Stability of Heroin Metabolites and Oxycodone in Rat Hair and Liver During Decomposition

By

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Abstract

The aim of this study was to investigate the influence of soft tissue decomposition on the stability of drugs incorporated in hair antemortem. Two burial trials were conducted: in the first trial, rats were administered oxycodone over five days; in the second trial, rats were administered heroin over nine days then drug abstinent for another nine days. After each respective treatment the rats were sacrificed and buried in controlled burial microcosms. Concentrations of oxycodone and selected metabolites or the metabolites of heroin; 6-monoacetylmorphine and morphine, incorporated within rat hair and liver were measured before and during the decomposition process. Oxycodone was analysed in hair and liver samples, while morphine and 6-monoacetylmorphine were analysed in hair samples by ultra-performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS). Oxycodone concentrations in both hair and liver decreased as the interment period and decomposition of the carcasses progressed. 6-Monoacetylmorphine was not detected in any hair samples collected from the exhumed carcasses.

Hair; Soil; Oxycodone; Heroin; Decomposition; Rat; Postmortem

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Table of Contents

List of Tables

List of Figures

List of Abbreviations

6-MAM	6-Monoacetylmorphine
amu	Atomic mass unit
ATP	Adenosine-triphosphate
BGE	Background electrolyte
CE	Capillary electrophoresis
CRO	Controlled release oxycodone
CYP	Cytochrome-P450
DCM	Dichloromethane
EI	Electron impact
ESI-MS	Electrospray ionization-mass spectrometry
GC	Gas chromatography
HPLC	High performance liquid chromatography
im	Intramuscular
LC	Liquid chromatography
LOD	Limit of detection
LOQ	Limit of quantification
M-3-G	Morphine-3-glucuronide
M-6-G	Morphine-6-glucuronide
MRM	Multiple-reaction mode
MS	Mass spectrometry
MS/MS	Tandem mass spectrometry
m/z	mass-to-charge ratio
$[M+H]^+$	Molecular species (positive) ion
PMR	Postmortem redistribution
QC	Quality control
SD	Standard deviation
SIM	Selected-ion mode
S/N	Signal-to-noise
SRM	Selected-reaction mode
SPE	Solid phase extraction
UOIT	University of Ontario Institute of Technology
UPLC	Ultra performance liquid chromatography
UV	Ultraviolet
V_d	Volume of distribution
WHC	Water holding capacity

Chapter 1
Introduction

1.0 Forensic Taphonomy

Taphonomy is the study of processes and variables which involve the decomposition, dispersal, erosion, burial and re-exposure of organisms after death (Ururahy-Rodrigues, Rafael, Wanderley, Marques, & Pujol-Luzv, 2008). Forensic taphonomy is a subfield that examines the postmortem fate of remains and how taphonomic forces have altered evidence that may be the subject of medico-legal investigations. Forensic taphonomy incorporates aspects of decomposition chemistry, which is a branch of chemistry that investigates the chemical degradation processes that occur in soft tissue as decomposition proceeds. The decomposition of an organism after death is a complex chemical process that also alters the microenvironment immediately surrounding the organism. Such alterations in the burial environment and changes to the organism have the potential to be used by forensic investigators in locating clandestine graves or in establishing the circumstances surrounding death.

1.1 Forensic Toxicology

Forensic toxicology is an interdisciplinary field, which applies toxicology, pharmacology and other disciplines including analytical chemistry to aid in medico-legal investigations. In instances where the presence or absence of alcohol, drugs or poisons is important to a forensic investigation, a toxicological examination is usually conducted, i.e. to aid in the investigation of impaired driving, sexual assault, fire death, homicide, or other crimes involving drugs.

Postmortem forensic toxicology focuses on determining if drugs or poisons may have contributed to the cause or manner of death of an individual, which may not be readily diagnosed at autopsy. Deaths may be attributed to direct drug intoxication or indirectly due to drugs where an individual under the pharmacological effects of drugs has a greater chance of committing or falling

victim to foul play (Levine, 2003). Both forensic toxicology and taphonomy can serve to determine these unknowns in an interdisciplinary approach.

1.2 Biological Specimens of Interest

The body can provide a wealth of information regarding the manner and cause of death in many instances. Collecting and analysing bodily fluids and tissues from a body for the presence or absence of drugs can be a valuable investigative aid. Traditional matrices of analyses are blood and urine, however if the decomposition of the body has progressed to a point where such samples are no longer available or have been contaminated by decomposition by-products, there are alternative specimens that can be of interest to the forensic toxicologist such as liver and hair. An important issue in forensic toxicology analyses is the potential for changes in drug concentrations to occur, which may be induced by decomposition from surrounding soft tissue and the burial microclimate

1.2.1 Liver

The liver is the largest organ within the human body aside from skin and muscular tissue (Williams, James, & Franklin, 2000). This highly perfused organ, which lies below the diaphragm in the thoracic cavity, is responsible for carbohydrate storage and metabolism, metabolism of hormones, synthesis of blood proteins, urea formation, bile formation, as well as lipid and protein metabolism. The liver is also responsible for the metabolism, bioactivation and detoxification of endogenous and exogenous analytes. Liver cells, known as hepatocytes, secrete an alkaline substance known as bile that aids in digestion by emulsifying lipids (Williams et al., 2000).

The liver is divided into two lobes; the right lobe is composed of the anterior and posterior segments and is found in the upper right abdomen; the smaller left lobe is divided into the medial and lateral segments and partially overlays the stomach. The liver receives approximately 30% of the cardiac output and is approximately five percent of the total body weight (Williams et al., 2000).

Drugs and chemicals absorbed from the gastrointestinal tract flow to the liver, where xenobiotic metabolizing enzymes are present in high concentrations; and regardless of how an analyte is absorbed into the bloodstream, it is subject to extraction and metabolism by the liver (Williams et al., 2000).

The enzymes within the liver are capable of a large variety of reactions, meant to help in the detoxification and elimination of many xenobiotic compounds. Cytochrome-P450 (CYP) enzymes are one class of drug-metabolizing related hemoproteins. They can be found in high concentrations in the endoplasmic reticulum of liver cells and at lower concentrations in the lungs, kidney, intestinal mucosa, and brain (Williams et al., 2000). CYP enzymes catalyze oxidation reactions and reduction reactions, while hydrolysis reactions are catalysed by esterases and amidases. Such reactions are commonly referred to as phase I reactions. Phase I reactions generally introduce a polar group to the parent drug to increase its water solubility, thus aiding in drug elimination.

Phase II reactions typically involve the conjugation or addition of a specific chemical compound to the parent drug and involve covalent bonding. This may include glucuronidation, sulfation, and glutathione conjugation to form a highly water soluble conjugate. An example of a phase II reaction is the glucuronidation of compounds, which involves the transfer of glucuronic acid from a uridine diphosphate glucuronic acid cofactor to a carboxyl, hydroxyl, or amine group of the analyte, which is mediated by a group of isozymes, uridine diphosphate-glucuronosyltransferases (Williams et al., 2000).

As the liver is a main site of drug metabolism, parent drugs and metabolites may be present in the liver at higher concentrations than in blood. Toxicological analyses on a body exposed to fire and/or severe trauma, or a body found to be in an advanced degree of decomposition, can be difficult to achieve due to the absence of blood and/or urine. In cases such as this, liver may be the most appropriate tissue sample available at autopsy.

1.2.2 Hair

Mammalian hair consists of three layers; the outermost layer is known as the cuticle, the middle layer is known as the cortex, and the innermost layer is the medulla (De Cássia Comis Wagner, Kiyohara, Silveira, & Joekes, 2007). The cuticle surrounding the individual hair shaft consists of 6 to 10 layers of cuticle cells and is approximately 1 µm thick. The cuticle is colourless and protects the cortex. The cortex makes up the bulk of the hair and provides colour and texture to the hair. Keratinous protein found in cortical cells is the major component of hair and is insoluble and relatively resistant to proteolytic enzymes (Dawber, 1996).

The hair root is located below the surface of the skin and is enclosed within a hair follicle, which produces an epidermal outgrowth called hair. Located at the base of the hair follicle is the vascularized dermal papilla, which supplies nutrients to the growing hair. Drugs present in the blood stream can be taken up by the papilla. The membranes of the matrix cells in the growing hair follicle are localized around the papilla and are exposed to the circulating blood, lymph, and extracellular fluids (Dawber, 1996; Pötsch, Skopp, & Moeller, 1997). Weakly acidic or basic drugs that are present in the surrounding fluids of the papilla can diffuse across cellular membranes in its free non-ionized forms. Factors such as the concentration gradient across membranes, the pH of the microenvironment of the blood and sweat around the hair shaft, and the partition coefficient of the drug will influence how much drug becomes incorporated into the hair shaft. Drugs

with greater partition coefficients will diffuse into cells at a faster rate and accumulate in larger concentrations (Pötsch et al.,1997).

Hair growth occurs in cycles of growth and dormancy referred to as the anagen, catagen, and telogen stages. During the anagen stage, the follicle develops and hair is produced over a span of four to eight years at a rate of approximately 0.22 to 0.52 mm per day (Kintz, 2004). The rate of hair growth is important when chronicling drug use history through hair analysis as drugs can become incorporated into a growing hair shaft. An accurate case history should be used to confirm results of any drug testing in hair. After the anagen stage, the hair will enter the catagen stage, a regression period where cell division stops and the follicle begins to degenerate. During the latter telogen stage, the hair shaft stops growing completely. In humans, each hair follicle has its own cycle, independent of its neighbouring hair follicles. After the telogen stage another growth cycle commences. Approximately 85% of the hair on the scalp of an adult is in the anagen phase and the remaining 15% is in catagen or telogen (Kintz, 2004; Kronstrand & Scott, 2007).

In a growing hair shaft, matrix cells at the base of the hair follicle synthesize proteins, which determine the durability and strength of the hair shaft. The majority of the protein synthesized in hair is keratin. Matrix cells may also acquire pigment as they differentiate to form hair, which determines the colour of the hair shaft. Melanocytes, the cells responsible for hair colour, are located at the base of the hair follicle and produce melanin, a coloured pigment, which is incorporated into keratinocytes of the hair shaft as it is formed. The colour of hair; black, brown, red or blonde; is a result of the type and proportions of melanin produced by melanocytes. White hair is due to the absence of melanin-producing melanocytes. Grey hair is a mixture of coloured hair with white hair and is the result of either dormant melanocytes, which no longer produce melanin or the inability of keratinocytes to incorporate melanin into the growing hair (Rothe, Pragst, Thor, & Hunger, 1997).

Although the exact mechanism in which chemicals are bound into hair is not known, drugs may be incorporated into hair by active or passive diffusion from the bloodstream to the dermal papilla, trapping drugs during keratinisation of newly formed cells; diffusion from sweat and sebaceous glands whose secretions bathe the hair shaft; or through external contamination by drugs present in the atmosphere from vapours or powders (Henderson, 1993; Rothe et al., 1997; Tsatsakis, Psillakis, & Paritsis 2000; Kronstrand & Scott, 2007).

Keratin and melanin contain polar groups, which may be utilised as binding sites for drugs and metals. Lipids in hair may also serve as binding sites for drugs; however the exact binding sites in hair have not been determined. Amino acids, including aspartic acid, glutamic acid, arginine, lysine, and histidine, contained in keratin provide free carboxyl, amide, phenolic hydroxyl, aliphatic hydroxyl, and sulfhydryl groups as potential binding sites for drugs (Breuer, 1981; Pötsch et al., 1997).

Because hair can function as a minor excretory organ and is free from enzymatic metabolism, levels of drug found in hair may be proportional to drug ingestion. The dose-concentration relationship in hair has been examined and the results have been mixed. Cone (1990) examined the drug levels in beard hair in two subjects after morphine or codeine administration and found that the concentrations appeared to be dose-related. However a larger study that examined hair collected from subjects participating in a controlled heroin-maintenance program by Kintz, Bundeli, Brenneisen, and Ludes (1998) demonstrated that using quantitative drug measurements in hair to determine the amount of drug ingested was not applicable due to the large degree of observed sample variability. Other studies have suggested using hair to monitor for determination of drug use, tolerance, and abstinence, as well as the establishment of the type of drug use, rather than trying to correlate the amount of drug ingested to drug concentrations found in hair (Valente, Cassini, Pigliapochi, & Vansetti, 1981; Tagliaro, De Battisti, Smith, & Marigo, 1998; Darke, Hall, Kaye, Ross, & Duflour, 2002; Kugelberg et al., 2007).

Drug testing in hair allows for a cumulative and retrospective reflection of long-term drug use or abuse. Hair analysis in postmortem toxicology has several advantages over other methodologies employing body fluids, including ease of sample collection, sample stability and availability, as well as an increased window of detection; ranging from weeks to months, compared to hours in blood or days for urine (Kintz, 2004). This window of detection varies between matrices due to the body's ability to metabolize and eliminate itself of drugs in blood, urine, saliva or sweat. Drugs incorporated into hair and nails are unique from other matrices including teeth and bone in that there are no metabolic or excretion mechanisms present to remove drugs once deposited (Guillot, de Mazancourt, Durigon, & Alvarex, 2007; Kronstrand & Scott, 2007). Drugs may be deposited in bone or teeth for a longer period of time than in blood and urine, however drugs may be eliminated from these samples by ion exchange or dissolution of bone during normal bone resorption (Jenkins, 2006).

Hair analysis should be used in conjunction with other matrices for toxicology testing in forensic investigations, including blood and urine (Tebbett, 1999; Kronstrand & Scott, 2007). However bodies that have been subjected to decompositional changes or scavenging may only have hair and bone available for forensic examination. Alternatively, any remaining blood and tissues may be contaminated by postmortem redistribution, decompositional by-products or scavenging (Watterson, 2006).

1.3 Postmortem Changes Associated with Biological Specimens of Interest

1.3.1 Decomposition of Soft Tissue

The decomposition process of a carcass or cadaver begins immediately after death as cells within the organism begin the process of self-digestion or autolysis. For carcasses buried in soil, the microbial biomass of the soil can

contribute to the biodegradation of soft tissue (Tibbett, Carter, Haslam, Major, & Haslam, 2004). The enteric flora and enzymes naturally present in the intestines and respiratory tract invade body tissues to breakdown soft tissue and alter protein, carbohydrate and fat constituents (Gill-King, 1997; Dent, Forbes, & Stuart, 2004).

The onset of autolysis is rapid in cells with high levels of hydrolytic enzymes, including the gastric and intestinal mucosa, while it is slower in heart and liver. The hydrolysis and transformation of lipids, proteins and carbohydrates can elevate blood pH, resulting in the formation of amines, including phenethylamine, tyramine, and tryptamine. The process of autolysis is visualized by skin slippage and blistering. Microorganisms from the intestine migrate into local tissues, through the lymphatic system and blood spreading throughout the entire body. The production of gases and liquids, such as hydrogen sulphide, carbon dioxide, methane, and ammonia, through the catabolism of tissues by enteric and soil microorganisms, results in bloating or inflation of body cavities and internal organs. It is the bloating or inflation of the body in its entirety that marks the putrefaction stage of decomposition. Gas and fluid accumulation within internal organs are capable of rupturing with sufficient force to cause postmortem injuries. The by-products of putrefaction include the release of volatile fatty acids (Janaway, Percival, & Wilson, 1997; Vass, 2001; Vass et al., 2002).

Active decay is the next stage of decomposition, which is marked by the breakdown of muscle tissue composed of proteins and amino acids. Amino acids readily form additional volatile fatty acids while adipose tissue decomposes to form phenolic compounds and glycerols (Forbes, Stuart, Dadour, & Dent, 2004). In warm and moist environments, adipocere, a solid material composed of fatty acids, can develop from adipose tissue. Evidence of adipocere formation can range from weeks to months (Vass, 2001; Vass et al., 2002).

The late stage of the decomposition process is advanced decay, which is marked by a release of decomposition fluids into the surrounding environment. Such fluids are composed of the liquefaction and disintegration products of soft tissue, which are highly concentrated sources of carbon and nutrients. With advanced decay, an intense localized pulse of nutrients is released from the body into the soil. This can result in the formation of a “cadaver decomposition island”, which is marked by blackened earth beneath the body (Carter, Yellowlees, & Tibbett, 2007).

The last stage of decomposition is the dry remains or skeletonisation stage. The remaining hair and skin become dehydrated or desiccated, resulting in mummification of the body. Complete skeletonisation of a human body buried at shallow depths can range from approximately 6 months to over a year (Rodriguez, 1997; Wilson et al., 2007a). Factors that can influence the rate of decomposition include the pH of soil and moisture. Remains subjected to wet or extreme pH burial environments can degrade at a quicker rate than if subjected to dry, neutral-pH environments. The moisture provided by the soil, air, and body itself can promote the growth of bacteria and fungi (Rodriguez, 1997; Wilson et al., 2007a).

Changes in pH of the soil and the body affect the decomposition process. Degradation of biological molecules lower intracellular pH as a result of organic acids produced through the breakdown of lipids and proteins. As oxygen availability decreases, carbohydrates ferment to form a variety of acids. Anaerobic bacteria in the soil and intestinal bowels also contribute to a decrease in pH through fermentation. Buried bodies can create an acidic environment in surrounding soils early in the decomposition process that later becomes alkaline as proteolysis occurs, which may take weeks or months (Gill-King, 1997).

1.3.2 Decomposition of Hair

Hair is synthesized in the hair follicle and is composed primarily of proteins (65 - 95%), lipids (1 - 9%), pigments including melanin (0.1 - 5%) and small amounts of trace elements, polysaccharides and water (Clarke & Rogers, 1970; Steinert &

Rogers, 1973; Harkey, 1993; Dawber, 1996; Kronstrand & Scott, 2007). The hair shaft does not undergo biogenic change post-keratinisation and is a robust non-skeletal tissue in decomposed bodies of forensic interest (Wilson, Dodson, Janaway, Pollard, & Tobin, 2007).

There are a few published studies regarding microscopic changes that occur within the hair and hair root still embedded in the decomposing scalp or body (Petraco, Fraas, Callery, & De Forest, 1988; Tafaro, 2000; Linch & Prahlow, 2001; Wilson et al., 2007). Two types of microscopic observations of the hair root include postmortem banding and brush-like proximal hair ends, also known as putrid roots (Petraco et al., 1988; Tafaro, 2000; Linch & Prahlow, 2001). When hairs originate from a body in a state of decomposition, a dark band may appear near the root of the hair to form a putrid root, which has a tapered or brush-like appearance of the proximal end (the portion of the hair closest to the root). Postmortem root banding typically occurs in the proximal area of the hair shaft where the sebaceous gland duct enters the follicle. Sebaceous gland secretions are the product of lysosomal enzymes, which may play a role in the formation of bands postmortem. Decompositional changes of hair are due in part to the burial microclimate, microbial actions and autolytic changes within the body (Petraco et al., 1988; Linch & Prahlow, 2001).

Linch and Prahlow (2001) suggested that the decomposition process of hair roots mimic the natural apoptosis process at the hair root stem, with the exception that the telogen club is not formed during decomposition. The root of hair undergoing telogen may thus be resistant to banding as the keratin, which is part of the telogen club, is resistant to decompositional changes in the surrounding tissues (Hordinsky, Sawaya, & Scher, 1999). Thus root banding is not observed in all postmortem hair samples and may reflect the growth stage of the individual hair shaft.

1.4 Fate of Drugs in Postmortem Remains

1.4.1 Postmortem Redistribution of Drugs in Soft Tissue

Drug degradation and drug formation, through the breakdown of drug conjugates formed during antemortem drug metabolism, have been observed during the postmortem interval (Stevens, 1984; Hearn, Keran , Wei, & Hime, 1991; Skopp, 2004). Because of the changing pH and degradation of cellular compartments, the effect of postmortem redistribution (PMR) and drug stability may affect the detection and quantification of drugs within the decomposing body.

PMR is the process by which the movement of fluids and degradation of cellular components of the body result in the migration of drugs to produce a change in drug concentrations after death. PMR can include the redistribution of drugs into blood from solid organs, including the lungs, liver, and myocardium (Yarema & Becker, 2005).

The death of a cell results in a variety of fates; including the cessation of aerobic respiration and adenosine-triphosphate (ATP) production; an increase in anaerobic metabolism; and the release of the cellular contents, including any drug present in the cell. Lactic acid, inorganic phosphates and sodium accumulate within the cell to decrease intracellular pH. With cellular edema, organelles are destroyed with enzymatic leakage into the cytoplasm, leading to the leakage of cellular contents into the extracellular space. The extracellular molecules then enter the remnants of the cell (Yarema & Becker, 2005).

Factors that affect PMR can be attributed to the characteristics of the drug itself or to the changes that are occurring within the body after death. The volume of distribution (V_d) of a drug, which refers to the apparent volume of bodily fluid into which a drug dose is dissolved, is small for drugs that are highly bound to plasma proteins and not to tissue components; as a result the V_d for such drugs is approximately equal to the plasma volume. Drugs that distribute into muscle and adipose tissue have a higher V_d than drugs confined to the bloodstream. Using a

rat model, Hilberg, Ripel, Slørdal, Bjørneboe, and Mørland (1999) demonstrated that drugs with a V_d greater than 3 L/kg is a good predictor that a drug will undergo PMR. The concentration for such drugs are greater in postmortem blood samples when compared to samples collected antemortem.

Lipophilic drugs and organic bases can concentrate in solid organs, including the liver, lungs, and myocardium. After death, passive diffusion occurs from these sites to the rest of the body due to a concentration gradient. The pH of a dead cell also decreases to create an acidic environment, thus a basic drug will become ionized. As a result of cell lysis, basic drugs will distribute more readily throughout the body as they are transported in the acidic fluid in which they are dissolved (Hilberg, Bugge, Mørland, & Bjørneboe, 1992).

Koren and Klein (1992) demonstrated using a rat model that there is a substantial redistribution of morphine (V_d: 2 to 5 L/kg) in blood sampled from the cardiac chamber (heart blood) of Wistar rats treated with 4 mg/kg of morphine (i.m.) within the first 24 - 96 hours after death. No substantial increases in morphine concentrations were observed after 96 hours. Because the concentration of morphine was determined by radioimmunoassay, the authors reported that endogenous substances may have skewed the reported levels of morphine as the cross-reactivity of decompositional products to morphine was not examined. However in a more recent study by Gerostamoulos and Drummer (2000), the authors demonstrated that there was no significant evidence of PMR for morphine and its metabolites in human autopsy subjects when antemortem morphine levels were compared to autopsy samples in which the postmortem interval ranged from 11 to 122 hours (mean: 59 hours).

Another consideration in the analysis of drugs in soft tissue is that as most organs are not homogeneous and PMR can lead to different drug concentrations within an organ, thus it may be difficult to determine the average concentration of a drug within any one organ without analysing the entire organ (Gerostamoulos & Drummer, 2000).

1.4.2 Postmortem Stability of Drugs in Hair

Although hair samples from archaeological burials appear inert, they are subject to biodegradation and weathering (Wilson et al., 2001). Much of the destruction, including fungal tunnelling, destruction of keratin and the breakdown of external lipids, are due to microbial activity. In contrast to the readily degradable keratinaceous structures of hair, Wilson et al. (2007) reported that melanin pigment granules are broadly resistant to microbial degradation.

In ideal storage conditions, ambient temperature and dry atmosphere, it is possible to detect drugs in hair many years after death (Kintz, 2004). The scalps of eight Chilean and Peruvian mummies dating from 2000 BC to 1500 AD tested positive for benzoylecgonine, the primary breakdown product of cocaine (Cartmell, Aufderhide, & Weems, 1991). The stability of incorporated drugs in hair has been investigated under different conditions including: after cosmetic treatment, such as bleaching and perming (Pötsch, Skopp, & Becker, 1995; Jurado, Kintz, Menéndez, & Repetto, 1997); in various environmental conditions, such as exposure in water and soil (Pötsch et al., 1995) as well as sunlight (Skopp, Pötsch, & Mauden, 2000).

Pötsch et al. (1995) subjected autopsy hair samples to a variety of treatments including bleaching, and partial exposure to water or soil for 6 months. Formerly positive drug hair samples tested negative for opioids upon exposure to water or soil for periods of six months. Pötsch and Skopp (1996) also demonstrated in controlled studies that drug concentrations in hair samples, incubated and saturated with known solutions of morphine, codeine and dihydrocodeine, decreased to 2 - 18% of their initial concentrations after bleaching and to 20 - 30% after perming.

