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Detection and quantitation of cocaine and benzoylecgonine in skeletal tissue using laminar flow triple quadrupole mass spectrometry

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DETECTION AND QUANTITATION OF COCAINE AND BENZOYLECGONINE IN SKELETAL TISSUE USING LAMINAR FLOW TRIPLE QUADRUPOLE MASS SPECTROMETRY

by

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PASCALE LISE YACAVONE

ABSTRACT

Toxicological analysis can be a critical factor in determining the cause of an individual's death especially when no other physical signs of trauma are present. However, there are cases where human remains are discovered several years after a person's death, leaving toxicologists with very few biological matrices to choose from for analysis. In the last 20 years, developments in the field of forensic toxicology have led to several promising methods for the use of human bone as a matrix for drug analysis. This presents a unique challenge for toxicologists due to the fact that bone is a hard tissue, requiring extensive homogenization and extraction procedures. Further, there is very little reference material available on the topic of skeletal remains as an alternative drug matrix. The objective of this study was to develop a quick and efficient method of extraction as well as subsequent analytical detection and quantitation of cocaine and benzoylecgonine in the complex matrix of skeletal remains following a 3-month period of chronic drug administration.

All rodent specimens utilized in this research were in accordance with the Institutional Animal Care and Use Committee (IACUC) protocol at Boston University. The 16 rats used for this study underwent a 12-week chronic self-administration of cocaine, followed by a 5-week period of abstinence, and then a 3-week period of resumed consumption before euthanasia. Twelve of the sixteen rat specimens received a pericardial infusion of paraformaldehyde and phosphate buffered saline at the time of death before being frozen. The carcasses were allowed to return to room temperature before undergoing dissection to remove the left humerus, left femur, and 13th thoracic vertebrae from each rat for toxicological analysis.

Segments of each bone were taken, and all internal bone marrow was removed. Fragments of each bone in the weight of 0.25g were then homogenized using a bead mill, methanol, and deionized water. Solid phase extraction was conducted on the homogenates using a mixed mode, reverse phase/ion exchange column. The resulting product was evaporated and reconstituted in 180 µL 10mM ammonium formate in DI water and 20 µL methanol prior to LC-MS/MS analysis employing the use of gradient elution, and electrospray ionization. The MS was operated in positive ion mode, with MRM ion detection.

The linear dynamic range for cocaine and benzoylecgonine was found to be 0.25 ng/mL – 200 ng/mL with a limit of quantitation (LOQ) of 0.25 ng/mL. Calibration curves were generated using 8 spiked human urine calibrators providing R^2 values greater than 0.98 for both compounds. This procedure utilized bead mill homogenization, mixed mode solid phase extraction, and LC-MS/MS analysis utilizing a laminar flow, triple quadrupole mass spectrometer to obtain drug concentrations in skeletal tissue in as short as 1 hour.

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LIST OF ABBREVIATIONS

APCI	Atmospheric Pressure Chemical Ionization
BZE	Benzoylecgonine
CSA	Controlled Substances Act
CYP450	Cytochrome P450
DNA	Deoxyribonucleic Acid
DOC	2,5-Dimethoxy-4-chloroamphetamine
DOD	Department of Defense
ELISA	Enzyme Linked Immunosorbent Assay
EM	Electron Microscopy
EME	Ecgonine Methyl Ester
ESI	Electrospray Ionization
g	Grams
HCl	Hydrochloric Acid
HPLC	High Performance Liquid Chromatography
HSID	Hot Source Induced Desolvation
LOD	Limit of detection
LOQ	Limit of Quantitation
LC	Liquid Chromatography
N	Concentration
ng/mL	Nanogram per Milliliter

mg	Milligrams
mg/kg	Milligram per Kilogram
mL	Milliliter
M	Molar
mM	Millimolar
MRM	Multiple Reaction Monitoring
MS	Mass Spectrometry
MS/MS	Mass Spectrometry/Mass Spectrometry
m/s	Meter per Second
m/z	Mass to Charge
PFA	Paraformaldehyde
PBS	Phosphate Buffered Saline
RPM	Revolutions Per Minute
TLC	Thin Layer Chromatography
UHPLC	Ultra-High-Performance Liquid Chromatography
v/v	Volume of Solute/Volume of Solvent
µL	Microliter
6-MAM	6-Monoacetylmorphine

1. INTRODUCTION

1.1 Postmortem Toxicology

Postmortem toxicology can be best defined as the analysis of bodily fluids after death to determine whether a foreign substance was the cause of death, a contributor to the death, or caused impairment (1). In a forensic context, toxicology is performed on any decedent whose death is believed to have been the result of unnatural or suspicious circumstances. Samples are typically taken during autopsy and can include but are not limited to peripheral and cardiac blood, urine, vitreous fluid, stomach contents, bile, and tissue samples from the liver, lungs, spleen, and kidneys. Several factors can impact the concentration of a substance within bodily fluids/tissues both antemortem and postmortem (2). These factors include the pharmacokinetic and pharmacodynamic properties of the drug, postmortem redistribution, and the effects of putrefaction and autolysis (1). Pharmacokinetics refers to the effects the body has on a drug, and pharmacodynamics refers to the effects a drug has on the body (1). Both processes are drug specific and time dependent. After ingestion, a drug undergoes absorption, distribution, metabolism, and excretion (1). During this process, drug concentrations within the various sites of the body change. The pharmacokinetic properties of a drug are dependent upon factors such as pKa, lipophilicity, and molecular size (3). These factors are critical considerations for forensic toxicologists when determining if a substance was consumed at a therapeutic, toxic, or lethal dosage (2). Postmortem redistribution is another important factor for toxicologists when selecting a biological matrix for drug analysis. Postmortem redistribution refers to site dependent changes in drug concentration within the body after death (3). These concentration changes are the result of passive diffusion from areas of high concentration to areas of low concentration as decomposition progresses (3). Putrefaction, which is the decay process of biological materials, and autolysis, which is the digestion of biological material by enzymes are two components of decomposition that alter drug concentrations. These factors can complicate interpretation of antemortem drug levels and require an alternative investigative route (1).

The selected fluids or tissues then undergo a two-step analysis that includes a screening test followed by a confirmatory analytical method (1). The screening, or presumptive test, is intended to identify if any foreign substances are present within the sample. The most common screening techniques used in forensic toxicology are immunoassay or catalytic color tests. Based on the results of the screening test, a confirmatory method is then used to identify and quantify the components within the sample. These methods utilize a separation instrumentation coupled with a detection system. The most common methods of separation are liquid or gas chromatography, followed by mass spectrometry or flame ionizing detection. Values obtained from this testing, in combination with considerations of pharmacokinetics and postmortem redistribution, allow for the interpretation of what role these substances may have played in an individual's death (2).

Postmortem drug analysis can become more complicated when fluids and soft tissues are no longer present due to severe decomposition. Cases of partial or full decomposition present a unique issue to investigators, being that cause of death may not be readily apparent during a medical examination of the remains (2). Without skin or vital organs that present physical damage, toxicological analysis may be the last remaining tool not only for determining a possible cause of death, but also to provide insight into events that occurred before death.

1.2 Non-Fluid Matrices for Drug Testing

1.2.1 Drug Testing in Tissue

When biological fluids such as blood and urine are no longer available, forensic toxicologists often turn to tissues/organs to test for foreign substances. An organ is defined as a structure containing at least two different tissue types that performs a specific function within the body (4). The four major tissue types that exist within the human body are epithelium, muscle, nervous, and connective tissue (4). Each organ within the body contains unique components that require consideration when it comes to method selection for toxicological analysis. As the matrix being used becomes more complex, so does the sample preparation. With tissue there is the extra step of homogenization, which is the process of blending the solid tissue into a viscous liquid. Typically, these samples must then be diluted with some form of buffer or solution due to their viscous nature. Tissue has been shown to be a highly suitable alternative for toxicological testing. One such study determined that postmortem tissue could be used from racehorses who died while competing to detect the presence of butorphanol, pentobarbital, and ergotamine via solid phase extraction (SPE) and gas chromatography-mass spectrometry (GC/MS) (5). Several other studies have demonstrated the ability to detect drugs of abuse in bodily organs postmortem using liquid chromatography-mass spectrometry (LC/MS) including heroin and its major metabolites, tramadol, morphine, cocaine, marijuana, and designer drug 2,5-

Dimethoxy-4-chloroamphetamine (DOC) in the brain, heart, lungs, kidney, and liver (6-9). These substances can persist in bodily tissue much longer than in blood in postmortem cases, which is extremely valuable to forensic analysis in situations when blood and urine are no longer available for testing.

1.2.2 Drug Testing in Skeletal Tissue

Skeletal tissue, known more commonly as bone, is a form of dense connective tissue composed of calcium hydroxyapatite. The exterior surface of bone is densely packed, which is what gives bones their strength and ability to support the surrounding structures. Below the outer layer is a spongy layer known as cancellous bone. Long bones have a hollow center called the medullary shaft, which is highly porous and filled with vessels and bone marrow (10). The long bones of the body are home to two types of bone marrow, red and yellow. Yellow marrow is composed of fatty connective tissue and is stored within the marrow cavity of the bone. Red marrow in certain bones is the production site for replacement blood cells that have been destroyed by the liver. These cells include erythrocytes, leukocytes, and platelets, which are then passed into the circulatory system. The body also uses bone as a place to store minerals, and in times of mineral deficiency, releases them into circulation. All bones within the body are covered by a thin layer of fibrous tissue called the periosteum. Bones serve as the major structural support for all muscles in the body, making them vital in all vertebrate organisms.

Bones possess properties of both hard and soft tissues, with a calcified exterior layer and a highly vascularized protected interior. Based on their intrinsic connection to the circulatory system, it is reasonable to speculate that substances ingested into the body could be stored within one or both present tissue types. Several studies have examined this question and successfully demonstrated that certain substances along with their primary metabolites, are deposited into bone and bone marrow (11-21). Some researchers have evaporated methanol extracts and reconstituted them without further sample preparation, while others have chosen to use liquid-liquid or solid phase extraction prior to analysis with either gas or liquid chromatography followed by mass spectrometry. Studies seeking only to detect and quantify levels of drugs in bone have determined that methamphetamine, prescription benzodiazepines, and methadone were detectable in bone samples postmortem (11-13).

Other researchers have examined possible confounding factors to determine what impacts the levels of xenobiotic accumulation within skeletal remains. James Watterson, a researcher at the Laurentian University in Ontario Canada has dedicated several experiments to examining the environmental impact of drug levels in bone, and the relationship between the type of bone to the concentration present (14-18). One of these experiments focused on dose-death interval and bone type following acute ketamine exposure via enzyme linked immunosorbent assay (ELISA) and liquid chromatography-tandem mass spectrometry (LC-MS/MS). Results indicated that ketamine was detectable in both bone and bone marrow, however ketamine's primary metabolite norketamine, was only present in bone marrow (14,16). Further research conducted by Watterson demonstrated the distribution of diazepam, amitriptyline, and pentobarbital in 12 different bone types utilizing solid phase extraction followed by GC/MS (15). The study yielded mixed results in terms of which bone type contained the highest concentration of the different substances analyzed. This indicates that the location of the bone itself within the body may not determine accumulation, rather that the metabolic properties of the drug determine its skeletal distribution (15). In another study involving ketamine levels in bone, the relationship between decomposition time and bone type were examined using solid phase extraction and GC/MS (17-18). Interestingly, the difference in concentrations over time were less varied than the difference in concentrations between samples of the same bone type, raising the important issue of the variability between two subjects who received the same dose of a drug (18). This is one of the many unanswered issues of drug analysis in bone, being that it is still considered a novel matrix.

Despite the increasing number of published research regarding drug detection and quantitation in bone, there are still issues with utilizing skeletal tissue in a forensic laboratory. The biggest disadvantage to these previous studies is the length of time needed for sample incubation (11-18). One example of this is seen in the previously mentioned study regarding methadone detection in skeletal tissue, which utilizes a 72-hour incubation in methanol before further sample preparation. Despite almost all documented methods including some sort of incubation step, none of the authors explain the need of this step in the extraction process. In a 2017 study conducted at Boston University, the efficiency of a method for the detection of cocaine and its two primary metabolites (benzoylecgonine and ecgonine methyl ester) in bone without the use of an incubation period was examined (19). Rat carcasses were allowed to decompose for a period of one year following chronic cocaine exposure. Collected bones then underwent sample preparation using a bead mill and methanol/water for homogenization, then solid phase extraction and subsequent analysis via two-dimensional liquid chromatography tandem mass spectrometry (2D-LC-MS/MS) for a total analysis time for one sample of 1 hour. This study utilized several bones from within the body including vertebrae, femur, humeri, and innominate bones to examine if cocaine was detectable in certain bones within the body following a period of decomposition (19). It was found that cocaine and benzoylecgonine were present and detectable even after a lengthy period of decomposition. Ecgonine methyl ester (EME) was not detectable in any of the decomposed bone samples, however one rat carcass was analyzed immediately after death, and EME was present within this sample set. These findings indicate that with further experimentation, there may be alternative methods to drug extraction from bone without a lengthy period of incubation (19).

In addition to research studies involving drug detection in skeletal remains, applications of this information to real forensic cases have also been addressed in literature (20). In 2016 the completely skeletonized remains of a Greek man were found near his home after what appeared to be a motorcycle accident (20). Due to very little soft tissue being present on the body, bone was selected for drug testing. A sample was taken from the central shaft on the medullary cavity of the femur for analysis by ultra-high performance liquid chromatography-tandem mass spectrometry (UHPLC-MS/MS). Findings were consistent with the known prescription history of the decedent, identifying quantifiable levels of alprazolam and zolpidem (20). This method has a remarkably shorter incubation period of approximately 6 hours when compared to previously published methods (11-18) but is still quite lengthy.

