URINE DRUG TESTING VIA VOLUMETRIC ABSORPTIVE MICROSAMPLING TIPS

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Abstract: Presently, laboratories typically perform urine drug testing with collected urine specimens using the dilute and shoot extraction technique. While the dilute and shoot technique is a functional means of evaluation for drugs, the development of Volumetric Absorptive Microsampling (VAMS) may improve the process of drug testing urine samples. This research compared urine analysis using dilute and shoot extraction to that of dried VAMS tips for drugs using liquid chromatography – tandem mass spectrometry (LC-MS/MS). Most drugs included in the panel were from the benzodiazepine and opioid class. The first element of evaluation was the ability to recover glucuronides. A hydrolysis method was devised that extracted the tips with methanol, followed by dry down and resuspension in enzyme and buffer, allowing for glucuronide conversion to parent drug and subsequent analysis. Following the development of the method, it was applied to anonymized specimens and the results were compared with a clinically validated dilute and shoot assay. It was determined that the techniques provided qualitatively similar results, suggesting that with appropriate validation, the VAMS methodology may be suitable for clinical analysis of drugs in urine specimens.

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CHAPTER I

INTRODUCTION

Pharmacies in the United States filled over 4 million prescription medications in 2016.[1] Painkillers, antidepressants, and lipid-lowering agents accounted for the majority of prescribed therapeutic medications.[2] While there are individuals that take their medication as prescribed, others misuse their prescription, so prescription compliance monitoring is necessary. This misuse can come in various forms, such as by taking a dose higher or lower than prescribed. Another option is taking the medication with additional substances when directed not to, such as alcohol. Other individuals may decide not to take the prescription altogether, potentially giving away or selling the medication to other people. To ensure patients are taking their prescriptions as directed many physicians and work environments require some form of drug testing, typically urine drug testing.

Urine drug testing has traditionally been performed by using an aliquot of wet urine in a technique termed dilute and shoot (DS), with analysis performed using liquid chromatography-mass spectrometry (LC-MS) or liquid chromatography-tandem mass spectrometry (LC-MS/MS). DS has gained popularity primarily due to its ease of use, where an aliquot of urine is diluted before being "shot," or injected, onto the instrument. There are slight variations in the DS technique, where some facilities only dilute the sample before injecting it onto the instrument,

while other laboratories prefer to treat the sample with β-glucuronidase to cleave off glucuronides that may be present in the urine to make the method more sensitive to parent drug.

Volumetric absorptive microsampling (VAMS) is an alternative means of collecting samples for analysis. While research has been conducted using VAMS, the majority of studies have focused on blood and plasma. At the time of conducting this particular study, there was one published article using VAMS with urine samples, completed by Mercolini et al.[3] VAMS tips are adapted swabs that absorb a specific amount of sample fluid and are then allowed to dry at room temperature, which has several advantages over wet specimens. First, the size of the VAMS tips is significantly smaller than that of a urine specimen cup. In fact, a box of 96 tips takes up a little more counter space than that of a 3 × 5-in notecard, while that same amount of space fits only about 4 (2 × 2) specimen cups. Additionally, wet urine samples need to be stored in a refrigerator or freezer to prevent degradation, and because specimen cups take up more space, it can be challenging to find adequate cold storage space for them. Care must be taken when transporting wet samples to ensure they remain cold and do not spill. On the other hand, dry urine samples do not have these problems, since the drug residues are dried on the sorbent and are no longer subject to enzymatic degradation in transport. Therefore, transportation would be much easier and cheaper with dry VAMS samples, as opposed to wet samples.

The purpose of this study was to determine if dried urine drug testing yields the same results as that of wet urine drug testing, when technique specific sample preparation methods are performed and followed by sample analysis using LC-MS/MS. Samples were prepared and analyzed at Oklahoma State University – Clinical Laboratory Services (OSU-CLS) in Tulsa, OK. The technique performed on dry urine samples was extraction in methanol, with subsequent dry-down followed by reconstitution with hydrolysis enzyme, while wet urine samples were treated with the clinically validated DS technique employed by the OSU-CLS facility.

Once the wet and dry urine samples were drug tested using LC-MS/MS, the data was analyzed so that the following research questions could be evaluated: 1) Is there a difference in glucuronide efficiency based on using wet or dry urine samples? 2) In unknown samples, will wet and dry urine samples identify the same drugs? Answers to these questions will allow laboratories to determine if the transition from wet urine samples to dry urine samples for drug testing would be beneficial to their facility.

CHAPTER II

LITERATURE REVIEW

2.1 Introduction

Medications have become a common part of society. Allergy sufferers may use over-the-counter medications or for more severe allergies obtain a prescription from their doctor. Anxiety sufferers are prescribed sedatives that provide calming effects needed to carry on with daily activities. After recovering from surgery, patients may be prescribed pain killers. Even when medications are obtained legally there is still a chance the medications may be misused. Over-the-counter medicines containing amphetamine and ephedrine are now monitored because amphetamine and ephedrine are the precursors in the production of methamphetamine. Additionally, some individuals may abuse medications by taking them with the intention of getting high.

Drug tests are conducted to ensure an individual is taking legal and prescribed medications. Urine has become the most common bodily fluid used for analysis due to ease of collection.[4] Liquid chromatography- tandem mass spectrometry (LC-MS/MS) provides a sensitive and specific means of qualifying and quantifying drugs and drug metabolites in urine samples.[5] Dilute and shoot (DS) is the preparation technique typically used for LC-MS/MS drug urine analysis. To perform DS, an analyst first receives a specimen cup of the patient's urine. A portion of the urine sample and the internal standard are added together, diluting the sample.

Rather than transporting and receiving bulky, wet urine samples in specimen cups, volumetric absorptive microsampling (VAMS) tips could be used. These tips are manufactured to absorb a fixed volume of fluid. Once allowed to dry, the tips can easily be transported and take up far less space than specimen cups.

2.2 Background

2.2.1 History of drugs in panel

Drugs, of the legal or illegal variety, have been used by individuals for centuries.[6] America, in particular, has had an ongoing problem with drug use since the introduction of cocaine, heroin, and morphine in the 1800s.[6] In 1814, 6 classes of drugs distinguished by their toxic effects were documented by M. J. B. Orfila.[7] Orfila grouped drugs into the following classes: amphetamine-like stimulants, barbiturates, benzodiazepines, opioids, illicit, and other.[8] New regulations in the middle of the 20th century aided in a significant decline in drug abuse, the 1960s featured a revival of drugs that were now more accessible: amphetamines, hallucinogens, and marijuana.[6] As the years have progressed, new drugs have entered the market, by legal means or otherwise.

Opioids are drugs derived from opium poppy or commercially manufactured with pain relief characteristics.[9] Certain opioids are legal through prescriptions; however, other opioids, such as heroin, have no legal therapeutic use. A 2016 report by the American Society of Addiction Medicine regarding opioids cited drug overdoses as "the leading cause of accidental deaths in the US" for 2015.[10] Prescription pain killers accounted for 20,101 deaths, with heroin-related overdoses totaling 12,990.[4]

Benzodiazepines are central nervous system depressants prescribed for the treatment of anxiety, muscle relaxation, obsessive-compulsive disorder, and more.[11] Bachhuber et al. reviewed the change in benzodiazepine prescriptions and overdoses resulting in death, finding that from 1996 to 2013 the rate of benzodiazepine overdose mortalities increased at a faster rate than that of benzodiazepine prescriptions being filled.[12]

Cocaine became a Schedule II drug in 1970 because while cocaine had medical value, there was a concern for abuse.[13,14] Reviewing the statistics of national overdose deaths it is evident there was cause to be concerned about cocaine's potential for abuse, with nearly 7,000 deaths in 2015.[15] Benzoylecgonine is known to be a primary metabolite of cocaine when excreted from the body. [14]

Gabapentin was originally prescribed as a treatment for seizure disorders. However, gabapentin is now also prescribed to treat drug addiction due to gabapentin's neurological effects.[16] From 2008 to 2011 there was a "nearly five times" increase in emergency room visits resulting from the "misuse or abuse of gabapentin" in metropolitan areas.[17]

Tetrahydrocannabinol (THC) is a type of cannabinoid that is the primary active ingredient in the Cannabis plant, which may also be referred to as marijuana, weed, pot, Mary Jane, and dozens of other names. At the time of research, 29 states and the District of Columbia have laws legalizing the use of THC.[18] The Federal government has designated THC as a schedule I substance with no medicinal value, and Oklahoma, the state in which this research study was conducted, currently considers THC to be an illegal substance.

2.2.2 Urine drug testing purposes

Drug testing may be conducted in a variety of fashions. The type of test used is determined by the substance and timeframe of interest. Breath tests are useful for evaluating the amount of alcohol in an individual's system at the specific time they are blowing into the breathalyzer. Blood samples, which must be collected by a licensed phlebotomist, provide results of what is in an individual's system at the time the sample is drawn and can identify a variety of substances. Urine samples can easily be collected with a specimen cup and a restroom. Due to known metabolic pathways and elimination rates of medications, most urine samples provide a 2- to 3-day history of drug usage.[19] As a result of the ease of sample collection, urine is often the body fluid of choice for drug testing.

Urine drug tests are conducted for various reasons. Pain management clinics require testing for prescription compliance, or to ensure the client is taking the prescription as directed and that any previously unreported substances are not interfering with the efficacy of the prescribed medication.[20] In 2015, it was reported that upwards to 75% of patients in pain management programs were noncompliant with the proper usage of their prescription; 11% of individuals in these programs were positive for one or more illegal substance.[5] Some pain management clinics have a clause noting that if the individual is found to be taking illegal substances or substances not prescribed, the patient may be dismissed from the program.

Pharmaceutical and clinical trials require testing. As part of the trials it is important to know and understand how and the rate at which the body metabolizes xenobiotics. Certain trials may also account for multiple medications being taken simultaneously. In these instances, potential drug-drug interactions must be observed and potentially monitored.

