Method Validation of Methamphetamine and its Metabolites in Equine Urine

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THESIS

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Contents

List of Tables

List of Figures

List of Abbreviations

NCU	Negative control urine
LLE	Liquid-liquid extraction
SPE	Solid-phase extraction
MA	Methamphetamine
AP	Amphetamine
OHAM	Hydroxymethamphetamine
OHAP	Hydroxyamphetamine
PPA	Phenylpropanolamine
PO	By mouth

Summary

Various precision studies were performed to validate this analysis method using negative control equine urine and standards of the target compounds; methamphetamine, amphetamine, hydroxyamphetamine, hydroxymethamphetamine, and phenylpropanolamine. Initial studies also led to changing the extraction method from liquid-liquid to solid-phase.

Once the precision studies were acceptable three independent sets of administered samples obtained from horses dosed with methamphetamine, hydroxyamphetamine, and phenylpropanolamine respectively, were extracted and analyzed using the method. The samples were taken over a range of hours after the dosing, from pre-dose (0 hours) to 48 hours. The method detected all the target compounds except phenylpropanolamine in the methamphetamine set, while the hydroxyamphetamine and phenylpropanolamine sets only had their respective compounds. This research has provided data that demonstrates this method is capable of detecting and quantifying methamphetamine and some of its metabolites in equine urine.

Introduction

The primary goal of this project was to establish a method to identify appropriate methamphetamine metabolites to support a change in the Association of Official Racing Chemists drug testing guidelines to require the presence of at least one or more metabolites in cases of suspected methamphetamine usage. In this project, I selected, developed, and validated a method for identifying methamphetamine metabolites in equine urine to convey that the methamphetamine found in a urine sample originated from within the urine and the horse itself, rather than from outside contamination. At present, the American Graded Stakes Committee drug testing list only includes methamphetamine and amphetamine as required analytes to be found in positive samples. Metabolites are not considered in the list. Currently, parties accused of having a positive test for amphetamines in equine urine samples are currently winning cases by claiming the positive result could possibly be due to outside contamination during testing of the sample.

The first aim was to identify the compounds of interest amphetamine, methamphetamine, hydroxyamphetamine, hydroxymethamphetamine, and phenylpropanolamine using liquid chromatography and triple-quad mass spectrometry analysis of negative control equine urine samples spiked with the compounds of interest.

The second aim was to conduct ion suppression studies and determine the limit of detection for each compound by analyzing negative control equine urine spiked pre- and post-extraction. The third aim was to create a quantitative curve by spiking negative control equine urine with the target compounds and serially diluting the sample to create the curve points. Six replicates of low- and high-quality control samples were used to validate the curve using appropriate statistical evaluations. Inter- and intra-day precision, accuracy, and uncertainty were calculated using single-factor ANOVA tables.

The final aim was to use the validated method to identify the target compounds in urine samples from horses administered parent amphetamines. Urine samples for this project were provided by the Interstate Drug Testing Alliance. The samples were collected from horses administered by the IDTA with either methamphetamine, phenylpropanolamine, or hydroxyamphetamine. The samples were collected at 0, 2, 4, 8, 24, and 48 hours after administration and analyzed by the validated method. Sample dilutions were performed to fit on the curve as needed. The data were then analyzed to determine the time course of the presence of metabolites. A subset of this aim was to determine the amount of metabolite conjugation occurring in the samples by preparing samples with and without ß-glucuronidase.

Literature Review

Background

Methamphetamine and amphetamine are stimulants that act as indirect sympathomimetic drugs. They are substrates of the monoamine transporter found in the cell membrane of neurons related to the neurotransmission of dopamine, norepinephrine, and epinephrine. They are also substrates for the transporter found in storage vesicle membranes (Anzenbacher & Zanger, 2012). Neither drug has affinity to the adrenoceptors or dopamine receptors. Neither stimulant is extensively metabolized in humans, and they are also excreted in urine as unchanged parent compounds. The metabolic pathways of both drugs are aromatic hydroxylation, aliphatic hydroxylation, N-demethylation, oxidative deamination, N-oxidation, and conjugation of nitrogen.

The ring hydroxylation of amphetamine and methamphetamine and the *N*-dealkylation of methamphetamine are mainly catalyzed by the CYP2D6 isozyme in humans. The two are both substrates and competitive inhibitors of CYP2D6. Amphetamine is also oxidatively deaminated by monoamine oxidase. CYP2C catalyzes the deamination to phenylacetone. (Anzenbacher & Zanger, 2012).

Studies have been conducted examining the metabolism of amphetamines in mammals. Caldwell studied the rat, mouse, guinea pig, rabbit, dog, cat, horse, monkey species, and man (Caldwell, 1976). In his examination of amphetamine usage on horses, he acknowledges that amphetamines and similar drugs are used to "improve the performance of horses in sporting events", warranting the need to examine how the drugs travel in a horse's system. Amphetamine's, along with ephedrine's, effects have been well documented. At the time of Caldwell's writing, only those two compounds had been examined in the horse.

70% of amphetamine goes through deamination. In the horse, deamination is still the main metabolization process for amphetamine. Caldwell stated that the products "are excreted both as acid-labile conjugates of the corresponding ketone and carbinol and as benzoic acid. Aromatic hydroxylation has also been shown to occur", the latter forming the hydroxyamphetamine metabolite. Small amounts of amphetamine are excreted unchanged, with part of it in the form of a conjugate – likely the N-glucuronide. P-hydroxyamphetamine is excreted principally as an O-glucuronide.

Ephedrine, in comparison, is structurally closely related to amphetamine, and is completely metabolized in the horse, also principally by deamination. Its major metabolite is norephedrine, also known as phenylpropanolamine (PPA). Norephedrine results from the N-demethylation of ephedrine and is another metabolite of methamphetamine.

Caldwell observed that the pattern of metabolism seen in the horse seemed to be like that of the rabbit's, which he had observed in the same study. In both animals, the two drugs are metabolized extensively, mainly by deamination, with some ring hydroxylation. They both also create benzoic acid and its conjugates. Smith and Dring (Dring, Smith, & Williams, 1970) posited that the metabolism of amphetamines in different species was possibly connected to their diets. This could explain why the horse and rabbit have similar metabolisms despite being so evolutionarily separated. Both species are herbivores, and herbivorous species were noted to have metabolized amphetamines primarily through

oxidative deamination. Caldwell noted that lipid solubility does not seem to be major determining factor in the extent of metabolism for the compounds in the guinea pig, rabbit, and horse. In a later study, Caldwell found that there was still a lack of enough information to be able to build a predictive framework for the differentiation of drug metabolism between species (Caldwell, 1987).

Effects on Urinary pH on Amphetamine Metabolism

Davis, Kopin, Lemberger, and Axelrod knew that urinary pH influences the rate of urinary excretion of amphetamine in man (- Davis, - Kopin, - Lemberger, & - Axelrod,). A study was conducted on the loss of amphetamine from plasma as a function of urinary pH, as well as on the metabolic result of amphetamine. They found that when the urinary pH is acid, amphetamine is cleared from the blood and "excreted into the urine more rapidly", and then excreted unchanged or unconjugated by the kidneys. However, alkaline pH showed amphetamine being metabolized by deamination, with more metabolization occurring the longer amphetamine was in the blood. They found that the excretion levels of the parent, both conjugated and unconjugated, and deaminated metabolites was about the same under alkaline conditions.