Looking beyond the field of forensic drug testing, xenobiotic analysis of skeletal remains shows promise in being useful for identification of biological and chemical weapons. A study conducted in 2020 at the University of Florida in partnership with the Department of Defense sought to determine if metabolites of chemical nerve agents are detectable in skeletal remains (21). The major metabolites of VX were analyzed using SPE and LC-MS/MS. Both femurs of 12 minipigs who had received injections of VX antemortem were used to test the efficacy of this method when applied to real, in vivo samples. Only 2 of the 12 samples yielded results above the limit of detection (21). Interestingly, these results came from the two minipigs that died as a result of exposure before being euthanized. This is the first time that nerve agents have been documented within skeletal remains, indicating that they do interact with bones in some way (21). With further research into the ways xenobiotics interact with the skeletal structure, this kind of testing could prove to be a highly useful tool in military and DOD investigations.

1.3 Paraformaldehyde Fixation of Tissue

1.3.1 Perfusion of Paraformaldehyde in Phosphate Buffered Saline

When conducting a scientific study on a once living organism, researchers often need to employ chemical methods of preservation to maintain the most lifelike state of the tissues and internal organs. In the field of clinical pathology, 10% buffered formalin is the standard selection for histopathologic analysis (22). From the moment of removal, tissue begins the process of decomposition, and thus, this kind of preservation cannot portray the exact appearance of cells and tissue structures in vitro. In situations where this kind of preservation is critical to the intended analysis, a paraformaldehyde perfusion can be used to preserve cellularity just as it was during life. Paraformaldehyde (PFA) is the polymerization product of formaldehyde and is a solid powder substance. PFA is the preferred method of fixation when electron microscopy (EM) is going to be performed on the cellular material (22). In standard formalin solution, methanol is added to stabilize the solution and prevent polymerization. This however can cause clustering of fine cellular structures and make them unsuitable for EM. It is prepared into a perfusion solution by dissolving the powder in heated PBS. When conducting this type of fixation on rodents, the animal is placed under general anesthesia and a tube is placed into the ascending aorta of the heart. Using an infusion pump, the PFA and PBS are injected through the tube under fixed pressure until the desired fixation is achieved (22). Formalin is an aqueous formaldehyde solution that can be unbuffered at formaldehyde's standard pH of 3-3.5, or it can be buffered to a neutral pH of 7. Paraformaldehyde has a similar pH to that of buffered formalin averaging between 7-7.6. To date there have been no published works regarding the stability of drugs in paraformaldehyde fixed tissues. There have however been several studies examining the stability of formaldehyde fixed tissues and formalin solution fixed tissues (23). One such study examined cocaine and benzoylecgonine stability in brain and liver tissue fixed with buffered and unbuffered formalin solutions. This study concluded that benzoylecgonine is relatively stable at pH 3.5 and 7.4, but cocaine was only stable at a pH of 3.5. The authors concluded that these results can be explained by cocaine's ester structures that are labile under alkaline conditions (23).

1.4 Rodent Anatomy

1.4.1 Metabolism

Rattus Norvegicus, more commonly known as the albino rat, is a species of rodent frequently used in scientific research to act as a model for understanding how drugs may act or affect the human body. Adult male rats weigh an average of 450-520 g and have a lifespan of 2.5-3.5 years (24). It has long been believed that rats were the perfect surrogate for humans in research due to the ease of reproducibility as well as their biologic similarities to humans. Most available research regarding comparison between rat and human metabolism suggests that the role of cytochrome P450, the enzyme responsible for the breakdown of xenobiotics, is similar between the two species (25). The only documented contention to this is that rodents eliminate substances affected by CYP450 more rapidly than humans due to a higher concentration of the hepatic enzyme in relation to their body weight. This discrepancy, however, is not believed to be a factor in the postmortem accumulation of foreign substances within the body (25).

1.4.2 Comparison of the Anatomic Structures of Rats and Humans

Very few differences exist between the anatomic structures of rats and humans, hence why they are favorable subjects for clinical research (26). One such difference is that rats have more vertebral bodies within their spine than humans do. Humans have 12 thoracic vertebrae followed by 5 lumbar vertebrae, whereas rats have 13 thoracic vertebrae followed by 6 lumbar vertebrae (27). Both humans and rodents also possess sesamoid bones, which are small, rounded bones that are located within tendons for stability purposes

(28). Rats however have a specific sesamoid bone that is rarely seen in humans. This bone is known as the fabella and is located within the lateral head of the gastrocnemius muscle behind the lateral femoral condyle (28). With regards to internal organ structures, the only major organ that humans have, and rats do not is a gallbladder (28). Despite difference in the shape and lobal structures of the rat liver to a human liver, a study examining the anatomy of 20 rat livers and 78 human cadaver livers determined that the locations and connections of portal and hepatic veins within the respective lobes are considered similar (29).

1.5 Cocaine

1.5.1 Cocaine and Use Statistics

Cocaine is an alkaloid substance in the form of an odorless, white crystalline powder and is derived from the leaves of the Erythroxylon Coca plant, which is most commonly native to certain regions of South America (30). Albert Niemann, a German graduate student, was the first to isolate cocaine from the coca leaves in the mid-19th century after being given the project by his chemistry professor, Carl Wohler (30). Based on the widely held belief that cocaine had “magical” properties, it began appearing in commercially available products such as wine and most famously, Coca Cola™ (30). It wasn’t until 1914 that the serious potential for abuse of cocaine was recognized within the United States, and over the counter sales were discontinued as a result of the Harrison Narcotic Act (30). Eventually in 1970 cocaine was added to the Controlled Substances Act under Schedule II, labeling it as having some legitimate medicinal purposes but a high potential for abuse (31). To date this is the category that cocaine remains under within the CSA.

On the street cocaine is sold in two major forms, hydrochloride salt (HCl) and crack cocaine (31). HCl salt is commonly ingested through insufflation, whereas crack cocaine, being the free base form of HCl salt cocaine, is smoked (32). When purchased illicitly, cocaine is rarely found in its pure form (32). Distributors add bulking agents that fall into two categories, adulterants, and diluents, to create more product (31). Adulterants are substances added to the drug product that produce a pharmacologic effect in the user (31). This can include substances like caffeine, diphenhydramine, and benzocaine as well as other substances. A diluent is a substance that is added to the product that does not produce any effects in the user, such as sucrose, flour, or ascorbic acid (31).

Cocaine is one of the most highly addictive and widely abused substances within the United States (32). In 2019, it is estimated that 20 million people had used cocaine within the past year (33). It is reported that from 2000 to 2006, cocaine related overdose deaths in the US increased from 1.26 to 2.50 per 100,000 people and has remained at an elevated level ever since (33). The DEA warns that the level of cocaine related overdoses may reach the same prevalence as opioid related deaths if use and abuse continues to increase at the same rate (33).

1.5.2 Chemical Properties of Cocaine and Benzoylecgonine

Cocaine is an ester of benzoic acid and the amino alcohol methylecgonine, containing a tropine moiety (31). The tropine moiety is what distinguishes cocaine from other anesthetic substances. Like other anesthetic substances however, cocaine possesses both a hydrophobic and hydrophilic region (31). The hydrophobic region consists of a benzene ring and the hydrophilic region consists of a secondary or tertiary amine group (31). It has two optical active centers, and this has four isomer products. It has a molecular weight of 303.35 g/mol and has a pKa of 8.7 (32). A diagram of cocaine's chemical structure can be seen in Figure 1.

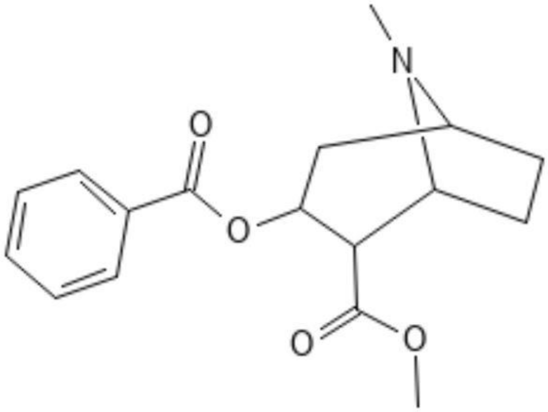


Figure 1: Chemical Structure of Cocaine (34)

Benzoylecgonine is one of the two primary metabolites of cocaine (35). Due to the rapid conversion of cocaine into metabolite products, benzoylecgonine is commonly seen in higher concentrations within blood plasma when compared to the parent drug (30). It has a molecular weight of 289.33 g/mol and a pKa of 10.82 (35). A diagram of benzoylecgonine's chemical structure can be seen in Figure 2.

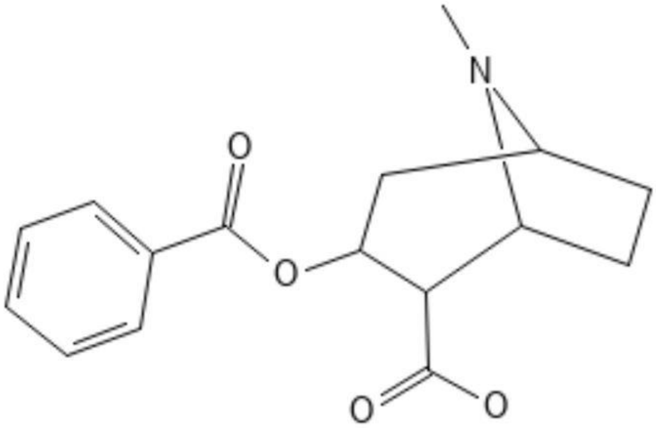


Figure 2: Chemical Structure of Benzoylecgonine (35)

1.5.3 Consumption of Cocaine

1.5.3.1 Pharmacokinetics

Pharmacokinetics refers to what the body does to a drug, including its ingestion, absorption, bioavailability, distribution, metabolism, and excretion (30). There are three factors that dictate how a drug will be absorbed into the body which include the physicochemical properties of the substance, the chemical formulation, and the route of ingestion (30). Cocaine can be ingested nasally, orally, intravenously, or through smoking (31). In all modes of ingestion excluding intravenous injection, the drug must first diffuse across the cell membrane before entering the circulatory system (36). Cocaine permeates the cell via passive diffusion, which is defined as the motion across a membrane from an area of high concentration to an area of low concentration (36). This is true for both the salt and the free base (crack) form of cocaine, because even in its ionized salt form, cocaine is quickly de-ionized by the normal physiological pH of blood and tissues, which is 7.4 (36). The route of administration dictates the relative bioavailability of cocaine, which has an estimated half-life of 78 minutes when taken nasally or via injection, and 69 minutes when smoked (37). Once cocaine has entered the body, it is metabolized primarily into benzoylecgonine and ecgonine methyl ester. Benzoylecgonine is produced by spontaneous chemical hydrolysis of cocaine catalyzed by liver methylesterase. Ecgonine methyl ester is formed by enzymatic hydrolysis of cocaine by pseudocholinesterase (30). Both metabolites can also result from the hydrolysis of cocaine via liver carboxylesterase (30). Cocaine and its primary metabolites are predominantly excreted through urine; however, a very small amount is excreted within fecal matter (37). The percentages of these three substances in excreted urine is dependent on the route of administration, but is generally 1-9% cocaine, 26-54% benzoylecgonine, and 18-41% ecgonine methyl ester (37).

1.5.3.2 Pharmacodynamics

Pharmacodynamics refers to the effects that a drug has on the body, such as its interaction with receptors in the brain, the effects of receptor binding, and its chemical interactions within the body (30). Cocaine users experience a variety of both psychological and physical effects during and after ingestion (32). The physical effects include topical anesthesia, vasoconstriction (shrinking of the blood vessels), hypertension (elevated blood pressure), tachycardia (elevated heart rate), and mydriasis (pupil dilation) (32). Users also report experiencing an elevation in mood, energy, and sexual arousal along with a sense of euphoria (30). Within the brain cocaine inhibits the reuptake transporters of multiple neurotransmitters, including dopamine, norepinephrine, and serotonin (37). Inhibition of these transporters results from cocaine molecules binding with the 5-HT transporter located in the mesolimbic region of the brain, causing increased synaptic levels of these neurotransmitters (37). This is the reason chronic users of the drug tend to binge when using it and will continue to self-medicate with the substance (32). Periods of sobriety often result in severe depression and subsequent cravings for the drug (37). The most common cause of death associated with cocaine consumption is myocardial infarction, more commonly known as a heart attack (37). This is due to cocaine's vasoconstricting properties which result in blood flow to the heart being limited causing a lack of oxygen in the heart muscles. There is also an increased risk of mortality when cocaine is consumed with ethanol (37). A unique metabolite is formed as a result of co-consumption, known as cocaethylene (37). Studies have indicated that the median lethal dose of cocaethylene is significantly lower than that of cocaine alone, estimated at 62 mg/kg and 95.1 mg/kg respectively (37).

1.5.4 Postmortem Redistribution of Cocaine

When a person dies, drugs or other substances ingested prior to death undergo a dispersion process known as postmortem redistribution (38). During this process the concentration of any drugs in bodily fluids or tissues changes over time based on certain factors. These factors can include the physicochemical properties of the drug, the tissue or fluid being tested, the time between death and sample collection, and the conditions in which the body was found (38). This redistribution can complicate toxicologist interpretation of antemortem concentrations. There are many mechanisms by which postmortem redistribution can occur, but with respect to cocaine there is a specific phenomenon that results in blood and tissue concentration changes (38). It has been proposed that there may be continuing metabolic activity after death causing a decrease in cocaine levels and an increase in metabolite levels in blood and tissues (38). This hypothesis was addressed by monitoring the activity of drug metabolizing enzymes in rat liver microsomes. They observed enzymatic activity steadily decreasing for up to 48 hours after death, indicating that there could be a postmortem metabolism process causing level fluctuations over time (38). To date there has been no research investigating the relationship between drug dose and postmortem levels in skeletal remains of humans, thus this concept is still just a theoretical explanation for observed results.

