COMPARISON OF HPLC-UV, GC-FID, AND GC-MS FOR THE ANALYSIS OF COCAINE AND BENZOYLECGONINE FROM VITREOUS HUMOR AND BRAIN TISSUE

By

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COMPARISON OF HPLC-UV, GC-FID, AND GC-MS

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NOMENCLATURE

COC	Cocaine
BE	Benzoylecgonine
EME	Ecgonine Methyl Ester
VH	Vitreous Humor
IV	Intravenously
IN	Intranasally
SM	Smoking
PO	Oral Ingestion
NE	Norepinephrine
BBB	Blood Brain Barrier
DAT	Dopamine Transporter
DA	Dopamine
CNS	Central Nervous System
PNS	Peripheral Nervous System
Na^+	Sodium Ion
Cl^-	Chloride Ion
SERT	Serotonin Transporter
VTA	Ventral Tegmental Area
ROS	Reactive Oxygen Species
ATP	Adenosine Triphosphate

ATPase	Adenosine Triphosphatase
HPLC-UV	High Performance Liquid Chromatography-Ultraviolet Detection
GC-FID	Gas Chromatography-Flame Ionization Detection
GC-MS	Gas Chromatography – Mass Spectrometry
IP	Intraperitoneal
BSTFA	*bis* – trimethylsilyltrifluoroacetamide
TMCS	trimethylchlorosilane
SPE	Solid Phase Extraction
ANOVA	Analysis of Variance
ISTD	Internal Standard
LLE	Liquid-Liquid Extraction
LC-MS	Liquid Chromatography – Mass Spectrometry

I. Introduction

As opposed to analyzing clinically derived specimens, the nature of the postmortem environment has led to difficulty in interpreting the significance of drug levels observed due to drug instability after death (Drummer, 2004). There is little known about predicting antemortem drug levels in the body from the postmortem drug levels. Factors like redistribution, postmortem environment in the body, such as temperature and pH, have hindered the development of a rate at which antemortem drug levels can be extrapolated from the postmortem levels (Hearn et al., 1991). Also, artifacts from poor body condition and poor sampling can limit the interpretation of the drug analysis (Drummer, 2004). Each drug or toxin introduced into the body has different types or mechanisms for breakdown and redistribution (Hearn et al., 1991). It is important to know the stability of the drug being analyzed in a postmortem setting since the biological matrices being used may have been exposed to the environment for a long period of time (Drummer, 2004). Assumptions about the relationship between the concentrations of substances found postmortem in autopsy specimens to the concentration that existed at or near the time of death are made by toxicologists or pathologists (Hearn et al., 1991). These assumptions could lead to an inaccurate determination of antemortem drug levels. Inaccurate results could then lead to a wrongful interpretation of the cause and mechanism of death.

Cocaine (COC) continues to breakdown and undergo hydrolysis postmortem. COC, in ante- and postmortem samples, undergoes degradation and metabolism by

esterases in plasma and the liver to benzoylecgonine (BE) and ecgonine methyl ester (EME). COC can also be stored in tissue antemortem and subsequently released by redistributive processes postmortem (Mackey-Bojack et al., 2000). Several tissues allow for the release of drugs following death such as, the gastrointestinal tract, lungs, liver, and myocardium. Later, processes like cell autolysis and putrefaction all contribute to redistribution (Pelissier-Alicot et al., 2003). The continuous degradation of COC postmortem and the redistribution of COC from stored tissue to blood makes it difficult to estimate antemortem COC levels (Mackey-Bojack et al., 2000).

In performing toxicological analysis and screening for an unknown compound in the living person, the most common specimens for drug analyses are blood and urine. The detection of the unknown compound or drug in postmortem specimens can pose some difficulty because of redistributive processes and altered specimens, which may limit the capabilities of screening processes (Drummer and Gerostamoulos, 2002). But, in a postmortem setting several specimens, such as liver, muscle, fat, bone, brain, vitreous humor (VH), bile, hair, and nails can be used for analysis for special circumstances (Drummer, 2004). Blood, plasma, or serum can be used for screening processes when looking for a target analyte, but urine is still the sample of choice for any non-target screen of unknown drugs (Maurer, 2005b). Urine is the sample of choice because the concentrations of drugs are higher in urine in comparison to blood or saliva. Blood continues to be used for analysis since the unchanged drug is present and detectable in the homogenous matrix (Moeller and Kraemer, 2002).

Given the widespread use and availability of COC, it is remarkable that a relationship between the postmortem COC blood levels and toxicity of antemortem levels

has not yet been established (Karch et al., 1998). The aim of the present studies is to determine whether COC metabolism can be backtracked from the postmortem COC concentration in the VH and brain tissue to arrive at the antemortem concentration. If the antemortem COC level can be extrapolated from the postmortem level, it would aid toxicologists and pathologists in determining if a person was under the influence at or near the time of death, and if the drug played any role in the death.

A. Cocaine

$C_{17}H_{21}NO_4$, commonly known as COC is an alkaloid found in two major forms: COC hydrochloride and crack COC. COC hydrochloride is a white powder and crack is COC hydrochloride processed into a free base form by a reaction with bicarbonate or ammonia (Lewis et al., 2004). Structurally COC contains a hydrophobic region represented by a benzene ring and a hydrophilic region that contains a secondary or tertiary amine (Levine, 2003) (Figure 1). COC comes from the leaves of the *Erythroxylum* coca plant found in the Andes mountain region of South America. This plant can reach nine feet tall, prefers high elevations, and the leaves are available for harvesting up to three times a year (Levine, 2003). For over 2,000 years the leaves of the coca plant have been chewed by the Indians of Peru, Columbia, and Bolivia (Benowitz, 1993). This plant was believed to be created by Inti, the sun god of the Incas, as a gift to aid in relieving the hunger and thirst of the Incas. COC was used experimentally as well as clinically after Albert Neimann in 1859 isolated and extracted the alkaloid from the leaves of the coca plant (Billman, 1990). COC was added to elixirs, wine, potions, and Coca Cola™ by using the leaves for a flavoring agent (Knuepfer, 2003). As COC was used on a widespread basis, addiction concerns began to grow. COC is classified under

the Controlled Substances Act as a Schedule II drug meaning it has some medicinal value with a high potential for abuse (Levine, 2003).

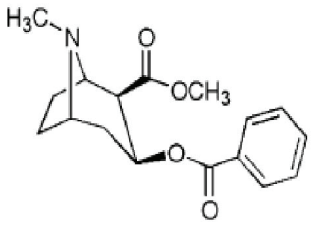

Figure 1. Structure of COC (Carrera et al., 2004).

COC is one of the most widely used and abused illicit drugs and has transcended all social, economic, and racial boundaries (Lewis et al, 2004). In the 19th century COC was believed to be safe and was used for the treatment of headaches, melancholy, hysteria, digestive disorders, alcoholism, and other drug addictions. Passage of the Harrison Narcotics Act of 1914 was prompted by the frequent abuse and growing addiction problems to COC (Benowitz, 1993). Abuse of COC then dropped since this Act only allowed prescription distribution of COC. COC abuse then remained low until the 1960's when it became popular again among young Americans. In the late 1960's into the early 1970's, affluent users of COC were sports stars and entertainers because of COC's image as an expensive party drug (Benowitz, 1993). COC abuse became epidemic in the United States in the 1980's and heroin and alcohol were replaced by COC in 1988 as the most frequent cause of drug-related deaths (Karch, 1991). COC was able to reach all classes and races at epidemic proportions as popularity and availability rose. COC is one of the most abused psychoactive drugs and the National Household Surveys on Drug Abuse reported in 2000 that 25 million people in the United States have used COC at some point and 1.5 million people use it currently (Knuepfer, 2003). COC

use has accounted for a larger percentage of hospital admissions compared to any other illicit or illegal drug (Knuepfer, 2003). The National Institute of Health reported a cost estimation of $97.7 billion for hospital admissions, loss of income, and criminal acts relating to illicit drug use in 1992 alone. The occurrence of COC toxicity seems to be substantially under reported since several deaths do not present as such, and therefore are not classified as being related to COC (Knuepfer, 2003). Incidences are also under reported due to the fact that some COC deaths may present as vascular problems instead of being directly related to COC. Also, even though there have been continued efforts of drug enforcement and treatment, COC and crack are still a large problem in the United States as well as the world (Jenkins, 1999). Epidemiological studies have suggested that about 17% of COC users become dependent on the drug (Nader and Czoty, 2005).

COC can be administered intravenously (IV), intranasally (IN), by smoking (SM), and by oral ingestion (PO) (Levine, 2003). The only route to produce 100% bio-availability is the IV route. The SM route is able to produce the same euphoria as the IV route because of the rapid delivery to the brain and it has bioavailability ranging from 57% to 70%. IN bioavailability seems to be dose-dependent and can range anywhere from 25% to 94% depending on the individual. The PO route of administration is least used since the bioavailability is only about 20% (Levine, 2003). The low bioavailability in comparison to other routes is due to first-pass effects. Low euphoric effects are also seen from the PO route due to a low efficiency for delivery to the brain. The high or euphoria related to COC exposure is dependent on plasma concentrations, not dose. Peak plasma concentrations develop rapidly after IV and SM routes and the peak plasma concentrations are delayed after IN and PO routes (Levine, 2003). A range of 20-100mg

is the common IN dose, which can be a lot higher in chronic or heavy users (Verstraete, 2004).

B. Metabolites

COC is rapidly metabolized in the living body ($t_{1/2}$ = 0.7 to 1.5 h) to EME and ecgonine by liver esterases and serum cholinesterase, and to BE via chemical hydrolysis (Levine, 2003; Spiehler and Reed, 1985) (Figure 2). COC is cleared from the bloodstream by two mechanisms: spontaneous hydrolysis to BE and enzymatic hydrolysis to EME via plasma pseudocholinesterase (Karch, 1991). BE and EME have been used to reveal past use of COC when there is no parent drug left in the blood (Drummer, 2004).

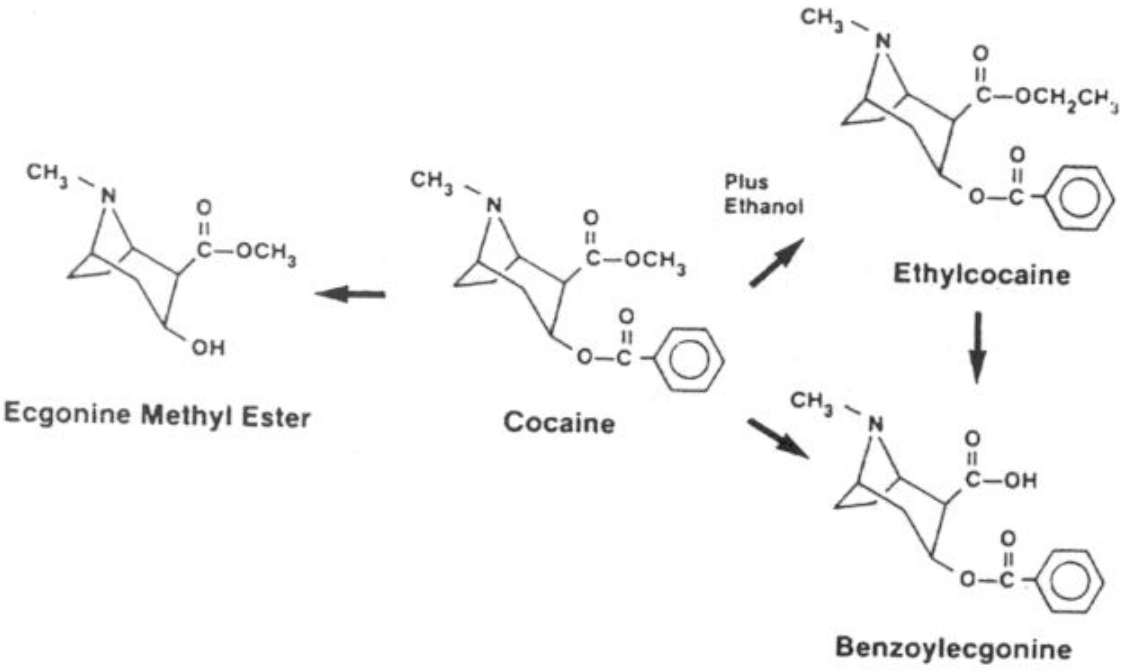

Figure 2. COC Metabolism Products (Benowitz, 1993).

COC's half-life does not seem to be dose dependent and elimination of COC follows first order elimination after IV and SM routes using one- and two-compartment

models. BE is measurable in the plasma 15-30 minutes after COC administration (Levine, 2003). BE's elimination rate is slower in comparison to its formulation rate, which provides a longer half-life and a higher accumulation in plasma when COC concentrations are decreasing (Levine, 2003).

Metabolism of COC is rapid and less than 5% is excreted in the urine unchanged (Benowitz, 1993). The hydrolysis to BE accounts for about 45% of COC metabolism and enzymatic hydrolysis to EME accounts for about 40% (Benowitz, 1993). COC contains ester linkages, similar to ester-linked local anesthetics, which allow for the hydrolysis and deactivation of the drug in the body. The ester group also becomes vulnerable to *in vitro* hydrolysis (Levine, 2003). Since COC can rapidly be metabolized to BE, the COC concentration found postmortem may not be indicative of initial exposure (Spiehler and Reed, 1985). COC is unstable in several biological matrices and analysis of its initial concentration becomes complicated due to vulnerability to hydrolysis in cholinesterase containing specimens like blood and plasma (Cingolani et al., 2004). Spontaneous hydrolysis of COC is known to occur after death, which further confounds postmortem COC findings and the problem of trying to relate postmortem to antemortem drug levels (Mackey-Bojack et al., 2000). These metabolic reactions complicate any interpretation of COC and its metabolite concentrations in the various biological specimens (Levine, 2003).

C. Systemic and Local Effects

COC causes several cardiovascular effects and potentiates cardiovascular diseases. COC use can result in myocardial infarction, arrhythmias and angina pectoris (Mittleman and Wetli, 1987). Myocardial infarction results from increased myocardial

work due to a demand for oxygen, coronary vasospasm, and thrombosis. Arrhythmias result from sympathetic nerve stimulation and myocarditis. The most common type of arrhythmia observed in COC users is sinus tachycardia (Benowitz, 1993).

COC is a potent vasoconstrictor and can severely constrict the cerebral vasculature. These adrenergic effects are due to the blocking of reuptake of the catecholamine, norepinephrine (NE) (Billman, 1990). COC also causes vasculitis (an inflammation in the brain vessel walls) and decreases cerebral blood flow (Carrera et al., 2004). COC increases heart rate and decreases skin temperature, consistent with the release of catecholamines (Benowitz, 1993). First time users may experience the complications of COC like cerebral vasculitis, ischemic stroke, brain hemorrhages, and intestinal ischemia. These complications are related to the high numbers of mortality seen in young, inexperienced adult COC users (Strickland et al., 1993). Cardiovascular diseases like contraction bands, cardiomyopathy, accelerated atherosclerosis, and endocarditis are all complications related to COC use. Chronic use of COC causes the same complications as acute exposure as well as an acceleration of atherosclerosis. Both arrhythmias and sudden death from COC use can be seen in chronic and acute exposure (Kloner et al., 1992). COC can also increase platelet aggregation, which can lead to stroke or other vascular disorders (Benowitz, 1993).

