Development and Validation of a Blood Micro Sampling Methodology to Facilitate Pharmacokinetic Studies (Drug Exposure) in Pregnancy and Postpartum Patients

by

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Pregnancy is associated with several physiological changes that can alter the pharmacokinetics (PK) and necessitate dosing regimen changes for certain drugs in order to achieve drug exposures that are comparable to non-pregnant population. However, limited data is available in pregnant women in order to make rational drug dosing recommendations. Therefore, dosing recommendations for drugs, including treatment for opioid addiction, are usually extrapolated from studies carried out in non-pregnant patients. Among the reasons for lack of data is the difficulty associated with collection of multiple blood samples in opioid addicted pregnant women. Permanent damage caused to veins due to chronic IV drug use in these patients makes it especially challenging to obtain venous blood samples. Application of micro blood sampling as a tool for remote blood sampling can offer a convenient way for performing studies in this population. A novel micro blood sampling device called Volumetric Absorptive micro sampling (VAMS) developed in 2014 has resolved sample inhomogeneity issues inherent with previous dried blood Spot (DBS) techniques. The VAMS technique has also demonstrated improved precision in the volume of sample collected. Proper experimental design and optimization of the extraction of drugs and metabolites of interest are the key parameters in the application of a VAMS approach. Our study objective is to evaluate the utility of a micro sampling approach for self-collection of blood samples in order to monitor buprenorphine

concentrations and to optimize the dosage of buprenorphine in pregnant and post-partum patients.

In this study, we have developed and validated an LC-MS/MS assay to measure Buprenorphine and attempted to quantify metabolites of the parent drug in whole blood samples simultaneously from the VAMS device, that can be of help in future studies in pregnant and postpartum subjects.

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Abbreviations

TDM	Therapeutic Drug Monitoring
LLOQ	Lower Limit of Quantification
RT	Retention Time
PBPK	Physiologically Based Pharmacokinetic Model
BUP	Buprenorphine
BUPG	Buprenorphine Glucuronide
NBUPG	Norbuprenorphine Glucuronide
NBUP	Norbuprenorphine
IS	Internal Standard
LC	Liquid Chromatography
LLE	Liquid-Liquid Extraction
NAS	Neonatal Abstinence Syndrome
FDA	Food and Drug Administration
CV	Coefficient of Variation
CYP	Cytochrome P 450
AUC	Area Under the Curve
GFR	Glomerular Filtration Rate
RSD	Relative Standard Deviation
Vd	Volume of Distribution
OUD	Opioid Use Disorder
MAT	Medication Assisted Treatment

Preface

I would like to express my deepest gratitude to my academic advisor, Dr. Raman Venkataramanan for his guidance, support, and motivation. The knowledge and training I have gained from him is an invaluable asset throughout my life.

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1.0 Introduction

1.1 Opioid Use Disorder

Opioids are substances that interact with the opiate receptors in the body. The ability of opioids to cause addiction goes hand in hand with their analgesic benefit, leading to a conflict among clinicians whether to prescribe them to relieve pain and cause long term damage due to addiction. After binding to opioid receptors, these agents also induce release of dopamine and activate the "reward circuits" in the brain, leading to a euphoric effect. On repeated exposure to the drug, brain cells become less responsive and higher doses are required to produce the same level of dopamine, hence tolerance develops. Long term use of opiates can potentially result in damage to the brain and lead to drug seeking behavior, making them highly addictive[6].

The chronic use of opioids leading to an overwhelming desire to obtain such drugs despite professional or social consequences, marked by increased tolerance and withdrawal upon discontinuation are hallmarks of opioid use disorder (OUD). It is an epidemic in the United States and costs the nation over $600 billion annually. Out of the 20 million Americans who abuse substances, two million individuals use prescription opioids including oxycodone, hydrocodone, and fentanyl and 500,000 use heroin[7]. The disorder prevalence varies by age and gender, however women who become pregnant are not spared in this healthcare crisis. According the Centers for Disease Control, prevalence of OUD during pregnancy has more than quadrupled from 1999 to 2014[8].

1.2 Current Treatments for opioid addiction

Treatment of OUD requires long term management, which is carried out through use of medications as well as psychosocial and behavioral treatment. However, psychosocial interventions or behavioral interventions alone are associated with poor outcomes for patients[9]. Currently, the best opportunity for treatment from opiate addiction is through medication-assisted treatment (MAT), in combination with counseling therapy. The Food and Drug Administration has approved three medications for long term treatment of addiction: methadone, buprenorphine, and naltrexone.

1.2.1 Methadone

In 1964, Vincent Dole pioneered the use of methadone to treat opioid addicts. [10] The drug was then approved for treatment under strict supervision of opioid treatment clinics under the Narcotic Addict Treatment Act of 1974[11]. Methadone is currently the gold standard of therapy for OUD.

Many clinical studies have proven its effectiveness in maintenance therapy for reducing illicit opioid use, reducing cravings, and improving patient's social productivity[12].

Methadone has properties similar to morphine, and binds to mu-, delta-, and kappa-opioid receptors. The drug has very slow onset of action with a half-life ranging from 24 to 36 hours and is administered as a racemic mixture of both (R) and (S)-methadone. Since it is a long-acting opioid, it has a better safety profile with less severe withdrawal syndrome compared to short-acting opioids like heroin[11, 13, 14].

Upon oral administration, the drug is well absorbed with a bioavailability of approximately 75%. As a basic drug, it is predominantly (88%) bound to alpha-1-acid glycoprotein[15]. Methadone undergoes N-demethylation to form inactive metabolites by CYP3A4, CYP2B6 and CYP2C19[16].

However, because it is a full agonist of the mu- opioid receptor, the risk of abuse is greater. There can also be significant interindividual variability in methadone exposure at a given dose[17, 18].

1.2.1.1 Methadone Use in Pregnancy

Methadone is the standard of care for MAT in pregnancy. If a patient is already receiving care in a methadone clinic, pregnancy may not interrupt this regimen. There is strong evidence, however, that dose alterations have to be made during pregnancy for methadone. Only 8% of patients are on the same dose of the drug throughout pregnancy[19]. As gestation advances, the dose of the drug has to be increased. Since the drug is eliminated very slowly, it is advisable to increase the dose gradually[20].

Plasma levels of methadone decrease, and renal clearance increases as gestation advances[21]. Therefore, cravings may become apparent before each dose. Split dosing or twice daily dosing every 12 hours has also been suggested but is not possible for all the patients since it would require take-home doses. Methadone is import regulated by state law and requires long standing proof of compliance (up to 2 years-meeting specific criteria) , therefore take-home doses are not feasible for most patients[22]. There exists high interindividual variability during pregnancy in the metabolism of methadone, signs and symptoms of withdrawal and increased maternal opioid cravings as well[23].

1.2.2 Buprenorphine

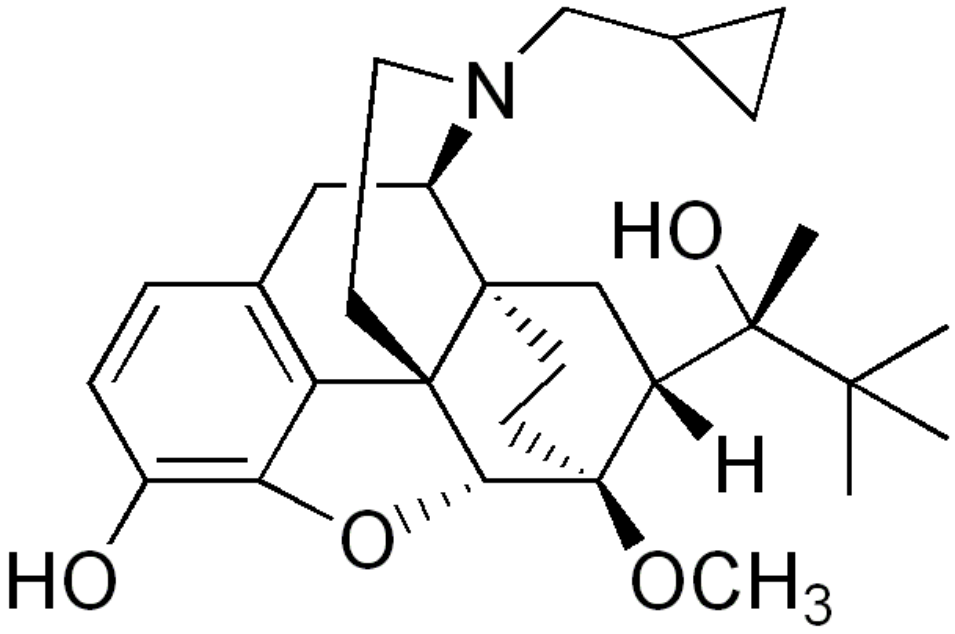


Figure 1: Structure of Buprenorphine

Buprenorphine is a semi-synthetic derivative of the alkaloid thebaine. It was developed by Reckitt and Coleman since they were searching for a morphine-like compound which retained the analgesic property of morphine while demonstrating less potential for addiction and withdrawal. The FDA approved buprenorphine for treatment of opioid addiction in 2002.

Being a partial agonist, it has a very unique pharmacological profile compared to that of methadone and morphine. Buprenorphine has high binding affinity for the mu-opioid receptor but low intrinsic activity. Therefore, even at high doses of the drug, when it binds all the mu receptors, it does not show maximum opioid agonist effect.

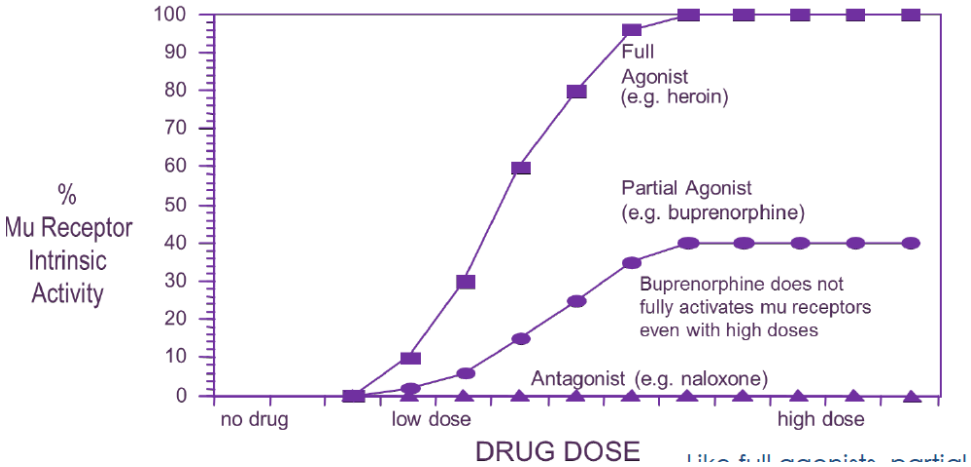


Figure 2: Partial Agonist activity

This has been supported by the bell-shaped dose response curve of anti-nociception effect in animal studies, where the agonist activity shows a ceiling effect at high doses of the drug[24].

Apart from binding to the mu-receptor, buprenorphine also binds to other opioid receptors. It has no intrinsic activity on the delta-opioid receptor and low activity on the kappa opioid receptor[25, 26].

Buprenorphine undergoes extensive first-pass metabolism, therefore has very low oral bioavailability. It is administered through parenteral, sublingual, transmucosal, subcutaneous or transdermal routes and not orally for this reason[27]. Buprenorphine is commonly formulated in combination with Naloxone. Since naloxone acts as a full opioid antagonist, it reduces risk of abuse and decreases the attractiveness to inject the drug in addicted patients.

Many studies have reported high interindividual variability in buprenorphine pharmacokinetics following sublingual administration. Ciraulo et al. found that the coefficients of variation in maximum plasma concentration (Cmax) and the area under the curve (AUC) ranged from 40-64%[28]. Absorption after sublingual dose is rapid, however the time to reach plasma peak concentration (Tmax) ranges from 1 to 6 hours. The longer time to reach plasma

peak concentration might be due to the slow release because of depot effect by sequestration of the drug in the oral mucosa[29].

Buprenorphine is lipophilic drug with a logarithm of the octanol to water partition coefficient (LogP) of 4.98[30]. Because of this, it readily penetrates tissues and crosses the blood brain barrier. It is a basic drug (pKa1=9.62, pKa2=8.31), which is highly bound to alpha and beta globulin(96%)[30] The apparent volume of distribution at steady state following intravenous administration is 335 L. Buprenorphine has a long plasma half-life ranging from 24-42 hours, following sublingual or transmucosal administration[31].

Table 1: Advantages and Disadvantages of Buprenorphine

Advantages	Disadvantages
1. Sublingual formulation 2. Allows for once daily or three times weekly dosing 3. Potent as an analgesic 4. Does not affect immune/endocrine system 5. Ceiling effect for respiratory depression 6. Wide safety margin regarding dose 7. Slow dissociation leads to reduced withdrawal symptoms	1. Expensive 2. Slow onset 3. Drug-drug interactions with CYP3A4 substrates 4. Need high doses of naloxone for reversal 5. Can cause QTc prolongation at high doses

After intravenous administration, plasma clearance of BUP is around 50L/hr in healthy volunteers[32]. When we compare BUP blood clearance with hepatic blood flow in healthy subjects, the estimated extraction ratio of the drug is 0.9 which indicates it to be a high hepatic clearance drug.

The primary metabolic pathway of the drug is via CYP3A4 mediated N-dealkylation to form norbuprenorphine. Norbuprenorphine undergoes further conjugation to form norbuprenorphine glucuronide through UGT1A1 and UGT1A3[33]. A part of buprenorphine is also directly conjugated to buprenorphine glucuronide. Therefore, overall CYP3A4, CYP2C8, UGT1A1, UGT1A3 and UG2B7 are involved in metabolism of the parent drug buprenorphine.

