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Method Development and Validation for Drug Identification and Confirmation by LC/MS-MS for Limited-specimen Cases

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Method Development and Validation for Drug Identification and Confirmation by LC/MS-MS for Limited-specimen Cases

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APPROVED FOR THE FORENSIC SCIENCE INSTITUTE

APRIL 2017

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Abstract

Driving under the influence of drugs (DUID) cases represent the largest portion of cases handled in most forensic toxicology laboratories. Blood is a commonly used specimen and is often analyzed using gas chromatography-mass spectrometry (GC/MS). A common extraction for this method requires two milliliters of blood. If more than one extraction is necessary, a larger volume of blood is required. Recently, laboratories have started using liquid chromatography-mass spectrometry (LC/MS) to obtain a lower limit of detection and extractions which require less blood to complete. Currently, the Oklahoma State Bureau of Investigation (OSBI) Laboratory operates LC-based extractions which require 250 to 500 microliters (µL) of sample to complete, but these are limited to specific drug classes. A general drug screen for forty drugs has been developed and validated using 250 microliters of blood. Even with this reduction of volume requirements, there are still instances in which less than one milliliter (mL) of blood is available for use by the analyst. An additional validation has been completed which required 100 microliters of sample to confirm the presence of thirty-nine drugs. A comparison between these methods was completed to verify the sensitivity of the 100 microliter method.

Introduction

Breath and blood are commonly encountered specimens associated with the assessment of driving under the influence of alcohol or drugs, hereinafter referred to as DUI cases, at this time. Urine is not a practical sample for DUI cases due to the fact that it does not establish time of impairment. Breath is only a viable specimen in the detection of volatile compounds. This leaves blood as the only suitable option to confirm the presence or absence of other intoxicating drugs. Limited sample can be a problem when it comes to obtaining results for DUI cases. With extractions that may require up to two milliliters of blood to perform, the completion of confirmatory testing could be prevented by lack of sample. Not to be forgotten, there must be enough sample available for independent testing if it is requested by the defendant. With all of these requirements, an extraction requiring significantly less blood is something that would allow a larger number of limited-sample cases to be more completely analyzed.

Literature Review

There are two types of instrumentation which are commonly used for drug confirmation in the field of forensic toxicology. They are gas chromatography-mass spectrometry (GC/MS) and liquid chromatography-mass spectrometry (LC/MS or LC/MS-MS). The main difference is apparent in the names of the instruments. However, there are many similarities between the two. The extracted sample is introduced into the instrument through the injector. It then moves through the chromatographic column, which separates the components of a mixture. After the components have traveled through the column, they pass through the mass spectrometer. As they pass through this portion of the instrument, they are broken into

reproducible fragments. The abundance of these fragments is then recorded by the detector, which uses this information to produce a mass spectrum or "picture" of the fragments (see Figure 1). The time from injection to detection, known as the retention time, is recorded for the fragments of the components. The retention time and mass spectrum can be used to compare unknowns to knowns in a library if one is available (Agilent, 2007).

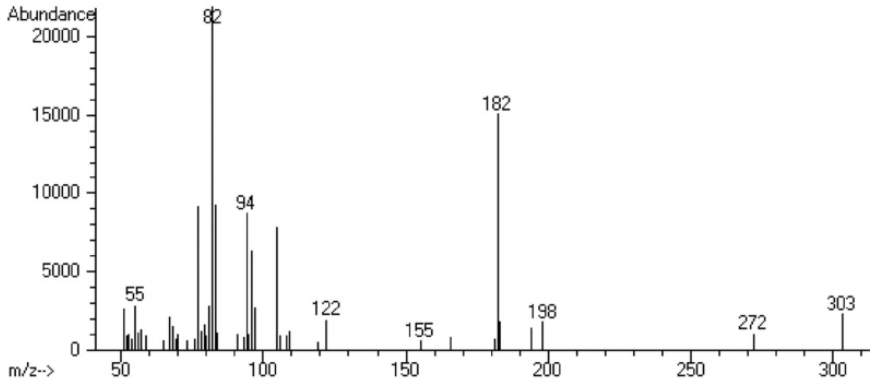


Figure 1. Cocaine spectrum.

A GC/MS uses a carrier gas to transport the injected mixture through the column and to the detector, which necessitates that the sample be turned into a gas from a liquid. This requires the analyte to be volatile or amenable to chemical derivatization to render the compound volatile. The sample is introduced into the instrument and is taken from atmospheric pressure to the system pressure, vaporized, and all or part of the resulting gas is introduced into the column. Helium is often used as the carrier gas, which moves through the instrument at a constant pressure and flow rate designated by the method being used. GC/MS is appropriate for analytes that do not ionize well using LC/MS techniques. Electron ionization (EI) is a commonly used ionization technique. EI is a very robust and reproducible technique which does not suffer from ion suppression caused by a co-eluting compound (Agilent, 2007).

In this technique, sample components collide with electrons emitted from a filament and are ionized. The molecules are broken down at the same time creating fragments. Although EI is a more common technique, there is another termed chemical ionization (CI). This technique involves ionization of reagent gas molecules which usually produces ions of the analyte by collision. Unlike EI, a preponderance of the ions created under the CI process result in the ability to determine the molecular weight due to the fact that most remain intact and few fragment ions are produced. GC/MS instruments also cost less than their LC/MS analogues.

A LC/MS can separate metabolites, products of the breakdown of drugs in the human body that are not volatile or derivatized. This allows for a wider range of chemical species to be analyzed. The commonly-used ionization techniques are electrospray ionization[1] (ESI) and atmospheric pressure chemical ionization[2] (APCI). Both of these techniques allow for ionization suppression which can cause co-eluting compounds to be underestimated or not detected at all. However, LC/MS almost always produces a molecular ion that can be used to limit the possible identities of a given analyte. Often, selected ion monitoring (SIM)[3] or multiple reaction monitoring (MRM)[4] are used which allows for a lower limit of detection (LOD) or limit of quantitation (LOQ) (Agilent, 2007).

In the last decade, the LC/MS has grown in popularity in forensic toxicology. With this increased popularity, new methods are constantly being developed, often for specific drugs or drug classes. In 2012, a method to detect twenty-five designer cathinones, part of the

[1] High voltage is applied to a liquid to create an aerosol.
[2] Utilizes gas-phase ion-molecule reactions at atmospheric pressure.
[3] Ions in a certain mass-to-charge ratio range are scanned for and are detected by the instrument eliminating unwanted ions.
[4] Used to target compounds of interest and lower background noise by moving only the ions for the drugs of interest through the instrument to the detector.

amphetamine class, was developed by Ammann, Mclaren, Gerostamoulos, and Beyers. This extraction requires one hundred microliters of blood, one milliliter of 1-chlorobutane containing ten percent isopropanol, one hundred microliters of internal standard, and two hundred microliters of trizma buffer (pH 9.2). The solvent layer is evaporated to dryness and reconstituted with 500 microliters of a 95:5 mixture of 50 millimoles per liter (mmol/L) aqueous ammonium formate and acetonitrile containing 0.1 percent formic acid. Ante-mortem blood was used for verification and calibration standards. A signal-to-noise ratio of at least 10:1 for the setting of all lower limits of quantitation (LLOQ) was also required. The researchers followed internationally accepted recommendations[5] and were successfully able to validate the method for all twenty-five compounds.

Clarkson, Lacy, Flignrt, Thiersch, Howard, Harruff, and Logan (2004) collected data from all death investigation and impaired driving cases which tested positive for tramadol between 1995 and 2000 at the Washington State Toxicology Laboratory. In total, there were 75 cases. Copies of the records containing details surrounding individual deaths, death certificates, and autopsy findings were also obtained for all death investigation cases. Cases were excluded if any of this information was missing or if an autopsy was not completed. This left a total of 66 cases. 46 cases were found to be one of the following: a tramadol-caused death (n=4), tramadol-contributed to cause of death (n=27), or an incidental appearance of tramadol (n=15).

In both 2007 and 2013-2014, the National Highway Traffic Safety Administration (NHTSA) conducted national surveys to estimate the prevalence of alcohol consumption and/or drug use and driving in the United States. In the 2007 study, only weekend nighttime drivers'

[5] Guidelines provided by the US Department of Health and Human Services as well as multiple peer reviewed recommendations. These have been widely accepted in the toxicology community.

data was collected. In the 2013-2014 survey, data collection included weekend nighttime and weekday daytime drivers. It was revealed in both surveys that there was a significant decline in alcohol-impaired driving since the initial NHTSA survey in 1973. However, noted was an increase in drivers having taken illegal drugs as well as an increase in drivers taking lawful medications, although some without the benefit of the associated prescription. The results of blood tests showed an increase from 9.8 percent to 14.3 percent for drivers with illegal drugs in their systems while driving. The increase was smaller for those with legal medications in their systems, 4.0 percent to 4.9 percent. The illegal drug category includes all drivers with any illegal drug in their system even if they had prescription and/or over-the-counter (OTC) medications in their system as well. The incidents of the use of THC also rose between the 2007 and 2013-2014 surveys. In 2007, the incidents of THC were 7.6 percent and have increased to 11.7 percent in the latest survey (Compton & Berning, 2009). Since there were two time periods used in the 2013-2014 survey, weekday daytime drivers and weekend nighttime drivers, NHTSA was able to compare this information. It was determined that there was a difference between these time frames regarding illegal drugs versus legal medications, but that there was not a difference between overall drug prevalence. For the weekday daytime period, 11.3 percent of those in the survey group had illegal drugs in their system and 10.3 percent had only legal medications. For weekend nighttime drivers, 14.3 percent had illegal drugs in their systems and 6.9 percent had only legal medications. This translates to an overall incident rate of 21.6 and 21.2 percent, respectively (Berning, Compton & Wochinger, 2015).

To understand the importance of the present study, it is important to understand the significance of the drugs selected for inclusion. A few are new additions to the OSBI laboratory

LC/MS protocols and GC/MS libraries due to their appearance in at least one DUID case in recent years. Most are drugs which have been shown to cause impairment in the average person and are either seen often in casework or are not easily confirmed by GC/MS. Drugs are commonly grouped into classes based on similarities in the structures and intended uses. Due to this classification, similar psychomotor and cognitive affects are observed within a given class.

In 2015, the most common drug class in OSBI Laboratory case work was benzodiazepines. This group is one of the most widely prescribed in the world, replacing barbiturates as the major central nervous system (CNS) depressant drugs. The most common of these are alprazolam, lorazepam, clonazepam, diazepam and temazepam. There are currently approximately twenty benzodiazepines approved by the United States Federal Drug Administration (FDA). Benzodiazepines are prescribed as muscle relaxants, anesthetic adjuncts, anticonvulsants, treatment for obsessive-compulsive disorder, and for anxiety. The time it takes for the concentration of the drug in the blood to reduce by one-half, due to metabolism in the body, is known as the half-life. The half-lives for these drugs range from one hour to four days. The therapeutic range varies from two nanograms per milliliter (ng/mL) to four milligrams per milliliter (mg/mL) in the blood.

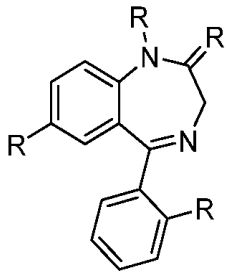
The core chemical structure for the benzodiazepine class consists of a benzene ring fused with a diazepine ring. This structure, as well as the structures for the benzodiazepines included in the developed methods can be seen in Figure 2. Benzodiazepines can have significant effects on psychomotor function, even at the recommended dose. These effects include: prolonged reaction times and impaired judgment, coordination, alertness,

concentration, and/or short-term memory. Clinical studies have shown that typical doses of many benzodiazepines can impair some necessary driving skills. It has been noted that some of the more polar drugs[6] in this class do not elute well from common GC/MS columns. As LC/MS has become more readily available, it is often selected for benzodiazepine analyses due to its high sensitivity and high specificity for the more polar and thermally reactive compounds (Levine & Jufer-Phipps, 2013).

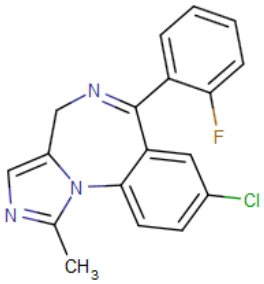
Amphetamines, as well as other phenethylamine compounds, are commonly encountered in Oklahoma[7]. The base structure for this class is amphetamine. The structure for amphetamine as well as all other amphetamines included in these methods can be seen in Figure 3. This growing class of compounds stimulates the sympathetic nervous system. These drugs were originally used as CNS stimulants for the treatment of narcolepsy and depression. Their ability to alleviate fatigue, improve performance of simple mental and physical tasks, elevate mood, and increase confidence has led to their abuse. Methamphetamine is easily synthesized in home (clandestine) laboratories, resulting in easy procurement and a larger abuse problem. Slight changes in the molecular structure have created "designer" amphetamines which include, 3,4-methylenedioxylmethamphetamine (MDMA), methylone, ethylone and methiopropamine. MDMA is one of the oldest, whereas methylone, ethylone, and methiopropamine have appeared in the past quarter century. This class of drugs is known as CNS stimulants. Some classic symptoms of these drugs include: tachycardia, hypertension, insomnia, nausea, and anxiety. Currently, amphetamine is still commonly prescribed to treat

[6] a compound in which the electric charge is not symmetrically distributed, so that there is a separation of charge or partial charge and formation of definite positive and negative poles

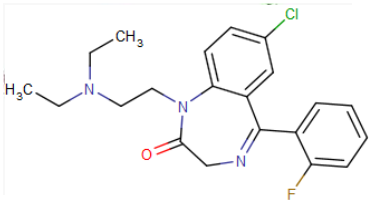
[7] This is based on 2015 OSBI Laboratory statistics where at least 21% of all DUID cases had, at a minimum, a presumptive positive for phenethylamines.



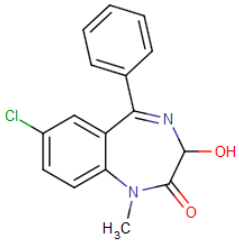
Base structure for benzodiazepines



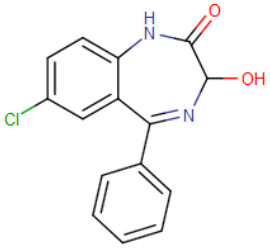
Midazolam



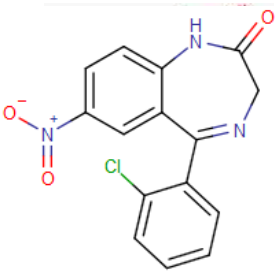
Flurazepam



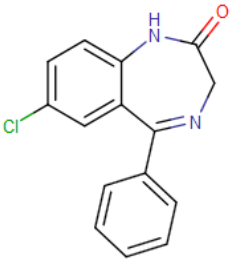
Temazepam



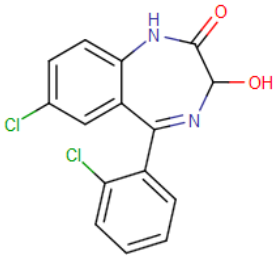
Oxazepam



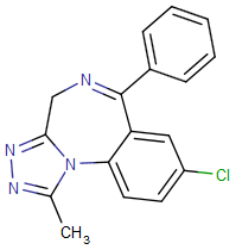
Clonazepam



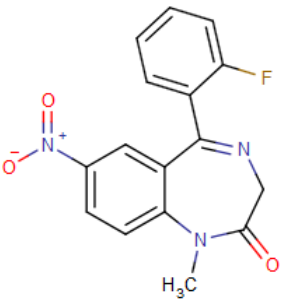
Nordiazepam



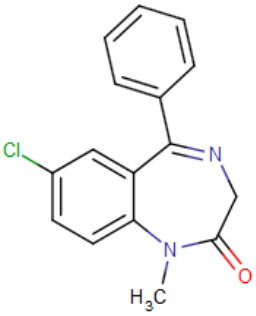
Lorazepam



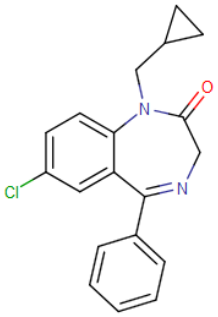
Alprazolam



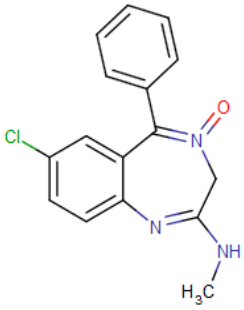
Flunitrazepam



Diazepam



Prazepam



Chlordiazepoxide

Figure 2. Benzodiazepine chemical structures.

attention deficit disorder (ADD) and attention deficit hyperactive disorder (ADHD). Drugs in this class are also commonly used for appetite suppression. At low doses methamphetamine induced CNS stimulation manifests as euphoria, alertness, intensified emotions, increased feeling of self-esteem and well-being, and sensations of extreme physical and mental power. After peak concentration is reached, the user may feel exhausted, disorganized, tense and paranoid (Merves & Moore, 2013).

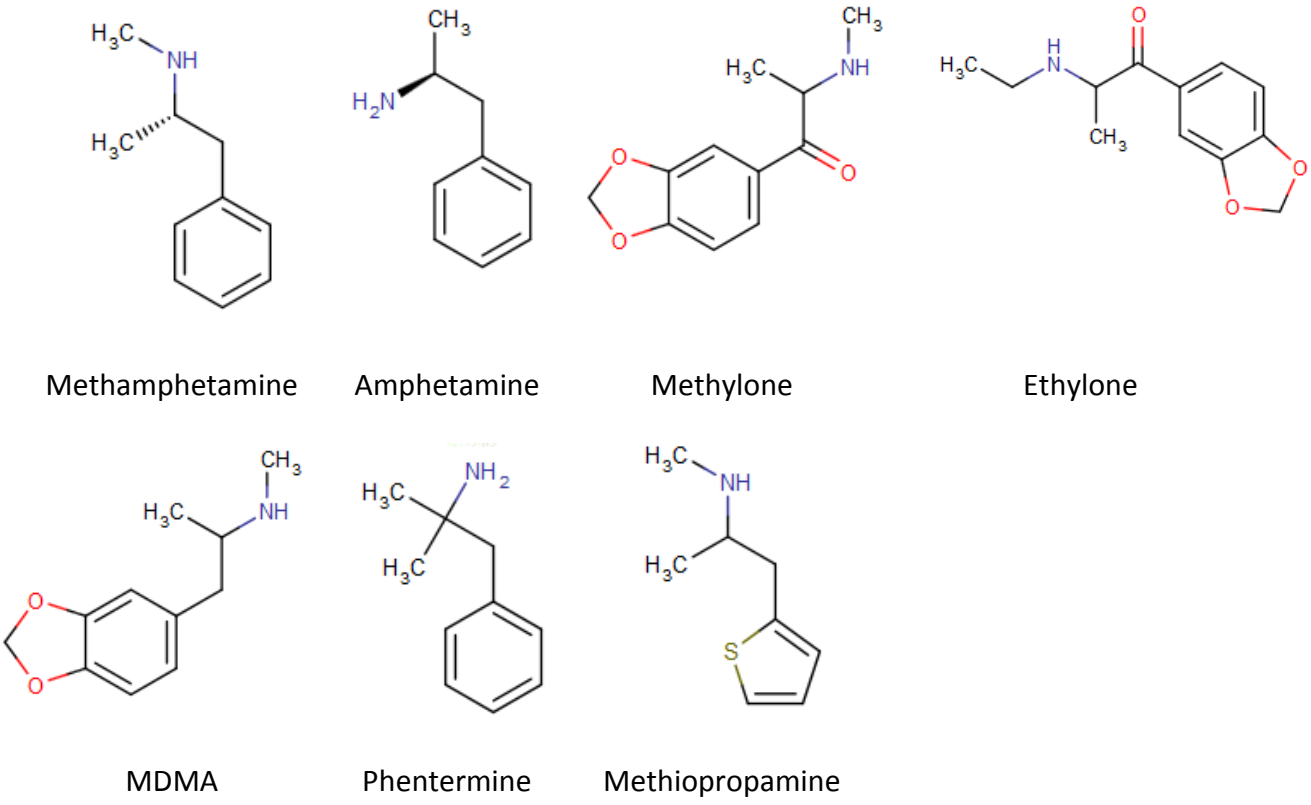


Figure 3. Amphetamine chemical structures.

Opiates, also known as opioids, have been in use for over two thousand years and are naturally occurring analgesics from the opium poppy, *Papaver somniferum*. Opioids work by

blocking the transmission of pain stimuli from the spinal cord to the brain and can also produce euphoria in some cases. In the 20th century, synthetic opioids were synthesized to replace morphine. Methadone, dextromethorphan and tramadol are examples of synthetic opioids (see figure 4). They are often used for the management of chronic pain related to cancer and terminal illness and can also be used for their sedative properties. It is common for those taking opioids to become physically dependent on them and develop increasing tolerance necessitating higher doses to obtain the same therapeutic effect. Methadone is often used for detoxification of heroin addicts due to the milder withdrawal symptoms associated with this compound.

Dextromethorphan is an analogue of codeine and is only used for its antitussive (cough relief) effects. Tramadol is used in a similar fashion to codeine but is said to have less abuse potential. Due to their CNS depressant effects, opioids have been shown to cause some level of impairment in the average driver. These effects include but are not limited to: drowsiness, lethargy, altered sensory perception, pupil constriction, droopy eyelids, slow driving, poor coordination, delayed reactions and difficulty following instructions. LC/MS extractions allows for the separation of both the free and deuterated drug[8] without derivatization, and simultaneous measurement of the parent drug and its metabolites. All of this is accomplished with minimum sample preparation (Kerrigan & Goldberger, 2013, Baselt, 1982).

[8] A compound in which the ordinary isotope of hydrogen has been replaced with deuterium.

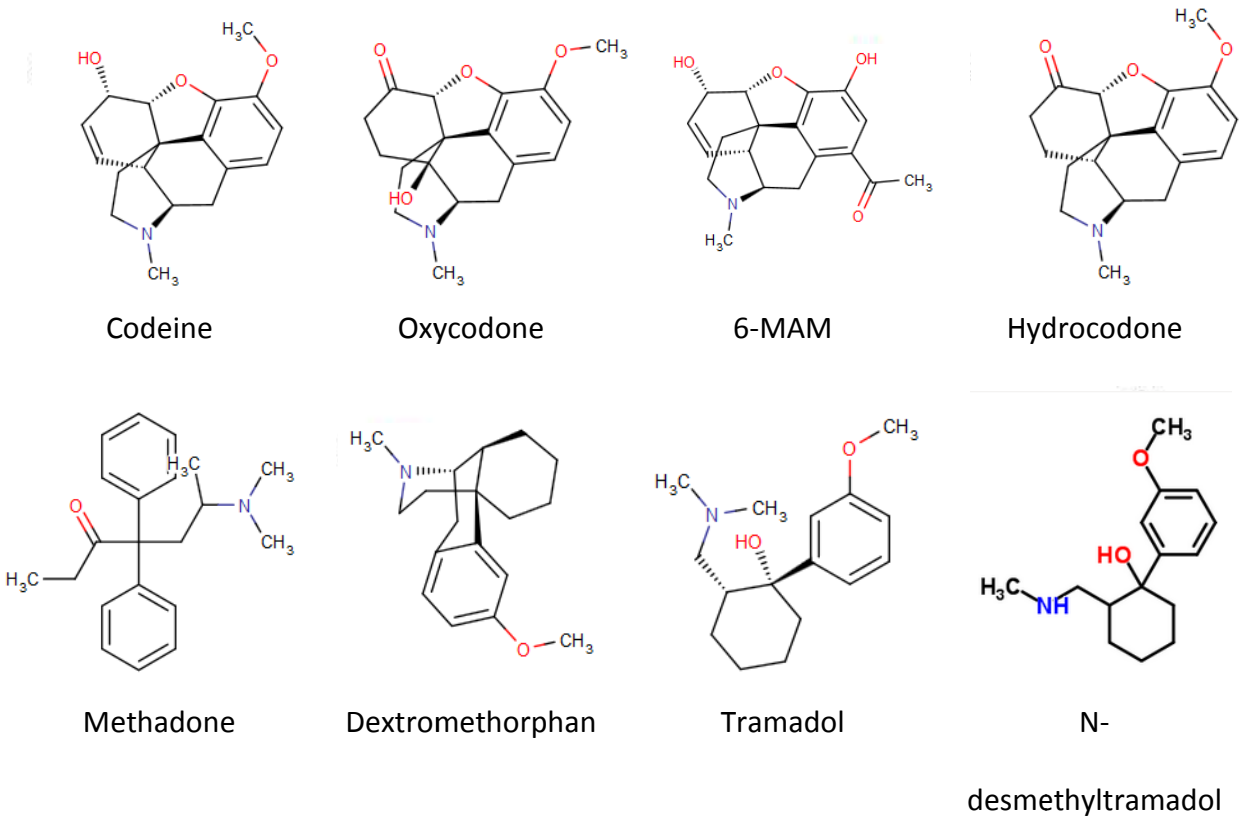
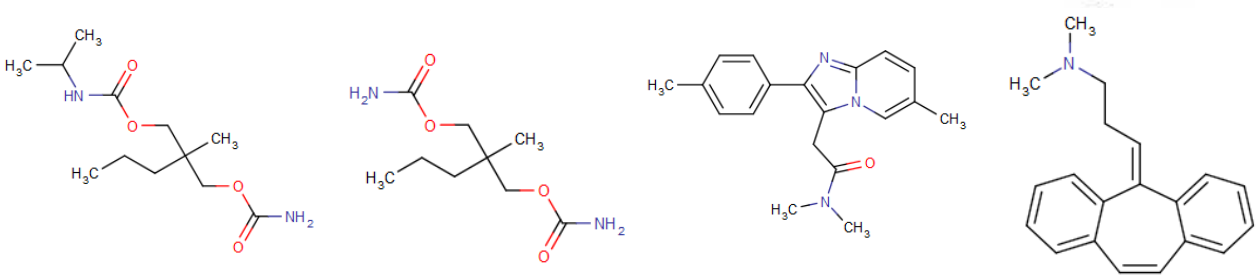


Figure 4. Opioid chemical structures.

