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Achieving the standard for the analytical
scope and sensitivity of forensic
toxicology urine testing in drug
facilitated crime investigations via
laminar flow tandem mass spectrometry

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BOSTON UNIVERSITY

SCHOOL OF MEDICINE

Thesis

**ACHIEVING THE STANDARD FOR THE ANALYTICAL SCOPE AND
SENSITIVITY OF FORENSIC TOXICOLOGY URINE TESTING IN DRUG
FACILITATED CRIME INVESTIGATIONS VIA LAMINAR FLOW TANDEM
MASS SPECTROMETRY**

by

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B.S., Loyola University of Chicago, 2013

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requirements for the degree of

Master of Science

2021

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ABSTRACT

Drug-facilitated sexual assaults are a public health and safety concern. Liquid chromatography paired with tandem mass spectrometry is theoretically capable of detecting the scope of drugs commonly encountered in these types of cases. An analytical method was developed for the quantitative analysis of 40 drugs designated by Academy Standards Board 121 “Standard for the Analytical Scope and Sensitivity for Forensic Toxicological Testing of Urine in Drug Facilitated Crime” (ASB 121). The targeted analytes spanned a range of drug classes including antidepressants, antihistamines, barbiturates, benzodiazepines, cannabinoids, stimulants, and opioids.

The final method utilized supported liquid extraction, followed by liquid chromatography tandem mass spectrometry with electrospray ionization in simultaneous positive and negative mode. Multiple reaction monitoring allowed quantification of analytes along with stable isotope internal standards. Validation parameters assessed included linearity, bias, precision, limit of detection, lower limit of quantitation, interference, and ion suppression or enhancement. The utilized sample preparation method

was able to extract 36 of the 40 target analytes and the developed analytical method was able to detect and quantify all analytes to the sensitivities required by ASB 121.

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LIST OF ABBREVIATIONS

%	Percent
±	Plus or minus
°C	Degrees Celsius
μL	Microliter
μm	Micrometer
Å	Angstroms
AAFS	American Academy of Forensic Science
ASB	AAFS Standards Board
Avg _m	Average slope
Cal	Calibrator
CC	Collision energy
CCL2	Collision cell lens 2
COOH	Carboxylic acid
CV	Coefficient of variation
DFSA	Drug-facilitated sexual assault
ESI	Electrospray ionization
EV	Entrance voltage
GC	Gas chromatography
GC-MS	Gas chromatography mass spectrometry
GHB	Gamma hydroxybutyrate

HED	High-energy dynode
HPLC	High pressure liquid chromatography
LC	Liquid chromatography
LC-MS	Liquid-chromatography mass spectrometry
LLOQ	Lower limit of quantitation
LOD	Limit of Detection
m/z	Mass to charge
mCPP	Meta-chlorphenylpiperazine
MDA	Methylenedioxyamphetamine
MDMA	Methylenedioxymethamphetamine
mL	Milliliter
mm	Millimeter
MRM	Multiple reaction monitoring
MS	Mass spectrometer
Ng	Nanogram
Psi	Pounds per square inch
R ²	Correlation coefficient
SLE	Supported Liquid Extraction
SOFT	Society of Forensic Toxicologists
s _y	Standard deviation of the y intercept
THCCOOH	Carboxy-tetrahydrocannabinol

UP	Ultra-performance
USA	United States of America
v/v	Volume to volume

1. INTRODUCTION

1.1 Drug Facilitated Sexual Assault

Drug-facilitated crimes are a public health and safety concern. A sexual assault is considered drug-facilitated if the victim is subjected to nonconsensual acts while incapacitated due to the effects of drugs, and is therefore unable to consent (1). The drug ingestion may be voluntary or involuntary, so long as the effects of the drugs deprive a person of the mental capacity to consent to sexual acts. Drug-facilitated sexual assault (DFSA) cases may present with clinical patterns of sudden intoxication, intoxication out of proportion with consumption, waking in an unexpected location, clothing removed or on inside out, clothing stained with biological fluids, genital soreness, and loss of memory (2). Drugs commonly encountered in DFSA have clinical effects that include strong sedation, lack of inhibition, muscle relaxation, and memory loss. Drugs purposefully used for DFSA may have no odor, color, or taste, have a rapid onset of action and are generally easily obtained (1).

The true incidence of DFSA is difficult to determine for several reasons. Victims may have limited memory of the events which can prevent or delay reporting. This in turn can lead to a delay in collection of biological samples and impact toxicological detection. While DFSA can occur through involuntary ingestion of a drug (“drink spiking”), the majority of DFSA cases occur after the voluntary ingestion of drugs or alcohol (2). In one study of drug screens of 76 cases of DFSA: voluntary alcohol use was reported by 77% of subjects, voluntary prescription drug use was reported in 49%, and

26% reported recreational drug use (3). Drugs unknowingly consumed were detected in 20% of the 76 subjects (3). DFSA may include a combination of drugs consumed voluntarily and involuntarily. The detection of more than one drug in DFSA cases is common, with ethanol being the most commonly identified substance in samples with multiple analytes (4,5,6,7). In an analysis of 1000 cases of suspected DFSA, a single drug was detected in just over a quarter of the cases, while more than half of the cases had two or more drugs detected (6).

Toxicological detection of drugs used in DFSA cases can be very challenging. Analyte concentration may be very low because of the short elimination half-lives typical to some of the drugs used combined with delays between the offense, reporting and sampling. In addition, the types of analytes encountered can wildly vary. A single method capable of detecting the majority of the scope of drugs utilized in DFSA must be amenable to drugs of varying acidity, polarity, and size while maintaining the sensitivity necessary to detect the low levels of drugs typical for DFSA cases. Liquid-chromatography mass spectrometry (LC-MS) is one methodology capable of meeting the needs of DFSA case samples.

1.2 LC-MS Instrumentation

Liquid chromatography-mass spectrometry (LC-MS) consists of a liquid chromatograph used to separate analytes from each other and a mass spectrometer used as a detector to provide quantitative measurement of the analytes.

1.2.1 High Pressure Liquid Chromatography

High pressure liquid chromatography (HPLC) rapidly separates analytes from complex mixtures and matrices (8). A high-pressure solvent delivery system utilizes a pump to inject the sample through an analytical column packed with small diameter porous particles. The physical properties, such as pore size, and the chemistry of the stationary phase impacts the retention and separation of target analytes. The stationary phase particles are coated in a chemically bonded phase that interacts with the sample. A binary pump delivers high pressure mobile phase consisting of an aqueous portion and an organic portion. The composition of the total mobile phase can change over the course of the run (gradient elution) or remain constant (isocratic elution). Based on the strength of each analyte's affinity for the stationary phase in the column, the analytes remain on the column until mobile phase conditions become more favorable. Selecting the proper analytical column and mobile phase conditions is critical for achieving meaningful separation of the target analytes. Consideration should be given to the functional groups available for interaction and differentiation of a method's target analytes. Ultimately the analytes are separated, ideally eluting at a unique time before undergoing electrospray ionization (8). While many co-eluting analytes can be differentiated according to mass and fragmentation patterns, separation of compounds with identical parent masses and similar fragmentation chemistry is critical for confident identification and quantitation.

1.2.2 Electrospray Ionization

The mass spectrometer requires gaseous charged ions for detection. Electrospray ionization (ESI) is one way to vaporize and charge a sample so it can be analyzed by the MS (9). Electrospray ionization begins as the eluent from the LC column flows through a metal capillary and where it is nebulized. A potential difference imparts electrical charge on the droplets. Heated drying gas de-solvates the droplets, increasing the charge density of the droplets. Once the electric repulsion of the charged droplet exceeds the surface tension, Coulombic fission occurs forming gas phase ions. As a soft ionization method, ESI results in protonated or de-protonated ions with little to no fragmentation. Using a soft ionization method allows for sensitive detection of the parent ion. Drawbacks to using ESI include ion suppression or enhancement from co-eluting analytes or matrix and the potential for ions with multiple charges (9).

1.2.3 Mass Spectrometry

Mass spectrometry separates and ionized gaseous ions based on their mass-to-charge (m/z) (8). While there are several different types of mass analyzers, one of the most common for quantitation is a triple quadrupole mass spectrometer (MS). The triple quadrupole MS is a tandem in space spectrometer that allows for the selection and fragmentation of a precursor ion and detection of the product ion(s) produced. Gas phase ions enter the mass analyzer through the sampling orifice. The ions follow an ion guide to enter the first mass analyzer which uses electrostatic fields to deflect ions according to their m/z ratios (8). The selected ion travels to the second quadrupole, or the collision

cell, where the precursor ion is fragmented via the introduction of a collision gas. Fragmentation is utilized to provide information about the chemical structure of the parent ion. Certain bonds break more readily than others, resulting in a characteristic fragmentation pattern based on the physio-chemistry of the parent ion. The third quadrupole acts as a fragment mass filter using electrostatic fields to deflect the selected fragment ions to the detector. At the detector, a current signal is generated based on the abundance of incident ions. Converting that signal intensity concentration requires a known relationship current signal intensity and ion concentration that is often established with a calibration curve. Because the response of the detector can fluctuate based on several variables, an internal standard experiencing identical conditions should be used to accurately convert signal intensity to a concentration value (10). Because the instrument selects for a specific parent mass in the first quadrupole and further selects for characteristic fragment masses selected in the third quadrupole, confident identification and quantification can be performed.

1.2.4 Advantages

LC-MS is advantageous over other methods in its combination of selectivity, sensitivity, and efficiency (11). The combination of two selective techniques allows analytes in highly complex mixtures to be separated and analyzed. LC separates compounds according to physicochemical properties while MS differentiates compounds according to their mass to charge ratio. Analytes that may interact similarly with the column and thus be co-eluted can be differentiated by their characteristic precursor ion

and subsequent unique fragment ions (the precursor ion, the quantifier ion, and the qualifier ion).

LC-MS analysis facilitates chromatographic resolution of isobaric species, or species with the same precursor ions. Samples with different masses can coelute and be differentiated in the mass spectrometer. Because coelution is acceptable, methods can have a much shorter run time. Many analytes are large, non-volatile, or thermally unstable thus they require derivatization to be compatible with small, thermally labile, and volatile GC-MS method requirements. LC-MS methods have been developed to forgo derivatization, decreasing sample preparation time (12).

1.2.5 Disadvantages

Optimization of LC-MS methods can be challenging given the “complex co-dependent synergy” between the two (11). LC-MS can have a limited range where the detector signal is linear in response to concentration, known as the dynamic range. An important aspect of developing an LC-MS method is determining the linear range. Often the range of concentrations should not exceed 500-fold (11). LC-MS methods, especially those utilizing ESI, are impacted by ion suppression and enhancement effects that can decrease the sensitivity of the method.

1.3 LC-MS Method Development Considerations

Method development is guided by information from four main categories: industry guidelines, analyte structure and chemical properties, matrix considerations, and previously developed methods (11).

1.3.1 Industry Guidelines

The American Academy of Forensic Science Standards Board (ASB) is a standard developing organization for forensic sciences accredited by the American National Standards Institute (13). Recognizing the critical nature of toxicological testing in drug-facilitated crimes, ASB standardized the analytical scope and sensitivity for toxicological examination of urine in DFSA cases (14). The Toxicology Subcommittee of the Organization of Scientific Area Committees for Forensic Science examined the availability and prevalence of drugs in the United States, as well as the recommendations of the Society of Forensic Toxicologists (SOFT), a global toxicology organization (15). The SOFT DFSA committee published a list of 100 commonly encountered DFSA drugs, their target analytes, and the recommended minimum performance limit for analytical urine testing (16). Flunitrazepam, commonly known as Rohypnol, was excluded from ASB 121 because several comprehensive case reviews of DFSA in the United States have found that it is infrequently detected due to its limited availability (6).

Table 1. Required Minimum Analytical Scope and Sensitivity for Testing of Urine in Drug Facilitated Crime Investigations (14).

