hold 0.2 minutes, 20˚C / minute to 280˚C, 70˚C / minute to 310˚C). The GC run time was 12.6 minutes. MS/MS utilized a 230˚C ion source temperature, electronic ionization, and collision induced dissociation. Selected reaction monitoring was employed for the analysis- THC: 371→ 289*, 265; OH-THC: 371→ 289 * 265; COOH-THC: 371→289, 265 *; CBD: 290→375, 301*; THC-d_3: 374→292*,268; OH-THC-d_3: 374→292*,268; COOH-THC-d_3: 374→292, 268*; CBD-d_3: 293→378, 304* (*- quantitation transition).

Results and Conclusions: The three analytes that have the same transitions - THC, OH-THC, and COOH-THC were chromatographically separated. Matrix selectivity studies using six different blank plasma sources showed endogenous chromatographic peak area ratios at the analyte retention times were generally less than 20 % of the analyte limit of quantitation peak area ratio. The intra-run accuracy ranged from 83.7 to 111.2 % of target and the intra-run precision ranged from 2.0 to 19.1 %. The inter-run accuracy ranged from 88.3 to 107.8 % of target and the inter-run precision ranged from 4.1 to 10.5 %. The analytes were stable up to 24 hours at room temperature and after 3 freeze-thaw cycles. The extraction recoveries were 76.8% at 5 ng/mL THC, 80.9% at 5 ng/mL OH-THC, 67.1% at 25 ng/mL COOH-THC, and 85.2% at 5 ng/mL CBD. This procedure is capable of quantitating 4 essential cannabinoid compounds over a wide concentration range. Because of interest in CBD as an anticonvulsant, it is important to include it in plasma cannabinoids analyses.

Funding: Supported in part by NIDA Contract N01DA-14-7788

Keywords: Cannabinoids, GC-MS/MS, Plasma

P-31

Chromatographic Separation Development for the Analysis of Synthetic Cannabinoids and Their Metabolites in Human Urine by LC-MS/MS

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Background/Introduction: The determination of cannabinoids and their metabolites, from a natural or synthetic source, has become routine in many forensic toxicology laboratories. The optimization of analysis time, resolution between metabolites, and method robustness 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 Ultra Selective Liquid Chromatography™ (USLC™) technology to produce simple dilute and shoot methods with an analysis time of 5 minutes, and a cycle time of 7 minutes for various relevant synthetic cannabinoids, including 17 parent compounds, 12 metabolites and 5 internal standards.

Objective: Provide a fast and easy method solution for synthetic cannabinoids and their metabolites resulting in complete resolution of isobars and separation from matrix interferences in diluted urine.

Methods: The chromatographic method development investigation was performed on a Waters Acquity I-class equipped with a Xevo TQ-S using electrospray ionization in positive ion mode. Chromatographic optimization resulted in complete resolution of isobars and separation from major matrix interferences of a representative pooled urine sample using water and acetonitrile mobile phases modified with 0.1% formic acid under gradient conditions on a Restek Raptor™ Biphenyl 2.7µm, 50 x 3.0mm column.

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. These isomers form because each parent compound has many sites available for hydroxylation. 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. Representative chromatograms of a 5 ng/mL standard in urine for 17 synthetic cannabinoids and a 10 ng/mL standard in urine for 12 synthetic cannabinoids metabolites are shown in Figures 2 and 3, respectively. All the isomeric analytes included in this method were resolved on the Raptor™ Biphenyl column. By chromatographically separating these isomers, the most abundant metabolites from a given parent compound can be identified in authentic samples and methodology can be further optimized specifically for metabolites of clinical significance.

Conclusion/Discussion: The analysis of synthetic cannabinoids and their metabolites can be a difficult and challenging task. Many laboratories face the difficult task of developing and validating methods while keeping up with the ever-growing list of synthetic cannabinoids illicit drug makers produce.

The Raptor™ Biphenyl provides solutions to many issues surrounding this analysis. It has the ability to provide highly retentive, selective, and rugged reversed-phase separations, allowing for the simultaneous analysis of 17 synthetic cannabinoids and 12 metabolites. 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: Cannabinoids, Metabolites, LC-MS/MS, Chromatographic Separation, Raptor™ Biphenyl

P-32

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

P-33

Method Validation for an Quantitative Method of Five Cannabinoids with an Automated SLE and LC-MS/MS

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Introduction: After alcohol, marijuana is the most commonly abused drug in the United States. The ability to quickly and accurately quantitate THC and its two metabolites, carboxy-THC and hydroxy-THC, in driving under the influence of drugs cases is becoming increasingly important for interpretation. Two additional cannabinoids present in marijuana, cannabinol and cannabidiol, are now being isolated and used for medicinal purposes and as well as drug facilitated sexual assaults.

Objective: A new automated extraction and LCMSMS method was developed and validated with higher throughput, lower solvent waste and less sample volume in blood and urine using the SWGTOX Standard Practices for Method Validation in Forensic Toxicology Guidelines.

Method: A Tecan Freedom EVO 200 was used to pipet all samples, standards and reagents. Prior to extraction the urine samples were hydrolyzed in five minutes using ICMS® β-glucuronidase. In a 96-well pretreatment plate, 175 µL blood or urine samples was added with 175 µL of 0.1% formic acid and 25 µL of deuterated internal standard for all five cannabinoids. A vacuum manifold applied sufficient vacuum to pull sample onto the sorbet where it sat for five minutes. The drugs were then eluted with 1.4 mL of MTBE. After the MTBE 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 five minute gradient that elutes all drugs with baseline separation. A Waters XeVo-TQS collected MRM data for quantitations.

Results / Discussion: Extraction recoveries for the method were performed for all drugs, except internal standards, resulting in all five of the drugs having an extraction recovery above 60% across all sample matrices. Limit of detection and quantitation, ion suppression/enhancement, carry-over, and possible interference studies were performed for all of the drugs in the method following the SWGTOX guidelines to streamline the validation. A quadratic model, weighted $1/x^2$ with no forcing through zero was deemed the best calibration model for all drugs over the desired concentrations. The concentration ranges of the calibration curves for THC, OH-THC, cannabinol, cannabidiol were 0.5 – 50 ng/mL and was 5 – 500 ng/mL for carboxy-THC. The uncertainty of measurement budget for all drugs quantitated were completed during the validation, using over 100 runs and five analysts. For the final validation, four fully trained analysts extracted 50 previously analyzed casework samples, comprised of all matrices, to determine if their results would be within 20% of each other. No deviations greater than 20% were seen

Conclusion: A quick quantitative extraction and LCMSMS method has been developed and validated for five cannabinoids that allows analysts to place samples on an instrument deck and walk away. The method has been validated using the SWGTOX guidelines and is currently the method being used at the Orange County Crime Lab for antemortem and postmortem casework. All drug concentration uncertainties are less than 10% when based on a 95% confidence level.

Keywords: Cannabinoids, Automated SLE, Method Development, LC-MS/MS

P-34

Method Development and Validation for Hair Ethyl Glucuronide Quantitation Using Both Liquid Chromatography Tandem Mass Spectrometry and Gas Chromatography Tandem Mass Spectrometry

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Introduction: Ethyl glucuronide (EtG) is a direct metabolite of ethanol and is formed by the enzymatic conjugation of ethanol with glucuronic acid. It's a stable compound that is detectable in the urine, blood, hair and post-mortem tissue for an extended time period after the complete elimination of ethanol from the body.

O**bjective:** Develop and validate a selective and sensitive method for the detection and quantification of EtG in hair using both liquid chromatography tandem mass spectrometry (LC-MS/MS) and gas chromatography tandem mass spectrometry (GC-MS/MS).

Methods: Hair samples obtained from anonymous volunteers and previously confirmed positive for cocaethylene by an alternate LC-MS/MS method, were used to validate the quantification of EtG in hair by LC-MS/MS and GC-MS/MS. Samples were extracted in methanol and sonicated using a water bath. The extract supernatant was removed, dried under nitrogen, reconstituted and/or derivatized prior to analysis. LC-MS/MS analysis was performed using a Shimadzu HPLC connected to an AB Sciex 5500 QTRAP mass spectrometer operated in negative electrospray ionization (ESI) and multiple reaction monitoring (MRM) modes. The chromatographic column was an Agilent Zorbax Sb-phenyl column (150 x 4.6 mm x 5 µm) and the mobile phase consisted of water and methanol with 0.1% (v/v) formic acid. GC-MS/MS analysis was achieved using pentafluoropropionic anhydride (PFPA) chemical derivatization and an Agilent Ion Trap Quadrupole GC-MS/MS instrument operated in negative ion chemical ionization mode. Chromatographic separation was achieved using a DB-1MS LTM column (15m x 250µm x 0.25 µm) and a DB-17MS LTM column (15m x 250µm x 0.25 µm). Both the LC-MS/MS and GC-MS/MS EtG assays were fully validated and included a determination of the limit of detection (LOD), limit of quantitation (LOQ), linearity, intra- and inter-day precision, accuracy, analyte carryover and the effect of matrix on quantitation.

Results: Method validation studies for EtG analysis in hair demonstrated a LOD and LOQ of 6 pg/mg hair respectively for both the LC-MS/MS and GC-MS/MS methods using 10 mg of hair sample. Both methods produced excellent linearity (R^2 >0.99) over the calibration range of 0 to 300 pg/mg. The precision and accuracy studies demonstrated acceptable performance (e.g., bias below ±20% and percent coefficient of variance (%CV) less than 20% (N = 5)). The effects from sample matrix were negligible for both the LC-MS/MS and GC-MS/MS methods.

Discussion: Simple and sensitive methods were developed and validated for the analysis of EtG in hair using LC-MS/MS and GC-MS/MS. The hair EtG extraction procedure deployed in this study proved to be relatively more complex and time-consuming than an equivalent procedure for urine EtG extraction. Hair is a unique matrix which often requires additional sample pre-treatment (e.g., extraction through sonication in solvent, pre-concentration, reconstitution, and chemical derivation) relative to urine. This method is intended to be used in conjunction with methods dedicated to EtG quantification in other biological matrices (e.g., oral fluid, blood or urine). The advantage of testing EtG in hair is that it offers a greater retrospective window of detection for alcohol consumption; relative to oral fluid, blood or urine.

Keywords: Ethyl Glucuronide, LC-MS/MS, GC-MS/MS, Hair Analysis

P-35

LC-MS/MS Analysis of Ethyl Glucuronide and Ethyl Sulfate in Urine: A Comparison of Sample Preparation Techniques

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Introduction: Ethyl glucuronide (EtG) and ethyl sulfate (EtS) are phase II metabolites of ethanol formed by conjugation of ethanol with glucuronic or sulfonic acid. These metabolites can be detected in urine for several days after last alcohol ingestion and are used as biomarkers of alcohol use in treatment or compliance programs. These compounds can be challenging analytically due to their small molecular weight, high water solubility and their propensity to form negatively charged ions under electrospray conditions.

Objective: Compare three different sample preparation techniques to determine if any provided the cleanest sample for LC-MS/MS analysis considering analyte recovery and minimal ion suppression.

Methods: We compared two solid-phase extraction (SPE) chemistries and a dilution protocol. The matrix was a pool of EtG/EtS-free urine collected from ten different donors. SPE #1 was a strong anion exchange sorbent (EVOLUTE AX, Biotage), SPE #2 was an amino-propyl sorbent (ISOLUTE NH2, Biotage). The dilution protocol consisted of raw urine diluted into purified water. The preparation protocols were optimized to provide equivalent amounts of analyte injected onto the LC-MS/MS system to ensure equivalent comparison. The LC-MS/MS system was an Agilent 1200 HPLC stack with a CTC-DLW autosampler coupled to an API4000 LC-MS/MS (Applied Biosystems). The HPLC column was a Phenomenex Synergi HydroRP (100Å, 2 x 100mm), The TurboSpray source was operated in the negative ion mode and all electrospray and CID parameters were optimized for MRM analysis of ethyl glucuronide, ethyl sulfate and their pentadeuterated analogs.

Results: All sample preparations allowed reproducible, quantitative analysis of EtG and EtS in human urine. Fortified calibration curves were linear from 100 – 10000 ng/mL. Ion suppression was evident in each preparation, but we controlled this with the use of deuterated internal standards for each analyte. A large endogenous peak eluted adjacent to EtS (*m/z* 125 → 80) with each preparation. The AX SPE preparation (reconstituted in mobile phase) was able to resolve this peak most efficiently. The NH2 SPE preparation (eluted with 10mM NH_4HCO_2) provided the cleanest chromatogram with the least amount of endogenous background. The straight dilution protocol provided the greatest amount of signal intensity compared to the SPE preparations.

Conclusions: No preparation was clearly superior to the other, but each offered distinct advantages. The dilution approach was by the far the fastest, but runs the risk of introducing unwanted matrix into the LC-MS/MS system. The AX SPE preparation offers the advantage of varying the reconstitution volume of the sample extract prior to analysis offering more control of the amount of material injected on the column. The NH2 SPE preparation provided a clean sample that did not require dry-down or reconstitution of the extract.

Keywords: Ethyl Glucuronide, Ethyl Sulfate, LC-MS/MS, Solid-Phase Extraction

P-36
Measurement of Uncertainty for Breath Alcohol Simulator Solutions

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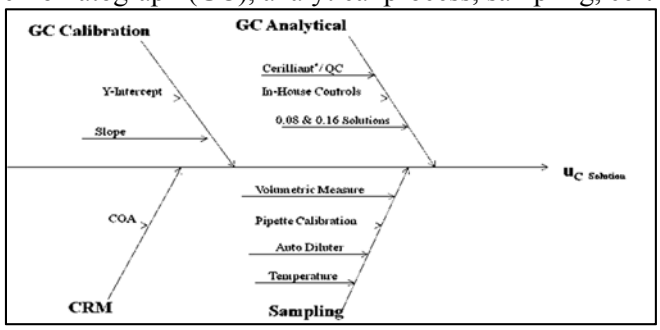
Introduction / Objective: Breath Alcohol Analysis is used by law enforcement agencies to determine the breath alcohol concentration of individuals suspected of impairment. Breath alcohol measurements have a finite and measurable amount of uncertainty characterized by the dispersion of values attributed to a particular measurement. This laboratory has conducted a measurement of uncertainty for the simulator solutions that are made at the laboratory and provided as calibration checks for breath alcohol analyses.

Methods: Four major groups were identified as significant sources of solution uncertainty: calibration of the gas-chromatograph (GC), analytical process, sampling, certified reference materials (CRM).



The measurand is represented by the following formula of four components:

$$U_{combined} = \sqrt{CRM^2 + Cal^2 + A^2}$$

Where: $U_{combined}$: combined uncertainty of alcohol in sample

CRM: certified reference material

Cal: gas-chromatograph calibration

Analytical (A) : analytical process

The GC calibration and the GC analytical fell into the Type A category, indicated by a normal (Gaussian) distribution. The calibration (Cal) of the GC was performed by calculating the slopes and corresponding y-intercepts that were generated by plotting response ratio versus concentration. The statistical dispersion of the slopes and y-intercepts comprised the calibration uncertainty. The analytical calculation relied on the data derived from the historic culmination of the quality controls utilized by the laboratory. The external controls were obtained through Cerilliant® and the in-house controls were prepared internally. The data were analyzed using two GC instruments. The data for the 0.080 and the 0.160 solutions were pulled from validation runs on the GC. The external, in-house, and 0.08/0.16 solutions comprise the analytical portion of the uncertainty. Since the method had been under statistical control, the analytical information included information from previous analyses and encompasses any uncertainty attributed to the analysts. The certified reference materials (CRM) fell into the Type B category, as indicated by a rectangular distribution and/or the use of a manufacturer-reported uncertainty measurement. Certificates of Analysis (COA) for the certified reference materials (CRM) report the uncertainty measured by the manufacturer. Sampling uncertainty is dependent on the uncertainty of the tools used for sample preparation. The sampling uncertainty was *not* used in the calculation since it was considered to be a reasonable contributor to the GC Calibration Study Uncertainty.

Combined Solution Uncertainty (%)	
Slope u	***0.540398327***
y-intercept u	***0.146409829***
External Control u	***0.138641671***
In-House Control u	***0.666298416***
0.08/0.16 Solution u	***0.072179201***
Cerilliant COA u	***0.246912158***
Combined $u_{C\ Solution}$	***0.918050507***
Expanded U@ 95% k=2	***1.836101013***

Results: The calculated uncertainties for the GC calibration study, the GC analytical study, and the certified reference materials were combined using the method of root-sum-squares to give $u_{combined}$. The overall combined uncertainty was expanded to reflect a confidence interval of 95% by multiplying by k= 2 obtained from the Student's t-table. Thus, for the simulator solutions, the laboratory reports an expanded uncertainty of ±1.8% at a confidence interval of 95% (e.g.: This simulator solution is 0.08 g/210 L ±1.8% at a confidence interval of 95%).

Keywords: Measurement of Uncertainty, Breath Alcohol, Simulators

P-37

Measurement of Uncertainty for Blood Alcohol Analyses Using Headspace Gas-Chromatography

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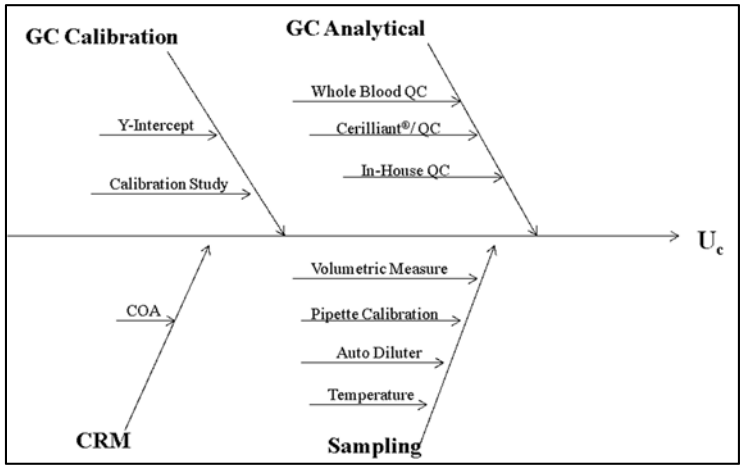
Introduction: Blood Alcohol Analysis using dual column capillary headspace gas-chromatography (GC) is used by the Toxicology Bureau to determine the amount of ethanol, methanol, acetone or 2-propanol in samples submitted by law enforcement agencies in human performance investigations or by the Office of the Medical Investigator (OMI) in death investigations. The measured blood alcohol concentration is reported for comparison to legal limitations regarding whether it is a significant contributor to the unlawful impairment and/or death of an individual.

Objective: This laboratory has conducted a study to estimate a reasonable uncertainty for the measurement of blood alcohol concentrations.

Methods: The measurand is the mass concentration of ethanol, methanol, 2-propanol, and acetone (g/100mL) determined through dual column capillary headspace GC/FID analysis calibrated with certified reference materials (CRMs) and verified with internal controls. The uncertainty of the measurand is a function of the combined uncertainties of certified reference materials, instrumental analysis, laboratory tools, and the inherent error associated with their function and use.



The measurand is represented by the following formula of four components:

$$U_{combined} = \sqrt{CRM^2 + Cal^2 + A^2}$$

Where:

$U_{Combined}$: combined uncertainty of alcohol in sample

CRM: certified reference material calibration

Cal: gas-chromatograph calibration

Analytical (A): analytical process

The calibration (Cal) of the GC was performed by calculating the slopes and corresponding y-intercepts that were generated by plotting response ratio versus concentration. The analytical (A) calculation relied on the data derived from the historic culmination of the quality controls utilized by the laboratory. Since the method had been under statistical control, the analytical information includes information from previous analyses and encompasses any uncertainty that may be attributed to scientists performing the analysis. Sampling uncertainty is dependent on the uncertainty of the tools used for sample preparation. This uncertainty is reflected in the GC Analytical Study and was deemed not to be a major contributor to the overall combined uncertainty, hence it was not used.

Results / Discussion: The calculated uncertainties for the GC calibration study, the GC analytical study and the certificates of analysis (COAs) for the certified reference materials (CRMs) were combined using the method of root-sum-squares to give $U_{Combined}$. The overall combined uncertainty was expanded to reflect a confidence interval of 95% by multiplying by k= 2.05. Thus, for the blood alcohol method, the laboratory reports an expanded uncertainty of 5.2% at a confidence level of 95% (e.g.: 0.08 g/100mL ± 5.2% at a confidence level of 95%). This value is constant for all results from 0.01 g/100mL to 0.400 g/100mL.

Variable	Source of Uncertainty	Uncertainty
u_{cal}	*GC Calibration*	*2.299628064*
u_A	*GC Analytical*	*0.701465231*
u_{CRM}	*CRMs*	*0.536*
u_C		***2.5230884***
U_E @ 95% CI k=2.05		***5.172332122***

Keywords: Measurement of Uncertainty, Blood Alcohol, Headspace GC/FID

P-38

Extraction of Barbiturates from Oral Fluid by ISOLUTE SLE+ after Collection with the Quantisal, Intercept & Oral-Eze Collection Devices Prior to GC/MS Analysis

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Introduction: Oral fluid testing is gaining approval in the forensic toxicology community as a suitable tool to supplement urine and blood testing where misuse of drugs is suspected. A quick, dignified specimen can be obtained from a person relatively easily in workplace applications, drug driving incidents and other applications. Barbiturates are a group of compounds which were originally developed to alleviate anxiety and nervousness. Today they have more relevance prescribed as anticonvulsant and migraine treatments in the form of phenobarbital and butalbital, respectively.

Objective: The objective was to develop a GC/MS assay for the determination of barbiturates from oral fluid using supported liquid extraction (SLE), after collecting specimens from a variety of collection devices. The devices evaluated were: Orasure Intercept, Immunalysis Quantisal and Quest Oral-Eze. Percentage analyte recovery was evaluated for Intercept, Quantisal and Oral-Eze by pooling multiple devices after sampling. Calibration curves were formed by spiking individual devices for Intercept and Quantisal only.

Methods: Butalbarbital, Butabarbital, Amobarbital, Pentobarbital, Secobarbital, Hexobarbital and Phenobarbital were spiked into blank oral fluid collection devices post-collection. No deuterated barbiturates were included. Extraction conditions were evaluated on ISOLUTE SLE+ 400 µL capacity columns for all three manufacturers and 1 mL capacity columns for Intercept and Quantisal devices, at pH environments native to the device and also modified with ammonium hydroxide. The final optimized procedure involved modifying the pH of the sample before extraction with 10 µL 0.5% ammonium hydroxide per Intercept device, 15 µL concentrated ammonium hydroxide per Quantisal device and 10 µL 4% ammonium hydroxide per Oral-Eze device. Extraction solvents used in development included methyl *tert*-butyl ether (MTBE), dichloromethane (DCM), 95/5 dichloromethane/isopropanol (DCM/IPA) and ethyl acetate (EtOAc). For all manufacturers, the optimum elution solvent was 95/5 DCM/IPA. For Intercept and Quantisal devices, MTBE was proven to be a suitable solvent where chlorinated solvents are prohibited. Samples were evaporated with air at 40°C and derivatization was performed using 80 µL EtOAc and 20 µL 0.2M trimethylanilinium hydroxide (TMAH). Samples were analyzed using an Agilent 7890 GC with a 5975 MSD. Chromatography was performed on a Phenomenex Zebron ZB-Semivolatiles capillary column; 30 m x 0.25 mm ID x 0.25 µm using 1.2 mL/min helium. Three ions were acquired for each analyte in SIM mode using electron ionization.

Results: Analyte peak areas from samples spiked before and after extraction were compared to determine percentage recovery. Recoveries were greater than 91% using Intercept, greater than 84% using the Quantisal and greater than 95% using Oral-Eze. RSDs were below 10% across 400 µL and 1 mL formats using an optimized method. Analyte limits of quantitation were measured to be between 5 and 25 ng/mL using the 400 µL format, and between 2 and 10 ng/mL when using the 1 mL format. Linearity experiments over concentration levels 2 to 500 ng/mL demonstrated coefficients of determination between 0.995 and 0.999.

Conclusions: This poster provides a quick, simple and reliable protocol for the extraction of barbiturates from three oral fluid collection devices prior to GC/MS, demonstrating high, reproducible extraction efficiencies and acceptable limits of quantitation.

Keywords: SLE+ (Supported Liquid Extraction), Oral Fluid, Barbiturates, GC/MS

P-39

Evaluation of Drugs of Abuse Extraction from Oral Fluid using Supported Liquid Extraction Prior to UPLC/MS-MS Analysis

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Introduction: Drug screening using oral fluid has gained popularity over recent years due to its simple, non-invasive collection. 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 a UPLC/MS-MS assay for the determination of a range of drugs of abuse from oral fluid using supported liquid extraction (SLE), after collecting specimens from a variety of collection devices. The devices evaluated were Intercept from Orasure and Quantisal from Immunalysis. The SLE extraction mechanism is very efficient, delivering higher analyte recoveries and cleaner extracts than equivalent LLE methods.

Methods: Negative oral fluid samples for method development purposes were obtained using the Immunalysis Quantisal and Orasure Intercept collection devices. To ensure maximum oral fluid extraction on SLE+, rather than dilute with weak pre-treatment buffer, samples from Quantisal and Intercept collection devices were modified in pH terms using 10-15 µL of concentrated NH_4OH. The target for elevated pH was between 8.2-8.5 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 15 µL of neat NH_4OH and 10 µL 0.5% NH_4OH resulting in loading pHs of 8.3 and 8.5 for the Quantisal and Intercept devices, respectively. Extraction was performed loading 300 µL on ISOLUTE SLE+ 400 µL capacity columns, followed by elution with 2x 1 mL of either MTBE, DCM, 95/5 DCM/IPA or EtOAc. Benzodiazepines, z drugs, amphetamines, cathinones, opiates, cocaine, buprenorphine, THC-COOH, fentanyl and ketamine spiked oral fluid extracts were analysed using a Waters ACQUITY UPLC system coupled to a Quattro PREMIER XE triple quadrupole mass spectrometer. Positive ions were acquired using electrospray ionisation operated in multiple reaction monitoring mode.