The major CNS depressant categories are barbiturates and benzodiazepines. However, other CNS depressants which do not fit in these categories have been developed (see Figure 5). Such drugs were originally created because they were believed to have advantages over barbiturates and benzodiazepines. Carisoprodol, meprobamate, zolpidem, and cyclobenzaprine all fall in this category. Carisoprodol is used as a muscle relaxer. Meprobamate was originally developed as an alternative for barbiturates, but was found to produce toxic effects similar to sedative-hypnotic drugs. It is also a metabolite of carisoprodol. Cyclobenzaprine is also used as a muscle relaxer with a therapeutic range of ten to thirty nanograms per milliliter of blood.

Zolpidem was a prototype for a class of sedative-hypnotic drugs. It possesses similar effects to benzodiazepines, but is not considered one due to structural differences. Zolpidem is currently used for short-term management of insomnia with an elimination half-life of a few hours (Levine, 2013).

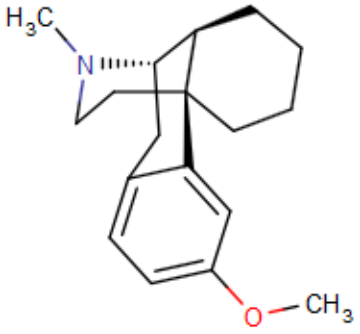


Carisoprodol Meprobamate Zolpidem Cyclobenzaprine

Figure 5. Miscellaneous CNS depressant structures.

Antihistamines are common place in almost any location. These drugs are used to relieve or prevent symptoms of allergies by preventing the effects of histamine. Some of these can cause the average person to feel impaired while not affecting others. Diphenhydramine, also known as Benadryl, is one of the most commonly encountered in the forensic toxicology world (see Figure 6). Diphenhydramine can cause CNS depression, slowed response, reduced attention, and drowsiness. The average therapeutic range for these drugs is 20 to 30 nanograms per milliliter of blood (Levine, 2013).

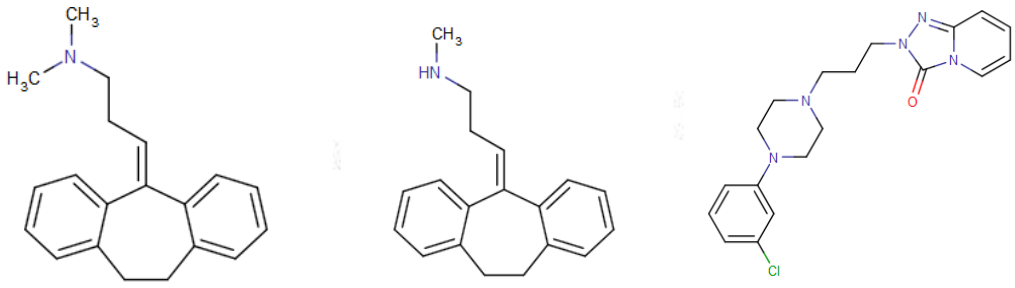
Approximately nineteen million people are affected by depression every year (Anderson, 2013). This would suggest that antidepressants are widely used and require different therapeutic doses to meet patent needs. Tricyclic antidepressants (TCAs), for example



Diphenhydramine

Figure 6. Antihistamine chemical structure.

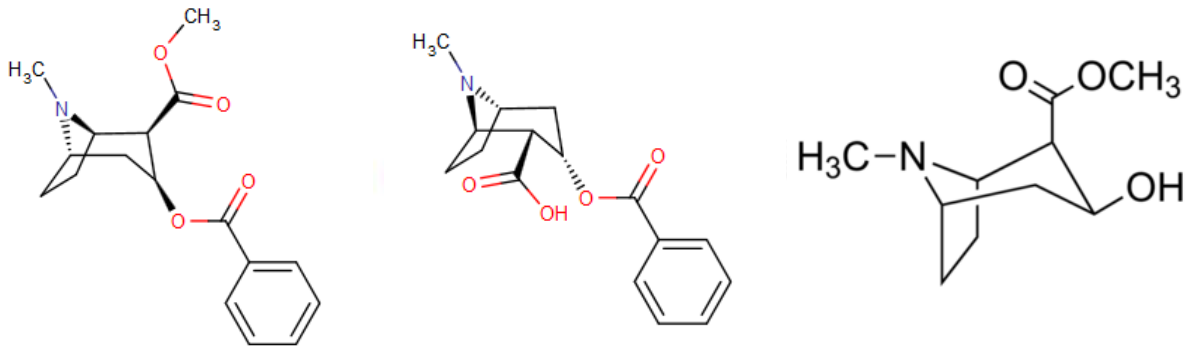
amitriptyline, have significant side-effects, which include: dizziness, sedation, blurred vision, and short-term memory impairment, as well as narrow therapeutic indices (see Figure 7). Second-generation antidepressants are represented by trazodone in the current study. Trazodone can be quite sedating in vivo and can cause drowsiness, confusion, incoordination, and fatigue. All antidepressants are well absorbed and reach peak levels between two and twelve hours after introduction. Due to the polarity of these medications, LC/MS allows for their detection at low levels (Baselt, 1982).



Amitriptyline Nortriptyline Trazodone

Figure 7. TCA chemical structures.

Cocaine is a psychotropic[9] drug that has been used for approximately two thousand years. In 2004, it was found that almost fifteen percent of Americans had tried cocaine and that approximately two million were current users. Cocaine hydrochloride is usually introduced by insufflation, whereas crack cocaine, a form of the base, is often smoked to maximize the high. The drug is used legitimately as a topical local anesthetic or vasoconstrictor. Cocaine can affect coordination skills, reaction time, risk taking, mental health and result in fatigue (Isenschmid, 2013). It is known to continue to be metabolized into benzoylecgonine (BE) and ecgonine methyl ester (EME) which are evident in a blood draw (see Figure 8). Many laboratories refrigerate samples and request that samples are collected in tubes containing sodium fluoride and potassium oxalate to slow down the hydrolysis[10] process which results in methyl benzoate. LC/MS is beneficial for the analysis of cocaine and benzoylecgonine without derivatization in a single analysis (Isenschmid, 2013).

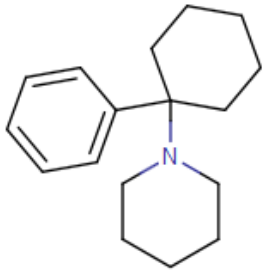


Cocaine Benzoylecgonine Ecgonine Methyl Ester

Figure 8. Psychotropic drug chemical structures.

[9] A psychotropic drug is one that changes the brain function and results in alterations in perception, mood, or consciousness.
[10] Chemical breakdown of a compound due to a reaction with water.

There are many drugs that can cause hallucinations at high concentrations, however; there is also a group of drugs that often result in a state of altered perception when taken. This group of drugs is known as psychedelic agents and contains the well-known drug phencyclidine (PCP). PCP was first synthesized in 1926, but its use as an anesthetic was not discovered until 1956 (see Figure 9). Shortly after, the adverse psychological reactions which include, delusions, delirium, hallucinations, and seizures were observed. Currently, PCP can be taken orally, intravenously, and has also been seen as a liquid that tobacco or marijuana cigarettes are dipped in before smoking. When smoked, peak effect is reached around thirty minutes with residual effects for four to six hours. PCP exhibits stimulant, depressant, hallucinogenic, and analgesic properties, but is classified as a dissociative anesthetic[11]. Some common side-effects experience by those using PCP include: tremors, incoordination, dissociation, amnesia, repetitive motor movements, hypnotic state, and tunnel vision (Jenkins 2013). It has also been reported that people under the influence of PCP tend to remove their clothing because they feel hot due to an increase in body temperature resulting from metabolism of the drug.



Phencyclidine

Figure 9. PCP chemical structure

[11] A form of general anesthesia, but not necessarily complete unconsciousness, characterized by catalepsy, catatonia, and amnesia.

Materials and Methods

Chemicals and Reagents

All compound standards were purchased from Cerilliant Corporation (Round Rock, Texas). All reference standards were of ≥ 98% purity. Formic Acid and Acetonitrile were purchased from Fisher Scientific (Pittsburgh, Pennsylvania). 100 millimolar (mM) phosphate buffered saline (PBS - pH 7.0) was purchased from Immunalysis (Pomona, California). The bovine blood used for method development was obtained from Lampire Biological Laboratories (Pipersville, Pennsylvania).

Preparation of Standard Solutions

Drugs that required optimization[12] were diluted in deionized (DI) water to a concentration of either 10 micrograms per milliliter (µg/mL) or 100 ng/mL and stored between 2-8°C. A working solution in deionized water containing all but the deuterated compounds at concentrations ten times those listed in Table 3 was made and stored between 2-8°C. Appendix 5 includes a copy of the first draft of the OSBI protocol for the 250 µL method. It also includes a copy of a deviation that outlines how the low and high positive controls were made. The high positive control was originally set at twenty times the low positive control. It was observed that this concentration overloaded the column, so the high positive control concentration was reduced to ten times the low positive control in the deviation. An internal standard[13] (ISTD) solution, including the three deuterated standards, was prepared in acetonitrile and stored

[12] An act, process, or methodology of making something (as a design, system, or decision) as fully perfect, functional, or effective as possible. In this context, it refers to determining the most efficient parameters for each drug when analyzed by LC/MS-MS.

[13] A chemical substance that is added in a constant amount to all samples, besides blanks, to verify that the instrument is working properly. This substance should not be one that is being analyzed for. Deuterated compounds are often used.

between -18 and -22°C following the protocol in Appendix 5. These standards were chosen due to their retention times – one early, one middle, and one late eluter. This demonstrates that the instrument is functioning properly throughout the entire analysis. The concentration of ISTD in the solution is approximately 100 ng/mL. This was diluted 1:3 with acetonitrile for the 100 µL extraction to adjust for the lower concentration of drugs. The ISTD concentration was set based on the lower limit set for the drugs in the method. 100 ng/mL was decided on because it would not be too high or too low compared to the other compounds, which range from 10 to 500 ng/mL of blood.

LC /MS-MS Conditions

The LC/MS-MS used was a Schimadzu LCMA-8030 triple quadruple system. The chromatographic column was a Kinetex C18 column (75mm x 2.1mm x 2.6µm), which was maintained at a temperature of 60° C with a flow rate of 0.800 milliliters per minute (mL/min). The mobile phase[14] was composed of solvents A (0.1% formic acid in deionized water) and B (0.1% formic acid in acetonitrile). The gradient[15] used is shown in Table 1. The sample injection volume was 20 µL. The nebulizing gas[16] flow was 2 liter per minute (L/min). The desolvation line[17] and heat block[18] temperature was 225°C. The drying gas[19] flow was 20 L/min and the entire run time was six minutes. All conditions were selected based on their use in the previously validated benzodiazepine and opiate methods currently in use at the OSBI. The

[14] Solvent used to move the sample through the column.
[15] The concentration of the solutions in the mobile phase changes over time.
[16] Gas used to transfer ions from the liquid phase to the gas phase before entering the mass spectrometer.
[17] Used to introduce the vaporized ions into the mass spectrometer.
[18] Used to heat the desolvation line.
[19] Used to prevent solvent clusters and promote better chromatography and complete vaporization of the sample

Table 1

Gradient used in current study

Time (min)	% A	% B
0.25	95	5
1.00	80	20
3.00	71.2	28.8
4.50	20	80
5.00	20	80
5.01	95	5

internal standard used for each compound, multiple reaction monitoring (MRM) transitions[20], volts used for each compound, and retention times are shown in Table 2. MRM transitions are used to differentiate drugs from one another by using a parent and product ion that is normally unique to each drug. For example, the MRM transitions for codeine are 300.10 to 198.80 and 165.15. 300.10 is the parent ion, while 198.80 and 165.15 are the product ions. If two drugs have similar molecular weights, they may have one similar product ion. For example, the parent ion for methamphetamine is 150.20 and the parent ion for phentermine is 150.10. The first product ion for both is 91.10 and 91.00, respectively. If this is the case, then a unique product ion will also be required. The unique product ion for methamphetamine is 119.10 and 133.10 for phentermine.

[20] Parent ion to product ion used for identification.

The parent and product ions for each internal standard can be seen in Table 2. The internal standard is used to standardize quantitation results between injections to negate differences in injection efficiency. They are also used to verify that the instrument is functioning properly throughout the analysis. This is accomplished by comparing the retention time of the internal standards in the case samples to the retention time of the internal standards in the control samples. If there is a significant change in the retention time, then maintenance is likely needed and the case and control samples will need to be reanalyzed. The information for columns 4, 5, and 6 in Table 2 will be discussed in more detail on pages 26, 27, and 28.

Table 2

Parameters for each drug in the current study.

Compound	Retention Time (min)	MRM Transitions (m/Z)	Q1 (Volts)	Collision Energy (Volts)	Q3 (Volts)	Internal Standard
Pseudoephedrine	0.77	166.10>148.05	-15	-13	-14	Methamphetamine-d8
		166.10>91.00	-15	-33	-19	
		166.10>132.95	-15	-23	-13	
Methiopropamine	0.78	156.20>96.95	-13	-23	-21	Methamphetamine-d8
		156.20>58.00	-13	-13	-10	
		156.20>125.10	-13	-16	-28	
Methylone	0.88	208.00>160.05	-17	-2	-16	Methamphetamine-d8
		208.00>132.05	-17	-27	-29	
		208.00>190.05	-17	-13	-19	
Codeine	0.88	300.10>198.80	-16	-32	-15	Methamphetamine-d8
		300.10>165.15	-16	-43	-15	

Compound	Retention Time (min)	MRM Transitions (m/Z)	Q1 (Volts)	Collision Energy (Volts)	Q3 (Volts)	Internal Standard
Amphetamine	0.93	136.20>91.10	-11	-18	-15	Methamphetamine-d8
		136.20>119.10	-12	-15	-12	
		136.20>65.10	-12	-41	-24	
Oxycodone	1.01	316.10>174.90	-16	-35	-14	Methamphetamine-d8
		316.10>212.00	-16	-45	-21	
Methamphetamine-d8	1.03	158.00>92.95	-14	-23	-14	Methamphetamine-d8
		158.00>66.10	-13	-46	-28	
		158.00>124.00	-13	-15	-12	
Methamphetamine	1.04	150.20>91.10	-12	-22	-17	Methamphetamine-d8
		150.20>119.10	-12	-15	-11	
Ethylone	1.06	222.20>173.95	-12	-20	-17	Methamphetamine-d8
		222.20>204.00	-12	-14	-21	
		222.20>146.00	-12	-28	-15	
6-MAM	1.07	328.10>165.00	-17	-46	-17	Methamphetamine-d8
		328.1>211.20	-17	-26	-22	
Hydrocodone	1.09	300.10>198.85	-15	-30	-20	Methamphetamine-d8
		300.10>171.00	-15	-40	-20	
MDMA	1.09	194.00>163.10	-11	-13	-16	Methamphetamine-d8
		194.00>105.15	-11	-27	-22	
		194.00>77.10	-11	-45	-14	
Caffeine	1.11	195.00>138.00	-17	-22	-29	Methamphetamine-d8
		195.00>42.00	-17	-40	-16	
		195.00>110.00	-11	-25	-25	
Phentermine	1.18	150.10>91.00	-12	-22	-20	Methamphetamine-d8
		150.10>133.10	-12	-14	-29	
Benzoylecgonine	1.33	290.00>168.00	-15	-20	-16	PCP-d5
		290.00>105.00	-15	-35	-21	
Tramadol	1.49	264.30>58.15	-11	-35	-13	PCP-d5
		264.30>42.00	-15	-50	-15	

Compound	Retention Time (min)	MRM Transitions (m/Z)	Q1 (Volts)	Collision Energy (Volts)	Q3 (Volts)	Internal Standard
N-desmethyltramadol	1.50	250.00>44.00	-10	-13	-18	PCP-d5
Cocaine	1.58	304.00>182.05	-16	-20	-19	PCP-d5
		304.00>81.95	-16	-33	-17	
		304.00>105.10	-16	-35	-23	
Zolpidem	1.75	308.00>235.00	-23	-35	-24	PCP-d5
		308.00>263.00	-23	-25	-27	
Meprobamate	1.80	219.10>158.15	-16	-10	-16	PCP-d5
		219.10>97.15	-12	-16	-21	
Chlordiazepoxide	1.86	299.90>227.10	-25	-25	-23	PCP-d5
		299.90>283.15	-25	-14	-19	
Trazodone	1.9	372.10>176.2	-15	-25	-17	PCP-d5
		372.10>148.05	-16	-38	-15	
		372.10>78.15	-15	-54	-17	
PCP-d5	2.00	249.30>86.15	-10	-25	-21	PCP-d5
PCP	2.01	244.20>86.20	-13	-13	-18	PCP-d5
		244.20>91.15	-13	-32	-20	
		244.2>159.20	-22	-15	-16	
Dextromethorphan	2.18	272.15>147.05	-14	-35	-15	PCP-d5
		272.15>171.05	-14	-40	-17	
Diphenhydramine	2.3	256.00>167.10	-14	-12	-17	PCP-d5
		256.00>152.10	-14	-40	-15	
		256.00>165.10	-14	-40	-16	
Midazolam	2.31	326.00>291.00	-12	-30	-30	PCP-d5
		326.00>244.00	-12	-25	-26	
Flurazepam	2.49	387.90>315.00	-15	-25	-21	PCP-d5
		387.90>288.00	-15	-25	-30	
Cyclobenzaprine	3.06	276.05>215.10	-22	-40	-22	PCP-d5
		276.05>84.10	-11	-25	-16	
Temazepam	3.17	301.20>255.00	-24	-25	-18	Prazepam-d5
		301.20>283.00	-24	-15	-21	
Nortriptyline	3.21	264.15>233.05	-21	-15	-26	PCP-d5
		264.15>218.00	-21	-26	-25	
		264.15>91.00	-21	-23	-10	
Oxazepam	3.27	287.00>241.00	-15	-24	-27	Prazepam-d5
		287.00>269.00	-15	-18	-19	
Amitriptyline	3.33	278.40>105.00	-14	-20	-22	Prazepam-d5
		278.40>233.05	-14	-15	-26	

Compound	Retention Time (min)	MRM Transitions (m/Z)	Q1 (Volts)	Collision Energy (Volts)	Q3 (Volts)	Internal Standard
Clonazepam	3.42	316.00>270.00	-12	-30	-29	Prazepam-d5
		316.00>214.00	-12	-45	-22	
Methadone	3.44	310.02>265.15	-17	-16	-27	Prazepam-d5
		310.02>105.00	-13	-28	-10	
		310.02>57.10	-13	-25	-23	
Nordiazepam	3.45	270.9>140.00	-20	-30	-14	Prazepam-d5
		270.90>165.00	-20	-30	-18	
Lorazepam	3.51	321.00>275.00	-12	-20	-29	Prazepam-d5
		321.00>229.00	-12	-30	-23	
Carisoprodol	3.62	261.00>158.20	-22	-10	-16	Prazepam-d5
		261.00>97.10	-14	-20	-22	
Alprazolam	3.64	309.00>281.00	-12	-30	-29	Prazepam-d5
		309.00>205.00	-12	-45	-22	
Flunitrazepam	3.75	314.00>268.00	-12	-30	-29	Prazepam-d5
		314.00>239.00	-12	-45	-22	
Diazepam	3.93	285.00>154.00	-12	-30	-29	Prazepam-d5
		285.00>193.00	-12	-45	-22	
Prazepam	4.32	325.00>271.05	-24	-25	-28	Prazepam-d5
		325.00>140.00	-24	-40	-28	
Prazepam-d5	4.33	330.00>276.00	-10	-24	-29	Prazepam-d5
		330.00>140.00	-10	-39	-14	

Sample Preparation

For the original extraction: in a micro-centrifuge tube (Figure 10), whole blood samples (225µL) were spiked with 25µL of low or high positive control[21] to serve as controls. 250 µL of sample was transferred to a micro-centrifuge tube for all test samples. Acetonitrile containing internal standards[22] (500 µL) is then added, vortexed well for thirty seconds, then centrifuged at approximately 13,000 rotations per minute (RPM) for five minutes. The acetonitrile layer

[21] Contains a known amount of analyte and verifies that the instrument is capable of identifying each compound. The low control is set at the bottom of the therapeutic range and the high control is twenty times higher.

[22] The internal standard/acetonitrile mixture is created by pipetting 50 microliters of each 100 µg/mL primary deuterated standard into a 250 milliliter volumetric flask and filling to the line with acetonitrile.

was then transferred to a centrifuge tube and evaporated at 40°C with a stream of dry nitrogen. Finally, the sample was reconstituted with 100 µL of reconstitution solvent.

Figure 10. Micro-centrifuge Tube

For the low-sample-volume extraction: in a micro-centrifuge tube (Figure 10), whole blood samples (90 µL) spiked with 10 µL of low or high positive control to serve as controls. 100 µL of sample was added to a micro-centrifuge tube for all test samples. 100 µL of PBS buffer (pH 7) was added. Acetonitrile containing internal standards (500 µL) is then added, vortexed well for thirty seconds, then centrifuged at approximately 13,000 RPM for five minutes. The acetonitrile layer was then transferred to a centrifuge tube and evaporated at 40°C with a stream of dry nitrogen. Finally, the sample was reconstituted with 100 µL of reconstitution solvent.

Method Development and Validation

The original goal for this extraction was to be able to take a portion of the sample used for a presumptive drug screen using enzyme-linked immunosorbent assay (ELISA) and extract it for use as a confirmatory test using the LC/MS-MS. However, this was revamped when it was found that the *SOFT/AAFS Forensic Toxicology Laboratory Guidelines* recommended that

separate aliquots of sample should be used for presumptive and confirmatory testing, to lessen the chance of reporting out contaminated results.

> 8.2.7 It is good practice to confirm the identity of an analyte in a different extract of the sample specimen from that used for the test, or in a second specimen. However, confirmation of a drug or toxin in the same original extract of a single specimen would not normally be regarded as acceptable, since that would not rule out the possibility that the extract became contaminated during the extraction or that the wrong sample was tested (2007).

The choice of drugs for the exploration of this method were chosen by both the OSBI Toxicology Unit Technical Manager as well as a survey of the most common drugs seen in casework at the OSBI in recent years. This resulted in an initial list of forty-six drugs including three deuterated internal standards. During the optimization process, three of the drugs were dropped, zaleplon, phenazepam, and estazolam, due to poor response. The product ions were too small to be used for the MRM and, therefore, were not viable options for this method. The process of optimization will be discussed in detail later. During the validation process for the lower sample volume extraction, it was noted that zopiclone did not extract with the proposed method so it was also dropped. This was verified by opening the window for analysis to the length of the entire run to confirm that it was not eluting earlier or later than previously determined in the 250 µL extraction method. The end result was a list of forty drugs and three internal standards that could be detected using the 250 µL extraction method and thirty-nine drugs and three internal standards using the 100µL extraction. In addition, caffeine is also in the method, but was not validated because it is not regularly reported in toxicology cases and there was a lack of blank blood for validation purposes.

The limit of detection for the instrument is lower than the therapeutic range, so the concentration for the low positive control for each drug was administratively assigned based on known therapeutic ranges and suggestions from articles authored by well-known toxicologists (Logan, Lowrie, Turri, Yeakel, Limoges, Miles, & Farrell, 2013; Winek, Wahba, Winek Jr., & Winek Balzer, 2000). These are listed in Table 3.

Bovine blood was originally used for method development in accordance with OSBI policy. Once the method was developed, blank human blood case samples that had been set to be destroyed were used for validation and method comparisons. All identifying information was not recorded to maintain anonymity. These samples were first analyzed by GC/MS and the methods in development to verify that they did not contain any of the drugs of interest. The 250 µL extraction procedure was developed first to meet case load requirements at the OSBI. Once this method was validated following the Scientific Working Group for Forensic Toxicology (SWGTOX) guidelines, a method was validated using a smaller sample volume mixed with phosphate buffer.

All new compounds were optimized. This was accomplished by injecting the sample and checking for the precursor ion[23]. The new compounds were injected as a neat sample at a concentration of 100 ng/mL. An example of this can be seen in Figure 11. This was achieved by diluting a 1 mg/mL standard at a 1:99 ratio with deionized water to create a 10 µg/mL solution. This solution was then diluted again using a 1:99 ratio with deionized water to create a 100 ng/mL solution. Next, the most efficient voltage for focusing this ion is selected and the

[23] Also known as a parent ion which may be a molecular ion or an electrically charged fragment of a molecular ion.

Table 3.

Drugs of interest and low positive control concentration.

Drug	Concentration ng/mL	Drug	Concentration ng/mL
Pseudoephedrine	100	Phencyclidine	10
Methylone	20	Dextromethorphan	20
Codeine	10	Diphenhydramine	25
Amphetamine	10	Midazolam	50
Oxycodone	10	Flurazepam	10
Methamphetamine-d8	80	Cyclobenzaprine	10
Methamphetamine	20	Temazepam	20
Ethylone	20	Nortriptyline	25
6-MAM	5	Oxazepam	20
Hydrocodone	10	Amitriptyline	25
MDMA	20	Clonazepam	10
Caffeine	50	Methadone	20
Phentermine	20	Nordiazepam	20
Benzoylecgonine	50	Lorazepam	10
Tramadol	20	Carisoprodol	500
N-desmethyltramadol	20	Alprazolam	10
Cocaine	10	Flunitrazepam	10
Zolpidem	10	Diazepam	20
Meprobamate	500	Prazepam	10
Chlordiazepoxide	50	Prazepam-d5	80
Trazodone	25	Methiopropamine	10
PCP-d5	80	Zopiclone	50

collision energy is determined through testing multiple options and selecting the most efficient (between 0 and -50 volts). Examples of this can be seen in Figures 12 and 13. If the most efficient focusing energy for Q1 is set correctly, then only the parent ion will be visible in the spectra in Figure 12. Figure 13 illustrates how the parent ion breaks at each collision energy level. The analyst will then determine which product ions are the most common among all collision energies and those will be chosen for use in the method. The focusing voltage will

allow only the parent ion to continue into the collision cell and the collision energy is what will break the parent ion into the product ions. This is indexed in 5 volt increments. Afterward, the analyst picks the product ions and the instrument focuses the product ions in the final quadrupole. An example of this can be seen in Figure 14. This requires a voltage determination for the final quadrupole as well and an example can be seen in Figure 15. The final results of the optimization can be seen in Figure 16. The top line shows the peak area of the transition between the parent ion and the most abundant product ion. In this case, it is 222.20 to 173.95. The next line down is the second most abundant which would be 222.20 to 204.00. The blue line is the third most abundant which is 222.20 to 146.00. The rest do not have enough of a response to warrant their use in the method. The voltages used in each step for the individual compounds can be seen in Table 2.