Thus while the above listed studies have examined the effects of decomposition on the morphological structure of hair; the rate of drug incorporation into a growing hair shaft; the stability of drugs in collected hair samples of known addicts; and the stability of drugs in hair samples collected from mummified

bodies, there is a knowledge gap regarding the effects of soft tissue decomposition on the stability of drugs incorporated in hair.

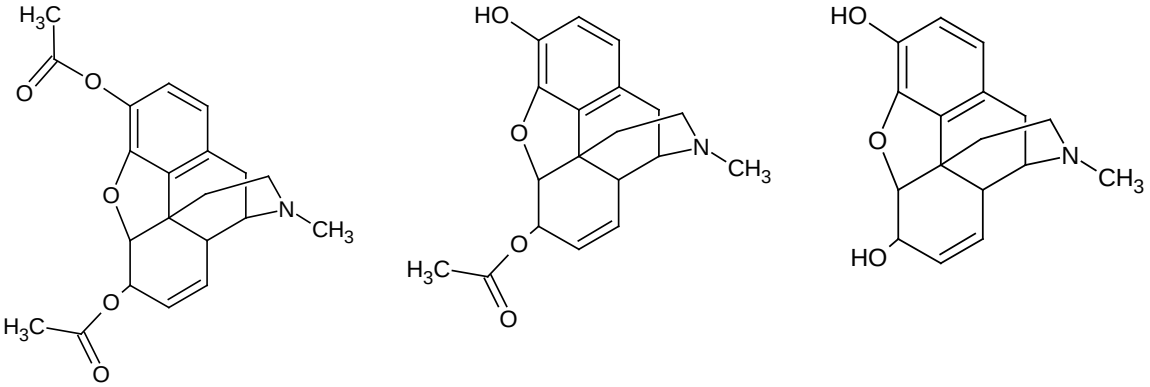
1.5 Drugs of Interest: Opioids

There are many different drugs that may be encountered in a forensic toxicological context. One common class of drugs which has been a subject of abuse are prescription and illicit opioids. Opiates is a term used to describe drugs that are derived from the opium poppy (*Papaver somniferum*), while the term opioid refers to drugs which have morphine-like pharmacological properties (Kalant, 1998). As morphine is derived from the opium poppy it is considered to be an opiate. The sap of the poppy capsules is dried to make powdered opium, which contains a mixture of alkaloids of two main types: benzylisoquinoline and phenanthrene. Phenanthrene alkaloids include morphine, codeine, and thebaine (Kalant, 1998).

1.5.1 Heroin and Morphine

Morphine is a potent analgesic commonly used for the treatment of severe pain. It is regarded as the prototypical opioid and is the standard by which all other opioids are characterized. The primary actions of morphine are mediated by binding to the μ-opioid receptor. Activation of the μ-opioid receptor is associated with analgesia, sedation, euphoria, physical dependence, and respiratory depression (Kalant, 1998). Morphine exerts its principal pharmacological effects on the central nervous system and gastrointestinal tract.

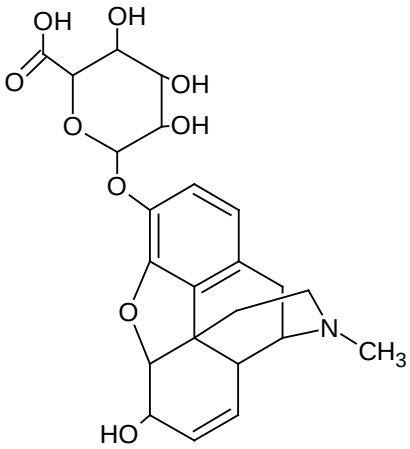
Morphine is metabolized by conjugation with glucuronic acid to morphine-3-glucuronide (M-3-G) and morphine-6-glucuronide (M-6-G) (Figure 1). M-3-G in humans is an inactive metabolite and M-6-G shows an analgesic activity greater than the parent compound (Stuart-Harris, Joel, McDonal, Currow, & Slevin, 2000). Minor metabolites of morphine metabolism include morphine-3-sulphate and normorphine.



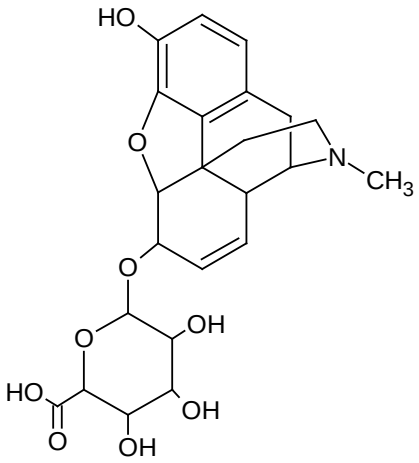
Diacetylmorphine (Heroin)

6-Monoacetylmorphine (6-MAM)

Morphine



Morphine-3-glucuronide (M-3-G)



Morphine-6-glucuronide (M-6-G)

Figure 1: Structures of heroin (diacetylmorphine) and selected metabolites.

Modifications to the morphine structure can result in semi-synthetic opiate derivatives. The most important parts on the morphine molecule are the phenolic hydroxyl at position 3, the alcoholic hydroxyl at position 6, and at the nitrogen atom (Figure 1). Diacetylmorphine, also known as diamorphine or heroin, is made from morphine by esterification with acetic anhydride of the hydroxyl groups at the 3- and 6- positions to produce a diacetyl ester (Girod & Staub, 2001; Phillips & Allen, 2006).

Heroin has a half-life of approximately 2 to 6 minutes and is rapidly deacetylated by blood esterases to 6-monoacetylmorphine (6-MAM). 6-MAM is hydrolyzed by liver enzymes to form morphine and further conjugated to its glucuronide metabolites (Baselt, 2008). Illicit heroin may contain acetylcodeine, which is metabolized to codeine and codeine glucuronide; hence morphine, its glucuronides, and codeine are the main metabolites found in urine after heroin administration (Phillips & Allen, 2006). Heroin metabolism to morphine and metabolites may be complete within a few hours (Baselt, 2008). Deacetylation of 6-MAM to morphine can be inhibited in blood samples with the addition of one percent sodium fluoride (Dolinak, 2005).

To differentiate heroin use from morphine use, the presence of 6-MAM should be identified (Figure 2). This metabolite may be detected in blood, saliva, sweat, and hair after consumption of heroin. Because of the short half-life (6 to 25 minutes (Phillips & Allen, 2006)) of 6-MAM in blood, it may not be found. Heroin itself is rarely found in conventional biological matrices such as urine, blood, or saliva, but may be detected in hair (Pichini, Altierii, Pellegrini, Zuccaro, & Pacifici, 1999).

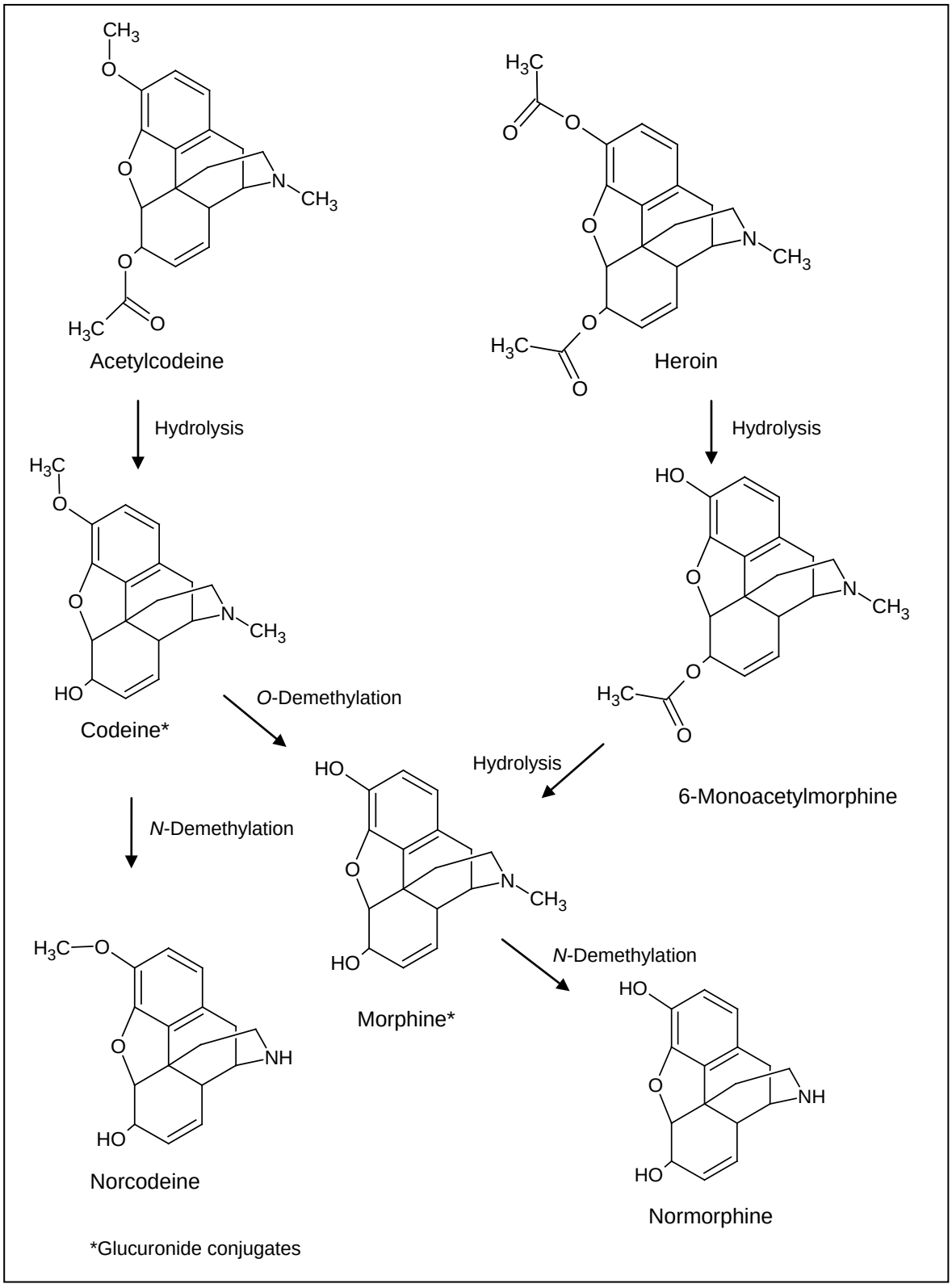


Figure 2: Metabolic pathway of codeine, heroin, and morphine.

The presence of 6-MAM and morphine in hair can be used to access the drug history of a subject. The issue of tolerance and the short half life of heroin can lead to difficulties in determining the manner and cause of death even if morphine and 6-MAM are detected in postmortem samples. Hair analysis can be used to determine if the descendent was drug-abstinent for a prolonged period of time, an active addict, or a naïve user.

1.5.2 Oxycodone

Oxycodone is a semi-synthetic opioid that is structurally related to codeine and morphine, and is derived from thebaine. Oxycodone is an opioid receptor agonist, which acts upon the kappa receptor and to a lesser degree, the mu and delta receptors (Ordóñez Gallego, González Barón, & Espinosa Arranz, 2007; Chan, Edwards, Wyse, & Smith, 2008). Its pharmacological effects are similar to other opioid analgesics; pain relief, sedation, nausea, vomiting and respiratory depression (Chan et al., 2008). Oxycodone has an oral bioequivalence to morphine in a ratio of 1:2 (Curtis et al., 1999; Ordóñez Gallego et al., 2007).

Oxycodone is a low molecular weight (316 g/mol) opioid with greater lipophilicity and oral bioavailability than morphine (Ordóñez Gallego et al., 2007). With chronic opiate exposure, an excitatory effect emerges which can contribute to analgesic tolerance and opioid-induced hyperalgesia (Wang, Friedman, Olmstead, & Burns, 2005). Oxycodone is metabolized to form noroxycodone by *N*-demethylation and oxymorphone by 3-*O*-demethylation (Lalovic et al., 2006; Chan et al., 2008) (Figure 3).

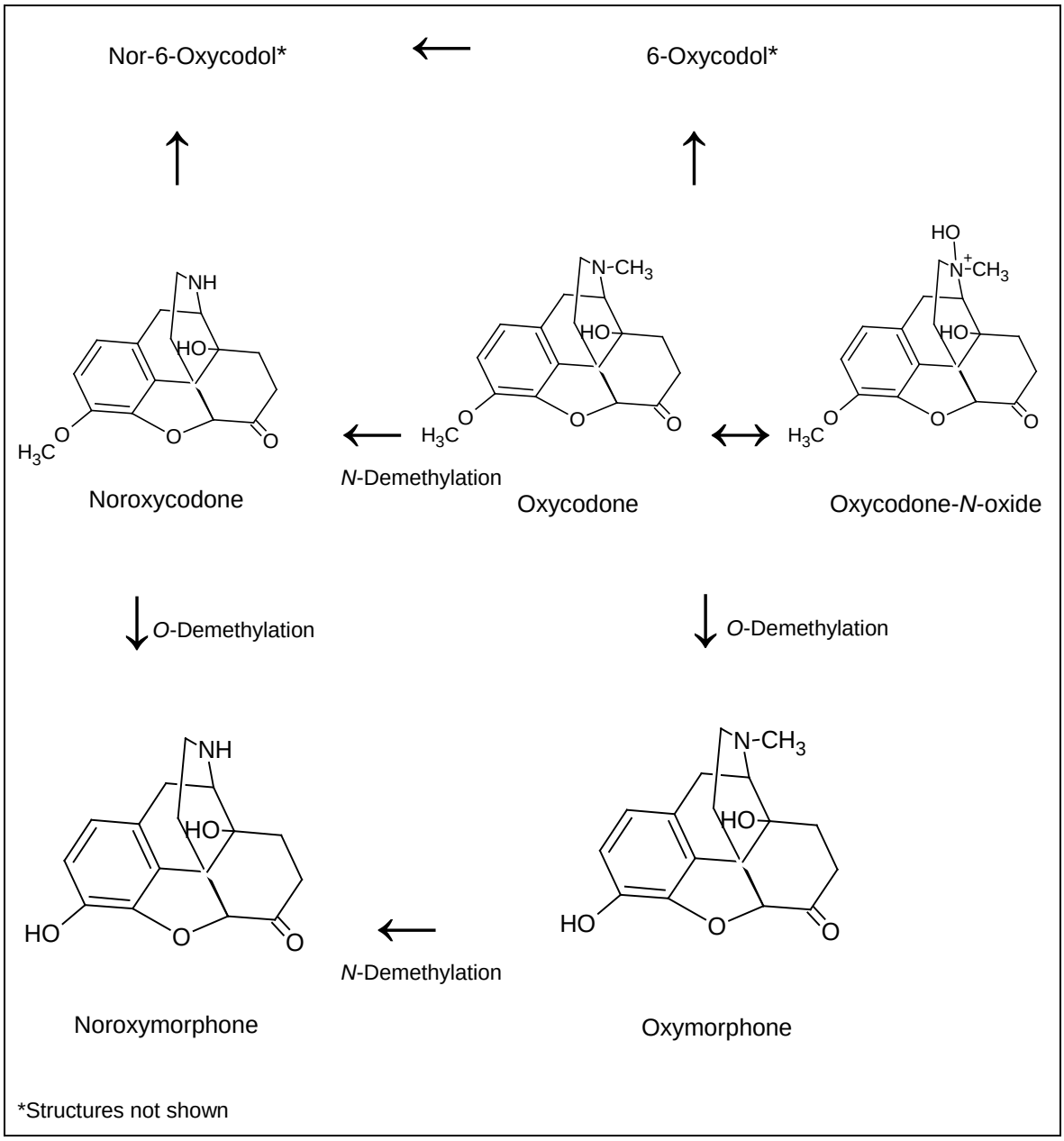


Figure 3: Metabolic pathway and structures of oxycodone and selected metabolites. Adapted from Baldacci et al. (2004)

Oxycodone is the active ingredient in OxyContin®, from Purdue Pharma, which contains a mechanism to release the drug slowly over a 12 hour period. OxyContin® was originally developed to provide analgesia in a similar manner to normal-release oxycodone but with improved compliance as it is administered once daily, compared to two to three times daily. The controlled release oxycodone (CRO) formulation requires each individual tablet to have a greater

quantity of oxycodone as the dosing regimen is reduced to once daily. However chewing or crushing the tablets is a method of bypassing the extended release mechanism, creating one larger dose of oxycodone that can increase the addiction and/or overdose potential (Cone et al., 2003; Davis et al., 2003; Tschakovsky, 2008). CRO is available in doses ranging from 10 to 80 mg. A 160-mg dose was withdrawn from the market in 2001. The former formulations are comparable to multiple doses of 5-mg tablets of immediate release oxycodone, Roxicodone® and Percocet®, a combined formulation of acetaminophen and oxycodone (Cicero, Inciardi, & Muñoz, 2005).

1.6 Analytical Instrumentation

High performance liquid chromatography (HPLC) and ultra performance liquid chromatography (UPLC) are analytical techniques used to identify and characterise compounds. Liquid chromatography (LC) separates mixtures of substances using packed columns, containing microspheres coated with the stationary phase, and the liquid mobile phase is pumped through the column with a high-pressure pump. In reverse-phase liquid chromatography, a polar liquid flows through a column packed with a nonpolar stationary phase and substances are separated based upon their differential interactions with the stationary and mobile phases.

Capillary electrophoresis (CE) is a separation technique, which combines aspects of gel electrophoresis and HPLC. Separation of analytes in CE is dependent upon differential migration in an electrical field, however unlike gel electrophoresis, separation is performed in free solution and not in cast gel. Similar to HPLC, as separation progresses, the resolved zones produce an electronic signal as they migrate past a detector, eliminating the need for staining.

Both CE and LC can be coupled to a variety of detectors, including spectrophotometric detectors, i.e. diode-array which can identify and quantify analytes based upon their characteristic UV spectra, as well as electrochemical detectors which identify and quantify analytes based upon their redox potential. However the utility of such detectors are limited by their sensitivity and specificity.

Mass spectrometry (MS) is an analytical tool capable of determining the chemical structure of molecules. The principle behind MS consists of ionizing chemical compounds to generate charged molecules or molecular fragments and measuring their mass-to-charge ratios (*m/z*). This can be accomplished by a variety of methods, including impacting the compound after it enters a vacuum with a beam of electrons, resulting in the formation of charged particles. The *m/z* is calculated from the motion of the ions as they travel through electromagnetic fields. An MS instrument consists of three modules: an ion source, which ionizes the compounds; a mass analyzer, which sorts the ions by their masses; and a detector, which measures the abundance and variety of each ion present.

Traditionally MS has been coupled to gas chromatograph to form gas chromatograph-mass spectrometers (GC-MS). After a sample has been vaporized and separation has been achieved through a gas chromatograph, the separated compounds in their gaseous form enter into the ion source, a metallic filament to which voltage is applied in an ionisation technique known as electron impact (EI). The filament emits electrons to ionize the compounds, which may fragment into a predictable pattern. Intact and fragment ions pass through to the mass analyzer to the detector.

Electrospray ionization-mass spectrometry (ESI-MS) is a technique used to produce intact ions from large analytes in solution (Fenn, Mann, Meng, Wong, & Whitehouse, 1989). The development of ESI-MS has resulted in the ability to pair MS detectors with liquid-phase chromatographic techniques, including LC and CE. A solution containing the analyte to be studied is dissolved in a volatile solvent. The analyte exists as an ion in solution and after it has been separated

by either LC or CE, it can undergo ionization and fragmentation within the MS to determine its identity and abundance.

When utilizing ESI-MS, liquid is nebulized to form highly charged droplets, which are desolvated as they pass through the atmospheric-pressure region of the source towards a counter electrode with a flow of pure nitrogen in the counter-current direction being continually passed through the spraying region. The evaporating droplets quickly decrease in size until ions of the appropriate analyte are produced and are directed by an electrical field from the needle towards the source sampling cone and through two differentially pumped regions into the source of the mass spectrometer (Pichini et al., 1999). The specificity and sensitivity of LC-MS makes it well suited for the analysis of hair as one pitfall of using hair as a matrix to determine the levels of drugs in the human body are the low concentrations incorporated into hair (ng/mg) (Srogi, 2006).

Screening of heroin or oxycodone in hair and tissue extracts may be done by -immunoassay, however to quantify and identify drugs in hair, chromatographic techniques such as CE-MS and/or LC-MS are preferred due to the separation ability, detection sensitivity, and specificity of the instrumental system. Quantification by GC-MS is also possible; however this often requires sample hydrolysis and derivatization prior to the extraction of conjugated metabolites, as not all analytes are thermally stable and separate on the GC column.

The interface chosen for method development is ESI as the metabolites of heroin and oxycodone are relatively polar, of high molecular weight, and may be thermally labile. Each standard should be introduced into the LC-MS individually to determine retention times, ions to monitor, and if the analytes are suitable for analyses for the specific instrumental setup. Ideally at least two unique ions for each analyte should be chosen to monitor for definitive identification of each analyte (Commission of the European Communities, 2002). It is preferable to monitor for such ions in the selected-ion-mode or multiple-reaction-mode, rather than in the scan mode when there may be large background noise.

Selected-ion mode (SIM) or selected-reaction mode (SRM) is used when a small number of ions specific to the compounds of interest need to be monitored. Multiple-reaction mode (MRM) can be utilised with a tandem MS or MS/MS and involves fragmenting the sample inside the instrument and analyzing the products generated.

ESI is a soft ionization technique, which means the resulting mass spectrum of a sample may consist of a single ion ($[M+H]^+$, the molecular species ion in positive ion mode). If the retention times for the individual analytes are very similar, the mobile phase composition and flow rates may require adjustment.

1.7 Objectives

Concentrations of heroin metabolites and oxycodone in rat hair and liver were examined to determine if the decomposition process of the carcass would influence their stability. A microenvironment or burial microcosm was developed to investigate the decomposition process of rats. As the buried rats carcasses progressed through various stages of decomposition, the carcasses were sequentially harvested and the concentrations of oxycodone and heroin metabolites were determined in hair and liver. Validated extraction methods were applied to isolate the drugs in hair and liver, including solid-phase and liquid-liquid extraction techniques. The concentrations of heroin metabolites and oxycodone in the samples were determined by UPLC-MS/MS. These concentrations were compared to determine if the decomposition of the carcasses affected the stability of heroin metabolites and oxycodone.

Chapter 2
Materials and Methods

2.0 Introduction

Animal subjects were obtained and prepared by the University of Guelph, Department of Psychology Laboratory of Dr. Francesco Leri (Guelph, ON). Rat cadavers treated chronically with heroin or oxycodone prior to sacrifice were buried in controlled laboratory soil environments. The carcasses were then sequentially harvested to determine the effects of soft tissue decomposition on the stability of oxycodone and its metabolites, and heroin metabolites incorporated in hair. The levels of oxycodone and its metabolites, and heroin metabolites extracted from hair samples before and during the decomposition process were determined. Soft tissue, including liver and heart muscle, was also collected throughout the decomposition process and examined.

2.1 Animal Experiments

Sprague-Dawley rats (350 - 540 grams, Charles River, QC) were implanted with intravenous silastic catheters (Dow Corning, Midland, MI) in the right jugular vein and administered intravenous oxycodone for a period of five days of 100/infusions/day of 1 mg/kg/infusion before sacrifice by decapitation in the first trial. Negative controls consisted of drug-free hair collected from Sprague-Dawley rats. Rats utilised in the second trial were administered heroin over a period of nine days at a dosage of 0.5 mg/kg/infusion; each rat received an average of 30 infusions per day (15 mg/kg of heroin per day). Rats were sacrificed nine days after the last day of heroin administration by cervical dislocation. Negative controls consisted of a separate group of six rats that were not administered any drugs.

All rats were stored at -20°C until the start of the decomposition trials. Twenty-four hours prior to the commencement of trials, the rats were stored at 4°C. The carcasses were then brought to room temperature for a period of three hours prior to burial.