Of the known metabolites, norbuprenorphine is the major metabolite which also exhibits agonist activity. In vitro studies have found that norbuprenorphine has high affinity to the mu-receptor, comparable to that of buprenorphine[34]. In rat studies, norbuprenorphine led to respiratory depression and showed up to 10 times more potency than buprenorphine[35].

While norbuprenorphine is a potent mu-receptor agonist, brain concentrations of the compound are very low. Norbuprenorphine is more hydrophilic than the parent drug, so it cannot pass through to the brain easily. This indicates that the metabolite may not contribute to the clinical effects of the parent drug.[36] It has been generally accepted that the glucuronide metabolites are inactive, however Brown et al. conducted a study which showed that buprenorphine-3-glucouronide had mild antinociceptive activity in a mouse model, and norbuprenorphine-3-gluconide had sedative effect and decreased respiratory tidal volume[37]. These findings add to the complexity of buprenorphine pharmacology and make it difficult to specifically identify the contribution of each metabolite towards the clinical effects of the parent drug.

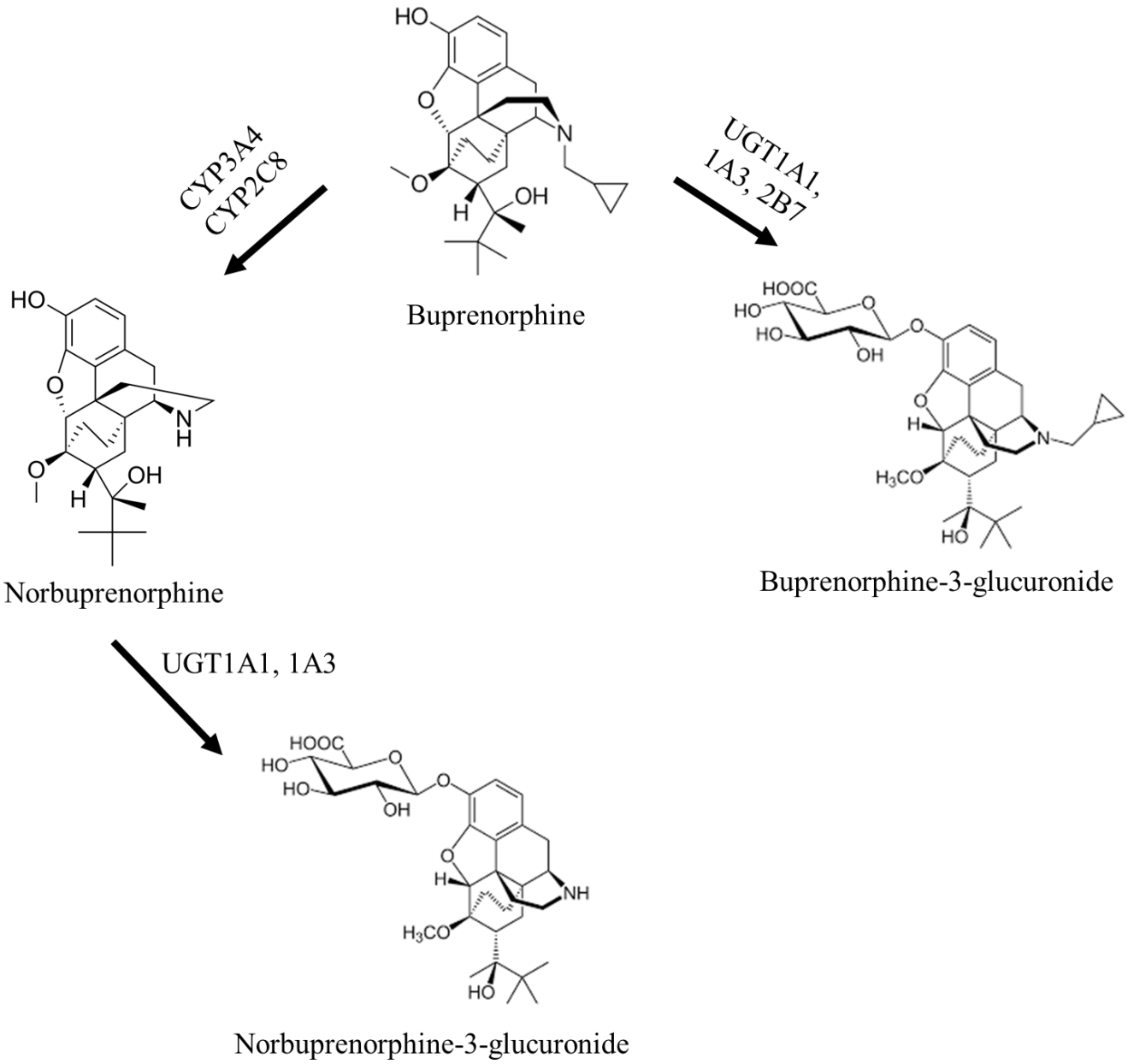


Figure 3: Buprenorphine Metabolic Pathway

1.3 Medication Assisted Treatment during Pregnancy

The 2010 National Survey on Drug Use and Health by the National Institutes of Health (NIH) reported a 47% increase in illicit drug use during pregnancy since 2002 (4.4% in 2010 versus 3% in 2002 for the age group of 15 to 44 among pregnant women)[38]

Use of opioids during pregnancy can lead to a drug withdrawal syndrome in newborn babies known as neonatal abstinence syndrome (NAS). Apart from the development of NAS, maternal opioid use is associated with many complications such as maternal death, cardiac arrest, intrauterine growth restriction and placental abruption, birth defects, still birth and preterm labor.[39, 40]

Ideally, the woman should abstain from opioids completely during pregnancy and many women are motivated to reduce illicit opioid use to provide the best outcome for their children. However, there are significant risks associated with abrupt stopping of opioids since the pregnant women would undergo intense withdrawal symptoms during detoxification which would lead to intrauterine stress and preterm labor, directly impacting fetal development[41].

Currently, the treatments for OUD in pregnant women are medication assisted maintenance therapy using long-acting mu-opioid receptor agonists such as methadone or buprenorphine[42]. Although methadone has been used for many decades and many studies have shown its effectiveness in pregnancy, there are several issues with this drug. Women prescribed methadone need to be under strict monitoring and attend a clinic daily to obtain the medication. In the US, women who suffer from severe opioid addiction tend to be prescribed methadone while women who have lower risk of relapse are prescribed buprenorphine[43].

Altered metabolism of methadone has been reported in several studies in pregnant women[44, 45]. Patients taking higher doses of methadone still show low trough plasma concentrations of the drug during pregnancy[45]. Moreover, the higher maternal methadone dose is associated with increased incidence of NAS[46].

Buprenorphine has comparable efficacy to methadone and shows significantly lower severity and incidence of NAS[43] Neonates exposed to BUP require up to 89% less morphine to treat NAS, a 43% shorter hospital stay, and a 58% shorter duration for treatment compared to methadone[47]. Moreover, buprenorphine is theorized to be a safer alternative to methadone due to its partial agonist property itself. As mentioned previously, the long half-life of buprenorphine is convenient, and it may be dosed 3 times/week compared to the daily dosing of methadone.

There is limited data on the alteration of BUP pharmacokinetics and pharmacodynamics during pregnancy, and therefore the dosing regimen for pregnant women is based on the recommendations derived for non-pregnant women and men. In order to understand the efficacy of BUP and methadone in pregnant populations, Jones et al. conducted a study and found that 33% of patients in the buprenorphine group terminated treatment early compared to 18% in the methadone group. More than 71% of the dropout was because of patient dissatisfaction with BUP. There was no such difference in retaining patients for the non-pregnant population, however[48].

Studies conducted at Magee Women's Hospital Pittsburgh also indicate an increase in BUP dose requirement during pregnancy[49]. Therefore, the lack of clear data and broad dosing range may be causing a dosing bias among individual physicians and might be affecting the patient satisfaction on BUP therapy. Since both intrinsic clearance and hepatic blood flow can

impact BUP clearance, it is clear that pregnancy is associated with increased clearance and decreased exposure of the drug.

Women with addiction also need to continue their opioid agonist therapy postpartum. The postpartum period is a time of heightened vulnerabilities, and the risk of relapse for women with opioid use disorder is much higher in the postpartum period than during pregnancy[50]. There tends to be limited access to adequate postpartum psychosocial support including relapse prevention programs or overdose training. Therefore, there is a need to gain better understanding of pregnancy and postpartum changes in the elimination of BUP. The main challenge in dosing the drug in pregnancy is to maintain drug levels at an optimum range, keeping the patients satisfied, while minimizing drug exposure to the fetus. Currently there are several studies on going to optimize BUP therapy in pregnancy and postpartum.

1.4 Pregnancy induced physiological changes and impact on pharmacokinetics

Pregnancy brings about many physiological changes and adaptation of organ systems in order to accommodate for the development of the fetus. Such pregnancy-induced physiological changes can significantly impact drug absorption, distribution, metabolism, and elimination[51].

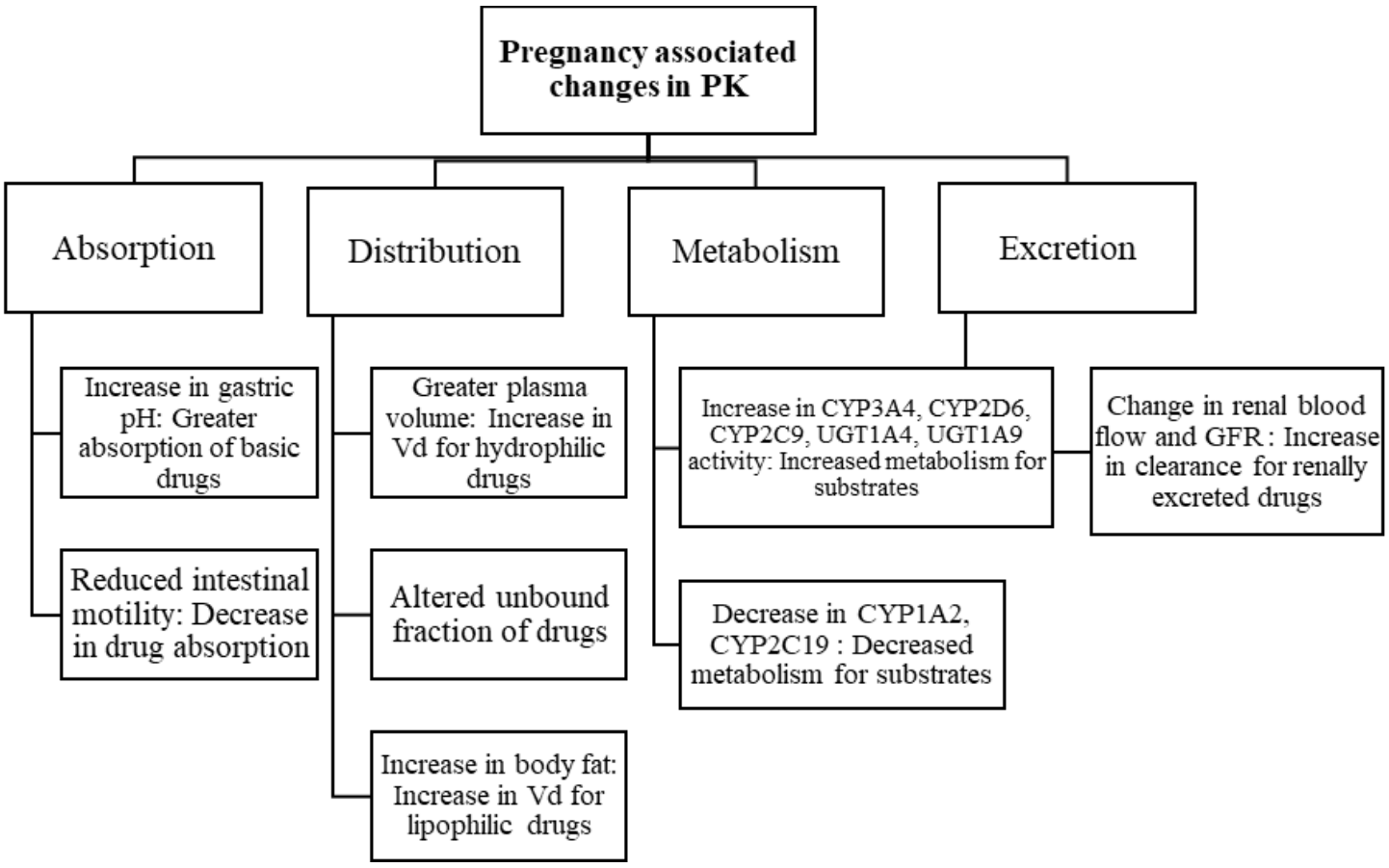


Figure 4: Pregnancy Associated PK changes (Adapted from Chaphekar N. et al)[5]

1.4.1 Absorption

The movement of drug from the site of administration into the bloodstream is the process of absorption. During pregnancy, there is an increase in gastric pH which can lead to ionization of acidic drugs and affect their absorption into the gut wall. Similarly, as a result of decreased gastric emptying and intestinal motility during pregnancy, there may be a decrease in drug absorption rate[52, 53].

