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PHARMACOKINETIC MODELING OF METONITAZENE AND ISOTONITAZENE USING IN VITRO AND IN VIVO MODELS AND COMPARISON TO HUMAN POSTMORTEM CASEWORK

A dissertation submitted in partial fulfillment
of the requirements for the degree of

DOCTOR OF PHILOSOPHY

to the faculty of the

DEPARTMENT OF PHARMACEUTICAL SCIENCES

of

COLLEGE OF PHARMACY AND HEALTH SCIENCES

at

ST. JOHN'S UNIVERSITY
New York
by
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Date Submitted 4/2/2024 Date Approved 6/6/2024

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ABSTRACT

PHARMACOKINETIC MODELING OF METONITAZENE AND ISOTONITAZENE USING IN VITRO AND IN VIVO MODELS AND COMPARISON TO HUMAN POSTMORTEM CASEWORK

Justine P. Sorrentino

Metonitazene and isotonitazene are two benzimidazole opioids that are contributing to the opioid crisis nationwide. Limited pharmacokinetic data has been published on these nitazene compounds, therefore this study sought to determine if these two structurally similar opioids would have comparable half-lives and clearance utilizing *in vitro* and *in vivo* models. Using the data determined in the pharmacokinetic study, and by testing human postmortem cases, this study aimed to inform interpretation of these drugs detected in human casework. Sample analysis for all aspects of the study was performed utilizing LC/MS/MS.

In vitro pharmacokinetics of both drugs were examined using a human liver microsomes (HLM) model. Drug solutions at 2.5 ug/mL were incubated with HLM at 1 mg protein/mL. Metonitazene and isotonitazene showed identical rates of metabolism in this model when comparing both mean half-lives (22.6 and 21.9 min) and intrinsic clearance rates (36.5 and 37.2 mL min^{-1} kg^{-1}) respectively. These half-lives were both significantly shorter than the *in vivo* study.

Sprague Dawley rats were administered a single dose of drug intravenously at 2.5 μg/kg or intraperitoneally at 25 μg/kg. Blood and urine were collected over six hours, and brain and liver were dissected. Metonitazene (88.5 min IV and 117.5 min IP) exhibited a

shorter half-life compared to isotonitazene (44.7 min IV and 56.4 min IP) in both administrations. The mean calculated volume of distribution for isotonitazene was higher than metonitazene, 59.6 mL and 37.3 mL respectively, which likely contributed to the longer half-life. In rat urine samples, two metabolites were detected.

In human postmortem cases, blood levels for isotonitazene in ten cases, ranged from 0.11 – 12.0 ng/mL and metonitazene in six cases ranged from 0.10 – 1.5 ng/mL. Metonitazene was more likely to be detected in urine. All four metabolites tested were detected in human samples, therefore species differences were exhibited as compared to rats.

It has been proven from *in vivo* studies that despite their structural similarities, metonitazene and isotonitazene show significant differences in clearance from the body. Nitazenes continue to emerge on the illicit market, and this study provides information on how this class of drugs behaves in the body.

DEDICATION

Dedicated to my grandmother, Louise M. Pardi, who never got to see me complete this degree, but who I know is cheering me on from above.

ACKNOWLEDGEMENTS

I want to take this opportunity to thank the many people that were with me through this project, without whom I would not be at this point today.

To Dr. Ford, thank you for your support and guidance throughout the many steps of this project. You have helped me grow as a person and a scientist through this process. I cannot explain enough how grateful I am that you allowed me to choose two controlled substances to study, and for your efforts in obtaining a drug license, which you easily could have decided against.

Thanks to Dr. Cooper for your encouragement to pursue this degree while working full time, always reassuring me I am capable of it, and for your understanding when I needed to set work aside and take time off to focus on this project. You are always a listening ear when I need it, open and understanding to discuss any challenges, and I am very grateful for that. Sincere thanks to the rest of my committee, Drs. Billack, Patel, Trombetta, and Yoganathan for your feedback and guidance.

I am also extremely grateful to the staff in the ACC, namely Eileen, Nancy, and Ernie, that taught me to work with animals both confidently and with care. I never would have been able to accomplish this project without you and your encouragement. Thank you to my classmates, lab mate Belkys, and the other students that worked in the imaging suite that made the hard days more bearable.

To all my friends and family, thank you for cheering me on through the ups and downs of this process. Thank you for your understanding when this project had to be my priority and I was not able to be as present as I would like to be – both physically and

mentally. To my parents, John and Janel, you mean the world to me, and I am so thankful to have your guidance and support always.

Finally, my utmost gratitude to my husband, Joseph. You met me when I was in the middle of this endeavor, and yet I cannot imagine finishing it without you. Your love, reassurance, and delicious home cooking got me through the lows of this process, and your celebration with me through the highs are both equally acknowledged and appreciated. I love you.

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CHAPTER 1: INTRODUCTION

1.1. The Opioids Crisis

According to the Centers for Disease Control and Prevention (CDC), more than one million people have died due to drug overdose since 1999 in the United States (U.S.) (1). The first wave of increasing opioid-related deaths began in the 1990s when the number of opioid prescriptions written for Americans began to rise (2). The second wave started when drastic increases of heroin deaths occurred beginning in 2010, which is when the opioids crisis switched from a prescription opioids problem, to an illicit opioids one (2, 3). Changes in the practices of prescribing opioids drove people with opioid dependence disorders to turn to illicit opioids (3). Then the third wave in the rise of opioids deaths began in 2013 with non-methadone synthetic opioids, mostly due to fentanyl, as foreign-produced illicit fentanyl became available (2, 3). A summary of the three waves of the opioid epidemic is shown in Figure 1.

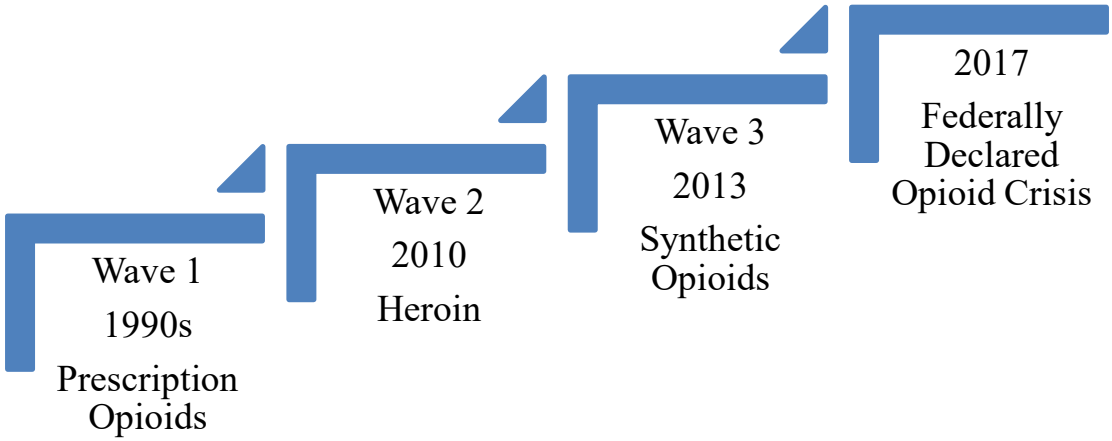

Figure 1. Timeline of the three waves of opioid overdose deaths. They are classified by when deaths started to rise and when the U.S. federally recognized the opioid crisis.

In October 2017, the federal government declared a nationwide public health emergency as a result of the opioid crisis, and this formal declaration has been renewed quarterly since 2017 as opioid-related deaths continue to rise (4). In 2017, the opioids epidemic cost the U.S. economy 1.02 billion dollars due to healthcare and treatment costs, lost productivity, and reduced quality of life (5). Synthetic opioid deaths are continually increasing as deaths in 2021 were 23x those in 2013, when the synthetic opioid wave of the crisis began (6). In comparing 2020 to 2021 data of the current drug climate, total opioid-involved death rates continued to increase by over 15%, while heroin-related deaths decreased and prescription opioid-related deaths stayed the same, and synthetic opioid deaths increased by over 22% (2).

1.2. Epidemiology of Opioids Abuse

Geographically, the eastern half of the U.S. overall sees higher incidences of drug overdose mortalities. The highest numbers of deaths per 100,000 people (age-adjusted) in the country are reported in West Virginia with 90.9 followed by Tennessee with 56.6 (7). In 2021, 106,699 drug overdose deaths occurred in the U.S., 75% of which were due to an opioid (1).

Opioid overdose deaths in New York (NY) state since 2010 mirror those in the U.S. as a whole. (Figure 2). After federal resources were put in place to combat the opioid crisis in 2017, for two years the numbers of deaths in NY decreased, dropping from 3,224 in 2017 to 2,939 in 2019 (8). However, due to many factors, the COVID-19 undid this small improvement, and opioid mortality rates have continued increasing ever since (8). In 2021, about 30 per 100,000 residents of the state of New York died of a drug overdose, 25 of those due to an opioid (9). Between 2021 and 2022, there was a 12%

increase in drug overdose deaths in New York City (NYC), and rates are the highest since reporting began in the year 2000 (10). In NYC in 2022, the top five neighborhoods with the highest rates of drug overdose deaths were all located in the Bronx (10). Statewide, opioid overdose rates in 2021 were also highest in Bronx county (11). Similar to the U.S. data, heroin deaths decreased in NYC from 53% of overdose deaths involving heroin in 2019 to 32% in 2022. In NYC, opioids continue to drive overdose deaths with 85% of all overdoses involving an opioid, 81% of those involving fentanyl (10).

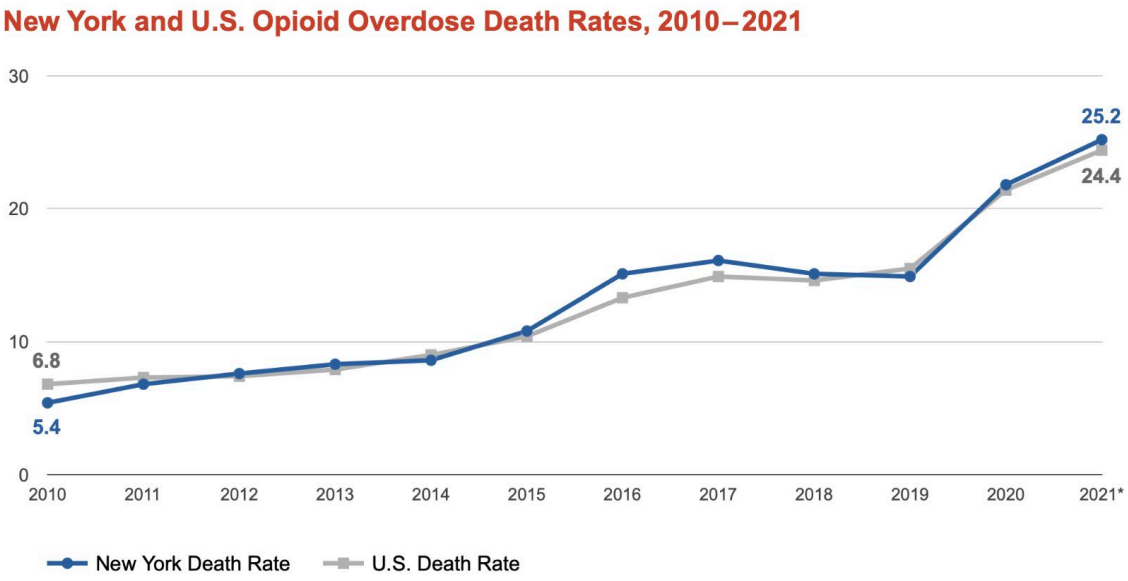

Figure 2. New York and US Overdose Death Rates, 2010 to 2021 age-adjusted per 100,000 population. Source: CDC, National Center for Health Statistics.

A compilation of reported benzimidazole opioid cases showed that like overall drug mortality trends, nitazenes are mostly confined to the Central and Eastern U.S. (12). Worldwide, nitazenes have been reported in Canada, across Europe, and are specifically trending in the U.K., and the Baltic States (13, 14, 15).

The Montanari et al. review also found that worldwide, isotonitazene cases (n=65) were reported between 2019 and the first half 2020, and metonitazene cases were detected after November 2020 (n=20) (12). They argued this indicated a shift in drug trends where metonitazene took the place of isotonitazene on the illicit drug market (12). Similar claims that isotonitazene was replaced on the drug market were made by other publications, including a 2022 report that isotonitazene would be replaced by brorphine, another novel synthetic opioid (16). They also reported the total lifecycle of isotonitazene to be 12 to 16 months with the peak positivity rates in the first half of 2020 (16). This report indicated the possibility of metonitazene taking the place of brorphine as its positivity rate dwindled (16). As will be reported in our study, these drug trends do not match NYC data for metonitazene and isotonitazene detection.

1.3. Benzimidazole Opioids

Substances that are naturally occurring and derived from the opium poppy are classified as opiates, while any natural or synthetic compound that interacts with opioid receptors are called opioids (3). The structures of three representative opioid drugs are seen in Figure 3 (17). Most naturally-derived opioids are part of the 4,5 expoxymorphinans structural class as well as some semi-synthetic opioids which include morphine, hydromorphone, codeine, hydrocodone, oxycodone, and oxymorphone (17). The phenylpiperidines structural class includes fentanyl and fentanyl analogs and derivatives, and the diphenylheptylamines include methadone (17). While the 2-benzybenzimidazole opioids are structurally dissimilar to the other classes of opioids, they are opioids in activity as they target the mu opioid receptor (MOR) (18).

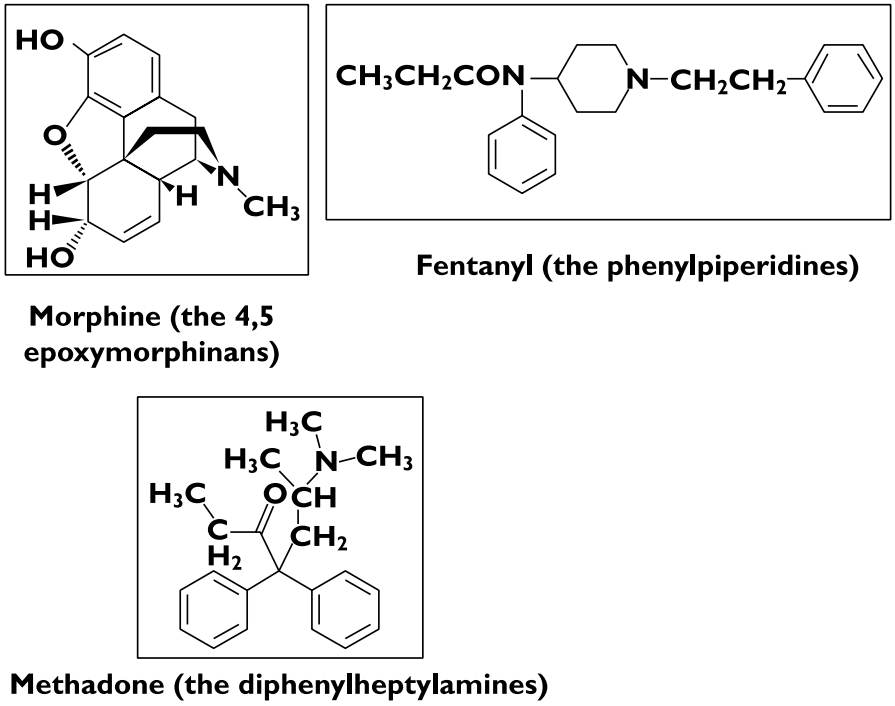

Figure 3. The three main structural classes of opioids represented by morphine, fentanyl, and methadone. Source 17, used with permission.

Benzimidazole, or 1-h-benzimidazole, is a compound composed by the fusion of a benzene and imidazole ring, the structure of which is seen in Figure 4 (19). Research on this compound and its derivatives started in the 1940s and today they are used in numerous pharmaceutical applications from antiviral to antihypertensive drugs (20). The "nitazene" drug class are derivatives of this compound as 2-benzylbenzimidazoles, also known as benzimidazole opioids. Benzimidazole opioids were first synthesized in the 1950s for their analgesic effects but were never approved for clinical use (21). Examples of these include etonitazene, clonitazene, and metonitazene which were reported to have selective affinity for the MOR and morphine-like effects (13, 23). A clinical trial utilizing metonitazene for post-operative pain showed 10x the potency to produce analgesia compared to morphine,

but also showed severe side effects such as confusion, nausea, respiratory depression, and cyanosis and was excluded from further clinical investigations (12).

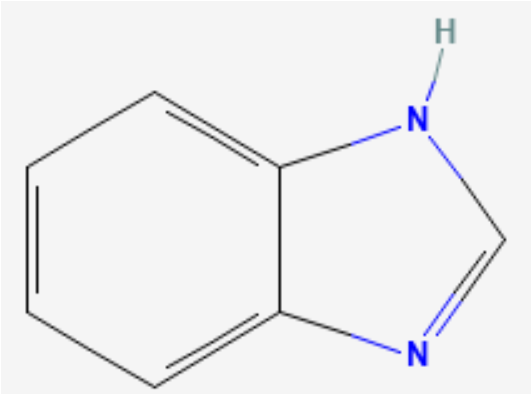

Figure 4. Benzimidazole, core structure of the nitazene drug class.

In 1960, another benzimidazole opioid, isotonitazene, was synthesized by Hunger et al., and reported to have 500x the potency of morphine in mice, and subsequently no clinical trials were initiated for this drug (23). In 1975, Shulgin predicted the benzimidazole opioid class as having potential use as heroin substitutes in the future due to the ease of illicit production and potent effects (24). Outside of limited reports on etonitazene from the 1960s to early 2000s, these compounds were not discussed again until isotonitazene emerged on the illicit drug market in 2019 (13, 22).

Isotonitazene (N,N-diethyl-2-[(5-nitro-2-{[4-(propan-2-yl)oxy]-phenyl}methyl)-1H-benzimidazol-1-yl]ethan-1-amine), the structure of which is seen in Figure 5, was the first nitazene to be available in online markets (25). It was first reported in August 2019 after detection in drug material seized in Belgium. The first reports in the U.S. came from a published alert on eight cases originating in Illinois and Indiana between August

through October 2019 (26). Isotonitazene was temporarily scheduled as Schedule I by the Drug Enforcement Agency (DEA) in August 2020 where it has remained (27). Drug effects as described in online drug forums include strong analgesia and long-lasting euphoria, and reports of administration include intravenous injection, sublingual, vaping, snorting, and smoking (12). Physical dependence symptoms have also been reported after recreational use (12).

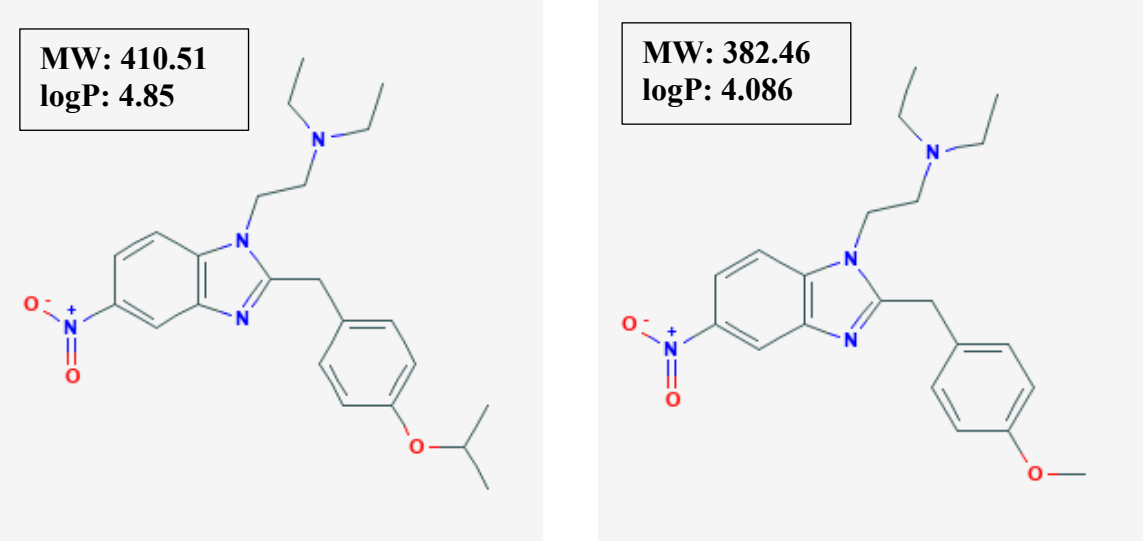

Figure 5. Structures of isotonitazene (left) and metonitazene (right) with associated molecular weight (MW) and octanol/water partition coefficients (logP).

