DETECTION AND QUANTIFICATION OF KETAMINE AND CLOMIPHENE IN COMPLEX MATRICES BY LC AND ESI-MS^N

A Thesis by

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The following faculty members have examined the final copy of this thesis for form and content, and recommend that it be accepted in partial fulfillment of the requirement for the degree of Master of Science, with a major in Chemistry.

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ABSTRACT

The research presented here details the development of two separate methods for ESI-MS and LC. The first chapter gives an introduction to the first of the methods presented here, the detection of ketamine in alcoholic drinks. The second chapter gives a brief introduction into the relevance of developing a method for investigating clomiphene in biological samples. The third and fourth chapters outline the experimental parameters for the ketamine and clomiphene detection methods, respectively. Included are the parameters used for the mass spectrometer, liquid chromatography, and UV/Visible absorbance spectroscopy as well as the methods for extraction and sample work up. Chapter five outlines the development of a method for detecting and quantifying the date rape drug Ketamine HCl in alcoholic drink matrices. The sixth chapter outlines the development of LC and MS methods for detecting and quantifying clomiphene citrate in fetal calf serum and the initial results for the extraction and detection of clomiphene citrate in meconium. Chapters seven and eight present the conclusions for the ketamine and clomiphene studies, respectively, with directions for future studies.

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LIST OF ABBREVIATIONS

1	µg	Microgram
2	µL	Microliter
3	µM	Micromolar
4	C	Celsius
5	CC	Clomiphene citrate
6	CID	Collision induced dissociation
7	cm	Centimeter
8	CV%	Co-efficient of Variance as a percent
9	D_2O	Deuterium oxide
10	DES	Diethylstilbestrol
11	E%	Percent error
12	ESI	Electrospray ionization
13	FDA	Food and Drug Administration
14	FSH	Follicle stimulating hormone
15	g	Gram
16	GC	Gas chromatography
17	GHB	Gamma-hydroxy butyrate
18	GI	Gastro-intestinal
19	GMP	Good Manufacturing Practice
20	GnRH	Gonadotropin releasing hormone
21	HCl	Hydrochloric acid
22	HPLC	High performance liquid chromatography

23	ICH	International Conference of Harmonisation
24	KE	Ketamine
25	kV	Kilovolts
26	L	Liter
27	LC	Liquid chromatography
28	LH	Leutinizing hormone
29	M	Molar
30	m/z	Mass to charge ratio
31	mg	Milligram
32	min	Minute
33	mL	Milliliter
34	mm	Millimeter
35	mM	Millimolar
36	MS	Mass spectrometry
37	ms	Millisecond
38	N_2	Nitrogen gas
39	nm	Nanometer
40	PCP	Phencyclidine
41	pM	Picomolar
42	RF	Radio frenquency
43	rpm	Revolutions per minute
44	S/N	Signal to noise

45	tBME	*tert*Butylmethylether
46	TC	Tamoxifen citrate
47	UV/Vis	Ultraviolet/Visible
48	V	Volt

SYNOPSIS AND OBJECTIVES

This thesis entitled “Detection and Quantification of Ketamine and Clomiphene in Complex Matrices by LC and ESI-MS^n” details the development of two different methods to detect and quantify drugs in complex matrices.

Many forensic labs rely on LC-UV and LC-MS methods to detect and quantify the levels of drugs in different types of matrices including biological and non-biological matrices. It can be a challenging task to develop methods that are specific, precise, and accurate, but it is important for these labs to have a wide variety of methods readily available for use. Two more challenges are that the methods need to be reliable and cost effective. The first objective for the ketamine study was to develop a simple alternative method for detecting and quantifying ketamine using MS. The method needed to be simple and effective yet also needed to be precise, accurate, and be able to quantify at levels lower than would normally be found at a crime scene. The second objective was to develop a method for detecting and quantifying ketamine using solely LC-UV analysis. This method also needed to be simple, effective, precise and accurate. The third major objective was to develop methods with the capability for delayed testing since current methods for detecting date rape drugs usually require testing within 48 hours. Here, a method for detecting date rape drugs in alcoholic beverages is presented. This method gives an alternate way to test for date rape drugs from the normal urine analysis and gives a way for delayed testing.

While clomiphene citrate is given to thousands of women each year, little is known about the effects of clomiphene on the development of the fetus. There is some evidence to suggest that clomiphene acts as an endocrine disruptor similar to

diethylstilbestrol. DES had many detrimental effects on the women that took it and the daughters of the women that were on a DES fertility treatment. In an initial study by our collaborator, Dr. May, clomiphene was shown to have similar effects on the ovaries of hamsters as does DES. One major hurdle in determining the effects of a drug on the developing fetus is to actually determine if the fetus is being exposed to the drug. The first objective was to validate the half life of clomiphene in vivo and determine to what extent clomiphene stays in the system of the women taking the drug. The second objective of the study was to actually determine fetal exposure by extracting and measuring clomiphene levels in the meconium. For both of these objectives to be completed, a method is required to be able to extract and quantitatively analyze the drug. This is not such a trivial task as the biological matrix can easily interfere with the analyte. Here we outline a method to be used to extract and analyze clomiphene from serum and meconium using a liquid phase extraction and LC and ESI-MS^n. The first objective for the method development was to be able to specifically extract the clomiphene from the biological matrices – serum and meconium. The second objective was to develop a precise and accurate quantitative method for analyzing the clomiphene extract on the MS with as low detection limits as possible. The third objective was to develop a precise and accurate LC method to be able to separate the drug from the rest of the matrix. The contents of the chapters are as follows.

Chapter 1: Ketamine Introduction

The first chapter starts by giving a brief background on date rape drugs and ketamine role in such cases. Date rape is defined as rape by a person the victim is acquainted with. Some date rape cases involve the use of drugs to facilitate the crime.

Some of the more widely used of the date rape drugs are gamma-hydroxy butyrate, flunitrazepam (also known as rohypnol), and ketamine hydrochloride. Ketamine was originally developed as an anesthetic, and is still used widely to sedate animals. A major drawback of ketamine for use as an anesthetic is the side effects when emerging from anesthesia. If a slightly smaller dose than what is required to enter into full anesthesia is taken, ketamine causes hallucinations, out-of-body experiences, analgesia, and amnesia.

It is crucial that drug exposure be able to be determined in drug facilitated date rape cases. Since ketamine is rapidly metabolized in the body, it is important to be able to obtain a urine sample from the victim within 48 hours of the incident. This is often difficult, but methods are available to measure ketamine and its metabolites in urine using LC-MS and GC-MS.

Chapter 2: Clomiphene Introduction

The second chapter gives a brief overview of endocrine disruptors and clomiphene's action as an endocrine disruptor. The endocrine system is the hormone system in the body. It acts by releasing hormones into the blood stream which bind to specific receptors elsewhere in the body and illicit a response. Endocrine disruptors are so called since it is believed they act as hormones and disrupt the natural action of the endogenous hormones. Since hormones are released in very small amounts in the body, it is suggested that only very small amounts of the disruptors are needed to change the normal hormonal balance in the body.

Certain fertility drug endocrine disruptors such as DES have proven to have many detrimental effects not only on the mothers, but also on the daughters and sons

born out of the fertility treatment. DES was eventually removed from the market due to the studies that linked it to the increases in rates of certain types of cancers, increased rates of reproductive tract structural defects, and more. DES also showed to produce an abnormally large amount of polyovular follicles.

Clomiphene is structurally related to DES and has also shown some of the detrimental effects on the daughters from the clomiphene fertility treatments. It has also been shown that an abnormally large amount of polyovular follicles are produced in hamsters, similar to that of DES. Here we aim to determine the extent of human fetal exposure to clomiphene by extracting it from meconium and analyzing the extract with LC and ESI-MS^n.

Chapter 3: Ketamine Materials and Methods

Chapter 3 discusses the preparation of the ketamine samples and standards. It also explains the instrument parameters for the ESI-MS and the CID experiments. Further, it discusses the parameters for UV/Vis detection and the instrument parameters, chemicals, and solvents used for LC analysis.

Chapter 4: Clomiphene Materials and Methods

Chapter 4 discusses the preparation of the clomiphene samples and standards. It also explains the instrument parameters for the ESI-MS and the CID experiments. Further, it discusses the parameters for UV/Vis detection and the instrument parameters, chemicals, and solvents used for LC analysis.

Chapter 5: Detection and Quantification by LC and ESI-MS^n of Ketamine HCl in Alcoholic Beverages

Ketamine HCl was first studied in water and ethanol. Ketamine has a m/z of 238 and upon CID loses water and methylamine. CID of deuterium labeled ketamine confirmed the fragmentation pathway. Positive and negative mode spectra were collected for a number of distilled alcohols, mixers, and mixed drinks. Each drink showed distinct spectra making it possible to distinguish between the different beverages. As well, different brands of a similar alcohol showed different spectra making it possible to distinguish between different brands of alcohol. Positive and negative mode spectra were collected for a second lot of several of the alcohols and the spectra were similar. The two lots of alcohols showed a “fingerprint” region where the spectra were the same.

The drinks were spiked with ketamine to study the matrix effects on the ability to detect and quantify ketamine. For all of the alcohols, mixers and mixed drinks, it was possible to detect and quantify ketamine using ESI-MS^n. Using a series of standards and plotting the peak intensity versus concentration, a calibration curve was constructed. The method is precise and accurate. Detection limits were down as low as picoM range for some alcohols, but was at 100 picoM range for all drinks tested. Stability testing was conducted and the ketamine stays stable in the beverages for at least 14 days under bench-top conditions. This included ketamine in acidic conditions in cola and lemon juice.

Using an isocratic LC set up, ketamine can be separated from the alcohol matrix with a total run time of 9 minutes. Quantitative analysis was carried out by UV

absorbance spectroscopy. A calibration curve was constructed by plotting absorbance versus concentration of a series of standards. The LC method was accurate and precise.

Chapter 6: Extraction, Detection and Quantification by LC and ESI-MS^n of Clomiphene Citrate in Fetal Calf Serum and Meconium

Clomiphene citrate was first studied in water and methanol. Clomiphene has a m/z of 406 and upon CID loses the terminal amine and HCl. The fragmentation pathway was confirmed by subjecting the fragments to MS^3 and using a large isolation width during MS/MS to be able to follow the Cl^{37} isotope. Extraction of clomiphene from serum and meconium was carried out by a liquid phase extraction using tBME. Extraction efficiency was 65% for serum. Quantitative analysis using the MS was possible by using tamoxifen citrate as an internal standard. The MS method was both precise and accurate. Detection limits were in the picoM range with quantitation limits at about the 10 picoM range. There was a linear response from mM to nM range using this method.

Using an isocratic LC set up, clomiphene can be separated from the serum and meconium extract with a total run time of 5 minutes. Quantitative analysis was carried out by UV absorbance spectroscopy. A calibration curve was constructed by plotting absorbance versus concentration of a series of standards. The LC method was accurate and precise.

Chapter 7: Ketamine Summary

Chapter 7 gives a brief overview of the results of the ketamine studies presented in Chapter 5. It ends with a short discussion of the advantages of this method as well as some direction for future studies.

Chapter 8: Clomiphene Summary

Chapter 8 gives a brief overview of the results of the clomiphene studies presented in Chapter 6. It ends with a short discussion of the advantages of this method as well as some direction for future studies.

CHAPTER 1

KETAMINE INTRODUCTION

1.1: Date Rape and Date Rape Drugs

The department of justice reports that between 2001 and 2005 the annual rate of sexual assault on women was about 1 out of 2000. Included in this category are date rape and drug-facilitated date rape. Date rape is defined as forced involuntary sexual intercourse by an acquaintance or friend and it makes up about 73% of all rape cases [1]. Drug facilitated date rape is a date rape case where a drug was used to put the victim in a comatose or dissociative state so that they are unaware that they are a victim of a rape crime. The most widely used of the date rape drugs are gamma-hydroxy butyrate, flunitrazepam (also known as rohypnol), and ketamine hydrochloride. Some compounds such as 1,4-butanediol and gamma-Butyrolactone exhibit the same effects as GHB after the drug is metabolized and are also commonly used compounds [2]. All of these drugs have been implicated in cases of date rape, with GHB being the most widely used of the drugs because of its ease of acquisition. However, due to increases in the regulation of GHB and its derivatives over the last several years, the use of other drugs are on the rise as GHB becomes increasingly more difficult to obtain. Illegitimate use of ketamine has seen increased use as a recreational drug among young adults at clubs as well [3,4,5]. In Hong Kong in 2002, 15,000 ketamine tests were performed compared to only 10 in 1999 [6,7]. The increasing popularity of the drug in club environments coincides with higher risk for cases of ketamine-facilitated date rape. GHB puts the victim in a comatose-like state, while drugs like ketamine will cause

the victim to enter a dissociative state. A dissociative state is described as a trance-like state where one separates perception from sensation. In this state, the date rape victim can experience extreme analgesia, amnesia, hallucinations, and “out of body” or “near death” experiences while still being awake [8,9]. Examples of dissociative drugs other than ketamine are nitrous oxide and phencyclidine. The dissociative drugs are desirable to the assailant since the victim is still awake yet incapable of any sort of self defense attempt and the victim often times experiences amnesia. KE HCl is also nearly tasteless, nearly colorless, and nearly odorless when dissolved in water. It readily dissolves in water and alcohol; therefore it can easily be dissolved in an alcoholic drink and given to the victim. In fact, this is the most common delivery method for the involuntary consumption of date rape drugs.