Methods of Detection

In 1998, Kraemer and Maurer (Kraemer & Maurer, 1998) reviewed various procedures detailed in papers from 1991 to 1997 for determining the presence of amphetamine, methamphetamine, and amphetamine-derived designer drugs in human blood and urine. In their review, immunoassays were noted for their frequent use in urine screening for amphetamines. Any positive results from the immunoassay then needed to be confirmed by a second independent method that had to, at minimum, be as sensitive as the immunoassay screening. The second method also needed to be a definitive test for the presence of a compound. Gas chromatography-mass spectrometry was the most common confirmation method of choice, as well as being required by the Guidelines for Federal Workplace Drug

Testing in the United States. High-performance liquid chromatography, along with high-performance thin-layer chromatography and capillary electrophoresis, were also viable methods. Kraemer and Maurer noted that the large number of publications on amphetamine analysis published in the years leading up to their paper indicated a need to improve the methods by which amphetamines are extracted and identified, largely inspired by reports on GC-MS methods resulting in false methamphetamine positives.

A 2008 review paper by Holcapek, Kolarova, and Nobilis (Holcapek, Kolarova, & Nobilis, 2008) examined tandem mass spectrometry techniques paired with high-performance liquid chromatography – HPLC -- for identifying and determining phase I and phase II drug metabolites. The papers reviewed were published largely between 2002 and 2007. In the review, the authors described drug processing through an organism and how it interacts with macromolecules in the body, dividing it into four parts: absorption, distribution, metabolism, and excretion. When discussing metabolism, it was noted that one can preliminarily verify the presence of a phase II metabolite by using enzymes such as β-glucuronidase (Nobilis et al., 2004). These enzymes cleave the bond between glucuronic or sulfuric acid and the phase II drug metabolite. HPLC analysis of treated and untreated samples offer both qualitative and quantitative information about phase I and phase II metabolites. They note that glucuronidation is a "major phase II metabolic pathway for vertebrates", excepting the cat family.

Holcapek et al. noted that at the time of writing, a decade after Kraemer and Maurer's paper, mass spectrometry is still generally considered as one of the most universal identification methods, while requiring separation techniques to precede it. When discussing HPLC, they noted that the metabolic changes in a drug lead to an increased polarity in the metabolites, and thus a decreased retention time in reversed-phase HPLC systems, when compared to the parent drug. Another analytical trend noted in the review is the focus on decreasing the analysis time while increasing the sample throughput, while not losing separation selectivity. Various parameters can be adjusted to make those

changes, and one of the more vital parameters is temperature. Performing separation at higher temperatures reduces the analysis time while also decreasing the column backpressure due to the decreased viscosity that results from increased temperatures (Plumb et al., 2006).

HPLC-MS/MS has played a large role in drug analysis due to how easily MS can be coupled to gas- and liquid-phase separation techniques, as well as its high sensitivity, low sample consumption, and relative ease of information retrieval. The established method for HPLC quantitation is based upon the use of tandem-MS and internal standards (Xu, Fan, Rieser, & El-Shourbagy, 2007). Choosing appropriate internal standards based on the target compounds is essential. The best approach to choosing internal standards is to use isotopically labeled analogs of the target compounds. It is also noted that deuterated analogs can go up to six deuterium atoms without having any observable differences in their chromatographic retention (Murphy & Huestis, 2005).

Liquid-Liquid Extraction versus Solid-Phase Extraction

In a 2009 paper by Matthew P. Juhascik and Amanda J. Jenkins, both liquid-liquid and solid-phase extraction methods for compared for their efficacy concerning alkaline drugs (Juhascik, 2009). At the time, liquid-liquid extraction, or LLE, was the traditional method. It can be used on many matrices, and any sort of drug (acidic, alkaline, or neutral) can be extracted. However, they did note that LLE has notable disadvantages. One such disadvantage is that is requires the usage of “relatively large volumes” of organic solvents. The larger problem is that the technique requires good hand-eye coordination to pipet the immiscible layers for transfer. The quality of transfer will differ from analyst to analyst, and no matter how well-trained or naturally capable the analyst is, loss of the sample as you extract between an aqueous and organic layer is inevitable. (Levine, 2006)

Solid-phase extraction, or SPE, has been performed since the 1960s, originally with the toxicologists making their own columns with materials such as sodium sulfate and cotton balls. By the

time of this paper, columns are now made and selected for use based on their chemical properties, like being hydrophobic or hydrophilic, or based on cation- or anion-exchange, or even mixed modes. Targeted drug analysis selects a column's stationary phase based on the target compound's chemical properties. Juhascik and Jenkins note that forensic toxicology laboratories quickly took to using SPE for target drug analysis. Not many used the columns for general unknowns, however, although mixed mode columns could be used for that purpose. The reasons given were "well validated and tested LLE methods, and the lack of time and resources to develop alternative procedures". With that in mind, Juhascik and Jenkins developed this study to "evaluate the feasibility of switching" from LLE to SPE without losing the ability "to detect a similar range of drugs at comparable concentrations".

The matrix used in their study was blood. For the LLE, they used a traditional extraction method that had been used in their laboratory for around ten years. The SPE method was modified from the 2006 United Chemical Technologies application manual. They chose this method because the manufacturer stated the method had the ability to extract multiple drugs. The columns used were mixed mode.

In their evaluation of the extraction procedures, Juhascik and Jenkins noted that each LLE produced seven milliliters of organic solvent waste and eight milliliters of aqueous buffer. SPE waste amounted to 20 mL of buffer and methanol waste, and 2 mL of organic solvent waste from hexanes. The SPE waste was noted to be easier to dispose of, given that the waste was contained within the extraction material in the column. However, the authors noted that they had to factor the cost of the purchasing the extraction apparatus for SPE into the comparison. Both methods required the usage of centrifuges and a "evaporation apparatus", while LLE also required the availability of something that would allow for the rotation or mixing of test tubes. Juhascik and Jenkins declared that the biggest financial difference between the two methods was the cost of the columns used in SPE. Each column cost about two dollars, and are not reusable, so that cost must be factored in per extraction.

When comparing the amount of time required to perform each extraction, the authors stated that LLE took them about three hours to perform the extraction of twenty-four samples. They also noted that it is a more labor-intensive method, in addition to the high skill required for efficient pipetting of the organic solvent. They were able to perform extraction on twice as many samples using SPE, in less than the three hours required for LLE, using two extraction manifolds. In comparison to LLE, SPE does not require as much labor, and the most rigorous requirement was performing the column preparation and cleaning steps in the correct order to ensure the success of the extraction. Due to not requiring as much technical precision as LLE, the possibility of a failed extraction using SPE was decreased. The lack of pipetting also made it so that SPE had a more consistent internal standard response. When looking at chromatogram responses, the authors noted that the SPE chromatogram did not have six "junk" peaks that were present in the LLE chromatogram, including cholesterol.

To evaluate the limits of detection (LOD) of analytes for both methods, Juhascik and Jenkins tested forty-one drugs under the methods. LLE gave lower LODs for eight drugs across a spectrum of drug classes. There was a significant difference in LODs between methods for a few drugs. SPE had lower LODs than LLE for sixteen drugs, including one that was lower by tenfold. Seventeen drugs had similar LODs between both techniques. In addition to evaluating and comparing the techniques, the authors sought to further validate the SPE method by determining the LOD for sixty-five more drugs, solely using SPE. This was to make sure that the drugs could be detected, and further, detected at concentrations seen in the casework done by their lab after extraction by SPE. Amphetamine and methamphetamine were included on this list, both at LODs of 100 ng/mL.