Analyte	Drug Class	Minimum Required Sensitivity
α -hydroxyalprazolam	Benzodiazepine	5 ng/mL

Analyte	Drug Class	Minimum Required Sensitivity
7-aminoclonazepam	Benzodiazepine	5 ng/mL
Amitriptyline	Antidepressant	10 ng/mL
Amphetamine	CNS Stimulant	25 ng/mL
Benzoylcegonine	CNS Stimulant	50 ng/mL
Brompheniramine	Antihistamine	10 ng/mL
Butalbital	Barbiturate	100 ng/mL
Carisoprodol	Miscellaneous	100 ng/mL
Chlorpheniramine	Antihistamine	10 ng/mL
Codeine	Opioid	10 ng/mL
Cyclobenzaprine	Miscellaneous	10 ng/mL
Desipramine	Antidepressant	10 ng/mL
Dextromethorphan	Miscellaneous	10 ng/mL
Diphenhydramine	Antihistamine	10 ng/mL
Doxylamine	Antihistamine	10 ng/mL
Ethanol	High-Dose Sedative	0.01 g/dL
Fentanyl	Opioid	1 ng/mL
Gamma hydroxybutyrate (GHB)	High-Dose Sedative	10 µg/mL
Hydrocodone	Opioid	10 ng/mL
Hydromorphone	Opioid	10 ng/mL
Imipramine	Antidepressant	10 ng/mL
Lorazepam	Benzodiazepine	5 ng/mL
Meta-chlorophenylpiperazine (mCPP)	Antidepressant	10 ng/mL
Methylenedioxyamphetamine (MDA)	CNS Stimulant	25 ng/mL
Methylenedioxymethamphetamine (MDMA)	CNS Stimulant	25 ng/mL
Meprobamate	Miscellaneous	100 ng/mL
Methamphetamine	CNS Stimulant	25 ng/mL
Morphine	Opioid	10 ng/mL
Norchlorcyclizine	Antihistamine	10 ng/mL
Nordiazepam	Benzodiazepine	10 ng/mL
Norfentanyl	Opioid	1 ng/mL
Norketamine	Miscellaneous	10 ng/mL
Nortriptyline	Antidepressant	10 ng/mL
Oxazepam	Benzodiazepine	10 ng/mL
Oxycodone	Opioid	10 ng/mL
Oxymorphone	Opioid	10 ng/mL
Phenobarbital	Barbiturate	100 ng/mL
Temazepam	Benzodiazepine	10 ng/mL
Carboxy-tetrahydrocannabinol (THCCOOH)	Cannabinoid	10 ng/mL
Tramadol	Opioid	10 ng/mL
Zolpidem carboxylic acid (COOH)	Miscellaneous	10 ng/mL
Zopiclone	Miscellaneous	10 ng/mL

1.3.2 Chemical and structural information about the analytes

1.3.2.1 Antidepressants

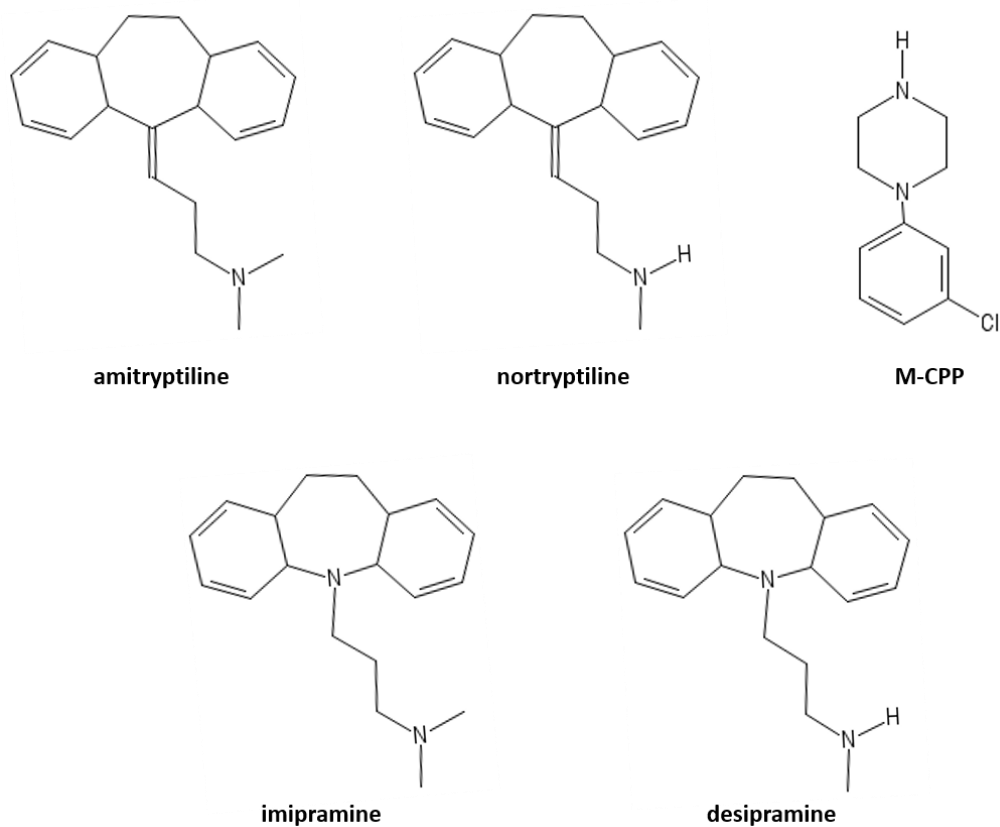


Figure 1. Molecular Structure of Antidepressant Analytes

Antidepressant drugs are most commonly prescribed for depression management but can also be used to treat anxiety, migraines or sleep disorders (17). Tricyclic antidepressants consist of three hydrocarbon rings with a central alkylamine chain (18). Amitriptyline and imipramine are tertiary amine tricyclic antidepressants, while desipramine and nortriptyline are secondary amine tricyclic antidepressants (Figure 1).

Their pKa values range from 9.4 to 10.3. In the body, antidepressants work by inhibiting the reuptake of both serotonin and norepinephrine leading to higher levels of serotonin and norepinephrine available and an elevated mood, but can cause sedation depending on dose size and tolerance levels (19). Trazadone's major urinary metabolite is mCPP. It is a centrally active 1-arylpiperazine that promotes serotonin release. Upon ingestion, mCPP can produce euphorogenic effects similar to alcohol (20).

1.3.2.2 Antihistamine

Antihistamines are typically used to treat allergy symptoms. Brompheniramine, chlorpheniramine, diphenhydramine, doxylamine, and chlorcyclizine are first generation antihistamines that are available over the counter and can cause extreme sedation (21).

The drugs function in the body to block histamine binding at H1-receptors (22).

Alkylamine H1-receptor agonists cause significant sedative action. As a class, the drugs are lipophilic thus are readily able to cross the blood brain barrier (22). The major urinary metabolite of chlorcyclizine is norchlorcyclizine. The chemical structure of antihistamine analytes is depicted in Figure 2.

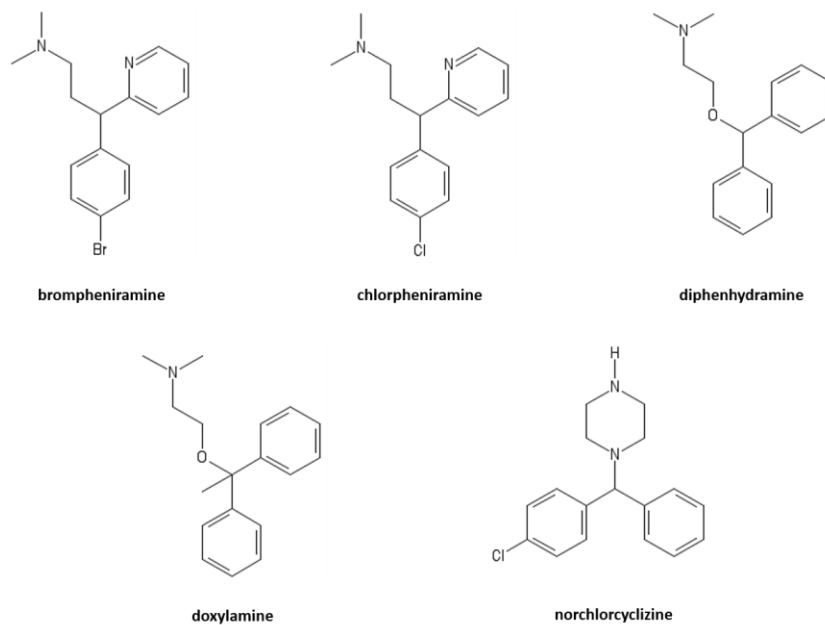


Figure 2. Molecular Structure of Antihistamine Analytes

1.3.2.3 Barbiturate

Barbiturates are derived from 5,5-di-substituted barbituric acid and act as a central nervous system depressant (23). Barbiturates can produce a dose dependent range effects from mild sedation to complete anesthesia (24). While they were previously prescribed therapeutically as antiepileptic drugs or sedatives, they have been largely replaced by benzodiazepines (25). Barbiturates ionize more readily in negative-ESI, with the 4-position oxygen likely carrying the negative charge (24).

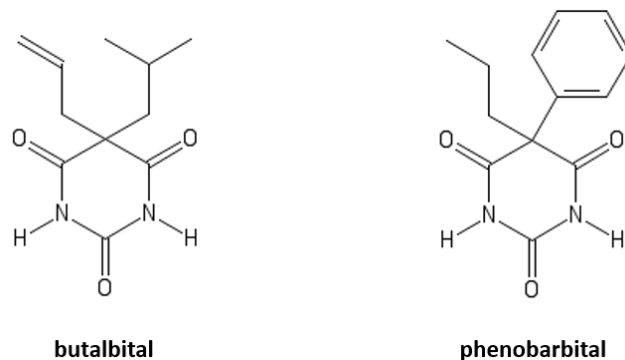


Figure 3. Molecular Structures of Barbiturate Analytes

1.3.2.4 Benzodiazepines

Benzodiazepines are central nervous system depressants that are prescribed for sleep aid, anxiety management, or seizure prevention (26). The relaxing and sedative effects of benzodiazepines have led to their increased use in drug facilitated crimes (27).

Benzodiazepines can be consumed as capsules, tablets, or via injection. Specific effects of benzodiazepine ingestion include confusion, memory loss, drowsiness, impaired coordination, and dizziness. Benzodiazepines have a shared chemical structure of a fused benzene and diazepine ring with an attached phenyl ring (26). They are lipophilic, and thus less soluble in ethanol and water (28). The chemical structures of benzodiazepine analytes are depicted in Figure 4.

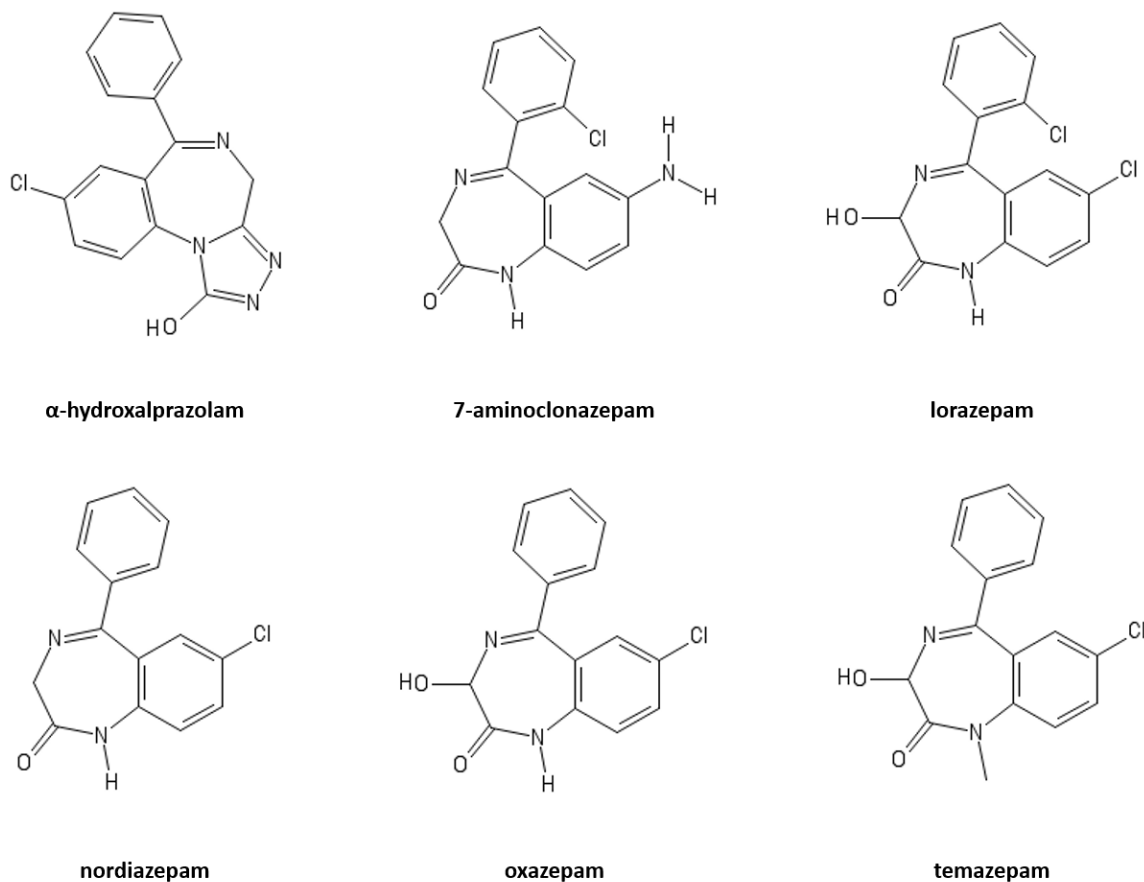
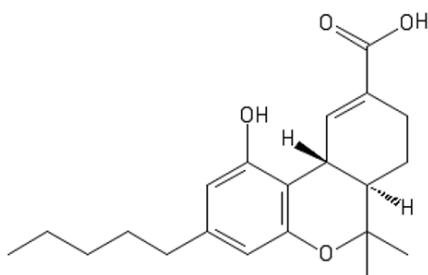


Figure 4. Molecular Structures of Benzodiazepine Analytes.

1.3.2.5 Cannabinoids

Cannabis sativa is a plant commonly referred to as marijuana and contains a family of drugs referred to as cannabinoids. Marijuana's major urinary metabolite is 11-nor- Δ^9 -tetrahydrocannabinol-9-carboxylic acid, or THCCOOH and its glucuronide ester (29). Unbound THCCOOH has a pKa of 4.4. THCCOOH decarboxylates to its corresponding phenol over time, under alkaline conditions, or upon heating. Upon intake, cannabis acts as a central nervous system depressant and can induce physiological effects of nausea, dry mouth, euphoria, and hallucinations (29). While typically used for

its psychoactive properties, marijuana has been used to treat pain, nausea, asthma, depression and insomnia. The molecular structure of THCCOOH can be found in Figure 5.