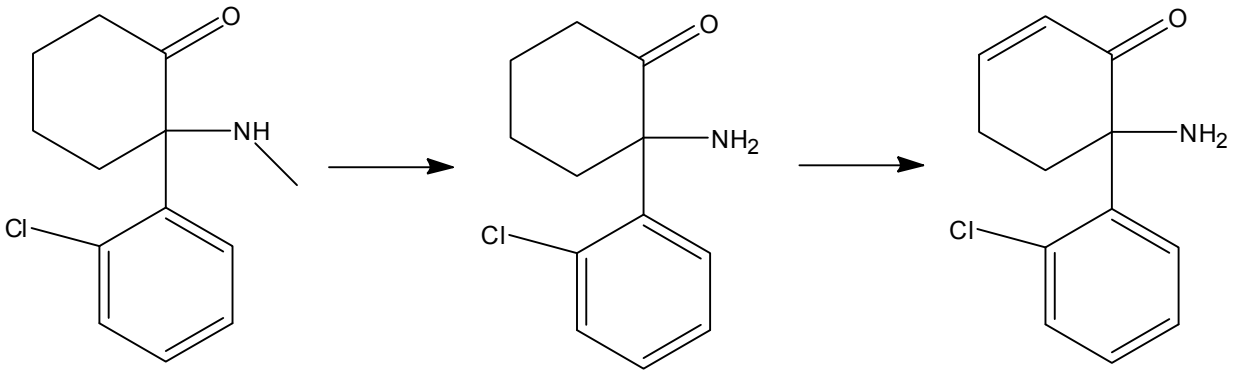
1.2: Ketamine HCl

Ketamine was originally synthesized in 1962 by Calvin Stevens at Parke Davis Labs [10] as an attempt to replicate the anesthetic qualities of phencyclidine but diminish the undesirable hallucinogenic affects that patients experienced [11,12]. Ketamine HCl was patented in 1966 and became available for use in humans and animals in Europe and China under the name Ketalar®. In 1970, the Food and Drug Administration approved ketamine for use in humans in the United States of America. Ketamine was a marginal success as hallucinations and delirium in patients emerging from anesthesia were not as common as with PCP and it had a shorter recovery time of 1-5 hours. Typically, children and elderly patients experience less of the side effects than adults patients do [13]. However, the risk of discomforting psychological symptoms has caused ketamine to be more commonly used in veterinarian clinics than

hospitals [14,15,16,17]. Ketamine is still widely used to sedate animals for surgery, travel, and euthanasia. Besides anesthesia, there are a number of legitimate medicinal uses for ketamine, including treatment of depression, migraines, chronic pain and use as an anticonvulsant for epileptic seizures [18,19,20,21,22,23].

1.3: Ketamine Metabolism

Ketamine is highly bioavailable from intravenous or intramuscular injections, but undergoes extensive first pass metabolism when consumed orally. Ketamine is metabolized with a half life of about 3-4 hours. The cytochrome P450 system leads to the *N*-demethylation of ketamine to produce norketamine. Norketamine is active and produces similar effects as ketamine. Norketamine is further dehydrogenated to produce dehydronorketamine (Figure 1.1). Both metabolites and the parent compound are further changed by hydroxylation and conjugation prior to excretion [24,25,26]. Excretion is 90% renal, 5% by feces, and 4% through the urine.



Ketamine Norketamine Dehydronorketamine

Figure 1.1: Metabolites of Ketamine

1.4: Ketamine HCl as a Date Rape Drug

The goal of a typical ketamine abuser is to experience and draw out the psychedelic effects of pre-anesthesia which is done by taking a slightly smaller dose

than would be required to cross the line into full anesthesia. This dose, called the 'line dose' or a 'bump,' of orally consumed KE is about 200-400 mg and the effects are noticed within 15-45 minutes and typically last for 1-5 hours [27,28,29,30]. Physical symptoms include muscle rigidity, slurred speech, bronchodilation, and loss of coordination. As well the victim can experience psychological effects including feelings of invulnerability, near-death or out of body experiences, hallucinations, and aggressive behavior. Generally, though, the victim will be uncommunicative but appear awake and eyes may or may not be open. These symptoms are particularly detrimental to the victims of sexual assault as they may not recall or realize the extent of the crime committed.

Detection of the drug is paramount for the prosecution of drug-facilitated date rape cases. Current methods for detecting ketamine and its metabolites in the body are done with urine analysis, commonly with GC-MS and LC-MS [31,32,33,34,35,36,37,38]. Since ketamine is metabolized rapidly by the body into norketamine and dehydronorketamine, methods have been developed for detection limits as low as 5ng/ml of these metabolites in urine as well. However, because urine analysis needs be completed within two days of use, there is a significant need for supplemental or alternative methods to detect ketamine that have a higher tolerance for delayed testing [39,40]. In this study, we have developed analytical methods using LC-UV and MS^n for identification and quantification of ketamine when found in a variety of different alcohols and mixed drinks and have shown a minimum stability of ketamine analysis of two weeks.

CHAPTER 2

KETAMINE MATERIALS AND METHODS

2.1: ICH Guidelines

The method presented here will address, in whole or in part, the International Conference of Harmonisation guidelines to the validation of analytical procedures. There are eight different areas that must be tested for a method to be valid for use on pharmaceuticals or human use. Since these methods are not used in Good Manufacturing Practice work environments or used for pharmaceutical use, the methods do not have to undergo as stringent testing. Nevertheless, these guidelines set in place were followed. The nine different areas of testing include: specificity, linearity, range, accuracy, precision, detection limits, quantification limits, and robustness. Specificity is the ability to assess the analyte among the presence of other compounds that would normally be expected to be present. Accuracy is the measure of the closeness of agreement between the true value and the experimental value. Precision is the closeness of agreement between a series of measurements from the same sample under the same conditions. The detection limit for a procedure is the lowest detectable amount of the compound in question. The quantification limits is the lowest amount of analyte that can by quantitatively determined with suitable precision and accuracy. Linearity is defined as the ability to obtain results which are directly proportional to the concentration of the sample. Range is the upper and lower concentrations of an analyte that gives suitable linearity, precision, and accuracy. Robustness is the ability of the method to remain unaffected by small, deliberate changes in the testing parameters.

2.2: Sample and Standard Preparation

Standard (1mg/mL) ketamine HCl in methanol was synthesized by the Cerilliant Corporation and purchased through Fisher Scientific. Deuterium oxide, 99.9% D, was purchased from Fisher Scientific. All alcoholic beverages were obtained from Dave's Liquor in Wichita, KS. Mixers were obtained from Dillon's in Wichita, KS. A 10^{-4} M stock ketamine HCl standard was prepared by adding 685.0 µL of 1 mg/mL standard ketamine HCl in methanol to a 25.0 mL volumetric flask and diluting to volume with deionized water. Subsequent standards were prepared by serial dilution from the 10^{-4} M stock standard. Alcoholic beverages used for determining matrices were analyzed with no sample work up. Spiked beverage samples were prepared by vortex mixing 500 µL of the alcohol and 500 µL of the stock ketamine standard to produce a 0.05 mM ketamine concentration in the drink. Deuterium labeling was carried out by mixing 0.5 mL of the ketamine stock standard with 0.5 mL of D_2O for 30 minutes. All samples and standards and reagents, except for the standard KE HCl, were stored under benchtop conditions. Standard KE HCl was stored at 4º C until dilution.

2.3: ESI-MS^n Parameters

Electrospray ionization mass spectra were collected using a Finnigan LCQ-DECA^{TM} ion trap mass spectrometer (San Jose, CA, USA). Samples were infused into the ESI-MS instrument using the incorporated syringe pump with a flow rate of 5 mL/min. The atmospheric pressure ionization stack settings for the LCQ (lens voltages, quadrupole and octapole voltage offsets, etc.) were optimized for maximum transmission to the ion trap mass analyzer by using the auto-tune routine within the LCQ Tune program. Following the instrument tune, the spray needle voltage was

maintained at +5 kV, and the N_2 sheath gas flow was maintained at 25 units (arbitrary for the Finnigan systems, corresponding to approximately 0.375 L/min). The capillary (desolvation) temperature was held at 180º C. The ion trap analyzer was operated at a pressure of approximately 1.5 x 10^{-5} Torr. Helium gas, admitted directly into the ion trap, was used as the bath/buffer gas to improve trapping efficiency and as the collision gas for collision induced dissociation experiments.

The CID experiments were performed as follows. The ions were isolated using an isolation width of 1.5 mass to charge units. The normalized collision energy, which defines the amplitude of the radiofrequency energy applied to the end-cap electrodes in the CID experiment, was set to 28%, which corresponds to approximately 0.85 V. The activation Q (used to adjust the q_z value for the precursor ion) was set at 0.30. The activation time was set to 30 ms.

2.4: UV/Vis Spectroscopy Parameters

Ultraviolet/Visible spectroscopy was carried out on a Hitachi U-2010 dual beam spectrophotometer with a tungsten-iodine visible spectrum lamp and a deuterium UV spectrum lamp. A wavelength scan was performed from 1100 nanometers to 190 nm with a scan rate of 200 nm/min and a lamp change at 370 nm. Quartz cells with a path length of 1 cm were used for absorbance measurements. Samples were prepared by diluting 100 µL of the stock standard solution with 3400 µL of deionized water. Samples were run against a deionized water baseline.

2.5: LC Parameters

Isocratic liquid chromatography was performed on a Varian ProStar system. The system consisted of one binary Varian model 210 pump, a Varian model 320 UV/Vis detector, a 20 µL loop, and a reverse phase Waters µ Bondapak^{TM} C18 3.9 x 300 mm column. The mobile phase consisted of 80% acetonitrile (HPLC grade), 20% deionized water and 0.1% trifluoroacetic acid (99.9+%, Reagant Plus grade). Mobile phase flow rate was set to 1.6 mL/min. Total run time was 9 minutes with ketamine eluting at 3.8 minutes. UV absorbance detection was used with the lamp set at 268 nm. Samples and standards were prepared similar to the preparation of samples and standards for analysis with mass spectrometry.

2.6: Quantitative Analysis

Quantification of MS samples was carried out by running a series of standards and constructing a calibration curve by plotting the Intensity of the ketamine peak versus the concentration of ketamine in molarity. A linear trend line in the form of y=mx + b (where m=slope, b=y-intercept, and y and x are the y and x values on the graph, respectively) was obtained using least squares linear regression. Concentrations of known samples were confirmed using the equation of the linear trend line. Precision was determined by analysis of multiple replicates of a known concentration of a sample. Precision was measured as the percent coefficient of variance (CV%) which is found by:

$$CV\% = (\sigma)/(\bar{x}) *100$$

where σ is the standard deviation and $\bar{x}$ is the mean. Percent error (E%) was calculated by:

$$E\% = (\text{experimental value} - \text{theoretical value})/(\text{theoretical value}) *100$$

E% gives an idea about the accuracy of the method by comparing the experimental value to the theoretical value.

Quantification of LC samples was carried out by running a series of standards and integrating under the peak to obtain the area under the peak. Integration was performed by the Varian ProStar software suite. A calibration curve was constructed by plotting the Area of the clomiphene standard versus the clomiphene concentration in molarity. A linear trend line was obtained using least squares linear regression. A linear trend line was obtained using least squares linear regression. Concentrations of known samples were confirmed using the equation of the linear trend line. Precision was determined by analysis of multiple replicates of a known concentration of a sample. Precision was measured as the %CV. E% was calculated to determine accuracy.

CHAPTER 3

DETECTION AND QUANTIFICATION BY LC AND ESI-MS^N OF KETAMINE HCL IN ALCOHOLIC BEVERAGES

3.1: ESI-MS^n of Ketamine HCl

Initially, ketamine hydrochloride (Figure 3.1) was studied in water by collecting ESI-MS and ESI-MS^n spectra of the 0.1mM stock liquid standard solution.

Figure 3.1: Structure of Ketamine

The dominant peak in the ESI-MS spectrum is at 238.1m/z which corresponds to the ketamine $[M+H]^+$ parent ion. As well, there is a ^{37}Cl isotope peak at 239.9m/z at approximately 30% abundance relative to the 238.1m/z peak. Upon CID of ketamine (238.1m/z), the dominant ion is a loss of 18 mass units at 220.0m/z. There is also a second ion with a loss of 31 mass units at 207 m/z. Further CID (MS^3) of the dominant 220.0m/z fragmentation peak showed the major loss of 57 mass units at 162.9m/z and also a smaller, about 20% RI, loss of 29 mass units at 190.9m/z (Figure 3.2). This CID profile of ketamine was used to positively identify ketamine in the alcohol samples. The loss of 18m/z corresponds to a loss of H_2O via a keto-enol tautomerization on the

benzene ring and a transfer of one hydrogen from the nearby nitrogen. The loss of 31m/z is the loss of methylamine (Figure 3.3).

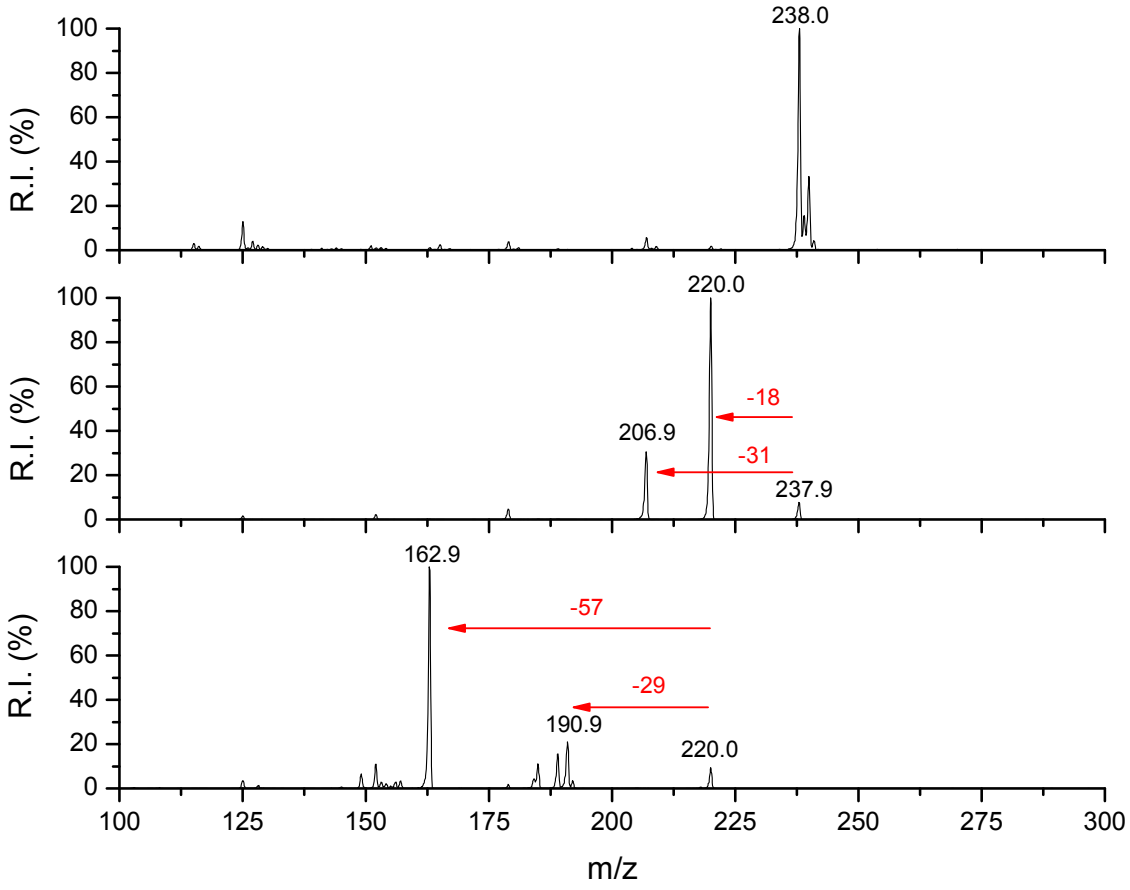


Figure 3.2: Positive ion mode full MS of Ketamine HCl in water (top spectrum), MS/MS of Ketamine HCl - 238.0m/z (middle spectrum), and MS^3 of 220.0m/z (lower spectrum)