Results: The DOA multisuite showed optimum extraction at pH conditions between 8-8.5. No degradation of 6-MAM was observed in this pH environment. EtOAc and MTBE performed well for the majority of analytes. However, 95/5 DCM/IPA was selected due to its optimum extraction of benzoylecgonine. Using this elution option, Quantisal recoveries were greater than 83% for 45 out of 46 analytes and Intercept recoveries were greater than 92% for all 46 analytes. Calibration curves were constructed from 1-500 ng/mL and good linearity was achieved for the analytes, demonstrating coefficients of determination > 0.99. When utilizing the elevated pH control for each oral fluid device and extraction with 95/5 DCM/IPA it was possible to extract a wide range of drugs of abuse with varying logP and pKa values, demonstrating the possibility of a single extraction, using UPLC/MS-MS as the endpoint.

Conclusions: This poster demonstrates the extraction of a range of drugs of abuse prior to UPLC/MS-MS analysis. The target analyte list includes benzodiazepines, z drugs, amphetamines, cathinones, opiates, cocaine, buprenorphine, THC-COOH, fentanyl and ketamine.

Keywords: SLE+ (Supported Liquid Extraction), Oral Fluid, Drugs of Abuse, UPLC/MS-MS

P-40

Extraction of Propofol from Whole Blood Using Supported Liquid Extraction (SLE) Prior to GC/MS Analysis

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Introduction: Propofol is used in clinical environments to induce states of anesthesia or reduced sensitivity during surgical procedures. However it also has the potential to be abused for short-term hallucinations and euphoric effects, and with a very small therapeutic range, unanticipated fatal results are possible.

Objective: The objective was to develop a GC/MS assay for the determination of propofol from whole blood using supported liquid extraction (SLE). The SLE extraction mechanism is very efficient, delivering higher analyte recoveries and cleaner extracts than equivalent LLE methods.

Methods: Blank human whole blood was spiked with propofol and propofol-d17 was used as the internal standard. Extraction conditions were evaluated using spiked whole blood pre-treated 1:1 (v/v) with HPLC grade water. Sample preparation was performed on ISOLUTE SLE+ 400 µL capacity columns using 300 µL of pre-treated whole blood. Extraction was evaluated using heptane, ethyl acetate or MTBE as the solvent of choice. Prior to evaporation under an air stream, 10 µL of 0.5% tetrabutylammonium hydroxide was added to each sample after extraction to evaluate stability during evaporation. Samples were evaporated under air below 25°C and derivatization was performed using 100 µL heptane. The samples were vortex mixed and transferred to glass vials with non-split caps prior to GC/MS analysis. All samples were analyzed using an Agilent 7890 GC with a 5975 MSD. Chromatography was performed on a Phenomenex Zebron ZB-Semivolatiles capillary column; 30 m x 0.25 mm ID x 0.25 µm using 1.2 mL/min helium as the carrier. Positive ions were acquired using electron ionization operated in SIM mode.

Results: The presence of tetrabutylammonium hydroxide (TBAH) proved to be essential in comparison evaporation experiments. In the absence of TBAH, it is believed the -OH on propofol becomes ionized during evaporation and analyte losses are almost total. Propofol peak areas from samples that were spiked before and after extraction were compared to determine percentage recovery when using the SLE procedure. Recovery profiles were determined to be greater than 95% and corresponding relative standard deviation (RSD) below 6%. The limit of quantitation was measured to be 2.5 ng/mL on the 400 µL format. In order to increase the sensitivity, the reconstitution solvent volume could be evaluated. Linearity experiments demonstrated coefficients of determination greater than 0.999 over concentration levels 2.5 ng/mL to 1000 ng/mL.

Conclusions: This poster provides a quick, simple and reliable protocol for the extraction of propofol from whole blood prior to GC/MS, demonstrating high, reproducible extraction efficiencies and acceptable limits of quantitation.

Keywords: SLE+ (Supported Liquid Extraction), Whole Blood, Propofol, GC/MS

P-41

Extraction of Δ^9-THC, THCA and 11-nor-9-carboxy-THC from Oral Fluid by ISOLUTE SLE+ After Collection with the Quantisal, Intercept & Oral-Eze Collection Devices Prior to GC/MS Analysis

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Introduction: Oral fluid testing is gaining approval in the forensic toxicology community as a suitable tool to supplement urine and blood testing where misuse of drugs is suspected. A quick, dignified specimen can be obtained from a person relatively easily in workplace applications, drug driving incidents and other applications. Cannabis misuse continues widely all over the world, and this has led to the necessity for rapid and reliable methods for the analysis and quantitation of THC and its main metabolite 11-nor-9-carboxy-THC (THC-COOH). In addition, THCA is marker which can show a distinction between *Cannabis Sativa* use and synthetic medicinal Dronabinol.

Objective: The objective was to develop a GC/MS assay for the determination of THC, THCA and THC-COOH from oral fluid using supported liquid extraction (SLE), after collecting specimens from a variety of collection devices: Orasure Intercept, Immunalysis Quantisal and Quest Oral-Eze.

Methods: THC, THCA and THC-COOH were spiked into oral fluid collection devices following collection of blank oral fluid and THC-d3 and THC-COOH-d3 were spiked as internal standards. Extraction conditions were evaluated on ISOLUTE SLE+ 400 µL capacity columns for all three manufacturers and 1 mL capacity columns for Intercept and Quantisal devices, at pH environments native to the device and also modified with ammonium hydroxide. The final optimized procedure involved modifying the pH of the sample before extraction with 10 µL 0.5% ammonium hydroxide per Intercept device, 15 µL concentrated ammonium hydroxide per Quantisal device and 10 µL 4% ammonium hydroxide per Oral-Eze device. Extraction solvents used in development included methyl *tert*-butyl ether (MTBE), dichloromethane (DCM), 95/5 dichloromethane/isopropanol (DCM/IPA) and ethyl acetate (EtOAc). For all manufacturers, the optimum elution solvent was 95/5 DCM/IPA. For Intercept and Quantisal devices, MTBE was proven to be a suitable solvent where chlorinated solvents are prohibited. Analytes were spiked into blank elution solvent following extraction to determine SLE efficiency. Samples were evaporated under an air stream at 40°C and derivatized using 50 µL EtOAc and 25 µL BSTFA:TMCS. Samples were analyzed using an Agilent 7890 GC with a 5975 MSD. Chromatography was performed on a Phenomenex Zebron ZB-Semivolatiles capillary column; 30 m x 0.25 mm ID x 0.25 µm using 1.2 mL/min helium as the carrier. Positive ions were acquired using electron ionization operated in SIM mode.

Results: Analyte peak areas from samples that were spiked before and after extraction were compared to determine percentage recovery when using the SLE procedure. Recoveries using 95/5 DCM/IPA were greater than 76% using Intercept, greater than 78% using the Quantisal and 104-109% using Oral-Eze. Relative standard deviations (RSDs) were below 10% across 400 µL and 1 mL formats using an optimized method. Analyte limits of quantitation using the 1 mL format were measured to be 4 ng/mL for THC, 10 ng/mL for THCA and 20 ng/mL for THC-COOH. Linearity experiments over concentration levels 4 ng/mL to 800 ng/mL, demonstrated r^2 between 0.9920 and 0.9997.

Conclusions: This poster provides a quick, simple and reliable protocol for the extraction of delta-9-THC, THCA and 11-nor-9-carboxy-THC from three oral fluid collection devices prior to GC/MS, demonstrating high, reproducible extraction efficiencies.

Keywords: SLE+ (Supported Liquid Extraction), Oral Fluid, THC, GC/MS

P-42

Comparison of SPE Cartridges for the Extraction of 25 New Psychoactive Substances

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Introduction: New psychoactive substances (NPS's) have appeared on the recreational drug market at an unprecedented rate. Sample clean-up is a critical step in toxicological analysis; not only does it improve sensitivity and selectivity of results, but it also increases the lifetime of the instruments.

Objective: The aim of this study was to determine the most suitable SPE cartridge to use for the extraction of various NPS's from a range of different matrices: blood, urine, plasma and serum.

Method: Blank methanol, urine, blood, plasma and serum samples (1 mL) were all spiked with 200µL of 10µg/mL solutions of various different NPS's (methiopropamine, flephedrone, mephedrone, MDPV, 2-DPMP,butylone, ethylone, naphyrone, 5-APB, 6-APB, 3-MeO-PCE, methoxetamine, benzedrone, 25B-NBOMe , 25C-NBOMe , 25D-NBOMe , 25E-NBOMe , 25H-NBOMe, 25I-NBOMe, Mescaline-NBOMe, 25N-NBOMe, 25P-NBOMe, 25T2-NBOMe, 25T4-NBOMe and 25T7-NBOMe). To each sample 1mL of 0.1M phosphate buffer (pH6) was added before centrifugation for 10 minutes at 4000rpm. Samples were then extracted using various different solid phase extraction cartridge; UCT's XCEL1, ZSDAU020, CSDAU133, XRDAH206, XRDAH502, XRPCH50z as well as WatersOasis. ZSDAU020, CSDAU133, XRDAH206, XRDAH502, XRPCH50z cartridges were conditioned using CH_3OH, D.I. H_2O and phosphate buffer before loading samples. Columns were washed using D.I. H_2O, 0.1M acetic acid and CH_3OH. Samples were eluted using methylene chloride; iso-propanol; ammonium hydroxide (78:20:2). Samples were loaded directly to the XCEL I cartridges and washed with 2%acetic acid/ 98% methanol before elution with methylene chloride; iso-propanol; ammonium hydroxide (78:20:2). Oasis cartridges were conditioned with CH_3OH and D.I. H_2O prior to loading samples. These were then washed using 2% acetic acid and CH_3OH prior to elution with 95% CH_3OH with 5% NH_4OH.Internal standards (mephedrone-D_3, methylone-D_3, ethylone-D_5, MDPV-D_8 and 25I-NBOMe-D_3) were added to the collection tubes prior to elution. Post extraction, samples were evaporated using a stream of nitrogen, derivatized using 50µL of PFPA:ethyl acetate at 70^oC for 40 minutes, before being evaporated again and reconstituted in 100µL of ethyl acetate. Samples were analysed by GC-MS.

Results: No drugs were detected (LOD <5 – 30 ng/mL) using the XRPCH50z cartridge. For urine samples, overall the UCT CSDAU cartridge had the best recovery with an average recovery rate per drug of 71% (ranging 9 - 163% excluding methoxetamine and benzedrone which were undetected). Overall, blood samples had the best recovery rates with CSDAU SPE cartridges, averaging 60% per drug (ranging 8 - 112%)(excluding methoxetamine, benzedrone and mescaline-NBOMe which were undetected). Plasma and serum samples were best extracted using the ZSDAU cartridges with average recovery rates per drug of 71% (ranging 5%- 156%) and 70% (ranging 12-167%) respectively. All recovery rates relate to the level of drug detected in comparison to unextracted samples.

Conclusion: This study has shown that when analysing urine and blood samples the smaller bed size of theCSDAU cartridges is preferable and when analysing plasma and serum samples ZSDAU cartridges should be used. XRPCH50z cartridges, using the method tested, are not recommended for sample clean-up of NPS compounds.

Keywords: SPE, Novel Psychoactive Substances, NBOMes, Cathinones

P-43
In-Vitro Conversion Between GHB and GBL and the Conversion Trom 1,4-BD to GHB

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Introduction: Gamma-butyrolactone (GBL) and 1,4-butanediol (1,4-BD) are structurally related to GHB and rapidly convert to GHB in-vivo. Despite the speed of this process GBL and 1,4-BD have both been identified in post-mortem cases. For this reason, it is important to not only know what factors effect GHB concentrations but also which may have an effect on GBL and 1,4-BD for a more accurate toxicological interpretation of all substances found.

Objective: The aim of this study was to determine the extent of in-vitro conversion between GHB, GBL and 1,4-BD in blood and urine over a period of 5 weeks. Parameters investigated were temperature, initial drug concentration and sample pH.

Methodology: One lot of blank ante mortem blood was split into 2 aliquots. One aliquot was left with its natural pH (7.20) and the other aliquot was pH adjusted to 6.60 using pH 5.5 phosphate buffer. One lot of antemortem urine was split into 3 aliquots. One aliquot was left at its natural pH (6.96), one was pH adjusted to 4.17 using 3M HCl and the other adjusted to 8.08 using 0.1M NaOH. Each aliquot was spiked with the three analytes (GHB, GBL and 1,4-BD) at concentrations of 5, 10, 25, 50 and 100mg/L and stored at -20°C, 4°C or 23°C for 5 weeks. Analysis was carried out in duplicate.
Liquid-liquid extraction was carried out prior to GC-MS analysis (GHB and 1,4-BD) or GC-FID analysis (GBL). GHB-D6 (50µL, 10µg/mL) was added as I.S. for GHB and 1,4-BD analysis. Benzyl alcohol (100 □L, 1mg/mL) was added as I.S. for GBL analysis. For GHB/1,4-BD the samples were derivatized with BSTFA (1% TCMS) prior to analysis. The precision for the GC-MS method used has a CV below 20%.

Results: GHB concentrations in acidic samples decreased whereas GHB concentrations in alkaline samples increased. The opposite was seen for GBL. 1,4-BD concentrations decreased regardless of pH although at a slightly faster initial rate in acidic samples with 100mg/L blood samples decreasing 26% after week 1 in acidic conditions but remained steady in neutral conditions. Samples stored at -20°C were the least affected by in-vitro conversion. In the 100mg/mL sample GHB concentrations dropped 17% over the 5 week period at pH6.60. Samples stored at 23°C were the most affected with GHB concentrations increasing 431% at pH7.2. In-vitro conversion was most apparent in lower concentration samples with 5mg/mL blood samples at pH6.60 showing an 86% decrease in concentration. Blank samples did not have a noticeable change over the 5 week period and remained below the method cut off level of 0.8mg/L

Conclusion: This study has shown that under acidic conditions GHB will convert in-vitro to GBL and vice versa in more alkaline conditions. 1,4-BD converts regardless of pH to GHB, although it is suggested that the rate at which this occurs is pH dependent. In-vitro conversions from 1,4-BD to GHB and between GHB and GBL were most significant at room temperature (23°C), and as a result all samples should be stored at -20°C in order to prevent further conversion.

Keywords: GHB, GBL, 1,4-BD, In-Vitro Conversion, Stability

P-44
Analysis of Fentanyl and Its Analogues in Human Urine by LC-MS/MS

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Background / Introduction: Synthetic opioid drugs, such as fentanyl and sufentanil, have very high analgesic potency. Abuse of these prescription opioids and their illicit analogue, acetyl fentanyl, is a growing public health problem. In this study, a simple dilute and shoot method was developed with an analysis time of less than 3.5 minutes for fentanyl, norfentanyl, acetyl fentanyl, and sufentanil in human urine by LC-MS/MS using the Raptor™ Biphenyl column.

Objective: Provide a simple, fast, and sensitive measurement of fentanyl, its metabolite, and analogues in human urine.

Methods: Pooled human urine was fortified with the analytes. The urine sample was diluted 5-fold in a water/methanol solution with the addition of internal standards (fentanyl-d5, norfentanyl-d5, sufentanil-d5, acetyl fentanyl-$^{13}C_6$) prior to injection on the Raptor™ Biphenyl column (50x2.1mm, 5□m). The mobile phases used were 0.1% formic acid in water (aqueous phase) and 0.1% formic acid in methanol (organic phase) and the chromatographic separation was achieved with a gradient elution of 30% - 80% organic phase in 2 minutes. The analysis was performed on a Waters ACQUITY UPLC® I-Class System coupled with a Waters Xevo TQ-S mass spectrometer using electrospray ionization in positive ion mode.

Results: All four analytes were completely resolved on the Raptor™ Biphenyl column with a 2-minute gradient elution. No matrix interference was observed for quantitation. The calibration linearity ranged from 0.05 to 50 ng/mL for fentanyl, acetyl fentanyl, and sufentanil; and 0.25 to 50 ng/mL for norfentanyl with % deviation of less than 10.0% and the R^2 of ≥ 0.999. The LLOQ was 0.25 ng/mL for norfentanyl, and 0.05 ng/mL for fentanyl, acetyl fentanyl, and sufentanil in urine. Three levels of QC samples were analyzed for accuracy and precision. Based on three independent experiments conducted on multiple days, the mean accuracy values ranged from 94 to 110% of the nominal concentrations for all compounds and the %RSD ranged from 0.2 to 9.2%.

Conclusion / Discussion: An easy dilute and shoot method was developed for the quantitative measurement of fentanyl, its metabolite, and analogues in human urine. The analytical method was demonstrated to be fast and sensitive with great accuracy and precision for urine sample analysis. It also shows that the Raptor™ Biphenyl column is well suited for the analysis of synthetic opioid compounds for forensic toxicology.

Keywords: Raptor, Biphenyl, Opioid, Fentanyl, Norfentanyl, Acetylfentanyl, Sufentanil, Human Urine

P-45

Accurate Quantitation of Amitriptyline and Cyclobenzaprine in Urine Using IMCSzyme™ for Hydrolysis

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Introduction: The use of a new enzyme (IMCSzyme™) for hydrolysis of urine samples lead to increased concentrations of both amitriptyline and cyclobenzaprine in case samples. We believe that the observed increase in concentrations was due to a more efficient hydrolysis procedure using the new enzyme.

Objective: To develop a hydrolysis method for analysis of amitriptyline and cyclobenzaprine.

Methods: All drug standards (amitriptyline, amitriptyline-d_3, and cyclobenzaprine) were purchased from Cerilliant Corporation (Round Rock, TX, USA). DPX-RP-S tips were purchased from DPX Labs, LLC (Columbia, SC).
Hydrolysis of 200 µL urine using IMCSzyme™ was performed by adding 100 µL enzyme solution (containing phosphate buffer at pH 7.4 and internal standard). The samples were heated and mixed at 60 °C for 30 minutes (using the vortemp). Subsequently, 500 µL acetonitrile was added to each sample, and the solution was extracted using DPX-RP-S tips. Then 300 µL of the top acetonitrile layer was transferred to a vial, 700 µL water was added, and the solution was mixed and injected. Hydrolysis studies with three other enzymes, Helix pomatia, Patella vulgata, and abalone, were performed similarly except the hydrolysis was carried out for one hour at a pH of 4.5. All analyses were performed using a Thermo TSQ Vantage triple quadrupole system with an Agilent 1100 HPLC equipped with an Agilent Poroshell EC-C18 column (3.0 x 50mm, 2.7 µm). Sample injections of 20 µL were made using a six port (0.25mm) Cheminert C2V injection valve incorporated on a dual rail GERSTEL MPS autosampler.

Results and Discussion: Table 1 shows data from the analysis of amitriptyline and cyclobenzaprine in patient urine samples with and without hydrolysis of IMCSzyme™. The two samples were run in duplicate with and without the enzyme, and the numbers listed indicate the area of the peaks associated with the glucuronide conjugate, the parent drug (amitriptyline or cyclobenzaprine) and internal standard. The IMCSzyme™ converted almost all of amitriptyline glucuronide to amitriptyline, and showed complete conversion of cyclobenzaprine conjugate.

Table 1. Hydrolysis results for IMCSzyme™ for two case samples containing amitriptyline and cyclobenzaprine.

	Sample	No Hydrolysis (1)			IMCS		
		Gluc	Parent	ISTD	Gluc	Parent	ISTD
Amitriptyline	PS42748 A	1,328,842	258,916	130,784	1280	4,111,906	102,698
	PS42748 B	1,352,454	254,307	124,294	1593	3,586,773	94,254
Cyclobenzaprine	PS49187 A	1,313,484	239,648	63,449	0	4,574,032	89,613
	PS49187 B	1,267,501	266,753	82,123	0	3,738,485	71,125

Negligible conversion of the glucuronide conjugates were noted for other enzymes analyzed, including Helix promatia, Abalone and Patella vulgata. There was little difference in the intensities for the glucuronides with and without hydrolysis, even though the hydrolysis time was doubled as compared to IMCSzyme™.

Conclusion: The IMCSzyme™ demonstrates efficient hydrolysis of amitriptyline and cyclobenzaprine glucuronides. The glucuronide hydrolysis did not work with H. pomatia, abalone, and P. vulgata.

Keywords: IMCSzyme™, DPX, LC/MS/MS, Amitriptyline, Cyclobenzaprine, Glucuronide Hydrolysis

P-46

Accurate Quantitation of 6-Monoacetylmorphine in Urine Using IMCSzyme™ for Hydrolysis

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Introduction: Analysis of heroin metabolite (6-MAM: 6-monoacetylmorphine) is complicated due to conversion into morphine following hydrolysis of gluruconides. A significant problem lies with esterase contaminants in several commercial beta-glucuronidase products that are utilized for removal of the glucuronide moiety from morphine glucuronide. Here we report the use of a purified B-glucuronidase (IMCSzyme™) that provides effective glucuronide removal without esterase activity. This new enzyme product maintains the acetyl moiety of 6-MAM, while rapidly cleaving the glucuronides in a time-effective manner to allow for simultaneous LC-MS/MS detection of 6-MAM and morphine.

Objective: To develop an accurate method for quantitation of 6-MAM and morphine in urine.

Methods: All drug standards (6-monoacetylmorphine, 6-monoacetylmorphine-d_3, morphine, morphine-$d_3$and morphine-d_6) were purchased from Cerrilliant Corporation (Round Rock, TX, USA). DPX-RP-S tips were purchased from DPX Labs, LLC (Columbia, SC). Hydrolysis of 200 µL urine using IMCSzyme™ was performed by adding 100 µL solution (containing IMCS rapid hydrolysis buffer, B-glucuronidase and internal standard). The samples were heated and mixed at 55°C (LabNetVortemp 56). Subsequently, 500 µL acetonitrile was added to each sample, and the solution was extracted using DPX-RP-S tips. This extraction results in separation of acetonitrile (containing drugs of interest) from water (containing salts and many interferences), and 50 µL of the top acetonitrile layer was transferred to a 96-well plate, solvent evaporated and reconstituted with 200 uL of water/methanol (95/5). Hydrolysis with 3 other enzymes was performed similarly, except the hydrolysis was carried out at 55°C using NaOAc buffer (167 mM, pH of 4.5).

Results and Discussion: Table 1 shows data from the analysis of 6-MAM in drug-free urine samples before and after hydrolysis with IMCSzyme, *Patella vulgata* (Sigma Aldrich, St. Louis, MO), Abalone extract (Campbell science), and *Helix pomatia* (Sigma Aldrich). While the concentration of 6-MAM remains approximately constant throughout the varying incubation times for the IMCSzyme, it's apparent that the concentration of 6-MAM decreases with increasing incubation time for the other enzymes. This indicates that the other enzymes convert 6-MAM to morphine increasingly with incubation time. Also worth noting is that a concomitant increase in intensity of morphine is found as 6-MAM intensity decreases. In the case of *Helix pomatia* hydrolysis of one particular patient sample, almost half of the initial detected 6-MAM is converted to morphine in the time necessary for the enzyme to hydrolyze morphine glucuronides.

Table 1. Concentrations (ppb), of 6-MAM and morphine after incubation with the respective enzyme.

	IMCSzyme		*Helix pomatia*		*Patella vulgata*		Abalone	
Hours	6-MAM	Morphine	6-MAM	Morphine	6-MAM	Morphine	6-MAM	Morphine
0	581.5	0	513.7	0.05	545	0	620.8	0
1	453.6	3.8	339.2	90.6	476.9	57.7	527.6	14.7
2	565.6	10.6	268.2	120.9	377.7	75.4	552.9	28.1

Conclusion: The IMCSzyme™ demonstrates efficient hydrolysis of morphine glucuronide without converting 6-MAM to morphine, whereas other beta-glucuronidase products convert 6-MAM to morphine.

Keywords: IMCSzyme™, DPX, LC/MS/MS, Hydrolysis, Morphine, 6-MAM, Heroin

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Optimization of Enzyme Hydrolysis of Synthetic Cannabinoid Glucuronides in Urine Using β-Glucuronidase and the Detection by Orbitrap Mass Spectrometer

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Background / Introduction: In recent years, the abuse of new psychoactive substances (NPS) has started to increase in Singapore. Among the NPS, synthetic cannabinoids and cathinones are the two categories that have been detected in the seized exhibits. In May 2014, NPS have been generically listed as Class A Controlled Drugs under the Misuse of Drugs Act. Hence, in order to curb the NPS abuse in Singapore, it is crucial for the laboratory to develop a sensitive and effective method for the detection of NPS in urine. Synthetic cannabinoids are known to be extensively metabolized and excreted almost exclusively in urine as glucuronide conjugates and carboxylic acids which are often present at low levels. So far, the detection of the parent drugs in urine has been rare. In order to analyse the metabolites of synthetic cannabinoids in urine, it is necessary to hydrolyze the urine prior to the instrumental analysis.

Objectives: The aim of this study is to obtain the optimal enzyme hydrolysis condition for the glucuronides of synthetic cannabinoids in urine using Helix Pomatia β-glucuronidase which is followed by the detection of the hydrolysed products using Orbitrap mass spectrometer.

Methods: JWH-018 N-(5-hydroxypentyl) β-D-Glucuronide was purchased from Cayman Chemical (Ann Arbor, MI). β-Glucuronidase from Helix pomatia was purchased from Sigma-Aldrich (St. Louis, MO). The hydrolysis of JWH-018 N-(5-hydroxypentyl) β-D-Glucuronide was carried out in spiked urine (20, 50 and 100 ng/ml) using Helix pomatia β-glucuronidase at various temperatures, durations and pH to obtain the optimal hydrolysis condition. To obtain the optimal conditions, the spiked sample (20 ng/ml) was hydrolysed at 37°C and 60°C in a water bath, and at intervals of 0h, 0.25h, 0.5h, 1h, 2h, 4h, 8h and 16hr at pH 5. All the spiked urine samples were tested in triplicates at pH 4, 5 and 6, at 60°C for 2h. After hydrolysis, the free drug was analysed using Thermo Scientific Q-Exactive Hybrid Quadrupole-Orbitrap Mass Spectrometer with dilute-and-shoot method. The chromatographic separation was performed on an Accucore PFP 2.1 x 100 mm (2.6 µm) column using linear gradient elution with ammonia formate (with 0.1% formic acid) and acetonitrile (with 0.1% formic acid). Under these instrument conditions, both the glucuronide and free drug were monitored through their accurate mass detection.

Results: Based on the preliminary results, approximately 80% of the glucuronide was hydrolyzed after 1 hr and almost full conversion to the free drug was achieved after 2 hr of incubation at 60°C with buffer condition at pH 5. At 37°C under the same conditions, the glucuronide conjugate was still detectable after 2 hr of hydrolysis and complete conversion was attained at 4hr. Testing under the same optimal conditions are in progress using urine samples obtained from 11 suspected drug abusers.