When COC is administered through the nasal cavity it is able to cause ischemia, necrosis, and infections of the nasal sinuses and mucosa. It can also cause several pulmonary complications like pulmonary hemorrhages and edema, obliterative bronchiolitis, asthma, and abnormalities in gas-exchange (Perper and Van Thiel, 1992). Because of the fact that smoking crack COC leads to a rapid euphoric effect caused by

rapid absorption into the pulmonary circulation, it has largely replaced IN and IV routes of administration. Smoking crack COC is able to produce several pulmonary complications because smoking exposes the lungs to several impurities, contaminants, pryloysis products, and vaporized alkaloidal cocaine. All of these products can cause airway and lung injury (Tashkin, 2001).

D. Nervous System Effects

COC readily crosses the blood brain barrier (BBB) because it has a low molecular weight (303.35) and is highly lipophilic. Rapid entry into the brain can cause the acute effect of euphoria by the stimulation of the dopaminergic system. The euphoric effect felt by COC users seems to be due to the occupancy of COC on 50% of the striatal dopamine transporter (DAT) binding sites (Kalasinsky et al., 2000). COC also produces an increase in energy, alertness, and self-confidence. At higher doses it causes restlessness, paranoia, anxiety, agitation, and aggressive behavior (Buttner et al., 2003). COC in moderation is able to produce arousal, a sense of friendliness, psychomotor stimulation, loquaciousness, and tremor (Benowitz, 1993), which are similar to the effects of amphetamine. Amphetamines and COC share toxicological features, i.e. a potent nerve stimulant. COC can also produce seizures and movement disorders. Seizures manifest after a decrease in the seizure threshold or because of an induction of cardiac arrhythmia. Movement disorders are commonly present in COC users and are due to dopaminergic transmission alterations in the nigrostriatal motor system (Drummer, 2004).

COC has been shown to augment sympathetic action in cardiac and other tissues. It also increases the adrenergic response by inhibiting the uptake of catecholamines into

nerve terminals (Billman, 1990). COC is able to block the reuptake of catecholamines like NE and dopamine (DA) at the presynaptic level in both the central nervous system (CNS) and the peripheral nervous system (PNS) (Kloner et al., 1992). COC primarily acts on the DAT in the brain. COC indirectly acts as a monoamine agonist at DA, serotonin, and NE receptors by binding to the respective transporters (Nader and Cztoy, 2005). The net results of DAT blockade are heightened levels of DA in the synapse as well as stimulation of the DA receptors (Benowitz, 1993). These heightened levels of activate the two families of DA receptors, D1- and D2- like. The D2-like receptors have been linked to drug abuse (Nader and Cztoy, 2005). Also, because of increased extracellular DA via the blockade of DAT, COC is able to cause the activation of motor and reward pathways in the midbrain, heighten locomotor activity, and heighten the euphoria felt by the user (Mortensen and Amara, 2003).

COC seems to be the only naturally occurring local anesthetic (Knuepfer, 2003). COC exhibits its anesthetic properties by blocking sodium (fast) channels through binding to the inside of the sodium ion (Na^+) channel membrane. This in turn inhibits action potentials in nerve and cardiac tissue and inhibits further conduction of the Na^+ (Billman, 1990; Knuepfer, 2003). By blocking the Na^+ channels, COC slows neuronal transmission (Benowitz, 1993). This blocking occurs in the myocardium and results in a depression of depolarization and conduction velocity. All of the excess catecholamines at the postsynaptic sites cause sympathomimetic stimulation of the CNS, heart muscle, and vascular smooth muscle (Kloner et al, 1992). COC is able to cause a physiological response resembling sympathetic nervous system actions by blocking the reuptake of catecholamines.

When COC binds to the NE transporter and DAT, there is a marked increase in the synaptic concentrations of these monoamines (NE and DA). As previously mentioned, COC blocks the reuptake of NE, which results in autonomic effects and it blocks the reuptake of DA, which results in CNS effects. DA has receptors on both the presynaptic and postsynaptic dopamine and non-dopamine axon nerve terminals (Harvey, 2004). Nearly 80% of DA in the brain is found in the corpus striatum (Siegel et al., 1999). DA as a neurotransmitter is able to affect the functioning of locomotor activity, motivation, the reward system, and cognitive processes (Bannon, 2005). Since COC is able to affect the DAT and reuptake of DA into the cell, it is able to exert its affects at several places in the CNS on process involved with DA.

E. Dopaminergic System

The neurotransmitter DA controls locomotor activity and is involved in reward and goal oriented behavior pathways. The DAT is seen specifically in dopaminergic neurons of the substantia nigra and the ventral tegmental brain areas. The striatum, prefrontal cortex, and nucleus accumbens contain dopaminergic neuron projections. DAT is present and expressed throughout the nerve cell on the axons, soma, and dendrites at the plasma membrane of these projections. DAT is responsible for regulating DA signal amplitude and duration, as well as the activation of extrasynaptic receptors by clearing DA from the extracellular spaces (Mortensen and Amara, 2003). DA release is also associated with the rewarding effects of COC in the mesolimbic and mesocortical pathways of the brain (Benowitz, 1993). DA projections in the brain contain the above-mentioned areas as well as the neostriatal pathway, mesolimbic pathway, mesocortical pathway, and tuberohypophysial pathway. The neostriatal pathway regulates motor

movement and the mesolimbic pathway regulates mood and reward systems. The last two pathways, mesocortical and tuberohypophysial, area able to mediate cognitive processes and inhibit prolactin release, respectively. Any disruption of these pathways could result in a disruption of motor skills, mood, cognitive abilities, and endocrine balance (Harvey, 2004).

DAT is a part of the Na^+ and chloride ion (Cl^-)-dependent neurotransmitter transporter family. Norepinephrine and serotonin are also included in this transporter family. DA transmission is mediated by receptors on the membrane belonging to a seven transmembrane domain, G-protein coupled family of receptors, with five receptor subtypes (Anderson and Pierce, 2005). DA is transported against the concentration gradient inwardly across the plasma membrane using the sodium gradient. The stoichiometry of DA transport is one DA, two Na^+, and one Cl^-, which results in transport that is potentially electrogenic. DAT is able to transport DA in the reverse direction (into extracellular space) following exposure to amphetamine, COC, and related drugs (Bannon, 2005). COC inhibits DAT in the nucleus accumbens and striatum (Itzhak et al., 1999). COC is a DAT binding transport inhibitor, which inhibits the uptake of released DA, causing and increase in extracellular DA. Since DAT is able to regulate and limit DA signaling, it is able to modify human behavior (Bannon, 2005). For most abused drugs, the dopaminergic system is a primary mediator of the euphoric effects and reward systems (Stephens et al., 2004). DA projection to the prefrontal cortex, nucleus accumbens, and amygdala are primary sites of action for COC (Kalivas, 2004). When COC binds to DAT and inhibits uptake of extracellular DA, the result is increased DA.

This then activates the reward pathways of the brain, increases locomotor activity, and heightens euphoria associated with COC use (Mortensen and Amara, 2003).

F. Mechanisms of Cocaine Toxicity

COC has a specific binding site on the DAT (Benowitz, 1993). When an acute dose of COC is administered, both NE and DA concentrations in the brain are briefly elevated and then the concentrations drop to levels below normal; represented by the rush and crash felt by the user (Levine, 2003). Also after an acute dose of COC, potentiation in DA cells and glutaminergic input is sensitized from the prefrontal cortex. Glutamate transmission is also a primary contributor to enduring neuroplasticity in the brain and to development and expression of COC addiction (Kalivas, 2004). The DA reuptake system, or pump, is the primary target of COC in the brain, thereby blocking the DAT and preventing the reuptake of DA in the presynaptic dopaminergic neuron (Benowitz, 1993; Levine, 2003). DAT is inhibited by COC binding to the sodium-binding site on the transporter. By binding to the sodium-binding site on DAT, cocaine is then able to alter the shape of the chloride-binding site, resulting in inhibition of binding for both ions. Since reuptake is inhibited, there is increased extracellular DA. This produces long-term stimulation of the DA receptor in the postsynaptic neuron since the translocation of DA across the presynaptic neuron membrane is inhibited (Levine, 2003).

Limiting catecholamine action and clearing them from the synaptic cleft is the primary goal of neuronal uptake (Levine, 2003). COC blocks the serotonin transporter (SERT) in a similar way as the DAT. COC blocks serotonin reuptake by binding to the SERT, which causes increased serotonin in extracellular spaces. This increase then causes activation of the serotonin receptors (Muller and Huston, 2006). When COC

blocks neuronal uptake of catecholamines, it is able to intensify the effects of those catecholamines. Changes in the DAT in the mesolimbic areas of the brain are observed following chronic COC exposure. Chronic users of COC continuously expose themselves to COC to compensate for the up-regulation of COC binding sites, which results in the need for more COC for continuation of its rewarding effects (Levine, 2003). After an overdose, chronic users show higher levels of ionotropic glutamate receptor subunits in the ventral tegmental area (VTA). In the VTA site, increased DA release causes a cascade facilitating cellular changes elsewhere in the brain (Kalivas, 2004). Uptake inhibitors, like COC, are able to up-regulate transporter surface expression (Mortensen and Amara, 2003). Cell culture studies by Daws et al (2002) have shown that an increase in DAT transport activity upon COC dose is accounted for by a parallel increase in DAT cell surface expression. Studies have shown that DAT blockers like COC are able to increase DAT cell membrane expression while increasing extracellular DA levels (Kahlig and Galli, 2003). COC's toxicity comes from the fact that it is able to block catecholamine reuptake as well as produce free radicals.

Free radicals like reactive oxygen species (ROS) can be generated after *in vivo* exposure to COC. COC-induced ROS may be linked to teratogenicity and genotoxicity (Yu et al., 1999). The mechanism of ROS formation from COC is the N-demethylation of COC by oxidative enzymes producing N-hydroxynorcocaine. N-hydroxynorcocaine can then quickly convert to a free radical metabolite, norcocaine nitroxide. This free radical metabolite is able to deplete glutathione, resulting in lipid peroxidation. Also, during biotransoformation of COC, superoxides produce hydroxyl radicals. The metabolites of COC may possibly induce hydroxyl radical production when hydrogen

peroxide is metabolized to superoxide ion in the presence of a water molecule (Yu et al., 1999). Overexposure to ROS is linked to degenerative diseases, like aging and cancers; therefore, chronic COC use can lead to excessive exposure to ROS and increasing the likelihood of developing cancers (Yu, 1999). The autoxidation of DA is a second source of ROS. DA can form reactive quinone species that then can modify and damage macromolecules such as DNA, lipids, and proteins. DA has the potential to be toxic forming the DA-quinone when a pair of oxygen atoms on the DA catechol ring consist of unpaired electrons on the outer orbitals (Stokes et al., 2000).

G. Redistribution of Cocaine

Drug redistribution is defined as the movement of drugs and other chemical toxins between bodily fluids, organs, and tissues postmortem (Cook et al., 2000). Redistribution is a complex process and phenomenon resulting from the disruption of cell membranes, diffusion of a drug from a higher concentration to a lower concentration, postmortem metabolism, or chemical changes of the compounds, thereby affecting the ability to determinate drug concentrations in postmortem samples (Drummer, 2004; Teixiera et al., 2004). Since COC is subject to redistribution, this process may allow for release of COC out of the respective organ it was stored in into the blood. Then because spontaneous hydrolysis and postmortem metabolism of COC do occur, the net effect might cause COC blood concentrations to increase, decrease, or stay the same (Hearn et al., 1991). The rate and extent of redistribution can vary. Factors like the nature of the drug and postmortem interval affect the extent of redistribution (Cook et al., 2000).

During the early stages postmortem, there are several mechanisms that contribute to the phenomenon of redistribution. In a hypoxic state there is a rapid decrease in

available adenosine triphosphate (ATP) and adenosine triphosphatase (ATPase), which results in a failure of the sodium/potassium pumps. Mitochondria and plasma membranes then become damaged allowing the drug accumulated in the cell to be released into the surrounding tissues. Also, both intracellular and extracellular pH values decrease due to lactate accumulation via anaerobic metabolism. These conditions occurring postmortem may allow for the leakage of intracellular drugs into extracellular spaces. Even though these mechanisms are known to transpire postmortem and contribute to redistribution, it is not known if one mechanism is predominant (Moriya and Hashimoto, 1999).

COC is able to store and accumulate into tissues, like the brain and liver, with moderate affinity (volume of distribution = 2 – 3 L/ kg). These tissues can then serve as a depot from which COC can be released into the blood. Increases in COC concentration seen in the blood of the heart, aorta, and femoral vein suggest that the postmortem redistribution or release of COC from tissue stores may overwhelm the effects of spontaneous hydrolysis (Benowitz, 1993; Hearn et al., 1991). The redistribution process is of significance to COC since it is of high lipid solubility and can store in tissues in higher concentrations in relation to blood (Drummer, 2004). Cook et al, (2000) used other drugs like amitriptyline, methadone, and salicylate to show the dangers of correlating postmortem drug concentrations to antemortem concentrations. They revealed that in every case studied, the postmortem concentration was as high as or higher than the original antemortem concentration at time of death due to drug redistribution (Cook et al., 2000). It is important to note that precaution must be taken when trying to extrapolate an antemortem drug level from the found postmortem

concentration. There has not yet been a standard extrapolation procedure set since several mechanisms are involved with postmortem redistribution.

H. Brain and Vitreous Studies

Brain tissue has been used for determining substance concentrations by toxicologists for several years. Brain tissue has been shown to be less susceptible to spontaneous hydrolysis and postmortem redistribution, which yields another matrix for analysis of COC than simply using blood alone (Drummer, 2004). Spiehler and Reed (1985) showed that brain tissue can be a better specimen for analyzing COC than postmortem blood since at peak cocaine plasma concentrations, the brain cocaine concentration was over four times the plasma concentration. They also stated that since COC is found with heterogeneous distribution throughout the brain, the regional source for a tissue sample used for postmortem analysis would not produce a source of variation in concentration.

VH has been found to be useful when analyzing COC and its metabolites and has been used for several years for the detection of ethanol in the body. VH can be helpful when blood is not available for analysis (Drummer, 2004; Mackey-Bojack et al., 2000). VH is less likely to undergo the same postmortem changes as blood, i.e. redistribution. The VH is basically like a buffer solution, a salt solution with very little protein present (Drummer and Gerostamoulos, 2002). VH is a clean fluid since it contains less protein than urine, is easily collected, and shows a high stability (Fernandez et al., 2006). Like the brain, VH limits transport across its membrane and drug transport is restricted by the lipid solubility of that drug. VH can be used for sampling in place of blood when the body has been subjected to burning, decomposition, or a considerable amount of bleeding

(Scott and Oliver, 2001). Since VH is a simple matrix, it is suitable as a complimentary specimen to blood when needed. If blood samples are decomposed or absent, the use of VH in forensic toxicology could be helpful to quantify toxic substances (Teixeira et al., 2004). VH analysis allows for certification of the presence of COC and metabolites when no blood is available, but cannot be used to estimate drug levels in the blood (Fernandez et al., 2006).

I. Use of Blood/Plasma for Analysis

The relevant matrices for analysis of drugs are whole blood, plasma, and serum. Blood is used for drug analysis since it is a homogenous matrix and drugs can be detected just after exposure and prior to any metabolism (Moeller et al., 1998). Even though urine is a very common specimen for drug analysis, blood allows for a suggestion that the person was not simply exposed to the drug, but the drug could have played a part in the person's impairment. A drug does not need to exceed toxic amounts to play a role in the person's death (Levine, 2003). For the most part, even if a drug observed in blood analysis is the only remarkable finding at death, the detected drug(s) will not be deemed the cause of death. COC, however, may be an exception to this general rule. When COC is determined in the blood, if there are no other findings present, death can be certified as a result of COC intoxication (Jenkins, 1999).