THCCOOH

Figure 5. Molecular Structure of Cannabinoid Analyte

1.3.2.6 Stimulants

Stimulants are commonly encountered in DFSA because they lower inhibition, increase susceptibility to suggestion, and can increase sex drive (28). Stimulants are often mixed with central nervous system depressants to enhance the high or compensate for undesired side effects (30). Cocaine quickly metabolizes to its main urinary metabolite, benzoylecgonine which is used as a target analyte for toxicological analysis (31). While cocaine has historically been used medicinally as a local anesthetic, it has been replaced by safer alternatives and is now mainly encountered in recreational use (32). MDA and MDMA are ring-substituted amphetamine analogues with psychotropic affects that are typically used recreationally. Amphetamine and methamphetamine can

be prescribed for attention deficit disorder management but are often diverted or synthesized for recreational use (32).

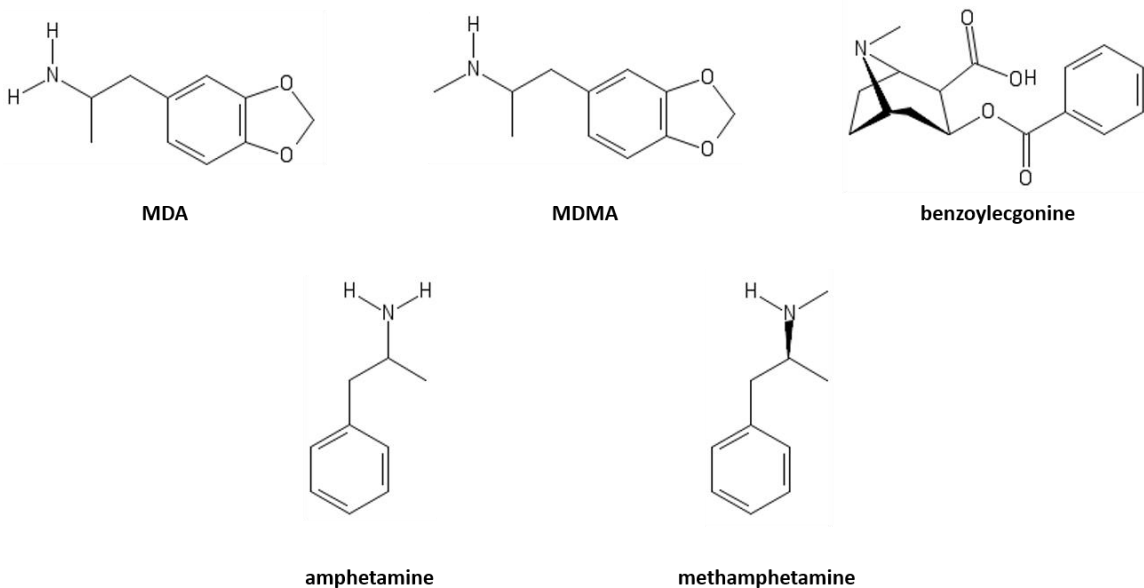


Figure 6. Molecular Structures of Stimulant Analytes.

1.3.2.7 Opioids

Opioids are natural alkaloids derived from opium poppy resin and their semi-synthetic and synthetic counterparts. They are often prescribed to treat acute pain or relieve moderate chronic pain. Opioid intake can cause dizziness, fatigue, sedation, confusion and blurred vision. Opioids are extensively glucuronidated in urine (33). They are typically weak bases with pKa values ranging from 8 to 10 (34). The molecular structures of opioid analytes are depicted in Figure 7.

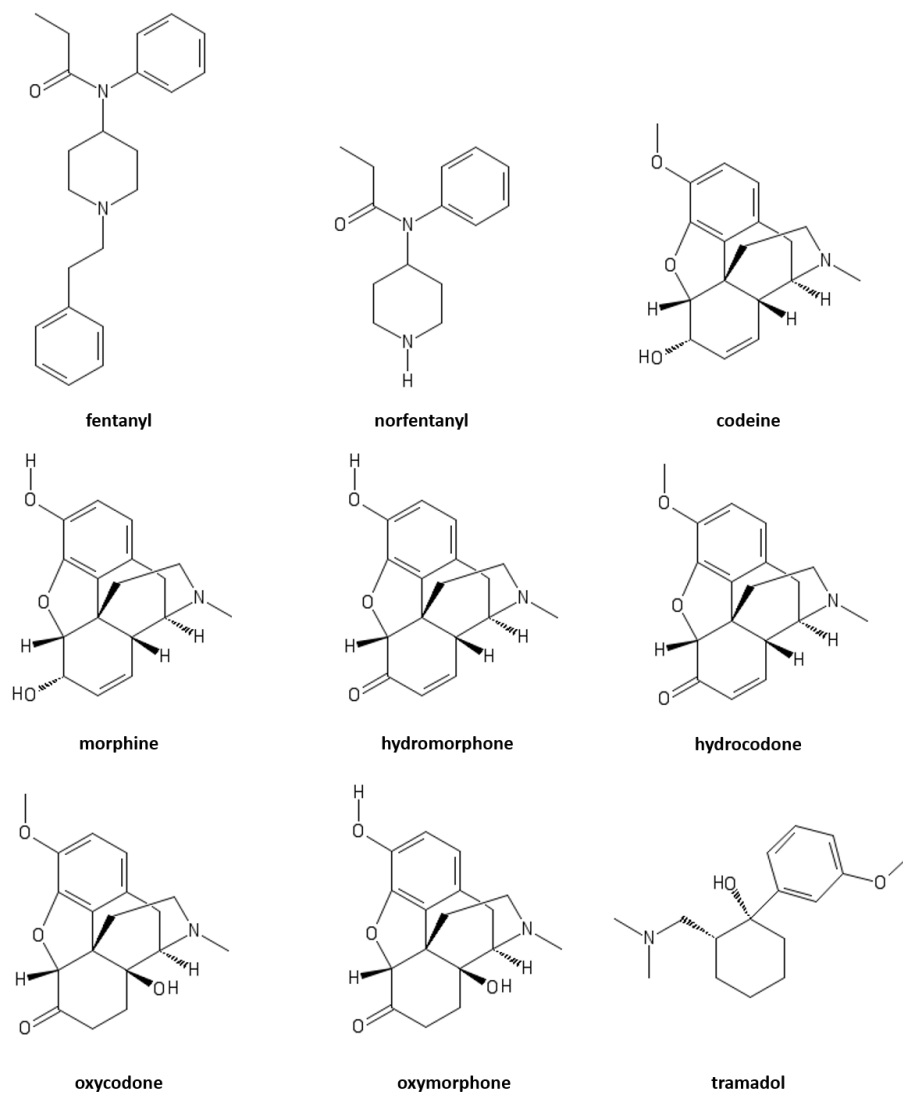


Figure 7. Molecular Structures of Opioid Analytes.

1.3.2.8 Miscellaneous

The last group of analytes include drugs that do not neatly fit into the other classes but have similar sedating effects. Dextromethorphan is an antitussive found in over the counter cough suppressants. In high doses, dextromethorphan can produce euphoria, hallucinations, and disorientation (35). Zolpidem and zopiclone are termed “z-

drugs” and are prescribed as sleep aids. Zolpidem’s major urinary metabolite zolpidem carboxylic acid. Ketamine, a non-competitive N-methyl-D-aspartate receptor antagonist, can cause psychotomimetic and dissociative effects. Norketamine is the major urinary metabolite of ketamine (36). Carisoprodol and meprobamate are muscle relaxants. Meprobamate is a metabolite of carisoprodol but can exist as its own pharmaceutical preparation (37). The molecular structures of the miscellaneous analytes are depicted in Figure 8. Ethanol and GHB are high dose sedatives, requiring higher doses than the other drugs discussed induce memory loss and sedative effects. Ethanol is the most commonly encountered substance in DFSA cases and can cause unconsciousness and memory disruption in high doses (38). GHB has memory-impairing effects, induces strong sedation, and is rapidly eliminated from the body (39).

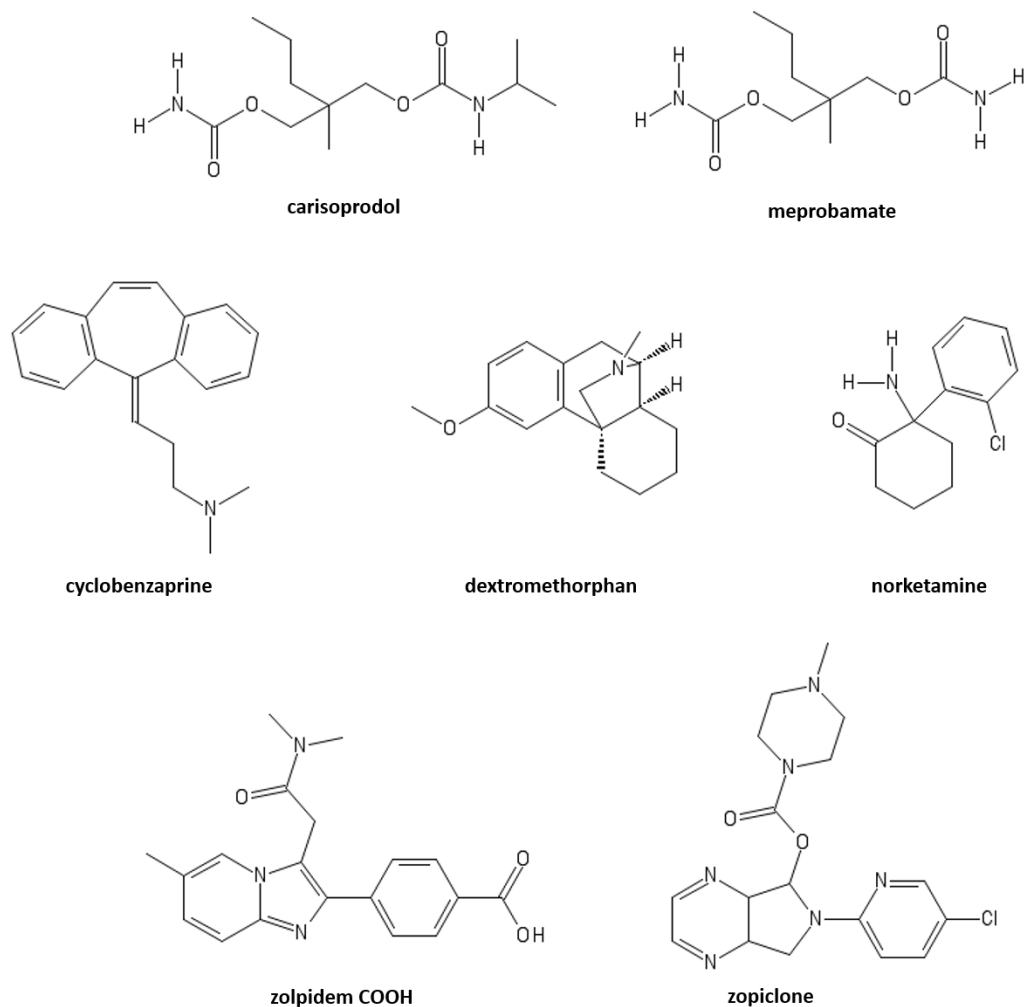


Figure 8. Molecular Structures of Miscellaneous Analytes

1.3.3 Information about the type and condition of sample that will be measured

Urine is a typical sample of choice in DFSA cases, as it may extend the window of drug detection up to 120 hours (5 days) after the alleged incident (40). Urine provides a longer detection window than blood. In one study, 9.3% of 155 cases where both blood and urine were collected, at least one substance was detected in urine but not in blood (6). However, urine results must be interpreted carefully. Metabolites of previously ingested

substances such as benzodiazepines, marijuana, or cocaine may be detectable in urine while no longer contributing to intoxication (41). When developing analytical methods for urine, it is important to consider whether the drug of interest will be present in urine and if species of similar mass to charge ratio as the targeted will interfere with analysis. The drug may undergo significant metabolism such that there will be little to no unchanged drug present in urine. For example, less than 1% of a zolpidem dose is detectable as unchanged drug in urine (42). Thus it is important that urine analysis target the major urinary metabolite, zolpidem 4-phenyl carboxylic acid which represents 33-48% of an ingested Zolpidem dose depending on pharmacokinetic dynamics (42).

Drugs are excreted in urine in free-base and glucuronidated forms. The percent of drugs in glucuronidated form varies by class, however benzodiazepines, opiates, and THCCOOH have been found to exhibit high levels of glucuronidation (43). Hydrolysis, whether chemical or enzymatic, can be performed during sample preparation to allow more drug to be available in free base form for examination.

1.3.4 Previously developed methods

Many current analytical approaches for suspected DFSA samples involve several methodologies and instrumentation types. For example, the Victorian Institute of Forensic Medicine performs analysis on DFSA urine samples as follows: alcohol testing via gas chromatography (GC), an immunoassay screen for amphetamines, benzodiazepines, cannabis, cocaine, and opiates, a liquid GC-MS screen for basic and neutral drugs, a confirmatory GC-MS method for any screening positives, and a method for the presence

of GHB with a 10 mg/L cut-off (44). In 2007, Feng et al developed one of the first LC-MS/MS methods for quantitative analysis of 30 drugs from multiple classes including opioids, barbiturates, stimulants, cannabinoids, and benzodiazepines (45). Their method, however, involved a separate positive and negative mode analysis with different LC gradients and MS parameters. Simultaneous quantitation of 78 drugs via LC-MS with a dilute-and-shoot sample preparation has been developed, but again with separate positive and negative mode LC gradients and MS parameters (46). Ultra-performance (UP) LC-MS methods utilizing positive and negative ESI mode have been successfully validated for the analysis of several drug classes simultaneously in equine urine, including all of the benzodiazepine and barbiturate analytes included in ASB 121 (47).

