2022

Monitoring the stability of cocaine and benzoylecgonine in postmortem tissues using laminar flow tandem mass spectrometry

BOSTON UNIVERSITY

SCHOOL OF MEDICINE

Thesis

MONITORING THE STABILITY OF COCAINE AND BENZOYLECGONINE IN POSTMORTEM TISSUES USING LAMINAR FLOW TANDEM MASS SPECTROMETRY

by

SIMONE NOELLE RUMPH

B.A., Bowdoin College, 2019

Submitted in partial fulfillment of the

requirements for the degree of

Master of Science

2022

Approved by

First Reader

Sabra Botch-Jones, M.S., M.A., D-ABFT-FT
Assistant Professor of Anatomy & Neurobiology
Program in Biomedical Forensic Sciences

Second Reader

Brendan Schweitzer, M. S.
Research Scientist
The University of Washington, Department of Neurology

Third Reader

Albert Elian, M.S.
President and CEO – A to Z Consulting

ACKNOWLEDGMENTS

I want to thank my wonderful family and friends for their never-ending support and motivation. I especially want to thank my mom, Mary, for always being there for me and encouraging me to keep going. I could not have done this without you.

Thank you to my thesis advisor, Professor Sabra Botch-Jones, for all of your guidance and patience. I would also like to thank my committee members, Brenden Schweitzer and Albert Elian, for their helpful insight and thoughtful comments. I am grateful for your time and input. Continually, thank you to Dr. Kathleen Kanak from the Boston University Psychological and Brain Sciences department, for providing the rat specimens used in this study. I want to thank Ken Crawston for taking time out of his schedule to provide rat dissection training for myself and several other students. And a special thank you to Jamie Foss for always being available to answer any questions I had and helping troubleshoot problems that arose with the instrumentation.

Thank you to all the professors and staff in the Boston University Biomedical Forensic Sciences program for sharing their wealth of knowledge, insight, and advice. I truly appreciate all the constant support and advice I received throughout my time in the program.

MONITORING THE STABILITY OF COCAINE AND BENZOYLECGONINE IN POSTMORTEM TISSUES USING LAMINAR FLOW TANDEM MASS SPECTROMETRY

SIMONE NOELLE RUMPH

ABSTRACT

In postmortem toxicology, certain cases require the examination of embalmed biological specimens to investigate the presence and potential role drugs may have played in a person's death. Key factors, like postmortem distribution, which can be greatly affected by temperature, may alter drug concentrations in different areas of the body. In the United States, the involvement of cocaine in overdose deaths has significantly increased between 2012 and 2019 (1). The purpose of this project was to examine the stability of cocaine and its primary metabolite, benzoylecgonine, in perfused postmortem rat tissues stored at different temperatures over a one month.

Twelve frozen cocaine positive rat specimens, intracardially perfused with a saline and formaldehyde solution, were received from a chronic cocaine rat study at Boston University Department of Psychological and Brain Sciences (Dr. Kathleen Kantak, Boston, MA, USA). The specimens were dissected, and the spleen, one kidney, and one lung were removed from each specimen. A fine-needle aspiration biopsy was performed on each organ to collect a time zero (T_0) sample. One set of four rat specimens were stored at room temperature (20-22°C), another four were stored at refrigerator temperature (4°C), and another four were stored at freezer temperature (-20°C). A section of each organ was collected for analysis at two weeks (T_1) and one

month (T_2). Samples underwent solid-phase extraction before liquid chromatography tandem mass spectrometry (LC-MS/MS) analysis using a QSight® 220 CR Laminar Flow Triple Quadrupole Mass Spectrometer with electrospray ionization, operated in positive ion mode (PerkinElmer, Shelton, CT, USA). Simplicity 3Q^{TM} software (PerkinElmer) was used for all data collection, analysis, and quantification.

All calibration curves generated for each analyte had acceptable R^2 values greater than 0.98 using a weighted linear regression model (1/x). Between T_1 and T_2, eight samples demonstrated a 15-873% increase in cocaine concentration and four samples had a 13-45% decrease in cocaine concentration. For benzoylecgonine, nine samples demonstrated an 18-289% increase in concentration between T_1 and T_2 and six samples had a 3-57% decrease in concentration. In samples collected at one month, concentration values for cocaine were highest in samples stored at freezer temperature (-20°C) and lowest in samples stored at refrigerator temperature (4°C). The highest benzoylecgonine values were found in samples stored at freezer temperature (-20°C) as well, and the lowest concentrations were in samples stored at room temperature (20-22°C). Due to the variability in analyte concentration in the organs of the intracardially perfused specimens, the impact storage conditions had on analyte stability could not be determined.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

±	Plus/minus
%	Percent
°C	Degrees Celsius
ALOQ	Above the limit of quantitation
Cal	Calibrator
CC	Collision energy
CCL2	Collision cell lens 2
DI	Deionized
ESI	Electrospray ionization
EV	Entrance voltage
g	Grams
GC	Gas chromatography
GC-MS	Gas chromatography-mass spectrometry
HPLC	High pressure liquid chromatography
INC	Inconclusive
LC	Liquid chromatography
LC-MS	Liquid-chromatography mass spectrometry
LOD	Limit of detection
LOQ	Limit of quantitation
m/z	Mass to charge
mg/mL	Milligram/milliliter

M	Molar
M/s	Meters/second
min	Minute
mL	Milliliter
mm	Millimeter
mM	Millimolar
MRM	Multiple reaction monitoring
MS	Mass spectrometer
MS/MS	Tandem mass spectrometry
ng/mL	Nanograms/milliliter
Psi	Pounds per square inch
R^2	Correlation coefficient
Q1	First quadrupole in triple quadrupole configuration
Q2	Second quadrupole in triple quadrupole configuration
Q3	Third quadrupole in triple quadrupole configuration
QC	Quality control
RPM	Revolutions per minute
SPE	Solid phase extraction
TIC	Total ion chromatogram
T_1	Time point 1, 2 week
T_2	Time point 2, 1 month
µg/mL	Microgram/milliliter

µL	Microliter
µm	Micrometer
USA	United States of America
v/v	Volume to volume

1 INTRODUCTION

1.1 Postmortem Forensic Toxicology

Postmortem forensic toxicology is used to determine the presence and absence of foreign substances in the body after death. Through toxicological analysis, forensic toxicologists can assist medical examiners with determining whether drugs were a contributing factor to the cause and manner of death. A myriad of biological specimens can be used for the detection and quantitation of drugs in the body such as urine, blood, spinal fluid, brain, liver, kidney, vitreous humor, and hair.

Decomposition of the body can skew the results of toxicological analysis. There is natural postmortem movement of drugs through the blood which is associated with changes from decomposition, which includes autolysis, putrefaction, and postmortem redistribution (2, 3). Autolysis is an enzyme-driven process that leads to the softening and liquefying of body tissue (2). Putrefaction is the breakdown of cells and tissues by microorganisms in the body (2, 4).

However, postmortem toxicological analyses of drug concentrations may not be consistent with perimortem levels due to postmortem drug redistribution (5). Postmortem drug redistribution is the change in drug concentrations in the body after death due to the diffusion of drug deposits in the body (6). The diffusion of drugs is a result of physicochemical changes in the body during autolysis and putrefaction. The rate and movement of drugs vary per person and is influenced by the nature of the drug, time between death and collection, changes in pH, cell membrane integrity, and the tissue-

binding characteristics of the drug (6, 7). Most drugs of abuse are lipophilic and lipophilic drugs and organic bases are known to concentrate in the solid organs and undergo passive diffusion into the blood after death (6, 8). Another factor contributing to postmortem redistribution is site variability. Site-dependent changes in concentration can occur due to incomplete drug distribution before death, passive diffusion from the lumen of a body cavity into surrounding organs, and release from drug binding sites (3).

1.1.1 Embalmed Specimens

The embalming of tissues is a means of preservation commonly used for burials. Embalming involves the infusion of formalin, an aqueous solution of formaldehyde, throughout the body to preserve it, removing blood from the body. Usually, embalming fluid is comprised of a 10% buffered or non-buffered formalin solution (9, 10). In toxicological situations where a body has already embalmed, the specimens available for toxicological testing are limited to formalin-fixed tissues, the formalin solution a tissue was stored in, and frozen tissues (9, 11). A decrease in drug concentrations can occur as a result of dilution from the introduction of embalming fluids (12).

Formaldehyde is a thermally unstable and highly reactive aldehyde that acts as a disinfectant and preservative (12). It may react to drugs present in the body through hydrolysis, degradation, or methylation and as a result, products of these reactions can appear during analysis and impact results (12, 13). Specifically, formaldehyde is known to easily react with nitrogen-containing compounds (14).

1.2 Cocaine

Cocaine is a tropane alkaloid, also known as methylbenzoylecgonine, and is derived from the coca plant, *Erythroxylum coca*, which is indigenous to the northern region of the South American Andes (15, 16). It is a psychoactive substance that has been used for both medicinal and illicit purposes. In 1970, the Drug Enforcement Administration classified it as a Schedule 2 substance, meaning there is some medicinal value, but it has a high potential for abuse (15). Medicinally, it has been used topically as a local anesthetic in ear, nose, and throat surgeries (15). It can block the reuptake of dopamine, serotonin, and norepinephrine neurotransmitters (15, 17).

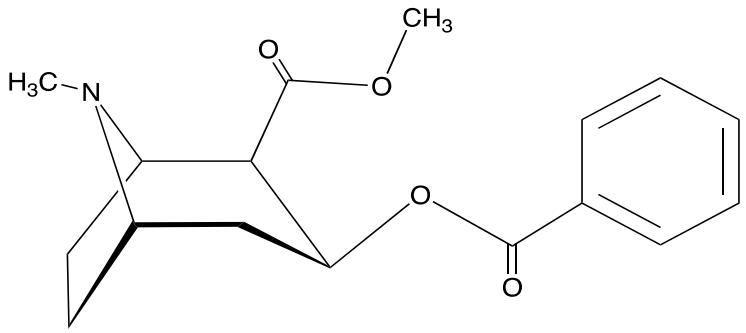
1.2.1 Effects on Mind and Body

Cocaine is abused for its desirable effects, which are primarily a result of dopamine. Some desirable effects include heightened sexual excitement, elevated mood, intense euphoria, psychic energy, and increased self-confidence (18). However, it can also cause paranoia, hallucinations, dysphoria, hyperthermia, delirium, and cardiorespiratory arrest (19). After use, users will often experience a “rush” and “crash” effect due to the quick increase in dopamine concentration, followed by a sharp decrease in concentration (15).

