A COMPARISON OF SAMPLE PREPARATION METHODS FOR FORENSIC OPIATE ANALYSIS BY LIQUID CHROMATOGRAPHY-TANDEM MASS SPECTROMETRY (LC/MS/MS)

By

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A COMPARISON OF SAMPLE PREPARATION METHODS FOR FORENSIC OPIATE ANALYSIS BY LIQUID CHROMATOGRAPHY-TANDEM MASS SPECTROMETRY (LC/MS/MS)

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Abstract: Qualitative and quantitative methods for the analysis of opiates on the liquid chromatograph-tandem mass spectrometer (LC/MS/MS) were developed and validated. The qualitative method was developed for the analysis of seven opiate compounds: 6-acetylmorphine (6-MAM), codeine, hydrocodone, hydromorphone, morphine, oxycodone, and oxymorphone. A quantitative method was developed for the quantitation of four opiate compounds: codeine, hydrocodone, morphine, and oxycodone. The validation was based on draft forensic validation Blood specimens were prepared using a dilute and shoot (D&S) method and a solid phase extraction (SPE) method. While the comparison data showed that the SPE was more sensitive for the analysis of opiates, the D&S method also produced accurate and reliable results at the levels required for forensic purposes

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CHAPTER I

INTRODUCTION

Opiates, naturally occurring alkaloid analgesics, are the most abused drugs in every socioeconomic level in today's society. These compounds have the ability to provide analgesia without loss of consciousness. The common effects are described as euphoria, sedation, and mental clouding. Opioid tolerance after chronic use can result in overdosing to feel the beneficial effects. Despite being highly addictive, these drugs are commonly prescribed for pain relief after surgeries or for chronic pain. As a result of frequent abuse, identifying opiates has become increasingly demanded in suspected driving under the influence of drugs (DUID) cases. [1-4]

There are several possible liquid chromatography-tandem mass spectrometry (LC-MS/MS) sample preparation methods employed in forensic laboratories to determine if an individual was under the influence of drugs while driving, including solid phase extraction (SPE), liquid-liquid extraction (LLE), and dilute and shoot (D&S) techniques. While the SPE method is considered more labor intensive and requires costly materials such as columns, it is considered to give cleaner extracts and better results. The LLE is a less expensive option and is potentially less labor intensive, depending how many

steps are required. The D&S method is the most rapid method because it skips the extraction process completely, although filtration may be required. [5] For state crime laboratories that are on a budget and have backlog, it is appealing to determine whether cheaper and quicker sample preparation methods may yield comparable results to SPE using the LC/MS/MS.

Recently published reviews of the literature have compiled various extraction methods into tables. The methods are usually separated by sample matrix (blood, urine, serum and hair) and by analytical instrument. The main analytical instruments employed are the gas chromatography-mass spectrometer (GC-MS) and the liquid chromatography-mass spectrometer (LC-MS). Most of the reviews cover a wide variety of drugs and are not specific to opiates. Methods specific for opiates were analyzed for only one or two opiate compounds. Often the papers reviewed lacked information needed for comparative purposes, such as linearity, limit of quantification (LOQ) and limit of detection (LOD).[1, 5-7]

Two analytical methods for the analysis of opiates on the LC/MS/MS were developed at the Oklahoma State Bureau of Investigation (OSBI). The qualitative method was developed for the analysis of seven opiate compounds: 6-acetylmorphine (6-MAM), codeine, hydrocodone, hydromorphone, morphine, oxycodone, and oxymorphone. A quantitative method was developed for the quantitation of four opiate compounds: codeine, hydrocodone, morphine, and oxycodone. The samples to be analyzed by LC/MS/MS were prepared using a D&S method. Both the qualitative and quantitative methods were validated at the OSBI lab. [8]

For the comparison study, samples were prepared using either SPE or D&S, and then analyzed by the quantitative LC/MS/MS method .The results were compared to determine which extraction method yielded better results. The goal of this study was to show demonstrate that the D&S method yields results with sufficient quality to avoid costly SPE preparation methods for the analysis of opiates. It would encourage future research to focus on cheaper and less time-consuming methods for other illicit drugs.

CHAPTER II

REVIEW OF LITERATURE

2.1 Overview

Opiates, naturally occurring alkaloid analgesics, are the most abused drugs in every socioeconomic level in today's society. Although the term opiate is used to refer to any compound derived from the poppy plant, *Papaver somniferum*, compounds that are naturally derived from opium are considered opiates, while compounds that are synthetically created are referred to as opioids. These compounds have the ability to provide analgesia without loss of consciousness. The common effects are described as euphoria, sedation and mental clouding. Opioid tolerance after chronic use can result in overdosing to feel the beneficial effects. Overdosing can lead to death through the suppression of respiratory drive. Despite being highly addictive, these drugs are commonly prescribed for pain relief after surgeries or for chronic pain. As a result of frequent abuse, identifying opiates has become increasingly demanded in suspected driving under the influence of drugs (DUID) cases. Use of opiates are known to affect driving in the following ways: slow driving, weaving, poor vehicle control, poor coordination, slow response to stimuli, delayed reactions, difficulty following instructions and falling asleep at the wheel.[1-4]

There is an abundance of literature specific to opiate analysis due to the combined efforts of clinical and forensic researchers. The main analytical instruments employed are the gas chromatography-mass spectrometer (GC-MS) and the liquid chromatography-mass spectrometer (LC-MS), with an increasing focus on the LC-MS. The most common sample preparation methods utilized are solid phase extraction (SPE), liquid liquid extraction (LLE), and dilute and shoot (D&S) techniques.[1-4] With all the literature available, a review of opiate compounds, DUID legislature, and LC/MS/MS instrumentation was conducted. In addition, a comparison of analytical instruments, sample preparation methods and chromatographic conditions was performed to determine which sample preparation protocol produces better results.

2.1 Opiates

Seven opiates and opioids were reviewed in this study, including: codeine, heroin, hydrocodone, hydromorphone, morphine, oxycodone, and oxymorphone. Their chemical structures are shown in Figure 1.

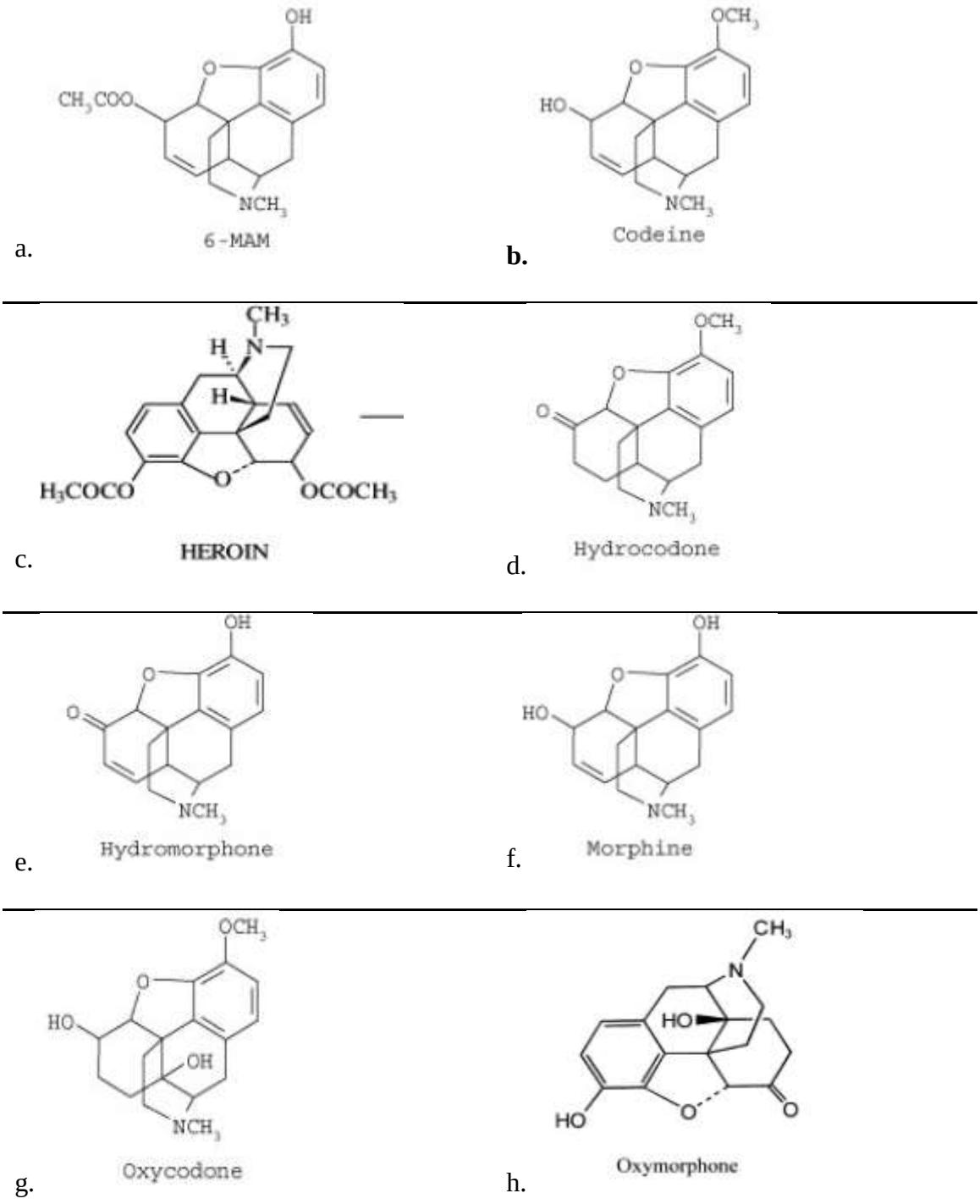

Figure 1. Chemical Structures of Opiates
The chemical structures of 6-MAM, codeine, heroin, hydrocodone, hydromorphone, morphine, oxycodone and oxymorphone are listed in a-h, respectively. Images from Lewis et al., Quary et al., and Capella-Peiro et al. [9-11]

2.1.1 Codeine

Codeine, a semisynthetic narcotic agonist, is naturally occurring in the juice of the poppy plant. It is created commercially by the methylation of morphine. Codeine is considered a low-potency analgesic, being approximately 1/10 to 1/6 as potent as morphine. It is metabolized by demethylation to morphine and norcodeine. Therapeutic doses found in toxicological samples range from 10 to 100 ng/ml. The wide range is due to the body building up tolerance to the drug, which makes higher concentrations of the drug necessary for any therapeutic effects. This opiate is often sold in over-the-counter medicines in combination with other non-opioid analgesics like aspirin or acetaminophen in the form of phosphate or sulfate salts. Adverse side-effects include nausea, vomiting, sedation, dizziness, rash, respiratory depression. [4] The chemical structure of codeine is shown in Figure 1.

2.1.2 Heroin

Heroin is synthesized from morphine and is currently a Schedule I substance in the United States. It currently has no known accepted medical use and is usually administered as the hydrochloride salt for intravenous injection or by nasal insufflation. The illicit compound undergoes deacetylation to 6-monoacetylmorphine (6-MAM), which is in turn hydrolyzed to morphine. The presence of 6-MAM with morphine is conclusive evidence of heroin use, because heroin is the only compound that metabolizes to 6-MAM.[4] The chemical structure of heroin and its metabolite 6-MAM are shown in Figure 1.

2.1.3 Hydrocodone

Hydrocodone is a semisynthetic narcotic analgesic agonist derived from codeine. It is considered to have 6 times the analgesic potency of codeine and a greater addiction liability. Therapeutic doses found in toxicological samples range from 2 to 24 ng/ml. It is usually administered as the bitartrate salt in combination with non-opioid analgesics and is widely present in cough syrup. The toxic effects include stupor, muscle flaccidity, respiratory depression, cold and clammy skin, and coma. While it is excreted as mostly unchanged drug, it does undergo demethylation to produce norhydrocodone, and hydromorphone.[4] The chemical structure of hydrocodone is shown in Figure 1.

2.1.4 Hydromorphone

Hydromorphone, a semisynthetic narcotic analgesic agonist, is a metabolite of hydrocodone. It is approximately 7 to 10 times more potent than morphine, but it is similar in addiction liability. Therapeutic doses found in toxicological samples range from 1 to 30 ng/ml. It is supplied as the hydrochloride salt and is commonly used in cough syrup. It undergoes conjugation to produce conjugated hydromorphone and conjugated hydromorphol.[4] The chemical structure of hydromorphone is shown in Figure 1.

2.1.5 Morphine

Morphine is the principle alkaloid of opium. It is a popular drug for moderate to severe pain. Therapeutic doses found in toxicological samples range from 10 to 80 ng/ml. The wide range is due to the body building up tolerance to the drug which makes higher concentrations of the drug necessary for any therapeutic effects. It is often supplied as the

sulfate salt for perinatal injection. It is metabolized by undergoing demethylation to normorphine.[4] The chemical structure of morphine is shown in Figure 1.

2.1.6 Oxycodone

Oxycodone, a semisynthetic narcotic agonist, is derived from thebaine. It is used for the relief of moderate to severe pain. Given subcutaneously, the drug is equipotent with morphine, but it has a higher oral/parenteral efficacy ratio. It is frequently used in combination with non-opioid analgesics as the hydrochloride or terephthalate salt. The compound undergoes demethylation, 6-keto reduction and conjugation to oxymorphone and noroxycodone. Therapeutic doses found in toxicological samples range from 10 to 100 ng/ml. The wide range is due to the body building up tolerance to the drug which makes higher concentrations of the drug necessary for any therapeutic effects. [4] The chemical structure of oxycodone is shown in Figure 1.

2.1.7 Oxymorphone

Oxymorphone, a metabolite of oxycodone, is a semisynthetic narcotic opiate agonist, also derived from thebaine. The compound is used for the relief of moderate to severe pain and for preoperative medication. It has about 6 to 8 times the analgesic potency of morphine. It is often supplied as the hydrochloride salt through oral dosing or parenteral injection. Oxymorphone undergoes extensive metabolism by reduction and conjugation to produce conjugated oxymorphone and 6-β-oxymorphol.[4] The chemical structure of oyxmorphone is shown in Figure 1.