After concluding the LOD studies, Juhascik and Jenkins' laboratory chose to switch their methodologies from LLE to SPE. After analyzing over 2000 case specimens with it, the laboratory had detected drugs that they previously had not under LLE. The authors concluded that the benefits of SPE made it useful extraction technique for forensic toxicology labs. It allows the lab to analyze more

samples at once, and its detection of additional drugs allows the lab to qualitatively identify cocaine and heroin use without using selective GC-MS. The conclusion was that SPE is "a suitable substitute for the more time-consuming LLE".

Materials

For this project, the compounds of interest were parent drugs methamphetamine and amphetamine, and their metabolites hydroxyamphetamine, hydroxymethamphetamine, and phenylpropanolamine. A multi-standard stock solution was prepared by adding 100 µL of each compound from a 1mg/mL stock to 500 µL methanol, creating a 100 µL/mL multi-standard solution to be used for spiking in samples. The stock compounds were supplied by Cerilliant, Sigma, and USP, and the methanol, when applicable, was supplied by Fisher Scientific.

Both the liquid-liquid extraction and solid-phase extractions shared many of the same reagents – enzyme buffer (UCT 1M Acetate pH 5.0 buffer), β-glucuronidase (UCT), and 0.5M potassium carbonate (Fisher) pH 6 phosphate buffer (UCT PKG10), prepared in-lab. The solid-phase extraction required the usage of methanol and acetic acid (Fisher) as part of column preparation. The liquid-liquid extraction utilized 8:2 DCM:IPA, while the solid-phase extraction utilized 78:20:2 DCM:IPA:NH_4OH. The columns used were UCT XRDAH13Z bonded solid phase extraction columns. These columns are mixed mode, designed for reverse phase and ion exchange. The extraction was done in a UCT positive pressure manifold. The LC instruments used were Agilent 1200 Series and 1260 Infinity Series with a 1100 Series diode array detector. The instruments respectively had a 6410 Triple Quadrupole LC/MS and 6460 Triple Quadrupole LC/MS attached.

All spiked samples were prepared by adding the multi-standard solution containing all five compounds of interest to one milliliter of negative control equine urine (NCU). The NCU was obtained from a horse that was known to not be given any of the compounds of interest and was owned by the

Analytical Forensic Testing Lab. The specific amounts added varied between steps of the research and will be detailed in later sections.

A total of 17 equine urine samples were provided by the Interstate Drug Testing Alliance: six from a horse administered with methamphetamine, six administered phenylpropanolamine, and five administered hydroxyamphetamine. Methamphetamine from Ovation Pharmaceuticals was dosed at 100mg/horse by mouth (PO) to two horses. Phenylpropanolamine from Sigma was dosed to two horses at 1.1mg/kg PO. The samples were collected from the horse at 0 hours (pre-dose), 2 hours, 4 hours, 8 hours, 24 hours, and 48 hours from administration. The set for hydroxyamphetamine did not include a 48 hours sample.

Methods

Determination of Ion Suppression and Enhancement

Liquid-Liquid Extraction

The first round of ion suppression experiment samples was prepared for a base liquid-liquid extraction process. Seven samples were prepared for this experiment: three samples spiked with 5 µL of the multi-standard solution for pre-extraction data, three samples spiked post-extraction, and a negative control. 5 µL each of two internal standards – amphetamine-d11, and methamphetamine-d5 – were then added to the three pre-extraction spiked samples. 250 mL of the enzyme buffer was added to all seven samples, followed by 50 µL of β-glucuronidase to all samples. The samples were then incubated for two hours at 65^{o} C, or overnight at 40^{o}C.

After incubation, 1 mL of 0.5M pH 6.0 potassium carbonate was added to all samples, followed by 3 mL of 8:2 DCM:IPA. All samples were then rotated in a multipurpose test tube rotator for 10 minutes, then spun in a centrifuge for 10 minutes at 4000 rpm. After centrifuging, the aqueous layer

that formed from each sample, using a vacuum pump, was aspirated. The remaining bottom layer was transferred to new tubes. 5 µL of the multi-standard solution and 5 µL of the internal standards were added to three of the new tubes to represent as spiked post-extraction samples.

After the transfer to new tubes, the samples were dried down using nitrogen in a 40^{o} C water bath. Once dry, the samples were reconstituted in 100 µL 95:5 0.2% formic acid in water:acetonitrile, which were then transferred to a well-plate to be analyzed under tandem liquid chromatography and triple-quad mass spectrometry.

Solid Phase Extraction

The initial hydrolysis steps of the solid-phase extraction were identical to that of the liquid-liquid extraction, up to adding the enzyme buffer, β-glucuronidase, and incubating the samples. After incubation, 2 mL of 1 M pH 6.0 phosphate buffer was added to each sample. The samples were then centrifuged at 4000rpm for 10 minutes.

While the samples were centrifuged, the SPE columns were conditioned with 1 mL of methanol, followed by 1 mL of the phosphate buffer solution, at a flow rate of 1 to 2 mL/minute in the positive pressure manifold. Once the columns were conditioned and the samples centrifuged, the samples were added to the columns and left at a flow rate of 1 to 2 mL/min. The columns were then washed with 1 mL of 0.5M acetic acid at the same flow rate. Once the acetic acid had flowed through the column, the flow rate was adjusted to 40 psi to dry the columns for 5 minutes. Following that, the columns were washed with 1 mL of methanol.

After the methanol wash, all samples were eluted into clean glass tubes with 1mL of 78:20:2 DCM:IPA:NH_4OH. Samples were then evaporated to dryness under nitrogen in a 40^{o} C water bath and reconstituted with 100 µl 95:5 0.2% formic acid in water:acetonitrile, followed by transfer to a well plate and injection on LC/MS-QQQ.

Determination of Limits of Detection

To determine the limits of detection of the method, a negative control urine sample was spiked with the multi-standard compound mix, and then serially diluted to create a range of concentrations to extract and inject on LC/MS-QQQ. The starting concentration was set at 100 ng/mL. This was created by adding 4 µL of the multi-standard mix to 4 mL of the NCU. The next concentration was 50 ng/mL, made by taking 2 mL of the 100 ng/mL mixture and adding it to 2 mL of the NCU. The serial dilution continued down in the same fashion, adding 2 mL of the new mix to 2 mL of the NCU. This was done until the full range of concentrations was 100, 50, 25, 12.5, 6.2, 3.1, 1.5, and 0.75 ng/mL. Then 1 mL of each concentration was taken for extraction. As with the ion suppression experiments, this experiment was initially done with liquid-liquid extraction, and later with solid-phase extraction.

Concentration (Nanograms/mL)	Tube	Starting Amount of Urine (mL)	Amount Transferred
100	1	4 mL	-
50	2	2 mL	2 mL from tube 1
25	3	2 mL	2 mL from tube 2
12.5	4	2 mL	2 mL from tube 3
6.2	5	2 mL	2 mL from tube 4
3.1	6	2 mL	2 mL from tube 5
1.5	7	2 mL	2 mL from tube 6
0.75	8	2 mL	2 mL from tube 7

Table I - Serial dilution

Chromatographic Conditions

For this project, a method “Methamphetamine Quantitation” was created for use on liquid chromatography/triple-quad mass spectrometry instruments. The stationary phase was water with 0.2% formic acid. The mobile phase was 10% water in acetonitrile. The first step of the method started at 95% stationary phase, and 5% mobile phase. Those conditions were held for one minute, then the method shifted to 50% of both phases at four minutes. The method then switched to 100% mobile phase at time

4.01 minutes, through 5 minutes, and then switched back to 95%:5% for the final 5.01 step. The injection runtime was set at 8 minutes to give the compounds enough time to be detected. For the entire run, the flow was set at 0.500 mL/minute.