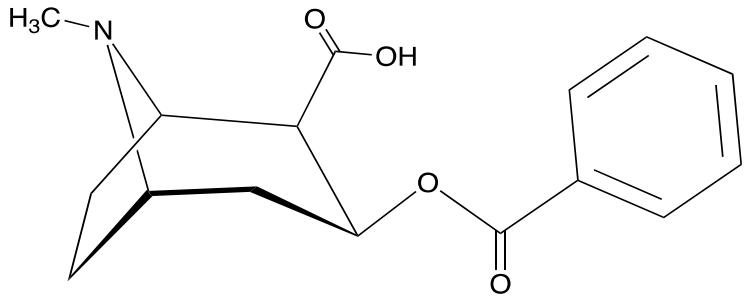
1.2.2 Metabolism and Elimination

Cocaine is quickly metabolized into its two major inactive metabolites, benzoylecgonine and ecgonine methyl ester (17). It is metabolized into benzoylecgonine

at an alkaline pH through spontaneous hydrolysis and into ecgonine methyl ester by enzymatic hydrolysis by pseudocholinesterase (15). The half-life of cocaine is approximately between 0.5-1.5 hours, which may make it difficult to detect in postmortem samples (20, 21). Benzoylecgonine has a longer half-life than its parent compound, of approximately 5-8 hours (8). The conversion of cocaine occurs quickly after death, due to the ester linkages in its structure (15). In postmortem specimens, cocaine will continue to be degraded through nonenzymatic hydrolysis into benzoylecgonine and into ecgonine methyl ester through enzymatic action (22). Continually, metabolic conversion of cocaine will occur at high temperatures in alkaline conditions (8, 23). Cocaine is known to undergo postmortem redistribution as well (3).



Cocaine



Benzoylecgonine

Figure 1. Chemical Structures of Cocaine and Benzoylecgonine (ChemDraw, PerkinElmer, Waltham, MA, USA).

1.2.3 Storage Temperature Impact

The stability of cocaine is greatly affected by storage temperature. A study tested the stability of cocaine in urine when stored at -20°C for twelve months and found a 37% average decrease in cocaine concentrations (24). Similarly, a study reported the concentration of benzoylecgonine decreasing an average of 19% in urine stored at -20°C for an average of 2.3 months (25). Using human plasma, another study examined the stability of cocaine when stored at 4°C and 20°C for 15 days in both fresh unpreserved samples and preserved samples, stored in 0.25% potassium fluoride (26). Results of the

study demonstrated the degradation of cocaine depended on the storage time and temperature (26). In comparing the fresh plasma samples and preserved samples, they found the concentration of benzoylecgonine was slightly higher in preserved plasma than in unpreserved plasma at both storage temperatures (26).

1.2.4 Sample Preparation

Cocaine and its metabolites are susceptible to undergo hydrolysis at high temperatures and in alkaline conditions (15). Therefore, the analytes must be separated from biological matrix samples immediately. The extraction methods often used for biological samples are liquid-liquid extraction and solid-phase extraction (SPE). In most studies, analyte elution is performed using a strong alkaline organic solvent such as a methylene chloride, isopropanol, and ammonium hydroxide mixture (15, 27, 28).

1.2.5 Common Detection Methods

Toxicological detection and analysis of cocaine and benzoylecgonine are often performed using hyphenated chromatography methods such as gas chromatography-mass spectrometry (GC-MS) or liquid chromatography-mass spectrometry (LC-MS). Often LC-MS is used for the analysis of cocaine and its metabolites rather than GC-MS because it does not require a derivatization step, which is needed for GC-MS analysis (15, 18). For postmortem analysis, the stability of cocaine over time in embalmed tissues has been shown to be very different compared to that in tissues taken at the time of autopsy (28).

Thus, liquid chromatography tandem mass spectrometry (LC-MS/MS), is extensively used because of its high sensitivity and selectivity (29).

1.3 Instrument Theory

1.3.1 Liquid Chromatography

Liquid chromatography is a technique used to separate compounds using a liquid mobile phase and a solid stationary phase to perform chromatographic separations. The sample is dissolved in the mobile phase and loaded into the analytical column, where, based on the affinity of the analyte for each phase, the sample will separate into its individual components, by eluting at different times. The column and mobile phase are selected to optimize separation based on the degree of analyte interaction between the mobile and stationary phases (30).

High-performance liquid chromatography (HPLC) linked to a mass spectrometer is often used in forensic toxicology. It has the ability to combine solvents, analyze a combination of different drug classes in one run, and consumes less biological matrix, which makes it a versatile instrument (31, 32). Analysis with HPLC is also ideal for analytes that do not volatilize easily and are thermally unstable compounds. High-pressure liquid chromatography involves the instrument operating at high pressure and forcing the analyte and the mobile phase through the metal column packed with the stationary phase, which consists of micron-sized particles (33). The size and chemical properties of the stationary phase in addition to the column dimensions can impact the efficiency of the column, retention time, and separation of the target analytes.

1.3.2 Mass Spectrometry

Often mass spectrometers are coupled with an instrument for chromatographic separation such as GC or LC. After a compound elutes off an analytical column, the compound is introduced to the ion source of the mass spectrometer under vacuum. At that point, the compound is ionized and fragmented (32). The ions are then detected by the mass spectrometer based on their mass to charge ratios (m/z) (34). Based on the ionization method, mass spectrometers can produce largely intact molecules or more fragmented molecules.

1.3.3 Electrospray Ionization

Electrospray ionization (ESI) is one of the most effective, and commonly used ionization techniques used for LC-MS analysis (34). It is an atmospheric ionization method, meaning ions are produced at atmospheric pressure, instead of under vacuum (35). Electrospray ionization is a soft ionization technique creating both positive and negative ions depending on the polarity of the applied charge. The ion source consists of a counter electrode that creates a charged electric field. As the liquid sample elutes off the column, it flows through a coated capillary tube into the ion source, which imparts a charge on the surface of the droplets. A heated drying gas is then applied, de-solvating the droplets and increasing their charge density. When the charge density of the droplets becomes too great, a coulombic explosion occurs, forcing the droplets to explode into smaller charged droplets that then flow into a mass analyzer (34).

1.3.4 Tandem Mass Spectrometry

In tandem quadrupole mass spectrometry, three quadrupoles are aligned linearly, allowing the user to select multiple ions to undergo fragmentation (32, 36). In the first quadrupole (Q1) the analyte of interest, also known as the parent/precursor ion, is selected by Q1 and then moves to the second quadrupole (Q2) to undergo fragmentation. The second quadrupole is referred to as the collision cell. The fragmented ions produced in Q2 then travel to the third quadrupole (Q3) where they are sorted before being sent into the detector (36). Essentially, Q1 and Q3 are mass spectrometers and Q2 is the collision cell designated for fragmentation. Due to its successive ion mass filtrations, the tandem mass spectrometer provides high selectivity (37). The use of LC-MS/MS has become a standard instrumentation for the analysis and quantitation of drugs in biological matrices because of the sensitivity of the tandem mass spectrometer (MS/MS) coupled with the versatility of LC.

1.5 Research Objective

This research aimed to examine the stability of cocaine and its primary metabolite, benzoylecgonine, in perfused postmortem rat tissues when stored at different temperatures for one month.

2 MATERIALS AND METHODS

2.1 Materials

2.1.1 Standards/Reagents

All solvents, including methanol, acetonitrile, isopropanol, methylene chloride, ammonium hydroxide, and hydrochloric acid were obtained from Fisher Scientific (Waltham, MA, USA). Cocaine, benzoylecgonine, cocaine-d3, and benzoylecgonine-d8 certified reference standards were purchased from Cerilliant (Round Rock, TX, USA). The standard solutions of cocaine and benzoylecgonine were at concentrations of 1.0 mg/mL in acetonitrile and methanol, respectively. Deuterated internal standards, cocaine-d3 and benzoylecgonine-d8, were at concentrations of 100 ug/mL in acetonitrile and methanol, respectively. A Milli-Q water purification system from Millipore Sigma (Burlington, MA, USA) produced all the water used throughout the study.

2.1.2 LC-MS/MS Instrumentation

All analyses were performed on a QSight® 220 CR Laminar Flow Triple Quadrupole Mass Spectrometer with an ESI source, operated in positive ion mode (PerkinElmer, Shelton, CT, USA). Simplicity 3Q^{TM} software (PerkinElmer) was used to perform all data collection, analysis, and quantification.

2.1.3 Matrix Samples:

Human urine was collected in compliance with Boston University's Biomedical Forensic Sciences Biological Donation Program and approved by the Boston University School of Medicine Institutional Review Board (Boston, MA, USA). The donated human urine samples were analyzed using GC-MS (Agilent, Santa Clara, CA, USA) to screen the absence of cocaine and benzoylecgonine.

2.2 Methods

2.2.1 LC-MS/MS Parameters

The method utilized in this project was adapted from a previous compound method optimization process. Analytes were detected using multiple reaction monitoring (MRM) in positive ESI mode. The use of MRM produces high sensitivity for a panel of analytes (35). The compound and source method parameters adapted are shown in Tables 1 and 2 below. Table 1 is the MRM table for all analytes and lists the parent ion masses, product ion masses, dwell time, and collision energy (CE) for each ion. Table 2 lists the ion source parameters.

Table 1. MRM Table for All Compounds

Compound	Precursor Ion (m/z)	Product Ions (m/z)	Dwell Time (ms)	CE
Cocaine	304.2	182.2	100	-50
	304.2	150.1	100	-50
Benzoylecgonine	290.2	168.1	100	-50
	290.2	105.1	100	-50
Cocaine-d3	306.8	185.0	100	-50
Benzoylecgonine-d8	298.2	171.0	100	-50

Table 2. Instrument Parameters Internal Standards

Drying Gas	HSID Temperature	Nebulizer Gas	ElectroSpray V1	Source 1 Temperature
100	250°C	150	5500	500°C

A Kinetex® Phenyl-Hexyl 50 x 4.6mm (2.6 μm) column (Phenomenex®, Torrance, CA, USA) was used for compound separation and was kept at 40ºC for the duration of the method. Mobile phase A was aqueous and consisted of 10mM ammonium formate in Millipore water and mobile phase B was organic and comprised of 0.1% formic acid in methanol. The injection volume was set at 7 μL with a flow rate of 0.50 mL/min for the duration of the method. The LC method run time was 12 minutes using a linear gradient elution (Table 3).

Table 3. LC Gradient Elution Program

Time (min)	Flow Rate (mL/min)	Mobile Phase A (%)	Mobile Phase B (%)
0.00	0.50	90	10
0.50 3.00	0.50	85 50	15 50
4.00 7.00	0.50 0.50	5 5	95 95
8.00 9.00	0.50 0.50	90 90	10 10

2.2.2 Rat Specimens

The bodies of twelve frozen cocaine-positive rat specimens were received from a chronic cocaine-rat study at Boston University Department of Psychological and Brain Sciences (Dr. Kathleen Kantak, Boston, MA, USA). All twelve specimens were intracardially perfused with a solution of saline and paraformaldehyde. The specimens were dissected, and the spleen, one kidney, and one lung were removed from each specimen. Fine-needle aspiration biopsies were performed on each organ to collect a time zero (T_0) sample. Then each organ was weighed, cut into thirds, and placed in separate open glass tubes. The organs from the 12 rat specimens were split into three groups. Organs from four rat specimens were stored at room temperature (20-22°C), another set of organs from four specimens were stored at refrigerator temperatures (4°C), and another set of organs were stored at freezer temperature (-20°C). A section of each organ was taken at the following time points: two weeks (T_1) and one month (T_2).