1.4.2 Distribution

The process of drug movement into various tissues once it has entered the body, is referred to as distribution. Physicochemical properties of the drug can affect the extent to which drug distributes within the body. For instance, the drug's lipid solubility (LogP), pKa and partition coefficient can significantly impact its passage through biological membranes. Since only the unbound fraction of the drug can cross cell membranes, the fraction of drug bound to plasma is an important factor that affects its distribution. Volume of distribution (Vd) is a parameter to determine the extent of drug distribution within the body. During pregnancy, increases in total body water, body fat, plasma volume and blood volume impact the drug Vd. Therefore, increase in body fat would impact the Vd for lipophilic drugs and cause it to increase. Whereas, increase in plasma volume and total body water cause increase in Vd for hydrophilic drugs as well[5, 54].

1.4.3 Metabolism

Conversion of drugs via enzymes to form more polar, hydrophilic compounds that can be easily eliminated is the process of metabolism. Factors that affect the hepatic clearance of the drug include intrinsic clearance, blood flow and unbound fraction of drug in blood.

In terms of pregnancy, the effect on metabolism is drug specific. Metabolism of drugs mediated by CYP3A4, CYP2C9, CYP2D6, UGT1A4 and UGT2B7 is increased whereas metabolism of drugs mediated by CYP1A2 and CYP2C19 is decreased[5, 51, 55].

1.4.4 Excretion

The process of drug removal from the body is excretion. One of the primary organs involved in elimination is the kidney. Mechanisms of renal excretion are glomerular filtration, tubular secretion, and reabsorption. Since only unbound fraction of drugs is filtered by nephrons, it is one of the factors that affects renal clearance. Therefore, for a drug that only undergoes filtration as mechanism for renal clearance, the renal clearance is defined as product of glomerular filtration rate (GFR) and unbound drug fraction (fu). Since pregnancy causes increase in fu and GFR is also increased from 97 ml/min in non-pregnant women to 180 ml/min in pregnancy, renal clearance of drugs such as lithium is increased in pregnancy[56].

1.5 Difficulty in performing pharmacokinetic studies in pregnant women and alternative approaches to evaluate pharmacokinetics in pregnancy

Since there are significant anatomical and physiological changes throughout the course of pregnancy, pharmacokinetics of several drugs is expected to be impacted in pregnancy. In order to properly characterize the pharmacokinetics of a drug, sufficient number of samples need to be collected to determine the time course of absorption, distribution, metabolism, and excretion from each subject.

A typical PK study involves taking 9-12 blood samples over 3-5 half lives of the drug from each patient and requires them to be hospitalized or use Clinical Research Centers. The subjects have to agree to repeated invasive blood sampling approaches or insertion of a blood sampling catheter (heparin lock) over an 8–12-hour time period. Any study visits in the months following birth are challenging to the mother and this reality is a disincentive for participation in any research study. Such intensive blood sampling and the long duration of the study adds to the practical issue in studying pharmacokinetics of drugs in pregnant women.

Moreover, pregnant women with opioid addiction are likely to suffer from coexisting mental health problems including depression, mood disorders, trauma, posttraumatic stress disorder and anxiety. They are at risk of abuse of other substances such as alcohol, tobacco, cocaine, and cannabis. Prolonged use of intravenous drug use also leads to permanent damage to the veins for these patients. This makes repeated venous blood sampling extremely difficult since there are not any perceptible veins to draw blood from. Most of these women suffer from inadequate nutrition and have disturbed support systems leading to social service needs. Frequent trips to the clinic can be a significant barrier for women without transportation or those from rural areas[20]. The ability for women to participate in research studies is limited with such

socio-economic disadvantages. In order to combat these barriers, several approaches may be considered as alternatives to a full PK study.

Table 2: Overview of Strategies to Optimize Clinical studies(Adapted from Balevic et.al[57])

Outcome	Innovative Approach
Clinical Trial Optimization	
Optimizing Data collection and implementation	Bioanalytical Optimization: micro sampling
Reducing risk for participants	Sparse Sampling Microdosing Opportunistic Study Design
Simulation and Modeling	
Optimizing Study Design	Simulations of Clinical Trials
Predicting Effects of Organ Dysfunction	PBPK
Individualizing dose	Population PK Bayesian Analysis

There have been significant advances in analytical chemistry that have allowed the use of miniscule volumes of blood to be utilized for bioanalysis. As of yet, these sampling methods are underutilized due to lack of suitable assays developed for the same.

Obtaining the same PK/PD information from minimally invasive approaches such as micro sampling can significantly reduce the burden on pregnant women to participate in research

studies. Such microliterμ blood samples can be taken by the patients themselves, dried under ambient conditions and sent to the lab for analysis.

2.0 The Micro sampling Approach

There has been a trend towards precision medicine with an emphasis on personalized approach. In order to support this method, it is necessary to develop tools that improve sample collection and enable patients to actively participate in monitoring their own therapy. Micro sampling is a technique that utilizes very small amounts of biological matrix, typically less than 50 μL, for quantitative analysis.

The origins of micro sampling can be traced back to 1913, where filter papers were used for the analysis of glucose. Micro sampling was first demonstrated by Ivar Bang[58] and further developed by Dr. Robert Guthrie. He presented the method of Dried Blood Spot(DBS) sampling for newborn screening of phenylketonuria[59], using filter papers for detection of the enzyme phenylalanine hydroxylase, which is a marker for phenylketonuria[60]. Since then, the use of DBS has become common for detecting sickle cell disease and human immunodeficiency virus, especially for mass studies in developing countries[61].

2.1 Traditional Method of Sampling and its Limitations

The traditional venous blood sampling methodology requires patients to be in a health care facility or a clinical laboratory. This can be problematic when samples are to be taken from populations such as children and geriatric patients[62].Collection of samples in patients living in remote areas also becomes a challenge. A special application of micro sampling to opioid addicted pregnant populations is what we propose here.

The procedure for blood testing normally requires a phlebotomist to collect a substantial blood volume (typically 3 to 5 mL) intravenously using a needle attached to an evacuated blood collection tube[62].Vein puncture is carried out on the forearm of the patient while using a tourniquet to constrain the flow of blood. After collection, the samples will be sent to the central laboratory for analysis[63].

The venipuncture method provides accurate diagnosis; however, it is costly and time-consuming due to numerous logistical requirements. For instance, in a laboratory setting, the time needed for blood testing for a pharmacokinetic study ranges from hours to days depending on the drug and the study procedures[63].

The major potential sources of variation in plasma or serum include specimen collection method, transport, processing, and storage. There is evidence that the reliability of an analytical method is conditional on rapid separation of plasma and serum from blood. The need to have a centrifuge at the point of sample collection or soon after sample collection in a lab to separate the plasma and serum is may also present an issue in some cases.

Blood as a matrix avoids several of the above concerns. With improvements in sensitivity of analysis, there has been a significant reduction in the sample size that is necessary for disease diagnosis. A smaller sample size overcomes the issues in conventional techniques thereby leading to potential for a minimally invasive diagnostic technique[64]. Repeated, large-volume sampling is uncomfortable for patients, especially for those with chronic conditions who require regular blood testing. The micro sampling approach is relatively less painful, with fewer requirements on handling and storage. Therefore, the use of micro sampling can cause a paradigm shift in patient care and drug monitoring.

Table 3: Overview of Micro sampling Challenges (Adapted from Parker et al. [65])

Collection and sample parameters	Blood	DBS	VAMS
Routinely used for bio-analysis	✓		
Measure total drug concentrations	✓	✓	✓
Measure unbound drug concentrations	✓		
Minimally invasive sampling		✓	✓
Low Sampling volume (<100 µL)		✓	✓
Ease of sample handling		✓	✓
Cost saving on transport and storage		✓	✓
Simple extraction procedure		✓	✓

3.0 Dried Blood Spots

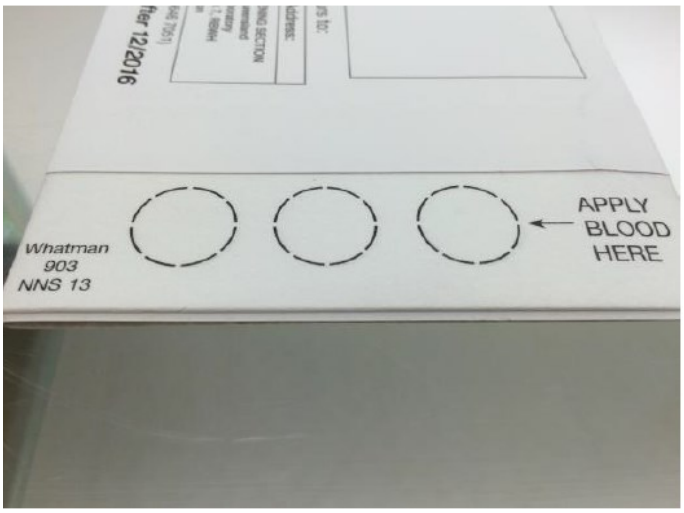


Figure 5: Dried Blood Spotting Card

The DBS technique involves the use of a blood lancet and a sample collection card or filter paper. After performing a finger prick, a few drops of the blood are adsorbed onto the filter paper. The sample is then dried for a period of 2-3 hours over an open non-adsorbent surface. These conditions can differ depending on the filter paper used, temperature and humidity of the surroundings[66] The DBS samples are then placed in plastic sealable bags containing desiccant and humidity card markers. The impact of sunlight, heat and moisture can thereby be minimized and avoid potential degradation of the analyte. Thereafter, a fixed diameter disc is punched from the sample. The punch size may vary from 3-6 mm to the whole spot, depending on the method used. The disc can be considered to provide a volumetric measurement, comparable to the usage of a pipette for liquids. After extraction, the analyte is measured using a suitable analytical method.

The demand for sampling for DBS is a lot less than for phlebotomy, and the experimental set up does not require any centrifugation or sub-aliquoting of plasma[67]. Moreover, shipment

and storage requirements are simple and can be carried out at room-temperature for most tests. The DBS samples can be transported with no reasonable expectation of exposure to blood or other possibly infectious materials by the handlers unlike the shipment of liquid samples. It can be sent via normal post without specialized mailing cartons or packaging. Most analytes are more stable in DBS than even in frozen samples[68].

3.1 Applications and Drawbacks of Dried Blood Spots

The application of this technique is widespread in toxicology, including epidemiology, environmental and forensic toxicology[69]. The stabilizing effect of a dried sample in slowing down degradation has proven to be beneficial. It also offers the potential to analyze biomarkers of exposure, including DNA adducts and protein adducts.

Therapeutic Drug Monitoring is a field gathering increasing attention in the application of minimally invasive blood sampling strategies. The possibility for at home-sampling, along with the noncontagious nature of DBS, makes it appropriate for TDM. There have been publications of DBS-based TDM for multiple drug classes-including antiepileptics, antiretrovirals, immunosuppressants, antimalarials, antibiotics and others.

However, the DBS technique has major drawbacks in drug quantification and analysis. Extensive validation of the assay protocol is required.

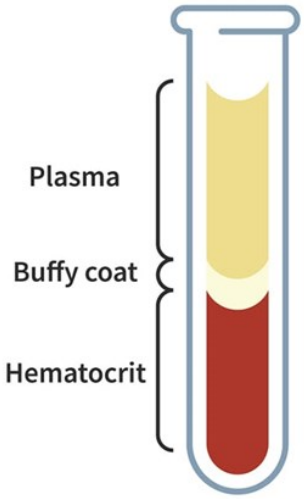


Figure 6: Composition of Blood

Hematocrit (HCT) has been currently identified to be the single most important parameter affecting the spread of blood on DBS cards. This can have an effect on the spot size, drying time and eventually the robustness and reproducibility of the assays. Since HCT levels are correlated with blood viscosity, a higher HCT value would lead to the blood spot spreading to a lesser extent, with a smaller diameter on the paper. The influence of differences in hematocrit values on the spot size and homogeneity has to be accounted for in the assay performance. For instance, there is an increased risk of negative bias in the result when the blood spots are smaller in size[70]

Other factors that affect the DBS assays include the environmental conditions and storage temperature at the time of collection. For example, when samples were stored over a year at 20ºC, they showed greater deterioration compared to samples stored at a lower temperature of 4ºC for the same time period[71]

3.2 Volumetric Absorptive Micro Sampling

Another approach is to make use of devices, which help with collection of a fixed volume of blood avoiding any impact of HCT. In 2014, the company Neoteryx came up with a proprietary micro sampling device called Mitra® based on the principle of Volumetric Absorptive micro sampling (VAMS). It allows for collection of an accurate volume of blood each time it is dipped into a drop of blood after finger pricking with a lancet. Many studies have been carried out using this device, documenting lack of any hematocrit bias with samples having hematocrit values

ranging from between 20 to 70% with a 10 µL blood volume capture[72]. The Mitra device has been designed to provide all the benefits of the DBS technique, while overcoming the hematocrit bias as well as providing simpler workflow for analysis of blood samples. It has also been shown that the VAMS method is accurate and reproducible when sampling is carried out at-home[73]. Practically, the use of VAMS also facilitates sample identification during analysis, which is not the case with DBS, since it cannot be identified once it has been punched[65].

The distinct advantage of using VAMS is that it complies with the 3Rs principle which helps reduce the number of animals tested as well as help curb the pain that animals face during sampling. Collection of smaller blood sample volumes from rodents in PK studies permits a larger number of samples to be collected from a single animal.

For VAMS, we would only need to take 10 µL sample at a single time point compared to approximately 200-500 µLs in using other approaches for PK studies. Considering the blood volume of rodents (55-70 mL/kg body weight), a cumulative amount of 0.3 mL blood can be withdrawn each day for a rat weighing 350-500 g. Therefore, multiple blood samples can be obtained in a single day from a rodent without sacrificing the animal, leading to improved pharmacokinetic profiles from one animal [74].