1.6 Instrumental Theory

1.6.1 Bead Mill

The first step when analyzing any hard tissue or solid substance in forensic toxicology is sample homogenization which ensures that all components of the sample are uniformly mixed. Bead mills are a fast and efficient method of sample homogenization (39). These instruments operate using high velocity motion under fixed pressure that is applied to sealed tubes containing the sample and some form of beads. The tubes are shaken at a certain speed measured in meters per second (m/s), causing the sample and the beads to collide. Beads can be made from glass, ceramic, metal, carbide, and garnet, and can range in size from 0.1 mm to 6.5 mm (39). Selection of bead material and size is dependent on the sample matrix as well as the target component of the testing. Beads made of glass, garnet, or carbide, are useful for soft matrices, while ceramic and metal are useful for solid or fibrous materials. The smaller the bead size, the finer the resulting particles will be due to increased collision frequency (39). This makes the bead mill an ideal tool for homogenizing solid skeletal tissue.

1.6.2 Mixed Mode Solid Phase Extraction

Solid phase extraction (SPE) is one of several sample preparation techniques utilized in forensic toxicology to isolate target substances for analytical testing (30). SPE is the process of selectively partitioning analytes contained in a solution onto a stationary phase. Separation of the analytes within solution results from the varying degrees of interaction that occur between individual components of the mixture and the stationary phase (40). Selection of the stationary phase is dependent on the properties of the analyte of interest, such as the pKa of the target analytes and properties of the sample matrix that could impact extraction efficiency (40). Commercially available SPE columns contain packed silica bonded with differing R-groups depending on the type of cartridge (40). The silica material is porous allowing for maximum interaction between the mobile solution and the bonded R-groups. Cartridges can be designed for normal phase, reverse phase, strong cation exchange, strong anion exchange, or mixed mode extraction (41). Before samples are loaded onto the column, it must be rinsed with a solvent or buffer similar to the pH of the sample to pre-wet the packing material. The sample is then loaded to adsorb onto the column either by gravity or using positive pressure. The pH of the system is then altered through one or more wash steps intended to remove weakly retained interfering compounds within the sample. The retained target analyte is eluted from the column by using an organic solution to disrupt hydrophobic interactions between the analyte and the packing material (42). The eluted substance can then either be evaporated and reconstituted for further analysis or injected directly depending on the instrument being utilized.

1.6.3 Liquid Chromatography

The term chromatography refers to the analytical technique of separating a mixture into its individual components for the purpose of further analyzing, identifying, purifying, and quantifying any substances present (43). Within analytical chemistry, there are two primary methods of chromatography: gas, and liquid. Chromatographic separation is achieved by applying the substance of interest to a stationary phase and passing a mobile phase, in the form of a gas or liquid, through the stationary phase (43). Individual components of the mixture are separated based on their affinity for the mobile and stationary phases (43). Liquid chromatography involves the use of a liquid mobile phase and a solid stationary phase, often silica based and bonded to the inner lining of a column (43). The chemical properties of the mobile and stationary phase are selected based on the targeted substance of analysis. The properties of the column can differ based on the target substance which includes pore size, particle distribution, and length of the column (43).

Samples are dissolved or reconstituted in a solution similar to the initial mobile phase and can then be introduced to the system manually or through the use of an autosampler (43). After a certain volume of sample is drawn into the sampler loop, it is then carried onto the column. As the mobile phase is passed through the column, individual sample components interact with the stationary phase and are retained until the mobile phase composition causes them to elute and exit from the column. Elution can be achieved in one of two methods, isocratic or using a gradient (43). Isocratic elution employs the use of one mobile phase that does not change composition during the chromatography process. Gradient elution employs the use of two or more solvents that differ significantly in their polarities and the ratio of the two solvents is changed at a fixed interval during the elution process (43). This method of separation is commonly used in tandem with a detection system to accurately identify and quantify the separated components within the sample.

1.6.3.1 Ultra-High-Performance Liquid Chromatography

UHPLC, or ultra-high-performance liquid chromatography is fundamentally similar to HPLC but offers several advancements to the process (43). The typical particle size in the stationary phase of an HPLC column is between 3 and 20 microns for the silica coating and 80 to 4,000 Angstrom in diameter for the internal pore size (43). In UHPLC the particles are much smaller, on average around sub-2 microns (43). This increases the back pressure within the column when the mobile phase is introduced. This is beneficial because it reduces the run time needed to separate the components within a sample and reduces the volume of solvents consumed during the process (43). This type of system can be particularly useful when many samples need to be analyzed, such as in research settings or a high work volume laboratory.

1.6.4 Mass Spectrometry

After a sample has been separated into its various components, the next step in the analytical process is to identify and quantify what is present in the sample. Mass spectrometry is the analytical process of separating molecular ions based on their mass to charge ratios (m/z) and determining the relative abundance of those ions (43). The instrument can then analyze the produced ions to determine the chemical structure and molecular weight of the substance. The three primary components of an MS system are the ionization source, the quadrupole system, and the detector system (43).

1.6.4.1 Electrospray Ionization

As samples elute off the LC column, the next function is to ionize the separated components (43). Electrospray ionization (ESI) is considered to be a soft ionization technique as it utilizes an electrically charged fine mist which results in very minimal fragmentation of the precursor ion (43). A high voltage current is applied on one end to the capillary inlet and on the other to the MS orifice. This current results in the characteristic formation of what is called a Taylor Cone, as the liquid sample exits the capillary in a fine, cone shaped mist (43). A drying gas, most commonly nitrogen, is applied to this region to facilitate the evaporation of the liquid mobile phase as ionization occurs. As the mobile phase evaporates, and the droplet sizes within the spray begin to decrease, the charge repulsion of the ions exceeds the surface tension of the droplets, resulting in the formation of smaller and smaller charged droplets until only the analyte ions remain and are drawn towards the oppositely charged MS orifice (43).

1.6.4.2 Triple Quadrupole Mass Analyzer

A triple quadrupole mass analyzer contains 3 sets of 4 diagonally opposed charged rods, two positively charged and two negatively charged within each set (43). The charged rods in quadrupole 1 (Q1) and quadrupole 3 (Q3) are combined with applied resonance frequency and pre-set to filter out all ions that don't have a specific m/z ratio. As the analytes pass through the quadrupoles, all ions that have the correct m/z ratio will proceed towards the detector, and those that do not will collide with the rods, become neutral, and will not reach the detector (43). Quadrupole 2 (Q2) acts as a collision cell in which the analyte ions will collide with a collision gas that results in ion fragmentation (43). The purpose of this multi-quadrupole design is to allow for multiple reaction monitoring (MRM) of ions (30). Q1 is set to detect the presence of the most common ion of the target analyte, known as the precursor ion. Q2 then fragments these precursor ions into product ions before they travel into Q3, which acts as a mass filter and only allows ions of interest to travel to the detector. This type of system increases selectivity and specificity of the ions detected by filtering out other compounds that may have the same m/z ratio as the precursor ion of the target analyte, enhancing confidence in quantitative interpretation (43).

1.6.4.3 Detector Systems

The final step of mass spectrometry is the detection of produced ions and the creation of data regarding the presence and abundance of ions. The most common type of detector system used with triple quadrupole mass analyzers are electron multiplier systems (44). Ions exit the mass analyzer and pass into the detector containing copper-beryllium dynodes oriented diagonally from one another causing the initial ions and emitted electrons from the first dynode to bounce off of each dynode as they pass through the detector (44). The emitted electrons are counted by the detector and converted into mass spectra graphs by the software interfaced with the given instrumentation being used. The resulting spectra depicts a vertical line graph with m/z ratios on the x-axis and the corresponding relative abundance of those ratios on the y-axis (43).

1.6.4.4 QSight 220 Technology

PerkinElmer has developed an innovation in mass spectrometry that employs the use of a vertical, coaxial flow-based ESI technique, laminar flow ion guide, and high-performance mass filter (45). The QSight® 220 uses a dual source coaxial flow ESI ionization technique where the nebulizing gas is applied coaxially to the sample exiting the spray needle creating a mixing region between the hot gas and the sample. This is advantageous because it enhances sample desolvation and maximizes ionization efficiency by minimizing the dispersion of charged ions in front of the MS orifice (45). At the MS orifice, to transition the produced ions from atmospheric pressure to the vacuum of the mass analyzer, a hot source induced desolvation (HSID) interface is used. This system is oriented in a multi-orthogonal ion flow path, designed to increase collision between the hot carrier gas and the formed ions, increasing desolvation and minimizing chemical background noise (45). The heated carrier gas prevents the ionized particles from colliding with the interior walls of this region creating a self-cleaning environment that also reduces risk of carryover or contamination from previous samples. A laminar flow ion guide is then used to transport the ions from the HSID interface to the quadrupole region (45). This ion guide has 3 sequential pump systems with set pressure gradient regions that are at values of 2 torr, 150 mtorr, and 1 mtorr respectively (45). This means that all ions regardless of their mass, travel at the velocity of the current. The overall impact of the QSight® 220's unique features result in a highly sensitive, quick, and adaptable means of sample separation, detection, and quantitation.

1.7 Research Objectives

This research has four major objectives. The first is to determine if cocaine and benzoylecgonine can be detected in skeletal remains with no period of decomposition. The second is to quantify these substances in skeletal remains. The third is to determine whether there is a difference in these levels between specimens that have been perfused with paraformaldehyde, and those that have no preservative treatment. The final objective is to determine which bone type within the body retains the largest concentration of these two substances following chronic use.

2. MATERIALS AND METHODS

2.1 Materials

2.1.1 Instrumentation

The bead mill homogenizer utilized in this research was an OMNI Bead Ruptor 12 purchased from OMNI International (Bedford, MA, USA). Centrifuges utilized included a Sorvall™ ST 8 and a Sorvall™ Biofuge Primo both purchased from Thermo Scientific (Waltham, MA, USA). Solid phase extraction was carried out with a Positive Pressure Extraction Manifold from United Chemical Technologies (Bristol, PA, USA). The nitrogen evaporator used was obtained from Organomation Associates, Inc. (Berlin, MA, USA). Instrumental analysis was carried out using a PerkinElmer QSight® 220 CR Triple Quadrupole Mass Spectrometer using an electrospray ionization probe, operated in positive ion mode, coupled with an LX-50 Liquid Chromatography system using a Kinetex® Phenyl-Hexyl 50x 4.6mm (2.6 µm) column (Phenomenex, Torrance, CA, USA). PerkinElmer® Simplicity 3Q Software was used for the interpretation and analysis of all chromatograms generated in this research.

2.1.2 Consumables

Disposable reinforced screw top Hard Tissue Homogenizing Mix tubes with 2.8 mm ceramic beads and a sterilized lysing mixture were purchased from OMNI International. Borosilicate glass vials and Vivaspin 6® Centrifuge Concentrator Tubes were purchased from Fisher Scientific (Waltham, MA, USA). Clean Screen® DAU solid phase extraction columns were purchased from United Chemical Technologies. Waters

Certified clear glass vials with screw cap pre-slit PTFE/silica septum lids and vial liners were LC/MS certified and purchased from Waters Corporation (Milford, MA, USA).

2.1.3 Standards and Reagents

Analytical reference standards for cocaine and benzoylecgonine in the form of 1 mg/mL in methanol were obtained from Cerilliant through Millipore Sigma (Burlington, MA, USA). Internal reference standards for cocaine-d3 and benzoylecgonine-d8 in the form of 100 µg/mL in methanol were also obtained from Cerilliant through Millipore Sigma. All solvents utilized in this research including methanol, acetone, ammonium hydroxide, isopropanol, hydrochloric acid formic acid and methylene chloride were LC/MS/MS grade purchased from Fisher Scientific. All deionized water used was obtained from a Millipore Milli-Q® Integral 5 Water Purification System purchased from Millipore Sigma. Solid chemicals utilized in this research include ammonium formate, disodium phosphate, and monosodium phosphate were purchased from Fisher Chemical.

2.2 Methods

2.2.1 Rodent Dissection/Bone Removal

All 16 rodent carcasses used in this research were received whole with the torso and abdominal cavity intact. Following a 2-week period of storage at –20° C, the carcasses were thawed to room temperature in preparation for dissection. A vertical superficial incision was made from the superior aspect of the sternum to the inferior aspect of the pelvis using an Excelta™ Safety Scalpel (Fisher Scientific). Using Cen-Med© Tissue

Forceps (Fisher Scientific), the outer pelt was then peeled away from the underlying skin layer. A second vertical incision was then made following the same path as the first to expose the inner body cavity. Using the tissue forceps and dissecting scissors (Sigma Aldrich), the anterior plate of the ribcage was removed. All internal organs were then evacuated from the chest and abdominal cavity to expose the vertebral spine. Oblique incisions were then made from the medial aspect of the left upper and lower extremity to the lateral aspect to expose the femur and humeri. Once enough of the soft tissue surrounding the bones was removed, the joints connecting the femur and humeri to the surrounding bones were manually severed and the bones were extracted. To remove a portion of the spine, a vertical incision was made on the posterior side of the carcass directly over the spine extending from thoracic vertebrae 11 to lumbar vertebrae 3. The surrounding muscle was cut away from the spine and DR Instruments© Heavy Duty Cutter bone clippers (Fisher Scientific) were used to remove the previously mentioned vertebral section from the carcass. All bones were then scraped using an Excelta™ scalpel to remove any remaining soft tissue as well as the periosteum. The bones were then sequentially rinsed with deionized water, methanol, and acetone respectively. To remove the bone marrow from the medullary cavity, the epiphyseal portions of each long bone were removed, and the cavity was rinsed following the same procedure. The final sample yield consisted of 16 spinal vertebrae, 16 femurs, and 16 humeri. Of each bone type, 4 had no preservative administered and 12 had been treated with paraformaldehyde.