Immunoassays are commonly used as a screening tool for toxicology samples, but require confirmation by other methods, and typically have a limited range of substances. Additionally, many commercial immunoassays do not have the requisite sensitivity to reliably detect the low drug levels common in DFSA cases (40). The analytical methods currently available do not meet the scope of drugs outlined in ASB 121. The aim of the presented research was the development and validation of a LC-MS/MS quantitative assay for the simultaneous identification of 40 analytes in DFSA urine samples.

2. MATERIALS AND METHODS

2.1 Chemicals, Reagents, and Standards

All certified reference standards and isotope-labeled internal standard solutions were purchased from Cerilliant (Round Rock, TX, USA), Cayman Chemical Company (Ann Arbor, MI, USA) or Lipomed (Cambridge, MA, USA). Methanol (Optima LC/MS grade), ethyl acetate, ammonium hydroxide, and ammonium formate were obtained from Fisher Scientific (Fair Lawn, NJ, USA). Formic acid (LC/MS grade, 98%) was purchased from Sigma-Aldrich (St Louis, MO, USA). Deionized water was generated with a Milli-Q water purification system from Millipore (Billerica, MA, USA). Drug-free urine was obtained via donations approved by Institutional Review Board (IRB) requirements at Boston University School of Medicine (Boston, MA, USA).

2.2 Instrumentation

2.2.1 Liquid Chromatography

Analysis was performed with a QSight® LX50 UHPLC system consisting of two UHPLC pumps, an autosampler, and a column oven from PerkinElmer (Waltham, MA, USA). Chromatographic separation was achieved with a 50 x 4.6 mm, pore size 100 Å, 2.6 µm core-shell Kinetex® phenyl-hexyl HPLC column from Phenomenex® (Torrance, CA, USA). The column was kept at 40 °C for the entirety of the method. The aqueous mobile phase A was 0.1% formic acid in Millipore water and the organic mobile phase B was 0.1% formic acid in methanol. The flow rate was 0.6 mL/min with a maximum pressure of 10,000 psi. The LC method run time was 11 minutes using linear gradient

elution (Table 2). A Rheodyne® diverter valve (Rohnert Park, CA, USA) was used to direct sample from the column to the mass spectrometer between 2-9 min. All other eluent off the column was directed to waste to minimize contamination of the MS.

Table 2. LC Gradient Time Program.

Time (min)	Flow (mL/min)	%A	%B
0.00	0.600	95	5
0.50	0.600	95	5
2.50	0.600	85	15
6.00	0.600	50	50
7.00	0.600	5	95
9.00	0.600	5	95
10.00	0.600	95	5
11.00	0.600	95	5

The samples were kept at 5°C in the autosampler to prevent sample degradation and sampled at a 10 µL injection volume. The autosampler needle was washed with a 250 µL weak wash of 50% (v/v) aqueous methanol, then a 250 µL strong wash of 90% (v/v) aqueous methanol, then a final 250 µL weak wash to prevent carryover in between sample injections.

2.2.2 Mass Spectrometry

Analytes were detected using a QSight® 220 series triple quadrupole MS detector with ESI source, HSID™ interface, and UniField Detector™. Data collection, analysis, and quantification was performed using Simplicity 3Q™ software (PerkinElmer).

Analytes were detected by mass spectrometry using time-managed multiple reaction monitoring (MRM) in either positive or negative ESI modes. The precursor and

identifying fragment ions were determined via compound optimization using purified standards to find the optimum signal for the entrance voltage (EV), collision cell energy (CC) and collision cell lens 2 voltage (CCL2). Experiments utilizing MRM allow for targeted analyte detection by allowing the first MS to transmit ions with the precursor ion m/z to the collision cell. After being fragmented with Nitrogen gas, the second quadrupole allowed transmission of all product ions to the third quadrupole, where only the products of interest were selected to be monitored by the detector.

Table 3. Optimized Source Conditions.

Drying Gas	HSID Temperature	Nebulizer Gas	Electrospray V1	Source 1 Temperature
100	250°C	150	5500	500°C

2.3 Method Development

2.3.1 Compound Optimization

Optimized MRM transitions for each analyte were determined by directly infusing certified reference standards diluted to 100 ng/mL in 50% aqueous methanol fortified with 0.1% formic acid into the mass spectrometer at a flow rate of 30 μ L/min. The precursor ion mass was determined by performing an MS full scan. Next, a product ion scan was performed of the precursor ion while the collision energy (CC) was ramped from -200 to zero. Two product ions were selected based foremost on intensity, but considerations for avoiding isobaric transitions and low background noise in urine were also considered. Adducts were avoided per the ASB Standard for Mass Spectral Data Acceptance in Forensic Toxicology, as they are less structurally diagnostic (48). The most abundant

MRM transition was defined as the quantifier, and the lesser abundant defined as the qualifier. For internal standards, only one MRM transition was monitored. Entrance voltage (EV), CCL2 (collision cell lens 2), and collision energy (CC) were determined for each MRM transition (Table 4) using the automatic optimization feature of the instrument software.

Table 4. Optimized MRM Parameters.

Analyte	Q1 m/z	Q2 m/z	CC	EV	CCL2	Polarity
α -hydroxyalprazolam	325.1	297	-33	32	-76	+
α -hydroxyalprazolam	325.1	216.1	-33	38	-320	+
α -hydroxyalprazolam-d5	330	302.2	-33	19	-96	+
7-aminoclonazepam	286.21	121.1	-34	23	-100	+
7-aminoclonazepam	286.21	222.2	-34	29	-76	+
7-aminoclonazepam-d4	290.8	121.2	-34	16	-108	+
Amitriptyline	278.3	202.3	-81	26	-160	+
Amitriptyline	278.3	191.1	-81	26	-160	+
Amitriptyline-d3	281.1	202.1	-81	26	-160	+
Amphetamine	136	119	-11	15	-24	+
Amphetamine	142.2	93.1	-46	10	-52	+
Amphetamine-d6	136	91	-60	12	-45	+
Benzoylcegonine	290.2	105.1	-28	0	-64	+
Benzoylcegonine	290.2	168.1	-27	0	-56	+
Benzoylcegonine-d8	298.2	171	-27	14	-60	+
Brompheniramine	321.1	276.1	-32	22	-84	+
Brompheniramine	321.1	167.1	-36	19	-220	+
Butalbital	223.1	180	16	0	36	-
Butalbital	223.1	42.2	16	0	44	-
Butalbital-d5	228.1	41.9	15	0	52	-
Carisoprodol	261.2	176	-13	10	-44	+
Carisoprodol	261.2	55.3	-59	13	-64	+
Carisoprodol-d7	268.2	183.1	-13	10	-44	+
Chlorpheniramine	275.1	167.1	-24	16	-316	+
Chlorpheniramine	275.1	230.1	-24	1	-56	+
Chlorpheniramine-d6	281.1	230.2	-23	4	-54	+
Codeine	300.1	153	-52	33	-136	+
Codeine	300.1	165	-52	30	-84	+
Codeine-d6	306.1	165	-52	39	-112	+
Cyclobenzaprine	276.2	215.1	-35	30	-68	+
Cyclobenzaprine	276.2	191.1	-31	40	-56	+

Analyte	Q1 m/z	Q2 m/z	CC	EV	CCL2	Polarity
Cyclobenzaprine-d3	279.1	191.1	-31	39	-60	+
Desipramine	267.2	236	-21	0	-56	+
Desipramine	267.2	72.1	-19	13	-48	+
Desipramine-d3	270.2	75.3	-19	13	-48	+
Dextromethorphan	275	213.2	-35	24	-92	+
Dextromethorphan	272.2	147.1	-35	24	-76	+
Dextromethorphan-d3	272.2	213.1	-35	24	-92	+
Diphenhydramine	256.2	152.1	-70	14	-112	+
Diphenhydramine	256.2	165.1	-70	13	-108	+
Diphenhydramine-d3	259.1	165.1	-70	14	-112	+
Doxylamine	271.2	182.1	-33	13	-64	+
Doxylamine	271.2	167.1	-50	13	-108	+
Doxylamine-d5	276.2	187.1	-50	13	-108	+
Fentanyl	337.1	188.2	-33	30	-80	+
Fentanyl	337.1	105.1	-53	30	-88	+
Fentanyl-d5	342.5	79.2	-69	15	-68	+
Hydrocodone	300.3	199.1	-34	43	-104	+
Hydrocodone	300.3	171	-35	43	-288	+
Hydrocodone-d3	303.3	199	-34	31	-100	+
Hydromorphone	286.1	157	-37	0	-252	+
Hydromorphone	286.1	185.1	-36	45	-112	+
Hydromorphone-d6	292.2	185	-36	12	-112	+
Imipramine	281.1	58.1	-23	25	-63	+
Imipramine	281.1	86.1	-23	6	-52	+
Imipramine-d3	284.2	89.1	-23	17	-58	+
Lorazepam	321.1	275	-27	26	-76	+
Lorazepam	321.1	229.1	-48	19	-116	+
Lorazepam-d4	325	279.1	-24	9	-76	+
mCPP	197.1	154.1	-26	27	-44	+
mCPP	197.1	91	-51	18	-72	+
mCPP-d8	205.1	158.1	-35	33	-52	+
MDA	180.2	163.1	-15	2	-40	+
MDA	180.2	105.1	-41	3	-64	+
MDA-d5	185.1	168	-15	5	-36	+
MDMA	194.2	163.2	-16	16	-36	+
MDMA	194.2	105	-44	13	-68	+
MDMA-d5	199.1	165.2	-17	16	-36	+
Meprobamate	219.2	97.1	-22	15	-43	+
Meprobamate	219.2	158.3	-13	1	-35	+
Meprobamate-d3	222	161.3	-13	1	-35	+
Methamphetamine	150	119.1	-16	18	-28	+
Methamphetamine	150	91	-32	17	-36	+
Methamphetamine-d5	155.1	92.2	-42	32	-36	+

Analyte	Q1 m/z	Q2 m/z	CC	EV	CCL2	Polarity
Morphine	286.1	153	-51	0	-104	+
Morphine	286.1	181.1	-50	15	-100	+
Morphine-d6	292.1	181	-50	21	-120	+
Norchlorcyclizine	287.1	166.1	-36	7	-228	+
Norchlorcyclizine	287.1	201.1	-25	9	-36	+
Nordiazepam	271	165	-35	22	-88	+
Nordiazepam	271.1	140	-34	18	-76	+
Nordiazepam-d5	276.1	140	-34	5	-72	+
Norfentanyl	233.2	177.1	-18	5	-48	+
Norfentanyl	233.2	84.1	-18	22	-44	+
Norfentanyl-d5	238.2	84.1	-18	28	-48	+
Norketamine	224	125	-13	19	-350	+
Norketamine	224	207.1	-14	16	-44	+
Norketamine-d4	228.2	129	-13	19	-350	+
Nortriptyline	264.1	117	-32	1	-60	+
Nortriptyline	264.1	233.2	-17	11	-60	+
Nortriptyline-d3	267.2	191.1	-32	1	-60	+
Oxazepam	287.2	269.1	-24	11	-60	+
Oxazepam	287.2	241.1	-24	34	-68	+
Oxazepam-d5	292.2	246.2	-24	36	-80	+
Oxycodone	316.2	298.2	-20	0	-64	+
Oxycodone	316.2	241.2	-33	27	-104	+
Oxycodone-d6	322.1	304.1	-20	18	-68	+
Oxymorphone	302.1	284.2	-23	0	-76	+
Oxymorphone	302.1	227.1	-24	0	-112	+
Oxymorphone-d3	305.1	287.1	-22	3	-76	+
Phenobarbital	231	188	14	-21	44	-
Phenobarbital	231	42.1	15	-11	48	-
Phenobarbital-d5	236.1	42.2	15	-11	44	-
Temazepam	301.2	255.2	-30	25	-72	+
Temazepam	301.2	283.1	-19	27	-52	+
Temazepam-d5	306.1	260	-27	9	-84	+
THCCOOH	343.2	245.2	34	-35	164	-
THCCOOH	343.2	191.2	51	-34	156	-
THCCOOH-d3	346.3	194.1	49	-21	104	-
Tramadol	264.32	58	-15	16	-48	+
Tramadol	264.32	264.32	-14	11	-56	+
Tramadol-d3	268.2	58.3	-15	24	-44	+
Zolpidem COOH	338.1	293.2	-43	34	-72	+
Zolpidem COOH	338.1	265.1	-47	42	-104	+
Zolpidem COOH-d4	342.1	269.2	-47	42	-104	+
Zopiclone	389	217.1	-49	1	-68	+
Zopiclone	389	245.1	-27	8	-68	+

Analyte	Q1 m/z	Q2 m/z	CC	EV	CCL2	Polarity
Zopiclone-d4	393.2	245.1	-49	1	-68	+

2.3.2 Sample preparation

Calibrators and quality control samples were prepared by spiking certified reference material for each analyte at the desired concentration in analyte-free human urine. Samples were additionally fortified, where appropriate, with each analyte's stable isotope internal standard to a final concentration of 150 ng/mL.