2.2.3 Homogenization

Organ samples were collected at the respective time points, weighed, and then placed in Soft tissue Homogenizing Mix 1.4 mm Ceramic Nuclease Free 2 mL tubes (Omni International, Bedford, NH, USA) with 1 mL of 0.1M phosphate buffer (pH 6). Homogenization was performed using an Omni International Bead Mill Ruptor 12 (Bedford, NH, USA) for 25 seconds at a speed of 6 M/s. Samples went through a minimum of three cycles on the homogenizer, although certain samples required more cycles to fully homogenize.

Following homogenization, samples were transferred to 15 mL tubes and diluted with 0.1M phosphate buffer (pH 6) at a 1:5 dilution ratio based on the weight of each organ segment at the time of collection. Then 20 μL of a 1 μg/ml internal standard working stock solution, consisting of cocaine-d3 and benzoylecgonine-d8, was added to each sample. The 15 mL tubes were centrifuged at 5000 revolutions per minute (RPM) for five minutes in a Thermo Scientific Sorvall ST 8 Centrifuge (Thermo Fisher Scientific, Waltham, MA, USA).

2.2.4 Calibrators

Calibration curves were generated using calibrators (Cal) prepared in analyte-free human urine at the concentrations listed in Tables 4 and 5. Quality control (QC) samples were also prepared in analyte-free human urine at low, middle, and high concentrations for each set of calibrators and were analyzed concurrently with each sample batch. Table 4 lists the calibrator and QC concentrations prepared and run alongside the samples at T_0

and T_1. The calibrator and QC concentrations prepared and run alongside the T_2 samples are listed in Table 5. Human urine was used to prepare the calibrators, QCs, negative controls, and double blanks because of the ease of access to the biological matrix and its similarities in sample preparation process to the tissue samples.

Calibrators and QC samples were prepared by combining a set of concentrated stock solutions with analyte-free human urine to reach 1 mL. The stock solutions contained the cocaine and benzoylecgonine standards at the following concentrations: Stock 1 = 200 ng/mL, Stock 2 = 10 ng/mL, and Stock 3 = 1 ng/mL. These three stock solutions were used to prepare the calibrators, QCs, negative controls, and double blank samples. All calibrators and QCs were spiked with 20 μL of a 1 μg/ml internal standard working stock solution. Negative controls consisted of 1000 μl of drug-free human urine with 20 μL of internal standard working stock solution and double blank samples consisted of 1000 μL of drug-free human urine without the addition of the internal standard working stock solution. Then all calibrators and controls were centrifuged for 5 minutes at 5000 RPM in a Thermo Scientific Sorvall ST 8 Centrifuge (Thermo Fisher Scientific). After centrifuging, all calibrators and controls underwent SPE alongside the homogenized tissue samples as listed in section 2.2.5.

Table 4. Calibrators and Quality Control Samples for T_0 and T_1.

Calibrators/ Quality Controls	Concentration (ng/mL)
Cal 1	0.25
Cal 2	1
Cal 3	5
Cal 4	15
Cal 5	25
Cal 6	50
Cal 7	100
Cal 8	200
Low QC	0.5
Medium QC	20
High QC	50

Table 5. Calibrators and Quality Control Samples for T_2 Samples.

Calibrators/ Quality Controls	Concentration (ng/mL)
Cal 1	0.1
Cal 2	0.25
Cal 3	2.5
Cal 4	5
Cal 5	10
Cal 6	25
Low QC	0.5
Medium QC	1
High QC	7.5

2.2.5 Solid Phase Extraction (SPE)

All specimens, including calibrators and controls, were prepared for instrumental analysis using Clean Screen (CSDAU206) SPE cartridges (United Chemical Technologies, Bristol, PA, USA). The columns were conditioned with 3mL methanol, 3mL deionized (DI) water, and 3mL 0.1M phosphate buffer (pH 6). The supernatant of the centrifuged samples was loaded onto the columns and allowed to flow through under the force of gravity. Each column was then washed with 3 mL DI water, 3 mL 0.1M hydrochloride, and 3 mL methanol, and then dried under vacuum for approximately 10 minutes. Analytes were eluted using 3 mL of methylene chloride/isopropanol/ammonium hydroxide (78:20:2, *v/v*), followed by evaporation at 40^{o}C using Organomation Multivap Nitrogen Evaporator (Organomation Associates, Inc, Berlin, MA, USA). The evaporated samples were then reconstituted in 90 μL of 10 mM ammonium formate in water and 10 μL of methanol and transferred into LC vials with a flat-bottom liners for analysis. All samples were then stored in the freezer at -20^{o}C until the time of instrumental analysis.

2.2.6 Quantification of Samples

Deuterated internal standards, cocaine-d3 and benzoylecgonine-d8, were used to quantify cocaine and benzoylecgonine, respectively. The linear regression (1/x) calibration model was generated using Simplicity 3Q^{TM} software (PerkinElmer). The calibration curve correlation coefficient (R^2) had to be equal to or greater than 0.98 to be

accepted. All positive QCs, negative controls, and at least five calibrators had to pass to be deemed acceptable. Analytes were identified if the retention time of the analyte was within ±2% of the corresponding deuterated internal standard and within 0.5 minutes of the average internal standard retention time of the calibrators.

3 RESULTS

3.1 Limit of Detection (LOD) and Limit of Quantitation (LOQ)

With each batch of samples analyzed, a calibration curve was created and used to determine the limit of detection (LOD) and limit of quantitation (LOQ) for each analyte. The LOD was determined for each analyte by examining the lowest calibrators to determine if the signal was greater than 3.3 times the background signal. The LOD at T_0, T_1, and T_2 was 0.25 ng/mL for cocaine and benzoylecgonine.

The limit of quantitation (LOQ) was determined as the lowest concentration that could be quantified with acceptable ion ratios, which were determined by the Simplicity 3Q^{TM} software used for data analysis. For T_0, T_1, and T_2, the LOQ for cocaine and benzoylecgonine was determined to be 0.25 ng/mL.

3.2 T_0: Time Zero

3.2.1 Calibration Model

A calibration curve ranging from 0.25 ng/mL to 200 ng/mL was analyzed alongside all samples and controls collected at T_0. Calibrators were prepared in analyte-free human urine at the following concentrations: 0.25 ng/mL, 1 ng/mL, 5 ng/mL, 15 ng/mL, 25 ng/mL, 50 ng/mL, 100 ng/mL, and 200 ng/mL. The low, middle, and high QC concentrations were at 0.5 ng/mL, 20 ng/mL, and 50 ng/mL, respectively.

The calibration curve R^2 value for the cocaine qualifier was 0.9988 (see Figure 2). The calibration curve R^2 value for the benzoylecgonine quantifier was 0.9989 (Figure 3).

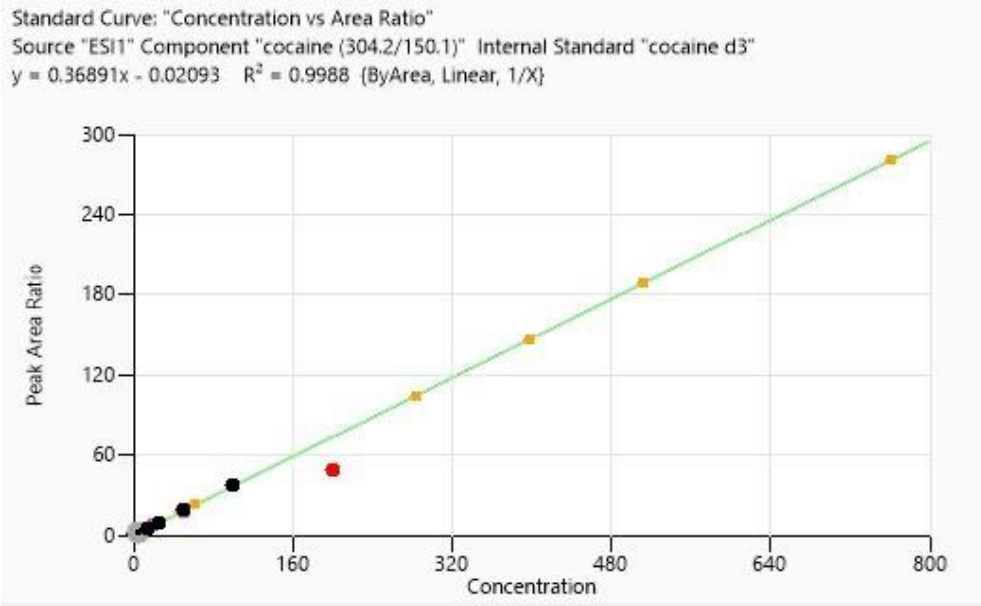


Figure 2. Linear Calibration Curve of Cocaine for T_0. The calibration curve R^2 value for the cocaine qualifier was 0.9988.

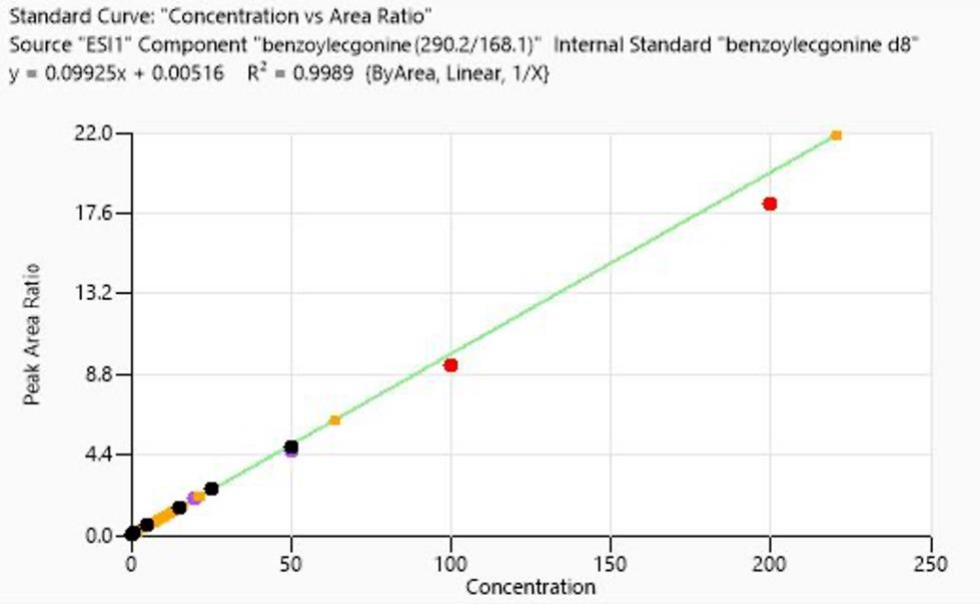


Figure 3. Linear Calibration Curve of Benzoylecgonine for T_0. The calibration curve R^2 value for the benzoylecgonine quantifier was 0.9989.