3.2.1 Sampling Process

The device is available in configurations that allow for accurate sampling of 10, 20 and 30 µL. The device consists of a proprietary adsorbent tip attached to a plastic handle with grooves for ease in gripping. A finger or heel prick has to be carried out, following which, the adsorbent sampling tip is held and dipped at an angle of 45º, allowing only the leading surface of the tip into the drop of blood which enables the uptake due to capillary action. The tip may be held in contact with blood pool for an additional 2-3 seconds to ensure complete filling. However, issues with overfilling of the tip have been reported. Double-dipping or keeping the tips in contact with blood longer than 6 seconds are likely to cause variations in volume.[75]

Re dipping the tip while they are still wet does not cause any major impact on the volume sampled. As time between the first and second dip increases, there is an impact on the result. This situation however should not occur since the practical application of VAMS is related to finger/heal prick and re dipping would be occurring within seconds of sampling. Utmost care needs to be taken that the tips do not touch surrounding surfaces. Once the tips have been suitably loaded with blood, they need to air dried prior to further processing. The dried tips can also be placed at RT in plastic pouches with desiccant[72].The device is self-indicating, since the tip turns completely red which signals it is filled to its capacity. Since the tip wicks the blood, it acts like a pipette absorbing the accurate blood volume which eliminates volume related variations to a major extent. Moreover, the tip is attached to a plastic handler, so it does not come in contact with any surface.

3.3 Objective of the Present Study

Between 2000 and 2009, the rate of opioid use among pregnant women increased 5-fold. The efficacy of buprenorphine to suppress withdrawal symptoms in these women is comparable to that of methadone. However, a large cohort of clinical trials that compared the efficacy of buprenorphine and methadone in pregnant patients reported that 30% of patients converted to buprenorphine stopped initially, and 71% of them dropped out due to dissatisfaction with the drug. This is likely due to the various physiological changes during pregnancy that potentially affect buprenorphine disposition and impact its efficacy.

Use of medications during pregnancy based on dosing recommendations for non-pregnant populations, without titration of its dose to account for the altered PK changes may lead to therapeutic failure or toxicity for the patient as well as the fetus. We predict that buprenorphine exposure will decrease due to an increase in the total body clearance of the drug during pregnancy. Previous studies carried out in our lab have documented lower exposure of BUP during pregnancy[49]. However, further data is needed to characterize the pharmacokinetic profile of buprenorphine in a larger patient population. The use of a micro sampling method to collect blood samples from these pregnant patients will eliminate the inconvenience encountered due to long hours spent in the clinic for multiple sample time points.

We hypothesize that a micro sampling tool such as the VAMS device will help facilitate conduct of pharmacokinetic studies in pregnant women and can provide substantial data to predict alterations in the clearance and exposure of buprenorphine during and after pregnancy.

It is necessary to first develop a sensitive and specific assay for determining buprenorphine and its metabolites in the 20 µL VAMS blood sample. To date, no method has

been developed to simultaneously quantify buprenorphine and its metabolites using 20 μL VAMS samples.

The goal of this study is to develop and validate a highly sensitive and reproducible LC-MS/MS micro sampling assay for buprenorphine and its metabolites and apply this method to process VAMS samples from clinical pharmacokinetic studies in pregnant women. The ultimate application is to optimize buprenorphine dosing in treating opioid addiction in pregnancy and post-delivery through a better understanding of the effect of pregnancy on BUP exposure.

Through the proposed experiment, we have attempted to develop and validate a simple and sensitive liquid chromatography-tandem mass spectrometry (LC-MS/MS) assay to quantify buprenorphine and three primary metabolites (norbuprenorphine, buprenorphine glucuronide, norbuprenorphine glucuronide) in 20 μL VAMS blood samples.

4.0 Materials and Methods

4.1 Chemicals and Reagents

Chemical structures of buprenorphine, norbuprenorphine, buprenorphine glucuronide, norbuprenorphine glucuronide and respective deuterated internal standards, buprenorphine-D4, norbuprenorphine-D3, buprenorphine-D4-3-β-D-glucuronide, norbuprenorphine glucuronide-D3 are shown in Figure 7. They were purchased from Sigma-Aldrich (St. Louis, MO). Ammonium acetate (99.9999 trace metals basis) and OptimaTM LC/MS grade acetonitrile, formic acid, methanol, and water were obtained from Fisher Scientific (Fair Lawn, NJ, USA). Human blood samples were procured from central blood bank of Pittsburgh (Pittsburgh, PA, USA). 20 µL VAMS devices were obtained from Neoteryx, Torrance CA, USA.

4.2 Chromatographic Conditions

The LC system used for the analysis of buprenorphine and three of its metabolites was a Waters Acquity H class model (Waters Corporation, MA, USA). Separation of all components of interest was achieved on Acquity LC BEH C18 1.7 µm, 2.1 x 100 mm column. The mobile phase A was 5% acetonitrile in water containing ammonium acetate (2 mM) and formic acid (0.1%) and mobile phase B was 95% acetonitrile in water containing ammonium acetate (2 mM) and formic acid (0.1%) at a flow rate of 0.3 mL/min. A sample volume of 5 µL was injected on column. A gradient method for the mobile phase was used to separate buprenorphine and its

three metabolites. The gradient started at 25% of B, maintained for 1.0 min, then increasing to 35% of B from 1.0 min to 1.1 min, maintaining for 2 min, then increased to 100% of B from 3.1 to 4.1 min, maintaining for 2 min, and decreased to 25% of B from 5.1 to 5.2 min, then maintained at 25% of B until 7 min. The gradient method is summarized in Table 4. The total run time for each injection was 7 mins.

Table 4: Gradient Method used to separate BUP and its metabolites

Run Time (min)	Mobile Phase A %	Mobile Phase B %
Up to 1.0	75	25
1.1	65	35
3.1	65	35
4.1	0	100
5.1	0	100
5.2	75	25
7	75	25

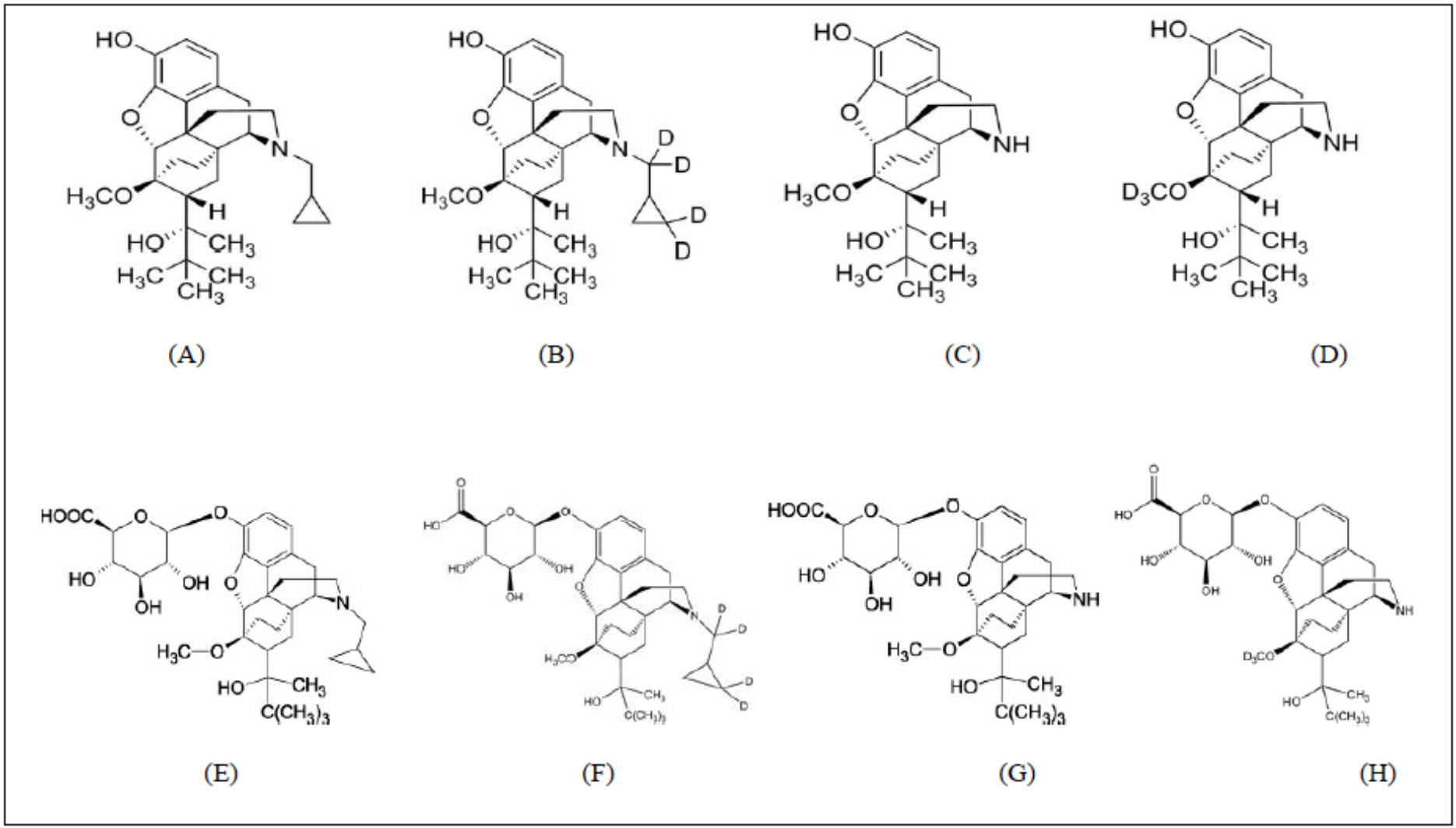


Figure 7: Chemical structure of buprenorphine (A), buprenorphine-D4 (B), norbuprenorphine (C), norbuprenorphine-D3 (D), buprenorphine-3-β-D-glucuronide (E), buprenorphine-D4-3-β-D-glucuronide (F), norbuprenorphine glucuronide (G), norbuprenorphine glucuronide-

4.3 Mass spectrometric conditions

Mass spectrometric analysis was carried out using a XEVO TQS triple quadrupole mass spectrometer (Waters, Milford, MA, USA) with positive electric spray ionization mode using multiple reaction monitoring (MRM). Multiple reaction monitoring used the precursor to product ion pairs for quantification of compounds used by MRM are summarized in Table 5 below.

Table 5: Product ion pairs-MRM Method

Compound	Parent (m/z)	Daughter (m/z)
Buprenorphine	468.5	396.3
Buprenorphine-D_4	472.2	400.2
Norbuprenorphine	414	101
Norbuprenorphine-D_3	417.3	101.1
Buprenorphine-3-β-D-glucuronide	644.1	468.2
Buprenorphine-D_4-3-β-D-glucuronide	648.6	472.5
Norbuprenorphine glucuronide	590.1	414.2
Norbuprenorphine glucuronide-D_3	593.5	417.4

The settings of MRM were as follows: capillary voltage, 2.8 kV; source temperature, 150°C; desolvation temperature, 500 °C; cone gas flow, 150 L/h; desolvation gas flow, 800 L/h. The LC–MS system was controlled by Masslynx® software version 4.2, and data were collected with the same software

5.0 Studies Conducted

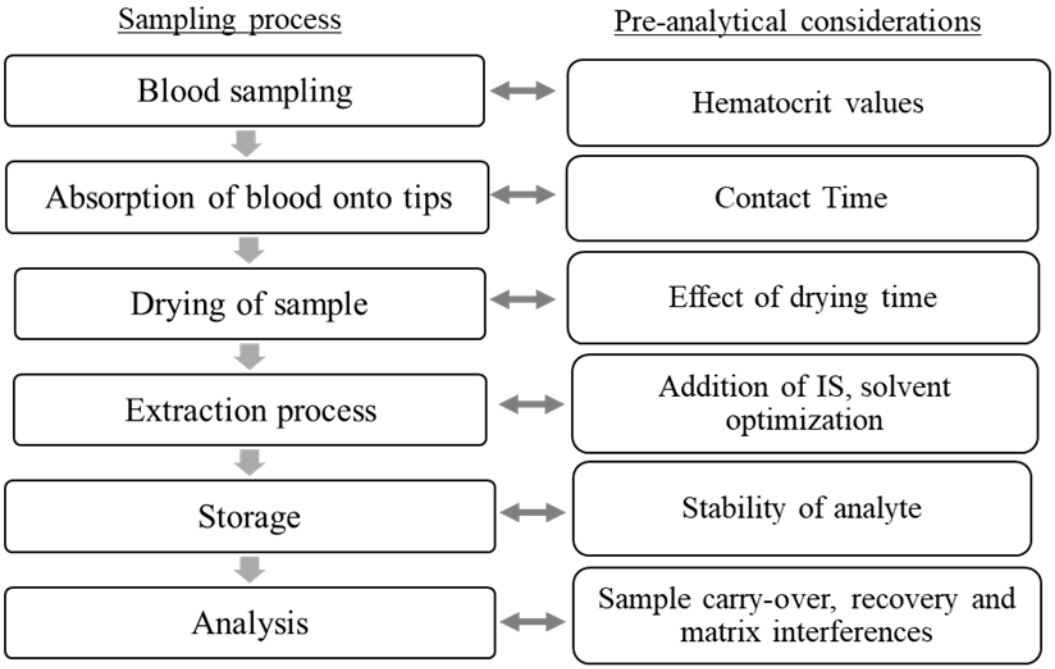


Figure 8: Preanalytical Considerations

The ideal solvent for extraction provides maximum analyte recovery, with high reproducibility and minimal interference from other compounds. Assay performance is impacted by the blood absorption onto tips, analyte recovery as well as the matrix effect.

