New and Emerging "Nitazene" Analogues Appearing in Medicolegal Death Investigations: *N*-Pyrrolidino Protonitazene & *N*-Desethyl Isotonitazene

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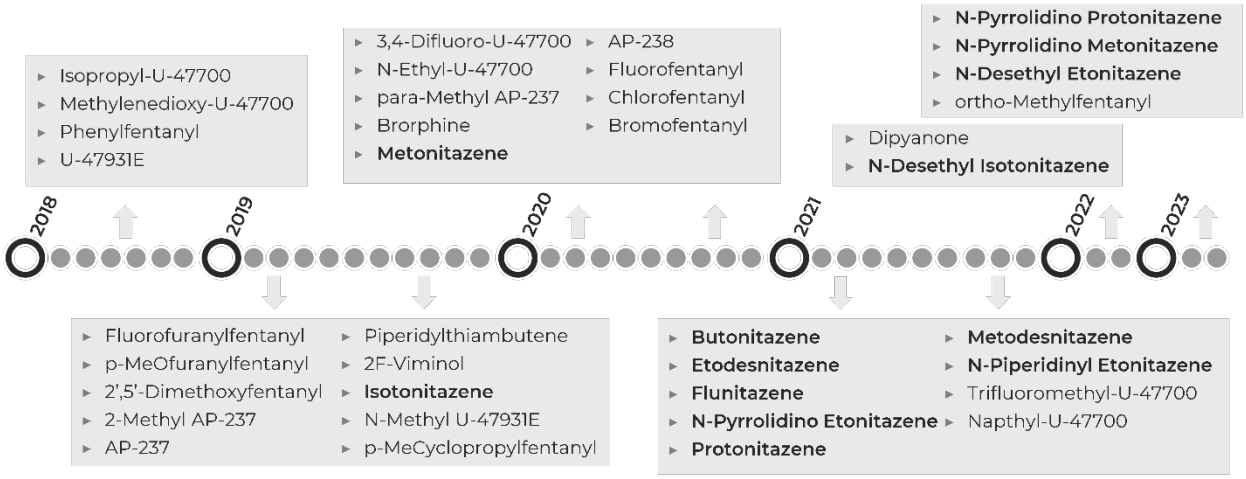
The "nitazene" sub-class of novel synthetic opioids has been increasingly identified since the emergence of isotonitazene in 2019. The emergence of this novel opioid, repurposed from pharmaceutical research, occurred after domestic legislation was enacted to curb the proliferation of fentanyl related substances, but it was only the first benzimidazole opioid to hit the recreational drug market (1). The "nitazene" subclass backbone is structurally distinct from fentanyl to evade the fentanyl-related compound scheduling, but compounds of this subclass still retain opioid receptor activity. Preclinical studies have estimated that the potency of nitazene analogues vary per substance, but many have been studied to be as potent or more potent than fentanyl (2). The "nitazenes" have infiltrated the illicit drug supply, and many end users may be unwitting in their exposure, as the "nitazene" analogues are often reported in seized drug and toxicology casework with fentanyl and/or designer benzodiazepines, in additional to other substances.

Similar to fentanyl-related analogues, slight chemical modifications to the core structure have resulted in new compounds within the subclass emerging, especially in response to scheduling efforts to curb these novel opioids. Isotonitazene was the first "nitazene", followed by other nitazene analogues that emerged in waves but also varied in both prevalence and geographical distribution. Some of the more prevalent substances included the following: metonitazene (2020), etodesnitazene (2021), *N*-pyrrolidino etonitazene (2022) and protonitazene (2022) **(Figure 1)**. The nitazene analogues have been identified globally but appear to be most detected in the midwestern part of the United States. More recently, *N*-desethyl isotonitazene and *N*-pyrrolidino protonitazene are two additional nitazene compounds that have emerged, further snowballing this category of novel opioids as even the "legacy" nitazene compounds continue to be detected in forensic casework.