The injections themselves were set at an amount of 5 µl, as an injection with a needle wash. The draw speed as set at 200 µl/minute, eject speed at 200 µl/minute, draw position at 0.5 mm, equilibration time 0.0 seconds, and the sample flush out factor at five times the injection volume. The column temperature was set at 40^{o} C. A blank sample consisting of 95:5 HCN:H_2O was injected under the method prior to every run. The column was set for reverse phase chromatography. For the triple-quad portion, the start time 0 was set to MRM scan, with the div valve going to waste and a delta EMV (+) of 400. Start time 1 was also MRM, but the div valve there was sent to mass spec. Start time 6 was sent to waste.

Results

Ion Suppression and Enhancement

Ion suppression and/or enhancement for each target compound was studied by making pre- and post-extraction spiked samples using the multi-standard mix containing all compounds. After extraction, data analysis was conducted to compare ratios of the pre- and post-extraction data and arrive at a percentage of ion presence. In one set of data, three replicates each of pre- and post-extraction spiked samples were used for statistical significance. In the data analysis, averages were first calculated of solely the pre-extraction spike data, using the areas of response. This was done for every target compound – amphetamine, hydroxyamphetamine, hydroxymethamphetamine, methamphetamine, and phenylpropanolamine. The average of the post-extraction spike data was then calculated in the same manner. The average of the pre-extraction spike data was then divided by the post-extraction spike data to calculate the ion suppression ratio. This chosen set gave the results seen in Table II.

Limit of Detection

To determine the limit of detection, blank matrices were spiked with the analytes of interest. To determine the LOD for the target compounds, one sample was prepared by spiking negative control urine with the compound mixture, and then serially diluting the mixture to create a suitable range of concentrations from which the LOD could reasonably be chosen, based on instrument restrictions. The qualitative LOD was determined by the concentration that could give a signal-to-noise ratio of 5, and the quantitative LOD a ratio of 10. For the set displayed in Table III, 5 µL of the compound mixture was added to 5 mL of NCU to create a 1:100 dilution. 50 µL of that dilution was then added to 4.95 mL NCU to create a sample at a concentration of 1 ng/mL. 2 mL of that sample was then added to 2 mL NCU, and 2 mL of every subsequent sample to 2 mL, until there were five samples across a range of concentrations. Those concentrations were 1, .5, .25, .125, and .0625 ng/mL. 1 mL of each sample, plus a negative control, were then processed through solid phase extraction and analyzed through LC/MS-QQQ.

Average of Pre-extraction spikes Area of Response (Resp.)					
AP	OHAP	OHMA	MA	PPA	
3555075	16398418	36059808	1186321	9723922	
Average of Post-extraction spikes Area of Response (Resp.)					
AP	OHAP	OHMA	MA	PPA	
4257717	45127406	70644181	1422002	19553994	
Ratio of Pre:Post (AvgPre/AvgPost)					
AP	OHAP	OHMA	MA	PPA	
0.83	0.36	0.51	0.83	0.49	
% 83	36	51	83	49	

Table II - Ratio calculations from ion suppression study

	AP	OHAP	OHMA	MA	PPA
Sample	S/N Ratio	S/N Ratio	S/N Ratio	S/N Ratio	S/N Ratio
1ng	21.5	53.7	105.8	267.1	167.7
.5ng	28	85.1	66.5	51.3	1.9
.25ng	2.1	71.1	34.0	27.4	153.7
.125ng	5.7	83.0	44.6	12.3	125
.0625ng	1.1	48.1	62.3	7.7	55.1
LOD					
Qualitative	.5ng	.5ng	.5ng	.125ng	.0625ng
Quantitative	.5ng	.5ng	.5ng	.125ng	.0625ng

Table III - Signal to noise ratio for limit of detection purposes

Validation for Quantitative Methods

For creating a calibration model, a calibration curve was designed with six points. The points chosen were from a range of 1ng/mL to 500 ng/mL: 1ng, 10ng, 50ng, 100ng, 200ng, and 500 ng/mL. The r^2 value for the curve must be 0.98 or higher, which is the generally accepted criteria for the fit of a quantitative line. The r^2 value was 0.99, with the curve staying linear. The curve points were created by spiking a blank NCU with a certain amount of the target compounds mixture to create a concentration of 500 ng/mL, and then transferring a set amount of that mixture to the next tube of NCU, to create a series of dilutions. The curve points and amounts of mixture, NCU, and transfers are displayed in Table IV.

Curve Point	**Concentration**	**Amount of 100 µg/mL analyte mixture (µl)**	**Negative Control Urine (mL)**	**Amount Transferred**
6	500 ng/mL	25	5	
5	200 ng/mL	-	3	2 mL from 6
4	100 ng/mL	-	2.5	2.5 mL from 5
3	50 ng/mL	-	2.5	2.5 mL from 4
2	10 ng/mL	-	4	1 mL from 3
1	1 ng/mL	-	4.5	.5 mL from 2

Table IV -Serial dilution to create quantitative curve

Precision Studies

For precision studies, high- and low-quality control samples were created by spiking NCU with the analyte mixture for two different levels. The high sample was 300 ng/mL – 15 µL of the multistandard to 5 mL of NCU -- and the low was created by adding 0.5 mL the high sample to 4.5 mL NCU to have a concentration of 30 ng/mL. Six replicates were created for each concentration for usage in the precision calculations.

The precision studies were calculated for both the high and low concentrations for each analyte of interest. Analysis of variance (ANOVA) tables were created using the quantitative results from the concentration replicates, and those tables were used to calculate the intra-day, inter-day, and total precision, along with accuracy and uncertainty.

High Concentration	**Low Concentration**
Intra-Day Precision	Intra-Day Precision
16.8	8.8
Inter-Day Precision	Inter-Day Precision
5.5	0.7
Total Precision	Total Precision
18.1	13.1
Accuracy	Accuracy
0.7	0.1
Expanded Uncertainty: 26%	

Table V – Amphetamine precision studies

High Concentration	**Low Concentration**
Intra-Day Precision	Intra-Day Precision
4.9	13.9
Inter-Day Precision	Inter-Day Precision
4.2	1.7
Total Precision	Total Precision
6.6	21.7
Accuracy	Accuracy
21.3	20.3
Expanded Uncertainty: 21%	

Table VI – Hydroxyamphetamine precision studies

High Concentration	**Low Concentration**
Intra-Day Precision	Intra-Day Precision
16.2	13.7
Inter-Day Precision	Inter-Day Precision
8.5	0.5
Total Precision	Total Precision
18.8	19.7
Accuracy	Accuracy
-9.2	-6
Expanded Uncertainty: 33%	

Table VII – Hydroxymethamphetamine precision studies

High Concentration	**Low Concentration**
Intra-Day Precision	Intra-Day Precision
14.0	9.5
Inter-Day Precision	Inter-Day Precision
6.1	1.6
Total Precision	Total Precision
15.7	16.3
Accuracy	Accuracy
1.9	3.9
Expanded Uncertainty: 25%	

Table VIII - Methamphetamine precision studies

High Concentration	**Low Concentration**
Intra-Day Precision	Intra-Day Precision
17.2	7.7
Inter-Day Precision	Inter-Day Precision
4.7	2.1
Total Precision	Total Precision
18.4	15.7
Accuracy	Accuracy
13.4	12.2
Expanded Uncertainty: 25%	

Table IX - Phenylpropanolamine precision studies

IDTA Samples

The first experiment run with the IDTA samples was to compare the amount of free drug available in the samples once the conjugates have been cleaved by the β-glucuronidase. To do this, two extractions were performed with an aliquot of each sample: one with the use of β-glucuronidase, and one without.