Metonitazene (N,N-diethyl-2-{2-[(4-methoxyphenyl)methyl]-5-nitro-1H-benzimidazol-1-yl}ethan-1-amine), seen in Figure 5, was first illicitly detected in seized drug powder in July 2020 (28). The first detection in human postmortem samples were reported between August and December 2020 in Illinois, Indiana, Ohio, Tennessee, and West Virginia. Metonitazene was categorized as Schedule I by the DEA in April 2022 (29). Metonitazene has been described on drug forums to be an exhilarating analgesic

drug that increases sociability and has been reportedly administered in multiple ways by ingestion, injection, snorting, smoking, vaping, and intranasally (11, 19).

One of the dangers of these drugs is their combination in drug products in unspecified amounts, or unknown to the users themselves (31). Metonitazene and isotonitazene have been reported in multiple formulations such as tablets and liquids, but are most commonly found as powders (19, 21). They can be found in drug product combinations with heroin, fentanyl, and benzodiazepines (22). Isotonitazene was reported in multiple instances as being falsely marketed as another drug. In Canada in 2019, Dilaudid pills of apparent pharmaceutical quality were seized and discovered to be isotonitazene with no presence of hydromorphone whatsoever (18). Another incidence in Canada was reported by CBC News in Montreal in January 2024, of a father warning the public after the death of his 15-year-old son who overdosed on isotonitazene after thinking he had purchased oxycodone pills (33). Another parent was interviewed in Annapolis, Maryland who revealed her 29-year-old son died due to an overdose of isotonitazene and designer benzodiazepine, bromazolam, after taking a pill thought to be Xanax (34).

The structures of these compounds also play into the risk of use. It has been shown that even subtle alteration to the structures of novel opioids can greatly impact receptor selectivity, duration of action, and their potency (35). This phenomenon has been demonstrated in the multitude of fentanyl analogs, where minor structural modifications result in significant changes to both opioid receptor affinities and metabolic pathways (37, 38). Metonitazene with a partition coefficient (logP) of 4.086 and isotonitazene with a logP of 4.85 show high lipophilicity which results in rapid entry

into the central nervous system (CNS) (15). Comparatively, morphine has a logP of 0.9, heroin 1.58, and fentanyl of 3.8 (26, 27). This rapid penetration causes severe respiratory depression and makes intervention difficult (31). Respiratory depression leads to apnea and cardiac arrest, which can result in hypoxic brain damage in both fatal and nonfatal overdoses of synthetic opioids (3, 20). Naloxone is used as an antidote for opioid overdoses and can reverse respiratory depression as it acts as a competitive antagonist at all opioid receptors, most strongly at the MOR (3). However, naloxone has shown to have its limitations. Naloxone has a 60-to-90-minute duration of action which can be much shorter than the duration of action of abused synthetic opioids, including fentanyl (3, 28). Therefore, the reversal of the effects of the opioid can be short lived leading to renarcotization (3, 20, 28). Higher or additional naloxone doses are required, and also indicated when used against highly potent opioids, like the nitazenes, that more avidly bind to the MOR (3, 20, 28).

Recent research has confirmed the dangers of nitazenes from historic reports in many studies on pharmacodynamics (PD) and potency at the MOR. Vandeputte et al. in 2021 performed *in vitro* potency studies for fourteen nitazene compounds including metonitazene and isotonitazene (21). This study found that N-desethylisotonitazene was the most potent of all fourteen analytes, including its parent compound, isotonitazene. Isotonitazene was the next most potent after N-desethylisotonitazene and etonitazene. Another study into isotonitazene potency at the MOR was conducted by Blanckaert et al., utilizing a drug powder sample purchased online in comparison to an analytical standard of isotonitazene and hydromorphone as a reference. They found that the seized drug powder concentration-response curve overlapped with the isotonitazene standard,

showing a high-purity product available for purchase online (25). They also determined that the potency and efficacy of isotonitazene was higher than fentanyl, EC_{50} of 11.1 nM and 18.7 nM respectively, and E_{max} of 180% and 155% compared to hydromorphone respectively (25).

A summary of *in vivo* studies was compiled by Ujváry et al, including research of antinociceptive activity utilizing the tail-flick tests in mice with a comparative dose of 5 mg/kg morphine. The results showed as relative to morphine, 100x potency for metonitazene and 500x for isotonitazene (18). A study of respiratory depression in rabbits showed that etonitazene and metonitazene had the most significant effect of all the nitazenes tested, after intravenous (IV) doses at 0.5 and 10 μg/kg respectively, 50% decrease in respiratory frequency was observed (18). Pharmacodynamic effects of isotonitazene in Sprague-Dawley rats reported by Walton et. al. utilizing subcutaneous (SC) injection at multiple doses, 3, 10 or 30 μg/kg showed that high doses (30 μg/kg) induced hypothermia. They showed hot-plate latency increases at all three doses tested, continuing for up to 120 minutes post-injection in the 30 ug/kg group (41). Catalepsy was scored based on appearance of splayed limbs, flattened posture, and immobility. The highest dose showed the maximum score up to 60 min post injection, whereas 10 ug/kg showed increased catalepsy up to 30 min post-injection, and the 3 ug/kg dose showed no catalepsy compared to the vehicle control (41). This study reported their results in comparison to other rat antinociception studies and determined that isotonitazene is approximately 1000-fold more potent than morphine and five-fold more potent than fentanyl (41).

1.4. Use of *In Vitro* and *In Vivo* Models in Pharmacokinetics

Pharmacokinetics (PK), also known as toxicokinetics, is the movement of a drug from its site of administration to site of action and describes the absorption, distribution, metabolism, and excretion (ADME) of compounds through experimental studies (40, 41). PK parameters that can be experimentally determined include the volume of distribution, half-life, clearance rate, and elimination rate constant. The volume of distribution is defined as the apparent volume into which a substance is diluted, with low volume of distribution corresponding to hydrophilic substances that do not distribute into tissues or those that are protein bound in plasma. Whereas higher volume of distribution are characteristic of hydrophobic substances that widely distribute into fat and tissue stores in the body (42). The half-life is the time taken for the concentration of a substance in the body to decrease by half (42). The clearance is the apparent volume of plasma from which a drug is removed over time, and is determined using IV drug administration (44). The total removal of a substance from systemic circulation is the sum total of clearance from all tissues (40, 42). The elimination rate constant is defined as the fraction of the substance removed from the body per unit time (42). In evaluation of the PK behavior of novel substances, both *in vitro* and *in vivo* models have been used and can be useful for forensic toxicologists in interpretation of the significance of these novel drugs.

In vitro models of drug metabolism assess *in vitro* half-life and intrinsic clearance (CL_{int}) (45). Intrinsic clearance is the ability of the liver to metabolize a substance without any restrictions such as blood flow or protein or cell binding (45). The use of hepatocytes for PK studies has been documented as a primary screening tool to the effects of first-pass metabolism in drug discovery, for calculation of intrinsic clearance,

and in metabolite elucidation (44, 45). Human liver microsomes (HLM) are also utilized as an *in vitro* PK modeling system. HLM are prepared using differential centrifugation of homogenized liver, resulting in subcellular fractions derived from the smooth endoplasmic reticulum (45). Human liver microsomes have been reported to be an ideal system due to their CYP enzyme concentration, long-term stability, and reproducibility (48). *In vitro* PK parameters are calculated using HLM by incubating the drug with HLM and determining drug depletion over time (45).

In vivo PK studies in a rodent model are a critical part of the novel drug discovery process and is often performed after novel drugs have arisen on the illicit drug market to better understand how these compounds are affecting humans (43). Rodents are the ideal model for these studies due to their genetic similarity to humans (>97%), their size, ability to reproduce rapidly, and adaptability. PK studies of novel opioids in a rat model have been extensively reported, with some examples in recent years including cyclopropylfentanyl PK in male rats by Bergh et al. in 2021 and U-47700 PK in male rats by Truver et al. in 2020 (47, 48).

1.5. Pharmacokinetics of Opioids

Overall statements regarding the ADME of opioids are challenging due to the structural differences between this diverse drug class. The majority of opioids show high gastrointestinal permeability, which makes them effective orally administered drugs (17). Some exceptions include fentanyl and buprenorphine which show high first pass metabolism, and therefore are not therapeutically administered orally (17). Illicit fentanyl is administered orally via tablets but due to the potency of these preparations, sometimes in doses considered lethal, effective amounts of fentanyl are still able to reach systemic

circulation (1, 2). Other common methods of administration for opioids, especially those that cannot be given orally, include using transdermal patches, intravenous injection, and sublingual routes (17). The main target for distribution of opioids therapeutically as analgesics, and the reason for the desired euphoric and sedative effects sought by illicit users, is their action in the central nervous system (CNS) (3, 5).

Opioids are generally excreted by the kidneys as metabolites (17). As an example of this, less than 8% of fentanyl is excreted unchanged (39). In persons with decreased kidney function, a buildup of active metabolites can cause differences in pharmacological effects compared to people that more efficiency excrete these substances, including the actions of morphine-6-glucuronide (17).

Metabolism of opioids can show notable inter-individual variability in the extent of first-pass and successive metabolism due to multiple factors such as history of drug exposure and genetics (35). First-pass metabolism is when a drug is metabolized prior to reaching systemic circulation which reduces the bioavailability of the opioid (5, 49). This metabolism after oral administration occurs mainly in the liver but the gastrointestinal tract can also contribute (52). Phenylpiperidines are metabolized by CYP3A4 and usually the metabolites generated are not pharmacologically active. These compounds, such as fentanyl, also show little inter-subject variability when it comes to pharmacologic action (17). 4,5-Epoxymorphinans metabolism occur by O-dealkylation catalyzed by CYP2D6 enzymes, i.e. codeine to morphine, hydrocodone to hydromorphone, and oxycodone to oxymorphone (17). These metabolites are pharmacologically active and often have higher potencies than the parent drugs, classifying the parent drugs as prodrugs (3,17). Heroin is also a prodrug as once it crosses the blood-brain-barrier (BBB) it is hydrolyzed to 6-

monoacetylmorphine (6-MAM) then to morphine in the CNS and periphery (3). Some of the enzymes responsible for opioids metabolism, the cytochrome p450 (CYP) family, exhibit polymorphisms which can affect the physiological effects of the drug and duration of action (17). As an example of genetic polymorphisms, therapeutic doses of codeine could result in morphine overdoses in persons characterized as ultrarapid metabolizers with multiple functional copies of the CYP2D6 gene (53).

Metabolites for isotonitazene and metonitazene were reported by Krotulski et al. by analyzing authentic case samples positive for the parent drug using liquid chromatography high resolution mass spectrometry (LC-HR-MS) instrumentation. In 2020, isotonitazene metabolites were reported. By evaluating urine specimens, isotonitazene undergoes dealkylation to form N-desethylisotonitazene, N-desethyl-O-desalkyl-isotonitazene, and 4'-hydroxy nitazene (54). 5-Aminoisotonitazene, formed by reduction of the nitro group, was only found in 2 out of 18 urine samples, but was found in almost all blood samples (54). Metonitazene metabolism was reported in 2021 by the same group. They reported metabolism similar to isotonitazene where dealkylation resulted in prominent urinary metabolites N-desethyl metonitazene, N,N-didesethyl metonitazene, and 4'-hydroxy nitazene (55). 4'-Hydroxy nitazene has been found to be a shared metabolite between multiple nitazene drugs. Metonitazene also has a nitro reduction metabolite, 5-aminometonitazene, which is detected primarily in blood samples (55).

While ample research has been reported on the pharmacokinetics of opioids, as well as the pharmacodynamics of isotonitazene and metonitazene specifically, limited data has been published on the pharmacokinetics of these compounds. At the inception of

this project, no data on pharmacokinetics had been published except for metabolite elucidation for both compounds. Still today, no *in vivo* pharmacokinetic data on metonitazene has reported, and limited data has been published on isotonitazene which will be summarized and discussed.

1.6. Analysis of Novel Opioids in Toxicology Casework

One of the biggest challenges facing forensic toxicology labs today is keeping up with the ability to detect emerging novel psychoactive substances (NPS). Public health and early warning reports of NPS in biological sample detection or seized drug constituents can alert labs to gaps in their testing scope, but it can take significant time to be capable of comprehensive reporting of the novel drugs (16). Additionally, resources in laboratories vary greatly in regard to their ability to spend analyst time to validate new methods and/or having funding to purchase and maintain sensitive analytical instrumentation to detect these substances.

Screening techniques in forensic toxicology are the first tests performed on an unknown sample to determine the presence of classes of drugs or an individual compound, depending on the technique used. It has become clear that immunoassays, historic and frequently used screening methods, have limited cross-reactivity with commercially available kits and are unable to detect novel opioids (56). Screening techniques utilizing gas chromatography mass spectrometry (GC/MS) are also very common, but have proven to have inadequate sensitivity for detection of synthetic opioids at low concentrations such as fentanyl and fentanyl analogs (57). Targeted screening methods utilizing liquid chromatography tandem mass spectrometry (LC/MS/MS) have been reported for synthetic opioids and expansive NPS panels by

multiple laboratories utilizing 0.2 – 0.5 mL sample volumes (55, 57, 58). Laboratories with high resolution mass spectrometry (HR/MS) technologies like time-of-flight mass spectrometry (TOF/MS) have reported success with non-targeted screening for NPS opioids including nitazenes, as well as the benefit of the ability to data mine previously analyzed samples for novel compounds without re-analysis (53, 54, 59).

The New York City Office of Chief Medical Examiner (OCME) forensic toxicology lab began screening for metonitazene and isotonitazene in March 2021. The protocol for detection is targeted screening by LC/MS/MS for all cases where an opioid screened positive, or where there is case history or suspicion of novel opioids use. Cases are reported qualitatively as compared to matrix-matched calibration samples at 0.10 or 1.0 ng/mL. Results are conveyed in the form of toxicology reports to medical examiners to aid in death investigations.

1.7. Hypothesis and Research Objectives

The aim of this study is to determine the pharmacokinetic parameters of two illicit benzimidazole opioids that are contributing to overdose deaths in New York City in collaboration with OCME. The first hypothesis is that isotonitazene and metonitazene metabolic profiles will be significantly different despite their structural similarities, like is observed in the case of fentanyl analogs. This hypothesis is further supported by their differences in partition coefficients which affect the ability of the drugs to distribute into tissues. The second hypothesis is that intravenous administration will show longer detection windows and elimination rates compared to intraperitoneal injection due to the first-pass metabolism effect. The third hypothesis is that metonitazene and isotonitazene will be detected in rat brain samples due to their action in the CNS. Finally, we aim to

inform the interpretation of human postmortem casework by retesting authentic casework and utilizing the results of drug distribution in animal samples, and two pharmacokinetic models. Therefore, the following objectives have been proposed for this research:

- To utilize a human liver microsomes model to calculate pharmacokinetic parameters to compare to *in vivo* data and determine if the two analytes show similar metabolic stability *in vitro*.
- To utilize a Sprague-Dawley rat model to experimentally determine pharmacokinetic parameters for both drugs utilizing two different administrations, intravenous and intraperitoneal. Over the course of the study, limited pharmacokinetic data has been reported on isotonitazene, which will be compared and discussed to this research. No data has been published on the pharmacokinetics of metonitazene apart from metabolite elucidation. Therefore, metonitazene pharmacokinetics will be novel to this study. The results from both administration types will be compared to each other, as well as to the *in vitro* results.
- To determine the distribution of both drugs into brain, liver, and urine matrices in rat samples after a single administration.
- To retest human postmortem cases received at OCME previously reported qualitatively using the validated quantitative method for all biological matrices available. This will aid in understanding of concentrations detected in forensic toxicology casework and give insight to the best samples to collect and analyze in suspected nitazene cases.
- To develop and validate a method for the detection of isotonitazene and metonitazene in blood, plasma, brain, liver, and urine, utilizing a low sample volume. It was determined at the inception of this project that the limiting factor for sample volume in this study would

be the rat plasma samples. A method utilizing 50 μL of sample will be attempted, which is lower than any sample volume for analysis that has been published. The validated method will be utilized for sample analysis for all phases of the study.

CHAPTER 2: MATERIALS AND METHODS

2.1. Chemicals and Reagents

All analytical reference standards utilized for this study were purchased from Cayman Chemical (Ann Arbor, MI). These included metonitazene citrate (1 mg, catalog no. 31303) and isotonitazene (1 mg, catalog no. 27255), the metabolites: N-desethyl isotonitazene hydrochloride (1 mg, catalog no. 30216), N-desmethyl metonitazene hydrochloride (1 mg, catalog no. 38178), 5-aminoisotonitazene (1 mg/100 µL, catalog no. 29318), and 4'-hydroxy nitazene (1 mg, catalog no. 30218) and internal standards (IS), metonitazene-d_3 citrate and isotonitazene-d_7 at 1 mg (catalog no. 31648 and 29319).

LC/MS grade acetonitrile, deionized water (diH2O) and methanol were purchased from ThermoFisher Scientific (Waltham, MA); LC/MS grade ethyl acetate from Sigma-Aldrich (St. Louis, MO, USA); and LC/MS grade formic acid for mobile phase was purchased from TCI America (Portland, OR). Sodium chloride was purchased from VWR International (Radnor, PA). Dimethyl sulfoxide, monobasic potassium phosphate, and dibasic potassium phosphate was purchased from ThermoFisher Scientific (Waltham, MA). Ultrapure water (>18.0 MΩ-cm resistivity) was acquired utilizing a Milli-Q IQ 7005 Water Purification System (Millipore Sigma, Burlington, MA). Deionized water was acquired utilizing a Culligan commercial filtration system (Culligan International, Rosemont, IL). NADPH Regenerating System Solutions A, $NADP^+$ and Glc-6-PO_4 (catalog no. 451220), and B, G6PDH (catalog no. 451200), were purchased from Corning Life Sciences (Corning, NY).

Negative matrices were purchased as follows; negative calf blood, brain, and liver from O. Ottomanelli & Sons (New York, NY), certified drug free urine from UTAK

Laboratories Inc. (Valencia, CA, catalog no. 88121-CDF), and negative calf plasma in sodium heparin from Rockland Immunochemicals (Royersford, PA, catalog no. D500-07-0050). Human liver microsomes (1.0 mL) were purchased from XenoTech (Kansas City, KS, catalog no. H2610) and consisted of 200 mixed gender donors at a protein concentration of 20 mg/mL.

2.2. Animal Care

Animal housing and experiments took place in St. Albert's Hall. Male Sprague Dawley rats (Taconic Biosciences, Germantown, NY) were received at seven weeks old and acclimated to the Animal Care Center (ACC) for a minimum of 72 hours before handling began. Animals were handled at least one time prior to the study day and the study was performed one week after animal receipt. Animals were fed a standard diet until the study began. During the six-hour experiment, animals were fasted while housed in metabolism cages but had free access to water. Throughout the course of the experiment, animals were monitored continuously for any signs of distress. All described procedures were reviewed and approved on August 21, 2021, by the Institutional Animal Care and Use Committee (IACUC) at St. John's University under protocol number 1996.