Dilute-and-shoot sample preparation was evaluated by transferring 100 μ L of sample to a glass vial and centrifuging for 3 minutes at 10,000 revolutions per minute in an Eppendorf 5430 centrifuge (Enfield, CT, US). Sample supernatant was transferred to an LC vial and diluted with starting phase mobile conditions (95% A: 5% B) referred to as diluent. Three different supernatant-to-diluent ratio preparations were evaluated: 10 μ L supernatant with 990 μ L diluent; 10 μ L supernatant with 490 μ L diluent; and 50 μ L supernatant with 150 μ L diluent.

Supported liquid extraction (SLE) sample preparation was evaluated by mixing 250 μ L of fortified urine samples in a disposable glass vial with 250 μ L of 0.5 ammonium hydroxide and 75 μ L of ICSMSzymeTM RT with buffer solution. The samples were gently vortexed and incubated at room temperature for 15 minutes. After incubation, 400 μ L of each sample was transferred to an individual Biotage Isolute[®] SLE+ (Salem, NH, USA) column and allowed to absorb for 5 minutes. Two separate 2 mL ethyl acetate elutions were performed, allowing 5 minutes after each wash for elution. Low positive pressure was applied to collect all the eluent in a glass tube. The

eluent was completely evaporated under nitrogen gas at 40°C and the sample was reconstituted in 100 µL of starting mobile phase conditions. The entire sample was transferred to an LC vial with a flat-bottomed liner for analysis.

2.4 Method Validation

The final method was validated for quantitation by assessing linearity, calibration model, limit of detection (LOD), lower limit of quantitation (LLOQ), bias, precision, carry over, interference, and ionization suppression or enhancement effects per the ASB Standard Practices for Method Validation in Forensic Toxicology, which delineates minimum requirements for validating analytical methods in forensic toxicology (49). As an additional measure, recovery was evaluated. Per ASB 036, the validation could have been limited to carryover, interference, limit of detection, and ionization suppression or enhancement if the method were to be used only qualitatively.

2.4.1 Analyte identification criteria

An analyte was considered identified if the retention time of an analyte was within $\pm 2\%$ of its corresponding deuterated internal standard with acceptable diagnostic ratios. Diagnostic ion ratios were required to agree with the ratios calculated from the reference material within 20% if the relative abundance was greater than 50%, within 25% if the relative abundance was between 20 and 50%, and within 30% if the relative abundance was less than 20% (48).

2.4.2 Calibration Model

Calibrator (Cal) samples of eight different concentrations were used to determine the working range of the method. Calibrators were prepared in a one stock methanol solution based on a percentage of the minimum required sensitivity for each analyte as follows: 50% (Cal 1), 100% (Cal 2), 300% (Cal 3), 500% (Cal 4), 700% (Cal 5), 1000% (Cal 6), 1500% (Cal7), and 2000% (Cal 8). The calibration model was established over five separate runs by plotting the ratio of the peak area of the analyte to the peak area of its internal standard against the known concentration at each calibrator concentration. Calibration curves with a correlation coefficient (R^2) of .980 or better were deemed acceptable.

Table 5. Analyte Calibration Model Concentrations.

Analyte	Cal 1 (ng/mL)	Cal 2 (ng/mL)	Cal 3 (ng/mL)	Cal 4 (ng/mL)	Cal 5 (ng/mL)	Cal 6 (ng/mL)	Cal 7 (ng/mL)	Cal 8 (ng/mL)
α -hydroxyalprazolam	2.5	5	15	25	35	50	75	100
7-aminoclonazepam	2.5	5	15	25	35	50	75	100
Amitriptyline	5	10	30	50	70	100	150	200
Amphetamine	12.5	25	75	125	175	250	375	500
Benzoyllecgonine	25	50	150	250	350	500	750	1000
Brompheniramine	5	10	30	50	70	100	150	200
Butalbital	50	100	300	500	700	1000	1500	2000
Carisoprodol	50	100	300	500	700	1000	1500	2000
Chlorpheniramine	5	10	30	50	70	100	150	200
Codeine	5	10	30	50	70	100	150	200
Cyclobenzaprine	5	10	30	50	70	100	150	200
Desipramine	5	10	30	50	70	100	150	200
Dextromethorphan	5	10	30	50	70	100	150	200
Diphenhydramine	5	10	30	50	70	100	150	200
Doxylamine	5	10	30	50	70	100	150	200
Fentanyl	0.5	1	3	5	7	10	15	20
Hydrocodone	5	10	30	50	70	100	150	200
Hydromorphone	5	10	30	50	70	100	150	200
Imipramine	5	10	30	50	70	100	150	200

Analyte	Cal 1 (ng/mL)	Cal 2 (ng/mL)	Cal 3 (ng/mL)	Cal 4 (ng/mL)	Cal 5 (ng/mL)	Cal 6 (ng/mL)	Cal 7 (ng/mL)	Cal 8 (ng/mL)
Lorazepam	2.5	5	15	25	35	50	75	100
mCPP	5	10	30	50	70	100	150	200
MDA	12.5	25	75	125	175	250	375	500
MDMA	12.5	25	75	125	175	250	375	500
Meprobamate	50	100	300	500	700	1000	1500	2000
Methamphetamine	12.5	25	75	125	175	250	375	500
Morphine	5	10	30	50	70	100	150	200
Norchlorcyclizine	5	10	30	50	70	100	150	200
Nordiazepam	5	10	30	50	70	100	150	200
Norfentanyl	0.5	1	3	5	7	10	15	20
Norketamine	5	10	30	50	70	100	150	200
Nortriptyline	5	10	30	50	70	100	150	200
Oxazepam	5	10	30	50	70	100	150	200
Oxycodone	5	10	30	50	70	100	150	200
Oxymorphone	5	10	30	50	70	100	150	200
Phenobarbital	50	100	300	500	700	1000	1500	2000
Temazepam	5	10	30	50	70	100	150	200
THCCOOH	5	10	30	50	70	100	150	200
Tramadol	5	10	30	50	70	100	150	200
Zolpidem COOH	5	10	30	50	70	100	150	200
Zopiclone	5	10	30	50	70	100	150	200

2.4.3 Limit of Detection

The LOD was estimated for each analyte by calculating the standard deviation of the y intercept (s_y) and the average slope (Avg_m) from 3 independent calibration curves across the working range of the method. In addition to the eight calibrators prepared earlier, three additional calibrators were added at concentrations of 25%, 12.5%, and 6.125% of the minimum required sensitivity. The estimation of the LOD according to Equation 4 has been found to be more realistic than using three times the signal-to-noise ratio (50).

$$LOD = (3.3 s_y) / Avg_m$$

Equation 1. LOD calculation.

2.4.4 Lower Limit of Quantitation

The lowest non-zero calibrator (Cal 1) was used to estimate the LLOQ. Three analyte-free urine samples from different sources were fortified with each analyte at 50% of the minimum required sensitivity and analyzed for detection, identification, bias, and precision. If the samples were within $\pm 20\%$ accuracy, Cal 1 was an acceptable estimated LLOQ.

2.4.5 Bias

Bias was measured in pooled fortified urine using quality control samples at 200% (low), 600% (medium), and 1200% (high) the minimum required sensitivity. All samples used for bias calculations fell within the working range for each analyte evaluated. Each concentration was analyzed in triplicate over five different runs. Bias was calculated via Equation 1, with a maximum acceptable bias of $\pm 20\%$.

$$\text{Bias (\%)} \text{ at } \text{Concentration}_x = \left[\frac{\text{Grand Mean of Calculated Concentration}_x - \text{Nominal Concentration}_x}{\text{Nominal Concentration}_x} \right] \times 100$$

Equation 2. Bias calculation.

2.4.6 Precision

Intra- and inter-run precision were measured concurrently with bias utilizing the three quality control sample concentrations. Intra-run precision was calculated for each of the five runs at the three different concentrations via Equation 2. Inter-run precision was calculated at low, medium, and high concentration via Equation 3. A grand mean precision was calculated by averaging the inter- and intra-run precisions.

$$\text{Intra - run CV (\%)} = \left[\frac{\text{standard deviation of a run}}{\text{mean calculated value of a run}} \right] \times 100$$

Equation 3. Within-run precision calculation.

$$\text{Inter-run CV}(\%) = \left[\frac{\text{standard deviation for each concentration}}{\text{grand mean for each calculation}} \right] \times 100$$

Equation 4. Between-run precision calculation.

2.4.7 Carryover

Carryover was evaluated by analyzing a blank urine sample following the highest calibrator for each analyte over five separate runs. Carryover was deemed acceptable if signal intensity did not exceed 10% of the lowest calibrator signal.

2.4.8 Interference

Matrix and internal standard interferences were evaluated. Commonly encountered analyte interferences were not evaluated in this validation.

2.4.8.1 Matrix Interferences

Ten analyte-free urine samples were analyzed without the addition of an IS to determine if common matrix interferences were present. Signal greater than the calculated limit of detection and within 2% of the known retention time were labeled as interferences.

2.4.8.2 IS Interference

Stable-isotope internal standard interference was assessed by analyzing an analyte-free urine sample spiked with IS for each analyte and observing for analyte signal above the LOD. Additionally, a urine sample fortified with 2000% of the minimum required sensitivity for each analyte but no internal standards, was analyzed for internal standard

signal. Signal greater than the calculated limit of detection and within 2% of the known retention time were labeled as interferences.

2.4.9 Ionization Suppression and Enhancement

A sample of 100 μL of mobile phase at starting conditions (95% A and 5% B) containing 150 ng/mL of all internal standards and all analytes at 200% the minimum required sensitivity was prepared. A similar sample was prepared with all analytes at 1000% the minimum required sensitivity. The neat samples were injected six times to obtain the average peak area of each analyte at high and low concentrations.

Ten analyte-free urine samples from different sources were extracted via the SLE protocol. After drying, the samples were reconstituted with starting condition mobile phase spiked with all analytes at 200% their minimum required sensitivities and 150 ng/mL of all internal standards. An additional ten samples were prepared in the same manner and spiked with all analytes 1000% of their minimum required sensitivities. The samples were run to obtain the average peak area of each analyte at high and low concentrations.

The neat sample average peak area was compared to the urine sample average peak area at the corresponding concentration to calculate ion enhancement or suppression (Equation 5).

Ion enhancement or suppression was deemed acceptable if it was within $\pm 25\%$.

$$\text{Ion Suppression (\%)} = \left(\frac{\text{neat standard average peak area}}{\text{urine standard average peak area}} - 1 \right) \times 100$$

Equation 5. Ion Suppression/Enhancement Calculation.

2.4.10 Recovery

Analyte recovery was determined by comparing samples fortified post-extraction with samples fortified pre-extraction according to Equation 5. 10 pre-extraction and 10 post-extraction samples were compared at low concentration (200% minimum required sensitivity) and high concentration (1200% minimum required sensitivity).

$$Recovery (\%) = \frac{\text{pre-extraction average peak area}}{\text{post-extraction average peak area}} \times 100$$

Equation 6. Recovery Calculation.

3. RESULTS

3.1 Analyte Detection

Analytes eluted between 2 and 9 minutes (Figure 9). Morphine eluted first at 2.12 minutes and THCCOOH eluted last at 8.14 minutes (

Table 6).

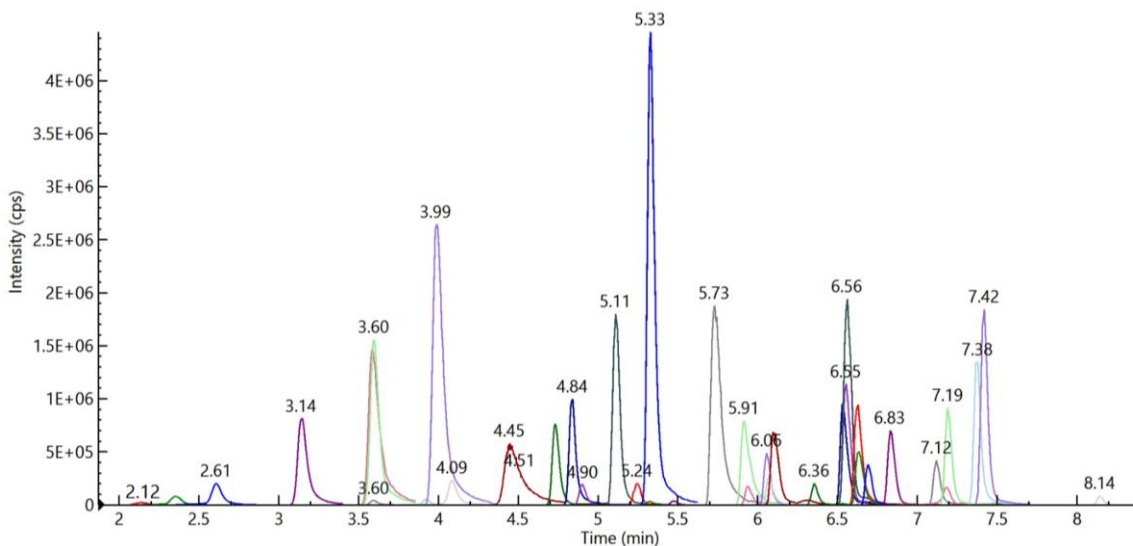


Figure 9. Complete MRM chromatogram of all Analytes at Cal. 7 Concentration

Analytes were eluted in the following order: morphine, oxymorphone, hydromorphone, amphetamine, codeine, methamphetamine, MDA, oxycodone, hydrocodone, MDMA, doxylamine, norketamine, zolpidem COOH, norfentanyl, tramadol, 7-aminoclonazepam, m-CPP, benzoylecgonine, zopiclone, chlorpheniramine, meprobamate, brompheniramine, fentanyl, phenobarbital, dextromethorphan, butalbital, cyclobenzaprine, desipramine, imipramine, amitriptyline, nortriptyline, norchlorcyclizine, carisoprodol, lorazepam, α -hydroxyalprazolam, oxazepam, nordiazepam, temazepam, and THCCOOH.