Next was to extract the samples in replicate, with diluting the samples as needed to fit within the established quantitation curve. For the methamphetamine set, the samples from 2, 4, and 8 hours were diluted by a factor of 10. All hours were run in a replicate of six to establish a mathematical certainty. The analysis produced the following metabolite readings: amphetamine was found in the methamphetamine set from 2 to 8 hours after administration. Hydroxyamphetamine was found from 2 to 24 hours, hydroxymethamphetamine 2 to 24, and no phenylpropanolamine was detected at high levels. Methamphetamine was found unchanged from hours 2 to 8. Provided is one instance of the amphetamine data generated from one of the 2-hour methamphetamine replicates.

Compound	ISTD	RT	Resp Ratio	Final Conc	
Amphetamine	Amphetamine-d11	3.052	11.7518	280.7402	ng/ml

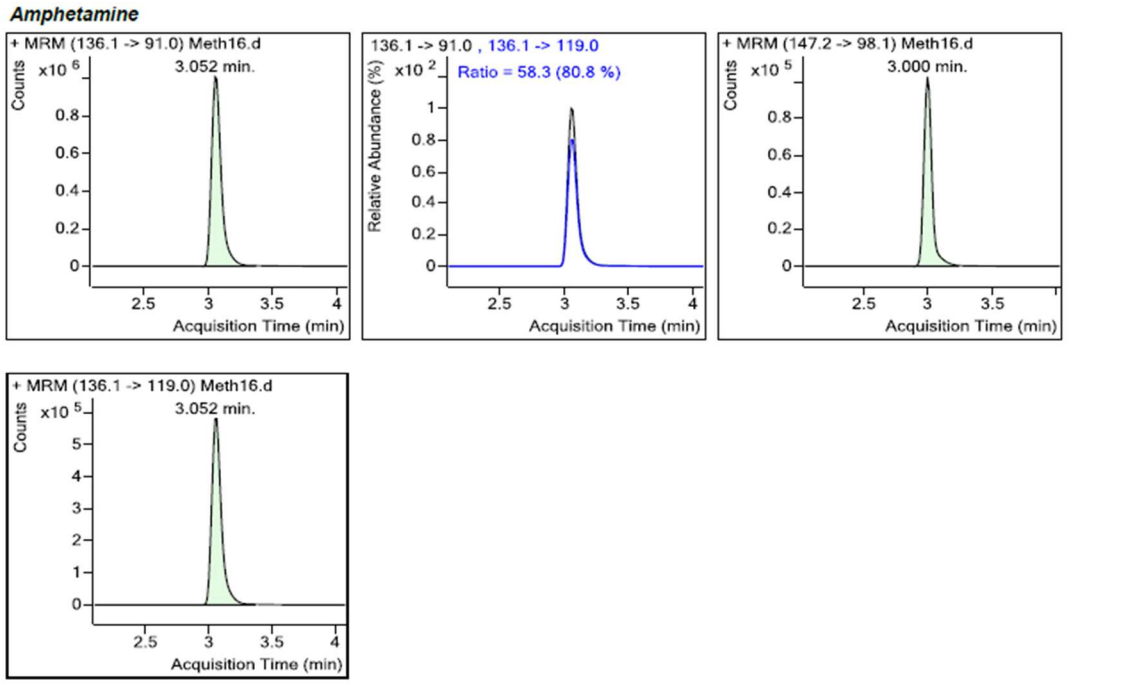

Figure 1 – Methamphetamine 2-hour sample

The phenylpropanolamine set was diluted by a factor of 10 for hours 2, 4, and 8. No drug except unchanged phenylpropanolamine was detected in the samples, from hours 2 to 48. Figure 2 is an example of phenylpropanolamine detected in a 4 hours post-dose sample.

Compound	ISTD	RT	Resp Ratio	Final Conc	
Phenylpropanolamine	Amphetamine-d11	1.581	10.9227	1451.0085	ng/ml

Phenylpropanolamine

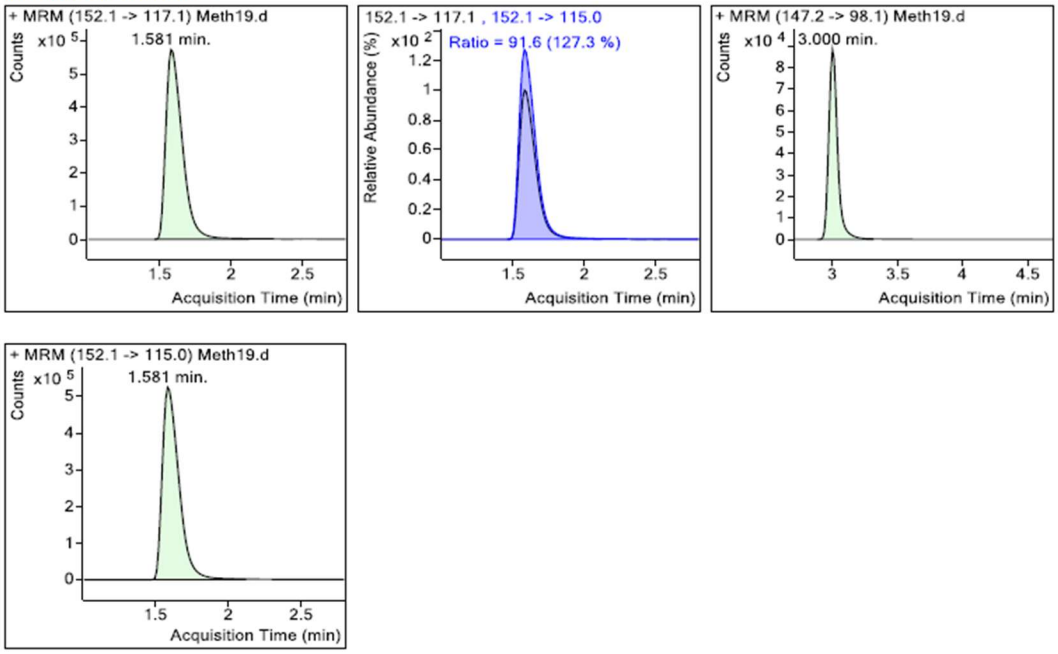

Figure 2 – Phenylpropanolamine 4-hour sample

The hydroxyamphetamine set was diluted by a factor of 10 for hours 2, 4, 8, and 24. No drug except unchanged hydroxyamphetamine was detected in the samples, from hours 2 to 48. Figure 3 shows hydroxyamphetamine detected 8 hours post-dose.