2.3. Standard and Control Preparation

Metonitazene, both internal standards, N-desethyl isotonitazene, N-desethyl metonitazene, and 4'-hydroxy nitazene were received as solid drug standards. A stock solution was prepared at 1 mg/mL. A working calibrator solution of metonitazene and isotonitazene in methanol was first prepared at 10 mg/L and serial diluted to 1.0 and 0.10 mg/L. The working solutions were then spiked into seven separate 5 mL aliquots of negative urine and blood matrices at the calibration range of 0.10 to 100 ng/mL. Each

prepared 5.0 mL calibrator was then aliquoted into 50 µL samples in microcentrifuge tubes and frozen at <0°C until use. Quality control working solutions were prepared at 1.0, 10, 100 and 1000 ng/mL in methanol. Working solutions for the four metabolites were prepared separately in the same manner as the parent drugs. The IS working solution was prepared at 25 ng/mL in methanol. All solutions were stored at <0°C when not in use.

2.4. Sample Preparation and Extraction Procedure

Tissue specimens required homogenization prior to aliquoting and analysis. Tissue samples are first weighed and diluted into deionized water (diH_2O) at specified dilution factors. Liver specimens are prepared as 1:5 homogenates and brain specimens at 1:3. The dilutions are calculated by 1 part tissue to the total weight of tissue in water. Human postmortem liver used in this study was submitted as a section which was then dissected into a 3.0 g sample, and 9 g of diH_2O was added for a total weight of 12 g. For the animal samples, whole brain tissues were weighed and then deionized water (diH_2O) was added for a 1:3 w/w ratio of brain:total weight brain and diH_2O. Approximately 2 g sections of whole rat livers were dissected and used for analysis. The liver sections were weighed and then diH_2O was added for a 1:5 w/w dilution ratio. Samples were homogenized using a Fisherbrand™ 850 Homogenizer (ThermoFisher Scientific, Waltham, MA).

The calibration samples (0.10 - 100 ng/mL) were removed from the freezer and allowed to thaw prior to extraction. Next, 50 µL of quality control (QC) samples in all matrices, blood, plasma, urine, and homogenates of brain and liver were aliquoted for each extraction and fortified with appropriate drug concentrations. Remaining negative

controls (negative matrix + IS) and casework or animal samples were aliquoted accordingly. The IS working solution was fortified at 10 µL into each sample for a final concentration of 5 ng/mL. Samples were capped and vortexed. Samples were then extracted into 150 µL cold acetonitrile for all matrices except liver which utilized 150 µL cold ethyl acetate. After addition of organic solvent, samples were capped and vortexed for 30 s. Vortexed samples were centrifuged for 10 min at 10,000 rpm. The supernatant was transferred to a test tube and dried down under nitrogen at 40°C. Samples were reconstituted in 100 µL of initial mobile phase mixture. The metabolites were analyzed separately in urine specimens only. Reconstituted samples were vortexed and transferred to autosampler vials with inserts, capped, and injected onto the instrument. Sample analysis was performed at OCME.

2.5. LC/MS/MS Conditions

Sample analysis was performed using an Agilent 1200 Infinity Series Liquid Chromatograph (LC) and chromatographic separation was achieved using a Poroshell 120 EC-C18 (2.1 x 100 mm, 2.7 µm) column combined with a guard column at (2.1 x 5 mm, 2.7 µm) purchased from Agilent Technologies (Santa Clara, CA). Mobile phase A consisted of 0.1% formic acid in LC/MS grade water and mobile phase B was 0.1% formic acid in LC/MS grade acetonitrile. The method used a 10 µL injection volume and flow rate of 0.5 mL/min with a gradient elution. The run started at 5% B and increased to 15% B over 2 min, then to 95% B at 3.5 min, and to 100% B at 5.0 min and was held at 100% B for 1.5 minutes. A post-run of 2 min allowed the instrument to equilibrate back to starting conditions. The column compartment was held at 55°C for the entirety of the 8.5 min run-time.

An Agilent 6460 Triple Quadrupole mass spectrometer (MS/MS) (Agilent Technologies, Santa Clara, CA) was run in electrospray ionization positive (ESI+) mode. The four analytes were optimized using MassHunter Optimizer software (Agilent Technologies) and data collected using dynamic multiple-reaction-monitoring (dMRM). dMRM allows collection of ions within specific retention time windows which helps increase instrument sensitivity. The dMRM parameters including precursor and product ions with their collision energies (CE) are listed below (Table 1). All compounds utilized a cell accelerator of 3 volts (V). MassHunter Quantitative Analysis software (Agilent Technologies) was used for data processing.

Table 1. dMRM parameters

Analyte	Precursor Ion	Fragmentor (V)	Product Ions (CE in V)
Isotonitazene	411.2	166	100.1 (21), 72.1 (49)
Isotonitazene-d_7	418.3	132	100.1 (25), 72.2 (57)
Metonitazene	383.2	130	100.1 (21), 72.1 (45)
Metonitazene-d_3	386.2	98	100.1 (21), 72.2 (49)

2.6. Pharmacokinetics in a Human Liver Microsomes Model

2.6.1. Study Design & Protocol

Human liver microsomes (HLM) were incubated with either metonitazene or isotonitazene in a method adapted from Feasel et al. to evaluate *in vitro* PK of another

synthetic opioid, carfentanil. (47). In order to individually evaluate the metabolism, the experiments were conducted for each drug. Fifty microliters of HLM were pre-aliquoted into microcentrifuge tubes and stored at -80°C until use. In duplicate, three types of samples were prepared in each of the experiments (n=6), HLM/drug mixture, HLM control, and regeneration system control. The drug solution contained 100 μg/mL of metonitazene or isotonitazene. The final concentration of the drug in all experimental samples was 2.5 μg/mL. Higher concentrations were attempted in initial trial experiments, at 10 and 5 μg/mL, but the potential of saturation and inadequate enzyme capacity was observed by the very slow to no metabolism of the drugs over the 75-minute period. HLM control was prepared by boiling the HLM aliquot for 5 min on a hot plate prior to the experiment. The regeneration system control involved preparing the sample as outlined in the procedure, while omitting the regeneration system reagents. See Table 2 for the experiment protocol which is listed in the order each reagent was added to the 50 μL thawed HLM aliquots. The total volume of each system was 1 mL and final protein content was 1 mg/mL.

Table 2. HLM experiment protocol

Reagent (Initial Concentration)	HLM/Drug Mixture	HLM Control	Regeneration Control
HLM (1 mg/mL protein)	50 μL	50 μL (boiled)	50 μL
Potassium Phosphate buffer (0.1 M, pH 7.4)	100 μL	100 μL	100 μL
Ultra-pure diH2O	765 μL	765 μL	825 μL
$NADP^+$ and Glc-6-PO_4	50 μL	50 μL	0 μL
G6PDH	10 μL	10 μL	0 μL
Drug Solution (100 μg/mL)	25 μL	25 μL	25 μL

After the addition of the last reagent, the drug solution, the specimens were mixed thoroughly, and the time zero (t_0) aliquot was immediately taken. The mixture was then poured into a 10 mL beaker and placed into the Dubnoff incubator where it was shaken at 100 rpm at 37°C for the entirety of the experiment. At each designated time point, 50 µL aliquots of the mixture were pipetted into a fresh microcentrifuge tube. The time points were 0, 5, 10, 20, 30, 45, 60, and 75 minutes. To each time point aliquot, 150 µL of cold ethyl acetate was added, and the sample was vortexed thoroughly to quench the reaction. Samples were then stored in a cooler and transported to OCME for analysis. Then, 10 µL of IS working solution was fortified into each sample for a final concentration of 5 ng/mL. Samples were centrifuged for 10 min at 10,000 rpm. A 100 µL aliquot of the supernatant was added to 400 µL of initial mobile phase mixture in a LC vial, which was capped and vortexed and run on the instrument. Samples were analyzed for their drug area responses and concentrations when run against the same calibration curve.

2.6.2. Data Analysis

Statistical analysis was performed using Microsoft Excel and GraphPad Prism 10 software. Data was visualized on graphs generated from GraphPad Prism 10 software. The duplicate area responses determined from the MassHunter Quantitative Analysis software at each timepoint for each experiment were averaged for both analytes. The drug area responses of the duplicate analyses were averaged in each experiment, then compared to the other experiments. Percent remaining was calculated by dividing the response of the time point sample, t_x, by the t_0 area response and multiplying by 100. Half-life in min was calculated using the slope of the graph of natural log (ln) % remaining v time in min (61).

$$\text{Equation 1: } t_{1/2} = \frac{0.693}{\text{slope (k)}}$$

Microsomal intrinsic clearance in μL min^{-1} mg^{-1} was calculated using the half-life and amount of protein in total incubation volume (44, 65).

$$\text{Equation 2: } CL_{int,\ micro} = \frac{0.693}{t_{1/2}} \text{ x } \frac{1000\ \mu L \text{ of incubation}}{1\ mg \text{ of protein}}$$

Estimated intrinsic clearance in mL min^{-1} kg^{-1} was calculated utilizing the microsomal intrinsic clearance (Equation 2) and average body and liver weight reference values from Davies et al. (65, 66).

$$\text{Equation 3: } CL_{int} = CL_{int,\ micro} \text{ x } \frac{1800\ g \text{ of liver weight}}{70\ kg \text{ of body weight}} \text{ x } \frac{45\ mg \text{ of microsomal protein}}{g \text{ of liver weight}}$$

All results were expressed as mean ± standard error of the mean (SEM). A student's t-test post hoc test was conducted to determine if there was significant difference in half-life or intrinsic clearance between metonitazene and isotonitazene ($p<0.05$).

2.7. Pharmacokinetics in a Sprague-Dawley Rat Model

2.7.1. Study Design

The study was designed to evaluate the pharmacokinetics of both novel opioids in rats when the drugs were administered either intravenously (IV) via tail vein or intraperitoneal (IP) injection. IV drug administration was used to mimic a commonly

utilized administration type by humans when abusing these drugs (12). IP administration is often used as an alternative to oral administration to evaluate the effect of first-pass metabolism as substances administered primarily enter the portal system, passing through the liver, before entering systemic circulation (63,64). IP administration has proven experimentally to result in faster and more complete absorption of low MW drugs compared to SC and oral routes, closely mimicking the systemic exposure observed after IV administration (63). IP was chosen as the second method of administration to directly evaluate the effect of first-pass metabolism of isotonitazene and metonitazene in comparison to results obtained via the IV route.

The originally proposed dose was 300 μg/kg based on two other synthetic opioid rat studies. Truver et. al. published a study of PD and PK of a novel opioid, U-47700, in male Sprague-Dawley rats. Their study utilized a SC injection of 300, 1,000 and 3,000 μg/kg doses (50). A study by Ohtsuka et. al. evaluated sex differences in fentanyl PK using an IV dose of 300 μg/kg (65). These two drugs' potencies were estimated as similar to or slightly more than fentanyl, therefore the fentanyl IV dose from the Ohtsuka study was utilized in trial studies. This dose also aligned with the lowest SC dose attempted in the Truver study with a drug known to be less potent than fentanyl.

During trial studies, significant catalepsy was observed after IP administration. IV isotonitazene injection in an animal at 300 μg/kg resulted in overdose and expiration. Doses were therefore decreased gradually in subsequent trials. It was clear in these studies that isotonitazene was the more potent drug, therefore the effects of the isotonitazene drug on trial animals were the determining factor for experimental doses. Trial studies also elucidated that IP and IV doses could not be equal as originally desired.

At equal doses, animals in the IV groups showed cataleptic effects, and lower IP doses resulted in undetectable drugs in plasma samples. Final experimental doses chosen were 25 µg/kg for IP administration and 2.5 µg/kg for IV. These doses allowed for the ability to find a detectable amount of drug in rat plasma, but one that did not give the animal a visible intoxicating effect.

Each drug was first prepared at 10 mg/mL in dimethylsulfoxide (DMSO). Individual drug solutions were then serial diluted into 0.9% sterile saline to 25 and 2.5 µg/mL for a final DMSO concentration of < 2%. DMSO is a solvent miscible with water into which many polar and nonpolar smaller compounds are dissolvable (66). DMSO has uses in pharmaceutical preparations as an excipient and has been recommended for use in percentages up to 10 to 20% total solution in oral and IV drug formulations (67).

Eight-week-old male Sprague Dawley rats weighing between 240 to 360 g were given a single injection of the drug at a dose (1 mL/kg) of 2.5 µg/kg for IV or 25 µg/kg for IP administrations. Rats were weighed using a Taconic Farms rat scale (Germantown, NY) so their individual doses could be determined. The volume of drug administered ranged from 0.24 to 0.36 mL. Animals were assigned groupings by which route of administration they were given, and by receiving either metonitazene, isotonitazene, or vehicle only, for a total of 6 groups. A total of 23 animals were included in this study. The visualization of the animal groupings is shown in Table 3.

Table 3. Sprague-Dawley rat study experimental groupings

Group	Number of Animals	Drug (Dose)	Route of Admin.	Blood Collection Time Points (min)
1	1	Vehicle only (2% DMSO in saline)	IV	5, 360
2	1	Vehicle only (2% DMSO in saline)	IP	5, 360
3	6	Isotonitazene (2.5 μg/kg)	IV	5, 30, 90, 240, 360
4	5	Isotonitazene (25 μg/kg)	IP	5, 30, 90, 240, 360
5	5	Metonitazene (2.5 μg/kg)	IV	5, 30, 90, 240, 360
6	5	Metonitazene (25 μg/kg)	IP	5, 30, 90, 240, 360

2.7.2. Study Protocol

Rats were manually restrained using a Broome-style restrainer (Plas-Labs, Inc, Lansing, MI) for IV injection and blood collections. The drug solutions were allowed to come to room temperature prior to administration. Tail veins were visualized by warming and massaging the tail and injections made using a 26g needle. IP injections were made by holding the animal with the legs and tail anchored in the crook of the nondominant arm with their stomachs exposed. The injection was made using a 25g needle into the lower right quadrant of the animal. After animals were dosed accordingly, they were housed in metabolism cages, 1 animal per cage, until the end of the study. Animals were housed in the metabolism cages for a maximum of 8 hours and were given free access to water.

Blood collections at specified time points, 5 to 360 min, were performed via tail snip. Topical anesthetic (benzocaine or lidocaine) was applied after the initial snip to limit pain to the animal. Approximately 200 µL of blood was collected at each timepoint into BD Microtainer^{TM} plasma collection tubes with sodium heparin (BD, Franklin Lakes, NJ). Milking of the tail was usually necessary to obtain the required amount of blood. A snip was made before the first blood collection, after which the clot that formed was removed for subsequent collections. After blood collection, heparinized tubes were centrifuged at 8,000 rpm for 4 min and the plasma was decanted into a separate cryovial which was frozen until analysis. Over the course of the study, urine was collected through the metabolism cage into 15 mL Falcon tubes and stored refrigerated until analysis. Multiple urine samples were collected if available. After all blood collections were completed for the set of animals performed per day, animals were transported for euthanization. The animals were euthanized via decapitation by guillotine and utilizing DecapiCones (Braintree Scientific, Inc., Braintree, MA) for animal restraint. Whole brain and liver tissues were dissected and collected for analysis. Specimens were then transported for analysis at OCME.

It became apparent as the study progressed that isotonitazene and metonitazene were not detected in many animal urine specimens. A modification to the study was initiated and all animal urine samples available were retested for two known metabolites of isotonitazene, N-desethylisotonitazene, and 5-aminoisotonitazene, one metabolite for metonitazene, N-desethylmetonitazene, and one shared metabolite, 4'-hydroxy nitazene. Other known metabolites of metonitazene, N,N-didesethyl metonitazene and 5-aminometonitazene were not analyzed due to the unavailability to purchase reference

material standards. Since the four available metabolites were not originally part of the method validation, they were analyzed qualitatively when run against a standard calibration curve with QCs at the same concentrations as the parent drugs.

2.7.3. Data Analysis

Area responses and calculated concentration values for all biological matrices were determined using MassHunter Quantitative Analysis software. Calculations were performed using Microsoft Excel and GraphPad Prism 10 software. Data was visualized using plots generated by GraphPad Prism 10 software. Mean plasma concentration ± SEM are plotted v time as well as ln mean concentration v time. Linear regression was performed on the semi-log graph to determine r^2, slope, and y-intercept. The elimination rate constant (k_e) and half-life ($t_{1/2}$) were calculated utilizing the slope of the semi-log graph.

Equation 4: $k_e = -(\text{slope})$

Equation 1: $t_{1/2} = 0.693 / k_e$

The amount of drug administered in ng was calculated for each animal based on their weight and the IV dose.

Equation 5: Individual animal wt. (kg) * 2.5 µg/kg * $\frac{1000\ \text{ng}}{1\ \mu\text{g}}$

The plasma concentration at time 0 (C_0) was calculated by taking the antilog of the y-intercept of the semi-log graph. Volume of distribution (V_d) in mL and clearance rate (CL) in mL min^{-1} kg^{-1} were calculated for IV groups only.

Equation 6: $V_d = \text{IV Dose} / C_0$

Equation 7: $CL = (k_e * V_d)/ \text{animal wt}$

All results were expressed as mean ± SEM. A student's t-test post hoc test was conducted to determine if there was significant difference in half-life of each analyte in

comparison to each other, and between the different administration routes ($p<0.05$). A student's t-test was conducted to determine if there was significant difference in the volume of distribution and clearance rates between the two drugs ($p<0.05$).

2.8. Human Postmortem Sample Analysis

2.8.1. Study Design

Cases that had previously been reported qualitatively in routine casework at OCME for metonitazene and isotonitazene were selected to be retested using the developed quantitative method. The positive cases were received between December 2020 to December 2023. The validated method encompasses multiple matrix types, however many of these were unavailable to be tested in the authentic cases. As a result of the COVID-19 pandemic, autopsy protocols changed to decrease the number of samples provided to the laboratory. Therefore, for most of these cases, only paired blood and urine were available as tissue samples had not been collected. A total of eighteen cases were pulled for analysis, seven that previously tested positive for metonitazene and eleven for isotonitazene. All available urine samples were also analyzed for the four metabolites of isotonitazene and metonitazene. All samples had been frozen at -20°C from the time of original testing completion to when they were identified for this study. This study was submitted to the Institutional Review Board (IRB) for approval (IRB-FY2022-13) and deemed exempt.

2.8.2. Determination of Case Demographics

In order to determine any patterns in concurrent drug use, or drug user demographics, toxicology case folders and the OCME Case Management System (CMS) database were reviewed, and case information was compiled. Cases were de-identified by

assigning arbitrary case numbers and no identifying information was reported to protect the privacy of the decedents.

2.9. Validation of an Analytical Method for Analysis of Plasma, Blood, Urine, Brain and Liver Specimens

2.9.1. Study Design

In anticipation of the animal study, a low sample volume extraction procedure was adapted and validated for quantification of metonitazene and isotonitazene. A protein precipitation reaction was utilized for sample clean-up. Protein precipitation reactions disrupt drug-protein binding to extract the drug from the sample matrix and can be accomplished using an organic solvent, salt, metal, or acid (68). Organic solvent was used as is standard practice for this extraction type at OCME. When an organic solvent is added to the biological matrix, it lowers the dielectric constant of the solution, which enables electrostatic protein interactions and displaces water from the protein surface, allowing for protein precipitation (68). Acetonitrile is a commonly used solvent for this purpose and was first attempted during method validation. Liver samples in trial studies showed poor chromatography and extensive matrix interferences, so a stronger solvent was utilized, ethyl acetate, for that matrix only. After determination of the extraction procedure, method validation was planned.