All analytes eluted within 2% of their corresponding stable isotope internal standard. Analytes not completely resolved via HPLC were easily distinguished by their distinctive MRM transitions.

Table 6. Retention Times.

Analyte	Retention Time (min)	IS	IS Retention Time (min)
α -hydroxyalprazolam	7.19	α -hydroxyalprazolam-d5	7.17
7-aminoclonazepam	5.33	7-aminoclonazepam-d4	5.28
Amitriptyline	6.65	Amitriptyline-d3	6.63
Amphetamine	3.16	Amphetamine-d6	3.12

Analyte	Retention Time (min)	IS	IS Retention Time (min)
Benzoyllecgonine	5.33	Benzoyllecgonine-d8	5.30
Brompheniramine	5.94	Chlorpheniramine-d6	5.74
Butalbital	6.36	Butalbital-d5	6.33
Carisoprodol	6.83	Carisoprodol-d7	6.83
Chlorpheniramine	5.74	Chlorpheniramine-d6	5.74
Codeine	3.61	Codeine-d6	3.55
Cyclobenzaprine	6.53	Cyclobenzaprine-d3	6.53
Desipramine	6.56	Desipramine-d3	6.56
Dextromethorphan	6.12	Dextromethorphan-d3	6.12
Diphenhydramine	6.08	Diphenhydramine-d3	6.08
Doxylamine	4.46	Doxylamine-d5	4.36
Fentanyl	6.06	Fentanyl-d5	6.06
Hydrocodone	4.09	Hydrocodone-d3	4.08
Hydromorphone	2.62	Hydromorphone-d6	2.60
Imipramine	6.56	Imipramine-d3	6.56
Lorazepam	7.12	Lorazepam-d4	7.12
mCPP	5.26	mCPP-d8	5.22
MDA	3.61	MDA-d5	3.58
MDMA	4.01	MDMA-d5	3.97
Meprobamate	5.94	Meprobamate-d3	5.94
Methamphetamine	3.61	Methamphetamine-d5	3.58
Morphine	2.12	Morphine-d6	2.12
Norchlorcyclizine	6.70	Amitriptyline-d3	6.63
Nordiazepam	7.38	Nordiazepam-d5	7.35
Norfentanyl	4.91	Norfentanyl-d5	4.88
Norketamine	4.74	Norketamine-d4	4.71
Nortriptyline	6.63	Nortriptyline-d3	6.63
Oxazepam	7.20	Oxazepam-d5	7.19
Oxycodone	3.93	Oxycodone-d6	3.89
Oxymorphone	2.37	Oxymorphone-d3	2.35
Phenobarbital	6.03	Phenobarbital-d5	6.00
Temazepam	7.43	Temazepam-d5	7.40
THCCOOH	8.15	THCCOOH-d3	8.14
Tramadol	5.12	Tramadol-d3	5.10
Zolpidem COOH	4.84	Zolpidem COOH	4.83
Zopiclone	5.47	Zopiclone-d4	5.47

3.2 Method Validation

3.2.1 Calibration Model, LOD, and LLOQ

Linearity was determined using the ratio of the analyte peak area to the internal standard against the known concentration of standards by weighted linear regression (1/x). In this study, correlation coefficients at or above .98 were considered acceptable. Most analytes displayed linearity with a correlation coefficient greater than .98 (Table A 12. R squared Values for Calibration Model Runs. Table 7). Zopiclone and zolpidem COOH as well as their corresponding internal standards were not detected in any calibration samples. THCCOOH had an average correlation coefficient of .94, with a range of .906 to .991 between the five runs analyzed (Table 7). ASB 036 recommends the use of residual plots to assess the homoscedasticity of each analyte across the working range instead of relying on calibration coefficients. The theoretical limit of detection was below the required minimum sensitivity for all analytes except THCCOOH. The lower limit of quantitation was determined to be equal to 50% the minimum required sensitivity for all analytes except doxylamine, dextromethorphan, brompheniramine, and norchlorcyclizine. For these analytes, 100% the minimum required sensitivity was an acceptable lower limit of quantitation. THCCOOH displayed a LLOQ of 200% the minimum required sensitivity.

Table 7. Working Range, Weighted Linear Regression, and R-squared Average in Urine.

Analyte	Working Range	Regression Model	R ² Average	LOD (ng/mL)	LLOQ (ng/mL)
α -hydroxyalprazolam	2.5-100 ng/mL	linear	0.989	1.37	2.5
7-aminoclonazepam	2.5-100 ng/mL	linear	0.993	4.84	2.5
Amitriptyline	5-200 ng/mL	linear	0.989	3.85	2.5
Amphetamine	12.5-500 ng/mL	linear	0.995	6.04	12.5

Analyte	Working Range	Regression Model	R ² Average	LOD (ng/mL)	LLOQ (ng/mL)
Benzoylcegonine	25-1000 ng/mL	linear	0.990	21.11	25
Brompheniramine	5-200 ng/mL	linear	0.993	4.63	10
Butalbital	50-2000 ng/mL	linear	0.990	27.40	50
Carisoprodol	50-2000 ng/mL	linear	0.994	18.29	50
Chlorpheniramine	5-200 ng/mL	linear	0.993	4.30	5
Codeine	5-200 ng/mL	linear	0.995	1.92	5
Cyclobenzaprine	5-200 ng/mL	linear	0.992	3.5	5
Desipramine	5-200 ng/mL	linear	0.994	2.56	5
Dextromethorphan	5-200 ng/mL	linear	0.996	2.47	10
Diphenhydramine	5-200 ng/mL	linear	0.992	2.75	5
Doxylamine	5-200 ng/mL	linear	0.993	6.85	10
Fentanyl	0.5-20 ng/mL	linear	0.995	0.392	0.5
Hydrocodone	5-200 ng/mL	linear	0.992	2.91	5
Hydromorphone	5-200 ng/mL	linear	0.993	3.10	5
Imipramine	5-200 ng/mL	linear	0.993	2.57	5
Lorazepam	2.5-100 ng/mL	linear	0.993	1.42	2.5
mCPP	5-200 ng/mL	linear	0.994	3.92	5
MDA	12.5-500 ng/mL	linear	0.995	11.5	12.5
MDMA	12.5-500 ng/mL	linear	0.994	5.09	12.5
Meprobamate	50-2000 ng/mL	linear	0.995	44.8	50
Methamphetamine	12.5-500 ng/mL	linear	0.994	5.25	12.5
Morphine	5-200 ng/mL	linear	0.995	3.31	5
Norchlorcyclizine	5-200 ng/mL	linear	0.987	7.35	10
Nordiazepam	5-200 ng/mL	linear	0.994	1.60	5
Norfentanyl	0.5-20 ng/mL	linear	0.995	.14	0.5
Norketamine	5-200 ng/mL	linear	0.993	4.22	5
Nortriptyline	5-200 ng/mL	linear	0.993	3.18	5
Oxazepam	5-200 ng/mL	linear	0.993	2.57	5
Oxycodone	5-200 ng/mL	linear	0.996	4.76	5
Oxymorphone	5-200 ng/mL	linear	0.995	4.93	5
Phenobarbital	50-2000 ng/mL	linear	0.990	17.77	50
Temazepam	5-200 ng/mL	linear	0.994	1.78	5
THCCOOH	5-200 ng/mL	linear	0.949	5	30
Tramadol	5-200 ng/mL	linear	0.994	4.20	5

3.2.2 Bias and precision

Bias was analyzed to determine accurate quantitation. Bias was calculated for each analyte at 200%, 600%, and 1200% the minimum required sensitivities. Each concentration was assessed for acceptability and the samples were averaged. Most analytes displayed acceptable bias of $\pm 20\%$ (Table 8). Brompheniramine, butalbital, and doxylamine were calculated to have bias values greater than 20%. Zolpidem COOH and zopiclone could not be analyzed for bias because they were not detected.

Precision was concurrently analyzed to determine the repeatability of results within run and between runs. Precision was determined for each analyte with triplicate samples on five independent runs at 200%, 600%, and 1200% the minimum required sensitivities. Intra-precision results for each of the five runs were averages and evaluated at the low, medium, and high concentration for acceptability. All the runs displayed intra-run precision within $\pm 20\%$. Inter-run precision was within $\pm 20\%$ for all analytes at all concentrations. Zolpidem COOH and zopiclone could not be analyzed for precision because they were not detected.

Table 8. Bias and Precision Calculations

Analyte	Average Bias	Average Precision %CV		
		<i>Intra-run</i>	<i>Inter-run</i>	<i>Grand Mean</i>
α -hydroxyalprazolam	4.46	9.41	11.00	10.21
7-aminoclonazepam	12.50	8.05	10.02	9.04
Amitriptyline	0.14	9.16	9.99	9.57
Amphetamine	7.47	6.06	6.46	6.26
Benzoylcegonine	-2.50	8.78	9.47	9.12
Brompheniramine	20.55	11.94	15.42	13.68
Butalbital	46.34	11.52	12.93	12.23
Carisoprodol	5.44	5.96	6.38	6.18

Analyte	Average Bias	Average Precision %CV		
		<i>Intra-run</i>	<i>Inter-run</i>	<i>Grand Mean</i>
Chlorpheniramine	7.62	5.23	5.83	5.53
Codeine	12.77	6.17	6.60	6.39
Cyclobenzaprine	8.19	6.78	7.21	7.00
Desipramine	4.57	5.87	6.43	6.15
Dextromethorphan	10.69	13.56	15.64	14.60
Diphenhydramine	10.96	4.95	5.45	5.20
Doxylamine	28.96	11.10	12.21	11.66
Fentanyl	6.730	4.63	5.12	4.87
Hydrocodone	3.02	7.58	9.49	8.54
Hydromorphone	4.07	5.25	5.76	5.51
Imipramine	6.13	5.25	5.56	5.41
Lorazepam	5.89	5.74	6.02	5.88
mCPP	7.68	7.43	7.36	7.40
MDA	9.49	5.02	5.61	5.32
MDMA	11.21	4.90	5.68	5.29
Meprobamate	3.73	5.41	6.42	5.92
Methamphetamine	10.97	6.14	6.38	6.26
Morphine	1.78	7.94	8.37	8.16
Norchlorcyclizine	4.67	13.18	15.20	14.19
Nordiazepam	9.76	5.02	5.41	5.22
Norfentanyl	4.89	4.92	5.50	5.21
Norketamine	8.94	5.11	5.48	5.30
Nortriptyline	6.27	5.24	5.746	5.50
Oxazepam	9.97	8.81	10.39	9.60
Oxycodone	19.76	8.68	11.57	10.12
Oxymorphone	4.28	6.43	6.82	6.63
Phenobarbital	4.93	14.78	15.65	15.22
Temazepam	6.61	5.19	5.47	5.33
THCCOOH	18.77	9.48	9.52	9.50
Tramadol	5.69	5.95	6.29	6.12

3.2.3 Carryover

Carryover was observed with signal intensity of 25% of the lowest calibrator for benzoylecgonine. All other analytes were determined to be free from significant carryover.

3.2.4 Interference

No matrix interference peaks were observed in blank urine samples which fell within 2% of a known analyte retention time and had a signal intensity greater than the calculated LOD. Low levels of internal standard appeared for meprobamate, cyclobenzaprine, lorazepam, and desipramine in a sample with all analytes at 2000% their minimum required sensitivity but no spiked internal standard. No interference peaks were observed in samples spiked with only internal standard and no analyte.

3.2.5 Ion suppression or enhancement

The method displayed significant ion suppression/enhancement among several analytes. Carisoprodol, desipramine, hydromorphone, imipramine, lorazepam, morphine, oxazepam, and oxymorphone were the only compounds to exhibit ionization suppression or enhancement effects in the acceptable range of 25% or less.

