METABOLISM AND ANALYSIS OF DESOMORPHINE

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Doctor of Philosophy

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by

Jessica N. Winborn

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DEDICATION

"You must do the things you think you cannot do."
-Eleanor Roosevelt

To my advisor, Dr. Sarah Kerrigan, for her unwavering faith in me and for all of her words of advice and encouragement.

To my co-advisor, Dr. Donovan Haines, for helping me look at problems from a different perspective.

To my PhD cohort, for all of their encouragement and the laughter when I needed it most.

To my parents, for their continued support and belief in me.

To my grandparents, who saw me start this journey but couldn't be here to see me finish it.

ABSTRACT

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Desomorphine is a semi-synthetic opioid that is ten times more potent than morphine, with a faster onset but shorter duration of action. It is a major component of the drug referred to as "Krokodil", which is used as a heroin substitute. Its prevalence is difficult to estimate due to a lack of analytically confirmed cases, which may in part be due to the limited studies regarding its pharmacology or methodology to detect the drug in biological specimens. This research seeks to further the understanding of both desomorphine's metabolism and its detection in biological specimens, to facilitate its identification in clinical and forensic toxicology laboratories.

Six commercially available enzyme-linked immunosorbent assays were evaluated to determine their effectiveness with respect to desomorphine detection. Cross-reactivities were highly variable between assays, ranging from <2.5-77%. Recombinant human cytochrome P450 enzymes (rCYPs) and recombinant uridine 5'-diphospho-glucuronosyltransferases (rUGTs) were used to investigate the biotransformational pathways involved in desomorphine metabolism. Phase I metabolism could be attributed to seven rCYPs (rCYP2B6, rCYP2C8, rCYP2C9, rCYP2C18, rCYP2C19, rCYP2D6 and rCYP3A4), producing a total of nine phase I metabolites (nordesomorphine, desomorphine-*N*-oxide, two norhydroxydesomorphine isomers, and five hydroxylated isomers). During phase II metabolism, desomorphine-glucuronide was produced by nine rUGTs (rUGT1A1, rUGT1A3, rUGT1A8, rUGT1A9, rUGT1A10, rUGT2B4, rUGT2B7, rUGT2B15, and rUGT2B17).

Chemical and enzymatic hydrolysis of conjugated metabolites were investigated using desomorphine-glucuronide generated *in situ* using rUGT enzyme. Acid hydrolysis and five β-glucuronidase sources (BGTurbo™, IMCSzyme™, *Escherichia coli*, *Helix pomatia* and *Patella vulgata*) were evaluated. Acid hydrolysis produced complete hydrolysis of desomorphine-glucuronide, and under optimal conditions, each enzyme produced complete or near complete hydrolysis (≥96%), with BGTurbo™ and IMCSzyme™ offering the shortest incubation times. Under simulated challenging conditions, *P. vulgata* was the most effective enzyme evaluated.

Desomorphine was analyzed in blood and urine samples using gas chromatography-mass spectrometry (GC-MS), and urine samples were additionally analyzed using liquid chromatography-tandem mass spectrometry (LC-MS/MS) and liquid chromatography-quadrupole/time of flight-mass spectrometry (LC-Q/TOF-MS). Each method was validated in accordance with published guidelines for forensic use. Additionally, LC-Q/TOF-MS was used to analyze desomorphine metabolites, which in the absence of commercially available reference material or authentic urine specimens, were generated *in-vitro*.

KEY WORDS: Desomorphine, Immunoassays, Metabolism, Gas Chromatography, Liquid Chromatography, High Resolution Mass Spectrometry

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CHAPTER I

INTRODUCTION

Background

The recreational abuse of opioids has a long and vivid history, from the discovery of the medicinal properties of *Papaver somniferum* thousands of years ago to the present-day opioid epidemic (1). In recent years that abuse has skyrocketed, particularly in the United States where the widespread abuse of both illegal and prescription opioids has led to the declaration of an "opioid crisis". It is an unfortunate fact that while measures can be taken regarding prescribed opioids, such as compliance testing and incentives to prescribe alternative medications, curtailing the clandestine manufacture and consumption of opioids is more difficult, particularly with the rise in popularity of so called 'designer drugs', whose legality is variable between jurisdictions. This challenge is compounded by the fact that the chemical modifications undertaken may render designer drugs undetectable by previously existing analytical methods.

Much attention has been placed upon the designer opioids with high prevalence, such as the fentanyl analogs. Considerably less attention has been given to more obscure opioids with unknown prevalence, such as desomorphine. Arguments can be made that resources should not be dedicated to the research of drugs unless there is evidence of their abuse. However, the number of analytically confirmed cases does not always represent actual prevalence. When drug users continue to self-report lesser known drugs such as desomorphine, the absence of confirmed cases should instead increase attention. Self-reported drug use and entries on internet drug forums are utilized as part of early warning

and surveillance systems. If a drug is being reported but not identified in casework, research should be initiated to resolve the discrepancy.