2.2 Chemicals and Reagents

Methanolic solutions of 1.0 mg/mL or 100 µg/mL oxycodone, noroxycodone hydrochloride, oxymorphone, oxycodone-D_6, noroxycodone-D_3, and oxymorphone-D_3, morphine, and morphine-D_3 were obtained from Cerilliant (Austin, TX). Morphine-3-β-D-glucuronide (1.0 mg/mL solution in methanol with 0.05% NaOH), morphine-6-β-D-glucuronide-D_3 (100 µg/mL in methanol: water (1:1)), 6-monoacetylmorphine (1.0 mg/mL in acetonitrile), and 6-monoacetylmorphine-D_3 (1.0 mg/mL in acetonitrile) were also sourced from Cerilliant. All reagents were stored at -18ºC, and used after dilution to the required concentrations.

All solvents were HPLC grade; methanol, dichloromethane, hexane, isopropanol (all from Fisher Scientific, Pittsburgh, PA), acetonitrile (JT Baker, Phillipsburg, NJ), ethyl acetate, diethyl ether, and chloroform (Caledon Laboratories, Georgetown, ON). All other reagents were reagent grade unless otherwise specified. Ammonium acetate and formic acid were obtained from Sigma-Aldrich (St. Louis, MO); ammonium hydroxide, potassium phosphate monobasic, sodium chloride, sodium hydroxide, hydrochloric acid and sulphuric acid were ACS grade and were purchased from Fisher Scientific (Pittsburgh, PA).

2.3 Blank Specimens

Calibrator and quality control (QC) samples were prepared using hair and liver obtained from rats without administered oxycodone or heroin. Collected hair roots were submerged for 30 seconds in approximately 2mL of dichloromethane with agitation to remove any external contaminants. The decontaminated samples were finely cut (approximately 0.5 to 1.0 mm length plucked from the back of the drug free rats) with a sterile razor blade. Processed samples were then allowed to air-dry overnight before transferring weighed samples to disposable borosilicate culture tubes (Fisher Scientific, Pittsburgh, PA).

Liver samples were collected in 60 mL polypropylene screw top containers and stored at -20ºC until analysis. The mass of each liver sample was measured upon collection.

2.4 Burial Microcosms

2.4.1 Trial 1: Oxycodone

Thirty burial taphonomic microcosms consisting of approximately 8-9 kg of loam topsoil were set up in the laboratory. Each microcosm consisted of a twenty-litre capacity high-density polyethylene container with a gasket-fitted lid. The containers were filled to a depth of 10 cm with non-sterile sieved loam soil collected from the Decomposition Facility at UOIT and maintained at a total equivalent water level of 50 to 60%, and an initial pH level of 6.8 as determined with the Kelway Soil Acidity and Moisture Tester (Model HB-2, Kel Instruments Co., Inc, Wyckoff, NJ). The non-sterile loamy soil was loose, granular, alfisol/luvisol with a loam texture and a colour of 2.5Y 3/2 (The Globe Soil Color Book, 2nd Ed, 2005).

Oxycodone-administered Sprague-Dawley rats were placed in individual microcosms with additional soil placed on top of each rat to create a shallow grave to a depth of approximately 5 cm. Oxycodone-free rats were not available for this portion of the study to act as a negative control. Instead control microcosms contained drug-free rat hair contained within mosquito netting to allow the microbial organisms within the soil to directly interact with the hair.

The headspace of each microcosm was maintained at 50% of the entire volume and replenished every 24 to 48 hours by removing the lid for five minute-periods to ensure adequate gas exchange for optimal microbial activity.

2.4.2 Trial 2: Heroin

Twenty-two burial taphonomic microcosms as set up in Trial 1 were utilised with an observed initial soil pH level of 6.2. Heroin administered Sprague-Dawley rats were placed in individual microcosms with additional soil placed on top of the rat to create a shallow grave of approximately 5 cm deep. Six control microcosms contained one rat each, which did not receive the heroin treatment.

The headspace of each microcosm was maintained at 50% of the entire volume and replenished every 48 hours by removing the lid for five minute-periods to ensure adequate gas exchange for microbial activity.

2.5 Sequential Harvest

For Trial 1 oxycodone-treated rats (n=3) in the burial microcosm were sequentially harvested at days 3, 6, 9, 12, 15, 18, 21, 24, 27 and 30. For Trial 2, heroin treated rats (n=3) in the burial microcosm were sequentially harvested at days 7, 14, 28, 42, and 54, and a control rat was also exhumed with each harvest. For each trial, the burial surface was photographed prior to exhumation of carcasses to note the presence of any fungal growth. The carcass was also photographed *in situ* and visual observations were made with regards to the degree of inflation, marbling and/or expulsion of bodily fluids through any orifices of the carcasses.

2.6 Sample Collection

Approximately 20 mg of hair roots were collected, decontaminated in a dichloromethane rinse solution, dried at room temperature, and finely cut from each subject and stored in a sealed borosilicate culture tube at room temperature until analysis. Hair samples were collected prior to burial and again upon

exhumation. The liver from each subject was also collected and mass recorded upon exhumation and stored at -20°C until analysis. In trial 2, the heart was also collected with the intent of comparing the different concentrations of heroin metabolites in different organs (liver compared to heart), however it was later recognized that its use was limited based upon the dosing regimen. Instead the pH of the heart and liver were examined to determine if there were site dependent changes related to the decomposition process.

The hair in a small area of the back of each rat was trimmed to 3-5 mm postmortem and approximately 20 mg of hair and root were collected at Day Ø. The drug content of the hair samples from each subject was then compared between its initial concentrations at Day Ø and the concentration at Harvest.

Soil pH and moisture levels were measured with a Kelway Soil Acidity and Moisture Tester (Model HB-2, Kel Instruments Co., Inc, Wyckoff, NJ), which was extended 8 cm into the soil base. Measurements were taken on Day Ø and the day of harvest for each individual microcosm. Soil was also collected from beneath the carcasses, the decomposition island, and stored at -20°C until further analysis.

2.7 Instrumentation

2.7.1 Capillary Electrophoresis-Mass Spectrometry (CE-MS)

CE-MS analysis for oxycodone and its metabolites was performed on an Agilent 1200 LC/MSD instrument (Agilent, Mississauga, ON) equipped with an electrospray ionization interface run in the positive mode (4.0 kV). An Agilent CE equipped with a 50 µm i.d. fused-silica capillary (Part no. G1600-67311, Agilent Technologies, DE, Germany) of 125cm length was connected to the electrospray ionization interface of the MS. Sheath gas consisted of purified nitrogen (99.5%), supplied by a Mistral Nitrogen Generator (Neuheim, Switzerland). The sheath

liquid consisted of a mixture of water: methanol (50:50 v/v) with 1% formic acid to support the formation of positively charged ions. The sheath liquid was infused into the interface at a flow rate of 4.0 µL /min. The sample was electrokinetically injected by applying a positive voltage for 10 kV for 20 s.

An adapted method from Tagliaro, Manetto, Crivellente, Scarcella, & Marigo (1998) was used to separate and identify oxycodone, oxymorphone, and noroxycodone. The background electrolyte (BGE) of 25 mM ammonium acetate, pH 9 adjusted with 6M NH_4OH was prepared daily. Prior to analysis, the capillary was conditioned by flushing with 0.1N NaOH, distilled water, then the BGE for 5 minute intervals. The applied voltage during separation was 27 kV. Full scan mass spectra were collected in the mass range *m/z* 100 to 700 amu. Fragmentation voltage was adjusted between 65 to 120 V with a gain of 3 to determine unique major ions *m/z* for each analyte.

2.7.2 Ultra Performance Liquid Chromatography-Tandem Mass Spectrometry (UPLC-MS/MS)

The UPLC-MS/MS analysis was performed on a Waters Acquity UPLC system consisting of a Waters Acquity Sample Organizer Model UPC, Binary Solvent Manager, Micromass Quattro Ultima MS/MS TM API QAA 890 (Micromass Manchester, UK) instrument equipped with an electrospray interface (Turbo Ion Spray) with a Zorbax Eclipse XDB-C18 (150 mm x 4.6 mm, 5 µm; Agilent Technologies, Mississauga ON) with no guard column.

2.7.2.1 Trial 1: Oxycodone

The organic solvent was run through line A while the aqueous solvent was run through line B. Mobile phase A consisted of acetonitrile with 0.1% formic acid and mobile phase B was MilliQ water with 0.1% formic acid. The system was run in a linear gradient with an initial mobile phase composition of 10% mobile phase A (see Table 1 for gradient program). The total flow rate was maintained at 0.9

mL/min for a total run time of 10 minutes, the flow to the MS/MS was split 1:10 with a post-column flow splitter.

Table 1: Gradient elution program for oxycodone and selected metabolites on the UPLC-MS/MS

Time (minutes)	**% Mobile Phase A Acetonitrile with 0.1% formic acid**	**% Mobile Phase B MilliQ water with 0.1% formic acid**
0.00 – 1.00	10	90
1.00 – 2.00	20	80
2.00 – 8.00	70	30
8.00 – 9.00	100	0
9.00 – 10.00	10	90

For detection and identification, multiple reaction monitoring measurements were made in positive ion mode at 250 desolvation temperature, 100˚C source temperature, 50 L/hr cone gas flow, 500 L/hr drying gas flow and collision cell pressure at $2.0e^{-3}$ bar Argon. The capillary voltage was maintained at 3.5 kV and the cone voltage at 35 V. The collision energy varied between 19 to 29 eV. The dwell time for all channels was set to 0.1s. The MS/MS parameters were optimized by infusing each analyte in methanol with 0.1% formic acid at a rate of 10 µL/min, via a Hamilton 500 µL gastight #1750 syringe pump. The most abundant ion transition was utilised for each analyte.

Table 2: MRM transition ions to identify oxycodone and selected metabolites and respective internal standards

Substance	Transition Mode Ion (amu)	Internal Standard
Oxycodone	316.00→241.11	Oxycodone-D_6
Oxymorphone	302.05→227.10	Oxycodone-D_6
Noroxycodone	302.05→227.10	Oxycodone-D_6
Oxycodone-D_6	322.11→247.20	
Oxymorphone-D_3*	305.00→231.10	
Noroxycodone-D_3*	305.00→231.10	

**Oxymorphone-D_3 and noroxycodone-D_3 were initially part of the internal standard solution; however inconsistent recovery later resulted in their exclusion from the study.*

2.7.2.2 Trial 2: Heroin

Mobile phase A consisted of methanol with 0.1% formic acid and mobile phase B was MilliQ water with 0.1% formic acid. The flow rate and gradient program was optimized to minimize co-elution of morphine-3-glucuronide and morphine-6-glucuronide, which have identical molecular masses and fragmentation patterns. The system used a linear gradient program (Table 3) and the flow was maintained at 0.7 mL/min and split 1:10 to the MS.

Table 3: Gradient elution program for heroin metabolites on the UPLC-MS/MS

Time (minutes)	% Mobile Phase A Methanol with 0.1% formic acid	% Mobile Phase B MilliQ water with 0.1% formic acid
0.00 – 2.00	20	80
2.00 – 3.00	50	50
3.00 – 3.80	80	20
3.80 – 10.00	100	5
10.00	25	75

For detection and identification, multiple-reaction-monitoring (MRM) mode was used in positive ion mode. The measurements were made at 350˚C desolvation temperature, 150˚C source temperature, 50 L/hr cone gas flow, 500 L/hr drying gas flow and collision cell pressure at $2.0e^{3}$ bar Argon. The capillary voltage was maintained at 3.5 kV and the cone voltage at 35 V. The collision energy varied between 25 to 58 eV. The dwell time for all channels was set to 0.1 s.

The MS/MS parameters were optimized by direct infusion of standard solutions of each analyte into the MS via a Hamilton 500 µL gastight #1750 syringe pump. The MRM of the molecular ions of each analyte and the two most predominant fragments were utilised (Table 3). The most abundant ion will be used as the quantifying ion, which is the ion transition that will be used to calculate the calibration curve and determine the concentration of morphine and 6-MAM. The second ion transition will be the qualifier ion, which is used to confirm that the correct target compound has been correctly identified.

Table 4: MRM transition ions to identify heroin metabolites and respective internal standards

Substance	Transition Mode 1 Quantifying Ion (amu)	Transition Mode 2 Qualifying Ion (amu)	Internal Standard
Morphine	285.92→152.00	285.92→165.00	Morphine-D_3
6-Monoacetylmorphine	327.87→165.00	327.87→211.00	6-Monoacetylmorphine-D_3
Morphine-3-glucuronide	461.88→199.00	461.88→286	Morphine-6-glucuronide-D_3
Morphine-D_3	288.96→152.00	288.96→201.00	
6- Monoacetylmorphine - D_3	330.97→165.00	330.97→211.00	
Morphine-6-glucuronide-D_3	464.88→201.00	464.88→289.00	

2.8 Preparation of Calibrators and QC Samples in Hair

Drug free calibrator and quality control (QC) hair samples were prepared using hair obtained from rats without oxycodone or heroin.

2.8.1 Trial 1: Oxycodone

A methanolic mixed stock solution containing oxycodone, noroxycodone, and oxymorphone was prepared. To twenty milligrams of finely cut hair roots from the negative control rats in 10-mL borosilicate glass tubes, twenty-five microlitres of working methanolic mixed calibrator standard solutions (0.625 to 5.00 mg/L) were added to result in concentrations of 0.78 to 6.8 ng/mg of hair. To each

sample, twenty-five microlitres of internal standard (3.3 mg/L deuterated oxycodone-D_6 in methanol) was also added.

Separate ampoules of drug solutions were used to prepare QC samples solutions. Twenty-five microlitres of mixed QC solutions containing oxycodone, oxymorphone and noroxycodone at concentrations of 0.8, 1.6 and 3.2 mg/L in methanol were added with twenty-five microlitres of internal standard to twenty milligrams of prepared blank hair to yield QC samples containing a mixture of all three analytes with concentrations of 1, 2 and 4 ng/mg in hair.

2.8.2 Trial 2: Heroin

Twenty-five microlitres of acetonitrile working solutions of 6-MAM and morphine (0.25 to 4.0 mg/L; 0.0625 to 1.0 mg/L, respectively) were added to twenty milligrams of finely cut hair roots to yield concentrations of 0.31 to 5.0 ng/mg and 0.08 to 1.25 ng/mg of hair respectively. Twenty-five microlitres of internal standard (2.5 mg/L deuterated internal standard, 6-MAM-D_3 and morphine-D_3 in acetonitrile) were also added to each sample.

Twenty-five microlitres of mixed QC solutions prepared at concentrations of 0.16, 0.32 and 0.64 mg/L of morphine and 0.64, 1.28 and 2.56 mg/L of 6-monoacetylmorphine were added with twenty-five microlitres of internal standard to twenty milligrams of prepared blank hair samples to yield a set of three QC samples with concentrations of 0.2, 0.4 and 0.8 ng/mg morphine and 0.8, 1.6 and 3.2 ng/mg 6-monoacetylmorphine in hair, respectively. A fourth QC sample was prepared at a concentration of 2.5 ng/mg 6-MAM in hair with no morphine present to determine the stability of 6-MAM during the extraction process.

2.9 Preparation of Hair Sample

Collected hair roots from exhumed rats in both trials were treated with the same method as described for blank hair samples. Twenty-five microlitres of internal standard (either 3.3 mg/L of oxycodone-D_6 in methanol or 2.5 mg/L 6-MAM-D_3 and morphine-D_3 in acetonitrile) was added to each sample. All hair samples were mixed with a vortex mixer while the solvent evaporated at room temperature.

Hair samples were digested with the addition of 0.5 mL of methanol: water: formic acid (25:74:1; v/v/v), vortexing for 1 minute and sonication in a warm water bath for 24 hours at 37°C. After sonication samples were centrifuged at 2600 x g for 10 min (Model 225A, Fisher Scientific, Pittsburgh, PA) and three hundred microlitres of the extract was filtered through 0.45 µm 4 mm Teflon syringe filters (National Scientific, Rockwood, TN) and analysed by UPLC-MS/MS.

Calibration standards and QC samples were analysed with each set of samples, with an internal standard curve constructed using linear regression. The limit of detection (LOD) was defined as a signal to noise (S/N) ratio of 3:1, while the lower limit of quantification (LOQ) was defined by the lowest calibrating standard. The precision and accuracy of calibrating standards and QC samples was within 20% of the target value.

2.10 Preparation of Liver Samples

Two grams of liver was homogenized in 3.0 mL of normal saline (0.9 g/100mL NaCl in deionised water) and centrifuged for 30 min at 3600 x g. The supernatant of each sample was analysed for the presence of either oxycodone and selected metabolites or heroin metabolites by UPLC-MS/MS after sample extraction.

Supernatants of the drug-free livers were utilised in the method development and quantification process as blank matrices for calibrators and both negative and positive controls.

2.11 Liver Extraction Development

2.11.1 Trial 1: Oxycodone

Various sample preparation techniques were investigated after a review of the available literature, including liquid-liquid extraction, and protein precipitation.

Method I: Liquid-liquid extraction

Method I was adapted from Koves and Wells (1985) for basic drugs in postmortem blood. Drug-free or blank supernatant samples of 1 mL were spiked with 25 µL of 10 mg/L solution of oxycodone, oxymorphone, and noroxycodone and 25 µL of internal standard solution (3.3 mg/L oxycodone-D_6) and vortexed. Five millilitres of hexane and 200 µL of 6 M NH_4OH were added to each sample, vortexed for 30 sec, shaken for 10 min, and centrifuged for 20 min at 3100 x g. The supernatant was discarded and 2 mL of 1 M H_2SO_4 was added to the hexane layer. Samples were then vortexed, centrifuged, and the hexane layer was discarded. One drop of bromothymol blue indicator solution was added to the remaining acidic aqueous layer which was made alkaline with 5 M NaOH (drop-wise addition until basic) and 1.5 mL of hexane was added. After vortexing and centrifuging for 5 min at 3100 x g, the hexane layer was transferred and dried down at room temperature under nitrogen. Samples were reconstituted in 300 µL of mobile phase and analysed using UPLC-MS/MS.

Method II: Liquid-liquid extraction

Method II was adapted from Cheremina et al. (2005) for oxycodone and noroxycodone in human plasma. Spiked 1 mL supernatant samples, prepared as

described above, were vortex mixed with 1.0 mL of 1M NaOH and 4.0 mL of diethyl ether. Samples were capped, shaken for 10 min, and then centrifuged at 3100 x g for 20 min. The organic layer was transferred and evaporated under nitrogen at room temperature. Samples were reconstituted in 300 µL of mobile phase and analysed using UPLC-MS/MS.

Method III: Liquid-liquid extraction

Method III was adapted from Adams, Pieniaszek, Gammaitoni, and Ahdieh (2005) for oxymorphone in human plasma. Spiked 1 mL supernatant samples, as described above, were vortex mixed with 1 mL of 0.1 M NaOH and 4.0 mL dichloromethane (DCM). Samples were then capped, shaken for 10 min and centrifuged at 3100 x g for 20 min. The organic layer was transferred and 2 mL of 0.5 M sulfuric acid was added. After mixing and centrifugation, the organic layer was discarded and 400 µL of 6 M NH_4OH and 4 mL of DCM were added to the aqueous layer. Samples were then vortexed and centrifuged again and the organic layer was collected and evaporated under nitrogen at room temperature before reconstituted in 300 µL of mobile phase.

Method IV: Protein precipitation

Method IV was adapted from Edwards and Smith (2005) for oxycodone in rat serum. Spiked 1 mL supernatant samples were vortexed while 50 µL of 6 M NH_4OH and 1 mL of acetonitrile were added. Diethyl ether (4 mL) was added to the acetonitrile-supernatant mixture, vortexed, and centrifuged at 3100 x g for 20 min. The ether was transferred and evaporated under nitrogen before reconstituted in 300 µL of mobile phase.

Method V: Protein precipitation

Spiked 1 mL supernatant samples were precipitated with 3.0 mL acetonitrile while vortexing then sonicated for 20 min and centrifuged at 3100 x g for 20 min. The liquid fraction of the samples was transferred and dried down under nitrogen before reconstitution in 300 µL of mobile phase.

2.11.2 Trial 2: Heroin

After a review of the current literature regarding the extraction of heroin metabolites, specifically morphine and 6-MAM, the following solid-phase extraction and liquid-liquid extraction methods were examined.

Method I: Solid Phase Extraction (SPE)

Method I was adapted from United Chemical Technologies Product Application Guide (2008) for opiates in whole blood for GC-MS. Spiked supernatants were analysed for morphine, 6-monoacetylmorphine and morphine-3-glucuronide using 200 mg/3 mL EZ-Extract CSDAU203 (octyl-benzyl sulfonic acid) (Diagnostix, Mississauga, ON). The original application guideline analysed extracted samples on the GC-MS, thus the method was adapted for extract analysis on the UPLC-MS/MS and steps pertaining to sample derivatization and volumes of extraction solvents were omitted and/or adjusted.

One of three sample preparation procedures was examined: A) To 1 mL of liver supernatant, 3 mL of acetonitrile was added with vortexing to precipitate proteins. Samples were centrifuged and the acetonitrile was transferred and evaporated to approximately 0.5 mL. Four millilitres of 0.1 M phosphate buffer (pH 6.0) was added to the extract. B) Either 3 mL of distilled water or C) 3 mL of 0.1 M phosphate buffer was added to 1 mL of liver supernatant and the remaining procedure outlined above repeated.

Cartridges were conditioned with 3 mL of methanol, 3 mL distilled H_2O, and 1 mL of 0.1 M phosphate buffer (pH 6.0). The sample was added to the cartridge then washed with 3 mL of distilled H_2O, 3 mL of 100 mM acetate buffer (pH 4.0), and then 3 mL methanol. The cartridge was dried with compressed air for 5 min and heroin metabolites were eluted with 4 mL of dichloromethane: isopropanol: NH_4OH (78:20:2, v/v/v). The eluent was dried under nitrogen and reconstituted in 200 µL mobile phase.

Method II: SPE

Method II was adapted from Rook, Hillebrand, Rosing, van Ree, and Beijnen (2005) for the quantification of heroin and its metabolites in human plasma by HPLC-MS/MS. Spiked supernatants were analysed for morphine, 6-MAM and morphine-3-glucuronide using a mixed mode sorbent column, Oasis MCX (Waters, Mississauga, ON). Columns were conditioned with 3 mL of methanol, 2 mL of distilled water, followed by 2 mL of 10 mM citrate buffer, pH 3.0. Saline supernatants were then loaded onto the column and washed with 1 mL of water adjusted to pH 3.0 with acetic acid and dried down for 1 min with compressed air. Analytes were eluted with 1.5 mL of 0.5% ammonium acetate in methanol and evaporated to dryness and reconstituted in 200 µL mobile phase.

Method III: SPE

Method III was adapted from Oasis Product Application Guide (2008) for morphine in rat plasma. Oasis MCX columns were conditioned with 1 mL of methanol and 1 mL distilled water before samples were loaded and washed with 1 mL of distilled water and 0.5 mL of 0.1 M HCl. Columns were dried with compressed air for 2 min, rinsed with 1 mL of methanol and dried again for 4 min before the sample was eluted with 3 aliquots of 250 µL of acetonitrile: isopropanol: ammonia (38:57:5, v/v/v), evaporated to dryness and reconstituted in 200 µL mobile phase.

Method IV: SPE

Method IV was adapted from an Oasis Product application guide (2008) for morphine in rat plasma. Oasis HLB columns were conditioned with 1 mL of methanol and 1 mL distilled water before samples were loaded and washed with 1 mL of methanol: distilled water (5:95, v/v). The column was dried with compressed air for 2 min before the sample was eluted with 3 aliquots of 250 µL of acetonitrile: isopropanol: formic acid (40:58:2, v/v/v), evaporated to dryness and reconstituted in 200 µL of mobile phase.

2.12 Soil Extraction Experiment

Soil from beneath each carcass was collected and stored at -20˚C until analysis. Soils were analysed for the presence of oxycodone and selected metabolites or heroin metabolites. Soil was weighed (1.0 gram), dissolved in 1 mL of deionised water or 0.1 M phosphate buffer (pH 6.0), spiked with 25 µL of internal standard solution, and sonicated at 37˚C in capped culture tubes overnight. The supernatant was collected after samples were centrifuged at 1000 x g for 15 min. All samples were extracted twice to ensure the highest recovery possible.