Conclusion / Discussion: Enzymatic hydrolysis condition of 60°C for two hours at pH 5 using β- glucuronidase was found to be effective in complete hydrolysis of the glucuronide conjugates. This method is applied to the suspected abusers' urine samples and the results will be discussed.

Keywords: JWH-018, Synthetic Cannabinoids, β-Glucuronidase, Orbitrap

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Enzymatic Hydrolysis Efficiency of Glucuronide Conjugates in Human Urine

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Background / Introduction: Opioid and benzodiazepine compounds are commonly conjugated with glucuronic acid to be liphilic for efficient elimination by the kidneys. Incomplete hydrolysis will lead the false-negative results for drug screening. Thus, finding the optimal conditions to hydrolyze these conjugated compounds becomes crucial for urine sample analysis. Previous studies were done by using both acid and enzymatic hydrolysis. Acid hydrolysis has numerous pros but its drastic condition, overestimation and compound destroy risk makes enzymatic hydrolysis another alternative to hydrolyze urine samples. Different types of β-glucuronidases such as E.*coli*, H. *pomatia*, P.vulgata have been tested for their hydrolysis efficiency. However, the hydrolysis using these enzymes are time consuming with low efficiency. Recently, abalone β-glucuronidases become commercial available for enzymatic hydrolysis. This study utilizes E.*coli*, abalone and H. *pomatia* β-glucuronidases to hydrolyze five glucuronide conjugates.

Objective: This investigation was designed to study the hydrolysis efficiencies of β-glucuronidases used for hydrolyzing five common glucuronide conjugates presented in urine samples.

Method: Three types of commercial available β-glucuronidases, E.*coli*, abalone and H. *pomatia,* from six sources were used to hydrolyze five glucuronide conjugates, morphine-3-β-D-glucuronide, codeine-6- β-D-glucuronide, oxazepam glucuronide, buprenorphine glucuronide and norbuprenorphine glucuronide. The testing samples were prepared by spiking drug conjugated standards to pre-screened negative human urine. The hydrolysis reactions were performed under three temperatures (37 ^{o}C, 60 ^{o}C and 65 ^{o}C) in pH 5.0 and 6.8 hydrolysis buffers. The hydrolyzed urine samples were collected at different time points to investigate the hydrolysis efficiency. The collected samples were analyzed by a Waters® Acquity® UPLC coupled with an AB Sciex® Triple Quad 5500 mass spectrometer.

Results: For E.*coli* β-glucuronidases under 37 ^{o}C at pH6.8, more than 90% hydrolysis conversion was achieved with 2 hours for all four glucuronide conjugates except codeine-6- β-D-glucuronide which needs 6 hours reaction time. Both abalone and H *pomatia* β-glucuronidases prefer higher incubation temperature (60 ^{o}C or 65 ^{o}C) in pH 5.0 buffer. The hydrolysis reaction using abalone and H *pomatia* β-glucuronidases can reach near completion within 2 hours and 1 hour for all four glucuronide conjugates except codeine-6- β-D-glucuronide, respectively.

Conclusion / Discussion: The results reveal that codeine-6- β-D-glucuronide is the most difficult one to be hydrolyzed among all five glucuronide conjugates. For all six enzymes, only one abalone enzyme can complete the hydrolysis reaction for codeine-6- β-D-glucuronide within two hours under 60 ^{o}C at pH 5.0.

Keywords: Enzymatic Hydrolysis, β-Glucuronidases, Glucuronide, Morphine, Codeine, Oxazepam, Buprenorphine, Norbuprenorphine

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Microwave-Assisted Enzymatic Hydrolysis of Phase II Conjugates in Human Urine

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Background / Introduction: Enzymatic hydrolysis compared to acid hydrolysis is more environmental friendly and there is no overestimate and compound decomposed concerns. However, most of the enzymatic hydrolysis needs longer hydrolysis time than acid hydrolysis. Thus, microwave incubation system might be a solution to promote the hydrolysis enzyme efficiency. A microwave incubation system (Rapid Enzyme Digestion System from Hudson Surface Technology, Inc.) keeps the reaction temperature at certain temperature by using a water bath chamber. Then low-frequency microwave is transferred continually to sample solution to increases the effective collisions. This increases the rate of hydrolysis reaction to result a shorten reaction time. In this study, three types of β-glucuronidases were used to hydrolyze five common phase II conjugates. The investigation of microwave effect was done by comparing the results of the same time point human urine control samples under microwave-free and microwave-assist conditions. The hydrolysis conditions of these two sets of samples were the same.

Objective: The purpose of this study was to determine the effectiveness of microwave for enzymatic hydrolysis using various β-glucuronidases

Method: Five phase II conjugates, morphine-3-β-D-glucuronide, codeine-6- β-D-glucuronide, oxazepam glucuronide, buprenorphine glucuronide and norbuprenorphine glucuronide, were hydrolyzed by three types β-glucuronidases (E. *coli*, abalone, and H. *pomatia*) from six sources. The hydrolysis samples were separated into two sets which were then incubated under the same hydrolysis temperature and pH buffer. Microwave-free sample set was incubated in a regular incubator and microwave-assist sample set was incubated in a microwave incubation system. The hydrolysis samples were collected at same time points and the samples were extracted and analyzed by a Waters® Acquity® UPLC coupled with an AB Sciex® Triple Quad 5500 mass spectrometer.

Results: For oxazepam glucuronide and buprenorphine glucuronide using six sources of β-glucuronidases, the hydrolysis completion can be achieved within 10 minutes with and without microwave. For norbuprenorphine glucuronide hydrolyzed by E.coli and abalone β-glucuronidases, microwave energy did not improve the hydrolysis efficiency. Norbuprenorphine hydrolysis time using H. *pomatia* β-glucuronidase decreased by 67% when microwave energy was added. For morphine-3-β-D-glucuronide hydrolyzed by abalone and H. *pomatia* β-glucuronidases, microwave shortens the hydrolysis incubation time by 67% and 50%, respectively. For codeine-6- β-D-glucuronide, the most difficult hydrolyzed conjugate, microwave did not promote the hydrolysis efficiency if E. coli and H. *pomatia* β-glucuronidases were used. However, microwave increased the codeine-6- β-D-glucuronide hydrolysis rate when abalone glucuronidase was used. The reaction time was decreased by 50%.

Conclusion / Discussion: The comparison results indicate that microwave may or may not assist the hydrolysis efficiency. It depends on target phase II conjugates and different β-glucuronidases utilized. In this study, microwave has no effect on E.coli β-glucuronidase but promotes some hydrolysis reactions when abalone and H. *pomatia* β-glucuronidases were used.

Keywords: Enzymatic Hydrolysis, Microwave, β-Glucuronidase, Glucuronide, Morphine, Codeine, Oxazepam, Buprenorphine, Norbuprenorphine

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Withdrawn

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Automated Sample Hydrolysis for a Forensic Toxicology Urine Screening LC-MS/MS Method

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Introduction: (For research use only, not for use in diagnostic procedures). Here we present an automated, novel and rapid beta glucuronidase hydrolysis sample preparation method integrated with LC-MS/MS analysis.

Objective: Evaluate the effectiveness of a new beta glucuronidase enzyme to efficiently hydrolyze glucuronide drugs in less than 30 minutes followed by a dilute and shoot LC-MS/MS analysis and so provide for a high throughput analysis.

Methods: Automated 30 minute IMCS β-Glucuronidase hydrolysis was performed on spiked urine, containing 8 glucuronidated drug compounds. For a more comprehensive panel the procedure was also performed on spiked urine containing 64 non-glucuronidated drug compounds. Samples were loaded onto a GERSTEL MPS2 equipped with an incubator that allowed for the automated hydrolysis followed by dilution and direct injection on to the LC-MS/MS system. The LC-MS/MS system consisted of an Eksigent ekspert™ ultraLC 100 interfaced to an AB SCIEX QTRAP® 4500 LC/MS/MS System. LC separation was performed on a Phenomenex Kinetex 2.6 µm Phenyl Hexyl 100 Å, 50 x 4.6 mm column at 30 °C; 10 minute gradient of water and methanol with ammonium formate buffer; 0.7 mL/min flow rate 10 µL injection volume.

Results: Urine spiked with known glucuronide drug standards were subjected to an automated 30 minute beta glucuronidase hydrolysis. In order to demonstrate that the hydrolysis reaction had gone to completion within this short time period compared to the traditional 2 hour hydrolysis protocol, the urine standards were analysed for the presence of the glucuronide. This was achieved by monitoring for MRM transitions corresponding to both the glucuronidated compound as well as the parent drug. In all cases only the parent drug was detected and no presence of the glucuronide was observed. The reproducibility of the automated protocol was assessed by preparing and analyzing replicates of each calibration standard, and the measured CVs were less than 15% over the entire concentration range covered by the assay. The method displayed good linearity for all analytes, with $R>0.99$. The method was shown to be robust after monitoring response of the analytes over 100 injections. Not only does method automation eliminate human error, thus increasing reproducibility, it also eliminates subjectivity during data processing, and furthermore has the potential to save a great deal of time.

Conclusion: An automated, rapid urine hydrolysis LC-MSMS forensic toxicology screening method using the GERSTEL MPS2 as the sample preparation workstation and LC autosampler, has been investigated and has been shown to provide a viable approach to provide automated sample analysis from start to finish.

Keywords: Automated 30 Minute Hydrolysis, Drug Screening, LC-MS/MS

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Monitoring a Daily Low Dose of Oxycodone in Nail Clippings by Liquid Chromatography-Tandem Mass Spectrometry: A Case Study

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Background / Introduction: Nail clippings are becoming an acceptable way of testing for drugs of abuse in forensic toxicology because of their long detection window and ease of collection. Oxycodone is a semi-synthetic opioid Schedule II controlled substance, which is used for treatment of moderate to severe pain. It has a high risk of dependence, abuse and addiction. There is need for alternative methods of monitoring long term oxycodone use.

Objective: The **o**bjective of this study was to demonstrate that a low dose daily oxycodone can be monitored in nail clippings.

Methods: After adding internal standard (Oxycodone-d_3), the nail clippings were *pulverized*, sonicated in 3 ml methanol for 3 hours, and incubated overnight at 53 ^{o}C in 3 ml 0.1 N HCL solution. The extracts were subjected to solid phase extraction and the eluates were analyzed using liquid chromatography tandem mass spectrometry. Separation was achieved on an Agilent 1100 HPLC System using Phenomenex Synergi Hydro - RP column with 2.0 µm particle size. Mobile phases used were A = 10 mM Ammonium Acetate and 0.1% Formic Acid B= Acetonitrile and 0.1 % Formic Acid. Analytes were detected on an ABSciex 3200 Tandem Mass Spectrometer equipped with ESI source in the positive ionization mode. Mass transitions monitored were *m/z* 319.2→ *m/z* 301.2 for internal standard (D3- oxycodone) and *m/z* 316.3→ *m/z* 298.3, 241.2 for oxycodone. The lower limit of quantitation (LLOQ) was 12.05 pg/mg and the limit of detection (LOD) was 4.08 pg/mg. The subject was a 60-69 year old Caucasian male, 1.78 meter tall, weighing 95 kilograms, (BMI = 30) and was taking 5mg of oxycodone per day starting on 09/01/12. The subject has been followed since and the study is ongoing.

Results: Base line nail clippings were collected on 9/1/2012 and the results were less than the limit of detection (LOD). Subsequently, we collected six nail clippings over a 20 month period. The measured concentrations ranged from zero to 80, (mean = 41.0 ± 30.66 pg/mg; median = 40 pg/mg)

Conclusion: Oxycodone was detectable in nail clippings of a subject taking a 5 mg daily dose for over a year and 8 months. Nail clippings show promise as a means to monitor use and abuse. Further studies, which will include multiple patients, doses and negative controls, need to be undertaken.

Keywords: Oxycodone, Nail Clippings, Fingernails, LC-MS/MS, Liquid Chromatography Tandem Mass Spectrometry

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Fentanyl, Norfentanyl and Sufentanil Quantitation in Human Hair by Liquid Chromatography Tandem Mass Spectrometry

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Background / Introduction: Fentanyl is a synthetic opioid analgesic often used in the management of patients suffering from severe and chronic pain. It is similar to morphine but exhibits 50-100 times greater potency with high addictive tendencies. Use of fentanyl therefore presents a high risk for abuse. The urine detection window for fentanyl is relatively short, ranging from ~48-72 hours post-dose. Determining the use and/or abuse of fentanyl can therefore be challenging if samples are not collected soon after dosing. Alternatively, the detection window of drug use in hair is significantly wider and can provide an extended window for detecting the use and/or abuse of fentanyl.

Objective: Develop and validate a selective and sensitive method for the simultaneous detection and quantification of fentanyl, norfentanyl and sufentanil in hair using liquid chromatography tandem mass spectrometry (LC-MS/MS).

Methods: Fentanyl, norfentanyl and sufentanil were extracted from hair through pulverization followed by solvent extraction. The LC-MS/MS system consisted of an Agilent 1200 HPLC system coupled with an AB Sciex 3200 QTRAP mass spectrometer. Chromatographic separation of all three compounds was performed on a Phenomenex Kinetex C18 column (100 x 4.6 mm, 2.6 µm). The mobile phase consisted of 2mM ammonium formate with 0.2% (v/v) formic acid in water (A) and methanol with 0.1% (v/v) formic acid (B). Analyte separation was achieved using a mobile phase gradient program. Electrospray ionization in positive ion mode was used to monitor MRM transitions for each analyte. For quantification, peak area ratios of the analytes to their corresponding deuterated analogues were calculated as a function of the concentration of the substances. Method parameters were fully validated and included the determination of LOD and LOQ, linearity, precision and accuracy, carryover, and matrix effects.

Results: LC-MS/MS method validation studies for the quantification of fentanyl, norfentanyl and sufentanil in hair demonstrated a limit of detection (LOD) and limit of quantification (LOQ) of 30 pg/mg and 90 pg/mg hair respectively for each drug using 10 mg of a hair sample. Each drug was linear over the calibration range 0 to 2400 pg/mg with a coefficient of correlation (R^2) of 0.98 or greater for all drugs. Precision and accuracy were within the acceptable range of ±20% (n=5) and matrix effects were negligible.

Discussion: A simple and sensitive LC-MS/MS method was developed and validated for the simultaneous analysis of fentanyl, norfentanyl and sufentanil in hair. This method is applicable as an alternative to urine analysis for the detection and quantification of fentanyl, its metabolite, and sufentanil after dosing has occurred.

Keywords: Fentanyl, Norfentanyl, Sufentanil, Opioids, LC-MS/MS, Hair Analysis

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Validation of Immunoassays for the Screening of Quantisal™ Collected Oral Fluid According to SWGTOX Guidelines

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Background: Based on recommended validation parameters set forth in the Standard Practices for Method Validation in Forensic Toxicology (SWGTOX), immunoassay screening parameters were fully validated for drugs in oral fluid collected with the Quantisal™ device at recommended cutoffs.

Objective: To determine whether Immunalysis ELISA kits are capable of performing under the recommended guidelines and to identify any limitations during the course of validation.

Methods: All kits were tested for intraday precision and accuracy by calculating the grand mean absorbance, standard deviation (SD) and CV% of 5 independent runs with 3 replicates per run (n=15). The calculated 2X SD was applied to the grand mean and an absorbance range was determined. Acceptance criteria for each validation included CV ≤20% and absorbance range for one calibration level must not overlap with another calibration level. All immunoassays were fully validated at 50% (low cal), 100% (cut-off cal), and 150% (high cal) of the recommended cutoffs according to the SWGTOX guidelines. Sample volumes were adjusted to give the best possible separation with the most difficult aspect being adequate distinction between 100% and 150% concentrations. All kits were tested according to the manufacturer's protocol including Δ9-THC with an additional 1 hour pre-incubation of sample only.

Results*:* the results are shown in the table. Cutoff in ng/ml				**Absorbance range**		
Drug	**Cut-off**	**CV(%)**	**Samp vol (µL)**	**Neg – Low cal**	**Low cal –Cutoff**	**Cutoff – High Cal**
Δ9-THC	4	< 4	25	1.776-1.894	1.298-1.386	0.908-1.056
Amphetamine	20	< 9	10	1.217-1.574	0.907-1.052	0.548-0.770
Methamphetamine	20	< 4	10	1.567-1.777	1.295-1.482	1.076-1.197
MDMA	20	< 4	10	1.234-1.330	0.963-1.079	0.773-0.891
MDA	20	< 7	10	1.143-1.365	0.616-0.765	0.374-0.484
Cocaine	20	< 7	10	1.241-1.376	0.956-1.120	0.718-0.919
Benzoylecgonine	20	< 5	10	1.395-1.684	1.071-1.287	1.035-1.065
Oxazepam	5	< 5	100	1.355-1.590	0.876-1.058	0.731-0.769
Carisoprodol	100	< 4	10	1.900-2.158	1.579-1.660	1.415-1.530
Meprobamate	100	< 6	10	1.428-1.748	1.172-1.324	0.909-1.148
Zolpidem	10	< 6	40	1.489-1.744	1.310-1.354	1.049-1.299
Butalbital	50	< 3	10	1.827-1.965	1.545-1.690	1.353-1.507
Phenobarbital	50	< 5	50	1.392-1.638	1.218-1.373	1.111-1.204
Methadone	50	< 7	10	1.195-1.548	0.642-0.701	0.427-0.480
Morphine	20	< 5	10	1.697-1.989	0.966-1.152	0.712-0.806
Oxycod/Oxymor	20	< 7	10	0.565-0.723	0.399-0.479	0.332-0.391
Phencyclidine	10	< 5	10	0.723-0.825	0.510-0.615	0.390-0.472

Conclusion: Immunoassay screening parameters for the analysis of drugs in oral fluid collected with the Quantisal™ device were successfully validated according to the SWGTOX guidelines at the recommended cutoff concentrations. Limitations were predominantly associated with large drug groups not having adequate cross-reactivity for all analytes (e.g. benzodiazepines, barbiturates etc.)

Keywords: Oral Fluid, ELISA, SWGTOX Validation Guidelines

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Validation of Immunoassays for the Screening of Whole Blood According to SWGTOX Guidelines

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Background: Based on recommended validation parameters set forth in the Standard Practices for Method Validation in Forensic Toxicology (SWGTOX), immunoassay screening parameters were fully validated for drugs in whole blood specimens at recommended cutoffs.

Objective: To determine whether Immunalysis ELISA kits are capable of performing under the recommended guidelines and to identify any limitations during the course of validation.

Methods: All kits were tested for intraday precision and accuracy by calculating the grand mean absorbance, standard deviation (SD) and CV% of 5 independent runs with 3 replicates per run (n=15). The calculated 2X SD was applied to the grand mean and an absorbance range was determined. Acceptance criteria for each validation included CV ≤20% and absorbance range for one calibration level must not overlap with another calibration level. All immunoassays were fully validated at 50% (low cal), 100% (cut-off cal), and 150% (high cal) of the recommended cutoffs according to the SWGTOX guidelines. Sample volumes were adjusted to give the best possible separation with the most difficult aspect being adequate distinction between 100% and 150% concentrations. With the exception of zolpidem (dilution factor 1:10), all controls were diluted 1:20 in 100mM phosphate buffer pH7.0 containing 0.1% w/v bovine serum albumin (BSA) before being tested according to the manufacturer's protocol.

Results: The results are shown in the table. Cut-off in ng/ml.

				Absorbance range		
Drug	**Cut-off**	**CV(%)**	**Sample vol(µL)**	**Neg – Low cal**	**Low cal –Cutoff**	**Cutoff –High cal**
THC-COOH	10	< 6	100	1.200 – 1.45	0.959 – 1.114	0.585 – 0.72
Amphetamine	20	< 4	25	0.780–1.989	1.421–1.558	1.154–1.240
Methamphetamine	20	< 4	25	1.121–1.281	1.287–1.417	1.121–1.281
Benzoylecgonine	50	< 7	20	1.791–2.069	1.430–1.564	1.087–1.389
Oxazepam	50	< 6	40	1.915–2.210	1.571–1.779	1.160–1.467
Carisoprodol	500	< 5	10	1.527–1.696	1.239–1.461	1.041–1.231
Meprobamate	500	< 5	10	1.229–1.352	0.967–1.054	0.782 – 0.929
Zolpidem	10	< 3	100	1.661–1.800	1.502–1.634	1.413–1.500
Butalbital	300	< 3	25	1.622–1.741	1.415–1.500	1.255–1.316
Phenobarbital	300	< 3	25	2.268–2.516	2.008–2.253	1.903–1.997
Methadone	50	< 9	20	1.855–2.625	1.351–1.681	0.949–1.252
Morphine	10	< 4	20	1.657–1.943	1.470–1.617	1.268–1.442
Oxycod/Oxymor	10	< 13	10	0.476–0.795	0.346–0.466	0.305–0.338
Phencyclidine	10	< 4	20	1.670–1.903	1.232–1.372	1.014–1.131

Conclusion: Immunoassay screening parameters for the analysis of drugs in whole blood were successfully validated according to the SWGTOX guidelines at the recommended cutoff concentrations. Limitations were predominantly associated with large drug groups not having adequate cross-reactivity for all analytes.

Keywords: Whole Blood, ELISA, SWGTOX Validation Guidelines

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A Review of 1,1-Difluoroethane Detected in Postmortem and DUID Cases

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Introduction: 1,1-Difluoroethane (DFE) is a colorless and odorless gas that is commonly used as the main ingredient in aerosol propellants such as Dust-off® cleaner. Although the incidence of inhalant abuse is relatively low as compared to oral administration of other drugs of abuse, inhalant use remains popular especially among young adults. It has been reported that inhalation of DFE can cause lightheadedness, disorientation, psychomotor impairment, cardiac dysrhythmias, and sudden death.

Objective: To obtain statistical information on DFE concentrations in blood samples from both postmortem and DUID investigations.

Method: In a three year period at NMS Labs (January 2011 through December 2013), 987 cases were analyzed for DFE using headspace gas chromatography/mass spectrometry (HS-GC/MS). The goal was to determine the concentration of DFE in blood at or above the reporting limit of 0.14 mcg/mL. The cases were submitted from medical examiners, coroners, and law enforcement agencies throughout the United States. The results were divided into two categories, death investigations and DUID investigations.

Results: Of the 987 cases examined, 776 were postmortem cases of which 94% of them (n=729) were positive for DFE; the remaining 211 cases were DUID cases with 87% of them (n=183) positive for DFE. DFE is not included in a routine scope of analysis in either type of case; therefore testing for DFE was performed only at the request of the investigators working the case or on cases in which the laboratory detected the presence of DFE in the routine alcohol analysis by headspace gas chromatography (HS-GC).

Table 1: Median concentration, range and median age of postmortem cases positive for DFE by year (n=729).

	2013 (n=212)	2012 (n=222)	2011 (n=295)
Median DFE Concentration (mcg/mL)	35	38	34
Range (mcg/mL)	0.15 - 460	0.24 - 480	0.21 - 390
Median Age (years)	31	32	31
Range (years)	16 - 62	16 - 64	12 - 68

Table 2: Median concentration, range and median age of DUID cases positive for DFE by year (n=183).

	2013 (n=53)	2012 (n=62)	2011 (n=68)
Median DFE Concentration (mcg/mL)	8.0	4.5	5.2
Range (mcg/mL)	0.16 - 290	0.25 - 130	0.18 - 330
Median Age (years old)	25	25	31
Range (years old)	17 - 54	18 - 55	16 - 62

Conclusion: The data clearly shows that the median concentration of DFE in postmortem cases is significantly higher than the concentration found in DUID cases. Although the observed range overlaps in both types of cases, higher DFE levels were seen in postmortem cases. The data also suggests that DFE users are typically young adults. Over the past three years, the requests to perform DFE analysis have remained consistent. Also consistent over the three year period is the median concentration of DFE.

Keywords: DFE, DUID, Postmortem Blood, HS-GC/GC-MS

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A Fatality Involving Nitrous Oxide, MDMA, Methoxetamine, and Opiates

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Introduction: This case illustrates a fatality in which a 27 year old male inhaled nitrous oxide (N_2O) in conjunction with MDMA (Ecstasy), methoxetamine (MXE), morphine and oxycodone.

Objective: The synergistic effects of multiple drugs can be difficult to predict. This case provides an interesting account of an alleged drug abuser's fatal combination of multiple intoxicating agents, including designer drugs, and illustrates the challenges involved in interpreting cause of death when multiple drugs are present.

Method: Suitable peripheral blood was not available for testing. Heart blood was screened for volatiles by headspace GC, and heart blood drug screening was performed using Enzyme Multiplied Immunoassay Technique (EMIT) and liquid-liquid alkaline extraction followed by GC/MS. All quantitations were performed in heart blood by an external laboratory: methoxetamine (LC-MS/MS), MDMA and MDA (LC-MS/MS), nitrous oxide (GC/MS), morphine and oxycodone (GC/MS).

Results: The decedent was discovered in a kneeling position, holding a white canister in his hand. Dozens of small N_2O containing capsules were found in the home and multiple drug paraphernalia were scattered about. Heart blood GC/MS drug screen analysis indicated the presence of MDMA, opiates, oxycodone, benzodiazepines, cannabinoids, benzoylecgonine and diphenhydramine. No volatiles were detected and carboxyhemoglobin was measured at 2.9 %. Opiate confirmation testing confirmed the presence of oxycodone at 63 ng/mL and morphine at72 ng/mL. 6-MAM was not detected. Nitrous oxide was determined to be present at 27 mcg/mL, and MDMA confirmed positive at 330 ng/mL with MDA at 45 ng/mL. Given the evidence recovered at the crime scene, the medical examiner requested special testing for bath salts, synthetic cannabinoids and NBOMe compounds. No bath salts, NBOMe compounds or synthetic cannabinoids were detected, however MXE was detected at 160 ng/mL. Diphenhydramine was not quantitated, and benzoylecgonine quantitated at less than 150 ng/mL.

Conclusion: Fatalities independently involving nitrous oxide, opiates, and MDMA have been established in the literature, but fatalities involving methoxetamine are relatively new. Methoxetamine first appeared in Europe in 2010 as a safe alternative to ketamine, but deaths involving this compound have since been reported with levels ranging from 30 ng/mL to 1100 ng/mL (EMCDDA-Europol). The MDMA in this case was detected at a level which is typically not toxic by itself, but its presence is interesting. A 2001 DEA report indicates that when users want to "come down" from the effects of MDMA, they will often use other drugs including GHB, marijuana, nitrous oxide, and ketamine. Though the decedent's intent in this case is unknown, combinations of the aforementioned drugs can be very dangerous and unpredictable. The cause of death was determined to be methoxetamine, MDMA, nitrous oxide, morphine, and oxycodone intoxication. The manner of death was ruled accidental.