Optimization of the extraction recovery is critical to eliminate the extractability-mediated stability bias as well as the hematocrit effect[76].

While developing an assay method for VAMS, two stages of sample extraction need to be considered. The first stage is desorption where the dried blood needs to be desorbed from the polymeric tip by use of an aqueous solution along with high energy sonication and vortexing in

order to ensure all of the blood has been displaced from the dried matrix. The composition of liquid blood that has been desorbed from VAMS tips is different from original liquid blood[77]. The second stage is the analyte extraction stage, wherein protein precipitation or liquid-liquid extraction are utilized.

There are differences in opinion about whether the IS should be added to fluid matrix before VAMS sampling or if the IS should be soaked into the tips after test tube sampling. According to Marasca et al, the results are overlapping either way. However, the extraction process described by Mohamed et al. emphasizes on addition of IS during the VAMS treatment step, rather than to the blood before the tips are sampled. This also reflects the real-life scenario where the blood is sampled by finger prick and then processed in IS solution later.[78-80]

5.1 Gravimetric Volume estimation

Typically, the devices are weighed before and after blood absorption in order to determine the volume of blood collected within the VAMS tip by considering the density of the blood matrix. This volume sampling test was carried out in the determination of Acetylsalicylic acid by Kim et al. using the given formulae[81]:

Average density of matrix-

Matrix density (mg/μL) = Mean blood weight in 20 μL aliquot (mg)/20

Volume of blood absorbed onto the VAMS tip-

Mean matrix volume in VAMS tip (μL) = [Mean blood weight in VAMS tip (mg)/ mean matrix weight in 20 μL aliquot (mg)] × 20

Assuming density of blood: 1060 kg/m3 or 1.06 g/ml

Table 6: Gravimetric Volume Estimation of VAMS device

Dry weight	Wet Weight	Difference	Volume	Bias (%)
g	g	g	Mean (µLs)	
1.19	1.21	0.03	24.8	24.2
1.19	1.21	0.02	20.3	1.5
1.20	1.23	0.02	22.8	13.9
1.13	1.16	0.02	23.2	16.0
1.16	1.18	0.02	22.4	11.8
1.19	1.21	0.02	21.3	6.5

We can conclude that majority of samples (83%) have a bias within 16% of the theoretical volume, which is acceptable for our studies.

5.2 VAMS-Internal Standard Interaction

The next step that we had to verify was whether presence of VAMS led to adsorption of IS. In order to understand this, we tested the internal standard response in the presence of three solvents along with the VAMS device tip.

1. 5% Methanol
2. 100% Acetonitrile
3. 100% Water

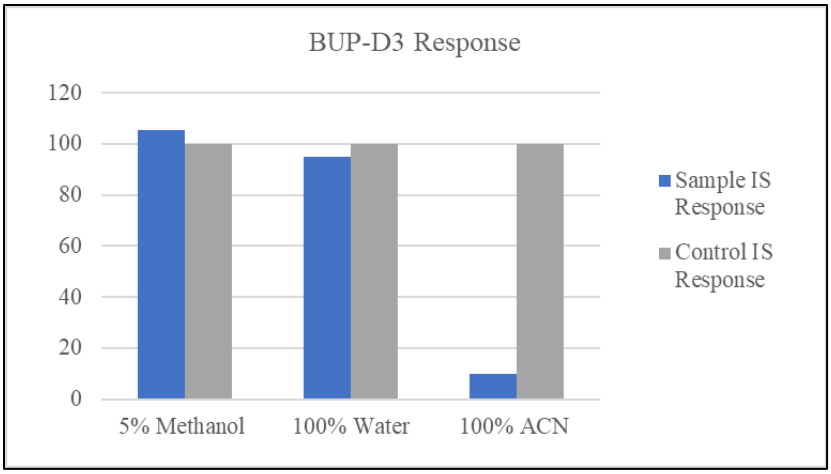


Figure 9: Internal Standard Response

In Figure 9, the control sample is denoted in grey which refers to the blood standard extracted using each of the solvents mentioned, without the presence of VAMS tip. The test sample is denoted in blue wherein the blood standards were absorbed by VAMS tip and subjected to the same extraction process. As we can conclude from the figure, extraction of VAMS with commonly used solvents such as 5% Methanol as well as 100% aqueous solution did not significantly affect the IS response. 100% Acetonitrile was ruled out as an extraction solvent due to poor response compared to that of the control. Moreover, since the Internal Standard Solution itself contains Acetonitrile, it would not be suitable as an extraction solvent regardless. Very high proportion of acetonitrile would lead to protein precipitation within the tip, entrapping the analyte within the polymer matrix, resulting in poor recovery.

5.3 Aqueous vs Organic Solvent for Extraction

Table 7: Results for VAMS-BUP Standard conc 100 ng/mL

Name	Retention Time	Area	IS Area	Response= Area/IS Area
BUP 10% Methanol	3.14	96433	28284	3.5
BUP 25% Methanol	3.1	60813	16293.	3.7
BUP 50% Methanol	3.1	60789	16806	3.6
BUP 100% Methanol	3.11	58903	31891	1.8
BUP 100% Methanol	3.1	55493	29038	1.9

Table 8: Methanol-Water Solvents Tested

Solvent used-Methanol:Water	**Effect on Peak Area**	**Effect on Internal Standard Response**
5%	BUP shows unacceptable response at lower standard concentrations, metabolites have appropriate response	IS response is consistent
10%	BUP response is unacceptable, other compounds have high recoveries	IS consistent
25%	Parent compound shows low response, metabolites unaffected	IS response is lower than control
30%	No major improvement, BUP and NBUP response is poor	IS consistent
50%	Metabolite recovery is high, BUP is unimproved	IS consistent

As shown in Table 7, we tested 10%, 25%, 50% and 100% Methanol solutions for extraction of BUP and its metabolites from the VAMS device.

After testing pure organic solvents such as 100% Methanol and 100% Acetonitrile, it was clear that an aqueous medium would be necessary in order to desorb blood from the VAMS device and diffuse into solution, since blood is mainly composed of aqueous media along with proteins and lipids.

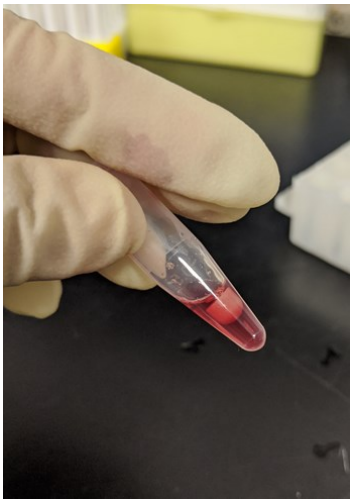
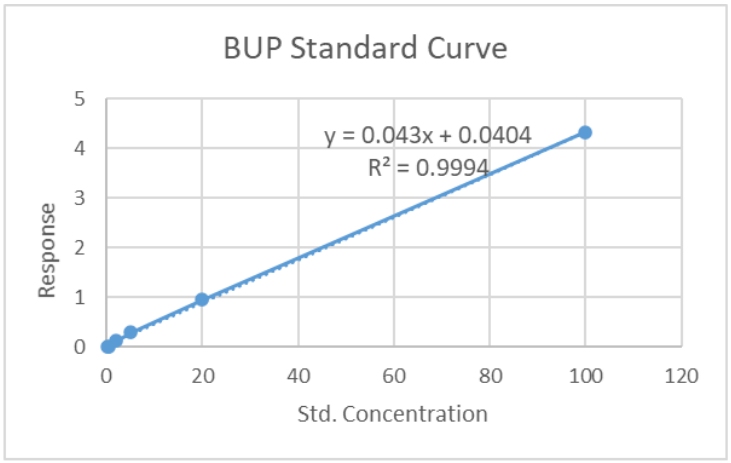
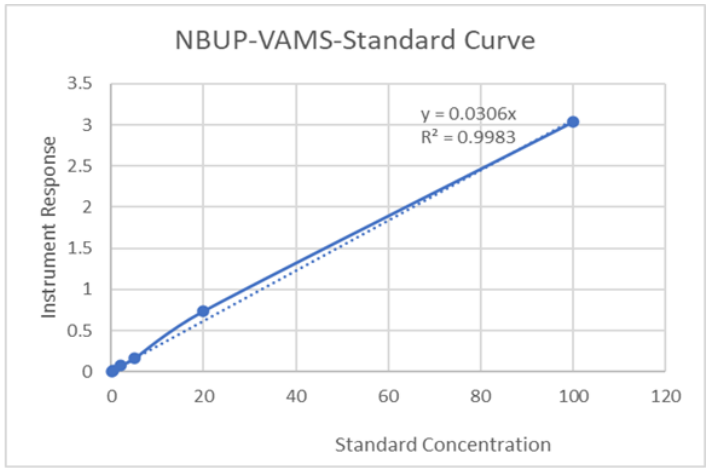


Figure 10: Aqueous Solvent to desorb blood

Since increasing the proportion of methanol in the solution did not show significant improvement in response, 5% Methanol was selected as optimum extraction solvent.



(A)



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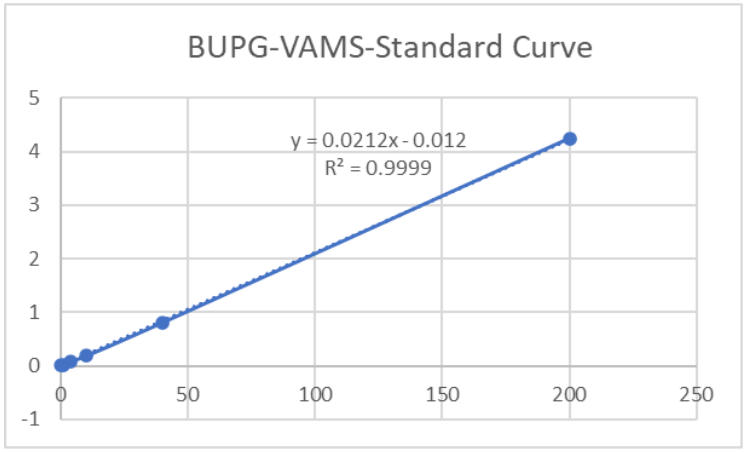

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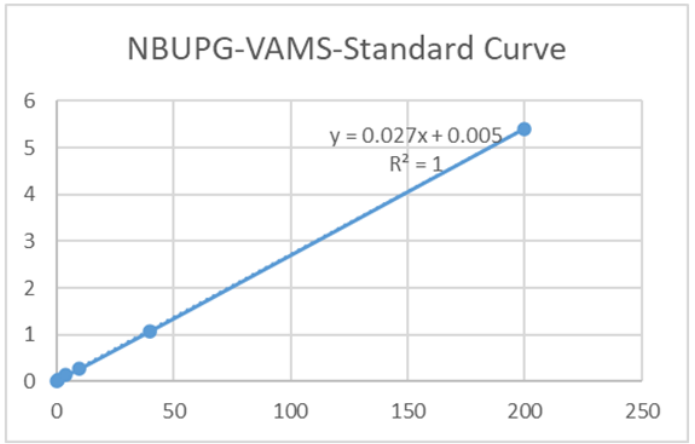


(D)

Figure 11: Standard Curves for (A) Buprenorphine (B) Norbuprenorphine (C) Buprenorphine-3-glucouronide (D) Norbuprenorphine-3-glucouronide

Table 9: Standard Curve Data for Buprenorphine, Buprenorphine G, Norbuprenorphine, Norbuprenorphine G

Name	RT	Area	IS Area	Response
BUP-100 pg mL	3.16	24.5	5615.8	0.004
BUP-500 pg mL	3.17	74.6	4891.3	0.015
BUP- 2 ng mL	3.17	575.9	4607.1	0.125
BUP-5 ng mL	3.16	1324.5	4380.2	0.302
BUP-20 ng mL	3.16	4808.6	5028.5	0.956
BUP-100 ng mL	3.16	29351.2	6781.4	4.328

Table 9: (Continued)

Name	RT	Area	IS Area	Response
BUPG-200 pg mL	1.36	192.8	43323.2	0.004
BUPG-1 ng ml	1.35	1050.9	49799.7	0.021
BUPG- 4 ng mL	1.35	4303.7	52055.1	0.083
BUPG-10 ng mL	1.35	9354.3	49499.5	0.189
BUPG-40 ng mL	1.35	41238.8	50994.1	0.809
BUPG-200 ng mL	1.35	206376.9	48665.7	4.241

Table 9: (Continued)

Name	RT	Area	IS Area	Response
NBUP- 100 pg mL	1.65	5.1	3994.3	0.001
NBUP- 500 pg mL	1.65	56.8	3198.5	0.018
NBUP- 2 ng mL	1.64	276.9	3528.2	0.078
NBUP-5 ng mL	1.64	527.3	3318.9	0.159
NBUP-20 ng mL	1.64	2614.3	3548.4	0.737
NBUP-100 ng mL	1.63	14960.8	4934	3.032

Table 9: (Continued)

`Name	RT	Area	IS Area	Response
NBUPG-200 pg mL	0.85	44.1	6092.8	0.007
NBUPG-1 ng mL	0.86	196.9	6646.1	0.03
NBUPG- 4 ng mL	0.86	833.4	6369.6	0.131
NBUPG-10 ng mL	0.87	1759.1	6457.3	0.272
NBUPG-40 ng mL	0.86	6590.1	6140.9	1.073
NBUPG-200 ng mL	0.86	33963.4	6282.1	5.406

While testing for standard curve concentrations of 0.1, 0.5, 2, 5, 20, 100 ng/ml of Buprenorphine, response for BUP as well its metabolites were consistently suitable using 5% Methanol as extraction solvent.