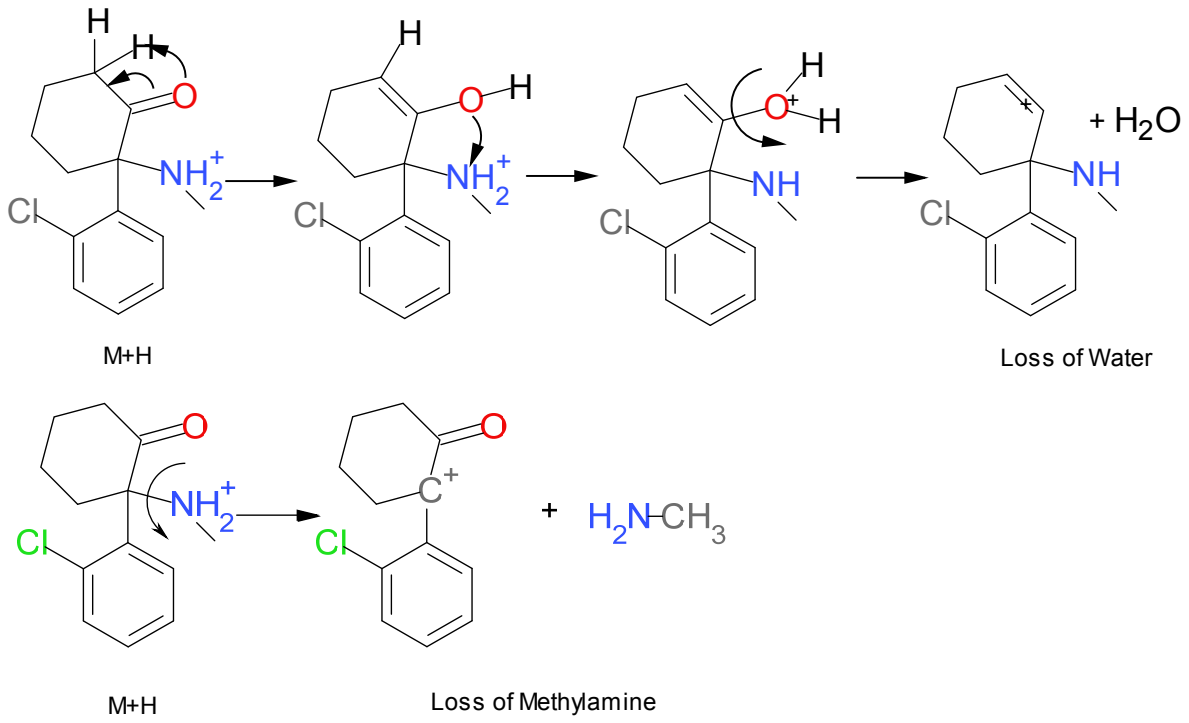


Figure 3.3: Mechanism of fragmentation for the loss of H_2O (upper scheme) and the loss of methylamine (lower scheme) for the MS/MS of Ketamine

MS/MS of deuterium labeled ketamine was also performed to confirm the fragmentation scheme. There are 2 exchangeable sites on ketamine - one being the hydrogen on the nitrogen and the other the hydrogen on the oxygen after tautomerization. The full MS of the deuterium labeled ketamine showed peaks at 238.1m/z (ketamine), 239.1m/z (ketamine with 1 deuterium atom), and 240.1m/z (ketamine with 2 deuterium atoms), and 241.1m/z (^{37}Cl isotope of 240.1m/z). The mass of 240.1 does have a portion of its peak made up of the ^{37}Cl isotope of the previous mass, but the mass peaks were much higher in intensity than would be expected for the normal isotope peak (Figure 3.4). Upon CID of 238.1m/z there is the expected loss of 18 and 31m/z. CID of 239.1m/z shows a loss of 19 and 32m/z indicating the loss of 1 deuterium atom. Upon CID of 240.1m/z, there is a loss of 20 and 33m/z indicating the

loss of 2 deuterium atoms. Since the CID of both the 239.1m/z and 240.1m/z molecules show the loss of deuterium, the hydrogens lost in the fragmentation must be coming from the H/D exchangeable sites as in Figure 3.4.

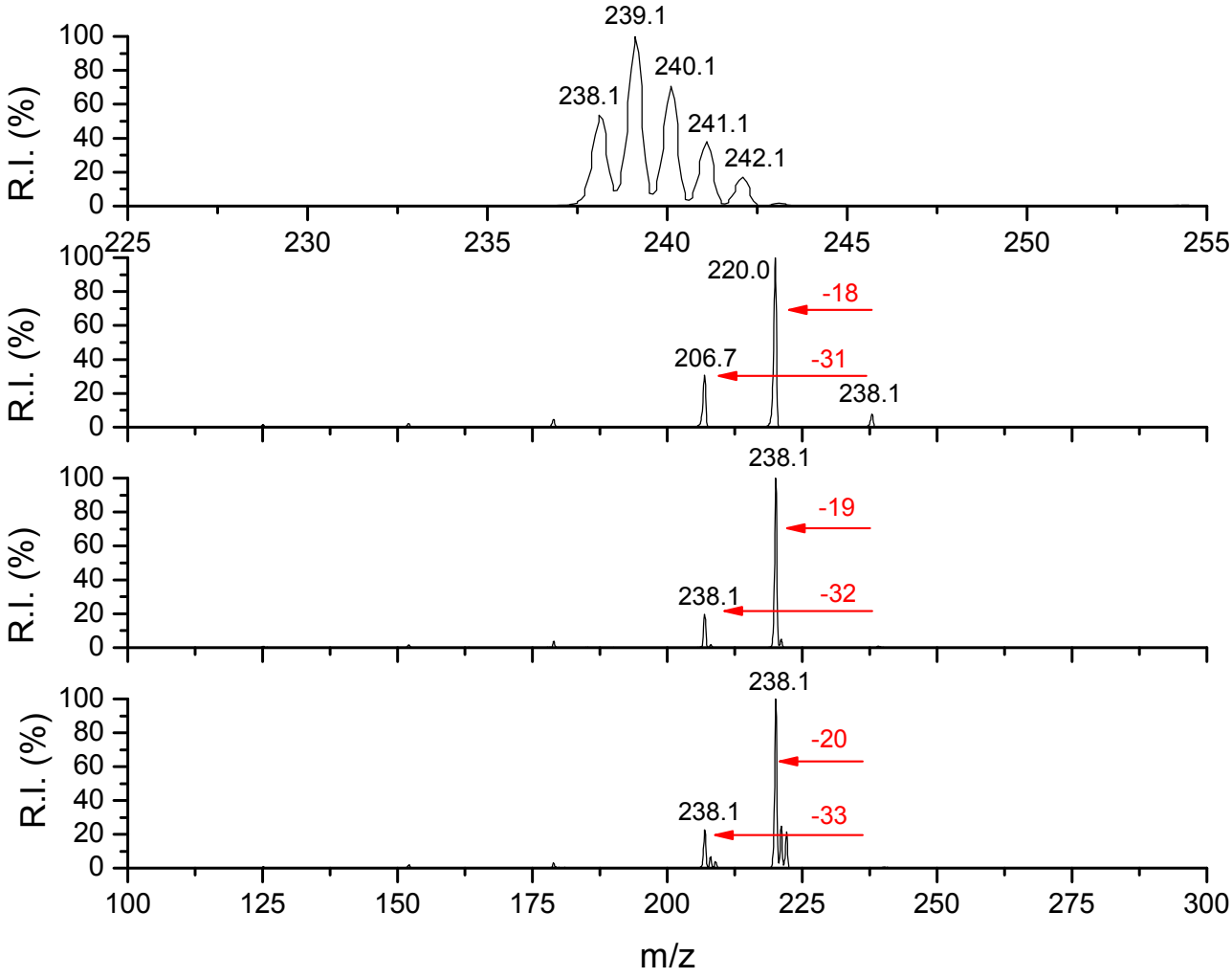


Figure 3.4: Positive mode MS of Deuterium labeled Ketamine (top spectrum), MS/MS of 238.1m/z (upper middle spectrum), MS/MS of 239.1m/z (lower middle spectrum), and MS/MS of 240.1m/z (lower spectrum)

3.2: ESI-MS of Alcohol Matrices

The matrices of distilled alcohols, mixers used in mixed drinks, and mixed drinks were determined by collecting positive and negative mode ESI-MS spectra. The spectra were collected with no sample work up. The alcohols used were Jim Beam™ Kentucky straight whiskey, Most Wanted™ Pioneer whiskey (bourbon style), Seagrams™ extra dry gin, Gordon's™ London dry gin, Ron Rico™ rum, Bacardi™ Peurto Rican rum, Martini and Rossi™ dry vermouth, Casco Viejo™ tequila, and Burnett's™ citrus Vodka. Other beverages tested were Big K™ Cola, Golden Crown™ tonic water, and lemon juice. The mixed drinks used were Jim Beam™ Kentucky straight whiskey and Big K™ Cola, Most Wanted™ Pioneer whiskey (bourbon style) and Big K™ Cola, Seagrams™ extra dry gin and Golden Crown™ tonic water, Gordon's™ London dry gin and Golden Crown™ tonic water, Burnett's™ citrus Vodka and lemon juice, Martini and Rossi™ dry vermouth and Seagrams™ extra dry gin, and Martini and Rossi™ dry vermouth and Gordon's™ London dry gin. The spectra show a distinct matrix for each alcohol and mixed drink making it possible to distinguish not only between different types of alcoholic drinks but also distinguish different brands from one another. Figure 3.5 shows an example of the difference between the matrices of two different brands of whiskey. Also, different lots of the same alcohol were bought and the matrices of each alcohol were run and compared to the original spectrum. Two different lots of Seagram's™ Extra Dry Gin, Jim Beam™ Kentucky Straight Bourbon Whiskey, Bacardi™ Puerto Rican Rum, and Casco Viejo™ Gold Tequila were tested. "Lot 1" of the alcohols was bought in May of 2008 and "Lot 2" was bought in April of 2009. While the spectra for each alcohol were similar, they were not exactly the same

between each lot. Interestingly, though, there are "fingerprint" regions in each alcohol that are the same between lots. In Figure 3.6, it is evident that Seagram's exhibits very similar mass spectra. Both of the negative spectra show large peaks at 89, 103, and 301m/z. Both positive spectra show major peaks at 249, 403, 425, 441, and 459m/z. The major difference comes in the positive spectra for this alcohol, where the intensities are different for the major peaks. This was typical for the other three alcohols tested - there are similarities between mass peaks between the spectra with differences in intensities of the peaks.

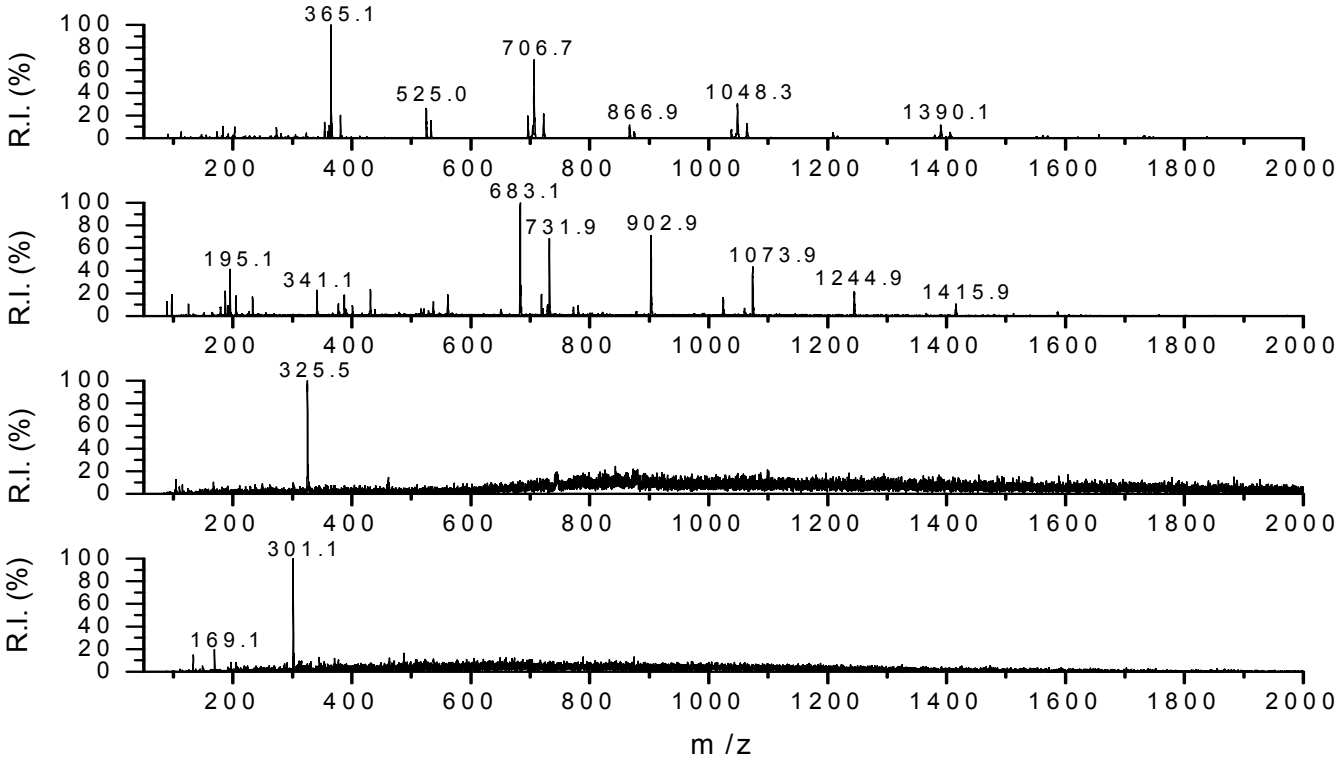


Figure 3.5: Positive mode (top spectrum) and negative mode (upper middle spectrum) ESI mass spectrum of Most Wanted™ Pioneer whiskey and positive mode (lower middle spectrum) and negative mode (lower spectrum) ESI mass spectrum of Jim Beam™ Kentucky straight whiskey (bourbon style)