Method validation experiments are performed to ensure a method is fit-for-purpose and to highlight any limitations prior to implementation in a laboratory workflow (69). Validation was performed according to ASB Standard 036, Standard Practices for Method Validation in Forensic Toxicology, which outlines minimum standards for

validation criteria broken down by scope of the method (69). This method is utilized for quantitative analysis therefore the parameters evaluated were calibration model, bias, precision, limit of detection, limit of quantitation, interference studies, ionization suppression/enhancement, carryover, and dilution integrity. All experimental procedures were carried out for all five biological matrices unless otherwise stated. All validation results in this study have been published (70). The validated method was utilized for sample analysis for all phases of the study.

2.9.2. Calibration Model

For quantitative methods, the calibration model is established to determine the mathematical relationship between the analyte concentration and instrument response (69). The model was examined over a predetermined working range of 0.10 -100 ng/mL that was prepared using analytical reference materials. This range was chosen based on the previously validated method for quantitation of synthetic opioids currently in use at OCME. The method consisted of a total of seven calibration points at concentrations of 0.10, 1.0, 5.0, 10, 20, 50 and 100 ng/mL. Reference materials were spiked into negative calf blood to calibrate blood, plasma, brain, and liver samples. Urine samples required a separate calibration curve utilizing negative human urine. After five days of calibration, the residuals at each concentration were calculated to determine the best-fit model as well as the most acceptable weighting. With the best model applied, the coefficients of determination (r^2) were observed for all runs.

2.9.3. Limit of Detection

The limit of detection (LOD) is the estimation of the lowest detectable concentration able to be distinguished from a blank matrix sample (69). LOD was

evaluated using the lowest calibration concentration of the method, which is also the limit of quantitation (LOQ). Matrix samples were spiked with reference materials at the determined concentration and analyzed over three runs. Criteria used for acceptance include target analyte signal to noise (S:N) 3.3x greater than S:N in blank matrix samples, acceptable retention time (RT), ion ratios, and peak shape for all replicates tested.

2.9.4. Bias and Precision

Bias is defined as the difference between the mean of replicate measurements as compared to the "true" value (69). Precision is the evaluation of closeness between replicate measurements (69). Bias and precision studies are conducted concurrently. Bias of three QC concentrations, low (LQC), mid (MQC) and high (HQC) were evaluated in triplicate over five days for a total of 15 replicates at each concentration, as well as at the limit of quantitation (LOQ). The LOQ was chosen using the lowest calibrator concentration and was tested for blood, plasma, and brain at 0.10 ng/mL, and at 1.0 ng/mL for liver and urine. The LQC was 0.50 ng/mL for blood, plasma, and brain, and 2.0 ng/mL for liver and urine. The MQC was 8.0 ng/mL for all matrix types. The HQC concentration for each matrix was 80 ng/mL. The percent bias was calculated by taking the average of the 15 replicates, and finding the percent difference from the target concentration, which should not exceed ± 20%. To evaluate precision of the method, fifteen replicates of LOQ, LQC, MQC, and HQC samples over five runs for all matrix types were analyzed using one-way analysis of variance (ANOVA). The within-run and between-run percent coefficient of variation (%CV) were calculated, with an acceptable value of ≤ 20%.

2.9.5. Ion Suppression and Enhancement

Ion suppression or enhancement is a phenomenon that occurs when coeluting compounds interfere with the instrument response of the target analytes (69). This occurrence is most commonly seen in LC/MS/MS instruments. Ion suppression or enhancement occurs when the percentage of ions generated in the ESI source is subject to matrix-dependent changes as compared to running a neat sample (71). Ion enhancement is expressed as a positive percentage and ion enhancement is reflected by a negative percentage. Matrix effects from varied sources were evaluated to determine ion suppression and enhancement using two separate sets of samples for both the LQC and HQC concentrations. Set 1 was composed of neat samples prepared in duplicate and injected onto the instrument three times for a total of six results per concentration. Set 2 included 10 matrix sources of each type (n=50) that were extracted and then fortified with LQC and HQC into the supernatant, prior to the evaporation step. The mean area response of each set for each matrix are averaged and the mean response of set 1 was subtracted from the mean response of set 2, then divided by the mean response of set one and multiplied by 100 to get the percentage of matrix effects.

Equation 8: $ME = (X_{\mathbf{Set\ 2}} - X_{\mathbf{Set\ 1}} / X_{\mathbf{Set\ 1}}) \times 100$

An acceptable level of matrix effects is ± 20% with a percent coefficient of variation (CV%) between the responses for all replicates of each concentration is within 20%. If the results exceed that cutoff, other parameters of the validation (i.e. LOD/LOQ) must not be affected by the high ion suppression or enhancement.

2.9.6. Interferences

Interference studies were completed in three parts: interferences from the matrix,

target and IS interferences, and interferences from commonly encountered analytes. Matrix interferences were tested by extracting 10 different sources of each matrix type (n=50) and running on the analytical method. The blank matrices were extracted with no addition of internal standard or target analytes. The processed data was evaluated for any interfering peaks present in the detection windows for metonitazene, metonitazene-d_3, isotonitazene, and isotonitazene-d_7. Potential interference from the target analytes and IS were evaluated. Three aliquots of each matrix were extracted with target analytes fortified at 100 ng/mL and no IS to determine if the target analytes resulted in detection of the IS. Three aliquots with 5 ng/mL IS and no target analytes were extracted to determine if the IS had any contamination from the target analytes present.

The third interference study involved fortifying a LQC sample in triplicate with commonly encountered analytes to determine if these other analytes affected chromatography or quantitation of the target compounds. Blood, plasma, brain, liver, and urine MQC samples (8 ng/mL) were fortified with 83 commonly encountered analytes. The samples comprised of 20 ng/mL fentanyl and fentanyl analogs, 200 ng/mL cocaine, ethylbenzoylecgonine, benzodiazepines, opiates, and opioids, 1000 ng/mL benzoylecgonine, amphetamine, methamphetamine, MDMA, MDA, PPA, phentermine, and 2000 ng/mL methadone and EDDP.

2.9.7. Dilution Integrity

The effect of sample dilution was explored for instances where not enough blood or serum volume was collected for analysis, or if high concentrations of drug were detected in urine. Dilution integrity was evaluated in three matrices: blood, plasma, and urine. Brain and liver sample homogenates were already diluted 1:3 and 1:5 respectively and

were unlikely to need any further dilutions and expected to be submitted with sufficient sample volume. This experiment utilized a 160 ng/mL sample diluted 1:2 which resulted in a concentration within the validated analytical range. Each matrix was tested in triplicate, and the accuracy of concentrations was evaluated, as well as the coefficient of variation between the replicates.

2.9.8. Carryover

Analyte carryover into subsequent injected samples can affect the accuracy of results (69). Carryover was evaluated by injecting negative QC samples after the highest calibrator concentration in triplicate for all matrix types (100 ng/mL), except for urine which was tested at twice the highest calibrator concentration (200 ng/mL). Samples deemed free from carryover showed no concentrations above the LOD of the method.

CHAPTER 3: RESULTS

3.1. Pharmacokinetics in Human Liver Microsomes

After analysis of HLM samples were completed for each experiment, the peak area responses were compiled, and the percent remaining was calculated. The results for the two control systems utilizing boiled microsomes and omission of the regeneration system reagents for metonitazene and isotonitazene showed little to no metabolism in all experiments as seen in Figures 6 and 7. The experimental systems showed metabolism over time in similar fashions as confirmed by Figure 8, which shows the behavior of both drugs in the experimental system represented as natural log of percent remaining v time. A linear regression analysis was performed for this graph and is shown in Table 4. The calculated elimination rate constant, half-life, microsomal intrinsic clearance, and intrinsic clearance rates are calculated using this analysis and are shown in Table 5. There was no significant difference between any of the calculated parameters when comparing isotonitazene to metonitazene at the 95% confidence interval. According to McNaney et.al. classification of intrinsic clearance and in vitro half-life rates, both isotonitazene and metonitazene fall within the intermediate clearance classification (61).

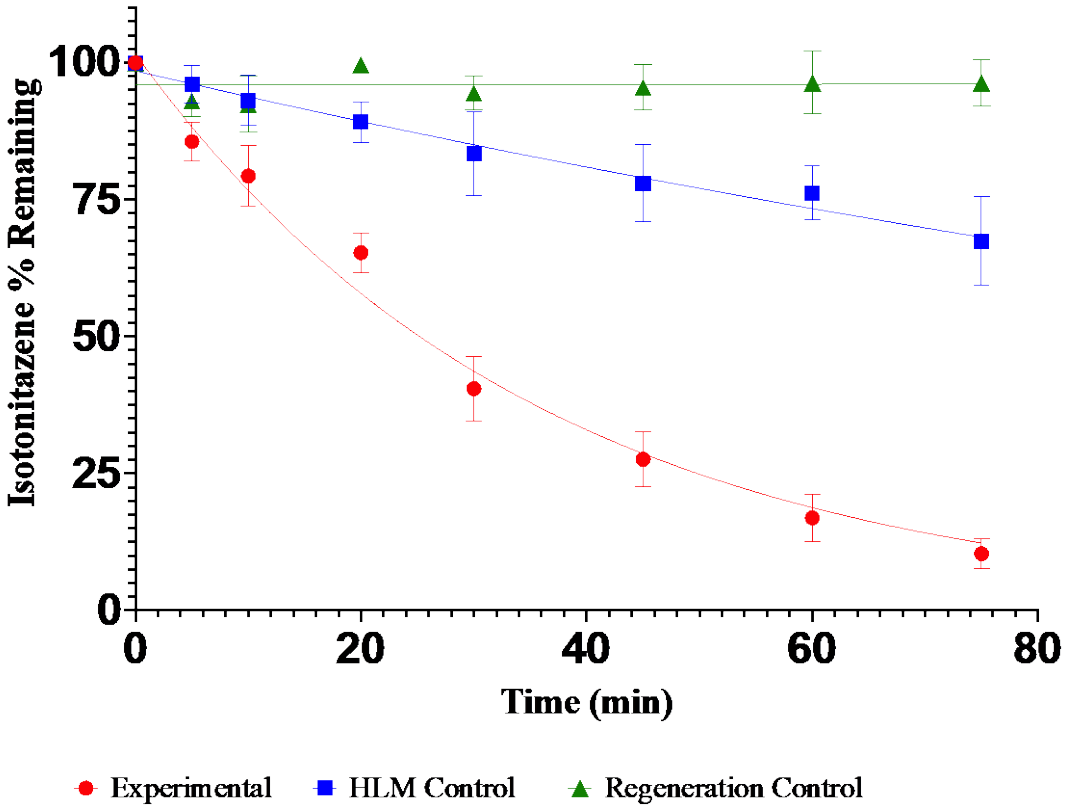

Figure 6. Percent remaining of isotonitazene in HLM shows metabolism in the experimental system and little to no metabolism in both control systems. The experimental system has all components added while the HLM control utilized boiled microsomes and the regeneration control omitted $NADP^+$, Glc-6-PO_4, and G6PDH. Data expressed as mean ± SEM.

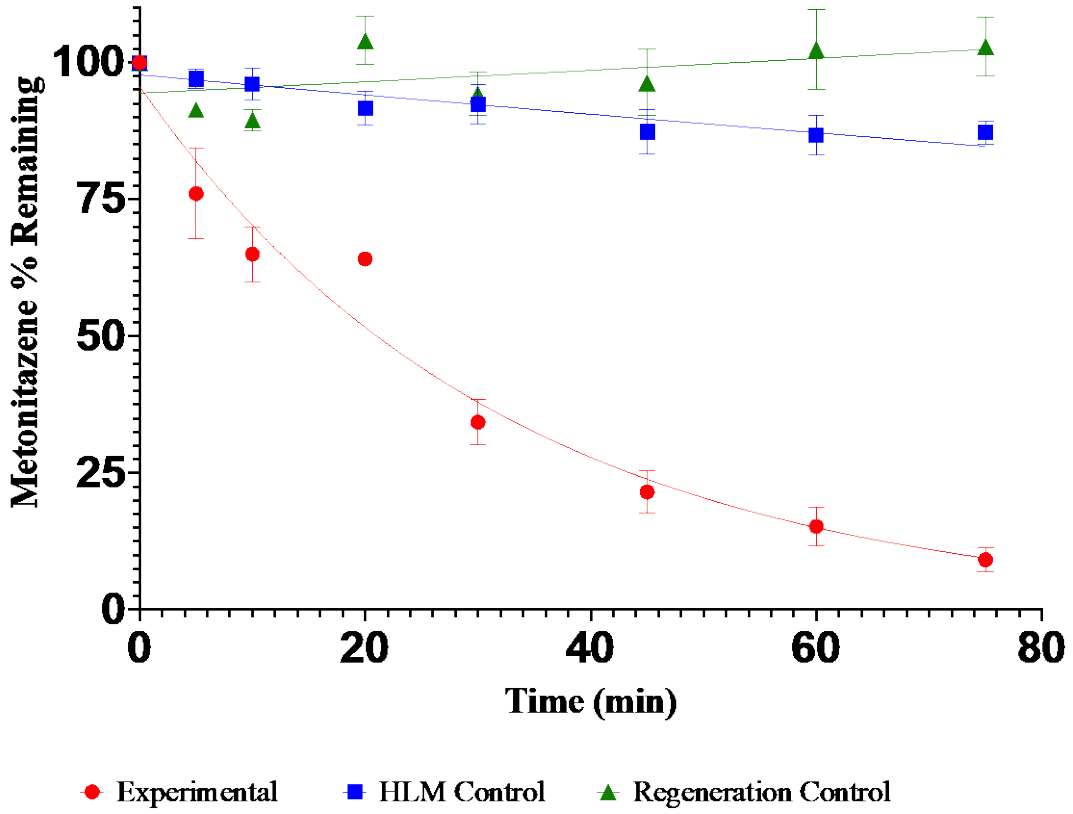

Figure 7. Percent Remaining of metonitazene in HLM shows metabolism in the experimental system and little to no metabolism in both control systems. The experimental system has all components added while the HLM control utilized boiled microsomes and the regeneration control omitted $NADP^+$, Glc-6-PO_4, and G6PDH. Data expressed as mean ± SEM.

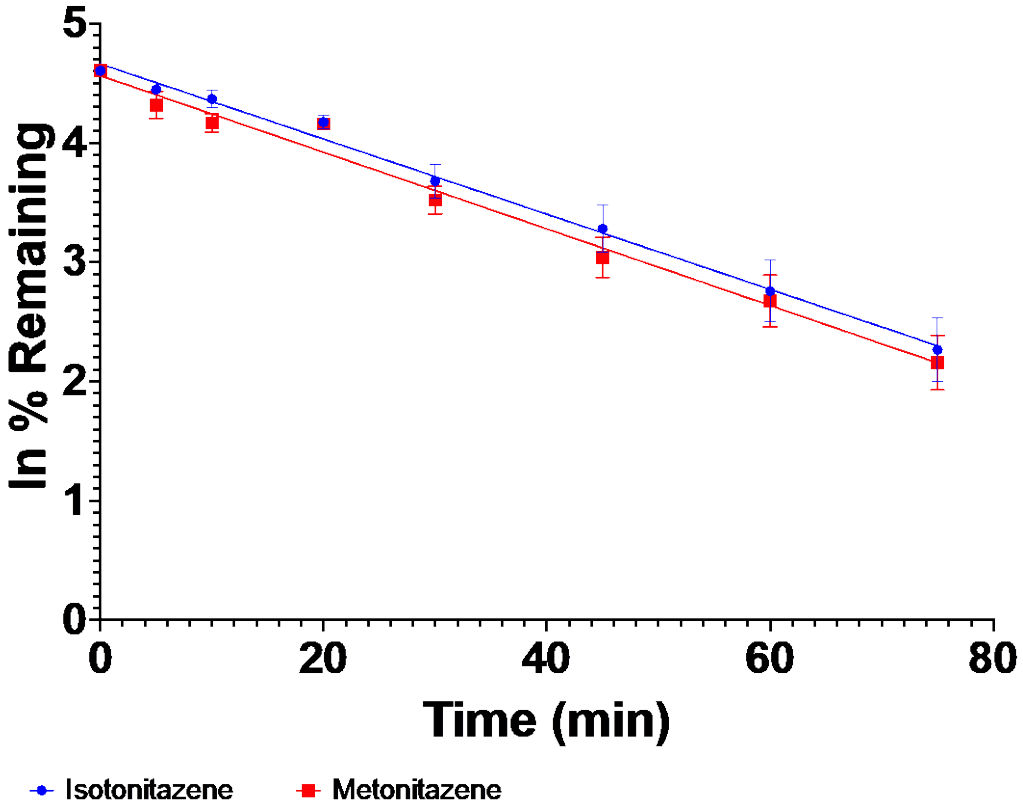

Figure 8. Semi-log of percent remaining over time for isotonitazene and metonitazene in HLM. Data expressed as mean ± SEM.

Table 4. Linear regression analysis of Figure 8, semi-log percent remaining of isotonitazene and metonitazene in HLM shown by correlation coefficient (r^2), slope, and y-intercept. Values expressed as mean ± SEM.

	Isotonitazene	Metonitazene
r^2	0.992 ± 0.003	0.980 ± 0.011
Slope	-0.03157 ± 0.003	-0.03216 ± 0.003
Y-intercept	4.66 ± 0.034	4.56 ± 0.047

Table 5. Calculated elimination rate constant, half-life, microsomal intrinsic clearance, & estimated intrinsic clearance of metonitazene and isotonitazene using a HLM model. Values expressed as mean ± SEM. There was no significant difference ($p<0.05$) between any parameter when comparing isotonitazene to metonitazene.

	Isotonitazene	Metonitazene
k (min^{-1})	0.03157 ± 0.003	0.03216 ± 0.003
$t_{1/2}$ (min)	22.6 ± 2.7	21.9 ± 2.2
$CL_{int, micr}$ ($\mu L\ min^{-1}\ mg^{-1}$)	31.5 ± 3.7	32.1 ± 3.0
CL_{int} ($mL\ min^{-1}\ kg^{-1}$)	36.5 ± 4.3	37.2 ± 3.5

3.2. Pharmacokinetics in a Sprague-Dawley Rat Model

3.2.1. Intravenous Administration Plasma Pharmacokinetics

All available animal samples were analyzed, and concentrations detected for parent drugs in all matrices were compiled for all experiments. The results for each drug were plotted as the mean ± standard error of the mean of all animals as seen in Figures 9 and 10. Linear regression analysis of each animal concentration time profile in Figure 11 were calculated, and the results are summarized in Table 6. The calculated PK parameters utilizing the data in Table 6 are shown in Table 7. There was a statistically significant difference between the mean elimination constant ($p=0.001$) and mean half-life ($p=0.001$). A more rapid half-life for metonitazene is visually observed and confirmed by the significantly larger calculated elimination constant, and significantly shorter half-life as compared to isotonitazene.