Various organic solvents were chosen to try to maximize the extraction potential. Dichloromethane, ethyl acetate, or diethyl ether (3 mL of each solvent) was added to each sample. The tubes were vortex mixed for 60 sec and centrifuged at 2600 x g for 15 minutes. The organic layer was then collected and evaporated in a Savant SC210A SpeedVac Concentrator (Thermo Electron Corporation, Asheville, NC) at medium dry rate and reconstituted in 300 µL of mobile phase and analysed on the UPLC-MS/MS. The remaining supernatant was made alkaline with the addition of 100 µL of 6 M NH_4OH, vortexed and either 3 mL of DCM, diethyl ether, or ethyl acetate was added to each tube to be re-extracted, dried down and reconstituted. The organic layer of the basic fraction was then analysed on the UPLC-MS/MS for oxycodone and selected metabolites or

morphine and 6-MAM. Ethyl acetate showed the highest recovery based on peak area for all analytes and was chosen as the extract solvent.

2.13 Statistical Analysis

Statistical analysis was conducted using Genstat© 9th edition and SPSS® 13th edition statistical packages. Linear regression was used to determine relationships between variables of drug concentration and interment period. One-way ANOVA analysis determined differences between the soil and tissue pH with the post-burial interval.

Chapter 3
Validation Procedure

3.0 Capillary Electrophoresis-Mass Spectrometry (CE-MS) Method

Consistent separation was achieved with the CE-MS for oxymorphone, oxycodone, and noroxycodone (retention times: 5.46; 5.69; 6.41 min respectively; Figure 4) with a total run time of ten minutes. An ultraviolet (UV) diode array detector was initially used in conjunction with the MS; however the capillary experienced a high rate of breakage at the non-silanated portion of the capillary, which served as a window for the UV detector. To extend the life of the capillary, the UV detector was by-passed, decreasing the tension placed on the non-silanated portion of the capillary and routed directly to the MS detector.

Due to the use of a single quadrupole, collision induced fragmentation was required to produce a unique fragmentation pattern to confirm the presence of oxycodone, however consistent fragmentation was not achieved. Sodium and water adducts were produced, however due to the lack of sensitivity and the other technical difficulties related to the nitrogen generator required to operate the instrument, an alternate analytical method was developed on the UPLC-MS/MS.

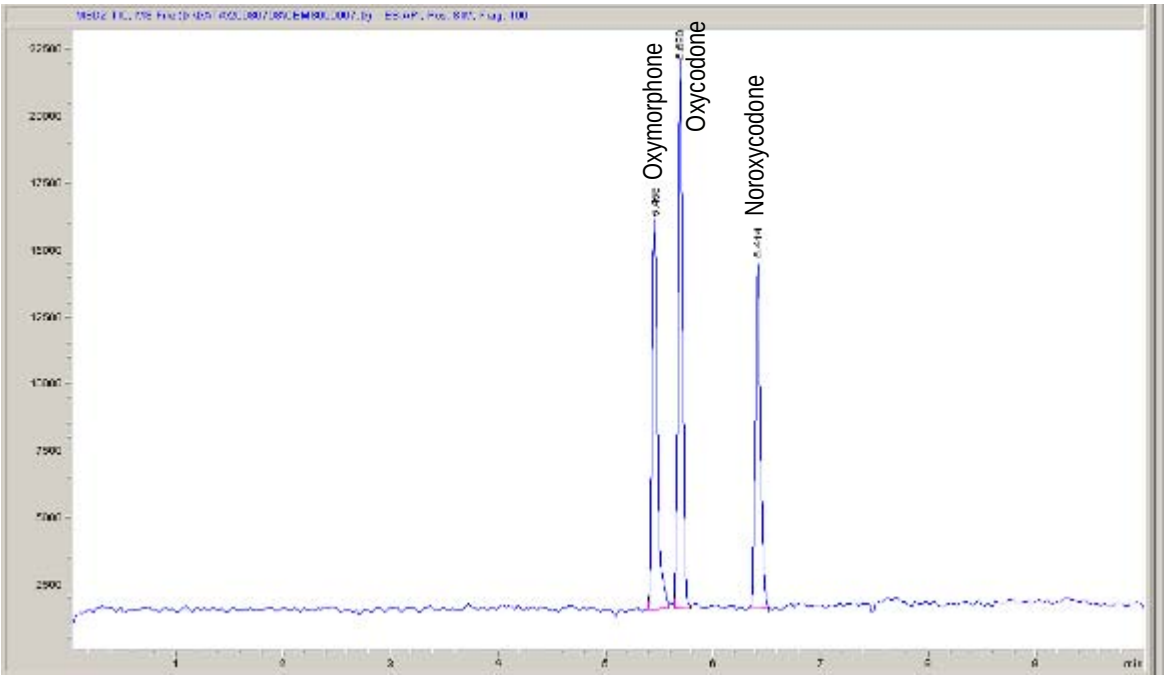


Figure 4: Total ion electropherogram of oxymorphone, oxycodone and noroxycodone standards in 25 mM ammonium acetate, pH 9 with sheath liquid of water: methanol (50:50, v/v) with 1% formic acid. The sample was electrokinetically injected by applied voltage for 10kV for 20 s, with an applied voltage of 27 kV.

3.1 Ultra-Performance Liquid Chromatography-Tandem Mass Spectrometry (UPLC-MS/MS) Method Validation

After optimization of the extraction and instrumental analysis on the UPLC-MS/MS, the methods for oxycodone and heroin analysis were validated both in hair and liver matrices.

3.1.1 Linearity

Calibration curves were obtained by analyzing drug-free hair roots or liver extracts fortified with working calibrator solutions. Calibration curves were constructed using a non-weighted linear regression based on 5 calibrator peak area ratios. The limit of quantification was defined by the lowest calibrator for each analyte with a QC precision and accuracy within 20%, while the limit of

detection had a minimum signal to noise ratio of 3:1. Intraassay linearity was assessed by analysing five sets of calibrators on one occasion. Interassay linearity was assessed by analysing different sets of calibrators on five separate occasions.

3.1.2 Accuracy and precision

To assess accuracy and precision, multiple QC samples at low, medium and high concentrations were analysed on five separate runs. Accuracy is a measure of how close the calculated value is to the expected value, expressed as a percentage (Accuracy = (mean conc. – nominal conc.)/ nominal conc. x 100%). Precision is defined as the ability of a measurement to be consistently reproduced (Precision = (SD of mean/mean) x 100%). The acceptance criteria of precision and accuracy were less than 20% deviation.

3.1.3 Matrix effects

To determine any ion-suppression and extraction efficiency, three sets of samples were prepared as follows: set 1 was neat solutions of the standards, dried down and reconstituted in mobile phase; set 2 consisted of blank hair or liver samples which were extracted, then fortified with QC and internal standard solutions; and set 3 composed of blank hair or liver samples fortified with QC and internal standard solutions added before extraction. Each set had five replicates and extraction efficiency was calculated as the ratio of the average peak area in set 3 to 2, expressed as a percentage. Matrix effect was defined as the percentage ratio of the average peak areas for sets 2 and 1.

3.1.4 Extract stability

Stability of extracted samples were evaluated after freeze-thaw with a full set of calibrators and QC samples, which consisted of freezing at -20°C after analysis for two weeks and reanalyzing the samples after unassisted thawing at room temperature. To confirm the within assay reproducibility of the UPLC-MS/MS, a calibrator was reinjected at the end of each assay.

3.2 Method Validation for Oxycodone

Multiple ion transition monitoring (MRM) in electrospray ionization was selected for the identification and quantification of oxycodone and its metabolites, noroxycodone and oxymorphone. One unique ion transition for each analyte was chosen; the most abundant ion transition that did not involve the loss of water (*m/z* = 18) was chosen.

All analytes eluted within four minutes, however the gradient program was run for 10 minutes to allow for column equilibration before the next injection. At least five different blank hair, liver and soil samples were analysed to determine that there were no interferences with the analytes of interest. Seven other opiates (codeine, hydrocodone, meperidine, methadone, morphine, 6-MAM, and hydromorphone) were also analysed to demonstrate that the ion transitions and retention times were specific to the analytes of interest. Initial method development included the use of oxymorphone-D_3 and noroxycodone-D_3 as internal standards, however the recovery and response of these deuterated internal standards were not consistent among different spiked blank-livers, thus oxycodone-D_6 was utilised as an internal standard for all three analytes.

3.2.1 Hair Validation

Hair samples were analysed for the presence of oxycodone, noroxycodone and oxymorphone. Hair root samples were briefly rinsed in approximately 2 mL of HPLC grade dichloromethane prior to extraction. The rinse solutions for the samples were dried down, reconstituted in mobile phase and analysed on the UPLC-MS/MS to demonstrate that there was no external contamination of collected samples (Figure 5).

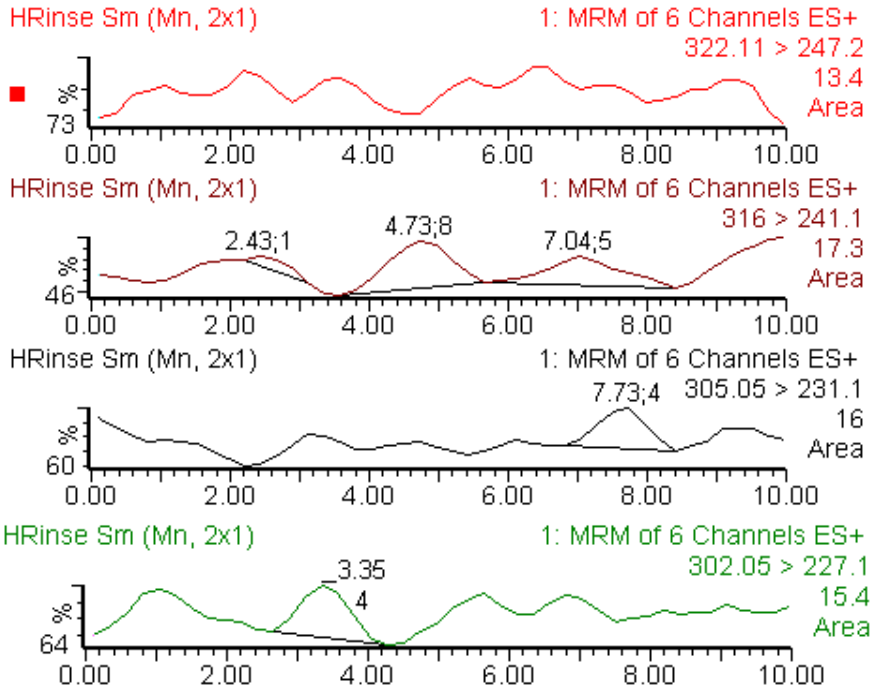


Figure 5: Extracted ion chromatograms of dried down and reconstituted rinse solution. Channel 322.11/247.2 for oxycodone-D_6 (RT: 3.64 min); 316/241.11 for oxycodone (RT: 3.64 min); 305/231.1 for oxymorphone-D_3 and noroxycodone-D_3 (RT: 3.54 and 2.45 min, respectively); 302.05/227.1 for oxymorphone and noroxycodone (RT: 3.54 and 2.45 min, respectively).

The hair method was linear (r^2 > 0.985) for oxycodone, oxymorphone, and noroxycodone from 15.6 to 125 ng/mL which for a ten-milligram sample of hair is equivalent to 0.78 - 6.25 ng/mg. The LOQ was the lowest point of the calibration curve (15.6 ng/mL) and the LOD was 0.78 ng/mL or 0.39 ng/mg of hair. The percentage deviation for the calibrators range did not exceed ± 20%. Both intra- and interassay linearity were assessed as described in Section 3.1.1 (Table 5, Table 6).

Table 5: Intraassay linearity assessed with five different blank hair matrices for oxycodone and selected metabolites

Analyte	Retention Time (minutes)	Slope	Intercept	r^2
Oxycodone	3.64	2.54 ($\pm$6.83 x10^{-1})	-4.28 ($\pm$ 2.75 x10^{1})	0.993 ($\pm$ 1.25 x10^{-2})
Oxymorphone	3.54	1.88 ($\pm$1.08 x10^{-1})	-0.44 ($\pm$ 2.39 x10^{1})	0.995 ($\pm$ 6.30 x10^{-3})
Noroxycodone	2.45	1.39 ($\pm$2.59 x10^{-1})	6.23 ($\pm$ 6.71 x10^{1})	0.999 ($\pm$ 5.64 x10^{-3})

Calibration range for all analytes: 0.78 to 6.25 ng/mg. Mean ($\pm$ S.D.)

Table 6: Interassay linearity assessed over five different assays in hair for oxycodone and selected metabolites

Analyte	Slope	Intercept	r^2
Oxycodone			
Blank Hair 1	2.19	-6.89	0.999
Blank Hair 2	2.69	1.61	0.987
Blank Hair 3	2.10	1.50	0.999
Blank Hair 4	2.72	1.69	1.00
Blank Hair 5	2.43	4.54	0.986
Mean ($\pm$ S.D.)	2.43 ($\pm$ 2.81x10^{-1})	0.487 ($\pm$ 4.32x10^{1})	0.994 ($\pm$ 7.05x10^{-3})
Oxymorphone			
Blank Hair 1	1.88	0.799	1.00
Blank Hair 2	1.76	-2.77	0.995
Blank Hair 3	1.81	-1.50	0.999
Blank Hair 4	1.99	-3.20	0.988
Blank Hair 5	1.78	1.09	0.989
Mean ($\pm$ S.D.)	1.84 ($\pm$ 9.49x10^{-2})	-1.120 ($\pm$ 1.98x10^{1})	0.994 ($\pm$ 5.54x10^{-3})
Noroxycodone			
Blank Hair 1	1.64	0.126	0.999
Blank Hair 2	1.11	12.076	0.986
Blank Hair 3	1.65	-8.174	0.987
Blank Hair 4	1.59	0.717	0.996
Blank Hair 5	1.23	12.010	0.996
Mean ($\pm$ S.D.)	1.44 ($\pm$ 2.52x10^{-1})	3.351 ($\pm$ 8.68x10^{1})	0.993 ($\pm$ 5.89x10^{-3})

To evaluate intraassay accuracy and precision, three QC samples at various concentrations within the calibration curve were prepared and analysed with each assay. The accuracy and precision data are summarized in Table 7.

Table 7: Intraassay and interassay accuracy and precision of QC samples at various concentrations in hair for oxycodone and selected metabolites

Analyte	**Concentration (ng/mg)**	**Intraassay (n = 5)**		**Interassay (n = 1)**	
		Accuracy (%)	**Precision (%CV)**	**Accuracy (%)**	**Precision (%CV)**
Oxycodone					
QC Low	1.0	82-97	8.6	80-93	6.5
QC Med	2.0	80-118	18.0	81-103	10.7
QC High	4.0	80-113	15.0	92-108	8.0
Noroxycodone					
QC Low	1.0	91-103	4.8	81-112	12.0
QC Med	2.0	89-114	11.5	83-95	6.6
QC High	4.0	92-114	8.7	95-120	10.2
Oxymorphone					
QC Low	1.0	81-109	11.5	88-104	6.5
QC Med	2.0	84-102	9.5	82-93	5.1
QC High	4.0	97-114	7.6	104-118	4.4

Ion enhancement was observed as the matrix effect for oxycodone, noroxycodone, oxymorphone and the internal standard, oxycodone-D_6 ranged from 130% (±11.5), 115% (±6.1), 103% (±4.1) and 113% (±1.1) respectively. The extraction efficiency for oxycodone, noroxycodone, oxymorphone and the internal standard, oxycodone-D_6 was 61% (±6.1), 90% (±1.2), 85% (±11), and 100% (±6.1) respectively.

3.2.2 Liver Validation

The analytical extraction chosen for liver sample analysis was protein precipitation by acetonitrile. The limit of detection (LOD) and lower limit of quantification (LOQ) were 3.9 ng/mL and 7.9 ng/mL and the calibration curves were linear over a range from the LOQ to 125 ng/mL. A one-gram sample of liver was homogenized with 1.5 mL of saline and 0.5 mL of supernatant was spiked and extracted, this calibration curve is equivalent to 5.85 to 187.5 ng/g liver. The percentage deviation for the calibrators range did not exceed ± 20%.

The mean calibration curve slope and intercept and mean retention time for each analyte over 5 batches in liver are given in Table 8. All analytes were calibrated using non-weighted linear regression with the origin excluded.

Table 8: Intraassay linearity assessed with five different blank liver matrices for oxycodone and selected metabolites

Analyte	**Retention Time (minutes)**	**Slope**	**Intercept**	r^2
Oxycodone	3.64	0.954 (± 1.25 x10^{-3})	0.348 (± 9.17 x 10^{-4})	0.999 (± 2.80 x10^{-4})
Oxymorphone	3.54	0.647 (± 1.02 x10^{-2})	0.627 (± 4.55 x10^{-1})	0.992 (± 5.59 x10^{-4})
Noroxycodone	2.45	0.512 (± 3.28 x10^{-1})	0.278 (± 6.10 x 10^{-1})	0.997 (± 1.93 x10^{-3})

Calibration range for all analytes = 5.9 to 188 ng/g. Mean (± SD)

Interassay linearity was assessed over 6 different days with 6 different sources of blank matrix. The linearity data is summarized in Table 9.

Table 9: Interassay linearity assessed over six different assays in liver for oxycodone and selected metabolites

Analyte	**Slope**	**Intercept**	r^2
Oxycodone			
Blank Liver 1	0.852	2.390	1.00
Blank Liver 2	0.956	0.469	1.00
Blank Liver 3	0.969	0.699	0.999
Blank Liver 4	0.968	0.111	1.00
Blank Liver 5	0.942	1.150	0.999
Blank Liver 6	0.833	2.282	0.999
Mean (± SD)	0.920 ($\pm$ 6.11x10^{-2})	1.183 ($\pm$ 9.54x10^{-1})	1.00 ($\pm$ 5.48x10^{-4})
Oxymorphone			
Blank Liver 1	0.632	0.627	0.999
Blank Liver 2	0.663	-0.027	0.998
Blank Liver 3	0.636	0.768	0.999
Blank Liver 4	0.648	0.351	0.999
Blank Liver 5	0.640	-0.275	1.00
Blank Liver 6	0.444	4.04	0.994
Mean (± SD)	0.611 ($\pm$ 8.23x10^{-2})	0.914 ($\pm$ 1.58)	0.998 ($\pm$ 2.14x10^{-3})
Noroxycodone			
Blank Liver 1	0.453	1.787	0.996
Blank Liver 2	0.493	0.834	0.998
Blank Liver 3	0.495	0.555	0.998
Blank Liver 4	0.569	-0.576	0.994
Blank Liver 5	0.492	0.297	0.998
Blank Liver 6	0.198	4.00	0.999
Mean (± SD)	0.450 ($\pm$ 1.29x10^{-1})	1.15 ($\pm$ 1.59)	0.997 ($\pm$ 1.84x10^{-3})

For intraassay accuracy and precision, quality control (QC) samples at various concentrations within the dynamic range of the calibration curve for each analyte were run with five replicates in one assay. For interassay accuracy and precision, each QC sample was run once each assay on five different occasions (Table 10).

Table 10: Intraassay and interassay accuracy and precision of QC samples at various concentrations in liver for oxycodone and selected metabolites

Analyte	Concentration (ng/g)	Intra Assay (n = 5)		Inter Assay (n = 1)	
		Accuracy (%)	Precision (%CV)	Accuracy (%)	Precision (%CV)
Oxycodone					
QC Low	30	96-106	4.6	91-104	5.5
QC Med	75	83-105	10.4	98-106	3.4
QC Med-High	98	89-117	10.9	-	-
QC High	150	90-102	6.1	84-115	11.1
Noroxycodone					
QC Low	30	95-101	2.7	91-99	3.2
QC Med	75	102-104	1.4	85-97	4.7
QC Med-High	98	78-117	14.9	-	-
QC High	150	91-114	9.6	89-107	6.4
Oxymorphone					
QC Low	30	98-116	7.5	86-96	4.5
QC Med	75	98-103	3.3	91-98	2.8
QC Med-High	98	88-115	11.9	-	-
QC High	150	99-101	1.2	90-106	7.0

The matrix effect for each analyte and internal standard were assessed at three concentrations (as described above at 30, 75 and 150 ng/mg). Ion suppression was observed as the matrix effect for oxycodone ranged from 85% (±11); for oxymorphone from 49% (±8); and for noroxycodone from 40% (±9); for all concentrations. The extraction efficiency for oxycodone, noroxycodone, oxymorphone and the internal standard, oxycodone-D_6 was 90% (±7.8), 86% (±7.3), 84% (±13), and 99% (±4.9), respectively.

3.2.3 Oxycodone in Soil

The soil directly beneath the carcasses, otherwise known as grave soil, was collected and analysed to determine if the presence of oxycodone and its metabolites could be determined. A qualitative method was validated for the determination of oxycodone and oxymorphone in soil; however the method used was not successful in the extraction of noroxycodone. Blank soil samples fortified with the three analytes were extracted and only oxycodone and oxymorphone were detected by UPLC-MS/MS. Both enzymatic and liquid-liquid extraction techniques were examined. The extraction solvent that showed the highest recovery from spiked samples of blank soil was ethyl acetate. The analysis of soil for the presence of drugs can be beneficial if the body is removed by scavenging or decomposition has proceeded to the stage where hair or bones are not available for analysis (Dunnett, Ashton, & Osselton 1979).

A modified validation procedure was utilised for this method as it was used to only determine the presence/absence of oxycodone in soil (Peters, Drummer, & Musshoff, 2007). Six different blank soil samples were analysed, with and without the addition of internal standards to check for the absence of interfering signals. Blank soil samples were spiked with known amounts of oxycodone, oxymorphone and noroxycodone at concentrations ranging from 0.78 to 2.5 ng/g and analysed along with a negative control sample and three positive control samples (1.56, 3.125 and 6.25 ng/g). The samples were analysed and a limit of detection was determined to be 6.25 ng/g for oxycodone and oxymorphone, however a linear response ($r^2 > 0.985$) could not be reliably achieved. Thus as a quantitative result would not be reflective of the amount of oxycodone administered or present in the body at the time of death, a simplified qualitative method was developed. Ten positive control samples were spiked, extracted and analysed at a concentration of 6.25 ng/g of soil (Figure 6).

The internal standard, oxycodone-D_6 had an extraction recovery of 38% (±6.9) while oxycodone and oxymorphone had extraction recoveries of 25% (±7.8) and 18% (±4.7). The positive controls were identified as oxycodone and oxymorphone positive in all ten samples and had an average response ratio of 158.3 (±22.1) with a coefficient of variation of 14.0% for oxycodone. The average response ratio for oxymorphone was 81.3 (±24.9) with a coefficient of variation of 30.6%.