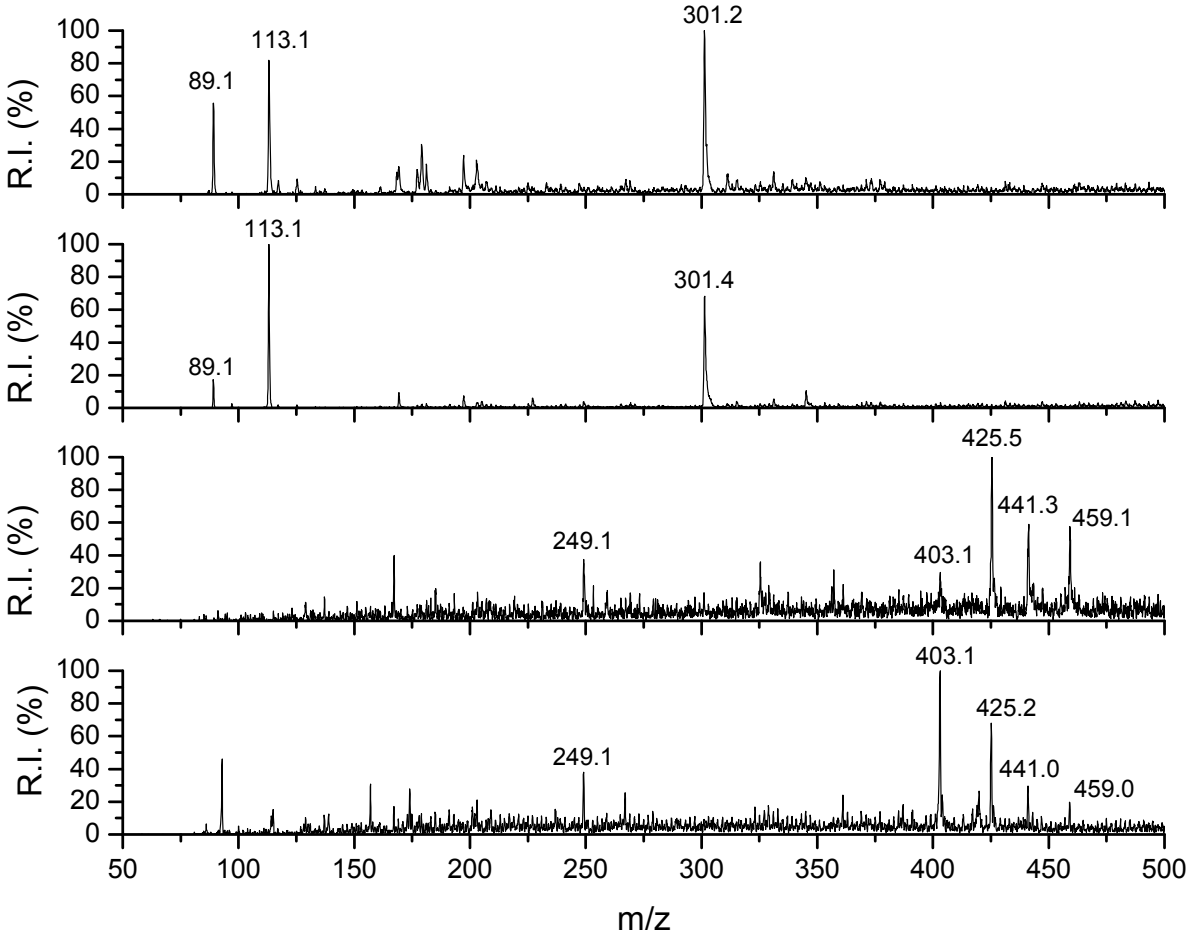


Figure 3.6: Negative ion mode MS of Lot 1 Seagram's™ extra dry gin (upper spectrum), negative ion mode MS of Lot 2 Seagram's™ extra dry gin (upper middle spectrum), positive ion mode MS of Lot 1 Seagram's™ extra dry gin (lower middle spectrum), and positive ion mode MS of Lot 2 Seagram's™ extra dry gin (lower spectrum)

3.3: ESI-MS^n of Ketamine in Alcohol Matrices

Once the matrices of the alcohol were collected, the alcohol samples were then spiked with ketamine hydrochloride. As seen in Figure 3.7, the spectra show the presence of the 238 m/z ketamine ion in the matrices of the alcoholic beverages. ESI-MS/MS spectra were collected on the ketamine peak and the CID spectra showed the

initial loss of 18 and 31 mass units to confirm the presence of ketamine. Alcohol solutions were then prepared with varying amounts of ketamine added, and ESI-MS results show a direct correlation between signal intensity and the concentration of the ketamine added to the solution. A calibration curve was constructed by plotting signal intensity versus concentration and was used to quantify samples of known concentration. The concentration of the samples calculated from the calibration curve had less than a 2% error. As well, 10 mL of the stock standard solution was mixed 1:1 with the different alcohols and mixed drinks and was allowed to stand in a glass cup. After 20 minutes, the alcohol/ketamine solution was discarded and the residue was allowed to air dry overnight. A cotton swab was used to swab the inside of the cup and was placed in 1mL of 1:1 ethanol:water solution and shaken for 15 minutes. Ketamine was present in the extract, allowing the analysis of residue left in a bottle or glass. A similar experiment was conducted after discarding the ketamine and rinsing the glass once with water before allowing drying. The resulting spectrum did not show the presence of ketamine. Therefore, ketamine analysis is not feasible if the glass has been washed.

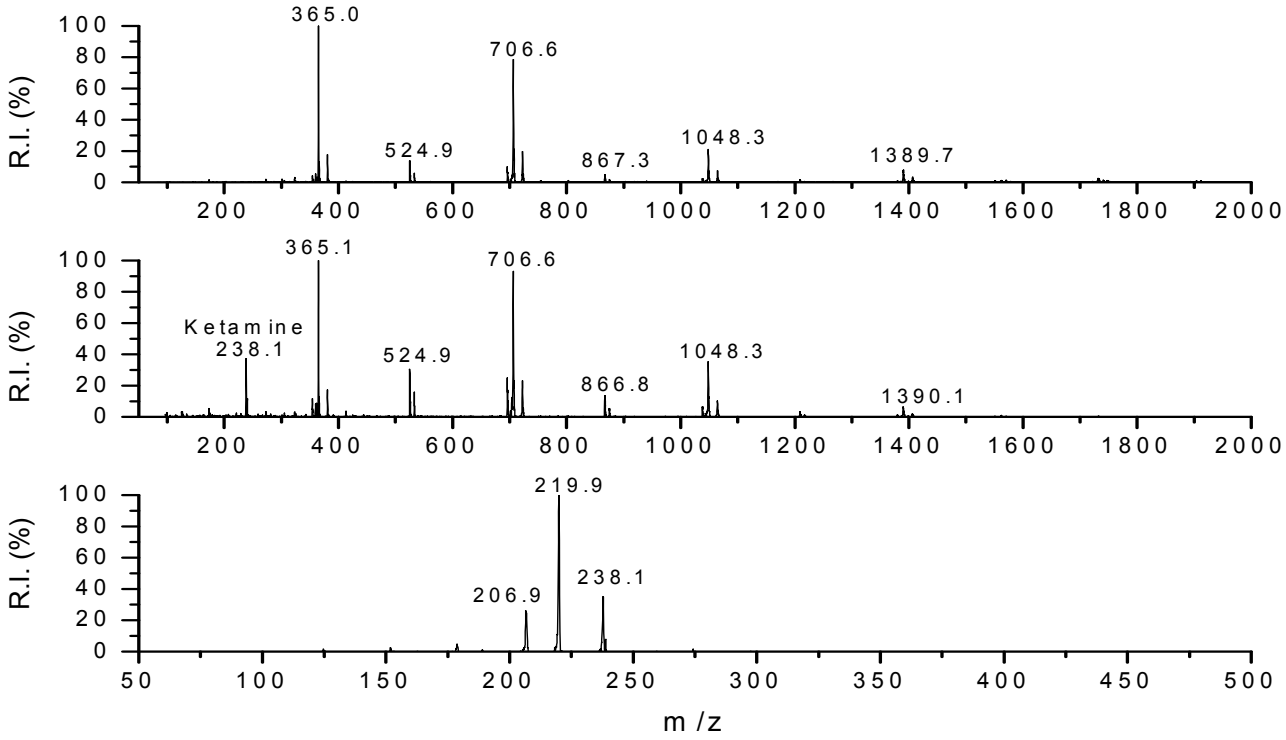


Figure 3.7: Positive mode ESI-MS of Most Wanted™ Pioneer whiskey bourbon style (upper spectrum) and ketamine spiked Most Wanted™ Pioneer whiskey bourbon style (middle spectrum) and CID of ketamine in Most Wanted™ Pioneer whiskey bourbon style (lower spectrum)

Detection limits were determined by running a series of dilute spiked alcohol samples as well as a series of dilute standards. Since a normal dose of ketamine is 200-400mg in an alcoholic beverage, the concentration of ketamine will be at least on the millimolar scale. Detection of ketamine was shown down to 100 picomolar concentrations in all of the alcoholic beverages studied as well as for the stock standard solution. This represents an increase of detection limits from the previous urine analysis methods by 2 orders of magnitude (by molarity). Stability studies were also conducted under ambient conditions. Samples and standards were prepared and kept in capped volumetric flasks on the lab bench. New standards were made for each

stability point. Typically samples obtained and sent to a forensics lab will be analyzed in 48 hours or less. Standards and samples showed stability for 14 days with less than a 5% decrease in concentration (molarity) under bench top conditions. Bench top conditions for this study are defined as solutions in a clear, capped vial or volumetric flask sitting on the laboratory bench top under normal lighting, pressure, and temperature conditions.

This method can be applied to other date rape drugs as well. Below in Figure 3.8, you can see the presence of flunitrazepam (roofies) in the Most Wanted™ Pioneer whiskey. Test for 1,4-butanediol has also shown the presence of the drug distinguishable from the matrix.

3.4: LC of Ketamine in Alcohol Matrices

Using an isocratic LC system, ketamine was successfully separated from the alcohol matrices. A gradient LC was not used due to lack of availability, but it is suspected that faster run times may be achieved with a gradient system. Under the conditions described in Chapter 3, ketamine eluted at roughly 3.8 minutes (Figure 3.9) and showed no interference from the alcohols and mixed drinks used. Alcohol solutions were then prepared with varying amounts of ketamine added. A calibration curve was constructed by plotting absorbance versus concentration and was used to quantify samples of known concentration. The concentration of the samples calculated from the calibration curve had less than a 2% error.

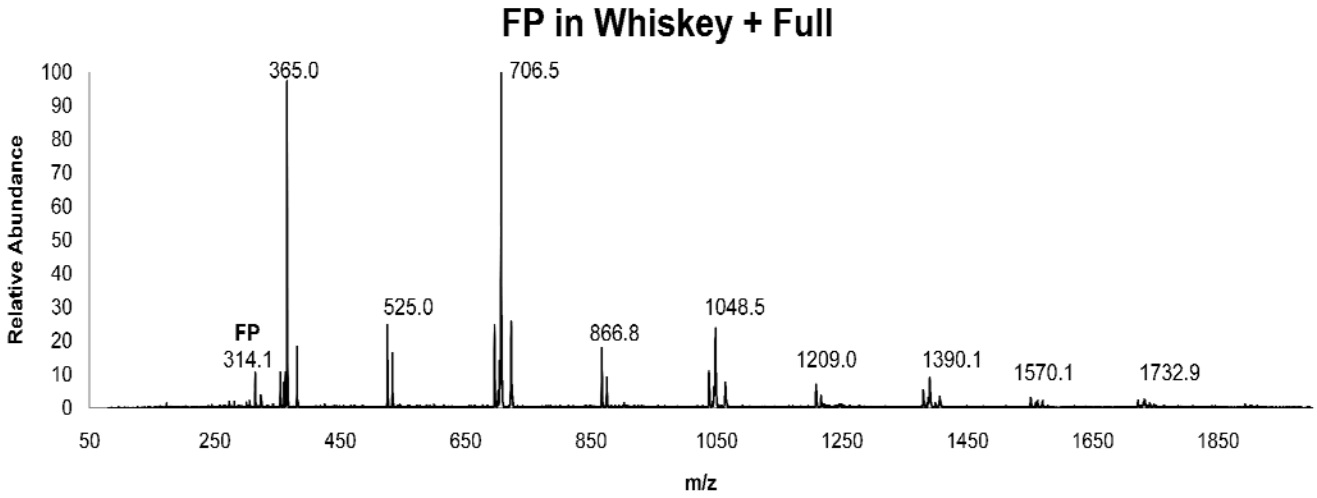


Figure 3.8: Flunitrazepam in Most Wanted™ Pioneer Whiskey

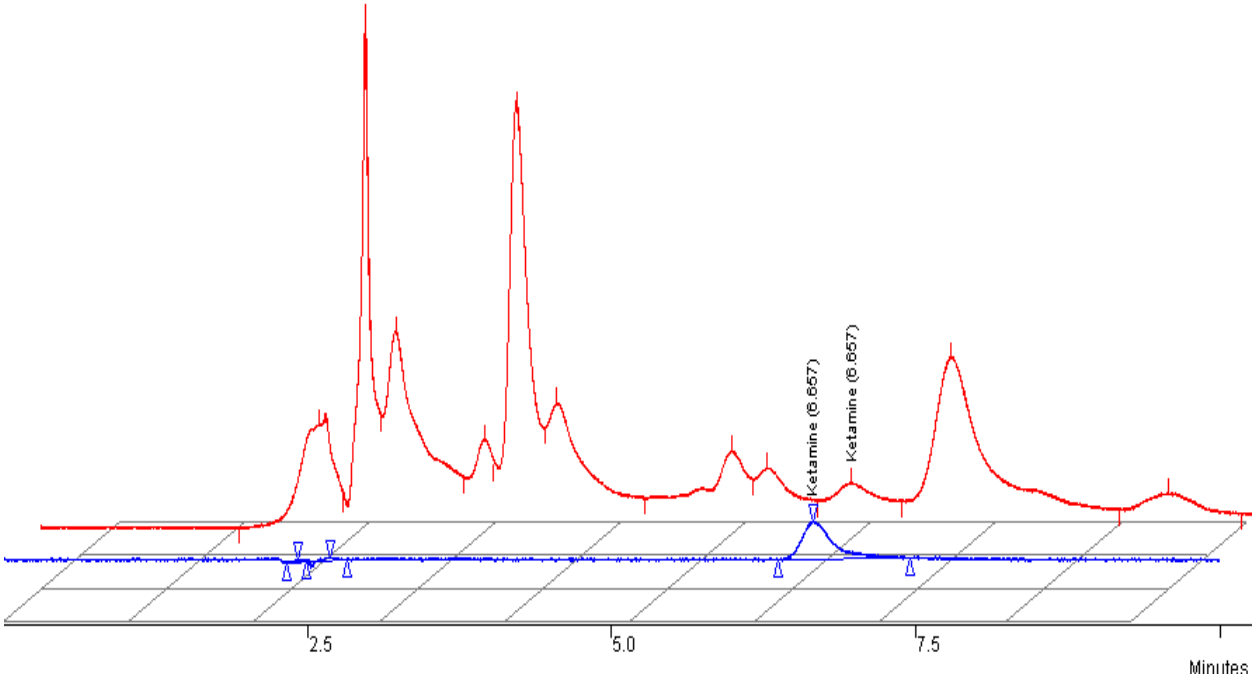


Figure 3.9: Chromatogram of ketamine standard (blue) and ketamine spiked Jim Beam™ Whiskey (red)