2.2.2 Homogenization and Solid/Liquid Extraction

To extract the compounds of interest from the bone samples, the next step was to homogenize each individual bone into a fine powder. Due to the size of the long bones, a segment was taken from the central shaft in the weight of approximately 0.25 g. It was determined that a singular vertebra with the transverse and spinous processes removed weighed approximately 0.25 g, and thus the 13th thoracic vertebra from each rodent was used for the spinal sample. Figure 3 depicts the segments used in each bone type respectively. Each bone sample was then broken into fragments and placed in a 2 mL Hard Tissue Homogenizing Mix Pre-Filled Tubes with 2.8 mm ceramic beads (OMNI International). An OMNI Bead Ruptor 12 was used to shake the tubes for two cycles of 90 seconds at a speed of 6 m/s to grind the bone fragments into a fine powder. After shaking the tubes were removed from the bead mill and tapped to ensure that the powder was not stuck on the lid. Methanol was then added to each tube in the amount of 1.25 mL, and shaken again for two cycles of 90 seconds at 6 m/s. After homogenization, the methanol/bone mix was then centrifuged for 5 minutes at 4000 rpm, after which the supernatant was collected. The original tubes containing the solid remaining bone powder were then shaken again in the bead mill with 1.25 mL of deionized water, following the same procedure. Samples were once again centrifuged, and the two supernatants were pooled together. The pooled mixture was then transferred into a 6 mL Vivaspin 6® Centrifuge Concentrator Tube (Sigma Aldrich) and centrifuged for 5 minutes at 6000 rpm to filter out any remaining particles of bone within the samples. The result of this process was approximately 1 mL of concentrated sample in a 1:1 mixture of methanol and deionized water. All samples were then diluted with 2 mL of phosphate buffer at pH 6. An internal standard mix was added to all samples before SPE in the amount of 20 µL containing cocaine-d3 and benzoylecgonine-d8 (Millipore Sigma).

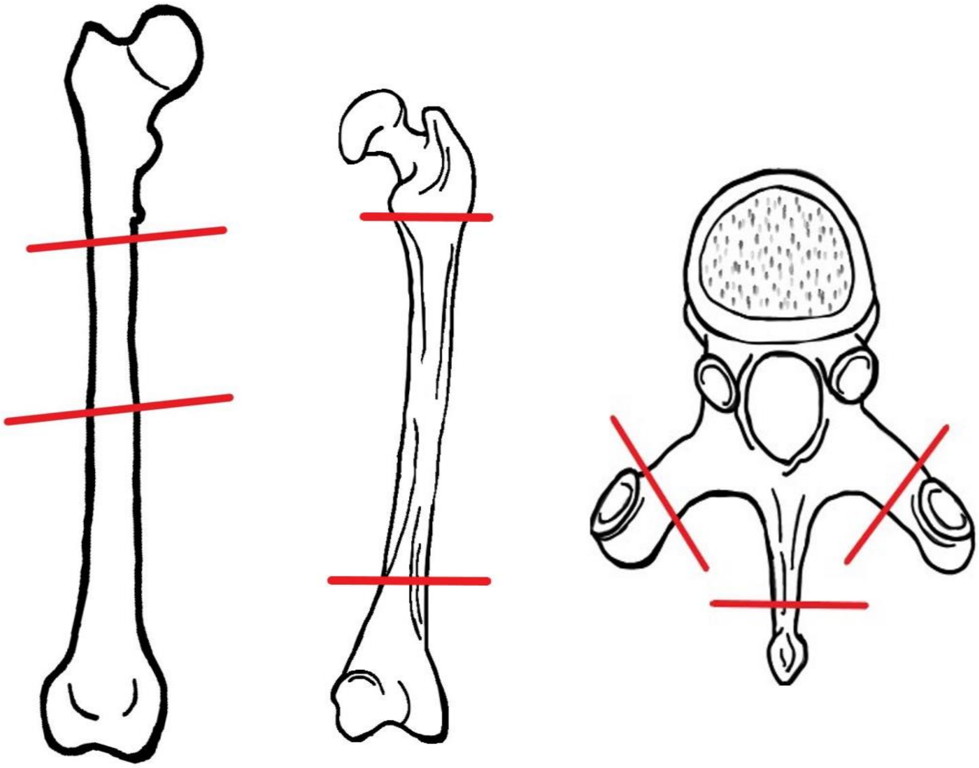


Figure 3: Femur, Humerus, and Vertebrae Segments (from left to right) (28)

2.2.3 Compound Extraction

Solid phase extraction was selected as the sample preparation method for this procedure using Clean-Screen® DAU 6 mL, 200 mg mixed mode, co-polymerized column containing a purified silica gel packing was selected. This column was chosen due to its versatility in that it can be used to extract acidic, basic, and neutral drugs of abuse. It contains two functional groups, a reverse phase, and an ion exchanger (benzenesulfonic acid). Column conditioning steps were chosen based on the recommended protocol for cocaine and benzoylecgonine extraction of blood, urine, and tissue samples using Clean Screen DAU Extraction Columns (46). First 3 mL of methanol were added and allowed to drip through by gravity, then 3 mL of phosphate buffer were added and allowed to pass through by gravity. The bone extracts were then loaded onto the columns and allowed to flow through by gravity, and after 5 minutes positive pressure was applied using a positive pressure manifold. Samples then underwent a two-step wash procedure, the first with 0.1 N HCl in deionized water, and the second with methanol. Both washes were at a volume of 2 mL and low positive pressure was used to pass the liquids through the column. The last step was to elute the substances of interest from the columns using a mixture of methylene chloride, isopropanol, and ammonium hydroxide in a 78:20:2, v/v ratio, which was loaded onto the columns in volume of 3 mL and allowed to pass through by gravity. Eluted samples were then evaporated to dryness under a 40° C stream of nitrogen (Organomation Associates, Inc.) and then reconstituted using 180 µL of 10 mM ammonium formate in deionized water and 20 µL of methanol.

2.2.4 LC-MS/MS Method

Due to its high selectivity and ability to detect low analyte levels, the PerkinElmer QSight® 220 LC-MS/MS was selected for the analytical portion of this experiment. A Kinetex® Phenyl-Hexyl column 50 x 4.6 mm (2.6 um) with a pore size of 100 Angstrom was utilized. The elution gradient consisted of mobile phase A being a 10 mM ammonium formate in deionized water and mobile phase B being 0.1% formic acid in methanol. Samples were injected using an LX-50 autosampler and pump at a volume of 7 µL and an oven temperature of 40° C with the gradient ramp set for 9 minutes per sample. Mobile phase percentages can be seen below (Table 1). Tables 2-3 detail information regarding operation of the mass spectrometer.

Table 1: QSight 220 CR Gradient Table. Changes in LC mobile phase composition over the duration of the run.

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

Table 2: MS Source Conditions. Conditions of the MS ionization source.

Parameters	**Settings**
Ionization Mode	ESI Positive
Drying Gas Setting	100
Nebulizer Gas Setting	150
HSID Temperature	250 °C
Electrospray Voltage (V)	5500
Source Temperature	500 °C

Table 3: MRM Table for all Compounds. Ion values, MS source conditions, and component identification for cocaine, benzoylecgonine, and their deuterated standards.

Compound	**Cocaine**		**Benzoylecgonine**		**Cocaine-D3**	**Benzoylecgonine-D8**
Entrance Voltage	30		30		30	30
Collison Energy	-50		-50		-50	-50
Collison Cell Lens 2	-120		-120		-120	-120
Collison Pressure	400		400		400	400
Ion Mode	ESI +		ESI +		ESI +	ESI +
Precursor Ion	304.2		290.2		306.8	298.2
Product Ion(s)	182.2	150.1	168.1	105.1	185	171
Component Type	Quant	Qual	Quant	Qual	Internal Standard	Internal Standard

2.3 Long Term Exposure of Cocaine in Rats

All rat specimen utilized in this research were obtained from the Boston University Behavioral Neurosciences Department (Boston, MA, USA). These rats underwent a 6-month period of treatment in which they were allowed to self-administer cocaine for 3 months, abstained from use for 5 weeks, and then resumed self-administration for 1 month before being euthanized. The average amount of cocaine consumed across the entire experiment among the 16 rats included in this research was 245 mg/kg. Sixteen rats were utilized in this research, 12 pericardially infused with paraformaldehyde at the time of death and 4 received no preservative at the time of death.

3. RESULTS

3.1 Calibration Models

Working stock solutions were prepared using certified reference standards for cocaine and benzoylecgonine to make 8 calibrators of known concentration using clean human urine obtained from the Biomedical Forensic Sciences Biological Donation Program in accordance with the Institutional Review Board requirements. The working stocks were prepared in the following concentrations: working stock 1 (200 ng/mL), working stock 2 (10 ng/mL), working stock 3 (1 ng/mL). Due to the lack of reference material regarding drug levels in bone, a wide range was initially selected for the calibration curves. Spiked urine samples were created at 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 with three quality control samples at 0.5 ng/mL, 20 ng/mL, and 150 ng/mL. An internal standard mix was prepared using 2.94 mL methanol, 30 µL cocaine-d3 reference material, and 30 µL benzoylecgonine-d8 reference material. All bone samples, calibrators, quality controls, and a negative control sample made of drug-free urine, were spiked with 20 µL of internal standard mix before undergoing the previously described solid phase extraction method. Calibrator and control preparation details can be found in Tables 4-5. A double blank sample composed of 1 mL of drug-free urine was run as a quality control and methanol blanks were placed between each bone sample. Urine was used as a substitute biological matrix due to a lack of drug free bone samples.

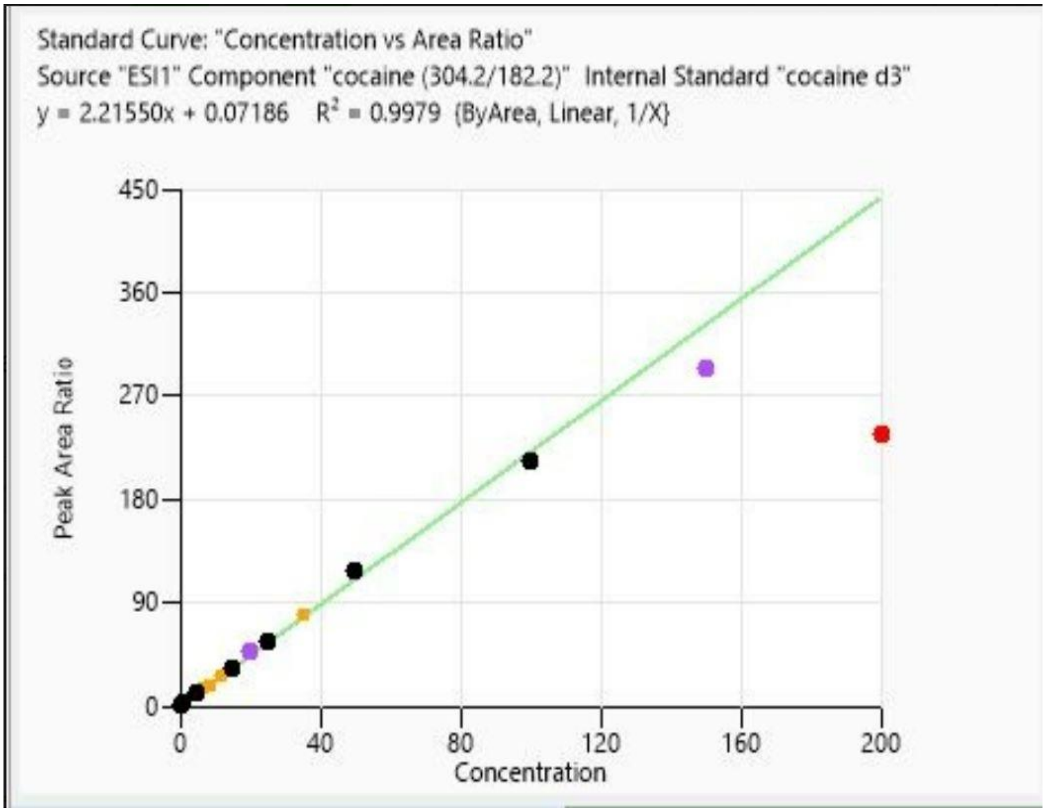
Table 4: Working Stock 1 Calibrators and Controls Run #1. Preparation instructions for calibrators 4-8, MQC, and HQC using working stock #1.

Sample	Volume WS1 (µL)	Volume Clean Urine (µL)	Final Concentration (ng/mL)
Cal 4	75	925	15
MQC	100	900	20
Cal 5	125	875	25
Cal 6	250	750	50
Cal 7	500	500	100
HQC	750	250	150
Cal 8	1000	0	200

Table 5: Working Stock 2 Calibrators and Controls Run #1. Preparation instructions for calibrators 1-3 and LQC using working stock #2.