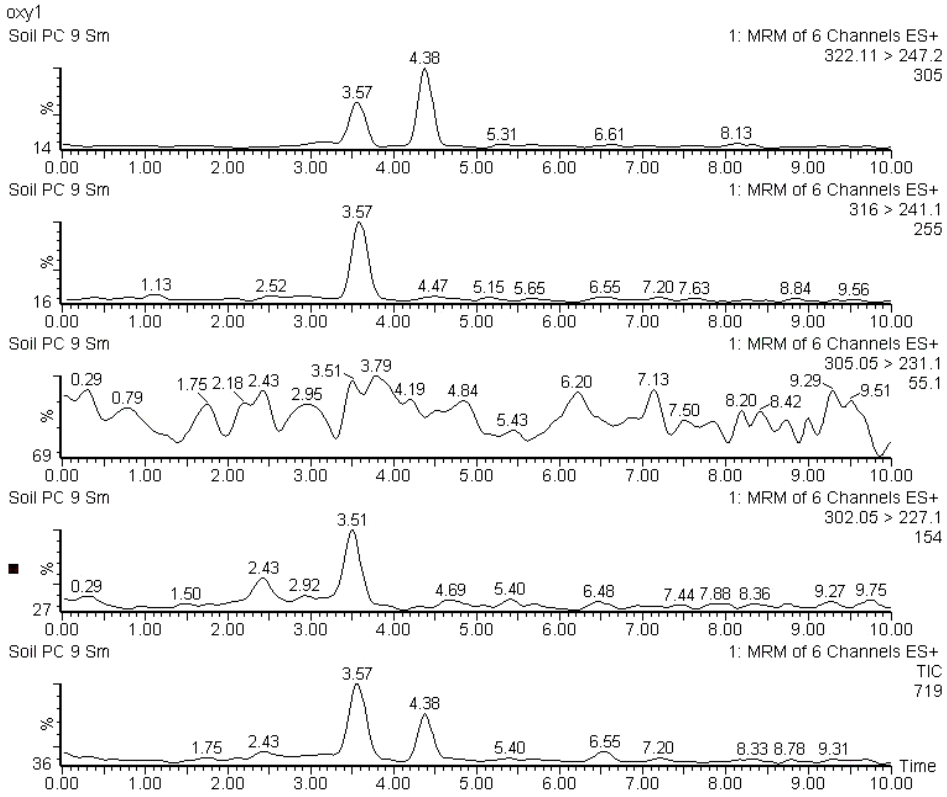


Figure 6: Total ion chromatograms and extracted mass chromatograms obtained from blank soil fortified with 6.25 ng/g of oxycodone (RT: 3.52 min, m/z 316/241.1) and oxymorphone (RT: 3.52 min, m/z 302.05/231.1). Internal standard: oxycodone-D_6 (RT: 3.57 min, m/z 322.11/247.2)

3.3 Method Validation for Morphine and 6-Monoacetylmorphine

The two most abundant MRM transitions for morphine and 6-monoacetylmorphine (6-MAM), excluding the loss of water, were chosen to quantify and qualify each analyte (Figure 7, Figure 8). Mass spectrometric conditions for each MRM transition were individually optimized. The mean relative abundance ratios of the two MRM transitions for morphine averaged 1.00 in all calibrators, with percent coefficient of variance (%CV) of less than 9% observed over five different assays. The mean relative abundance ratio for 6-MAM averaged 1.39 in calibrators with a %CV of less than 10% observed over five different assays.

All analytes eluted within four minutes and the analytical run time was ten minutes. The flow was adjusted after the elution of the last analyte (6-MAM) to allow the column to re-equilibrate and fully elute any other potential interfering compounds from the column before the next injection.

Blank liver and hair from five different sources had minimal interferences with the analytes of interest, with the exception of morphine-3-glucuronide and its internal standard morphine-6-glucuronide-D_6. Morphine-3-glucuronide and the deuterated internal standard were added to all calibrators, however it was not consistently detected by any of the extraction methods and it was excluded from the study. Validation of the method was completed with a second set of working standard solutions which excluded the glucuronide standards to eliminate the possibility that the glucuronide moiety could be cleaved during the extraction process to produce morphine, thereby possibly resulting in an increased morphine and/or deuterated morphine concentration.

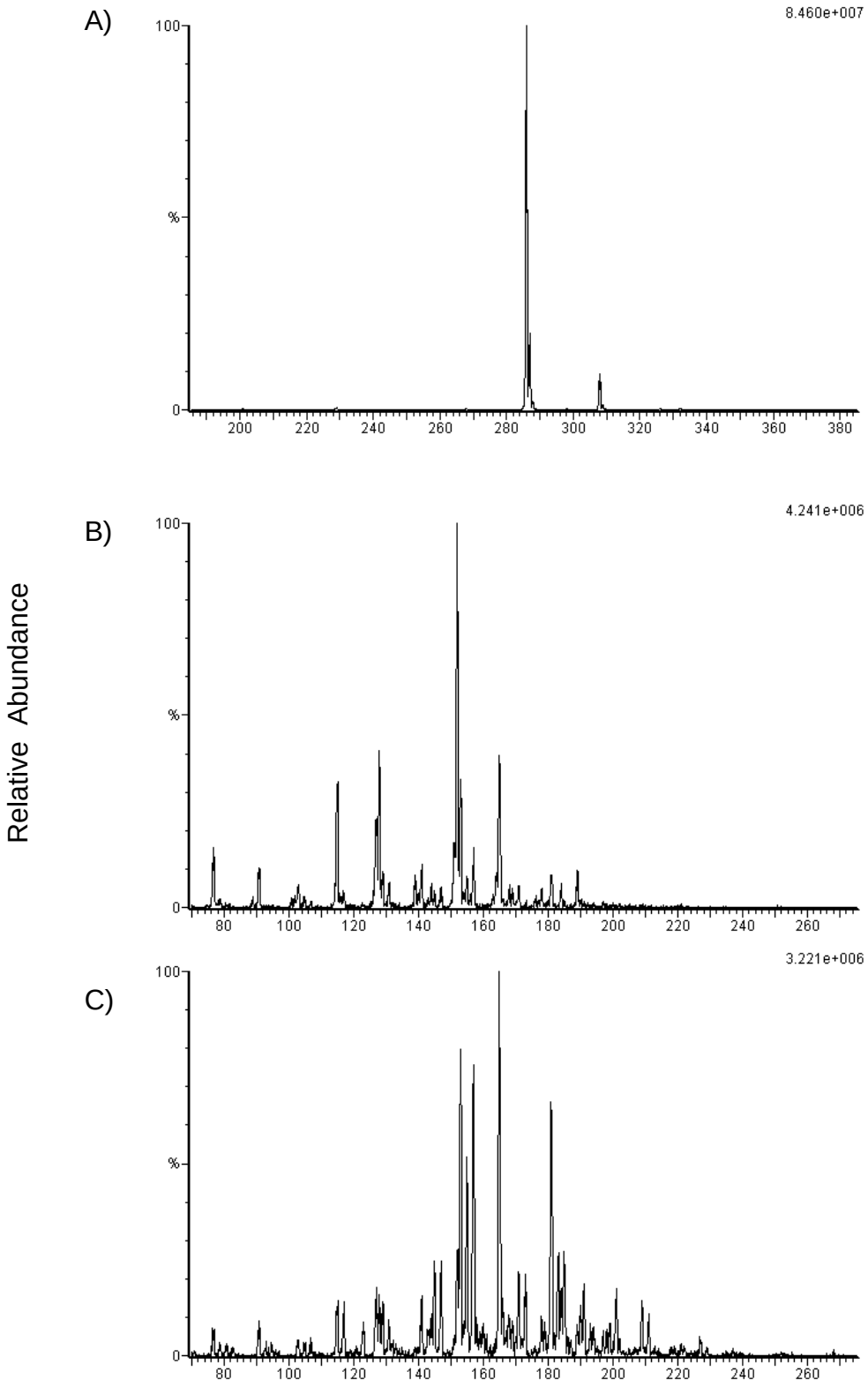


Figure 7: Mass spectra of morphine obtained from a MRM autotune. A) Parent mass of 285.92 and B) Daughter masses of 152.0 and C) 165.0 with a cone voltage of 50 V and collision energy for the formation of daughter ions at 57 and 37 eV, respectively.

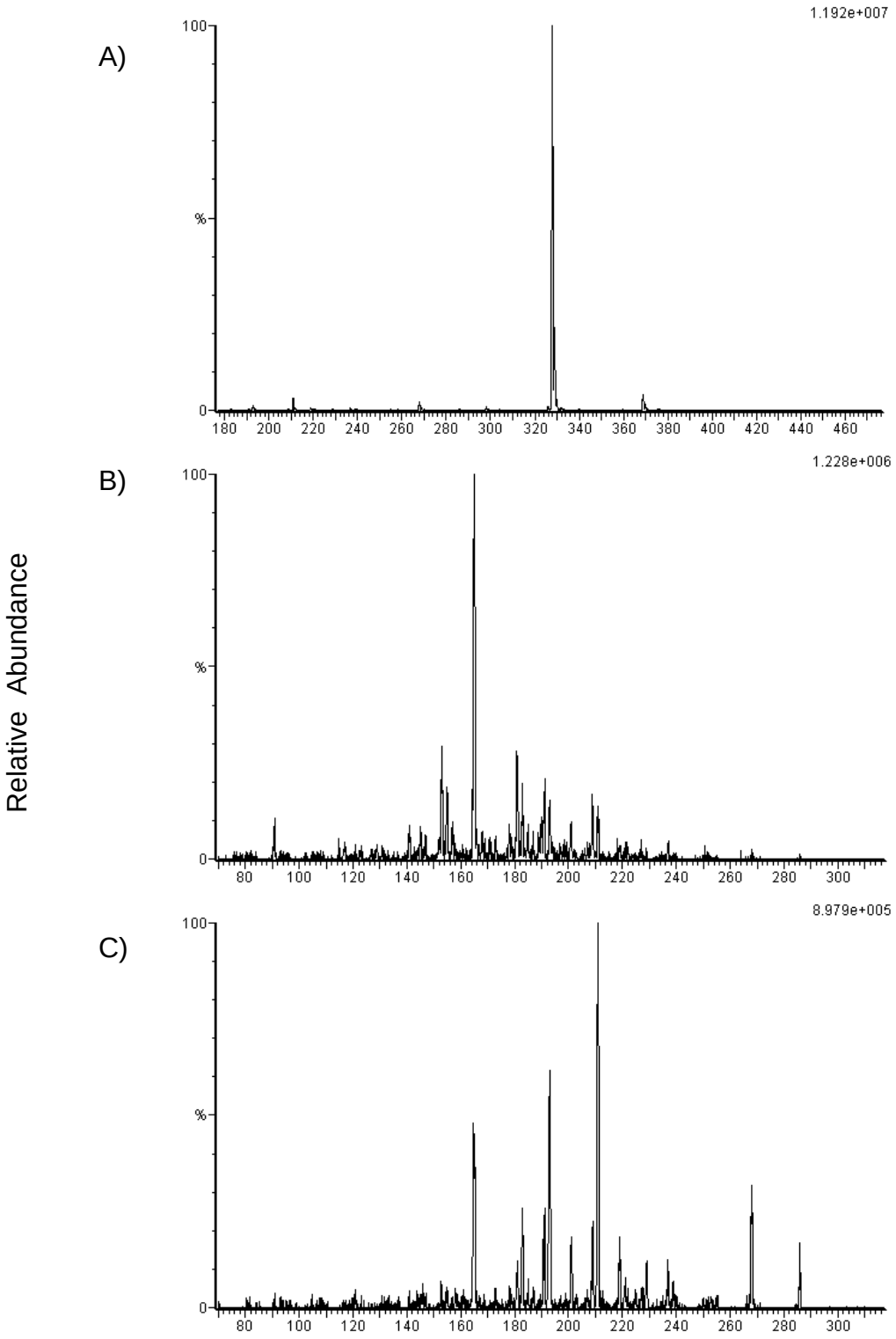


Figure 8: Mass spectra of 6-MAM obtained from a MRM autotune. A) Parent mass of 327.87 and B) Daughter masses of 165.0 and C) 211.0 with a cone voltage of 52 V and collision energy for the formation of daughter ions at 39 and 25 eV, respectively.

3.3.1 Hair Validation

For morphine and 6-MAM in hair, two transition ions were monitored for all analytes and internal standards. Non-weighted linear regression using least squares regression was used to calculate the slope, intercept and coefficient of correlation. Linear regressions using the primary ion and the secondary ion were calculated (Table 11). Although all the intraassay coefficients of correlation were equal to or greater than 0.999 for both modes of quantification, the interassay variability (as measured by the standard deviation) was greater and the coefficient of correlation was not consistently greater than 0.985 when using the secondary ion; thus case and quality control samples were quantified using the primary transition ion response ratio and the secondary transition ion as a qualifier (Table 11 and Table 12).

Table 11: Intraassay linearity assessed with five different blank hair matrices for morphine and 6-MAM

Analyte	**Slope**	**Intercept**	r^2
Morphine			
Primary ion	7.27 (± 0.31)	3.36 (± 1.30)	0.999 (± 0.001)
Secondary ion	8.28 (± 0.46)	1.14 (± 4.20)	1.00 (± 0.002)
6-MAM			
Primary ion	16.16 (± 1.30)	0.55 (± 0.86)	1.00 (± 0.001)
Secondary ion	24.08 (± 2.50)	52.95 (±13.03)	0.999 (± 0.001)

Calibration range for morphine = 0.08 to 1.25 ng/mg; for 6-MAM = 0.31 to 5.0 ng/mg. Mean (± SD)

Table 12: Interassay linearity assessed with five different blank hair matrices for morphine and 6-MAM

Analyte	**Slope**		**Intercept**		***r*^{2}**	
	1st ion	**2nd ion**	**1st ion**	**2nd ion**	**1st ion**	**2nd ion**
Morphine						
Blank Hair A	6.87	9.60	5.39	-19.41	1.00	0.972
Blank Hair B	6.60	8.27	6.05	5.01	0.992	0.975
Blank Hair C	7.90	8.34	0.68	4.55	1.00	0.957
Blank Hair D	7.69	7.72	1.32	2.03	1.00	0.959
Blank Hair E	7.72	7.46	0.98	8.26	1.00	0.987
Mean (± SD)	7.36 (± 0.58)	8.28 (± 0.83)	2.88 (± 2.61)	0.088 (± 11.12)	0.998 (± 0.004)	0.970 (± 0.012)
6-MAM						
Blank Hair A	15.90	19.57	11.25	311.19	0.997	0.884
Blank Hair B	14.78	20.17	40.92	78.69	0.989	0.983
Blank Hair C	16.90	20.88	-30.30	93.46	1.00	0.996
Blank Hair D	15.88	28.25	6.67	-12.38	0.999	0.991
Blank Hair E	17.03	27.03	-15.08	52.03	1.00	0.983
Mean (± SD)	16.10 (±0.91)	23.18 (±4.12)	2.69 (±27.18)	104.60 (±122.40)	0.997 (±0.006)	0.967 (±0.047)

To quantify morphine and 6-MAM in hair and liver, all calibrators used in each calibration were within ± 20% of their target concentrations. The relative abundance ratios of the qualifier MRM transition ion to quantification MRM transition ion for morphine and 6-MAM were calculated based on the calibrators. Morphine has a relative abundance ratio of 1.0 and 6-MAM has an abundance ratio of 1.39. The internal standards had a relative abundance ratio of 1.02 for morphine-D_3 and 2.29 for 6-MAM-D_3. All QC and case samples had a minimum relative abundance of ± 20% for the analyte to be considered detected.

The method was evaluated with five replicates of low, medium, and high concentration quality control (QC) samples for each analyte in one assay for intraassay accuracy and precision. Interassay accuracy and precision were evaluated once with low, medium, and high concentration quality control samples over 5 different assays (Table 13).

To evaluate the in-vitro stability of 6-MAM, an additional QC sample in hair was made up to contain only 6-MAM at a concentration of 2.5 ng/mg. The absence of morphine in the extracted sample demonstrated that 6-MAM was not hydrolyzed to morphine during the incubation, extraction and ionization process. Authentic samples underwent the same extraction process with a preparatory rinse step. Drug free hair samples used to make up calibrators and QC samples were rinsed prior to the addition of calibrator, QC, and internal standard solutions. All samples were rinsed in approximately 2 mL of HPLC-grade dichloromethane and the solutions were dried down and analysed on the UPLC-MS/MS to demonstrate that there was no external contamination of collected samples (Table 13).

Table 13: Intraassay and interassay accuracy and precision in hair for morphine and 6-MAM

Analyte	Target Concentration (ng/mg)	Intraassay (n = 5)		Interassay (n = 1)	
		Accuracy (%)	Precision (%CV)	Accuracy (%)	Precision (%CV)
Morphine					
QC Low	0.2	80 - 93	6.7	89 - 94	7.0
QC Med	0.4	85 - 103	7.8	94 - 108	11.2
QC High	0.8	83 - 100	9.6	83 - 115	15.1
QC 6-MAM	0	not detected	not detected	-	-
6-MAM					
QC Low	0.8	92 - 100	4.0	90 - 100	5.5
QC Med	1.6	78 - 113	16.8	80 - 115	17.6
QC High	3.2	93 - 98	2.2	91 - 103	5.5
QC 6-MAM	2.5	80 - 87	3.7	82 - 120	18.9

Note: QC 6-MAM contained 2.5 ng/mg of 6-MAM; the sample did not contain morphine. This sample was prepared to assess the stability of 6-MAM.

The extraction efficiency and matrix effect for each analyte and internal standard were assessed across all calibrators. The extraction efficiency in hair was 82% (±9.4) for morphine; 85% (±6.8) for 6-MAM; 87% (±3.3) for morphine-D_3; and 87% (±5.1) for 6-MAM-D_3. Ion suppression was observed in the non-deuterated drug standards as the matrix effect for morphine was 85% (±8.3); and 6-MAM was 70% (±6.2). The matrix effect for the internal standards was 116% (±9.6) for morphine-D_3 and 101% (±3.4) for 6-MAM-D_3.

3.3.2 Liver Validation

The same chromatographic and MS conditions were utilised for the analysis of morphine and 6-MAM in liver. Calibration curves utilising the secondary ion as the quantification ion for intraassay analysis yielded linear curves with an r^2 value of 0.996, while the r^2 value for the primary ion was 0.983 (Table 14). However, interassay testing demonstrated that the use of the primary ion for quantifying morphine resulted in a consistent linear curve with an r^2 of 0.985 or greater for all 5 different matrices. Thus the secondary ion was used as a qualifier or confirmation ion (Table 15) and the primary ion was the quantifying ion. The linearity for 6-MAM was comparable utilising either ion transition as the quantifying ion, however as 6-MAM in hair was quantified using the primary ion, the same transition ion was selected to quantify 6-MAM in liver, while the second ion was used as a qualifying ion.

Table 14: Intraassay linearity assessed with five different blank liver matrices for morphine and 6-MAM

Analyte	**Slope**	**Intercept**	**r^2**
Morphine			
Primary ion	0.78 (± 0.41)	4.30 (± 2.90)	0.983 (± 0.006)
Secondary ion	0.67 (± 0.13)	2.83 (± 1.56)	0.996 (± 0.009)
6-MAM			
Primary ion	1.54 (± 0.21)	-2.50 (± 2.81)	0.999 (± 0.005)
Secondary ion	1.11 (± 0.20)	-3.87 (± 7.61)	1.00 (± 0.005)

Calibration range for morphine and 6-MAM = 23 to 375 ng/g. Mean (± SD)

Table 15: Interassay linearity assessed with five different blank liver matrices for morphine and 6-MAM

Analyte	**Intercept**		**Slope**		**r^2**	
	1^{st} ion	2^{nd} ion	1^{st} ion	2^{nd} ion	1^{st} ion	2^{nd} ion
Morphine						
Liver A	0.812	2.587	1.360	0.758	0.987	0.987
Liver B	18.452	12.449	0.397	0.470	0.985	0.774
Liver C	1.207	15.611	0.692	0.466	0.987	0.784
Liver D	-1.140	4.607	0.692	0.631	0.997	0.905
Liver E	1.332	2.158	0.621	0.644	0.983	0.992
Mean (±SD)	4.132 (±8.067)	7.482 (±6.151)	0.7525 (±0.361)	0.594 (±0.125)	0.988 (±5.40x10^{-3})	0.888 (±1.06x10^{-1})
6-MAM						
Liver A	-4.373	-0.675	1.827	1.292	0.985	0.994
Liver B	5.305	1.911	1.398	1.021	0.997	0.998
Liver C	3.258	-13.230	1.648	1.178	0.999	0.994
Liver D	-3.780	0.190	1.302	0.973	0.995	0.998
Liver E	-1.050	1.950	1.515	1.112	0.994	0.998
Mean (±SD)	-0.128 (±4.278)	-1.971 (±6.395)	1.538 (±0.207)	1.115 (±0.127)	0.994 (±5.00x10^{-3})	0.996 (±2.00x10^{-3})

The accuracy and precision of the assay were assessed by analysing three QC samples at concentrations of 30, 120, and 240 ng/g of both morphine and 6-MAM during intra- and interassay linearity testing. For intraassay accuracy and precision, the QC samples were run with five replicates in one assay, n = 5. For interassay accuracy and precision, the QC samples were run once each assay on five different occasions. All QC samples were within 20% of the expected concentration (Table 16).

Table 16: Intraassay and interassay accuracy and precision in liver for morphine and 6-MAM

Analyte / Sample	**Target Concentration (ng/g)**	**Intraassay (n = 5)**		**Interassay (n = 5)**	
		Accuracy (%)	**Precision (%CV)**	**Accuracy (%)**	**Precision (%CV)**
Morphine					
QC Low	30	90-119	14.3	101-120	9.4
QC Med	120	82-96	8.0	85-97	8.7
QC High	240	92-114	11.8	94-99	8.6
6-MAM					
QC Low	30	80-113	17.2	82-120	18.7
QC Med	120	116-120	2.0	101-120	8.8
QC High	240	95-116	10.8	96-117	10.2

Ion suppression was observed with the matrix effect for morphine in liver at 65.1% (± 3.0) and 55.4% (± 2.5) for morphine-D_3. The extraction efficiency of the assay for morphine and morphine-D_3 was 70.2% (± 13.0) and 59.0% (± 7.0), respectively. For 6-MAM and 6-MAM-D_3, ion suppression was not as great as the matrix effect was calculated to be 94.2% (± 8.3) and 101.0% (± 14.3), respectively. The extraction efficiency for 6-MAM and 6-MAM-D_3 was 67.9 (± 7.0) and 83.1% (± 1.5), respectively.

3.3.3 Morphine and 6-Monoacetylmorphine in Soil

As with the soil extraction method developed for oxycodone and oxymorphone, the soil extraction method for heroin metabolites was qualitative only. The same procedure for method validation was followed. The limit of detection for morphine and 6-MAM was 3.13 ng/g soil. The extraction efficiency for morphine was 25% (± 7.8) and 24% (± 5.6) for morphine-D_3. The recovery for 6-MAM was 36 (± 10.3) and the recovery for 6-MAM-D_3 was 40% (± 7.4).

Chapter 4
Results

4.0 Postmortem Changes of Trial 1: Oxycodone

The burial trial of 30 rats administered oxycodone for 5 days prior to sacrifice consisted of a post burial interval that ranged from 0 to 30 days (Figure 9). With each harvest (every 3 days), the state of decomposition steadily progressed from the Fresh, Bloated/Inflated, to Deflated or Active Decay stage. At the end of the 30 days, the remaining rats had not yet progressed to the Advanced Decay or Dried Remains stage (Figure 9, Day 30).

The first stage (Fresh) was the period between death and initial bloating and was observed at the initial harvest (Day 3). At Day 3, the surface soil was partially covered with a very thin coat of white fungi, which was not identified (Figure 10, Day 3). The carcasses were intact and the organs were bright red in colour and solid in texture with liquid in the abdominal cavity resembling blood.

Between Day 6 and Day 9 all the exhumed carcasses were fully inflated at the abdomen and scrotum (Figure 9, Day 6 and Day 9). One of the three carcasses exhumed on Day 12 was also observed to be in the second stage of decomposition. The degree of inflation from decomposition gases against the abdominal walls resulted in displacement of surface soil in one of the microcosms and there was a thick white coat of fungus covering the soil above the carcass. An incision at dissection to the abdominal cavity resulted in deflation of the carcasses. Bloating was not evident on the appendages, however internal organs were yellow coloured and the colon and stomach were inflated. Translucent yellow liquid in the abdominal cavity was observed and the underside of the carcasses exposed to the grave base was darker in colour compared to the upper surface.