CHAPTER 4

KETAMINE SUMMARY

ESI mass spectra were collected directly from samples of gin, rum, vermouth, vodka, whiskey, and tequila as well as from samples of mixers used to make up mixed drinks (cola, tonic water, and lemon juice) and mixed drinks. The ESI mass spectra derived from the range of samples are distinct, thus demonstrating the ability to use ESI-MS to distinguish between different types of alcoholic beverages. Different brands of the same type of liquor show spectra that are distinct from each other. Two different lots of the alcohols were tested and the results showed similar mass spectra between each lot, which makes it possible to differentiate not only the type of alcohol but also the brand. The CID profile of ketamine showed the initial loss of water and methylamine. This CID profile along with MS^3 allowed for indisputable determination of ketamine in the matrix of the alcohols, mixers, and mixed drinks. Quantification is possible using ESI-MS since there is a direct correlation between signal intensity and the concentration of ketamine added to the solution. A calibration curve was constructed using signal intensity versus concentration of ketamine and showed less than a 2% error on samples of known concentration. Ketamine can also be detected in the 100 pM range for all samples and is stable for at least 14 days under benchtop conditions. Analysis of residue left on a glass after the alcohol also showed the presence of ketamine, making it possible to test for ketamine from a glass that has not been washed. Using a simple isocratic LC system, ketamine was successfully separated from the alcohol matrices using a reverse phase C18 column and an acetonitrile/water/TFA mobile phase. Total run time for the LC run was 9 minutes with ketamine eluting at roughly 3.8 minutes. UV

absorbance at 268nm can be used to quantify ketamine in alcohol samples by constructing a calibration curve of absorbance versus concentration. Quantification of samples of known concentration showed less than a 2% error. In general, our study shows that ESI-MS and LC can be used to detect and quantify ketamine at levels much lower than a normal dose given by a spiked drink, and do so with minimal sample preparation.

One of the major advantages of this method compared to previous methods is the ability to delay testing for up to two weeks. Even in the acidic environments of the cola and lemon juice, the ketamine was fairly stable. Also, this method demonstrates a significant improvement in detection limits over previous methods – two orders of magnitude lower (in molarity). Since the method is simple, effective, and fast and most forensic labs have an LC, this method is also cost effective. The biggest downfall to the method is the requirement to obtain a sample of the drink or the glass before it is washed.

CHAPTER 5

CLOMIPHENE INTRODUCTION

5.1: Endocrine Disruptors

The human body uses two main channels to send communications from the brain to the rest of the body – the nervous system and the endocrine system. The nervous system sends electrical impulses through nerves to allow the body to rapidly respond to stimuli. The endocrine system acts more slowly by releasing hormones into the blood stream that act as messengers. Hormones travel through the blood stream until they bind to a specific receptor and the binding of the hormone causes a targeted response. It has been suggested that some exogenous chemicals, called endocrine disruptors, can interfere with the endocrine system by binding to the hormone receptors and either inhibiting the binding of the endogenous hormone or by acting in a similar fashion to endogenous hormone. Since hormones are released in very small quantities naturally, it would only require a small amount of an endocrine disruptor to interfere with the endogenous hormones [41,42].

Compounds such as diethylstilbestrol, bisphenol A, and genistein are classified as estrogens and endocrine disruptors [43]. DES was first synthesized in 1938 and was approved for use in clinical settings by the FDA in 1941 and was used until the late 1980's [44,45,46]. DES was prescribed for many things including estrogen replacement therapy and to prevent miscarriages. DES was generally considered safe for the mother and the developing baby, but in 1971 the FDA published a FDI Drug Bulletin stating that DES was linked to a rare form of vaginal cancer in the daughters that were

exposed to DES in-utero [47]. DES has since been linked to many detrimental side effects for both first and second generation exposure. First generation users of DES show a slight increase in breast cancer rates. Most side effects of DES are experienced by second generation DES exposure, though. Women exposed to DES in the womb are termed DES daughters, and men exposed to DES in the womb are designated as DES sons. DES daughters have increased rates of reproductive tract structural abnormalities, infertility, pregnancy complications, and certain types of cervical and vaginal cancer, and auto-immune diseases [48,49]. Moreover, in studies conducted by a May and co-workers, exposure of DES has shown to increase the number of polyovular follicles in the ovaries (Figure 5.1). The follicle is the basic unit of female reproductive biology. Each follicle normally contains a single ovum, but as can be seen in Figure 5.1, each of the three drugs produced follicles that contain more than a single ovum [50].

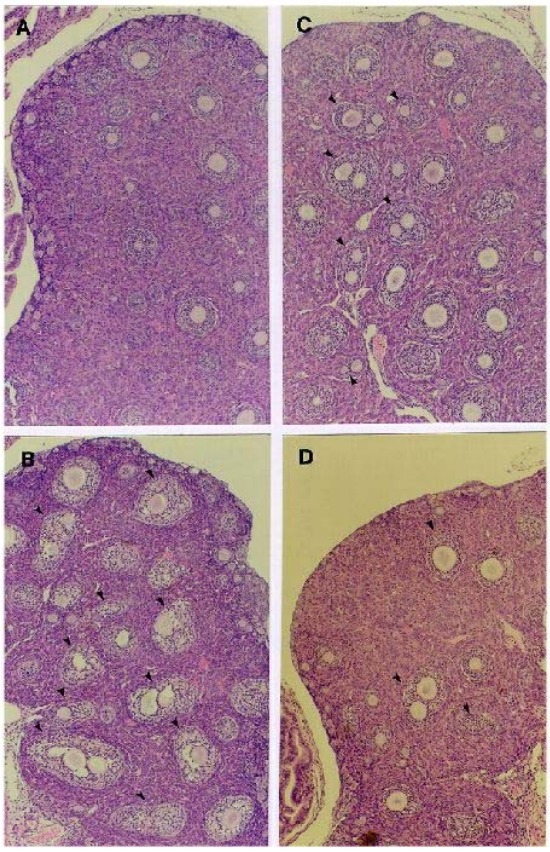


Figure 5.1: Hamster ovaries from animals exposed to A) control, B) DES, C) bisphenol A, and D) genistein [50]

5.2: Clomiphene Citrate

Clomiphene is a selective estrogen receptor modulator that is structurally related to DES (Figure 5.2).

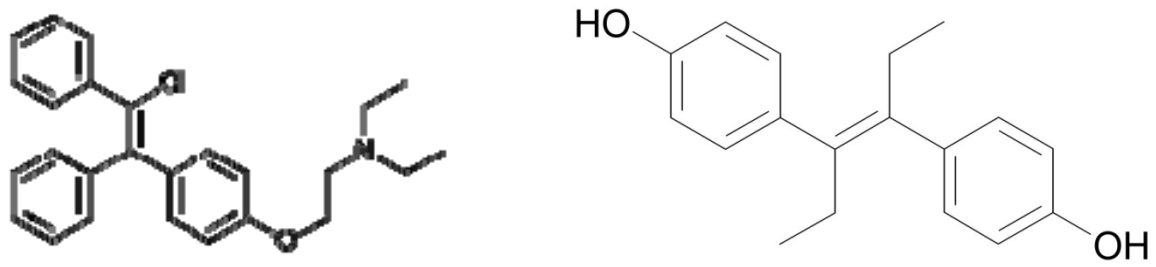


Figure 5.2. Structures of Clomiphene (left structure) and DES (right structure)

CC was synthesized in 1956 and was approved for clinical use in 1967 and has been used extensively as a first tier infertility treatment to treat irregular or anovulation [51]. It consists of racemic mixture of its two isomers, zuclomiphene (z-form) and enclomiphene (e-form) [52]. Zuclomiphene has shown to be the more active isomer. CC binds to an estrogen receptor at the hypothalamic/pituitary axis where it inhibits the binding of the more potent endogenous estradiol-17β [53]. This causes the hypothalamus to sense that there is too little estrogen and release gonadotropin releasing hormone. GnRH acts by stimulating the pituitary to release gonadotropins. The gonadotropins, leutinizing hormone and follicle stimulating hormone, then drive ovarian follicle development [54,55,56]. By this action, clomiphene acts as an anti-estrogen and coupled with intercourse or insemination is an effective infertility treatment.

5.3: Clomiphene Citrate acts as an Endocrine Disruptor

Although CC has been used extensively as an infertility treatment for the past 40 years and the action of CC at the hypothalamus is known, little is known about how it affects the ovary. Also, it is not known to what extent a developing fetus is exposed to the drug as it is not known how long CC remains in the mother's system. Since CC is related to DES, it could possibly be classified as an endocrine disruptor and it is important to understand the affects CC has on a developing baby. In preliminary study by May and co-workers [57], female hamster pups were injected with differing concentrations (0, 1, 10, or 100 μg) of CC within 6 hours of birth. The hamster pups were euthanized at 21 days and the ovaries were isolated and analyzed. As can be

seen in Figures 5.3 and 2.4, there is an increase of polyovular follicles in the hamster ovaries. Comparing Figure 5.4 to Figure 5.2 the ovaries from hamster treated with CC show a striking resemblance to those treated with DES, bisphenol A, and genistein. This suggests that CC may act as an endocrine disruptor and may have similar detrimental side effects as the other estrogens.

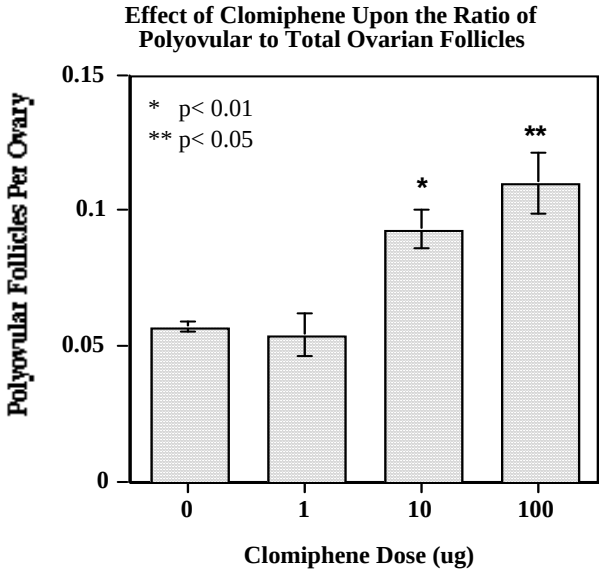


Figure 5.3: Polyovular follicles in hamster ovaries exposed to CC [57]

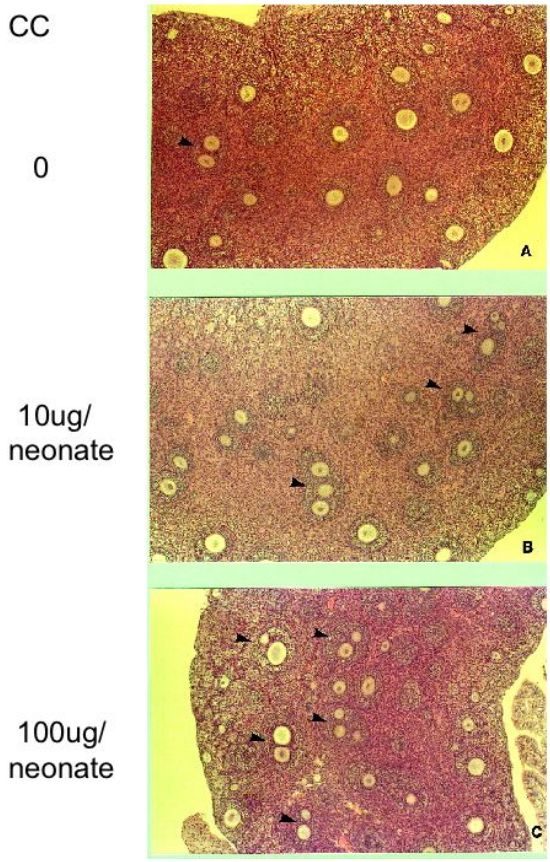


Figure 5.4: Typical 21 day ovary follicle content of control ovary (A), ovary from a hamster treated with 10 µg CC (B), and ovary from a hamster treated with 100 µg CC (C) [57]

CC has been shown to have disruptive behavior in the animal model, but it is difficult to directly correlate these findings to a human model. One major problem is that there is little known about how much CC a fetus is actually exposed to. In this study, we aim to determine the extent of fetal exposure to clomiphene and to determine how long clomiphene stays in the mother's system. A recently published pharmacokinetic study found the half life for a single 50 mg dose of clomiphene to be five days. However, detectable levels of clomiphene were still found in the feces after twenty-one days. Since a normal clomiphene regiment includes the consumption of 50 – 250 mg pills for

5-7 days in the early stages of the menstrual cycle for up to 6 continuous months, significant fetal exposure seems likely [58]. Extraction and analysis of clomiphene from serum samples should be viable to validate the half life of clomiphene in vivo. Meconium analysis will be used to determine the extent of fetal exposure. Meconium is the material contained in the GI tract that is normally excreted after the baby is born. Since the GI tract of a fetus is basically inert, the meconium represents most of what the fetus was exposed to while in the womb. Meconium analysis has already been used to determine exposure to different drugs, pesticides and heavy metals, and it is expected that meconium analysis should be feasible for the determination of fetal CC exposure [59,60,61,62,63,64].