Many work environments have begun to impose a "drug free workplace" policy. Workplace drug testing is conducted to ensure that employees are unimpaired, assuring the individual is fully capable of performing required tasks, maintaining the safety of themselves and those around them. At the time of hiring, an individual may be required to take a urine drug test. Depending on state laws and employer's policies, the individual may be subject to additional drug testing throughout their employement.[21] Most companies choose to test for illegal drugs using urine because sample collection is more convenient and less invasive than blood samples, with a broader window of detection.[22]

In 1981, the Drug Demand Reduction Program (DDRP) was mandated to "deter and detect illicit drug use by [Department of Defense] military and civilian personnel."[23] Before the mandate of DDRP, stimulant drugs had been provided to combat personnel as a means to lessen fatigue and reduce pain.[24] In order to counter the drug use, the Department of Defense (DoD) implemented educational training and random urine drug tests for those who violated the 'zero tolerance' mandate.[23] In 2011, the DoD analyzed 5.145 million specimens.[23] From 2007 to 2011

the DoD saw an increase in urine samples testing positive for marijuana, d-amphetamine, d-methamphetamine, codeine, morphine, heroin, oxycodone, and oxycodone.[23] Marijuana was the primary illicit drug DoD personnel tested positive for from 2007 to 2011.[23] In 2016, Larson et al. published an article about their research of drug usage in enlisted Army personnel after their deployment. Larson et al. used a urine drug test panel that included: metabolites of cocaine, metabolites of heroin, metabolites of delta-9-Tetrahydrocannabinol (THC), d-amphetamine, d-methamphetamine, Methylenedioxyamphetamine (MDA), Methylenedioxyethylamphetamine (MDEA), and Methylenedioxymethamphetamine (MDMA).[25] They initially tested the individual 0 to 6 months after return from deployment and then 6 months to 3 years postdeployment.[25] For both active duty and national guard/reserve personnel there was an increase in drug usage when the 6 month to 3 year urine samples were tested.[25] One pitfall as to how the data was reported, was that it did not break down the statistics for the particular drugs. Professional athletes are subjected to urinary drug testing to assess if performance-enhancing drugs are taken. Each year the World Anti-Doping Agency publishes a comprehensive list of prohibited substances.[26] Known drug abusers are routinely drug tested, and the frequency of the drug tests is based on the individual's treatment plan and/or the court's sentencing.

2.2.3 Urine drug excretion

Kidneys perform multiple tasks; however, the one of interest for this research is the kidneys' ability to filter blood to excrete drugs and drug metabolites through the urine.[27] For urine to be formed from filtered blood, the following 3 processes must occur: filtration, secretion, and reabsorption.[27,28] Filtration occurs based on the charge and size of molecules. Since opposites attract, the negatively charged filtration system draws through positively charged molecules, leaving negatively charged molecules behind.[27] Smaller molecules are permitted to pass through, while bulkier substances remain in the system for the time being.

Secretion assists in the elimination of the bulkier substances, particularly those that are protein bound. Some carrier proteins, which reside within the epithelial wall, can separate drugs

bound to plasma. Once the drug has been removed, the carrier protein transfers the drug to the kidney tubular fluid.[28] Carrier proteins transport either acidic or basic drugs.[28]

Reabsorption may also occur. The reabsorption process is necessary for retaining plasma and interstitial fluid the human body needs to function.[29] However, certain drugs may be reabsorbed based on pH and lipid solubility.[28] Drugs with pH and solubility similar to that of plasma and interstitial fluid will be transported across the membrane, and these drugs will return to the bloodstream, eventually returning to the kidney. Between the time the drug cycles back through the blood system, it may have had structural changes that result in it not being filtered or secreted out of the kidney into the urine.

2.3 Glucuronides and β-glucuronidase

For certain xenobiotics to be excreted from the body, a glucuronide must be attached. Glucuronides are a sugar group that are covalently bonded to the xenobiotic through a process called glucuronidation, making the xenobiotic more water soluble.[30] As the water solubility of the xenobiotic is increased, it is more readily excreted from the body in the urine.

Prior to analyzing a sample using liquid chromatography-tandem mass spectrometry (LC-MS/MS), it is common to remove the conjugated glucuronide. Removal of the glucuronide is completed using an acidic solution or an enzyme called β-glucuronidase. It is also important to consider if the analytical method is looking for analytes with or without glucuronides. The approximate molecular weight of the analyte of interest is used for LC-MS/MS, so in the event the method file only accounts for the molecular weight of the specific parent analyte, the glucuronides must be cleaved off. However, if the analytical method is looking for the glucuronide conjugate, then it would not need to be converted back to parent compound.

2.4 Dilute and Shoot Method

Dilute and shoot (DS) is the most common technique of urine sample preparation for LC-MS/MS analysis. DS gained its popularity due to the ease of sample preparation.[5]As the name suggests, a urine sample is diluted before “shooting” the sample on the instrument. Facilities that

use the DS technique have their variations of the technique. Cao et al performed DS by centrifuging the urine sample, removing an aliquot, adding internal standard and sample diluent to the aliquot, and then injecting the sample for analysis.[5] Conversely, Kong et al. chose to combine an aliquot of sample with internal standard, centrifuge, and pipet off the supernatant to be injected for analysis.[31] Deventer et al varied the DS technique based on the drug of interest; samples with internal standard were either only centrifuged, only filtered, or centrifuged and filtered.[32] While there is variation among these DS techniques, ultimately each dilutes the sample in some fashion and includes the addition of internal standard.

Urine, naturally excreted through urination, is filled with various substances that might interfere with LC-MS/MS analysis. Much of urine is water (91-96%), with the remaining portion consisting of organic solvents, inorganic ions, fatty acids, enzymes, carbohydrates, hormones, mucins, and pigments.[33] The amount of these other items within urine are based on kidney function. Therefore, there are some urine samples that have more components, at higher concentrations, than others. Though the additional components of urine are necessary for life, they are not beneficial to the analysis of urine samples for drugs. Samples are often vortexed to create a pellet of the unnecessary components of urine at the bottom of the tube. When unnecessary components have been excluded from the overall urine sample, the remaining liquid is a cleaner form, ready to be injected onto the instrument for analysis.

2.5 Volumetric Absorptive Microsampling (VAMS)

The device used for Volumetric Absorptive Microsampling (VAMS) is a plastic holder with a porous absorptive tip, which is manufactured to draw up a specific amount of sample by way of capillary action. Tips can be purchased to have an absorptive volume of 10 or 20 µL.[34] VAMS tips are cited to be compatible with 8 fluid types; however, blood and plasma are the most widely researched.[34] At the time research was conducted, one article was published that evaluated

urine and oral fluid.[3] At the time thesis writing was being completed, a second article was published that assessed urine.[35]

2.5.1 Body fluids most researched with VAMS- Blood and Plasma

VAMS has been being researched as an alternative to dried blood sampling (DBS) since 2014.[36] DBS consists of spotting a blood droplet onto a filter card and allowing the blood to dry, and has been previously established as feasible for drug detection..[36] When it comes to transportation and storage, DBS has advantages to that of wet blood samples. Because the blood is dried, there is no need for cooling and hazardous transportation arrangements. Additionally, the cards can more easily be stored and packaged than specimen vials. Despite these advantages to DBS, there are still flaws. The greatest concern is obtaining a consistent amount of sample, as there are no specific requirements for the volume of blood that must be spotted on the card or specific technique used for spotting.[36] Hematocrit (HCT), the number of red blood cells, affects the consistency of blood, which will factor into how much sample volume is being spotted.[36,37] When there is a lower concentration of HCT, the blood is less viscous, which in turn means the sample is spread more easily, making for a larger blood spot.

Comparative research has been conducted to assess if VAMS is a viable alternative to DBS. Denniff and Spooner appear to be the first to publish their research comparing DBS and VAMS in terms of HCT concentration in 2014.[36] Using the 10 µL VAMS tips, Denniff and Spooner found that the average volume of sample drawn up, regardless of HCT concentration was 10.5 ± 0.1 µL.[36] Additionally, a test was conducted to evaluate the variability of VAMS between users. It was found that, "…VAMS has similar volume errors to other already accepted and established techniques." [36] Another element of VAMS that Denniff and Spooner evaluated was the amount of additional volume that would be collected in the event of the microsampling tip being fully submerged in the blood sample. When fully submerged, the volume increased by

26% or greater than the control average of 10.5 µL.[36] This finding emphasizes the importance of proper training using VAMS.