As illustrated in Figure 17, quadrupoles use an electro-magnetic field to isolate the target compound and move it through the mass spectrometer. The collision chamber breaks the precursor ions into reproducible fragments. They then travel into the final quadrupole where the selected product ion(s) are directed to the detector. The information obtained through optimization is used to create a method that will then be modified regarding retention times, gradients, and cycle times throughout the method development phase.

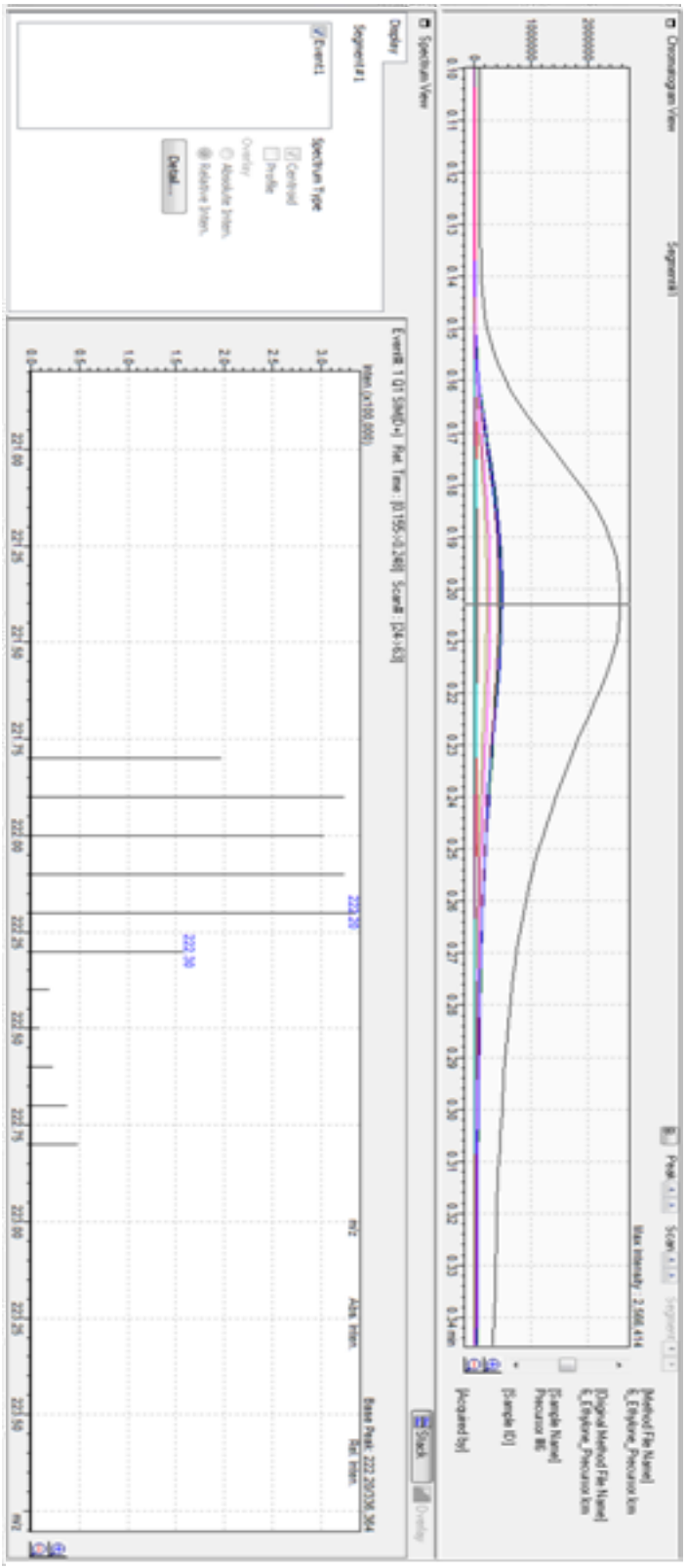


Figure 11. Step 1 of optimization, determination of parent ion.

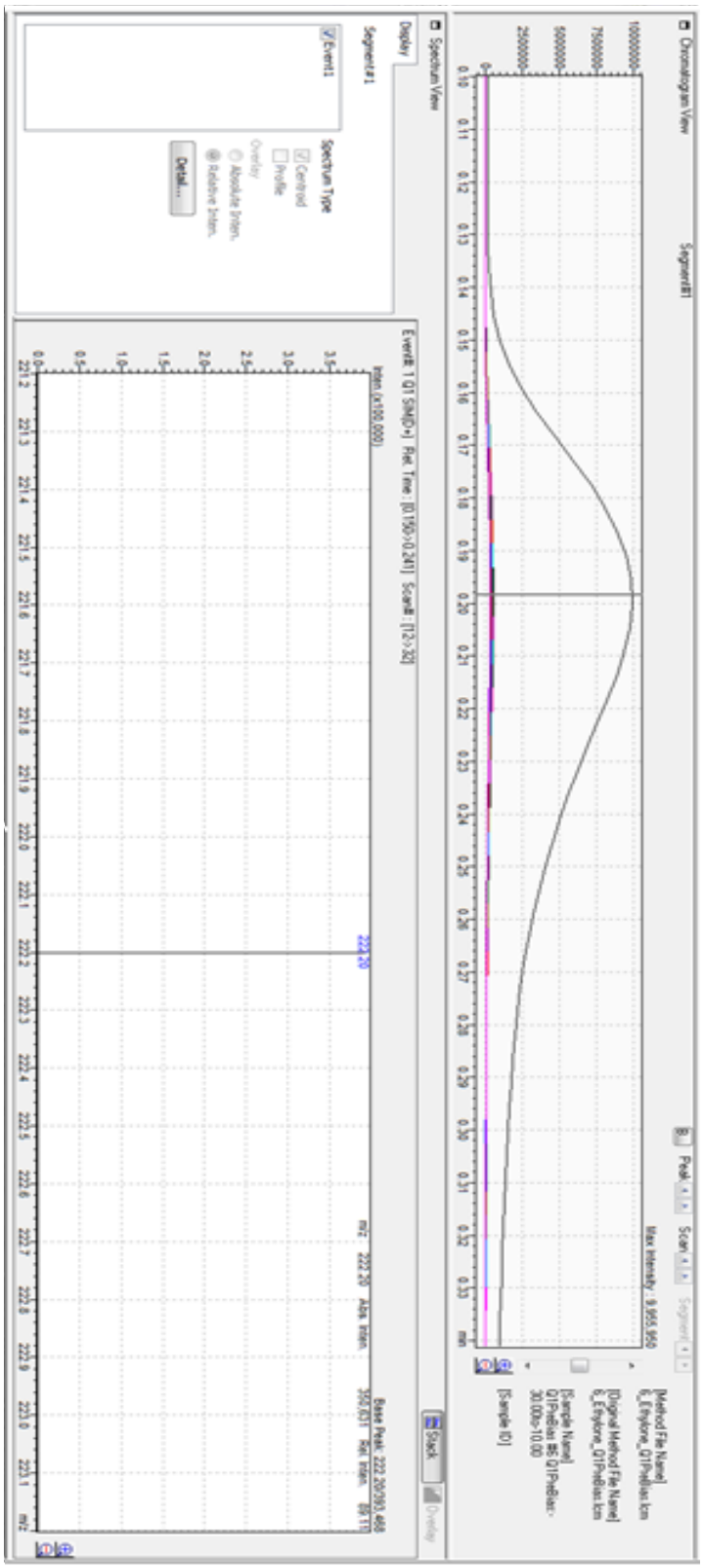


Figure 12. Step 2 of optimization, determination of Q1 voltage.

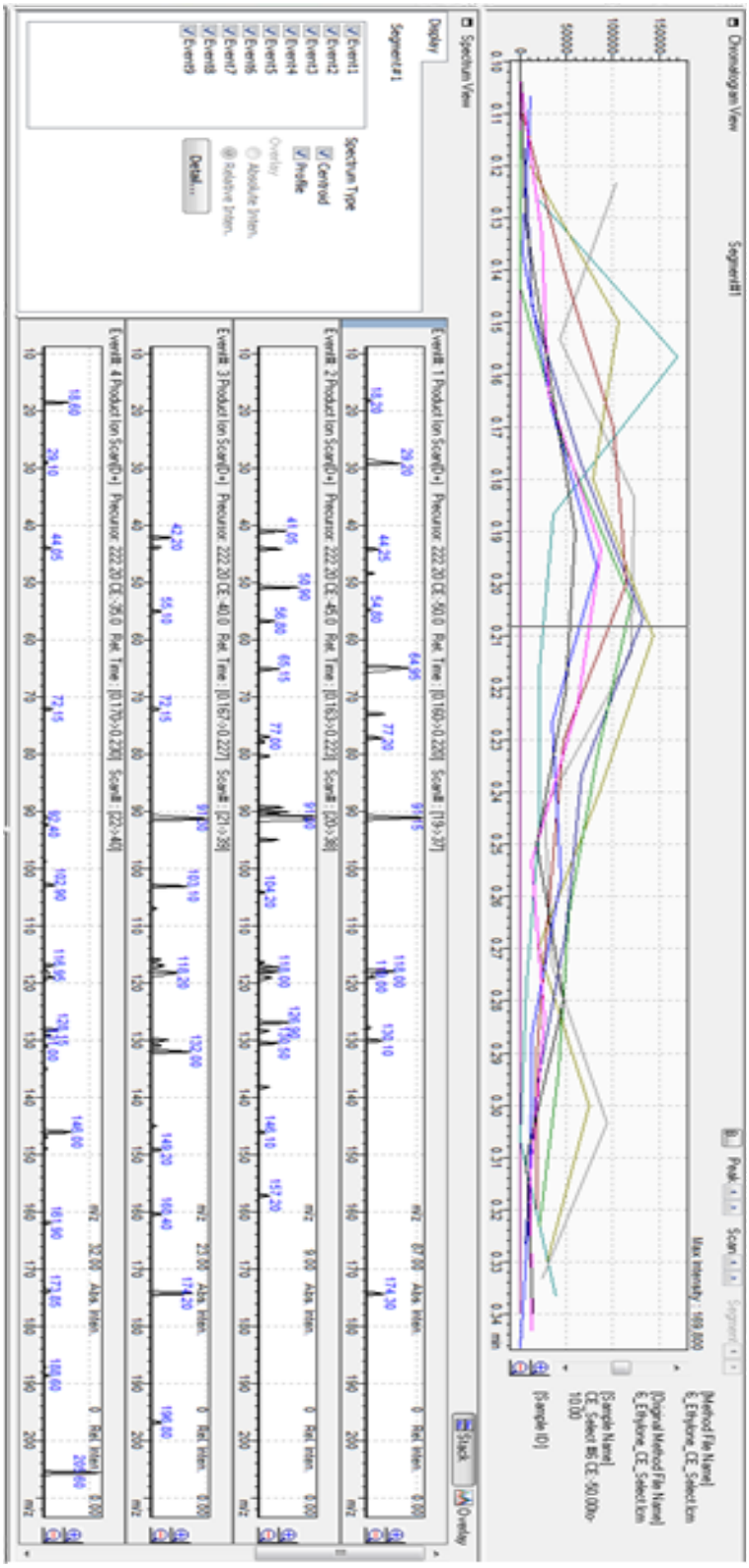


Figure 13. Step 3 of optimization, determination of collision energy voltage.

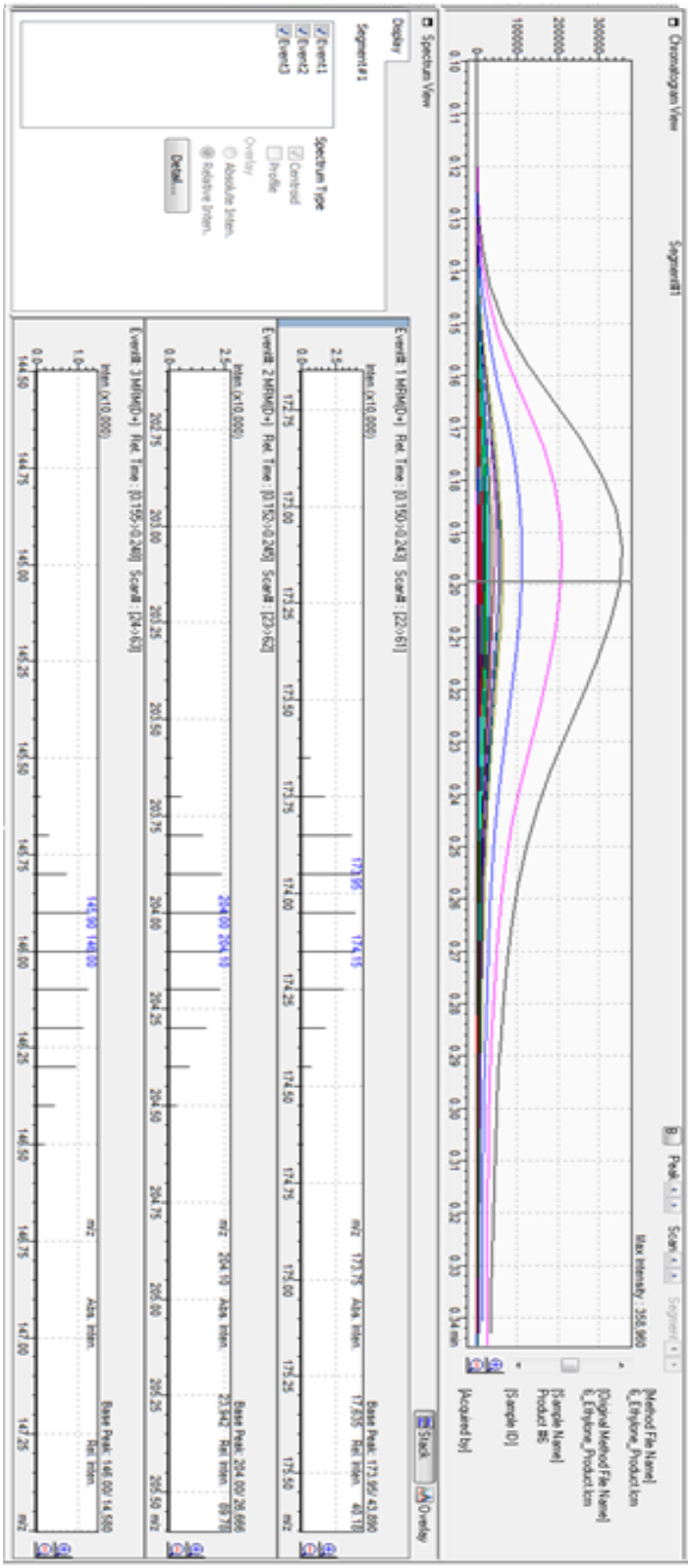


Figure 14. Step 4 of optimization, selection of most common product ions.

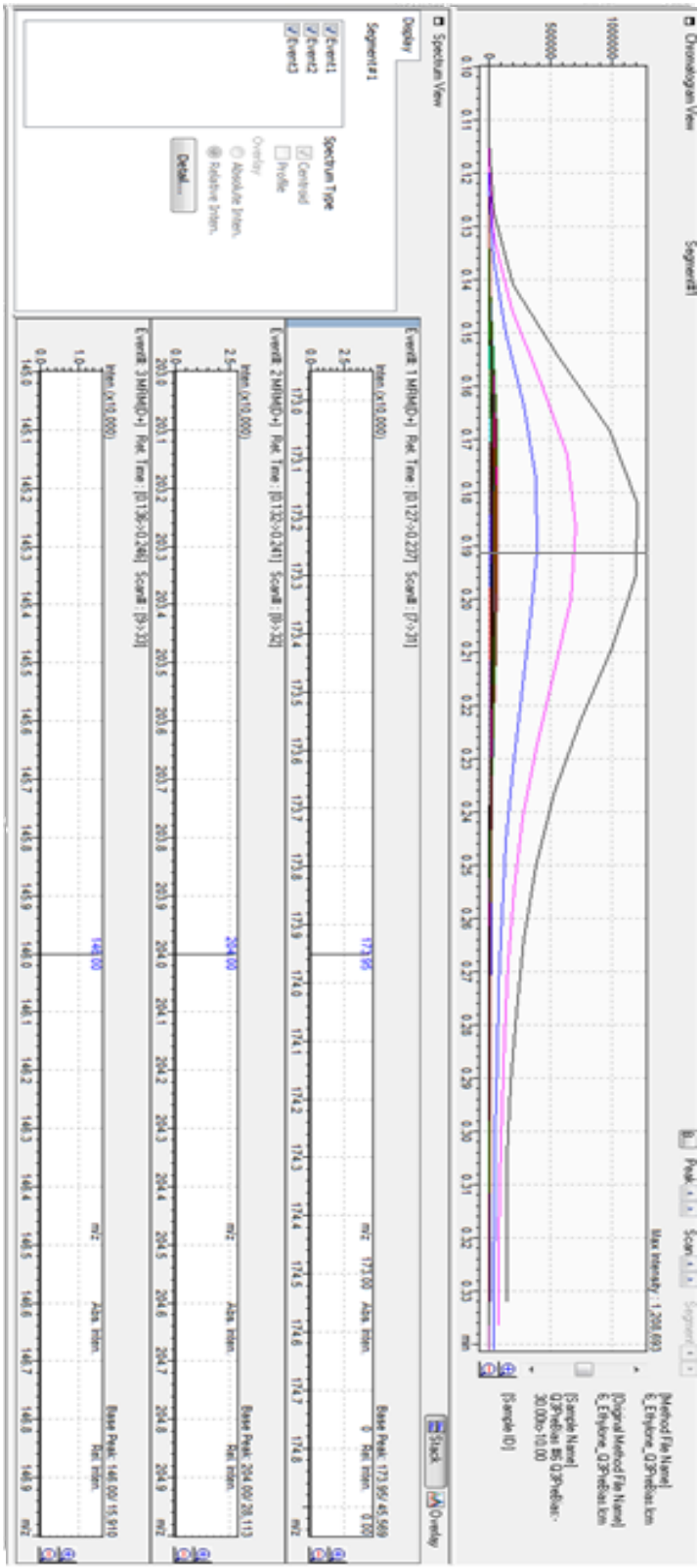


Figure 15. Step 5 of optimization, determination of Q3 voltage.

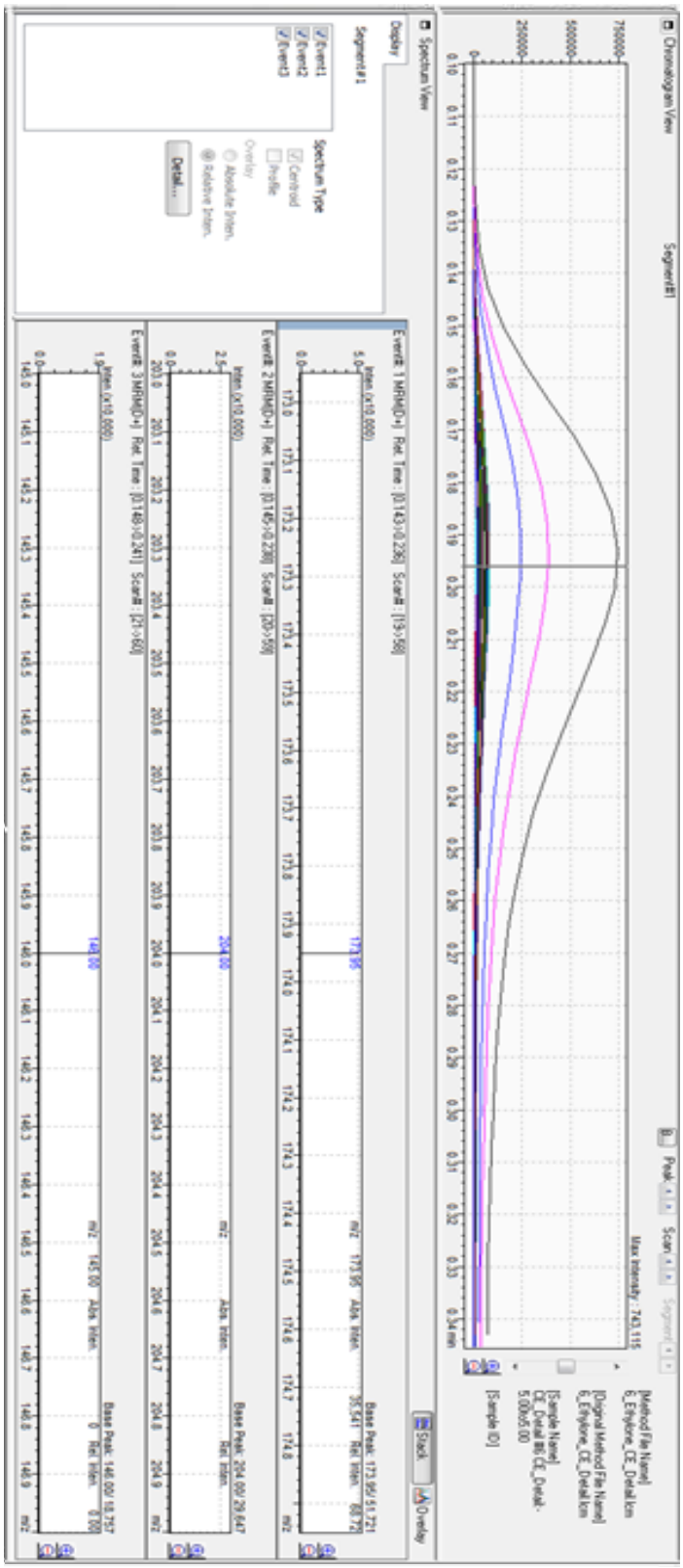


Figure 16. Results of optimization.

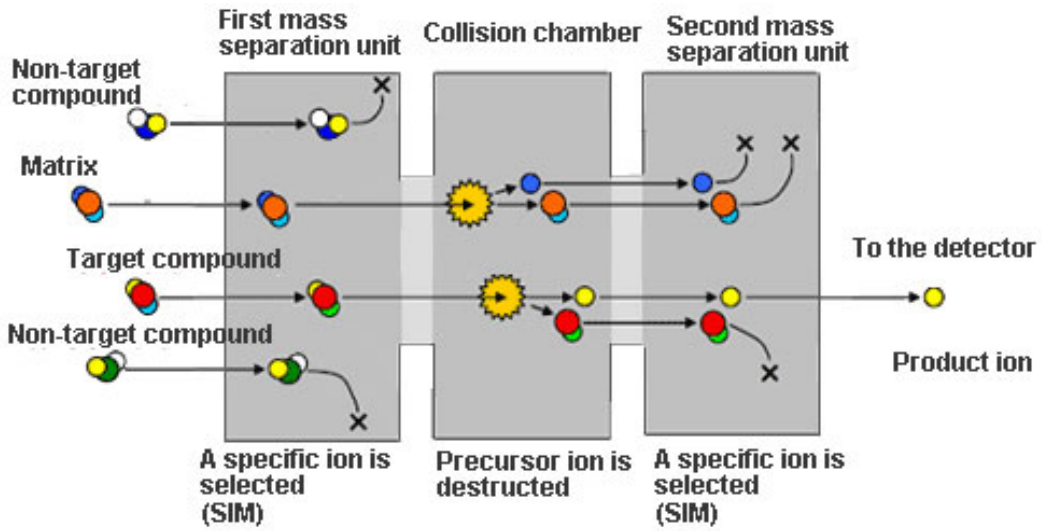


Figure 17. Schematic of a LC/MS-MS. Based on Schimadzu (n.d.)

Once a starting gradient and cycle time have been selected, all drugs are analyzed using this method to determine their retention time and to observe their location compared to other compounds. The starting gradient and length of analysis for these methods was based off of a previously developed benzodiazepine method since all compounds from that method were to be analyzed in the methods currently being developed. It was noted that methamphetamine and phentermine did not have sufficient separation (Figure 18). The red line represents methamphetamine and the blue line represents phentermine. As mentioned previously, both have similar parent and first product ions which means that adequate separation is required if the method is to be used for quantitation in the future. The gradient was adjusted to allow for better separation. The gradient developed for the method being validated can be seen in Table 1. A gradient can be modified in two ways. The first option is by lengthening the time taken to reach a new mixture percentage, i.e. setting the instrument to require two minutes instead of one minute to adjust from a 95:5 mobile phase A:mobile phase B mixture, to an 80:20 mixture.

The second is to changing the percentage of the mixture in a certain time period, i.e. instead of moving from 95:5 mobile phase A:mobile phase B mixture to an 80:20 mixture, adjust to a 75:25 mixture. To obtain the most efficient gradient, multiple samples must be analyzed while adjusting the gradient slightly between each injection until separation is achieved. Figure 19 illustrates the results of the gradient adjustment regarding methamphetamine and phentermine, the first and second peak, respectfully.