2.2 DUID legislature

2.2.1 *Per se* law

Per se laws are implemented in an effort to deter drunk or drugged driving. These laws make it an offense to drive with a drug concentration past a set limit. The per se law is most commonly implemented for the detection of alcohol. Without per se laws for drugs, most of the evidential basis for a conviction for drugged driving is based on behavioral observations. Behavioral observations of impairment are often vague and easily challenged in courts. The zero tolerance law makes it an offense to drive with a certain drug at any detectable amount. This is often seen for alcohol present in individuals under the legal drinking age of 21. Studies on the effects of these laws have found that there are some beneficial effects on traffic safety. This may be due to general deterrence, where someone who might otherwise drink and drive are deterred from doing so due to knowledge of the law and resulting repercussions by violating it. [12, 13]

2.2.2 Oklahoma

The Oklahoma statues for driving under the influence (DUI) of alcohol or intoxicating substances (47 O.S. §,11-902) states that it is unlawful and punishable to have a breath or blood alcohol concentration of 0.08 or more within two hours after an arrest. It is unlawful to have any amount of a Schedule I chemical present in a person's system within two hours after an arrest. It is also unlawful to have any controlled substance present in a person's system within two hours after an arrest, meaning a Schedule II or III chemical without a physician's prescription. If an officer suspects DUI and any intoxicating substance is detected (level not specified), then the individual is in violation of the statute.

2.3 Sample preparation method

Sample preparation methods are employed for purposes of removing potential interferents from the sample, increasing the amount of the analyte recovered, converting the analyte into a more suitable form for detection/separation, providing a robust and reproducible method for dependable analysis. [14]

2.3.1 Solid Phase Extraction

SPE is a frequently used sample preparation method in toxicology laboratories. SPE involves column conditioning, sample application, column washing, column drying, and analyte elution. [15] The SPE column is a material-packed cartridge and there is a large variety available for specific trapping mechanisms of the analyte. Polarity, hydrophobicity, or ionization are often used as trapping mechanisms on the column, which requires matrix pH modifications to make the analyte either non-polar or aqueous. The trapped analyte is then released from the column by altering the polarity or pH in an eluting solvent. [14]

The benefit of using SPE columns is the wide variety of columns available and numerous methods with several combinations of cartridge material combined with eluent/sample matrices available in the literature. The use of disposable SPE columns minimizes the biohazard risk by reducing the handling of body fluids, such as urine, plasma, and blood. While the consumable cost of single-use cartridges and filters is quite a considerable amount, it may be more cost-effective than purchasing chemicals necessary for other sample preparation methods like LLE. The SPE method is considered more labor intensive, but it considered to yield cleaner extracts and better results.[5, 14]

2.3.2 Liquid-liquid extraction

LLE is also a frequently used sample preparation method in toxicology laboratories. LLE involves adjusting the pH of a liquid sample with a buffer, acid, or base and then extracting analytes with an organic solvent. Back extraction may also be used to remove the analyte from the organic solvent to further increase selectivity and sensitivity. [14, 15]

The benefit of using the LLE sample preparation method is the vast amount of literature available, which provides information on organic solvents and on the pH type, and concentration of reagents.[16] The LLE is a less expensive option and is potentially less labor intensive, depending on how many steps are required.[5]

2.3.3 Dilute and shoot

The D&S sample preparation method involves protein precipitation by diluting a sample with an organic solvent, such as acetonitrile. It is the fastest method, because it skips the extraction process altogether. In the literature, this sample preparation method is known to experience matrix effects during LC-MS analysis. There is minimal cleanup of the biological samples due to the non-selective nature of the D&S method. While it is one of the least labor intensive methods, the results may be affected due to inadequate sample cleanup.[5, 17]

2.4 Analytical Instrument Comparison

Within the last few decades, there have been tremendous technological advancements in the field of mass spectrometry (MS), resulting in the integration of the LC-MS in toxicological analysis.[1] Use of the LC-MS is favored over use of the gas chromatography-mass spectrometry (GC-MS) because of its increased sensitivity and

specificity.[2] In the recent literature, researchers have compared the LC-MS opiate method developed to a former GC-MS opiate method.

Sample preparation is necessary to remove interfering matrix compounds; thereby improving both selectivity and sensitivity.[1] Overall, the LC-MS has a simpler sample preparation protocol and allows the exclusion of steps that are otherwise necessary for the GC-MS. Coles et al. developed a LC-MS method for the simultaneous analysis of six opiates in various specimens. The sample preparation for the LC-MS excluded the derivitization and glucuronide hydrolysis necessary in the described GC-MS sample preparation. The reduction of steps in the sample preparation dramatically reduced extraction time and use of expensive eluents.[2]

Gustavsson et al. described a sample preparation procedure for the LC-MS which involved a straight injection of the urine specimen for the analysis and quantification of morphine, codeine and 6-MAM.[18] This is much simpler than the multi-step preparation necessary for the GC-MS, which included hydrochloric acid hydrolysis, solid-phase extraction and the formation of silyl derivatives.

Most of the literature shows that the LC-MS method developed is comparable to the GC-MS analysis in accuracy and precision. In qualitative methods, both of these instruments were in agreement with regard to the identification of drug analytes. But in quantitative methods, the LC-MS method was even more sensitive and selective.[1, 18, 19] In the Coles et al. article, the LC-MS had a 100-fold increase in analytical sensitivity with the lower limit of quantitation of 2.0 ng/ml, while the GC-MS had a lower limit of quantitation at 200 ng/ml. The LC-MS method had an increased specificity with an

interference rate of 3.9% compared to the higher interference rate of 13.6% for the GC-MS method.[2]

2.5 Sample Preparation Comparison

Coles et al. described a SPE method for use with urine, serum, plasma, and whole blood. To 1.0 mL of specimen, 2.0 mL of 0.1M sodium phosphate buffer was added. The sample was mixed and then centrifuged at 0°C, 3500 rpm for five minutes. The samples were loaded onto Trace-B columns at 4 drops/s and washed with 1 ml each of sodium bicarbonate buffer, water, 0.1M acetic acid, methanol, and ethyl acetate at 1 drops/s. The columns were dried at 25 psi for a minute and then the samples were eluted into recovery vials with 1 ml of a 70:25:5 mixture of ethyl acetate/isopropanol/ammonium hydroxide. These samples were dried down at 40°C for 12 minutes, reconstituted with 200μL acetonitrile and analyzed on the LC-MS.[2]

Cailleux et al. described an LLE method that was used for urine, plasma and blood. First, 25 μL of 2.5 mg/L internal standards were added to 250 μL of specimen. Then, 100 μL of 1M ammonia buffer and 1.25 mL of $CHCl_3$/isopropanol (95:5) mixture were added to the specimen, which was then rotated and centrifuged for 10 minutes. The organic phase was dried down and reconstituted in a 5:1 mixture of water/acetonitrile.[6]

In comparing the quantitative results, the SPE method has a lower limit of quantitation at 2.0 ng/ml, while the LLE method had a lower limit of 10 μg/ml. Because the SPE method involves a multiple-step washing process, the specimen analyzed is theoretically cleaner than the LLE specimen.[2, 6]

2.5.1 Sample preparation of blood, serum and plasma

Musshoff et al. described a type of SPE method for plasma specimens. First, 1.96 ml of a pH 9 buffer and 40 uL of the internal standards were added to 1 ml of plasma. The sample was then extracted using the Caliper Life Sciences Rapid Trace Workstation, which involved conditioning the column, loading the sample, washing the column and air-drying the column. The sample was then eluted and collected off the column. The collected sample was dried down and reconstituted.[20]

Bouzas et al. described an LLE method for serum specimens. Protein precipitation was performed by adding 200 μL of a 4:1 mixture of methanol/zinc sulphate (0.1 M) to 100 uL of serum. The specimen was then centrifuged at 13,200 rpm for 10 minutes and the supernatant was evaporated to dryness at 40°C. The specimen was then reconstituted in 100 μL of 0.1% formic acid and analyzed.[19]

The results of sample preparation specific to blood, serum, and plasma specimens differed from the general specimen sample preparation methods discussed in the last section. In serum and plasma, the LLE method has a lower limit of quantitation that varies from 0.5 to 2.8 ng/ml. The lower limit of quantitation for the SPE method varied from 1.0 ng/ml to 10 ng/ml.[19, 20]

2.5.2 Sample preparation of urine

Most of the sample preparation methods found for specimens in urine were created for the clinical purpose of identifying drug abuse in patients. The sample preparations described were very simple LLE methods. Shakleya et al. mixed 100 μL of urine by vortexer and centrifuged the sample for five minutes to remove larger particles. Then 10 μL of the supernatant was placed into injection vials and analyzed. Researchers

found that the lower limit of quantitation was at 10 to 100 μg/ml.[11] Gustavsson et al. described a similar LLE method that involved mixing 20 μl of urine with 80μl of internal standard solution. The lower limit of quantification was much lower than the Shakleya et al. article, with a variation from 1.0 ng/ml to 126 ng/ml.[18, 21]

2.6 Chromatographic conditions

Chromatographic conditions of the LC can drastically alter the results of a drug analysis. Column and eluent selection are the most variable aspects in effecting chromatographic conditions.[5]

2.6.1 Type of column

Using the right type of column can shorten analysis time and improve chromatographic conditions. Controlling the temperature of the column assists in reproducible retention times of the drug analytes.[5] Many of the opiate methods described in the literature used Allure PFP or Phenomenex columns. Most were packed with anywhere from 3 μm to 5 μm particles, although Musshoff et al. used a Phenomenex column packed with 400 μm particles. The oven temperature ranged from 30°C to 40°C.[2, 5, 18-20] There is most likely a lack of diversity concerning column types, because the Allure PFP and Phenomenex columns give consistent and reliable results.

2.6.2 Eluents

Eluent composition used for the LC-MS method is chosen based on the mode the drugs are ionized in. The electrospray ionization (ESI) probe is more commonly used for positive mode and atmospheric pressure chemical ionization (APCI) is used more often

for negative mode, although this is not always the case. All the papers reviewed used ESI, possibly because it produced a stronger response than using APCI.[2, 5, 18-20, 22] When using the ESI mode, aqueous eluents with an additive concentration of less than 10 mM are recommended. A lower flow rate of 0.1 mL/min or less is also recommended to improve signal intensity on the LC-MS.[5]

Most of the articles reviewed used a greater flow rate than 0.1 mL/min. Lower flow rates may increase sensitivity, but they take longer to analyze on the LC. Lower flow rates also tend to have poorer chromatography compared to higher flow rates. [4] Most methods included eluents with an additive concentration of formic acid or ammonium formate at less than 10 mM, except the Gustavsson et al. method.[2, 5, 18, 19, 22] that employed an eluent additive at a concentration of 25 mM, but the higher concentration did not noticeably affect results.[18]

2.7 Findings

The LC-MS method specific to opiates is well-researched, with many studies conducted within the last fifteen years. Review of the literature has found that the use of the LC-MS has several advantages over the GC-MS for opiate methods. The sample preparation for the LC-MS is faster and uses less eluents. The LC-MS also produces more selective and sensitive results than the GC-MS. The chromatographic conditions employed in the literature did not vary much, with most methods using similar eluents and columns types. Most of these conditions are chosen specifically for the compounds being analyzed, so that would explain the lack of variation.

Most of the published literature reviewed covered a wide variety of drugs and was not specific to opiates. The methods that were specific to opiates only analyzed one or two opiate compounds. Often the papers reviewed were lacking the information needed for comparative purposes, such as linearity, limit of quantification (LOQ) and limit of detection (LOD).[1, 5-7]

While articles are available on the results of SPE and LLE sample preparation, the extraction methods seem to depend on the specimen type. There is not enough research to confidently determine which extraction method produces better results. Comparison studies are necessary to determine how different sample preparations and specimen types affect results.

CHAPTER III

METHODOLOGY

3.1 Introduction

At the OSBI in Edmond, Oklahoma, a qualitative and quantitative method for the liquid chromatograph-mass spectrometer (LC-MS) was developed for the analysis of opiates. The qualitative method was developed for the analysis of 6-acetylmorphine, codeine, hydrocodone, hydromorphone, morphine, oxycodone and oxymorphone. A quantitative method for the was developed for the analysis of codeine, hydrocodone, morphine, and oxycodone. A D&S sample preparation method for blood was developed and validated according to the protocols set by the OSBI. A SPE for the quantitative analysis of codeine, hydrocodone, morphine, and oxycodone in blood was developed for comparison to the D&S sample preparation method.

3.2 Instrumentation

All samples were analyzed with a Shimadzu LC-MS instrument. The LC-MS-8030 was equipped with the system controller CBM-20A, solvent delivery unit LC-30AD, autosampler SIL-30AC, column oven CTO-20AC, and column Kinetex 2.6u PFP 100A. The Kinetex column had the dimensions of 75mm in length and 2.1mm in

diameter. The LC-MS instrument is pictured in Figure 2.

Figure 2. Liquid Chromatography- Mass Spectrometer Used in Study Shimadzu LC-MS 8030 system in the Oklahoma State Bureau of Investigation Laboratory

3.3 Materials

Methanol (OmniSolv) and acetonitrile were both LCMS grade; formic acid was ACS grade. Ammonium acetate was obtained in solid form. UltraPure water was obtained from a Millipore Water Purification System (Synergy, Thermo Scientific). Drug standards (6-acetylmorphine, 6-acetylmorphine-D6, codeine, codeine-D6, hydrocodone, hydrocodone-D6, hydromorphone, hydromorphone-D3, morphine, morphine-D6, oxycodone, oxycodone-D6, oxymorphone and oxymorphone-D3) were purchased from Cerilliant (Cerilliant Corporation, Round Rock, TX). Listed in Table 1 are the concentrations and solvents for the drug standards as obtained from Cerilliant.