Figure 1: Timeline of the emergence of novel synthetic opioids after the regulation of fentanyl and fentanyl analogues. "Nitazene" analogues are listed in bold print.



N-Desethyl isotonitazene was first identified by the CFSRE in a drug material submitted in December 2022, while *N*-pyrrolidino protonitazene was first detected in a biological sample submitted in January 2023 (3, 4). Due to these subsequent new emergences, a standard addition method was developed for the identification and quantitation of the "new generation" nitazene analogues, including *N*-pyrrolidino protonitazene, *N*-pyrrolidino metonitazene, *N*-pyrrolidino isotonitazene, *N*-desethyl isotonitazene and *N*-desethyl protonitazene. Chromatographic separation is necessary for structural isomers, especially for *N*-desethyl isotonitazene and *N*-desethyl protonitazene as their precursor and product ions are identical, with no unique fragment ions to target for quantitation. These drugs are both metabolites of the respective compounds isotonitazene and protonitazene, which are also isomers. Isotonitazene-D7 was used as the internal standard. Samples were prepared via a basic single-step liquid-liquid extraction. The quantitative range was assessed from 0.2-50 ng/mL. Quantitation was performed using a Waters Xevo TQ-S Micro liquid chromatograph tandem quadrupole mass spectrometer (LC-QQQ-MS). The method was successfully validated using standard addition based on ASB standards 036 and 054 (5, 6).

N-Pyrrolidino protonitazene and *N*-desethyl isotonitazene **(Figure 2**) were commonly detected substances of the "nitazene" subclass from forensic cases in 2023 and continue to be reported in 2024. Both drugs were qualitatively and quantitatively identified in postmortem blood across the United States (primarily northeast and Midwest) and United Kingdom (UK). *N*-Pyrrolidino protonitazene was qualitatively identified in 39 samples and quantitated in 26 postmortem samples with concentrations ranging from 0.3 to 55 ng/mL (mean ± standard deviation: 8.0±17, median: 1.2 ng/mL). *N*-Desethyl isotonitazene was identified in 16 samples and successfully quantified in 9 postmortem samples ranging from 0.82 to 8.3 ng/mL (mean ± standard deviation: 4±2, median: 3.4 ng/mL). In one case from the UK, *N*-pyrrolidino protonitazene was identified at a concentration of 55 ng/mL in subclavian blood, with no other traditional or synthetic opioids detected (other findings: bromazolam, caffeine, cotinine, naloxone). *N*-Pyrrolidino protonitazene and *N*-desethyl isotonitazene were commonly detected alongside other novel psychoactive substances (e.g., bromazolam, para-fluorofentanyl), traditional drugs (e.g., fentanyl, psychostimulants) and common adulterants (e.g., quinine, xylazine).

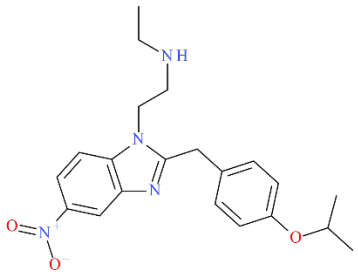
Between October 2023 and April 2024, NMS Labs reported *N*-desethyl isotonitazene qualitatively in 24 postmortem blood samples in the absence of isotonitazene, with the earliest collection date listed in August 2023. In addition, NMS Labs has reported *N*-pyrrolidino protonitazene qualitatively in 29 postmortem bloods, with the earliest collection date listed as October 2023. For these two compounds, samples were submitted from 20 different US states, four Canadian provinces, and the UK. The 11 different states were geographically diverse, including states from across the country. The Miami-Dade Medical Examiner Dept (MDME) reported two postmortem cases in October 2023 that were positive for *N*-pyrrolidino protonitazene and one case in January 2024 positive for *N*-desethyl isotonitazene, without the presence of isotonitazene. Of note, all three cases were also positive for bromazolam

While *N*-desethyl isotonitazene is a known active metabolite of isotonitazene, it was identified in this case series in the absence of isotonitazene. As *N*-desethyl isotonitazene has since been detected in drug materials as the primary drug and has not been identified as a metabolite without the presence of isotonitazene, it is assumed that *N*-desethyl isotonitazene was the drug consumed in these cases (7, 8). *N*-desethyl isotonitazene was commonly found alongside designer benzodiazepines, specifically bromazolam, while *N*-pyrrolidino protonitazene was commonly discovered with other nitazene analogues, such as metonitazene, protonitazene, and *N*-pyrrolidino metonitazene.