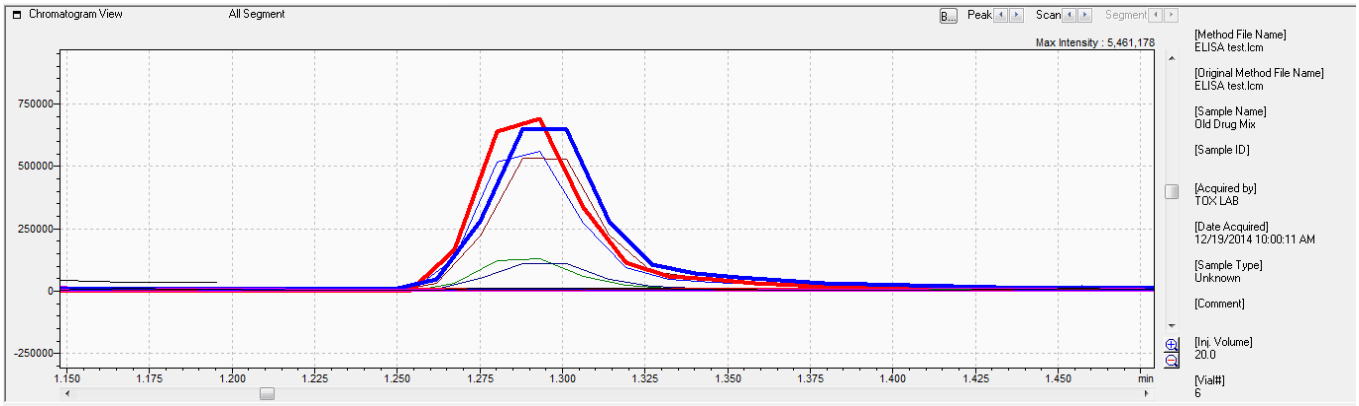


Figure 18. Coelution of methamphetamine and phentermine

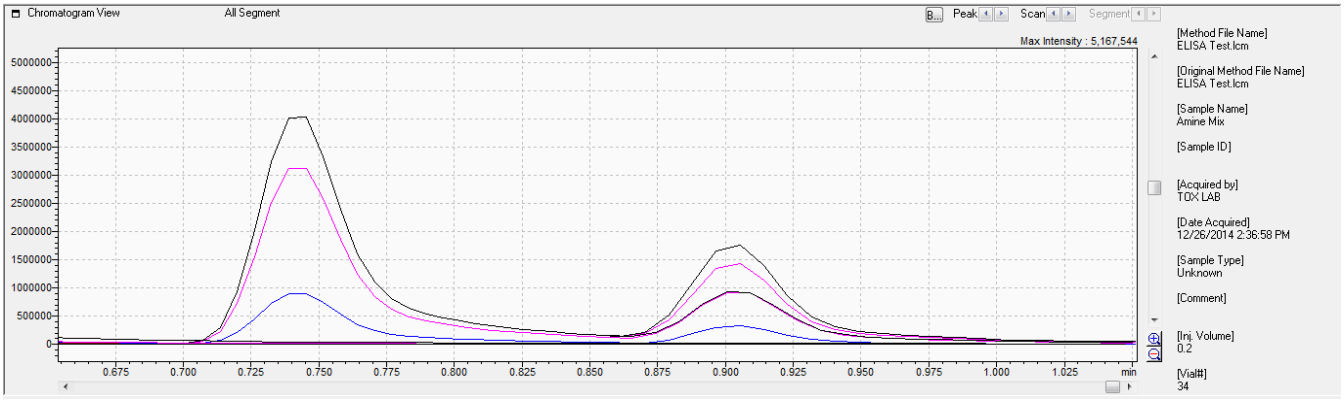


Figure 19. Separation of methamphetamine and phentermine.

The percent of each component making up the mobile phase (mobile phase components can be found on page 18) was determined using previously developed methods as

well as troubleshooting injections. This consisted of adjusting the percentages slightly and injecting neat standards until the most efficient mixture was determined. Once this was completed, the retention times were obtained for all compounds. Retention times are determined by setting the window of analysis for each compound to the entire length of the run. An extracted spiked sample was analyzed, the MRM transitions for each compound were reviewed and the retention time recorded. The window of analysis of each compound was then established. According to OSBI policy, the retention time for each control and sample must be within 0.15 minutes of the retention time observed for the low positive control. It is important to note the retention time window is something that can be adjusted based on drift that may result from the age of the column or slight differences between mobile phase batches, but it should not drift more than 0.15 minutes.

At this point, the potential blank human blood samples were extracted using the developed method to verify that they were indeed blank. It was determined that not all of these samples were drug free so more case samples that were set to be destroyed were obtained and analyzed as mentioned above. Some samples were originally analyzed by GC/MS only. When they were examined using the more sensitive LC/MS method, it was observed that some had low concentrations of drugs that were undetectable when using a GC/MS scan method.

The make-up of the reconstitution solvent was tested while the samples were being procured. There were three original options with the ability to try more if necessary. These consisted of de-ionized water and 0.1% formic acid with either a 9:1 or a 19:1 de-ionized water to acetonitrile mixture. This was assessed by using all possible reconstitution solvents mixtures,

to reconstitute an extraction of 225 µL bovine blood spiked with 25 µL of positive control. They were each analyzed using the developed method for the LC/MS and the peak areas for each compound were compared. There were no noticeable differences for each drugs of interest so only the ones with significant differences were reviewed to determine the most efficient reconstitution solvent. It was found that 0.1% formic acid in 9:1 de-ionized water to acetonitrile mixture worked best based on peak area response. It was also verified that the temperature of the acetonitrile used for protein precipitation did not matter. Protein precipitation will be discussed in more detail on page 39. This was tested by extracting samples using room temperature acetonitrile as well as acetonitrile stored in the freezer. There were no apparent differences in the resulting peak area.

It was noted that the benzodiazepines appeared to be suppressed due to their retention time being the same as carisoprodol which had a much higher cutoff concentration. The dwell time was adjusted to try and remedy this issue. The dwell time refers to the amount of time that the instrument analyzes for each drug individually. These are often very short periods of time. For the drugs that have a much higher concentrations, i.e. carisoprodol and meprobamate, the dwell time was set for 5 milliseconds (msec). For the benzodiazepines that eluted around the same time as carisoprodol, the dwell time was set for 50 milliseconds (msec). For all other drugs, the dwell time was set for 25 msec.

Once the method was developed, three internal standards were selected to span the range of anticipated retention times. Methamphetamine-d8, phencyclidine-d5, and prazepam-d5 were selected as an early, middle and late eluter, respectively. Methamphetamine-d8 elutes at approximately 1.00 minutes, phencyclidine-d5 elutes at approximately 1.95 minutes and

prazepam-d5 elutes at approximately 4.25 minutes. The length of the method developed was approximately 5.5 minutes. This placed an internal standard in the group of early eluters, one near the middle of the method and one right at the end of the method which allowed the compounds of interest to be compared to an internal standard that eluted near the same time. The use of internal standards is important because it allows for standardization between injections for quantitation purposes. This works by using a ratio of the peak area of the internal standard divided by the peak area of the drug of interest. Quantitation was not pursued in this validation so additional information regarding this process was not relevant or included in this paper. Internal standards also provide the ability to verify that there was no retention time drift during analysis. Drift could be caused by a clog, a leak, or another maintenance issue. Having internal standards in each control and sample allows the analyst to verify that none of these issues occurred during analysis.

To verify that an acetonitrile protein precipitation extraction[24] was the most appropriate for this method a liquid-liquid extraction[25] using borate buffer and chlorobutane was completed. This extraction mixture was used for comparison because it is the validated alkaline drug[26] extraction for GC/MS used at the OSBI currently. This extraction was compared directly to the protein precipitation extraction and it was found that 250 µL of sample extracted using the protein precipitation method was both simpler and provided the best recovery. With this completed, the method was validated by assessing the interference, carryover, limit of

[24] The addition of an organic solvent, acetonitrile in this instance, causes the proteins in the samples to precipitate out and create a "plug" at the bottom of the vial while keeping the compounds of interest in the liquid sample which can then be poured off.

[25] Extraction that uses basic and acidic reagents to move the drugs to a cleaner solvent that can be analyzed by GC/MS or LC/MS.

[26] This is a drug that has a pH greater than 7. Also known as a basic drug. This extraction also works for higher concentrations of neutral drugs, carisoprodol and meprobamate for example.

detection, and ion suppression and enhancement as acceptance criteria. These criteria are listed in Table 4.

Table 4

Assessed validation parameters.

Parameter	Acceptance Criteria:
Interference Studies	Evaluate interference from compounds in all current LC/MS-MS methods as well as other drugs commonly identified in the toxicology laboratory.
Carryover	Carryover after a high concentration sample must be less than 20% of the mean decision point peak area.
Limit of Detection	The LOD is defined as the decision point.
Ionization Suppression/ Enhancement	Less than 25% suppression or enhancement and <15% %CV due to matrix (if not, evaluate impact on LOD)

Possible matrix interferences were assessed by using ten previously analyzed blank whole blood case samples which were extracted using the developed method. No internal standards were added to these samples and no interferences were detected. Neat samples of commonly encountered drugs were analyzed at a concentration of 100 ng/mL to verify that there was no interference. Interference would be considered anything that results in a false positive for a drug of interest. Carryover was tested by injecting an extracted spiked sample that contained 20 times the concentrations listed in Table 3. Carryover results when a drug

contained in one sample can be seen in a later sample. This can be the result of the drug requiring more time to pass through the column causing it to show in the next sample instead of the original sample, contamination during any portion of the extraction or injection, a high concentration of the drug which results in the compound remaining in the column and being detected in other samples or any combination of the three. The extracted sample was injected three times, each was immediately followed by a blank extraction. No carryover was observed. The carryover extractions were prepared as described in sample preparation.

As mentioned previously, the limit of detection (LOD) was administratively set. This was tested by completing three analytical runs, analyzed on different days or extracted by different analysts, which consisted of three replicates. The matrices used were spiked blank blood samples from previously analyzed cases. As per OSBI policy, the ion ratios must be within 30% of the values set by the first sample of the run and the %RSD for retention times is less than four percent (Appendix 2). The table in Appendix 2 contains the peak area and retention time for all compounds for each replicate, as well as the ion ratio evaluated for precision.

For ionization suppression and enhancement, a post-extraction addition approach was used. Ionization suppression and enhancement is a result of either a reduced or increased response resulting from the extraction process. Two sets of ten blank samples were extracted. A low and high concentration reconstitution solvent was created. The low concentration used was double the concentrations listed in Table 3 and the high concentration was twenty times the concentrations listed in Table 3. The neat reconstitution solvent was also injected six times on the instrument. Results for each drug are listed in Appendix 2. Set one consists of the neat standards and set two consists of the blank blood sources.

$$Ionization\ suppression\ or\ enhancement\ (\%) = \left(\frac{Average\ Area\ of\ Set\ 2}{Average\ Area\ of\ Set\ 1} - 1\right) x\ 100$$

The example below is the calculation for methamphetamine:

Ionization suppression or enhancement (%) = [(3529187/4589072) – 1] * 100

Ionization suppression or enhancement (%) = [(0.7690415404) – 1] * 100

Ionization suppression or enhancement (%) = [-0.2309584596] * 100

Ionization suppression or enhancement (%) = -23.10

The method passed all of these requirements and was approved by the OSBI technical manager to be used for casework. The validation plan and completed validation report can be found in Appendices 1 and 2.

The development of a variation of this method facilitating smaller sample volumes was the next step in this project. First, the amount of sample and buffer needed was explored alongside the same portion of blood without buffer, and one with water instead of buffer. The extractions are shown in Table 5. It was found that 100 µL of sample mixed with 100 µL of phosphate buffer provided the best recovery regarding pivotal compounds. This conclusion was based on a comparison of the peak areas of each drug of interest for each extraction. There were not significant differences between extractions for all compounds, so only those that did have significant differentiation were considered in the decision making process (see Table 6). It was noted at this time that zopiclone did not extract under these conditions. A chromatogram showing the low positive control for the 100µL extraction can be seen in figures 20, 21, 22, and 23. Codeine, oxycodone, and 6-MAM are not visible in the chromatogram due to their low peak height. However, their peak area can be seen in Table 7. The instrument also provides a close up of each compound using the post-run software. This allows the analyst to

Table 5

Extraction possibilities for 100 µL method.

Amount of Blood (µL)	**Amount of Phosphate Buffer (µL)**	**Amount of Deionized Water (µL)**	**Amount of Acetonitrile (µL)**
100 µL	100 µL	_	500 µL
100 µL	_	100 µL	500 µL
100 µL	400 µL	_	1000 µL
100 µL	_	400 µL	1000 µL
100 µL	900 µL	_	1000 µL
100 µL	_	900 µL	1000 µL
100 µL	_	_	250 µL

Table 6

Peak areas for key compounds for 100 µL method.

Compound Name	**Peak 1:1 PBS**	**Peak 1:1 Water**	**100µL Blood 50µL Recon**	**100µL Blood 100µL Recon**
Methiopropamine	110451	16917	3549	8683
Codeine	6957	11613	13016	6240
Meth–d8	779488	105698	46367	70017
Methamphetamine	438323	55038	56867	72128
Phentermine	302219	29519	30227	41708

verify the existence of a peak for each compound in the controls. An example of this for oxycodone can be seen in Figure 24. After this verification was completed, the method passed all validation requirements set by the SWGTOX guidelines. The validation plan and completed validation report can be found in Appendices 3 and 4.

Once the validation was complete, the method was tested for use for synthetic cannabinoids and organic cannabinoids. It was found that this method was not appropriate for these compounds. This was determined by spiking blank blood samples with a high concentration of all synthetic and organic cannabinoids currently tested for by the OSBI. They were extracted and analyzed using the 250 µL method. It was found that both synthetic and organic cannabinoids do not extract using the developed methods. These compounds were previously optimized for other OSBI methods which allowed them to be easily added to the current method. The instrument was then programmed to analyze for all of the compounds for the entire length of the method. The only observed peaks were those of the internal standards. This would suggest that the synthetic and organic cannabinoids did not extract from the samples using this particular method.

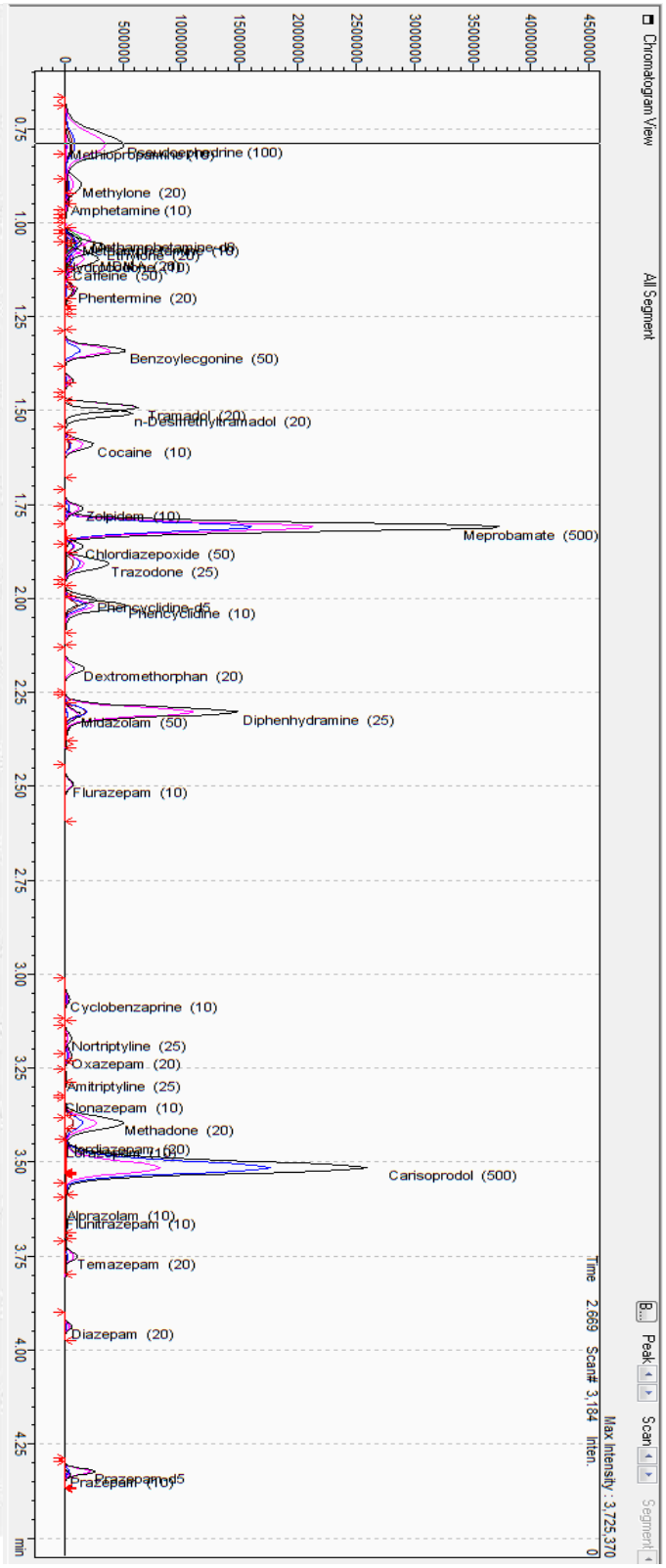


Figure 20. Low positive control for 100 µL extraction.

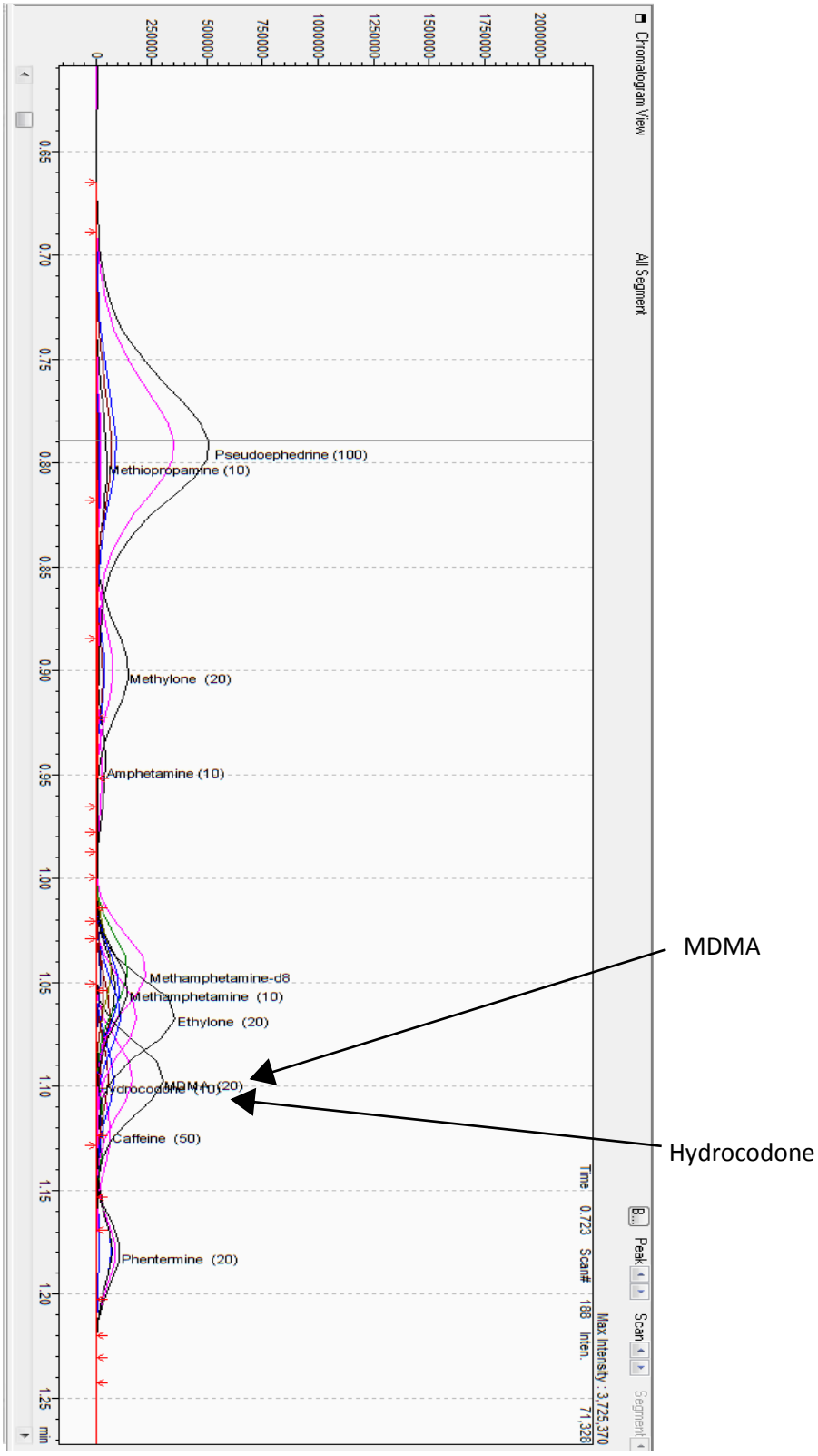


Figure 21. Close up of front third of Figure 20.

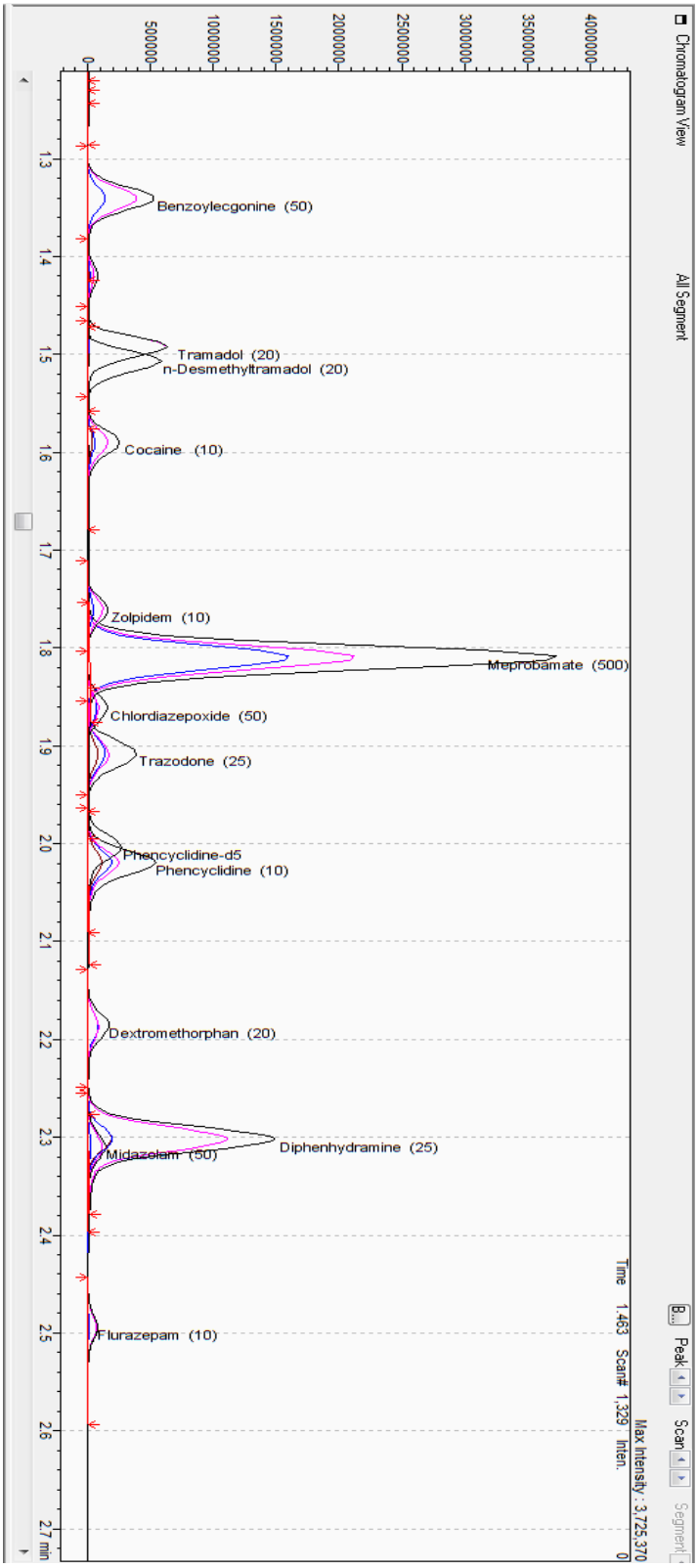


Figure 22. Close up of middle third of Figure 20.

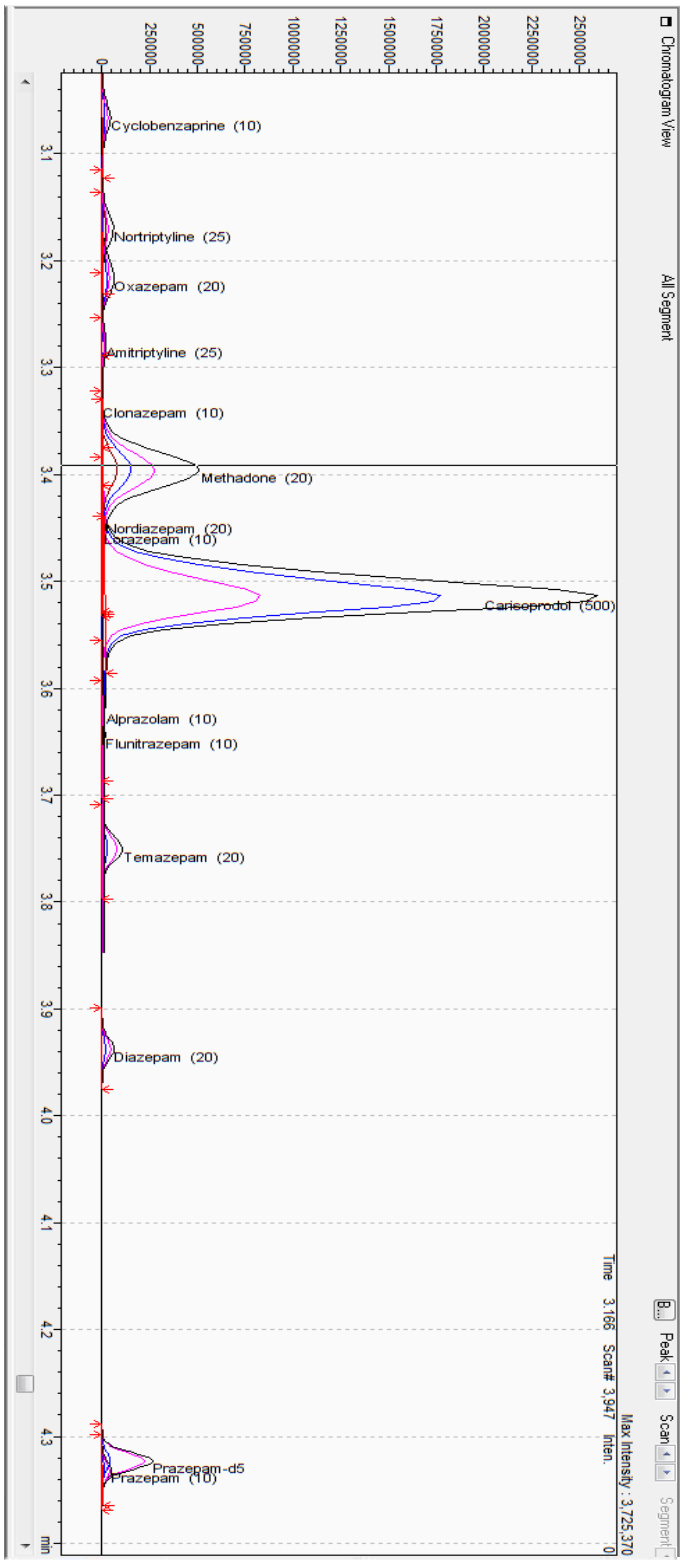


Figure 23. Close up of last third of Figure 20.