J. Summary and Significance of Study

COC, a psychostimulant, has been found to be in widespread use in the United States and can cause several cardiovascular, PNS, and CNS complications. It is found with heterogeneous distribution in the brain and is able to cross the BBB rapidly. It is broken down into its inactive metabolites, BE and EME, through chemical and enzymatic

hydrolysis respectively. Because of the phenomenon of redistribution and spontaneous hydrolysis to its metabolites postmortem, a rate has yet to be established to extrapolate the antemortem drug concentrations at or near the time of death from postmortem findings.

Since the rate at which COC is metabolized has not been established and it is difficult to relate antemortem COC levels to postmortem specimens, this study is aimed at comparing high performance-liquid chromatography with ultraviolet detection (HPLC-UV), gas chromatography-flame ionization detection (GC-FID), and gas chromatography-mass spectrometry (GC-MS) for the analysis of COC and BE in VH and brain tissue. Previous studies focused on prescription drugs, other drugs of abuse, and site differences in drug levels in the specimen (Clauwaert et al., 2000; Cook et al., 2000; Hearn et al., 1991). This present study deals specifically with analyzing COC and BE and comparing three analytical methods. Non-fatal doses of COC are being used to resemble a user under the influence at or near the time of death. Rats are being used as the animal model since they have previously been found to mimic the postmortem drug concentration changes that occur in humans (Hilberg et al., 1993).

Case studies have found it difficult to find a valid relationship between postmortem and antemortem drug levels because time of death and antemortem drug levels were not known (Karch et al., 1998). This study will compare known COC levels injected into the rat while comparing which analytical method is best for detecting adequate COC and BE levels. If a better analytical method emerges for analyzing VH and brain tissue, this will aid toxicologists and pathologists to better determine antemortem COC levels from postmortem drug levels and possibly draw correlations to previously studied drugs.

- *AIM # 1:* Comparison of extraction techniques and assay validation.

  Solid phase extractions will be done on J.T. Baker Narc-2 SPE columns and Varian Bond Elut Certify SPE columns. Liquid-liquid extractions with hexane will also be performed to determine the best extraction method.

- *AIM # 2:* In vivo analysis of COC metabolism in the brain and VH.

  Male Sprague-Dawley rats (4-6 mo) will receive intraperitoneal (IP) injection of 15mg/kg COC at time zero (N = 4). After 10-15 minutes (peak plasma COC concentration following IP injection), they will be euthanized in order to examine the brain tissue and tap the VH. Both the VH and brain tissue samples will be analyzed on the HPLC-UV, GC-FID, and GC-MS.

The research questions are as follows: Are COC and BE levels in the VH and brain measurable by HPLC-UV, GC-FID, and GC-MS? and can these levels help determine the rate at which COC metabolizes? The hypothesis is that GC-MS will be a better analytical tool to quantitate COC and BE in VH and brain tissue than HPLC-UV and GC-FID. Also, VH and brain tissue will be useful samples for finding adequate levels of COC and its metabolites.

II. Materials and Methods

A. Chemicals and Drugs

COC hydrochloride and BE were obtained from Isotec (Miamisburg, OH). The internal standard used for HPLC was bupivacaine from Sigma-Aldrich (St. Louis, MO). Internal standards from Isotec (Miamisburg, OH) for GC-MS were d_3-cocaine and d_3-benzoylecgonine. The derivatizing agent used for GC analysis was *bis*-trimethylsilyltrifluoro-acetamide (BTSFA) and trimethylchlorosilane (TMCS), (99:1). Concentrations of 165ng/ml, 500ng/ml, 750ng/ml, and 1500ng/ml for both BE and COC were made in mobile phase and analyzed on the HPLC to produce a standard curve. 100µg/ml of bupivacaine was used as the internal standard spike and 30µg/ml of COC was used as the standard for analysis. For GC-FID, 7.5µg/ml of COC and 100µg/ml of bupivacaine were used as the standards. GC-MS internal standards were 30µg/ml d_3-cocaine and 30µg/ml d_3-benzoylecgonine.

B. Animal Model

Male Sprague-Dawley rats (4-6 mos) were the animal model used for *in vivo* analysis of COC metabolism. At this age range, rats are in their young adult period. The animal insurance number is A367901. The protocol is 96-97-03, effective 1/20/2000.

C. In vivo analysis

Rats were given an IP injection of 15mg/kg COC at time zero. After 15 minutes when BE first appears in the plasma, they were euthanized (Levine, 2003). VH was removed and preserved with 1% sodium fluoride in a micro-centrifuge tube and stored at

-20°C prior to analysis. The whole brain was removed and sectioned into 4mm slabs containing the ventral and dorsal striatum areas. These areas were frozen in liquid nitrogen and stored at -80°C prior to analysis.

D. Solid Phase Extraction

1. HPLC

BAKERBOND spe™ narc™-2, 3ml (125mg) extraction columns from J.T. Baker were used for the assay validation (Phillipsburg, NJ). Varian (Palo Alto, CA) Bond Elut Certify, 130 mg, columns were used for solid phase extraction (SPE). The protocol was adapted from the Varian manual for extraction of COC and BE from VH and brain tissue instead of serum, plasma, or whole blood. For VH sample preparation, each sample was thawed and then centrifuged for three minutes. The supernatant was removed and added to 4ml of 100mM potassium phosphate buffer (pH 6.0) containing 100µg/ml bupivacaine. In order to prepare the brain sample for extraction, the sectioned brain was thawed and placed in 2ml of 100mM potassium phosphate buffer (pH 6.0). It was then homogenized by a hand held tissue homogenizer and centrifuged for three minutes. The supernatant was removed and added to 4ml potassium phosphate buffer containing 100µg/ml bupivacaine. The brain, buffer, and internal standard were then taken up into a 5ml syringe and filtered through a 0.45µ filter. The SPE column was conditioned first with 2ml methanol and 2 ml of 100mM potassium phosphate buffer (pH 6.0). The sample was then added at approximately 2 ml/minute, followed by a wash of 6mL HPLC grade water and 3ml of 1M acetic acid. The column was then allowed to dry under vacuum for five minutes at ambient temperature. After five minutes of drying, 6ml of methanol was added to the column. The last step, elution, was done by adding 2ml of

methanol/ammonium hydroxide (98:2) while collecting it at approximately 2ml/min. All extractions were performed on a BAKER spe-12G Glass Column Processor (J.T. Baker, Phillipsburg, NJ). After extraction, the liquid was evaporated by speed vacuum and the resulting COC or BE was reconstituted in mobile phase to run on the HPLC for analysis.

2. GC-FID

For the GC-FID, the protocol was the same as the HPLC methods. After the eluate was dried in the speed vacuum, the resulting COC or BE was reconstituted in 40µl BSTFA, TMS (99:1), vortexed, and held at 100°C for 25 minutes. The liquid was then evaporated off again in a speed vacuum and reconstituted in 40µl methlyene chloride. The sample was then run through the GC-FID, producing a chromatograph for each sample.

3. GC-MS

Bond Elut Certify SPE columns (Varian, Palo Alto, CA) were also used for extraction prior to GC-MS analysis. The protocol was adapted from the Varian manual for COC and BE extraction from VH and brain tissue instead of serum, plasma, or whole blood. Both the VH and brain tissue sample preparation were the same as the HPLC method except for the internal standards. 30µg/ml of d_3-cocaine and 30µg/ml of d_3-benzoylecgonine were added to the 4ml of potassium phosphate buffer (pH 6.0) for each sample. The column was conditioned with 2ml of methanol and 2ml of 100mM potassium phosphate buffer (pH 6.0). The sample was loaded onto the column at a rate of approximately 1-2ml /minute. The column was rinsed with 6ml HPLC grade water, 3ml of 1M acetic acid, and allowed to dry under vacuum for five minutes. Following drying, 6ml of methanol was added to the column. Elution was accomplished by adding

2ml methylene chloride/isopropyl alcohol (80:20) containing 2% ammonium hydroxide while collecting at approximately 1-2ml/minute. The eluate was placed in a speed vacuum and evaporated to dryness. The derivatization step consisted of adding 50µl BSTFA (with 1% TMCS), vortexing, and held at 70ºC for 20 minutes.

E. Chromatography

1. HPLC

The mobile phase consisted of 80% acetonitrile and 20% 0.01M potassium phosphate adjusted to a pH of 3.0. All chemicals used were of HPLC grade from Sigma-Aldrich (St. Louis, MO). The 0.01M potassium phosphate was filtered by the Millipore filtration system through a two-micron filter. It was then degassed for ten minutes with helium. Chromatography was performed at ambient temperature on a Supelcosil™ LC-Si 5µm, 15cm x 4.6mm column (Supelco, PA). The HPLC consisted of a Beckman System Gold autosampler 507 with a 100µl loop, System Gold 126AA solvent module, and System Gold 166 detector. The flow rate was set at 1ml/minute and the UV wavelength was set to 254 nm. The Gold Chromatography Data System analyzed the samples to produce a chromatograph.

2. GC-FID

Analysis was performed on an Agilent 6890N Network GC System with an on board FID (Palo Alto, CA). An Agilent HP-5MS capillary column (30m X 0.25mm i.d., 0.25µm film thickness) was used for chromatographic separation (Palo Alto, CA). The GC-FID oven operated at an initial temperature of 120°C with a ramp of 7°C/min to end at a final temperature of 225°C. Helium was the carrier gas flowing at 2ml/min with a hydrogen flame in the detector. The total run time was 15 min. It was run in split less

mode with an injector temperature of 250°C. The FID temperature was set at 300°C. Before each injection the needle was pre-cleaned with 2µl of the sample. 5µl of each sample was injected and then the needle was washed with 3µl of methlylene chloride (Sigma-Aldrich, St. Louis, MO).

3. GC-MS

Analysis was performed on an Agilent 6890N Network GC System (Palo Alto, CA) interfaced with an Agilent 5873 Inert Mass Selective Detector (Palo Alto, CA). An Agilent HP-5MS capillary column (30m X 0.25mm i.d., 0.25µm film thickness) was used for chromatographic separation (Palo Alto, CA). The oven temperature profile was the protocol of Lewis et al, (2004). The ramps, rates, and temperatures are shown in Table 1.

Initial	Final	Rate (C/min)
70	130	30
130	140	5
140	210	35
210	222	4
222	290	45

Table 1. GC Oven Ramp Temperature and Rates. All values are in Centigrade.

There was a final hold time of 0.49 min with a total run time of 11 min. It was run in the split less mode with an injector temperature of 250°C with helium as the carrier gas and a flow of 1ml/min. Before each injection the needle was pre-cleaned with 1µl of the sample. 5µl of each sample was injected and then the needle was washed with 3µl of methanol (Sigma-Aldrich, St. Louis, MO).

F. Statistical Analysis

All statistical analyses were performed on GraphPad Prism, version 4. Linear regression, 2-way analysis of variance (ANOVA), and unpaired corrected t-tests were run

on the respective results in order to statistically analyze the appropriate data. Significant differences were based on the p value, $p<0.05$ for all analyses done.

III. Results

A. Preliminary Studies

1. HPLC Validation

Four different mobile phases (A-D) were tested to optimize detection and quantitation limits for HPLC analysis. Three of the four (A-C) consisted of 45mM ammonium acetate. Each one was modified by changing the organic solvent strength. The composition of A is 10% methanol and 10% acetonitrile adjusted to pH 6.0 with 0.1M HCl. B contained 40% methanol and 40% acetonitrile adjusted to pH 6.0 with 0.1M HCl. The composition of C is 20% methanol and 20% acetonitrile adjusted to pH 3.0 with 0.1M HCl. Lastly, the mobile phase used for the injections (D) was 80% acetonitrile and 20% potassium phosphate adjusted from pH 4.5 to pH 3.0 with 0.1M HCl. Reduction in pH permitted the best separation on the chromatograph, shown in Table 2 and 3. Also, this mobile phase was suggested by Isotec (Miamisburg, OH) on their certificate of analysis sheet that came with the preparation COC standard.

2. Assay Validation

In order to validate the Narc -2 columns for use on the HPLC, standard concentrations for both COC and BE (165-1,500 ng/ml) were prepared and analyzed for separation and consistency. Four concentrations of COC and BE (165ng/ml, 500ng/ml, 750ng/ml, and 1,500ng/ml) were analyzed (Figure 3A and B). Retention times are listed in Table 2 and 3 and the resulting chromatographs are shown in Figures 4 and 5. The results demonstrated that responses, or peak heights, did not significantly differ from a

slope of 1. These findings suggest that both COC and BE standards yield linear responses at the concentrations tested, for both sets of standards following Narc-2 extraction.

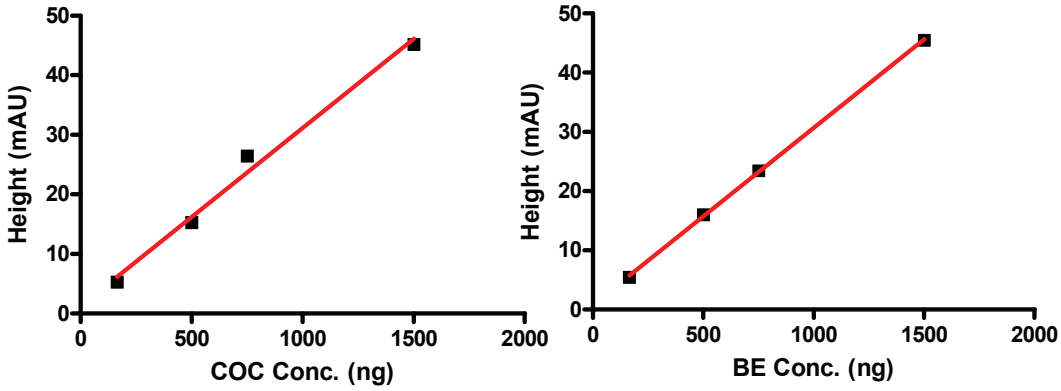

Figure 3. Standards Analysis. (A) COC Concentration vs. Height and (B) BE Concentration vs. Height.

COC Conc. (ng)	Height (mAU)	Retention Time (min)
165	5.265	5.700
500	15.286	5.683
750	26.431	5.683
1500	45.171	5.667

Table 2. COC Standards. Chromatography Results

BE Conc. (ng)	Height (mAU)	Retention Time (min)
165	5.424	6.508
500	16.037	6.45
750	23.435	6.508
1500	45.476	6.433

Table 3. BE Standards. Chromatography Results.