Table 1: Drug Standards Used in Study

	Concentration	Solvent
6-Acetylmorphine	1.0 mg/ml	1 ml Methanol
6-Acetylmorphine-D6 (internal standards)	1.0 mg/ml	1 ml Methanol
Codeine	1.0 mg/ml	1 ml Methanol
Codeine-D6 (internal standards)	1.0 mg/ml	1 ml Methanol
Hydrocodone	1.0 mg/ml	1 ml Methanol
Hydrocodone-D6 (internal standards)	1.0 mg/ml	1 ml Methanol
Hydromorphone	1.0 mg/ml	1 ml Methanol
Hydromorphone-D3 (internal standards)	1.0 mg/ml	1 ml Methanol
Morphine	1.0 mg/ml	1 ml Methanol
Morphine-D6 (internal standards)	1.0 mg/ml	1 ml Methanol
Oxycodone	1.0 mg/ml	1 ml Methanol
Oxycodone-D6 (internal standards)	1.0 mg/ml	1 ml Methanol
Oxymorphone	1.0 mg/ml	1 ml Methanol
Oxymorphone-D3(internal standards)	1.0 mg/ml	1 ml Methanol

3.4 Preparation of Standards

3.4.1 Dilute and Shoot sample preparation

For the qualitative analysis, a multi-drug standard containing 6-acetylmorphine, codeine, hydrocodone, hydromorphone, morphine, oxycodone, and oxymorphone was prepared at a 10 µg/ml concentration using Cerilliant drug standards diluted in acetonitrile. The 10 µg/ml qualitative multi-drug standard was made up by combining 100 µl of each 1 mg/ml opiate standard to a 10 ml volumetric and filling to the line with acetonitrile. A multi-drug internal standard containing the seven internal standards (6-acetylmorphine-D6, codeine-D6, hydrocodone-D6, hydromorphone-D3, morphine-D6, oxycodone-D6, and oxymorphone-D3) were prepared at 15 ng/ml, using Cerilliant drug standards diluted in acetonitrile. First, a 1.0 µg/ml internal standard mix was made up by

adding 10 µl of each 1.0 mg/ml internal standard to a 10 ml volumetric and filling to the line with acetonitrile. Next, 3.75 ml of the 1.0 µg/ml internal standard mix was added to a 250 ml volumetric and filling to the line with acetonitrile to make the 15 ng/ml multi-drug internal standard.

For quantitative analysis, a multi-drug standard containing codeine, hydrocodone, morphine, and oxycodone was prepared at a 10 µg/ml concentration using Cerilliant drug standards diluted in acetonitrile. The 10 µg/ml quantitative multi-drug standard was made up by combining 100 µl of each 1.0 mg/ml opiate standard to a 10 ml volumetric and filling to the line with acetonitrile. A multi-drug standard containing the internal standards of codeine-D6, hydrocodone-D6, morphine-D6, and oxycodone-D6 were prepared at 50 ng/ml and diluted in acetonitrile. First, a 10 µg/ml internal standard mix was made up by adding 100 µl of each 1.0 mg/ml internal standard to a 10 ml volumetric and filling to the line with acetonitrile. Next, 2.5 ml of the 10 µg/ml internal standard mix was added to a 500 ml volumetric and filling to the line with acetonitrile make the 50 ng/ml multi-drug internal standard.

3.4.2 Solid Phase Extraction sample preparation

For quantitative analysis, the same 10 µg/ml qualitative multi-drug standard made for the quantitative analysis for the D&S preparation method was utilized. A multi-drug standard containing the internal standards of codeine-D6, hydrocodone-D6, morphine-D6, and oxycodone-D6 were prepared at 1.0 µg/ml diluted in acetonitrile. The 1.0 µg/ml multi-drug internal standard was made up by adding 10 µl of each 1.0 mg/ml internal standard to a 10 ml volumetric and filling to the line with acetonitrile.

3.5 Sample Collection

For the validation of the qualitative and quantitative opiate method using the D&S sample preparation method, the OSBI provided drug-free whole blood and blood specimens from adjudicated criminal cases for analysis in this study. Pig blood treated with 2% w/v of sodium fluoride and 75% w/v potassium oxalate was utilized for the comparison of the SPE and D&S sample preparation methods.

3.6 Method Development on the Liquid Chromatograph-Mass Spectrometer

3.6.1 Qualitative Opiate Method

A qualitative method was developed for the analysis of seven opiates (6-acetylmorphine, codeine, hydrocodone, hydromorphone, morphine, oxycodone, and oxymorphone).

3.6.1.1 Drug Standard Optimization

Each drug standard (6-acetylmorphine, 6-acetylmorphine-D6, codeine, codeine-D6, hydrocodone, hydrocodone-D6, hydromorphone, hydromorphone-D3, morphine, morphine-D6, oxycodone, oxycodone-D6, oxymorphone and oxymorphone-D3) was optimized on the mass spectrometer (MS) for identification purposes in analysis. The mass-charge ratio (m/z) values of the precursor (Q1) ions were manually inputted into the multiple reaction monitoring (MRM) event table and optimized automatically by the MS. This allowed for multiple user-defined ion fragments to be monitored. The drug standards were analyzed separately at 1.0 µg/ml concentrations and injections of 1.0 µl. Product (Q3) ions and voltage of collision energies were manually selected for each drug standard

based on sensitivity and selectivity. Table 2 lists the MRMs selected for each drug standard.

Table 2. Multi Drug MS Parameters

Name	Q1 Mass (Da)	Q3 Mass (Da)	Q1 Pre Bias (volts)	CE (volts)	Q3 Pre Bias (volts)
6-MAM	328.30	165.00	17	46	17
	328.30	211.20	17	26	22
6-MAM-D6	334.10	165.00	17	46	17
Codeine	300.10	198.80	16	32	20
	300.10	165.15	16	43	15
Codeine-D6	306.10	218.00	16	25	22
Hydrocodone	300.10	198.85	15	30	20
	300.10	171.00	15	40	20
Hydrocodone-D6	306.10	202.05	18	35	20
Hydromorphone	286.10	157.15	15	43	16
	286.10	184.70	15	35	19
Hydromorphone-D3	289.10	184.95	16	40	15
Morphine	286.10	165.00	15	45	20
	286.10	151.00	15	45	20
Morphine-D6	292.10	153.00	16	50	25
Oxycodone	316.10	174.90	16	35	14
	316.10	212.00	16	45	21
Oxycodone-D6	322.10	304.20	20	40	42
Oxymorphone	302.10	284.15	15	21	30
	302.10	226.80	15	29	24
Oxymorphone-D3	305.00	287.15	10	20	20

3.6.1.2 Liquid Chromatograph Parameters

An aqueous mobile phase (Eluent A: 1% formic acid 5 mM ammonium acetate in deionized water), as well as an organic mobile phase (Eluent B: 1% formic acid 5 mM ammonium acetate in 50:50 Methanol:Acetonitrile) were used to carry the sample through the column at an oven temperature of 30°C. The organic mobile phase (Eluent B) was increased as a gradient from 5% to 95% over the first 3.25 minutes of the run and

then returned to 5% for the remainder of the 4.25 minute run. The sample was injected at a volume of 20 µl with a total flow rate of 0.6 ml/min.

3.6.2 Quantitative Opiate Method

The same liquid chromatograph and mass spectrograph parameters used for the qualitative method was used for the quantitative method, except the method only included the MRM events for codeine, hydrocodone, morphine, oxycodone, and their respective internal standards.

3.7 Preparation of Samples

3.7.1 Qualitative Samples

The qualitative samples were fortified at a concentration 10 ng/ml, the proposed limit of detection. These samples were made by adding 50 µl of the 10 µg/ml of the multi-drug standard (containing all seven opiates) to 5 ml of blood.

3.7.2 Quantitative Samples

There were seven point calibration curve with concentrations at: 1000, 500, 250, 100, 50, 25, and 10 ng/ml. There was a high quality control (QC) at 400 ng/ml, a medium QC at 200 ng/ml and a low QC at 100 ng/ml concentrations. A 1000 ng/ml working blood solution was made up daily for the purpose of generating the calibration curve. The working blood solution was made by combining 200 µl o the 10 µg/ml quantitative multi-drug standard to 1.8 ml of blank blood. Table 3 lists how each calibration level was made up. The QCs were made up separately from the calibration curve and were used throughout the method validation process. The high QC at 400 ng/ml was created by mixing 1.0 ml of the 10 µg/ml quantitative multi-drug standard to25 ml of blood. The

medium QC at 200 ng/ml was created by mixing 0.5 ml of the 10 µg/ml quantitative multi-drug standard to25 ml of blood. The low QC at 100 ng/ml was created by mixing 250 µl of the 10 µg/ml quantitative multi-drug standard to 25 ml of blood.

Table 3. Calibration Curve

Calibration Level	Working Blood Solution (µl)	Blank Blood (µl)	Final Concentration (ng/ml)
1	500	-	1000
2	250	250	500
3	125	375	250
4	50	450	100
5	50	950	50
6	25	975	25
7	10	990	10

3.7.3 Dilute and shoot sample preparation

Samples were extracted using a D&S sample preparation method as described by Dahn et al. A 1.0 ml aliquot of cold acetonitrile, spiked at a 50 ng/ml concentration for each internal standard (6-acetylmorphine-D6, codeine-D6, hydrocodone-D6, hydromorphone-D3, morphine-D6, oxycodone-D6 and oxymorphone-D3), was added to test tubes containing 0.5 ml of blood. Test tubes were capped, vortex mixed for 20 seconds, and centrifuged for 10 minutes at 2550 x G (Sorvall T3 centrifuge, Thermo Scientific). Approximately 200 µl of the organic phase was transferred to a clean tube and was evaporated to dryness under a steady stream of nitrogen. The sample was reconstituted in 100 µl of UltraPure water, briefly vortex mixed and transferred to an autosampler vial.[8][8]

3.7.4 Solid Phase Extraction

The SPE was adapted to the method developed from the SPEware Corporation (Baldwin, Ca) protocol titled "Extraction of Opiates from Whole Blood: For GC or GC/MS Confirmations". The protocol called for Trace-B35 mg, TB-335 extraction columns. Approximately 1.0 ml of 100 mM phosphate buffer, at pH=6.0, was added to a 0.5 ml whole blood sample in a test tube. The sample was vortex mixed for 30 seconds, sonicated for 15 minutes, and then centrifuged for 5 minutes at 3000 rpm. The supernatant was drawn through the column at a flow of 2-5 ml/minute. The column was then washed with 1.0 ml of deionized water, 100 mM of hydrochloric acid, 1.0 ml of methanol, and 1.0 ml of ethyl acetate. The column was then dried for 2 minutes at 25 psig. In a clean test tube, the eluate was collected by using 0.8 ml of ethyl acetate containing 2% ammonium hydroxide at 1.0 ml/minute. The eluate was evaporated to dryness with a steady stream of nitrogen at 40°C. The sample was reconstituted in 100 µl of UltraPure water, briefly vortex mixed and transferred to an autosampler vial.

3.8 Method validation

3.8.1 Validation of the Qualitative Method

Validation of the qualitative method utilizing the D&S sample preparation method followed the method validation plan for qualitative drug analysis by LC-MS as proposed by the OSBI. The LC-MS parameters remained the same for the quantitative method, so the data from the matrix effect study from the qualitative method was utilized.

3.8.1.1 Selectivity and Sensitivity

Selectivity of a method is determined by evaluating the extent of interference caused by endogenous and exogenous compounds. To evaluate the interference of endogenous compounds multiple sources of blank matrix were analyzed. Twenty sources of blank matrix (old case samples that were to be destroyed) were obtained. One aliquot from each blank matrix was analyzed without the addition of any drug standard and one aliquot from each blank matrix was spiked at the LOD (10 ng/ml), but without the internal standard. The absolute peak area for each of the twenty blanks was compared to the mean peak areas of the twenty LOD samples. The method was considered free from matrix interference if the peak area in each blank is less than 20% of the mean LOD peak area.

To evaluate the interference of exogenous compounds, the possibility of carryover must be determined. A blank matrix sample spiked to a high concentration (approximately the high therapeutic concentration) and a blank matrix sample were prepared and analyzed one after the other. The method was deemed free from carryover if the analyte peak area of the blank matrix sample is less than one-half of the mean LOD peak area.

3.8.1.2 Limit of Detection

The LOD is the lowest amount of a drug analyte that can be differentiated from any noise present in a blank matrix. The LOD may be the lowest amount that can be detected at a level greater than or equal to 3 times the noise in a blank matrix or it may be administratively set. If administratively set, the LOD must be determined over a course of multiple days, and may be chosen as the lowest calibrator in a quantitative method.

The LOD may also be calculated by using data generated from multiple runs of linear calibration models. The standard deviation of the y-intercepts from each calibration model and the mean average of the slopes from each calibration model are first determined and then used in the following equation:

$$LOD = (3.3 \times SD_{Yint}) / mean$$

For this method, the LOD concentration for the qualitative method was administratively set at 10 ng/ml. The LOD was evaluated in at least five different runs. In each run, ten replicate aliquots were spiked at 10 ng/ml of the multi-drug standard and 15 ng/ml of the multi-drug internal standard, followed by sample preparation and analysis on the LC-MS. In order for the LOD to be accepted, the mean signal for each run must be greater than five times the mean blood blank signals that were determined in the selectivity study. [23]

3.8.1.3 Matrix Effects

Ion suppression or ion enhancement may occur in LC-MS methods due to co-eluting compounds. Two concentrations were evaluated for matrix effects; one concentration is at two times the LOD (20 ng/ml) and the other is ten times the LOD (100 ng/ml). Set 1 consists of the injection solvent (1% formic acid 5 mM ammonium acetate in deionized water) spiked at the appropriate concentration and analyzed a minimum of six times each. Set 2 consists of samples from twenty sources which are spiked to the appropriate concentration, extracted and then analyzed. The responses within each set were averaged. The coefficient of variation (CV) was calculated to evaluate the

variability of peak area for each concentration. The coefficient of variation should be less than 15% at each concentration.