Compound	ISTD	RT	Resp Ratio	Final Conc	
Hydroxyamphetamine	Amphetamine-d11	1.256	3.8618	406.4597	ng/ml

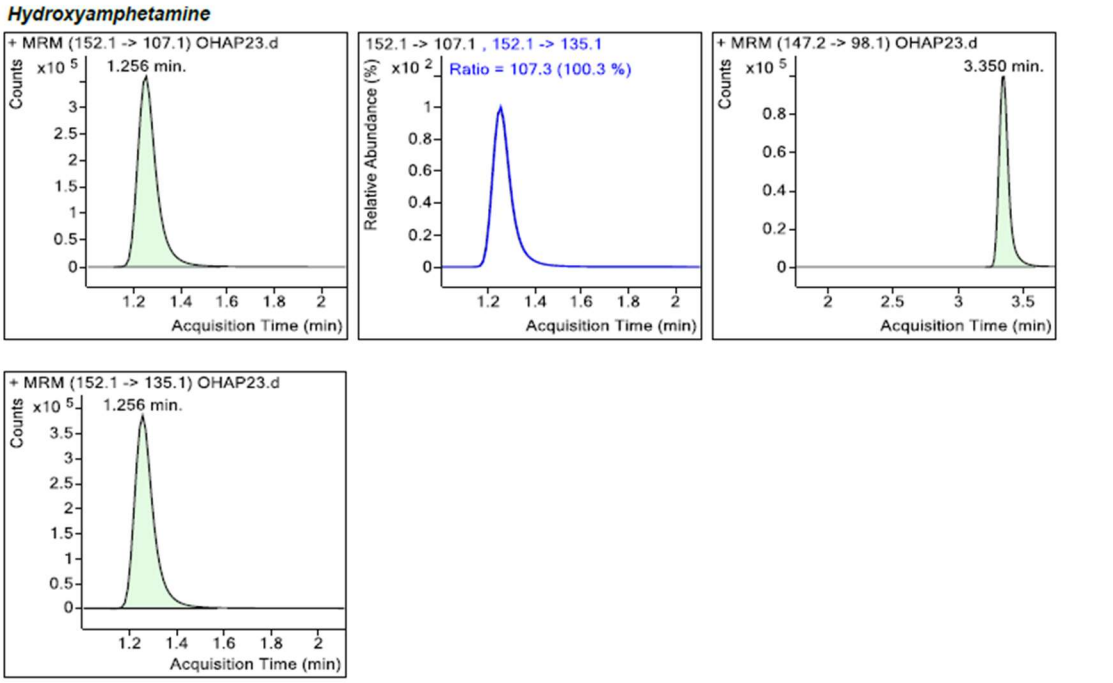

Figure 3 – Hydroxyamphetamine 8-hour sample

Discussion

The goal of this project was to successfully validate a method for extracting and analyzing methamphetamine and its metabolites amphetamine, hydroxyamphetamine, hydroxymethamphetamine, and PPA. This goal was met.

Another goal, conducting ion suppression studies along with determining the limit of detection, was met through spiking negative control urine to create pre- and post-extraction samples.

Ion Suppression and Enhancement

Ion suppression is a common occurrence in liquid chromatography. In my suppression studies, suppression was detected in all analytes of interest. This was expected, and the suppression is

compensated by the usage of internal standards. Using internal standards is the most widely used technique to allow for accurate analysis even in the presence of ion suppression (Volmer & Jessome, 2006). An internal standard helps to normalize the analyte's response, which compensates for variations at any point of the process: sample preparation, injecting, the chromatography, and so on.

The internal standards used in this research were methamphetamine d-5 and amphetamine d-11. The presence of the internal standards ensures that despite the ion suppression, accurate quantification by the method is still achieved – in this case, all compounds of interest here can be quantified to at least a LOD of .5ng/mL.

Limits of Detection and Quantification

Declaring a limit of detection is determined when the signal-to-noise ratio of any of the identification ions falls below 5-to-1. The quantitative LODs were chosen with the ratio being at 10-to-1, as well as for the quantitative methods. This was because target compounds could not be reasonably quantified if they could not be identified. The LOD was determined by finding the lowest concentration that had a signal to noise ratio for a product ion was 5:1, and 10:1 for the LOQ.

Regarding creating a quantitative curve, when preparing the high- and low-quality control samples, modification had to be done for the high QC sample specifically. Originally, the sample was prepared by spiking 25 µL of the 100 µg/mL analyte mixture into 5 mL of NCU to create a 500ng/mL mixture, and serial diluting from there. However, after a few runs, the calculated concentrations of the six replicates were not staying within a consistent range of each other. To remedy this, a composite of an even higher concentration was created by aliquoting 15 µL of each 1mg/mL stock into 5 mL of NCU to create a 3000 ng/mL mixture. That mixture was then diluted to make a 300ng/mL mixture. The calculated concentrations of the replicates were then more consistent.

I hypothesize that this occurred because the initial 300ng/mL mixture was prepared in a 6mL glass tube, which did not allow for efficient mixing throughout the tube. Given that the low-quality control samples, created from the first aliquot taken from the high QC mixture, were always more consistent in both range and expected concentration, it seems that the first aliquot was able to be an accurate sample of the mixture. This is perhaps due to the small amount of the aliquot itself. The larger aliquots, for the replicates, were what had the inconsistencies.

An avenue I would explore in future research would be to replicate the high QC mixture in a variety of glassware to determine which is best suited for proper mixing.

Choosing Solid Phase Extraction

The first instance of performing base pH solid-phase extraction was to compare the results of the solid-phase extraction to those of the liquid-liquid extraction. This was first done when comparing the results of a quantitation curve. In addition to the SPE analysis yielding a higher area of response, the liquid extractions had lower recovery percentages and higher signal suppression. This led to higher LODs and difficulty with calculating precision and accuracy. When compared to LLE, SPE had superior results and thus was chosen as the extraction method moving forward.

The results for LLE were also found to have poor reproducibility for the compounds examined in this project. When I first considered changing the extraction method to SPE, I ran two sets of samples concurrently: one set extracted through LLE, and the other through SPE. The LLE set had multiple points of concern once analyzed for data quantitation. First, the linearity of quantitation curve for each

compound of interest was not established to be reproducible. The curve for amphetamine from that day is shown in Figure 4.