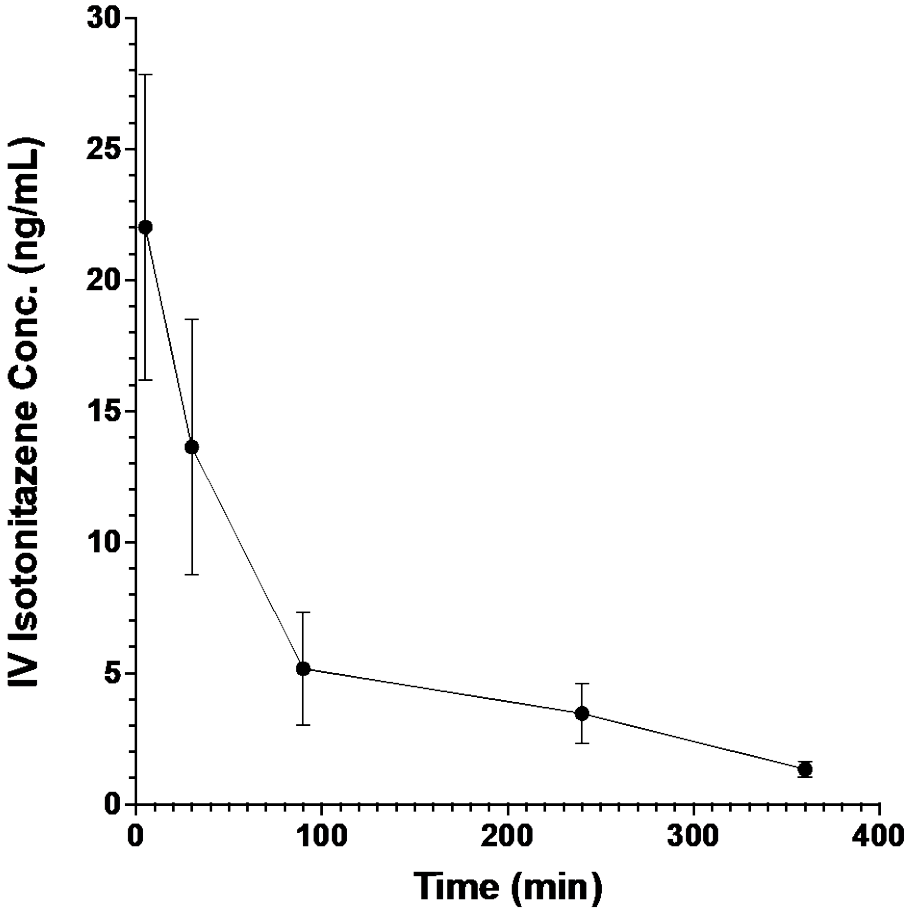

Figure 9. Isotonitazene plasma concentration after single IV dose at 2.5 μg/kg in all animals (n=6). Data expressed as mean ± SEM.

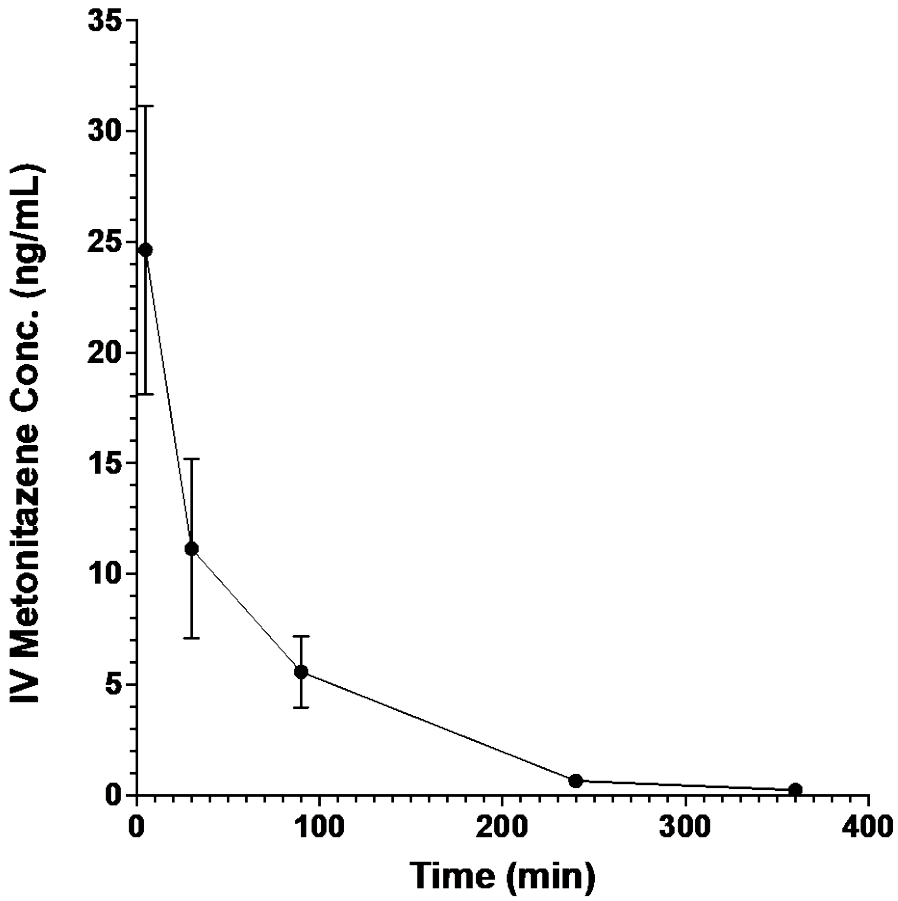

Figure 10. Metonitazene plasma concentration after single IV dose at 2.5 μg/kg in all animals (n=5). Data expressed as mean ± SEM.

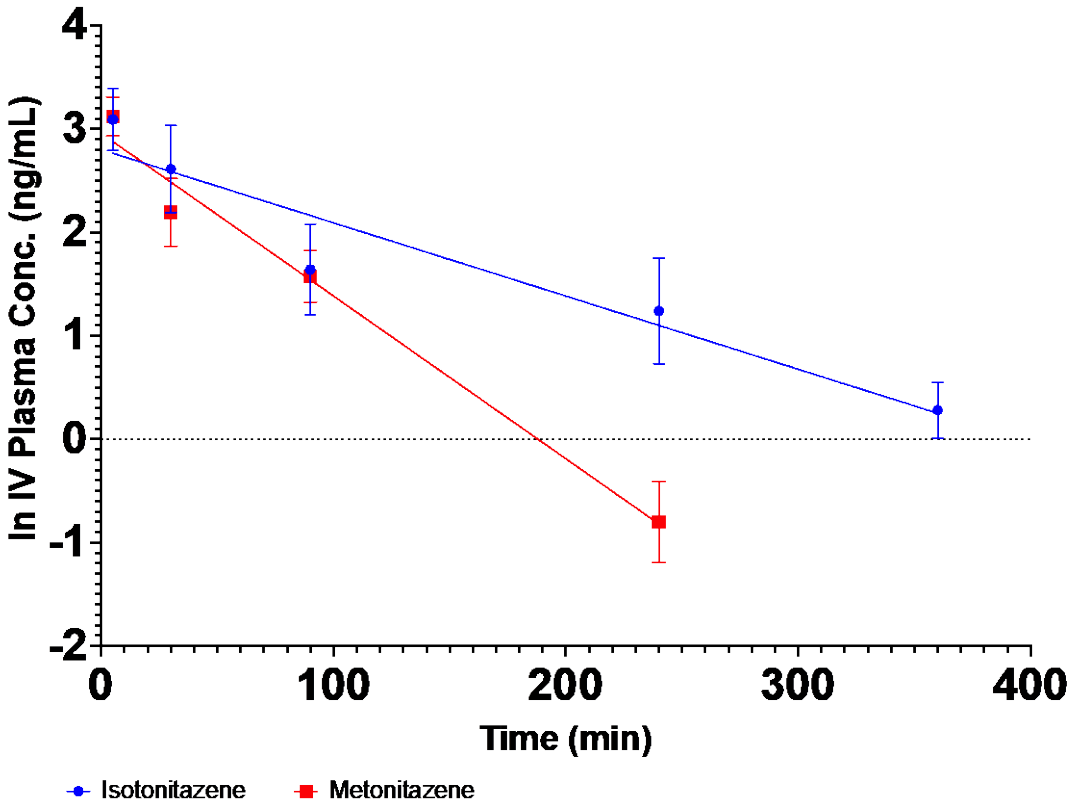

Figure 11. Semi-log of plasma concentration of isotonitazene and metonitazene after a single IV dose at 2.5 µg/kg. Data expressed as mean ± SEM.

Table 6. Linear regression analysis of Figure 11, semi-log plasma concentration of isotonitazene and metonitazene after IV administration shown by correlation coefficient (r^2), slope, and y-intercept. The slope also represents the elimination constant (k_e). Values expressed as mean ± SEM.

	Isotonitazene	Metonitazene
r^2	0.948 ± 0.022	0.971 ± 0.015
Slope ($-k_e$)	-0.0083 ± 0.0009	-0.0159 ± 0.001
Y-intercept ($\ln C_0$)	2.73 ± 0.34	2.97 ± 0.26

Table 7. Calculated elimination rate constant, half-life, volume of distribution, and clearance of isotonitazene and metonitazene after intravenous administration. There was a significant difference ($p<0.05$) in calculated k_e and half-life of isotonitazene compared to metonitazene. There was no significant difference ($p<0.05$) between the other parameters. All data expressed as mean ± SEM.

	Isotonitazene	Metonitazene
k_e (min^{-1})*	0.0083 ± 0.0009	0.0159 ± 0.0013
Half-life (min)*	88.5 ± 8.2	44.7 ± 3.9
Volume of Distribution (mL)	59.6 ± 20.2	37.3 ± 10.4
Clearance (mL min^{-1} kg^{-1})	2.1 ± 1.0	2.3 ± 0.7

*Statistically significant difference at the 95% confidence interval ($p<0.05$) when comparing the results of the two drugs.

3.2.2. Intraperitoneal Administration Plasma Pharmacokinetics

All available animal samples were analyzed, and concentrations detected for parent drugs in all matrices were compiled for all experiments. The results for each drug were plotted as the mean ± standard error of the mean of all animals as seen in Figures 12 and 13. Isotonitazene had varied plasma concentrations after IP injection, and only one animal had a positive t_{240} sample. The typical curve of concentrations decreasing over time is not observed for this drug. This is partially due to the very low concentrations detected at all time points, partially due to the limited number of plasma samples that were positive for isotonitazene, and a result of the variability at the t_{30} timepoint as seen by the wide error bars. A semi log graph was not generated for this data. The elimination constant and half-life were manually calculated using three out of the five runs that had at least three positive samples. This indicates the animals that did not clear isotonitazene

from the plasma before 90 min. The PK equation utilized to calculation the elimination constant can be seen below as Equation 9. The calculated k_e can then be plugged into the half-life calculation, Equation 1. Where c_f and c_i are final concentration and initial concentration respectively, and t_f and t_i are time final (90 min) and time initial (5 min) respectively. The results for elimination constant and half-life were added to Table 9 for comparison with metonitazene.

$$\text{Equation 9: } k_e = \frac{\ln\frac{c_f}{c_i}}{(t_f - t_i)}$$

$$\text{Equation 1: } t_{1/2} = 0.693 / k_e$$

Metonitazene showed a more typical response (Figure 12), and therefore linear regression analysis of the ln concentration time profile was calculated (Figure 13), and the results are summarized in Table 8. Calculated PK parameters utilizing the data in Table 8 are shown in Table 9. There was not a statistically significant difference in the calculated elimination constant or half-life when comparing isotonitazene and metonitazene at the 95% confidence interval. This is likely due to the highly variable results of isotonitazene after IP injection.

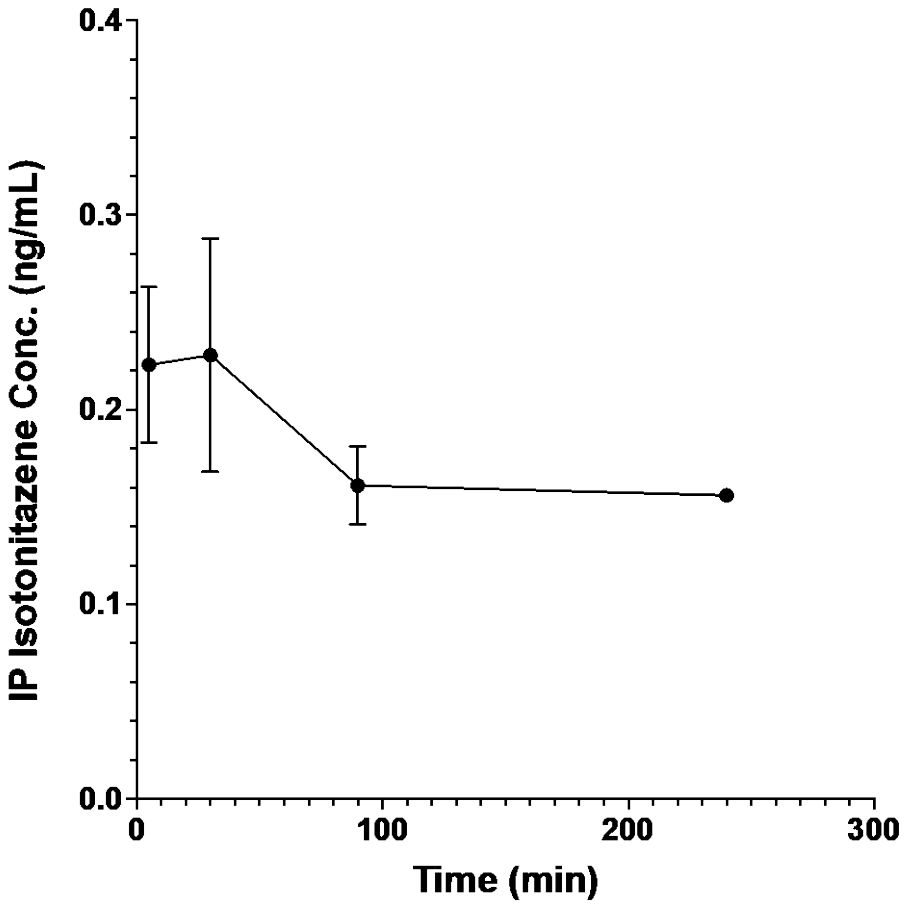

Figure 12. Isotonitazene plasma concentration after single IP dose at 25 μg/kg in all animals (n=5 at all timepoints except t_{240}, n=1). Data expressed as mean ± SEM.

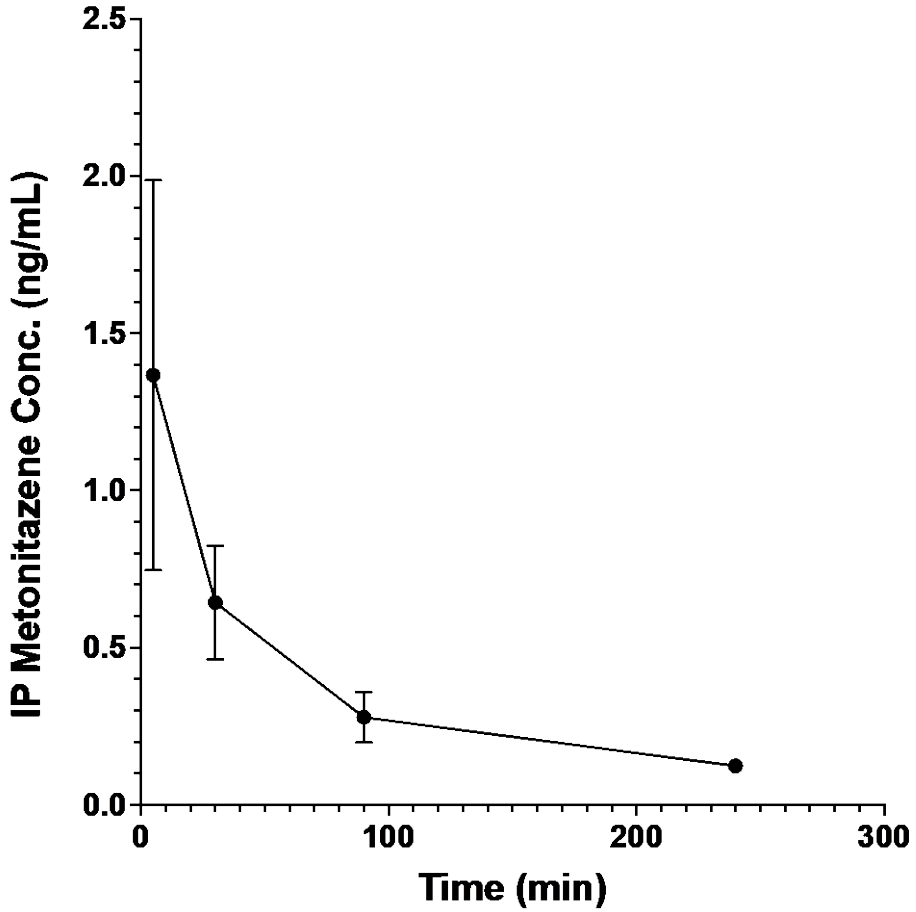

Figure 13. Metonitazene plasma concentration after single IP dose at 25 μg/kg in all animals (n=5 at all timepoints except t_{240}, n=3). Data expressed as mean ± SEM.

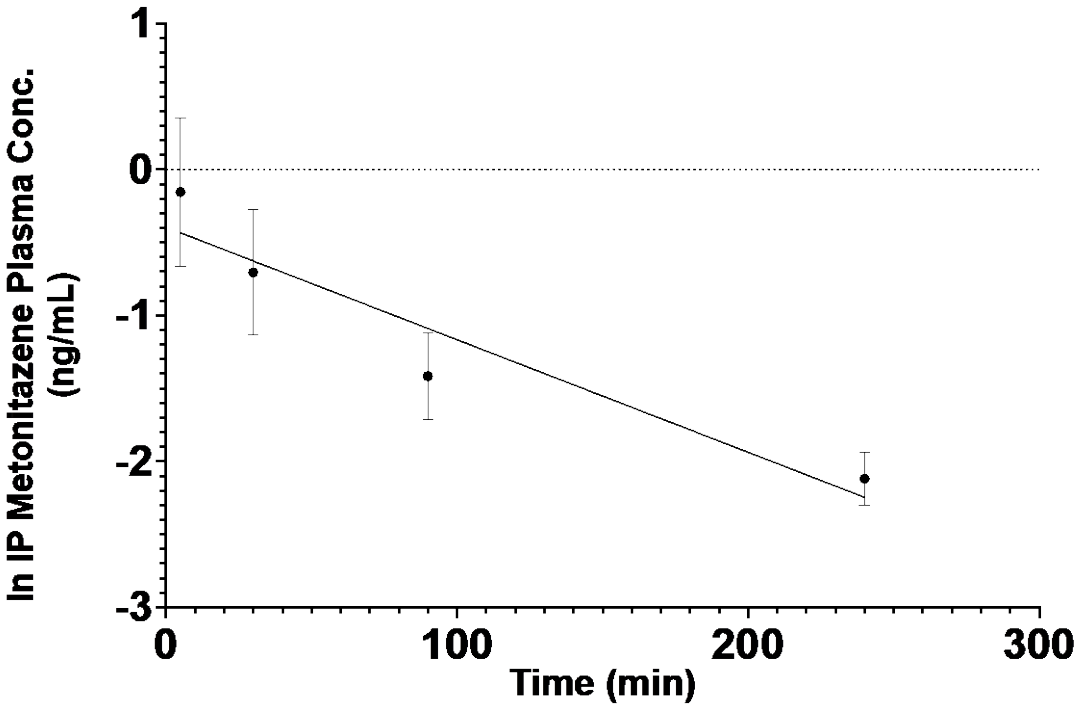

Figure 14. Semi-log of plasma concentration of metonitazene after a single IP dose at 25 μg/kg. Data expressed as mean ± SEM.

Table 8. Linear regression analysis of Figure 14, semi-log plasma concentration of metonitazene after IP administration, shown by correlation coefficient (r^2), slope, and y-intercept. Values expressed as mean ± SEM.

r^2	0.893 ± 0.045
Slope	-0.0161 ± 0.004
Y-intercept (ln C_0)	-0.224 ± 0.41

Table 9. Calculated elimination rate constant and half-life isotonitazene and metonitazene after IP injection. There was not a significant difference ($p<0.05$) in mean value for either parameter. All data expressed as mean ± SEM.