3.8.1.4 Co-Administered Drugs

To evaluate whether other drugs reasonably expected to be present in a sample will cause interference with the opiates analyzed, high concentrations of other drugs were analyzed. Each opiate was analyzed at a concentration of 1 ug/ml, which far exceeds the expected concentration of an authentic sample. A 1 ug/ml benzodiazepine solution was analyzed, containing the following benzodiazepines: zolpidem, alprazolam, diazepam, nordiazepam, flurazepam, temazepam, oxazepam, flunitrazepam, midazolam, lorazepam and clonazepam. Another solution containing 1 ug/ml of drugs from various drug classes was analyzed as well. The solution contained: methamphetamine, 3,4-MDMA, acetaminophen, ibuprofen, diphenhydramine, delta-9-THC, 9-carboxy-11-nor-delta-9-THC and caffeine. The responses observed must be less than 20% of the mean LOD signal from the selectivity study for an acceptable performance in light of co-administered drugs.

3.8.2 Validation of Quantitative Method

Validation of the opiate sample preparation method for the analysis of morphine, codeine, oxycodone, and hydrocodone followed the suggested protocol for validating quantitative methods at the OSBI. The data generated from the selectivity and sensitivity, limit of detection, and matrix effects was included in validating the quantitative method. Additional analyses were conducted to prove the specificity and stability of the calibration curve.

3.8.2.1 Accuracy

Five consecutive runs were conducted; each run included the sample preparation of the seven point calibration curve and two quality controls, followed by analysis on the LC-MS. The % error was calculated to evaluate the closeness of the calibrator (1000, 500, 250, 100, 50, 25, and 10 ng/ml), high QC (400 ng/ml), and low QC (100 ng/ml) to the true value. The method was considered to be sufficiently accurate if the ratios were within 20%.

3.8.2.2 Limit of Quantitation

The LOQ is the lowest amount of a drug analyte that can be accurately measured and is typically set at the lowest calibrator. The LOQ may be the lowest amount that can be quantitated at a level greater than or equal to 10 times the noise in a blank matrix. The LOQ may also be calculated by using data generated from multiple runs of linear calibration models. The standard deviation of the y-intercepts from each calibration model and the mean average of the slopes from each calibration model are first determined and then used in the following equation:

$$LOQ = (10 \times SD_{Yint}) / \text{mean}$$

While the LOQ was administratively set as the same concentration as the LOD at 10 ng/ml, data from the accuracy study was utilized to calculate the LOQ.

3.8.2.3 Precision

Precision was evaluated to measure the repeatability of several measurements of the same sample. The high (400 ng/ml), medium (100 ng/ml), and low (20 ng/ml) quality control were analyzed in replicates of six over the course of five runs. Using the

calculated means, the within-run precision and between-run precision was calculated for each opiate analyte at each concentration. The method was considered to be sufficiently precise if the within-run and between-run percentages were within 20%.

3.8.2.4 Benchtop Stability

Benchtop stability was evaluated to determine whether analytes degrade while sitting in unrefrigerated conditions during sample preparation. Six replicates of both high and low quality controls were prepared and analyzed quickly. Meanwhile, sufficient volumes of both the high and low quality controls were left on the countertop for 4 hours. After the four hours, 6 replicates of both high and low quality controls were prepared and analyzed. The acceptable ratio of mean responses of the room temperature and samples to the refrigerated samples was between 85 and 115%.

3.8.2.5 Processed Sample Stability

Processed sample stability was evaluated to determine the stability of processed samples as they sit in the autosampler tray while awaiting analysis on the LC-MS. Ten replicates of both the high and low quality controls were prepared, the remaining reconstitutes were pooled together at the end (high and low quality controls still separate). The pooled extracts were then divided into nine autosampler vials and placed into the autosampler tray. Over a 24 hour period, these samples were evaluated in triplicates, meaning three injections per vial, at three hour increments (0, 3, 6, 9, 12, 15, 18, 21 and 24 hours). The data was plotted as the ratio of the analyte area to internal standard area versus time. Slopes at zero were desired, but values between -0.01 to 0.01 indicated processed sample stability up to 24 hours.

3.9 Comparison of Sample Preparation Results

For both the D&S and SPE sample preparation method, six consecutive runs were conducted with each run including the seven point calibration curve and a blank, followed by quantitative analysis on the LC-MS. The ratio of the averages of the drug standards to the internal standards was calculated. The accuracy and precision of both sample preparation methods were compared.

3.10 Statistical Analysis

All statistical analyses were performed using Microsoft Excel 2007 and GraphPad Prism®.

CHAPTER IV

RESULTS

4.1 Method development

Before the Dahn et al. sample preparation method was considered for validation at the OSBI, other sample preparation methods were attempted and abandoned due to poor area counts or poor protein cleanup. The same column (Kinetex 2.6u PFP 100A) was utilized. The eluents were 5 mM ammonium acetate and 50:50 methanol: acetonitrile.

4.1.1 Dilute and shoot sample preparation

A simple D&S sample preparation was first attempted. A 3.0 ml solution of a 49:49:2 hexane: ethyl acetate: ammonium hydroxide was added to a 1.0 ml of blood. The sample was vortex mixed for 10 minutes and centrifuged for 5 minutes. The supernatant was evaporated under a steady stream of nitrogen. The sample was reconstituted in a 20 µl of methanol and 80 µl of the running buffer (5 mM ammonium acetate), vortex mixed and transferred to an injection vial.

The chromatogram of the first sample prepared with the D&S method is seen in Figure 3.

The injection amount, the amount the sample was reconstituted in (from 100 µl to 50 µl), dwell scanning times, and the time-program on the LC-MS were modified in attempt to get better results. Figure 4 displays the chromatogram of a D&S prepared sample, after the changes.

When run in replicates, there were issues with the column clogging, retention times shifting when not all of drug analytes moved off the column. Figure 5 displays a chromatogram of the fifth 10 ng/ml sample replicate in a run of ten replicates. After changes to the time program, the replicates were reanalyzed but the column clogged (Figure 6) before all ten samples were analyzed. The area counts of the first sample analyzed and the fifth sample analyzed are listed in Table 4.

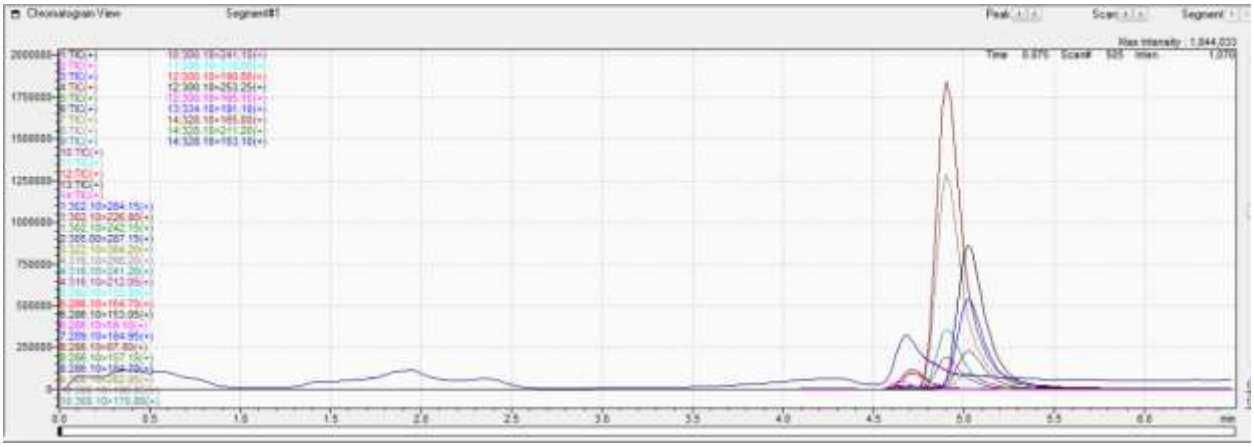

Figure 3. 500 ng/ml dilute and shoot chromatogram

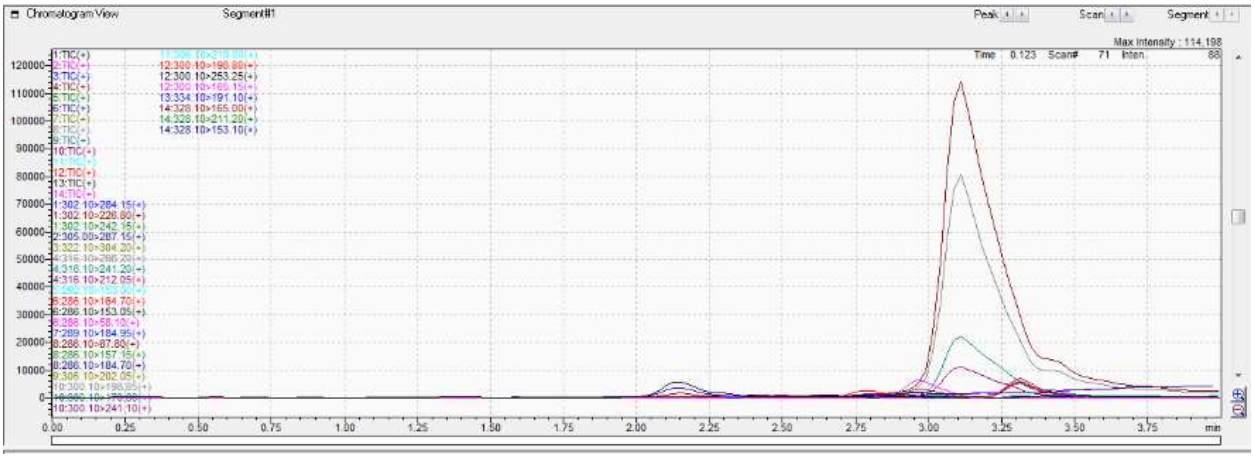

Figure 4. 10 ng/ml dilute and shoot sample chromatogram after method modification

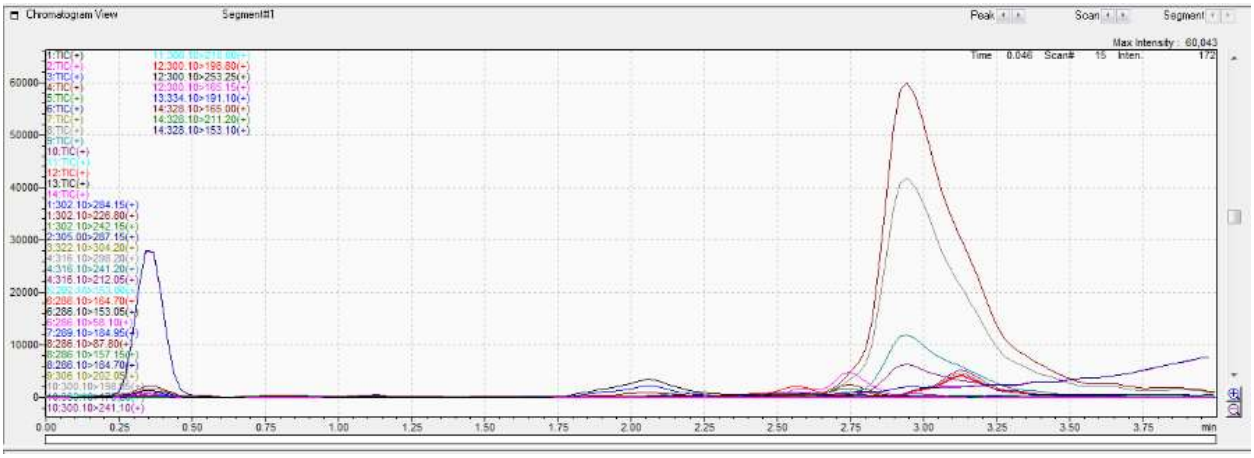

Figure 5. 10 ng/ml dilute and shoot sample chromatogram, fifth replicate analyzed

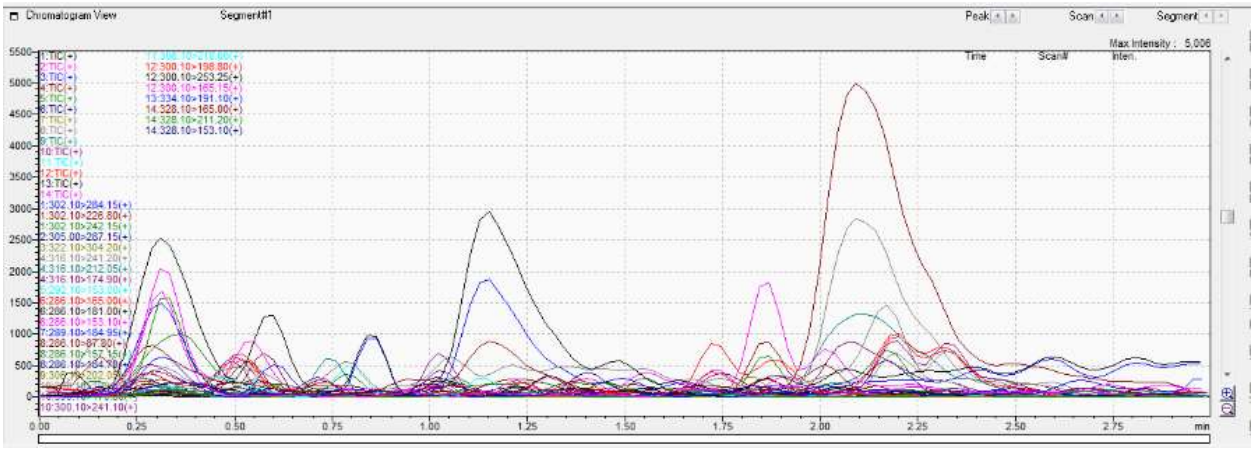

Figure 6. 10 ng/ml dilute and shoot sample chromatogram, fifth replicate reanalyzed

Table 4. Dilute and shoot replicate areas

Drugs	Rep 1 Area	Rep 5 Area
Morphine	3147	505
Oxymorphone	27714	113
Hydromorphone	260	-
Codeine	8806	142
Oxycodone	384205	423
6-MAM	18084	852
Hydrocodone	31642	53346

4.1.2 Application of syringe and centrifugal filters

Syringe and centrifugal filters were applied to the D&S method in an attempt to clean up the samples and prevent clogging. A syringe filter was used to filter the supernatant after vortex mixing and centrifugation. The filtered sample was then evaporated under a steady stream of nitogren. Figure 7 displays a chromatogram of the 10 ng/ml sample. A 0.2 µm nylon membrane centrifugal filter was used to clean up supernatant after vortex mixing and centrifugation. The filtered sample was then evaporated under a steady stream of nitrogen. Figure 8 displays a chomratogram of the 10 ng/ml sample. A 0.2 µm nylon membrane centrifugal filter was used to clean up the sample after the dry-down with nitrogen and reconstitution. The sample was then transferred to an injection vial. Figure 9 displays a chromatogram of the 10 ng/ml sample. All attempts lacked the sensitivity desired, so a different method was employed to determined if it produced more sensitive results.