The following year, De Kesel et al performed similar experimentation to evaluate how the HCT concentration would factor in to blood sample collection using VAMS. De Kesel et al was more interested in the concentration of caffeine and paraxanthine, a caffeine metabolite, than the volume of sample collected.[38] At the time of their research, the VAMS manufacturer reported the 10 µL tip absorbed an average of 10.7 µL. While Denniff and Spooner had only compared DBS and VAMS, De Kesel et al expanded out to compare DBS and VAMS to wet whole blood samples.[38] It was reported there was less variation in drug concentrations when using VAMS regardless of the concentration of HCT when analyzed using LC-MS/MS.[38] Additionally, it was observed that "recovery was somewhat lower for the IS than for the analyte..." when using VAMS.[38]

In 2016, Bolea-Fernandez et al evaluated VAMS using whole blood as an alternative to venipuncture and DBS testing for metals in individuals that received metallic prosthetics.[39] When it came to ultra-trace amount testing, Al, Cr, and Ni, were the metals in the study that were found to not be suitable for VAMS blood testing.[39] The authors speculated that those particular metals may have been used in the production process of making the VAMS tips, which resulted in the increased concentrations of those metals.[39] However, the remaining metals were successfully qualified and quantified through inductively coupled plasma – mass spectrometry (ICP-MS).[39]

Barco et al compared the concentration of four antibiotics in DBS, dried VAMS, and wet plasma through LC-MS/MS testing.[40] It was found that the concertation of the antibiotics across the three methods were highly comparable, with a relative standard deviation of 12%.[40] In addition, an evaluation of analyte stability for VAMS was performed. Over short term storage of 72 hours, all antibiotics had minimal loss when stored at -20°C or 4°C. [40] When stored at room temperature for short term, one antibiotic experienced 20-35% loss. [40] Over long term storage of 1

month, all antibiotics had minimal loss when stored at -20°C. Conversely, all antibiotics experienced 22-36% loss when stored at 4°C or room temperature for long term.[40]

While more research must still be conducted, several studies have so far found VAMS possess the potential to be an alternative, if not replacement, of DBS.[36,38,39,40,41,]

2.5.2 VAMS in Urine

At the time of this research project, there had been one publication about the use of VAMS for drug identification in dried urine samples. Mercolini et al. compared dried urine, plasma, and oral fluid to the wet matrix for the quantification of cathinone analogues.[3] Cathinones are a psychoactive stimulant similar to amphetamines. [3] They are more commonly referred to as "bath salts." [3] Through their research, Mercolini et al. found cathinones to degrade in wet samples. [3] In order to properly identify and quantify cathinone analogues a dried urine sample is ideal. Ultimately, Mercolini et al. found that "the sampling/processing methods demonstrated a good equivalence between conventional wet samples and dried samples collect on VAMS devices."[3]

During the writing process of this thesis, a second journal article was published regarding dried urine with VAMS. Protti et al. compared wet plasma to dried blood spots (DBS) and dried plasma spots (DPS), and wet urine to dried VAMS and dried urine spots (DUS).[35] Protti et al.'s research focused on oxycodone, oxymorphone, and noroxycodone. [35] Similarly to Mercolini et al., Protti et al. found that certain analytes of interest were more stable in dried matrices rather than wet.[35] However, when it came to comparing the dried samples to each other with their respective matrix, Protti et al. found there to be "no clear winner..."[35]

At this time there is limited information when it comes to VAMS being used with dried urine samples for drug testing. Therefore, additional studies are necessary to establish the validity and practicality of using VAMS.

2.6 Liquid Chromatography – Tandem Mass Spectrometry

Liquid chromatography – tandem mass spectrometry (LC-MS/MS) combines the polar separation capabilities of LC with that of tandem MS to ionize and sort molecules based on mass-to-charge (m/z) ratios.[42]

2.6.1 Liquid Chromatography

Liquid chromatography may be used in either planar or column techniques. Column LC is what was used in this research. The column provides a stationary phase, for the molecules to flow through, with the application of mobile phases of varying hydrophilicity. In the case of a non-polar stationary phase, polar molecules will elute off the column with an aqueous (polar) mobile phase at a faster rate. The more polar the molecule, the faster it will come off the column. A gradual transition from aqueous to organic (non-polar) mobile phase occurs, creating an increased non-polar environment. Non-polar molecules that remained in the column will now elute off in the non-polar mobile phase. With high pressure liquid chromatography (HPLC), a high pressure system is used to assist in moving the mobile phases, and thus the analytes of interest, through the column.[43]

The time at which molecules elute off the column is termed the retention time. The retention time is specific to the molecule, mobile phases used, and the concentration gradient of the mobile phases. However, it is possible for molecules to coelute, meaning they come off the column at the same retention time. In instances of coelution, it is important to have a secondary analysis is completed to ascertain what the molecule is, such as a mass spectrometer.[42]

2.6.2 Mass Spectrometry

Mass spectrometry measures ionized analytes.[44] In the case of a triple quadrupole MS/MS, as seen in Figure 1, sorting and measurement of ions take place in three stages. Quadrupole typically refers to the fact that there are four rods or poles arranged around the ion

flow to act as mass filters. Radio frequencies and direct current voltages applied to the poles allow only ions with a specified m/z ratio through to the detector. [44] Figure 1 shows the configuration of a tandem mass spectrometer, or triple quadrupole instrument, in which the first and third quadrupoles act as mass filters and the second quadrupole serves as a collision cell.

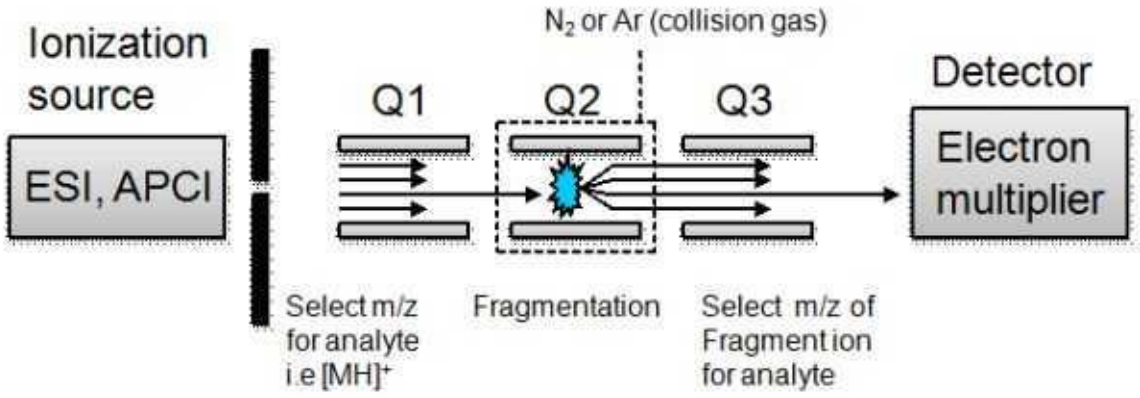

Figure 1. Triple quadrupole of tandem mass spectrometer. (Taken from Ni J, Ouyang H, Aiello M, et al. Microdosing Assessment to Evaluate Pharmacokinetics and Drug Metabolism in Rats Using Liquid Chromatography-Tandem Mass Spectrometry. *Pharm Res*. 2008;25(7):1572-1582. doi:10.1007/s11095-008-9555-x)

Specifically, in the first quadrupole (Q1) the spectrometer is set to filter only ions of a specific m/z ratio. For example, oxycodone is known to have a m/z ratio of 315.90. Ions with the specified m/z ratio are allowed through to Q2, where a specific amount of energy is applied, resulting in ion collision and fragmentation of the target analyte. Continuing with the example of oxycodone, when collision energy is applied to oxycodone it is known to fragment into 298.15 and 241.10 m/z. Q3 of the spectrometer will then allow only these specific m/z ratios to pass to the detector. The combination of the Q1 and Q3 m/z targets are called a transition, and the resultant chromatograms have a peak for each transition that appear in a predictable ratio.

2.7 Conclusion

While doctors can be careful about for who and how they write prescriptions, there is still the need to ensure prescription compliance for the safety of the patient being treated. Urine is ideal for drug testing because the collection is noninvasive and easy, and it has a wider detection window for drugs than blood. Additionally, urine drug testing using LC-MS/MS allows for a variety of drugs to be detected.

Dilute and shoot (DS) has traditionally been the technique of choice for urine sample extraction. The popularity of DS is due in part to the fact that until VAMS, there had not been a viable technique available for testing dry, rather than traditional wet, urine samples. The goal of this research is to compare the wet dilute and shoot technique to dry VAMS technique for urine drug testing for analysis on LC-MS/MS. From this research, it may be determined whether or not dry urine testing with VAMS is as reliable for drug identification in urine samples as that of the wet dilute and shoot technique.

CHAPTER III

METHODOLOGY

3.1 Introduction

The purpose of this research project was to determine the effectiveness and efficiency of two urine drug identification techniques, wet urine using dilute and shoot and dry urine using VAMS tips. The research was conducted as a two-part study. The first part of the study involved the development of a hydrolysis method for the VAMS tips and to determine the percent recovery of hydrolyzed glucuronides. This was accomplished by calculating the hydrolyzing efficiency of glucuronidase enzymes using known amounts of glucuronide conjugates across multiple drug classes. The second part of the study pertained to anonymized samples that had been analyzed clinically using a validated DS technique. These samples were used to compare each technique's ability to identify the drugs present in the specimens at various concentrations. All samples were analyzed using liquid chromatography-tandem mass spectrometry (LC-MS/MS) at Oklahoma State University – Clinical Laboratory Services (OSU-CLS).

3.2 Materials

All drugs in Tables 1 - 3 were purchased from Cerilliant (Cerilliant Corporation, Round Rock, TX) in the specified concentrations. Methanol was purchased from JT Baker (Avantor

Performance Materials Inc., Center Valley, PA). Formic acid and glacial acetic acid were purchased from EDM (EDM Millipore Corporation, Billerica, MA). Isopropanol was purchased from Honeywell (VWR International, Randor, PA). HPLC grade water was obtained from a Barnstead Nanopure water system (Thermo Scientific, Waltham, MA). Ammonium formate was purchased from Alfa Aesar (Alfa Aesar, Ward Hill, MA). Sodium acetate was purchased from BDH (VWR International, Randor, PA). Drug-free urine was purchased from UTAK (UTAK Laboratories Inc., Valencia, CA). β-glucuronidase was purchased from Campbell Science (Campbell Science, Rockford, IL). IMCSzyme and rapid hydrolysis buffer were purchased from IMCS (IMCS, Irmo, SC). Mitra ® microsampling devices were obtained from Neoteryx for a collection volume of 20 µL (Neoteryx, Torrance, CA).

Table 1. Standards purchased from Cerilliant at 1.0 mg/mL.

6-Acetylmorphine	Morphine
Alpha-Hydroxyalprazolam*	Norbuprenorphine*
Amphetamine	Nordiazepam
Benzoylecgonine	Norhydrocodone
Clonazepam	Noroxycodone
Codeine	Oxazepam
Diazepam	Oxycodone
Gabapentin	Oxymorphone
Hydrocodone	Temazepam
Hydromorphone	(-)-11-nor-9-Carboxy-delta9-THC (THCA)
Lorazepam	Tramadol

* Concentration at 100.0 µg/mL

Table 2. Glucuronide conjugates purchased from Cerilliant at 100.0 µg/mL.