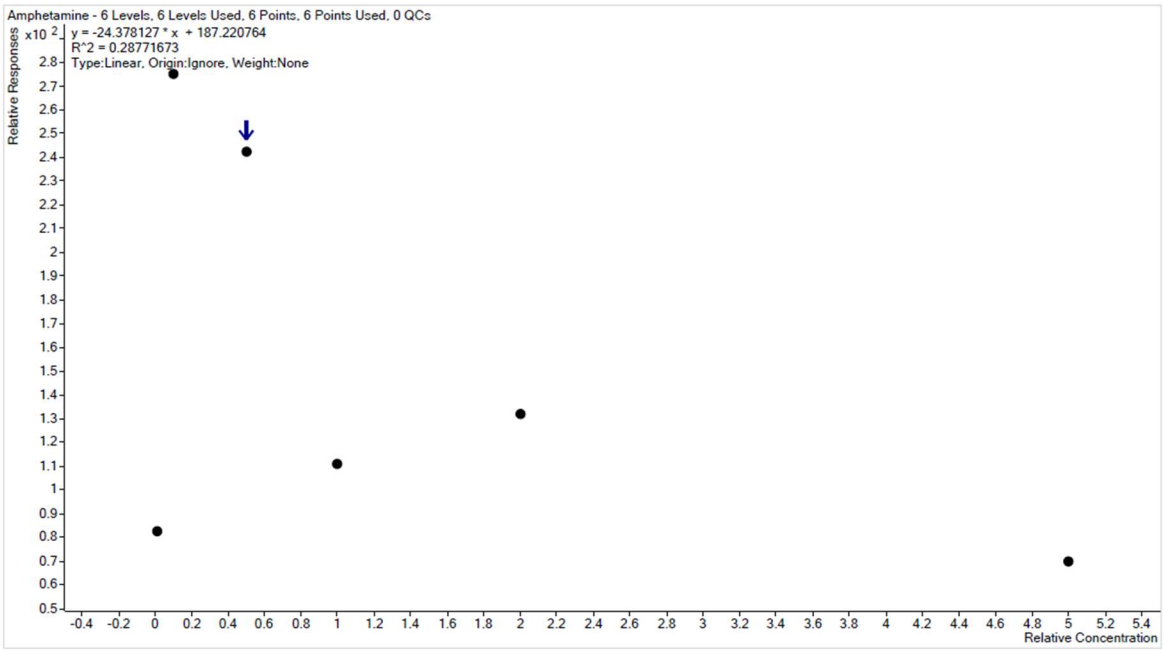

Figure 4 – LLE quantitative curve

In comparison, the curve from the same day's SPE of amphetamine:

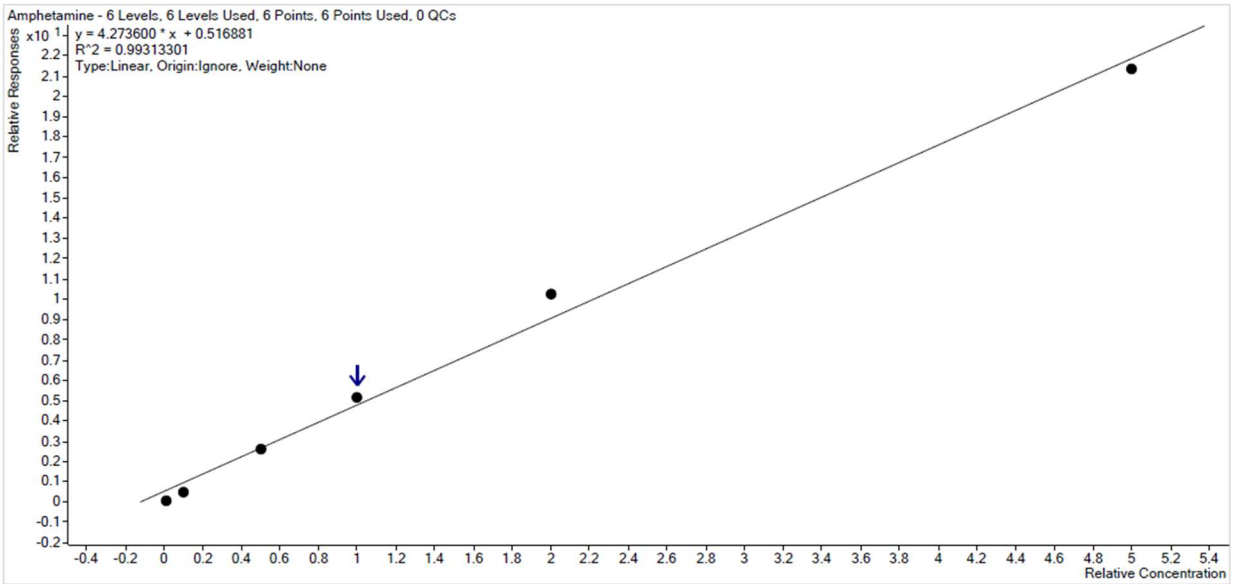

Figure 5 – SPE quantitative curve

The quantitation curve was not the only issue with the LLE set. The quantitative response of the ISTDs used in the samples, particularly for the curve, were not consistent, which possibly indicated that the extraction itself was not behaving reproducibly. Using amphetamine once more as an example, compare the responses:

Liquid-Liquid Extraction	**Solid Phase Extraction**
46208	2737648
31158	3142817
39568	3289569
15758	3701611
12389	3572568
53279	3988299
Relative Standard Deviation	
0.454442354	0.118901926

Table X – ITSD extraction

The unacceptable linearity combined with the inconsistency of ISTD peak responses indicated either the extraction reproducibility was an issue, or the possibility that the peaks produced in the analysis were not the correct peaks to be analyzing, with no way to confirm which were correct. It is difficult to say that LLE results would not have been potentially improved if I had explored other solvent combinations or different buffers, but the initial results from SPE demonstrated that it was a more viable path to pursue for the purposes of this project. These factors are what led me to change the extraction method over to SPE.

Precision

For the high and low concentrations for all compounds except hydroxyamphetamine, the precision values were less than 20%. However, after analyzing the replicates, the total precision for hydroxyamphetamine was found to be 21%, so an extra margin of error must be considered for that compound. Accuracy bias was found to be less than 25%. At a 95% confidence level, the expanded uncertainty ranges from 21-33%, depending on the compound.

IDTA Samples

As expected, neither methamphetamine nor any of its metabolites appeared in the pre-dose samples. The hydroxyamphetamine and phenylpropanolamine samples contained only their respective drugs, as expected. The methamphetamine samples displayed concentrations of methamphetamine along with hydroxymethamphetamine and hydroxyamphetamine, with no phenylpropanolamine detected. This shows that the method can detect the parent drug and metabolites in one sample, although phenylpropanolamine appears to not be a product of methamphetamine metabolism in the horse.

Hydroxyamphetamine was present as a metabolite in the sample collected 24 hours post-administration, indicating that methamphetamine usage can be detected in the urine for a up to a day. Hydroxyamphetamine and phenylpropanolamine as the parent drugs were present in the samples collected 48 hours post-administration, indicating that they can be detected in urine for an extended period.

As briefly mentioned earlier, the first extraction done with the IDTA samples was one where one extraction was performed on the samples without the inclusion of β-glucuronidase, and one extraction performed including it. This was so that the results could be compared across samples and calculate the percentage of free drug that is present in every sample. This was done by dividing the area of response

from the unhydrolyzed set over the area of response from the hydrolyzed set, multiplied by 100. The results are in the tables below. In each table, the first column lists one replicate of each IDTA sample, starting with methamphetamine at 0 hours and ending with PPA at 48 hours. The next two columns are the peak area responses for each sample in both the unhydrolyzed and the hydrolyzed set. The final column is calculated by dividing the unhydrolyzed peak area by the hydrolyzed peak area and multiplying by 100 to find the percentage.