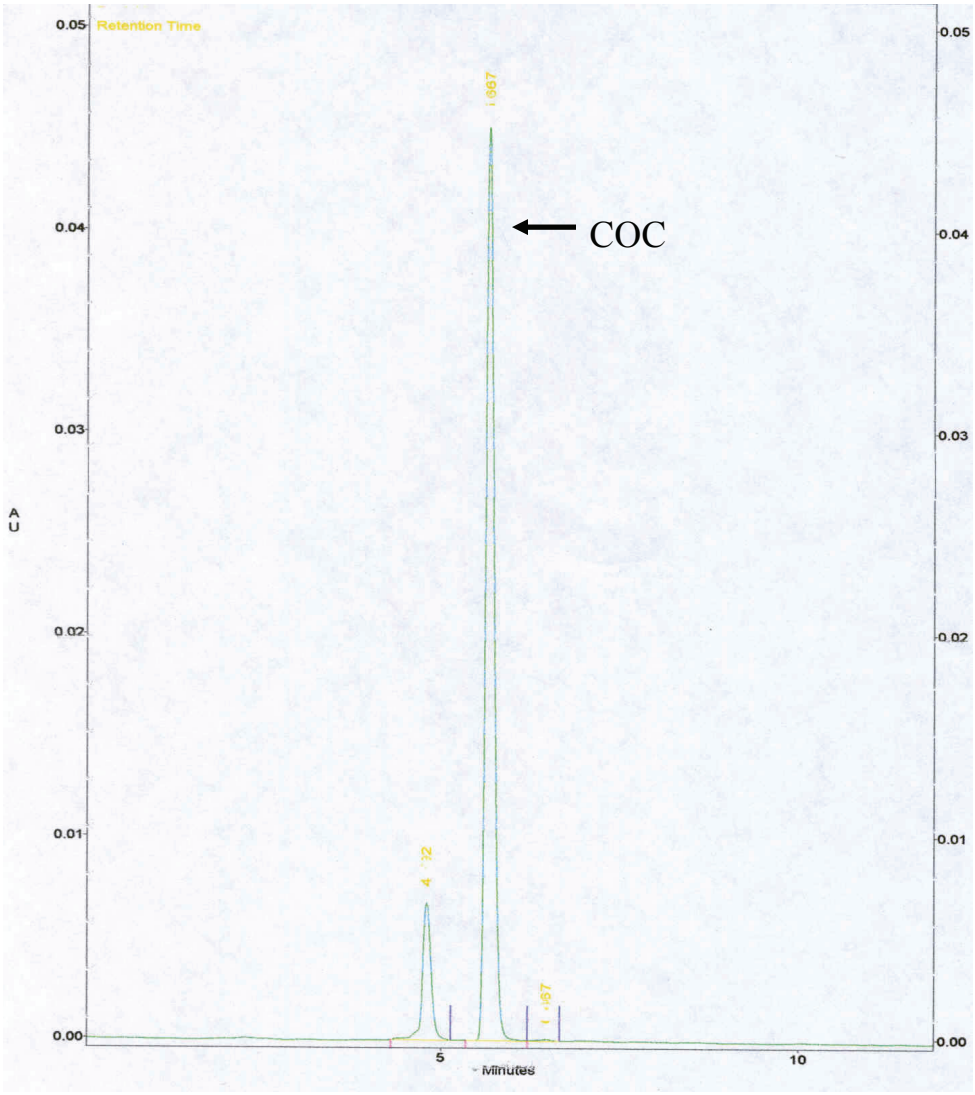

Figure 4. HPLC Chromatograph – COC Standard, 1500ng/ml.

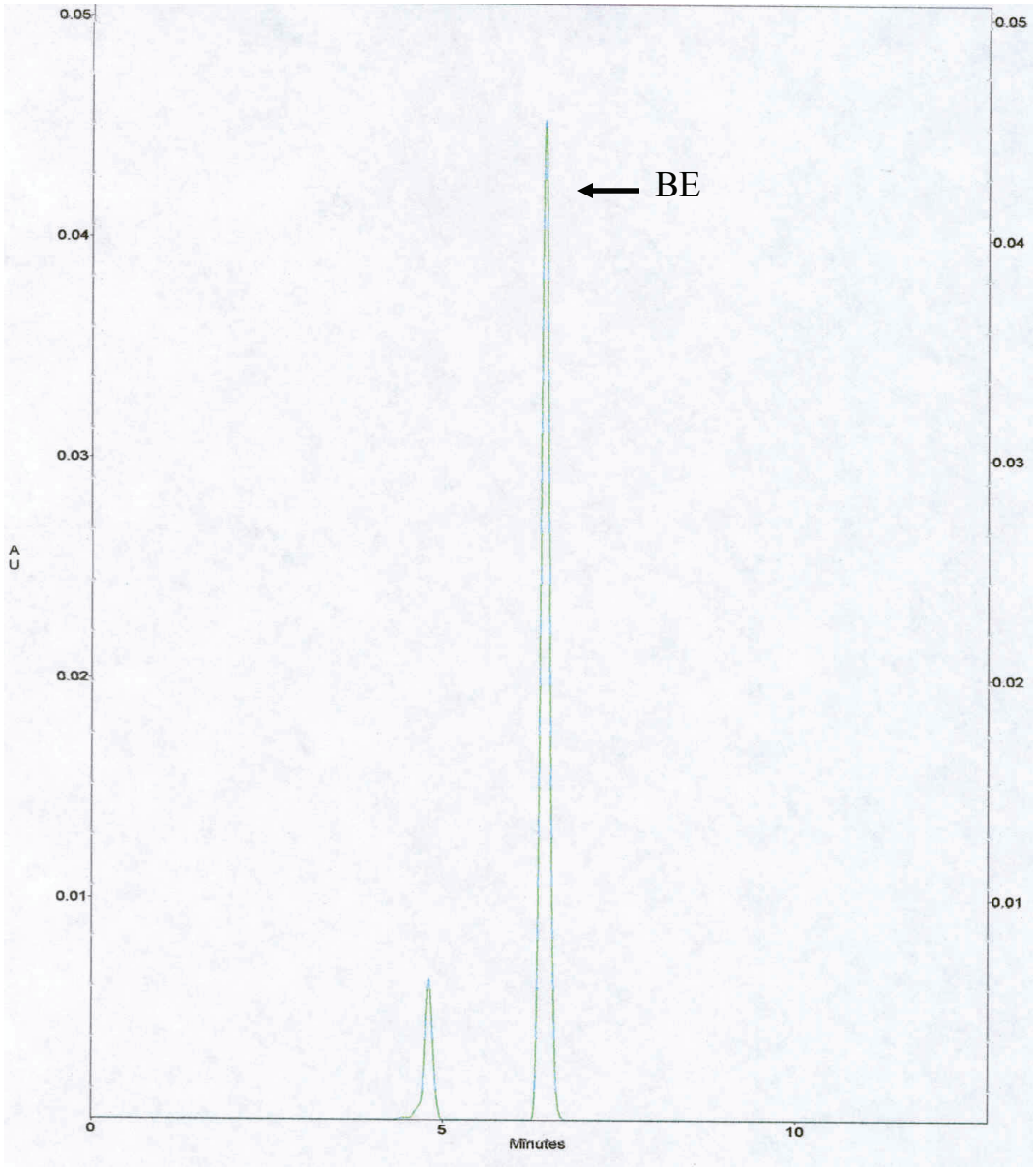

Figure 5. HPLC Chromatograph – BE Standard, 1500ng/ml

3. Narc-2 vs. Hexane Extractions

Six different hexane extractions were done. Each one was spiked with 2µg/ml bupivacaine and 2µg/ml COC. The average recovery for bupivacaine from hexane was 23.65%. The average recovery for COC from hexane was 9.623%. Ten different Narc - 2 extractions were run for comparison. The average bupivacaine recovery was 94.07%. The average COC recovery was 229.38% and BE was 104.38%.

Due to the greater than 100% recovery for COC and BE, Narc-2 extractions with no drug standard were run and the chromatograph showed extraneous peaks. The COC and BE percent recovery of greater than 100% is due to 1) an unknown elution peak interference or 2) an problem with the evaporation/reconstitution of the sample leading to inclusion of a contaminant.

4. Narc-2 Column Variability and Time Course Variability

To determine the reproducibility of Narc-2 extractions, four different extractions were run using bupivacaine, COC, and BE standards. Peak heights were compared between the four columns. Also, in order to determine the stability of COC post-extraction, four time points were used after extraction. The results of column variability over time (Figure 6) and COC degradation post-extraction (Figure 7) are shown. No significant difference on column variability shows that these SPE columns can be used for analysis with confidence that no variability will be introduced due to column effect.

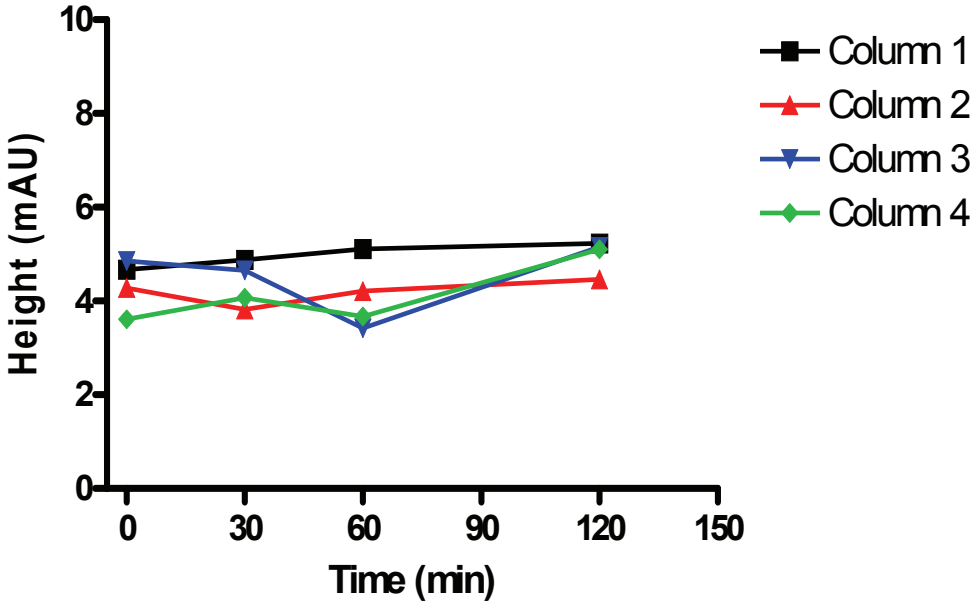

Figure 6. Narc-2 Column Variability. On each of the four columns a COC standard extraction was done and the eluent collected was run on the HPLC for analysis. A 2-way ANOVA demonstrated there was no effect of column on COC standard peak heights (p=0.0923).

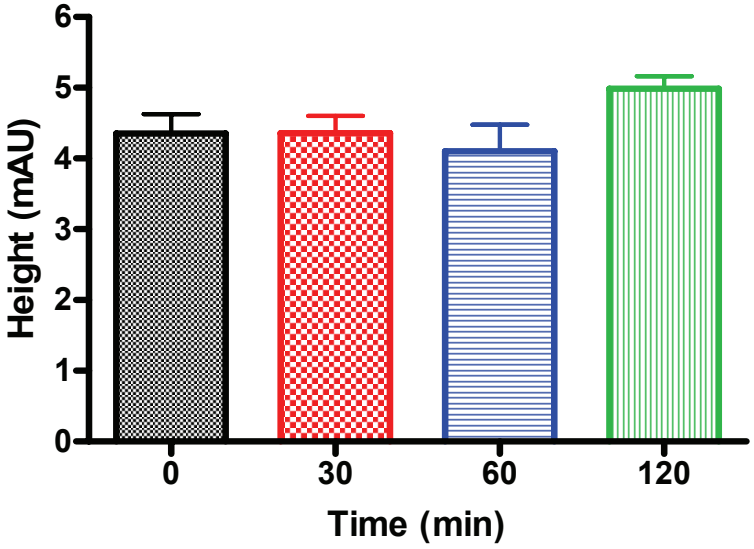

Figure 7. Degradation of COC Post-Extraction. From the same four columns used in Figure 5, a time test was run. The eluent collected from the extractions was analyzed on the HPLC at time zero, 30 min, 60 min, and 120 min. A 2-way ANOVA demonstrated there was no effect of post-extraction time degradation on COC standard peak heights(p=0.1055).

Data analysis using a 2-way ANOVA demonstrated that there was no interactive effect of column by time ($p=0.1922$) on peak height of a COC standard. Thus, samples could be analyzed up to 120 minutes after SPE with no effect on peak height. Therefore, samples can be extracted up to two hours following sample preparation with no loss of sensitivity.

B. Chromatography

1. HPLC

Chromatographs of VH and brain extractions are shown in Figures 8 and 9. In order to find the amount of COC or BE recovered from each tissue, calculations were as follows:

Equation 1: **extracted internal standard (ISTD) / unextracted ISTD = % recovery**

Equation 2: **(unknown peak height) / (% recovery) = calculated height of unknown**

Equation 3: **(Calculated height of unknown) X (STD height expressed as [µg/ml]mm) = unknown in µg/ml**

Equation 4: **VH value X 10 = µg/ml for VH; brain value / 2 = µg/ml for brain**

These values are shown in Figure 10 and Table 4. The ratio of the COC standard to the BE standard is 1.138, almost a 1:1 ratio.

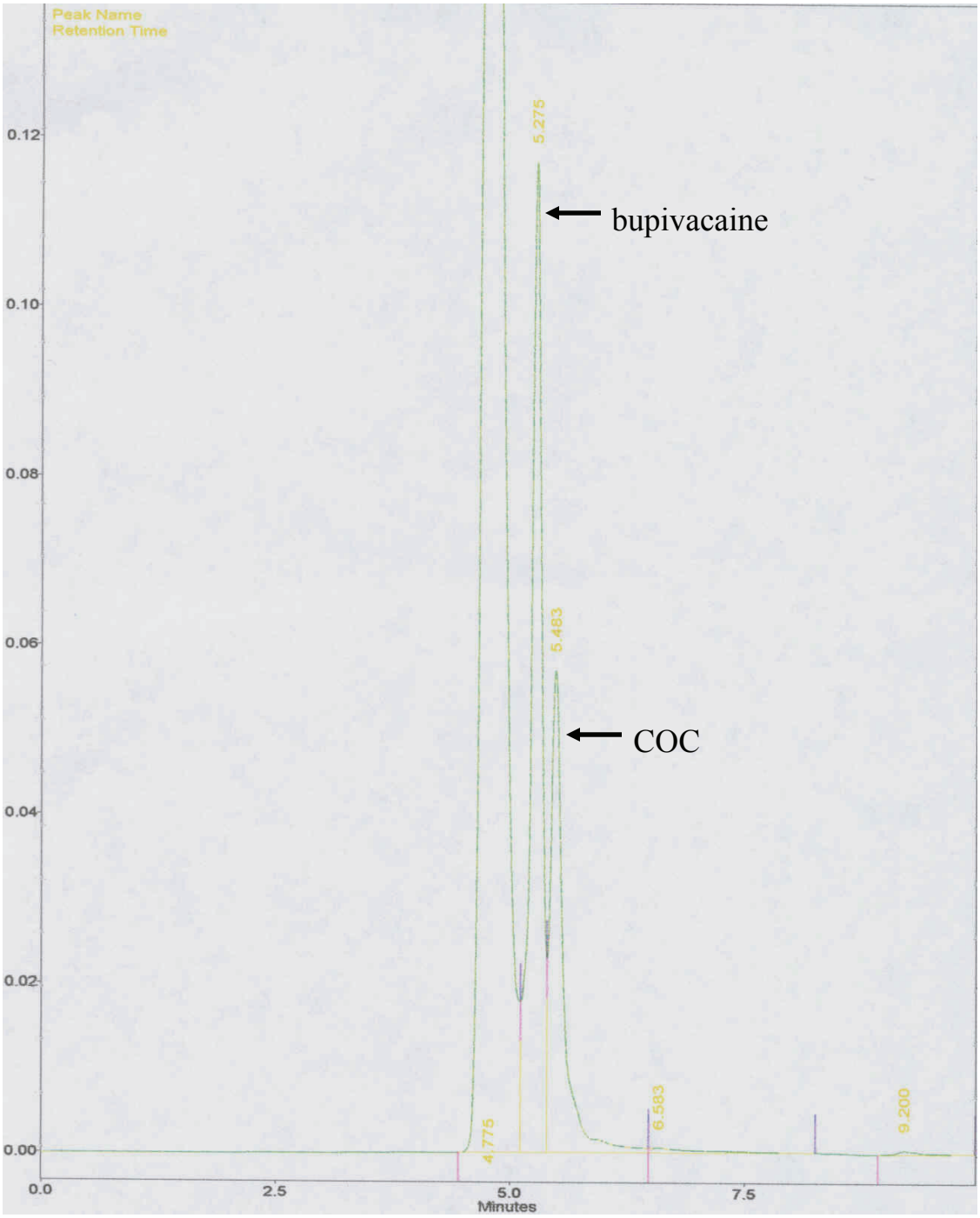

Figure 8. HPLC Chromatograph of VH Extraction. Yellow label = Retention time.