	Isotonitazene	Metonitazene
k_e (min^{-1})	0.0068 ± 0.001	0.0161 ± 0.004
Half-life (min)	117.5 ± 42.5	56.4 ± 13.4

3.2.3. Brain and Liver Analysis

Brain and liver samples were analyzed for all animals in all trial studies and experimental studies. Isotonitazene and metonitazene were not detected in any animal liver sample collected throughout the course of this research. Brain samples were also typically negative except for one animal given an IP dose of isotonitazene which had a concentration of 1.3 ng/g. Unlike the liver however, multiple trial animals administered higher doses (150 µg/kg IV or 300 µg/kg IP) were positive in brain for both analytes. These trial doses were 500% higher for IV administration and 20% higher for IP administration as compared to the experimental dose.

3.2.4. Urine Analysis

Due to animals voiding during blood collection while outside the metabolism cages, urine was not collected in all experiments. In all animals with urine available, one group four (IP injection) animal had isotonitazene detected in urine at 1.4 ng/mL. No other animals had a positive isotonitazene urine sample. Three animals had metonitazene <1.0 ng/mL, one from the IV group, and two from the IP group. All three were in the first urine collection available. In trial studies, most animals had no urine collected. This was due to animals voiding during blood collections, or because in most of the trials, shorter

study timeframes were used, and therefore animals were not in the metabolism cages long enough for them to void. Therefore, we are unable to report on the effect of dose differences on urine positivity rates.

Of the four total metabolites of isotonitazene and metonitazene tested, N-desethylisotonitazene, N-desethylmetonitazene, 5-aminoisotonitazene, 4'-hydroxynitazene, two of them were detected in animal samples. No metabolites were detected in the urine samples for either drug in the IV administration groups. N-desethylisotonitazene was detected in three animals from group four (IP injection), and all urine samples collected from those animals were positive. N-desethylmetonitazene was detected in one animal from group 6 (IP injection) in all three urine samples collected for that animal. 4'-hydroxy nitazene and 5-aminoisotonitazene were not detected in any animal urine samples. Overall, metabolite positivity rates were low in the animal samples.

3.3. Comparison of Pharmacokinetics from *In vitro* and *In vivo* Models

A comparison of the calculated half-life for each drug in all aspects of the study are summarized in Table 10. Statistical evaluation was completed to compare *in vivo* to *in vitro* half-life results and IV to IP for each drug. IP half-life compared to either IV data ($p= 0.50$) or in vitro ($p=0.11$) data showed no significant difference for isotonitazene at the 95% confidence interval. IP half-life differences for metonitazene as compared to IV ($p=0.44$) and in vitro ($p=0.064$) also was not significant at the 95% confidence interval. In comparing IV half-life to in vitro half-life, both isotonitazene ($p=0.0002$) and metonitazene ($p=0.002$) showed significant differences at the 95% confidence interval.

Table 10. Comparison of *in vitro* (HLM) and *in vivo* (Sprague Dawley rat) pharmacokinetic parameters for both drugs. Significant differences in mean half-life for in vitro compared to IV *in vivo* data were seen for both drugs ($p<0.05$). There is not a significant difference in *in vitro* half-life compared to IP half-life for either drug ($p<0.05$).

	Isotonitazene			Metonitazene		
Parameter	In vitro	In vivo - IV	In vivo - IP	In vitro	In vivo - IV	In vivo - IP
$t_{1/2}$ (min)	22.6 ± 2.7	88.5 ± 8.2	117.5 ± 42.5	21.9 ± 2.2	44.7 ± 3.9	56.4 ± 13.4
k_e (min^{-1})	0.0315 ± 0.003	0.0083 ± 0.0009	0.0068 ± 0.0015	0.0321 ± 0.005	0.0159 ± 0.001	0.0161± 0.004
CL (mL min^{-1} kg^{-1})	36.5 ± 4.3 (intrinsic)	2.1 ± 1.0 (total)	N/A	37.2 ± 3.5 (intrinsic)	2.3 ± 0.7 (total)	N/A
V_D (mL)	N/A	59.6 ± 20.2	N/A	N/A	37.3 ± 10.4	N/A

3.4. Human Postmortem Sample Analysis

All matrices available for the eighteen positive cases retested for this study are summarized in Table 11 including the presence or absence of urine metabolites. Of these cases, numbers 1-14 were summarized in a publication along with the validation data as explained in Section 3.5. (70). Those cases reflected the period of those tested before publication submission in March 2023, in deaths from December 2020 until October 2022. The remaining four cases, numbers 15-18, were detected since the publication and reflect more recent positives of deaths up to December 2023. In all case submissions included in this study, no brain samples were collected.

Table 11. Nitazenes detected by case in all samples.

Case	Sex, Age (years)	Nitazene Detected	Conc in fem (ng/mL)	Conc in matrix #2 (source)	Conc in matrix #3 (source)	Urine Metabolites Present
1	M, 30	ITZ	NDT	<1.0 (urine)	N/A	NDT
2	M, 49	ITZ	0.13	NDT (urine)	N/A	N-DI
3	M, 51	ITZ	3.4	N/A	N/A	N/A
4	M, 24	ITZ	0.11	N/A	N/A	N/A
5	M, 43	ITZ	1.0	N/A	N/A	N/A
6	M, 36	ITZ	0.17	1.1 (urine)	NDT (liver)	N-DI, 4'-HN
7	F, 29	ITZ	3.3	NDT (urine)	N/A	NDT
8	F, 36	ITZ	12	8.4 (urine)	N/A	N-DI, 4'-HN, 5-AI
9	M, 44	MTZ	NDT	30 (urine)	N/A	NDT
10	M, 61	ITZ	0.43	0.76 (heart blood)	<1.0 (urine)	N-DI
11	M, 42	ITZ	0.16	NDT (urine)	N/A	NDT
12	M, 32	MTZ	0.49	14 (urine)	N/A	N-DM, 4'-HN
13	M, 49	MTZ	0.10	1.0 (urine)	N/A	N-DM
14	M, 57	MTZ	1.5	<1.0 (urine)	N/A	N-DM
15	M, 50	ITZ	0.37	3.5 (urine)	N/A	N-DI, 4'-HN
16	M, 62	MTZ	<0.10	2.5 (urine)	N/A	4'-HN
17	F, 37	MTZ	0.11	NDT (liver)	N/A	N/A
18	M, 34	MTZ	1.2	<1.0 (urine)	N/A	NDT

Abbreviations: 4'-HN: 4'-hydroxy nitazene; 5-AI: 5-amino isotonitazene; conc: concentration; ITZ: isotonitazene; F: female; fem: femoral blood; N/A: not applicable; N-DI: N-desethylisotonitazene; N-DM: N-desethylmetonitazene; NDT: not detected; M: male; MTZ: metonitazene

Not applicable indicates that matrix was not available for testing in that case.
Not detected indicates the matrix was tested but the analyte(s) was negative.

Table 12. Summary of blood results for both analytes in human postmortem cases.

Blood Results	Isotonitazene	Metonitazene
Conc. range (ng/mL)	0.11 – 12.0	0.10 – 1.5
Median conc. (ng/mL)	0.43	0.49
Average conc. (ng/mL)	1.9	0.69

3.4.1. Isotonitazene Analysis

For isotonitazene, there were ten cases with concentrations > 0.10 ng/mL in blood, a summary of which is seen in Table 12. The blood concentrations spanned the range of 0.11 to 12.0 ng/mL with a median concentration of 0.43 ng/mL, and average concentration of 1.9 ng/mL. All blood sources were from femoral blood, except for one heart blood sample. To our knowledge, 12 ng/mL of isotonitazene in femoral blood is the highest reported concentration to date. One case that previously tested positive for isotonitazene in femoral blood tested negative in blood and < 1.0 ng/mL in urine. The original qualitative result had a blood concentration at the reporting limit, 0.10 ng/mL. Of the positive blood samples that had urine available for testing (n=8), only three were positive and one was < 1.0 ng/mL. Only one case had liver collected. The liver was poor quality and both the fluid in the specimen cup and a fresh liver tissue homogenate (at 1:5 dilution) were negative.

3.4.2. Metonitazene Analysis

There were seven positive metonitazene cases retested for quantitation. All seven cases had paired blood and urine specimens except for one case which had paired blood and liver. A summary of blood results in seen in Table 12. Metonitazene femoral blood

concentrations were 0.10, 0.11, 0.49, 1.20 and 1.5 ng/mL with an average concentration of 0.69 ng/mL. One case was <0.10 ng/mL in blood, therefore not included in the quantitative statistics, and one case was negative in blood but positive in urine only. Five of the blood positive cases also had urine available for testing. Of those, three were positive ≥1.0 ng/mL, and the other two were also positive below the LOQ. The one negative blood case had metonitazene detected in urine only, which was the specimen that was originally reported positive. This case had the highest level of metonitazene detected in urine in this study, at 30 ng/mL. The four urine positive case concentrations ≥1.0 ng/mL were 1.0, 2.5, 14, and 30 ng/mL.

3.4.3. Urine Metabolites

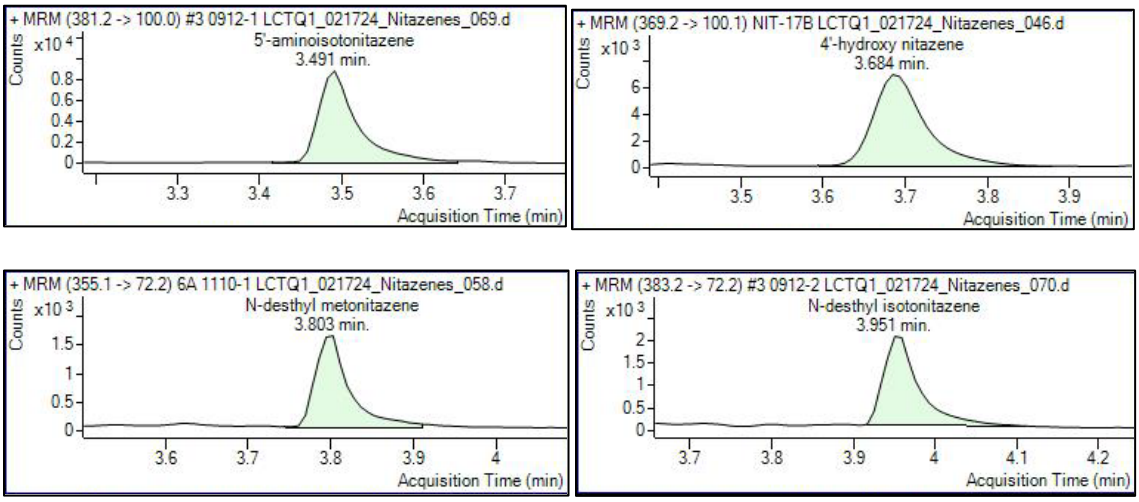

Figure 15. Chromatography in authentic postmortem urine specimens for all four metabolites.

The presence of four metabolites were evaluated in all available urine specimens. All four metabolites tested were detected in human casework (Figure 15). All three metabolites of isotonitazene that were tested were detected in urine from the case with the highest blood concentration. No other isotonitazene case had all three metabolites

present. Out of the eight urine samples available in isotonitazene cases, six of them were not detected for metabolites. N-desethylisotonitazene had the highest incidence as it was positive in 5 out of 8 urine samples. 5-aminoisotonitazene was the metabolite found in lowest incidence at 1 case. Two of the six metonitazene cases that had urine available were not detected for metabolites. The highest incidence of metabolite positivity for metonitazene was N-desethylmetonitazene which was present in half the cases. We observed the presence of 4'-hydroxynitazene in human postmortem urine samples in both isotonitazene and metonitazene cases. It was detected in 2 of 6 cases for metonitazene, and 3 of 8 isotonitazene cases.

3.4.4. Case Demographics and Concurrent Drug Findings

Further case details including a detailed list of drug findings that were determined using toxicology case files and the OCME CMS are included in Appendix 1. A summary of these findings is shown in Figure 16. Isotonitazene and metonitazene were not found together in any reported case and were found in the presence of at least one other intoxicant in all cases. Etazene, another nitazene, was found with isotonitazene in one case. All but one case had the presence of fentanyl, many also with the presence of fentanyl analogs. The next most encountered analyte was xylazine in 14 out of 18 cases.

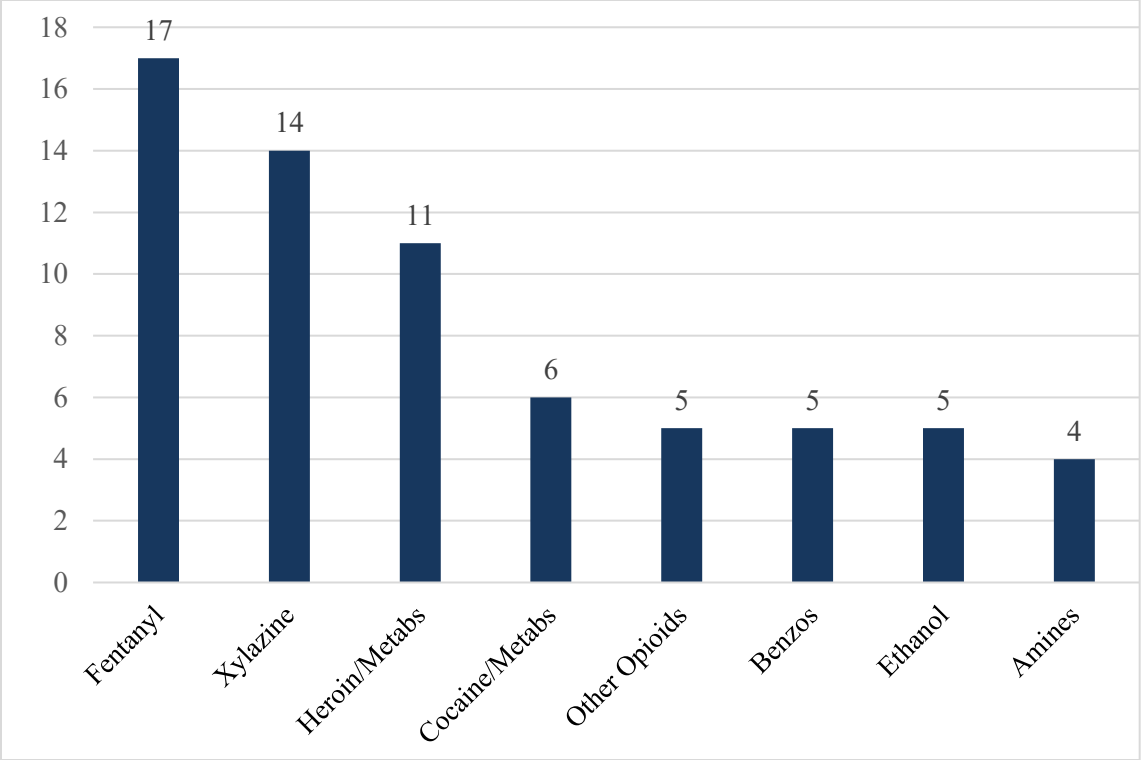

Figure 16. Concurrent toxicological findings by individual drug or drug class, out of eighteen total cases. Abbreviations used are amines: sympathomimetic amines, benzos: benzodiazepines, and metabs: metabolites.

In the nitazene cases reported, male decedents (n=15) ranged in age from 24 to 62 years, while female decedents (n=3) were 29, 36 and 37 years old. The mean and median age of all nitazene users was 42 years. Age and biological sex of users are summarized in Figure 17. The manner of death for all cases was "accident". The positive nitazene analog was reported as contributory to death in all cases except two. Of the two cases where metonitazene was not included on the death certificate, one case had metonitazene absent from blood, so unlikely to be contributory to death due to delayed use. The other case was due to multiple-blunt force injuries as a result of subway train impact. Cause and manner of death for each case are included in Appendix 1.

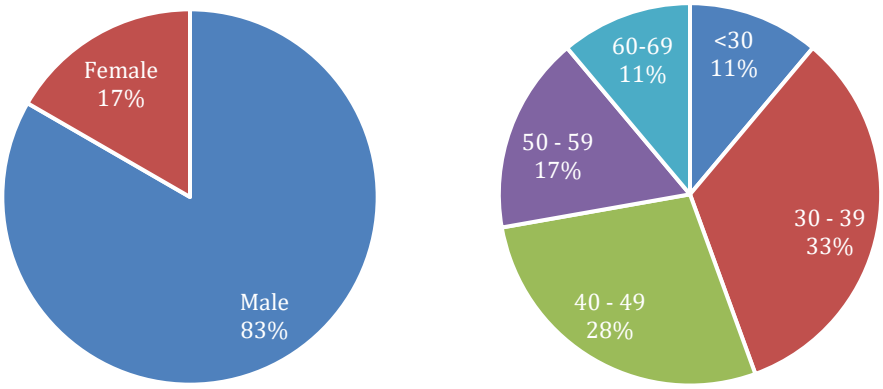

Figure 17. Biological sex (left) and age by decade (right) of nitazene users in OCME postmortem cases. Females made up 17% of all cases, and males 83%. The majority of users (33%) were in the age range of 30-39 years. The mean and median ages were 42 years.

3.5. Method Validation

3.5.1. Calibration Model

Both isotonitazene and metonitazene had residual values within ± 2 standard deviations (SD) for both linear and quadratic plots. There was an insignificant difference in residual values when an inverse calibration model was compared to an inverse squared model. Residuals were improved when the curve was not forced through the origin. Therefore, the simplest model that fit criteria was chosen for the two analytes. The calibration model for both analytes in blood, and isotonitazene in urine was determined to be linear, non-forced, with a 1/x weighting. The best model that fit the metonitazene urine data was quadratic, non-forced, with 1/x weighting. After these calibration models were applied to the five days of experiments, the r^2 values for both analytes in all runs were greater than 0.990.

3.5.2. Limit of Detection

Nine replicates of each matrix were tested at the LOD concentrations of 0.10 ng/mL (blood, plasma, brain) and 1 ng/mL (liver, urine). At least three separate sources were used of each matrix to make up the nine total replicates. The sources included the negative matrices used for calibrators and controls as well as postmortem samples. All matrices for both analytes met the designated acceptance criteria. Extracted ion chromatograms for isotonitazene in blood and liver at the LOD are shown in Figure 18. and metonitazene in blood and liver in Figure 19.

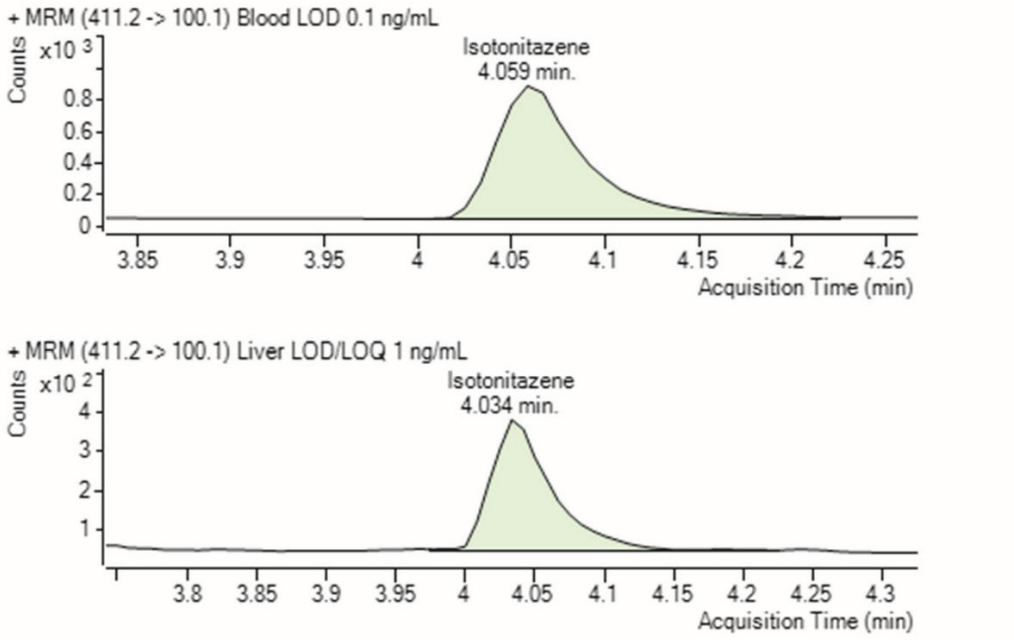

Figure 18. Extracted chromatograms of isotonitazene at the LOD in blood (0.10 ng/mL) and liver (1.0 ng/mL).