ID#	Name	Ret. Time	Area
1	Pseudoephedrine (100)	0.793	1599225
2	Methylone (20)	0.900	250592
3	Codeine (10)	0.910	2335
4	Amphetamine (10)	0.946	84902
5	Oxycodone (10)	1.026	8306
6	Methamphetamine-d8	1.044	368044
7	Methamphetamine (10)	1.053	271284
8	Ethylone (20)	1.066	465316
9	6-MAM (5)	1.084	2848
10	Hydrocodone (10)	1.098	17457
11	MDMA (20)	1.096	429329
12	Caffeine (50)	1.122	83767
13	Phentermine (20)	1.180	190449
14	Benzoylecgonine (50)	1.341	694374
15	Tramadol (20)	1.492	975942
16	n-Desmethyltramadol (20)	1.507	921804
17	Cocaine (10)	1.590	299322
18	Zolpidem (10)	1.761	217838
19	Meprobamate (500)	1.810	3923987
20	Chlordiazepoxide (50)	1.861	164164
21	Trazodone (25)	1.909	343545
22	Phencyclidine-d5	2.004	517422
23	Phencyclidine (10)	2.019	486626
24	Dextromethorphan (20)	2.186	167380
25	Diphenhydramine (25)	2.302	2084639
26	Midazolam (50)	2.310	236564
27	Flurazepam (10)	2.494	134899
28	Cyclobenzaprine (10)	3.067	63623
29	Nortriptyline (25)	3.170	67861
30	Oxazepam (20)	3.217	97192
31	Amitriptyline (25)	3.277	27870
32	Clonazepam (10)	3.336	11592
33	Methadone (20)	3.396	698757
34	Nordiazepam (20)	3.444	39128
35	Lorazepam (10)	3.452	22106
36	Carisoprodol (500)	3.515	4419356
37	Alprazolam (10)	3.622	14590
38	Flunitrazepam (10)	3.645	26366
39	Temazepam (20)	3.751	134665
40	Diazepam (20)	3.937	62635
41	Prazepam-d5	4.323	256337
42	Prazepam (10)	4.332	45889
43	Methiopropamine (10)	0.799	96915

Table 7. Compound table associated with Figure 20.

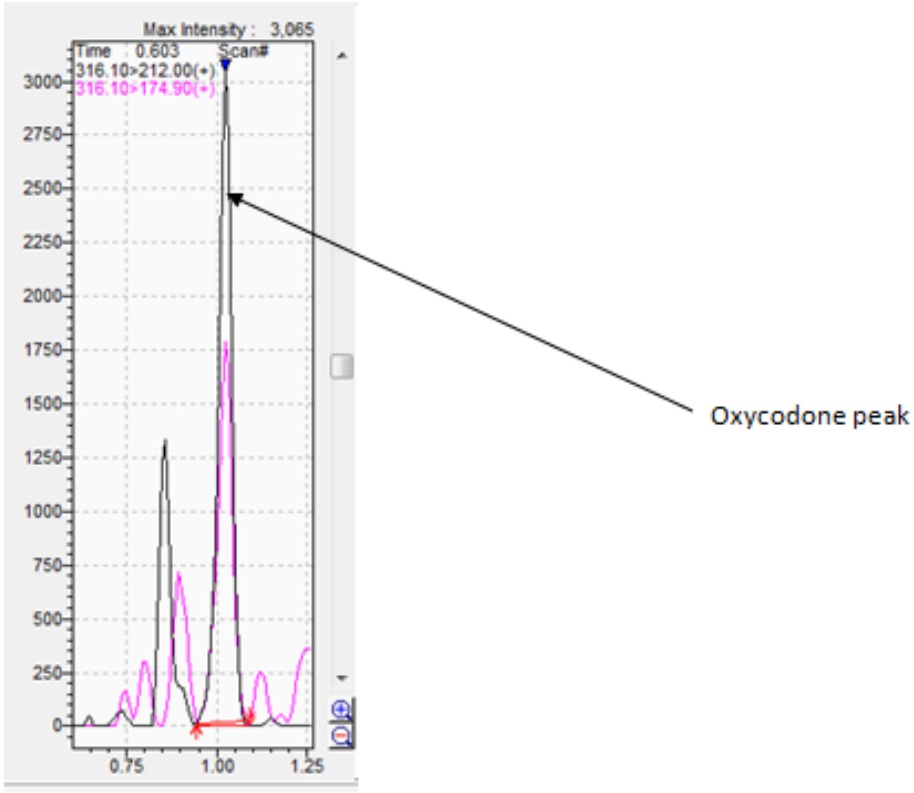


Figure 24. Oxycodone peak in association with Figure 20.

Results

Thirty case samples, i.e. DUI case specimens that were set for destruction, were analyzed using the newly developed methods for comparison with the results from the current OSBI GC/MS extraction. These case samples were selected due to positive presumptive and GC/MS results, or to verify negative presumptive results. Through this analysis, it was verified that the LC/MS-MS method would be sensitive enough to be used instead of the GC/MS method if required due to limited sample quantity. The results of this analysis are in Table 8. The results show that the LC/MS-MS is more sensitive than the GC/MS. This is due to a couple of factors. First, the GC/MS requires compounds to be semi-volatile, whereas the LC/MS does

not. Second, the developed LC/MS method uses MRM while the GC/MS uses a scanning method. MRM is discussed in more detail on page 4. By selecting to only analyze the sample for certain ions, the background noise, and consequently the limit of detection are much lower than those seen when using a scanning method.

The OSBI acceptance criteria for GC/MS is a 3:1 signal to noise ratio, a retention index that is within 25 units of the library known, and a Gaussian shaped peak. A retention index allows for comparison among instruments by using 24 straight chain hydrocarbons, known as a hydrocarbon ladder, to create a unit-less number assigned to each compound based on their retention time compared to the hydrocarbon ladder. This allows analysts to compare results between two instruments as long as both have the same type of column. This would not be possible if only retention time was used due to slight differences in the length of the column due to maintenance performed during the life of the column. Gaussian peak shape refers to a symmetrical peak shape. The OSBI acceptance criteria for LC/MS-MS is mentioned on pages 37 and 41. All compounds that appear to be present by GC/MS, but not LC/MS, were not part of the method because they are not known to cause impairment, are used as cutting agents, or were less commonly seen metabolites of a drugs of interest, and were not selected for the initial validation.

For the direct comparison study, many of the drugs were found in both LC/MS methods. There were some that did not meet criteria in the dilution method so they were not reported even if they appeared present. The current OSBI requirement to report LC/MS-MS results are symmetrical peaks, and that they meet the retention time and ion ratio requirements set in policy as mentioned on pages 37 and 41. There are multiple factors that could have caused

Table 8

Results of comparison study between GC/MS and LC/MS-MS methods.

Sample #	ELISA Results	EtOH Results	GC/MS Liquid-Liquid Extraction Results	General drug identification and confirmation by LC/MS Results (250µL)	Dilution Results (100µL)
1	Methadone	Negative	Diphenhydramine Chlorpheniramine Methadone	Diphenhydramine Methadone Lorazepam Cyclobenzaprine	Diphenhydramine Methadone Lorazepam Cyclobenzaprine
2	THC, Meth	Negative	Methamphetamine	Amphetamine Methamphetamine	Amphetamine Methamphetamine
3	Benzos	0.355	Diazepam Nordiazepam	Amphetamine Methamphetamine Nordiazepam Temazepam Diazepam Oxazepam	Amphetamine Methamphetamine Nordiazepam Diazepam Temazepam
4	Cocaine, Opiates	0.014	Hydrocodone	Hydrocodone Diazepam Benzoylecgonine	Benzoylecgonine
5	Carisoprodol	0.318	Negative	Meprobamate	Meprobamate
6	Benzo	0.259	Negative	Alprazolam Zolpidem	Alprazolam
7	Benzos	Negative	Citalopram Hydrocodone Alprazolam	Hydrocodone Alprazolam	Alprazolam
8	Barbs, Benzos, Opiates	Negative	Diphenhydramine Diazepam Hydrocodone Nordiazepam	Hydrocodone Diphenhydramine Oxazepam Nordiazepam Alprazolam Diazepam	Hydrocodone Diphenhydramine Nordiazepam Alprazolam Diazepam
9	Negative	0.184, 0.185	Negative	Hydrocodone	Negative

Sample #	ELISA Results	EtOH Results	GC/MS Liquid-Liquid Extraction Results	General drug identification and confirmation by LC/MS Results (250µL)	Dilution Results (100µL)
10	Negative	Negative	Cyclobenzaprine Citalopram	Hydrocodone Cyclobenzaprine Lorazepam	Cyclobenzaprine Lorazepam
11	Negative	0.092, 0.092	Negative	Amphetamine Methamphetamine Trazodone	Negative
12	Benzos, Meth, Opiates	Negative	Methamphetamine Citalopram Hydrocodone	Amphetamine Methamphetamine Hydrocodone Clonazepam	Amphetamine Methamphetamine Hydrocodone Clonazepam
13	Meth, Opiates	Negative	Methamphetamine Hydrocodone	Amphetamine Methamphetamine Hydrocodone	Amphetamine Methamphetamine Hydrocodone
14	Negative	0.053, 0.052	Negative	Negative	Negative
15	THC, PCP	Negative	Chlorpheniramine Dextromethorphan Dextrophan Citalopram	Dextromethorphan	Dextromethorphan
16	Benzos, Opiates	Negative	Citalopram Hydrocodone Alprazolam Trazodone	Hydrocodone Trazodone Alprazolam Meprobamate PCP Cyclobenzaprine	Hydrocodone Trazodone Alprazolam Cyclobenzaprine
17	Benzos, Opiates	0.122, 0.116	Diazepam, Hydrocodone, nordiazepam	Hydrocodone, Oxazepam, Clonazepam, Nordiazepam, Diazepam, Temazepam	Hydrocodone, Clonazepam, Nordiazepam, Diazepam

Sample #	ELISA Results	EtOH Results	GC/MS Liquid-Liquid Extraction Results	General drug identification and confirmation by LC/MS Results (250µL)	Dilution Results (100µL)
18	Benzos, Cari, Meth	Negative	Methamphetamine Meprobamate Carisoprodol Doxylamine Dextromethorphan Amitriptyline Nortriptyline Citalopram Diazepam Nordiazepam Temazepam	Amphetamine Methamphetamine Meprobamate Dextromethorphan Diphenhydramine Nortriptyline Amitriptyline Oxazepam Carisoprodol Methadone Nordiazepam Temazepam Diazepam	Amphetamine Methamphetamine Meprobamate Nortriptyline Oxazepam Nordiazepam Carisoprodol Temazepam Diazepam
19	Benzos, THC, Cari	Negative	Meprobamate Carisoprodol Alprazolam	Amphetamine Methamphetamine Hydrocodone Meprobamate Clonazepam Carisoprodol Alprazolam	Meprobamate Carisoprodol Alprazolam
20	Benzos, THC, Opiates, Oxy	Negative	Hydrocodone Alprazolam	Oxycodone Hydrocodone Cyclobenzaprine Alprazolam	Hydrocodone Cyclobenzaprine Alprazolam
21	THC, Meth	Negative	Methamphetamine	Amphetamine Methamphetamine Clonazepam	Methamphetamine Clonazepam
22	THC, Meth	Negative	Amphetamine Methamphetamine	Amphetamine Methamphetamine	Amphetamine Methamphetamine
23	THC, Cari, Meth	Negative	Amphetamine Methamphetamine Meprobamate	Amphetamine Methamphetamine Meprobamate Alprazolam	Amphetamine Methamphetamine Meprobamate Alprazolam

Sample #	ELISA Results	EtOH Results	GC/MS Liquid-Liquid Extraction Results	General drug identification and confirmation by LC/MS Results (250µL)	Dilution Results (100µL)
24	Benzos, Cari, Opiates	Negative	Meprobamate Carisoprodol Hydrocodone Alprazolam	Hydrocodone Meprobamate Carisoprodol Zolpidem Alprazolam	Hydrocodone Meprobamate Carisoprodol Alprazolam
25	Benzos, THC, Meth	0.039, 0.038	Amphetamine Methamphetamine Alprazolam	Amphetamine Methamphetamine Benzoylecgonine Alprazolam	Amphetamine Methamphetamine Alprazolam
26	Cari, Meth, Opiates, Oxy	Negative	Meprobamate Carisoprodol Hydrocodone Oxycodone	Oxycodone Hydrocodone Meprobamate PCP Carisoprodol saturated the detector	Hydrocodone Meprobamate Carisoprodol PCP
27	Benzos, THC, Meth, Opiates	Negative	Amphetamine Methamphetamine Citalopram Hydrocodone Clonazepam	Amphetamine Methamphetamine Hydrocodone Clonazepam Nordiazepam Alprazolam Diazepam	Amphetamine Methamphetamine Hydrocodone Clonazepam Nordiazepam Alprazolam
28	Benzos	Negative	Amitriptyline Nortriptyline Alprazolam Quetiapine	Nortriptyline Amitriptyline Alprazolam	Nortriptyline Amitriptyline Alprazolam
29	Benzos, Cocaine	0.020, 0.020	Methamphetamine Levamisole Cocaine Alprazolam	Amphetamine Methamphetamine Benzoylecgonine Cocaine Alprazolam	Methamphetamine Benzoylecgonine Alprazolam

Sample #	ELISA Results	EtOH Results	GC/MS Liquid-Liquid Extraction Results	General drug identification and confirmation by LC/MS Results (250µL)	Dilution Results (100µL)
30	THC, Cocaine	0.083, 0.080	Hydrocodone	Hydrocodone Benzoylecgonine Cocaine	Benzoylecgonine

this, including the fact that it is a dilution method as well as the fact that the samples used were whole blood and may have experienced more of a matrix effect due to the limited amount of sample

Concluding this section, two forms of a single method were developed, validated and either have been or will be put into policy at the Oklahoma State Bureau of Investigation Forensic Science Center (OSBI FSC). These methods will allow testing of limited case samples and can be used when a presumptive positive is unable to be confirmed by GC/MS.

Discussion and Future Research

The general drug identification and confirmation by LC tandem MS used for a sensitivity comparison for LC/MS-MS has been validated and approved for use. The methods were verified by completing all SWGTOX requirements and approved for use by the OSBI Technical Manager after all data was reviewed. A peak in the same range as pseudoephedrine was observed, but when the retention time was compared to the positive control, it was determined that it was not pseudoephedrine. This was remedied by narrowing the detection window. All compounds that appear to be present by GC/MS, but not LC/MS, were not part of the method because they are not known to cause impairment, are used as cutting agencies, or

were less commonly seen metabolites of a drugs of interest, and were not selected for the initial validation. They can be added in at a later date and include chlorpheniramine, citalopram, dextrophan, doxylamine, and levamisole.

There are drugs that elute at the same time, however, this is not a problem in these methods due to the fact the methods developed are qualitative in nature and each compound is analyzed for by using their unique parent and product ion combinations. This allows for differentiation between multiple drugs at the same time. The gradient was adjusted multiple times to remedy this to no avail.

It is obvious that the phosphate buffer had some impact on the recovery of most of the drugs in this method (see page 42). No testing was completed because the reasoning behind this enhancement was outside the scope of this validation. However, there are two possibilities that came to mind regarding this occurrence. Either the phosphate buffer helps partition the drug out of the blood into the acetonitrile or it is enhancing the solubility of the drug in the reconstitution solvent aiding in recovery.

This list of drugs currently in this method is by no means complete. New compounds are constantly appearing in DUID cases that could and should be added to this method. Each will require full validation, but should not require any change in method. Additional research could be completed to obtain the true limit of detection for each compound in these methods. There is the possibility of this method being used for quantitative purposes, but that would require a new validation with true limits of quantification. This is also a validation that could be costly due to the number of standards and deuterated standards that would be required for

completion. An experiment of why the phosphate buffer helped with the 100µL extraction could also be completed in the future.

There is a possibility that this method could be used in regards to dried blood samples. To test if this would be possible, four 250 µL samples were tested with some positive results. Two aliquots were spiked with the low positive control concentrations listed in Table 3 and two were spiked with the high positive control concentration. The sample was then spread on a designated portion of a plastic sheet and allowed to dry. Once the samples were dry one low and one high concentration were either swabbed or scraped. The swab was wet with DI water and then rinsed with 250 microliters of DI water that was placed in a micro-centrifuge tube for extraction. The flakes from the scraped samples were placed in 250 µL of DI water and vortexed. All samples were then extracted using the newly validated method. Most compounds were seen at both the low and high concentrations. It also appeared that scraping resulted in better recovery, but was also a messy process and would not allow for a control to be analyzed alongside. This could be followed up with more research and testing.

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VALIDATION PLAN

TOXICOLOGY UNIT // OSBI-FSC Laboratory

Scope: Drug Identification and Confirmation by LC/MS/MS

Matrix(ces):	Blood
Analyte(s):	See Attached List
Instrumentation:	LC/MS/MS
Analytical Method(s):	TX-34
Sample Preparation:	Protein Precipitation

Acceptable Limits

☐ Bias (accuracy):	N/A
☐ Calibration Model:	N/A
☒ Carryover:	Carryover after a high concentration sample must be less than 20% of the mean decision point peak area.
☒ Interference Studies:	Evaluate interference from compounds currently in TX-39 as well as other drugs commonly identified in the toxicology laboratory.
☒ Ionization Suppression/Enhancement:	Less than 25% suppression or enhancement and < 15% CV due to matrix (if not evaluate impact on LOD)
☒ Limit of Detection:	A minimum of three samples per run of a fortified matrix sample at the concentration of the decision points shall be analyzed over three runs to demonstrate that all detection and identification criteria are met. Decision point concentrations attached as an appendix to this document.
☐ Limit of Quantitation:	N/A
☐ Precision:	N/A
☐ Processed Sample Stability:	N/A
☐ Dilution Integrity (if applicable):	N/A

Other Information: These compounds will be evaluated for the above indicated performance areas according to the current Toxicology Quality Manual.

Lead Scientist: _____ Date: _____

Technical Manager Approval: _____ Date: _____

Drug	Concentration ng/mL
Pseudoephedrine	100
Methylone	20
Amphetamine	10
Methamphetamine	10
MDMA	20
Caffeine	50-100
Phentermine	20
Benzoylecgonine	50
Cocaine	10
Zolpidem	10
Meprobamate	500
Trazodone	25
Chlordiazepoxide	50
Phencyclidine	10
Midazolam	50
Diphenhydramine	25
Flurazepam	10
Oxazepam	20
Clonazepam	10
Methadone	20
Lorazepam	10
Diazepam	20
Nordiazepam	20
Alprazolam	10
Carisoprodol	500
Flunitrazepam	10
Tramadol	20
Prazepam	10
N-desmethyltramadol	20
Ethylone	20
Dextromethorphan	20
Methiopropamine	10
Cyclobenzaprine	10
Amitriptyline	25
Nortriptyline	25
Zaleplon	10
Zopiclone	50
Estazolam	25
Phenazepam	25

Drug Identification & Confirmation by LC/MS/MS
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Liquid chromatography-tandem mass spectrometry (LC-MS/MS) has proven to be a powerful tool for fast and reliable sample analysis in the OSBI toxicology lab. Furthermore, it allows the lab the flexibility to further refine and expand assay capabilities.

This document describes the validation for the identification and confirmation of many drugs that screen positive by enzyme-linked immunosorbent assay. This validation demonstrates that the procedure provides reliable results for the analysis of drugs identified and that meet the acceptable criteria set for this application. The concentration range of target compounds used in this validation was chosen to fit the recommended scope and cutoffs for identification and confirmation as outlined in "Recommendations for Toxicological Investigation of Drug-Impaired Driving and Motor Vehicle Fatalities", Journal of Analytical Toxicology 2013;37:552–558. Concentration ranges of target compounds not identified in this article were determined by commonly encountered range of compound concentrations seen in casework or literature review.

The sample preparation steps, as well as instrumental settings for use with blood matrices were assimilated from the TX-39 method. The validation parameters were assessed against the pre-defined requirements listed in ***Table 1***.

In brief, the procedure is outlined below:

- Pipet 250 µL blood to a microcentrifuge tube
- Add 500 µL of acetonitrile containing internal standards
- Vortex approximately 30 seconds
- Centrifuge at 13,200 rpm for 5 minutes
- Decant supernatant to conical tube
- Evaporate to dryness
- Reconstitute in 100 µL of reconstitution solvent and inject 20 µL.

Table 1: Validation parameters to be assessed

Parameter	Acceptance Criteria:
Interference Studies	No interfering signal from matrix, internal standard, common drugs of abuse, OTC drugs and prescription medication.
Carryover	Carryover after a high concentration sample must be less than 20% of the mean decision point peak area.
Limit of Detection	The LOD is defined as the decision point. Decision point concentrations are attached as an appendix to this document.
Ionization Suppression/ Enhancement	Less than 25% suppression or enhancement and less than 15% CV due to matrix (if not, evaluate impact on LOD)

Interference Studies

Matrix Interferences

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Ten independent sources of blank whole blood were secured from previously analyzed cases to evaluate matrix interferences. The blank matrix samples were extracted without the addition of internal standard and analyzed using the method.

Interference from Stable-Isotope Internal Standards

Isotopically-labeled compounds were assessed by analyzing a blank matrix sample fortified with the internal standards and monitoring the signal of the compounds of interest.

Interference from Other Commonly Encountered Compounds

This evaluation was accomplished by analyzing fortified matrix samples of the potential intereferences.

Opiates and Related	Tramadol, N-desmethyltramadol, methadone
Drugs of Abuse	Amphetamine, cocaine, benzoylecgonine, methamphetamine, phencyclidine, MDMA, ethylone,methiopropamine, methylone
Prescription Drugs	Antidepressants (amitriptyline, Nortriptyline, Trazodone), Benzodiazepines (lorazepam, alprazolam, midazolam, clonazepam, nordiazepam, diazepam, oxazepam, estazolam, flunitrazepam, temazepam, flurazepam, prazepam, phenazepam, chlordiazepoxide), CNS depressants (zopiclone, zaleplon and Zolpidem, carisoprodol, cyclobenzaprine, meprobamate), CNS stimulants (caffeine and phentermine)
OTC Drugs	Antihistamine (diphenhydramine), antitussive (dextromethorphan), decongestant (pseudoephedrine)

Carryover

To evaluate carryover as part of method validation, blank matrix samples are analyzed immediately after a high concentration sample (20 times the decision point concentration). The highest compound concentration at which no compound carryover is observed in the blank matrix sample is determined to be the concentration at which the method is free from carryover. This concentration was confirmed using triplicate analyses.

Ionization Suppression/Enhancement

The post-extraction addition approach was used to assess ionization suppression/enhancement. Two different sets of samples are prepared and the compound peak areas of neat standards are compared to matrix samples fortified with neat standards after extraction.

Set one consists of neat standards prepared at a high and low concentration for each compound (20x and 2x the decision point). The neat standards were injected a minimum of six

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times to establish a mean peak area for each concentration. Results of the two concentrations are presented in ***Table 2***.

Set two consists of ten different matrix sources. Each blank matrix sample was extracted in duplicate. After the extraction was complete, each blank matrix sample was then fortified to either the low or high concentration with each compound. Each concentration set sample was injected one time each.

The average area of each set was used to estimate the suppression/enhancement effect at each concentration. The following equation was used to calculate the percentage of ionization suppression or enhancement and % CV. ***Table 2*** list the percentage of ionization suppression or enhancement at each concentration for each compound.

[1] $$Ionization\ suppression\ or\ enhancement\ (\%) = \left(\frac{Average\ Area\ of\ Set\ 2}{Average\ Area\ of\ Set\ 1} - 1\right) x\ 100$$

[1] Strategies for the Assessment of Matrix Effect in Quantitative Bioanalytical Methods Based on HPLC−MS/MS, B. K. Matuszewski, M. L. Constanzer, and, and C. M. Chavez-Eng, Anal. Chem. 2003 75 (13), 3019-3030.