3.2.2 Quantitation of Unknown Samples

The results of the calculated concentrations of the tissues are displayed in Tables 6-8, divided by storage temperature. Quantitation for each sample was completed by using the linear calibration curve generated by the calibrators listed above. For interpretation purposes, the samples with concentration values below the LOQ could not be reliably used for comparison. In the total ion chromatograms (TIC) of the samples marked as inconclusive (INC) neither the internal standards, cocaine-d3 and benzoylecgonine-d8, nor the analytes, cocaine and benzoylecgonine, were detected (see Table 6-8). In addition, samples were marked as INC if the analyte concentration value was below the LOD.

Table 6. Quantitation Data of T_0 Samples Stored at Room Temperature (20-22°C).

Sample	Cocaine (ng/mL)	Benzoylecgonine (ng/mL)
3878 Kidney	INC	0.40
3878 Spleen	INC	INC
3878 Lung	INC	INC
3892 Kidney	3.98	INC
3892 Spleen	ND	INC
3892 Lung	ND	INC
3877 Kidney	0.37	INC
3877 Spleen	2.87	INC
3877 Lung	INC	INC
3868 Kidney	5.83	INC
3868 Spleen	INC	INC
3868 Lung	INC	INC

Table 7. Quantitation Data of T_0 Samples Stored at Refrigerator Temperature (4°C).

Sample	Cocaine (ng/mL)	Benzoylecgonine (ng/mL)
3879 Kidney	INC	1.01
3879 Spleen	INC	INC
3879 Lung	INC	INC
3865 Kidney	INC	INC

Table 7. Quantitation Data of T_0 Samples Stored at Refrigerator Temperature (4°C).

3865 Spleen	INC	INC
3865 Lung	INC	INC
3866 Kidney	INC	INC
3866 Spleen	INC	INC
3866 Lung	INC	INC
3895 Kidney	INC	INC
3895 Spleen	INC	INC
3895 Lung	INC	INC

Table 8. Quantitation Data of T_0 Samples Stored at Freezer Temperature (-20°C).

Sample	Cocaine (ng/mL)	Benzoylecgonine (ng/mL)
3870 Kidney	INC	INC
3870 Spleen	INC	INC
3870 Lung	INC	INC
3872 Kidney	INC	0.34
3872 Spleen	INC	INC
3872 Lung	INC	INC
3896 Kidney	INC	0.29
3896 Spleen	INC	INC
3896 Lung	INC	INC

Table 8. Quantitation Data of T_0 Samples Stored at Freezer Temperature (-20°C).

3876 Kidney	INC	INC
3876 Spleen	INC	INC
3876 Lung	INC	INC

3.3 T_1: Two Weeks

3.3.1 Calibration Model

A calibration curve ranging from 0.25 ng/mL to 200 ng/mL was analyzed alongside all samples and controls collected at T_1. Calibrators were prepared in analyte-free human urine at the following concentrations: 0.25 ng/mL, 1 ng/mL, 5 ng/mL, 15 ng/mL, 25 ng/mL, 50 ng/mL, 100 ng/mL, and 200 ng/mL. The low, middle, and high QC concentrations were at 0.5 ng/mL, 20 ng/mL, and 50 ng/mL, respectively.

The calibration curve R^2 value for the cocaine qualifier was 0.982 (see Figure 4). The calibration curve R^2 value for the benzoylecgonine qualifier was 0.9966 (see Figure 5).

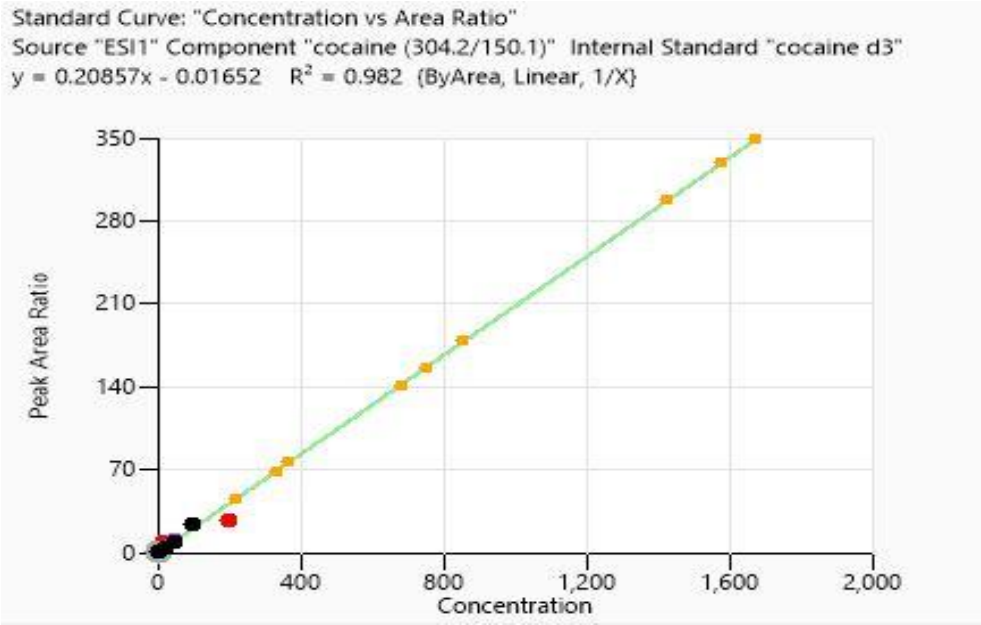


Figure 4. Linear Calibration Curve of Cocaine for T_1. The calibration curve r^2 value for the cocaine qualifier was 0.982.

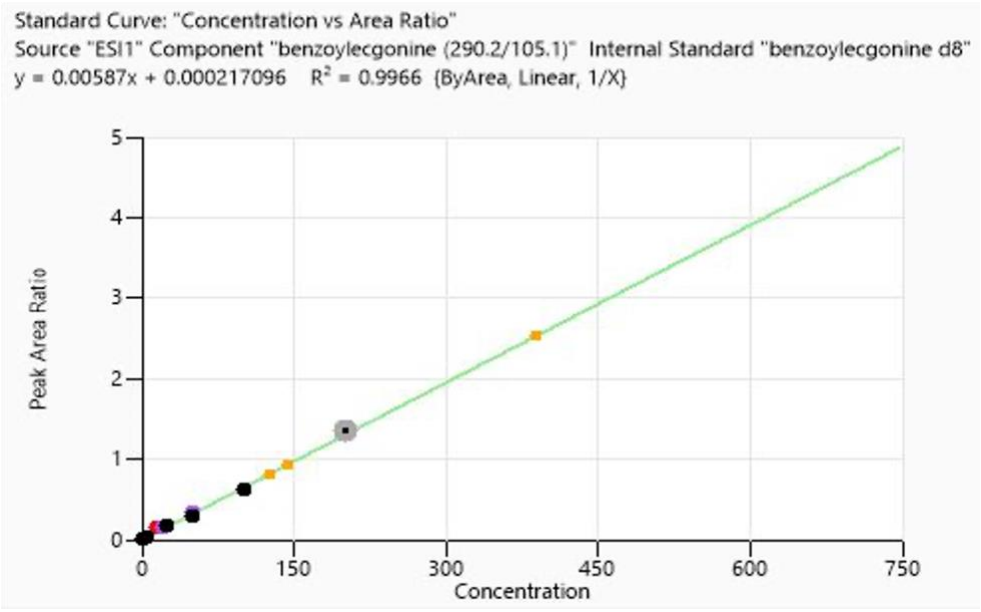


Figure 5. Linear Calibration Curve of Benzoylecgonine for T_1. The calibration curve R^2 value for the benzoylecgonine qualifier was 0.9966.

3.3.2 Quantitation of Unknown Samples

The results of the calculated concentrations of the tissues are displayed in Table 9-10, separated according to storage temperature. Quantitation for each sample was completed by using the calibration curve generated from the calibrators listed above. The T_1 samples stored at freezer temperature (-20°C) did not produce quantifiable analyte concentrations.

Table 9. Quantitation Data of T_1 Samples Stored at Room Temperature (20-22°C).

Sample	Cocaine (ng/mL)	Benzoylecgonine (ng/mL)
3878 Kidney	6.05	1.47
3878 Spleen	INC	0.83
3878 Lung	0.91	0.31
3892 Kidney	0.25	0.29
3892 Spleen	INC	0.96
3892 Lung	0.26	INC
3877 Kidney	0.82	3.03
3877 Spleen	INC	0.99
3877 Lung	1.17	0.33
3868 Kidney	0.30	0.39
3868 Spleen	0.29	0.46
3868 Lung	0.35	0.43

Table 10. Quantitation Data of T_1 Samples Stored at Refrigerator Temperature (-4°C).

Sample	Cocaine (ng/mL)	Benzoylecgonine (ng/mL)
3879 Kidney	0.47	1.15
3879 Spleen	INC	0.70
3879 Lung	0.51	0.30
3865 Kidney	0.93	1.73
3865 Spleen	0.61	0.64
3865 Lung	0.54	0.39
3866 Kidney	INC	INC
3866 Spleen	INC	INC
3866 Lung	INC	INC
3895 Kidney	INC	INC
3895 Spleen	INC	INC
3895 Lung	INC	INC

3.4 T_2: One Month

3.4.1 Calibration Model

A calibration curve ranging from 0.1 ng/mL to 25 ng/mL was analyzed alongside all samples and controls from T_2. Calibrators were prepared in analyte-free human urine at the following concentrations: 0.1 ng/mL, 0.25 ng/mL, 2.5 ng/mL, 5 ng/mL, 10 ng/mL,

and 25 ng/mL. The low, middle, and high QC concentrations were 0.5 ng/mL, 1 ng/mL, and 7.5 ng/mL, respectively.

The calibration curve R^2 value for the cocaine qualifier was 0.986 (see Figure 6). The calibration curve R^2 value for the benzoylecgonine qualifier was 0.9989 (see Figure 7.