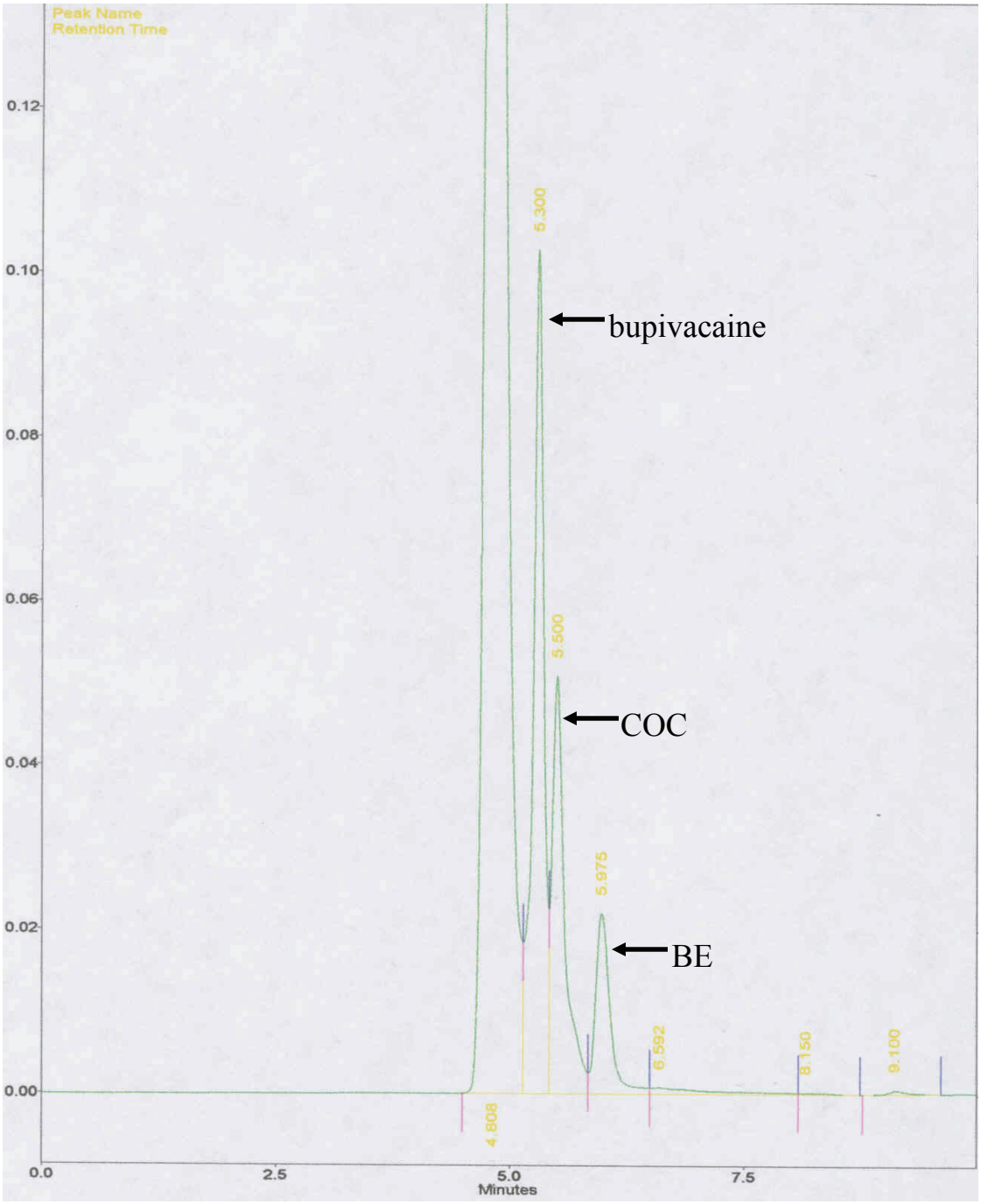

Figure 9. HPLC Chromatograph of Brain Extraction.

No BE was observed by HPLC analysis in any of the four VH extractions done. But, BE was seen in all of the brain tissue extractions by HPLC analysis. BE has an approximate retention time of 5.975 min, seen in Figure 9.

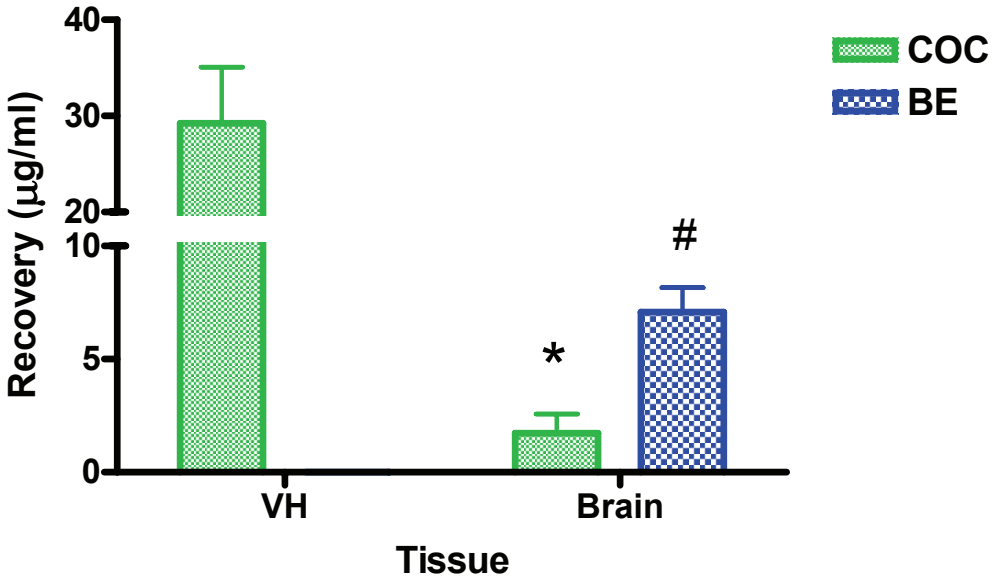

Figure 10. Analysis of Recovery of COC and BE from VH and Brain Tissue on HPLC. *p<0.005. #p<0.001.

COC VH	BE VH	COC Brain	BE Brain
44.694	0	0.4912	5.766
31.508	0	1.008	9.239
18.77	0	1.165	8.577
22	0	4.227	4.722

Table 4. Calculated Extraction Recoveries of COC and BE by HPLC. All values are in µg/ml.

Unpaired corrected t-tests were done to compare COC and BE levels in VH and brain. All four comparisons showed a significant difference. COC VH vs. COC brain ($p=0.0034$), COC brain vs. BE brain ($p=0.0081$), BE VH vs. BE brain ($p=0.0006$), and COC VH vs. BE VH ($p=0.0024$) had a significant difference between the means. COC in

the VH was significantly higher than COC in the brain. BE in the brain was significantly higher than BE in the VH.

2. GC-FID

The analysis performed on the GC-FID was unclear. There were several unresolved peaks detected on the chromatograph of the COC standard that could not be used for analysis as seen in Figure 11. No one peak could be determined to be the COC peak, therefore no quantitation analysis could be performed on the VH and brain samples. The bupivacaine internal standard also showed several unresolved peaks on the chromatograph, seen in Figure 12. The chromatography results for VH and brain tissue can be seen in Figures 13 and 14. There were no clear chromatograph peaks for the standards, no peaks could be matched to each other in the samples, and no quantitaion or statistics could be performed on these results.

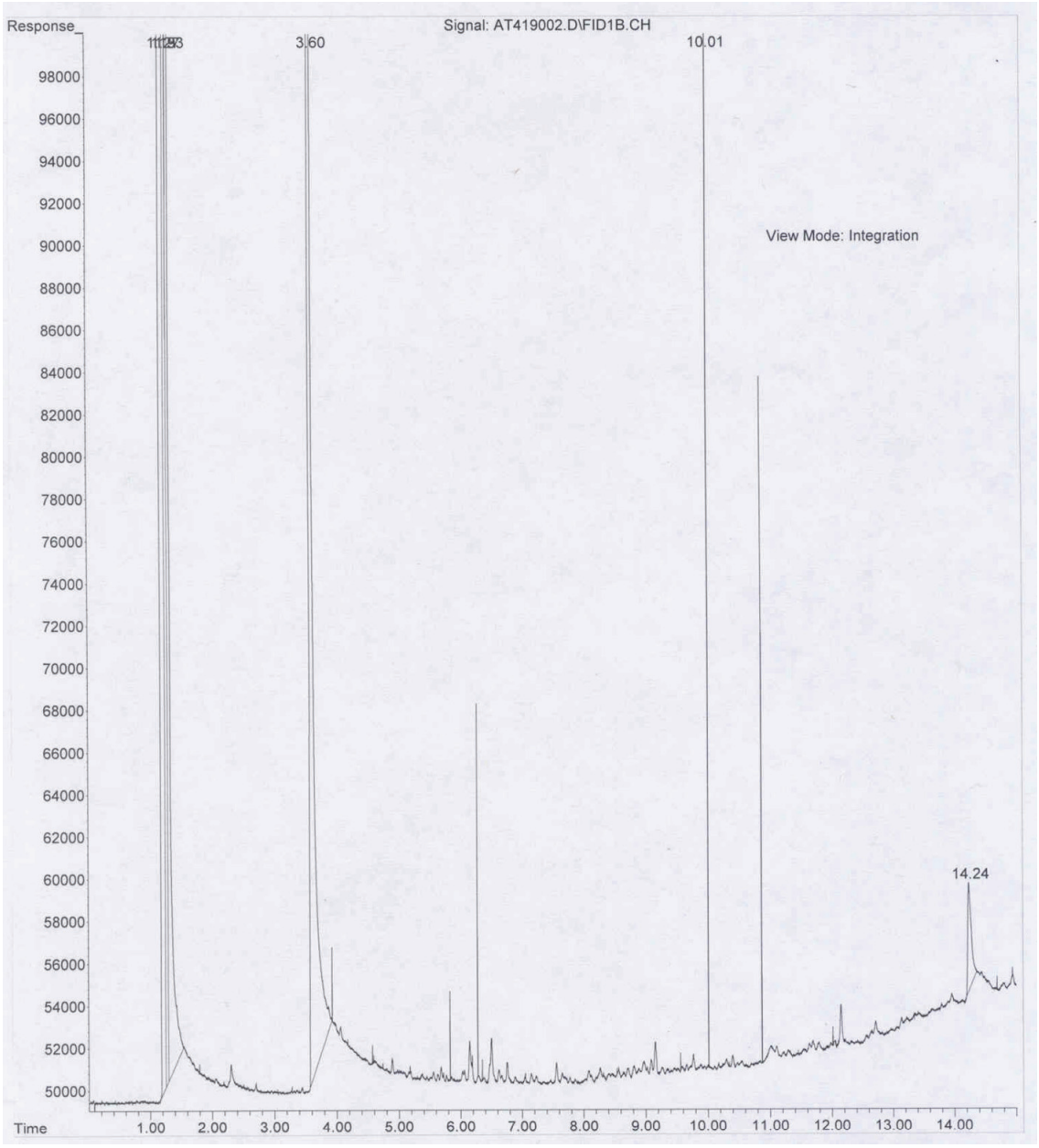

Figure 11. GC-FID Chromatograph – COC Standard, 40ug/ml.

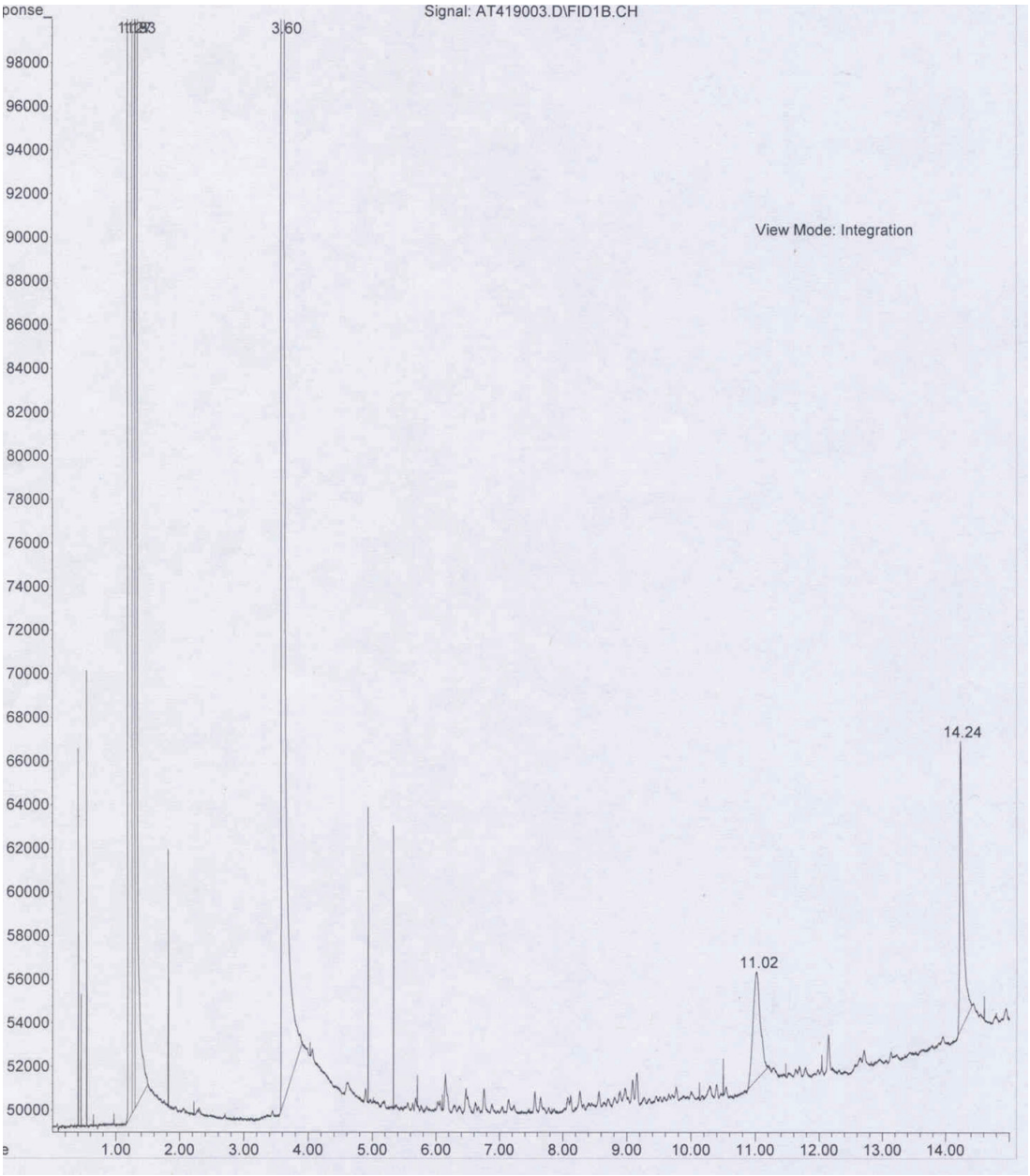

Figure 12. GC-FID Chromatograph – Bupivacaine Standard, 100ug/ml.