Table 2: Ionization Enhancement/Suppression

	Average Peak Areas		Average Peak Areas		Average Peak Areas		Average Peak Areas	
	Low	High	Low	High	Low	High	Low	High
	Pseudoephedrine		Methiopropamine		Methylone		Codeine	
Set 1	13852525.17	110349726	2296065	17622506	2290637	18234839	33918.5	254750.17
Set 2	12583398.9	82682647.7	1765213	12723779	1456584	11894243	19379.5	148279.6
[2] **% EE/(Suppr)**	-9.16	-25.07	-23.12	-27.80	-36.41	-34.77	-42.86	-41.79
% CV	5.47	5.89	6.34	3.91	21.94	9.19	21.35	11.02
	Average Peak Areas		Average Peak Areas		Average Peak Areas		Average Peak Areas	
	Low	High	Low	High	Low	High	Low	High
	Amphetamine		Oxycodone		6-MAM		Methamphetamine	
Set 1	1904546.5	16926401.5	73073.5	486663.7	24297.7	145306.7	4589072	33372765
Set 2	1133193.8	9820558.8	40985.1	289929	16426.4	97489.1	3529187	24733391
[2] **% EE/(Suppr)**	-40.50	-41.98	-43.91	-40.43	-32.40	-32.91	-23.10	-25.89
% CV	11.90	8.66	13.74	13.70	19.13	19.03	7.94	9.75
	Average Peak Areas		Average Peak Areas		Average Peak Areas		Average Peak Areas	
	Low	High	Low	High	Low	High	Low	High
	Ethylone		Hydrocodone		Phentermine		MDMA	
Set 1	3400360.667	21666147	133390	965641.8	1642267	13108964	2376922	20831581
Set 2	2757540.8	16595573.7	92832.1	664413.2	1230867	10057445	2646265	19709605
[2] **% EE/(Suppr)**	-18.90	-23.40	-30.41	-31.19	-25.05	-23.28	11.33	-5.39
% CV	9.57	32.79	20.82	11.55	34.93	21.00	12.11	9.08

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	Average Peak Areas		Average Peak Areas		Average Peak Areas		Average Peak Areas	
	Low	High	Low	High	Low	High	Low	High
	Caffeine		BE		Zolpiclone		Tramadol	
Set 1	263581.1667	2467634.17	629043	4319467	2188053	17103023	4307504	24048971
Set 2	3432164.7	4307611.7	757506	4560473	1874816	13794378	4452889	27107848
[2] % EE/(Suppr)	1202.13	74.56	20.42	5.58	-14.32	-19.35	3.38	12.72
% CV	86.36	64.04	7.80	3.87	7.69	8.33	4.39	12.76
	Average Peak Areas		Average Peak Areas		Average Peak Areas		Average Peak Areas	
	Low	High	Low	High	Low	High	Low	High
	n-Desmethyltramadol		Cocaine		Zolpidem		Chlordiazepoxide	
Set 1	4213810.667	26736781.5	2792170	22418767	1405156	12598208	1514264	13834679
Set 2	3272563	24454858.8	2806710	21340192	1372902	11418591	1301784	9444408.4
[2] % EE/(Suppr)	-22.34	-8.53	0.52	-4.81	-2.30	-9.36	-14.03	-31.73
% CV	28.38	8.85	3.24	3.67	2.44	3.21	5.00	4.99
	Average Peak Areas		Average Peak Areas		Average Peak Areas		Average Peak Areas	
	Low	High	Low	High	Low	High	Low	High
	Meprobamate		Trazodone		PCP		Dextromethorphan	
Set 1	4490350.5	29957402.5	2652914	15483975	3004426	18050022	1028149	8496406.5
Set 2	4571800.9	26995814.3	2159439	14058242	2839744	18209889	747476	6199230.1
[1] % EE/(Suppr)	1.81	-9.89	-18.60	-9.21	-5.48	0.89	-27.30	-27.04
% CV	8.54	3.74	31.80	3.57	8.46	30.77	6.57	31.64
	Average Peak Areas		Average Peak Areas		Average Peak Areas		Average Peak Areas	
	Low	High	Low	High	Low	High	Low	High
	Diphenhydramine		Midazolam		Flurazepam		Cyclobenzaprine	
Set 1	20035599.33	71829470.8	1248496	7416501	2171229	17481742	951042	8082041.5
Set 2	15918815.6	64209294.1	1005633	8118957	1944670	15561369	325448	5152256.6
[1] % EE/(Suppr)	-20.55	-10.61	-19.45	9.47	-10.43	-10.99	-65.78	-36.25
% CV	4.82	1.64	46.59	3.63	4.08	3.90	13.87	7.90
	Average Peak Areas		Average Peak Areas		Average Peak Areas		Average Peak Areas	
	Low	High	Low	High	Low	High	Low	High
	Nortriptyline		Oxazepam		Amitriptyline		Methadone	
Set 1	1465160.5	12606794	476323	3954652	330989	2091380	6388399	61860756
Set 2	367318.5	5925154.7	314571	2256772	106431	1845257	3954256	46495890
[2] % EE/(Suppr)	-74.93	-53.00	-33.96	-42.93	-67.84	-11.77	-38.10	-24.84
% CV	12.95	11.51	12.88	25.89	14.01	11.21	9.13	31.98

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	Average Peak Areas		Average Peak Areas		Average Peak Areas		Average Peak Areas	
	Low	High	Low	High	Low	High	Low	High
	Clonazepam		Carisoprodol		Lorazepam		Nordiazepam	
Set 1	51441.33333	263043.667	3737510	28882323	220439	2239290	254047	2168496
Set 2	33993.2	165929.5	3891659	26870569	198726	1598588	148501	1052152.7
[2] % EE/(Suppr)	-33.92	-36.92	4.12	-6.97	-9.85	-28.61	-41.55	-51.48
% CV	13.76	17.49	2.52	2.89	14.24	10.82	33.53	41.07
	Average Peak Areas		Average Peak Areas		Average Peak Areas		Average Peak Areas	
	Low	High	Low	High	Low	High	Low	High
	Alprazolam		Flunitrazepam		Temazepam		Diazepam	
Set 1	36675.66667	283536.333	130407	1257004	1226357	10578704	409482	1935233.3
Set 2	69031.2	367517.5	132050	1149772	1052148	3958305	223030	1826684.5
[2] % EE/(Suppr)	88.22	29.62	1.26	-8.53	-14.21	-62.58	-45.53	-5.61
% CV	8.59	6.02	3.14	4.23	21.52	72.93	5.96	2.75

	Average Peak Areas		Average Peak Areas		Average Peak Areas		Average Peak Areas	
	Low	High	Low	High	Low	High	Low	High
	Prazepam		Meth-d8		PCP-d5		Prazepam-d5	
Set 1	300870	2535919.17	5818796	43262546	4496532	34825444	1816668	15300894
Set 2	120665.1	1297061.9	564219	4087310	518492	3603258	144351	864161
[2] % EE/(Suppr)	-59.89	-48.85	-39.12	-63.67	-35.36	-63.93	-47.06	-74.12
% CV	11.81	11.45	15.92	26.01	17.83	28.68	15.01	21.82

[2] % Enhancement/Suppression

Limit of Detection

Using the decision point concentration as the limit of detection is useful for qualitative and quantitative methods. For the mission of this laboratory, it is sufficient to define the LOD as the value of an administratively-defined decision point to fit the recommendations for toxicological investigation of drug-Impaired driving and motor vehicle fatalities, when available.

A minimum of three samples per run of a fortified matrix sample at the concentration of the decision point shall be analyzed over three runs to demonstrate that all detection and identification criteria are met see ***Table 3***.

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Table 3: Decision Point Peak Area, Retention Times and Ion Ratios

	6-MAM		Alprazolam		Amitriptyline		Amphetamine	
Sample	Peak Area	Retention Time (min)	Peak Area	Retention Time (min)	Peak Area	Retention Time (min)	Peak Area	Retention Time (min)
Run 1 Rep 1	16864	1.045	47664	3.573	102625	3.167	294131	0.919
Run 1 Rep 2	18650	1.044	60080	3.574	124799	3.17	331195	0.914
Run 1 Rep 3	16365	1.053	59394	3.572	99313	3.177	638121	0.919
Run 2 Rep 1	18470	1.045	38485	3.574	79060	3.164	177270	0.917
Run 2 Rep 2	14048	1.041	48367	3.574	80991	3.168	189851	0.911
Run 2 Rep 3	14786	1.052	43432	3.574	96627	3.167	186016	0.912
Run 3 Rep 1	13688	1.05	62389	3.574	120868	3.17	261943	0.927
Run 3 Rep 2	16061	1.046	52966	3.57	95758	3.165	370705	0.92
Run 3 Rep 3	13486	1.055	63058	3.575	77283	3.172	346814	0.923
Std Dev.	1958	0.005	8891	0.002	17124	0.003	142800	0.005
Average	15824	1	52871	4	97480	3	310671	0.918
%RSD	12	0.454	16	0.042	17	0.124	45	0.563
	Benzoylecgonine		Carsioprodol		Chlordiazepoxide		Clonazepam	
Sample	Peak Area	Retention Time (min)	Peak Area	Retention Time (min)	Peak Area	Retention Time (min)	Peak Area	Retention Time (min)
Run 1 Rep 1	440554	1.301	3448441	3.458	1078718	1.821	48662	3.278
Run 1 Rep 2	466180	1.301	3468989	3.457	1262371	1.823	49199	3.278
Run 1 Rep 3	428655	1.3	3419365	3.456	11112632	1.824	51151	3.277
Run 2 Rep 1	476114	1.302	3064341	3.459	1135193	1.822	45964	3.278
Run 2 Rep 2	391275	1.301	3244407	3.459	1206963	1.822	43852	3.279
Run 2 Rep 3	513895	1.302	3550518	3.458	1249501	1.823	46788	3.279
Run 3 Rep 1	636523	1.302	3555857	3.456	1090740	1.821	48426	3.278
Run 3 Rep 2	553458	1.298	3369047	3.452	1046923	1.819	44280	3.273
Run 3 Rep 3	670872	1.303	3401113	3.457	1000558	1.825	43903	3.278
Std Dev.	95117	0.001	154753	0.002	3327483	0.001	2617	0.001
Average	508614	1	3391342	3	2242622	1	46913	3
%RSD	18	0.111	4	0.062	148	0.098	5	0.055

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	Cocaine		Codeine		Cyclobenzaprine		Desmethyltramadol	
Sample	Peak Area	Retention Time (min)	Peak Area	Retention Time (min)	Peak Area	Retention Time (min)	Peak Area	Retention Time (min)
Run 1 Rep 1	2264594	1.535	22125	0.887	318729	2.96	2910859	1.453
Run 1 Rep 2	2296968	1.537	18011	0.887	386097	2.962	2637883	1.455
Run 1 Rep 3	2241106	1.537	22800	0.89	304062	2.967	2611392	1.455
Run 2 Rep 1	2022308	1.536	15672	0.887	275186	2.958	2573722	1.453
Run 2 Rep 2	2143967	1.536	22761	0.881	284412	2.958	2606051	1.454
Run 2 Rep 3	2154520	1.538	18878	0.883	319608	2.96	2280326	1.456
Run 3 Rep 1	2558314	1.535	14773	0.899	327517	2.963	3175666	1.454
Run 3 Rep 2	2295055	1.533	22263	0.892	263566	2.956	3171614	1.451
Run 3 Rep 3	2436978	1.539	19686	0.893	249803	2.963	2740340	1.457
Std Dev.	159596	0.001	3070	0.005	41131	0.003	293447	0.001
Average	2268201	1	19663	0.888	303220	2	2745317	1
%RSD	7	0.116	15	0.613	13	0.113	10	0.122

	Dextromethorphan		Diazepam		Diphenhydramine		Ethylone	
Sample	Peak Area	Retention Time (min)	Peak Area	Retention Time (min)	Peak Area	Retention Time (min)	Peak Area	Retention Time (min)
Run 1 Rep 1	606840	2.092	197388	3.916	102625	3.167	294131	0.919
Run 1 Rep 2	678833	2.097	225362	3.916	124799	3.17	331195	0.914
Run 1 Rep 3	598566	2.1	223510	3.915	99313	3.177	638121	0.919
Run 2 Rep 1	557183	2.093	168469	3.917	79060	3.164	177270	0.917
Run 2 Rep 2	576977	2.094	196504	3.917	80991	3.168	189851	0.911
Run 2 Rep 3	565880	2.094	206488	3.916	96627	3.167	186016	0.912
Run 3 Rep 1	646750	2.093	208787	3.916	120868	3.17	261943	0.927
Run 3 Rep 2	611435	2.091	179931	3.911	95758	3.165	370705	0.92
Run 3 Rep 3	585398	2.098	191436	3.916	77283	3.172	346814	0.923
Std Dev.	39106	0.003	18722	0.002	17124	0.003	142800	0.005
Average	603095	2	199763	3.92	97480	3	310671	0.918
%RSD	6	0.143	9	0.046	17	0.124	45	0.563

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Sample	Flunitrazepam		Flurazepam		Hydrocodone		Lorazepam	
	Peak Area	Retention Time (min)	Peak Area	Retention Time (min)	Peak Area	Retention Time (min)	Peak Area	Retention Time (min)
Run 1 Rep 1	125652	3.606	1752485	2.393	104761	1.064	185656	3.383
Run 1 Rep 2	131185	3.607	1983516	2.396	94308	1.065	153341	3.383
Run 1 Rep 3	132321	3.604	1832331	2.4	81256	1.073	208136	3.382
Run 2 Rep 1	104350	3.607	1743501	2.392	87683	1.064	147397	3.385
Run 2 Rep 2	122292	3.608	1900068	2.394	85423	1.057	169934	3.385
Run 2 Rep 3	118800	3.607	2018435	2.394	71131	1.071	151198	3.384
Run 3 Rep 1	116687	3.606	1962393	2.392	98537	1.069	195799	3.383
Run 3 Rep 2	108241	3.6	1973993	2.39	94875	1.065	124027	3.377
Run 3 Rep 3	115350	3.606	1828037	2.397	73082	1.075	152356	3.384
Std Dev.	9542	0.002	103168	0.003	11392	0.005	26773	0.002
Average	119430	3	1888307	2	87895	1	165316	3
%RSD	7	0.066	5	0.126	12	0.519	16	0.071

Sample	Chlordiazepoxide		Clonazepam		MDMA		Meprobamate	
	Peak Area	Retention Time (min)	Peak Area	Retention Time (min)	Peak Area	Retention Time (min)	Peak Area	Retention Time (min)
Run 1 Rep 1	102625	3.167	294131	0.919	1433974	1.064	4534225	1.762
Run 1 Rep 2	124799	3.17	331195	0.914	1524385	1.068	3926219	1.763
Run 1 Rep 3	99313	3.177	638121	0.919	1538813	1.077	4002079	1.761
Run 2 Rep 1	79060	3.164	177270	0.917	1060027	1.063	4175818	1.763
Run 2 Rep 2	80991	3.168	189851	0.911	994562	1.055	4615533	1.763
Run 2 Rep 3	96627	3.167	186016	0.912	866735	1.027	3998668	1.764
Run 3 Rep 1	120868	3.17	261943	0.927	1566964	1.071	4214730	1.761
Run 3 Rep 2	95758	3.165	370705	0.92	1308875	1.069	3253384	1.758
Run 3 Rep 3	77283	3.172	346814	0.923	1651904	1.08	3934324	1.764
Std Dev.	17124	0.003	142800	0.005	285564	0.015	396993	0.001
Average	97480	3	310671	0.918	1327359	1	4072776	1
%RSD	17	0.124	45	0.563	21	1	9	0.107

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	Methadone		Methamphetamine		Methamphetamine-d8		Methiopropamine	
Sample	Peak Area	Retention Time (min)	Peak Area	Retention Time (min)	Peak Area	Retention Time (min)	Peak Area	Retention Time (min)
Run 1 Rep 1	3113086	3.288	947157	1.019	479631	1.012	428771	0.791
Run 1 Rep 2	3832463	3.29	889449	1.022	607528	1.014	288760	0.791
Run 1 Rep 3	3300553	3.305	1737933	1.035	928229	1.027	701459	0.792
Run 2 Rep 1	2907997	3.285	644294	1.017	339156	1.01	241126	0.789
Run 2 Rep 2	3004234	3.289	483475	1.014	351931	1.007	160550	0.782
Run 2 Rep 3	2975974	3.288	472024	1.028	341210	1.017	193179	0.774
Run 3 Rep 1	3260571	3.29	1001933	1.026	611273	1.018	268288	0.811
Run 3 Rep 2	3331431	3.284	1266546	1.024	756390	1.017	425867	0.796
Run 3 Rep 3	2913377	3.293	794816	1.043	483914	1.033	270796	0.797
Std Dev.	294043	0.006	400033	0.009	202802	0.008	166307	0.01
Average	3182187	3	915291	1	544362	1	330977	0.791
%RSD	9	0.187	43	0.888	37	0.807	50	1

	Methylone		Midazolam		Nordiazepam		Nortriptyline	
Sample	Peak Area	Retention Time (min)	Peak Area	Retention Time (min)	Peak Area	Retention Time (min)	Peak Area	Retention Time (min)
Run 1 Rep 1	1105604	0.886	1168861	2.223	261869	3.43	320752	3.145
Run 1 Rep 2	1223611	0.882	1286657	2.226	274026	3.432	482582	3.06
Run 1 Rep 3	1237416	0.884	12287439	2.211	257462	3.43	376873	3.068
Run 2 Rep 1	869530	0.884	1095501	2.222	252821	3.431	310748	3.055
Run 2 Rep 2	1032647	0.877	1224708	2.224	258504	3.432	326927	3.057
Run 2 Rep 3	856153	0.878	1269455	2.224	267472	3.431	389051	3.057
Run 3 Rep 1	651409	0.896	1201221	2.223	254660	3.43	400369	3.061
Run 3 Rep 2	838718	0.886	1216701	2.221	178470	3.426	306869	3.055
Run 3 Rep 3	869959	0.888	1167922	2.227	224068	3.429	246956	3.061
Std Dev.	196760	0.005	3694960	0.004	29416	0.001	68697	0.028
Average	965005	0.884	2435385	2	247705	3	351236	3
%RSD	20	0.637	151	0.208	11	0.053	19	0.94

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	Oxazepam		Oxycodone		**PCP**		**PCP-d5**	
Sample	Peak Area	Retention Time (min)	Peak Area	Retention Time (min)	Peak Area	Retention Time (min)	Peak Area	Retention Time (min)
Run 1 Rep 1	320752	3.145	38269	0.997	1055295	1.938	884736	1.923
Run 1 Rep 2	317546	3.146	35962	1	1388283	1.941	1254017	1.925
Run 1 Rep 3	314006	3.144	29054	1.008	1678613	1.943	1330489	1.928
Run 2 Rep 1	295750	3.146	34637	0.997	709795	1.939	653941	1.924
Run 2 Rep 2	306862	3.147	36167	0.99	506625	1.941	503701	1.926
Run 2 Rep 3	302759	3.147	27518	1.006	548849	1.941	500784	1.926
Run 3 Rep 1	333191	3.145	34930	1.002	1082093	1.938	983577	1.923
Run 3 Rep 2	273978	3.14	36508	1	978869	1.936	930973	1.921
Run 3 Rep 3	271149	3.146	39828	1.012	1042576	1.942	906471	1.927
Std Dev.	20840	0.002	4023	0.006	379885	0.002	294384	0.002
Average	303999	3	34763	1	998999	1	883187	1
%RSD	6	0.068	11	0.66	38	0.116	33	0.115
	Phentermine		Prazepam		Prazepam-d5		Pseudoephedrine	
Sample	Peak Area	Retention Time (min)	Peak Area	Retention Time (min)	Peak Area	Retention Time (min)	Peak Area	Retention Time (min)
Run 1 Rep 1	476484	1.141	133521	4.315	295723	4.305	6120972	0.79
Run 1 Rep 2	476437	1.143	182057	4.315	431679	4.305	6395152	0.784
Run 1 Rep 3	473493	1.148	167363	4.313	385418	4.304	7118602	0.79
Run 2 Rep 1	284380	1.141	112228	4.315	274058	4.306	4500941	0.784
Run 2 Rep 2	255396	1.133	146611	4.315	387529	4.306	4856466	0.775
Run 2 Rep 3	162946	1.148	154287	4.315	418938	4.306	3900756	0.771
Run 3 Rep 1	441361	1.144	137493	4.314	373748	4.305	5223610	0.806
Run 3 Rep 2	574494	1.142	122882	4.1	320767	4.3	5243693	0.791
Run 3 Rep 3	148361	1.15	117438	4.315	318415	4.306	5903876	0.792
Std Dev.	155161	0.005	23360	0.071	55749	0.001	1004716	0.01
Average	365928	1	141542	4	356252	4	5473785	0.787
%RSD	42	0.445	16	1	15	0.044	18	1

Drug Identification & Confirmation by LC/MS/MS

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	Temazepam		Tramadol		Trazodone		Zolpidem	
Sample	Peak Area	Retention Time (min)	Peak Area	Retention Time (min)	Peak Area	Retention Time (min)	Peak Area	Retention Time (min)
Run 1 Rep 1	1124179	3.711	2970320	1.439	1936537	1.835	1162355	1.7
Run 1 Rep 2	1298352	3.711	2912311	1.441	2249154	1.837	1208665	1.702
Run 1 Rep 3	1296265	3.71	3118931	1.441	2118852	1.84	1154086	1.703
Run 2 Rep 1	922972	3.711	2052569	1.439	2021507	1.835	1055524	1.7
Run 2 Rep 2	1043150	3.712	2175428	1.44	2115189	1.836	1145964	1.702
Run 2 Rep 3	1041672	3.711	2144107	1.442	2286934	1.837	1187418	1.701
Run 3 Rep 1	1271925	3.71	3014973	1.44	2243915	1.835	1300961	1.7
Run 3 Rep 2	1118305	3.705	3191591	1.437	2245110	1.833	1253091	1.698
Run 3 Rep 3	1060824	3.711	3326572	1.442	2129121	1.839	1217669	1.705
Std Dev.	131948	0.002	498693	0.001	117867	0.002	70200	0.002
Average	1130849	3	2767422	1	2149591	1	1187304	1
%RSD	11	0.055	18	0.112	5	0.118	5	0.12

	Zopiclone	
Sample	Peak Area	Retention Time (min)
Run 1 Rep 1	1717392	1.372
Run 1 Rep 2	1529671	1.373
Run 1 Rep 3	1490835	1.373
Run 2 Rep 1	1602586	1.372
Run 2 Rep 2	1596522	1.372
Run 2 Rep 3	1653834	1.372
Run 3 Rep 1	175354	1.372
Run 3 Rep 2	1525477	1.369
Run 3 Rep 3	1559353	1.375
Std Dev.	474840	0.001
Average	1427891	1
%RSD	33	0.113

Drug Identification & Confirmation by LC/MS/MS

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Table 4: Summary of validation results

Parameter	Acceptance Criteria:	Results
Interference Studies	No interfering signal from matrix, internal standard, common drugs of abuse, OTC drugs and prescription medication.	No observed interferences from matrix or from common drugs/metabolites
Carryover	Carryover after a high concentration sample must be less than 20% of the mean decision point peak area.	No significant carryover observed at 20x the decision point concentration.
Limit of Detection	The LOD is defined as the decision point. Decision point concentrations are attached as an appendix to this document.	All compounds were detected at the decision point concentration.
Ionization Suppression/ Enhancement	Less than 25% suppression or enhancement and less than 15% CV due to matrix (if not, evaluate impact on LOD)	Average suppression or enhancement exceeded ±25% or the % CV of the suppression or enhancement exceeded 15 for several drugs. The influence on the above parameters were assessed by evaluating the impact on the limit of detection. Further assessment demonstrated that there was no impact on other critical validation parameters.

Drug Identification & Confirmation by LC/MS/MS

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Table 5: Compounds with decision point concentrations

No.	Drug	Concentration ng/mL	2x	20x
1	Alprazolam	10	20	200
2	Amitriptyline	25	25	250
3	Amphetamine	10	20	200
4	Benzoylecgonine	50	100	1000
5	Caffeine	50-100		
6	Carisoprodol	500	1000	10000
7	Chlordiazepoxide	50	100	1000
8	Clonazepam	10	20	200
9	Cocaine	10	20	200
10	Cyclobenzaprine	10	20	200
11	Dextromethorphan	20	40	400
12	Diazepam	20	40	400
13	Diphenhydramine	25	50	500
14	Estazolam	25	50	500
15	Ethylone	20	40	400
16	Flunitrazepam	10	20	200
17	Flurazepam	10	20	200
18	Lorazepam	10	20	200
19	MDMA	20	40	400
20	Meprobamate	500	1000	10000
21	Methadone	20	40	400
22	Methamphetamine	10	20	200
23	Methiopropamine	10	20	200
24	Methylone	20	40	400
25	Midazolam	50	100	1000
26	N-desmethyltramadol	20	40	400
27	Nordiazepam	20	40	400
28	Nortriptyline	25	50	500
29	Oxazepam	20	40	400
30	Phenazepam	25	50	500
31	Phencyclidine	10	20	200
32	Phentermine	20	40	400
33	Prazepam	10	20	200
34	Pseudoephedrine	100	200	2000
35	Tramadol	20	40	400
36	Trazodone	25	50	500
37	Zaleplon	10	20	200
38	Zolpidem	10	20	200
39	Zopiclone	50	100	1000

VALIDATION PLAN

TOXICOLOGY UNIT // OSBI-FSC Laboratory

Scope: Drug Identification and Confirmation by LC/MS/MS

Matrix(ces):	Blood
Analyte(s):	See Attached List
Instrumentation:	LC/MS/MS
Analytical Method(s):	TX-34 addition
Sample Preparation:	Protein Precipitation

Acceptable Limits

☐ Bias (accuracy):	N/A
☐ Calibration Model:	N/A
☒ Carryover:	Carryover after a high concentration sample must be less than 20% of the mean decision point peak area.
☒ Interference Studies:	Evaluate interference from compounds currently in TX-39 as well as other drugs commonly identified in the toxicology laboratory.
☒ Ionization Suppression/Enhancement:	Less than 25% suppression or enhancement and < 15% CV due to matrix (if not evaluate impact on LOD)
☒ Limit of Detection:	A minimum of three samples per run of a fortified matrix sample at the concentration of the decision points shall be analyzed over three runs to demonstrate that all detection and identification criteria are met. Decision point concentrations attached as an appendix to this document.
☐ Limit of Quantitation:	N/A
☐ Precision:	N/A
☐ Processed Sample Stability:	N/A
☐ Dilution Integrity (if applicable):	N/A

Other Information: These compounds will be evaluated for the above indicated performance areas according to the current Toxicology Quality Manual.

Lead Scientist: _____ Date: _____

Technical Manager Approval: _____ Date: _____

Drug	Concentration ng/mL
Pseudoephedrine	100
Methylone	20
Amphetamine	10
Methamphetamine	10
MDMA	20
Caffeine	50-100
Phentermine	20
Benzoylecgonine	50
Cocaine	10
Zolpidem	10
Meprobamate	500
Trazodone	25
Chlordiazepoxide	50
Phencyclidine	10
Midazolam	50
Diphenhydramine	25
Flurazepam	10
Oxazepam	20
Clonazepam	10
Methadone	20
Lorazepam	10
Diazepam	20
Nordiazepam	20
Alprazolam	10
Carisoprodol	500
Flunitrazepam	10
Tramadol	20
Prazepam	10
N-desmethyltramadol	20
Ethylone	20
Dextromethorphan	20
Methiopropamine	10
Cyclobenzaprine	10
Amitriptyline	25
Nortriptyline	25
Zaleplon	10
Zopiclone	50
Estazolam	25
Phenazepam	25

Drug Identification & Confirmation by LC/MS/MS

Validation Report

Liquid chromatography-tandem mass spectrometry (LC-MS/MS) has proven to be a powerful tool for fast and reliable sample analysis in the OSBI toxicology lab. Furthermore, it allows the lab the flexibility to further refine and expand assay capabilities.