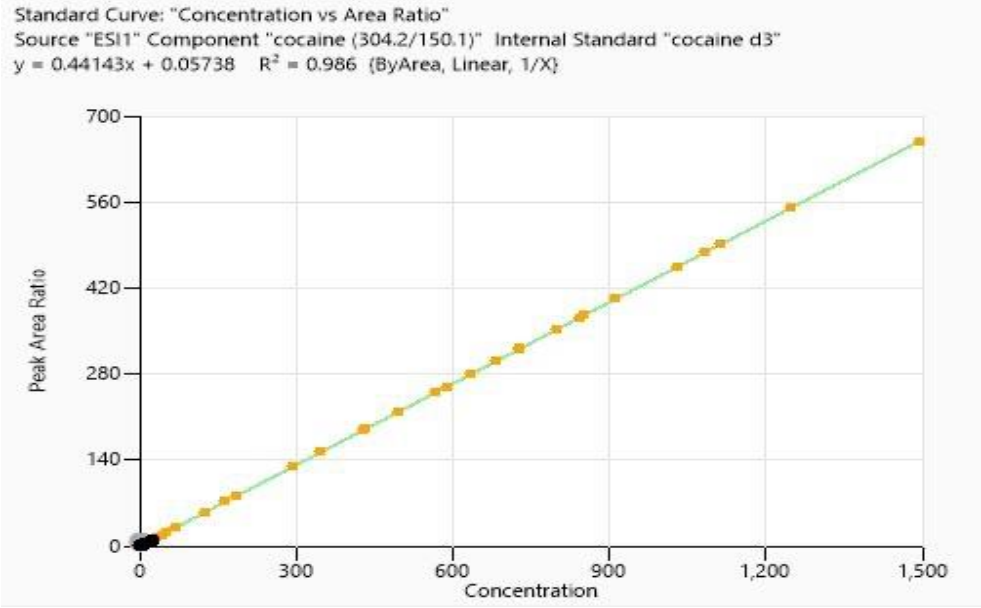


Figure 6. Linear Calibration Curve of Cocaine for T_2. The calibration curve r^2 value for the cocaine qualifier was 0.986.

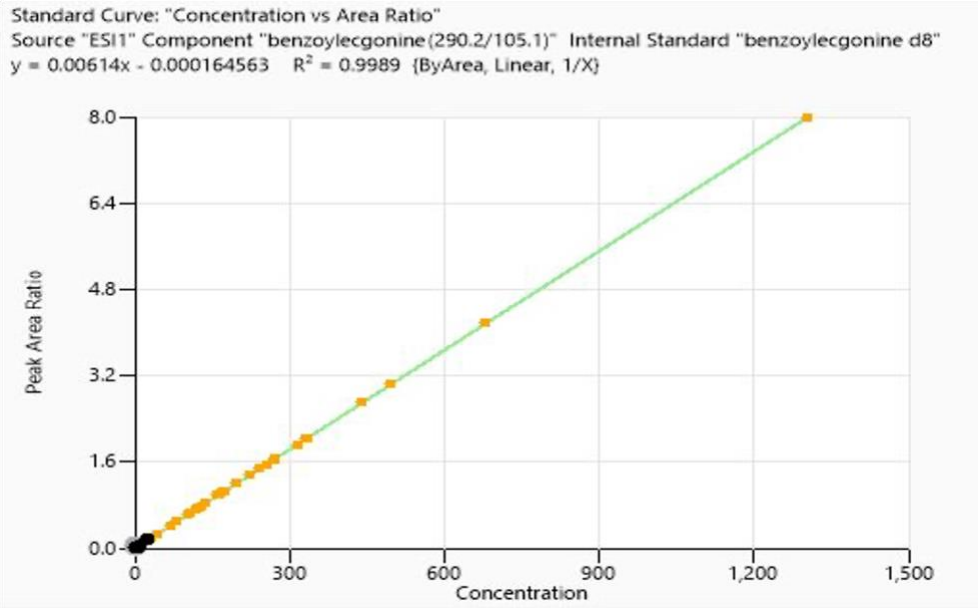


Figure 7. Linear Calibration Curve of Benzoylecgonine for T_2. The calibration curve R^2 value for the benzoylecgonine qualifier was 0.9989.

3.4.2 Quantitation of Unknown Samples

The results of the calculated concentrations of the tissues are displayed in Table 11-13, separated according to storage temperature. Quantitation for each sample was completed by using the calibration curve generated from the calibrators listed above. Concentration values that were detected but were above the limit of quantitation are listed as above the limit of quantitation (ALOQ).

Table 11. Quantitation Data of T_2 Samples Stored at Room Temperature (20–22 °C).

Sample	Cocaine (ng/mL)	Benzoylecgonine (ng/mL)
3878 Kidney	ABLOQ	2.19

Table 11. Quantitation Data of T_2 Samples Stored at Room Temperature (20–22 °C).

3878 Spleen	INC	1.92
3878 Lung	1.85	0.73
3892 Kidney	2.43	INC
3892 Spleen	INC	0.42
3892 Lung	0.33	INC
3877 Kidney	INC	2.13
3877 Spleen	INC	0.96
3877 Lung	0.76	0.31
3868 Kidney	1.54	1.51
3868 Spleen	0.68	1.03
3868 Lung	0.50	0.65

Table 12. Quantitation Data of T_2 Samples Stored at Refrigerator Temperature (4°C).

Sample	Cocaine (ng/mL)	Benzoylecgonine (ng/mL)
3879 Kidney	0.54	0.95
3879 Spleen	INC	0.36
3879 Lung	0.36	0.30
3865 Kidney	0.81	2.04
3865 Spleen	0.33	1.01
3865 Lung	0.90	1.11

Table 12. Quantitation Data of T_2 Samples Stored at Refrigerator Temperature (4°C).

3866 Kidney	0.81	0.41
3866 Spleen	INC	INC
3866 Lung	0.43	0.55
3895 Kidney	0.79	2.58
3895 Spleen	0.65	0.45
3895 Lung	1.54	0.60

Table 13. Quantitation Data of T_2 Samples Stored at Freezer Temperature (-20°C).

Sample	Cocaine (ng/mL)	Benzoylecgonine (ng/mL)
3870 Kidney	0.50	0.57
3870 Spleen	INC	INC
3870 Lung	0.34	INC
3872 Kidney	1.52	2.59
3872 Spleen	0.29	4.73
3872 Lung	0.71	1.13
3896 Kidney	0.66	3.20
3896 Spleen	INC	0.54
3896 Lung	0.69	0.67
3876 Kidney	0.50	1.51
3876 Spleen	0.29	1.04

Table 13. Quantitation Data of T_2 Samples Stored at Freezer Temperature (-20°C).

3876 Lung	2.89	0.70

4 DISCUSSION

4.1 Analyte Carryover

Analyte carryover was evaluated by monitoring the double blank samples and the blank samples run after the highest calibrators. The signal intensity of the analytes in the blanks and double blanks needed to be lower than the LOD of each analyte. Analyte carryover was not observed in any of the double blank samples or blank samples analyzed after the highest calibrators for T_0, T_1, and T_2.

4.2 Quantitative Analysis of Unknown Samples

Cocaine was detected and quantified in four T_0 samples, all were stored at room temperature: 3892 Kidney, 3977 Kidney, 3977 Spleen, and 3868 Kidney (see Table 6). The concentration of cocaine in these four samples ranged from 0.37 ng/mL to 5.83 ng/mL. Benzoylecgonine was detected and quantitated in four T_0 samples (see Tables 6-8). Samples 3878 Kidney, 3879 Kidney, 3872 Kidney, and 3896 Kidney had benzoylecgonine concentrations ranging from 0.29 ng/mL to 1.01 ng/mL.

The lack of quantifiable T_0 data may have been due to the extremely small sample volumes collected from the fine needle aspiration biopsy. Each organ sample collected ranged from 0.01 g to 0.05 g. After homogenization, each sample was diluted to 1 mL, which is another contributing factor to the lack of analyte detection in most of the T_0 samples.

Both analytes were detected in 18 of the 36 T_1 samples (see Tables 9-10). Cocaine was detected and quantified in fourteen samples. The concentration of cocaine

ranged from 0.25 ng/mL to 1.17 ng/mL. For cocaine, sample 3878 Kidney had the highest concentration value at 6.05 ng/mL. Benzoylecgonine was detected and quantified in 17 samples. For benzoylecgonine, sample 3877 Kidney had the highest concentration value of 3.03 ng/mL in comparison to the rest of the samples which had benzoylecgonine concentrations ranging from 0.29 ng/mL to 1.73 ng/mL.

The other 18 samples from T_1, marked 'INC' in Tables 9-10 were missing the peaks for both internal standards of the analytes as well as the analyte peaks. The samples were re-analyzed with new calibrators and QCs to determine if there was an instrumentation error or a problem with the sample extraction. However, results from the re-analysis of the samples produced the same results. It was determined that because the internal standard was not present in these samples, the most likely cause for the lack of detection was the result of errors made during sample preparation or ion suppression.

All samples collected at T_2 were successfully analyzed. As previously noted, the range of the calibrators was changed to include lower calibrators and QC concentrations to ensure the detection of both analytes at lower concentrations. Despite lowering the range of calibrators to include a calibrator at 0.1 ng/mL, the calibrator had poor ion ratios. Therefore, the LOD and LOQ remained at 0.25 ng/mL for both analytes. Cocaine was detected and quantitated in 27 samples. The calculated concentration of cocaine ranged from 0.29 ng/mL to 2.89 ng/mL. Benzoylecgonine was detected and quantitated in 31 samples. For benzoylecgonine, the calculated concentrations ranged from 0.30 ng/mL to 3.20 ng/mL.

4.3 Change in Concentration Over Time

Of the data collected from T_0, two samples had quantifiable cocaine analyte concentrations and could be compared to the T_1, and T_2 values: 3892 Kidney and 3868 Kidney (see Figure 8). The cocaine concentration values decreased for both samples between T_0 and T_1 and then increased between T_1 and T_2 as displayed in Figure 8. Sample 3892 Kidney had a 94% decrease between T_0 and T_1 followed by an 873% increase from T_1 to T_2. Similarly, sample 3868 had a 95% decrease in cocaine concentration between T_0 and T_1, followed by a 411% increase between T_1 to T_2. For both samples, the T_1 cocaine concentration was significantly different than the concentration values at T_0 and T_2. The increase in cocaine concentration between T_1 and T_2 could have been a result of sample degradation or errors made during sample preparation. In comparing the values at T_0 and T_2, there was an overall decrease in cocaine concentration for both samples 3892 Kidney and 3868 Kidney. The overall decrease in cocaine concentration over time agrees with literature, especially since both samples were stored at room temperature (23).