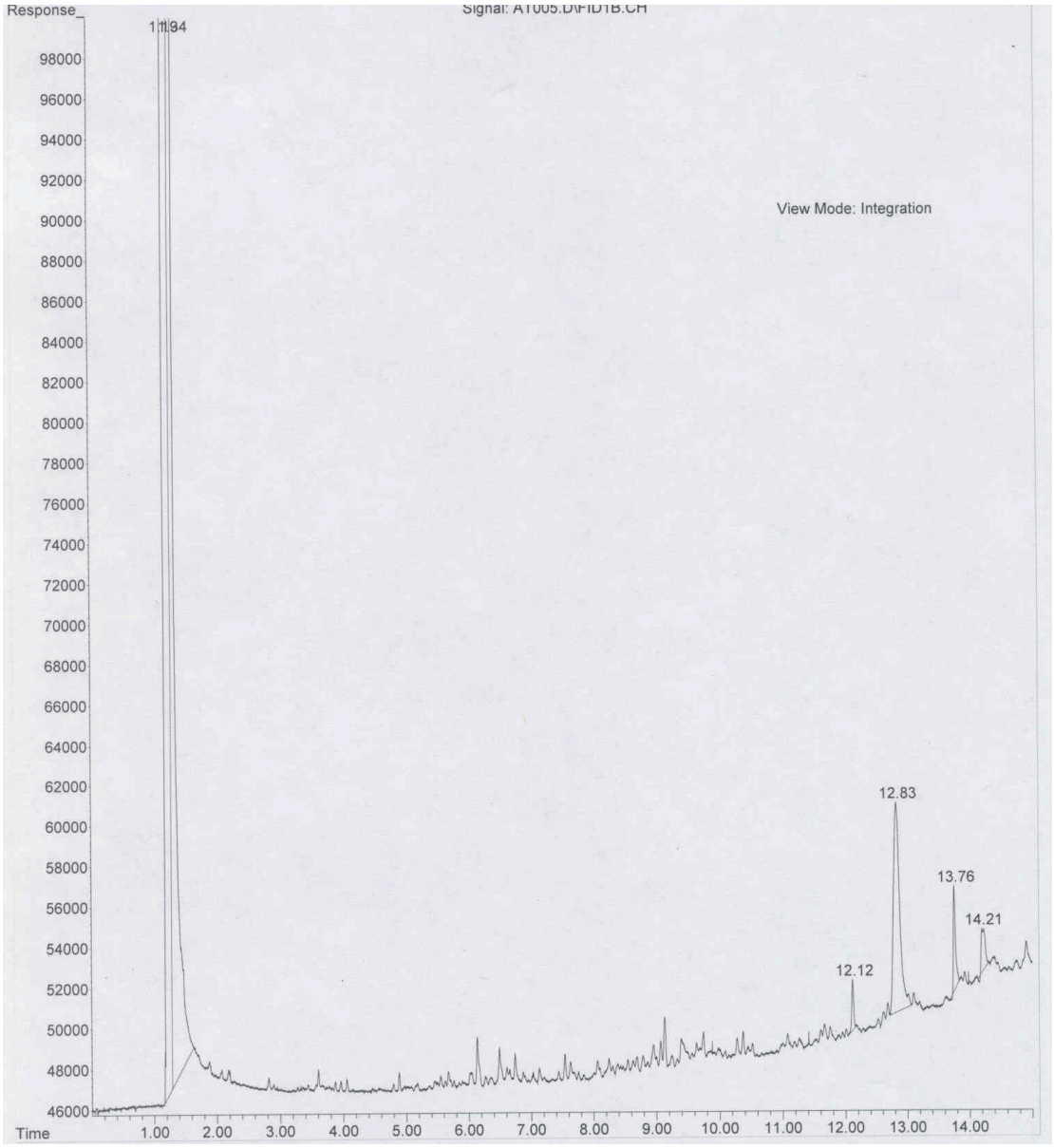

Figure 13. GC-FID Chromatograph of VH Extraction.

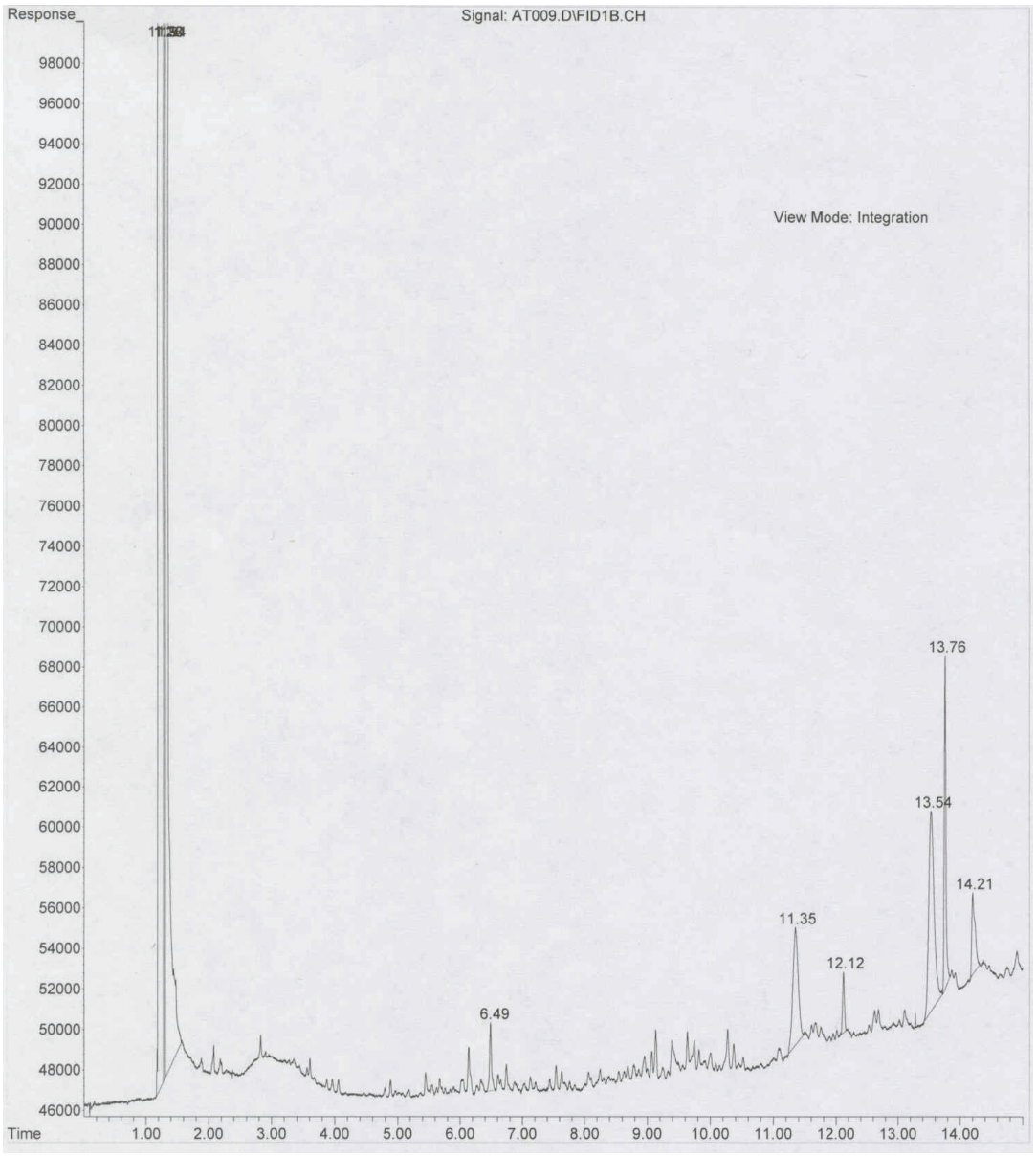

Figure 14. GC-FID Chromatograph of Brain Extraction.

3. GC-MS

In order to find the amount of COC and BE recovered from each tissue, calculations were performed using the HPLC equations. The only difference is that d_3-COC and d_3-BE were used as the internal standards in place of bupivacaine. These calculated values are shown in Figure 15 and Table 5. The chromatographs of these

extractions are seen in Figures 16-19. The ratio of the COC standard to the BE standard was 1.527.

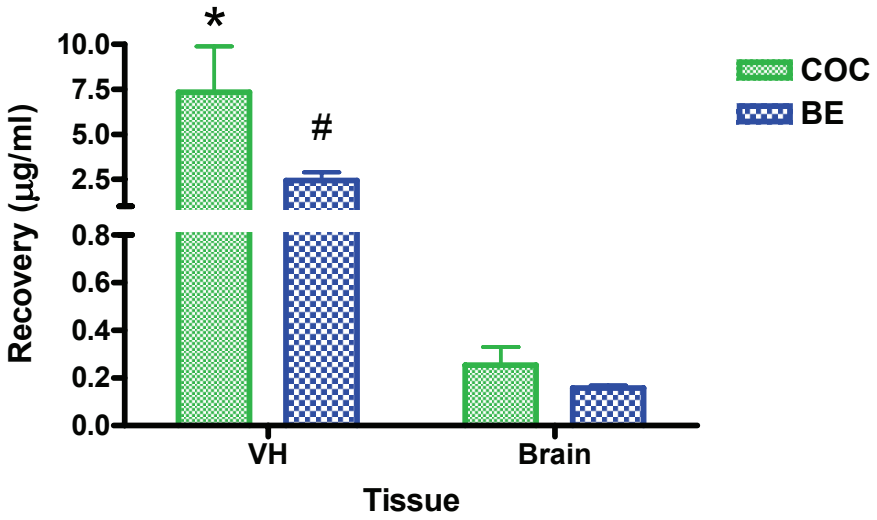

Figure 15. Analysis Recovery of COC and BE from VH and Brain Tissue on GC-MS. *p<0.05. #p<0.005.

COC VH	BE VH	COC Brain	BE Brain
10.766	1.993	0.4452	0.1635
3.5	1.986	0.1546	0.1542
12.614	1.967	0.3049	0.1329
2.47	3.803	0.1122	0.1838

Table 5. Calculated Extraction Recoveries of COC and BE by GC-MS. All values are in µg/ml.

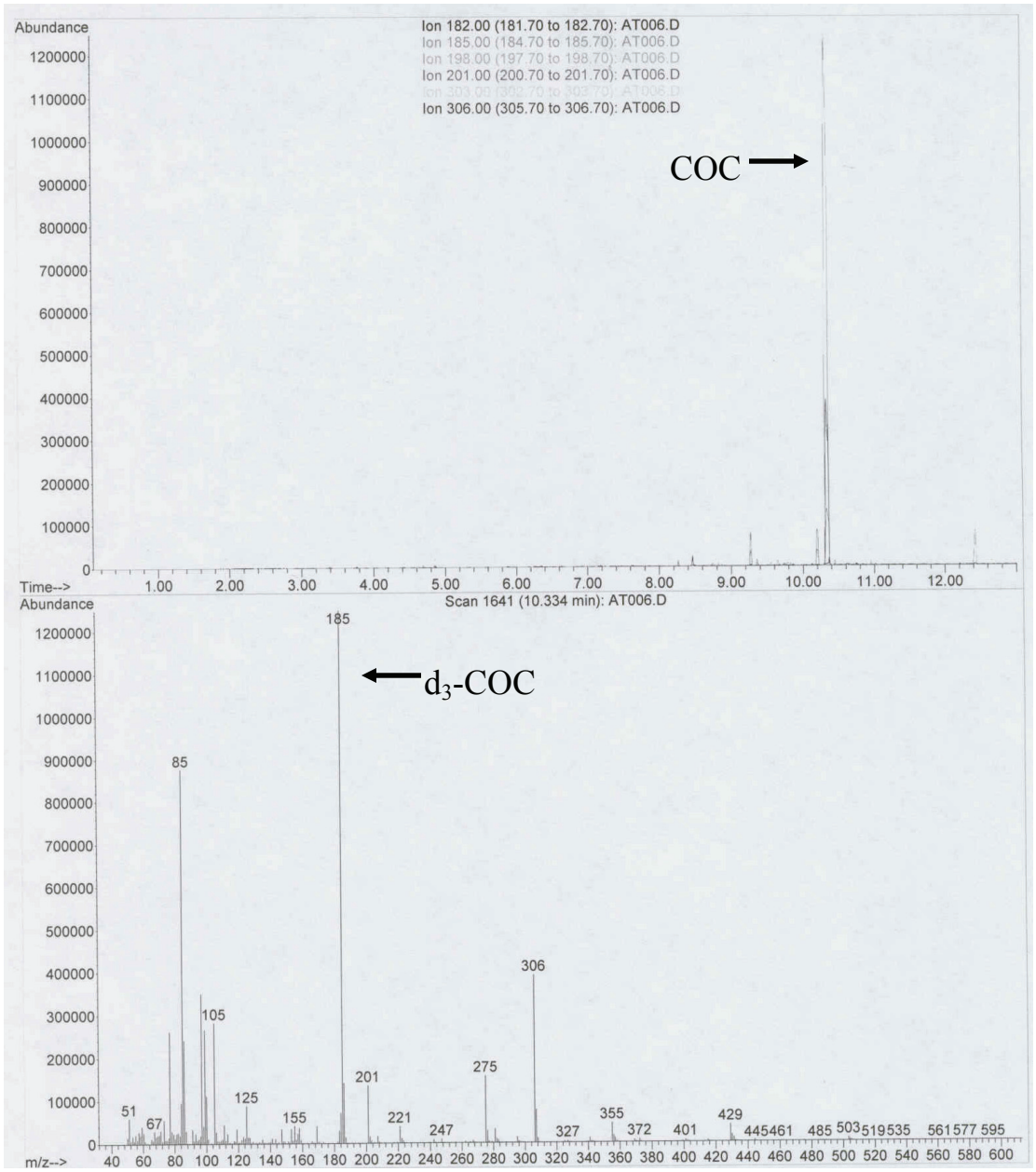

Figure 16. GC-MS Chromatograph of VH Extraction – COC and COC Ions.