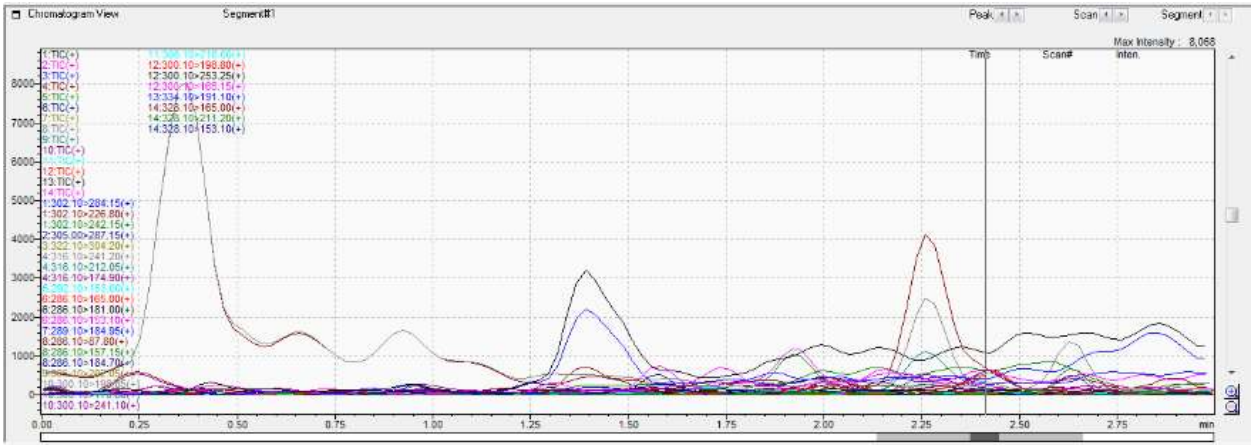

Figure 7. Chromatogram of a sample prepared with syringe filter

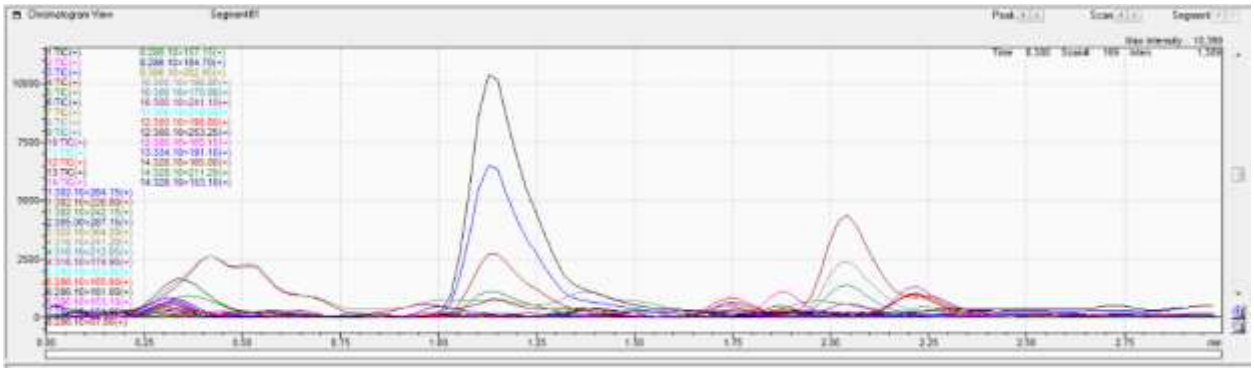

Figure 8.Chromatogram of a sample prepared with a centrifugal filter before dry-down with nitrogen

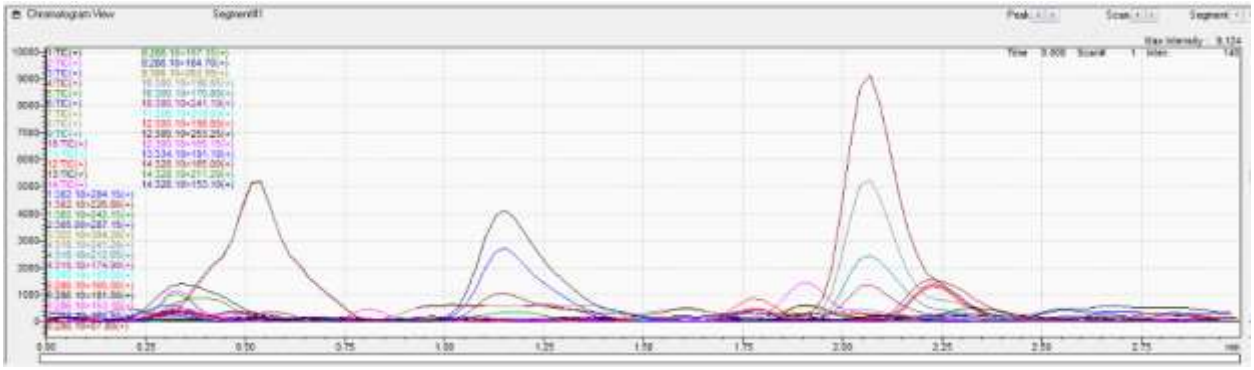

Figure 9. Chromatogram of a sample prepared with a centrifugal filter after dry-down with nitrogen and reconstitution

4.1.3 Bouzas et al. dilute and shoot sample preparation

A D&S method was adapted from Bouzas et al. A 200 µl 4:1 methanol: zinc sulphate (0.1 M) solution was added to a 100 µl blood sample. The sample was vortex mixed and centrifuged for 10 minutes. The supernatant was then evaporated under a steady stream of nitrogen and resuspended in 100 µl of the running buffer (5 mM ammonium acetate).[19] Ten microliters was injected onto the LC-MS, this amount was later increased to 50 µl. The sample volume was also increased to 0.5 ml and the 4:1 solution was increased to 1.0 ml. The peaks signals were too low and blended in with the background noise (Figure 10).

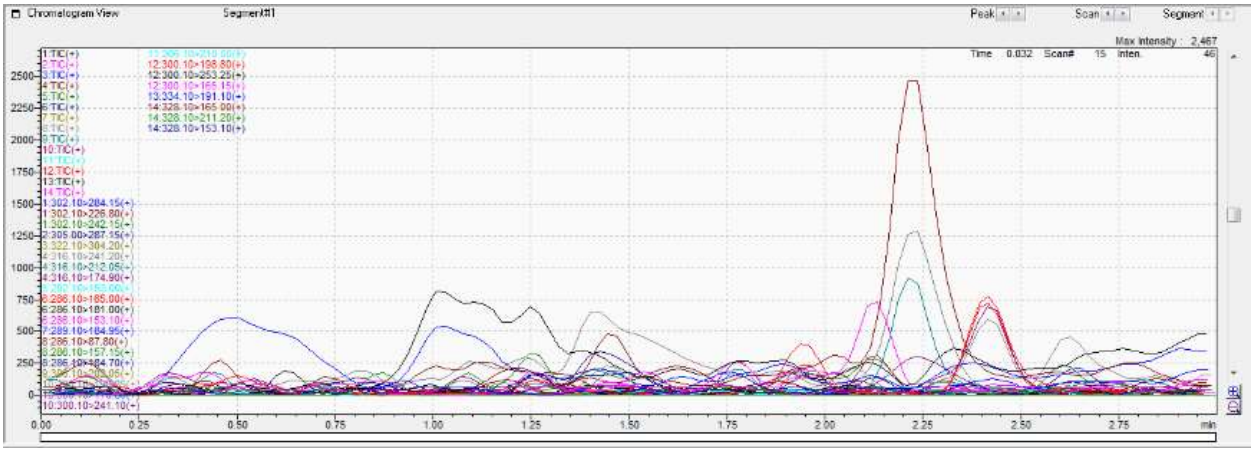

Figure 10. Chromatogram of a 10 ng/ml sample prepared with Bouzas et al. protocol

4.1.4 In-house gas chromatography-mass spectrometry sample preparation

An in-house (OSBI) opiate sample preparation protocol for analysis on the gas chromatography-mass spectrometry (GC-MS) produced promising results. Whole blood sample was diluted with 4.0 ml of deionized water and vortex mixed. The blood matrix was transferred to an Ultra-15 ultra-filtration tube and centrifuged for 15 minutes at 2550G. The ultrafiltrate sample was transferred to a ToxiLab-A tube and rotated for 20

minutes and centrifuged for 10 minutes. The supernatant was then evaporated under a steady stream of nitrogen and resuspended in 50 µl of the running buffer. A chromatogram of a 10 ng/ml sample is seen in Figure 11. The area counts for a 10 ng/ml sample are seen in Table 5.

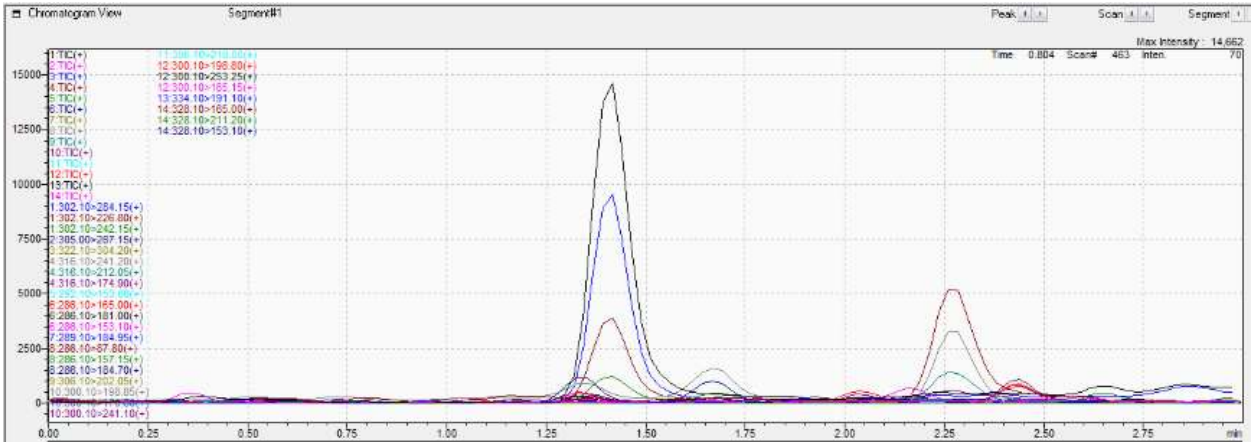

Figure 11. Chromatogram of a 10 ng/ml sample prepared using the GC-MS opiate protocol

Table 5. In-house GC-MS method

Drug	Area
Morphine	3456
Oxymorphone	64254
Hydromorphone	7382
Codeine	2074
Oxycodone	-
6-MAM	2537
Hydrocodone	-

4.1.5 Dahn et al. sample preparation

The Dahn et al. sample preparation method was the method selected for the analysis of opiates and was approved to undergo a method validation study. There was a time

program change and an eluent change to 0.1% formic acid, 5 mM ammonium acetate in deionized water and 5 mM ammonium acetate in 50:50 methanol: acetonitrile.

4.2 Qualitative Analysis Method Validation

4.2.1 Selectivity and Sensitivity

Twenty sources of blank matrix were prepared using the D&S sample preparation method. All sources showed no response for any of the seven opiates analyzed in the method. The same twenty sources were then spiked to 10 ng/ml concentrations for all seven opiates, prepared by the D&S method and analyzed by LC-MS. The mean peak areas for each opiate in the spiked sources are listed in Table 6.

Table 6. Mean Peak areas for spiked sources

Drug Analyte	Mean Peak Area of Blank Samples	Mean Peak Area of Spiked Samples
Morphine	No response	6321
Oxymorphone	No response	45822
Hydromorphone	No response	19245
Codeine	No response	8574
Oxycodone	No response	16169
6-MAM	No response	14887
Hydrocodone	No response	33169

To evaluate carryover, a blank matrix was analyzed immediately following the analysis of the high concentration sample (1000 ng/ml). No response was seen in the matrix blank.

4.2.2 Limit of Detection

Five separate analytical runs consisting of ten replicates each were analyzed. The acceptance criteria was set such that the mean peak area for each run had to be greater than five times the mean blood blank signals from the selectivity study. The mean peak area, quantitation ratios for each run is seen in Table 7. Table 8. Comparison of LOD peak area averages and blank sources lists the peak area averages of each compound for the entire LOD study and responses of each compound for the blank sources in the selectivity study.