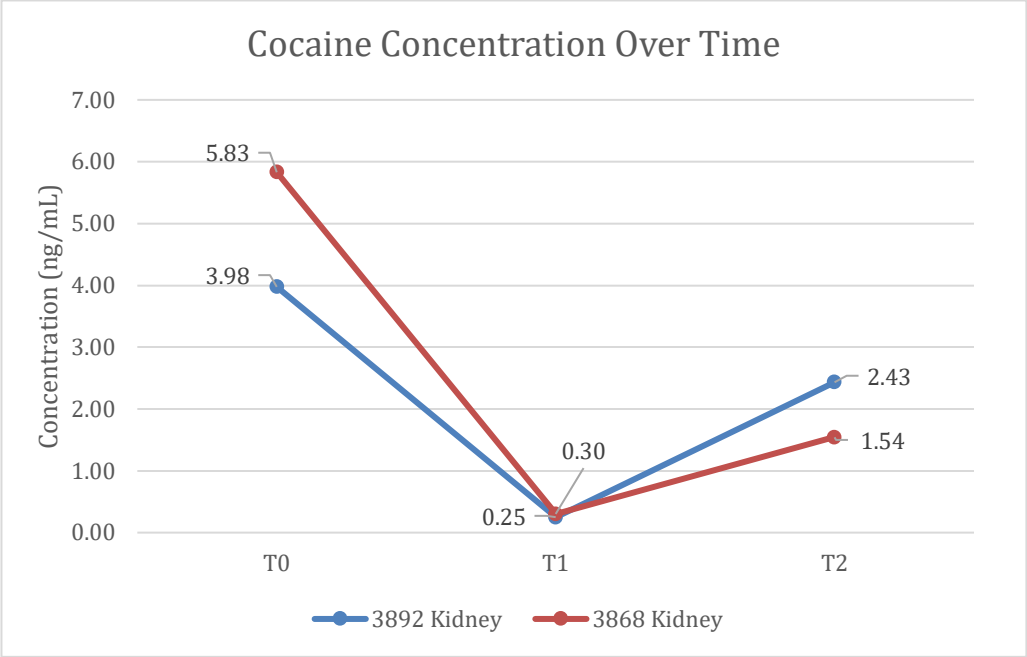


Figure 8. Change in Cocaine Concentration in Sample 3892 Kidney and 3868 Kidney Over One Month.

Two samples, 3878 Kidney and 3879 Kidney, had quantifiable benzoylecgonine analyte concentrations at T_0 and could be compared to their corresponding T_1 and T_2 samples. Sample 3878 Kidney demonstrated an overall increase in benzoylecgonine concentration (see Figure 9). Between T_0 and T_1, there was a 270 % concentration increase and between T_1 and T_2 there was a 49% increase. Contrastingly, sample 3879 Kidney showed an overall decrease in benzoylecgonine concentration between T_0 and T_2. From T_0 to T_1, there was a 14% concentration increase followed by a 17% decrease in benzoylecgonine concentration from T_1 to T_2. Both samples show opposite patterns of the change in benzoylecgonine concentration over time, but without other samples for comparison, samples 3878 Kidney and 3879 Kidney are not significant.

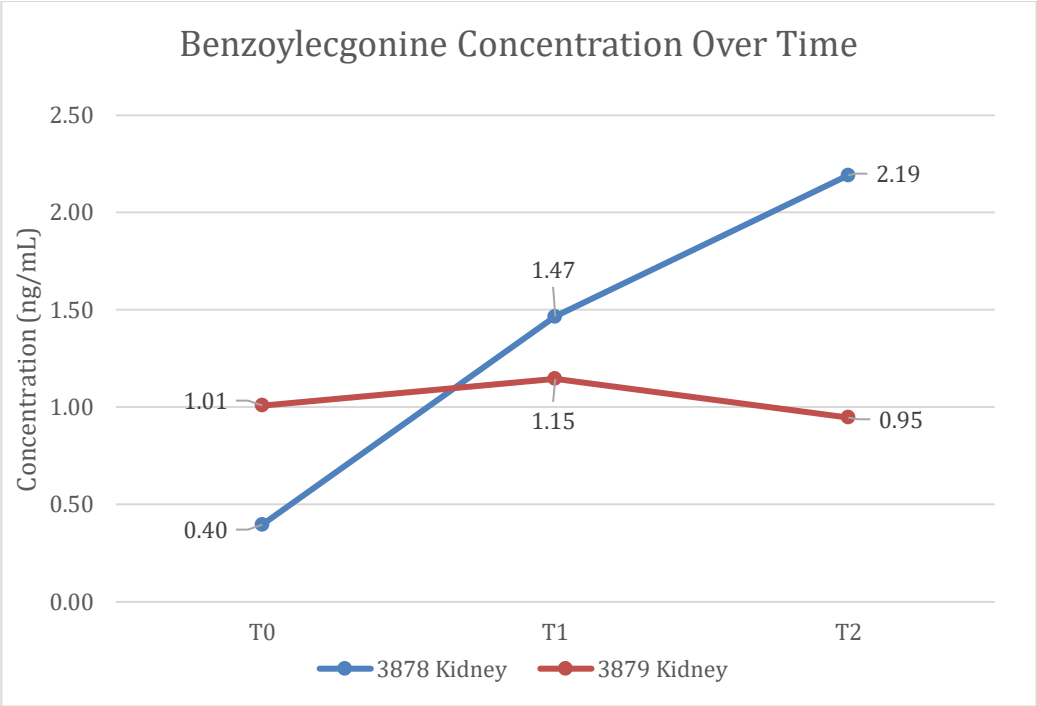


Figure 9. Change in Benzoylecgonine Concentration in Sample 3878 Kidney and 3879 Kidney Over One Month.

Several patterns were present when comparing the quantifiable data collected at T_1 and T_2. Eight samples exhibited a 15-873% increase in cocaine concentration between T_1 and T_2. In contrast, four samples exhibited a decrease in cocaine concentration. The four quantitated samples exhibited a 13-45% decrease in cocaine concentration. The eight samples with an increase in cocaine analyte concentration were unusual, especially in the seven samples stored at room temperature (20-22°C) because cocaine is known to undergo hydrolysis to benzoylecgonine faster at warmer temperatures (23). The decrease in cocaine analyte concentration over time was anticipated because cocaine is not a stable analyte over long periods, has a shorter half-life than benzoylecgonine, and studies have shown the concentration of cocaine in fixed tissues decreases over time (9, 21).

The decrease in cocaine concentration could have been caused by the pH of the formalin solution. In this project, the pH of the saline and paraformaldehyde solution used to fix the rat specimens was unknown, but it is worth noting studies have found the pH of the formalin solution used can impact and change the concentration of cocaine. In a formalin solution at pH 7.4, a 30-day study found that 80% of the cocaine analyte was hydrolyzed into benzoylecgonine within 15 days, but at pH 3.5, cocaine remained stable for 30 days (9).

Reviewing the concentrations of benzoylecgonine between T_1 and T_2, 15 samples were compared. Nine samples showed an 18-289% increase between T_1 and T_2, and six samples had a 3-57% decrease in concentration. The decrease in concentration could have been caused by a loss of analyte through the sample preparation process and from analyte degradation. In addition, storing the vialed samples in the freezer before instrumental analysis could have made the analytes unstable and caused further degradation.

The relationship between the change in cocaine and benzoylecgonine concentrations over time is an area with conflicting study results. Postmortem studies investigating analyte stability over time have demonstrated the concentration of cocaine decreases while the concentration of benzoylecgonine increases (13, 28). Yet, another study examining postmortem blood and body fluids found that although benzoylecgonine is a degradation product of cocaine, there was no correlation between the change in analyte concentration over time (38). More studies need to be conducted to investigate this discrepancy.

4.4 Storage Temperature

Analyte concentration values from samples stored at different temperatures could only be compared amongst the T_2 samples. Of the twelve samples stored at room temperature (20-22 °C), seven samples were able to be quantified for cocaine and had cocaine concentrations ranging from 0.33 ng/mL to 2.43 ng/mL (see Table 11). Ten samples stored at refrigerator temperature (4°C) had cocaine concentrations ranging from 0.33 ng/mL to 1.54 ng/mL (see Table 12). Ten samples stored at freezer temperature (-20°C) had cocaine concentrations ranging from 0.29 ng/mL to 2.89 ng/mL (see Table 13). The highest concentration values were in samples stored at freezer temperature (-20°C), while the lowest cocaine concentration values were in samples stored at refrigerator temperature (4°C). Literature states the rate of cocaine hydrolysis can be slowed at colder temperatures, which means the results from the T_2 samples are in concordance with the literature (5, 15, 23).

Of the twelve samples stored at room temperature (20-22 °C), ten were able to be quantitated for benzoylecgonine and ranged from 0.31 ng/mL to 2.19 ng/mL (see Table 11). Eleven T_2 samples stored at refrigerator temperature (4°C) had benzoylecgonine concentrations ranging from 0.30 ng/mL to 2.58 ng/mL (see Table 12). Ten samples stored at freezer temperature (-20°C) had benzoylecgonine concentrations ranging from 0.54 ng/mL to 3.20 ng/mL (see Table 13). Amongst the three storage temperatures, benzoylecgonine concentrations were highest in samples stored at freezer temperature (-20°C), followed by those stored at refrigerator temperature (4°C). The lowest benzoylecgonine concentrations were in samples stored at room temperature (20-22°C).

4.5 Possible Interference

All data collected for the cocaine analyte at each time point showed unidentified peaks extending from 6.5 to 7.5 minutes in all samples including unknown samples, solvent blanks, calibrators, quality controls, negative samples, and double blanks. In all data collected from each time point for benzoylecgonine, the irregular unidentified peaks were not present at either ion transition. This made it clear the irregular unidentified peaks were unique to the cocaine analyte. The peaks were occasionally present in TICs, but not consistently. Further investigation revealed that the peaks appear in the extracted ion chromatograms (EIC) +MRMs of the 150.1 ion transition of cocaine, but not in the EIC +MRMs of the 182.2 ion transition of cocaine. Figures 11 and 12 show the presence of the peaks in the cocaine 150.1 ion transition EIC +MRMs chromatograms of Sample 3896 Lung and Blank 9 from the T_2 samples.