History of desomorphine

Therapeutic use

Analgesia is defined as the inability to feel pain. Analgesics are classified as either non-narcotic or narcotic, with non-narcotic analgesics acting upon the peripheral nervous system (PNS) while narcotic analgesics act upon the central nervous system (CNS). The mechanism of action for non-narcotic analgesics is to suppress nociceptive signals in the PNS and prevent them from reaching the CNS. Conversely, the mechanism of action for narcotic analgesics is to suppress incoming nociceptive signals in the CNS. As a consequence, they typically suppress outgoing signals leading to side effects such as respiratory depression.

Common therapeutic uses of opioids include post-operative analgesia, chronic pain management, and medical emergencies. They can also be used as supplements for anesthesia and sedatives as well as antitussives and antidiarrheals. Their purpose is to provide analgesia without an accompanying loss of consciousness. Disadvantages associated with their use includes respiratory depression and addiction liability.

While desomorphine was first synthesized in the United States (2), it was never sold commercially in the country due to concerns about its addiction liability. In fact, its only recorded use as a therapeutic drug was in Switzerland, where it saw limited use as a post-operative analgesic. Beginning in 1940, the pharmaceutical company Hoffman-Roche produced desomorphine under the trade name Permonid, which was available in both ampule and suppository forms (3). Permonid was permanently withdrawn from the

market twelve years later in 1952. Despite no longer being sold, the production of Permonid continued until 1981. This small-scale production was for the use of a single individual suffering from an unspecified disease that resulted in specialized analgesic needs. After their death all production of desomorphine ceased permanently. The individual was reported to have consumed 80 ampules of Permonid daily. Each ampule contained 2 mg of desomorphine, making the total amount of desomorphine consumed per day 160 mg (3). No information exists in the literature regarding whether this was typical for patients receiving desomorphine, or a result of the individual's specific needs.

Illicit use and epidemiology

The lack of therapeutic applications for desomorphine can be attributed to its abuse potential, which is derived from its capacity to produce physical dependence, characterized by the emergence of classical opioid abstinence syndrome (4), including irritability, tremors, hypertension, nausea, vomiting and hyperthermia. The sought-after effects of opioid misuse include euphoria and analgesia, while undesirable effects include sedation, dizziness, nausea, vomiting, constipation, respiratory depression and mental clouding. Tolerance to opioids can develop within three weeks of repeated use and physical symptoms of withdrawal can begin in as little as six hours after the last dose. In addition to physical dependence, there is a psychological component to drug addiction, where the pleasurable memory of the experienced high creates a craving for a repeat of the experience. Chronic drug abuse is characterized by the cycle of positive reinforcement from desirable effects, and negative reinforcement associated with the symptoms of withdrawal.

The illicit use of desomorphine, in the form of the clandestine heroin replacement Krokodil, was first reported in the media in 2003 (3). Krokodil use began in eastern Russia before expanding into neighboring countries, and later central Europe. To date, approximately twenty case reports have been published in the scientific literature.

The earliest report of desomorphine abuse was in 2008 (5). Limited information included beyond its alleged abuse and its status as a controlled substance in Russia. It was not until 2012 that additional information regarding estimated prevalence was published, along with the first mention of the term Krokodil, which has been attributed to both the discoloration and texture of the skin from associated side effects like gangrene (3), as well as an intermediate product of one of the synthesis pathways for desomorphine, α-chlorocodide (6). According to media reports, approximately sixty-five million doses of Krokodil were seized in 2011 in Russia and one hundred thousand individuals were using Krokodil, many claiming to have switched from heroin due to the lower cost. The apparent spike in popularity coincided with a severe shortage in the heroin supply during the preceding year due to disease affecting poppy crops (7, 8). In response to the rise in Krokodil use, the Russian government implemented stricter controls on desomorphine's primary precursor codeine, which had previously been available without a prescription (9). During the same period, the Eurasian Harm Reduction Network conducted a survey of needle and syringe programs and pharmacies in seventeen countries regarding the preferences of intravenous drug users (10). The results from four countries (Russia and the Republics of Armenia, Georgia and Kazakhstan) included reports of Krokodil use. The first published case report linked to Krokodil, but not analytically confirmed, also appeared in 2012, originating from Germany (11).