Codeine-6β-D-glucuronide	Oxazepam glucuronide
Lorazepam glucuronide	(+)-11-nor-9-carboxy-delta9-THC glucuronide
Morphine-3β-D-glucuronide	

Table 3. Internal standards purchased from Cerilliant at 100.0 µg/mL.

Amphetamine-D5*	Hydromorphone-D3*
Benzoylecgonine-D3	Morphine-D6
Codeine-D6*	Normeperidine-D4
Diazepam-D5	Oxycodone-D6*
Fentanyl-D5	(+)-11-nor-9-Carboxy-delta9-THC-D3*
Gabapentin-D10	

* Concentration at 1.0 mg/mL

3.3 Solution Preparation

3.3.1 Standards

3.3.1.1 Internal standard. A total of 5.0 mL of the internal standard was prepared in methanol as described in Table 4. The total volume of internal standards came to 1040 µL, and the remaining 3960 µL consisted of methanol. Internal standard was prepared in advance and stored at 4°C until ready for use, expiring 1 year from the date it was made.

Table 4. Preparation of 5.0 mL of internal standard solution. Total internal standard volume was 1040 µL. Methanol volume was 3960 µL, for a total volume of 5 mL.

Internal Standard	**Spike Volume (µL)**	**Final Concentration (µg/mL)**
Amphetamine-D5	10.0	2.0
Benzoylecgonine-D3	50.0	1.0
Codeine-D6	10.0	2.0
Diazepam-D5	250.0	5.0
Fentanyl-D5	50.0	1.0
Gabapentin-D10	50.0	1.0
Hydromorphone-D3	10.0	2.0
Morphine-D6	500.0	10.0
Normeperidine-D4	50.0	1.0
Oxycodone-D6	10.0	2.0
(+)-11-nor-9-Carboxy-delta9-THC-D3	50.0	10.0

3.3.1.2 Calibration curve. A calibration curve was prepared to run alongside the glucuronide mix and unknown samples to quantify the drug concentrations. A calibration curve provides a range of quantitation. A 1 mL calibration stock solution was prepared for analytes to be at a final concentration of 1000 ng/mL in UTAK urine, the necessary spike volumes are stated

in Table 5. Of the prepared 1.0 mL of 1000 ng/mL stock, 500 µL was used to generate a stock of 500 ng/mL with 500 µL of UTAK urine. This 500 ng/mL solution was used as the starting concentration for preparing the curve. A total of 6 calibration points were made that ranged from a lower limit of quantitation of 20 ng/mL to an upper limit of quantitation of 500 ng/mL. The concentrations for each of the 6 calibration points are listed in Table 6. Calibration levels were prepared by using the previous calibration level and UTAK urine, with the specific volumes necessary for curve production as presented in Table 7. The prepared calibration curve was compared to a validated OSU-CLS calibration curve.

Table 5. Preparation of calibration stock solution for a final concentration of 1000 ng/mL. Total spike volume was 40 µL. UTAK urine volume was 960 µL.

Drug	**Spike Volume (µL)**
6-Acetylmorphine	1.0
Alpha-Hydroxyalprazolam	10.0
Amphetamine	1.0
Benzoylecgonine	1.0
Clonazepam	1.0
Codeine	1.0
Diazepam	1.0
Gabapentin	1.0
Hydrocodone	1.0
Hydromorphone	1.0
Lorazepam	1.0
Morphine	1.0
Norbuprenorphine	10.0
Nordiazepam	1.0
Norhydrocodone	1.0
Noroxycodone	1.0
Oxazepam	1.0
Oxycodone	1.0
Oxymorphone	1.0
Temazepam	1.0
(-)-11-nor-9-Carboxy-delta9-THC (THCA)	1.0
Tramadol	1.0

Table 6. Concentrations of each drug mentioned in Table 5 at the specific level.

Concentration (ng/mL)	Level
500.0	25.0 c
300.0	15.0 c
200.0	10.0 c
150.0	7.5 c
50.0	2.5 c
20.0	1.0 c

Table 7. Preparation of the 6 calibration points starting from a concentration of 500 ng/mL of the drugs listed in Table 5.

Sample Name	Calibrator Solution (µL)	25.0c (µL)	15.0c (µL)	10.0c (µL)	7.5c (µL)	2.5c (µL)	UTAK Urine (µL)	Total Volume (µL)	Remaining (µL)
25.0c	175						1575	1750	718
15.0c		1032					688	1720	700
10.0c			1020				510	1530	720
7.5c				810			270	1080	700
2.5c					380		760	1140	720
1.0c						420	630	1050	700

3.3.1.3 OSU-CLS quality control. Validated quality control (QC) standards were obtained from OSU-CLS. This was done as an additional measure to ensure the quality of calibration curve.

3.3.2 Glucuronide conjugate mix. The glucuronide conjugate mix used was prepared from a stock solution where each of the five glucuronide conjugates was at a final concentration of 500 ng/mL in methanol. An aliquot of the stock solution was diluted in half for a final concentration of 250 ng/mL in methanol.

3.3.3 Unknown samples. Unknown samples were anonymized samples. A total of ten individual samples were used.

3.4 Techniques

3.4.1 Dilute and Shoot

3.4.1.1 Overview. Dilute and shoot (DS) is a technique in which a sample is diluted before being injected (shot) onto an instrument. The DS procedure used in this study was the clinically validated procedure used at the OSU-CLS.

3.4.1.2 Methodology. Because the DS procedure used was a validated method of OSU-CLS, there was no need for method development. A fresh batch of hydrolysis solution was prepared for each sample batch. Hydrolysis is the act of cleaving off a sugar group to produce the parent drug. This hydrolysis solution consisted of 20 µL internal standard, 25 µL sodium acetate buffer pH 4.0, and 10 µL Campbell's β-glucuronidase per sample. Once the reagents are added together, it was vortexed for 10 seconds using a VWR signature mini vortexer (VWR International, Randor, PA), followed by centrifugation at 13 000 RPM for 10 minutes with a Heraeus Pico 17 centrifuge (Thermo Scientific, Waltham, MA).

Into each 1.5 mL Eppendorf tube, 50 µL of hydrolysis was added; then 50 µL of sample was added to its respective tube. Each tube was vortexed for 10 seconds. Samples were incubated at 55°C for two hours in a VWR mini incubator (VWR International, Randor, PA). When incubation was complete, 150 µL of 1:9 methanol to water sample diluent was added.

Each sample was vortexed for 10 seconds. Samples were then centrifuged at 30 000 RPM for 10 minutes in an Eppendorf Centrifuge 5424 (VWR International, Randor, PA). Upon completion of centrifugation, 200 µL of supernatant was placed into the respective injection vial. A lid to the injection vial was secured in place. Samples were then ready to be placed on the instrument for analysis. Samples were extracted once and injected 4 times in order to perform statistical analysis.

3.4.2 Microsampling Tips

3.4.2.1 Overview. The Neoteryx Mitra ® VAMS devices used in this research were designed to collect 20 µL of sample. Figure 2 is an image of the microtip. These VAMS devices absorb urine and are then allowed to dry for 3 hours at room temperature, resulting in a dry urine sample.

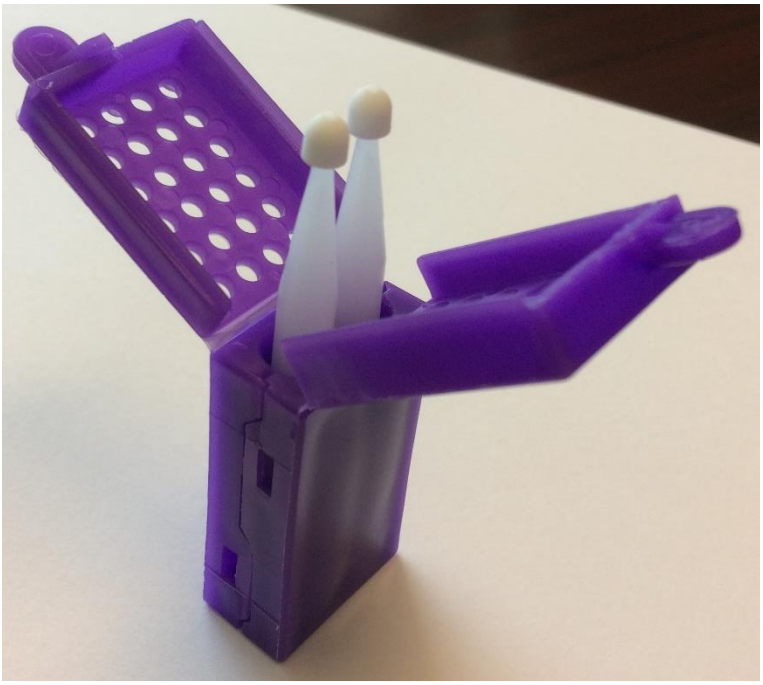

Figure 2. Neoteryx Mitra ® microsampling device, 20 µL.

3.4.2.2 Volume evaluation. The Neoteryx Mitra ® microsampling devices used in this research were manufactured to absorb 20 µL of a solution. A test was conducted to determine the amount of liquid that was truly absorbed by the tip in the time it took the solution to be absorbed plus an addition 6 seconds. This test was conducted using two mediums: deionized water and blank urine. The temperature of the work space was recorded at the start and end of the experiment. A 5-mL disposable polystyrene beaker (Fisher Scientific, Hampton, NH) was placed on the XS64 analytical balance (Mettler Toledo, Columbus, OH) and then tared. A 1000 µL pipet was used to mass 1 mL of deionized water 3 times. Between each addition the mass was recorded

in grams and tared before the next 1 mL was added. This same process was repeated using blank urine, collecting a total of 3 masses.