Table 9. Ion Suppression/Enhancement Values

Analyte	Low Concentration (%)	High concentration (%)
α -hydroxyalprazolam	24.83	40.96155
7-aminoclonazepam	19.96	36.18
Amitriptyline	-25.92	-5.32
Amphetamine	16.84	-32.27
Benzoyllecgonine	105.29	3.11
Brompheniramine	-77.07	27.81
Butalbital	-19.49	-36.60
Carisoprodol	16.16	0.06
Chlorpheniramine	-57.11	16.64
Codeine	87.35	-9.17
Cyclobenzaprine	-22.41	15.85
Desipramine	-14.74	6.75
Dextromethorphan	-70.21	1.47
Diphenhydramine	-74.07	-20.71
Doxylamine	-73.97	-48.63
Fentanyl	-47.42	10.41

Analyte	Low Concentration (%)	High concentration (%)
Hydrocodone	90.50	-0.14
Hydromorphone	9.83	1.46
Imipramine	-23.67	15.11
Lorazepam	-14.28	16.95
mCPP	86.76	1.46
MDA	58.73	-37.01
MDMA	55.82	-11.33
Meprobamate	-41.11	-15.97
Methamphetamine	32.83	-18.99
Morphine	5.79	10.02
Norchlorcyclizine	-16.53	32.75
Nordiazepam	24.34	37.44
Norfentanyl	70.80	45.48
Norketamine	57.53	-7.07
Nortriptyline	-65.01	0.56
Oxazepam	-7.08	12.52
Oxycodone	129.16	8.68
Oxymorphone	10.72	10.15
Phenobarbital	-69.59	-44.41
Temazepam	16.17	25.72
THCCOOH	-67.11	50.49
Tramadol	22.81	44.98
Zolpidem COOH	112.35	54.77
Zopiclone	135.67	9.29

3.2.6 Recovery

Recovery percentages using the SLE sample preparation are shown in Table 10. Recovery was moderate for amitriptyline, cyclobenzaprine, desipramine, imipramine, dextromethorphan, and diphenhydramine. Recovery was poor for benzoylecgonine and THCCOOH. Zolpidem COOH and zopiclone were not recovered after sample preparation.

Table 10. Recovery Values

Analyte	Low Concentration Recovery (%)	High concentration Recovery (%)
α -hydroxyalprazolam	66.72	70.66

Analyte	Low Concentration Recovery (%)	High concentration Recovery (%)
7-aminoclonazepam	79.26	87.27
Amitriptyline	54.52	32.64
Amphetamine	83.16	62.67
Benzoylcegonine	6.31	6.08
Brompheniramine	88.48	74.94
Butalbital	79.77	71.09
Carisoprodol	127.43	118.11
Chlorpheniramine	82.41	71.39
Codeine	103.98	68.95
Cyclobenzaprine	55.98	44.20
Desipramine	58.01	47.09
Dextromethorphan	22.96	41.00
Diphenhydramine	39.33	48.97
Doxylamine	127.36	101.52
Fentanyl	74.95	62.17
Hydrocodone	103.77	87.61
Hydromorphone	109.90	76.34
Imipramine	57.23	44.81
Lorazepam	66.35	69.95
mCPP	84.94	73.22
MDA	94.33	66.08
MDMA	101.21	79.75
Meprobamate	126.34	102.95
Methamphetamine	82.29	56.04
Morphine	99.70	72.19
Norchlorcyclizine	67.67	52.25
Nordiazepam	59.50	60.13
Norfentanyl	98.49	95.24
Norketamine	94.25	83.94
Nortriptyline	24.29	41.89
Oxazepam	64.28	66.83
Oxycodone	118.58	89.37
Oxymorphone	118.03	92.86
Phenobarbital	84.18	70.82
Temazepam	71.17	70.73
THCCOOH	5.84	5.37
Tramadol	101.26	90.98
Zolpidem COOH	0	0
Zopiclone	0	0

4. DISCUSSION

4.1 Method Development

4.1.1 Column

Initial attempts at chromatography utilized a C18 column resulting in several analytes co-eluting such that a decrease in sensitivity because of competition for ionization was seen. By implementing the phenyl hexyl column, more selective separation was observed allowing for greater signal intensity. Compounds such as doxylamine, chlorpheniramine, brompheniramine and dextromethorphan can overload residual silanol groups on a typical C18 column leading to poor peak shape (51). Interactions with the hexyl linkers in the phenyl-hexyl column allow for better separation and more defined chromatography (51).

4.1.2 Mobile phase

Methanol was chosen as the organic solvent because of its weaker elution strength as compared with acetonitrile (11). Analytes were retained in the column longer, allowing for matrix components to elute off in waste in the beginning of the run. The weaker elution strength of methanol ensured analytes were eluted in a mobile phase with a higher organic content resulting in more effective de-solvation and enhanced electrospray response (11).

Original analysis utilized 10mM ammonium formate in ultrapure water as the aqueous mobile phase resulting in low signal intensity when operating in negative mode. To improve signal intensity, the aqueous mobile phase was altered to .1% formic acid in

water. Theoretically, a weak acid could provide additional protons at the ESI capillary tip, facilitating the creation of excess negative charge, and ultimately promoting analyte deprotonation (52). Additional improvements in negative mode signal intensity could be made using acetic acid, which has a higher gas phase proton affinity (52).

4.1.3 Separation of isobaric compounds

Two analytes shared a precursor mass with one of their metabolites: morphine with hydromorphone, and codeine with hydrocodone. Because the analyte-metabolite pairs have similar structures, they also exhibited similar fragmentation chemistry. To ensure clear identification, different product ions were monitored, and the LC method was adjusted to ensure the pairs did not co-elute. Using the LC method outlined by Zheng et al did not initially resolve morphine and hydromorphone fully and the LC method was adjusted to allow for further separation between the peaks (46).

4.1.4 Optimizing for positive and negative ESI

The fast polarity switching capabilities of the UniField Detector™ facilitated the acquisition of data for positive and negative ESI within one method (53). The detector's design allows for the near-simultaneous detection of both negative and positive ions without the use of high-voltage switching. Negative ions are attracted to a fixed +6kV dynode. An electron is released when the negative ion strikes the dynode, and the electron cascades down the series of increasingly positive dynodes to produce a signal (Figure 10). Positive ions are attracted to a high-energy dynode (HED) fixed at -6kV. The positive ion collides with the HED, releasing an electron which is attracted to the +6kV dynode. The

electron then produces a signal in a similar manner as the negative ion: by cascading down the series of increasingly positive dynodes to the detector. Polarity switching occurs in microseconds allowing for the detection of positive and negative ions regardless of elution time within one analytical method (53).

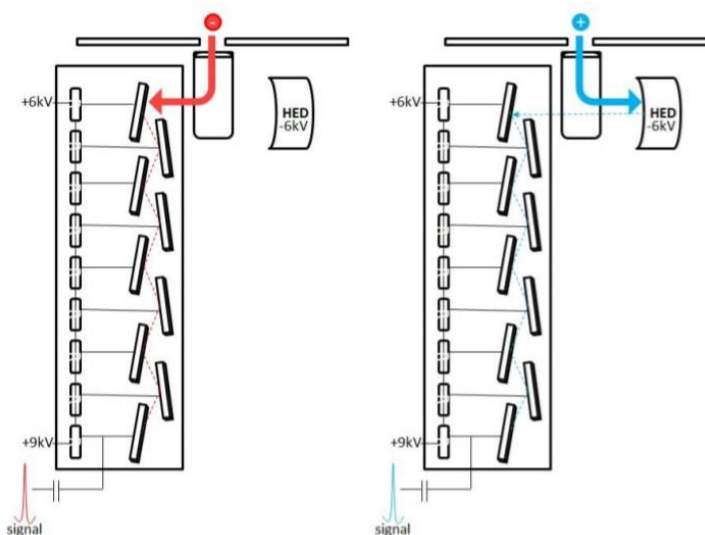


Figure 10. Schematic of the Unifield Detector.

(A) The negative ion path through the detector; and (B) the positive ion path through the detector.

Historically, barbiturates and THCCOOH exhibit a greater ionization efficiency in ESI negative mode and do not generate a significant response in ESI positive mode (24). Some analytes, such as THCCOOH, can be ionized in both positive and negative mode. Consideration must be given that an analyte measured in negative mode can still ionize and create interfering species for analysis in positive mode. While the QSight was able to detect drugs in both positive and negative ionization mode, creating conditions for optimal ionization in a singular method proved challenging. Butalbital displayed unacceptable bias of greater than 40% across low, medium, and high concentrations. THCCOOH did not

show an acceptable linear response. Inconsistent sample recovery could be contributing to THCCOOH's low calibration coefficient. Signal intensity for butalbital, phenobarbital, and THCCOOH was low considering their concentrations relative to other analytes. Even if signal intensity remains low for analytes in negative mode the method still could be suitable for DFSA sample analysis because the minimum required sensitivities for the barbiturates is especially high.

4.1.5 Scan time/duty-cycle optimization

Given the number of analytes in this method, optimization of dwell times was exceedingly important. MRM transitions require the MS to continuously scan the designated mass range for each transition. Creating windows of scan time based on each analyte's retention time can help maximize the dwell time for each analyte. Utilizing a time-managed MRM allows for more data points at the critical time an analyte is expected to elute, thus improving peak shape. The Simplicity software can calculate the optimal dwell time to provide sufficient points across a peak for acceptable bias and precision. At least twelve points were observed on acceptable analyte peaks for this analytical method.

Analytes that elute within thirty seconds of each other necessarily decrease the dwell time available for the machine to scan for each analyte's m/z transitions. Thus, as the number of closely eluting analytes increase, the dwell time for each transition decreases, and ultimately the sensitivity decreases. One way to address unacceptable bias or precision would be to create a longer method, allowing larger differences between retention times.

4.1.6 Sample Preparation

The dilute-and-shoot sample preparation failed to give acceptable chromatography at dilution levels of 1:100 and 1:50. Several drugs exhibited poor peak shape, including split peaks and peak broadening. For example, morphine exhibited peak splitting and low signal intensity with dilute-and-shoot preparation but gave a clean Gaussian shape at the same concentration with SLE sample preparation (Figure 11).

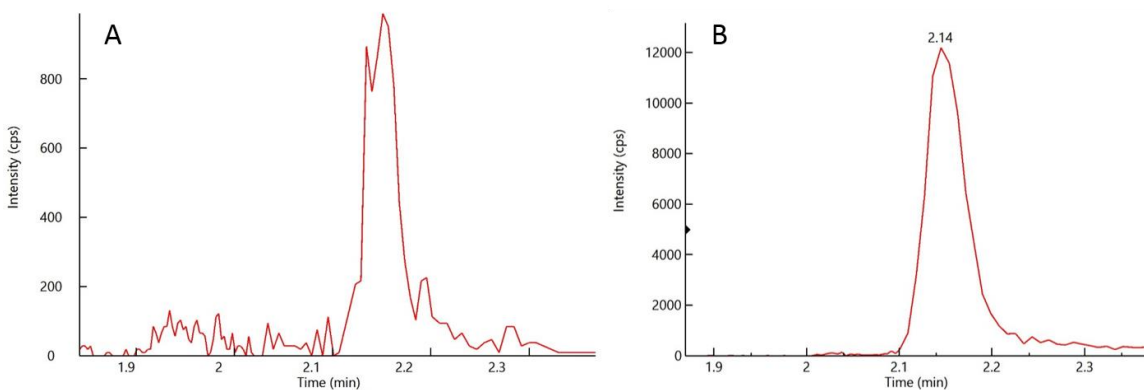


Figure 11. Morphine peak comparison. Morphine at 10 ng/mL concentration prepared via a 1:50 dilute and shoot (A) and SLE method (B)

Drugs that had low required minimum sensitivities suffered especially. The peak for norfentanyl was not found in Calibrator 1, Calibrator 2, and the LQC sample for both dilutions. This is not surprising, given Calibrator 1 contained norfentanyl at a concentration of 0.5 ng/mL, and would have been diluted to as little as 5 picograms/mL. The 1:3 sample preparation showed significant ion suppression or enhancement effects. However, the SLE sample preparation samples also suffered from unacceptable levels of ion suppression or enhancement.

4.2 Method Validation

4.2.1 Bias

While most of the analytes displayed acceptable bias, calculated bias values for brompheniramine, butalbital, and doxylamine were greater than 20%. The peak representing doxylamine was broad and consistently lacking in Gaussian shape. The hydrophobic properties of doxylamine can lead to overloading of the residual silanol groups on the column leading to poor peak shape, but interactions with the hexyl linker in the column should enhance. Similar interactions could impact chlorpheniramine, brompheniramine, and dextromethorphan. Separation of chlorpheniramine, diphenhydramine, and dextromethorphan has been achieved on a phenyl-hexyl column while employing a basic strong mobile phase (pH 9.0). Adjusting the pH of the organic mobile phase could help decrease the peak width and improve the precision results.

4.2.2 Ion suppression or enhancement effects

Analysis of urine samples via ESI is susceptible to ion suppression effects. Ion suppression occurs when more than one component in the ion source results in competition for ionization. This leads to a decrease in signal intensity which can negatively impact the method's limit of detection. Ion suppression can be difficult to predict, with physiological changes and other variable matrix components causing variation in suppression between samples (8). Salts and other urine components can increase ion suppression effects and were prominently seen in the 1:3 dilution. The SLE sample preparation technique removes salts and other ionizable matrix components by

binding them to the diatomaceous earth packed cartridge in the body of the SLE cartridge while the analytes are eluted, thus decreasing ion suppression and enhancement. Given the number of closely eluting analytes, competition for ionization could be significantly impacting the number of analytes effectively ionized and detected. By creating a method with a longer run time, the analytes would elute less closely, and ionization suppression or enhancement effects could be decreased. The pH also can have significant impact on ionization and should be modified in future work to optimize ionization.