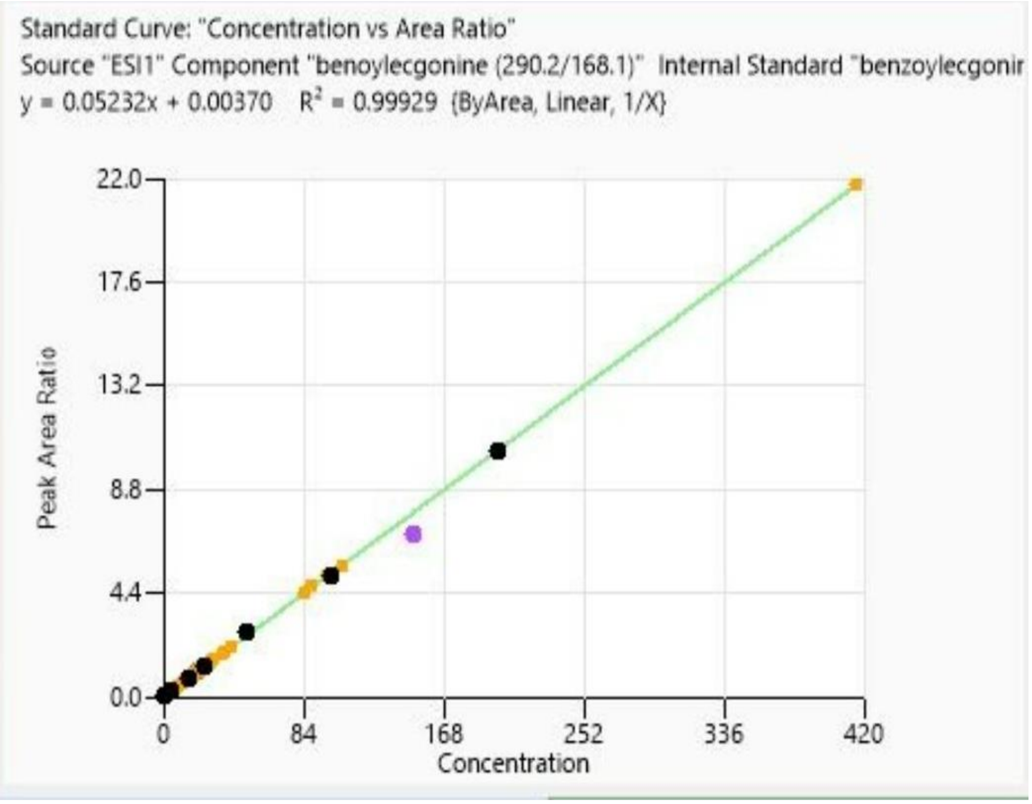
Sample	Volume WS2 (µL)	Volume Clean Urine (µL)	Final Concentration (ng/mL)
Cal 1	25	975	0.25
LQC	50	950	0.5
Cal 2	100	900	1
Cal 3	500	500	5

Cocaine and benzoylecgonine both demonstrated exceptional linearity with R^2 values of 0.9979 and 0.99929 respectively (see Figure 4-5). For both compounds, the 200 ng/mL calibrator (Cal 8) was dropped when creating the calibration curves due to failure to be within 20% of the target concentration. All remaining calibrators and controls were within 20% of their target concentrations. Based on this a new range of calibrators was selected at 0.01 ng/mL, 0.05 ng/mL, 0.1 ng/mL, 0.25 ng/mL, 2.5 ng/mL, 5 ng/mL, 10 ng/mL, and 25 ng/mL with quality controls at 0.5 ng/mL, 1 ng/mL, and 7.5 ng/mL. Preparation of new calibrators and controls can be seen below (Table 6-8). Bone samples were re-run with newly prepared calibrators and controls after 2 weeks of frozen storage.



Standard Curve: "Concentration vs Area Ratio"
Source "ESI1" Component "cocaine (304.2/182.2)" Internal Standard "cocaine d3"
y = 2.21550x + 0.07186 R² = 0.9979 (ByArea, Linear, 1/X)

Figure 4: Cocaine Calibration Curve Run #1. Calibration curve for cocaine using 0.25 ng/mL, 1 ng/mL, 5 ng/mL, 15 ng/mL, 25 ng/mL, 50 ng/mL, and 100 ng/mL calibrators with a cocaine-d3 internal standard. The equation of the line is $y = 2.2155x + 0.07186$ with $R^2 = 0.9979$. Red dots represent calibrators not included in the calibration curve, purple dots represent quality controls, black dots represent calibrators included in the curve, and orange dots represent unknown samples.



Standard Curve: "Concentration vs Area Ratio"
Source "ESI1" Component "benoylecgonine (290.2/168.1)" Internal Standard "benzoylecgonir
y = 0.05232x + 0.00370 R² = 0.99929 (ByArea, Linear, 1/X)

Figure 5: Benzoylecgonine Calibration Curve Run #1. Calibration curve for benzoylecgonine using 0.25 ng/mL, 1 ng/mL, 5 ng/mL, 15 ng/mL, 25 ng/mL, 50 ng/mL, and 100 ng/mL calibrators with a benzoylecgonine-d8 internal standard. The equation of the line is y = 0.0532x + 0.0037 with R² = 0.99929. Red dots represent calibrators not included in the calibration curve, purple dots represent quality controls, black dots represent calibrators included in the curve, and orange dots represent unknown samples.

Table 6: Working Stock 1 Calibrators and Controls Run #2. Preparation instructions for calibrators 7-8 using working stock 1.

Sample	Volume WS1 (µL)	Volume Clean Urine (µL)	Final Concentration (ng/mL)
Cal 7	50	950	10
Cal 8	125	875	25

Table 7: Working Stock 2 Calibrators and Controls Run #2. Preparation instructions for calibrators 5-6, MQC, and HQC using working stock 2.

Sample	**Volume WS2 (µL)**	**Volume Clean Urine (µL)**	**Final Concentration (ng/mL)**
MQC	100	900	1
Cal 5	250	750	2.5
Cal 6	500	500	5
HQC	750	250	7.5

Table 8: Working Stock 3 Calibrators and Controls Run #2. Preparation instructions for calibrators 1-4 and LQC using working stock 3.

Sample	**Volume WS3 (µL)**	**Volume Clean Urine (µL)**	**Final Concentration (ng/mL)**
Cal 1	10	990	0.01
Cal 2	50	950	0.05
Cal 3	100	900	0.1
Cal 4	250	750	0.25
LQC	500	500	0.5

Cocaine and benzoylecgonine demonstrated R^2 values of 0.9904 and 0.99977 respectively (see Figure 6-7). The 0.01 ng/mL, 0.05 ng/mL, and 0.1 ng/mL calibrators were excluded from the calibration curve due to failure to be within 20% of their target concentrations. All remaining calibrators and controls were within appropriate range of their target concentrations.

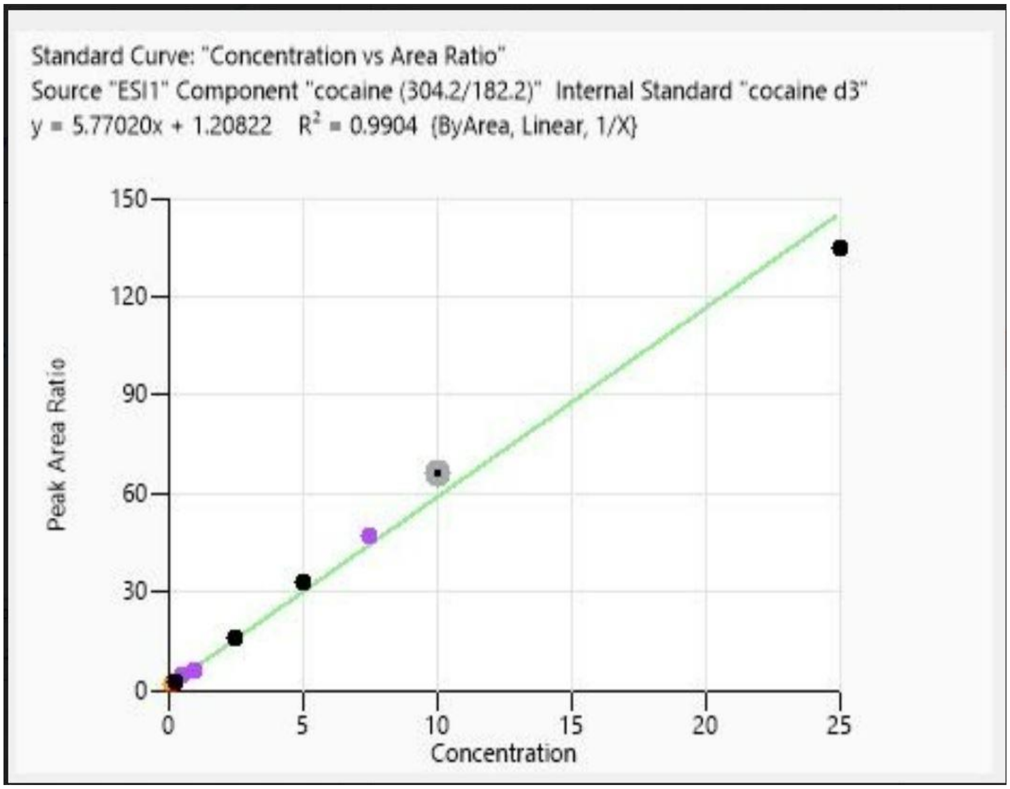


Figure 6: Cocaine Calibration Curve Run #2. Calibration curve for cocaine using 0.25 ng/mL, 2.5 ng/mL, 5 ng/mL, 10 ng/mL, and 25 ng/mL calibrators with a cocaine-d3 internal standard. The equation of the line is $y = 5.7702x + 1.20822$ with $R^2 = 0.9904$. Red dots represent calibrators not included in the calibration curve, purple dots represent quality controls, black dots represent calibrators included in the curve, and orange dots represent unknown samples.

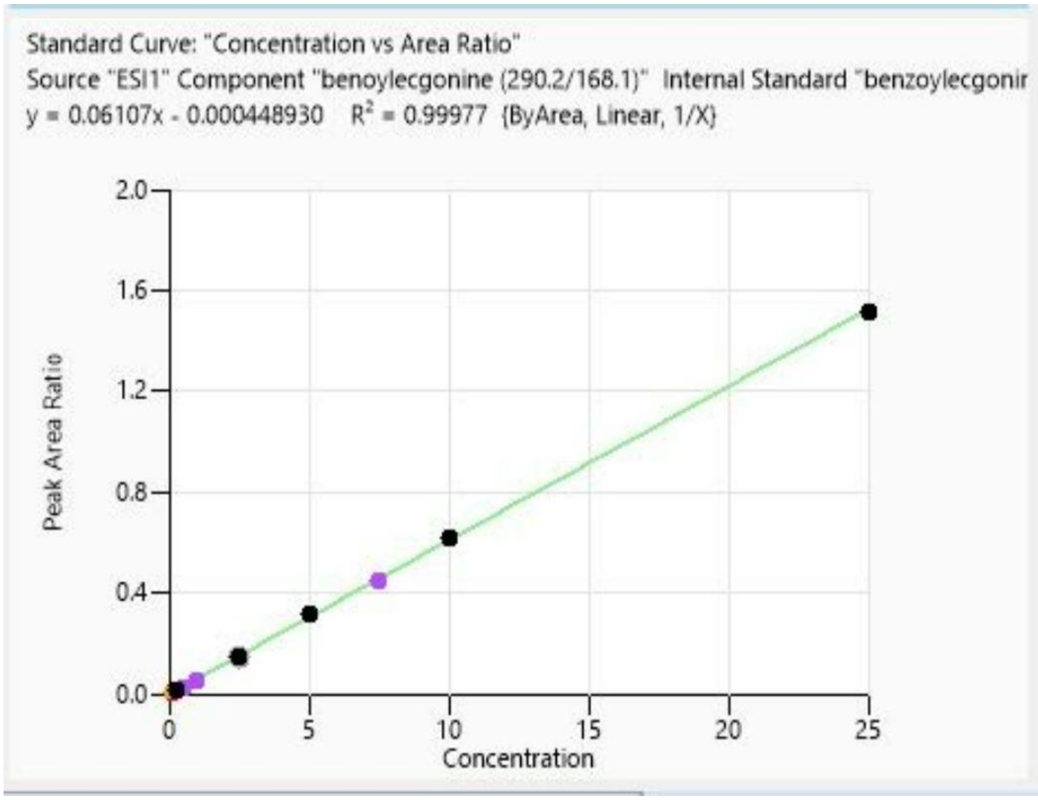

Standard Curve: "Concentration vs Area Ratio"
Source "ESI1" Component "benoylecgonine (290.2/168.1)" Internal Standard "benzoylecgonir
y = 0.06107x - 0.000448930 R² = 0.99977 {ByArea, Linear, 1/X}

Figure 7: Benzoylecgonine Calibration Curve Run #2. Calibration curve for benzoylecgonine using 0.25 ng/mL, 2.5 ng/mL, 5 ng/mL, 10 ng/mL, and 25 ng/mL calibrators with a benzoylecgonine-d8 internal standard. The equation of the line is $y = 0.06107x + 0.000448930$ with $R^2 = 0.99977$. Red dots represent calibrators not included in the calibration curve, purple dots represent quality controls, black dots represent calibrators included in the curve, and orange dots represent unknown samples.

3.2 Detection and Quantitation of Unknown Samples

For the purposes of this section, the high range calibrator analysis of bone samples will be referred to as "Run #1" and the low range calibrator analysis will be referred to as "Run #2". The calculated concentrations of unknown bone samples are displayed below, organized by bone type and run number (see Table 9-14). Calculated concentrations were ascertained using the respective calibration curves displayed above. The bone samples analyzed included 16 humeral homogenates, 16 femoral homogenates, and 16 spinal homogenates. The limit of detection (LOD) and the limit of quantitation (LOQ) were determined based on the calibration curves and were found to be the same for both calibrator ranges. The LOD was deemed to be 0.001 ng/mL and the LOQ was 0.25 ng/mL for both cocaine and benzoylecgonine. Samples with concentrations lower than the LOQ with appropriate retention times and corresponding peaks were estimated using the calibration curve to determine the LOD. Samples that fell below the LOD were designated as not detected (ND), and samples that were not included in Run #2 have been labeled as not applicable (NA). Values highlighted in yellow indicate that the sample was within the appropriate range for quantitation.