A 5-mL disposable polystyrene beaker with approximately 3 mL of deionized water was placed on the analytical balance and tared. A Neoteryx Mitra ® microsampling device was dipped into the solution enough to make contact with the water but not enough to fully submerge the tip. A very subtle color change was observed as the water was absorbed. The microtip was held in the water an additional 6 seconds after the tip experienced a complete change in color. The absolute values of the change in mass in grams were recorded and the analytical balance was tared. This was repeated 2 more times, using different microsampling tips. Another set of 3 microsampling tips were used when repeating the process with blank urine.

The volume absorbed by each tip was calculated using the mass absorbed and the average solution density of the respective solution. The three volumes were averaged to determine the average volume of deionized water and blank urine absorbed by the Neoteryx Mitra ® microsampling tips.

3.4.2.3 Method development. When developing a method for the Volumetric Absorptive Microsampling (VAMS) tips extraction technique, the hope was to use similar, if not the same, ratios of each reagent as that of the DS technique. VAMS samples were to be collected per the recommendation of the manufacturer.

To remove drugs from the tip, the tips were sonicated in solution containing an internal standard. Initially, the intent was to use 10 µL of internal standard and 30 µL of sodium acetate buffer pH 4.0, the same buffer used in the DS technique. However, 40 µL was not enough solution to fully submerge the tip in the Eppendorf tube. Therefore, the volume of acetate buffer was increased to 80 µL, making for a final volume of 90 µL. Samples were then sonicated for 15 minutes. After sonication, the solution was injected onto the LC-MS/MS. At this time, no β-glucuronidase enzyme was used because the recovery of drugs bound to glucuronides was not the objective. Instead, the objective was to determine if drugs and internal standard were able to be

seen using 80 µL of acetate buffer and 10 µL of internal standard. Upon analysis with LC-MS/MS it was determined that sodium acetate buffer pH 4.0 was not able to remove drugs from the VAMS tip. Additionally, identification of internal standards was minimal. From a literature review, it was determined that methanol and acetonitrile had been successfully used in other studies using VAMS during sonication in order to extract drugs from the VAMS tip. The experiment was repeated, one using methanol, the other using acetonitrile, and LC-MS/MS analysis was conducted. The results for the sample using methanol and the sample using acetonitrile were very comparable, as in both instances, drugs and internal standards were easily identifiable. It was decided to proceed with methanol since that is the basis of the chromatography mobile phase used in this research.

The next step was to determine the lower limit of quantitation for drugs. Many of the drugs in the OSU-CLS validated DS method were known to be identifiable down to 15 or 20 ng/mL, except for (-)-11-nor-9-Carboxy-delta9-THC (THCA) at 150 ng/mL. UTAK urine was fortified to 20 ng/mL for all the analytes, then samples were collected and allowed to dry for 3 hours. They were then submerged into 90 µL of 1:8 mix of internal standard to methanol and sonicated for 15 minutes. At this time, no β-glucuronidase enzyme was used, because the spiked urine did not contain glucuronide conjugates. Analysis with LC-MS/MS determined that aside from THCA, drugs were able to be seen down to 20 ng/mL. It was determined that the lower limit of the calibration curve would be 20 ng/mL for all drugs, realizing that THCA would not be able to be seen at that level.

The activity of β-glucuronidase is known to be disrupted by methanol. As methanol is being used to dislodge the drugs from the VAMS tip, it must be removed prior to the addition of the enzyme. A test was conducted by putting 20 µL of each of the calibration curve points or 20 µL of a 200 ng/mL glucuronide mix into individual Eppendorf tubes, then adding 90 µL of the 1:8 mix of internal standard to methanol to each tube. At this point in time, VAMS tips were not used because the goal was proof of concept. Samples were dried down on a SPEware CEREX 48

sample concentrator (Tecan SP, Inc., Baldwin Park, CA). Originally the thought was to just dry off the methanol. However, it proved challenging to determine exactly when the methanol was fully gone from the sample. Therefore, in order to achieve complete dryness, the drying took place for 1 hour with the nitrogen dry down gas at 60°C. Once drying was complete, 25 µL of IMCS mix, consisting of 10 µL enzyme and 15 µL of buffer, were added to the Eppendorf tubes. Samples were then incubated for 30 minutes at 55°C, 40 µL of a 1:9 methanol to water sample diluent was added, and the resultant samples were analyzed using LC-MS/MS. From analysis, the calibration curve and glucuronide mix appeared to provide results consistent with functional enzyme.

The idea of increasing glucuronide recovery by altering the amount of enzyme used was evaluated. The following was added to an Eppendorf tube: 90 µL of the 1:8 mix of internal standard to methanol and either 20 µL of the calibration curve points or 20 µL of a 200 ng/mL glucuronide mix. Three separate glucuronide mix tubes were prepared. Sonication occurred for 15 mins, followed by dry down on the SPEware CEREX for 1 hour at 60°C. The first glucuronide tube received 10 µL IMCS enzyme and 15 µL of IMCS buffer. The second glucuronide tube received 20 µL IMCS enzyme and 30 µL of IMCS buffer. The third glucuronide tube received 30 µL IMCS enzyme and 45 µL of IMCS buffer. All curve samples received 35 µL of HPLC grade water because there were no glucuronides to cleave. Samples were vortexed for 10 seconds and incubated for 30 minutes at 55°C. When incubation was complete, sample diluent was added to all tubes. The first glucuronide tube received 75 µL of sample diluent, second glucuronide tube received 50 µL of sample diluent, and the third glucuronide tube received 25 µL of sample diluent. The curve samples received 65 µL of sample diluent, bringing the total volume for all tubes to 100 µL. All samples were vortexed for 10 seconds and centrifuged for 10 minutes at 13,000 RPM. After LC-MS/MS analysis, minimal difference was observed in between the 3 glucuronide samples. The overall volume of the sample supernatant was raised from 65 µL to 100 µL to allow for additional injections on the instrument, should it be necessary.

Using methanol as the solvent for sonication worked; however, 1 hour was being spent drying down the sample. In an effort to speed up the dry down process, dichloromethane (DCM) was used in place of methanol. Internal standard and DCM were found to be immiscible with each other. Once more, 20 µL of each calibration curve points were added to their respective Eppendorf tubes. After 15 minutes of sonication no solvent was present in the Eppendorf tubes, therefore no dry down was necessary. In place of enzyme and buffer, 25µL of HPLC water was used and 75 µL of sample diluent was added. The sample was then injected onto the LC-MS/MS. Using DCM, not all of the drug analytes and internal standards were able to be recovered. Therefore, it was accepted that methanol and drying down was the most viable option for drug extraction from the VAMS tips for analysis using LC-MS/MS.

3.4.2.4 Urine VAMS Methodology. Labeled microtips were dipped into the sample, enough to break the surface of the urine, but not enough to fully submerge the tip. A slight color change was observed on the microtip as the urine was absorbed. The microtip was held in the urine an additional 6 seconds after the tip experienced a complete change in color. Samples were permitted to dry at room temperature for 3 hours.

A stock of internal standard/methanol mix was prepared at the start of each batch. Stocks were prepared at a ratio of 1:8 mix of internal standard to methanol. Once dried, samples were placed in the respective 1.5 mL Eppendorf tube that contained 90 µL of the internal standard/methanol mix. Figure 3 shows what the microtip looked like once in the Eppendorf tube. Care was taken to ensure no bubbles were trapped below or alongside the microtip.

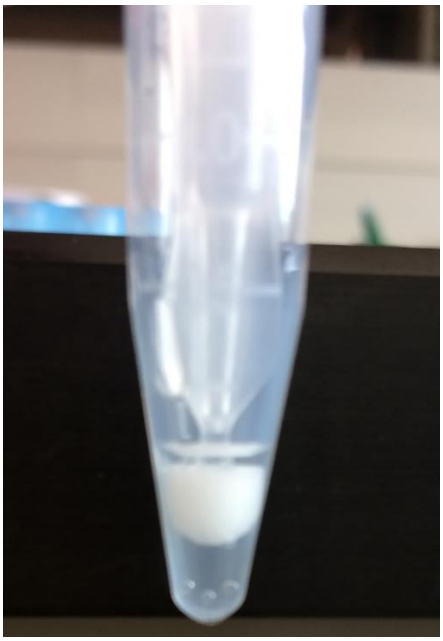

Figure 3. VAMS tip submerged in internal standard/methanol mix.

Samples were sonicated for 15 minutes using a B2500A-MTH ultrasonics cleaner (VWR International, Randor, PA). The lids of the Eppendorf tubes remained open because the microsampling tip extended past the top of the tube. Upon completion of sonication, the microsampling devices were removed from the tubes.

Samples were transferred to a drying rack and dried on a SPEware CEREX 48 sample concentrator (Tecan SP, Inc., Baldwin Park, CA). Samples were dried at with nitrogen gas at 45°C for 70 minutes, followed by 60°C until completely dry. The time at 60°C ranged from 11 minutes up to 35 minutes. The rate of drying was a function of the laboratory conditions; on humid days samples took longer to dry down.

A stock of IMCSzyme and rapid hydrolysis buffer was prepared 5 – 10 minutes before the dry down completion and stored at 4°C until ready for use. This stock was made so that each sample would receive 10 µL of enzyme and 15 µL of buffer. A total of 25 µL of IMCS mix was added to the now dried down samples in the Eppendorf tubes. All samples were vortexed for 10 seconds using a VWR signature mini vortexer (VWR International, Randor, PA), followed by incubation at 55°C for 30 minutes in a VWR mini incubator (VWR International, Randor, PA).

When incubation was complete, 75 µL of a 1:9 methanol to water sample diluent was added, and each sample was vortexed for 10 seconds. Samples were then centrifuged at 30,000 RPM for 10 minutes in an Eppendorf Centrifuge 5424 (VWR International, Randor, PA). Upon completion of centrifugation, 100 µL of supernatant was placed into an insert in the respective injection vial, and the lid to the injection vial was secured in place. Samples were then ready to be placed on the instrument for analysis. Samples were injected 4 times to allow the researchers to perform statistical analysis.