4.2.3 Recovery

While recovery was evaluated for this method, ASB 036 does not require it for method validation (48). Namely, a method may have low or moderate recovery but still capable of meeting the cutoffs indicated in ASB 121. A generic SLE procedure was selected for this method because of the mixture of acidic, basic, and neutral analytes in the assay. The initial addition of ammonium hydroxide was intended to increase the affinity of acidic analytes, such as opiates, for the SLE column. Zopiclone and zolpidem carboxylic acid displayed appropriate signal intensity and chromatographic separation in all post extraction samples and samples that underwent dilute and shoot preparation. However, both Z-drugs and their stable isotope internal standards were not detected in any sample that underwent the enzymatic hydrolysis and SLE preparation. Zolpidem carboxylic acid has been detected via LC-MS after enzymatic hydrolysis with IMCSzymeTM utilizing the same MRM transitions monitored in this work (54). Previous studies show that high (>85%) recovery levels of both drugs can be achieved using the

Isolute® Supported Liquid Extraction SLE+ cartridges (55). Successful elution of zolpidem carboxylic acid has been achieved using 2% formic acid preconditioning and methanol as the eluent (54). Zolpidem carboxylic acid and zopiclone most likely were not eluted by the ethyl acetate and remained bound in the diatomaceous earth matrix of the SLE cartridge. Changes to the SLE eluent to replicate the protocol of Feng, Cummings, and McIntire would likely allow recovery of zolpidem COOH and zopiclone but may also impact recovery of other analytes.

Recovery of volatile compounds like methamphetamine and amphetamine could be further optimized by adding a mixture of 0.01% nitric acid in methanol to the eluent samples before evaporating them to dryness (56). THCCOOH sample loss during the extraction process could lead to variability in calibrators and quality control samples, which could explain the poor linearity, bias, and precision results. However, THCCOOH has shown similarly poor bias and precision results in previous multi-drug LC-MS-MS methods (45). A challenging aspect of measuring urinary cannabinoids is their common adsorption to sample preparation materials, which could explain the poor recovery observed for this analytical method. (57). A comparison of sample extraction for THCCOOH and other cannabinoids found that C18 SPE provided higher sample recoveries than diatomaceous earth (57). Additionally, they utilized a 96-well plate sample preparation which limited the number of transfers required for sample preparation. Wei, Wang, and Blount were able to achieve a LOD well below the ASB 121 minimum requirement (57).

However, their analysis only examined cannabinoids, and their preparation would need to be tested for suitability with the other analytes in ASB 121.

4.2.2 Limit of Detection

Because the collection of DFSA samples is frequently delayed and many common DFSA drugs require low doses to induce the desired effects, an analytical method developed for DFSA samples must be highly sensitive. ASB 121 defines the limit of detection as an estimate of the lowest analyte concentration that can be reliably identified and differentiated from blank matrix (14). Each analyte's required minimum sensitivity was derived from the literature or an estimated concentration of single dose usage (14). For the developed analytical method, the theoretical limit of detection was below the required minimum sensitivity dictated by ASB 121 for all analytes except THCCOOH, zopiclone, and zolpidem COOH. The LC-MS method could detect all analytes below the required minimum sensitivity when added post extraction. Based on these results, poor sample recovery during the SLE protocol likely accounts for the high LOD values for THCCOOH. The previously discussed changes to sample preparation could improve recovery and allow the method to meet the ASB 121 required sensitivities.

4.2.2 Quantitative or Qualitative Application

While this method was validated for quantitative application, consideration should be given to whether a quantitative result is necessary. Quantification would be necessary to estimate the magnitude of the dose and infer the potential incapacitating effect of the drug. However, interpretation of this kind with urine samples must be done

with caution. Many unique variables impact the amount of a drug detected in urine, and metabolites of previously ingested substances can be detected while not contributing to intoxication (41). As such, quantitation in urine samples is not always recommended (58). Drug identification above the minimum required threshold may be sufficient for many applications.

5. CONCLUSIONS

A quantitative assay was developed to attempt to meet the minimum scope and sensitivity requirements laid out in ASB 121. The developed method included the detection of 37 analytes in positive mode, and 3 in negative mode. The LC-MS method was able to detect all analytes, with retention times between 2 and 9 minutes. Two analytes, zolpidem COOH and zopiclone, were lost during sample preparation, likely because they did not elute off the SLE cartridge. All analytes displayed linearity with correlation coefficients greater than 0.98 apart from THCCOOH and the two undetected Z-drugs. For the analytes displaying acceptable linearity, the method was able to meet the minimum sensitivity requirements. Significant ionization suppression and enhancement effects were observed for several analytes. Doxylamine and butalbital displayed unacceptable bias.

There is potential in the developed method for an effective quantitative assay for DFSA urine samples. For many analytes, the method is already effective and could be utilized in its current state. Further optimization of sample preparation could produce a method capable of meeting the ASB 121 scope and sensitivity requirements.

6. FUTURE DIRECTIONS

Several improvements can move this method toward a valid quantitative assay for DFSA urine samples. Changes to sample preparation to allow better recovery of zopiclone, zolpidem COOH, benzoylecgonine, and THCCOOH are necessary. Specifically, ethyl acetate is likely a poor eluent for zopiclone and zolpidem COOH. Methanol elution after a 2% formic acid preconditioning should be attempted based on the success of previous studies (54). Different mobile phase pH should be explored to narrow doxylamine's peak therefore improving bias and precision results for the analyte. Ion suppression or enhancement effects may be improved with a longer run time to decrease coelution and competition of ionization. Additional blank samples should be tested to determine the impact of the heightened ionization suppression/enhancement observed on each analyte's LOD.

Upon improvement, patient urine samples should be tested to validate the efficacy and recovery after enzymatic hydrolysis. Additional interference testing with commonly encountered analytes should also be performed.

APPENDIX A

Table A 11. Certified reference standard information.

Analyte	Provider	Lot Number
(-)-11-nor-9-carboxy- Δ 9-THC-D3	Cayman	0571148-3
(-)-11-nor-9-carboxy- Δ 9-THC	Cayman	0571148-3
(\pm)-Amphetamine	Cerilliant	FE04061701
(\pm)-Amphetamine-D6	Cerilliant	FE08301801
(\pm)-MDA	Cerilliant	FE05061901
(\pm)-MDA-D5	Cerilliant	FE01131506
(\pm)-MDMA	Cerilliant	FE06141804
(\pm)-MDMA-D5	Cerilliant	FE02211702
(\pm)-Methamphetamine	Cerilliant	FE12141602
(\pm)-Methamphetamine-D5	Cerilliant	FE03211801
(\pm)-Norketamine HCl	Cerilliant	FN10231801
(\pm)-Norketamine-D4 HCl	Cerilliant	FN10231801
1-(3-Chlorophenyl)piperazine (mCPP) HCl	Cerilliant	FN03212001
1-(3-Chlorophenyl)piperazine-D8 HCl	Cerilliant	FN03232014
7-Aminoclonazepam	Cerilliant	FN11171503
7-Aminoclonazepam-D4	Cerilliant	FN12081502
alpha-Hydroxyalprazolam	Cerilliant	FN07051601
alpha-Hydroxyalprazolam-D5	Cerilliant	FN01261703
Amitriptyline HCl	Cerilliant	FN06131706
Amitriptyline-D3 HCl	Cerilliant	FN04151601
Benzoylcegonine	Cerilliant	FE01061604
Benzoylcegonine-D8	Cerilliant	FE06071702
Brompheniramine maleate	Cerilliant	FN06301701
Butalbital	Cerilliant	FE08101701
Butalbital-D5	Cerilliant	FE02281072
Carisoprodol	Cerilliant	FE03032012
Carisoprodol-D7	Cerilliant	FE08111701
Chlorpheniramine maleate	Cerilliant	FN02061903
Chlorpheniramine-D6 maleate	Cerilliant	FN07071604
Cis-Tramadol HCl	Cayman	0572837
Codeine	Cerilliant	FE11021502
Codeine-D6	Cerilliant	FE06221701
Cyclobenzaprine HCl	Cayman	522139
Cyclobenzaprine-d3 HCl	Cayman	529241
Desipramine HCl	Cerilliant	FN06301705
Desipramine-D3 HCl	Cerilliant	FN03281701
Dextromethorphan	Cayman	0581522
Dextromethorphan-D3	Cerilliant	FN02031706
Diphenhydramine HCl	Cerilliant	FN02212011
Diphenhydramine-D3	Cerilliant	FN03152001
Doxylamine succinate	Cerilliant	FN11131904
Doxylamine-D5	Cerilliant	FN05132001
Fentanyl	Cerilliant	FE06151802
Fentanyl-D5	Cerilliant	FE07281604
Hydrocodone	Cerilliant	FE09091505

Analyte	Provider	Lot Number
Hydrocodone-D3	Cerilliant	FE07311801
Hydromorphone	Cerilliant	FE08011802
Hydromorphone-D6	Cerilliant	FE08271801
Imipramine HCl	Cerilliant	FN07021901
Imipramine-D3	Cerilliant	FN05311901
Lorazepam	Cerilliant	FE10151502
Lorazepam-D4	Cerilliant	FE10021702
Meprobamate	Cerilliant	FE03252001
Meprobamate-D3	Cerilliant	FE06301704
Morphine	Cerilliant	FE06231704
Morphine-D6	Cerilliant	FE10241701
Nordiazepam	Cerilliant	FE11181503
Nordiazepam-D5	Cerilliant	FE11201801
Norfentanyl oxalate	Cerilliant	FN10051802
Norfentanyl-D5 oxalate	Cerilliant	FN05131603
Norketamine HCl	Cayman	0572743
Nortriptyline- D3 HCl	Cerilliant	FN09161904
Nortriptyline HCl	Cerilliant	FN11021803
Oxazepam	Cerilliant	FE05261603
Oxazepam-D5	Cerilliant	FE02051602
Oxycodone	Cerilliant	FE08241701
Oxycodone-D6	Cerilliant	FE09201701
Oxymorphone	Lipomed	2406.1B6.1L1
Oxymorphone-D3	Lipomed	1229.1B3.1L2
Phenobarbital	Cerilliant	FE08021601
Phenobarbital-D5	Cerilliant	FE08091702
Temazepam	Cerilliant	FE04231903
Temazepam-D5	Cerilliant	FE09271701
Zolpidem Phenyl-4-carboxylic acid	Cerilliant	FN10031902
Zolpidem Phenyl-4-carboxylic acid-D4	Cerilliant	FN07251708
Zopiclone	Cayman	0582124
Zopiclone-D4	Cerilliant	FE03242001

Table A 12. R squared Values for Calibration Model Runs.

Analyte	Run 1 R ²	Run 2 R ²	Run 3 R ²	Run 4 R ²	Run 5 R ²
α-hydroxyalprazolam	0.9915	0.991	0.983	0.9945	0.987
7-aminoclonazepam	0.989	0.9918	0.9924	0.9941	0.9985
Amitriptyline	0.989	0.987	0.985	0.991	0.9918
Amphetamine	0.9941	0.9941	0.995	0.9975	0.9966
Benzoylcegonine	0.9935	0.9902	0.9935	0.981	0.9935
Brompheniramine	0.989	0.9945	0.9924	0.9979	0.99
Butalbital	0.9959	0.9906	0.9902	0.982	0.9934
Carisoprodol	0.9926	0.9901	0.992	0.999	0.9975
Chlorpheniramine	0.993	0.987	0.9907	0.9985	0.998
Codeine	0.9926	0.9938	0.994	0.9974	0.9975
Cyclobenzaprine	0.9907	0.989	0.989	0.9969	0.9963
Desipramine	0.9948	0.989	0.9916	0.9977	0.9978
Dextromethorphan	0.993	0.9956	0.9981	0.9987	0.9965
Diphenhydramine	0.987	0.985	0.9918	0.9972	0.9987
Doxylamine	0.985	0.9919	0.9958	0.9966	0.9945
Fentanyl	0.9922	0.9921	0.9971	0.9973	0.9982
Hydrocodone	0.9909	0.984	0.9915	0.9971	0.9974
Hydromorphone	0.993	0.981	0.9943	0.9989	0.9977
Imipramine	0.99	0.987	0.9926	0.9964	0.9969
Lorazepam	0.9905	0.9912	0.9916	0.9955	0.9973
mCPP	0.9904	0.9907	0.9967	0.9969	0.9971
MDA	0.9924	0.9907	0.9944	0.9983	0.997
MDMA	0.9917	0.993	0.9918	0.9969	0.997
Meprobamate	0.9943	0.9926	0.9922	0.99932	0.9969
Methamphetamine	0.9948	0.9903	0.9923	0.9972	0.9976
Morphine	0.9937	0.99	0.9952	0.9983	0.9969
Norchlorcyclizine	0.981	0.982	0.9978	0.99	0.983
Nordiazepam	0.9909	0.9924	0.9921	0.9975	0.997
Norfentanyl	0.9932	0.9938	0.9938	0.9973	0.9967
Norketamine	0.9907	0.989	0.9945	0.9968	0.9951
Nortriptyline	0.9903	0.9904	0.9937	0.9949	0.9974
Oxazepam	0.9913	0.9959	0.992	0.9945	0.9934
Oxycodone	0.9946	0.9945	0.9961	0.9967	0.9968
Oxymorphone	0.9921	0.9923	0.998	0.9949	0.997
Phenobarbital	0.994	0.994	0.985	0.9939	0.982
Temazepam	0.9905	0.9929	0.9918	0.9975	0.9957
THCCOOH	0.959	0.906	0.944	0.947	0.991
Tramadol	0.9923	0.9914	0.988	0.9986	0.9973
Zolpidem COOH	N/A	N/A	N/A	N/A	N/A
Zopiclone	N/A	N/A	N/A	N/A	N/A

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CURRICULUM VITAE