CHAPTER 6

CLOMIPHENE MATERIALS AND METHODS

6.1: ICH Guidelines

The method presented here will address in whole or in part the International Conference of Harmonisation guidelines to the validation of analytical procedures. There are eight different areas that must be tested for a method to be valid for use on pharmaceuticals or human use. Since these methods are not used in Good Manufacturing Practice work environments or used for pharmaceutical use, the methods do not have to undergo as stringent of testing. Nevertheless, these guidelines set in place were followed. The nine different areas of testing include: specificity, linearity, range, accuracy, precision, detection limits, quantification limits, and robustness. Specificity is the ability to assess the analyte among the presence of other compounds that would normally be expected to be present. Accuracy is the measure of the closeness of agreement between the true value and the experimental value. Precision is the closeness of agreement between a series of measurements from the same sample under the same conditions. Detection limits for a procedure is the lowest detectable amount of the compound in question. Quantification limits is the lowest amount of analyte that can by quantitatively determined with suitable precision and accuracy. Linearity is defined as the ability to obtain results which are directly proportional to the concentration of the sample. Range is the upper and lower concentrations of an analyte that gives suitable linearity, precision, and accuracy.

Robustness is the ability of the method to remain unaffected by small, deliberate changes in the testing parameters.

6.2: Sample and Standard Preparation

Tamoxifen Citrate was synthesized by Stuart Pharmaceuticals and purchased through Fisher Scientific. Clomiphene Citrate (43% cis isomer – enclomiphene - and 57% trans isomer - zuclomiphene) was obtained from Sigma-Aldrich. Fetal calf serum was obtained from Dr. Jeffrey May, Department of Biological Sciences, Wichita State University. Methanol (HPLC grade) and methyl *tert*-butyl ether (HPLC grade) were purchased from Fisher Scientific. A 10^{-3} M stock standard of Clomiphene citrate was made by adding 0.0598 g of CC to a 100.0 mL volumetric flask and diluting to volume with 50/50 methanol/water mixture. The 10^{-3} M tamoxifen citrate stock standard was made by adding 0.0563 g of TC to a 100.0 mL volumetric flask and diluting to volume with 50/50 methanol/water mixture. Subsequent standards were made by serial dilution of the stock standards. Extraction samples were prepared by vortex mixing 500 µL of fetal calf serum or meconium with 500 µL of the stock standard or a dilution of the CC stock standard to a 5 mL capped vial. Extraction was performed by adding 1000 µL of methyl *tert*-butyl ether to the 5 mL vial with the spiked fetal calf serum and mixing for 1 hour. After 1 hour of mixing, the sample was centrifuged for 15 minutes at 3300 rpm on a Fisher Scientific Centrific Model 330 centrifuge. The extract sample was then added to a separatory funnel. The tBME and the spiked fetal calf serum formed two layers with the organic layer on top. The organic layer was collected in a 10 mL test tube and the tBME was evaporated under a stream of argon gas. The solid clomiphene extract

was reconstituted in 1000 µL of 50/50 methanol/water. Sample was then analyzed by LC-UV or ESI-MS^n.

6.3: ESI-MS^n Parameters

Electrospray ionization mass spectra were collected using a Finnigan LCQ-DECA^{TM} ion trap mass spectrometer (San Jose, CA, USA). Samples were infused into the ESI-MS instrument using the incorporated syringe pump with a flow rate of 5 mL/min. The atmospheric pressure ionization stack settings for the LCQ (lens voltages, quadrupole and octapole voltage offsets, etc.) were optimized for maximum transmission to the ion trap mass analyzer by using the auto-tune routine within the LCQ Tune program. Following the instrument tune, the spray needle voltage was maintained at +5kV, and the N_2 sheath gas flow was maintained at 25 units (arbitrary for the Finnigan systems, corresponding to approximately 0.375 L/min). The capillary (desolvation) temperature was held at 200º C. The ion trap analyzer was operated at a pressure of approximately 1.5 x 10^{-5} Torr. Helium gas, admitted directly into the ion trap, was used as the bath/buffer gas to improve trapping efficiency and as the collision gas for collision induced dissociation experiments.

The CID experiments were performed as follows. The ions were isolated using an isolation width of 2.0 mass to charge units. The normalized collision energy, which defines the amplitude of the radiofrequency energy applied to the end-cap electrodes in the CID experiment, was set to 35%, which corresponds to approximately 1.06V. The activation Q (used to adjust the q_z value for the precursor ion) was set at 0.30. The activation time was set to 30 ms.

6.4: UV/Vis Spectroscopy Parameters

Ultraviolet/Visible spectroscopy was carried out on a Hitachi U-2010 dual beam spectrophotometer with a tungsten-iodine visible spectrum lamp and a deuterium UV spectrum lamp. A wavelength scan was performed from 1100 nanometers (nm) to 190 nm with a scan rate of 200 nm/min and a lamp change at 370 nm. Quartz cells with a path length of 1 cm were used for absorbance measurements. Samples were prepared by diluting 100 µL of the stock standard solution with 3400 µL of deionized water. Samples were run against a deionized water baseline.

6.5: LC Parameters

Isocratic liquid chromatography was performed on a Varian ProStar system. The system consisted of one binary Varian model 210 pump, a Varian model 320 UV/Vis detector, a 20 µL loop, and a reverse phase Waters Atlantis dC18 5µM 4.6x150mm column. The mobile phase consisted of 80% acetonitrile (HPLC grade), 20% deionized water and 0.1% trifluoroacetic acid (99.9+%, Reagant Plus grade). Mobile phase flow rate was set to 1.0 mL/min. Total run time was 5 minutes with clomiphene eluting at 3.86 minutes. UV absorbance detection was used with the lamp set at 235 nm.

6.6: Quantitative Analysis

Quantification of MS samples was carried out by running a series of standards and constructing a calibration curve by plotting the (Intensity of clomiphene peak)/(Intensity of tamoxifen peak) versus the concentration of clomiphene in molarity. A linear trend line was obtained using least squares linear regression. Concentrations of known samples were confirmed using the equation of the linear trend line. Precision was determined by analysis of multiple replicates of a known concentration of a sample.

Precision was measured as the percent coefficient of variance. Percent error (E%) was calculated to determine accuracy.

Quantification of LC samples were carried out by running a series of standards and integrating under the peak to obtain the area under the peak. Integration was performed by the Varian ProStar software suite. A calibration curve was constructed by plotting the Area of the clomiphene standard versus the clomiphene concentration in molarity. A linear trend line was obtained using least squares linear regression. A linear trend line in the form of y=mx + b (where m=slope, b=y-intercept, and y and x are the y and x values on the graph, respectively) was obtained using least squared linear regression. Concentrations of known samples were confirmed using the equation of the linear trend line. Precision was determined by analysis of multiple replicates of a known concentration of a sample. Precision was measured as the %CV. E% was calculated to determine accuracy.

CHAPTER 7

EXTRACTION, DETECTION AND QUANTIFICATION BY LC AND ESI-MS^N OF CLOMIPHENE CITRATE IN FETAL CALF SERUM AND MECONIUM

7.1: ESI-MS^n of Clomiphene Citrate

Initially, clomiphene in 50/50 methanol/water was studied by collecting ESI-MS on the 1mM stock standard. The dominant peak in the ESI-MS spectrum is at 406.0 m/z which corresponds to the clomiphene $[M+H]^+$ parent ion. As well, there is a ^{37}Cl isotope peak at 408.0 m/z at approximately 30% abundance relative to the 406.0m/z peak. Upon CID of clomiphene (406.0 m/z), the dominant ion is a loss of 109 mass units at 297.0m/z. There is also a second ion with a loss of 73 mass units at 333.0 m/z and a third ion loss of 36 mass units at 370.1 m/z that are at approximately 10% relative abundance (Figure 7.1). Further CID (MS^3) of the 333.0m/z fragmentation peak showed the major loss of 36 mass units at 297.0 m/z. MS^3 of the 370.1 m/z fragmentation peak showed the major loss of 73 mass units at 297.0 m/z. The loss of 36 mass units corresponds to the loss of HCl and the loss of 73 mass units corresponds to the loss of $C_4H_{11}N$ (Figure 7.2). The loss of 109 mass units would correspond to the loss of both HCl and the amine. Rationale for this fragmentation is provided by the MS/MS spectra and the MS^3 spectra for the 370 and 333 fragmentation peaks. Using an isolation width of 4, the MS/MS spectra of clomiphene shows the losses of 36, 73, and 109. However, the loss of 73, which corresponds to the loss of the terminal amine, still retains the mass peak 2 units heavier at 335 m/z that would correspond to the ^{37}Cl isotope peak – suggesting that this fragment retains the chlorine atom. The peaks

relating to the losses of 36 and 109 do not show the ^{37}Cl isotope peak which suggests that the fragments have lost the chlorine atom. As well, since the MS^3 of both the 370 and 333 fragments give rise to the 297 fragment, it is likely that the loss of 109 is the loss of both the chlorine and the terminal amine. This CID profile of clomiphene was used to positively identify clomiphene in the serum samples

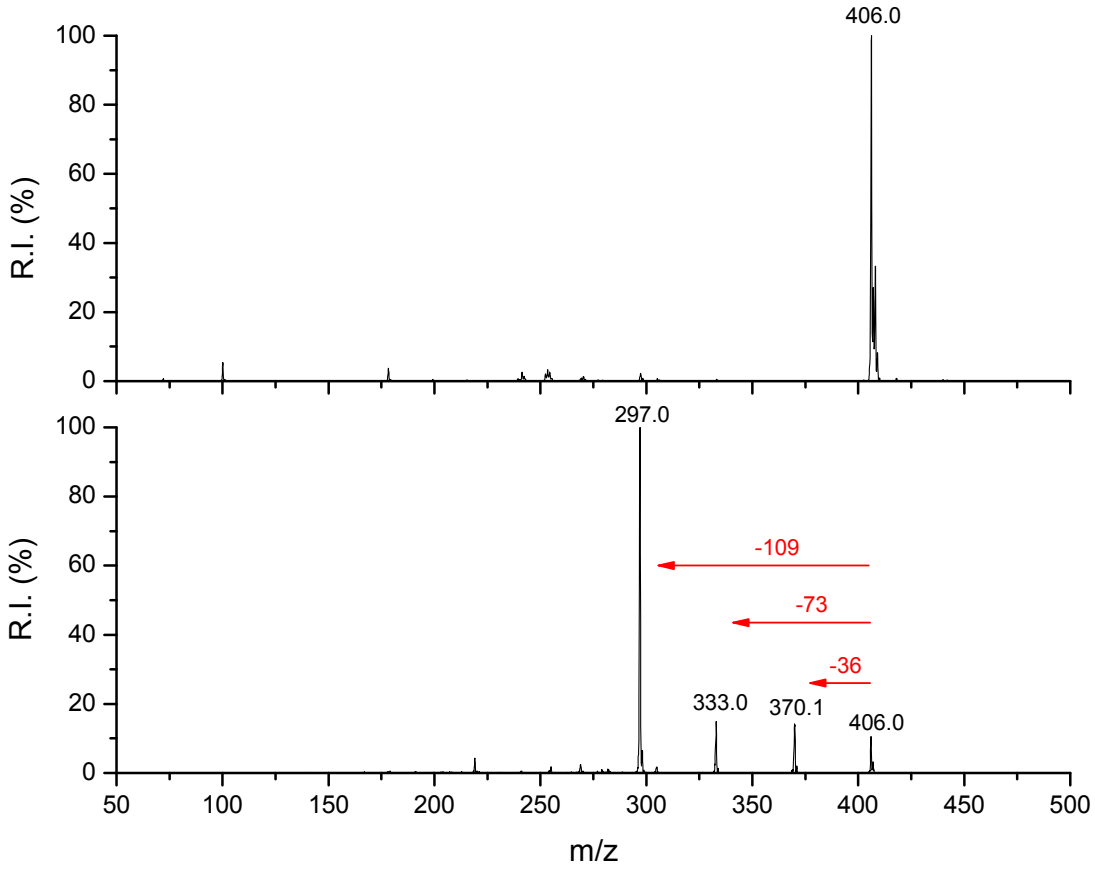


Figure 7.1: Full MS of clomiphene stock standard (top spectrum) and MS/MS of clomiphene stock standard (lower spectrum)

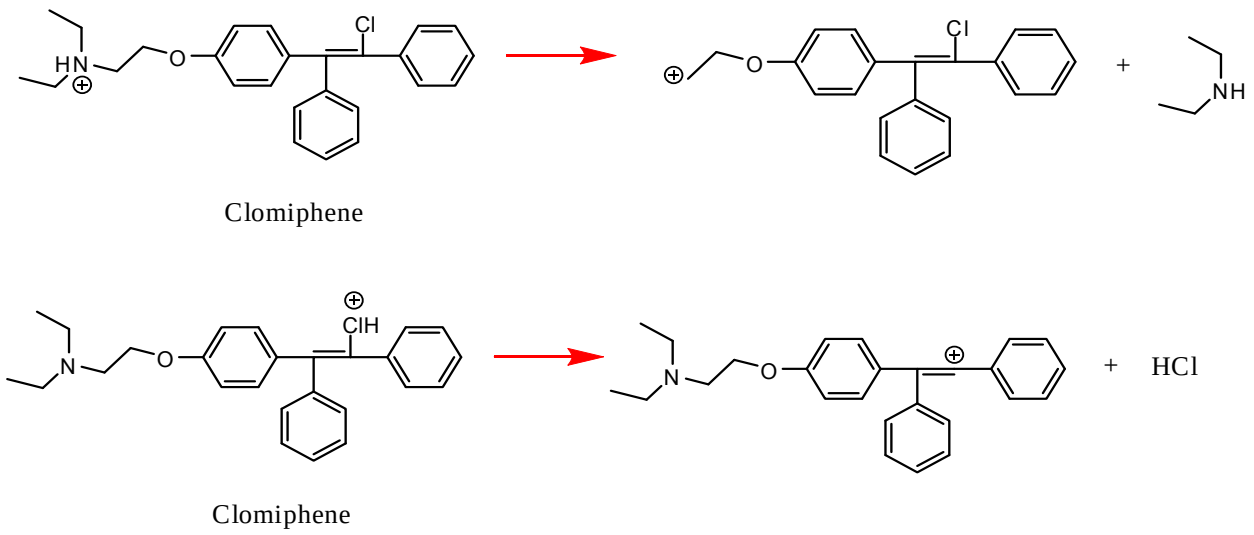


Figure 7.2: MS/MS fragmentation pathways of clomiphene

7.2: Extraction of Clomiphene Citrate from Fetal Calf Serum and Meconium

Attention was then shifted to developing methods for extracting clomiphene from fetal calf serum and meconium and the subsequent analysis by MS and LC-UV. Extractions were initially performed on a microscale level using 250 µL of serum vortex mixed with 250 µL of CC stock standard and 500 µL of tBME. The extraction method proved to be specific for clomiphene in fetal calf serum (Figure 7.3) and meconium (Figure 7.4).