3.5 Liquid Chromatography-Tandem Mass Spectrometry

Samples were analyzed using a Shimadzu HPLC system (Shimadzu Corporation, Kyoto, Japan) with a CBM-20A control module, 2 LC-AD solvent pump, SIL-20A HT autosampler, CTO-20A column oven, and FCV-20AH2 diverter valve. Liquid chromatography separation was completed using a Restek® Raptor™ Biphenyl guard column (2.7 µm, 5 x 3.0 mm) and column (2.7 µm, 50 x 2.1 mm) (Restek, Bellefonte, PA). The HPLC was attached to the front of a Shimadzu LCMS-8040 liquid chromatography tandem mass spectrometer (LC-MS/MS) system.

The aqueous mobile phase (MPA) consisted of 2 mM ammonium formate and 0.1% formic acid in HPLC grade water. The organic mobile phase (MPB) consisted of 2 mM ammonium formate and 0.1% formic acid in LCMS methanol. The LC pumps had a total flow rate of 0.35 mL/min. Total injection volume was set at 5 µL. MPB concentration began at 10%, ramped up to 35% by 1.90 minutes, ramped up to 100% by 3.90 minutes, held at 100% until 4.50 minutes, then dropped to 10% by 4.51 minutes, where it remained until the end of the 5-minute run time (Figure 4). The oven temperature was set at 30°C. Tables 8 and 9 are a comprehensive list of the internal standards and drugs in the panel, to include the ion transitions and instrument parameters for compound detection. Regardless of the technique used, all samples were analyzed using the same LC-MS/MS method parameters.

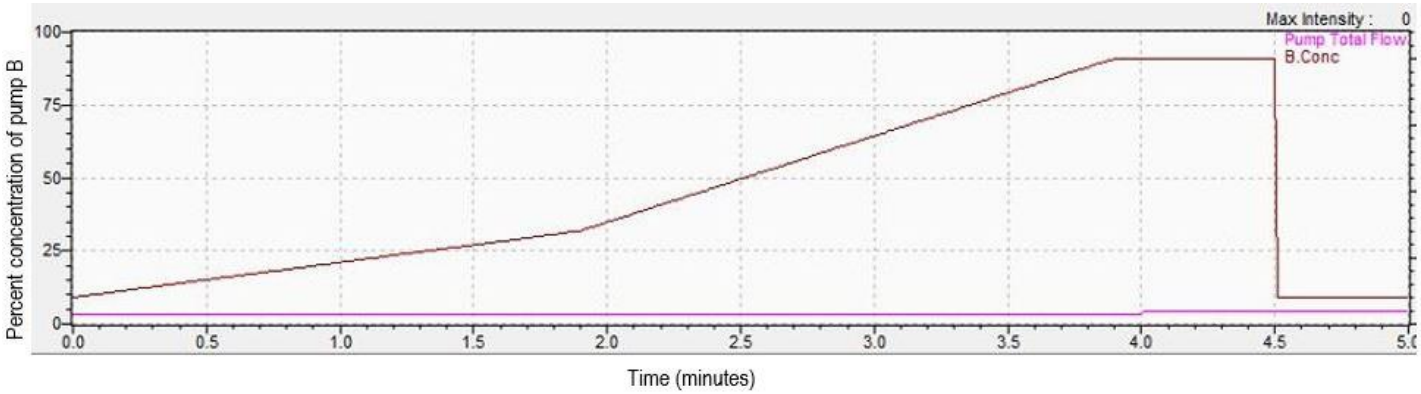

Figure 4. Graphical representation of the change in MPB concentration during the 5 minute run.

Table 8. Analyte internal standards in the panel. Includes LC-MS/MS parameters for ion transitions and instrument parameters for detection of compounds.

Analyte [a]	**Q1 Mass (Da)** [b]	**Q3 Mass (Da)** [c]	**DP (V)** [d]	**CE (V)** [d]	**CXP (V)** [d]
Amphetamine-D5	140.80	93.00	15.00	19.00	35.00
Benzoylegonine-D5	292.80	171.05	38.00	19.00	30.00
Codeine-D6	305.90	165.10	40.00	45.00	30.00
Diazepam-D5	289.90	153.95	50.00	29.00	28.00
Fentanyl-D5	341.85	105.10	46.00	42.00	38.00
Gabapentin-D10	182.10	55.05	12.00	27.00	20.00
Hydromorphone-D3	288.90	184.95	50.00	31.00	34.00
Morphine-D3	289.10	165.05	15.00	42.00	30.00
Normeperidine-D4	238.00	42.05	12.00	35.00	44.00
Oxycodone-D6	321.90	304.15	38.00	20.00	32.00
(+)-11-nor-9-Carboxy-delta9-THC-D3	348.20	330.20	20.00	15.00	35.00

[a] Drugs were identified using two mass ion fragments, internal standards were identified using one mass ion fragment.
[b] The molecular mass of each analyte measured in Daltons.
[c] Unique ion mass fragment measured in Daltons.
[d] "DP", "CE", and "CXP" refer to voltages used for declustering potential, collision energy, and collision cell exit potential.

Table 9. Analyte drugs in the panel. Includes LC-MS/MS parameters for ion transitions and instrument parameters for detection of compounds.

Analyte [a]	Q1 Mass (Da) [b]	Q3 Mass (Da) [c]	DP (V) [d]	CE (V) [d]	CXP (V) [d]
6-Acetylmorphine	328.20	165.05	10.00	40.00	30.00
	328.20	58.10	10.00	29.00	20.00
Alpha-Hydroxyalprazolam	324.90	297.05	16.00	27.00	32.00
	324.90	216.00	16.00	42.00	40.00
Amphetamine	136.00	91.10	20.00	17.00	15.00
	136.00	119.15	20.00	14.00	45.00
Benzoylecgonine	289.95	168.05	14.00	19.00	30.00
	289.95	104.95	20.00	29.00	18.00
Clonazepam	316.20	270.00	50.00	35.00	30.00
	316.20	214.00	50.00	35.00	45.00
Codeine	299.90	165.00	35.00	43.00	30.00
	299.90	215.00	35.00	28.00	20.00
Diazepam	284.90	153.95	36.00	28.00	28.00
	284.90	193.00	46.00	34.00	34.00
Gabapentin	172.20	154.00	30.00	15.00	15.00
	172.20	137.10	30.00	18.00	25.00
Hydrocodone	299.90	199.00	35.00	40.00	35.00
	300.30	171.10	50.00	40.00	30.00
Hydromorphone	285.90	185.00	32.00	32.00	34.00
	285.90	157.00	32.00	42.00	26.00
Lorazepam	320.80	274.90	40.00	23.00	50.00
	320.80	229.00	40.00	30.00	45.00
Morphine	286.10	165.00	45.00	42.00	30.00
	286.10	155.10	45.00	35.00	15.00
Norbuprenorphine	414.30	83.10	25.00	54.00	15.00
	414.30	101.20	24.00	40.00	40.00
Nordiazepam	271.20	139.90	18.00	29.00	24.00
	271.20	208.10	18.00	30.00	40.00
Norhydrocodone	285.90	198.95	32.00	30.00	36.00
	286.00	127.95	14.00	55.00	46.00
Noroxycodone	302.10	187.00	50.00	26.00	35.00
	302.10	227.10	50.00	28.00	15.00
Oxazepam	287.20	241.00	50.00	23.00	25.00
	287.20	104.00	15.00	34.00	40.00
Oxycodone	315.90	298.15	40.00	30.00	30.00
	316.30	241.10	50.00	30.00	45.00
Oxymorphone	301.90	227.00	34.00	30.00	40.00
	301.90	198.00	34.00	48.00	36.00
Temazepam	301.10	255.00	20.00	40.00	25.00
	301.20	176.90	20.00	40.00	30.00
(-)-11-nor-9-Carboxy-delta9-THC (THCA)	345.20	299.15	50.00	20.00	20.00
	345.00	193.10	45.00	26.00	35.00
Tramadol	263.60	264.10	30.00	7.00	30.00
	264.20	58.05	10.00	10.00	20.00

[a] Drugs were identified using two mass ion fragments, internal standards were identified using one mass ion fragment.

[b] The molecular mass of each analyte measured in Daltons.

[c] Unique ion mass fragment measured in Daltons.

[d] "DP", "CE", and "CXP" refer to voltages used for declustering potential, collision energy, and collision cell exit potential.

3.6 Data Analysis

Statistical analysis for quantification of analytes within samples was completed using Microsoft Excel (Microsoft Office, Microsoft Corporation, Redmond, WA) and GraphPad Prism 7.03 (GraphPad Software, La Jolla, CA). It was known that the concentration of the glucuronide conjugates was to be 250 ng/mL. Percent efficiency of drug recovery was calculated for each injection, as well as the average of the triplicate injections. Two-tailed t-tests were completed to determine if there was a statistically significant difference between the concentration of each drug in the samples based on the technique used.

3.7 Conclusion

Blank urine was fortified with a glucuronide conjugate mix at 250 ng/mL and unknown samples with drugs in the method panel were evaluated. The wet and dry techniques were performed on each of these samples. LC-MS/MS was used for identification and quantification of each analyte. Data analysis was performed to determine if there was a statistical difference between the two techniques.

CHAPTER IV

RESULTS

4.1 Glucuronide Conjugate Mix

The percent efficiency of the glucuronide conjugates recovered in each sample, from each injection, was determined by dividing the concentration reported from LC-MS/MS in ng/mL by the theoretical maximum of 250 ng/mL, then multiplying by 100. Mean percent efficiencies were then determined for DS and VAMS tips. The two-tailed t-test comparing the mean percent efficiencies of DS and VAMS tips for each of the 5 glucuronides are summarized in Table 10.