Table 7. Mean peak areas, quantitation ratios of ten 10ng/ml replicates

	Day 1		Day 2		Day 3		Day 4		Day 5			
	Mean Area	QR	Mean Area	QR	Mean Area	QR	Mean Area	QR	Mean Area	QR	AVG	STDEV
Morphine	3770	0.44	3885	0.56	5827	0.59	4145	0.59	1688	0.60	3863	0.07
Oxymorphone	28282	0.50	27794	0.60	39003	0.59	28521	0.59	15083	0.62	27737	0.05
Hydromorphone	12196	0.45	12632	0.54	16942	0.58	12610	0.54	5844	0.63	12044	0.07
Codeine	5379	0.39	5669	0.47	7660	0.47	5530	0.47	2351	0.47	5318	0.04
Oxycodone	9306	0.27	9377	0.20	14258	0.22	10182	0.21	4497	0.18	9524	0.03
6-MAM	8591	0.34	9082	0.40	11721	0.42	9363	0.40	3903	0.45	8532	0.04
Hydrocodone	20894	0.46	20522	0.52	28839	0.55	21310	0.53	8083	0.57	19930	0.04

Table 8. Comparison of LOD peak area averages and blank sources

	Blank sources from Selectivity Study	LOD Peak Area Average
Morphine	No response	3863
Oxymorphone	No response	27737
Hydromorphone	No response	12044
Codeine	No response	5318
Oxycodone	No response	9524
6-MAM	No response	8532
Hydrocodone	No response	19930

4.2.3 Matrix Effects

The CV percentages were calculated to evaluate the variability of peak area for each concentration (20 ng/ml and 100 ng/ml), and are listed in Table 9.

Table 9. CV percentages for matrix effects

	20 ng/ml		100 ng/ml	
Drug	% CV Set 1	% CV Set 2	% CV Set 1	% CV Set 2
Morphine	4.9	8.8	1.9	4.3
Oxymorphone	8.0	8.7	3.1	13.2
Hydromorphone	4.0	10.7	8.4	5.0
Codeine	5.2	14.9	1.4	9.2
Oxycodone	5.2	11.0	2.2	10.6
6-MAM	5.0	7.7	3.1	5.1
Hydrocodone	4.9	7.8	3.8	5.0

4.2.4 Co-Administered Drugs

The signal response for the compounds analyzed in the co-administered drug study is listed in Table 10 and the calculated percentage of the responses to the LOD peak areas are listed in Table 11.

Table 10. Co-Administered Drugs Area Response

	Reported Peak Areas						
Solution Analyzed	Morphine	Oxymorphone	Hydro-morphone	Codeine	Oxycodone	6-MAM	Hydrocodone
Morphine	595238	0	0	0	0	0	0
Oxymorphone	0	4067786	0	0	0	0	0
Hydromorphone	0	0	1758837	0	0	0	0
Codeine	305	0	66	618726	1317	2144	550
Oxycodone	0	1809	0	0	587584	0	1033
6-MAM	1287	0	0	0	0	800195	0
Hydrocodone	0	0	0	0	0	0	2238311
Benzo mix	0	0	0	0	1073	0	0
Drug mix	0	0	0	0	2457	0	0

Table 11. Percentage of response to LOD signal

	Percentage of response to LOD signal						
Solution Analyzed	Morphine	Oxymorphone	Hydromorphone	Codeine	Oxycodone	6-MAM	Hydrocodone
Morphine	9417						
Oxymorphone		8877					
Hydromorphone			9139				
Codeine	5			216	8	14	2
Oxycodone		4			3634		3
6-MAM	20					5375	
Hydrocodone							6748
Benzo mix					7		
Drug mix					15		

4.3 Quantitative Analysis Method Validation

4.3.1 Accuracy

Accuracy of the seven-point calibrator (1000, 500, 250, 100, 50, 25, and 10 ng/ml), high QC (400 ng/ml), and low QC (100 ng/ml) of each of the four drugs were examined and the results are shown in Table 12. Accuracy for the opiate method was sufficient in that the calculated calibrator values for morphine, codeine, oxycodone, and hydrocodone were all within 10% of their known values.

Table 12. Accuracy of calibration curve

Drug	% Error								
	1000 ng/ml	500 ng/ml	250 ng/ml	100 ng/ml	50 ng/ml	25 ng/ml	10 ng/ml	High QC	Low QC
Morphine	4.2	2.9	3.0	0.6	3.6	2.9	1.7	8.8	3.3
Codeine	8.5	0.3	1.8	3.9	4.9	3.9	2.8	6.4	5.3
Oxycodone	8.2	0.0	0.1	3.4	4.2	3.0	2.3	5.0	5.4
Hydrocodone	10.5	1.9	1.0	1.1	5.2	3.9	2.7	0.0	5.7

4.3.2 Limit of Quantitation

For the purposes of opiate analysis in the OSBI laboratory, the LOQ and lowest calibrator was set at 10 ng/ml. This calibrator displayed a high degree of accuracy and was considered sufficient for reliable concentration analysis. While the LOQ was administratively set, further analysis of data from the accuracy study was utilized to calculate actual LOQs of the opiates. The calculated LOQs, listed in Table 13, were well below 10 ng/ml.

Table 13.Weighting and calculated LOQ

Drug	Weighting	LOQ (ng/ml)
Morphine	1/x^2	0.0126
Codeine	1/x^2	0.0135
Oxycodone	1/x^2	0.0117
Hydrocodone	1/x^2	0.0120

4.3.3 Precision

The percentages for the within-run precision are listed in Table 14. The percentages for the between-run precision are listed in Table 15. The within-run precision

was below 17% for all the opiates at each quality control level. The between-run precision was below 12% for all the opiates at each quality control level.

Table 14. Within-run precision

		Within-run precision (%)				
Drug		Run 1	Run 2	Run 3	Run 4	Run 5
Morphine	High (400 ng/ml)	6.9	7.7	3.3	1.9	14.0
	Mid (200 ng/ml)	7.6	5.7	5.9	3.3	4.8
	Low (100 ng/ml)	17.3	7.4	9.1	3.2	3.0
Codeine	High (400 ng/ml)	6.9	7.2	1.7	2.2	15.8
	Mid (200 ng/ml)	4.7	8.9	7.0	3.0	2.1
	Low (100 ng/ml)	2.8	8.6	6.7	2.1	1.7
Oxycodone	High (400 ng/ml)	9.2	8.8	1.9	3.6	10.7
	Mid (200 ng/ml)	10.9	7.5	6.9	3.2	3.7
	Low (100 ng/ml)	5.4	4.3	11.2	3.5	4.6
Hydrocodone	High (400 ng/ml)	8.8	9.0	2.2	3.0	14.2
	Mid (200 ng/ml)	7.0	5.3	4.2	2.2	2.1
	Low (100 ng/ml)	4.5	9.2	8.9	3.2	2.1

Table 15. Between-run precision

Drug		Between-run precision (%)
Morphine	High (400 ng/ml)	11.4
	Mid (200 ng/ml)	6.0
	Low (100 ng/ml)	9.7
Codeine	High (400 ng/ml)	9.9
	Mid (200 ng/ml)	6.3
	Low (100 ng/ml)	6.4
Oxycodone	High (400 ng/ml)	9.9
	Mid (200 ng/ml)	7.5
	Low (100 ng/ml)	7.1
Hydrocodone	High (400 ng/ml)	8.8
	Mid (200 ng/ml)	6.6
	Low (100 ng/ml)	7.9

4.3.4 Benchtop Stability

The ratio percentages for the high and low quality controls are listed in Table 16.

Table 16. Benchtop stability

	Morphine	Codeine	Hydrocodone	Oxycodone
High QC (400 ng/ml)	106.1%	103.6%	103.0%	101.8%
Low QC (100 ng/ml)	108.4 %	85.8%	104.8%	90.1%

4.3.5 Processed Sample Stability

Over a 24 hour period, already prepared high and low quality controls were analyzed on the LC-MS. The data was plotted as the ratio of the analyte area to internal standard area versus time (Figure 12 and Figure 13). The trend line and calculated hours before a 20% signal decrease of the quantitation ratios for the low and high QC are seen in Table 17and Table 18.

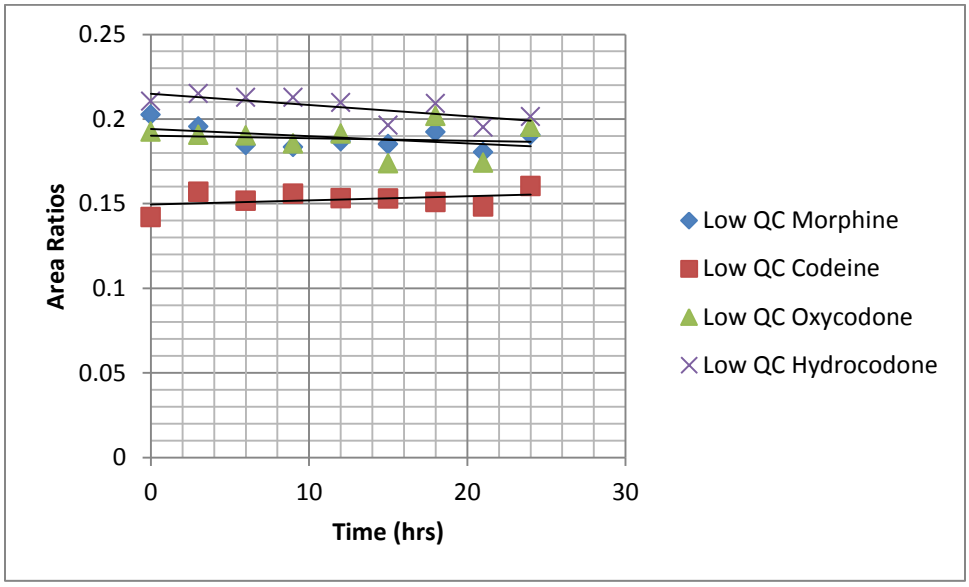

Figure 12. Processed sample stability of low quality control

Table 17. Trend line of low quality control

Drug Analyte	Trend line equation	Hrs at 20% signal decrease
Morphine	y = -0.0004x + 0.1941	1515
Codeine	y = 0.0002x + 0.1496	3252
Oxycodone	y = -0.0002x + 0.1902	3049
Hyrdrocodone	y = -0.0007x + 0.2149	836

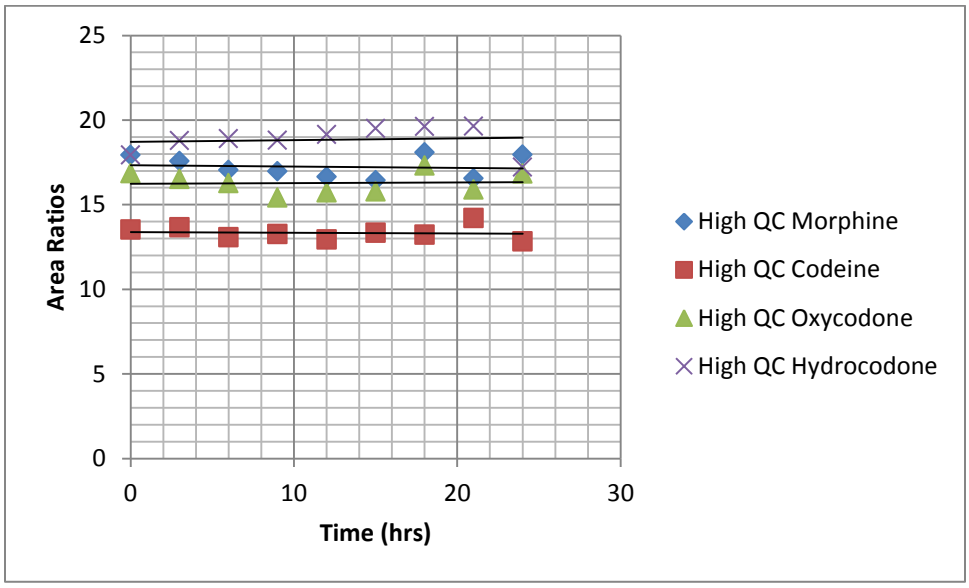

Figure 13. Processed sample stability of high quality control

Table 18. Trend line of high quality control

Drug Analyte	Trend line equation	Hrs at 20% signal decrease
Morphine	y = -0.0083x + 17.336	1992
Codeine	y = -0.0045x + 13.385	2797
Oxycodone	y = 0.0034x + 16.242	4542
Hyrdrocodone	y = 0.0091x + 18.712	1791

4.4Comparison of Sample Preparation Methods

4.4.1 Accuracy

4.4.1.2 D&S sample preparation

Accuracy of the 1000, 500, 250, 100, 50, 25, and 10 ng/ml calibrator of each of the four drugs was examined and the results are shown in. Table 19

Table 19. Accuracy: Dilute and shoot sample preparation

Drug	%Error						
	1000 ng/ml	500 ng/ml	250 ng/ml	100 ng/ml	50 ng/ml	25 ng/ml	10 ng/ml
Morphine	6.3	3.8	0.1	1.5	2.5	3.2	0.6
Codeine	3.2	4.6	1.7	1.9	7.6	1.5	14.0
Oxycodone	2.1	4.3	1.9	2.8	5.0	6.0	9.5
Hydrocodone	5.4	6.0	8.4	5.2	0.2	3.6	10.7

4.4.1.2 SPE sample preparation

Accuracy of the 1000, 500, 250, 100, 50, 25, and 10 ng/ml calibrator of each of the four drugs was examined and the results are shown in Table 20.