However, while checking response for low standard concentrations of 0.1-10 ng/ml of the drug which reflects clinical concentration levels, the BUP samples showed unacceptable recovery, as pictured in Figure 12.

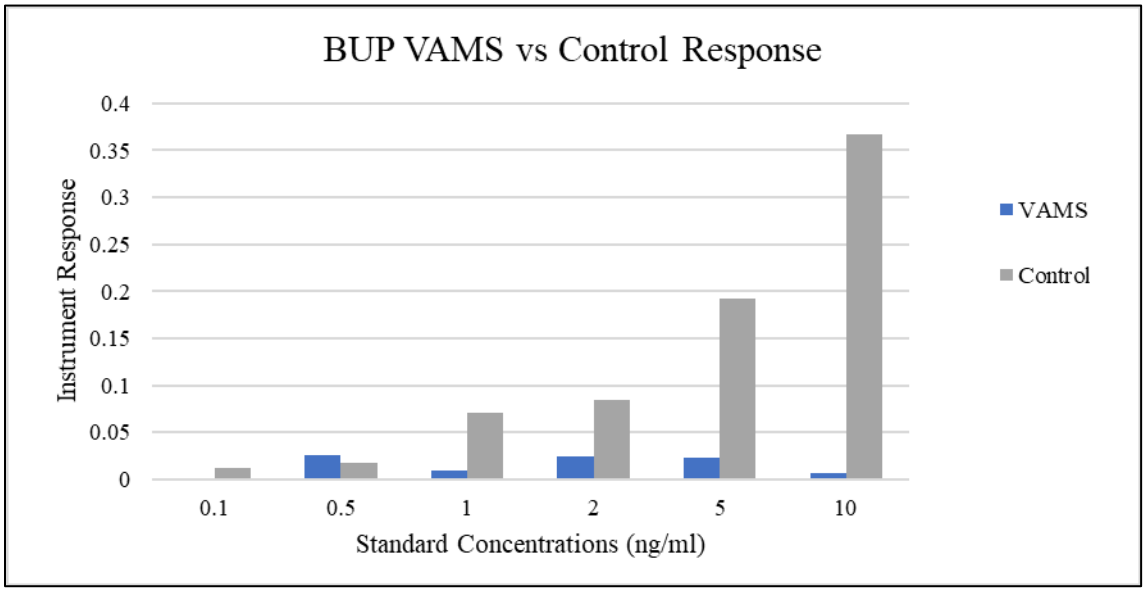


Figure 12: Unacceptable BUP Response

The issue of poor recovery of BUP has been consistent throughout assay development by use of 5% Methanol as extraction solvent, with NBUP, NBUP-G and BUP-G showing appropriate response while use of higher standard concentrations.

This has been hypothesized due to the parent compound being highly lipophilic and attaching to the VAMS device tip. Whereas its metabolites, being hydrophilic would get easily diffused into the aqueous-organic extraction solvent from the hydrophilic device tip.

5.4 Solvent Optimization

Table 10: Solvents tested for Optimization

Solvent/Method used-	Effect on Peak Area	Effect on Internal Standard Response
100% Methanol	Unacceptable recovery of all compounds	Change in IS area from control
100% Water	Unacceptable recovery for BUP+metabolites	IS area decreased from control
50% Methanol: 50% Acetonitrile	BUPG shows acceptable response, other compounds have low recovery	IS area unaffected
Prewetting tips (Rehydration step- add 50 microlitres water+vortex+ extraction solvent)	No considerable improvement in recovery for any compound	IS area unaffected
LLE using ethyl acetate+ammonium hydroxide	Unacceptable recovery	IS area is increased compared to control
Water+1% Formic Acid Solution	BUP response is low, metabolites show appropriate response	IS area unaffected
50% Methanol: 50% ethyl acetate	BUPG shows suitable response, other compounds have low recovery	IS area unaffected

In order to improve recovery of BUP, multiple other solvents were tested. Prewetting tips which incorporates a rehydration step prior to addition of extraction solvent was also tested, but no significant improvement in recovery was observed.

Use of LLE in order to improve extraction recovery of BUP was carried out using ethyl acetate and ammonium hydroxide. However, there was no significant improvement in peak response for the parent compound.

The use of formic acid (FA) solution in water improved Buprenorphine recovery, however potential issues with the formation of a colored solution as well as peak area enhancement for the glucuronide metabolites had to be tackled.

Upon review of literature, we found that Houbart et al. considered the proportion of organic solvent, acidic additive, and duration of shaking to optimize the extraction process for a VAMS method. Addition of formic acid improved recovery for a hydrophobic compound but at very low proportion[78]. Therefore, we reduced the proportion of acidic additive and tested the use of 0.1% formic acid instead of 1% formic acid solution. Since 5% Methanol showed good results for all the metabolites, addition of the 0.1% formic acid additive to the solvent was considered.

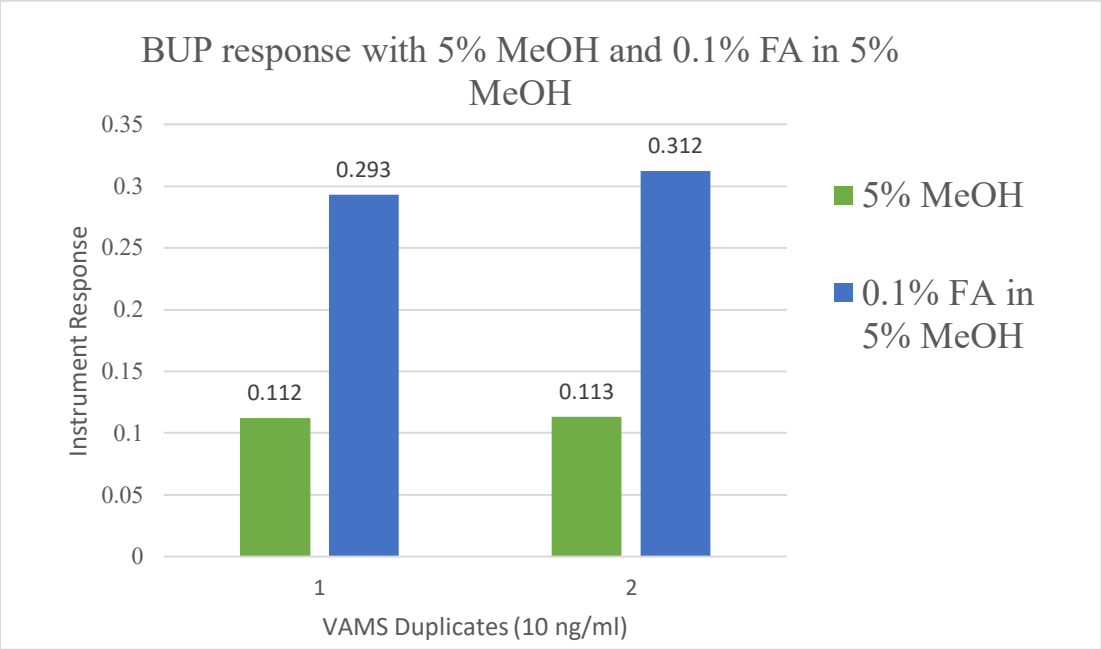


Figure 13: Improvement in BUP response using 0.1% FA

5.5 Vortexing/Sonication optimization

Along with this, the use of a more vigorous lateral shaker instead of sonication showed improvement in BUP recovery. With this, shaking for longer duration resulted in more effective blood desorption from the tips. Therefore, as pictured in Figure 14, use of a lateral shaker for 120 minutes was considered optimum.

Table 11: VAMS Samples Std.Conc 5 ng/ml- Subjected to Sonication

Name	Area	IS Area	Response
BUP-VAMS-Sonication-30 min	822.527	2864.75	0.287
BUP-VAMS-Sonication-30 min	542.281	3449.287	0.157

Table 12: BUP Response is Improved- Use of Lateral Shaker

BUP_LateralShaker_VAMS_120	3854.6	5485.06	0.703
BUP_LateralShaker_VAMS_120	3003.385	4629.668	0.649

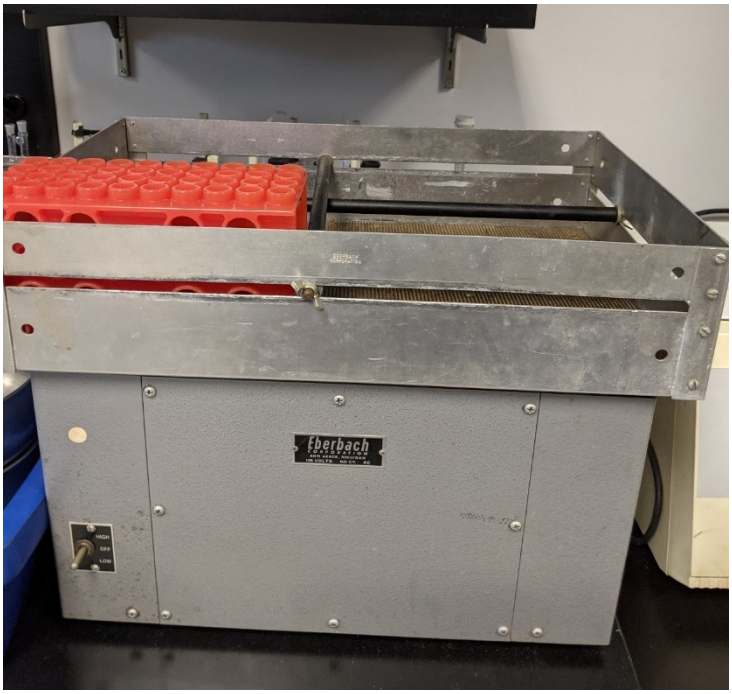


Figure 14: Eberbach Lateral Shaker

The primary challenge was to maintain the acceptable response for NBUP, NBUP-G and BUP-G while improving BUP recovery. Therefore, the final solvent used is 0.1% Formic acid in 5% Methanol, along with the use of a lateral shaker for a period of 120 minutes to achieve optimum recovery.

5.6 Sample preparation

Daily calibration standards, QC samples, and clinical blood samples were thawed at room temperature. Blood concentrations of buprenorphine, norbuprenorphine, buprenorphine glucuronide, norbuprenorphine glucuronide were determined by LC-MS/MS. VAMS and Blood samples were processed simultaneously in the same workflow.

1. Calibration standards and QC samples were prepared by spiking blank human blood with stock solution, the range is from 1 -20 ng/mL, QC from 3, 9 and 18 ng/mL for BUP and NorBUP, range is 2-40 ng/mL, QC from 9, 18, 36 ng/mL for BUPG and NorBUPG
2. VAMS tips were weighed once before spotting with blood (Dry weight) and once after absorption of blood (wet weight). This is for gravimetric volume estimation of the blood taken up by the device tip.
3. The 20 µL VAMS tips were inserted into 1.5 ml Eppendorf tubes using the cap of the tube to detach the tip from its plastic handler. Simultaneously, 20 µLs of blood of the same concentration was pipetted into the 1.5 ml Eppendorf tubes.
4. To this, 180 µL of extraction solvent was added. The solvent consisted of 0.1% formic acid in 5% Methanol solution.
5. Tubes containing extraction solvent were subjected to shaking using a lateral shaker on HIGH for 120 minutes.
6. Subsequently, 800 µL of 100% acetonitrile (ACN) which contains 500 pg/mL (buprenorphine-D_4, norbuprenorphine-D_3,), 1 ng/mL (buprenorphine-D_4-3-β-D-glucuronide, norbuprenorphine glucuronide-D_3) was added to the samples.
7. To this, the tube was vortexed for 30 s, then followed with centrifugation at 15,000 rpm for 12 min at 8 °C. 700 µL supernatant was transferred to a glass tube and was dried

under a stream of air.

8. The dried residues were reconstituted in 100 µL of mobile phase (consisting of 2A:1B), vortexed for 30 s, and then transferred to an Eppendorf microcentrifuge tube. The tube was centrifuged at 15,000 rpm for 12 min at 8 °C.
9. The supernatant was transferred to a sample vial for injection. 5 µL of the solution was injected on the column, the sampler set at 15 °C.

5.7 Bioanalytical method Validation

The LC-MS/MS method was developed and validated according to the guidance of bioanalytical method validation by the FDA in 2013.

Calibration curves

The calibration curves were generated by plotting the response ratio of buprenorphine to buprenorphine-D4, norbuprenorphine to norbuprenorphine-D3, buprenorphine-3-β-D-glucuronide to buprenorphine-D4-3-β-D-glucuronide, norbuprenorphine glucuronide to norbuprenorphine glucuronide-D3 against nominal concentration of the corresponding four analytes in blood samples. The calibration curves were fit by a quadratic equation using weighing factor of $1/x^2$. Concentration of analytes in the unknown blood samples were calculated from their peak area ratios and the calibration curve. The deviations of back calculated concentrations from the nominal concentrations of QC samples were used to check the assay performance over the concentration ranges on each sample run day. The acceptance criteria of accuracy and precision of QC samples are described below.

Accuracy and precision

Accuracy was investigated by intra- and inter-day coefficient of variation (CV). Quality control samples (3, 9, 18 ng/mL for buprenorphine and norbuprenorphine; 6, 18, 36 ng/mL for buprenorphine glucuronide and norbuprenorphine glucuronide) were tested. For intra-day accuracy, three samples of each QC concentration were analyzed on a single day; for inter-day accuracy, a total of three samples of each concentration were measured on three consecutive days. The back-calculated concentrations should be between 85% and 115% of the nominal concentrations.