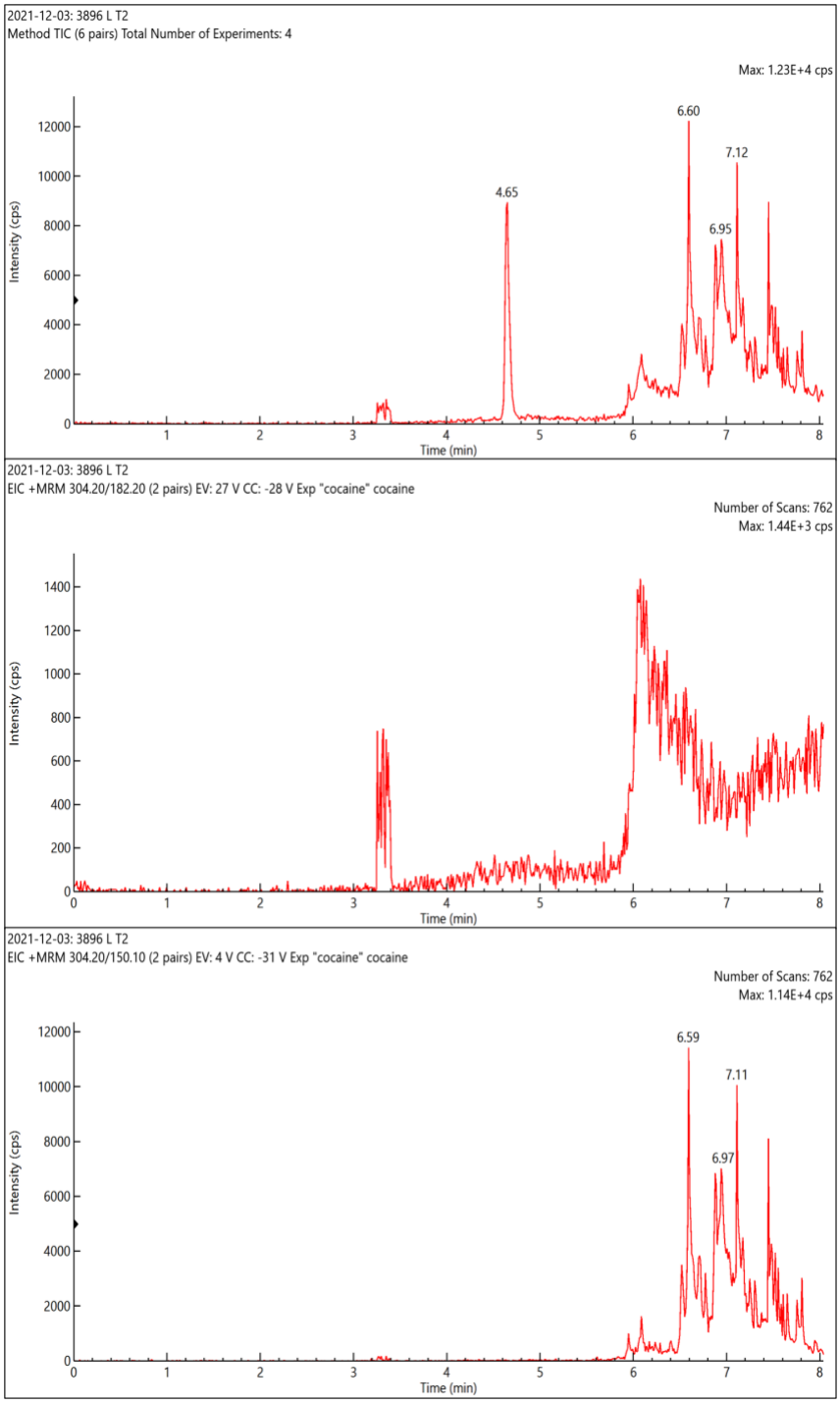


Figure 10. Total Ion and Extracted Ion Chromatograms + MRM of Cocaine in Sample 3896 Lung from T_2. The top image is the TIC of sample 3896 Lung, the middle image is the EIC +MRM of the 182.2 ion transition of cocaine, and the bottom image is the 150.1 ion transition of cocaine.

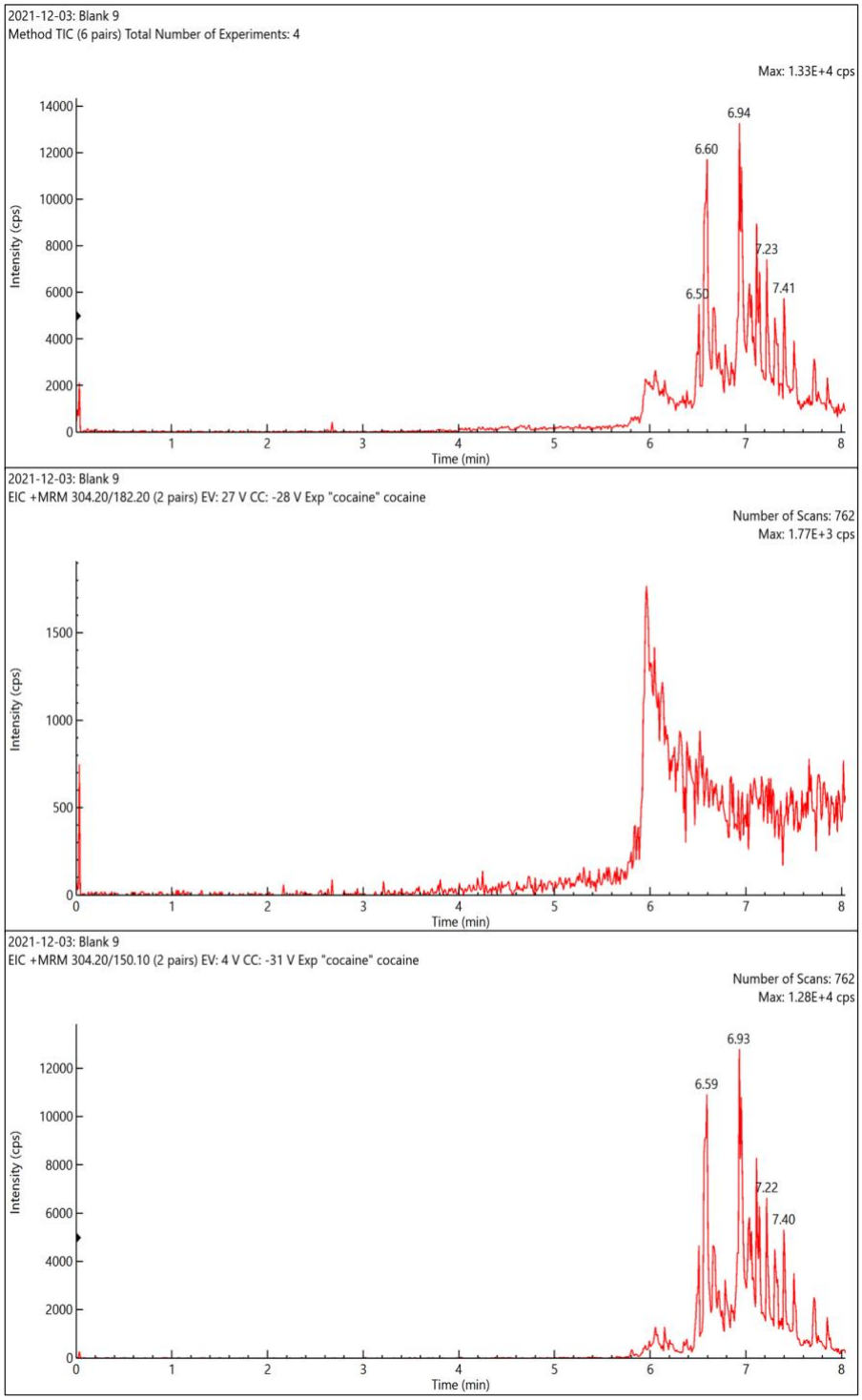


Figure 11. Total Ion and Extracted Ion Chromatograms + MRM of Cocaine in Blank 9 from T_2. The top image is the TIC of Blank 9, the middle image is the EIC +MRM of the 182.2 ion transition of cocaine, and the bottom image is the 150.1 ion transition of cocaine.

The peaks for the cocaine and benzoylecgonine analytes and their deuterated internal standards were at the correct retention times and ion transitions in all the quantitated samples. Therefore, the peaks present between 6.5 and 7.5 minutes are not the cocaine or benzoylecgonine analytes, nor their deuterated internal standards. The presence of the peaks in the EIC +MRMs of the 150.1 ion transition suggests possible matrix effect interference during the detection of the product ions of cocaine. Liquid chromatography tandem mass spectrometry has been known to produce inaccurate results due to matrix effects (37, 39). The presence of outside substances, other than the target analyte, can interfere with the ionization process, thereby interfering with the response of the instrument (40). The interference can cause ion enhancement or ion suppression.

Another possibility is that the peaks in the samples could be a result of a chemical interaction between cocaine and the formaldehyde from the embalmed tissues because formaldehyde is known to interact with nitrogen-containing compounds (14). However, this would not explain why the peaks were found in the calibrators and controls.

4.6 Limitations

Several limiting factors were identified throughout this project. The organs dissected from the rat specimens were shared between several students. This could have impacted sample volume and subsequent analyte concentration. In addition, due to the LC-MS/MS instrument maintenance, the vials were stored in the freezer at -20°C until they could be analyzed. Storing prepared samples in the freezer is not typical unless post-extraction stability is assessed, which was not part of this research. Therefore, it is

possible that storing the extracted samples could have impacted analyte stability and concentration.

To obtain a more accurate depiction of the change in analyte concentration over time at different storage temperatures, the organs from the rat specimens needed to be analyzed earlier. A T_0 sample should have been collected from the organs at the time of death. Knowing the concentration of both analytes at the time of death would have led to a clearer and more accurate investigation of analyte stability over time. In addition, the storing of rat specimens at -20°C immediately after embalming could have impacted the analytes' stability. The pH of saline and paraformaldehyde solution used to embalm specimens was not disclosed. As previously discussed, the pH of the embalming solution used is important as it can impact the stability and concentration of cocaine.

5 CONCLUSIONS

5.1 Summary of Findings

Despite many studies on drug stability in postmortem tissues, there is a lack of investigations into the degradation of drugs in formalin-fixed tissues. This project performed analysis using LC-MS/MS with ESI in positive ion mode and was able to detect and quantify analytes at concentrations as low as 0.25 ng/mL and 0.25 ng/mL, respectively. Cocaine and benzoylecgonine were detectable in kidney, spleen, and lung tissues after one month of storage at temperatures ranging from 20-22°C, 4°C, and -20°C. Most samples displayed a decrease in cocaine analyte concentrations in concordance with other studies. A clear pattern of benzoylecgonine stability over one month could not be determined due to the lack of quantifiable data from T_0 and T_1. Although the results of analyte stability based on storage conditions were inconclusive, the developed method showed good sensitivity for the detection and quantification of cocaine and benzoylecgonine in biological matrices.

5.2 Future Directions

Future studies should include analyzing samples collected at additional time points to provide more information on the stability of cocaine over time through comparison to data collected from T_1 and T_2. The project could be further extended by performing an interference study as other research studies have concluded formaldehyde does not chemically react with cocaine or benzoylecgonine (9, 28). Yet, the samples

from T_1 and T_2 had an unidentified peak which could be a metabolism product or a product from a different chemical reaction.

The sample preparation method could be improved in several ways. Based on the work of previous studies, using solutions with a low pH during sample preparation is best when working with cocaine as it will slow hydrolysis (41, 9). The sample preparation methods could be improved by knowing the pH of the saline and formaldehyde solution used to embalm the rat specimens. The addition of a protein precipitation step during sample preparation could assist with the purification of analytes in the biological matrix. One study investigating cocaine, benzoylecgonine, and other drugs used a protein precipitation step in their sample extraction method, which produced accurate and precise results with LC-MS/MS analysis (31).