This document describes the validation for the identification and confirmation of many drugs that screen positive by enzyme-linked immunosorbent assay. This validation demonstrates that the procedure provides reliable results for the analysis of drugs identified and that meet the acceptable criteria set for this application. The concentration range of target compounds used in this validation was chosen to fit the recommended scope and cutoffs for identification and confirmation as outlined in "Recommendations for Toxicological Investigation of Drug-Impaired Driving and Motor Vehicle Fatalities", Journal of Analytical Toxicology 2013;37:552–558. Concentration ranges of target compounds not identified in this article were determined by commonly encountered range of analyte concentrations seen in casework or literature review.

The sample preparation steps, as well as instrumental settings for use with blood matrices were assimilated from the TX-34 method. The validation parameters were assessed against the pre-defined requirements listed in ***Table 1***.

In brief, the procedure is outlined below:

- Pipet 100 µL blood to a microcentrifuge tube
- Pipet 100 µL 0.10 M Sodium Phosphate Buffer (pH=7.0) into microcentrifuge tube
- Add 500 µL of acetonitrile containing internal standards
- Vortex approximately 30 seconds
- Centrifuge at 13,200 rpm for 5 minutes
- Decant supernatant to conical tube
- Evaporate to dryness
- Reconstitute in 100 µL of reconstitution solvent and inject 20 µL.

Table 2: Validation parameters to be assessed

Parameter	Acceptance Criteria:
Interference Studies	Evaluate interference from compounds currently in TX-39 as well as other drugs commonly identified in the toxicology laboratory.
Carryover	Carryover after a high concentration sample must be less than 20% of the mean decision point peak area.
Limit of Detection	The LOD is defined as the decision point. Decision point concentrations are attached as an appendix to this document.
Ionization Suppression/ Enhancement	Less than 25% suppression or enhancement and <15% %CV due to matrix (if not, evaluate impact on LOD)

Drug Identification & Confirmation by LC/MS/MS

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Interference Studies – Blank Matrices

Ten independent sources of blank whole blood were secured from previously analyzed cases to evaluate matrix interferences. The blank matrix samples were extracted without the addition of internal standard and analyzed using the method. No interferences for were detected.

Carryover

To evaluate carryover, high concentrations (20 times the target decision point concentration) of blood fortified with the drug of interest were prepared and analyzed. A blank sample was also analyzed. The high concentration extract was analyzed with the instrument three times, each immediately followed by analysis of the blank extract. Analysis of the data showed that there is no carryover of the high concentration sample into the following blank injections.

Ionization Suppression/Enhancement

The post-extraction addition approach was used to assess ionization suppression/enhancement. Two different sets of samples are prepared and the analyte peak areas of neat standards are compared to matrix samples fortified with neat standards after extraction.

Set one consists of neat standards prepared at a high and low concentration for each compound (20x and 2x the decision point). The neat standards were injected a minimum of six times to establish a mean peak area for each concentration. Results of the two concentrations are presented in ***Table 2***.

Set two consists of ten different matrix sources. Each blank matrix sample was extracted in duplicate. After the extraction was complete, each blank matrix sample was then fortified to either the low or high concentration with each analyte. Each concentration set sample was injected one time each.

The average area of each set was used to estimate the suppression/enhancement effect at each concentration. The following equation was used to calculate the percentage of ionization suppression or enhancement and % CV. ***Table 2*** list the percentage of ionization suppression or enhancement at each concentration for each compound.

$$[1] \quad Ionization\ suppression\ or\ enhancement\ (\%) = \left(\frac{Average\ Area\ of\ Set\ 2}{Average\ Area\ of\ Set\ 1} - 1\right) x\ 100$$

[1] Strategies for the Assessment of Matrix Effect in Quantitative Bioanalytical Methods Based on HPLC−MS/MS, B. K. Matuszewski, M. L. Constanzer, and, and C. M. Chavez-Eng, Anal. Chem. 2003 75 (13), 3019-3030.

Drug Identification & Confirmation by LC/MS/MS

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Table 2: Ionization Enhancement/Suppression

	Average Peak Areas		Average Peak Areas		Average Peak Areas		Average Peak Areas	
	Low	High	Low	High	Low	High	Low	High
	Pseudoephedrine		Methiopropamine		Methylone		Codeine	
Set 1	30981430	81943693	2515110	7278922	6559520	16499973	67991	136459.3
Set 2	10481185.5	50856399	780351.6	3246010	2835914	13657024	34736.8	152119.8
% Enhncmnt/(Suppr)	-66.17	-37.94	-68.97	-55.41	-56.77	-17.23	-48.91	11.48
% CV	6.69	5.43	7.39	6.25	5.54	3.96	6.98	7.95
	Average Peak Areas		**Average Peak Areas**		**Average Peak Areas**		**Average Peak Areas**	
	Low	**High**	**Low**	**High**	**Low**	**High**	**Low**	**High**
	Amphetamine		**Oxycodone**		**6-MAM**		**Methamphetamine**	
Set 1	3829846.8	12658331	138304.5	304673	40978.7	117496.2	6036401	23365532.2
Set 2	1709188.6	9638625	73788.3	315056.1	22313.2	116904.9	3602658	19326843.1
% Enhancement/ (Suppression)	-55.37	-23.86	-46.65	3.41	-45.55	-0.50	-40.32	-17.28
% CV	9.08	4.81	14.48	7.78	15.47	7.93	8.20	4.20
	Average Peak Areas		**Average Peak Areas**		**Average Peak Areas**		**Average Peak Areas**	
	Low	**High**	**Low**	**High**	**Low**	**High**	**Low**	**High**
	Ethylone		**Hydrocodone**		**Phentermine**		**MDMA**	
Set 1	6712424	17701944	301545.7	892789	4727226	15599319	7013868	22448939
Set 2	3992561	14800727	161303.7	777113.2	2950330	13614037	4029273	19223507
% Enhancement/ (Suppression)	-40.52	-16.39	-46.51	-12.96	-37.59	-12.73	-42.55	-14.37
% CV	8.65	5.11	9.68	4.24	6.74	3.66	11.13	7.51
	Average Peak Areas		**Average Peak Areas**		**Average Peak Areas**		**Average Peak Areas**	
	Low	**High**	**Low**	**High**	**Low**	**High**	**Low**	**High**
	Caffeine		**BE**		**Tramadol**		**n-Desmethyltramadol**	
Set 1	1088004	4635441	7775807	31491353	9843307	28074537	14775005	38325669
Set 2	2127731	4810856	6398559	29611702	7896898	27991314	10819447	37040026
% Enhancement/ (Suppression)	95.56	3.78	-17.71	-5.97	-19.77	0.30	-26.77	-3.35
% CV	58.73	14.29	10.74	3.68	6.22	3.14	8.20	1.42
	Average Peak Areas		**Average Peak Areas**		**Average Peak Areas**		**Average Peak Areas**	
	Low	**High**	**Low**	**High**	**Low**	**High**	**Low**	**High**
	Cocaine		**Zolpidem**		**Chlordiazepoxide**		**Meprobamate**	
Set 1	5225668	10716941	3232078	10272182	3257821	10957646	33830417	50351760
Set 2	4061768	10131558	2505908	10343609	2399111	10289862	26350245	49256536
% Enhancement/ (Suppression)	-22.27	-5.46	-22.47	0.70	-26.36	-6.09	-22.11	-2.18
% CV	3.96	5.30	6.77	5.52	7.01	6.53	5.12	1.23

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	Average Peak Areas		Average Peak Areas		Average Peak Areas		Average Peak Areas	
	Low	High	Low	High	Low	High	Low	High
	Trazodone		PCP		Dextromethorphan		Diphenhydramine	
Set 1	3263820	8784639	8634551	33902960	2484416	8816129	32825661	59586931
Set 2	2381710	8558473	7806750	34681210	1701682	8478124	25071822	52870382
[1] % EE/(Suppr)	-27.03	-2.57	-9.59	2.30	-31.51	-3.83	-23.62	-11.27
% CV	4.01	4.92	7.24	4.70	6.58	4.73	8.17	1.48
	Average Peak Areas		Average Peak Areas		Average Peak Areas		Average Peak Areas	
	Low	High	Low	High	Low	High	Low	High
	Midazolam		Flurazepam		Cyclobenzaprine		Nortriptyline	
Neat	2062941	7015459	1759836.7	5450462	1711863	6462559	2849405	102915277
Blank Matrix	1560127	7552946	1416259.2	5535664	915907	551829.6	1172152	8140935
[1] % EE/(Suppr)	-24.37	7.66	-19.52	1.56	-46.50	-14.61	-58.86	-20.89
% CV	6.07	3.76	5.37	4.18	9.68	4.97	14.19	6.06
	Average Peak Areas		Average Peak Areas		Average Peak Areas		Average Peak Areas	
	Low	High	Low	High	Low	High	Low	High
	Oxazepam		Amitriptyline		Methadone		Clonazepam	
Neat	1565073	19650617	474839.7	2217793	12797464	506951212	82896.3	584155.7
Blank Matrix	1389991	7757968	269894.2	1832978	10487914	51369991	114871.6	750418.9
[1] % EE/(Suppr)	-11.19	-60.52	-43.16	-17.35	-18.05	1.33	38.57	28.46
% CV	5.13	3.83	11.24	4.50	6.05	4.72	6.83	3.88
	Average Peak Areas		Average Peak Areas		Average Peak Areas		Average Peak Areas	
	Low	High	Low	High	Low	High	Low	High
	Carisoprodol		Lorazepam		Nordiazepam		Alprazolam	
Neat	34352234	63699639	342641.8	1722496	582987.7	2529640.5	114965.2	530138.5
Blank Matrix	288374423	62445260	291090.4	1678787	275299.7	1552872.3	131295.8	623905.8
[1] % EE/(Suppr)	-16.05	-1.97	-15.12	-2.54	-52.78	-38.61	14.20	17.69
% CV	5.73	0.90	5.52	5.28	6.59	3.94	5.79	2.59
	Average Peak Areas		Average Peak Areas		Average Peak Areas		Average Peak Areas	
	Low	High	Low	High	Low	High	Low	High
	Flunitrazepam		Temazepam		Diazepam		Prazepam	
Neat	435591.8	1721663	1226351.8	6324941	786458.8	2810601.5	1093593	4460037.5
Blank Matrix	312727.3	1628222	1134084.8	6242084	564253.2	2716065.5	578295	3920800.4
[1] % EE/(Suppr)	-28.21	-5.43	-7.52	-1.31	-28.25	-3.36	-47.12	-12.09
% CV	6.70	3.74	11.17	5.22	5.83	2.83	9.11	4.41

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	Average Peak Areas		Average Peak Areas		Average Peak Areas	
	Low	High	Low	High	Low	High
	Meth-d8		PCP-d5		Prazepam-d5	
Neat	6979861.5	38678343	5675285	37880325	2925342	19262346
Blank Matrix	4211068	26757944	5484792	33939386	1611739	11261721
[2] % EE/(Suppr)	-39.67	-30.82	-3.36	-10.40	-44.90	-41.54
% CV	6.06	5.19	2.57	3.52	6.64	7.11

[2]% Enhancement/Suppression

Limit of Detection

The method limit of detection (LOD) was evaluated using the cutoff for each analyte. Three separate analytical runs consisting of three replicates each were analyzed. In all samples, the ion ratios were within 30% of the values set in the first sample of the LOD run and the retention times of each compound has % RSD of less than one percent (Table 4).

Table 4: LOD Peak Area, Retention Times and Ion Ratios

	6-MAM			Alprazolam		
Sample	Peak Area	Retention Time (min)	Ion Ratio (165/211)	Peak Area	Retention Time (min)	Ion Ratio (281/205)
Run 1 Rep 1	3529	1.06	48	14630	3.59	101
Run 1 Rep 2	3209	1.05	28	16407	3.59	102
Run 1 Rep 3	3046	1.05	64	15714	3.59	92
Run 2 Rep 1	2080	1.07	55	13368	3.62	124
Run 2 Rep 2	2848	1.08	53	14590	3.62	113
Run 2 Rep 3	3340	1.07	24	15707	3.61	89
Run 3 Rep 1	3497	1.09	39	14927	3.64	99
Run 3 Rep 2	2339	1.09	57	10865	3.65	91
Run 3 Rep 3	4258	1.05	22	11533	3.64	85
Std Dev.	654	0.016	16	1911	0.02	12
Average	3127	1.07	43	14193	3.62	100
%RSD	21	1.53	36	13	0.65	13

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Sample	Amitriptyline Peak Area	Amitriptyline Retention Time (min)	Amitriptyline Ion Ratio (105/233)	Amphetamine Peak Area	Amphetamine Retention Time (min)	Amphetamine Ion Ratio (91/65)	Amphetamine Ion Ratio (91/65)
Run 1 Rep 1	27008	3.21	82	50584	0.92	59	20
Run 1 Rep 2	34607	3.2	75	88638	0.92	36	21
Run 1 Rep 3	28290	3.27	79	80592	0.92	45	17
Run 2 Rep 1	27870	3.28	79	83820	0.93	44	20
Run 2 Rep 2	35768	3.27	75	250592	0.9	51	39
Run 2 Rep 3	36078	3.2	93	89950	0.93	41	18
Run 3 Rep 1	51048	3.28	82	118223	0.96	45	20
Run 3 Rep 2	48837	3.28	77	95249	0.96	43	21
Run 3 Rep 3	49042	3.28	70	87939	0.88	50	21
Std Dev.	9658	0.037	6	57291	0.03	7	7
Average	37616	3.25	79	105065	0.92	46	22
%RSD	26	1.14	8	55	2.76	14	30

Sample	Benzoylecgonine Peak Area	Benzoylecgonine Retention Time (min)	Benzoylecgonine Ion Ratio (168/105)	Carsioprodol Peak Area	Carsioprodol Retention Time (min)	Carsioprodol Ion Ratio (97/158)
Run 1 Rep 1	665912	1.32	35	3827573	3.48	48
Run 1 Rep 2	721917	1.32	35	4246625	3.47	45
Run 1 Rep 3	656057	1.32	36	4101558	3.47	49
Run 2 Rep 1	642433	1.33	35	4357265	3.51	46
Run 2 Rep 2	694374	1.34	36	4419356	3.52	47
Run 2 Rep 3	604621	1.33	36	4214706	3.51	47
Run 3 Rep 1	681135	1.35	39	4584646	3.54	47
Run 3 Rep 2	552710	1.35	38	3873151	3.55	47
Run 3 Rep 3	504087	1.33	37	3632540	3.54	47
Std Dev.	70256	0.012	1	309781	0.03	1
Average	635916	1.33	36	4139713	3.51	47
%RSD	11	0.90	4	7	0.88	2

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	Chlordiazepoxide			Clonazepam		
Sample	Peak Area	Retention Time (min)	Ion Ratio (227/283)	Peak Area	Retention Time (min)	Ion Ratio (270/214)
Run 1 Rep 1	197473	1.83	87	15837	3.29	30
Run 1 Rep 2	188391	1.82	84	13737	3.29	37
Run 1 Rep 3	201984	1.82	79	15076	3.29	25
Run 2 Rep 1	155511	1.86	84	10408	3.33	31
Run 2 Rep 2	164164	1.86	86	11592	3.34	40
Run 2 Rep 3	157858	1.85	87	12145	3.33	31
Run 3 Rep 1	203314	1.87	85	14875	3.36	28
Run 3 Rep 2	168472	1.88	83	13691	3.36	29
Run 3 Rep 3	171788	1.87	83	13971	3.35	38
Std Dev.	19138	0.02	2	1774	0.03	5
Average	178773	1.85	84	13481	3.33	32
%RSD	11	1.22	3	13	0.89	16

	Cocaine				Codeine		
Sample	Peak Area	Retention Time (min)	Ion Ratio (182/81)	Ion Ratio (182/105)	Peak Area	Retention Time (min)	Ion Ratio (165/198)
Run 1 Rep 1	260718	1.56	32	23	3310	0.89	33
Run 1 Rep 2	389636	1.56	30	21	3504	0.89	78
Run 1 Rep 3	363680	1.56	28	20	3158	0.88	92
Run 2 Rep 1	288106	1.58	35	25	3181	0.88	68
Run 2 Rep 2	299322	1.59	33	23			
Run 2 Rep 3	362144	1.58	27	21	3507	0.9	36
Run 3 Rep 1	378525	1.59	29	20	3883	0.93	26
Run 3 Rep 2	319294	1.6	27	20	4852	0.92	35
Run 3 Rep 3	283237	1.59	30	22	5465	0.84	41
Std Dev.	47177	0.02	3	2	851	0.03	25
Average	327185	1.58	30	22	3858	0.89	51
%RSD	14	0.97	9	8	22	3.08	48

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	Cyclobenzaprine			Desmethyltramadol		
Sample	Peak Area	Retention Time (min)	Ion Ratio (215/84)	Peak Area	Retention Time (min)	Ion Ratio
Run 1 Rep 1	68273	3	48	896807	1.48	
Run 1 Rep 2	73281	2.99	48	981378	1.48	
Run 1 Rep 3	91307	2.99	45	945665	1.48	
Run 2 Rep 1	75137	3.06	48	907799	1.5	
Run 2 Rep 2	63623	3.07	52	921804	1.5	
Run 2 Rep 3	59154	3.06	52	959393	1.5	
Run 3 Rep 1	93207	3.06	47	870687	1.51	
Run 3 Rep 2	73699	3.07	49	785738	1.52	
Run 3 Rep 3	74484	3.07	49	753316	1.5	
Std Dev.	11332	0.04	2	77093	0.01	
Average	74685	3.04	49	891398	1.50	
%RSD	15	1.19	5	9	0.94	

	Dextromethorphan			Diazepam		
Sample	Peak Area	Retention Time (min)	Ion Ratio (147/171)	Peak Area	Retention Time (min)	Ion Ratio (154/193)
Run 1 Rep 1	124025	2.13	100	58357	3.92	42
Run 1 Rep 2	151688	2.13	102	56421	3.92	46
Run 1 Rep 3	152283	2.13	103	58091	3.92	43
Run 2 Rep 1	146566	2.18	108	62591	3.93	43
Run 2 Rep 2	167380	2.19	98	62635	3.94	43
Run 2 Rep 3	159849	2.17	99	54754	3.93	43
Run 3 Rep 1	182580	2.19	99	20702	3.95	44
Run 3 Rep 2	161480	2.2	102	49994	3.95	42
Run 3 Rep 3	158040	2.2	101	45961	3.95	47
Std Dev.	15924	0.03	3	12989	0.01	2
Average	155987	2.17	101	52167	3.93	44
%RSD	10	1.41	3	25	0.34	4

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Sample	Diphenhydramine				Ethylone			
	Peak Area	Retention Time (min)	Ion Ratio (167/165)	Ion Ratio (167/152)	Peak Area	Retention Time (min)	Ion Ratio (173/204)	Ion Ratio (173/146)
Run 1 Rep 1	742824	2.24	18	18	303573	1.04	57	37
Run 1 Rep 2	1665219	2.24	16	18	397864	1.04	57	33
Run 1 Rep 3	1492537	2.24	17	19	318744	1.04	60	37
Run 2 Rep 1	1969879	2.3	17	18	476331	1.06	60	34
Run 2 Rep 2	2084639	2.3	17	18	465316	1.07	62	35
Run 2 Rep 3	2004757	2.29	17	19	499943	1.05	64	37
Run 3 Rep 1	2311344	2.3	17	18	487087	1.07	59	36
Run 3 Rep 2	2075993	2.31	18	18	425452	1.08	61	38
Run 3 Rep 3	1954781	2.31	17	18	381825	1.03	65	39
Std Dev.	466713	0.03	1	0.4	72319	0.02	3	2
Average	1811330	2.28	17	18	417348	1.05	61	36
%RSD	26	1.38	4	2	17	1.64	5	5

Sample	Flunitrazepam			Flurazepam		
	Peak Area	Retention Time (min)	Ion Ratio (268/239)	Peak Area	Retention Time (min)	Ion Ratio (315/288)
Run 1 Rep 1	30136	3.62	21	1555523	2.44	10
Run 1 Rep 2	34812	3.61	15	164083	2.43	10
Run 1 Rep 3	31474	3.61	18	154421	2.43	10
Run 2 Rep 1	26823	3.64	21	129404	2.49	11
Run 2 Rep 2	26366	3.65	21	134899	2.49	10
Run 2 Rep 3	26389	3.4	21	141233	2.48	10
Run 3 Rep 1	32708	3.66	21	147527	2.5	11
Run 3 Rep 2	27216	3.67	20	127444	2.52	10
Run 3 Rep 3	27240	3.66	18	127669	2.51	9
Std Dev.	3146	0.08	2	471733	0.03	1
Average	29240	3.61	20	298023	2.48	10
%RSD	11	2.30	11	158	1.40	6

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Sample	Hydrocodone Peak Area	Hydrocodone Retention Time (min)	Hydrocodone Ion Ratio (198/171)	Lorazepam Peak Area	Lorazepam Retention Time (min)	Lorazepam Ion Ratio (275/229)
Run 1 Rep 1	15261	1.07	27	32272	3.41	45
Run 1 Rep 2	19368	1.07	27	32542	3.4	37
Run 1 Rep 3	13578	1.07	58	30276	3.4	45
Run 2 Rep 1	9537	1.09	28	22883	3.45	42
Run 2 Rep 2	17457	1.1	29	22106	3.45	50
Run 2 Rep 3	15455	1.08	48	26173	3.44	43
Run 3 Rep 1	16443	1.1	37	30107	3.47	48
Run 3 Rep 2	16313	1.1	29	29593	3.5	23
Run 3 Rep 3	14691	1.06	27	28877	3.49	45
Std Dev.	2741	0.02	11	3794	0.04	8
Average	15345	1.08	34	28314	3.45	42
%RSD	18	1.44	33	13	1.08	19

Sample	MDMA Peak Area	MDMA Retention Time (min)	MDMA Ion Ratio (163/105)	MDMA Ion Ratio (163/77)	Meprobamate Peak Area	Meprobamate Retention Time (min)	Meprobamate Ion Ratio (158/97)
Run 1 Rep 1	218948	1.07	57	34	4477001	1.78	77
Run 1 Rep 2	327444	1.08	55	33	4198461	1.78	79
Run 1 Rep 3	283122	1.07	51	35	4320051	1.78	76
Run 2 Rep 1	404347	1.09	53	33	3619824	1.8	76
Run 2 Rep 2	429329	1.1	49	33	3923987	1.81	75
Run 2 Rep 3	361803	1.09	51	35	4017818	1.8	79
Run 3 Rep 1	410641	1.1	53	33	4744052	1.83	75
Run 3 Rep 2	356436	1.1	55	35	3991218	1.83	76
Run 3 Rep 3	323026	1.06	53	34	3929247	1.82	78
Std Dev.	66945	0.02	2	1	337796	0.02	2
Average	346122	1.08	53	34	4135740	1.80	77
%RSD	19	1.39	5	3	8	1.14	2

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	Methadone				Methamphetamine		
Sample	Peak Area	Retention Time (min)	Ion Ratio (265/105)	Ion Ratio (265/57)	Peak Area	Retention Time (min)	Ion Ratio (91/119)
Run 1 Rep 1	518672	3.34	55	28	132676	1.03	31
Run 1 Rep 2	841482	3.34	56	27	220902	1.03	28
Run 1 Rep 3	754845	3.33	59	27	213055	1.03	30
Run 2 Rep 1	766868	3.42	57	29	289630	1.04	28
Run 2 Rep 2	698757	3.4	58	28	271284	1.05	32
Run 2 Rep 3	751781	3.39	59	29	275338	1.04	28
Run 3 Rep 1	869963	3.4	58	28	327762	1.06	29
Run 3 Rep 2	816983	3.41	58	28	294294	1.06	33
Run 3 Rep 3	792855	3.42	58	29	232083	1.01	29
Std Dev.	103140	0.04	1	1	58059	0.02	2
Average	756912	3.38	58	28	250780	1.04	30
%RSD	14	1.08	2	3	23	1.56	6

	Methamphetamine-d8			Methiopropamine			
Sample	Peak Area	Retention Time (min)	Ion Ratio (92/124)	Peak Area	Retention Time (min)	Ion Ratio (97/58)	Ion Ratio (97/125)
Run 1 Rep 1	201185	1.02	47	36588	0.79	72	58
Run 1 Rep 2	293745	1.02	51	67386	0.78	76	52
Run 1 Rep 3	288043	1.02	42	73963	0.78	90	52
Run 2 Rep 1	390663	1.03	50	83797	0.78	96	66
Run 2 Rep 2	368044	1.04	49	96915	0.8	73	51
Run 2 Rep 3	372909	1.03	49	82970	0.79	93	63
Run 3 Rep 1	383104	1.05	50	79209	0.83	84	58
Run 3 Rep 2	418303	1.05	49	89536	0.82	78	47
Run 3 Rep 3	346553	1	48	85084	0.72	71	50
Std Dev.	67617	0.02	3	17464	0.03	10	6
Average	340283	1.03	48	77272	0.79	81	55
%RSD	20	1.57	5	23	3.95	12	12

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	Methylone				Midazolam		
Sample	Peak Area	Retention Time (min)	Ion Ratio (160/132)	Ion Ratio (160/190)	Peak Area	Retention Time (min)	Ion Ratio (291/244)
Run 1 Rep 1	172094	0.88	44	38	254006	2.26	22
Run 1 Rep 2	223776	0.88	46	45	251855	2.25	22
Run 1 Rep 3	277453	0.88	48	44	256457	2.25	21
Run 2 Rep 1	277453	0.88	48	44	241752	2.31	21
Run 2 Rep 2	250592	0.9	51	39	236564	2.31	21
Run 2 Rep 3	282617	0.89	49	43	235631	2.3	21
Run 3 Rep 1	312549	0.92	46	39	250879	2.31	22
Run 3 Rep 2	282705	0.92	43	42	235600	2.33	21
Run 3 Rep 3	232510	0.83	52	42	228261	2.32	21
Std Dev.	42120	0.03	3	3	10064	0.03	1
Average	256861	0.89	47	42	243445	2.29	21
%RSD	16	3.04	6	6	4	1.36	2