Precision was evaluated by intra- and inter-day reproducibility. Quality control samples (3, 9, 18 ng/mL for buprenorphine, 3, 9, 18 ng/mL for norbuprenorphine; 6, 18, 36 ng/mL for buprenorphine glucuronide and norbuprenorphine glucuronide) were tested. For intra-day precision, three samples of each concentration were assayed on a single day; for inter-day precisions, a total of three samples of each concentration were determined on three consecutive days. The intra-day and inter-day coefficient of variation should be within 15%.

Extraction recovery and matrix effects

The extraction recovery of buprenorphine and three of its metabolites was performed by comparing the responses obtained from neat standard QC samples (3, 9, 18 ng/mL for buprenorphine, 3, 9, 18 ng/mL for norbuprenorphine; 6, 18, 36 ng/mL for buprenorphine glucuronide and norbuprenorphine glucuronide) with the responses obtained from extracted blank human blood spiked with buprenorphine, norbuprenorphine, buprenorphine glucuronide and norbuprenorphine glucuronide and internal standards post extraction.

To evaluate the effect of endogenous matrix on the ionization of buprenorphine and three metabolites, responses of buprenorphine and three metabolites at the QC concentrations (3, 9, 18 ng/mL for buprenorphine and norbuprenorphine; 6, 18, 36 ng/mL for buprenorphine glucuronide and norbuprenorphine glucuronide) in triplicate were evaluated. The effect of blood matrix on analytes was defined by comparing the response obtained from extracted blank blood samples spiked with buprenorphine, norbuprenorphine, buprenorphine glucuronide and norbuprenorphine glucuronide post extraction with the absolute response of non-extracted methanolic solvent to which the same number of analytes were added.

Chemical Stability

It is crucial to understand the stability of VAMS samples since they would be subject to storage for a period of at least two days before reaching a clinical lab setting. Therefore, we tested the devices under multiple separate conditions reflective of clinical handling. They were stored at room temperature for a period of 24 hours, 3 days, and 4 days. One pair of samples was also stored at -80º C for a period of 4 days. The samples were taken in duplicate at mid-level 9 ng/ml QC concentration of BUP and NBUP, and 18ng/ml QC for BUPG and NBUPG.

6.0 Results

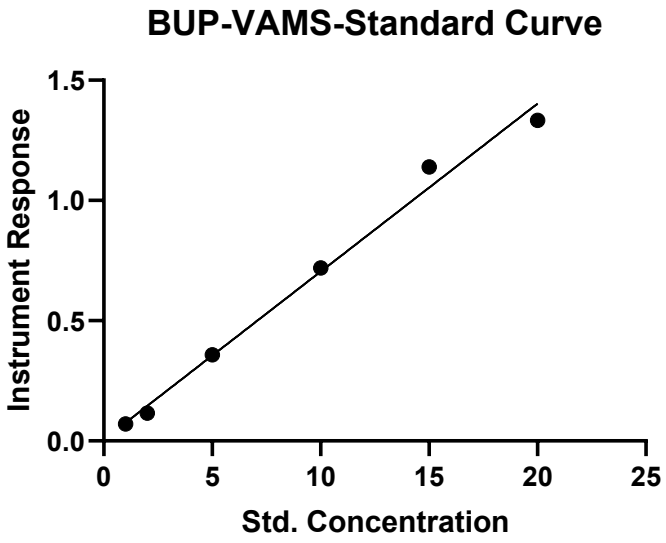
Following an injection of the reconstitution solution into LC-MS/MS system with positive ion electrospray ionization interface, the retention time of buprenorphine, norbuprenorphine, buprenorphine glucuronide, and norbuprenorphine glucuronide were 2.16, 1.28, 1.1 and 0.83 min, respectively. The assay did not show any significant interference with blood constituent at the retention times of analytes of each ion pair for MRM.

Typical chromatograms of blood samples spiked with buprenorphine at 2 ng/mL; norbuprenorphine, buprenorphine glucuronide, and norbuprenorphine glucuronide at 20 ng/mL, and their respective internal standards are listed in Figure 16. The regression coefficient (r2) of all calibration curves was higher than 0.98 for buprenorphine, buprenorphine glucuronide, and norbuprenorphine glucuronide. However, the standard curve for norbuprenorphine did not pass the linearity criteria.

6.1 Linearity

The ratio of mean peak area of the analytes to the internal standard was linearly related to the standard concentration of the compounds in the concentration range of 1-20 ng/mL for BUP and 2-40 ng/mL for BUPG and NorBUPG. However, NorBUP did not demonstrate linearity with significant Y intercept value of 0.75 and R-squared of 0.94. These are depicted in Figure 15.

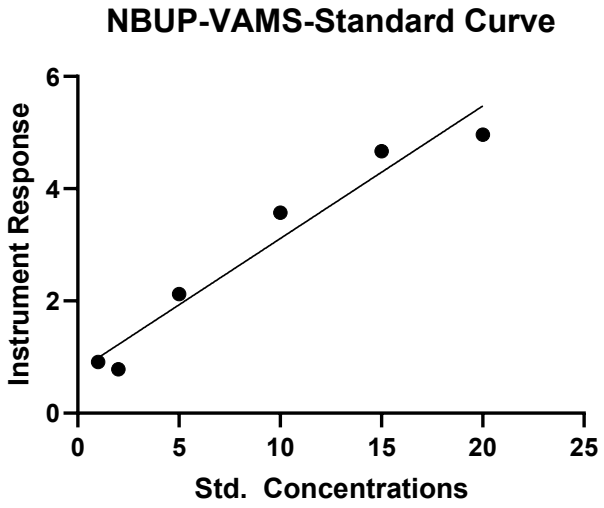
Figure 15: Standard Curves of VAMS samples for buprenorphine and its metabolites



Best-fit values	
YIntercept	0.005547
Slope	0.06984

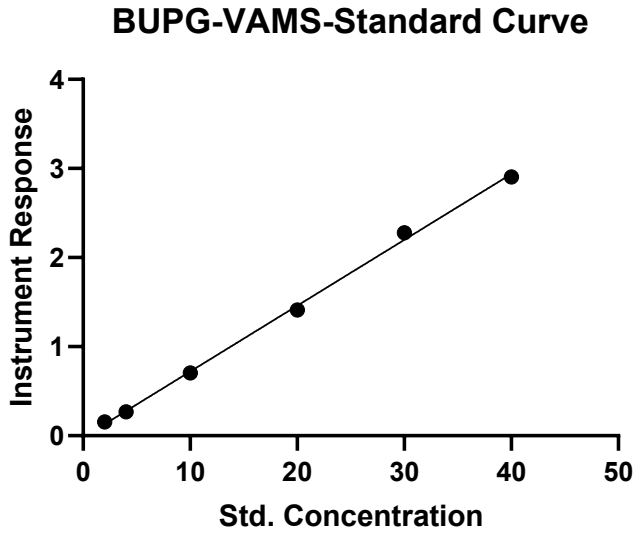
Goodness of Fit	
Degrees of Freedom	4
R squared	0.9905

Fig 15: (Continued)



Best-fit values	
YIntercept	0.7493
Slope	0.2362

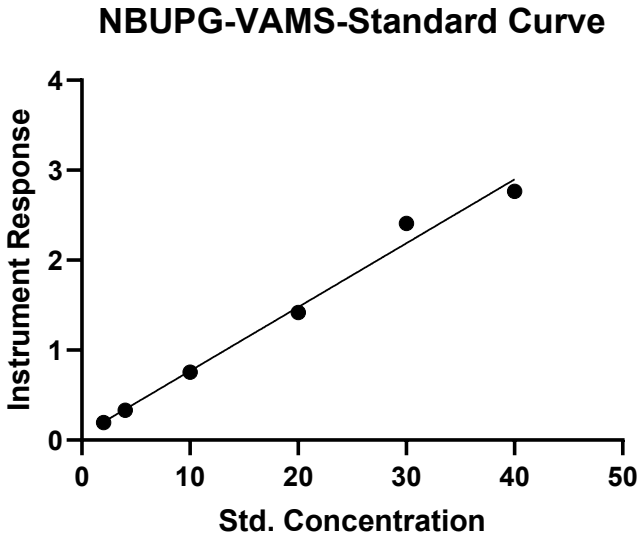
Goodness of Fit	
Degrees of Freedom	4
R squared	0.9495



Best-fit values	
YIntercept	-0.01828
Slope	0.07389

Goodness of Fit	
Degrees of Freedom	4
R squared	0.9983

Fig 15: (Continued)



Best-fit values	
YIntercept	0.05849
Slope	0.07097

Goodness of Fit	
Degrees of Freedom	4
R squared	0.9881

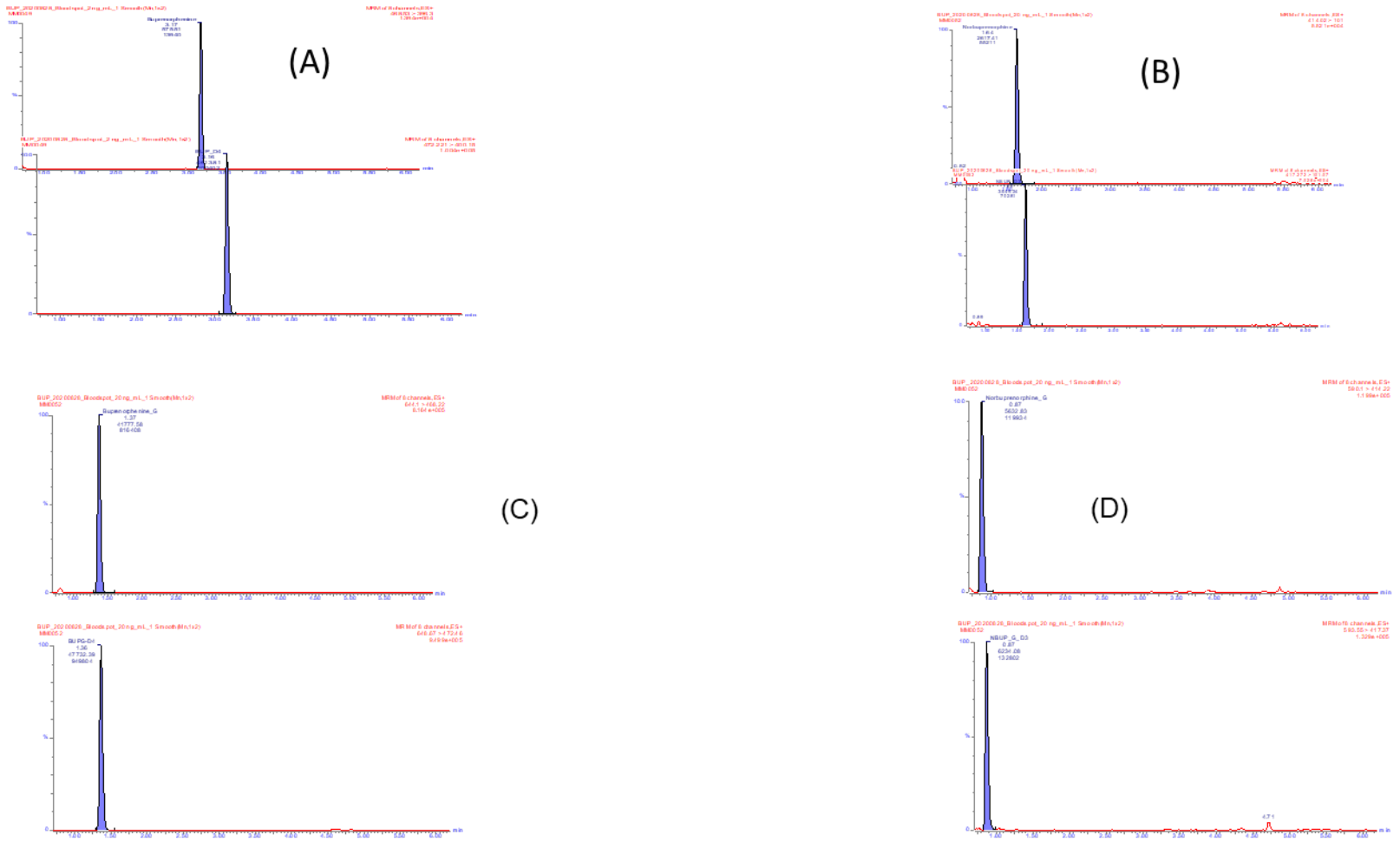


Figure 16: Representative chromatogram spiked with buprenorphine (2 ng/mL, A), norbuprenorphine (20 ng/mL, B), buprenorphine glucuronide (20 ng/mL, C), and norbuprenorphine glucuronide (20 ng/mL, D) with their respective deuterated internal standards

6.2 Accuracy and Precision

The LLOQ was determined as 1 ng/ml for BUP and NBUP. Intra-day and inter-day coefficients of variation (CV) were within acceptable limits according to the guidance on bioanalytical method validation for buprenorphine which is less than 15% for nominal concentrations and less than 20% for the lower limit of quantification (LLOQ). The inter and intraday accuracy and precision of the control samples quantified without the use of VAMS in the same volume of blood standards has also been depicted in Table 14 and 16.