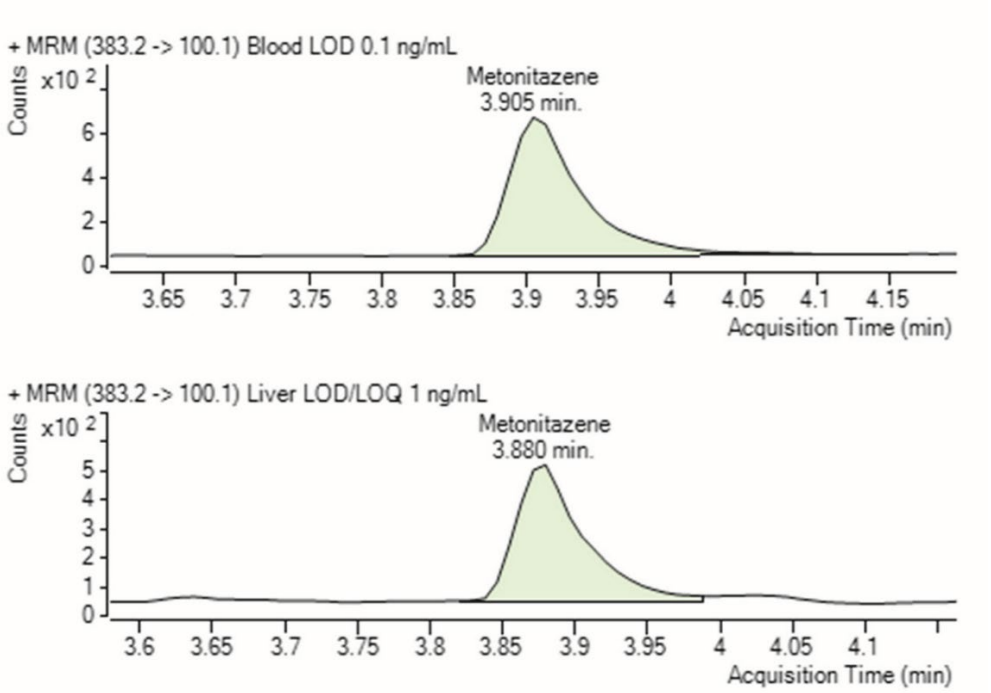

Figure 19. Extracted chromatograms of metonitazene at the LOD in blood (0.10 ng/mL) and liver (1.0 ng/mL).

3.5.3. Bias and Precision

QC replicates for each matrix (n=15) and LOQ (n=9) were evaluated, and percent bias and precision were calculated. The mean QC and LOQ results for each concentration were within 15% of the expected value for both analytes in all matrices except metonitazene in urine at the LOQ. The mean urine LOQ concentration after three runs and nine replicates for metonitazene was 0.83 ng/mL which was within 20% of 1 ng/mL. Bias was within ± 20% for all matrices for both isotonitazene and metonitazene for all concentrations. All bias percentages are summarized in Table 13. Precision %CV was calculated for within-run and between-run values using one-way analysis of variance (ANOVA). All matrices for both analytes had a %CV < 20%. Full precision data is outlined in Table 14.

Table 13. Bias (%) for isotonitazene and metonitazene in all five biological matrices at the LOQ, and three QC concentrations.

Compound name	Matrix	LOQ	Low QC	Mid QC	High QC
Isotonitazene	Blood	13.82	-2.93	-4.28	-0.19
	Plasma	9.97	-2.82	-7.36	-4.55
	Liver	-2.36	-10.64	-8.81	-6.37
	Brain	4.16	-10.59	-9.09	-2.23
	Urine	10.65	9.53	4.50	6.81
Metonitazene	Blood	2.71	-0.72	0.36	-1.46
	Plasma	-6.98	-6.67	-2.54	-6.11
	Liver	2.69	-6.28	-2.21	-1.45
	Brain	-1.97	-11.15	-5.49	-4.42
	Urine	-16.58	0.36	-0.51	-0.58

Table 14. Precision for isotonitazene (ITZ) and metonitazene (MTZ) in all five biological matrices at the LOQ, and three QC concentrations as within-run (W/R) and between-run (B/R) percent coefficient of variation (%CV).

	Matrix	LOQ %CV		LQC %CV		MQC %CV		HQC %CV	
		W/R	*B/R*	*W/R*	*B/R*	*W/R*	*B/R*	*W/R*	*B/R*
ITZ	Blood	9.55	10.52	12.44	11.98	4.61	5.57	7.78	9.35
	Plasma	6.98	11.66	19.14	18.60	4.98	6.02	7.51	7.05
	Brain	3.84	7.55	4.33	8.42	3.74	8.38	5.06	7.35
	Liver	11.09	11.97	6.06	10.02	5.55	6.78	9.20	10.37
	Urine	4.33	10.79	3.25	4.88	6.18	5.93	5.67	5.84
MTZ	Blood	10.54	13.59	12.40	11.70	3.56	4.89	5.33	8.27
	Plasma	13.31	19.46	10.67	11.01	4.71	5.10	7.14	6.66
	Brain	6.93	17.83	7.00	10.93	3.44	4.79	4.00	6.43
	Liver	5.97	6.62	11.40	11.77	7.61	8.42	2.40	4.58
	Urine	4.68	11.47	5.01	8.58	14.09	8.75	4.93	4.45

3.5.4. Ion Suppression and Enhancement

Tissue samples exhibited ion suppression for both analytes at the LQC and HQC concentrations. Ion suppression for brain was -35%, -64%, -55.2%, and -88% for metonitazene LQC and HQC and isotonitazene LQC and HQC, respectively. Plasma samples were least affected by matrix effects for both analytes at both concentrations exhibited by the results between -12% ion suppression to 2.6% enhancement. Urine matrix effects were 31%, -20%, 28% and -23% for metonitazene LQC, HQC, and isotonitazene LQC, HQC, respectively. High ion enhancement was observed in blood sources at 154%, 79%, 81%, and 41% for metonitazene LQC and HQC and isotonitazene LQC and HQC, respectively. Despite the observed matrix effects when analyzing these drugs, the ability to reliably detect the target analytes at the LOD concentrations and bias and precision at the LOQ was unaffected.

3.5.5. Interferences

Matrix interference studies demonstrated that all ten blank matrix samples of blood and plasma had no discernible peaks detected for any of the analytes. Isotonitazene, isotonitazene-d_7 and metonitazene had no discernible peaks in liver or brain samples, whereas metonitazene-d_3 showed some interferences. Liver had one sample where a peak was integrated for metonitazene-d_3 but had no qualifier ion present and did not meet acceptance criteria. Seven of the ten brain samples showed a non-Gaussian peak in the metonitazene-d_3 window with high baseline noise and low response. Urine samples showed no interferences for metonitazene-d_3, isotonitazene, or isotonitazene-d_7. There were peaks detected in the metonitazene RT window in two urine samples, both of which had RTs approximately 0.07 mins early from true metonitazene

peaks.

Interferences between the internal standard and matched target analytes were explored in all matrix types. Triplicate analyses of IS fortified at 5 ng/mL in negative matrix showed no positive identification of target analytes in any samples. Triplicate analyses of target analytes fortified at 100 ng/mL in negative matrix showed no interference with isotonitazene-d_7 in any matrix type. Metonitazene-d_3 was found in the presence of high amounts of metonitazene at responses of 8-10% of true fortified samples. This low-level contribution is not expected to affect quantitation of authentic metonitazene cases.

Blood, plasma, brain, liver, and urine MQC samples (8 ng/mL) that were fortified with 83 additional drugs were evaluated for chromatography and accuracy in quantitation of the 8 ng/mL metonitazene and isotonitazene. No interferences were observed for metonitazene in any matrix type exhibited by passing QC and ion ratio accuracy (± 20%) and Gaussian chromatography. Isotonitazene in all matrix types passed criteria when interference of drugs of abuse were tested in groups.

3.5.6. Dilution Integrity

Blood, plasma, and urine were evaluated for 1:2 dilution integrity of a 160 ng/mL sample. For metonitazene, all three replicates of each matrix type resulted in a quantitative value within 20% of the target concentration. The %CV for each matrix type was 2.9, 12.1 and 8.9 for blood, plasma, and urine, respectively. Isotonitazene had one urine replicate that failed concentration accuracy at 126 ng/mL, but all other replicates of urine, plasma and blood were within 20% of 160 ng/mL. The %CV was 6.0, 13.8 and 4.9 for blood, plasma, and urine, respectively.

3.5.7. Carryover

Carryover was evaluated using negative QC samples injected immediately after a 100 ng/mL sample in triplicate for blood, plasma, brain, and liver, and a 200 ng/mL sample for urine. All replicates for both analytes were shown to be free from carryover, with any integrated peaks quantitating below the LOD.

CHAPTER 4: DISCUSSION

4.1. Preface

One hypothesis in this study was that isotonitazene and metonitazene would have different metabolic profiles despite their similar chemical structures when compared using both *in vitro* and *in vivo* models. It was also hypothesized that the IP half-life would be shorter than the IV half-life due to the first pass metabolism effect. Additionally, it was hypothesized that both analytes would be detected in brain samples due to their action in the CNS. Finally, as a result of the pharmacokinetic study and retesting of authentic cases, this research sought to determine drug distribution to inform human postmortem case interpretation.

4.2. Comparison of Pharmacokinetics of Isotonitazene and Metonitazene Using An *In vitro* and *In vivo* Model

All pharmacokinetic parameters from both *in vitro* and *in vivo* studies are summarized in Table 10. Despite initial predictions, metonitazene and isotonitazene had the same half-life and intrinsic clearance in the HLM model. Reported PK classification based on *in vitro* intrinsic clearance between 15 and 45 mL min^{-1} kg^{-1} and half-life between 20 and 60 min are categorized as intermediate clearance compounds (61). Isotonitazene with a mean ± SEM for intrinsic clearance (mL min^{-1} kg^{-1}) and half-life (min) respectively of 36.5 ± 4.3 and 22.6 ± 2.7, and metonitazene intrinsic clearance and half-life of 37.2 ± 3.5 and 21.9 ± 2.2 indicates both drugs fall into the intermediate clearance category.

In vivo half-lives after IV administration were significantly different than each other, while calculated clearance rates were the same. The half-life of metonitazene was

almost exactly half that of isotonitazene at 44.7 ± 3.9 and 88.5 ± 8.2 min respectively. Original predictions were that these compounds would have distinctive PK parameters, therefore this data is more consistent with the hypothesis, but it does not match the *in vitro* data where the same half-life was determined for both analytes using microsomal incubation. This comparison of *in vitro* to *in vivo* IV half-lives for each drug were significantly different from each other. Isotonitazene exhibited a higher volume of distribution as calculated in this study, and higher logP than metonitazene at 4.85 compared to 4.086 (15). The logP of isotonitazene is higher than other illicitly abused opioids such as fentanyl and heroin as well. An increased logP value shows a higher lipophilicity, and therefore greater penetration into tissue compartments in the body. This phenomenon accounts for isotonitazene having a longer half-life than metonitazene due to its wider distribution outside of the plasma compartment. The lack of correlation with the *in vitro* study, where half-lives were equal and significantly shorter than in the IV study, is likely attributed to the lack of distribution possible in the *in vitro* system.

Intrinsic clearance in the *in vitro* model was calculated and compared to the total clearance calculated from the *in vivo* model. The lack of correlation between the values could be due to multiple factors. As previously described, intrinsic clearance is a parameter of drug elimination that does not account for liver perfusion or protein binding. Another reported cause of the discrepancies observed between intrinsic clearance and total clearance is a drug exhibiting nonlinear kinetics (72). Nonlinear kinetics have been observed in other reports after high SC doses of isotonitazene in male rats (>10 μg/kg) likely due to drug accumulation and delayed clearance (41). A similar effect was observed in male rats after SC administration of carfentanil at 1, 3, and 10 μg/kg, where

half-life increased at the 10 ug/kg dose, also indicating impaired clearance at high doses (73). While this study did not explore multiple doses to compare the kinetics, it is not expected that nonlinear kinetics are affecting the *in vivo* study due to the low concentrations of drugs detected in plasma samples. Clearance in the *in vivo* model was the same for both drugs while their half-lives were significantly different at the 95% confidence interval. This can be explained by the fact that clearance is directly proportional to both volume of distribution and elimination constant. While metonitazene has a higher elimination constant, it has a lower volume of distribution compared to isotonitazene, resulting in their nearly identical calculated clearances.

Drug accumulation in brain was demonstrated in this study in only one animal given an IP dose of isotonitazene. Both isotonitazene and metonitazene were detected in brain samples from trial animals administered higher doses utilizing both types of injection. Therefore, detectable distribution into brain tissue is not dependent on the administration (IV v IP) but rather appears to be concentration dependent. A study by Lee et al. included data on isotonitazene concentrations in homogenized whole brain samples after IP administration of 200 μg/kg in female mice. The mean isotonitazene concentration in brain was 3 ng/mL (40). This concentration is well above our limit of detection in brain at 0.10 ng/mL, however, their dose was 8x the dose in this study. Also, mice were sacrificed by guillotine 15 min post administration. Sacrifice was a minimum of six hours post injection in this study; therefore, the drug might have been eliminated from the brain by the time the samples were collected.

No animal liver samples were positive for either drug during the experimental or trial phases of this research. No reports of attempted testing of liver samples were mentioned

in the two published reports of isotonitazene *in vivo* pharmacokinetics, and ours is the first study reporting metonitazene data (40,41). Due to the sample preparation procedure, the 1:5 w/w homogenization in water could be diluting out low concentrations resulting in false negatives. However, in combination with the results of human cases, there is evidence that both drugs are not accumulating in liver tissue in detectable concentrations across multiple species.

Urine results mirrored the brain results in that only one animal administered isotonitazene IP had a concentration above the reporting limit, which in the case of urine, is 1.0 ng/mL. No urine samples were positive for metonitazene at concentrations >1.0 ng/mL. Many of the earliest urine sample collected had indications of the drugs < 1.0 ng/mL. This study is unable to corroborate the lack of urine positivity to the concentration of the dose as was possible with the brain data, due to lack of urine samples collected during trial studies.

As a result of the lack of parent drugs detected in urine, all urine samples were tested for four metabolites. One metabolite for each drug, N-desethylisotonitazene and N-desethylmetonitazene were positive in animal urine samples. Positive samples for both analytes were in the IP administration group only, and all urine samples taken from the associated animal (2 to 3 for each) were positive for the metabolite. 4'-Hydroxy nitazene and 5-aminoisotonitazene were not detected in animal urine samples. No data on urine metabolites in animals have been reported for comparison. Metabolites in blood were reported in the Walton et al. study and interestingly, no 5-aminoisotonitazene was detected in rat plasma samples despite the same group reporting it as the most common blood metabolite detected for isotonitazene in humans (30, 60). Our research observed

species differences in both 4'-hydroxy nitazene and 5-aminoistonitazene positivity in urine between rat and human samples.

IP injection was explored to determine if isotonitazene and metonitazene were especially susceptible to first-pass metabolism which would be exhibited as differences in PK parameters compared to the IV data. Specifically, if significant first-pass metabolism was occurring, it would be expected that the IP half-lives would be shorter than IV half-lives. This would be due to the absorption directly into the portal vein system and subsequent metabolism by the liver, prior to distribution into the rest of the body. We were unable to demonstrate the prevalence of first pass metabolism due to longer mean IP half-lives than IV for both drugs. The variability in plasma concentrations detected after IP injection were more evident in the case of isotonitazene compared to metonitazene, but both showed inconsistencies. Metonitazene half-life was shorter than isotonitazene, as demonstrated by the IV data as well, however not at a statistically significant level likely due to the variability observed. Since the drugs were detected in brain and urine after IP injection, it is evident the drugs were absorbed into the body of the animal, but it is possible that especially in the case of isotonitazene, there was a distribution issue when administered intraperitoneally. As mentioned in the brain data discussion, Lee et al. used IP injection of isotonitazene successfully, however they used a much higher dose than our study (40). As we aimed to limit any physical effects of the drugs, their goal was PD/PK analysis after administering a novel vaccine for benzimidazole opioids. Therefore, at higher doses, it is likely we also would have observed better distribution of the drug throughout the body. The occurrence of first-pass metabolism is a possibility due to low concentrations of circulating drug detected in the plasma after IP administration of both

drugs when compared to IV administration. However, first-pass metabolism was not able to be clearly demonstrated in this study.

The mean half-life of isotonitazene in this study was approximately 90 mins IV and approximately 120 mins after IP administration. Lee et al. published a study using naïve female BALB/cByJ mice that were administered 200 μg/kg of isotonitazene IP where the mean half-life for isotonitazene in serum was 1.64 h, or 98.4 min, with a maximum concentration of 3.11 ng/mL (40). They compared their results to other mice studies which showed both nitazene compounds have longer half-lives than fentanyl, 0.8 to 0.9 h, and naloxone, 0.5 h (40). The study by Walton et al. of isotonitazene pharmacodynamic effects in male Sprague-Dawley rats previously mentioned, also reported pharmacokinetic constants using SC injection at multiple doses, 3, 10 or 30 μg/kg. They were unable to calculate pharmacokinetic parameters for the 3 μg/kg dose due to limited samples with quantifiable drug amounts (41). Half-life (min) as mean ± SEM was reported as 34.1 ± 5.2 for 10 ug/kg dose and 56.4 ± 3.1 for 30 ug/kg. They found statistically significant differences in the parameters when comparing 10 to 30 μg/kg showing a dose-dependent response and the potential for delayed drug clearance of isotonitazene in doses >10 μg/kg (41).

Pharmacokinetics of other opioids in rats have been reported. Methadone and morphine have been shown to have similar half-lives in rats after SC injection, between 70-90 min despite their observed different durations of action (75). IV fentanyl half-life in male rats has been reported as 0.85 h (51 min) with standard deviation (SD) of 0.09. Metonitazene IV half-life in our study was similar at 44.7 ± 3.9 min. The half-life of two fentanyl analogs, cyclopropylfentanyl and carfentanil were reported by Bergh et al. as

mean ± SEM. Cyclopropylfentanyl half-life in rats was determined as 115 ± 11 min (SC 30 ug/kg) with k_e of 0.0063 ± 0.000 (49). Isotonitazene had a similar half-life after IP administration at 117.5 ± 42.5 min. Carfentanil half-life (min) in male rats at two doses was 55.1 ± 6.2 (SC 3 ug/kg) and 64.4 ± 8.4 (SC 10 ug/kg) (73). In our study, both metonitazene and isotonitazene showed similar elimination to reported values for fentanyl and fentanyl analogs.

4.3. Determination of Isotonitazene, Metonitazene, and Their Metabolites in Human Postmortem Casework

The concentrations determined using authentic cases in this study are compared with other published data on these analytes as summarized in Tables 15 and 16. This research has published the highest concentrations of isotonitazene reported yet at 12.0 ng/mL in blood and 8.4 ng/mL in urine with otherwise comparable concentrations detected. Like other reports, this research detected higher urine concentrations of metonitazene, with the highest case at 30 ng/mL. The metonitazene blood data was overall lower than the two other studies. These low ng/mL blood concentrations detected of nitazenes in this study and other reports also correlate with concentrations detected in cases of other potent opioids, like fentanyl and fentanyl analogs (39).

Table 15. Comparison of isotonitazene blood and urine concentrations detected in postmortem casework from this research to other published studies.