Table 10. Two-tailed t-test analysis of glucuronide efficiency.

	Mean % Efficiency	
Drug	**DS**	**Tip**
Codeine***	28.7	57.0
Lorazepam**	50.6	62.5
Morphine*	50.8	58.9
Oxazepam**	49.3	58.6
THCA*	66.1	88.0

* p-value ≤ 0.05 ** p-value ≤ 0.01 *** p-value ≤ 0.001
N = 4

4.2 Volume Evaluation

The microsampling tips obtained from Neoteryx was reported to absorb 20 µL of sample. The mean density of deionized water was determined to be 1.002 g/mL. The mean density of UTAK urine was determined to be 0.982 g/mL. Based on the mass of deionized water or UTAK urine absorbed by the microsampling tips, the mean volume of solution absorbed by the tip was 25.6 and 27.2 µL, respectively. A two-tailed t-test was conducted, determining there was not a

significant difference between the volume of deionized water and UTAK urine absorbed. A two-tailed t-test was conducted to evaluate the difference between the volume of deionized water and UTAK urine absorbed compared to the 20 µL the Neoteryx Mitra ® microsampling device was intended to absorb.

Based on the findings of the microsampling tip volume experiment, the experiment was repeated at a later date to determine the true volume taken up when pipetting 50 µL. The mean density of deionized water was determined to be 1.004 g/mL. The mean density of UTAK urine was determined to be 0.977 g/mL. Based on the mass of deionized water or UTAK urine drawn up by the pipet, the mean volume of solution was 49.8 and 51.2 µL, respectively. A two-tailed t-test was conducted using a p-value < 0.05, which determined there is not a significant difference between the volumes drawn up during the pipet volume test.

4.3 Internal Standard Comparison

A comparison of the areas of internal standards (IS) under the curve, QC's, and blanks were made. All ISs had significantly different areas given the two extraction techniques, as shown in Table 11. The ratio difference of sample to resuspension volume was minimal between the two techniques. DS used 50 µL of sample and had a resuspension volume of 200 µL, while VAMS used 27.2 µL of sample and had a resuspension volume of 100 µL.

Table 11. Two-tailed t-test analysis of average IS area in DS and microsampling tip extraction techniques.

	DS	Tip
Amphetamine-D5	11,900,336	7,290,158
Benzoylecgonine-D3	4,439,902	2,654,737
Codeine-D6	458,506	275,922
Diazepam-D5	1,698,232	897,732
Fentanyl-D5	4,023,548	2,156,112
Gabapentin-D10	958,379	554,722
Hydromorphone-D3	939,752	511,121
Morphine-D6	3,011,061	1,636,919
Normeperidine-D4	1,061,516	631,915
Oxycodone-D6	10,686,960	5,728,450
THCA-D3	59,580	1,152,184

All significantly different when two-tailed t-test conducted with p-value < 0.05 is used.
N = 23

4.4 Unknown Samples

The 10 unknown samples were evaluated for the 22 drugs using the two techniques. Drugs with a concentration greater than the lower limit of quantitation are considered positive for the particular drug. Drugs with a concentration less than the lower limit of quantitation are considered negative. The qualitative results of drugs present in the unknown samples by DS or VAMS are presented in Table 12.

Table 12. Unknown samples positive or negative for drugs based on being greater or less than the lower limit of quantitation, separated by extraction technique.

	1		2		3		4		5		6		7		8		9		10	
	DS	Tip	DS	Tip	DS	Tip	DS	Tip	DS	Tip	DS	Tip	DS	Tip	DS	Tip	DS	Tip	DS	Tip
6-MAM	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
α-Hydroxyalprazolam	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Amphetamine	-	-	-	-	+	+	-	-	-	-	-	-	-	-	-	-	+	+	-	-
Benzoylecgonine	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Clonazepam	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Codeine	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+
Diazepam	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Gabapentin	+	+	+	+	+	+	-	-	-	-	+	+	-	-	-	-	-	-	+†	+
Hydrocodone	-*	+	-	-	-	-	-	-	+	+	-	-	-	-	+	+	+	+	-	-
Hydromorphone	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+	+	+	+
Lorazepam	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Morphine	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+
Norbuprenorphine	-	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Nordiazepam	-	-	-	-	-*	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-
Norhydrocodone	+	+	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+	+	-	-
Noroxycodone	+	+	-	-	+	+	+	+	+	+	+	+	+	+	-	-	-	-	-	-
Oxazepam	-	-	-	-	+	+	+	+	-	-	+	+	-	-	-	-	-	-	-	-
Oxycodone	-	-	-	-	+	+	+	+	+	+	+	+	+	+	-	-	-	-	-	-
Oxymorphone	-	-	-	-	+	+	+	+	+	+	+	+	+	+	-	-	+	+	-	-
Temazepam	-	-	-	-	+	+	+	+	-	-	+	+	-	-	-	-	-	-	-	-
THCA	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Tramadol	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

DS: dilute and shoot. Tip: VAMS tip.
Italicized drugs are released from the body as glucuronides. A positive result is denoted by +. A negative result is denoted by -.
* Below lower limit of quantitation. † Drug was highly saturated.

4.5 Linearity

Initially, the linearity of the calibration curve for DS and VAMS had been set to be the same. Upon review the linearity equation for line of best fit for the DS technique is not the same as the equation for line of best fit for VAMS. Table 13 depicts the line fit, weighting, and R^2 value for the line generated line of best fit for DS and VAMS.

Table 13. Line fit, weighting, and R^2 for calibration curve line of best fit for DS and VAMS.

	DS			VAMS		
Analytes	**Line Fit**	**Weighting**	**R^2**	**Line Fit**	**Weighting**	**R^2**
6-MAM	Linear	1/C^2	0.9984	Quadratic	1/C^2	0.9984
Alpha-Hydroxyalprazolam	Linear	1/C	0.9981	Quadratic	1/C	0.9975
Amphetamine	Linear	1/C	0.9954	Quadratic	1/C	0.9997
Benzoylecgonine	Linear	1/C	0.9971	Quadratic	1/C	0.9993
Clonazepam	Linear	1/C^2	0.9972	Quadratic	1/C	0.9990
Codeine	Linear	1/C^2	0.9960	Quadratic	1/C	0.9994
Diazepam	Linear	1/C	0.9956	Quadratic	1/C	0.9983
Gabapentin	Linear	1/C^2	0.9979	Quadratic	1/C	0.9997
Hydrocodone	Linear	1/C^2	0.9943	Quadratic	1/C^2	0.9996
Hydromorphone	Linear	1/C^2	0.9967	Quadratic	1/C^2	0.9983
Lorazepam	Linear	1/C^2	0.9978	Quadratic	1/C	0.9999
Morphine	Linear	1/C^2	0.9956	Quadratic	1/C	1.0000
Norbuprenorphine	Linear	1/C^2	0.9974	Quadratic	1/C	0.9996
Nordiazepam	Linear	1/C^2	0.9965	Quadratic	1/C	0.9992
Norhydrocodone	Linear	1/C^2	0.9972	Quadratic	1/C	0.9983
Noroxycodone	Linear	1/C^2	0.9929	Quadratic	1/C	0.9999
Oxazepam	Linear	1/C^2	0.9977	Quadratic	1/C	0.9984
Oxycodone	Linear	1/C^2	0.9959	Quadratic	1/C	0.9996
Oxymorphone	Linear	1/C	0.9963	Quadratic	1/C	0.9986
Temazepam	Linear	1/C^2	0.9975	Quadratic	1/C^2	0.9982
THCA*	Quadratic	1/C^2	0.9844	Quadratic	1/C^2	0.9878
Tramadol	Linear	1/C^2	0.9966	Quadratic	1/C	0.9981

* THCA DS calibration curve did not include 20 and 50 ng/mL, lower limit was 150 ng/mL.

4.6 Cost Analysis

4.6.1 Specimens

Specimen cups that hold 4 ounces of sample are available through many retailers. For instances, Fisher Scientific offers 100 specimen cups for approximately $32.00.[45] The cost would calculate to be $0.32 per cup. This does not include product provider shipping costs. Mitra ® volumetric absorptive microsampling (VAMS) devices are only available from Neoteryx. According to a quotation from Neoteryx, a 6 pack of 96-rack 20 µL tips costs $1,625.00.[46] This cost would calculate to be approximately $2.82 per tip. This cost does not include shipping cost from the retailer.

4.6.2 Shipping

In the event the samples need to be shipped from one location to another there will be secondary shipping costs. Many shipping companies offer a type of flat rate shipment for different box sizes they provide. For example, FedEx offers a FedEX® Small Box at the size of 8-3⁄4 × 2-5⁄8 × 11-1⁄4-in with flat rate shipping for packages under 50 lbs based on the distance between the two locations.[47] Cost for shipping this small box ranges from $8.65 when 150 miles or less, up to $13.75 when traveling 601 miles or more.[47] A 96-rack of VAMS tips would easily fit inside this box with some extra padding.

While there are larger boxes available that could hold 96 specimen cups, the wet samples should be shipped cold. FedEx offers a variety of boxes that have a 48 or 96 hour cooling time at 2-8°C.[48] The exact sizes of specimen cups vary based on manufacturer. However, for this purpose it will be assumed the size is 3 × 3-in. A single layer of 96 specimen cups would best be laid out to be 12 rows with 8 cups each. This would require a box with an interior size of 36 × 24 × 3-in. Unfortunately, FedEx does not have a cool shipping box with those inner dimensions. Instead, 2 layers of 6 rows by 8 cups would require a box with inner dimensions of 18 × 24 × 6-in. Once again, this size is not available through FedEx. The largest size cool shipping box FedEx has inner dimensions of 9.5 × 8.7 × 4.8-in, priced at $133.00 for 48 hours of cooling time.[48] Therefore, it would be necessary to ship multiple boxes. Each of these large boxes would be able to transport a single layer of 3 × 2 specimen cups. This would mean shipping only 6 samples per box, shipping 16 boxes to transport all 96 samples. Cost for the boxes alone would be $2,128.00. The cost of shipping would still need to be added on.