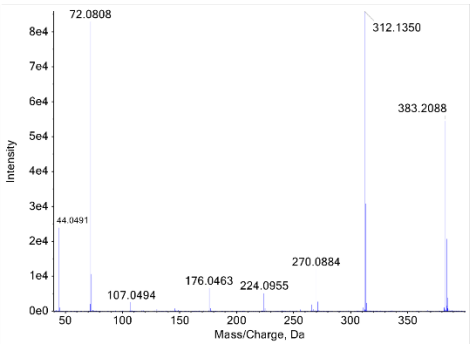
The Center for Forensic Science Research and Education (CFSRE) was able to identify and quantify low levels of *N*-pyrrolidino protonitazene and *N*-desethyl isotonitazene using standard addition methodology in postmortem medicolegal death investigations. For detailed method and case information, please visit the *Archives of Toxicology Journal* which recently accepted the manuscript entitled "*In Vitro* Structure-Activity Relationships and Forensic Case Series of Emerging 2-Benzylbenzimidazole 'Nitazene' Opioids (De Vrieze & Walton et al. 2024). The CFSRE and SOFT NPS Committee release quarterly scope recommendations, where *N*-desethyl isotonitazene and *N*-pyrrolidino protonitazene have been listed as recommended or strongly recommended since Q1 2023 and Q3 2022, respectively (9). The rapid identification of emerging nitazene analogues and isomers remains a constant challenge for forensic toxicologists, and the low to sub-ng/mL concentrations of these drugs forces the use of sensitive methodology and instrumentation, in addition to frequent scope updates in response to surveillance efforts.

Figure 2: Molecular structure, mass, and LC-QTOF-MS/MS spectra of **(left)** *N*-desethyl isotonitazene and **(right)** *N*-pyrrolidino protonitazene.

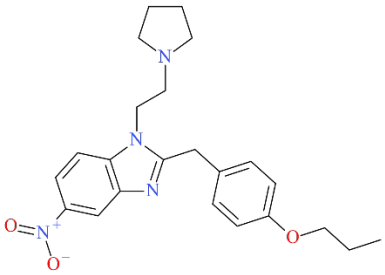
N-Desethyl Isotonitazene



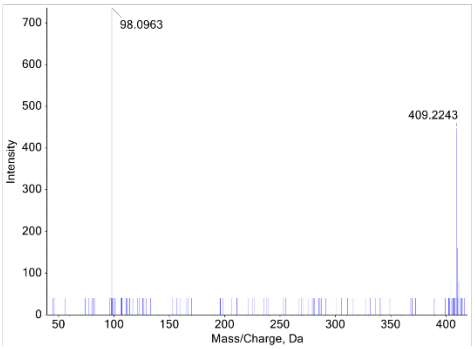
Synonyms: Desethyl Isotonitazene, “Des-Iso”
Molecular Weight (Nominal Mass): 382.5
[M+H]+: 383.2078
Pharmacological Drug Class: NPS Opioid, CNS Depressant
Suggested LOD: <1 ng/mL
LC-QTOF-MS/MS Spectrum:



N-Pyrrolidino Protonitazene



Synonyms: Protonitazepyne
Molecular Weight (Nominal Mass): 408.5
[M+H]+: 409.2234
Pharmacological Drug Class: NPS Opioid, CNS Depressant
Suggested LOD: <1 ng/mL
LC-QTOF-MS/MS Spectrum:



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