Keywords: Nitrous Oxide, Methoxetamine, Opiates, MDMA, Postmortem Toxicology

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Interpretation of Ethanol Detection in Postmortem Cases by Use of Ethyl Glucuronide and Ethyl Sulphate

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Introduction: Postmortem ethanol formation is a well-known problem in forensic toxicology. Traditional criteria to interpret postmortem ethanol detections cannot be used in all cases. Analysis of the non-oxidative ethanol metabolites, ethyl glucuronide (EtG) and ethyl sulphate (EtS) in blood, is used as a marker for the ingestion of ethanol. These metabolites have a high sensitivity and specificity to detect postmortem ethanol formation however EtG has been proven to be unstable whereas EtS is more stable.

Objective: The aim of this study was to interpret findings of ethanol in blood, in a large collection of forensic autopsy cases, by using EtG and EtS markers. We especially wanted to study the number of potentially false ethanol detections being produced by postmortem formations at each ethanol concentration level.

Method: All postmortem blood samples positive for ethanol during a 15 month period were included and results from EtG and EtS analyses in blood were used to interpret whether the ethanol was formed postmortem or ingested prior to death. In this study, according to previously published literature, antemortem ethanol ingestion was excluded in EtS-negative cases.

Results: Among 493 ethanol-positive forensic autopsy cases collected during the study period, EtS was not detected in 60 (12%) of the cases. Among cases with a blood alcohol concentration (BAC) $\leq$ 0.54 g/kg (5.7 g/dl), ante-mortem ethanol ingestion was excluded in 38% of the cases, while among cases with a BAC $\geq$ 0.55 g/kg, ante-mortem ethanol ingestion was excluded in 2.2% of the cases. For all cases where ethanol was measured at a concentration > 1.0 g/kg, EtS was detected. The highest blood ethanol concentration in which EtS was not detected was 1.0 g/kg. In this case, putrefaction of the corpse was reported.
The median concentration of ethanol in blood for all cases was 1.2 g/kg (range: 0.1 - 4.9). In the 60 cases where EtS tested negative, the median concentration of ethanol was 0.3 g/kg (range: 0.1- 0.99), while in the 433 cases where EtS tested positive, the median concentration of ethanol was 1.4 g/kg (range: 0.1 - 4.9). This difference was statistically significant ($p < 0.001$). The median concentrations of EtG and EtS in blood were 9.5 µmol/L (range: n.d - 618.1) and 9.2 µmol/L (range: n.d - 182.5), respectively.
There was a statistically significant positive correlation between concentration levels of ethanol and of EtG (Spearman´s rho=0.671, $p < 0.001$) and EtS (Spearman´s rho=0.670, $p < 0.001$), respectively.

Conclusion: In conclusion, this study showed that in a large number of ethanol-positive forensic autopsy cases, ethanol was not ingested before the time of death, particularly among cases where ethanol was present in blood in low concentrations. Routine measurement of EtG and EtS is therefore recommended, especially in cases with the BAC is below 1g/kg.

Keywords: Alcohol, Postmortem, EtG, EtS

P-59

Pharmacokinetic Interactions Between Ethanol and Heroin; A Study on Post-Mortem Cases

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Introduction: Poly drug use is a frequent finding in heroin related deaths, and ethanol is one of the substances that is most often detected. Combined use of ethanol and heroin is known to be particularly dangerous, due to both drugs having depressant effects on the central nervous system. A pharmacokinetic interaction between ethanol and heroin has also been suggested in previous studies, and both inhibition of the metabolism of 6-monoacetylmorphine (6-MAM) to morphine as well as inhibition of the glucuronidation of morphine has been demonstrated in smaller studies.

Objective: The aim of the present study was to investigate if there was a pharmacokinetic interaction between heroin and ethanol in a large selection of forensic autopsy cases.

Method: The case selection consisted of 1583 forensic autopsy cases, all containing 6-MAM, as evidence of heroin intake, in either blood or urine samples from the time period between the 1st of January 2000 and the 31st of December 2012. Due to the high risk of post-mortem ethanol formation in cases having blood ethanol concentrations between 0.01 -0.03 g/dl, these cases were excluded from the study, along with cases where the analysis for ethanol was missing. After this exclusion of cases, the remaining cases (n=1474) were divided into two groups; one group where ethanol was not detected in blood (n=1160), and another group where ethanol was detected in blood at or above the concentration of 0.04 g/dl (n=314). Furthermore, the selected cases were also divided into two other subgroups; one group where 6-MAM was detected in blood samples, indicating a very recent intake of heroin, and another group where 6-MAM was detected in the urine, but not in blood, indicating a historic heroin intake.

Results: The concentration ratios of morphine/6-MAM, morphine-3-glucuronide (M3G)/morphine, and morphine-6-glucuronide (M6G)/morphine in blood samples, were all significantly lower in the ethanol positive cases compared with that of the ethanol non detected cases. For the subgroup of cases revealing a very recent intake of heroin (n=645), only the morphine/6-MAM ratio was significantly lower in the ethanol positive cases than in the ethanol non detected cases. For the subgroup of cases with a historic heroin intake (n=817), lower M3G/morphine and M6G/morphine ratios were found among the ethanol positive cases.

Conclusion: The results support the proposal that ethanol inhibits two steps in heroin metabolism; the hydrolysis of 6-MAM to morphine, and the glucuronidation of morphine to M3G and M6G. Inhibition of the hydrolysis of 6-MAM was also observed in cases involving a very recent intake of heroin, suggesting that the findings were not caused by different time intervals between heroin intake and death. A pharmacokinetic interaction could, in addition to the well-known pharmacodynamic interactions, further increase the risk of a fatal outcome after combined use of heroin and ethanol. Post-mortem changes in blood concentrations of analytes are considered a minor problem in our cases due to the fact that approximately 90 % of the samples were from peripheral blood, 1% was from heart blood and the remaining 9% were from an unspecified source of blood.

Keywords: Post-Mortem, Pharmacokinetic, Ethanol, Heroin, Metabolism

P-60
Alcohol Hangover as a Cause of Impairment in Apprehended Drivers

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Introduction: Previous studies have already shown the possibility of impairment during a hangover phase after alcohol ingestion when the blood alcohol concentration has returned to zero. The prevalence of drivers being in a hangover phase and the relation to impairment relevant for traffic-safety has not been studied.

Objective: The aim of this study was to investigate the prevalence and the concentrations of the two ethanol metabolites, ethyl glucuronide (EtG) and ethyl sulphate (EtS) in blood from apprehended drivers, in which no psychoactive substances (including alcohol) were detected. EtG and EtS indicate very recent (within the last 12-24 hours) alcohol intake. The aim was also to study these findings in relation to the impairment observed in these drivers.

Method: Blood samples drawn from suspected drunk or drugged drivers were analysed for a broad repertoire of psychoactive substances, using a fully validated ultra-performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS) method. Ethanol was analysed using an enzymatic ADH method. A clinical test for impairment (CTI) was performed at the same time. The CTI included a number of tests on motor coordination, cognitive performance, degree of alertness, and appearance. The result of the CTI is an evaluation of the total performance of the driver made by a physician working for the police. The CTI results are used to determine whether the driver is; not-impaired, mildly impaired, moderately impaired, or considerably impaired. One-hundred-and-forty-six cases, in which no psychoactive substances were detected, and where a valid CTI was performed, were analysed for EtG and EtS in blood. The prevalence of EtG and EtS and the concentrations of these substances were then related to the conclusions of the CTIs.

Results: EtS and EtG were detected in a total of 19 of the 146 cases (13%). In 16 (18%) of these cases, the drivers were judged as “impaired”, while in 3 (5%) of the cases, the drivers were judged as “not impaired”. There were significantly more detections of EtS and EtG among the impaired group of drivers compared with the not-impaired drivers ($p=0.030$), and the concentrations of both EtG ($p=0.027$) and EtS ($p=0.026$) were significantly higher in the group of impaired drivers compared with the not-impaired drivers. There was a statistically significant positive correlation between the concentrations of EtG (Spearman´s $rho=0.170$, $p=0.041$) and EtS (Spearman´s $rho=0.189$, $p=0.022$) and the degree of impairment.

Conclusion: EtG and EtS were prevalent findings in blood collected from apprehended drivers testing negative for a broad repertoire of psychoactive substances. Their relation to impairment, as judged by the CTI, indicates that hangover symptoms may be relevant for traffic-safety.

Keywords: Alcohol, Hangover, Driving, EtG, EtS

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The Influence of Alcohol and Drugs at the Time of Injury Among Patients in a Norwegian Emergency Department

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Introduction: Prevalence of alcohol and drugs in injured patients is well studied, but less is known about the actual level of influence among these patients, especially at time of injury compared to time of admittance to the hospital.

Objective: The aim of this study was to obtain an estimate of level of influence at time of injury on the basis of blood concentrations of alcohol and drugs.

Method: The study was conducted among patients presenting to the emergency department of a university hospital. All adult patients admitted to the hospital within 6 hours of injury during one year (n=996) were included. Quantification in blood was done by an enzymatic method for alcohol and by liquid chromatography-mass spectrometry or gas chromatography-mass spectrometry for 28 other impairing drugs. Concentrations of alcohol and drugs in blood at time of injury were estimated by doing a back-calculation according to zero-order kinetics for alcohol, using a mean elimination rate of 0.015 % per hour, for the other drugs the concentrations were back-calculated according to first- order kinetics (using the measured concentration (C measured) multiplied by 2 elevated in the time span (Δt) divided by the half-life ($t1/2$) of the substance; $C = C \text{ measured} \times 2^{\Delta t / t1/2}$). The half-lives used in our study, are the mean values of the half-lives given in Disposition of Toxic Drugs and Chemicals in Man (Baselt). Tetrahydrocannabinol is the substance with the greatest variation in half-life, since its half-life to a large degree depends on time elapsed since intake. For tetrahydrocannabinol, we used the mean terminal half-life of 24 hours, and did not back-calculate the concentrations of tetrahydrocannabinol, as the measured concentrations were considered to reflect the concentrations at time of injury. This was also the case for the other substances with a mean terminal half-life of 24 hours or more (diazepam, methadone and nitrazepam). The level of influence at time of injury was assessed on the basis of the estimated blood concentrations using previously developed standardized criteria. The degree to which different substances will influence a person's behavior will vary between individuals. For several substances, such as benzodiazepines, zopiclone and zolpidem, amphetamines, tetrahydrocannabinol and codeine, a positive relationship between blood concentration and drug influence has been found. We gave the concentrations of alcohol and drugs one to six points according to four concentration intervals for each substance (see table below). Legal drugs were scored so that one point corresponded to the average peak blood concentrations after ingestion of the maximum recommended therapeutic dose, for example 5-10 mg of diazepam or zopiclone, while increasing supra-therapeutic concentrations corresponded to two, four or six points. Ethanol and illegal drugs were scored in the same way, with intervals giving a degree of impairment comparable to the aforementioned drugs. Comparing the different influence levels to BAC, 1 point will reflect a BAC of approximately 0.05 %. The threshold of influence was set at a blood alcohol concentration (BAC) of 0.05 %, or a drug concentration leading to influence similar to that of a BAC of 0.05 %. In Norway, impairment based legislative limits are established for most of the substances included in the study. These limits, representing drug concentrations in blood likely to be accompanied by an impairment in driving skills comparable to blood alcohol concentrations of 0.02, 0.05 and 0.12 %, are low compared to the concentration intervals for influence used in our study. Our concentration intervals should apply to regular users who might have developed tolerance to many of the drug effects, whereas the legislative limits are based on acute intake in naïve individuals.

Drug	Cut-off	1 point	2 points	4 points	6 points
Alcohol (ethanol) *	0.01	0.05-0.10	0.11-0.15	0.16-0.25	≥ 0.26
Illicit Drugs					
Amphetamines**	4.06/ 4.48	70-270	271-554	555-1052	≥ 1053
Cannabis (tetrahydrocannabinol)	0.16	0.6-2.8	2.9-4.7	4.8-9.7	≥ 9.8
Cocaine	6.07	60-243	244-515	516-789	≥ 790
Medicinal Drugs					
Alprazolam	4.63	15-46	47-97	98-147	≥ 148
Clonazepam	4.74	9-47	48-98	99-148	≥ 149
Codeine	2.99	90-286	287-603	604-920	≥ 921
Diazepam	28.5	85-313	314-654	655-996	≥ 997
Flunitrazepam	0.78	1.6-6	7-12	13-18	≥ 19
Methadone	31	123-433	434-896	897-1360	≥ 1361
Morphine	2.85	15-54	55-140	141-284	≥ 285
Nitrazepam	7.03	14-53	54-110	111-166	≥ 167
Oxazepam	143	583-1118	1119-2265	2266-3411	≥ 3412
Zolpidem	7.68	46-104	105-212	213-430	≥ 431
Zopiclone	9.72	35-66	67-136	137-206	≥ 207

* Values given in %. All other values given in ng/ml. ** Sum of amphetamine and/or methamphetamine

Results: Of the 996 patients included, a total of 324 patients (32.5 %) were influenced by one or more substances at the time of injury. In comparison, 394 patients (39.6 %) had one or more substances over the analytical cut-off value in blood at the time of admittance to the hospital. Among the 324 patients who were influenced, the total score of points ranged from one to twelve, with a mean (and median) score of four points. In patients with violence related injuries, almost 75 % were found to be influenced. Influenced patients were younger than patients who were not influenced, and men were more often determined to be influenced than women. More patients were influenced at nighttime and during weekends than during daytime and on weekdays. Alcohol was the most prevalent substance leading to influence at 25.9 %, followed by benzodiazepines at 6.8 % and cannabis (tetrahydrocannabinol) at 4.7 %. The number of substances found in the blood of patients who were not influenced ranged from 0 to 3, compared to 1 to 8 substances in patients who were determined to be influenced. Approximately 98 % of the patients with alcohol in their blood at time of admittance to the hospital were determined to be influenced at the time of injury. For patients with benzodiazepines and cannabis in their blood at the time of admittance to the hospital, the percentage determined to be influenced at the time of injury were about 70 % and 80 %, respectively.

Conclusion: About one third of the patients admitted to the hospital with injuries were determined to be influenced by alcohol and or drugs at the time of injury, with alcohol being the most prevalent substance. Violence related injuries, and injuries occurring at nighttime and on weekends, were particularly related to the influence of alcohol and drugs. The fact that assessment of influence was made solely on the concentrations in blood is a limitation in our study. Assessment of influence should preferably be supported by a clinical evaluation of signs and symptoms, which is the way it is generally done in forensic toxicology.

Keywords: Alcohol Impairment, Impairing Drugs, Injury, Emergency Department

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Analysis of Dextromethorphan and Dextrorphan in Decomposed Skeletal Tissues by Microwave Assisted Extraction, Microplate Solid Phase Extraction and Gas Chromatography-Mass Spectrometry (MAE-MPSPE-GC-MS)

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Introduction: Interpretation of toxicological measurements in bone is complex due to an absence of reference databases, standard methods and calibration challenges. Analysis of bone of rats exposed to various drugs facilitates the controlled study of drug and metabolite distribution under a wide range of conditions of dose, pattern of exposure, and interval between exposure and death, while providing for sufficient sample sizes to allow for statistical analysis. Sample preparation is laborious, and continued research in this area requires analysis of large numbers of samples obtained under various conditions of drug exposure and postmortem environment.

Objective: The objective was to develop a Microwave assisted extraction (MAE) followed by microplate solid phase extraction (MPSPE) for the semi-quantitative determination of dextromethorphan (DXM) and dextrorphan (DXT) in postmortem rat bone by GC/MS.

Method: Rats (n=5) received 100 mg/kg DXM (i.p), and were euthanized by CO_2 asphyxiation 20 min post-dose. Remains decomposed to skeleton outdoors and femoral bones were collected, rinsed lightly with methanol; phosphate buffer (PBS: 0.1 M; pH 6) and acetone (3 mL each) and air dried overnight. Bones were ground using grinder and ball mills. Samples (0.2 g) underwent MAE in 5 mL methanol at 80ºC in a MARS6 laboratory grade microwave (CEM Corp., Matthews, NC). Extracts were recovered, evaporated and reconstituted in 1ml PBS. Internal standard (D3-DXT; 200 ng), 100 uL glacial acetic acid (AA) and acetonitrile:methanol (1:1; 3 mL) was added to each extract, followed by storage at -20 ºC overnight. Following centrifugation, supernatants were evaporated to 1 ml and diluted with 3 mL PBS. Diluted supernatants underwent SPE (XCEL I, 130 mg, UCT Inc., Bristol PA) in a 48-well microplate format. Following conditioning and sample loading, wells were washed with PBS (3ml) and 0.1 M AA (3 mL); and dried (~5 in Hg, 5min). Wells were then washed with methanol (3 mL) and dried under vacuum (~10 in Hg; 10 min). Elution was done with 3% ammonium hydroxide in 20:80 isopropanol:ethyl acetate (3 mL). Extracts were evaporated and derivatized with MSTFA + 1% TMS-Cl (50 uL) in ethyl acetate (50 uL, 70 ºC, 1 hr). DXM and DXT levels were measured by GC-MS-SIM as the mass-normalized response ratios (RR/m), (RR: peak area of the analyte divided by that of the internal standard, using ions intended for quantitative analysis). Ions used for DXM, DXT and DXT-d_3 were: (quantitative ions in bold): DXM - m/z **271**, 214, 150 amu; DXT - **329**, 272, 150 amu; D3-DXT – **332**; 272, 153 amu.

Results: Analytes were stable for at least 60 min irradiation time. The majority (> 95%) of each analyte was recovered after 15min MAE. The concentration dependence of the assay response was best fit by a quadratic regression (R^2 =0.995-0.998, n=3, on each of three different days, 10-2000 ng/mL) with coefficients of variation <20% (n_i = 3). The limit of detection was 10ng/mL for each analyte. Following MAE for 30 min (80 ºC, 1200 W), MPSPE-GC-MS analysis of femoral bone of DXM-exposed rats detected DXM and DXT in all samples assayed. DXM levels ranged from roughly 0.9-1.5 ug/g, and DXT levels ranged from roughly 0.6-1.8 ug/g.

Conclusion: The MAE-MPSPE method developed was an efficient means of preparing skeletal tissues for analysis of dextromethorphan and its metabolite, dextrorphan.

Keywords: Dextromethorphan, Dextrorphan, Bone, Microwave, Extraction, Postmortem

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Reduced Sample Volume and Simplified Sample Preparation in the Simultaneous Measurement of Zinc, Antimony, Bismuth, and Manganese in Whole Blood Using Inductively Coupled Plasma-Mass Spectrometry

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Introduction: To achieve required sensitivity from whole blood samples with Inductively Coupled Plasma-Mass Spectrometry (ICP-MS), historic extraction methods often required a time-consuming chemical and/or microwave digestion. Our historic method for the analysis of Antimony (Sb), Bismuth (Bi), and Manganese (Mn) required 500uL of patient sample diluted with 500uL of concentrated nitric acid. These acidified samples were then placed in an 80°C heat block for 4 hours to digest the sample. Our newly developed method, described here, uses a simplified dilution for the simultaneous analysis of Sb, Bi, Mn, as well as Zinc (Zn) in whole blood.

Objective: To develop a simplified ICP-MS method for the analysis of Sb, Bi, Mn and Zn in whole blood.

Method: A 100uL sample was extracted with 4.9mL of basic diluent containing 1.75% EDTA, 0.1% Triton-X 100, 1% ammonium hydroxide, and indium (internal standard). Samples were quantitated using a standard addition curve; all calibrators were prepared in goat blood to achieve a matrix matched method.

Element	Standard 1	Standard 2	Standard 3	Standard 4
Sb (ug/L)	1	5	15	25
Bi (ug/L)	1	5	15	25
Mn (ug/L)	1	10	40	80
Zn (ug/dL)	50	500	1000	1500

Samples were analyzed with an Agilent 7700x ICP-MS (Agilent Technologies, Santa Clara, CA) in Helium (He) gas mode with a Cetac ASX-500 series auto-sampler (Teledyne Cetac Technologies, Omaha, NE) with Glass Expansion Niagara Plus upgrade (Glass Expansion, Pocasset, MA). The He gas is required for the elimination of polyatomic interferences found at the same masses as the elements of interest (Sb (121 m/z), Bi (209), Mn (55), Zn (66)).

Results: Inter and Intra-assay imprecision was less than 4.5% for all four elements. The sensitivity and linearity of each element was analyzed and the analytical measurement range (AMR) was established to match the calibration curve. The AMRs for Sb and Bi was 1-25ug/L, Mn was 1-80ug/L, and Zn was 50-1500ug/dL. To ensure accuracy of the assay 40 samples for each element were analyzed. These samples consisted of previously analyzed proficiency test and patient samples, as well as spiked samples. The Sb, Bi, and Mn patient samples were compared with the historic method while the Zn patient samples were compared with an outside reference laboratory. Sample stability, assay stability, carryover, recovery, and dilutions were also assessed and determined to be within laboratory established criteria.

Conclusion: The method described for Sb, Bi, Mn, and Zn whole blood measurements was validated based upon requirements for Clinical Laboratory Improvement Amendments (CLIA)-certified clinical laboratories. The method uses matrix-matched calibrators to ensure accurate elemental measurements and to comply with the College of American Pathologists, recommendations and guidelines.

Keywords: Antimony, Bismuth, Manganese, Zinc, ICP-MS, Whole Blood

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An Evaluation of the Immunalysis Buprenorphine Direct ELISA Kits for the Detection of Buprenorphines in Umbilical Cord

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Introduction: There has been an increase in maternal prescription opiate use resulting in an increase of treatment using buprenorphine. Infants exposed *in utero* to buprenorphine may display neonatal abstinence syndrome (NAS) shortly after birth. Therefore neonatal professionals need tools that are rapid, cost effective, and sensitive to identify newborns to refer them to appropriate treatment.

Objective: The objective of this study is to present a validated assay for the detection of buprenorphines in umbilical cord using Immunalysis Buprenorphine Direct ELISA kit.

Method: Umbilical cord (0.5 grams) in 3mL of acetone was homogenized in a Next Advance Bullet Blender™ Homogenizer. The specimens were centrifuged and the supernatants were decanted into 13x100 test tubes with 50µL of 0.2% succinic acid added (While succinic acid serves no purpose for this specific assay, succinic acid is used to reduce the volatility of other compounds in the profile such as methamphetamine.). After evaporation under N_2, the residues were reconstituted in 700µL Immunalysis Reconstitution Buffer and the extracts were analyzed using the Immunalysis Buprenorphine Direct ELISA Kit on an automated pipetting Tecan Freedom Evo®. The ELISA screening cutoff of 0.5ng/g is used to distinguish the positives and negatives. Twenty-eight (28) consecutive specimens from a high risk population received in a commercial reference laboratory were subjected to the assay. Additionally, all specimens were analyzed using a previously validated LC-MS/MS method for Buprenorphine and Norbuprenorphine for comparison. The limit of quantitation for Buprenorphine and Norbuprenorphine was 0.4ng/g and the limit of detection was 0.2ng/g. The assay was linear up to 20ng/g for both compounds.

Results: The ELISA screen was validated with five different concentration levels of Buprenorphine at 0.25ng/g, 0.375ng/g, 0.5ng/g, 0.625ng/g, and 0.75ng/g to determine precision and accuracy. The precision (CV %) intra-day was between 0.6-14.9% and inter-day was 4.6-24.0%. The mean plus 2 standard deviation ranges did not overlap. Of the twenty-eight authentic specimens, there were three true positives (3.14ng/g /6.16ng/g, 1.17ng/g /2.83ng/g, and 1.17ng/g /4.55ng/g for Buprenorphine/Norbuprenorphine respectively), two false positives (positive for screen but negative for LC-MS/MS), and twenty-three true negatives (sensitivity = 100%; specificity = 92%).

Conclusion: The recent increase of prescription opiate abuse has resulted in an increase of maternal buprenorphine use. The method presented here demonstrates that the Immunalysis Buprenorphine Direct ELISA Kit may be utilized as an initial test for the detection of *in utero* buprenorphine exposure.

Keywords: Buprenorphine, Norbuprenorphine, ELISA, Umbilical Cord, LC-MS/MS, Liquid Chromatography Tandem Mass Spectrometry

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Telling the Difference Between Buprenorphine and Buprenorphine-Naloxone in Drugs and Driving Cases

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Introduction: Buprenorphine testing is being requested more often in drugs and driving cases. Buprenorphine does not cross react with typical opiate immunoassays. It cannot be detected in a basic drug screen using gas chromatography-mass spectrometry (GC-MS) without derivatization. Liquid chromatography-tandem mass spectrometry (LC-MS/MS) offers an efficient alternative to GC-MS.

Objective: This study was initiated to produce an efficient, robust method of analysis using LC-MS/MS to differentiate between those cases submitted where buprenorphine had been prescribed legitimately, or via diverted samples (containing naloxone) where the medication had not been prescribed. The optimized enzymatic hydrolysis and solid phase extraction (SPE) conditions developed will help forensic analysts working in the area of drugs and driving testing to efficiently analyze samples containing buprenorphine and/or naloxone.

Method: 1 mL samples of urine (calibrators, controls, and test samples each containing deuterated internal standards) were hydrolyzed with β-glucuronidase reagent (1 hour, 65°C, pH 5). After this time, the samples were cooled and adjusted to pH 6 with aqueous 0.1M phosphate buffer. SPE was performed using mixed mode SPE columns (C8/SCX). The SPE columns were conditioned with methanol, deionized (DI) water and 0.1M phosphate buffer (3 mL, 3 mL, 1 mL, respectively). After loading samples at 1 mL/ minute, the SPE cartridges were washed with DI water, 1.0 M acetic acid and methanol (3 mL of each, respectively). The SPE columns were dried and eluted with 3 mL of a solution containing methylene chloride-isopropanol-ammonium hydroxide (78-20-2). The eluates were evaporated to dryness under nitrogen at 35°C. The residues were dissolved in 100 µL of mobile phase for LC-MS/MS. Liquid chromatography was performed in gradient mode employing a 50 mm x 2.1 mm (2.1 µm) aromatic phase LC column using mobile phase consisting of acetonitrile and 0.1% aqueous formic acid at a flow rate of 0.5 mL/ minute. Positive multiple reaction monitoring (MRM) mode was used for tandem mass spectrometry.