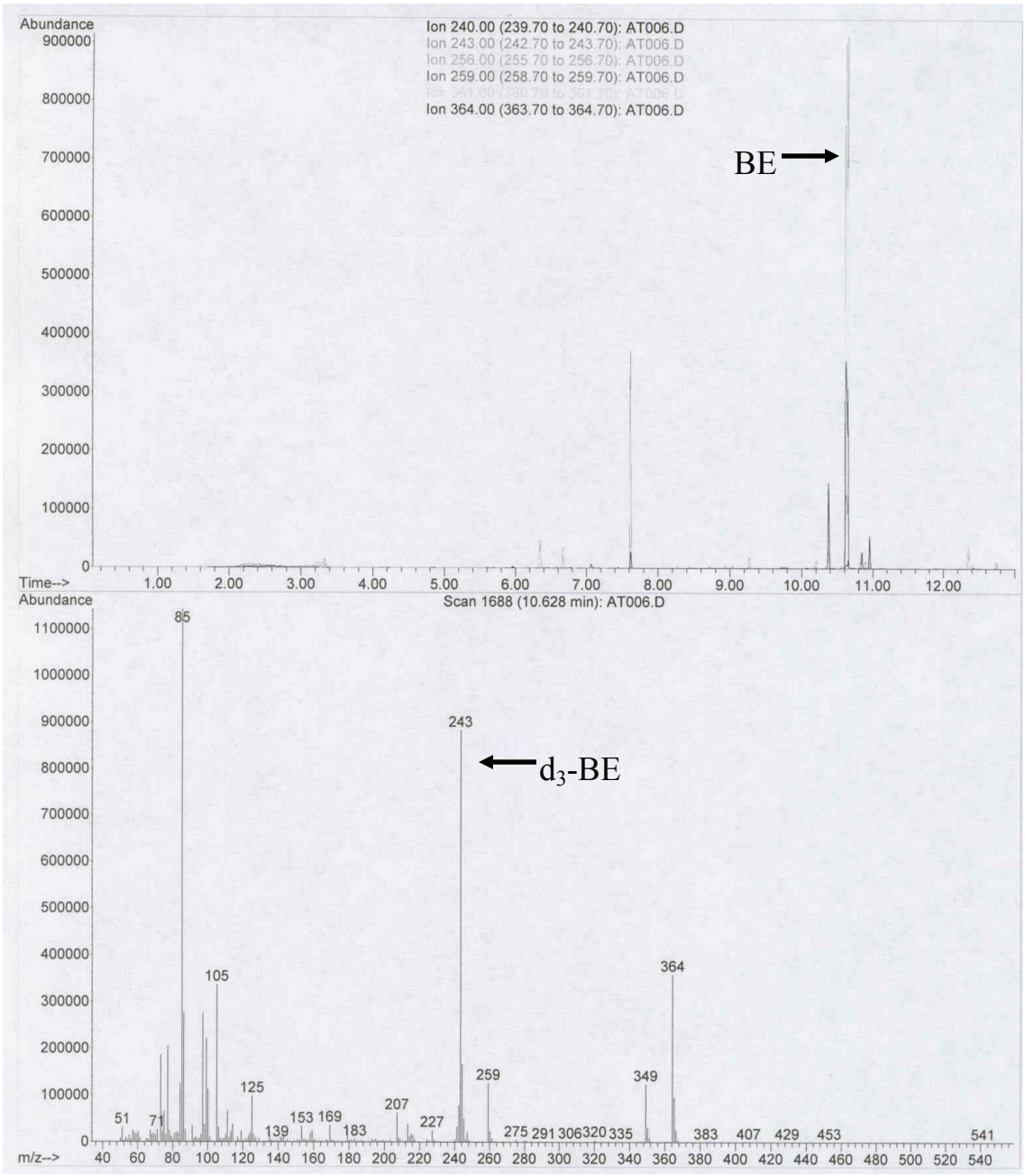

Figure 17. GC-MS Chromatograph of VH Extraction – BE and BE Ions.

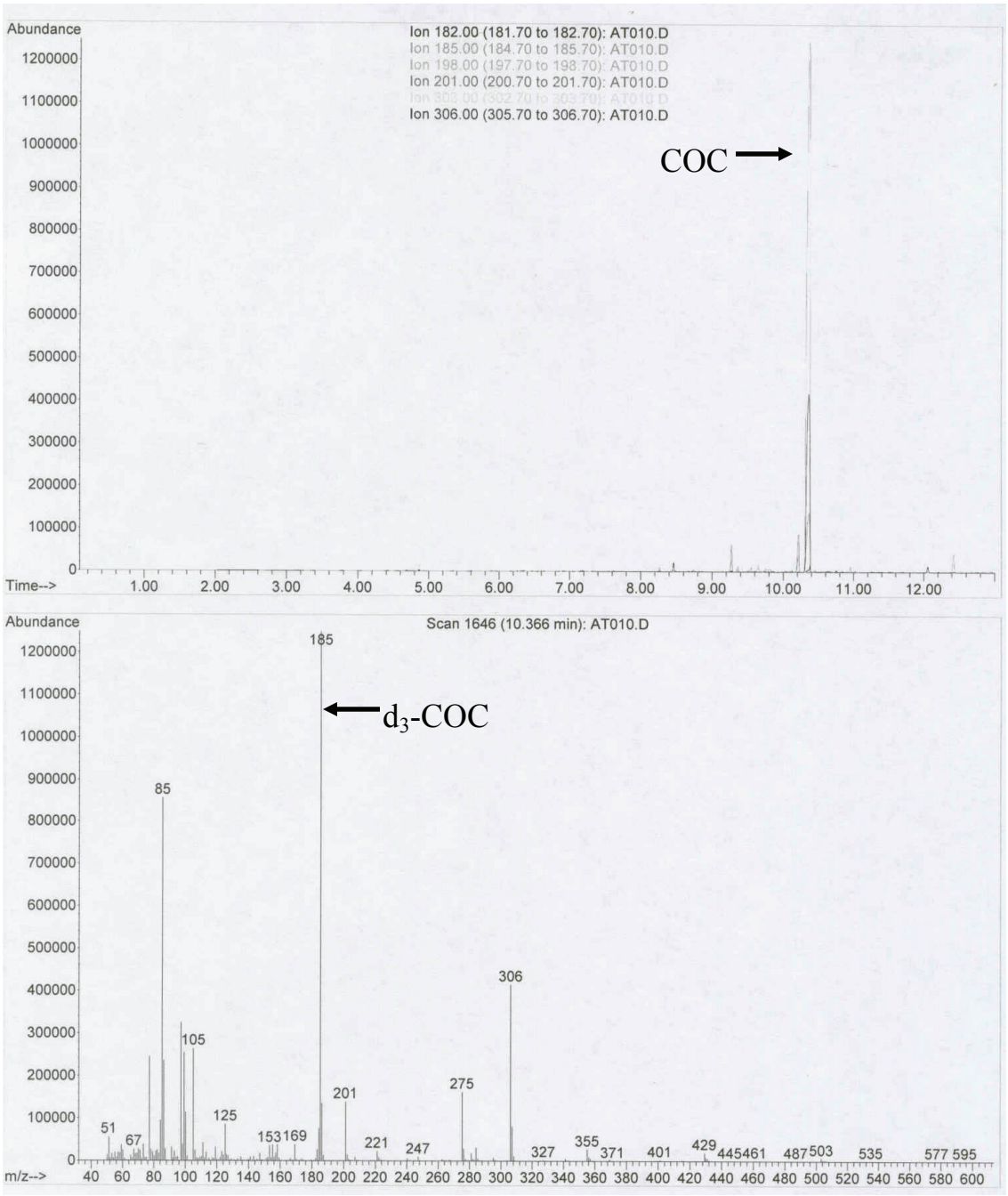

Figure 18. GC-MS Chromatograph of Brain Extraction – COC and COC Ions.

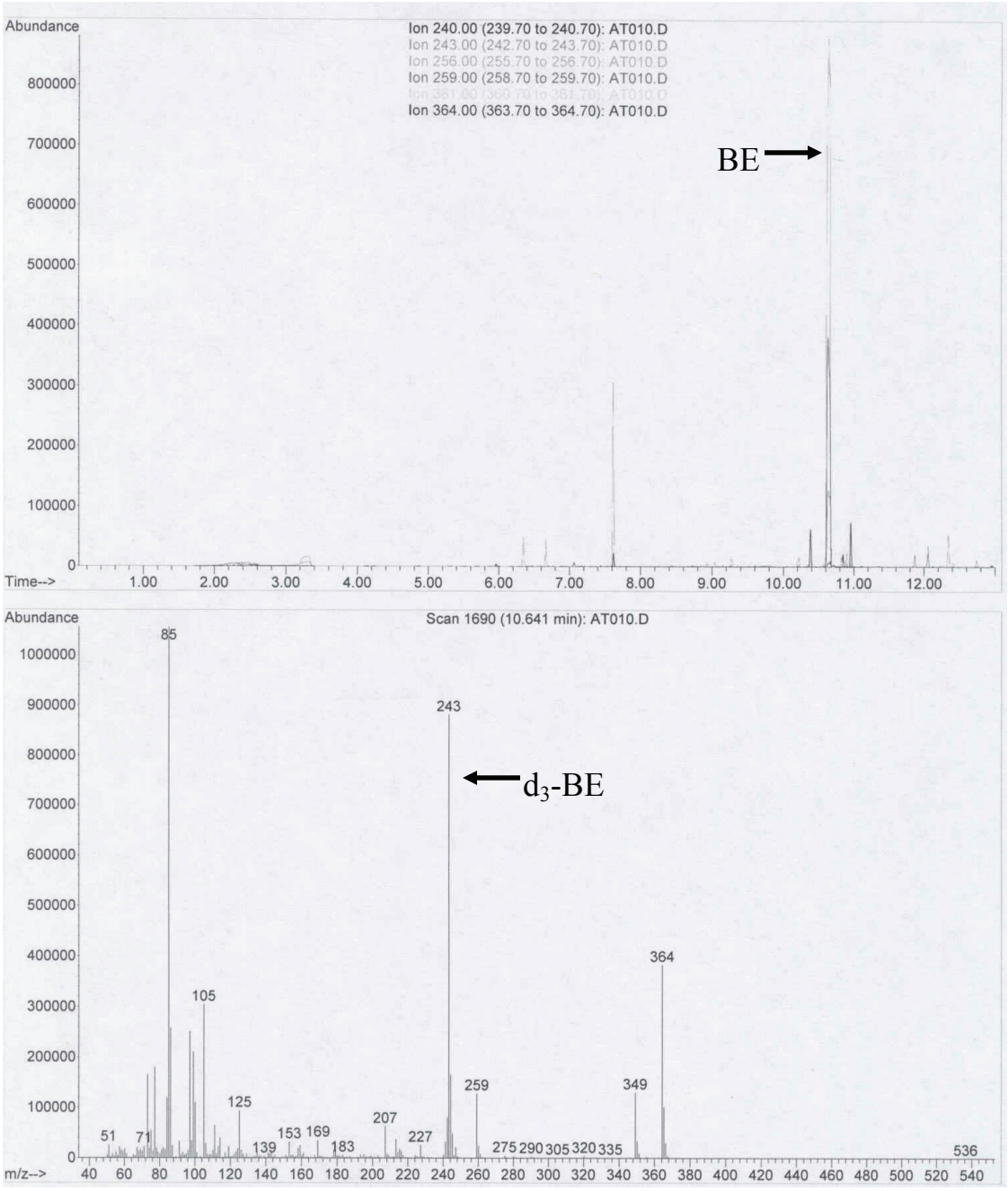

Figure 19. GC-MS Chromatograph of Brain Extraction– BE and BE Ions.

Unpaired corrected t-tests were performed on the same pairings as those from the HPLC t-tests. COC VH vs. COC brain means (p=0.0321) and BE VH vs. BE brain means (p= 0.0024) showed a significant difference. COC VH vs. BE VH had no significant difference for means (p=0.1074). Also, COC brain vs. BE brain showed no significant

difference between means (p=0.2586). COC and BE in the VH were significantly higher than COC and BE values in the brain.

IV. Discussion

Cocaine is used widely available and used worldwide. Due to the fact that its contribution to cause of death is a forensic issue, it was used as the model drug (Stephens et al., 2004). Frequently, forensic toxicologists are questioned about the role of COC in cause of death. They must assess the specimens provided to determine antemoretem levels and the degree to which a drug played a role in the death or impairment if any (Klingmann et al., 2001). Due to the postmortem changes such as redistribution and hydrolysis that occur to COC, a rate at which COC metabolizes postmortem has not yet been found. In order to aid the process of finding a rate at which COC degrades postmortem, this study was undertaken to find the method to best analyze COC and BE extracted from VH and brain tissue.

A. HPLC Validation

Each HPLC system as well as the column used for separation is different. The parameters and mobile phase chosen is dependent on the system, column, and analyte being separated. Four mobile phases were tested with the HPLC to investigate the chromatographic separation and level of detection of COC and its metabolite BE. Each mobile phase was altered in organic strength and pH to aid in this process. Increasing the acidity of the mobile phase resulted in a slowing of BE elution and a reduction in COC elution time as indicated by BE shifting to the right of COC on the chromatograph as well as shifting COC out of the bupivacaine peak. Since COC is slightly basic, the lower pH

allowed COC to be retained on the very polar, negatively charged silica packed in the HPLC column long enough for it to separate from the internal standard, bupivacaine. The mobile phase used for this study was at pH 3.0 which proved to be the best mobile phase for drug separation.

B. Assay Validation

Isolation of compounds from biological matrices is an important step in toxicological testing, which must be both reproducible and reliable (Bogusz et al., 1996). Mixed phase SPE columns were first commercially introduced in the late 1980's and now are proving to be a good method for the pretreatment of samples used for drug analysis. SPE columns clean the extracts of unwanted contaminants, have reproducible results, and exhibit high selectivity (Bogusz et al., 1996; Chen et al., 1992).

Bogusz et al, (1996) reported on four different commercial SPE cartridges concerning extraction efficiency and extract purity. Bond Elut Certify and Narc-2 columns were a part of the study. Bogusz et al, (1996) found that extractions from the Bond Elut Certify columns were almost free of interference peaks on the HPLC. On the GC-MS, plasticizer peaks were smaller for the Bond Elut Certify and Narc-2 in comparison to one of the other cartridges tested, Chromabond MN Drug columns (Macherey and Nagel, Düren, Germany), (Bogusz et al., 1996). However, SPE does have some drawbacks. Commercial products like Bond Elut Certify and Narc-2 have demonstrated low reproducibility in the packing material (Bogusz et al., 1996). Also, among the same and different lots of products, different recovery percentages and failure of extraction has been observed (Bogusz et al., 1996). SPE columns also tend to be time

consuming and expensive when compared to other extraction methods incorporated in clinical and toxicological labs.

Narc-2 columns were used for the assay validation to check for separation and consistency of the columns with four concentrations of both COC and BE standards. The results of this study were somewhat different than the results found in the Bogusz et al study (1996). When using the Narc-2 and Bond Elut Certify SPE cartridges, there were peak interferences seen on the HPLC analysis. A Narc-2 blank showed a peak that co-eluted with the COC peak and Bond Elut Certify had two peaks, one that co-eluted with the bupivacaine peak, and one that co-eluted with the COC peak. Even though there were extraneous peaks observed, linearity of drug concentrations validated the ability of Narc-2 to separate drugs from biological samples for analysis by HPLC. A blank extraction with no drug standards was run along with all sample extractions in order to subtract out the interference peaks seen on HPLC. No interference peaks in a blank extraction were seen by the GC-MS.

C. Narc-2 and Bond Elut Extractions vs. Hexane Extractions

Liquid-liquid extractions (LLE) are a direct method to extract the target analyte using only an organic solvent, providing an inexpensive and simple method for drug isolation. LLE and SPE are frequently done by laboratories to clean up and prepare a sample for analysis. These extractions are incorporated since they are inexpensive and not time consuming. These types of extractions are mainly done for identification purposes due to the usual low recovery percentages (Farina et al., 2002; Garside et al., 1997). LLE are frequently used in toxicological analysis since blood and urine are specimens looked at for drug determinations and these specimens can be easily

partitioned with an organic solvent. Solvent polarity and pH are factors are important considerations when performing LLE. Solvents like chloroform, toluene, ethyl ether, acetone, and hexane are frequently used for LLE. Narc-2 SPE columns were compared to LLE using hexane, an organic solvent, to see which extraction method was best for this study. Hexane was found to be a very simple, quick, but crude method of extraction. It showed low recovery percentages and it was decided it can be used for simple identification but not for quantification purposes. Both Narc-2 and Bond Elut SPE cartridges proved to be good for separating COC from VH and brain to produce relevant amounts. Since these cartridges showed good extraction recoveries, this study used Bond Elut for extractions. The percent recovered for Narc-2 columns (bupivacaine, 94.07%) outweighed the time consumption and expense since hexane extractions showed low recovery percentages (bupivacaine, 23.65%).