Table 13: VAMS- Inter-day and Intra-day accuracy of buprenorphine and three metabolites (expressed as a percentage of the nominal concentration)

	BUP	NBUP		BUPG	NBUPG
QC Concentration (ng/mL)	Intra-day accuracy (%, n=3)		QC Concentration (ng/mL)	Intra-day accuracy (%, n=3)	
3	98.7	43.5	6	87.3	80.3
9	108.7	74.7	18	97.5	98.5
18	115	82.7	36	114.1	117.7
QC Concentration (ng/mL)	Inter- day accuracy (%, n=3)		QC Concentration (ng/mL)	Inter-day accuracy (%, n=3)	
3	111.9	103.3	6	111.6	113
9	99.3	73.5	18	111.0	108
18	108	95.6	36	98.4	111

Table 14: Controls-Inter-day and Intra-day accuracy of buprenorphine and three metabolites (expressed as percentage of nominal concentration)

	BUP	NBUP		BUPG	NBUPG
QC Concentration (ng/mL)	Intra-day accuracy (%, n=3)		QC Concentration (ng/mL)	Intra-day accuracy (%, n=3)	
3	95.8	NA	6	107.2	164.9
9	105.5	88.3	18	121.2	135.6
18	78.9	82.2	36	87.6	89.7
QC Concentration (ng/mL)	Inter- day accuracy (%, n=3)		QC Concentration (ng/mL)	Inter-day accuracy (%, n=3)	
3	96.7	NA	6	108.8	123
9	118.3	121.3	18	117.6	113.8
18	101.9	69.7	36	117	92.36

Table 15: VAMS-Inter-day and Intra-day precision of buprenorphine and three metabolites (expressed as coefficient of variation)

	BUP	NBUP		BUPG	NBUPG
QC Concentration (ng/mL)	Intra-day precision (%, n=3)		QC Concentration (ng/mL)	Intra-day precision (%, n=3)	
3	6.4	6.3	6	22.8	10.7
9	11.0	27.8	18	7.0	11.1
18	1.3	NA	36	7.1	7.2
QC Concentration (ng/mL)	Inter- day precision (%, n=3)		QC Concentration (ng/mL)	Inter-day precision (%, n=3)	
3	3.7	1.3	6	10.8	14.5
9	8.3	12.6	18	5.5	1.5
18	7.5	7.4	36	13.0	6.0

Table 16: Controls-Inter-day and Intra-day precision of buprenorphine and three metabolites (expressed as coefficient of variation)

	BUP	NBUP		BUPG	NBUPG
QC Concentration (ng/mL)	Intra-day precision (%, n=3)		QC Concentration (ng/mL)	Intra-day precision (%, n=3)	
3	7.16	NA	6	11.8	12.1
9	14.2	15.6	18	10.4	8.5
18	13.5	1.7	36	9.6	2.4
QC Concentration (ng/mL)	Inter- day precision (%, n=3)		QC Concentration (ng/mL)	Inter-day precision (%, n=3)	
3	14.9	NA	6	17	35.4
9	5.1	9.6	18	8.2	9.1
18	7.6	41.5	36	17.4	7.7

6.3 Recovery

The QC blood samples at different concentrations were processed to examine the recovery of buprenorphine and three metabolites. The recoveries for all analytes in VAMS samples were above 100% over the concentration range tested, and BUP showed acceptable recovery within range. The control samples showed recovery >70% for the same concentrations for BUP. However, the recovery for NBUP, BUPG and NBUPG were not within acceptable limits and exceeded 300% which indicates significant overestimation using the VAMS method. Results are listed in Table 17.

Table 17: Recovery of buprenorphine and its metabolites in VAMS and Control Samples

	BUP	NBUP		BUPG	NBUPG
QC Concentration (ng/mL)	Recovery for VAMS (%, n=3)		QC Concentration (ng/mL)	Recovery for VAMS (%, n=3)	
3	109.2	NA	6	164.3	303.5
9	113.3	NA	18	152.8	264.8
18	105.7	297	36	150.4	267.3
QC Concentration (ng/mL)	Recovery for Control Samples (%, n=3)		QC Concentration (ng/mL)	Recovery for Control Samples (%, n=3)	
3	79.5	428.9	6	125.3	211.6
9	81.1	259.3	18	107.4	219.4
18	69.8	161.5	36	105.2	190.9

6.4 Matrix Effect

In order to evaluate matrix effect, the post extraction addition protocol was followed. The response obtained by injecting analyte in standard solution (neat solvent of 50% methanol) was compared to that of post extract blank blood spiked with analyte at the same concentration. It was calculated using the formula:

$$\text{Matrix Effect (ME)} = \frac{A-B}{A} * 100$$

Where A is the peak area obtained of analyte in neat solvent, B is the peak area of the analyte in sample which underwent sample preparation and extraction and later spiked with analyte of the same concentration as the standard solution.

Table 18: Matrix Effect for buprenorphine and its metabolites

	BUP	NBUP		BUPG	NBUPG
QC Concentration (ng/mL)	Matrix Effect (%, n=3)		QC Concentration (ng/mL)	Matrix Effect (%, n=3)	
3	-18	NA	6	35.6	22.4
9	-18.1	NA	18	30.5	20.3
18	-19.4	-18.3	36	19.4	26.4

6.5 Stability

The VAMS samples were loaded with QC 9 ng/ml in duplicates and subjected to drying at room temperature for a period of 24 hours, 3 days, and 4 days. Two duplicate QC VAMS samples were also stored for a period of 4 days at -80º C. All these samples were analyzed in a single run and compared to fresh VAMS samples from the same blood QC standard dried for a period of 3 hours on the day of analysis.

The changes in concentrations for buprenorphine and its three metabolites at the 9 ng/ml concentration of the QC samples were evaluated. There was no significant degradation of samples after storage at any of the conditions tested compared to the duplicates analyzed immediately after sampling, however the deviations between the duplicates were large. Results have been displayed in Table 19.

Table 19: Stability of VAMS samples

		BUP	NBUP			BUPG	NBUPG
QC Concentration (ng/mL)	Stability Condition	Mean % ± SD		QC Concentration (ng/mL)	Stability Condition	Mean % ± SD	
9	3 days RT	80.4± 1.5	99.4±21.6	18	3 days RT	87.1±5.2	95.9±14.9
9	Overnight RT	81.9±4.4	107.8±14	18	Overnight RT	92.3±7.2	104±21.6
9	4 days RT	77.4±2.6	98.4±12.5	18	4 days RT	87.4±4.4	90.1±15.3
9	4 days -80º C	81.3±10.9	98.5±1.6	18	4 days -80º C	92.6±2.8	89.4±7.6

7.0 Discussion

Studies to evaluate the pharmacokinetics of buprenorphine and its metabolites have been conducted, typically using plasma or blood samples of large volumes. An assay developed for the same compounds in our lab utilized a plasma UPLC-MS/MS assay and evaluated pregnant subjects during the second and third trimester and postpartum. They found that CYP and UGT activities were significantly increased as evidenced based on the metabolic ratios of BUP during pregnancy vs postpartum. Therefore, there was a need to increase dose of BUP in pregnant women to account for the increased metabolism and to reach the same drug exposure in these patients as in non-pregnant subjects[82].

In order to conduct a classical pharmacokinetic study, large volumes of 1-2 ml samples are required. These can represent a significant inconvenience to obtain from special populations including pregnant women, infants, and geriatrics. Advancements in bioanalytical technology has enabled determination of drug concentrations in extremely low volumes in the range of 20-100 μLs. There is a need for development of a multiplex assay that can determine concentrations of multiple compounds from one sample itself, especially with ultra-low volumes. This enables optimization in the amount of pharmacokinetic data obtained from the smallest possible volume of sample collected[57].

The specific special population we addressed in this study is opioid addicted pregnant women. Conducting pharmacokinetic studies in this population brings in unique challenges which need innovative strategies to combat. Data on drug use in pregnant women has been virtually nonexistent for decades. Some of the reasons for this include:

1. Ethical concerns in including pregnant women in research studies

2. Concerns in affecting organogenesis of fetus and potential long-term exposure to infants
3. Lack of financial incentive for conducting such studies
4. Logistical issues to obtain blood samples for studies

Therefore, dosing recommendations for pregnant women are extrapolated from non-pregnant data obtained for the same drug, and most of these medications are used "off-label"[83].

We propose to make use of an opportunistic study by obtaining clinical data from pregnant women already receiving the drug buprenorphine as part of their clinical care.

The objective of the current study was to develop and validate an LC-MS/MS method to simultaneously quantify buprenorphine and its metabolites using 20 µL blood VAMS samples. The method displayed linearity over a range of concentrations buprenorphine (1-20 ng/ml), buprenorphine glucuronide and norbuprenorphine glucuronide (2-40 ng/mL) with acceptable intra and inter day precision and accuracy for Buprenorphine. This is the first study conducted using Volumetric Absorptive Micro sampling to quantify buprenorphine and its metabolites.

Greenwald et al. has demonstrated a strong relation between the occupancy of the mu opioid receptor and concentrations of Buprenorphine. Clinically, the concentration range for pregnant patients has been recommended to be above 1 ng/ml to prevent symptoms of withdrawal[84, 85]. At current dose ranges, pregnant patients have levels below 1 ng/ml which needs to be quantified using the VAMS method.

The therapeutic blood concentrations of Buprenorphine are between 0.1 to 1 ng/ml, with a maximum of 2-3 ng/ml. This highlights the limitation of our study in poor sensitivity for detecting concentrations of the parent drug below 1 ng/ml. Further work has to be done in order to improve sensitivity of the method. We have to consider using a higher volume of blood to

suitably quantify the metabolites, either by use of a 30 µL VAMS device or by processing two separate 20 µL VAMS tips separately for Buprenorphine and the metabolites by different extraction methodologies.

Optimizing extraction of the compound was the most critical step in developing this assay.

In order to maximize recovery, we tested multiple solvents and vortexing/sonication/shaking conditions. Use of a methanol-water based solvent along with an acidic additive formic acid proved to give the best extraction recovery. Sonication was concluded to be ineffective since the VAMS tip structure would not allow ultrasonic frequencies to penetrate the pores, rather it would absorb the sound, making blood desorption difficult. For the current method, use of a lateral shaker for a long duration made a significant impact in improving recovery of the parent compound buprenorphine.

Protein precipitation, liquid-liquid extraction and solid-phase extraction are the three common methods used to remove interfering compounds from blood samples. Among these, the use of liquid-liquid extraction was tested. However, it is unsuitable due to the wide-ranging polarity of each of the four analytes. We employed protein precipitation using acetonitrile to maximize extraction recovery from the VAMS device tip.

As discussed in Section 5.3, we optimized the extraction recovery by utilizing aqueous solvent with organic component and an acid modifier. The use of formic acid in low proportion enhanced extraction recovery of all compounds, however it led to formation of colored solutions and enhancement in peak response for glucuronide metabolites. The mechanism by which the presence of 5% Methanol and 0.1% formic acid aids in improving extraction recovery is unknown but can be hypothesized as an effect of strong solvation ability of methanol coupled

with the aqueous concentration would lead to significant desorption of blood from the VAMS polymer tip.

Validation of the assay methodology was carried out to demonstrate acceptable accuracy and precision in both VAMS and Control samples for buprenorphine. However, there are significant inconsistencies observed for norbuprenorphine and the glucuronide metabolites for linearity, accuracy, precision, recovery, and matrix effect.

Stability was evaluated at the mid-level QC concentration in duplicate for four conditions: 24 hours at RT, 3 days and 4 days at RT, 4 days at -80º C. All samples were within an acceptable range compared to QC samples analyzed within 3 hours of drying on the same day, despite deviations between each duplicate sample being large. However, more rigorous long term stability studies need to be performed to make conclusive statements about VAMS integrity when stored under extreme temperatures.

In conclusion, further validation studies need to be conducted for norbuprenorphine, buprenorphine glucuronide and norbuprenorphine glucuronide. The assay demonstrates validity for the parent compound buprenorphine itself. Therefore, the assay methodology developed is suitable for analysis of Buprenorphine in VAMS samples.

8.0 Conclusions

We have developed and validated a rapid, sensitive, and robust UPLC-MS/MS assay with simple sample preparation to quantify the concentration of buprenorphine in 20 μL VAMS blood samples. The advantages of this analytical method include simple sample processing, ultra-low volume requirement, acceptable recovery of BUP and short sample run time.

Since our end goal for the study is to understand the time course of change in CYP enzyme expression throughout pregnancy and post-partum by looking at parent drug to metabolite ratios, further work is needed to quantify norbuprenorphine, buprenorphine glucuronide and norbuprenorphine glucuronide. However, majority of the clinical effect in mediating relief of withdrawal symptoms for opioid addicted patients is due to buprenorphine and not the metabolites. Therefore, it is far more important to quantify the parent drug compared to the metabolites for these patients, from a clinical point of view.

This assay enables us to quantify concentration time profiles of buprenorphine, after low dose of BUP using limited volume of blood samples.

9.0 Future Directions

Additional work has to be carried to validate the assay methodology for the metabolites of buprenorphine. We are currently collecting samples from pregnant patients at Magee Women's Hospital Pittsburgh to apply this assay methodology on 20 µL samples. The major aim is to enable at-home sampling without the need of a nurse, wherein patients can send in their own fingerpick samples to the lab. Use of VAMS obviates the need for special storage conditions, and needs simple air drying for a period of few hours before analysis.

We plan to utilize the data obtained from patient samples to characterize the pharmacokinetic profile of buprenorphine by comparing metabolite ratios during and after pregnancy. This enables dose optimization in these patients, and on a larger basis, monitoring of drug levels at the same time. Even though buprenorphine is not a drug that is routinely monitored, maintaining drug levels is critical to avoid adverse withdrawal symptoms or craving in these patients.

Moreover, the use of VAMS can be extended to perform other studies in pregnancy, wherein contributions of genotype within the enzyme change can also be quantified.

The primary goal is to establish simple research design to conduct studies within pregnancy such that the patient can perform sampling at home and demonstrate increased adherence to the study protocol.

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