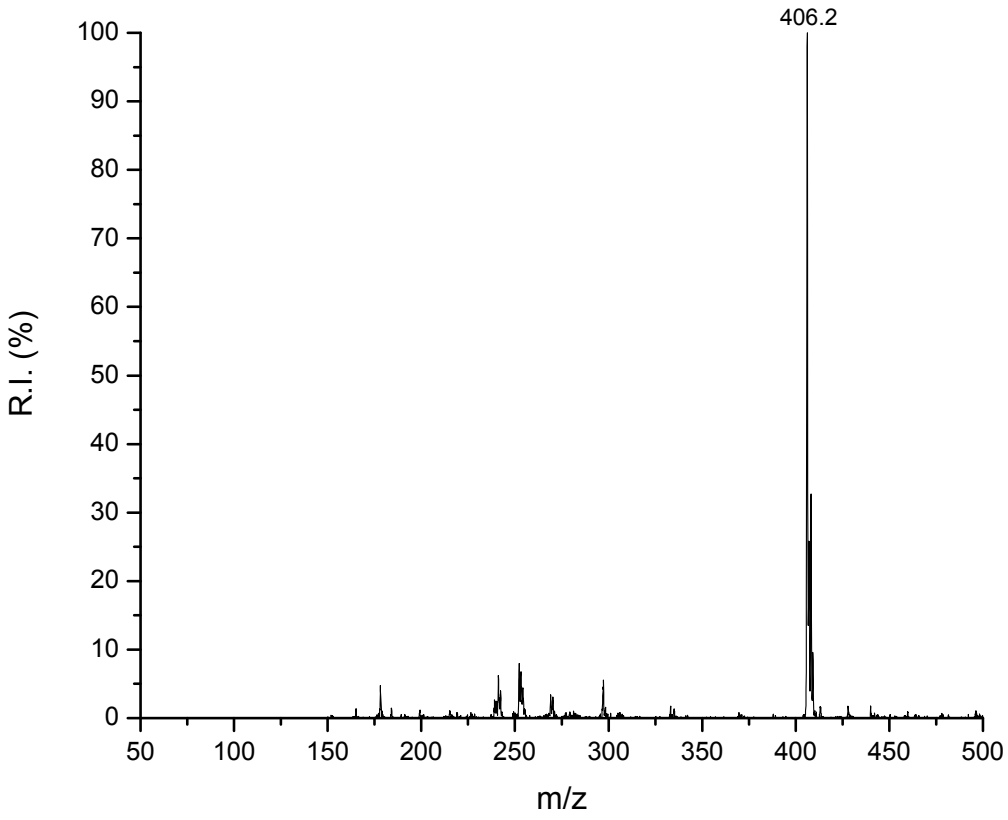


Figure 7.3: MS of clomiphene extracted from fetal calf serum

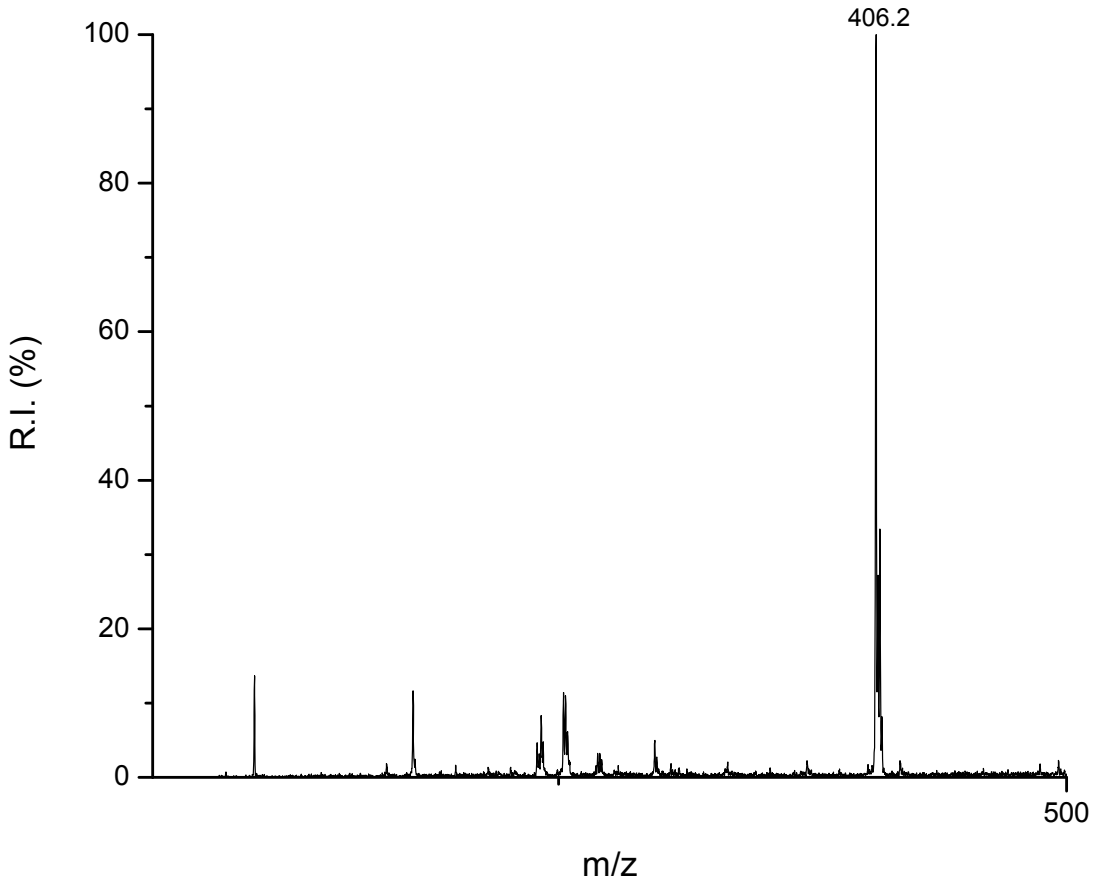


Figure 7.4: MS of clomiphene extracted from meconium

Differing shake times for the extraction process were tested. Times included were 5 minutes, 10 minutes, 15 minutes, 30 minutes, 60 minutes, 90 minutes, and 120 minutes. Also, sonication was substituted for shaking at the same time points. Using HPLC-UV quantitative analysis, the 60 minutes shake time showed to have the optimal extraction efficiency with the higher shake times giving no improvement to the extraction efficiency. Precision measurements for the microscale procedure were not acceptable by giving CV% of up to 40%, though, for the fact that during the transfer of the

extraction solution after centrifugation to a separatory funnel, it is easy to lose a small amount of the extract and any amount lost can lead to significant error due to the small amount of analyte in the extract to begin with. The macroscale procedure detailed in Chapter 6 proved to be much more precise (Table 7.1).

7.3: Quantitative Analysis of CC using ESI-MS

Quantification was then carried out by using tamoxifen citrate as an internal standard. A series of standards were made by holding the concentration of tamoxifen constant and changing the concentration of clomiphene in the standards. Several extraction samples were made by the macroscale extraction method, but were reconstituted in 1000μL of 50/50 methanol/water spiked with tamoxifen citrate. A calibration curve was constructed by plotting the (intensity of the clomiphene peak)/(intensity of the tamoxifen peak) versus the concentration of clomiphene (Figure 7.5).

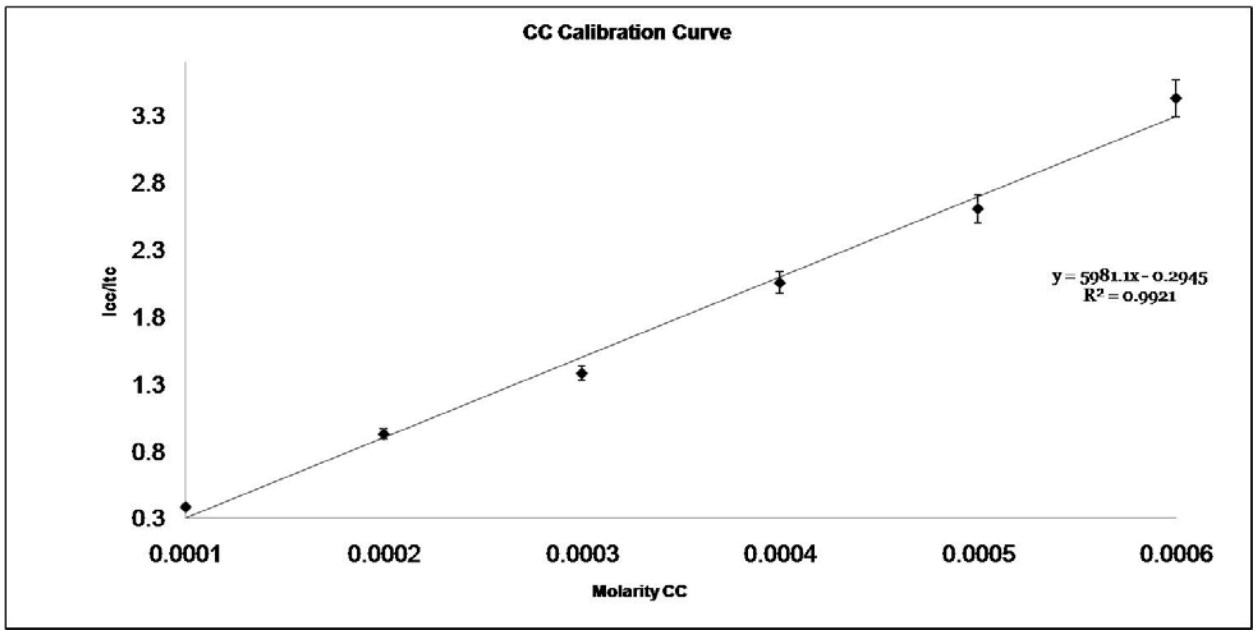


Figure 7.5: Typical calibration curve for the quantifying of clomiphene by MS using tamoxifen as an internal standard

Using tamoxifen as an internal standard showed to produce good precision and accuracy results. Table 7.1 shows typical precision and accuracy results for the quantification of clomiphene by MS. All results thus far have given CV% and % error of less than 10%. Linearity was also measured by using tamoxifen spiked clomiphene standards and samples. Thus far, clomiphene has given a linear response from the mM to the nM range. Detection limits and quantification limits were determined by making and running a series of standards and extraction samples from mM to pM ranges and comparing them to a blank sample or standard. Signal to noise ratio for the detection limit must be about 3:1 and 10:1 for quantification limits, and in this case the average signal to noise ratio for the 1 pM samples and standards was 6:1 and the average signal to noise ratio for 10 pM samples and standards was 9:1. Therefore, the quantification

limit for the MS was approximately 10 pM and the detection limits were at least 1 pM of clomiphene (Figure 7.6).

Clomiphene Concentration (mM)	Co-efficient of Variation (%)	% Error	Replicates
0.25	2.1	1.53	3
0.20	5.1	3.87	3
0.15	3.6	6.77	3

Table 7.1: Precision as co-efficient of variation (%) for the quantification of clomiphene by MS using tamoxifen as an internal standard

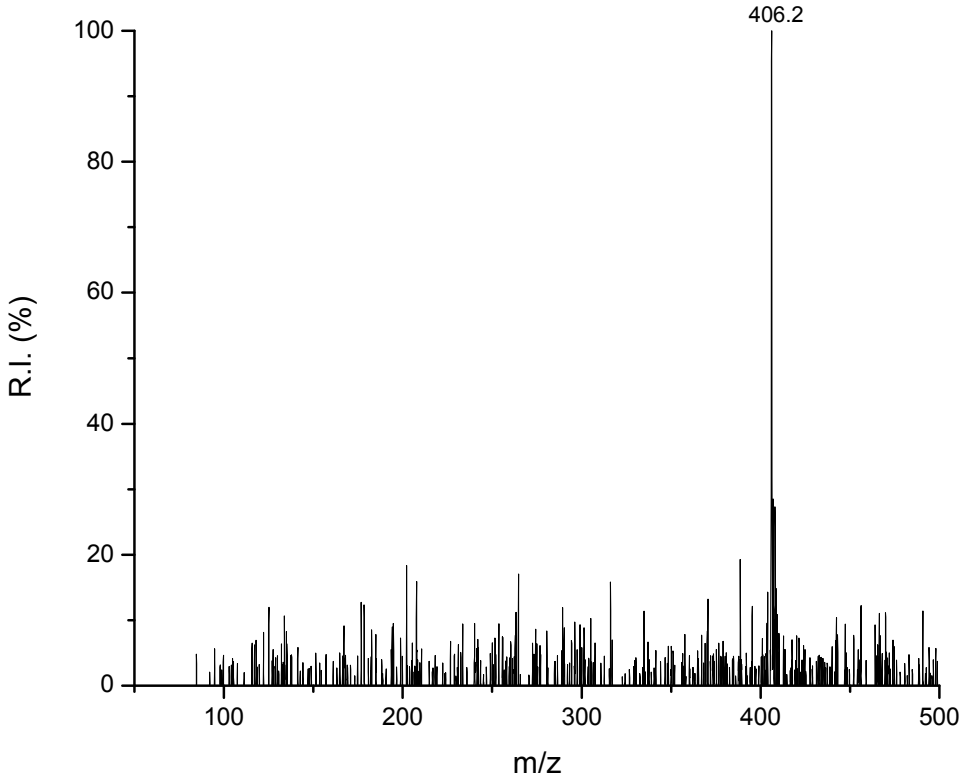


Figure 7.6: 1 picoM clomiphene standard with a signal to noise ratio of 6:1

7.4: LC Analysis of Clomiphene Citrate

Using isocratic LC, clomiphene was successfully separated from the rest of the fetal calf serum extract. Initially, the UV/Vis spectrum was obtained from clomiphene (Figure 7.7). A local maximum at 235 nm was used to quantify clomiphene in the chromatograms.