Table 9: Run #1 Humerus Calculated Concentrations. Calculated concentrations for cocaine and benzoylecgonine in humeral bone samples.

	Cocaine (ng/mL)	**Benzoylecgonine (ng/mL)**
Humerus 1	0.042	0.054
Humerus 2	0.036	0.025
Humerus 3	0.017	0.571
Humerus 4	0.093	0.101
Humerus 5	0.073	0.069
Humerus 6	0.019	ND
Humerus 7	ND	0.013
Humerus 8	0.022	0.021
Humerus 9	0.045	0.035
Humerus 10	0.015	0.015
Humerus 11	0.01	0.006
Humerus 12	0.001	ND
Humerus 13	0.017	0.011
Humerus 14	0.001	0.027
Humerus 15	0.046	0.003
Humerus 16	0.044	0.022

Table 10: Run #1 Femur Calculated Concentrations. Calculated concentrations for cocaine and benzoylecgonine in femoral bone samples.

	Cocaine (ng/mL)	**Benzoylecgonine (ng/mL)**
Femur 1	0.153	0.018
Femur 2	0.082	0.051
Femur 3	6.866	2.853
Femur 4	0.123	0.197
Femur 5	0.023	0.022
Femur 6	0.034	ND
Femur 7	0.017	0.093
Femur 8	ND	ND
Femur 9	0.025	0.038
Femur 10	ND	0.012
Femur 11	ND	0.019
Femur 12	0.011	0.006
Femur 13	ND	0.008
Femur 14	ND	0.013
Femur 15	0.289	0.037
Femur 16	0.001	0.022

Table 11: Run #1 Spine Calculated Concentrations. Calculated concentrations for cocaine and benzoylecgonine in spinal vertebrae samples.

	Cocaine (ng/mL)	**Benzoylecgonine (ng/mL)**
Spine 1	0.07	0.084
Spine 2	0.345	0.51
Spine 3	8.508	7.169
Spine 4	0.641	0.831
Spine 5	0.07	0.058
Spine 6	0.047	ND
Spine 7	0.044	ND
Spine 8	0.051	ND
Spine 9	0.035	0.026
Spine 10	0.081	0.046
Spine 11	0.159	0.099
Spine 12	0.108	0.042
Spine 13	0.07	0.07
Spine 14	0.292	ND
Spine 15	0.037	0.027
Spine 16	0.035	ND

Table 12: Run #2 Humerus Calculated Concentrations. Calculated concentrations for cocaine and benzoylecgonine in humeral bone samples.

	Cocaine (ng/mL)	**Benzoylecgonine (ng/mL)**
Humerus 1	0.068	0.143
Humerus 2	ND	0.122
Humerus 3	ND	0.109
Humerus 4	ND	0.22
Humerus 5	ND	0.167
Humerus 6	ND	0.062
Humerus 7	ND	0.078
Humerus 8	ND	0.113
Humerus 9	ND	0.131
Humerus 10	ND	0.086
Humerus 11	ND	0.07
Humerus 12	ND	0.067
Humerus 13	ND	0.097
Humerus 14	ND	0.071
Humerus 15	ND	0.063
Humerus 16	ND	0.104

Table 13: Run #2 Femur Calculated Concentrations. Calculated concentrations for cocaine and benzoylecgonine in femoral bone samples.

	Cocaine (ng/mL)	**Benzoylecgonine (ng/mL)**
Femur 1	0.127	0.129
Femur 2	ND	0.171
Femur 3	NA	NA
Femur 4	0.298	0.317
Femur 5	ND	0.107
Femur 6	0.073	0.041
Femur 7	ND	0.072
Femur 8	ND	ND
Femur 9	ND	0.141
Femur 10	ND	0.12
Femur 11	ND	0.062
Femur 12	ND	0.057
Femur 13	0.151	0.094
Femur 14	ND	0.074
Femur 15	0.314	0.147
Femur 16	ND	0.118

Table 14: Run #2 Spine Calculated Concentrations. Calculated concentrations for cocaine and benzoylecgonine in spinal vertebrae bone samples.

	Cocaine (ng/mL)	**Benzoylecgonine (ng/mL)**
Spine 1	ND	0.178
Spine 2	NA	NA
Spine 3	NA	NA
Spine 4	NA	NA
Spine 5	0.16	0.234
Spine 6	ND	0.047
Spine 7	ND	0.06
Spine 8	ND	0.049
Spine 9	ND	0.113
Spine 10	ND	0.14
Spine 11	ND	0.189
Spine 12	ND	0.136
Spine 13	ND	0.174
Spine 14	ND	0.076
Spine 15	ND	0.104
Spine 16	ND	0.067

Representative chromatograms of quantitated samples for each bone type and analyte are included in Appendix A. All retention times for quantitated samples were within +/- 2% of their deuterated internal standard retention times. For Run #1 the average retention time for cocaine was 5.5 minutes and for benzoylecgonine was 4.7 minutes. For Run #2 the average retention time for cocaine was 5.7 minutes and for benzoylecgonine was 4.6 minutes. Based on these results the spinal vertebrae appears to be the best bone type for detecting and quantitating cocaine and benzoylecgonine, however the results obtained from the femoral samples demonstrate a similar efficiency to the vertebral samples.

4. DISCUSSION

4.1 Matrix Interference

When conducting quantitative analysis on any low-level analyte, elimination of baseline interference is extremely important to ensure accurate quantitative results. Bone is a highly complicated matrix containing biologic and mineral components that make it a unique tissue type for toxicological analysis. During data review it became very clear that despite extensive sample preparation there was still significant noise visible within the baseline of all bone samples. One interesting find within the chromatograms of all unknown bone samples is a persistent, intense peak with a retention time between 6.9 minutes and 7.1 minutes that is not seen within the calibrator chromatograms. Further matrix interference testing would be required before any conclusions could be made regarding the origin of this peak. It is also worth noting that when comparing the four rats that were not preserved with paraformaldehyde to the twelve that were, concentrations of both compounds were higher in the unpreserved rat specimens (see Table 15). This indicates that there may be some loss in drug concentration as a result of postmortem infusion with a preservative like paraformaldehyde. Since the calibrators used in this procedure were not composed of the same matrix as the unknown samples, a single point peak height ratio calculation was conducted on one of each bone type. This comparison was used to determine if calibration mismatch played a role in the low quantitation values (see Table 16). Based on comparison between the calibration curve quantitation and the point-based calculation for benzoylecgonine in one of each bone type, it is possible that higher quantitative results could be achieved if the sample and calibrator matrix were the same. Further testing including compound optimization, extraction efficiency, and paraformaldehyde effects on recovery would be required to confirm these hypotheses.

Table 15: Concentration Averages by Bone Type in Perfused vs. Non-Perfused Samples. Average concentrations of cocaine and benzoylecgonine in bone samples which received no preservative (1-4) and those that received pericardial perfusion of paraformaldehyde in PBS (5-16).

	Cocaine (ng/mL)	**Benzoylecgonine (ng/mL)**
Non-Perfused Humerus	0.047	0.188
Perfused Humerus	0.027	0.019
Non-Perfused Femur	1.806	0.780
Perfused Femur	0.033	0.025
Non-Perfused Spine	2.391	2.149
Perfused Spine	0.086	0.031

Table 16: Comparison of Benzoylecgonine Calibration Curve Concentrations to Single Point Calculated Concentrations. A comparison of the quantitation values produced using the urine-based calibration curve versus a single point calculation of concentration within the unknown bone samples using the peak area ratio of the bones to the internal standard within a single sample

Sample	**Calculated Concentration (ng/mL)**	**Peak Area Ratio Calculated Concentration (ng/mL)**
Humerus 13	0.097	0.546
Femur 4	0.317	1.888
Spine 11	0.189	1.112

4.2 Low Level Detection

Despite the presence of baseline noise and several samples falling below the limit of detection, it is still clear that cocaine and benzoylecgonine deposit into the skeletal structure at detectable levels following chronic consumption of cocaine. Using the calculated concentration data from both runs, the average concentrations of cocaine and benzoylecgonine were calculated for each bone type (Table 17). This data reflects the previous statement that both cocaine and benzoylecgonine concentrations were highest in vertebral samples, followed by femoral and humeral. One possible cause for the consistently low concentrations of both compounds in this research could be the small sample size utilized. Within all three bone types only segments of the respective bones were taken, rather than using the whole bone. Using the bone in its entirety could potentially provide higher concentrations that are more representative of the concentrations within each tissue type.

Table 17: Average Concentrations in Skeletal Tissue. Mean values of cocaine and benzoylecgonine concentrations in humeral, femoral, and spinal vertebrae samples in Run #1 and #2.

	Cocaine (ng/mL)		**Benzoylecgonine (ng/mL)**	
	Run #1	Run #2	Run #1	Run #2
Humeral	0.032	0.004	0.061	0.106
Femoral	0.477	0.064	0.226	0.110
Spinal	0.662	0.012	0.560	0.121

5. CONCLUSIONS

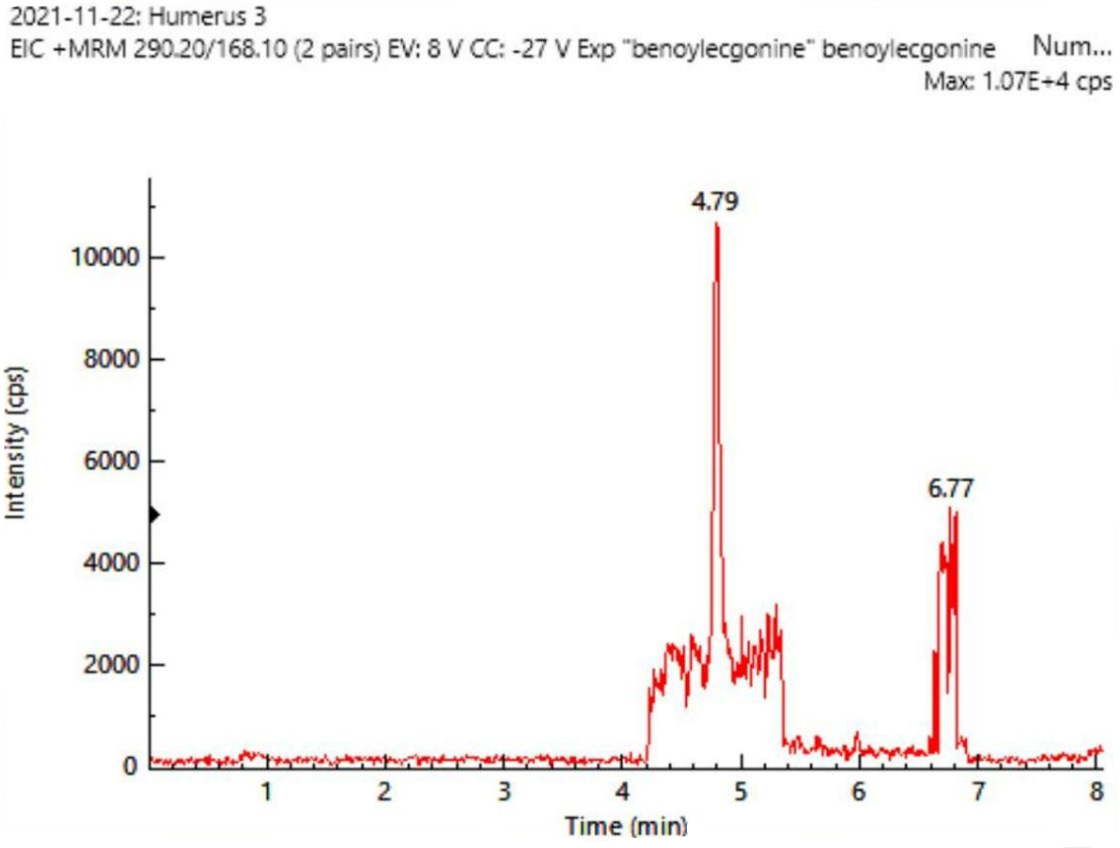
The purpose of this research was to develop an efficient method for the extraction and quantitation of cocaine and benzoylecgonine in preserved and unpreserved skeletal remains following chronic consumption of cocaine. Following extraction of humeral, femoral, and vertebral bone samples from deceased rodent carcasses, the bones underwent a liquid homogenization, solid phase extraction, and analysis using liquid chromatography-tandem mass spectrometry. Cocaine was detected in humeral, femoral, and spinal samples at ranges of 0.001 - 0.093 ng/mL, 0.001 - 6.866 ng/mL, and 0.035 – 8.508 ng/mL respectively. Benzoylecgonine was detected in humeral, femoral, and spinal samples at ranges of 0.003 - 0.571 ng/mL, 0.006 - 2.853 ng/mL, and 0.026 – 7.169 ng/mL respectively. Detectable and quantifiable levels of both compounds were found in all bone types both preserved and unpreserved. Utilizing a larger sample weight, more accurate levels of both compounds could be achieved. Further optimization of the utilized extraction protocol and inclusion of matrix-matched calibrators and controls could further improve the quality of the resulting concentrations and the overall analytical process. From start to finish this procedure was able to produce quantifiable levels of cocaine and benzoylecgonine in 1 hour. In forensic investigations where time and efficiency are of high importance, this method would prove to be extremely useful in cases were no other tissues or fluids are available for toxicological analysis.