Results: The limits of detection and quantification for this method were determined to be 5 ng/ mL and 10 ng/ mL, respectively for the buprenorphine, norbuprenorphine and naloxone. The method was found to be linear from 10 ng/ mL to 1000 ng/ mL ($r^2 > 0.999$). The analyte recoveries were greater than 95% for all of the noted analytes. Interday and intraday variation of the method was found to be < 8% and < 10 %, respectively. Matrix effects were determined to be < 6%. Details regarding the concentrations of buprenorphine, norbuprenorphine and naloxone confirmed in urine from 20 genuine cases are presented.

Conclusion: This method demonstrates the efficient use of both enzymatic hydrolysis and SPE coupled with the use of LC-MS/MS for the analysis of total buprenorphine, norbuprenorphine and naloxone in urine in cases of driving under the influence of drugs. The ability to differentiate the use of buprenorphine from the use of a combination product of buprenorphine and naloxone in case work will greatly assist toxicologists in offering the appropriate interpretation as the presence of naloxone can indicate the use of a combination product such as Suboxone®.

Keywords: Buprenorphine, LC-MS/MS, SPE

P-66

Case Report: Cross-Reactivity of Mesalazine with Salicylate Immunoassay and Trinder's Reagent

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Introduction: Mesalazine (5-aminosalicylic acid, mesalamine) is a salicylic acid derivative that is used to treat inflammatory bowel diseases. It is also an active metabolite of sulphasalazine. Although the action of mesalazine is not well defined, it appears to be topically active on the intestinal mucosa, where it exerts an anti-inflammatory effect. Because mesalazine has predominantly local actions in the gut, systemic side effects are minimal. The most commonly reported side effects include diarrhea, cramping, nausea, headache and flatulence. Mesalazine may be administered orally or rectally. Bioavailability following oral administration ranges from 20-30%; after rectal administration it ranges from10-35%. The absorbed mesalazine is excreted primarily in the urine as N-acetyl-5-aminosalicylic acid. The half-life of mesalazine is 7 hours at steady state. Very few reports of serious toxicity have been reported after mesalazine use or overdose.

Objective: To determine if postmortem specimens from an individual taking mesalazine will produce a positive result for salicylate testing by colorimetry and immunoassay.

Method: A 29-year old female was found unresponsive on a sofa in her residence. Her medical history included ulcerative colitis, Crohn's disease, fibromyalgia, migraines, seizures, asthma and substance abuse. The decedent's prescriptions that were recovered from the residence included omeprazole, ondansetron, morphine, etodolac, oxycodone, metaxalone, sertraline, lamotrigine, atenolol, gabapentin, buspirone, quetiapine and Asacol® (mesalazine). At autopsy, 14 minimally digested delayed release mesalazine tablets were present in her gastric contents. Occasional similar fragments were recovered from the large and small bowels. Specimens were submitted for toxicological analysis and analyzed for volatiles, acidic/neutral drugs, basic drugs and free morphine. Urine screened positive for multiple drugs, including salicylate (by Trinder's). Urine salicylate confirmation by immunoassay (ELISA) was positive. Heart blood toxicology results included cyclobenzaprine (0.09mg/L), sertraline (0.1 mg/L), desmethylsertraline (0.2 mg/L), lamotrigine (1.0 mg/L), oxycodone (0.1 mg/L) and free morphine (510 µg/L). The medical examiner ruled that the cause of death was mixed drug intoxication; the manner of death was undetermined. Initial postmortem screening for salicylate was performed by colorimetry (Trinder's). Immunoassay was used for urine salicylate confirmation. Quantitation of salicylate in blood and urine was completed by Trinder's/spectrophotometric analysis Additional confirmatory testing of blood and urine for salicylate was by LC-MS. Mesalazine analysis was performed by UPLC-MS-MS.

Results: Quantitation of salicylate in blood and urine by Trinder's/spectrophotometric analysis determined apparent salicylate concentrations of 220 mg/L in heart blood and 3700 mg/L in urine. Urine immunoassay confirmation was positive for salicylate. Confirmatory testing of blood and urine for salicylate by LC-MS was negative. Mesalazine was detected in both blood and urine by UPLC-MS-MS.

Conclusion: Because of the similar structures of 5-aminosalicylate, N-acetyl-5-aminosalicylate and salicylate, it is suspected that mesalazine and/or its metabolite cross-reacted with two frequently used methods of analysis for salicylate. If a significant concentration of salicylate is detected by immunoassay or spectrophotometry, it may be necessary to consider the contribution of 5-aminosalicylate drugs in individuals known to take a drug containing or metabolized to this compound or in individuals with a history of inflammatory bowel disease.

Keywords: 5-aminosalicylate, Salicylate, Postmortem, Mesalazine, Mesalamine

P-67
Case Report: An Unusual Case of LSD Poisoning

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Introduction: A family of four became ill after consuming a home cooked meal of steak fajitas. Initial symptoms included tingling in arms and legs, nausea, dizziness, tachycardia, dilated pupils and hallucinations. The family drove to a nearby hospital where they were admitted. Out of an abundance of caution, all four family members were intubated for airway protection and an emergency Caesarean section was performed on the mother who was in her ninth month of pregnancy. All victims (including newborn infant) recovered spontaneously within 24 hours after admission and were released within a few days.

Objective: To identify the chemical ingested by the family that led to the unusual symptoms and the family's subsequent hospitalization.

Method: The victims' hospital admission blood and urine specimens were screened using a routine liquid/liquid alkaline drug screen by Gas Chromatography and Mass Spectrometry (GC/MS). Other than caffeine, no drugs were detected in the blood and urine specimens. Based on case history, several pieces of the steak fajita meat were homogenized in water and extracted using a routine liquid/liquid alkaline procedure followed by analysis by GC/MS. A compound that eluted approximately one minute after cholesterol indicated a potential library match to Lysergic Acid Diethylamide (LSD). Subsequently, a standard of LSD and Lysergic Acid Methylpropylamide (LAMPA) were purchased for a definitive GC/MS identification and confirmation. In addition, the victims' urine specimens were sent to an outside reference laboratory for a targeted analysis of LSD by Immunoassay and confirmation by Liquid Chromatography and Tandem Mass Spectrometry (LC-MS/MS).

Results: LSD was identified on the steak fajita meat by GC/MS comparison to an underivatized standard of LSD as well as a GC/MS comparison to the BSTFA derivatized standard of LSD. Targeted analysis of LSD by LC-MS/MS confirmed the presence of LSD in three of the victims' urine samples (the fourth victim's urine specimen had insufficient volume for LSD analysis).

Conclusion: This case identifies an unusual poisoning by LSD in which the victims required hospitalization. A routine liquid/liquid alkaline extraction analyzed by GC/MS was able to identify and confirm LSD on the steak fajita meat. In addition, a targeted analysis of LSD by LC-MS/MS confirmed the presence of LSD in the victims' urine specimens. The source of the LSD in this case remains under investigation.

Keywords: LSD, Poisoning, GCMS

P-68
Four Odd Cases in the Valley of the Sun

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Introduction: In Phoenix, casework through the toxicology laboratory of the Office of the Medical Examiner contains the typical array of drugs, both illicit and prescribed, as seen across the nation. Methamphetamine, cocaine, oxycodone, heroin, and alprazolam are seen in the majority of casework, while other drugs are seen less frequently. Presented here are four cases in which either a new drug was observed for the first time, or a new drug trend was noticed. *Case #1*: A 20 year-old female was found unresponsive after a night of drinking, inhalants, and presumed "Molly" use. *Case #2*: Two males found unresponsive after completing a drug rehabilitation program. *Case #3*: A 19 year-old male was found unresponsive in his room by his father with drug paraphernalia in clear view. *Case #4*: A 54 year-old female with a past history of alcoholism was found unresponsive in her residence.

Objective: Basic drug screens are performed routinely at Maricopa County for cases in which drugs are suspected or in the absence of trauma. These cases demonstrate how broad screens can yield uncommon drugs and provide direction for additional testing.

Method: Case samples were subjected to the following analyses: volatiles testing by GC/FID, EIA testing by ELISA (Barbiturates, Benzoylecgonine, Benzodiazepines, Opiates, Fentanyl, Amphetamines, and Oxycodone/Oxymorphone), basic drug screen by GC/NPD followed with confirmation by GC/MS, acid/neutral drug screen by LC/MS/MS, and quantitation of confirmed drugs by various techniques (GC/NPD, GC/MS, LC/MS/MS).

Results: *Case #1*: The EIA analysis was positive for amphetamines, yet subsequent confirmation of amphetamines was undetected. The screening of the admission blood by GC/MS revealed methylone. This sample was sent to NMS for quantitation which was 1.4 mg/L. *Case #2*: Both decedents had a variety of illicit and prescription drugs present including cocaine, heroin, and diazepam. However, MDMA and mitragynine (kratom) were found and confirmed in the older male. *Case #3*: An unidentified fentanyl analog was observed in the GC/MS screen. After ruling out acetyl fentanyl, further investigation and cooperation with the DEA was sought. *Case #4*: Volatiles testing resulted in a BAC of 0.05 gm%, while GC/MS screening revealed an unidentified peak that was eventually identified by comparison to a standard of allocryptopine. Further investigation led to the identification of an allocryptopine metabolite, protopine.

Conclusion: All of these case reports reinforce the need for broad screens in post-mortem toxicology for determining cause of death. *Case #1*: The references to "Molly" in the investigative report led to the belief that the decedent had overdosed on MDMA (Ecstasy). However, current drug trends show that "Molly" is often not MDMA as seen in the past, but newer "bath salt" compounds such as methylone. *Case #2*: Although both decedents had illicit drugs in their system which could have easily led to their demise, the presence of the drug mitragynine in the older decedent was surprising. *Case #3*: The mass spectral information gained by our GC/MS screen allowed us to observe ions similar to fentanyl and direct our search for identification. The investigation of this compound is still on going. *Case #4*: Allocryptopine and protopine were previously unseen in our lab, and not much information is available about these compounds. The source has been linked to a product by Herb Pharm, Inc. called California Poppy Extract.

Keywords: Methylone, Fentanyl Aanalog, Allocryptopine, Mitragynine, Postmortem

P-69

Explanations for Puzzling Toxicology Results Detected in Fatal "Heroin" Overdoses

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Introduction: Cuyahoga County, Ohio has experienced a large increase in fatal heroin overdoses since 2007. In an attempt to combat this epidemic, local law enforcement now process heroin overdose scenes as crime scenes. The Cuyahoga County Prosecutor has begun adding "death specification" charges to individuals who have been accused of dealing drugs resulting in a fatal overdose. The toxicology results are an important piece of the puzzle when prosecuting these cases, and toxicologists may be called to court to clarify these results. Often the morphine levels detected in heroin cases are large enough to explain cause of death. The more problematic cases occur when the toxicology laboratory detects therapeutic levels of morphine in suspected heroin overdose deaths, or even more puzzling, when no morphine is detected at all.

Objective: This presentation examines possible explanations for unexpected toxicology results in heroin overdoses. The authors stress the importance of looking at a case in its entirety, as well as the value of open communication between all departments/agencies involved in death investigations.

Method: Recent heroin overdose deaths were reviewed for instances where case history seemed inconsistent with toxicology results. A handful of these cases were selected to use as examples for this presentation. Cases which were difficult to interpret were investigated more thoroughly by examining investigator case histories, autopsy reports, and police and medical records. These cases underwent thorough toxicological examination by the CCMEO toxicology department. Cases were organized into three groups for this study; **Group 1:** “Lingering” Heroin Deaths, **Group 2:** Acute Heroin Deaths Receiving Medical Intervention, **Group 3:** Non-Heroin Deaths.

Results: Morphine levels lower than 100 ng/mL were detected in the postmortem femoral and/or ante-mortem admission blood from decedents in **Groups 1 and 2**. 6-Acetylmorphine was not detected in these blood samples. Greater than 15 ng/mL fentanyl was detected in two of the decedents from **Group 3**, no other opioids were detected. Toxicology results from the additional two cases in **Group 3** remain inconclusive.

Conclusion: The cases in **Group 1** appear to be "lingering" opiate deaths. A “lingering” death occurs when the decedent remains alive, although unconscious, sufficiently long to metabolize a large portion of the heroin dose. Other signs of "lingering" deaths are heavy lungs due to pulmonary edema and occasionally acute bronchopneumonia. The low level of morphine present in hospital admission blood from the cases in **Group 2** may be a result of dilution of drug due to resuscitative efforts administered prior to drawing "admission" blood. The companions of decedents in **Group 3** incorrectly believed the drug they were using was heroin. Fentanyl was the substance administered by two of the decedents, the other two individuals injected unidentified substances which are still being evaluated. The cases presented here signify the importance of good communication between agencies in order to provide all the details needed to explain unexpected toxicology results. While the toxicology results are correct, the challenge is identifying the explanation.

Keywords: Heroin, Unexpected Toxicology Results, Opioids, Fentanyl

P-70

Methylone Induced Death: Distribution of Methylone in Postmortem Fluids and Tissues

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Introduction: Methylone (methylenedioxy-ß-keto amphetamine) is a psychoactive, synthetic analog of cathinone and is commonly known as a "bath salt". This drug is popular among all age groups and can be used by snorting, injecting, or adding it to food/drink. It exerts its pharmacologic effect by inhibiting the reuptake of dopamine and norepinephrine. Toxic effects observed include tachycardia, hypertension (vasoconstriction), insomnia, hyperthermia, mydriasis, panic attack, seizures, and aggressive behavior.

Objective: To report a case involving a high concentration of methylone and its distribution in various matrices.

Method: Case History: a 58-year-old white male was last known alive approximately 12 hrs. prior to his body being found, prone and nude on the floor of the bedroom in his locked residence. Concerns of foul play were raised because there was a large quantity of blood on his body and throughout the residence. No alcohol or illegal drugs were found at the scene. Last contact with the decedent was at 1430 hrs. when he stated he was not well and would not be attending a holiday party. Neighbors heard multiple loud bangs around 0330 hrs. the next morning. The external examination of the decedent revealed minor blunt force injuries to the head, neck, trunk and extremities. The blood at the scene was from a laceration of the right supraorbital ridge; he bled heavily because he was on Plavix® for a previous stroke. The autopsy findings were remarkable for hypertensive and atherosclerotic cardiovascular disease with cardiomegaly (heart - 475 grams) and atherosclerotic coronary artery disease. The left anterior descending coronary artery had 75% stenosis. Multiple specimens were collected and analyzed by the toxicology lab. Methylone was extracted using UCT Clean Screen ZSDAU020 extraction columns with a previously published and validated basic drug UCT extraction procedure. A whole blood matrix was used in the preparation of calibrators and controls for blood, vitreous humor and bile. A urine matrix was similarly used for the urine sample and the methylone was qualitatively identified in the liver. Analytes were separated and detected by an Agilent GC/EI- MS using a Zebron ZB-50 capillary column.

Results: Concentrations of methylone in the various matrices tested are shown in the table below. Samples were analyzed in duplicate. Butylone was also present in urine only. No other analytes were present in the blood. Initially, the specimens were sent to a reference laboratory and the result of the methylone analysis was used as a control for validation of the methylone calibration curve. The inter-laboratory results for the femoral and cardiac blood samples were within 8.4 and 4.3%, respectively.

Specimen	Femoral Blood	Heart Blood	Urine	Gastric per 150 mls	Bile	Vitreous	Liver (mg/kg)
Conc. (mg/L) unless otherwise noted	11.9	15.3	1600	65 mg	318	21.9	Present

Conclusion: Methylone was present in high levels and was distributed among multiple matrices with values ranging from 11.9 mg/L to 1600 mg/L. The heart to peripheral blood ratio was 1.42. This case was unique in that only methylone was present with no other drugs being found in the blood and these are the highest reported values to date in the specimens tested. Cawrse, Pearson, Kovacs (2012) and Carbone *et al*. (2013) reported lethal blood methylone levels from 0.272 – 3.3 mg/L and Pearson and Cawrse reported liver concentrations of 0.88 and 1.8 mg/kg, respectively. Cawrse reported liver-to-blood ratios from 1.19 – 4.66, average 2.68. Elliott *et al*. (2010) reported a postmortem blood methylone level of 11 mg/L and butylone of 1.7 mg/L. The cause of death in the presented case was ruled as acute methylone intoxication with the manner of death ruled as accidental.

Keywords: Bath Salts, Methylone Distribution, Postmortem

P-71

Repeated Administration of the Synthetic Cannabinoid CP47,497 Produces Behavioral Tolerance and Withdrawal Effects in Mice

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Introduction: Chronic use of synthetic cannabinoid (SCB) receptor agonists may lead to the development of tolerance and/or withdrawal symptoms upon cessation; however, limited data is available regarding the consequences of repeated SCB exposure. Due to severe adverse reactions reported in chronic users, the consequences of prolonged synthetic cannabinoid use need to be systematically investigated. To address this, CP47,497 (Schedule I) was evaluated in these studies as it is biologically active and has been a confirmed constituent in herbal incense products (HIPs).

Objective: To investigate whether repeated CP47,497 administration will elicit tolerance to characteristic cannabinoid behavioral effects in mice and determine if physical dependence, assessed by somatic withdrawal signs upon CB_1 receptor antagonist treatment, are produced in mice treated repeatedly with CP47,497 or THC.

Method: For tolerance or cross-tolerance and dependence experiments, ICR mice received twice daily (5.5 days) subcutaneous injections of CP47,497 (15 mg/kg), THC (50 mg/kg), or vehicle (ethanol:emulphor:saline in a ratio of 1:1:18). To examine tolerance development, mice were tested for catalepsy, antinociception, and hypothermia in a cumulative CP47,497 (1, 3, 10, 30 and 56 mg/kg) dose-response study twenty-four hours after the last drug injection (day 6). For dependence experiments, mice received rimonabant (10 mg/kg, i.p.) 30 minutes after the last drug dose (day 6), and somatic withdrawal signs (paw flutters/tremors/clapping, headshakes) were recorded for 1-hour. All experiments were approved by the Institutional Animal Care and Use Committee at Virginia Commonwealth University in accordance with the National Institutes of Health's Guide for the Care and Use of Laboratory Animals.

Results: Two-way analysis of variance (ANOVA) revealed CP47,497- experienced mice displayed significant less catalepsy ($p \leq 0.001$) when challenged with CP47,497 (10, 30, and 56 mg/kg) and THC-treated mice exhibited cross tolerance to CP47,497 at 10 mg/kg, 30 mg/kg, and 56 mg/kg versus animals repeatedly given vehicle injections. Significant antinociceptive tolerance ($p \leq 0.001$) was detected for CP47,497 and THC at cumulative doses of 30 and 56 mg/kg versus vehicle. Significant tolerance developed to hypothermia ($p \leq 0.05$) in CP47,497-experienced mice at 3, 10, 30, and 56 mg/kg with significant cross-tolerance observed for THC at 3 mg/kg, 10, 30, and 56 mg/kg compared to repeated vehicle controls. Following a one-way ANOVA, significant increases in head shakes were observed in CP47,497-treated and THC-treated mice ($p \leq 0.05$) versus vehicle-treated mice. Rimonabant-precipitated paw flutter withdrawal signs were significant in CP47,497- and THC-treated groups as compared to vehicle-treated groups. Overall, tolerance to CP47,497- induced catalepsy, antinociception, and hypothermia and physical dependence was produced, but statistical analyses revealed no significant differences in the magnitude of tolerance development or presentation of somatic withdrawal signs between mice repeatedly treated with CP47,497 or THC when compared to vehicle-treated mice.

Conclusion: Repeated SCB administration produces tolerance to the cataleptic, antinociceptive, and hypothermic effects in mice, with significant presentation of somatic withdrawal signs (paw flutter and head shakes) upon drug cessation. These findings are consistent with the incidence of adverse clinical reports. Similar to THC, long-term SCB users are likely to escalate their dosage to achieve desired effects and present with withdrawal symptoms upon drug abstinence.

Keywords: Synthetic Cannabinoid CP47,497**,** CB_1 Receptor, Mice, Behavioral Tolerance, Withdrawal

P-72

Applications of a New Headspace GC-MS Validated Method for Volatile Organic Compounds in Different Forensic Matrices

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Introduction: Volatile organic compounds (VOCs) are of great interest in forensic toxicology as they come from a variety of products commonly used in daily routines, both domestic and workplace. Because these products are extensively used, available and are an inexpensive/legal alternative to traditional street drugs, VOCs have significant potential for abuse with adverse effects on the central nervous system (CNS). Furthermore, a multitude of VOCs can also evolve in the early stages of human decomposition as well as in fire related deaths. In our laboratory, a headspace GC-FID method is used routinely for ethanol, isopropanol, acetone, methanol and n-propanol quantitation. A complementary method with an extensive list of VOCs is of great importance to expand our testing panel in impaired driving, sexual assault and death investigation cases.

Objective: A sensitive and specific qualitative method is required when an unidentified suspected VOC is detected using the routine headspace GC-FID method or when VOC use/presence is suspected in casework. This method has simplified sample preparation to minimize the loss of VOCs.

Method: This 30 minute method allows for the qualitative analysis of 65 VOCs, with tert-butanol as the internal standard, using an Agilent 6890/5973 GC-MS system with a DB-1MS column. Forensic biological (blood, urine, gastric content and brain) and non-biological matrices can be analyzed. A 1 mL or 1 g sample with 0.15 g of sodium chloride in a 5 mL headspace vial is prepared. The headspace is manually sampled and analyzed after a 20 minute incubation on a dry block at 60°C. An extensive list of VOCs contained in different products is covered by this method, such as aerosol propellants, adhesives, paints, removers, cleaning products, anesthetics, gases, (Freon®), nitrites (poppers), industrial solvents and fuel.

Results: This method was successfully validated according to SWG-TOX recommendations which include specificity, sensitivity, carryover, matrix effects and identification of gaseous VOCs. Examples of forensic case work includes the presence of isoflurane above the limit of detection of 25 ng/mL and identification of 1,1-difluoroethane in impaired driving cases. Chloroform, toluene, heptane and diethyl ether, have also been found in suspicious death and homicide cases above the limit of detection of 25, 50, 75 and 250 ng/mL, respectively. Validation results will be presented for all of the VOCs tested. In addition, different VOCs produced during the early stages of human decomposition are also identified as well as typical hydrocarbon profiles in fire related deaths.

Conclusion: This easy, fast and comprehensive qualitative validated method enhances our toxicological expertise in terms of volatiles analysis and expands our contribution to the police, coroners and pathologists' investigations. This extended volatiles qualitative method should be used in any forensic case where there is a suspicion of VOC use or preliminary detection in the routine headspace GC-FID method.

Keywords: Volatile Organic Compounds (VOCs), Biological Matrices, Headspace-GC-MS

P-73

Prevalence of Clinically Aberrant Urine Drug Test Results in an Obstetric and Gynecologic Patient Population

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Introduction: Reported rates of neonatal abstinence syndrome are increasing; however, obstetric and gynecologic patients are rarely subjected to urine drug testing despite the risks of drug use in this population. High urine drug test positivity rates observed in obstetric and gynecologic patients validate the utility of drug testing in effort to improve prenatal care and neonatal outcomes.

Objective: Determine the prevalence of clinically aberrant results in the obstetric and gynecologic patient population.

Method: A retrospective IRB approved review was conducted of urine specimens submitted from 41 obstetrics and gynecology clinics in 21 states across the United States including; AL, AR, AZ, CO, FL, IL, KS, LA, MD, MI, MO, NJ, NY, OH, OK, PA, SC, TN, TX, UT and VA from 01/03/2013 to 12/31/2013. Tests included and reporting thresholds in ng/mL consisted of the following: amphetamines (100-250), barbiturates (200), benzodiazepines (50), carisoprodol/meprobamate (200), cocaine (50), cotinine (500), ethyl glucuronide (500), fentanyl (5), heroin (10), marijuana (5), meperidine (100), methadone (200), opiates (100), synthetic cannabinoids (1), synthetic cathinones (25), and tramadol (100). All samples were tested directly by confirmatory mass spectrometry, except for amphetamines and synthetic cannabinoids which were screened by LDTD/MS/MS at thresholds of 250 ng/mL and 2 ng/mL, respectively. Data was sorted using JMP®, a statistical software program. Aberrant results were defined as being positive for an illicit substance, a prescription drug not indicated as prescribed, cotinine, ethyl glucuronide, and/or an abnormally high concentration of a prescription drug (i.e., a concentration more than two standard deviations from the mean drug concentration observed in a pain management population for the combination of parent drug and metabolites). Urine concentrations were normalized by specific gravity. An abnormally high urine concentration is defined as one that falls in the top 2.5% of the concentrations observed.

Results: A total of 2,528 specimens were tested. Of these, 1,033 demonstrated clinical aberrancies (41%). A total of 511 (20.2%) of specimens were positive for at least one of the following illicit drugs: marijuana (n=462), cocaine (n=35), synthetic cannabinoids (n=17), synthetic cathinones (n=2), methamphetamine (n=18), and heroin (n=3). A total of 602 (23.8%) specimens were positive for cotinine, 97 (3.8%) were positive for ethyl glucuronide, 274 (10.8%) were positive for a prescription drug that was not indicated as prescribed by the requesting provider, and 29 (1.1%) specimens were positive for an abnormally high concentration of a prescription drug. The most commonly identified non-prescribed medications are listed below:

Table 1: Prevalence of non-prescribed licit drugs in clinically aberrant specimens (n=1,033)

Drug	N (%)	Drug	N (%)
Hydrocodone	69 (6.7%)	Clonazepam	28 (2.7%)
Tramadol	53 (5.1%)	Butalbital	18 (1.7%)
Benzodiazepines (nordiazepam, oxazepam, temazepam)	51 (4.9%)	Carisoprodol/Meprobamate	16 (1.5%)
Oxycodone	30 (2.9%)	Morphine	16 (1.5%)
Alprazolam	29 (2.8%)	Phentermine	14 (1.4%)

Conclusion: Urine drug testing provides a relatively simple, non-invasive objective measure and is a valuable addition to prenatal care. A high prevalence of clinically aberrant results necessitates further follow-up in this patient population. Identifying substance use and misuse early during pregnancy may allow providers to counsel patients and improve neonatal outcomes. When implemented, drug testing should include a comprehensive profile to identify the substances used in this population.