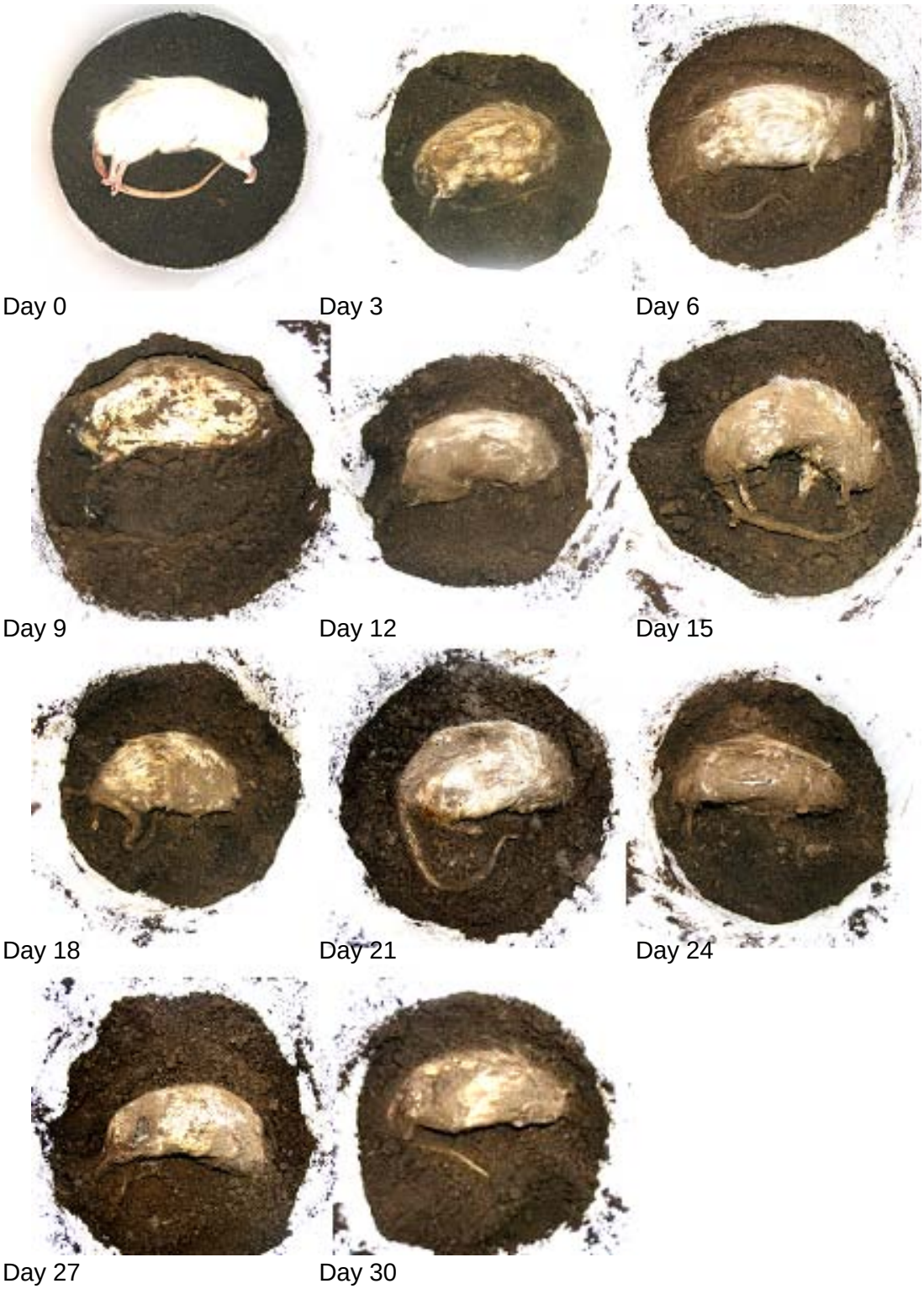


Figure 9: Oxycodone treated rats sacrificed and buried in soil microcosms. Exhumed carcasses in-situ from Day 0 to Day 30; carcasses harvested every 3 days, n = 3.

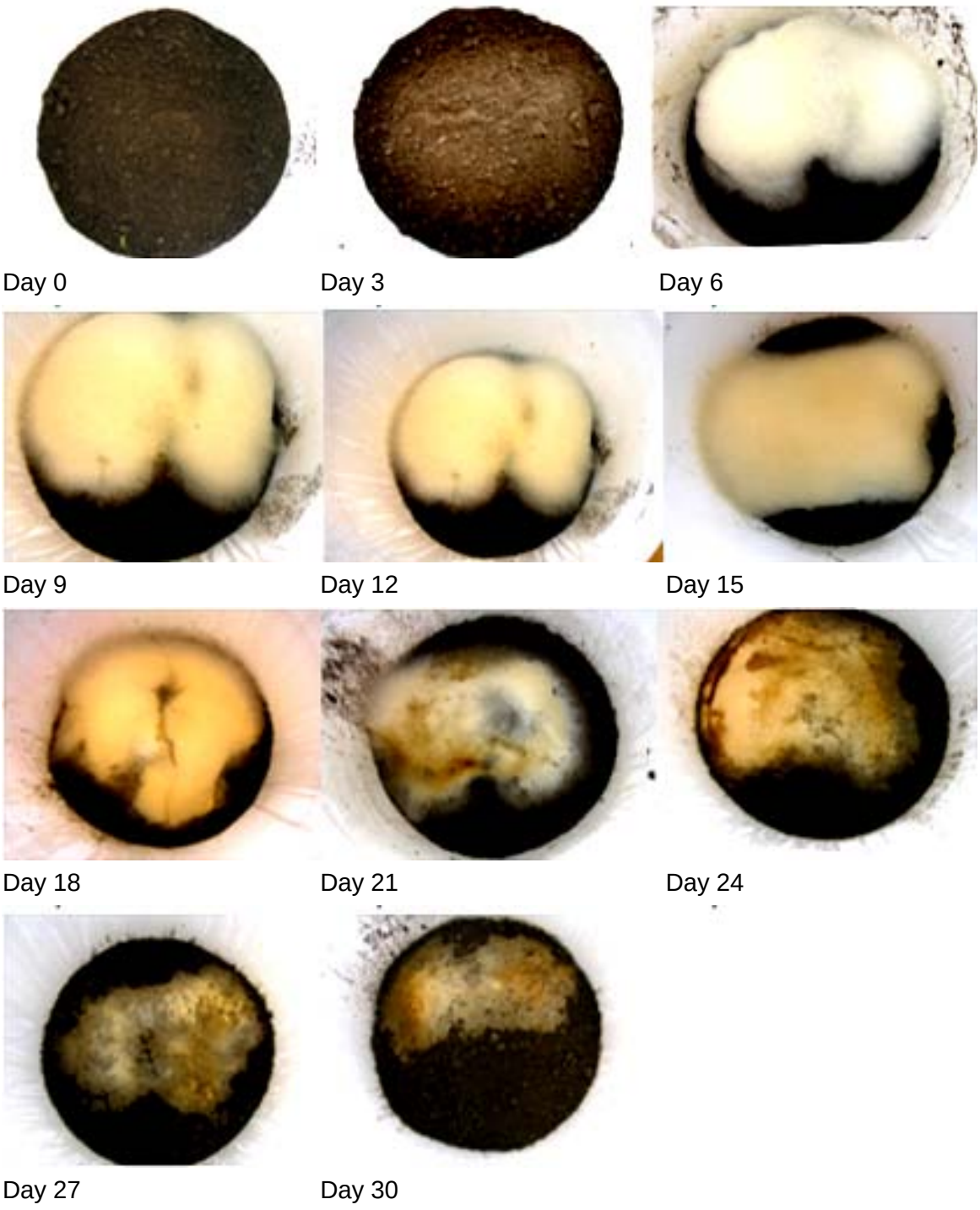


Figure 10: Undisturbed grave surface of burial microcosms from Day 0 to 30. Carcasses harvested every 3 days, n = 3.

At Day 12, the carcasses were deflated and the outer epidermal layers were sloughing off. Upon exhumation, the arms and tail of the carcasses adhered to the grave floor, and the surfaces of the tissue on the abdomen and back of each carcass were black in colour.

It can be observed from Figure 11 in the Day 21 and Day 27 photos, the stomach and part of the intestines ruptured and became shrivelled and partially dehydrated. The ability to distinguish between internal organs become more difficult as the post burial interval increased. At Day 30, the organs became liquefied (Figure 11, Day 30). A summary of the stage of decomposition reached by the rat carcasses is shown in Table 17.

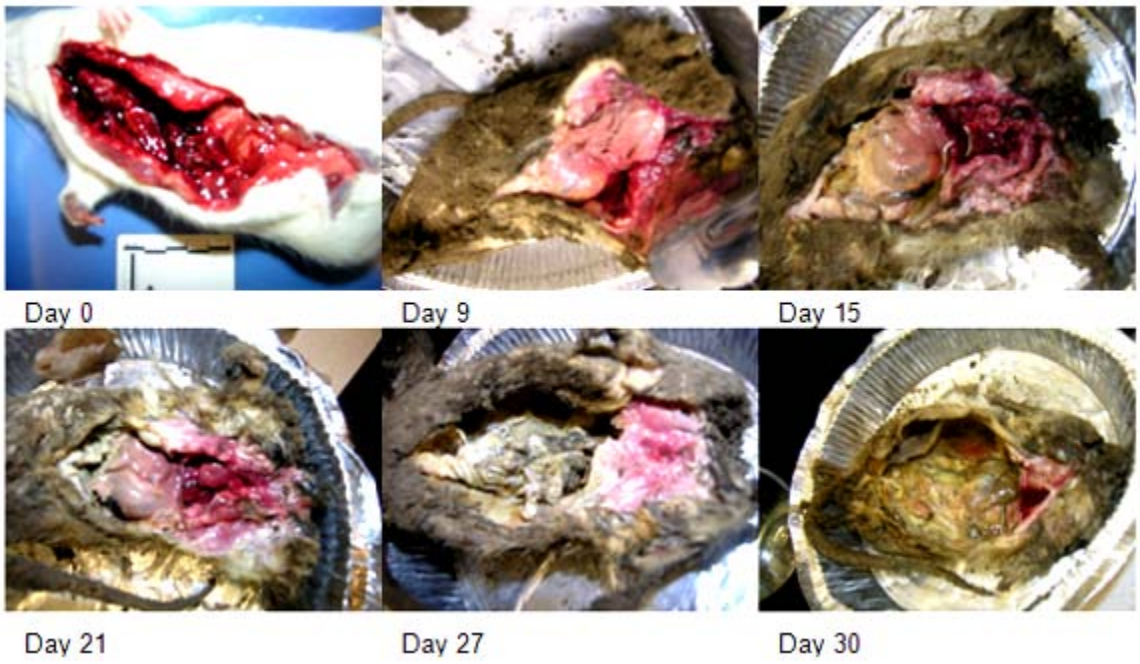


Figure 11: Internal organs of oxycodone-dosed rats upon exhumation.
Organs exhibited decomposition changes as the post burial interval increased.
Discolouration and distension of various internal organs can be observed; liquefaction of organs at Day 30.

Table 17: Stage of decomposition of oxycodone-dosed rat cadavers by post burial interval, n = 3.

Post Burial Interval (Days)	**0**	**3**	**6**	**9**	**12**	**15**	**18**	**21**	**24**	**27**	**30**
Stage of Decomposition	Fresh										
			Inflated								
					Active						

Stages were determined by visual observations.

As the post burial interval increased, the upper and lower surfaces of the carcasses became very distinct; the upper surfaces became desiccated and stiff while the lower surfaces remained moist and pliable due to the gravitational flow of decomposition fluids downwards towards the grave floor (Figure 12). The tissue on the lower surface was very fragile and was susceptible to tearing. Towards the end of the burial trial, a scalpel was not required to access the abdominal cavity.

Figure 12: Upper and lower surfaces of carcass at Day 24.
The soft and moist lower surface (abdomen) is distinct in texture and more pliable compared to the stiff and dried upper surface (back).

The moisture content of the soil increased from approximately 50% water holding capacity (WHC) to 60 to 65% over the thirty-day period; however the pH of the soil varied over time. The initial 6.8 pH of the soil decreased to 5.2 by Day 27. Conversely, liver pH increased as the post burial interval increased from mean pH 6.3 to 8.3 by Day 30 ($p < 0.001$, Figure 13).

Upon collection, all samples were stored at -20°C until analysis. The pH of liver samples collected did not change over time and subsequent analysis did not change with 5 freeze-thaw cycles.

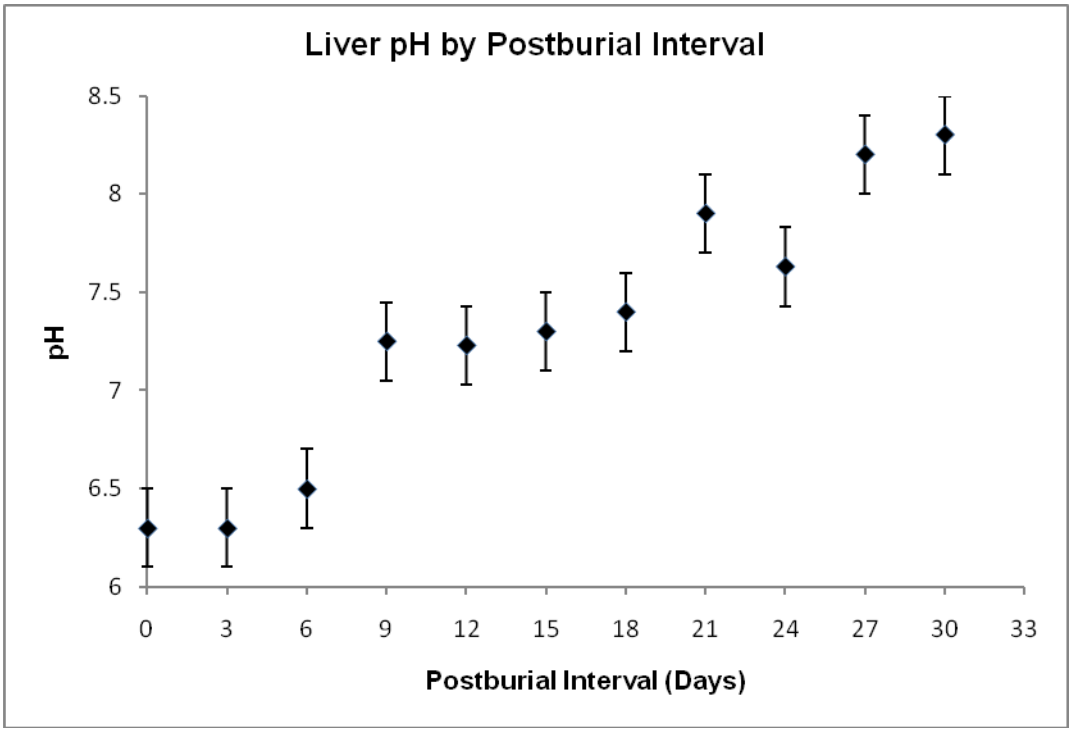


Figure 13: The pH of liver was measured upon exhumation.
ANOVA $F_{(10, 19)} = 11.49$, $p < 0.001$. There was a significant difference between Days 3 and 15 - 30; 6 and 24 - 30; 9 and 12 to 27 - 30.

4.1 Postmortem Changes of Trial 2: Heroin

During the second burial trial with heroin-administered rats, the post burial interval between exhumations was increased from every 3 days to an initial 7 days and then to every 14 days after the first two weeks to increase the total internment period from 30 to 56 days. Rats that were not dosed with heroin were designated as control rats; one control was exhumed with every harvest.

The first set of carcasses exhumed on Day 7 had progressed to the bloat/inflated stage of decomposition (Figure 14, Day 7). The carcasses were extensively inflated in the abdominal and thoracic cavities, upon dissection the internal organs were also inflated with mild discolouration. At Day 14, the carcasses had not progressed beyond the bloat/inflated stage (Figure 14, Day 14).

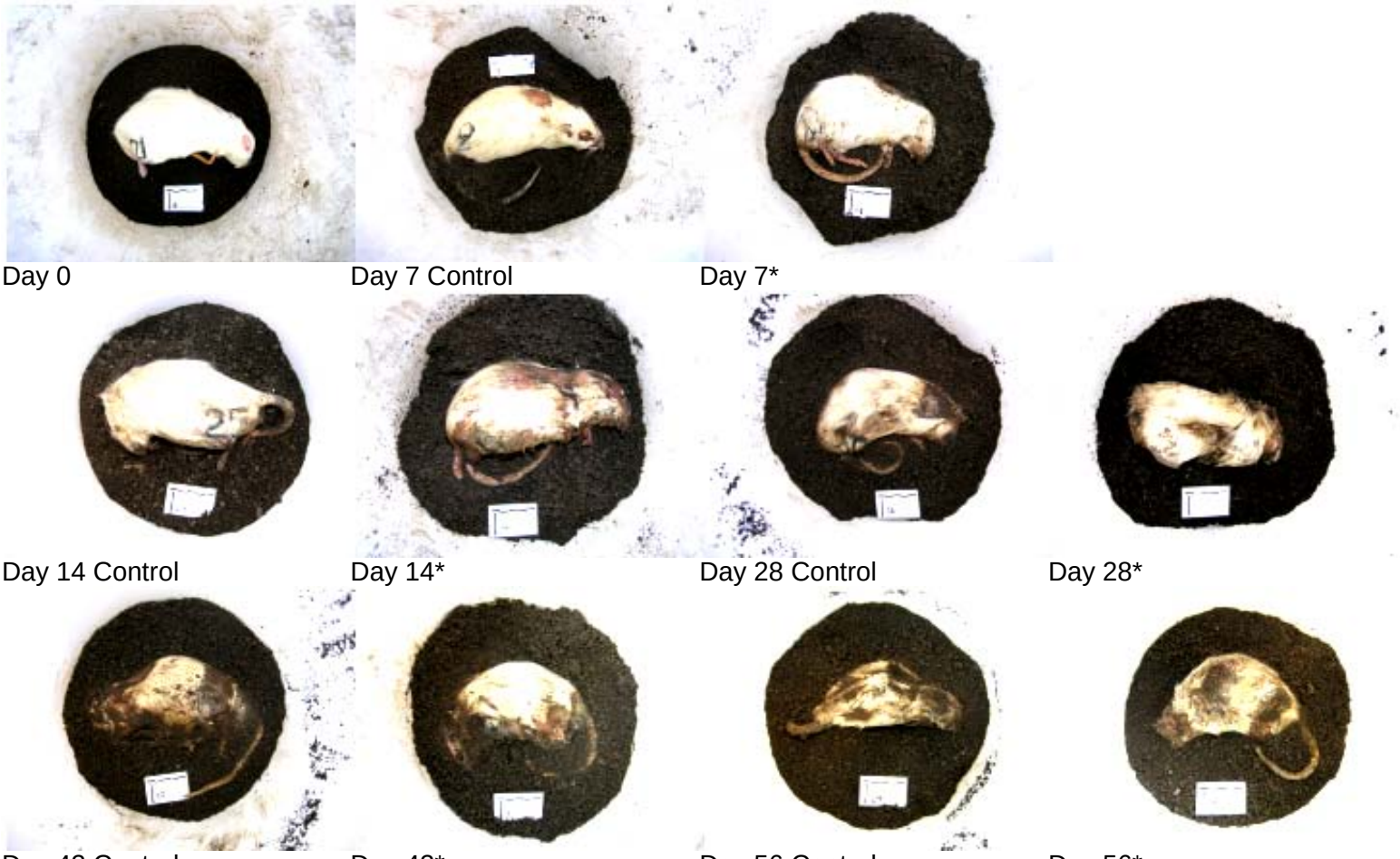


Figure 14: Control and heroin-treated rats (*) sacrificed and buried in soil microcosms.
One control and three heroin-treated carcasses were exhumed at Days 7, 14, 28, 42 and 56.

Active decay was observed in carcasses harvested at Day 28 with ruptures and tears on the surface of the abdominal cavity, presumably from the pressure of putrefactive gases (Figure 15). Disarticulation of the skull was also observed and despite ruptures to the abdominal cavity, the thoracic cavity remained intact.

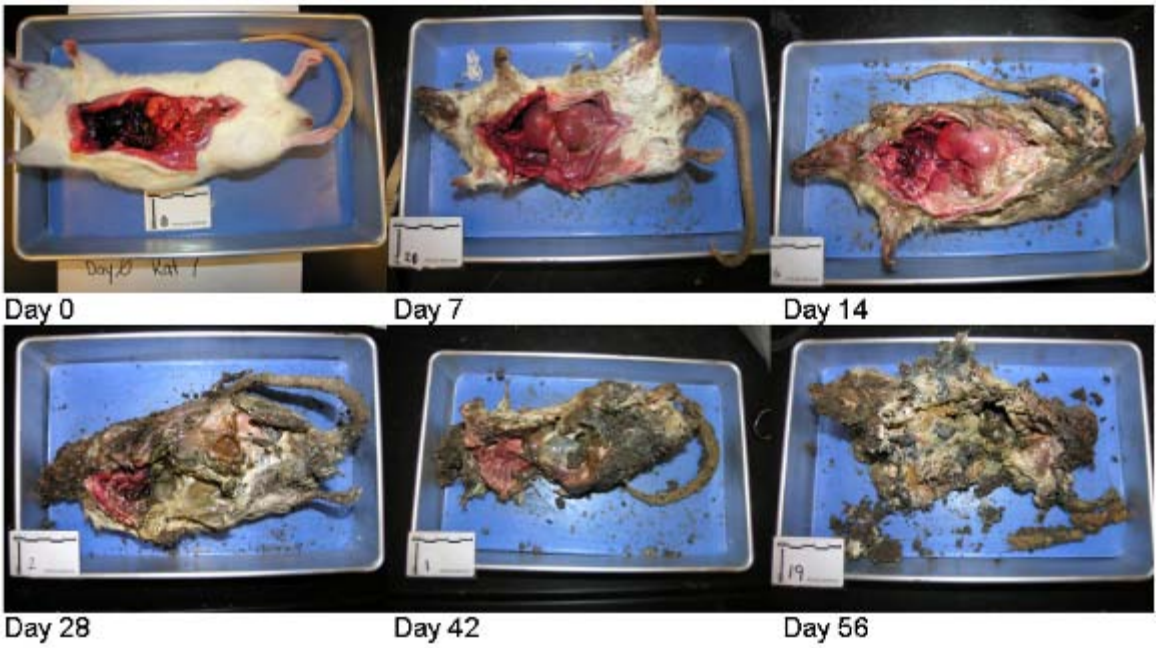


Figure 15: Internal organs of heroin-treated rats from Day 0 to Day 56. Abdominal cavity ruptures were observed from Day 28.

At Day 42, the carcasses were in advanced decay, as the tail and at least one limb of each carcass had either fallen off or tore off upon exhumation (Figure 16). At Day 56, partial mummification was observed on the upper surface of the carcass while the lower portion was moist and marbled in colour. A rupture of the abdominal wall exposed the internal organs, which were partially liquefied. The stomach and the portions of the large intestines ruptured, releasing its contents into the abdominal cavity.

Figure 16: Control Rat (10) and Heroin-treated Rat (1) exhumed on Day 42. Ruptured abdominal walls exposed internal organs.

It is important to note that there were no visible differences with respect to the rate and nature of decomposition between control and treatment rats throughout the decomposition trial (Figure 14). At each harvest, the stage of decomposition was visually observed and categorized as either: Fresh, Inflated, Active, or Advanced Decay (Table 18). Despite the increased interment period from 30 to 56 days, the carcasses did not reach the Dried Remains stages of decomposition.

Table 18: Stage of decomposition of control and heroin-dosed rat cadavers by post burial interval, n = 1 and n = 3, respectively.

Post Burial Interval (Days)	**0**	**7**	**14**	**28**	**42**	**56**
	Stage of Decomposition					
Heroin, n = 3	Fresh	Inflated		Active	Advanced	
Control, n = 1	Fresh	Inflated		Active	Advanced	

There were no observed differences between control and heroin-dosed rats.

Both the grave surfaces of the control rat and the heroin-treated rats (n = 3) with each harvest exhibited similar growth of white fungus, which was not identified (Figure 17). The fungal growth for the second trial followed a similar pattern of growth as observed in the first (oxycodone) burial trial (Figure 10).