Amphetamine			
Sample	Unhydrolyzed (peak area)	Hydrolyzed (peak area)	% Free Drug
MA0	477	4403	10.8
MA2	260924	7085454	3.7
MA4	914748	5641124	16.2
MA8	475161	3418684	13.9
MA24	9040	125698	7.2
MA48	6901	22179	31.1
OHAM0	273	3052	9
OHAM2	873	4417	19.8
OHAM4	429	3321	12.9
OHAM8	182	4864	3.7
OHAM24	1137	3544	32.1
PPA0	354	2922	12.1
PPA2	499	3560	14
PPA4	196	1451	13.5
PPA8	118	4769	2.5
PPA24	148	5051	2.9
PPA48	325	4926	6.6

Table XI - Amphetamine hydrolysis

Hydroxyamphetamine			
Sample	Unhydrolyzed (peak area)	Hydrolyzed (peak area)	% Free Drug
MA0	163	771	21.2
MA2	1943	656598	0.3
MA4	13607	903688	1.5
MA8	25287	971257	2.6
MA24	2566	84864	3
MA48	1409	6792	20.7
OHAM0	1911	238	801.
OHAM2	801169	2662802	30.1
OHAM4	403899	4381267	9.2
OHAM8	387277	3970078	9.8
OHAM24	12924	1244160	1
PPA0	230	817	28.2
PPA2	4634	669	692.7
PPA4	2126	327	648.8
PPA8	3006	333	902.5
PPA24	116	458	25.4
PPA48	132	303	43.7

Table XII - Hydroxyamphetamine hydrolysis

	Hydroxymethamphetamine		
Sample	Unhydrolyzed (peak area)	Hydrolyzed (peak area)	% Free Drug
MA0	3140	9665	32.5
MA2	5748	1461993	0.4
MA4	1554	1932231	0.8
MA8	32576	1819765	1.8
MA24	4757	181638	2.6
MA48	3033	25720	11.8
OHAM0	551	7492	7.4
OHAM2	6416	8291	77.4
OHAM4	20624	10311	200
OHAM8	5913	10562	56
OHAM24	4776	7194	66.4
PPA0	4176	10467	39.9
PPA2	10818	7709	140.3
PPA4	697	6987	10
PPA8	1183	10427	11.3
PPA24	3577	13452	26.6
PPA48	6602	10207	647

Table XIII - Hydroxymethamphetamine hydrolysis

Sample	Methamphetamine Unhydrolyzed	Hydrolyzed	% Free Drug
MA0	4407	10167	43.3
MA2	518813	2761227	18.8
MA4	1005295	2754066	36.5
MA8	999566	2336330	42.8
MA24	14016	80069	17.5
MA48	7154	19760	36.2
OHAM0	14579	7537	193.4
OHAM2	5948	9792	60.7
OHAM4	4399	6018	73.1
OHAM8	4516	8254	54.7
OHAM24	4349	7419	58.6
PPA0	4547	8197	55.5
PPA2	3510	6978	50.3
PPA4	3258	5197	62.7
PPA8	5315	9484	56
PPA24	6283	9324	67.4
PPA48	4748	20563	23.1

Table XIV - Methamphetamine hydrolysis

Sample	Phenylpropanolamine Unhydrolyzed	Hydrolyzed	% Free Drug
MA0	1152	3272	35.2
MA2	576	4820	12
MA4	4088	3847	106.2
MA8	893	4013	22.3
MA24	277	1650	16.8
MA48	775	1562	49.6
OHAM0	124	978	12.7
OHAM2	2038	1333	152.9
OHAM4	1352	2485	54.4
OHAM8	1686	3171	53.2
OHAM24	314	1149	27.4
PPA0	127566	29222	436.5
PPA2	6267897	2847971	220.1
PPA4	3705140	1319156	280.9
PPA8	4895648	2639787	185.5
PPA24	905921	351298	257.9
PPA48	716087	227581	314.7

Table XV - Phenylpropanolamine hydrolysis

This was a preliminary look that I would expand upon in further research. I would need to consider the calculated concentrations and specific samples, as non-zero values are present in this table for the 0-hour, pre-dose samples. The percentages for the PPA samples, when looking for PPA specifically, are also all above 100%, which would need to be further investigated. Performing the extraction with replicates, along with the previously mentioned steps, would create a clearer picture of free drug presence in a given sample.

Conclusion

A method to identify and quantify methamphetamine and its metabolites was developed and validated. The method validation demonstrated acceptable precision and accuracy thresholds and can competently quantify methamphetamine and related metabolites in administration samples.

Cited Literature

- Davis, J. M., - Kopin, I. J., - Lemberger, L., & - Axelrod, J.- *EFFECTS OF URINARY pH ON AMPHETAMINE METABOLISM* - Blackwell Publishing Ltd. doi:- 10.1111/j.1749-6632.1971.tb46926.x

Anzenbacher, P., & Zanger, U. M. (2012). In Anzenbacher P., Zanger U. M. (Eds.), *Metabolism of drugs and other xenobiotics*. Weinheim: John Wiley & Sons, Incorporated.

Caldwell, J. (1976). The metabolism of amphetamines in mammals. *Drug Metabolism Reviews, 5*(2), 219-280. doi:10.3109/03602537609029979 [doi]

Caldwell, J. (1987). **The current status of attempts to predict species differences in drug metabolism**. *Drug Metabolism Reviews, 12*(2), 221-237.

Dring, L. G., Smith, R. L., & Williams, R. T. (1970). The metabolic fate of amphetamine in man and other species. *The Biochemical Journal, 116*(3), 425-435.

Holcapek, M., Kolarova, L., & Nobilis, M. (2008). High-performance liquid chromatography-tandem mass spectrometry in the identification and determination of phase I and phase II drug metabolites. *Analytical and Bioanalytical Chemistry, 391*(1), 59-78. doi:10.1007/s00216-008-1962-7 [doi]

Juhascik, M. P. (2009). **Comparison of liquid/liquid and solid-phase extraction for alkaline drugs.** *Journal of Chromatographic Science, 47*(- 0021-9665 (Print); - 0021-9665 (Linking)), 553.

Kraemer, T., & Maurer, H. H. (1998). Determination of amphetamine, methamphetamine and amphetamine-derived designer drugs or medicaments in blood and urine. *Journal of Chromatography.B, Biomedical Sciences and Applications, 713*(1), 163-187. Retrieved from https://www.sciencedirect.com/science/article/pii/S037843479700515X

Levine, B. (Ed.). (2006). *Principles of forensic toxicology* (2nd ed.)

Murphy, C. M., & Huestis, M. A. (2005). LC-ESI-MS/MS analysis for the quantification of morphine, codeine, morphine-3-beta-D-glucuronide, morphine-6-beta-D-glucuronide, and codeine-6-beta-D-glucuronide in human urine. *Journal of Mass Spectrometry : JMS, 40*(11), 1412-1416. doi:10.1002/jms.921 [doi]

Nobilis, M., Holčapek, M., Kolářová, L., Kopecký, J., Kuneš, M., Svoboda, Z., & Květina, J. (2004). *Identification and determination of phase II nabumetone metabolites by high-performance liquid chromatography with photodiode array and mass spectrometric detection* doi:https://doi.org/10.1016/j.chroma.2004.01.031

Plumb, R., Rainville, P., Smith, B., Johnson, K., Castro-Perez, J., Wilson, I., & Nicholson, J. (2006). Generation of ultrahigh peak capacity LC separations via elevated temperatures and high linear mobile-phase velocities. *Analytical Chemistry,* (- 20), - 7278. doi:- 10.1021/ac060935j

Volmer, D. A., & Jessome, L. L. (2006). **Ion suppression: A major concern in mass spectrometry**. *LCGC North America, 24*(5), 498-510.

Xu, R. N., Fan, L., Rieser, M. J., & El-Shourbagy, T. A. (2007). *Recent advances in high-throughput quantitative bioanalysis by LC–MS/MS* doi:https://doi.org/10.1016/j.jpba.2007.02.006

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