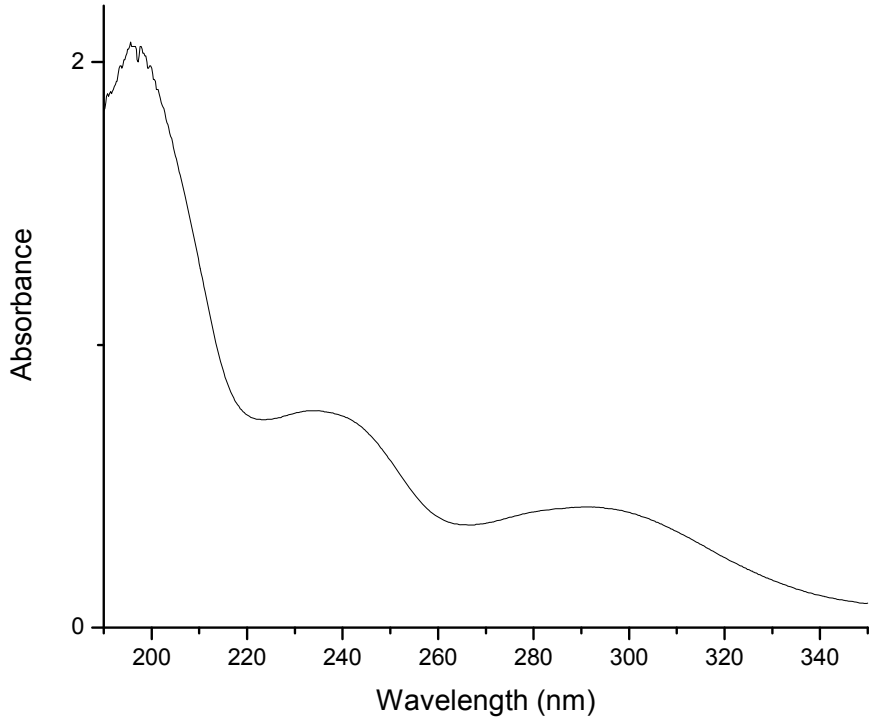


Figure 7.7: UV/Vis spectrum of Clomiphene citrate in 50/50 methanol/water

The LC method proved to be specific for clomiphene in fetal calf serum and meconium. Figure 7.8 shows the chromatogram of a FCS extraction sample with the integration of the clomiphene peak. Figure 7.9 shows the chromatogram of a meconium extraction sample. Quantifying clomiphene was carried out by running a series of clomiphene

standards and integrating the area under the clomiphene peak in the chromatogram. A calibration curve was constructed by plotting the area under the clomiphene peak in mV versus the concentration of clomiphene in molarity (Figure 7.10). By quantifying a series of samples, the LC method was linear over a range of 100 nM to 1 mM. Detection limits and quantification limits were carried out by running a series of standards and samples from 1 mM to 1 picoM. Detection limits were determined by signal to noise ratio as with the MS method. 10 nM concentrations gave an average signal to noise ratio of about 3:1, making 10 nM the detection limit. 100 nM samples gave an average signal to noise ratio of about 8:1, making 100 nM an approximate quantification limit. Precision and accuracy were determined by running a series of standards and quantifying several extraction samples. Precision for all samples for all runs was under 8%. The method showed to be very accurate. Percent error for all samples was under 2% (Table 7.2).

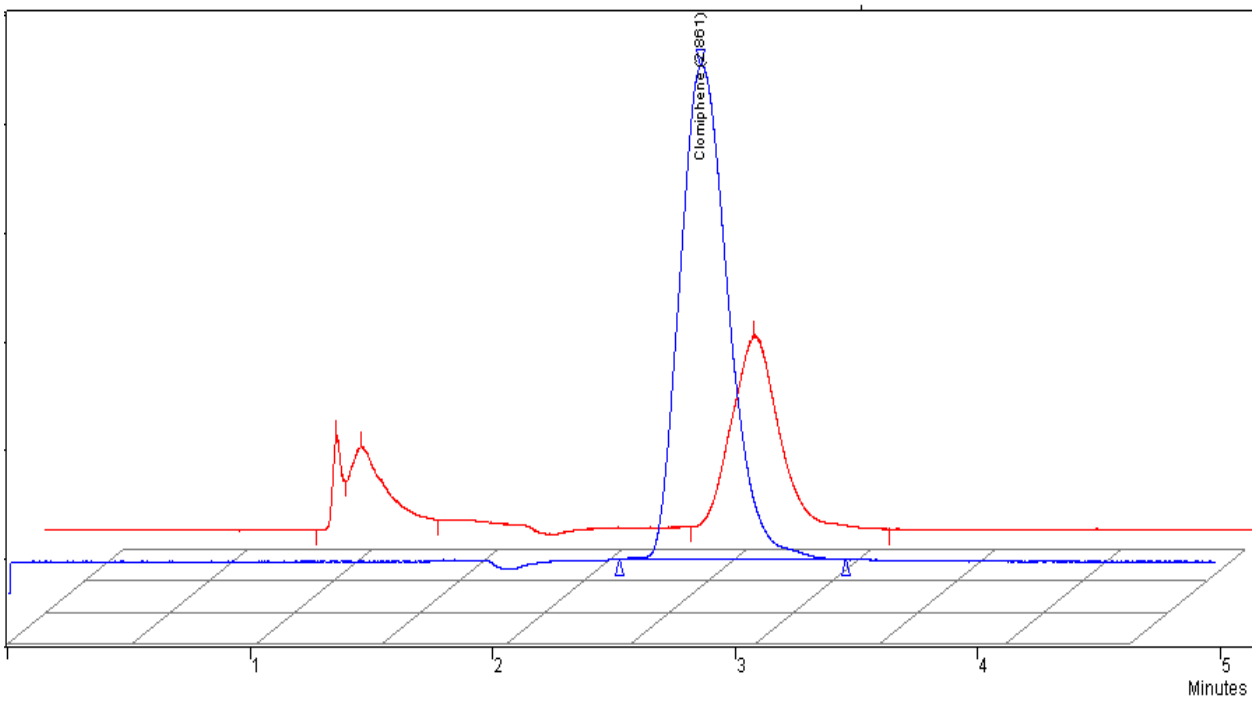


Figure 7.8: Chromatogram of a clomiphene standard (blue) and extraction sample from FCS (red)

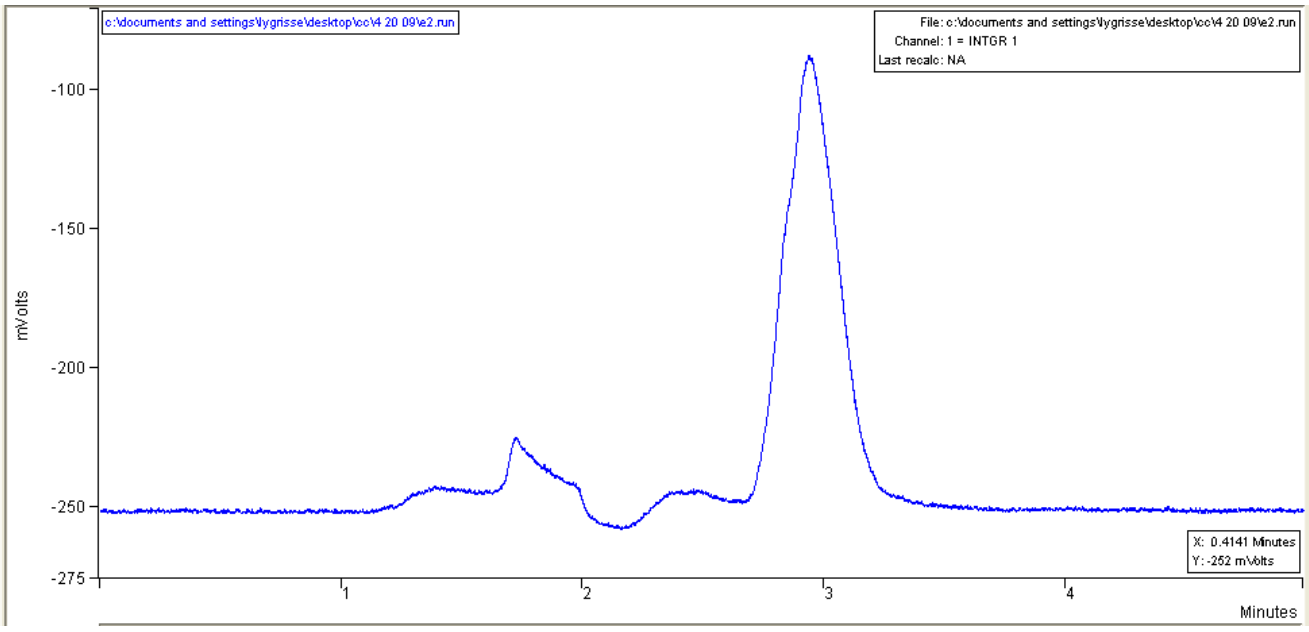


Figure 7.9: Chromatogram of a clomiphene extraction sample from meconium

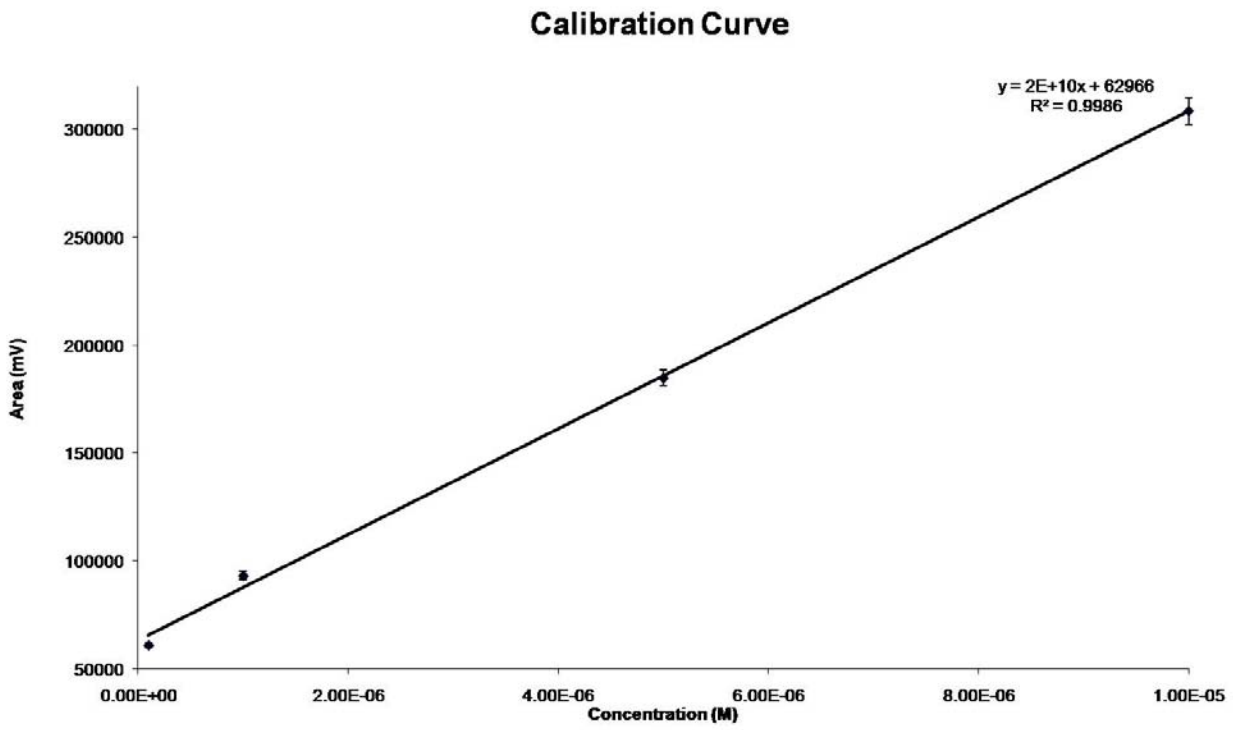


Figure 7.10: Calibration curve for quantifying by LC-UV

Clomiphene Concentration (mM)	Co-efficient of Variation (%)	% Error	Replicates
0.50	6.82	1.76	3
0.40	3.58	0.32	3
0.30	7.02	1.00	3

Table 7.2: Typical precision and accuracy results for the LC method of quantifying clomiphene.

CHAPTER 8

CLOMIPHENE SUMMARY

ESI mass spectra were collected for clomiphene in 50/50 methanol/water to show a dominant peak at 406 m/z with a ^{37}Cl isotope peak at 408 m/z. The CID profile shows the initial loss of HCl, $C_4H_{11}N$, and of both HCl and $C_4H_{11}N$. This profile along with MS^3 allows for the detection of clomiphene in the serum matrix. Using a liquid/liquid extraction method with tBME as the organic layer and clomiphene spiked serum as the aqueous layer and an optimum shake time of 60 minutes, clomiphene was extracted from the serum with little concentrations of other extracts present – showing the extraction to be specific for clomiphene in fetal calf serum. Tamoxifen was used as an internal standard for quantitative analysis on the MS. By constructing a calibration curve by plotting (Intensity of Clomiphene peak)/(Intensity of tamoxifen peak) versus the concentration of clomiphene, CV% and % Error were both under 10% for all samples sets run. Clomiphene was detectable to 1 pM and was available for quantitative analysis down to 10 pM. The samples gave a linear peak intensity response in the range of 1 mM to 1 μM.

Liquid chromatography coupled with UV absorbance spectroscopy was used to separate clomiphene from the serum matrix. Using a simple isocratic LC with a reverse phase C18 column, clomiphene was readily separated from the serum matrix with an elution time of about 2.8 minutes and total run time of 5 minutes. Quantitative analysis of clomiphene was carried out by running a series of clomiphene standards and integrating the area under the peak. A calibration curve was then constructed by plotting clomiphene peak area versus clomiphene concentration. Detection limits were

at 10 nM and clomiphene was available for quantitative analysis down to 100 nM using UV absorbance. With a CV% of less than 8% and a % error of less than 2% for all sample sets, the precision and accuracy were respectable. The UV absorbance showed a linear relationship with concentration from 1mM to the detection limit of 10 nM.

In general, we showed that clomiphene can be precisely and accurately detected and quantified using ESI-MS and LC-UV analysis. Further studies will include the extraction and quantitative analysis of clomiphene from meconium and serum from women that have been on a clomiphene regiment.

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