6. FUTURE DIRECTIONS

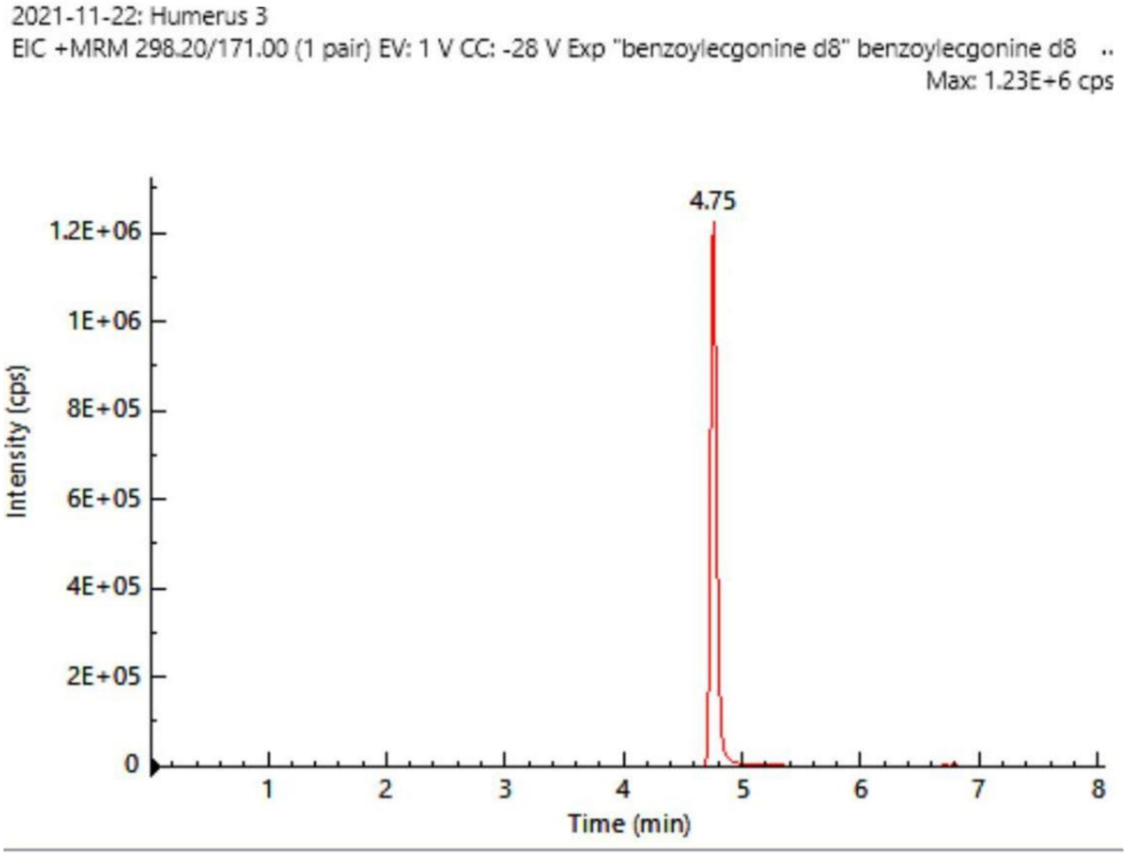
This project has many areas in which it could be expanded and further explored. This experiment did not include any period of decomposition for the skeletal remains. In real casework, any situation where skeletal remains would be utilized for toxicological analysis would likely be following a period of decomposition. It would be interesting to determine what the effect of decomposition would have on compound concentrations in preserved remains. Further, since long bones exist in pairs within the body, and there are many vertebrae present, a comparison study could be conducted at time zero and after a period of decomposition utilizing whole bone samples. A larger sample weight could also be utilized to determine if use of whole bones provides higher concentrations compared to only using segments, as was the case in this research.

Additionally, it would be interesting to utilize this instrumentation for the detection of other common drugs of abuse in skeletal remains that have different chemical properties from cocaine, such as heroin or amphetamine substances. This methodology could even be extended into detection of poisons, chemical weapons, and other environmental toxins.

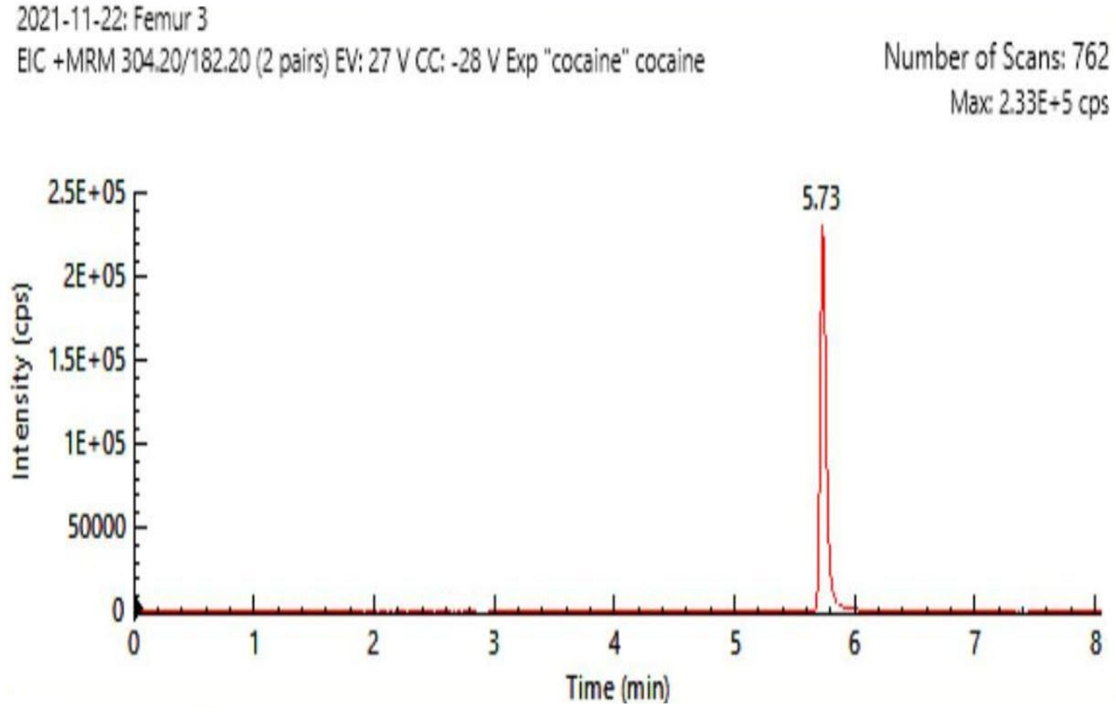
APPENDIX A: REPRESENTATIVE CHROMATOGRAMS OF RUN # 1 QUANTITATED SAMPLES



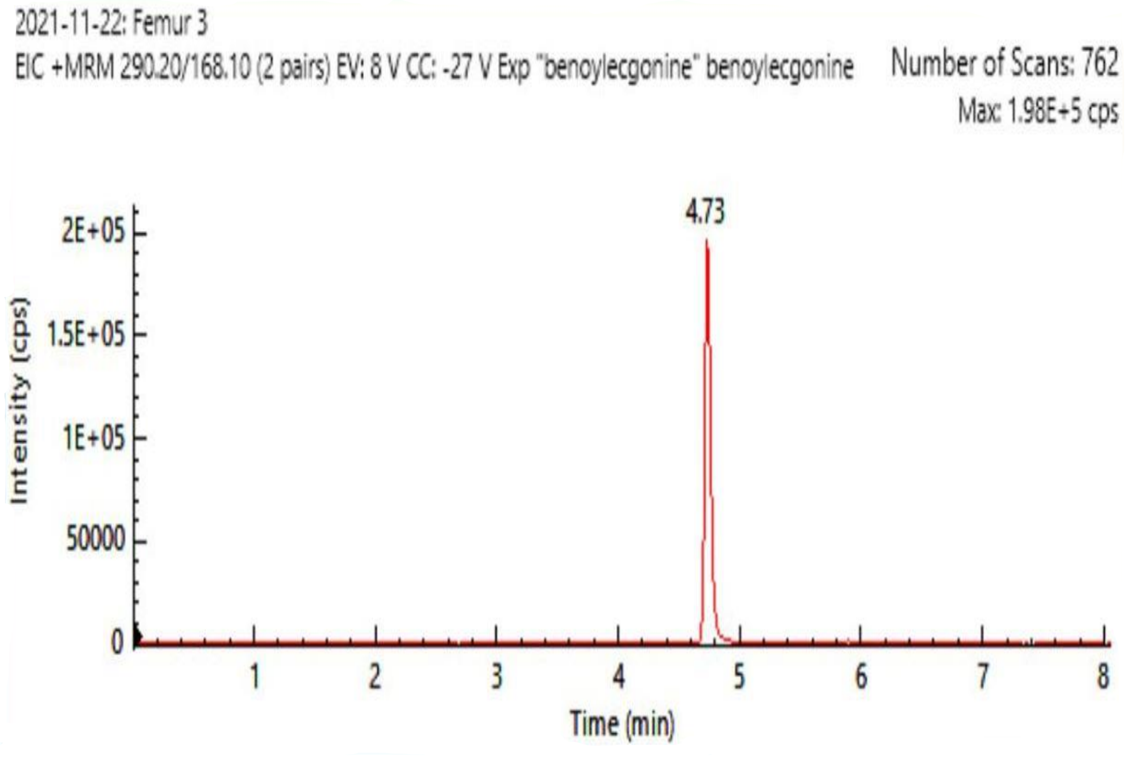
Humerus 3 Benzoylecgonine Extracted Ion Chromatogram with Retention Time 4.789 Minutes



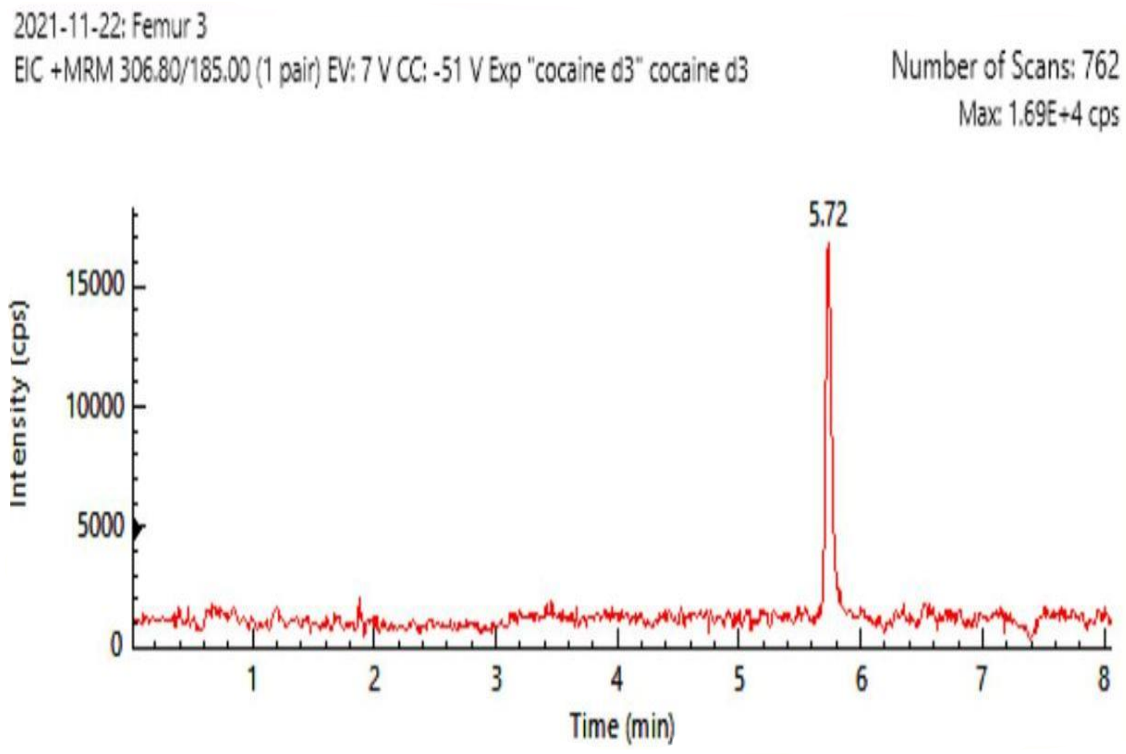
Humerus 3 Benzoylecgonine-D8 Extracted Ion Chromatogram with Retention Time 4.75 Minutes