In addition, performing a recovery study using negative rat organ tissues would be enlightening as to the effectiveness of the sample preparation method. It would provide additional details as to the challenges of working with tissue samples and it would help identify areas of improvement for sample preparation techniques to prevent loss of analytes. Carryover, ionization suppression or enhancement effects, and matrix interferences should be studied in drug-free rat organ tissues to prepare the calibrators as per the minimum requirements in ASB Standard Practices for Method Validation in Forensic Toxicology (42).

Another potential project would be to investigate the stability of cocaine in more organ types, specifically in the brain. Determining drug concentrations in the brain is highly beneficial in cocaine cases because the ratio of cocaine to benzoylecgonine is an

indicator of recent use (9, 43). Brain tissues have also been reported to be a more reliable sample for cocaine analysis in comparison to the liver and other biological matrices (17, 44).

BIBLIOGRAPHY

1. Hedegaard H MA, Warner M. Drug overdose deaths in the United States, 1999–2019. NCHS Data Brief, no 394. Hyattsville, MD: National Center for Health Statistics; 2020.

2. Zhou C, Byard RW. Factors and processes causing accelerated decomposition in human cadavers – An overview. Journal of Forensic and Legal Medicine. 2011 Jan 01;18(1):6-9.

3. Skopp G. Postmortem toxicology. Forensic Science, Medicine, and Pathology. 2010 2010/12/01;6(4):314-25.

4. Hyde ER, Haarmann DP, Petrosino JF, Lynne AM, Bucheli SR. Initial insights into bacterial succession during human decomposition. International Journal of Legal Medicine. 2015 May 2015;129(3):661-71.

5. Hearn W, EE Keran, H. Wei, and G. Hime. Site-Dependent Postmortem Changes in Blood Cocaine Concentrations. Journal of Forensic Sciences. 1991;36(3):673-84.

6. Pounder DJ. AUTOPSY, FINDINGS | Postmortem Drug Sampling and Redistribution. In: Payne-James J, editor. Encyclopedia of Forensic and Legal Medicine. Oxford: Elsevier; 2005;211-7.

7. Apple FS. Postmortem Redistribution of Drugs. In: Levine BS, Kerrigan S, editors. Principles of Forensic Toxicology. Cham: Springer International Publishing; 2020;595-601.

8. Yarema MC, Becker CE. Key concepts in postmortem drug redistribution. Clinical toxicology (Philadelphia, Pa). 2005;43(4):235-41.

9. Viel G, Nalesso A, Cecchetto G, Montisci M, Ferrara SD. Stability of cocaine in formalin solution and fixed tissues. Forensic Science International. 2009 Dec;193(1):79-83.

10. Takayasu T, Saito K, Nishigami J, Ohshima T, Nagano T. Toxicological analysis of drugs and poisons in formalin-fixed organ tissues. International Journal of Legal Medicine. 1994 01 Janurary;107(1):7-12.

11. Suma R, Kosanam H, Sai Prakash PK. Stability study of bupropion and olanzapine in formaldehyde solutions. Rapid Communications in Mass Spectrometry. [https://doi.org/10.1002/rcm.2458]. 2006;20(8):1390-4.

12. Spargo EA. Drugs in Embalmed Tissues. In: Levine BS, Kerrigan S, editors. Principles of Forensic Toxicology. Cham: Springer International Publishing; 2020;665-71.

13. Hilal A, Dağlioğlu N, Battal D, Yener F, Dağlioğlu K. Detection of cocaine and benzoylecgonine in formalin fixed rat tissues. Toxicology mechanisms and methods. 2009 Sep;19(6-7):422-6.

14. Nikolaou P, Papoutsis I, Dona A, Spiliopoulou C, Athanaselis S. Toxicological analysis of formalin-fixed or embalmed tissues: A review. Forensic Science International. 2013 Dec;233(1):312-9.

15. Isenschmid DS. Cocaine. In: Levine BS, Kerrigan S, editors. Principles of Forensic Toxicology. Cham: Springer International Publishing; 2020;371-87.

16. Trifilieff P, Martinez D. Cocaine: Mechanism and Effects in the Human Brain. In: Madras B, Kuhar M, editors. The Effects of Drug Abuse on the Human Nervous System. San Diego, CA: Elsevier Science & Technology; 2014;103-34.

17. Drummer OH. Postmortem toxicology of drugs of abuse. Forensic Science International. 2004 May;142(2):101-13.

18. Giroud C, Michaud K, Sporkert F, Eap C, Augsburger M, Cardinal P, et al. A Fatal Overdose of Cocaine Associated with Coingestion of Marijuana, Buprenorphine, and Fluoxetine. Body Fluid and Tissue Distribution of Cocaine and Its Metabolites Determined by Hydrophilic Interaction Chromatography-Mass Spectrometry (HILIC-MS). Journal of Analytical Toxicology. 2004;28(6):464-74.

19. Karch SB. Cocaine: History, Use, Abuse. Journal of the Royal Society of Medicine. 1999;92(8):393-7.

20. Logan BK, Smirnow D, Gullberg RG. Lack of predictable site-dependent differences and time-dependent changes in postmortem concentrations of cocaine, benzoylecgonine, and cocaethylene in humans. J Anal Toxicol. 1997 Jan-Feb;21(1):23-31.

21. Stephens BG JJ, Karch S, Mash DC, Wetli CV. Criteria for the Interpretation of Cocaine Levels in Human Biological Samples and Their Relation to the Cause of Death. American Journal of Forensic Medicine & Pathology. 2004;25(1):1-10.

22. Kennedy MC. Post-mortem drug concentrations. Internal Medicine Journal. [https://doi.org/10.1111/j.1445-5994.2009.02111.x]. 2010 Mar 01;40(3):183-7.

23. Carvalho V, da Matta Chasin, AA, and de Carvalho, DG A study on the stability of anhydroecgonine methyl ester (crack biomarker), benzoylecgonine, and cocaine in human urine. Revista de Psiquiatria Clínica. 2008;35:17-20.

24. Dugan S, Bogema S, Schwartz RW, Lappas NT. Stability of drugs of abuse in urine samples stored at -20 degrees C. J Anal Toxicol. 1994 Nov-Dec;18(7):391-6.

25. Romberg RW, Past MR. Reanalysis of forensic urine specimens containing benzoylecgonine and THC-COOH. J Forensic Sci. 1994 Mar;39(2):479-85.

26. Klingmann A, Skopp G, Aderjan R. Analysis of cocaine, benzoylecgonine, ecogonine methyl ester, and ecgonine by high-pressure liquid chromatography-API mass spectrometry and application to a short-term degradation study of cocaine in plasma. J Anal Toxicol. 2001 Sep;25(6):425-30.

27. Hernandez A, Andollo W, Hearn WL. Analysis of cocaine and metabolites in brain using solid phase extraction and full-scanning gas chromatography/ion trap mass spectrometry. Forensic Science International. 1994 May 13;65(3):149-56.

28. Cingolani M, Cippitelli M, Froldi R, Gambaro V, Tassoni G. Detection and Quantitation Analysis of Cocaine and Metabolites in Fixed Liver Tissue and Formalin Solutions. Journal of Analytical Toxicology. 2004;28(1):16-9.

29. Al-Asmari AI. Method for the identification and quantification of sixty drugs and their metabolites in postmortem whole blood using liquid chromatography tandem mass spectrometry. Forensic Science International. 2020 April;309:110193.

30. Northrop DM. Forensic Applications of High-Performance Liquid Chromatography and Capillary Electrophoresis. In: Richard Saferstein ABH, editor. Forensic Science Handbook. 3rd ed. Boca Raton: CRC Press; 2020;496-562.

31. Wagner R, Moses L. Validation of two methods for the quantitative analysis of cocaine and opioids in biological matrices using LCMSMS. Journal of Forensic Sciences. 2021;66(3):1124-35.

32. Bell S. Instrumentation. Forensic Chemistry. 2nd ed. Upper Saddle River, NJ: Pearson Education, Inc.; 2013;140-205.

33. Fanali S, Haddad PR, Poole C, Riekkola M-L, Riekkola M-L. Liquid Chromatography: Fundamentals and Instrumentation. Saint Louis, United States: Elsevier, 2017.

34. Adam B. Hall RS. Forensic Mass Spectrometry Analytical Advancements and Casework Applications. In: Richard Saferstein ABH, editor. Forensic Science Handbook. Boca Raton: CRC Press; 2020;563-611.

35. Pitt JJ. Principles and applications of liquid chromatography-mass spectrometry in clinical biochemistry. The Clinical biochemist Reviews. 2009;30(1):19-34.

36. Ho CS, Lam CWK, Chan MHM, Cheung RCK, Law LK, Lit LCW, et al. Electrospray ionisation mass spectrometry: principles and clinical applications. The Clinical biochemist Reviews. 2003;24(1):3-12.

37. Taylor PJ. Matrix effects: the Achilles heel of quantitative high-performance liquid chromatography–electrospray–tandem mass spectrometry. Clinical Biochemistry. 2005 April;38(4):328-34.

38. Logan BK, Smirnow D, Gullberg RG. Lack of predictable site-dependent differences and time-dependent changes in postmortem concentrations of cocaine, benzoylecgonine, and cocaethylene in humans. Journal of Analytical Toxicology. 1997 Jan-Feb;21(1):23-31.

39. Bonfiglio R, King RC, Olah TV, Merkle K. The effects of sample preparation methods on the variability of the electrospray ionization response for model drug compounds. Rapid Communications in Mass Spectrometry. [https://doi.org/10.1002/(SICI)1097-0231(19990630)13:12%3C1175::AID-RCM639%3E3.0.CO;2-0]. 1999 June 30;13(12):1175-85.

40. Peters FT, Remane D. Aspects of matrix effects in applications of liquid chromatography–mass spectrometry to forensic and clinical toxicology—a review. Analytical and Bioanalytical Chemistry. 2012 June;403(8):2155-72.

41. Garrett ER, Seyda K. Prediction of Stability in Pharmaceutical Preparations XX: Stability Evaluation and Bioanalysis of Cocaine and Benzoylecgonine by High-Performance Liquid Chromatography. Journal of Pharmaceutical Sciences. 1983;72(3):258-71.

42. Board AAS. ANSI/ASB Standard 036: Standard Practices for Method Validation in Forensic Toxicology. Colorado Springs, CO: AAFS Standards Board, LLC; 2019.

43. Alvear E, von Baer D, Mardones C, Hitschfeld A. Determination of cocaine and its major metabolite benzoylecgonine in several matrices obtained from deceased individuals with presumed drug consumption prior to death. Journal of Forensic and Legal Medicine. 2014 March;23:37-43.

44. Browne SP, Moore CM, Scheurer J, Tebbett IR, Logan BK. A rapid method for the determination of cocaine in brain tissue. Journal of Forensic Sciences. 1991 Nov;36(6):1662-5.

CURRICULUM VITAE