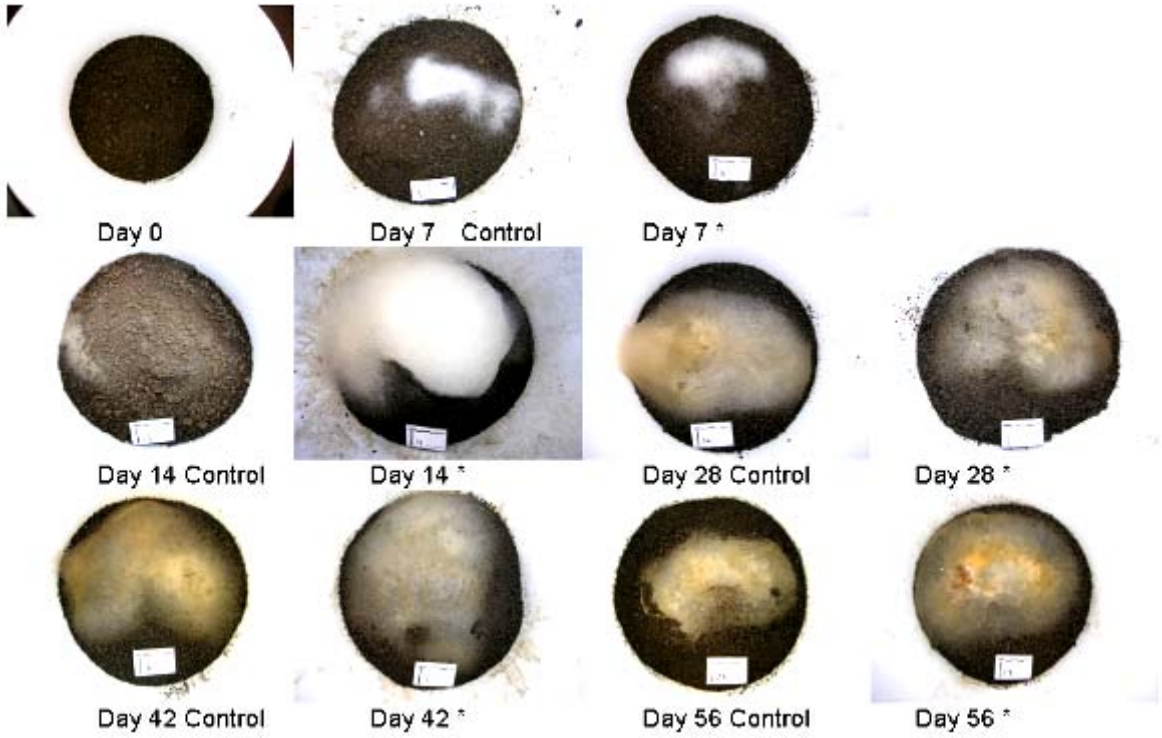


Figure 17: Undisturbed grave surfaces of control and heroin-treated (*) rats (n = 3) from Day 0 to Day 56. Carcasses were exhumed at Days 7, 14, 28, 42, and 56.

There was no significant difference in pH between livers collected from control and heroin-dosed rats. There was also no significant difference in the pH of livers collected from the initial burial trial and the livers collected in the second burial trial ($p = 0.819$). There was a difference of liver pH over time ($p < 0.001$) with the pH increasing as the burial trial progressed. The soil pH decreased as the interment period increased with a sharp drop at Day 14, returning to basal levels by Day 56 ($p < 0.001$).

4.2 Detection of Oxycodone in Samples

Liver samples collected from sequentially harvested exhumed rats were analysed for the presence of oxycodone and its major metabolites, noroxycodone and oxymorphone. The concentration of oxycodone in liver decreased as the post burial interval increased (r^2 = 0.48, $p < 0.001$) (Table 19, Figure 18). Oxymorphone concentrations also decreased as the post burial interval increased (r^2 = 0.47, $p < 0.001$), however there was no significant difference in noroxycodone concentrations. The large standard deviations for noroxycodone and oxymorphone liver concentrations reflect the small sample size, n = 3, and the drug was not detected in all samples analysed.

Table 19: Levels of oxycodone and selected metabolites in rat livers after sequential harvests

PBI Days	Oxycodone	Noroxycodone Mean (± SD) ng/g	Oxymorphone
0	21.94 (± 3.01)	ND	40.2 (± 5.15)
3	23.27 (± 5.61)	6.60 (± 11.43)	27.2 (± 7.81)
6	20.05 (± 4.23)	6.75 (± 11.69)	20.35 (± 5.98)
9	13.00 (± 1.80)	12.45 (± 10.94)	6.00 (± 10.39)
12	11.74 (± 1.70)	ND	12.60 (± 10.92)
15	12.49 (± 2.11)	ND	6.45 (± 11.17)
18	9.16 (±0.82)	trace	ND
21	8.93 (±0.16)	ND	trace
24	10.82 (± 0.40)	ND	trace
27	trace	ND	ND
30	ND	trace	ND

ND = not detectable; trace = below the LOQ (5.9 ng/g) and above the LOD (2.0 ng/g).

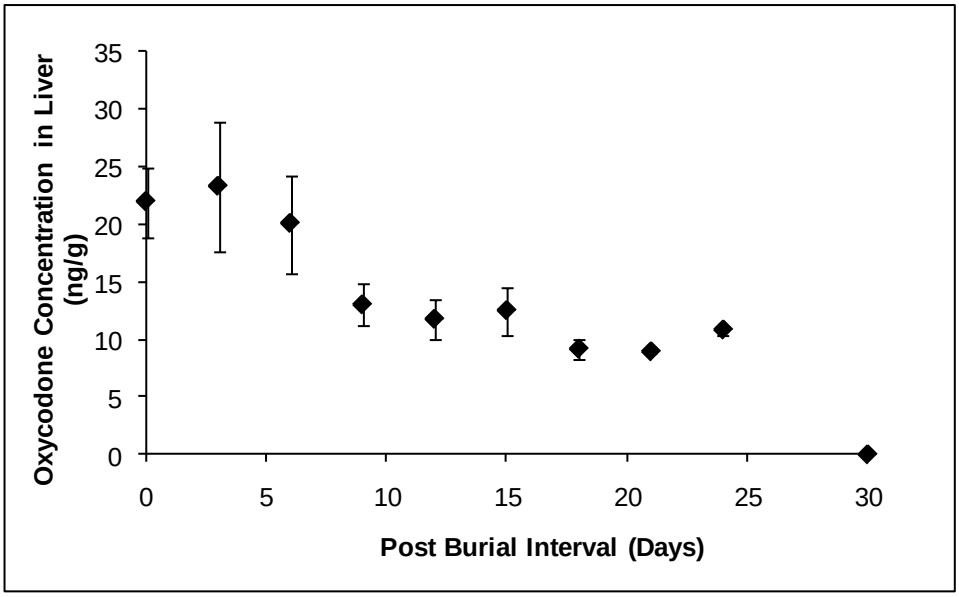


Figure 18: Oxycodone concentrations (mean) in liver in rats exhumed from burial microcosms, n = 3. Burial intervals ranged from 0 to 30 days.

Hair roots from the back of each rat were collected prior to burial. Each rat was then buried and exhumed after a number of days. Hair roots collected after exhumation were analysed and compared to the hair roots collected from the same rat prior to burial. The concentration of oxycodone and its metabolites in each sample are given in Table 20. Oxymorphone was detected in liver samples while the hair samples examined did not contain oxymorphone at quantifiable levels. This may be explained in part by the chemical nature of the drug and the fact that oxymorphone is a minor metabolite of oxycodone (Pöyhiä, Olkkola, Seppälä, & Kalso, 1991). Noroxycodone is the major metabolite formed in the metabolism of oxycodone and it was detected in only 5 of 10 sets of pre-burial hair samples (Day 18 to 30), with only one set at concentrations greater than the limit of quantification.

Table 20: Concentration of oxycodone and selected metabolites in rat hair (n =3)

Analyte	**Oxycodone**		**Noroxycodone**		**Oxymorphone**	
	Mean (±SD) (ng/mg)					
PBI (Days)	**Preburial**	**Postburial**	**Preburial**	**Postburial**	**Preburial**	**Postburial**
0	2.18 (± 1.81)	--	trace	--	trace	--
3	3.00 (± 1.39)	0.83 (± 0.76)	ND	ND	ND	ND
6	3.00 (± 4.44)	1.07 (± 0.93)	ND	trace	ND	ND
9	2.37 (± 0.64)	1.13 (± 0.99)	ND	ND	ND	ND
12	1.73 (± 1.27)	trace	ND	trace	ND	ND
15	2.50 (± 0.44)	ND	ND	ND	ND	ND
18	2.40 (± 1.49)	trace	trace	ND	ND	ND
21	1.67 (± 0.64)	ND	1.1 (± 0.56)	trace	ND	ND
24	0.97 (± 1.34)	ND	trace	ND	ND	ND
27	2.47 (± 3.06)	ND	trace	ND	ND	ND
30	trace	trace	trace	ND	ND	ND

Limit of Quantification= 0.78 ng/mg, Limit of Detection = 0.39 ng/mg; > LOD and < LOQ = trace; < LOD = ND. There is no "Postburial" analytical results for samples collected at Day 0, as those samples were not buried. Preburial = concentration of drug in hair samples collected from rats prior to burial in microcosms. Postburial = concentration of drug in hair samples collected from same rats after exhumation.

The concentration of oxycodone decreased or was not detectable in all hair samples after burial in soil ($r^2 = 0.14$, $p < 0.001$). Oxycodone was not detected in any of the hair of control microcosms. This was expected, as the microcosms did not contain any rats or hair dosed with oxycodone.

Soil collected from Day 3 exhumations did not contain any oxycodone or oxymorphone in two of the three replicates. This is reflective of the stage of decomposition of the rats as fresh rats were exhumed and minimal purging of bodily fluids from the carcasses was observed. From Day 6 to 24, and Day 30, collected soils contained oxycodone in at least 2 out of 3 microcosms. Oxymorphone was only detected in soil on Days 3, 12, and 15.

4.3 Detection of Heroin Metabolites in Samples

All liver samples collected from harvested carcasses were analysed for the presence of morphine and 6-MAM. However, morphine and 6-MAM were not detected in any of the liver samples of the rats studied in the second burial trial. This was not unexpected, as administration of heroin was discontinued 9 days prior to sacrifice and the elimination half-life of morphine and 6-MAM are approximately 2 - 3 hours and 6 - 25 minutes, respectively (Baselt, 2008). Because of the absence of heroin metabolites in liver, the heart tissue collected during this burial trial were not analysed for the presence of morphine or 6-MAM.

Hair collected from the back of rats administered with heroin intravenously for 9 consecutive days followed by nine days of abstinence prior to sacrifice, was collected prior to and after burial in a soil microcosm (Table 21). Preburial concentrations refer to the concentrations of morphine and 6-MAM in hair collected from the specific rats harvested prior to burial. The postburial concentration of morphine and 6-MAM refer to the concentration in hair collected from rats harvested on that specific day.

Morphine and/or 6-MAM were detected in hair samples collected prior to burial by UPLC-MS/MS by monitoring two transition ions utilising positive electrospray ionisation (Figure 19). There was no significant difference between the concentrations of 6-MAM and morphine observed before and after the burial intervals. However it should be noted that 6-MAM was only detected at trace levels after the first harvest interval of seven days and it was not detected in any samples collected from the harvested carcasses from day 14 to 56. Morphine was detected in four out of five sets of preburial samples; however analysis of samples collected from harvested carcasses from day 7 to 56 were all positive for morphine (Table 21). There was a large standard deviation of morphine and 6-MAM. This may be due to the small sample set (n = 3) and high variability of concentrations between samples.

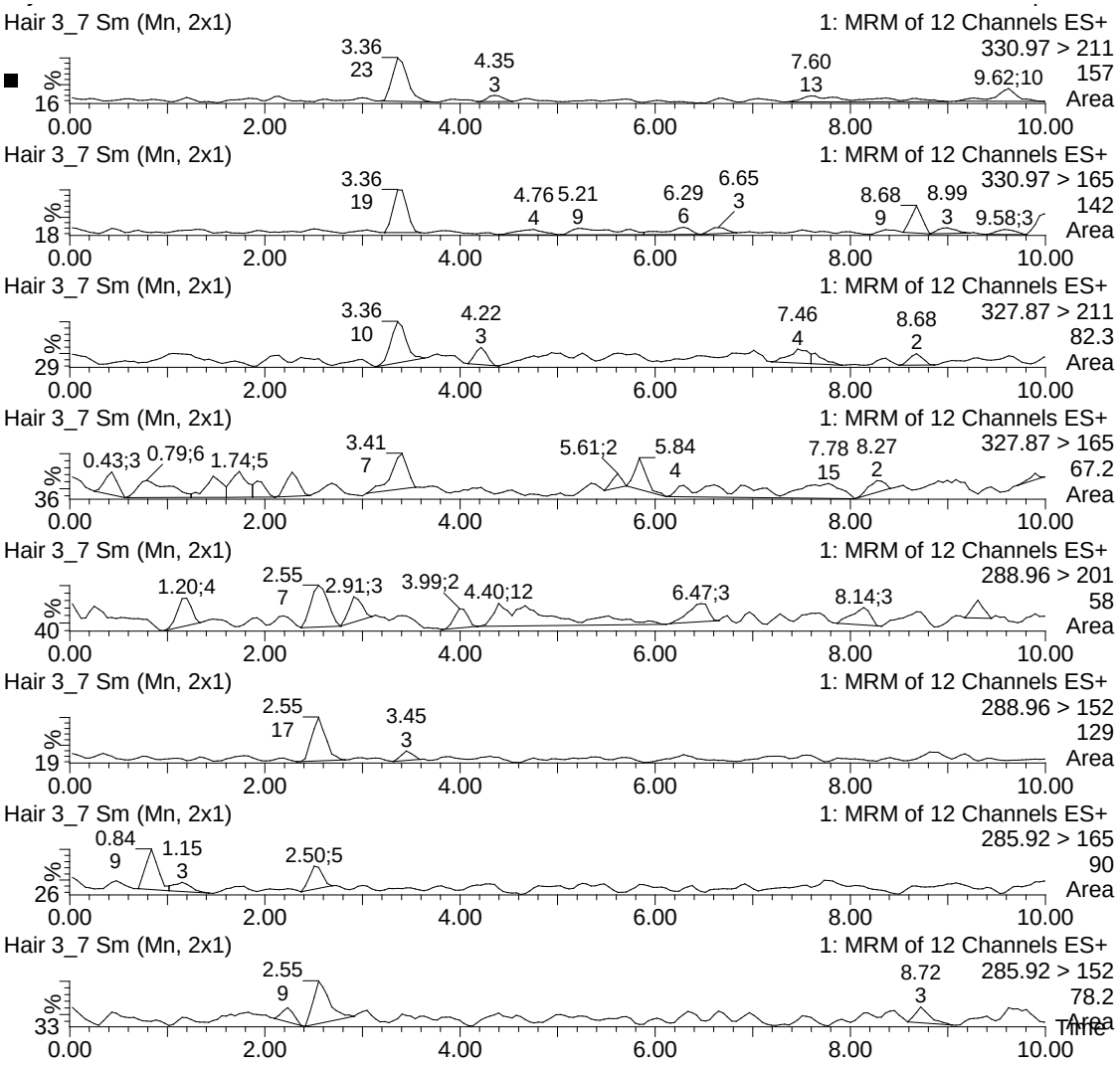


Figure 19: Extracted ion chromatograms of hair collected from a heroin-dosed rat, at postburial interval of 7 days, from 0 to 10 minutes.
Transition ions for 6-MAM-D_3: 330.97/165 and 330.97/211 at 3.36 minutes; 6-MAM: 327.87/165 and 327.87/211 at 3.36 minutes; morphine-D_3: 288.96/152 and 288.96/165 at 2.55 minutes; and morphine: 285.92/152 and 285.92/166 at 2.55 minutes.

Table 21: Concentration of morphine and 6-monoacetylmorphine (6-MAM) in rat hair

Post Burial Interval	**Morphine**		**6-MAM**	
	Mean (±SD) (ng/mg)			
(Days)	**Preburial**	**Postburial**	**Preburial**	**Postburial**
0	10.47 (± 18.13)	--	119.82 (± 206.27)	--
7	0.91 (± 1.57)	19.47 (± 32.00)	trace	trace
14	1.53 (± 2.66)	3.38 (± 5.23)	16.24 (± 23.67)	ND
28	ND	3.34 (± 5.78)	1.37 (± 1.37)	ND
42	1.53 (± 2.64)	24.31 (± 31.37)	16.61 (± 22.74)	ND
56	ND	1.37 (± 2.37)	3.14 (± 1.85)	ND
0 control	ND	--	ND	--
7 control	ND	ND	ND	ND
14 control	ND	ND	ND	ND
28 control	ND	ND	ND	ND
42 control	ND	ND	ND	ND
56 control	ND	ND	ND	ND

Morphine Limit of Quantification (LOQ) = 0.08 ng/mg, Limit of Detection (LOD) = 0.04 ng/mg; 6-MAM LOQ = 0.31 ng/mg, LOD = 0.15 ng/mg. > LOD and < LOQ = trace; < LOD = ND. There is no "Postburial" analytical results for samples collected at Day 0, as those samples were not buried. ND = not detected. Preburial = concentration of drug in hair samples collected from rats prior to burial in microcosms. Postburial = concentration of drug in hair samples collected from same rats after exhumation.

Morphine and 6-MAM were not detected in any of the grave soils. This is reflective of the absence of the drug analytes in collected liver samples. Drug and drug metabolites incorporated within the hair of the carcasses may have leached or diffused into the soil, however as the drug content was determined to be in the range of ng/mg of hair, the total drug content was minimal and below the limit of detection of this assay. Control rats were not administered heroin, thus the negative results for morphine and 6-MAM were expected.

Chapter 5
Discussion

5.0 Discussion

A model to study taphonomic and toxicological changes in rats exposed to a burial environment was developed. The present study examined hair roots collected from oxycodone and heroin dosed rats, which were collected prior to and after burial for a specified period of interment in a burial microcosm. The effects of the decomposition process on the stability of the drugs incorporated into the hair roots were examined. Although previous studies have examined the effects of decomposition on the morphological structure of hair (Pötsch et al., 1995; Tebbet, 1999; Wilson et al., 2001; Wilson et al., 2007)); the rate of drug incorporation into the hair shaft (Gygi, Wilkins, & Rollings, 1995; Gygi, Joseph, Cone, Wilkins, & Rollings, 1996; Rothe et al., 1997; Toyo'oka et al., 2001); the stability of the drugs in collected hair samples of known addicts (Gaillard & Pépin, 1998; Girod & Straub, 2001); or the stability of drugs in hair samples collected from mummified subjects (Cartmell et al., 1991), no other published studies have examined the effect of an actively decomposing body on the stability of incorporated drugs in hair.

5.1 Method Selection and Optimisation

To examine the drug content of hair samples, protocols on the collection, preparation, extraction, and analysis of samples were developed. The collected hair samples underwent a decontamination process prior to analysis to minimize any potential external bias. Samples were rinsed in a non-polar halogenated solvent, which were subsequently analysed for the presence of drugs. Methanol was chosen as the extraction solvent for hair as all the analytes of interest were soluble in methanol and was also amiable to sample reconstitution for analysis on the UPLC-MS/MS. Use of methanol as the choice of extraction solvent was supported by Stout, Ropero-Miller, Baylor, and Mitchell (2007). Their study demonstrated that methanol was capable of lifting the scales of the hair shaft and solubilising the cell membrane complex without damaging the structure.

Methanol also causes the hair shaft to swell, which enables the solvent to remove more lipid from hair than non-swelling solvents like dichloromethane (Robbins, 2002). Thus using dichloromethane as a decontamination rinse prior to a methanol extraction should have only removed external contaminants from the hair shaft. To ensure that the rinse solutions did not penetrate the hair shaft, the rinse solution was analysed for the presence of oxycodone or heroin metabolites.

When selecting hair for drug analysis, the site of hair collection was taken into consideration. The back or dorsal surface of the rat was chosen as the site of sample collection, despite the large surface area available. The hair on the abdomen, lower surface and extremities was avoided as the potential for external contamination was greater, i.e. such surfaces would have a greater likelihood of coming into contact with urine, fecal material, and saliva, which may have contained drug and drug metabolites. The pattern of decomposition may have an impact upon the microenvironment upon which the sampled hair was exposed to. For example, hair collected from the perianal region would be more susceptible to ante- and postmortem contaminants as the anus, an existing orifice, would be a route for fluid expulsion during active decomposition (Pinheiro, 2006). Another consideration is that decomposition may vary within the carcass itself, as was observed in the burial trials; one portion of the body was desiccated while another portion remained moist and putrefied (see Figure 12).

5.2 Experimental Approach

Postmortem redistribution (PMR) is a term used to describe the drug concentrations in samples collected postmortem, which may not accurately reflect antemortem drug levels as the concentrations of various drugs may increase or decrease after death. Many studies regarding the postmortem redistribution and stability of drugs have utilised human subjects obtained from autopsies conducted through medical examiner's or coroners' offices (Stevens, 1984; Pounder, Hartley, & Watmough, 1994; Moriya & Hashimoto, 1997; Hadidi

& Oliver, 1998; Moriya & Hashimoto, 1999). The use of animal models including the pig, rabbit and rat has also shown reasonable agreement with human cases when studying PMR (Hilberg et al., 1992; Pounder et al., 1994; Hedouin et al., 1999; Hilberg, Rogde, & Mørland, 1999; Moriya & Hashimoto, 1999), thus rats were utilised for this study. Another reason for using a human analogue was due to the ethical issues and availability of drug positive human hair and decomposed remains. The use of rats as human analogues has been well established in forensically relevant pharmacological as well as taphonomic studies (Tomberlin & Adler, 1998; Aturaliya & Lukasewycz, 1999; Hilberg et al., 1999; Casamatta & Verb, 2000; Carter et al., 2007). Sprague-Dawley rats administered oxycodone or heroin were used to study the stability of drugs incorporated in hair postmortem and controlled laboratory experiments. The use of the rats also helped to control the dose, route of drug administration, drug use history and manner of death, which are forensically relevant factors. The collection of hair from rats prior to burial in the soil microcosms allowed for direct comparison of how the decomposition process would alter the drug content in each individual rat, as hair was collected from the same specimen and tested for its drug content.

The burial microcosms utilised in this study attempted to control variables such as grave depth, soil type (including initial soil pH and moisture level), air exchange, and temperature. The control rats in the second trial demonstrated that the rate of decomposition was unaffected by previous drug exposure as visual observations were similar between the heroin-treated rats and the control rats. The heroin-administered rats had no heroin injected for a period of nine days prior to sacrifice, thus the drug and drug metabolites were cleared from the body's blood and urine (Baselt, 2008) as it was unknown if the presence of heroin may have had an effect on the activity of the bacterial microorganisms which utilised the body as a nutrient source.

5.3 Progression of Decomposition

For both burial trials, as the burial interval increased, the carcasses progressed through the Fresh, Inflated/Bloated, and Active stages of decomposition. In the second trial the carcasses progressed to Advanced Decay. The pH of the soil and tissue fluctuated as the rats decomposed; the soil pH was initially measured at 6.8 and decreased to 5.0 at day 27, where as the liver pH was initially measured at 6.1 and increased to 8.5 by the end of the burial trial at day 30 (see Figure 13). The changes in relative pH may be due to the activity of the autolytic enzymes present within the body and the formation of putrefactive amines. The total mass of the carcasses also decreased over time (see Appendix A and B) while the soil moisture increased, however the total mass of the burial microcosm did not change over time, and the loss in mass of the carcasses may be attributed to purging of bodily fluids and moisture into the soil.

The first burial trial with oxycodone had a harvest interval of 3 days, for a total decomposition period of 30 days. The rats in this trial did not progress beyond the active stage of decomposition. In an attempt to capture the later stages of decomposition (Advanced and Skeletonisation/Dried Remains) in addition to the early stages thereby examining the entire decomposition process, the harvest interval was increased to 7 then 14 days, for a total harvest interval of 56 days. The rats in the second trial did not progress beyond the initial portion of Advanced decomposition, which may last for months before progressing to dried remains (Rodriguez, 1997; Pinheiro, 2006; Ferner, 2008). It was expected that by extending the total burial interval, the pH of the soil may return to basal levels. The pH of soil in the second burial trial was initially measured at 6.2 and decreased to 5.0 by day 14 and returned to basal levels by day 56. However the liver pH did not return to basal levels as the soil pH did; instead the liver pH continued to increase from an initial 6.0 to 8.0 by day 56. It was hoped that soil pH could be linked to tissue pH and drug stability, however more work is required. Research conducted in 1995 and 1996 (Pötsch et al., 1995; Pötsch & Skopp,1996) examined the stability of incorporated drugs in hair upon exposure

to cosmetic treatments, including perming, bleaching, and colouration of hair, and incubation in soil and water. However the hair samples were all collected from known users (i.e. clipped from the scalp) before being subjected to various treatments. Although hair is essentially inert, it has the ability to incorporate drugs and various compounds through passive contamination and diffusion from the intact root after death (Kintz, 2004). The cosmetic treatments would have exposed hair specimens to different pH environments, including a basic solution for perming and an acidic solution for bleaching, both of which resulted in decreases of drug content. Soft tissue decomposition exposes the hair shaft to a unique mixture of chemical compounds not observed in just soil or water, i.e. blood and putrefactive gases/liquids. Although Pötsch et al. (1995) demonstrated that exposure of opiate positive hair to soil for a period of six months decreased the drug content, the rate of drug instability and etiology for the decrease in drug content were not identified. This research measured the drug content in hair, which was freshly plucked from decomposing carcasses buried in soil. The oxycodone concentrations decreased in hair as the burial interval increased in soil and this may be due to diffusion of drugs out of the hair shaft as it was exposed to the moisture of the soil and putrefactive fluids of the body.