Keywords: Substance Abuse Detection, Obstetrics, Urine Drug Testing

P-74

Method Development Study for Ethyl Glucuronide and Ethyl Sulfate in Postmortem Urine Samples

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Introduction: Ethanol is most commonly abused substance presented in most forensic cases. It can be found in postmortem blood samples due to alcohol consumption before death or as a result of postmortem decomposition. In many judicial cases, inebriation degree is important for the clarification of the circumstances of the case. Detection of ethanol in the body is possible only for a relatively short time. The analysis of stable ethanol direct metabolites, such as ethyl glucuronide (EtG) and ethyl sulfate (EtS) gives many advantages with respect to determining the past history of ethanol use. EtG is a non-oxidative metabolite of ethanol, arising from its conjugation with glucuronic acid. This non-volatile, stable, polar and hydrophilic conjugate represents about 0.5% of the total ethanol elimination and is detectable for up to 18 h in blood and up to 80 h in urine. Ethyl sulfate is the other stable metabolic product of ethanol undergoing sulfate conjugation through sulfotransferase activity. It is also a specific metabolite of alcohol consumption because it cannot be produced by endogenous alcohol.

Objective: Confirmation of alcohol intake in forensic cases has great importance, therefore EtG and EtS determination is very useful. They have been analyzed routinely for alcoholic patients and for certain occupational groups in some pain management laboratories recently, however for postmortem cases this application is not used widely in forensic laboratories. The aim of this study is to construct and validate a method for determining EtS and EtG in urine samples by liquid chromatography tandem mass spectrometry (LC/MS/MS) to confirm alcohol exposure of cases in forensic toxicology laboratories.

Method: The urine samples were prepared by protein precipitation, in which 100 uL of urine sample was mixed with 200 uL of acidified methanol (containing 200 ng/mL of EtG-D5 and EtS-D5 as internal standard in 0.5% formic acid in H_2O). The prepared samples were blown dry under nitrogen and reconstituted with 200 uL of mobile phase. Mobile phase A is 0.1% formic acid in water and mobile phase B is 0.1% formic acid in acetonitrile. The HPLC gradients are: 0-3.5 min mobile phase B increased from 0 to 15% at flow rate of 0.5 mL/min. An Agilent 6460 QQQ with JetStream technology LC/MS/MS System with Agilent Polaris C18-Ether, 3x150mm, 3µm Analytical Column and Agilent Polaris C18-Ether MetaGuard 2 mm, 3µm, Guard Column were used for instrumentation set up. The MRM transition are: EtG: m/z (221->74.9, 221->85); EtG-D5: m/z (226->74.9, 226->85) EtS: m/z (125->96.9, 125->80); EtS-D5: m/z (130->96.9, 130->79.9) in negative mode.

Results: The Calibration curve was established with 6 points (25-2000 ng/mL) with a linearity of $R2 > 0.99$. EtG was eluted at retention time of 2.86 min, followed by EtS of 2.80 min. The precision and accuracy were tested by carrying out 3-sets of low (100 ng/mL) and high (500 ng/mL) controls in 2 inter-day runs, respectively to yield CV < 10% for both EtG and EtS in the two control concentrations .

Conclusion: This method was utilized in urine samples in a forensic toxicological laboratory in order to confirm the history of ethyl alcohol use. For further study it will be useful to determine the correlation in different biological matrices with blood and vitreous humor.

Keywords: Ethyl Glucuronide, Ethyl Sulfate, Postmortem Urine, LC/MS/MS

P-75

Endogenous Concentrations of Gamma-Hydroxybutyrate (GHB) in Post-Mortem Blood from Deaths Unrelated to GHB Use

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Introduction: Gamma-hydroxybutyrate (GHB) is an endogenous compound but its presence in post-mortem blood presents a challenge when interpreting levels as it is misused recreationally, but is also produced post-mortem. The use of preservatives has been suggested to minimize this production and Kintz and Villain have also suggested the use of a cut-off of 50 mg/L for post-mortem blood samples to differentiate between post-mortem production and exogenous GHB use. This is of particular importance when other matrices are unavailable or decomposition has advanced. This study presents the largest data set of post-mortem cases where exogenous GHB use was excluded, decomposition changes were noted and samples were submitted for analysis in both preserved and unpreserved vials.

Objective: The aim of this retrospective study was to evaluate the concentrations of GHB found in post-mortem cases where alcoholic and/or diabetic ketoacidosis was suspected, with special focus on the relationship between GHB concentration and the advancement of decomposition.

Method: GHB was analysed using deuterated GHB as the internal standard (GHB-d6) within a calibration range of 5 - 500 mg/L. The analytical method was adapted from a method developed by Hassan and Cooper. Only cases submitted for analysis between 2010 and 2012 for investigations into the potential role of ketoacidosis were selected. Additional details, including age, sex, cause and manner of death, date of death, the last time the deceased was seen alive and the date of the autopsy were collated.

Results: A total of 387 post-mortem cases (273 male, 114 female) were submitted to the laboratory between 2010 and 2012 specifically requesting tests for suspected ketoacidosis. GHB was not detected at or below 10 mg/L in 18% of the cases (N=68), between 10 and 50 mg/L in 73% of the cases (N=283) and between 51 and 193 mg/L in 9% of the cases (N=36). The manner of death was classified as accidental (N=11), alcohol-related (N=237), drug-related (N=23), homicide (N=1), natural (N=91), suicide (N=9), medical related (N=1) and undetermined (N=14). Six cases had GHB concentrations in excess of 100 mg/L with advanced decomposition changes noted in five of these cases. Moderate to advanced decomposition was also noted in 50% (N=15) of the cases with GHB concentrations in excess of 50 mg/L but less than 100 mg/L.
Approximately one third of the blood samples tested contained a preservative and although a higher proportion of these samples had GHB concentrations < 10 mg/L or not detected (~30% preserved v 11% unpreserved), there were still cases with GHB concentrations > 51 mg/L (~6% preserved v 11% unpreserved).

Conclusion: The findings in this study support other published investigations highlighting the difficulties and dangers of only using a cut-off to establish endogenous levels compared with exogenous use of GHB in post-mortem blood, especially when decomposition has reached advanced stages. The use of a preservative may be advantageous but more research must be conducted.

Keywords: GHB, Postmortem, Decompostion, Blood

P-76

Development of a Homogeneous Enzyme Immunoassay for the Detection of Hydrocodone and Hydromorphone in Human Urine

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Introduction: Hydrocodone is a semi-synthetic opioid derived from codeine and thebaine. It is used to relieve moderate to severe pain and to treat cough. Hydrocodone is prescribed predominantly in the United States. Commercial hydrocodone preparations are always combined with another medication to increase efficacy and reduce adverse effects. It is available as a mixture with acetaminophen (Vicodin), aspirin (Lortab), ibuprofen (Vicoprofen) and antihistamines (Hyconine). Currently, four pharmaceutical companies are developing long acting formulations of hydrocodone. Of these, Zohydro ER has been submitted to FDA for new drug consideration. An increase in the abuse of the prescription pain management drugs hydrocodone and hydromorphone has been observed in recent years. The International Narcotics Control Board reported that 99% of the worldwide supply in 2007 was consumed in the United States. Analgesic action of hydrocodone begins 20–30 minutes after oral consumption and lasts 4–8 hours. Hydrocodone is rapidly metabolized to hydromorphone and its glucuronide and can be found in human urine for up to 2-3 days. Currently, no specific immunoassay methods are commercially available for the detection of hydrocodone/hydromorphone.

Objective: The objective of this study is to develop a homogeneous enzyme immunoassay for the specific detection of hydrocodone and hydromorphone in human urine.

Method: 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 Microgenics DRI® Hydrocodone/Hydromorphone Assay has a 300 ng/mL cutoff. The reagents and calibrators are in liquid form and ready-to-use. The assay range is 0 ng/mL to 1000 ng/mL. The assay uses a highly specific monoclonal antibody that can detect hydrocodone, hydromorphone and hydromorphone β-D-glucuronide.

Results: The within-run and total precision (CV) for the cutoff calibrator and ± 25% controls was ≤ 2.0% (qualitative) and ≤ 10.0% (semi-quantitative). The performance of the assay was evaluated on the Beckman Coulter AU680 analyzer. The assay demonstrated 100% cross-reactivity to hydromorphone and its glucuronide with minimal cross-reactivity to opiate compounds and other concomitantly taken drugs. No significant interference was observed from endogenous substances. Two hundred and thirty three patient samples were tested by immunoassay and the results showed >90% agreement with LC-MS/MS.

Conclusion: The Microgenics DRI® Hydrocodone/Hydromorphone Assay demonstrated excellent specificity and sensitivity to hydrocodone, its major metabolites hydromorphone, and hydromorphone β-D-glucuronide. The assay can be applied to various clinical chemistry analyzers.

Keywords: Hydrocodone, Hydromorphone, Immunoassay

P-77
Barbiturates in 2013: A Pain Medication Monitoring Perspective

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Introduction: Barbiturates are central nervous system depressants that once dominated the sedative-hypnotic market in the early 1900s. In the mid-1900s, barbiturates were commonly abused for their sedative properties and widespread accessibility. Barbiturates were eventually supplanted by benzodiazepines because of barbiturate's low therapeutic-to-toxic index and high risks for lethal overdose and physical dependence. Following federal law changes in 1970, the use, availability, and abuse of barbiturates steeply declined. They are still prescribed for insomnia relief and the treatment of epilepsy and withdrawal symptoms. Because of the associated risks and abuse potential, physicians often monitor patients prescribed barbiturates and other pain medications to assist with prescription adherence and possible co-ingestion of other drugs.

Objective: In a random cross-sectional study, the prevalence of barbiturates in a pain medication population during the 2013 calendar year was assessed.

Method: Urine specimens (n= 798,979) were collected from chronic pain patients ranging in age from 13 to 98 years with a median age of 50 and were screened for barbiturates including; butalbital, pentobarbital, phenobarbital and secobarbital by enzyme immunoassay (EIA) as part of a medication monitoring program. Non-negative screens were confirmed for the four target barbiturates by gas chromatography-mass spectrometry (GC/MS). Reporting cutoffs were 200 ng/mL for screening and 50 ng/mL for confirmation of all target compounds. Results were compiled and interrogated for trends, n= 783,149.

Results: Only 2.3% of all samples, 18,362, gave a presumptive positive result for barbiturates after EIA screening. Lower EIA cross-reactivities of butalbital (66.7%), pentobarbital (40.0%) and phenobarbital (33.3%) could have produced false negative screen results, however the distribution of confirmed positives implies low bearing. Of the set of presumptive positives, 98.8% were confirmed positive for one or more of the target compounds. The false positive screens may have presented from EIA cross-reacting interferents or a non-targeted barbiturate in the confirmation method. Of the confirmed positives, only 26.4% had an associated barbiturate prescription listed. While inconsistent and unexpected positive results may be due to clerical omission at sample collection, gross underreporting of barbiturates by patients is indicated. The GC/MS positive results were distributed among butalbital (77.87%), pentobarbital (0.2%), phenobarbital (22.05%) and secobarbital (0.06%). Butalbital being the most frequently detected barbiturate is not atypical, given one formulation contains codeine, which is a common pain medication. Of the butalbital positives determined, 1.2% also contained codeine, but no morphine; 11.3% also contained morphine, but no codeine; and 10.4% contained both codeine and morphine. Concentrations in positive specimens ranged from 50-97 ng/mL and 52-557 ng/mL for pentobarbital and secobarbital, respectively. The relatively low urinary concentrations and infrequency of pentobarbital and secobarbital is not surprising, given that their primary indications are as pre-anesthetics. Butalbital concentrations ranged 51-170,725 ng/mL with median of 825 ng/mL, while phenobarbital concentrations ranged 51-148,171 ng/mL with median of 1121 ng/mL.

Conclusion: As expected, barbiturates are not very commonly detected in urine from a pain management population. However, there is evidence of underreporting of barbiturate consumption. Butalbital was the most common barbiturate identified by urine drug testing for this patient dataset.

Keywords: Barbiturates, Urine, Prevalence

P-78
Opiate Hydrolysis by a Novel Recombinant Beta-Glucuronidase for Urinalysis

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Introduction: Opiates are widely prescribed drugs that are readily abused and as such are very frequently targeted in therapeutic drug monitoring. For many of these drugs, only trace amounts of parent drug are present in urine due to extensive metabolism and conjugation and their glucuronides are major urinary species. Hydrolysis to cleave the glucuronides prior to analysis is necessary for improved detection. Acid hydrolysis is a fast and effective option, but converts 6-AM to morphine. Alternatively, enzyme hydrolysis can be costly and time-consuming, with reported incubation times ranging from 0.5 to 16 hours.

Objective: The assessment and application of a novel recombinant beta-glucuronidase for hydrolysis of opiate glucuronides for urinalysis is presented.

Method: IMCSzyme™ recombinant beta-glucuronidase was buffered to the recommended optimum pH 6.8 and evaluated. Aliquots of drug-free urine fortified separately with glucuronides of morphine, codeine, oxymorphone and hydromorphone were treated 1:3 with a mastermix of enzyme, buffer and internal standard and hydrolyzed in triplicate. The hydrolysis efficiency was assessed at 55°C and 65°C, at incubation times from 0 to 60 mins. The ideal enzyme amount (275 µL of mastermix containing enzyme stock at 21.75%) was also determined. The optimized enzyme hydrolysis was applied to 20 randomly selected positive authentic urine samples for each analyte and compared to results from acid hydrolysis under validated optimized conditions. Hydrolyzed urine samples were analyzed without any further sample preparation on a TLX-4 Multiplexed HPLC with Agilent 1200 Series Binary Pumps coupled to a Thermo Scientific™ TSQ Quantum Ultra™ Triple-Stage Quadrupole Mass Spectrometer using a previously validated "dilute and shoot" method.

Results: The optimal amount of enzyme required for complete hydrolysis was determined to be 3000 Fishman units. As expected, the enzyme performed better for some compounds than others within the same drug class. Complete hydrolysis was only observed for some opiate glucuronides at 60 mins, even at maximum enzyme concentration and incubation temperature. Codeine glucuronide and hydromorphone glucuronide required heat and longer incubation times to achieve complete hydrolysis, which is consistent with observations for other enzyme sources. For morphine-3-glucuronide, complete hydrolysis (100%) was observed at room temperature in the transfer time between aliquoting and injection on instrument, with no additional incubation time, which was faster than has been reported. Hydrolysis efficiency was best at the maximum incubation time assessed (60 mins) for oxymorphone glucuronide (101%), morphine-6-glucuronide (92%), hydromorphone glucuronide (94%) and codeine glucuronide (82%). The efficiency did not increase for codeine glucuronide at the higher temperature tested. Since these opiates are analyzed in a single "dilute and shoot" method simultaneously, overall this was not an improvement to the more convenient acid hydrolysis. Also, total analyte in opiate patient samples subjected to recombinant beta-glucuronidase hydrolysis only compared fairly to targets from the acid hydrolysis method overall. However, there was no evidence of 6-AM conversion to morphine under the enzyme hydrolysis.

Conclusion: The IMCSzyme™ recombinant beta-glucuronidase was demonstrated to be less effective as acid for opiate hydrolysis, but still performed satisfactorily under the conditions investigated. Unlike conventional hydrolyses, this recombinant enzyme did not convert 6-AM to morphine.

Keywords: Enzyme, Hydrolysis, Opiates, Urine, 6-AM, Morphine, Glucuronides

P-79

Buprenorphine Hydrolysis Using a Novel Recombinant Beta-Glucuronidase for Urine Drug Testing

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Introduction: Buprenorphine is a synthetic opioid that is commonly prescribed for pain and addiction therapy with a high potential for abuse. As such, it is very frequently targeted in therapeutic drug monitoring. Only trace amounts of parent drug are present in urine due to extensive metabolism and conjugation. Thus, buprenorphine glucuronide and norbuprenorphine glucuronide are the major urinary species. Hydrolysis to cleave the glucuronides prior to analysis is necessary for improved detection when the intact glucuronides are not targeted in the analytical method. Chemical hydrolysis is ineffective on buprenorphine and norbuprenorphine. Alternatively, enzyme hydrolysis can be costly and time-consuming, with reported incubation times ranging from 1 to 16 hours.

Objective: The assessment and application of a novel recombinant beta-glucuronidase for hydrolysis of buprenorphine glucuronide and norbuprenorphine glucuronide for urinalysis is presented.

Method: IMCSzyme™ recombinant beta-glucuronidase was buffered to the recommended optimum pH 6.8 and evaluated. Aliquots of drug-free urine fortified separately with glucuronides of buprenorphine and norbuprenorphine were treated 1:4 with a mastermix of buffer, enzyme and internal standard and hydrolyzed in triplicate. The hydrolysis efficiency was assessed at 55°C and 65°C at incubation times from 0 to 60 mins. The ideal enzyme amount (400 µL of mastermix containing enzyme stock at 5%) was also determined. The optimized enzyme hydrolysis was applied to 20 randomly selected positive authentic urine samples for buprenorphine and/or norbuprenorphine and compared to results from a non-hydrolysis method monitoring the intact glucuronides. After incubation, hydrolyzed urine samples were analyzed without any further sample preparation on a Waters Acuity UPLC® TQD using a previously validated "dilute and shoot" method.

Results: The optimal amount of enzyme required for complete hydrolysis was determined to be 1000 Fishman units (lowest amount tested). Norbuprenorphine glucuronide required heat and longer incubation times to achieve complete hydrolysis, which is consistent with observations for buprenorphine hydrolysis by other enzyme sources. Buprenorphine achieved complete hydrolysis (100%) at room temperature in the transfer time between aliquoting and injection on instrument, with no additional incubation time. Complete hydrolysis (100%) was observed for norbuprenorphine glucuronide at 65°C with 30 min incubation, which is still faster than what has been reported for this compound by other hydrolysis methods. Total analyte in buprenorphine patient samples subjected to recombinant beta-glucuronidase hydrolysis compared well to targets from a method monitoring the intact glucuronides.

Conclusion: The superiority of the IMCSzyme™ recombinant beta-glucuronidase was demonstrated with fast buprenorphine hydrolysis leading to decreased processing time compared to other buprenorphine hydrolysis reagents.

Keywords: Enzyme, Hydrolysis, Buprenorphine, Norbuprenorphine, Urine

P-80

Monitoring of Baclofen and Metabolites by Liquid Chromatography Coupled to High Resolution Accurate Mass Spectrometry

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Introduction: Baclofen (Lioresal ®) is a centrally acting muscle relaxant and has been prescribed in France for more than 40 years for the treatment of cerebral spasticity. A number of studies also confirm the efficacy of baclofen for the treatment of alcohol addiction. Currently, psychiatrists at the Talwar (GH Cochin) centre treat alcohol-dependent patients using high doses of baclofen which can vary from 50 to 450 mg/day (average dose of prescription 170 mg/day). It is therefore of interest to explain this variability in dose observed in patients by monitoring baclofen and its major metabolites (de-aminated and glucuronidated).

Objective: We describe a method to determine plasma and urinary concentrations of baclofen and a semi-quantitative evaluation of the metabolic ratio of such metabolites by liquid chromatography coupled to high resolution mass spectrometry (LC-HR/MS).

Method: Plasma and urine samples from six patients treated with Baclofen were prepared using the following protocol. To 100 µL of plasma (or 100 µL of urine diluted 1/100) 10 µl baclofen-d4 (1 µg/mL) was added and then precipitated with acetonitrile. The organic phase was evaporated and re-suspended with 100 µL 80/20, water/acetonitrile. Chromatographic separation was achieved using an Accucore PFP column (100 x 2.1 mm , 2.6 µm, Thermo Scientific™) using gradient elution, the mobile phase consisting of water and acetonitirile (both containing 0.1 % formic acid). Mass spectrometric analysis was performed on an Exactive Plus (Thermo Scientific™) using electrospray ionization. Data was acquired in full MS, scanning positive and negative up to 4.9 minutes at a resolution of 17500 (mass m/z 200) and negative mode only at a resolution of 700000 (mass 200 m/z) from 5 to 8 minutes. The exact mass obtained for baclofen and its metabolites are: 214.0629 for baclofen $[M+H]^+$, 213.0324 for the hydroxylated metabolite $[M-H]^-$, 388.0805 for baclofen- glucuronide $[M-H]^-$, and 218.0880 for baclofen-d4 $[M+H]^+$.

Results: The metabolites were commercially unavailable therefore identified and confirmed using exact mass of the parent and fragment ions, within a 5 ppm search window. The method was successfully validated for the quantitation of baclofen in urine and plasma with linearity between 10 and 2000 ng/mL (LLOQ = 3 ng/mL , CV = 5.7%). Precision and accuracy were evaluated using QC samples (n=3) and was measured at < 10 % . The total extraction recovery was > 90 %. Matrix affects were evaluated in plasma spiked at the following QC levels: 10 ng/ml and 1500 ng/ml (n=6). The results were between 7.4 and 16.1 % and 0.3 to 9.3 % , respectively. For patients receiving doses around 100 mg for at least 1 month, concentrations of baclofen in plasma varied from 24 to 1039 ng/mL. De-aminated and hydroxylated metabolites were detected and the percentage ratio of these baclofen-related compounds detected in urine and plasma was measured at 35.10% and 8.55 % respectively. Baclofen-glucuronide was detected only in patients with elevated plasma concentrations (with a ratio in the urine of 0.13%).

Conclusion: A validated LC-HR/MS method was developed not only to quantify baclofen but also simultaneously monitor its phase I and II metabolites in urine and plasma for purposes of determining variability in dose observed in patients receiving treatment in alcohol addiction. High resolution and accurate mass capabilities allow simultaneous identification and quantitation of baclofen and its metabolites within a single run.

Keywords: Baclofen, High Resolution Accurate Mass Spectrometry, Metabolites, Quantitation

P-81

Urine Drug Screening Positivity Rates: Demographic and Seasonal Trends Within a Broad Patient Cohort

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Introduction: Urine drug screening is often performed to detect the use of prescribed, undisclosed or illicit substances by the specimen donor. Comprehensive and specific information regarding drug use within community-based clinical settings is often region-specific and not broadly available nor transferrable to other patient cohorts. Such knowledge may however be indirectly gained by examining urine drug screening positivity rates for a defined population within a specific geographical area.

Objective: Identify gender, seasonal and age-specific trends in urine drug screening positivity rates for patients tested within a community laboratory healthcare setting in Ontario, Canada.

Method: Results for approximately 9,400 male and 5,300 female patients, tested between January 1 and December 31, 2013, by Cloned Enzyme Donor Immunoassay (CEDIA) or Diagnostic Reagent Inc. (DRI) immunoassay for the presence of amphetamines (DRI, cut-off 1000 ng/mL), barbiturates (DRI, cut-off 300 ng/mL), benzodiazepines (CEDIA, cut-off 300 ng/mL), cannabinoids (DRI, cut-off 50 ng/mL), cocaine metabolite (DRI, cut-off 300 ng/mL), ethanol (DRI, cut-off 4 mmol/L), methadone (DRI, cut-off 300 ng/mL), methadone metabolite (CEDIA, cut-off 100 ng/mL), opiates (CEDIA, cut-off 300 ng/mL) and oxycodone (DRI, cut-off 100 ng/mL) were retrospectively reviewed to identify demographic and monthly trends within each test's respective positivity rate. These urine drug screening tests were requested by approximately 2,200 independent physician and treatment facilities located in 195 different cities or towns within 1,083 different postal codes across Ontario. Patients ranged in age from < 1 to 94 years-old (yo). The mean and median ages of both the male and female patients were 41 and 42 yo, respectively. No clinical history, including medication lists or the specific reason for why the testing was requested, was available for all patients. The monthly testing volume (mean ± SD) for these samples was roughly 1,200 ± 120 urine specimens per month.

Results: Overall screening positivity rates were: < 1%, barbiturates; 2%, amphetamines; 3%, ethanol; 9%, benzodiazepines and methadone; 10%, methadone metabolite and cocaine metabolite; 20%, oxycodone; 24%, opiates and 25%, cannabinoids. Gender differences (e.g., female vs. male) in normalized positivity rates were respectively observed for: ethanol (2% vs. 3%); benzodiazepines (11% vs. 8%); methadone and methadone metabolite (7% vs. 11%); cocaine metabolite (8% vs. 11%); oxycodone (22% vs. 18%); opiates (28% vs. 22%) and cannabinoids (21% vs. 28%) over this timeframe. The normalized monthly positivity rates, for both female and male patients, showed no statistically significant seasonal trends or variances for any of the screened drug/drug classes. Cannabinoid metabolite positivity was highest in the < 20 yo cohort (34% females and 46% males) and despite decreasing linearly with age, showed significant positivity within the 65 to 69 yo cohort (e.g., 10% females and 17% males). Benzodiazepines, opiates and oxycodone positivity generally increased with age and were more pronounced for females than males: 25 to 29 yo benzodiazepines, opiates and oxycodone positivity's were 7, 16, 11% and 4, 17, 12% for females and males, which respectively increased to 23, 49, 46% and 8, 34, 39% at 60 to 64 yo. 18% was the peak positivity rate for cocaine metabolite and was associated with both females and males between the ages of 40 and 44 years.

Conclusion: The overall positivity rates for cannabinoids and opiates were similar for the heterogeneous population studied and the relative prevalence of urine barbiturate, amphetamine and ethanol detection was low. No significant seasonal variations in drug positivity rates were identified. Urine drug screening positivity rates for pain management medications (e.g., benzodiazepines, opiates and oxycodone) were relatively higher in females than males and increased with age. Identifying and communicating demographic-specific trends in urine drug screening positivity rates, for both illicit and prescription drugs, to independent physicians and treatment facilities should broaden and enhance their understanding of drug use within community-based clinical settings.

Keywords: Urine Drug Testing, Positivity Rate, Drugs of Abuse, Demographic Trends, Seasonal Trends

P-82
A Four Year Retrospective Study of Methamphetamine in Los Angeles County

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Introduction: Methamphetamine use has been on the rise over the past ten years. Despite Federal and International intervention, the abuse of the drug has only become more popular as the years pass. The drug is primarily used in urban and metropolitan areas on the west coast, although its use has been spreading to more rural areas, with Los Angeles being one of the major cities with a history of methamphetamine abuse among its population.

Objective: In order to better understand the demographics of methamphetamine abuse in Los Angeles County, data from the past four years (2010-2013) was examined. Other interests were methamphetamine concentrations in non drug related suicide cases and how they related to those of other modes, as well as how much the drug has infiltrated Los Angeles County.