	Nordiazepam			Nortriptyline			
Sample	Peak Area	Retention Time (min)	Ion Ratio (140/165)	Peak Area	Retention Time (min)	Ion Ratio (233/91)	Ion Ratio (233/218)
Run 1 Rep 1	59139	3.4	46	78193	3.1	70	19
Run 1 Rep 2	47649	3.4	47	77061	3.1	70	20
Run 1 Rep 3	48840	3.4	53	96524	3.09	69	21
Run 2 Rep 1	43706	3.44	49	89800	3.17	70	17
Run 2 Rep 2	39128	3.44	43	67861	3.17	77	19
Run 2 Rep 3	45323	3.44	44	66851	3.16	70	21
Run 3 Rep 1	53473	3.47	47	124101	3.17	69	20
Run 3 Rep 2	48337	3.5	44	109068	3.18	66	21
Run 3 Rep 3	46727	3.49	45	92067	3.18	76	22
Std Dev.	5711	0.04	3	19034	0.04	3	1.5
Average	48036	3.44	46	89058	3.15	71	20
%RSD	12	1.11	7	21	1.21	5	7.5

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	Oxazepam			Oxycodone		
Sample	Peak Area	Retention Time (min)	Ion Ratio (241/269)	Peak Area	Retention Time (min)	Ion Ratio (212/174)
Run 1 Rep 1	129110	3.17	70	5734	1.01	25
Run 1 Rep 2	127059	3.16	65	4412	1	37
Run 1 Rep 3	144164	3.16	60	5149	1.01	56
Run 2 Rep 1	86653	3.21	68	9290	1.01	20
Run 2 Rep 2	97192	3.22	66	8338	1.03	67
Run 2 Rep 3	94964	3.21	70	5275	1.01	29
Run 3 Rep 1	132365	3.23	63	6738	1.04	24
Run 3 Rep 2	118781	3.24	66	6400	1.03	24
Run 3 Rep 3	112180	3.23	72	5915	0.98	22
Std Dev.	19477	0.03	4	1568	0.02	17
Average	115830	3.20	67	6361	1.01	34
%RSD	17	0.99	6	25	1.78	49

	PCP			PCP-d5		
Sample	Peak Area	Retention Time (min)	Ion Ratio (86/91)	Peak Area	Retention Time (min)	Ion Ratio
Run 1 Rep 1	147756	1.97	61	130002	1.96	
Run 1 Rep 2	419313	1.97	66	346284	1.95	
Run 1 Rep 3	399331	1.97	73	368027	1.95	
Run 2 Rep 1	525628	2.01	73	576361	2	
Run 2 Rep 2	486626	2.02	73	517422	2	
Run 2 Rep 3	573963	2.01	71	549486	1.99	
Run 3 Rep 1	673210	2.02	65	629665	2.01	
Run 3 Rep 2	557538	2.03	72	602137	2.02	
Run 3 Rep 3	519111	2.03	73	530785	2.01	
Std Dev.	148479	0.03	5	161162	0.03	
Average	478053	2.00	70	472241	1.99	
%RSD	31	1.30	6	34	1.37	

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Sample	Phentermine Peak Area	Phentermine Retention Time (min)	Phentermine Ion Ratio (91/133)	Prazepam Peak Area	Prazepam Retention Time (min)	Prazepam Ion Ratio (271/140)
Run 1 Rep 1	104165	1.15	18	49623	4.33	33
Run 1 Rep 2	160029	1.16	22	51729	4.32	33
Run 1 Rep 3	157513	1.15	23	53705	4.32	33
Run 2 Rep 1	138001	1.18	23	57419	4.33	32
Run 2 Rep 2	190449	1.18	17	45889	4.33	33
Run 2 Rep 3	193327	1.16	21	41722	4.32	31
Run 3 Rep 1	214905	1.18	22	60406	4.33	32
Run 3 Rep 2	201755	1.19	19	53219	4.34	32
Run 3 Rep 3	209156	1.15	20	50479	4.33	32
Std Dev.	37017	0.02	2	5633	0.01	1
Average	174367	1.17	21	51577	4.33	32
%RSD	21	1.36	11	11	0.15	2

Sample	Prazepam-d5 Peak Area	Prazepam-d5 Retention Time (min)	Prazepam-d5 Ion Ratio (276/140)	Pseudoephedrine Peak Area	Pseudoephedrine Retention Time (min)	Pseudoephedrine Ion Ratio (148/91)	Pseudoephedrine Ion Ratio (148/133)
Run 1 Rep 1	256773	4.32	20	784885	0.78	25	21
Run 1 Rep 2	264322	4.31	20	962081	0.77	24	20
Run 1 Rep 3	288954	4.31	19	1046753	0.78	22	19
Run 2 Rep 1	329531	4.32	19	1460158	0.77	25	19
Run 2 Rep 2	256337	4.32	19	1599225	0.79	26	20
Run 2 Rep 3	222766	4.32	20	1325620	0.78	25	21
Run 3 Rep 1	302099	4.32	20	1354951	0.82	26	20
Run 3 Rep 2	284984	4.33	19	1185172	0.81	25	19
Run 3 Rep 3	271026	4.32	19	998203	0.71	24	20
Std Dev.	30701	0.01	1	264443	0.03	1	1
Average	275199	4.32	19	1190783	0.78	25	20
%RSD	1	0.14	3	22	3.98	5	4

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Sample	Temazepam Peak Area	Temazepam Retention Time (min)	Temazepam Ion Ratio (255/283)	Tramadol Peak Area	Tramadol Retention Time (min)	Tramadol Ion Ratio
Run 1 Rep 1	167112	3.73	40	603331	1.47	
Run 1 Rep 2	162570	3.72	37	934143	1.46	
Run 1 Rep 3	164941	3.72	41	846859	1.46	
Run 2 Rep 1	118114	3.75	37	967566	1.49	
Run 2 Rep 2	134665	3.75	37	975942	1.49	
Run 2 Rep 3	129090	3.74	37	1013637	1.48	
Run 3 Rep 1	159279	3.77	38	1024858	1.49	
Run 3 Rep 2	154074	3.77	38	886884	1.5	
Run 3 Rep 3	148360	3.77	39	887414	1.49	
Std Dev.	17511	0.02	1	127957	0.01	
Average	148689	3.75	38	904515	1.48	
%RSD	12	0.55	4	14	0.98	

Sample	Trazodone Peak Area	Trazodone Retention Time (min)	Trazodone Ion Ratio (176/148)	Trazodone Ion Ratio (176/78)	Zolpidem Peak Area	Zolpidem Retention Time (min)	Zolpidem Ion Ratio (235/263)
Run 1 Rep 1	339845	1.86	97	43	237814	1.72	34
Run 1 Rep 2	356281	1.86	100	45	248504	1.72	36
Run 1 Rep 3	361254	1.86	99	48	237315	1.72	37
Run 2 Rep 1	355749	1.9	95	42	191778	1.75	39
Run 2 Rep 2	343545	1.91	86	45	217838	1.76	37
Run 2 Rep 3	338522	1.9	93	45	212096	1.75	38
Run 3 Rep 1	388189	1.91	92	44	254181	1.77	36
Run 3 Rep 2	319856	1.92	97	49	210161	1.78	36
Run 3 Rep 3	321335	1.92	96	46	209079	1.77	37
Std Dev.	21191	0.03	4	2	20948	0.02	1
Average	347175	1.89	95	45	224307	1.75	37
%RSD	6	1.37	4	5	9	1.35	4

Drug Identification & Confirmation by LC/MS/MS
Validation Report

Table 5: Summary of Validation Results

Parameters:	Desired Limit:	Results
Interference Studies	No interfering signal from matrix, internal standard, common drugs of abuse (including other common opiates/metabolites), OTC drugs, and prescription medications	No interferences noted.
Carryover	Carryover after a high concentration sample must be less than one-half of the mean LOD peak area.	No carryover noted.
Ionization Suppression/ Enhancment	Must be less than 25% and have a %CV of less than 15% at high (20 times LOD) and low (2 times LOD) concentrations. If these values are exceeded, it must be demonstrated that there is no negative impact on LOD.	Ionization suppression was determined to not prohibit detection of any validation compound at target LOD.
Limit of Detection	Target LOD concentrations are: 5 ng/mL – 6-acetylmorphine 10 ng/mL – alprazolam, amphetamine, clonazepam, cocaine, codeine, cyclobenzaprine, flunitrazepam, hydrocodone, lorazepam, methamphetamine, methiopropamine, oxycodone, phencyclidine, prazepam, zolpidem 20 ng/mL – dextromethorphan, diazepam, ethylone, MDMA, methadone, methylone, nordiazepam, n-desmethyltramadol, oxazepam, phentermine, temazepam, tramadol 25 ng/mL – amitriptyline, diphenhydramine, notriptyline, trazodone 50 ng/mL – benzoylecgonine, caffeine, chlordiazepoxide, midazolam, zopiclone 100 ng/mL – pseudoephedrine 125 ng/mL – carisoprodol, meprobamate	All target compounds detected at the listed concentrations.

Drug Identification & Confirmation by LC/MS/MS

Validation Report

No.	Drug	Concentration ng/mL	2x	20x
1	Alprazolam	10	20	200
2	Amitriptyline	25	25	250
3	Amphetamine	10	20	200
4	Benzoylecgonine	50	100	1000
5	Caffeine	50-100		
6	Carisoprodol	500	1000	10000
7	Chlordiazepoxide	50	100	1000
8	Clonazepam	10	20	200
9	Cocaine	10	20	200
10	Cyclobenzaprine	10	20	200
11	Dextromethorphan	20	40	400
12	Diazepam	20	40	400
13	Diphenhydramine	25	50	500
14	Estazolam	25	50	500
15	Ethylone	20	40	400
16	Flunitrazepam	10	20	200
17	Flurazepam	10	20	200
18	Lorazepam	10	20	200
19	MDMA	20	40	400
20	Meprobamate	500	1000	10000
21	Methadone	20	40	400
22	Methamphetamine	10	20	200
23	Methiopropamine	10	20	200
24	Methylone	20	40	400
25	Midazolam	50	100	1000
26	N-desmethyltramadol	20	40	400
27	Nordiazepam	20	40	400
28	Nortriptyline	25	50	500
29	Oxazepam	20	40	400
30	Phenazepam	25	50	500
31	Phencyclidine	10	20	200
32	Phentermine	20	40	400
33	Prazepam	10	20	200
34	Pseudoephedrine	100	200	2000
35	Tramadol	20	40	400
36	Trazodone	25	50	500
37	Zaleplon	10	20	200
38	Zolpidem	10	20	200
39	Zopiclone	50	100	1000

SUBJECT: **DRUG IDENTIFICATION & CONFIRMATION BY LC/MS/MS**

1. PURPOSE

In this procedure an extracting solvent is added to a sample, precipitating proteinaceous material. The sample is centrifuged and the supernatant is collected and evaporated to dryness. The sample is then reconstituted and injected onto the LC/MS/MS for identification and confirmation.

2. ASSOCIATED PROTOCOL(S)

2.1 OSBI Laboratory's Criminalistic Services Division Quality Manual

2.2 OSBI Policy #121.1 OSBI Chemical Hygiene Plan

3. SAMPLE(S)

Preferred samples are fluoridated blood collected from outside agencies intended for human performance testing.

4. REAGENTS

4.1 Acetonitrile, LCMS reagent grade

4.2 Methanol, LCMS reagent grade

4.3 0.1% Formic Acid

4.4 Bovine, synthetic or human drug-free blood

5. SUPPLIES

5.1 Disposable Microcentrifuge Tubes

5.2 Five mL Disposable Conical Centrifuge Tubes with PTFE Lined Screw Caps

5.3 Vortexer

5.4 Disposable Pasteur Pipettes

5.5 Eppendorf Pipettors - Fixed volume (10, 20, 25, 40, 50, 100, 200, 250 and 500µL)

5.6 Eppendorf Pipettor - Variable volume (500-5000 µL)

5.7 Nitrogen Evaporator

5.8 Microcentrifuge

5.9 Volumetric Flasks

6. APPARATUS AND MATERIALS

6.1 Liquid Chromatograph-Tandem Mass Spectrometer: Shimadzu LC-MS 8030

6.2 Column: Phenomenex, Kinetex 2.6u C18 100A, Size 75 x 2.10 mm

6.3 Nitrogen Generator

6.4 Argon Supply

7. SOLUTIONS

7.1 Mobile Phase A (dH_2O with 0.1% formic acid): Add 4 mL of formic acid to 3.996 L dH_2O. Stored at room temperature up to 6 months.

7.2 Mobile Phase B (ACN with 0.1% formic acid): Add 4 mL of formic acid to 3.996 L acetonitrile (ACN). Stored at room temperature up to 6 months.

SUBJECT: **DRUG IDENTIFICATION & CONFIRMATION BY LC/MS/MS**

7.3 Reconstitution solution, dH_2O: ACN (9:1) with 0.1% formic acid: Add 5 mL of acetonitrile and 45 mL dH_2O into a graduated cylinder and add 50µL of formic acid. Stored at room temperature up to one year.

8. INTERNAL STANDARDS AND QUALITY CONTROLS

Internal Standard

8.1 Primary internal standards (100 µg/mL): Methamphetamine-d8 (ISTD), PCP-d5 (ISTD) and Prazepam-d5 (ISTD).

8.2 Acetonitrile containing internal standards: Add 200 µL of each deuterated primary internal standard to 250 mL of acetonitrile. Store in the freezer for up to one year.

Negative Control

8.3 Negative Control: Add 250µL of drug-free whole blood in a microcentrifuge tube.

Working Multi-Drug Control

8.4 Primary standards (1.0 mg/mL): See ***Table 1*** for CRM's.

8.5 Secondary working solution: Transfer volume of all non deuterated primary standard(s) listed in ***Table 1*** into a 100 mL volumetric flask. Dilute to the mark with dH_2O.

8.6 Tertiary working solution: Transfer 5 mL of secondary solution to a 10mL volumetric flask and dilute to the mark with dH_2O.

8.7 Working multi-drug control: Transfer 25 µL of tertiary working solution to 225 µL of drug-free whole blood in a microcentrifuge tube.

Working Positive Control

8.8 Primary standards (1.0 mg/mL): See ***Table 1*** for CRM's.

8.9 Positive control secondary solution: Transfer volume of all primary standard listed in ***Table 1*** into a 100 mL volumetric flask. Dilute to the mark with dH_2O.

8.10 Positive control tertiary solution: Transfer 5 mL of positive control secondary solution to a 10mL volumetric flask. Dilute to the mark with dH_2O.

8.11 Working positive control: Transfer 50 µL of tertiary solution to 200 µL of drug-free whole blood in a microcentrifuge tube.

9. INDIVIDUAL STEPS OF PROTOCOL

9.1 Label clean disposable microcentrifuge tubes with controls and case sample IDs.

9.2 Prepare the multi-drug, positive and negative controls (see Section "Solutions, Standards and Controls"). Handle all controls in the same manner as case samples throughout extraction and analysis.

9.3 Transfer approximately 250µL of each case specimen to the appropriately labeled microcentrifuge tubes.

9.4 Add 500µL of cold acetonitrile (containing the internal standards) to each control and case specimen and vortex for approximately 30 seconds.

9.5 Centrifuge all samples at 13,000 rpm for approximately 5 minutes.

9.6 Transfer acetonitrile (top) layer to clean centrifuge tube.

9.7 Evaporate to dryness at approximately 40°C with a steady stream of nitrogen.

SUBJECT: **DRUG IDENTIFICATION & CONFIRMATION BY LC/MS/MS**

9.8 Add 100µL of reconstitution solution and vortex briefly.

9.9 Transfer sample to labeled autosampler vial and place into LCMS sample tray.

9.10 Inject 20µL of sample into LC/MS/MS.

9.11 Use the "TX-34.lcm" method for analysis.

10. QUALITATIVE IDENTIFICATION

10.1 The following are acceptable confirmatory practices in order of preference. At least one condition must be satisfied in order to identify and report a drug:

10.1.1 Identification is made by the substance class and specific identification of the substance in an aliquot of sample by a different chemical principle (e.g., immunoassay followed by LC/MS/MS).

10.1.2 Identification of the substance in one biological sample using two separate aliquots and one chemical principle (e.g., Clonazepam by LC/MS/MS).

10.2 Qualitative Chromatographic Criteria

10.2.1 The presence of the target analyte in the sample is indicated if:

10.2.1.1 The chromatographic peak shape is Gaussian.

10.2.1.2 The MRMs (i.e., precursor and products) being monitored line-up within their given retention time windows.

10.2.1.3 The area counts are equal to or greater than the area counts of the multi-drug positive control.

10.2.1.4 The ion ratios for the analyte in the sample, established by the multi-drug positive control, do not differ by more than ± 30%.

10.2.1.5 The retention time for the sample does not differ by more than ±2% of the multi-drug control.

11. QUALITY ASSURANCE REQUIREMENTS

11.1 The multi-drug, positive and negative control will be injected immediately prior to casework.

11.2 The relative ion ratios will be set for each day of analysis using the multi-drug control analyzed in each batch.

11.3 The negative control must be free of any drugs except internal standards. If not, re-inject or re-extract.

11.4 The positive and multi-drug control must be prepared from separate secondary control solutions.

11.5 The positive control areas must be greater than the cut-off peak areas.

11.6 If any problems cannot be remedied, stop casework and notify the technical manager.

11.7 The LCMS method associated with this protocol is TX-34.lcm. The TX-34.lcm method is found in the validation documents.

SUBJECT: **DRUG IDENTIFICATION & CONFIRMATION BY LC/MS/MS**

12. ANALYSIS DOCUMENTATION

A packet containing original data for all controls and standards will be prepared for each analysis run and stored with the batch on the BEAST.

13. RECOMMENDED REPORT WORDING

13.1 The result of the examination will be reported as in the following examples:

13.1.1 Results detected below the cut-off will be reported as "No drug(s) detected by LC/MS/MS."

13.1.2 A specimen is positive when its confirmatory drug test is equal to or greater than the cutoff. Positive results will be reported as "The following drug(s) were confirmed by LC/MS/MS: [*drug name*]."

14. ATTACHMENTS

Table 1: Analytical data for each of the 44 compounds in the LC/MS/MS database.

15. REFERENCES

15.1 Applications of LC-MS in Toxicology, ed. Aldo Polettini, (2006).

15.2 Analysis of Benzodiazepines in Blood by LC/MS/MS, Agilent Technologies, (2006).

15.3 The Mass Spectrometry Primer, Michael P. Balough, (2009).

16. APPROVAL

FTU Technical Manager _____ Date: 01-20-15

Matthew Stillwell

Assistant Director _____ Date: 1-20-15

Andrea Swiech

Issue Date	Revision No.	Revised By	Document History
1-20-15	0		Original Issue

SUBJECT: DRUG IDENTIFICATION & CONFIRMATION BY LC/MS/MS

Table 1: Analytical data for each of the 44 compounds in the LC/MS/MS database

Compound (Primary Stds)	Precursor ion	Product ion (Q1)	Product ion (Q2)	Product ion (Q2)	µL of 1° Std to Make 100 mL of 2° Standard
6-Acetylmorphine	328.1	165	211.2		10
Alprazolam	309	281	205		20
Amitriptyline	278.4	105	233.05		50
Amphetamine	136.2	91.1	65.1	119.1	20
Benzoylecgonine	290	168	105		100
Caffeine	195	138	42	110	100
Carisoprodol	261	97.1	158.2		250
Chlordiazepoxide	299.9	227.1	283.15		100
Clonazepam	316	270	214		20
Cocaine	304	182.05	81.95	105.1	20
Codeine	300.1	165.15	198.8		20
Cyclobenzaprine	276.05	215.1	84.1		20
Dextromethorphan	272.15	147.05	171.05		40
Diazepam	285	154	193		40
Diphenhydramine	256	167.1	165.1	152.1	50
Ethylone	222.2	173.95	204	146	40
Flunitrazepam	314	268	239		20
Flurazepam	387.9	315	288		20
Hydrocodone	300.1	198.85	171		20
Lorazepam	321	275	229		20
MDMA	194	163.1	105.15	77.1	40
Meprobamate	219.1	158.15	97.15		250
Methadone	310.2	265.15	105	57.1	40
Methamphetamine	150.2	91.1	119.1		20
Methamphetamine-d8 (IS)	158	92.95	124		
Methiopropamine	156.2	96.95	58	125.1	20
Methylone	208	160.05	208	132.05	40
Midazolam	326	291	244		100
N-Desmethyltramadol	250	44			40
Nordiazepam	270.9	140	165		40
Nortriptyline	264.15	233.05	91	218	50
Oxazepam	287	241	269		40
Oxycodone	316.1	212	174.9		20
PCP-d5 (IS)	249.3	86.15			
Phencyclidine	244.2	86.2	91.15		20
Phentermine	150.1	91	133.1		40
Prazepam	325	271.05	140		20
Prazepam-d5 (IS)	330	276	140		
Pseudoephedrine	166.1	148.05	91	132.95	200
Temazepam	301.2	255	283		40
Tramadol	264.3	58.15			40
Trazodone	372.1	176.2	148.05	78.15	50
Zolpidem	308	235	263		20
Zopiclone	389.1	244.9	216.85	139	100

Deviation Request Form

I. Explanation of Request

Name: Robert Weston Date: 02-03-2015

Applies to (Policy/Procedure): TX-34

Describe Requested Deviation: Change multi-drug preparation. Change positive control concentrations. The positive control will also be used to set the ion ratios for identification purposes.

See attached page for detailed changes. Cut-off and positive control concentrations are specifically listed.

Specify the Instance/Circumstance for which the Deviation is Requested:
This will apply to all casework.

Reason for Deviation: Ion ratios can be concentration dependent. Changing the concentration of the positive control and using that positive control to set the ion ratios for the method will better reflect what is normally encountered with actual case specimens.

II. Technical Review and Authorization

Merits:

Risks/Impact:

Duration of Authorization: Until next revision

Restrictions/Limitations:

Authorized/Rejected (signature) Date: 02-03-15

III. Quality Assurance Manager Authorization

Acceptability Within General Quality Assurance Principles? YES/NO

Significant Negative Impact to Division-Wide Quality Standards? YES/NO

Restrictions/Limitations:

Authorized/Rejected (signature) Date:

IV. Criminalistics Division Director Authorization

Authorized/Rejected (signature) Date: 2-3-15

Effective Date:

Deviations for Protocol TX-34 to be incorporated into the next revision:

8.5 Secondary Multi-drug standard: Transfer volumes of primary standards listed in Column B of Table 1 to a 100 mL volumetric flask. Dilute to the mark with water.

8.6 Tertiary multi-drug standard: Transfer 5.0 mL of the secondary multi-drug standard to a 10 mL volumetric flask. Add the volumes of primary standards listed in column C of Table 1 to the flask. Dilute to the mark with water.

8.7 Working multi-drug control: Transfer 25 uL of tertiary multi-drug standard to 225 uL of drug-free whole blood in a microcentrifuge tube.

8.9 Positive control secondary standard: Transfer volumes of primary standards listed in Column D of Table 1 to a 10 mL volumetric flask. Dilute to the mark with water.

8.10 Working positive control: Transfer 25 uL of positive control secondary standard to 225 uL of drug-free whole blood in a microcentrifuge tube.

8.11 - Rescind

10.2.1.3 The ratio of analyte peak area to internal standard peak area for the sample is greater than the same ratio in the multi-drug control.

10.2.1.4 The ion ratios for the analyte in the sample, established by the positive control, do not differ by more than ±30%.

11.2 The relative ion ratios will be set using the positive control analyzed in each batch.

11.5 – rescind

Column A	Column B	Column C	Column D	Column E	Column F
Compound (Primary Stds @ 1 mg/mL unless otherwise noted)	µL of 1° Std to Make 100 mL of 2° Multi-Drug Standard	µL of 1° Std to add to 5 mL of 2° Multi-Drug Standard, then dilute to 10 mL total volume	µL of 1° Std to Make 10 mL of Positive Control Working Solution	Cut-off concentration, ng/mL	Pos Cont concentration, ng/mL
6-Acetylmorphine (100 ug/mL)	10		50	5	50
Alprazolam	20		10	10	100
Amitriptyline	50		25	25	250
Amphetamine	20		10	10	100
Benzoylecgonine (100 ug/mL)		50	500	50	500
Caffeine	100		50	50	500
Carisoprodol		50	500	500	5000
Chlordiazepoxide	100		50	50	500
Clonazepam	20		10	10	100
Cocaine	20		10	10	100
Codeine	20		10	10	100
Cyclobenzaprine	20		10	10	100
Dextromethorphan	40		20	20	200
Diazepam	40		20	20	200
Diphenhydramine	50		25	25	250
Ethylone	40		20	20	200
Flunitrazepam	20		10	10	100
Flurazepam	20		10	10	100
Hydrocodone	20		10	10	100
Lorazepam	20		10	10	100
MDMA	40		20	20	200
Meprobamate		50	500	500	5000
Methadone	40		20	20	200
Methamphetamine	20		10	10	100
Meth-d8 (IS)					
Methiopropamine	20		10	10	100
Methylone	40		20	20	200
Midazolam	100		50	50	500
N-Desmethyltramadol	40		20	20	200
Nordiazepam	40		20	20	200
Nortriptyline	50		25	25	250
Oxazepam	40		20	20	200
Oxycodone	20		10	10	100
PCP-d5 (IS)					
Phencyclidine	20		10	10	100
Phentermine	40		20	20	200
Prazepam	20		10	10	100
Prazepam-d5 (IS)					
Pseudoephedrine	200		100	100	1000
Temazepam	40		20	20	200
Tramadol	40		20	20	200
Trazodone	50		25	25	250
Zolpidem	20		10	10	100
Zopiclone	100		50	50	500