The increase in liver pH observed in both burial trials may be due to cell autolysis and the putrefactive processes related to decomposition. Microorganisms present in the intestinal organs can multiply and spread to breakdown connective tissue to release cellular components, including pH sensitive autolytic enzymes. The decomposition of nitrogen containing proteins to form amines can lead to the increase of pH in tissue, as was observed in liver samples (Stevens, 1984; Gill-King, 1997; Skopp, 2004).

The organic matter released by decaying carcasses, including putrefactive gases and liquids such as methane, hydrogen sulphide and lactic acid, are incorporated into soil, to form a decomposition island, which is rich in nitrogen, carbon and phosphorus. The decomposition of carbohydrates from the decaying body in soil may have resulted in the observed initial decrease in soil pH.

5.4 Effect of Decomposition on Soft Tissue

The pH of liver samples increased from approximately 6.0 to over 8.0 as the decomposition trial progressed. Under physiological conditions, plasma pH is approximately 7.4 and cellular pH is approximately 5.0 (Ferner, 2008). Weak bases in their nonionized state in the plasma can permeate through cell membranes due to the pH gradient to accumulate in tissues. With the increase in pH, the weak bases, including oxycodone, may diffuse back out of the tissue and into the extracellular fluid of the body. As the liver pH increased, the oxycodone in the liver may have diffused out of the tissue and into neighbouring organs and the vascular compartments. With purging of bodily fluids from the carcass, the oxycodone would have diffused out of the body and into the surrounding environment, namely the soil. Oxycodone was detected in the majority of soil samples from day 6 and onwards, which would be consistent with visual observations of the carcasses undergoing bloating and purging from the inflated and active stages of decomposition.

In the second burial trial, both the liver and heart were collected from each carcass. Because of the negative drug findings of the liver samples, the collected heart samples were not analysed for the presence of morphine or 6-MAM. The samples were analysed for their pH, however the pH of the heart homogenates and liver homogenates were not significantly different (see Appendix B) from each other. Upon dissection, the diaphragm was found to be intact in most of the carcasses exhumed; at days 42 and 56 the structures of the diaphragms were compromised (see Figure 15, Day 42 and 56). The intact diaphragm can act as a physical barrier, to shield the heart and lungs from the putrefactive liquids and gases formed from the intestinal organs. When the carcasses progressed from the inflated stage to deflated/active decay stage, the heart remained largely protected, thus it was expected that there would be a difference between the pH of the heart and liver as the liver would have been exposed to a greater variety of putrefactive enzymes from its proximity to the digestive system and the enzyme load of the liver itself. That there was no significant difference between the pH of

the heart and liver was unexpected; however this may be due to the fact that the vascular system was relatively intact and it was exposed to the same burial environment.

5.5 Oxycodone in Liver

The burial microcosms were maintained at room temperature (20-22 degrees Celsius) and the livers of oxycodone-dosed rats were collected upon exhumation. The concentrations of oxycodone in liver could not be established in each carcass prior to burial in the soil microcosms as this would have required harvesting part of the organs. The analytical extraction chosen for liver sample analysis was protein precipitation by acetonitrile. Concentrations of oxycodone and its metabolites decreased as the post-burial interval increased. Oxycodone was not detected in the last set of replicates exhumed at Day 30, while noroxycodone and oxymorphone were not detected from days 12 and 18, respectively. This decrease in drug content may be due to postmortem degradation and/or metabolism by bacteria and other microorganisms present during the putrefactive process. Moriya and Hashimoto (1997) demonstrated with *in vitro* experiments that conjugated morphine converted to free morphine in liver samples at 18 - 22 and 37 degrees Celsius during a ten-day period, resulting in an apparent rise in free morphine concentration over time. Robertson and Drummer (1998) reported that nitrobenzodiazepines, including clonazepam, nitrazepam and flunitrazepam, are metabolised to their respective 7-amino metabolites in various tissues by enterobacteria containing oxygen-sensitive nitroreductase enzymes. These results are in good agreement with previously published studies regarding the stability of drugs in postmortem samples, as longer postmortem intervals can lead to unexpected findings following the analysis of liver (Stevens, 1984; Pounder & Davies, 1994; Pounder et al., 1994; Moriya & Hashimoto, 1997).

5.6 Oxycodone in Hair

Studies published by Gygi et al. (1995, 1996) have determined the distribution of codeine and morphine into rat hair after long-term daily dosing over a time course of 21 days, as well as plasma and hair concentrations of codeine and its metabolites in male Sprague-Dawley rats over a five-day dosing regime. Toyo'oka, Tano, Kato, and Nakahara (2001) established that a single intraperitoneal administration of morphine hydrochloride can be detected in hair shafts and roots of Dark Agouti rats. In this study, Sprague-Dawley rats were administered oxycodone or heroin over a five or nine-day period, respectively. The oxycodone-administered rats were sacrificed on their last day of treatment; however the heroin-administered rats were not administered any drug for a period of nine days prior to sacrifice. Both treatment periods were sufficient to incorporate the administered drug or drug metabolite into the hair shaft of the rat at a level that could be detected by the current method of analysis.

Oxycodone was detected in hair root samples of rats administered oxycodone for a period of five days intravenously prior to sacrifice; however its metabolites, noroxycodone and oxymorphone were not detected at quantifiable levels. The concentrations of oxycodone decreased in all samples regardless of the interment period (range: 3 – 30 days). In selected samples (Day 15 and onwards), oxycodone was no longer detected in quantifiable levels in hair after exhumation. Although there have been other studies regarding the analysis of postmortem hair samples for the presence of oxycodone (Deveaux & Pépin, 2006; Moore, Marinettii, Coulter, & Crompton, 2008), there have been no published reports regarding the stability of oxycodone in hair collected from decomposing bodies. Factors which may have contributed to the decrease in drug concentration include the presence of soil microbes, increased moisture content of the soil and changes to soil pH, and exposure of the hair roots to putrefactive liquids produced by the carcass as decomposition progressed. Water is a fundamental component of hair and the moisture content of hair alters in relation to the relative humidity of the environment (Downes, 1961). The

amount of moisture in hair can affect its physical properties. The peptide bonds and hydrophilic side chains of keratin fibers contribute to water sorption; at humidity below 25%, water is principally bound to hydrophilic sites of the fibre by hydrogen bonds. As the humidity increases, additional water is sorbed and as the humidity increases above 80%, multimolecular sorption (water binding to water) is evident (Robbins, 2002). As hair is exposed to liquid, the cuticles and hair shaft swell and open up. This creates a possible opportunity in which drugs present in the hair shaft may leach out to effectively decrease the concentration upon exhumation and analysis. The drug bound to hair may either be displaced by the water or diffuse out of the hair shaft as a result of a concentration gradient.

The unidentified white fungal growth on the carcasses observed from day 3 and onwards may have also contributed to the decrease in drug content (see Figure 10, Figure 17). Soil and intestinal microorganisms in addition to fungal growth and activity can alter the structure of hair (Wilson et al., 2007), thus altering any compounds which may be incorporated within the hair shaft. Fungal tunnelling of the hair shaft would have compromised the physical structure of the hair shaft allowing for greater access of soil microbes to utilize the hair as a nutrient source. Subsequently the drug content of the hair would be expected to decrease in relation to the structural integrity of the hair. The hair collected from exhumed cadavers was not examined at a microscopic level, however they were noticeably less pliable and rigid compared to samples collected prior to burial. The rigidity may be due to exposure to the fungal growth or decomposition fluids.

5.7 Heroin Metabolites in Hair

Heroin is quickly deacetylated to 6-monoacetylmorphine (6-MAM) and morphine in the body and the presence of 6-MAM in biological fluids and hair is required to distinguish between heroin and morphine administration (Society of Hair Testing, 2004). Because of the short half-life of heroin and its metabolites in blood and urine, hair is a better matrix to analyse when attempting to establish a history of

drug use or abuse, i.e. previous administration of heroin, as drug and drug metabolites can be detected in hair long after drug exposure (Polettini, Stramesi, Vignali, & Montagna, 1997; Girod & Staub, 2001; Darke et al., 2002; Jones, Tomlinson, & Moore, 2002; Druid et al., 2007; Kronstrand et al., 2007). The major metabolites of heroin, namely morphine and 6-MAM, have been positively identified in hair of known addicts (Girod & Staub, 2001). 6-MAM and morphine in the majority of samples, were identified in hair samples collected from preburial heroin-administered rats, thus confirming that the rats were administered heroin and not morphine, which is in good agreement with other published studies regarding the presence of 6-MAM in hair of known heroin users (Kronstrand, Grundin, & Jonsson, 1998) or in animal models administered heroin (Nakahara, Takahashi, Shimamine, & Saitoh, 1992; Nakahara, Kikura, & Takahashi, 1994; Gaillard & Pépin, 1998; Girod & Staub, 2001).

After nine consecutive days of heroin treatment, the rats in this study underwent a nine-day period of abstinence, thus with the short half-life of heroin and its metabolites, any trace of their drug use history would not be evident in tissue or fluid (blood or urine) analysis. In this study, all liver samples analysed for the presence of 6-MAM and morphine were negative, thus if trying to establish a drug history of these rats, liver analysis would not have been informative. Analysis of preburial hair samples on the other hand, provided evidence of past heroin administration, which may be helpful in a forensic investigation. However, the 6-MAM detected in preburial samples (trace levels in one set of samples) was not detected at quantifiable levels in samples collected from the same rats after burial. The degradation of 6-MAM is most likely due to non-specific hydrolysis upon exposure to the moist environment created by the decomposition process and autolytic enzymes present in the body.

Morphine was detected in hair samples collected from rats, which were exhumed on days 7, 14 and 42. The corresponding postburial samples collected were still positive for morphine. An interesting finding was that there were two sets of preburial samples in which morphine was absent or present at levels below the

limit of detection or quantification (Day 28 and 56), however the corresponding postburial samples were positive for morphine. This may be attributed to the decomposition of morphine's parent compounds, either heroin or 6-MAM and/or sample variability. It was observed that 6-MAM was not stable during the burial process, and as morphine is a product of 6-MAM hydrolysis, it is possible that the morphine was produced postmortem during the decomposition process. As part of the validation procedure, a QC sample containing only 6-MAM was analysed to demonstrate the stability of 6-MAM during the extraction and analysis process, thus any degradation attributed to 6-MAM in the samples was not due to the analytical method.

As putrefaction and decomposition progress with the postburial interval, the determination of 6-MAM's presence becomes more difficult. The ability to distinguish between heroin and morphine administration cannot be made if 6-MAM is not detected, thus when analysing samples from an exhumed individual, it may not be possible to confirm heroin use as part of their drug history through hair analysis. Because morphine was still detectable in samples after 56 days, the exposure to an opioid cannot be excluded.

5.8 Limitations

The hair of the rats was in different stages of growth and therefore the drug uptake would have been variable between individual strands. To account for this, in future research, the area of sample collection could be shaved. The rats would then be dosed with the drug and as the hair grew back it would be taken up by all of the hair at the same rate. This recommendation would eliminate this variable in the present research, which at this point cannot be accounted for.

Another confounding factor is that the dose of any particular drug has not been correlated to concentrations found in hair. The pharmacokinetics of drugs in hair has not been established or studied as extensively as the disposition of drugs in

blood and urine. Thus concentrations of oxycodone and heroin metabolites found in hair may not correspond to the dose administered, which may also account for the large variability in hair samples analysed for morphine and 6-MAM.

This study only examined the disposition of two opiates, oxycodone and heroin, in non-pigmented rat hair. Drugs with different chemical characteristics may have greater stability than the two examined. For example, drugs with greater lipophilicity may be more stable in hair as the hair shaft would act as a lipophilic reservoir, which would be resistant to hydrolytic actions of the decomposition process. Other drug characteristics which may affect stability include pKa and molecular size, as a drug with a simple small chemical structure may diffuse through cellular membranes more freely than a larger complex structure. .

The hair samples examined in this study were derived from Sprague-Dawley rats, which have nonpigmented (white) hair. As the drugs that were administered are weak bases (i.e. oxycodone) and amphoteric (i.e. morphine derived from heroin), it can be expected that pigmentation of hair would have an effect on the initial concentrations of drug as one study found that black hair incorporated codeine (a weak basic drug) up to 44-times greater than nonpigmented hair in rats (Gygi et al., 1996). The effect of decomposition on the stability of drugs may or may not change, as the exact mechanism and components of drug incorporation into hair has not been clearly elucidated. Thus it may be of interest to repeat the burial trials in pigmented hair, as it could lead to different results. Ideally, if the studies could be replicated in rats which contained both nonpigmented and pigmented hair, drug uptake and stability could be examined within the same animal. The stability of oxycodone and heroin metabolites may be greater, as melanin may be an important drug binding site (Hubbard, Wilkins & Rollins, 2000). If melanin is more resistant to degradation in a burial environment and it also serves as a significant binding site for drugs, it can be expected that there would be greater drug stability in such samples.

The first burial trial lasted 30 days, while the second burial trial was extended to 56 days. However with both trials, the rats did not progress beyond active decomposition. If the remains were allowed to progress to skeletonisation, where no tissue would be available for analysis, the results of hair testing may be more important as it would be the only specimen remaining other than bone. Oxycodone was not detected in samples exhumed at the later portion of the decomposition trial in levels above the limit of quantification after only 12 days. Morphine was detected in hair after 56 days, however because of the limited number of rats and the destructive nature of the harvest, it was not determined if morphine would be detected in hair samples from skeletonised remains.

5.9 Future Work

Factors such as body positioning, grave depth, absence/presence of clothing, and desiccation rate were not examined and were beyond the scope of this study; it is recognized that such factors may influence the rate of decomposition (Aturaliya & Lukasewycz, 1999). For example, an outdoor surface burial would have exposed the carcasses to insects and scavengers, resulting in an accelerated rate of decomposition which may have produced limited sample availability (Carter et al., 2007).

Further work should be explored to determine how the burial environment and/or the general process of decomposition altered the concentrations of drugs incorporated into hair. An outdoor burial trial would expose carcasses to an environment which would have fluctuating temperatures and moisture, as well as potential scavenging by insects and animals. The rate of decomposition should not be very different from the rate of decomposition observed with the indoor burial trials during the summer months; however in Southern Ontario, the winter season would result in exposing the carcasses to sub-zero ground temperatures, essentially halting the decomposition process.

Surface trials could be undertaken to determine the effects on drug stability. As the decomposition process would be expected to occur at an accelerated rate, compared to burial trials, the oxycodone and/or heroin metabolites may have decreased in concentration at a faster rate. Outdoor surface decomposition trials also have other variables to consider, including temperature fluctuations, moisture and humidity changes, scavenging and insect activity, as well as UV exposure from sunlight. Exterior environments expose the body to weathering. Weathering effects in human hair have been studied by comparing tip ends to root ends. Robbins (2002) examined the effects of ultraviolet light on hair and hair from root and tip sections to examine sulphur in hair. The data suggests that cystine and tryptophan in hair is sensitive to photochemical degradation (Robbins, 2002). As weathering can alter the basic structure of hair, it can be expected that any drugs incorporated within the hair structure would also be compromised, as demonstrated by Skopp et al. (2000). Their study collected cannabinoid positive hair and exposed them to various storage conditions; eleven different samples exposed to natural sunlight for ten weeks all decreased in cannabinoid content.

To study the effect of surface decomposition in an exterior environment, drug administered rats could be placed on a soil surface in an outdoor field environment and allowed to decompose. Hair could be collected from the decomposing carcasses at various stages of decomposition, while monitoring soil moisture, soil pH, precipitation, temperature, and UV radiation.

The fungal growth observed during both burial trials may have influenced the stability of drugs in hair, as fungal tunnelling can alter the basic structure of the hair shaft. Attempts to characterise the white growth should be undertaken in future work as it may play a role in hair integrity and drug stability. In future burial microcosms, any fungal samples should be collected and characterised. Drug positive hair could be inoculated with either soil or fungal samples to determine its effect on hair integrity and drug stability.

Because of the short dosing regime of the rats for both burial trials (five to nine days of drug exposure), segmental hair analysis was not performed. If the rats had been acutely and/or chronically exposed to a drug or mixture of drugs over a longer time period, segmental hair analysis could have been undertaken and the stability of drugs incorporated in the mid to distal ends of the hair shaft could be examined. The hair roots examined in this study were in closer proximity to the epidermis than the soil, thus the rate of drug stability may differ between different regions of the hair shaft.

Although hair is a matrix, which is generally considered to be a convenient sample of analysis because it is less intrusive and more readily available than urine or blood, utilising hair in postmortem toxicology analysis may have more pitfalls than advantages if the results are taken out of context.

Chapter 6
Conclusion

6.0 Conclusion

Methods for the detection and quantification of oxycodone, noroxycodone, oxymorphone, 6-monoaceytylmorphine and morphine using LC-MS/MS in soil, liver, and hair were developed and validated. One MRM transition was selected for the analysis of oxycodone and its metabolites. However with the second burial trial and set of method development, two MRM transitions were selected for the analysis of heroin metabolites for improved selectivity.

The oxycodone concentrations within the liver of treated rats decreased as the post mortem/burial interval increased. Over a 30-day period, the concentrations were assessed and oxycodone was not detected after 30 days. Heroin metabolites were not detected in any of the liver samples, thus the stability of morphine and 6-MAM in liver samples could not be examined.

The concentrations of oxycodone and heroin metabolites were assessed in hair. Hair samples collected before burial were compared to hair samples collected upon exhumation from burial microcosms. The concentrations of oxycodone decreased as the interment period increased and were not detected at levels above the limit of quantification after day 12. The concentration of 6-MAM was detected in preburial samples but not detected in postburial samples, whereas concentrations of morphine fluctuated and was detected in some previously negative samples and for as long as 56 days postburial, however the differences were not significant.

Heroin metabolites and oxycodone can be detected in hair even after dosing periods as short as five to nine days. Testing for the presence of drugs in hair can be more informative than testing in liver as the window of drug detection is greater in duration in hair than in liver as was the case for the heroin-administered rats. Hair analysis has the potential to be used to help establish a partial drug history of the decedent, which may also help in the identification of unknown remains. However as decomposition progresses, the absence of certain analytes should not be interpreted as abstinence of drug use as this study

has shown that 6-MAM and oxycodone within hair are not stable in burial environments. Within each set of hair samples, variations in drug concentrations were observed and the different drugs showed different degrees of stability in hair; 6-MAM was detected at only trace amounts after just seven days of burial; oxycodone was still detected in samples after two weeks of burial; whereas morphine was detected in samples exhumed after 42 days.

Chapter 7
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7.0 References

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Chapter 8
Appendices

8.0 Appendix A Trial 1 Oxycodone Rat Data

Trial 1: February 2008 to March 2008, n = 30

Table A-1: Oxycodone-dosed rats mass of body and mass of organs upon exhumation

Post Burial Interval (Days)	Initial Body Mass (grams)	Percentage Body Mass Change	Mass of Liver (grams)
0	427.38	N/A	13.69
3	472.59 (37.38)	5.88 (3.26)	9.39 (0.79)
6	438.43 (10.50)	5.96 (8.85)	8.64(1.85)
9	356.53 (22.27)	-3.82 (1.80)	4.74 (0.60)
12	379.50 (8.06)	-9.09 (4.21)	4.77 (1.72)
15	364.6 (14.48)	-11.33 (3.22)	5.42 (1.09)
18	367.79 (31.52)	-11.42 (4.25)	6.09 (2.23)
21	329.24 (13.07)	-14.72 (0.93)	4.91 (0.34)
24	385.35 (49.30)	-21.71 (4.82)	6.33 (1.67)
27	297.42 (11.71)	-20.58 (2.06)	5.23 (1.75)
30	313.67 (9.84)	-21.20 (3.76)	4.88 (0.29)

n = 3, Mean (± SD)

Table A-1: Moisture and pH of soil and liver of oxycodone-dosed rats upon exhumation

Post Burial Interval (Days)	Soil Moisture (%)	Soil pH	Liver pH
0	50.0	6.8	6.30
3	50.0	6.8 (0)	6.30 (0.20)
6	50.0	6.7 (0.1)	6.50 (0.20)
9	55.0	6.0 (0)	7.25 (0.10)
12	60.0	6.0 (0)	7.23 (0.67)
15	55.0	6.0 (0.1)	7.30 (0.20)
18	60.0	5.6 (0.2)	7.40 (0.20)
21	60.0	5.4 (0.2)	7.90 (0.20)
24	60.0	5.5 (0.1)	7.63 (0.60)
27	60.0	5.2 (0.2)	8.20 (0.20)
30	60.0	5.7 (0.2)	8.30 (0.20)

n = 3, Mean (± SD) Initial soil moisture and pH for all microcosms were standardized to 50% moisture and pH of 6.8.

8.1 Appendix B Trial 2 Heroin Rat Data

Trial 2: February 2009 to March 2009

Heroin Dosed Rats, n = 18; Control Rats, n = 6

Table B-1: Heroin-dosed rats (trial 2) mass of body and organs

Post Burial Interval (Days)	Initial Body Mass (grams)	Percentage Body Mass Change	Mass of Liver (grams)	Mass of Heart (grams)
Heroin-treated				
0	515.39 (±36.21)	N/A	18.53 (±0.79)	1.84 (±0.74)
7	514.86 (±45.12)	-0.75 (±0.93)	11.59 (±0.24)	1.96 (±0.21)
14	521.89 (±39.37)	-11.35 (±1.11)	7.81 (±0.43)	1.50 (±0.20)
28	538.84 (±49.63)	-14.04 (±1.47)	7.06 (±1.60)	1.59 (±0.13)
42	538.40 (±33.67)	-24.97 (±5.40)	5.81 (±1.04)	0.95 (±0.13)
56	539.14 (±30.48)	-35.68 (±1.50)	7.62 (±1.74)	1.38 (±0.45)
Control				
0	462.29	N/A	17.78	2.09
7	531.00	-0.44	14.94	1.69
14	510.34	-14.91	10.15	2.20
28	437.42	-19.73	5.54	1.57
42	509.21	-17.93	7.13	0.67
56	449.74	-35.61	6.06	0.78

At each harvest: Control, n = 1. Heroin treated rats, n = 3. Mean (± S.D.)

Table B-2: Moisture and pH of soil and liver of control and heroin-dosed rats upon exhumation

Post Burial Interval (Days)	Soil Moisture (%)	Soil pH	Liver pH	Heart pH
Heroin				
0	60.0	6.2	6.04 (±0.30)	6.17 (±0.29)
7	65.0 (±0)	5.3 (±0.1)	5.98 (±0.16)	7.00 (±0.50)
14	73.3 (±2.9)	5.1 (±0.1)	6.63 (±0.18)	7.00 (±0.0)
28	80.0 (±0)	5.6 (±0.0)	7.71 (±0.51)	7.83 (±0.29)
42	95.0 (±0)	5.9 (±0.1)	7.86 (±0.16)	7.67 (±0.29)
56	90.0 (±0)	6.1 (±0.1)	7.87 (±0.19)	8.00 (±0.0)
Control				
0	60.0	6.2	6.18	7.00
7	60.0	5.5	6.08	7.50
14	65.0	5.2	6.63	8.00
28	70.0	5.4	8.14	8.50
42	90.0	6.0	7.93	8.00
56	90.0	6.0	7.96	8.00

At each harvest: Control, n = 1. Heroin treated rats, n = 3. Mean (± SD)