Method: A retrospective study was conducted with data obtained from a departmental database that aggregates and displays information of interest such as cause and manner of death, gender, location, age, brief synopsis of the case, race, and concentration of methamphetamine.

Results: Confirmation and quantitation of methamphetamine accounts for approximately 10% of the toxicology casework conducted at the Los Angeles County Department of Medical Examiner - Coroner. On average there are approximately 4,550 postmortem cases analyzed per year, with about 450 decedents that screen positive for methamphetamine. In 2013, the Laboratory observed an increase of 27% in the number of positive methamphetamine cases from the previous year. A large portion of cases were determined to be accidental, followed by homicide, suicide, natural, and undetermined. In general, the vast majority of decedents were male (81.8%), the average age of decedents was 38 years old, and the average concentration of methamphetamine across all modes was 1.57 µg/mL (0.03 -116 µg/mL, median 0.55 µg/mL, n= 2172). The average methamphetamine concentration for the mode of accident was 1.86 µg/mL, homicide 1.12 µg/mL, suicide 1.17 µg/mL, natural 0.42 µg/mL, and undetermined 2.32 µg/mL. Furthermore, in 2013, there was a significant increase of suicidal deaths, 37%, where methamphetamine was detected in comparison to 2012. Moreover there was an average increase of 19.5% every year in cases where methamphetamine was detected from 2010-2013. A geographical analysis of the data utilizing the zip code of death was performed and it was found that methamphetamine positive deaths occurred across the entirety of Los Angeles County with the largest portions of methamphetamine positive deaths occurring in downtown Los Angeles, Long Beach, and South Los Angeles.

Conclusion: The Los Angeles County Department of Medical Examiner-Coroner conducted a four-year retrospective study of the decedent population in an attempt to understand the methamphetamine concentrations in relation to the causes and manners of death. It was apparent from the routine cases being analyzed that the volume of cases were definitely rising and the blood methamphetamine concentrations increasing as well. A broad correlation between the methamphetamine concentration and a cause of death could not be established. There are several cases in each mode where a significant amount of methamphetamine (> 1.0 µg/mL) was measured, however, it was considered an incidental finding in comparison to the actual anatomical cause of death. This happened most often in homicide and suicide cases. Lastly, it was evident that Los Angeles County undeniably has locations in which there could be considered ‘hot spots’ for methamphetamine deaths.

Keywords: Methamphetamine, Postmortem, Los Angeles County, Demographics

P-83

The Simultaneous Confirmation of 38 Stimulants and Psychoactive Compounds in Human Performance Toxicology Cases by LC-QTOF-MS and Trends in Use

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Introduction: In recent years, some drug users have modified their drug choices and now appear to be using drugs from the phenethylamines and cathinone drug families. While there have been methods published for the detection of these drugs, the panels of compounds tend to be limited. This creates a need for a comprehensive screening method that detects traditional stimulants and newer designer drugs in human performance cases. The Liquid Chromatography-Quadruple-Time of Flight-Mass Spectrometer (LC-QTOF-MS) has gained popularity as a screening technique due to its high mass accuracy, good sensitivity, and flexibility in adapting to new analytes. This instrumentation can be used to screen for traditional stimulants and newer designer drugs simultaneously.

Objective: The purpose of this study was to develop and validate a method for the rapid screening and confirmation of 38 stimulants and psychoactive compounds by LC-QTOF-MS and apply this method to casework. In addition, the frequency of occurrence of these stimulants and psychoactive compounds in recent driving under the influence (DUI) cases, alongside impairment profiles were evaluated.

Method: Urine samples with DRE profiles indicating stimulant use were extracted using a mix-mode solid phase extraction method. An Agilent 1260 High Performance Liquid Chromatography system coupled to an Agilent 6530 Quadrupole Time of Flight Mass Spectrometer in targeted MS/MS was used. A reverse phase gradient on a C18 column separated the analytes of interest (t = 9.5 min). Mass Hunter Qualitative Data Analysis and PCDL manager software were used for the identification of the compounds. The method was validated in accordance with UNODC and SWGTOX guidelines. Validation parameters evaluated included limit of detection (LOD), interference, ion suppression/enhancement and carryover. Authentic case samples were subjected to this analysis by this method. When available, DRE reports were evaluated to compare the impairment profile with the confirmed drugs.

Results: A targeted MS/MS method was successfully validated following UNODC and SWGTOX guidelines. Overall the assay demonstrated sensitivity of 10 ng/mL or less. Results of the case studies and drug use trends are presented in Table 1. General impairment profiles for these cases demonstrated dilated pupils, rapid pulse and fast internal clocks, increased body temperature, and agitated mood which are consistent with stimulant use.

Conclusion: A validated method that uses LC-QTOF-MS was developed to confirm the presence of 38 stimulants and psychoactive compounds in DUI cases. An increase in the number of confirmed synthetic cathinones has been observed in these cases. Only screening for routine stimulants would have produced a negative toxicology result and not support the observed DRE findings. This demonstrates the need for the inclusion of these newer compounds in routine screening.

Table 1: Drug trends in the use of stimulant drugs in human performance cases

	Year				
Drug	2010	2011	2012	2013	2014
MDMA	14	3	1	4	1
MDA	11	1	1	2	1
Methylone	0	2	4	23	7
α-PVP	0	0	0	0	2
Butylone	0	0	0	0	2
Ethylone	0	0	1	0	0

Keywords: LC-QTOF-MS, Synthetic Cathinones, Human Performance, Drug Trends, Stimulants

P-84
A Death Involving Hydrogen Sulfide Exposure from a Domestic Sink Drain

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Introduction: Often referred to as "pit gas", hydrogen sulfide is a highly toxic gas. Following exposure, clinical presentation includes headache, nausea, vomiting, unconsciousness, seizure and coma. Fatalities have been reported at concentrations greater than 150 ppm.

Objective: This case presents a fatality involving hydrogen sulfide exposure and demonstrates the importance of submitting complete case history when requesting toxicology testing.

Method: Antemortem blood, postmortem femoral blood, vitreous, urine, and lung tissue were submitted for toxicological analysis. The analysis of antemortem blood included enzyme immunoassays for drugs of abuse, gas-chromatography/flame ionization detection for volatiles (ethanol, methanol, isopropanol and acetone), and broad spectrum drug screening (>150 drugs) by GC/MS. The case history suggested hydrogen sulfide poisoning and as a result, thiosulfate and sulfhemoglobin testing was performed. Femoral blood was sent to a reference laboratory for the analysis of sulfhemoglobin and thiosulfate. Sulfhemoglobin was analyzed by spectrophotometry and thiosulfate was analyzed by ion chromatography. Lung tissue was also sent to the reference laboratory for a hydrocarbon and oxygenated volatiles panel by gas chromatography.

Results: The subject was a 44-year old white female who was reported to have been attempting to unclog a drain under a kitchen sink. The subject was discovered unresponsive on the kitchen floor with her head inside the sink cabinet and was pronounced one minute after hospital admission. The drain pipe had been removed and a solution known as "Liquid Fire", which contains sulfuric acid and rodine, had been poured into the drain. The subject had a history of COPD and chronic asthma. First responders noted a strong smell of sewer gas. External examination did not reveal any injury, but autopsy revealed a dusky gray-green discoloration to the gray matter of the cerebral hemispheres. Toxicology drug screening indicated the presence of amphetamines and cannabinoids. Amphetamines were confirmed as methamphetamine at 378 ng/mL in antemortem blood. Ethanol levels in antemortem blood and vitreous were 91 mg/dL and 101 mg/dL, respectively. The thiosulfate level in femoral blood was 15.5 mcg/mL and the urine thiosulfate was 9.4 mg/g creatinine. The sulfhemoglobin level in femoral blood was measured at 6.3%.

Conclusion: Hydrogen sulfide's toxic effects result from the inhibition of cellular respiration. Death is generally due to cardiovascular and respiratory failure. Thiosulfate concentrations reported in various fatalities range from 2.8 – 72.6 mcg/mL. According to the Occupational Safety and Health Administration's web site (osha.gov), exposure to a concentration of 2-5 ppm hydrogen sulfide can lead to bronchial restriction in some asthma patients. Based upon the circumstances surrounding the death and the findings at autopsy, the subject of this case report died as a result of hydrogen sulfide intoxication with methamphetamine abuse and asthma as contributing factors. The manner of death was accident.

Keywords: Hydrogen Sulfide, Thiosulfate, Sulfhemoglobin

P-85
Oral Fluid Testing for Opiates by "Dilute and Shoot" LC-MS/MS

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Introduction: Oral fluid (OF) sample collection and analysis is projected to grow in the near future. These samples are often stabilized upon collection through the addition of phosphate buffer, surfactants, and other additives. Hence, successful LC-MS/MS analysis traditionally has depended upon analyte extraction from this matrix along with concentration due to the relatively low levels of analytes in OF. However, in this work a unique "dilute-and-shoot" method is demonstrated for the analysis of eight different opiate compounds with acceptable limits of quantitation/limits of detection (LOQ/LOD) values and robust instrument performance that saves both time and cost in analyzing this sample matrix.

Objective: To develop a high-throughput method for opiate analysis in OF without the need for time-consuming clean up protocols.

Method: A Quantisal™ sampling device from Immunalysis was used for patient sample collection. This device uses a collection pad to collect 1mL (± 10%) of sample, which is then diluted in 3mL of buffer to stabilize the sample. Samples were diluted an additional 10X with internal standard and methanol/water and analyzed by LC-MS/MS on an AB Sciex 4500 platform using an Agilent 1290 chromatographic system and a Phenomenex Kinetex 2.6µm Phenyl-Hexyl 100Å, 50 x 4.6mm (00B-4495-E0) column. The run time for this method is 2.2 minutes. No sample clean-up or extraction was performed. This assay monitors two transitions for each of the following eight analytes: Codeine, Hydrocodone, Hydromorphone, Morphine, Norhydrocodone, Noroxycodone, Oxycodone, and Oxymorphone; and five internal standards: Codeine D3, Hydrocodone D6, Hydromorphone D3, Morphine D3, and Oxycodone D3.

Results: Preliminary data exhibited a reproducible quadratic fit with consistent peak shapes and ion ratios for Codeine, Hydrocodone, Hydromorphone, Morphine, Norhydrocodone, Noroxycodone, Oxycodone, and Oxymorphone. Daily 6-point calibration curves routinely demonstrate 100 ± 25% accuracy for each point and excellent linearity ($R^2 > 0.99$). Insignificant matrix effects were observed while some, albeit expected, interferences were detected from other common opioid medications; these will be discussed. Additionally, no appreciable carryover was observed following the highest curve point of 1000ng/mL. The LOD/LOQ for all analytes was determined to be 2.5 ng/mL. This concentration reflects the buffered oral fluid sample prior to the final 10X dilution which is then injected onto the column. Thus, the "in mouth" LOD/LOQ would be 4 times this value or 10 ng/mL. Despite the common view that buffered oral fluid samples cause serious instrument wear and failure, no appreciable time or money was spent performing extra maintenance or on replacement of consumables. The Phenomenex phenyl-hexyl column showed stable performance through greater than 2000 injections and the curtain plate on the AB Sciex 4500 was cleaned only every month despite consistent use with these types of samples.

Conclusion: Using these patient samples, the utility of a LC-MS/MS dilute-and-shoot method for analysis of oral fluid will be demonstrated for reduction of time and labor per sample, conservation of sample, and the preservation of required LOQ/LOD values for these diluted samples.

Keywords: Oral Fluid, LC-MS/MS, Opiates, Dilute and Shoot

P-86

Oral Fluid Testing of Pain Patients: Illicit Prevalence and Regional Patterns

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Introduction: Oral fluid is used as an alternative specimen matrix for compliance testing in pain patients. Collection under direct observation may limit opportunity for specimen adulteration, and studies have demonstrated higher prevalence of illicit drugs in oral fluid compared to urine.

Objective: Characterize prevalence patterns in oral fluid for five illicit drugs: methamphetamine (MAMP), 3,4-methylenedioxy-N-methylamphetamine (MDMA), 3,4-methylenedioxyamphetamine (MDA), cocaine (COC), and marijuana (THC).

Method: Oral fluid specimens (n=30,304) were collected using the Quantisal™ device (Immunalysis) from 849 pain clinics during 2013. Specimens originated from 39 states, later classified as four regions: Northeast (MA, NH, NJ, NY, PA), South (AL, AR, DC, DE, FL, GA, KY, LA, MD, MS, NC, OK, SC, TN, TX, VA, WV), Midwest (IA, IL, IN, KS, MI, MN, MO, NE, OH, WI), and West (AZ, CA, CO, NV, OR, UT, WA). Specimens were screened by ELISA kits for Oral Fluids (Immunalysis) for the following classes and thresholds: Amphetamines 50 ng/mL, Methamphetamine 50 ng/mL, Cocaine/Benzoylecgonine 20 ng/mL, and Cannabinoids 4 ng/mL. Non-negative specimens were tested by LC/MS/MS for the following analytes and limits of quantitation (LOQ): MAMP, MDMA, and MDA 8 ng/mL; COC, benzoylecgonine (BZE), THC, and 11-nor-9-carboxy-delta-9-tetrahydrocannabinol (THCCOOH) 2 ng/mL. MAMP was classified as an illicit drug if not marked as prescribed on the laboratory requisition.

Results: 3,614 specimens (11.9%) were positive for at least one illicit drug (MAMP, MDMA, COC, or THC); no specimens were positive for MDA. Of these, 263 specimens were positive for multiple illicit drugs. Prevalence rates were as follows: THC/THCCOOH (n=2,435, 8.0%), COC/BZE (n=1,160, 3.8%), MAMP (n=288, 1.0%), MDMA (n=3, 0.01%).

United States Regions and Illicit Prevalence in Oral Fluid

Region	# Samples	# Samples positive for illicit drugs (%)	# Samples positive for MAMP (%)	# Samples positive for MDMA (%)	# Samples positive for COC/BZE (%)	# Samples positive for THC/THCCOOH (%)
Northeast	3,959	802 (20.3%)	22 (0.6%)	2 (0.05%)	329 (8.3%)	517 (13.1%)
South	20,484	1,926 (9.4%)	167 (0.8%)	0	623 (3.0%)	1,264 (6.2%)
Midwest	4,525	690 (15.2%)	50 (1.1%)	1 (0.02%)	167 (3.7%)	529 (11.7%)
West	1,336	196 (14.7%)	49 (3.7%)	0	41 (3.1%)	125 (9.4%)

Conclusion: The illicit prevalence was highest in the Northeast, followed by the Midwest, West, and South. The highest prevalence rates were observed for MAMP in the West, and for COC and THC in the Northeast. Detection rates were higher in oral fluid than previously reported rates in urine for the same illicit drugs (< 9%). Oral fluid is a useful option for monitoring compliance in pain patients.

Keywords: Pain Management, Compliance Testing, Oral Fluid, U.S. Illicit Drug Demographics

P-87

Interpreting Test Results for Pain Management Practitioners Utilizing Clinical Pharmacy Services

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Introduction: Drug testing for chronic pain patients has become standard in recent years. However, practitioners' understanding of test results has been demonstrably lacking in published studies. If test results are not properly interpreted then noncompliance, possible diversion, and substance abuse or misuse may be missed or misidentified. This may adversely impact healthcare costs and patient outcomes.

Objective: Characterize the most frequent topics that prompt consultation with expert laboratory personnel during the course of pain management compliance testing.

Method: A team of clinical pharmacists electronically tracked 7,282 consults for toxicology result interpretation which originated from practitioners primarily treating patients with chronic pain. Questions were tracked over 30 months, from November 2011 through May 2014.

Results: The top 10 topics were identified as follows: which licit or illicit medications could have caused an unexpected positive result (18.1%); potential false positive findings, whether at the laboratory or at point-of-care (10.1%); reasons for detecting parent drug in urine in absence of tested metabolite(s) (10.0%); interpretation of amphetamine or methamphetamine positives (7.9%); reference ranges for specimen type tested (7.6%); potential unexpected positives due to metabolism of prescribed drugs (7.3%); evaluation of unexpected or potential false negative results (7.2%); period of detection (6.4%); the amount of drug ingested (4.4%); and questions about test methodology used (e.g., method type, thresholds, or specimen type) (4.3%).

Conclusion: These results may help guide training programs for laboratory scientists who assist practitioners with results interpretation. Knowledge of analytical toxicology and expertise in clinical pharmacy is necessary. Pharmacists should be trained in a broad range of topics, including: laboratory procedures, analytical methods (both those used by the laboratory and at point-of-care), targeted drugs and metabolites, thresholds, differences in specimen matrices, and limitations of testing. Pharmacists must also be proficient in pharmacotherapy options for pain medicine; clinical guidelines for patient assessment; drug pharmacokinetics (including impact of intra-thecal administration or various dialysis procedures) for a broad range of drugs; drug-drug and drug-disease interactions; and drug information and toxicology references. Clinical pharmacists, particularly those who are board-certified and/or residency trained, may offer interpretive assistance to practitioners conducting drug testing as part of routine compliance assessment. As abuse of prescription drugs has reached epidemic proportions and drug testing has become necessary in both chronic pain management and primary care, pharmacists may offer a unique contribution to the implementation of these patient care services.

Keywords: Pain Management, Results Interpretation, Clinical Pharmacists, Routine Compliance Assessment

P-88
Evaluation of Patients Prescribed Adderall® for the Presence of Methamphetamine

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Introduction: Adderall is a commonly prescribed medication in which compliance monitoring may be required as part of the regimen. After experiencing a challenge with interpretation, a study of 12 samples was completed. To be included, patients had to have a prescription for Adderall® and have an amphetamine concentration greater than 5,000 ng/mL. The study utilizes two different sample preparation, chromatographic, and mass spectrometry techniques to identify/quantify the drugs.

Objective: Evaluate if low concentrations of methamphetamine are present in patients prescribed Adderall® as described in peer reviewed literature.

Method: Deuterated internal standards were added to 1mL of urine followed by 0.35 N sodium periodate, 1 N sodium hydroxide, and extraction solvent (3.9:1:0.1 toluene:ethyl acetate:methanol). This step was followed by rotation, centrifugation, a N_2 dry down step and reconstitution with aqueous 0.1% formic acid (FA). The samples were analyzed by LC/MS/MS for amphetamine and methamphetamine. Chromatographic separation was achieved using a HSST3 column (1.7micron, 2.1x50 mm) with a gradient system consisting of aqueous 0.1% FA and 0.1% FA in acetonitrile with a flow rate of 0.6 mL/min. Criteria for acceptable results included the following; retention time must be within 0.02 minutes or ±2.5% (whichever is greater), and ion ratios must be within ±20% of calibrators, and positive controls must be within ±20% of the target concentration. The lower limit of quantitation for amphetamine and methamphetamine was 50 ng/mL and 5 ng/mL, respectively. Chiral analysis was completed by GC-ToF. Sample preparation for this analysis included the addition of internal standard (racemic propylamphetamine), 0.5mL of urine, 0.35 N sodium periodate, 1 N sodium hydroxide, and chlorobutane. This step was followed by rotation, centrifugation, and addition of N-trifluoroacetyl-*ℓ*-prolylchloride (0.1 M, 95% purity, Regis Technologies) to the organic layer. After 15 minutes of rotation, a back extraction was completed with 0.01 N sodium hydroxide. The organic layer was dried down, reconstituted in ethyl acetate, and analyzed. Chromatographic separation was achieved using a DB-5MS column (30m x 0.25mm x 0.25 µm). Criteria for acceptable results were as follows: relative retention time must be within 0.02 minutes of QC, accurate mass must be within 5mDa, and QC must be within ±2 standard deviations of the established target ratio. Enantiomer ratios were calculated as a percentage of each enantiomer of the total using integrated peak areas for each enantiomer. The limit of quantitation for both analytes was 5 ng/mL.

Results: Evaluation of the LC/MS/MS results of the 12 specimens yielded both amphetamine and methamphetamine concentrations that ranged from 5,671-425,777 ng/mL and 9-594 ng/mL, respectively. Dextroamphetamine isomeric distribution ranged from 69-79%, which correlate with published Adderall administration and excretion profiles. Dextromethamphetamine isomeric distribution ranged from 73-94%. Eight samples had similar amphetamine and methamphetamine isomeric distribution, which all had methamphetamine to amphetamine ratios less than 0.5%, as described in the literature. The remaining samples did not have similar isomeric distribution and the methamphetamine to amphetamine ratio was greater than 1.25%.

Conclusion: The data collected during this study supports the observations reported previously. The presence of dextromethamphetamine may suggest a pharmaceutical impurity or methylation during the metabolism phase of amphetamine. Current studies are underway to analyze different doses and manufactures of pharmaceutical preparations. Additional studies may be warranted in order to fully determine this phenomenon. This study will add to the existing knowledge surrounding this subject and further aid with interpretation.

Keywords: Methamphetamine, Adderall®, Amphetamine, Vyvanse®, Polydrug Use, Metabolism

P-89
Suicide from Azide Ingestion – a Case Report

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Introduction: A 35 year old graduate student had been living with his girlfriend for 14 years. She discontinued their relationship. He took a leave of absence from his graduate studies because of emotional problems. He was a smoker and had snuffed cocaine occasionally. Six months after their break-up, he bought a hamburger and coffee at 11 am, and then parked in a back lane at another friend's house. An hour later, the other female friend went out to the locked car and saw him slumped over in the driver's seat. She called the paramedics, who did a forced entry but he was already deceased. No resuscitation was performed. There was vomit on him and on the car floor. Some white powder was found in a plastic tube in his back-pack. The autopsy was performed 3 days after his death. The pathologist was unable to determine the cause of death. Blood, urine, vitreous and the vial containing a few milligrams of white powder was sent to the toxicology laboratory. His supervising professor told us that he had sodium azide in his laboratory. It was used to preserve his homemade reagents. Azide ingestion will oftentimes induce vomiting.

Objective: To determine if the cause of death of this subject was due to azide ingestion by analysis of biological specimens and powder.

Method: Alcohol analysis was performed on blood, urine and vitreous using gas chromatography. A urine drug screen was performed by gas chromatography – mass spectrometry. A blood drug screen was performed by tandem liquid chromatography – mass spectrometry. Carbon monoxide was performed by CO-oximetry. Cyanide was performed by GC-NPD. Azide was assayed in blood, urine, vitreous and the white powder by gas-chromatography with nitrogen-phosphorus detection. This is a published method by R. Meatherall and W. Palatnick, entitled; Convenient headspace gas chromatographic determination of azide in blood and plasma. J. Anal. Toxicol. **33**: 525-531 (2009).

Results: No alcohols were detected in the 3 fluids. Caffeine, nicotine and cotinine were detected in the urine and blood. Carboxyhemoglobin was 7.2%. Blood cyanide was 0.4 μg/mL (normal < 2 μg/mL). Azide was measured in the blood (1.1 μg/mL), urine (7.5 μg/mL) and the vitreous (43 μg/mL). The vial of white powder was analyzed and it was found to contain azide.

Conclusion: The graduate student died from azide ingestion. The azide was probably dissolved in his coffee. Azide consumption causes hypotension, nausea, vomiting, diarrhea, unconsciousness, respiratory depression, alternating tachycardia and bradycardia and cardiac arrhythmias. Azide is gradually converted to nitric oxide in the blood; hence some fatalities have no azide remaining in the blood. The blood azide is metabolized over time, and the urine will have a lower value. If the gastric content is vomited, the azide may not be detected in it. The vitreous humor is the best fluid for azide screening because it is not metabolized as quickly.

Keywords: Azide, Fatality, GC-NPD

P-90

Prescription and Illicit Drug Trends in Buprenorphine Positive Oral Fluid Specimens

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Introduction: Buprenorphine is prescribed for the treatment of substance abuse and pain. Increases in buprenorphine prescribing practices have coincided with reported escalations in buprenorphine abuse and misuse. In pain management compliance testing, the importance of assessing for prescribed buprenorphine is clear. However, buprenorphine may also be an important drug to include in testing when it is not prescribed. Though buprenorphine is thought to have a more favorable risk profile than other full agonist opioids, the risks for adverse drug events and overdose increase when buprenorphine is ingested in combination with other medications.

Objective: Assess positivity trends of prescription and illicit drugs in oral fluid specimens positive for buprenorphine.

Method: More than 30,000 oral fluid specimens from pain management clinics were collected with the Quantisal™ (Immunalysis) device and tested by ELISA kits for Oral Fluids (Immunalysis) and liquid chromatography/tandem mass spectrometry (LC/MS/MS) for amphetamine, methamphetamine, 3,4-methylenedioxy-N-methylamphetamine (MDMA), cocaine, benzoylecgonine, tetrahydrocannabinol (THC), 11-nor-9-carboxy-delta-9-tetrahyrdorcannabinol (THCCOOH), carisoprodol, meprobamate, buprenorphine, norbuprenorphine, methadone, 2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidene (EDDP), tramadol, N-desmethyltramadol, O-desmethyltramadol, codeine, morphine, hydrocodone, hydromorphone, dihydrocodeine, norhydrocodone, oxycodone, oxymorphone, noroxycodone, fentanyl, norfentanyl, alprazolam, clonazepam, diazepam, nordiazepam, temazepam, oxazepam, lorazepam, flurazepam, and butalbital.

Results: Evidence of buprenorphine ingestion was detected in 5.8% (n=1,769) of the specimens; of these, 56.1% (n=992) were positive for only buprenorphine or norbuprenorphine. Buprenorphine prescriptions were reported for 76.3% (n=1,349) of the specimens. At least one illicit drug was detected in 16.9% of buprenorphine-positive specimens. The illicit drugs detected, in order of descending frequency, were THC, cocaine, methamphetamine, and MDMA. Other opioids were detected in 20.6% of buprenorphine-positive specimens, with prevalence in descending order as follows: oxycodone, morphine, hydrocodone, codeine, tramadol, methadone, fentanyl, hydromorphone, and oxymorphone. Prescriptions for other opioids were not disclosed in 64.1% of these cases. Benzodiazepines were detected in 11.5% of specimens, in order of descending frequency: alprazolam, diazepam, nordiazepam, clonazepam, oxazepam, temazepam, and lorazepam. 4% of specimens with evidence of buprenorphine ingestion were also positive for another opioid in combination with a benzodiazepine.