CHAPTER V

CONCLUSIONS

5.1 Glucuronide Conjugate Mix

Based on the concentrations of the glucuronide conjugates determined in LC-MS/MS analysis, the recovery rate of each glucuronide was able to be calculated. Through statistical analysis, it was revealed there is a significant difference in the mean percent efficiency of the glucuronide recoveries using dilute and shoot (DS) and VAMS tips. Morphine (p-value: 0.226), THCA (p-value: 0.0127), lorazepam (p-value: 0.0030), oxazepam (p-value: 0.0033), and codeine (p-value: ≤ 0.001) were all found to be significantly different.

It is important to keep in mind, that while the extraction processes were different, the β-glucuronidase enzyme used in the two extraction processes were also different. DS used Campbell's with a buffer solution prepared in-house, while VAMS used IMCSzyme and rapid hydrolysis buffer, which is a buffer obtained through IMCS intended to be used with their enzyme. As a result of the two extraction methods using two different enzymes, the statistically significant differences cannot be directly attributed to the extraction methods alone. Future research may be of interest to determine exactly which aspect, extraction method or enzyme, may be credited for the difference.

5.2 Volume Evaluation

The DS and VAMS tips extraction processes called for the use of two different volumes of sample. DS was to use 50 µL, to be obtained using a calibrated pipet. Microsampling was to use 20 µL, to be obtained using a 20 µL Neoteryx Mitra ® VAMS device. A test was conducted to evaluate just how much sample was being used in each of the techniques. It was determined the volume of water and urine drawn up by the pipet was not significantly different than the expected 50 µL. This was not the case for the VAMS tip. While there was no significant difference between the volume of deionized water and UTAK urine absorbed by the tip, there was a significant difference in the amount the tip actually absorbed versus the expected 20 µL. In the case of deionized water, absorbing on average 25.6 µL, there was a p-value of 0.0031, indicating a significant difference. While UTAK urine, absorbing on average 27.2 µL, there was a p-value less than 0.0001, also indicating a significant difference. To absorb the intended 20 µL of solution, the time in which the VAMS tip is in contact with the solution would need to be lessened from the manufacturer's recommended 6 seconds.

5.3 Internal Standard Comparison

The concentration of a drug will be affected by the area of the internal standard (IS) associated with that particular drug. It was observed that DS had a greater IS area for 10 of the 11 ISs used in the method. THCA-D3 was the only IS that had a greater area associated with it in the VAMS extraction technique. Preferential absorption of ISs are evident, however the reason for this is currently unclear.

5.4 Unknown Samples

LC-MS/MS allowed for the qualification and quantitation in the 10 unknown samples that were used in both extraction techniques. As a whole, both techniques qualitatively identified the same drugs in each of the unknown samples. However, in 2 particular instances, there was a discrepancy in whether the unknown sample was positive or negative for an analyte. This was seen with Unknown 1 for hydrocodone and Unknown 3 with nordiazepam. While the analyte was able to be detected, the concentration fell below the lower limit of quantitation. In both instances, it was the DS extraction that was unable to be reported as positive for the drug.

Unknown 10 was positive for gabapentin using both extraction techniques. However, in the DS technique, the gabapentin was saturated to the point that the computer program used for analysis was not able to estimate a concentration. As the goal of the unknown samples study was to determine if the same drugs were able to be qualitatively identified, gabapentin is considered positive in Unknown 10 DS.

5.5 Linearity

It was observed that the line of best fit for the calibration curve is not the same for the DS and VAMS extraction techniques. Each facility should conduct their own linearity study to determine what line fit and weighting provides the best fit line.

5.6 Cost Analysis

A single specimen cup is approximately $2.50 cheaper than a single VAMS tip. At first glance, specimen cups seem like the cheaper option. However, in terms of shipping the samples from one facility to another, dried VAMS are well over $2,000.00 less than wet specimen cup samples. Keeping the substantial price difference in mind, facilities would need to consider if there is a need for shipping specimens from one place to another. If there is that need, the

question of how many samples would be shipped at a time should be addressed at each facility when considering switching from wet specimen cup samples to dried VAMS tips.

5.7 Comparisons to Other Research

This study looked to compared glucuronide drug recovery and qualitative detection of drugs in the benzodiazepine class, opioid class, and 3 other miscellaneous drugs. At the time of this study there was one publication about VAMS urine testing by Mercolini et al. While Mercolini et al's study compared wet and dry urine samples, the study only evaluated cathinones. At the time of writing, a second study using VAMS for urine was published by Protti et al. Protti et al's study focused only on oxycodone and its 2 major metabolites. While all studies compared wet DS to dried VAMS, there are several key differences between the 3 studies. The OSU study used β-glucuronidase enzyme, while the others did not, and the research completed at OSU included a wider variety of drugs than the other studies. Additionally, Mercolini et al. and Protti et al. set out to quantitate drug levels in the urine samples. The OSU research only did a quantitative analysis of glucuronide recovery. The goal of the unknown sample testing in the OSU study was qualitative in nature. However, all 3 studies demonstrated proof of concept for the use of VAMS tips for analysis of drugs in urine samples.

5.8 Future Research

Additional research should be conducted to determine the viability of VAMS for drug testing urine samples, especially if the hope is to replace wet DS and dried urine samples with VAMS. One such study should focus on altering the amount of time the VAMS tip is in contact with the urine to absorb the intended volume. It should be evaluated how samples of different consistencies are absorbed by the tip because the viscosity of urine is not identical for samples. It may also be possible for the VAMS device companies to include a dye to indicate adequate absorption of specimen at the appropriate volume.

The stability of urine samples on VAMS tips have not been studied in length. It is important to know how to maintain the integrity of the sample and ensure there is no drug degradation. Typically, validation studies have shown that wet samples are good for 3 days when stored in a refrigerator and good for a month when stored in a freezer. VAMS samples dry at room temperature, but the length of time they can be stored at room temperature is unknown, along with the appropriate storage conditions. It is important to know the answer to these questions before converting over to dried urine VAMS testing in a facility.

Another study should look to compare the recovery of glucuronides by trying different β-glucuronidase enzymes. This study used Campbell's for DS and IMCS for VAMS. The reason this study used Campbell's for DS was because that was the type of enzyme used in the already validated method. IMCS had been used for VAMS based on literature review and other research at OSU has identified this enzyme as being very effective at hydrolyzing the analytes of interest in this method.

Regular microcentrifuge tubes and tips were used throughout the research. It is possible that low binding tubes and tips will have "a bonded polymer technology that reduces protein and nucleic acid binding, resulting in better sample recovery."[49] It would be interesting to see if using low binding tubes and tips could potentially decrease any drug loss that is occurring.

5.9 Discussion

When initially doing the literature review, Volumetric Absorptive Microsampling (VAMS) appeared to be a promising alternative to wet urine drug testing. However, after completing research there are several elements that must be considered. While the research conducted at OSU and by other researchers have indeed proved VAMS is a viable option for dried urine drug testing, the technique does have some shortcomings. At this time, the volume of sample being absorbed by the tip is higher than expected. This runs the potential of having inconsistent volumes of sample absorbed. The viscosity of urine samples received in labs varies from the ideal

mostly water urine sample to highly turbid urine sample. It is unclear how the fluid viscosity factors in to the volume of sample absorbed by the tip.

Furthermore, there are several ways in which a sample could be collected incorrectly using VAMS tips. The most obvious mistake being fully submerging the tip into the urine sample, which will retain more urine. Next is the potential for holding the tip to the surface for too short or too long of a time, affecting how much sample is absorbed. Employees need to be properly trained on how to collect samples using VAMS. As part of their training, employees would also need to understand how to ensure there is no sample contamination. The contamination could be cross sample contamination, mislabeling the tip, improper drying, contact with other chemicals/samples, and other factors.

With VAMS, one tip goes to one specimen. However, in the event there is an error in the extraction process, there needs to be an additional sample available for repeat analysis. To circumvent this problem, at least 2 samples should be collected per specimen. That would mean that the cost per specimen will double, instead of $2.82 it would be $5.64 per specimen. This may not be an option financially for some laboratories. On the other hand, the dilute and shoot (DS) technique allows the laboratory technician to return to the wet specimen cup sample and re-extract the sample, provided it has been properly stored to prevent drug degradation.

While studies still need to be conducted to truly determine how to best store VAMS, at this time it is believe they would be able to be shipped in a regular, non-temperature controlled package. The size and cost of shipping a 96-tip rack would be much less than that of 96 specimen cups in cold storage. In the event there is no need to ship, then this would be a null point. For some labs, the number of samples they ship at a time may not be worth the cost of switching over to VAMS just to save on shipping costs. This is something each facility would need to evaluate for themselves.

5.10 Conclusion

This study set out to evaluate a wider range of drug classes than had been researched previously. From this research, proof has been supplied that VAMS is able to obtain valuable results through analysis. DS and VAMS both recovered glucuronides in the glucuronide experiment but VAMS had a higher recovery rate. When it came to unknown samples, both extraction techniques identified the same drugs. Findings of the research suggest VAMS has a higher sensitivity than that of DS. For the unknown samples, hydrocodone and nordiazepam were qualified below the lower limit of quantitation for DS, but were found to be above the lower limit of quantitation for VAMS. Additionally, the VAMS calibration curve was able to extend as low as 20 ng/mL in the case of (-)-11-nor-9-Carboxy-delta9-THC (THCA), whereas DS had a lower limit at 150 ng/mL. From the findings of this research, VAMS has demonstrated to be a viable option for dried urine drug testing of the drugs in the research panel. That being said, the decision to switch from a DS approach to a VAMS approach is something each laboratory needs to evaluate after more research has been conducted.

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