Table 20. Accuracy: Solid Phase Extraction preparation

Drug	%Error						
	1000 ng/ml	500 ng/ml	250 ng/ml	100 ng/ml	50 ng/ml	25 ng/ml	10 ng/ml
Morphine	0.0	1.0	1.5	3.6	9.9	2.5	9.4
Codeine	1.5	2.1	2.2	2.7	8.7	1.4	1.9
Oxycodone	12.9	16.2	9.0	5.1	30.1	14.3	11.5
Hydrocodone	9.5	15.5	12.2	3.6	33.6	10.2	10.3

4.4.2 Sensitivity

The LOD and LOQ for both the D&S and SPE sample preparation methods are seen in Table 21. A comparison of both sample preparation methods are seen in Figure 14, Figure 15, Figure 16, and Figure 17.

Table 21. Weighting, LOD and LOQ for D&S and SPE sample preparation

	Dilute and Shoot			Solid Phase Extraction		
Drug	Weighting	LOD (ng/ml)	LOQ (ng/ml)	Weighting	LOD (ng/ml)	LOQ (ng/ml)
Morphine	1/x^2	0.0073	0.0221	1/x	0.0022	0.0067
Codeine	1/x^2	0.0101	0.0305	1/x	0.0051	0.0154
Oxycodone	1/x^2	0.0010	0.0300	1/x^2	0.0044	0.0132
Hydrocodone	1/x^2	0.0072	0.0217	1/x^2	0.0046	0.0140

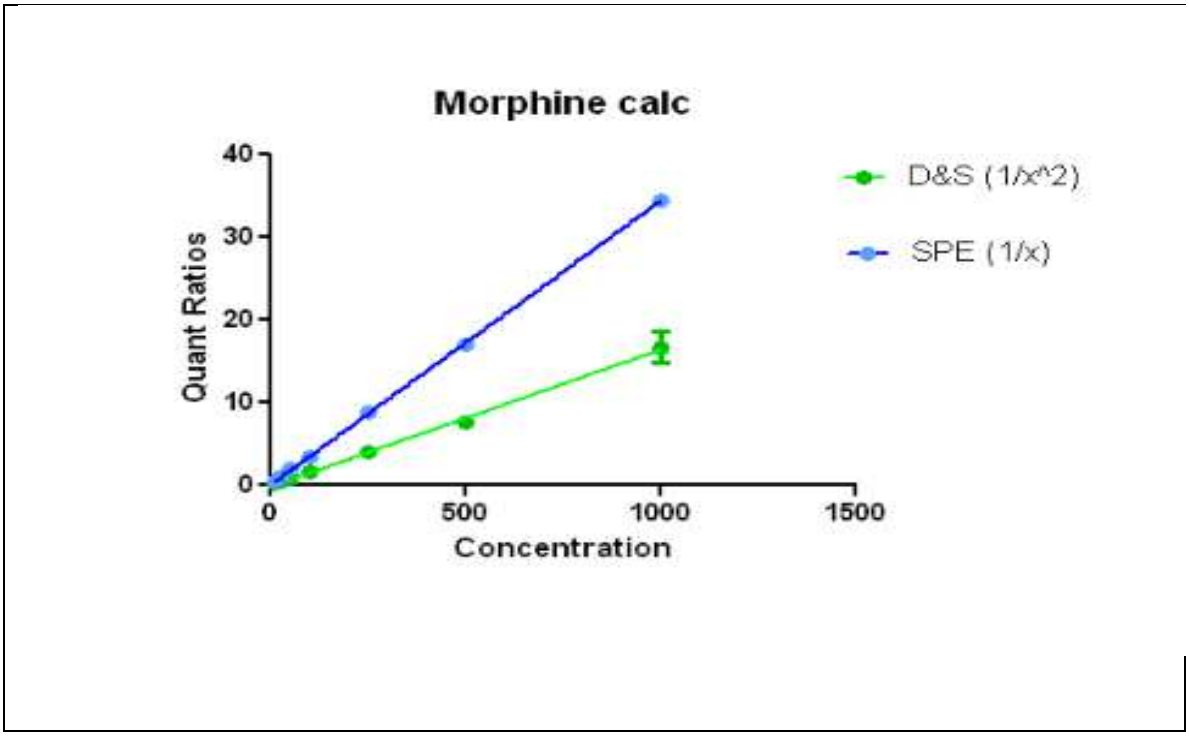

Figure 14. Sample preparation comparison graph for morphine

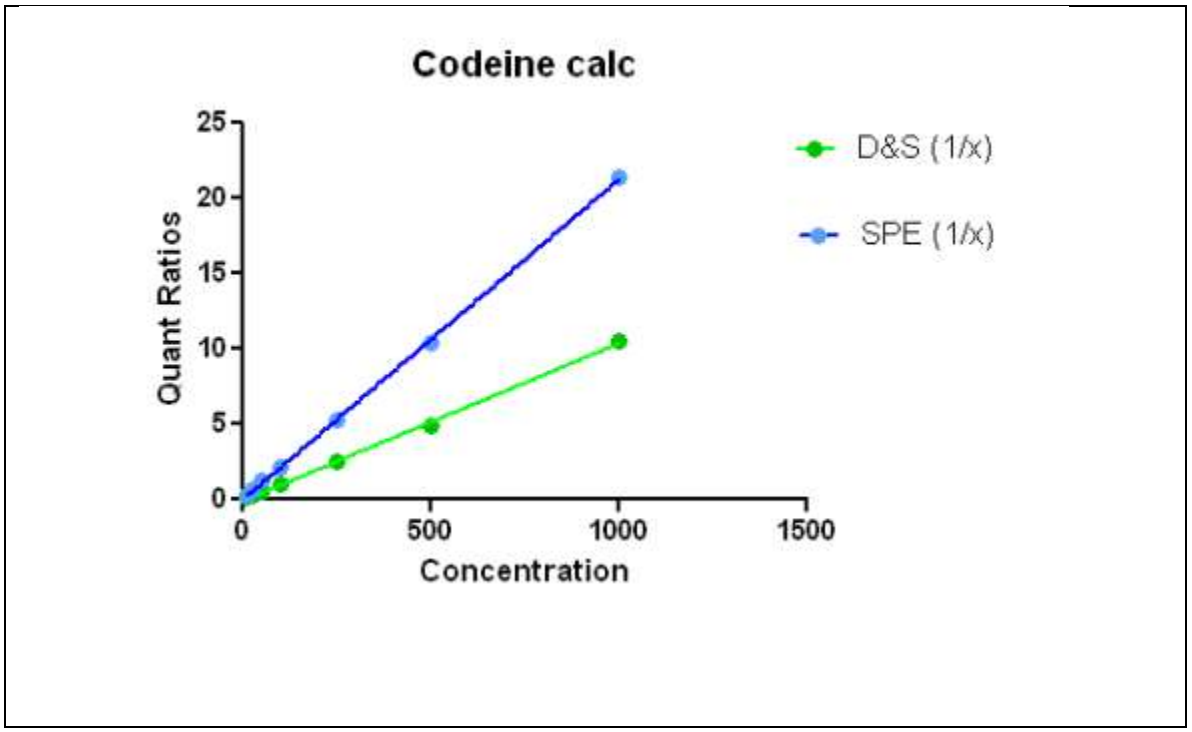

Figure 15. Sample preparation comparison graph for codeine

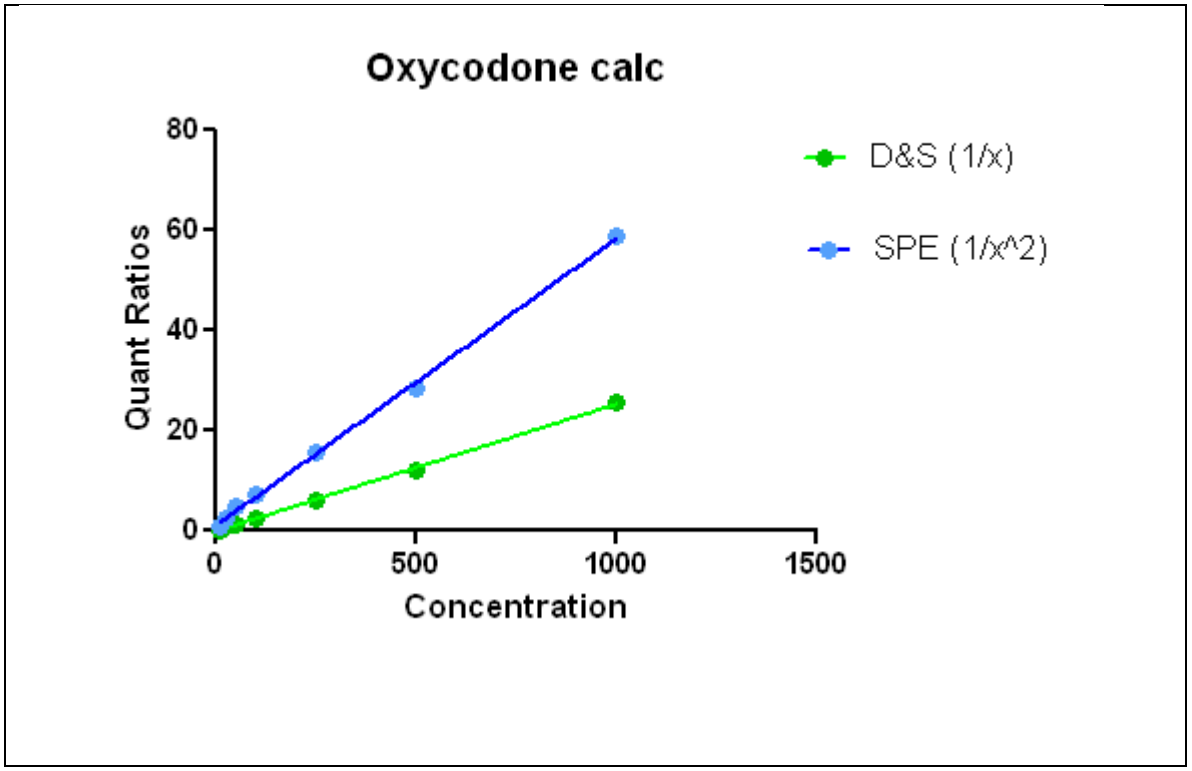

Figure 16. Sample preparation comparison graph for oxycodone

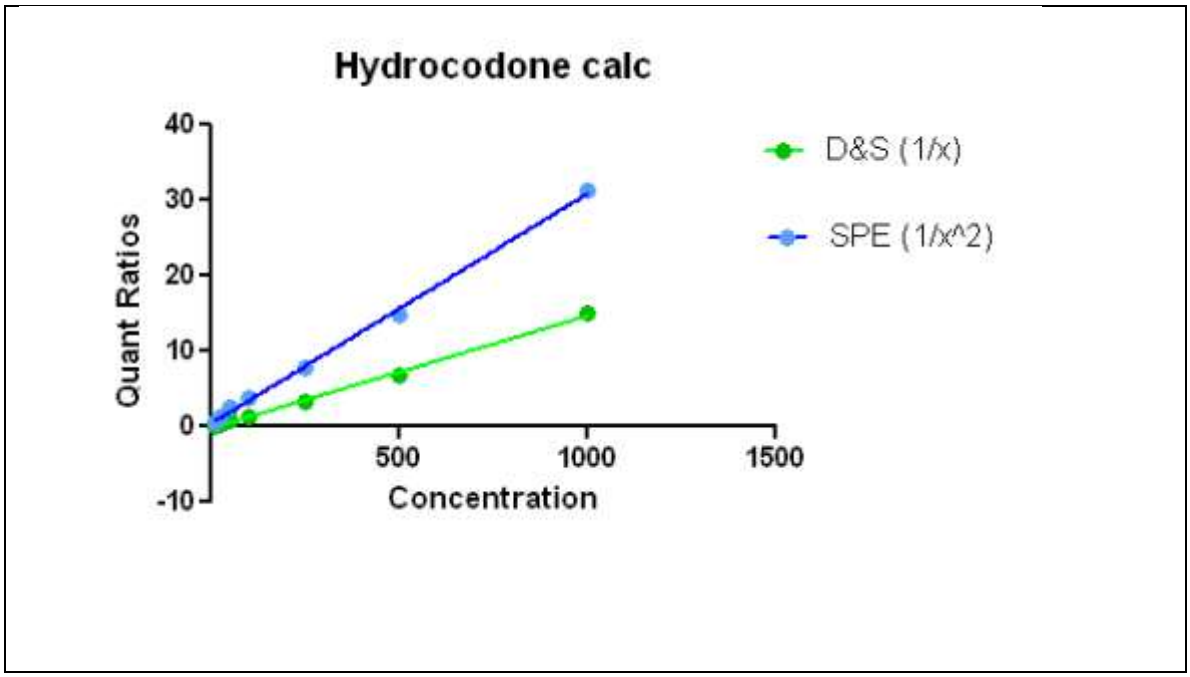

Figure 17. Sample preparation comparison graph for hydrocodone

4.4.3 Dilute and Shoot

A chromatogram of a sample prepared with the D&S procedure and spiked at 1000 ng/ml can be seen in Figure 18.