Conclusion: The results demonstrate that approximately 25% of specimens were unexpectedly positive for buprenorphine (no prescription for buprenorphine recorded). This illustrates the importance of considering inclusion of buprenorphine in toxicology testing even in cases when it is not prescribed. Additionally, the relatively high positivity rates of illicit drugs, other opioids, and benzodiazepines in specimens positive for buprenorphine is concerning given the increased risk for adverse drug events and overdose when buprenorphine is ingested in combination with other drugs. Toxicology findings provide objective information which may prompt practitioners to further assess their patients.

Keywords: Buprenorphine, Oral Fluid, Pain Management Compliance Testing (PMCT)

P-91
2012 and 2013 Trends in Cannabimimetic Urine Drug Testing

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Introduction: Cannabimimetic use in the U.S. was first documented in 2008, when these chemicals were detected on plant materials packaged and sold in gas stations, smoke shops, and on the internet. Subsequent legislation placed several cannabimimetic agents into Schedule I of the Controlled Substances Act. Few data are available describing recent cannabimimetic use trends and specific metabolites detected in urine.

Objective: To characterize urinary cannabimimetic trends in 2012 and 2013 from a diverse client population.

Method: Urine specimens (400 µL screen; 1 mL confirm) were hydrolyzed with β-glucuronidase at 60°C for 2h and extracted by solid phase extraction. Screening samples were reconstituted with ethylenediaminetetraacetic acid (EDTA) in acetonitrile/water/ammonium hydroxide and spotted onto LazWell plates. Analyte mass transitions were monitored by Phytronix laser diode thermal desorption (LDTD)-MS/MS with a1 ng/mL (2 ng/mL for UR-144 pentanoic acid) limit of quantitation (LOQ). HPLC confirmation was conducted by gradient elution with 10 mM ammonium acetate and 0.1% formic acid in water and 0.1% formic acid in acetonitrile. An AB Sciex 3200 MS/MS was operated in positive ionization mode with two MRM transitions monitored per analyte and a 1 ng/mL LOQ. JWH-073 and JWH-018 metabolites (except 4-hydroxypentyl) were monitored from January 2012 to September 2012. In September 2012, the LC-MS/MS assay was modified with 0.1% formic acid in methanol as organic mobile phase, scheduled MRM, and the inclusion of metabolites of 9 cannabimimetics (UR-144, JWH-250, AM-2201, JWH-122, JWH-210, MAM-2201, JWH-019, JWH-081, and JWH-398). Samples were received from pain management, sports, and workplace clients (includes drug-free workplace, healthcare professionals, criminal justice, and probation and parole testing). This study was approved by an internal Institutional Review Board.

Results: Sample volume increased by 52.2% between 2012 and 2013, from 108,951 to 165,825 samples. 890 samples confirmed positive by LC-MS/MS; 507 positive in 2012, with a 24% decrease to 383 positive samples in 2013. Males comprised the majority of positive samples reported (59.1%), with 8.8% of unknown gender. Some analyte metabolites were not detected (MAM-2201, JWH-019, JWH-081, JWH-398). Table 1 describes the number of confirmed positive urine samples and the percentage of the total positive samples from 2012 and 2013.

Table 1: Cannabimimetic Positive Urine Samples in 2012 and 2013

	JWH-073		**JWH-018**			**AM-2201**	**UR-144**			**JWH-122**		**JWH-210**	**JWH-250**	
	4HB	**BA**	**4HP**	**5HP**	**PA**	**4HP**	**4HP**	**5HP**	**PA**	**4HP**	**5HP**	**4HP**	**4HP**	**PA**
2012														
#confirmed	12	148	2	363	401	3	4	45	65	1	2	0	1	1
% of total	2.4%	29.2%	0.4%	71.6%	79.1%	0.6%	0.8%	8.9%	12.8%	0.2%	0.4%	0.0%	0.2%	0.2%
2013														
#confirmed	1	7	5	30	26	6	10	235	329	3	3	1	0	0
% of total	0.3%	1.8%	1.3%	7.8%	6.8%	1.6%	2.6%	61.4%	85.9%	0.8%	0.8%	0.3%	0.0%	0.0%

4HB- 4-hydroxybutyl; BA- butanoic acid; 4HP- 4-hydroxypentyl, 5HP- 5-hydroxypentyl; PA- pentanoic acid

Conclusion: Between 2012 and 2013, JWH-073 and JWH-018 metabolite prevalence substantially declined. UR-144 pentanoic acid was the analyte most commonly (85.9%) observed in positive samples in 2013. Legislative efforts, combined with increased public health warnings, may be one explanation for the decrease in positive samples in 2013. A second explanation involves the primary assay limitation, which targets only those metabolites with available reference standards at validation. Thus, it is likely that the emergence of additional third and fourth generation cannabimimetics in 2012 and the absence of available reference standards for urinary metabolites prevented detection. Monitoring cannabimimetic metabolites in urine remains a challenge. It is important for laboratories to report trends, particularly in raw products and blood/plasma, as parent analyte reference standards are more readily available. Once parent analytes are identified, metabolic studies may be conducted to determine appropriate urinary metabolites.

Keywords: Cannabimimetics, Synthetic Cannabinoids, LDTD, LC-MS/MS

P-92

Will the Real "Molly" Please Stand Up? A Comparison of MDMA and Synthetic Cathinone Deaths in Miami-Dade County, Florida

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Introduction: "How many people in this crowd have seen Molly?" was the question posed by Madonna in Miami at the Ultra Music Festival back in 2012. Historically, the term "Molly" referred to the powder or crystal form of MDMA. Over the last several years however, it is clear that they now contain synthetic cathinones, most commonly Methylone. Many users are ignorant to this fact and are convinced they are ingesting pure MDMA.

Objective: To identify all MDMA and synthetic cathinone positive cases from 1996 to present, to determine if the change in Molly composition has affected cause and manner of death. Demographic information will be analyzed to identify trends and differences between the two groups.

Method: The Miami-Dade County Medical Examiner Department (MDME) processes approximately 3400 cases per year. All case data is cataloged in LIMS which captures demographic information, terminal event data, social and medical history, toxicology results and autopsy reports. Utilizing the case information from LIMS, data will be presented in a visual manner to compare how drug trends have changed over the years in Miami-Dade County, and how these drugs may influence cause and manner of death in certain demographic groups.

Results: MDMA was detected in 173 cases from 1996 until 2011. Of these deaths, approximately 35% were homicides, 6% were suicides, 14% were accidents, 5% were natural and 40% were toxicology related. Synthetic cathinones were first detected at the MDME in 2011 and continue to be detected to this date, with 73 cases so far. Initially, Methylone was the predominant drug; however, in the past few months there has been a rise in Alpha-PVP and other cathinones. Of these deaths, approximately 63% were homicides, 11% were suicides, 7% were accidents and 19% were toxicology related. The demographics of the homicides in both groups were the same; with over 80% being young black males under the age of 40. Gunshot wounds were responsible for 93% of the homicide deaths in both groups.

Conclusion: It is clear that in Miami, Molly no longer contains MDMA, since there hasn't been a single case since 2011. The truth is that Mollies now contain Methylone or other synthetic cathinones such as Alpha-PVP. Interestingly, the number of accidents, and toxicology related deaths have decreased by half, compared to the MDMA group, however it is alarming that the rates of homicide and suicide have doubled in users of these synthetic cathinones compared to MDMA. Molly is no longer the love-drug "she" used to be, and appears to have a more sinister and violent side than "her" predecessor. She will only continue to change and evolve over time, presenting increasing challenges to toxicologists, drug chemists and law enforcement. The truth is that the users may never know who Molly really is, which could result in deadly consequences.

Keywords: Molly, MDMA, Methylone, Postmortem

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Identification and Characterization of Synthetic Cannabinoid Pyrolysis Products

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Introduction: The study of synthetic cannabinoid abuse is challenging because of the potential for exposure to a large number of known and theoretically possible compounds. Additionally, chemical changes in the exposure may be induced by smoking or vaporization, and the metabolism of these chemical constituents in vivo.

Objective: To generate data that will enhance understanding of chemical exposures and pharmacological effect profiles in users, thereby informing clinical strategies to combat use and dependence or withdrawal.

Method: A CDS Analytical 5250T pyrolysis autosampler and an Agilent 7890 GC with 7001B mass selective detector were used to analyze 35 synthetic cannabinoids. 8ug of standard material was loaded onto a quartz wool plug within a glass tube. The temperature was held at 50˚C for one second then ramped at 20˚C/s to 800˚C and held for 10 seconds. Pyrolysis products were collected at 50˚C and desorbed for two minutes at 300˚C. An Agilent DB-5MS capillary column (30 m x 0.25 mm x 0.25 µm) was used with a helium carrier gas flow of 1.0 mL/min, a split injection with a ratio of 1:50 at 300˚C, and the EI source set to 230˚C. Affinity and efficacy at CB1 and CB2 receptors of UR-144; XLR-11; PB-22 carboxyindole metabolite; and ring open degradants of UR-144, XLR-11, and A-834735 were measured using G-protein coupled signal transduction (GTP-γ-[^{35}S]) assays. Assay mixtures contained test compound (0.01 nM−10 µM), GDP (20 µM), GTP-γ-[^{35}S] (100 pM), and the desired membrane preparation (0.4 pM) in assay buffer. Nonspecific binding was determined in the presence of 100 µM unlabeled GTP-γ-S, and basal binding was determined in the absence of test compound. Samples were incubated with shaking for 1 h at 25°C and the assays were terminated by filtration under vacuum. Microscint 20 was added and filter-bound radioactivity was counted on a Packard scintillation counter.

Results: Of the 35 synthetic cannabinoids studied, only four retained more than 80% of the original dose after pyrolysis: JWH-018 (91%), JWH-018 adamantyl analog (85%), JWH-019 (84%), and THJ-018 (98%). All ester containing synthetic cannabinoids underwent significant thermally-induced structural changes resulting in less than 5% retention of the original dose: PB-22 (0%), 5F-PB-22 (0%), BB-22 (0%), A-834735 (2%), FDU-PB-22 (3%), and FUB-PB-22 (0%). For all synthetic cannabinoids containing a tetramethylcyclopropyl ring substituent, greater than 85% of the original dose was converted to a ring opened form of the parent compound. The ring opening change in structure does not appear to dramatically alter cannabinoid receptor affinity, selectivity and efficacy, with the ring-opened analogs retaining nM affinity and acting as full agonists at the CB1 and CB2 receptors as do their non-degraded, parent, analogs. However, the thermal degradation product and biological metabolite observed with PB-22 (1-pentyl-1H-indole-3-carboxylic acid) was unable to alter GTP-□-S binding at doses up 1000-fold higher than active concentrations of PB-22.

Table 1: Additional Synthetic Cannabinoids Analyzed and the % of Unchanged Drug Retained After Pyrolysis

AB-PINACA (41%)	ADB-FUBINACA (12%)	EAM2201 (41%)	THJ (65%)
AB-FUBINACA (18%)	ADBICA (0%)	JWH-122 (56%)	THJ-2201 (63%)
5F-AKB48 (74%)	ADB-PINACA (11%)	MAM2201 (67%)	UR-144 5-Bromopentyl Analog (3%)
5-Fluoro AB-PINACA (10%)	AKB48 (74%)	SDB-006 (34%)	UR-144 (14%)
5-Fluoro THJ (8%)	AM2201 (70%)	STS-135 (17%)	UR-144 5-Chloropentyl Analog (7%)
5-Chloro AB-PINACA (1%)	AM2201 Benzimidazole - Analog (79%)	JWH-018 Adamantyl Carboxamide Analog (26%)	XLR11 4-Pentenyl Analog (6%)
			XLR-11 (8%)

Conclusions: It is likely that users are unknowingly being exposed to novel synthetic cannabinoid structures from thermal degradation and pyrolysis, which may have unique pharmacological properties from the original chemical entities.

Keywords: Synthetic Cannabinoids, Pyrolysis Products, CB1 and CB2 Receptors

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Major Metabolites of Structurally Related Cannabimimetic Compounds by UPLC-QTOF Mass Spectrometry

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Introduction: Cannabimimetic compounds are found in herbal products and abused for their marijuana-like effects. Clandestine manufactures continue to produce structurally different compounds in an effort to avoid judicial consequences. Little is known about the pharmacological effects and metabolism of many of these novel cannabimimetic compounds.

Objective: This study aimed to identify major metabolites of structurally similar cannabimimetic compounds in order to provide the forensic community with suitable markers of use.

Method: In vitro samples were generated by incubating parent drugs at 10 μM in cryopreserved human hepatocytes. At 0, 15, 120, and 180 min, an aliquot was removed and quenched with acetonitrile containing 0.2% acetic acid. Samples were hydrolyzed with abalone beta-glucuronidase for 2 h at 60°C and filtered prior to LC analysis. Non-hydrolyzed samples were centrifuged and the supernatant was removed with no additional sample preparation. Data were collected on both the hydrolyzed and non-hydrolyzed samples using a Waters Acquity ultra performance liquid chromatography (UPLC) system coupled to a Synapt G2 HDMS quadrupole time-of-flight (QTOF) mass spectrometer. Liquid chromatography was carried out using an Acquity BEH C18 column (1.7 μm X 2.1 X 50mm). A gradient elution was used with mobile phase A consisting of water with 0.1% formic acid and mobile phase B consisting of acetonitrile with 0.1% formic acid.

Results: Major metabolites were identified for cyclopropyl ketone indoles; UR-144, XLR-11, A-834735 and their degradant compounds. Major metabolites of 3-indole ester linked cannabimimetic compounds;, PB-22, 5F-PB22, BB-22, FDU-PB22, and FUB-PB22, were also identified. Some metabolites were only tentatively identified but others were confirmed with reference standards. The majority of the non-conjugated metabolites were observed starting at 15 min and the conjugated metabolites were observed at longer incubation times. Monohydroxylated and its glucuronide and di-hydroxylated and carboxylated UR-144 and UR-144 degradant were detected in their respective incubations. Metabolic transformations detected for XLR-11 and its degradant were similar to those detected for UR-144 and it's degradant, and in addition, XLR-11 and its degradant underwent defluorination, resulting in several metabolites common to both parent compounds. A-834735 and its degradant both metabolized to hydroxylated, hydroxylated glucuronide and di-hydroxylated metabolites. Major metabolites observed for all ester linked compounds were 3-carboxyindoles and hydroxylated 3-carboxyindoles and their corresponding glucuronide conjugates. Due to different modifications on the indole moiety between PB-22, 5F-PB-22 and BB-22, these 3-carboxyindole metabolites were identifiable with different retention times. FDU-PB22 and FUB-PB22 do not have different indole moiety modifications therefore the retention times for the 3-carboxyindole related metabolites are the same. PB-22 hydroxylated metabolites were detected in both PB-22 and 5F-PB-22 incubation samples. Interestingly, no hydroxylated 5F-PB-22 or hydroxylated 5F-PB-22 3-carboxy indole was observed. FDU-PB22 and FUB-PB22 do not appear to undergo defluorination, and no defluorinated 3-carboxyindole was observed in incubations of either compound. For both compounds, the major metabolites were 3-carboxyindole and its glucuronide and hydroxylated 3-carboxyindole glucuronide.

Conclusion: This research provides forensic practitioners with the identification of major in vitro metabolites of novel designer drugs consisting of a tetramethylcyclopropyl group and their degradants as wells as compounds with 3-indole ester linkages.

Keywords: Metabolites, Cannabimimetic, UPLC-QTOF

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Designer Stimulant and Hallucinogen Use in Alabama: DOC, α-PVP, DMAA, 25C-NBOMe

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Introduction: According to the National Forensic Laboratory Information System (NFLIS), the use of designer stimulants and hallucinogens is a major problem across the nation. Behaviors associated with these drugs include aggression, paranoia, and suicidal thoughts. Communication between medical examiners, investigators, and forensic toxicologists is critical in cases where use of these drugs is suspected, so that appropriate testing may be conducted.

Objective: We present four designer drug cases from the Alabama Department of Forensic Sciences from 2012-2014.

Method: Routine drug screening of specimens was performed using either the Tecan or Randox immunoassay systems,and liquid-liquid extraction for basic drugs followed by GC/MS. In house quantifications were performed utilizing solid phase extraction followed by GC/MS, when indicated for amphetamine/methamphetamine, cocaine panel (cocaine, cocaethylene, and benzoylecgonine), and opiates panel (meperidine, methadone, hydrocodone, oxycodone, morphine, and codeine). The cannabinoid quantification uses a liquid-liquid technique followed by LC/MS/MS for delta-9-THC, 9-carboxy-delta 9-THC, and 11-hydroxy-delta 9-THC. If use of a designer drug was detected/suspected, samples may have been sent to a commercial laboratory for identification and/or confirmation, utilizing their Bath Salts and Stimulants Designer Drugs panel with analysis by LC/MS/MS.

Results:
Case 1: A 32 year old female was observed to sit down in the middle of the street at night, naked, after an argument with another person who left the scene. Before witnesses could offer assistance to the woman, two vehicles swerved to avoid hitting her, but she was struck by a third. The autopsy was performed approximately 12 hrs after death. Chest blood samples were collected in gray stopper tubes containing sodium fluoride and potassium oxalate. The toxicology results were: benzoylecgonine 63 ng/mL, 9-carboxy-11-nor-delta-9-THC 6.4 ng/mL, and 4-chloro-2,5-dimethoxyamphetamine (DOC) present. A reference standard had been obtained from Alltech Associates, Inc. for qualitative identification of the DOC. The cause of death was multiple blunt force trauma and the manner of death was accident.

Case 2: A stillborn fetus was delivered at 36 weeks gestational age. The mother arrived by personal vehicle at the hospital where she tested positive for amphetamines. She admitted to using "bath salts" earlier that morning. The autopsy was conducted approximately 24 hrs after the stillborn fetus was delivered. Cardiac blood samples were collected in gray stopper tubes containing sodium fluoride and potassium oxalate. An aliquot of the blood was sent to the commercial reference laboratory. Toxicology results were: methamphetamine 17 ng/mL, 3,4-methylenedioxypyrovalerone (MDPV) 130 ng/mL, and alpha-pyrrolidinovalerophenone (alpha-PVP) 29 mg/L. The cutoff -concentration for the MDPV and the alpha-PVP were 10 and 2.0 ng/mL, respectively. The cause of death was listed as stillborn, related to maternal drug use and the manner of death was listed as accident.

Case 3: Law enforcement arrived at the decedent's residence in response to a domestic/welfare call. The decedent, a 35 year old male, confronted the police with a gun and was acting in an aggressive manner. The decedent was instructed several times to drop the weapon, but he refused. The decedent was shot with a Conducted Electrical Weapon (CEW) once, but he quickly recovered and aggressively approached law enforcement, at which time he was shot with bullets and died as a result. The autopsy was performed approximately 8 hrs after death. Subclavian blood samples were collected in gray stopper tubes containing sodium fluoride and potassium oxalate. An aliquot of the blood was sent to the commercial reference laboratory. Toxicology results were: ethanol 0.161 g/100mL, amphetamine 32 ng/mL, 9-carboxy-delta-9-THC 7.6 ng/mL, and 1,3-dimethylamylamine (DMAA) 95 ng/mL. The cutoff-concentration for DMAA was 50 ng/mL. The cause of death was listed as gunshot wound, and the manner of death was homicide.

Case 4: A 39 year old male was having a party at his residence when he took some type of substance and immediately collapsed. Witnesses stated that he had ordered some items from the internet which they thought were synthetic marijuana or bath salts. The autopsy was conducted approximately 4.5 hrs after death. Iliac blood samples were collected in gray stopper tubes containing sodium fluoride and potassium oxalate. An aliquot of the blood was sent to the commercial reference laboratory for their qualitative NBOME panel, via LC/MS/MS with a cutoff-concentration of 0.50 ng/mL. The toxicology results were: amphetamine 160 ng/mL, benzoylecgonine 50 ng/mL, hydrocodone 150 ng/mL, 25C-NBOMe and 25H-NBOMe present. The cause of death was multiple drug toxicity, with atherosclerotic and hypertensive cardiovascular disease contributing, and the manner of death was listed as accident.

Conclusion: Though not true in all instances, unusual behavior can be the result of drug use. These cases emphasize the importance of critically reading case narratives and officer reports in order to direct testing. Indications of unexplained behavior, paranoia, aggression or excited delirium may warrant comprehensive testing for designer drugs. Method development to routinely detect these drugs should also be considered.

Keywords: Designer Drugs, DOC, α-PVP, DMAA, 25C-NBOMe**,** Alabama, Postmortem Toxicology

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Blood Urea Nitrogen (BUN) Contamination of Hyaluronidase Used for Vitreous Electrolyte Analysis

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Introduction: The quantitative values of vitreous electrolytes hold toxicological significance in the diagnosis of dehydration, renal failure, hyperglycemia, and alcoholic ketoacidosis. Vitreous has long been a prized specimen for forensic drug analysis for many reasons, including isolation from potential contaminants, chemical composition that closely reflects that of plasma, and lower occurrence of postmortem redistribution than blood or other matrices. The primary disadvantages to utilizing vitreous fluid are the low sample volume and the specimen viscosity. The viscous nature of the specimen creates analytical difficulties, leading to imprecision and inaccuracy. Hyaluronidase is an enzyme utilized to significantly reduce the viscosity of the vitreous fluid to facilitate chemical analysis. The addition of hyaluronidase to these samples before analysis has become commonplace, and hyaluronidase is generally considered to be contaminant-free because of its high purity. Many studies have been performed to verify that no clinical differences exist between samples treated with or without hyaluronidase. Our laboratory observed biased results for BUN in controls while other electrolytes remained consistent, including creatinine. An investigation demonstrated that a new lot of hyaluronidase contained a contaminant that caused elevated results for BUN in proportion to how much enzyme was added. This appears to be a lot-specific phenomenon.

Objective: This study describes an instance where a specific lot of hyaluronidase was found and proven to be contaminated with urea nitrogen.

Method: Analysis of electrolytes within vitreous fluid was performed through the use of a chemical analyzer with an electrode biosensor, NOVA Biomedical Stat Profile Critical Care Xpress. Electrolytes were measured in a panel containing creatinine, sodium, chloride, potassium, glucose, and blood urea nitrogen. BUN, creatinine and other electrolytes were determined in control samples that were treated with different amounts of Hyaluronidase. Since this enzyme is added in minute quantities as a powder, the amount was subjectively determined by a single analyst and categorized as “less”, “normal”, and “excess.” The same procedure was performed using a previous lot of hyaluronidase that was known to produce consistent results, regardless of the amount added.

Results: BUN varied with hyaluronidase amount in the new lot of enzyme, but remained constant in the old lot. The other electrolytes and creatinine remained consistent in both lots. Therefore, a validation and verification process of hyaluronidase is recommended before production introduction.

Conclusions: An example is presented here of a reason that the hyaluronidase enzyme should be screened for potential contaminates before entering the production environment. One lot of purchased Hyaluronidase shows a direct correlation of increasing BUN values in relation to the amount added to the patient or quality control sample. Test results as high as 100 mg/dL above the target concentration were noted throughout this study. In this case, hyaluronidase was identified as a source of contamination and falsely elevating the BUN results of quality control material. This issue is significant and required that hyaluronidase lots be checked at varying amounts prior to use in a production environment.

Keywords: Hyaluronidase, Vitreous Humor, Electrolytes

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Analysis of Zolpidem in Postmortem Fluids and Tissues Using Ultra-Performance Liquid Chromatography-Mass Spectrometry

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Introduction: Zolpidem is a non-benzodiazepine sedative hypnotic drug used for the short-term treatment of insomnia. While quite effective in producing sedation, zolpidem has potentially hazardous side effects when put in the context of complex tasks.

Objective: To more fully understand postmortem concentrations of zolpidem, our laboratory has developed a sensitive method for the quantitation of zolpidem in biological specimens. We have evaluated the distribution of zolpidem in various postmortem tissues and fluids from 10 aviation fatalities. Each of these cases had a majority of the desired biological tissues and fluids available for analysis.

Method: This method incorporated a modified acetonitrile "crash and shoot" extraction and a Waters Xevo TQ-S with an Acquity ultra-performance liquid chromatograph (UPLC). A calibration curve at concentrations ranging from 0.2 to 800 ng/mL was prepared with bovine blood and purchased standards. Controls, also prepared with bovine blood and purchased standards, were prepared at concentrations of 1, 10, 100, and 500 ng/mL and analyzed with the unknowns to verify the accuracy of the calibration curve. The UPLC was operated at 0.400 mL/min with a mobile phase gradient of 70:30 A:B (A - water with 0.1% formic acid, B - acetonitrile with 0.1% formic acid), to 5:95 A:B, with a 2 µL sample injection volume. Method validation included various studies for ion suppression or enhancement, accuracy and precision, carryover, extraction efficiency, and stability. No ion suppression or enhancement was observed. Accuracy was expressed as relative error (%E), and precision was expressed as the coefficient of variation (CV). The intra-day %E was 9% and the CV was 4%. The inter-day study was reproducible with a %E of 7% and CV of 4%. There was no carryover observed. The extraction efficiency was 78 ± 2-% at 1 ng/mL, 83 ± 2-% at 100 ng/mL, and 87 ± 2-% at 500 ng/mL. Short term and intermediate stability of zolpidem in bovine whole blood had a maximum deviation of 6% from the target. Long term stability of a 90 ± 6 ng/mL (3 year mean) blood control was analyzed during the study with an 86 ± 1 ng/mL mean.

Results: The linear dynamic range was 0.4 – 800 ng/mL. The extraction efficiencies ranged from 78 – 87%, depending on the concentration. Postmortem blood zolpidem concentrations in these 10 cases ranged from 7.6 – 76.5 ng/mL, with a mean of 26.9 ng/mL. The actual site from which the blood was collected at autopsy is unknown for most of these cases, but the majority were labeled as "cavity". The mean concentration (ng/mL, ng/g) of zolpidem in the other types of specimens analyzed were: urine 20.8, liver 62.7, spleen 41.8, brain 14.2, kidney 37.9, muscle 10.6, heart 27.3, vitreous humor 14.9, and lung 39.4.

Conclusion: A modified "crash-and-shoot" method was incorporated to minimize preparation time and sample and solvent volumes. Seventy-eight tissue and fluid samples, from a total of 10 aviation accident fatalities, were analyzed to determine the zolpidem concentrations and postmortem distribution. The blood concentrations were all in the sub-therapeutic to low therapeutic range. This methodology was demonstrated to be highly effective for the identification and quantitation of zolpidem in various postmortem fluid and tissue specimens.

Keywords: Forensic Toxicology, Zolpidem, Ambien, Postmortem Distribution, LC/MS/MS, UPLC