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By 2013, Krokodil had apparently spread to Belgium, the Czech Republic, France, Norway, Poland, Sweden, Ukraine and the United Kingdom as well (6, 12-14). This was attributed to the flow of immigrants from Russia, where Krokodil use was widespread. In Ukraine, an estimated twenty thousand people were using Krokodil (6), with data from one published survey showing approximately 25% of interviewees (n=550) self-reported Krokodil use (15). While no statistics are provided, the use of Krokodil is included by the European Monitoring Centre for Drugs and Drug Addiction (EMCCDA) in overviews prepared for the Republics of Georgia and Kazakhstan (16, 17).

In 2014, case reports involving Krokodil use were published from the United States (18) and the Republics of Armenia and Georgia (19, 20). Clinical case reports have since been published from Italy (21), Poland (22), Russia (23, 24), Spain (25) and the United Kingdom (14), as well as additional case reports from the Republic of Armenia (26, 27) and the United States (28-31). The first and only analytically confirmed case originated from Italy. **Table 1.1** summarizes the case reports in the literature, including the symptoms noted with each case. Based on these reports Krokodil use has been associated with necrotic ulcers, osteonecrosis (death of bone tissue) and osteomyelitis (infection of bone tissue) in multiple patients. In cases involving fatalities, respiratory distress, organ failure, paroxysmal hypoxia (reoccurring instances of low oxygen supply), dyspnea (shortness of breath) and tachycardia (elevated heartrate) have been reported.

Table 1.1

Published case reports of Krokodil use.

Origin	Year	Last Use of Krokodil	Other Drug Use	Symptoms	No. of Individuals	Fatality	Reference
United States (Missouri)	2014	unknown	heroin	necrotic ulcer	1	no	(18)
Unites States (Ohio)	2015	unknown	heroin	necrotic ulcers	2[a]	no	(28)
United States	2016	1 year prev.	heroin	necrotic ulcers	1	no	(29)
United States (Colorado)	2017	unknown	none	respiratory distress, fever, multi-organ failure	1	yes	(30)
United States (New York)	2018	4 days prev.	heroin, alprazolam, cocaine	necrotic ulcers	1	no	(31)
Republic of Armenia	2014	unknown	none	jaw osteonecrosis	40	no	(20)
Republic of Armenia	2017	unknown	none	jaw osteonecrosis	1	no	(26)
Republic of Armenia	2018	1-24 months	none	jaw osteonecrosis	6	no	(27)

(continued)

Republic of Georgia	2014	unknown	methamphetamine, benzodiazepines, barbiturates	pulmonary embolism[b]	160	yes[b]	(19)
Germany	2012	unknown	heroin	paroxysmal hypoxia; coma	1	yes[c]	(11)
Italy	2018	unknown[d]	cannabis	hyperthermia, tonsillitis, dyspnea, tachycardia, cardiac arrest	1	yes[e]	(21)
Poland	2018	unknown	none	necrotic lesions	1	no	(22)
Russia	2015	unknown	none	jaw osteonecrosis	165	no	(23)
Russia	2015	unknown	none	jaw osteomyelitis	52	no	(24)
Spain	2016	unknown	heroin, cocaine, benzodiazepines	fever, itchiness, altered breathing, headache, relaxation/sedation	1	no	(25)
United Kingdom	2013	3 months	heroin, amphetamines	hallucinations	1	no	(14)

[a]Only 1 reported Krokodil use.
[b]In 1 patient out of 160.
[c]No desomorphine in blood or urine; femoral blood findings: morphine (40 ng/mL), morphine-3-glucuronide (100 ng/mL), methadone (400 ng/mL), trimipramine (200 ng/mL) and *N*-desmethyltrimipramine (200 ng/mL).
[d]Last use was estimated to be 2-3 days based on the toxicology.
[e]Desomorphine (270 ng/mL) in urine with caffeine, acetominophen and phosphoric acid also detected.

As Krokodil is used as a clandestine heroin substitute, users also commonly report having used heroin, as can be seen in the case reports (**Table 1.1**) where it is the most frequent additional drug reported, though it is unclear whether these drugs are co-administered. Other drugs reported include: barbiturates and benzodiazepines, which are CNS depressants used clinically to treat anxiety and seizures; cannabis (marijuana), which is used medically for analgesia and appetite stimulation or illicitly for its hallucinogenic properties; cocaine, a CNS stimulant which also sees limited medical use as a topical anesthetic; and methamphetamine, which is used clinically both to treat attention deficit hyperactivity disorder under the brand name Desoxyn® and as a nasal decongestant (*l*-methamphetamine), with *d*-methamphetamine being used illicitly for its psychoactive properties. Additional drugs identified from toxicological testing in reported Krokodil cases are morphine, trimipramine (antidepressant), caffeine and acetaminophen (11, 21). In a study to detect desomorphine in authentic urine specimens, desomorphine's precursor codeine was also detected (5).