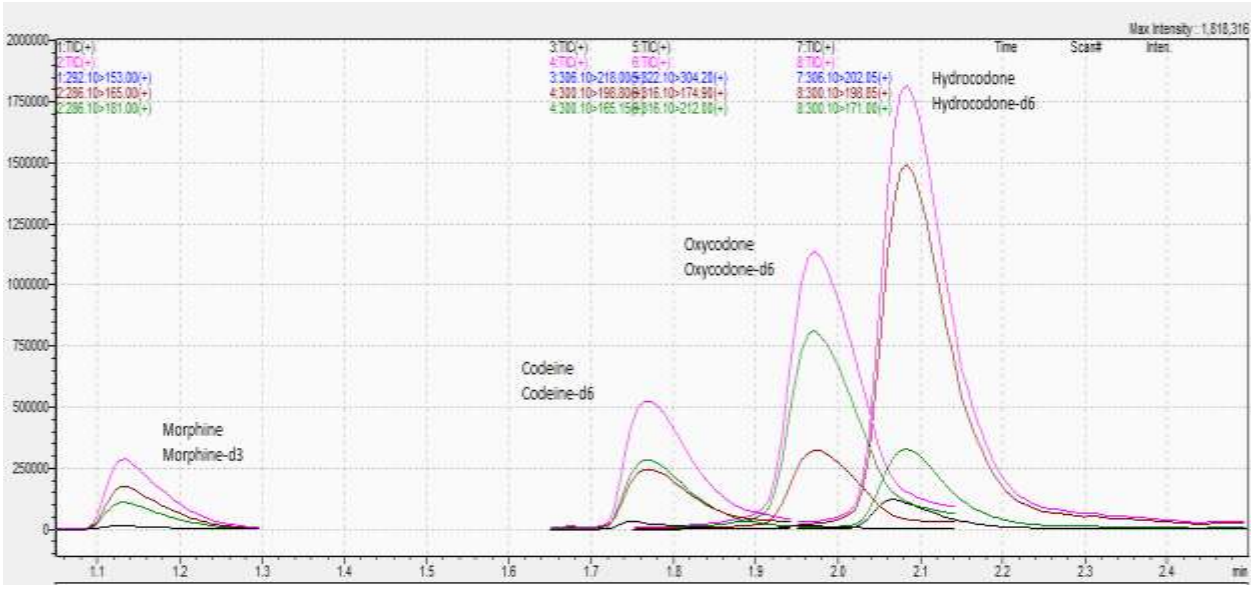

Figure 18. Chromatogram of a 1000 ng/ml prepared with dilute and shoot procedure

4.4.4 Solid Phase Extraction

A chromatogram of a sample prepared with the solid phase extraction procedure and spiked at 1000 ng/ml can be seen in Figure 19.

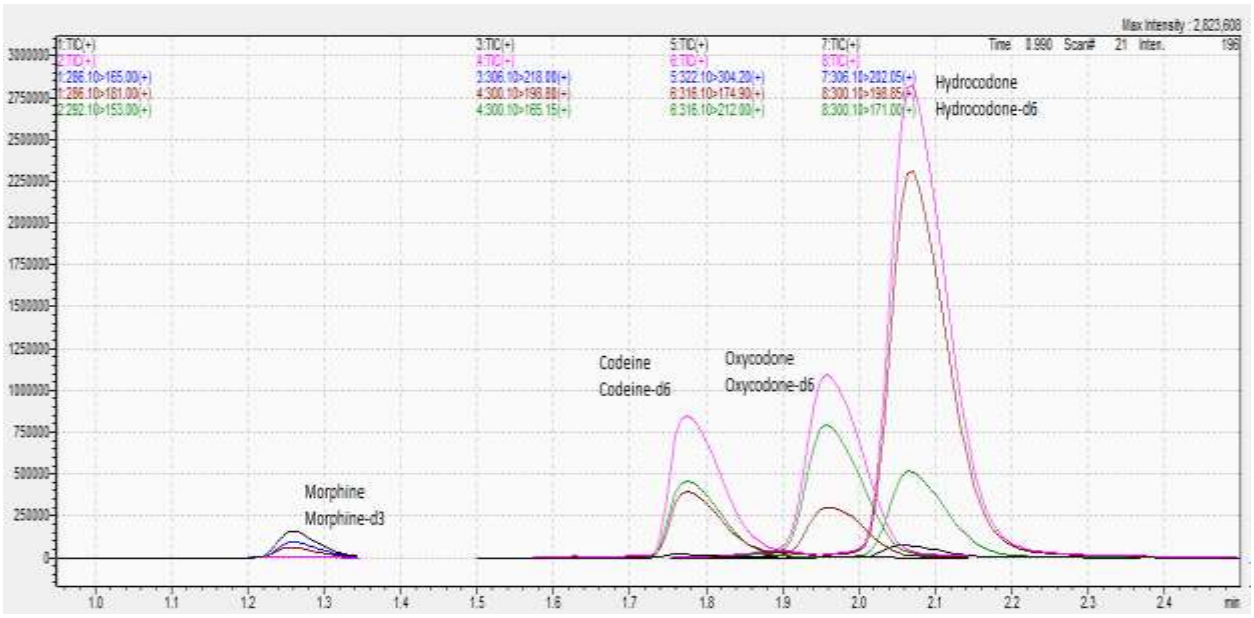

Figure 19. Chromatogram of a 1000 ng/ml prepared with Solid Phase Extraction procedure

CHAPTER V

DISCUSSION

5.1 Method development

5.1.1 Dilute and shoot sample preparation

Based on the chromatograms of the samples prepared with the hexane: ethyl acetate: ammonium hydroxide solution, the solution did not adequately separate the drug analytes from the protein in blood. Clogging of the column was seen when samples were run in replicates. Replicates showed a significant reduction in peak area counts and poor peak shape. The retention time of the peaks also shifted forward and there was an increase in background noise. By the sixth replicate, the clogged column caused a pressure increase high enough to automatically shut off the instrument.

5.1.2 Application of syringe and centrifugal filters

The application of syringe and centrifugal filters were added to the D&S method, for further clean-up of the samples. The use of the syringe and centrifugal filters were discontinued because there was no improvement in results. The use of centrifugal filters was also more labor- intensive. The centrifugal filters held only 0.5ml sample at a time

and the samples prepared were approximately 4.0 ml. So the time it took to filter one sample was quadrupled. The hexane: ethyl acetate: ammonium hydroxide solution melted other types of centrifugal filters, so only the 0.2 µm nylon membrane centrifugal filter was used.

5.1.3 Bouzas et al. dilute and shoot sample preparation

The Bouzas et al. D&S sample preparation method produced samples that were not clean enough for analysis. The method was originally intended for plasma, whole blood without the blood cells, which is a cleaner sample compared to whole blood. The methanol: zinc sulphate solution was most likely not strong enough to efficiently separate the protein from a sample in whole blood.

5.1.4 In-house gas chromatography-mass spectrometry sample preparation

The in-house opiate sample preparation for analysis on the GC-MS is an established method at the OSBI. Analysis of these prepared samples showed less background noise and more consistent peaks than previous sample preparation methods produced. While this sample preparation method produced cleaner and more precise results, it was not pursued, due to the costliness of the ultra-filtration tubes and ToxiLab tubes.

5.1.5 Preference for LLE or D&S sample preparation method

While the OSBI considered using a SPE sample preparation method, the LLE or D&S sample preparation method was desired over a SPE sample preparation method. This preference was due to the fact that SPE was believed to require more of the analyst's time and effort. Also, the OSBI laboratories did not have a SPE apparatus or the

cartridges necessary to carry out a SPE method. Working with items and solutions that were readily available in the laboratories sped up the method development process.

5.2 Qualitative Analysis Method Validation

5.2.1 Selectivity and Sensitivity

Selectivity was determined by comparing the mean peak areas for the spiked sources to the blank sources. The mean peak areas for each opiate in the spiked sources were easily differentiated from the lack of peaks in the blank matrix, demonstrating that the method is free from sample matrix interference. In evaluating sensitivity, no response was seen in the matrix blank, demonstrating that the method is free from carryover at samples with a concentration of 1000 ng/ml or more. The method was considered to be selective and sensitive.

5.2.2 Limit of Detection

The OSBI does not pursue DUID cases with opiate levels less than 10 ng/ml due to the difficulty in proving driving impairment. So the true LOD was not determined, but was administratively set at 10 ng/ml.

5.2.3 Matrix Effects

The method was considered free from matrix effects because all analytes at 20 and 100 ng/ml produced a CV of less than 15%.The CV of both concentrations for the samples in injection solvent were below 9% and the CV of both concentrations for the samples in whole blood were below 15%. While there is some occurrence of ion enhancement/suppression, the CV percentages are low enough that other validation studies, such as LOD and bias, are unaffected.

5.2.4 Co-Administered Drugs

Most of the unexpected responses for analytes were below 20% of the mean LOD signal. The analysis of the 6-MAM sample caused a response for morphine that was 20% of the mean LOD signal. Since these samples were injected at high concentrations that far exceed what is expected to be present in authentic case samples, the response at 6-MAM was considered acceptable. It is unlikely that a case sample containing other common drug analytes will cause interference. The method was considered free from co-administered drug interference.

5.3 Quantitative Analysis Method Validation

5.3.1 Accuracy

The calculated calibrator values for morphine, codeine, oxycodone, and hydrocodone were all within 10% of their known values. This validation study shows that this method accurately quantitates samples and is reliable for the quantitation of case samples.

5.3.2 Limit of Quantitation

Since the OSBI does not pursue cases with opiate concentrations of less than 10 ng/ml, the LOD was used as the LOQ and was administratively set at 10 ng/ml. The calculated LOQs were well below 10 ng/ml, indicating the possibility for a more sensitive method with use of a lower calibration curve and further validation. Although concentrations lower than 10 ng/ml are not utilized at the OSBI, clinical applications of the method may analyze opiates at lower concentrations.

5.3.3 Precision

The method was determined to be precise, considering that the within-run and between-run precision percentages were all below the suggested percentage of 20%. This shows that the quantitative method is capable of consistently analyzing samples quantitatively.

5.3.4 Benchtop Stability

All of the mean responses for the high QC fall within the range of 85 and 115%. This indicates that samples on the benchtop remain stable for up to four hours.

5.3.5 Processed Sample Stability

The plotted ratios of the high and low QC showed fairly flat trend lines with all slopes falling between -0.01 to 0.01. Samples that have been left on the LC-MS overnight are in stable enough conditions that analysis is reliable up to 24 hours after the sample is prepared. Using the trend line to calculate hours of stability up to 20% signal decrease in samples, these samples have potential to remain stable for much longer than 24 hours. Before these times are put into practice, they must first be studied and revalidated.

5.4 Comparison of Sample Preparation Methods

5.4.1 Accuracy

The calculated calibrator values for the D&S samples were within 14% of their known values for all drug compounds. All, except two of the calculated calibrator values for the SPE samples were within 16% of their known values. The 50 ng/ml calibrator for oxycodone and hydrocodone were within 33% of their known values. The high deviation

for just one of the calibrators, suggests a possible error when preparing the calibrators or is possibly due to an unfamiliarity to the SPE preparation method.

5.4.2 Sensitivity

The LOD and LOQ were calculated for both the D&S and SPE method to determine sensitivity. The SPE method was found to be more sensitive than the D&S method. The LOD calculated for the D&S method was 0.010 ng/ml and 0.005 ng/ ml for the SPE method. The LOQ calculated for the dilute and shoot method was 0.030 ng/ml and 0.015 ng/ml for the SPE method. Plotted quant ratios and chromatograms show that the SPE method produced higher peak areas than the D&S method. The calculated LOQs are theoretical values and would have to be proven with a series of calibration curves.

5.4.3 Price comparison

The price of a making up a SPE and a D&S sample was calculated (Table 22) based on the cost of solvents, materials, glassware, and analyst's time. The cost of making up a D&S samples is approximately 1/3 the cost of making up a SPE sample. The major expenses for the SPE samples are due to the use of extra solvents and a cartridge, but mostly due to the cost of the analyst's time. It takes roughly 2 hours to prepare a sample using the SPE sample preparation method, while the D&S sample preparation takes roughly 45 minutes.

Table 22. Sample cost comparison

	SPE	D&S
Solvents/materials	$4.35	$0.07
Glassware	$1.87	$1.87
Analyst's time	$52.00	$21.44
Total Cost	$58.22	$23.37

5.5 Comparison to other studies

There is a lack of studies over D&S sample preparation methods for the quantitative analysis of opiates in blood samples in the literature. But there several SPE sample preparation methods which are comparable to the SPE sample preparation method utilized for this study. The LOD's and LOQ's found in the literature were considerably lower than the administratively set LOD and LOQ of 10 ng/ml for the validation study.[2] The LOD for opiates were as low as 0.5 and 1.0 ng/ml and the LOQ for opiates were as low as 1.0 and 2.0 ng/ml. One study determined the LOD of codeine as 0.0002 mg/kg and the LOD of morphine as 0.0001 mg/kg.[24] The LLOQ of codeine was 0.0025 mg/kg and the LLOQ of morphine and 0.0025 mg/kg.[25] While these numbers are lower than the administratively set LOD and LOQ, they are quite close to the calculated LOD and LOQ values which were as low as 0.0022 ng/ml for the LOD and 0.0132 ng/ml for the LOQ.

5.6 Significance

Not many studies have extensively compared the results of different sample preparation methods using the same analytical method on the LC-MS, but there is agreement in that the SPE method produces cleaner samples which will then produce more accurate and sensitive results. While the results were in agreement that the SPE

sample preparation method has better sensitivity, the D&S method appeared to produce more accurate results. It is possible that since the analytical method created on the LC-MS was created for D&S samples, the LC-MS method was more specific to the analysis of D&S methods than the SPE samples.

5.7 Future work

Future work that could be included with this study, is adding another sample preparation method for the analysis of opiates. The resulting data would give more insight into how different sample preparation methods affect analytical results. Also, changing components of the LC/MS/MS analysis such as column type, eluent type or injection amount could show the importance of choosing a certain brand or type for the analysis of opiates.

Another study that could be conducted involves the comparison of sample preparation methods for the analysis of another drug class altogether. This study would give insight into how efficient the sample preparation methods are at extracting the drug analyte and how the sample preparation methods affect analytical results.

5.8 Conclusion

The comparison of the SPE and D&S sample preparation methods yielded results that were mostly expected from the literature, in that the SPE sample preparation method produced more sensitive results than the D&S method. Since the use of a column and multiple steps of solvent-washing of samples in the SPE typically produce cleaner result, it was unexpected that the D&S method produced more accurate results than the SPE method.

Data generated from the validation studies for the qualitative and quantitative method demonstrate a reliable and accurate dilute and shoot sample preparation method for the opiate analysis. While the comparison data shows that the SPE is the better sample preparation method for the analysis of opiates, the D&S method also produces results which are accurate and reliable for use in government crime labs.

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VITA

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