D. Narc-2 Column Variability and Time Course Variability

The variability in recovery seen by Bogusz et al (1996) between columns from the same lots prompted us to run four different extractions to test for variability. Since these columns are disposable and can only be used once for each extraction, this was an important step to make sure there was no effect introduced between columns. An assay validation was run on four separate columns of the same batch to ensure that there was no variability among the SPE cartridges used for this study. We found that these columns can be used for analysis with confidence since no significant variability will be introduced due to column effect. From the same four columns used for the column variability test, a time course was performed on the eluents to ensure there was no COC degradation post-extraction. Data analysis showed there was no effect of time post

elution on peak height of a COC standard. Lack of a time effect on COC peak height indicates the sample could be analyzed up to 120 minutes after SPE with no degradation of COC in the sample and no loss of sensitivity. Therefore, these columns could be used for extraction purposes with no effect introduced due to column and the eluent produced could be analyzed up to 2 hours following extraction with confidence of no loss of sample.

E. Chromatography

The analytical tool of choice is dependent on the question being asked and the problem being solved (Maurer, 2005a). The analytical methods used for determining unknown drugs in alternative specimens, i.e. VH, usually employ traditional techniques like GC-MS. But there are limitations due to small sample volume/mass seen in these alternative specimens or target analyte may react differently compared to traditional specimens used in toxicological analysis (Wood et al., 2005). This study compared three analytical methods for sensitivity and specificity in each tissue.

When results must be confirmed, GC-MS is the most widely used application. For high throughput screening, methods like thin layer chromatography, HPLC, and electrokinetic techniques can be used. Even so, GC-MS is still widely used for that type of screening (Maurer, 2005a). HPLC was a method of choice for comparison in the present study because of advantages over GC-MS, that include lower costs and the lack for a derivatization step prior to analysis (Phillips et al., 1996). Even though GC-MS is the most widely used confirmatory toxicological test, HPLC is beginning to be more widely used alternative because of these advantages (Fernandez et al., 2005). GC-MS and liquid chromatography-mass spectrometry (LC-MS) are sensitive methods for the

analysis of COC, but HPLC is more affordable and has been shown to successfully determine and quantitate COC and its metabolites (Jamdar et al., 2000). HPLC is also becoming more common even though GC-MS offers more sensitivity and is less susceptible to matrix interferences in comparison to HPLC (Phillips et al., 1996). LC-MS is emerging as an important tool for quantification and routine analysis (Maurer, 2005a). LC-MS has already been shown to determine COC, opiates, amphetamines, and LSD in plasma and is a good complement to GC-MS for quantification purposes (Concheiro et al., 2006; Maurer, 2005a). LC-MS demonstrates high specificity, short chromatography run times, and has the potential to reduce extensive sample preparation since it does not require derivatization. These points are making LC-MS emerge as a useful analytical tool for high-output confirmatory tests of drugs of abuse (Wood et al., 2005). Jeanville et al (2001) report that HPLC and HPLC-MS require extensive sample clean-up; while GC-MS analysis also requires same clean-up and a derivatization step. Their study employed SPE methods to minimize time spent on sample clean-up to then be used on these traditional analytical methods (Jeanville et al., 2001). This current study also employs the use of SPE for sample clean-up prior to analysis to aid in proper detection of COC and BE.

1. HPLC

HPLC was used because of the aforementioned reasons, lower cost and less sample preparation. HPLC detected COC in the VH and brain and BE in the brain. It was not able to detect BE in the VH, due to either the amount of BE in the VH fell below the HPLC level of detection (~100ng/ml) or low enzymatic activity in the VH caused incomplete metabolism of COC. The latter conclusion could have important implications

regarding use of VH. If the COC entering the VH via the blood-retina barrier does not undergo metabolism rapidly like it does in the brain or blood and is not subject to postmortem redistribution, the VH could be a compartment model for establishing a rate at which COC metabolizes. Once a rate is found, VH can then be used for indicating antemortem levels of the drug detected. Mackey-Bojack et al, (2000) reported that VH is useful for quantification of COC and its metabolites. Drug levels were substantial enough and had a strong correlation with blood to report that VH is an alternative specimen for postmortem analysis if blood is unavailable (Mackey-Bojack et al., 2000).

Benzoylecgonine detected in the brain was significantly higher than COC brain levels suggesting brain metabolism of COC. COC is able to rapidly enter the brain via passive diffusion through the BBB. It then is metabolized due to the abundant amount of enzymes in the brain. BE is unable to cross the BBB, therefore, any observed in the brain is due to the metabolism of COC that entered the brain (Kalasinsky et al., 2000). Because COC can be completely metabolized in the brain to its metabolites, it may not be as good as good a compartment model as VH when looking at a postmortem metabolism rate. However, it was able to be analyzed by HPLC following SPE with adequate levels of COC and BE present in the sample.

2. GC-FID

Gas chromatography-flame ionization detection has previously shown to analyze COC and its metabolites in VH, bile, blood, and urine (Fernandez et al., 2004; Fernandez et al., 2006). As opposed to using the more sensitive GC tool coupled with MS or another common type of analytical tool, HPLC, Fernandez et al (2006) chose to develop a

method for GC-FID. This study followed the similar procedures and conditions with only a few modifications (Fernandez et al., 2004, 2006).

The chromatographic conditions of the oven temperature, injector temperature, and carrier gas of this study were the same as Fernandez et al (2006). The total run time and internal standard used were two differences between this study and Fernandez et al study (2006). Their total run time was almost 22 minutes compared to 15 minutes for this study. This change in method was because the Fernandez et al study (2006) reported that COC and BE had retention times of less than 15 minutes. This study chose bupivacaine as the internal standard after a literature review. The internal standard used by Fernandez et al (2006), proadifen, was not come across in the brief literature search.

Even though this study was not able to determine a COC or bupivacaine peak based on GC-FID analysis, Fernandez et al (2006) determined that in VH, COC was seen at higher concentrations when compared to BE and EME. These results are similar to our findings on the HPLC and GC-MS, but no analysis or calculations could be performed on the GC-FID because of lack of resolution of peaks. Both the Fernandez et al studies (2004; 2006) were able to show a method for analyzing COC and its metabolites on a GC-FID, but this study is not able to say that COC can be detected by GC-FID. It is not clear why we were unable to properly detect COC, BE, and bupivacaine. This analytical method should be looked at again for analysis of COC and BE from VH and brain to see if the results are similar to what was observed with the HPLC and GC-MS.

3. GC-MS

Due to the fact that forensic analysis must be reliable with methods employed showing high sensitivity in order to detect small amounts of substances in matrices

analyzed, GC-MS is the most widely used analytical method to identify compounds (Maurer, 2005). Quantification of drugs and corresponding metabolites by analytical methods is a major part in interpretation of the data. The level of detection is important in interpreting the data as well. Level of detection is described as the smallest amount of drug able to be distinguished from the noise level (Bressolle et al., 1996). The level of detection of the GC-MS in our study was approximately 25ng/ml (Mark Boese, personal communication), which is four times more sensitive when compared to the level of detection of HPLC in this study. Since our GC-MS was more sensitive than the HPLC in relation to level of detection, BE was detected in the VH. Both COC VH and BE VH were significantly higher than COC brain and BE brain. COC concentrations in the VH compared to the brain may be different due to the barriers for each. Even though both the VH and brain have similar barriers that only let some molecules pass, higher COC concentrations in the VH may be due to allowance of COC to pass more freely in comparison to the brain. As previously mentioned, since BE cannot pass through the BBB and the VH barrier, and any BE seen in these matrices is resulting from COC metabolism, more BE in the VH compared to the brain may also be due to allowance of more COC into the VH (Kalasinsky et al., 2000). Stewart and Tuor (1994) reported that after using a vascular tracer in the rat eye and brain, the tracer amount found in the retina compared to the brain was almost four times in comparison in the same animal.

Structurally, the retina has a high density of junctions and endothelial vesicles, allowing for vascular permeability. The blood-retina barrier allows for more molecules to cross, making it almost as effective as the BBB (Stewart and Tuor, 1994). The BBB has a network of tight junctions formed by transmembrane proteins that deny free

diffusion across the membrane. The BBB only allows for active transport or small, lipophilic molecules to enter the brain (Alavijeh et al., 2005). Since COC is able to enter the VH, the amount of BE seen in VH may be due to spontaneous or chemical hydrolysis of COC occurring that was detected by GC-MS but not by HPLC.

Even though the results of GC-MS analysis have lower absolute values of recovered COC and BE when compared to the absolute values of HPLC, these values cannot be compared statistically since they are two separate means of analysis. Since GC-MS was four times more sensitive than HPLC, it allowed for a smaller concentration to be detected compared to background noise. GC-MS drug levels may look lower in absolute terms when comparing to HPLC, but in terms of which method is better for analyzing COC and BE from VH and brain, GC-MS gave the best detection and relevant amounts seen in the tissues based on greater sensitivity.

The derivatization step applied to GC analysis has not been looked at as a likely area for error in analysis. The possibility of incomplete derivatization could be a factor since any difficulty with this extra preparation step is also applied to the ISTD. But, when comparing to another analytical method, this may become a factor in the different recovery concentrations.

Since statistics cannot be applied when comparing the COC and BE values between GC-MS and HPLC, no statistical analysis was run between the two methods. Both the HPLC and GC-MS were able to detect COC and its major metabolite BE, and therefore lead to the calculation of relevant amounts in the VH and brain. Phillips et al (1996) compared HPLC and GC-MS for the analysis of COC in urine. They were able to say that HPLC was more precise for determining COC and GC-MS was more precise for

determining its metabolites. This could be why we see BE in the VH with the GC-MS and not with the HPLC. Observing BE in VH with GC-MS may be due to GC-MS being a more sensitive analytical tool than HPLC. They also state that HPLC can be used as a legitimate analysis method for some analytes (Phillips et al., 1996). HPLC was successful in the analysis of COC, BE, norcocaine, and EME extracted from plasma and urine based on a study by Jamdar et al (2000). This study cannot state that one analytical method is better than the other statistically, but it does agree with Phillips et al (1996) in that the HPLC method requires less pretreatment of samples before analysis since samples run on GC-MS and GC-FID must be derivatized. This study also agrees with the Jamdar et al study (2000) in that COC and BE could analyzed by HPLC methods. Even though GC-MS is the benchmark for analysis and identification of unknown substances in the forensic laboratory, this study showed that HPLC and GC-MS were able to detect and calculate adequate levels of COC and BE. Since HPLC is lower in cost than a GC-MS system, many forensic institutions might be able to afford HPLC and still be able to properly detect several drugs like COC, leading to quantification of that drug (Phillips et al., 1996).

Based on the collective results of this study, the research question was not completely answered. HPLC and GC-MS allowed for the detection of COC and BE in VH and brain tissue, but GC-FID showed unclear chromatographs which could not be used for analysis of drugs detected. GC-MS was the best analytical tool in this study based on the lower sensitivity of the instrument compared to HPLC. Both GC-MS and HPLC detected COC and BE leading to calculations of relevant amounts observed in the respective tissue. A rate could not be established since a postmortem time course study

was not done. However, this study has set the stage for future studies looking at rate by narrowing down the compartment to be looked at, which is VH.

F. Future Directions

Since we were able to observe that both HPLC and GC-MS were able to detect and calculate relevant amounts COC and BE in the VH and brain, the next step would be to look at postmortem degradation of COC in VH and brain. A time course study should be implemented by subjecting the animal model after euthanization to a set a time course study and look at the rate of degradation at set time points. It will be interesting to see how and if the ratios of COC to BE change over time postmortem especially in the VH, since it is a unique compartment in the body with low enzymatic activity. A temperature study should also be implemented. The animal model could be subjected to different temperatures along with the time points. Since it is known that the refrigeration or freezing process slows hydrolysis of COC, it would be interesting to look at the ratios of COC to BE at different time points as well as at different temperatures (Cingolani et al., 2004). All of these studies could be observed by either HPLC or GC-MS analysis.

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Candidate for the Degree of

Master of Science

Thesis: COMPARISON OF HPLC-UV, GC-FID, AND GC-MS FOR THE ANALYSIS OF COCAINE AND BENZOYLECGONINE FROM VITREOUS HUMOR AND BRAIN TISSUE

Major Field: Forensic Science

Biographical:

Personal Data: born in Augusta, KS on November 11, 1980, the daughter of Allen and Margaret Tiller.

Education: Received Bachelor of Science in Biology from Lenoir-Rhyne College, Hickory, NC in May, 2003. Completed the requirements for the Master of Science degree with a major in Forensic Science at Oklahoma State University in July 2006.

Name: Adrea Tiller Date of Degree: July, 2006

Institution: Oklahoma State University Location: Tulsa, Oklahoma

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Pages of Study: 65 Candidate for the Degree of Master of Science

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Scope and Method of Study: A rate at which cocaine metabolizes postmortem has not yet been established. Factors such as postmortem environment, metabolism, and redistribution have given rise to complications in extrapolating antemortem drug levels from postmortem findings. The purpose of this study is to look at *in vivo* cocaine metabolism in vitreous humor and brain tissue of male Sprague-Dawley rats 4-6 months of age employing the use of three analytical methods. Rats were injected with 15mg/kg cocaine (IP), euthanized, and vitreous humor and the dorsal and ventral striatal areas of the brain were collected for analysis. Solid-phase extractions on Bond Elut Certify (Varian, Palo Alto, CA) were done to prepare the samples for analysis. Samples were run on HPLC-UV, GC-FID, and GC-MS and calculations were done to determine if adequate levels of cocaine and benzoylecgonine were seen in vitreous humor and brain. This allowed for direct comparison of sensitivity and quantitative analysis from three analytical methods simultaneously.

Findings and Conclusions: HPLC detected cocaine in vitreous humor and brain and benzoylecgonine in the brain. Either benzoylecgonine levels in vitreous humor fell below the level of detection (~100ng/ml) or low enzymatic activity caused incomplete metabolism of cocaine. Incomplete metabolism of cocaine in the vitreous humor may warrant the use of vitreous humor as a compartment for establishing a rate of postmortem metabolism. Analysis on GC-FID showed several unresolved peaks and levels of cocaine and benzoylecgonine could not be determined. GC-MS detected cocaine and benzoylecgonine in the vitreous humor and brain. The level of detection for GC-MS was ~25ng/ml, four times more sensitive than HPLC. There was a significant difference, $p<0.05$, between the means of cocaine in vitreous humor and cocaine in brain. There was also a significant difference, $p<0.05$, between benzoylecgonine in vitreous humor and benzoylecgonine in brain. Since GC-MS was able to detect cocaine and benzoylecgonine in both tissues, future studies to establish a rate of cocaine metabolism postmortem should employ the use of GC-MS for analysis of cocaine metabolism in the vitreous humor.

Advisors Approval: Dr. David Wallace