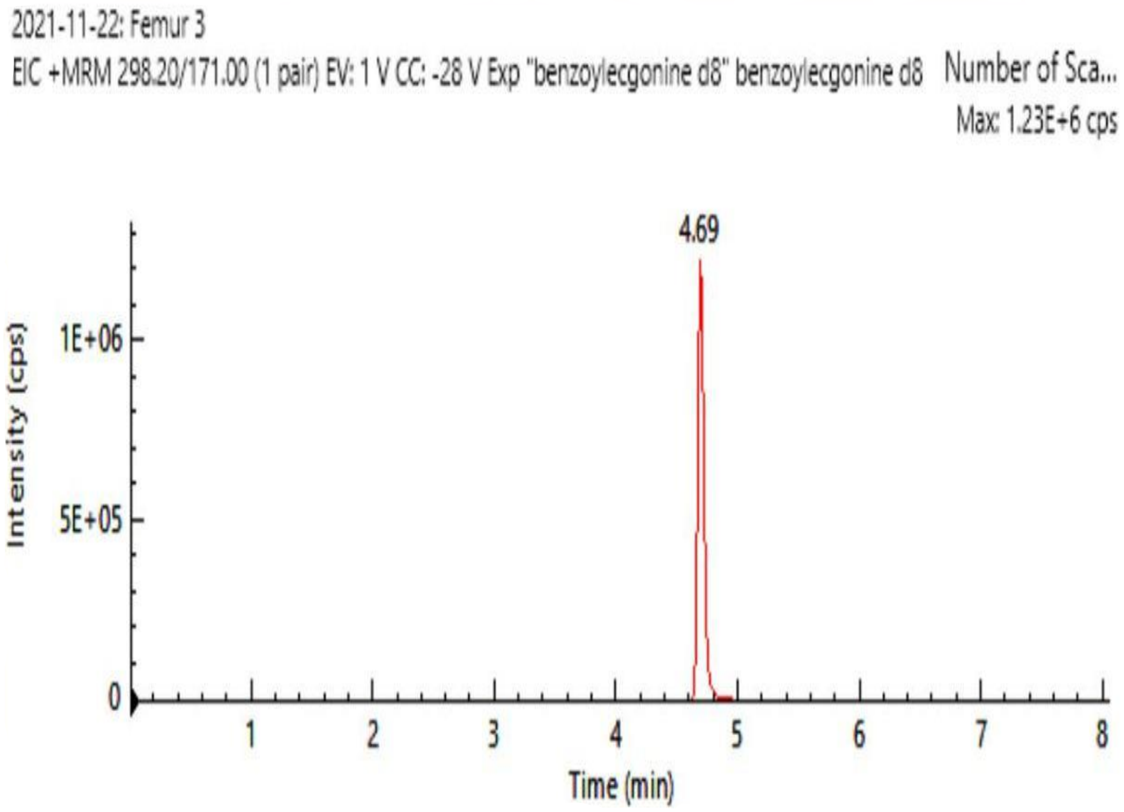
Femur 3 Cocaine Extracted Ion Chromatogram with Retention Time 5.726 Minutes



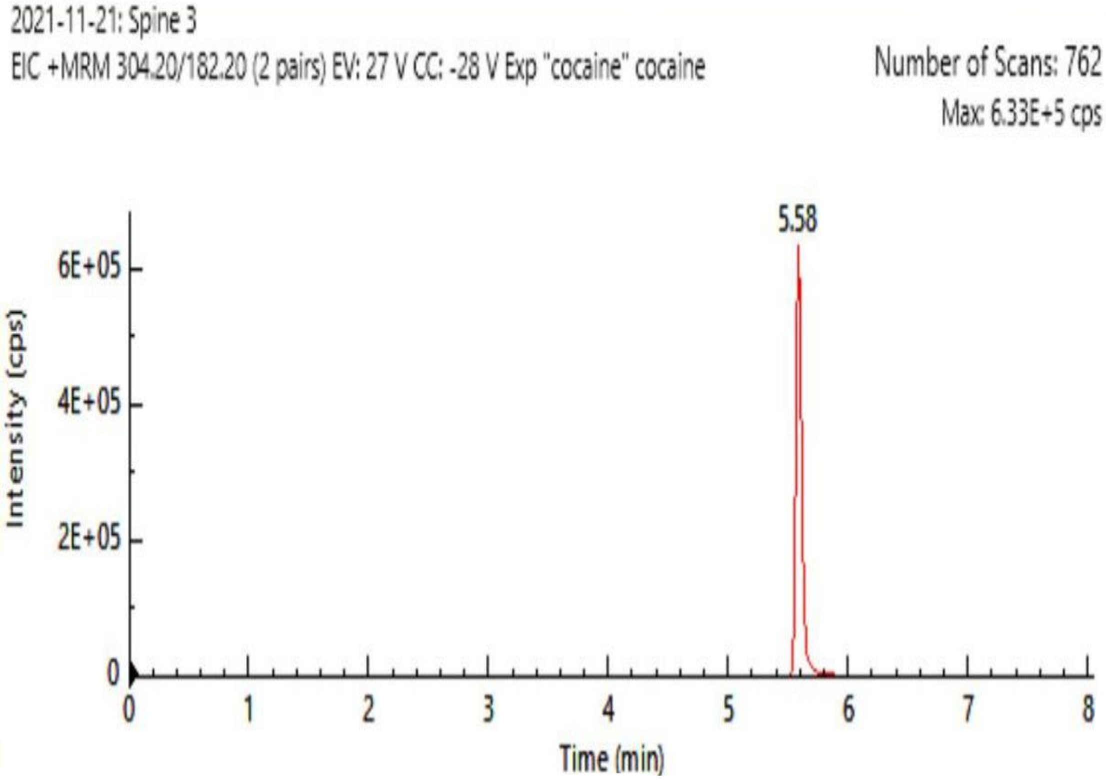
Femur 3 Benzoylecgonine Extracted Ion Chromatogram with Retention Time 4.726 Minutes



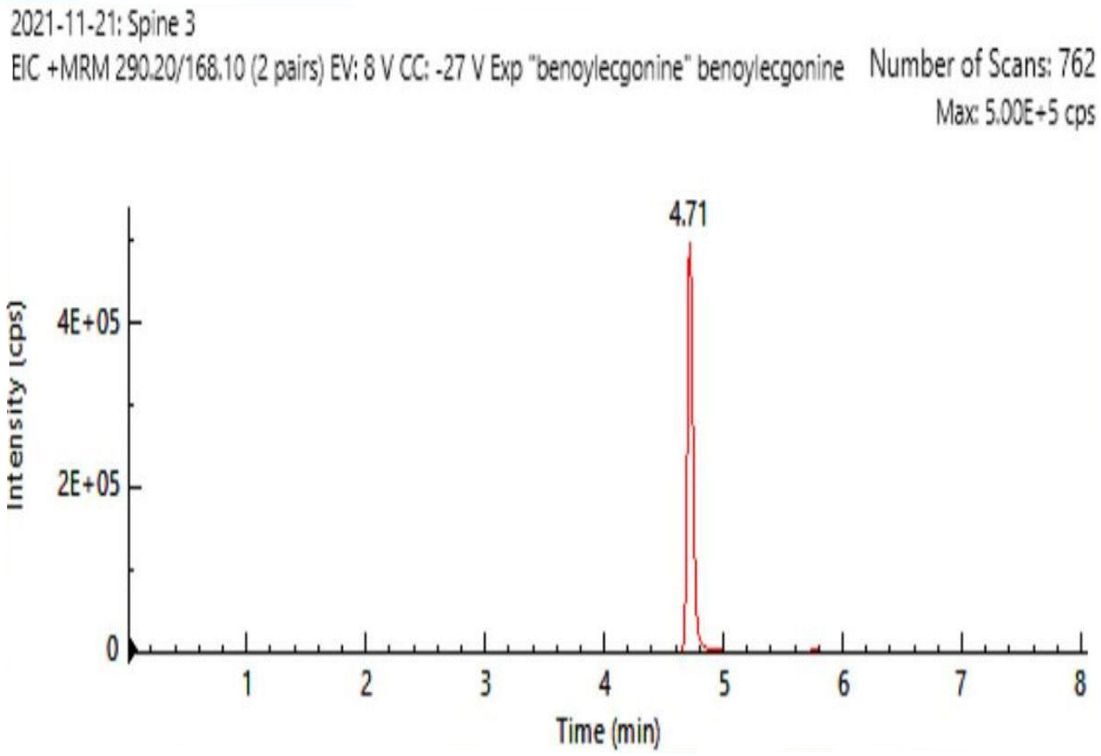
Femur 3 Cocaine-D3 Extracted Ion Chromatogram with Retention Time 5.724 Minutes



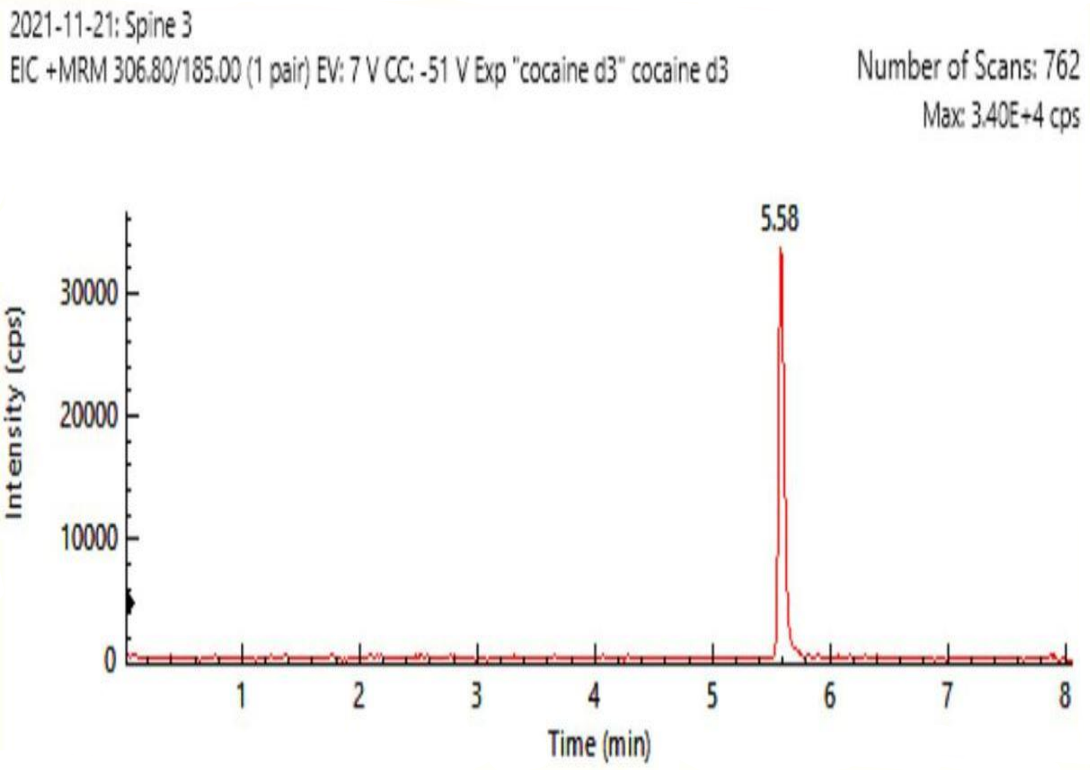
Femur 3 Benzoylecgonine-D8 Extracted Ion Chromatogram with Retention Time 4.69 Minutes



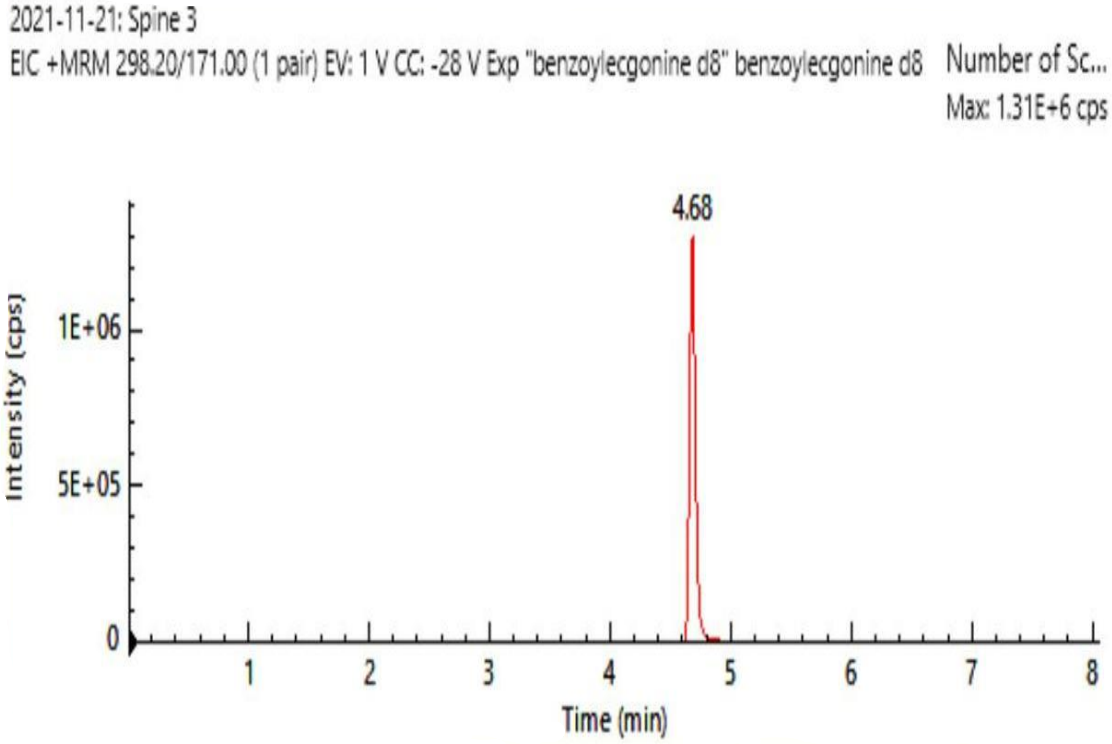
Spine 3 Cocaine Extracted Ion Chromatogram with Retention Time 5.578 Minutes



Spine 3 Benzoylecgonine Extracted Ion Chromatogram with Retention Time 4.715 Minutes



Spine 3 Cocaine-D3 Extracted Ion Chromatogram with Retention Time 5.576 Minutes



Spine 3 Benzoylecgonine-D8 Extracted Ion Chromatogram with Retention Time 4.676 Minutes

LIST OF JOURNAL ABBREVIATIONS

Am J Pub Health	American Journal of Public Health
ACS Chem Neurosci	ACS Chemical Neuroscience
Forensic Sci Int	Forensic Science International
Forensic Sci Med Pathol	Forensic Science, Medicine, and Pathology
J Anal Toxicol	Journal of Analytical Toxicology
J Drug Metab Toxicol	Journal of Drug Metabolism and Toxicology
J Forensic Sci	Journal of Forensic Sciences
J Hepatobiliary Pancreat Surg	Journal of Hepato-Biliary-Pancreatic Surgery
J Vis Exp	Journal of Visualized Experiments

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