Study	n	Blood data (ng/mL)	Urine data (ng/mL)
Sorrentino 2024	11	Mean: 1.9, max: 12.0	Mean: 4.3, Max: 8.4
Brooks-Lim 2019	1	Iliac blood: 0.12, 0.56	Mean: 2.3
Krotulski 2020	18	Mean: 2.2, max: 9.5	Mean: 2.4, max: 4.0
Mueller 2021	3	Median: 1.2, max: 2.28	Max: 3.3
Walton 2022	6	Mean: 2.0, max: 8.6	Mean: 1.4, max: 2.5

Table 16. Comparison of metonitazene blood and urine concentrations detected in postmortem casework from this research to other published studies.

Study	n	Blood data (ng/mL)	Urine data (ng/mL)
Sorrentino 2024	7	Mean: 0.69, max: 1.5	Mean: 12, Max: 30
Krotulski 2021	20	Mean: 6.3, max: 33	Mean: 15, max: 46
Walton 2022	16	Mean: 5.5, max: 12	Mean: 14, max: 33

All positive cases for metonitazene in blood that had a paired urine specimen also had a detectable amount of metonitazene in urine, five out of five cases. Out of the isotonitazene blood positives that had urine available, five out of the eight cases were positive in urine as well. The positive blood isotonitazene cases with negative urines were not the highest or lowest blood concentrations seen in the study. It is possible that the lack of correlation of isotonitazene urine positives is due to the longer half-life and therefore elimination rate. This potent drug is likely taken and immediately contributing to death, at which time it is still circulating in the blood and has not been eliminated yet. For the analysis of metabolites in urine, only four were tested due to availability of drug reference standards. All four metabolites were detected in human casework. 5-aminoisotonitazene was only detected in one isotonitazene case which is in-line with

published reports that is most commonly detected as a metabolite in blood rather than urine (54). 4'-Hydroxy nitazene was detected with both metonitazene and isotonitazene cases as a shared metabolite which is also consistent with other reports (12).

Due to changes in autopsy protocols at OCME, no brain samples were available for testing in nitazene-positive cases. In one case each for isotonitazene and metonitazene, we were able to analyze liver specimens, but both were not detected for the drug. With the exception of one report, no other liver positive cases have been reported in the literature due to both lack of testing and lack of detection in the liver (12, 53, 54, 60, 78). One report from Switzerland of testing for isotonitazene in all available biological matrices in three separate cases, included blood (femoral and cardiac), urine, vitreous humor, pericardial fluid, hair, brain, spleen, kidney, lung, heart, liver, bile, cerebral spinal fluid (CSF), and muscle (77). They found liver positive in the lowest concentration of all matrices tested in all three cases at < 0.05 ng/g. This concentration is substantially lower than the validated limit of detection in our method at 1.0 ng/g.

In our study, fentanyl was detected in all but one nitazene case, with the second most commonly found drug being xylazine. Fentanyl is widely used as an anesthetic and analgesic in a hospital setting, and is now the compound responsible for the most overdose deaths in the United States (32, 79). It can be diverted for misuse, but the majority of fentanyl detected in toxicology casework is now from illicitly manufactured fentanyl (78). Xylazine is an anesthetic used in veterinary medicine that is increasingly being found in illicit drug supplies. It is not approved for human use and therefore is not a scheduled substance (79). Xylazine is most commonly identified in combination with heroin, fentanyl, or cocaine as a presence in drug mixtures, however xylazine is also

being misused on its own (80, 81). Within OCME, in all cases where xylazine has been identified, it is combined with an opioid (81). The fact that all metonitazene cases and all but one isotonitazene case in our study were detected in the presence of an opioid can be attributed to the testing strategy at OCME. These analytes are not being evaluated in all cases but are screened only in cases where an opioid screened positive, or where targeted testing is completed due to suspicion of use. The pattern of incidence with other opioids that has been reported in other studies is variable.

In a study of isotonitazene cases from Cook County, IL and Milwaukee County, WI, flualprazolam, a novel benzodiazepine, was the most commonly co-identified analyte, with fentanyl as the next most common (76). Isotonitazene was reported with no other opioids present in 50% of cases by Krotulski et al. And the three isotonitazene fatality cases reported from Switzerland were not encountered with other opioids (77). Similar to the cases in our study, a report from Tennessee regarding cases from 2019 to 2021 also found fentanyl as the most commonly encountered analyte in nitazene cases, and all cases involved polydrug use (82). Previous publications involving isotonitazene have reported its presence with novel benzodiazepines (16, 60). The presence of nitazenes and novel benzodiazepines was consistent in this study. One isotonitazene case also had the presence of etizolam, and one metonitazene case contained bromazolam.

Nitazene-combination cases have also been reported including metonitazene in the presence of butonitazene and flunitazene (55). Etazene (etodesnitazene) was also detected with isotonitazene in one of our cases. Reports of emerging nitazenes contributing to overdose deaths continue to be reported, including drugs that are isomers of other nitazenes (22,41). Protonitazene was first reported in December 2021 by the

Center for Forensic Science & Research Education (CFSRE) in nine total cases from IA, TX, LA, TN, and NJ, but was the first case was detected at OCME in 2024. Protonitazene is a positional isomer of isotonitazene therefore has the exact same mass spectra and many analytical methods would be unable to distinguish between them (74).

The demographic profile of nitazene users in our study, 83% male with a mean and median age of 42 years, aligns with that reported by other laboratories. Middle-aged males tend to be the typical synthetic opioid users, which was true of our data as well (12). The compilation of all cases in 2022 by Montanari et al. of all published reports of nitazene deaths showed that approximately 80% of reported nitazene cases involved males (n=82), and the median age was 41 years old (n=39).

4.4. Use of Low Sample Volume in Quantitative Analysis of Metonitazene and Isotonitazene

An analytical method for the quantitative analysis of metonitazene and isotonitazene was validated in blood, plasma, brain, liver, and urine extracted by protein precipitation and analysis using LC/MS/MS. One challenge in the validation was the high matrix effects observed. Tissue samples showed high ion suppression with the brain isotonitazene HQC the largest suppression observed at -88%. Conversely, blood samples showed ion enhancement up to 154% at the LQC for metonitazene. This was not isolated to our study as other published studies also observed matrix effects with these analytes. A method published by Krotulski, et al. reported high ion enhancement of metonitazene (174%) without effect to other validation parameters (55). Mueller et al. reported ion enhancement >100% for isotonitazene in all matrix types tested which included blood, urine, liver, and brain (77). Garneau et al. reported isotonitazene enhancement of 291% in

blood and 149% in urine (83). The limit of detection at 0.10 ng/mL for blood, plasma and brain, and 1.0 ng/mL for liver and urine in our study was not affected by this phenomenon. Some interferences in urine for metonitazene were observed during validation. Therefore, in urine casework analysis, extra diligence must be taken to ensure RT windows are updated and compared to calibrator and controls when evaluating unknown samples. Chromatography and QC accuracy were affected when all 83 common drugs of abuse were combined into one sample when evaluating interfering with isotonitazene. This scenario would be unlikely to occur in routine casework; however, in multi-analyte targeted screening procedures, care should be taken when planning pooled drug combinations due to this potential interference in isotonitazene in the presence of many drugs.

This multi-matrix quantitative method utilized a 50 μL sample volume. Other published methods for analysis of these two drugs by LC/MS/MS utilized a minimum of 200 μL. In the animal study, a maximum of 200 μL of blood was collected at each time point, and many times the maximum volume was not obtainable, so significantly less than 200 μL of plasma was available for analysis, therefore making this low-volume method essential. Human postmortem blood samples are usually submitted with volumes adequate for analysis in much higher amounts than 50 μL, but often hospital samples drawn at the time of admission are submitted with limited quantity remaining. These low volume samples can be critical to test in death investigations after a person was treated in the hospital for days or weeks before death, as the postmortem samples would not be indicative of the intoxication that brought them to the hospital. Our published method has

applications in animal studies or human casework where only low quantity samples are available and accurate quantitation of isotonitazene and metonitazene is required.

4.5. Future Work

Potential areas for continuing research on metonitazene and isotonitazene have been illuminated as a result this study. While this research aimed to get as much data as possible from each animal, breaking up the drug distribution study from the plasma pharmacokinetics study would give more conclusive results for tissue concentrations. A shorter duration of study could allow for more definitive data on distribution of isotonitazene and metonitazene into brain and liver. Additionally, specific sites of the brain that are known to contain high concentrations of MOR, like the hippocampus, nucleus accumbens and the amygdala could be targeted for analysis rather than using whole brains (84). However, one published animal study also utilized whole brain analysis with sufficient concentrations at a higher dose than our study. Similarly, sectional segments of the liver tissue could be tested to determine if drugs only accumulate in specific lobes. Also, homogenization techniques could be optimized utilizing less dilution volumes to determine if low concentrations of drugs are being lost in the sample preparation process. Additionally, vitreous humor testing was not originally attempted in this study due to difficulty in obtaining this sample type from rats. However, vitreous is a valuable biological matrix in postmortem toxicology due to its stability from postmortem redistribution and decomposition processes compared to blood and tissues (85). This matrix could be explored in future studies.

This study focused on the presence of metabolites of isotonitazene and metonitazene in urine only. Future work could involve the presence of metabolites in

blood to compare with results in other published reports. Also, as more reference standards for metabolites become commercially available, they could be analyzed in animal and human samples. Finally, continued studies on emerging nitazene drugs could be performed and compared to our data to establish similarities and differences in pharmacokinetics across this potent drug class.

CHAPTER 5: CONCLUSION

This research found similar pharmacokinetic constants for isotonitazene and metonitazene in an *in vitro* HLM study which significantly differed from the *in vivo* rat data. *In vitro* metabolic studies are frequently used during drug development for a quick classification of elimination rate to select or reject compounds for further study (45). This application seems more valuable than attempting to do full elucidation of pharmacokinetics with expected correlation to *in vivo* data, especially when studying drugs that have high volumes of distribution.

Blood samples have been shown to be the most reliable specimen for detection in acute intoxications. *In vivo* data showed longer drug clearance of isotonitazene compared to metonitazene in both administration types. In human casework, higher incidences of metonitazene positivity was observed in urine as compared to isotonitazene. It is expected that isotonitazene will not always be detected in urine specimens in acute intoxications but its presence in urine can indicate delayed use. Metabolites of isotonitazene and metonitazene were both detected in the first urine collection from study animals and in human urine samples. 4'-Hydroxy nitazene was proven to be a shared metabolite of isotonitazene and metonitazene in human casework but was not detected in any animal samples. This finding shows species differences in metabolite formation across rats and humans.

This study was unable to fully determine the distribution of isotonitazene and metonitazene into tissues in animals, but evidence was found to show that the distribution is dose dependent. Neither drug was positive in the human liver samples or any animal liver sample throughout the study. In many death investigations of decomposed

individuals, tissues are the only remaining biological matrix suitable for toxicological testing. If a liver sample is negative for nitazenes, their contribution to an individuals' death cannot be ruled out due to the lack of ability to detect these compounds in this matrix in known positive cases.

The highest published isotonitazene femoral blood concentration was reported in this study. But overall, low ng/mL concentrations can be expected for both metonitazene and isotonitazene. In all cases, nitazenes were not detected as the sole intoxicant to date, and OCME and other labs alike are seeing them in combination with other established opioids. However, due to the incidence of nitazenes found without other opioids in some reports, the importance of updating the detection strategy at OCME has been highlighted. Rather than targeting testing to cases where another established opioid has screened positive, these analytes should be present in the screening techniques themselves. The importance of detection strategies of novel compounds is a crucial consideration to be made in forensic toxicology laboratories while balancing the challenges of time and resources. Sensitive screening procedures should be employed whenever possible to ensure that critical drug findings are not missed. Chromatographic methodologies should be able to separate isomeric compounds so that accurate drug reporting can be made, therefore specific method development and optimization is important. 4'-Hydroxynitazene has been proven to be a shared metabolite of multiple analytes in this drug class, therefore has potential to be utilized as a biomarker for detection of nitazenes, and to indicate the presence of a nitazene not yet included in testing panels.

An extraction procedure utilizing the lowest published sample volume, 50 µL, was successfully validated for testing five different biological matrices and utilized in all

aspects of this research. This sample volume allows for testing of limited postmortem specimens, low volume hospital specimens, and may also be utilized for clinical applications and other pharmacokinetic studies. This study was performed in collaboration with the NYC OCME to gain additional insight into how nitazenes are contributing to deaths in local communities.

Due to their potencies, and the possibility of their presence being unknown to users, these are dangerous drugs that are contributing to deaths in NYC and across the world. Multiple reports were published predicting the decline of isotonitazene as it was replaced by metonitazene after being scheduled in mid-2020. However, both drugs have been detected in OCME casework years after they were federally scheduled, with the most recent isotonitazene-related death in September 2023 and metonitazene death in December 2023. Even still, knowing the unpredictable nature of novel opioid trends, it is expected that additional nitazene drugs and other synthetic opioids will emerge. It is the challenge of toxicologists to continue studying and reporting information on emerging drugs and for laboratories to utilize testing strategies that can detect novel compounds for multiple reasons. Examples include to aid clinicians in understanding how these drugs affect the body, and so that pathologists are aware of their presence in various biological matrices and can understand their contributions to death in fatal cases. Finally, educating the public by accurate reporting of toxicological information on novel drugs, including their incidence in regional areas, is critical information to help to combat the next wave of the opioid crisis.

APPENDIX 1: NITAZENE POSITIVE CASE DETAILS

Case No.	Sex, Age (years)	Nitazene Detected (matrix)	Other Toxicological Findings	Cause of Death	Manner of Death
1	M, 30	Isotonitazene (urine)*	etizolam, naloxone, cannabinoids[1]	Acute intoxication by the combined effects of etizolam and isotonitazene	Accident
2	M, 49	Isotonitazene (femoral blood)	fentanyl, xylazine, norfentanyl, 4-ANPP, β-hydroxyfentanyl, morphine, naloxone, 6-MAM[2], codeine, cannabinoids[1]	Mixed intoxication (heroin, fentanyl, xylazine and isotonitazene)	Accident
3	M, 51	Isotonitazene (femoral blood)	fentanyl, acetylfentanyl, xylazine, norfentanyl, 4-ANPP, β-hydroxyfentanyl, morphine, codeine, tramadol, mirtazepine, sertraline, cotinine, 6-MAM, norbuprenorphine	Acute intoxication due to the combined effects of fentanyl, acetylfentanyl, isotonitazene, heroin, xylazine, tramadol, mirtazapine, and sertraline	Accident
4	M, 24	Isotonitazene (femoral blood)	fentanyl, 4-ANPP, xylazine, β-hydroxyfentanyl, amphetamine, methamphetamine	Toxic effects of fentanyl, xylazine, isotonitazene, and methamphetamine	Accident
5	M, 43	Isotonitazene (femoral blood)	fentanyl, norfentanyl, 4-ANPP, *p*-fluorofentanyl, etazene, β-hydroxyfentanyl, PPA, amphetamine, methamphetamine	Acute intoxication by the combined effects of fentanyl, *p*-fluorofentanyl, etazene, isotonitazene, phenylpropanolamine,	Accident

				amphetamine and methamphetamine	
6	M, 36	Isotonitazene (femoral blood, urine)	fentanyl, norfentanyl, 4-ANPP, valerylfentanyl, β-hydroxyfentanyl, xylazine, *p*-fluorofentanyl, 4-ANPP, tetrahydrofuran fentanyl, morphine, 6-MAM[2], benzoylecgonine, cocaine[2], amphetamine, methamphetamine, cannabinoids[1]	Multiple blunt force injuries	Accident
7	F, 29	Isotonitazene (femoral blood)	cocaine, benzoylecgonine, cannabinoids[1], fentanyl[2,3]	Acute intoxication by the combined effects of isotonitazene and cocaine	Accident
8	F, 36	Isotonitazene (femoral blood, urine)	fentanyl, β-hydroxyfentanyl, 4-ANPP, norfentanyl, morphine, codeine, 6-MAM[2], tramadol, xylazine, alprazolam, α-hydroxyalprazolam, benzoylecgonine, cocaine[2]	Acute intoxication due to the combined effects of heroin, fentanyl, isotonitazene, xylazine and alprazolam	Accident
9	M, 44	Metonitazene (urine)	Fentanyl, norfentanyl, *p*-fluorofentanyl, 4-ANPP, β-hydroxyfentanyl, morphine, methadone, EDDP, xylazine, tramadol[3],	Acute intoxication by the combined effects of fentanyl, *p*-fluorofentanyl, morphine, methadone, xylazine	Accident

			o-desmethyltramadol[3]		
10	M, 61	Isotonitazene (femoral blood, heart blood, urine)	fentanyl, norfentanyl, 4-ANPP, xylazine, morphine	Acute intoxication by the combined effects of fentanyl, isotonitazene, morphine, xylazine and ethanol	Accident
11	M, 42	Isotonitazene (femoral blood)	fentanyl, acetylfentanyl, 4-ANPP, *p*-fluorofentanyl, norfentanyl, n-methylnorfentanyl, benzylfentanyl, β-hydroxyfentanyl, xylazine, 6-MAM, morphine, tramadol, naloxone, norbuprenorphine, benzoylecgonine, cocaine[2]	Acute intoxication by the combined effects of fentanyl, acetylfentanyl, *p*-fluorofentanyl, isotonitazene, xylazine, heroin, tramadol and cocaine	Accident
12	M, 32	Metonitazene (femoral blood, urine)	fentanyl, norfentanyl, nordiazepam, alprazolam, α-hydroxyalprazolam, cannabinoids[1]	Acute intoxication by the combined effects of metonitazene, fentanyl, alprazolam, and diazepam	Accident
13	M, 49	Metonitazene (femoral blood, urine)	fentanyl, 4-ANPP, *p*-fluorofentanyl, norfentanyl, β-hydroxyfentanyl, benzylfentanyl[3], morphine, codeine, methadone, naloxone, bromazolam, nordiazepam, xylazine[3], cannabinoids[1]	Acute intoxication	Accident

14	M, 57	Metonitazene (femoral blood, urine)	fentanyl, 4-ANPP, norfentanyl, β-hydroxyfentanyl, morphine, amphetamine, methamphetamine, cocaine, benzoylecgonine, xylazine[2,3]	Acute intoxication by the combined effects of fentanyl, metonitazene, methamphetamine, cocaine, and ethanol	Accident
15	M, 50	Isotonitazene (femoral blood, urine)	fentanyl, norfentanyl, xylazine, 6-MAM[2], morphine, codeine, naloxone	Pending further study	Accident
16	M, 62	Metonitazene (femoral blood, urine)	Fentanyl, acetylfentanyl, fluorofentanyl, norfentanyl, 4-ANPP, β-hydroxyfentanyl, xylazine, 6-MAM, morphine, codeine, naloxone, benzoylecgonine, cocaine, cocaethylene, ethanol	Acute intoxication by the combined effects of fentanyl, acetylfentanyl, fluorofentanyl, xylazine, heroin, codeine, cocaine, and ethanol	Accident
17	F, 37	Metonitazene (femoral blood)	Fentanyl, acetylfentanyl, norfentanyl, 4-ANPP, xylazine, 6-MAM, morphine, codeine, phencyclidine	Acute intoxication by the combined effects of fentanyl, acetylfentanyl, metonitazene, xylazine, heroin, phencyclidine	Accident
18	M, 34	Metonitazene (femoral blood, urine)	Fentanyl, fluorofentanyl, norfentanyl, 4-ANPP, xylazine, ethanol	Acute intoxication by the combined effects of fentanyl, fluorofentanyl, metonitazene, xylazine, and alcohol	Accident

[*]Isotonitazene detected in femoral blood specimen originally, but not in upon reanalysis
[1]Cannabinoids reported as presumptive positive
[2]Analyte not detected in blood but detected in vitreous humor
[3]Analyte not detected in blood but detected in urine

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