With a lack of analytically confirmed case reports, the available information regarding the prevalence of Krokodil use suggests that it is primarily used in Russia and bordering states. In the United States in particular, there is contention over the validity of known reports of Krokodil use, with physicians arguing for and against the acceptance of reports of Krokodil use without analytical confirmation (29, 32-34). Desomorphine's precursor, codeine, is a schedule V drug and is available only with prescription. While this might diminish its abuse, it is still possible to obtain codeine illicitly. Of particular note, two exhibits submitted to forensic laboratories in 2004 were analytically identified as desomorphine, according to the National Forensic Laboratory Information System

(NIFLIS) (35). While there is no further information regarding these two exhibits, the report raises interesting questions regarding Krokodil use in the United States.

Legal Status

Today desomorphine is generally regarded as having no medical use and is controlled in multiple countries. The United Nations Single Convention of Narcotic Drugs (1961) classified it as a schedule IV drug (36). Russia included it on the List of Narcotics, Psychotropic Substances and Precursors Thereof, Subject to Control in the Russian Federation (5) and it is considered non-marketable in both Austria and Germany (3). In Brazil, desomorphine is a class F1 drug (37) and in the United Kingdom it is a class A drug (38).

In the United States desomorphine is a schedule I drug under the Federal Controlled Substances Act (CSA) (35), though its sale was prohibited even before the creation of the CSA. In 1935, the Committee on Drug Addiction recommended desomorphine be prohibited in the United States due to its addiction liability (39). This was largely based on early studies demonstrating that desomorphine produced respiratory depression and withdrawal symptoms similar to morphine (40). This was the first instance of addiction liability being the decisive factor in narcotics control. Indeed, the studies of desomorphine are regarded as being used to devise the first reliable test for addiction potential among the opioids (41).

Chemistry of desomorphine

Chemical properties

Opioids can be divided into seven classifications, including phenanthrenes, morphinans, phenylheptylamines, phenylpiperidines, benzomorphinans, cyclohexanols

and phenols. All have aromatic cores and many are considered amphoteric bases. Desomorphine belongs to the phenanthrene class, also called 4,5-epoxymorphinans, which includes the morphine-like opioid alkaloids found in *P. somniferum*. Derivatization of these natural alkaloids, also referred to as opiates, to produce semi-synthetic opioids has led to the phenanthrenes becoming the largest class of opioids. Morphine and codeine were the most commonly used starting materials for chemical alterations, each causing changes to the pharmacological action, which will be discussed in greater detail later. Synthetic opioids, while having similar mechanism of action, do not structurally resemble natural and semi-synthetic opioids. **Figure 1.1** shows the structure of desomorphine and morphine for comparison. Phenanthrene opioids are composed of five rings: the aromatic A ring, two six-membered carbon rings B and C, the nitrogen containing D ring and the oxygen containing five membered E ring. Desomorphine differs from morphine in the absence of the hydroxyl group at C6 and the saturation of the C7-C8 double bond. It is a solid at room temperature, with a molecular weight of 271.35 g/mol and a melting point of 189°C (2). It is soluble in water as a salt and partially soluble as a free base. It is also soluble in acetone and ethyl acetate (42).

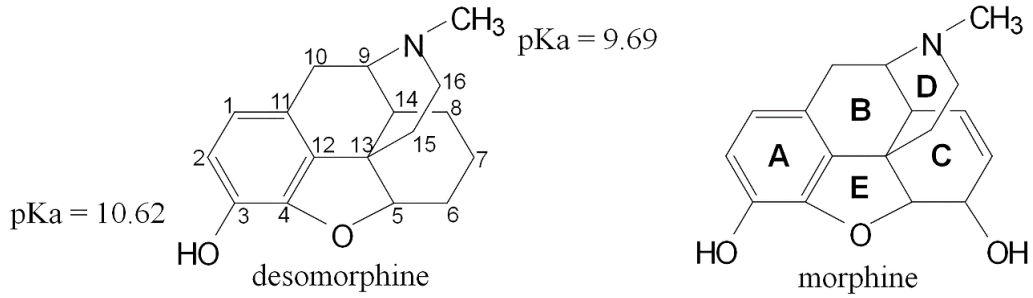

Figure 1.1. Structures of desomorphine and morphine. Carbon numbers are shown for desomorphine with the rings labeled for morphine.

Desomorphine's absolute structural conformation is a T-shape, with the C ring in chair confirmation. This is in contrast to morphine, whose C ring is in boat confirmation. Desomorphine's levorotary isomer has a specific optical rotation of $[\alpha]_D^{28}$ -77° (c 1.6, methanol) (42). Research with morphine has shown that the structural conformation of opioids can play an important role in terms of their analgesic potency. Levorotary isomers may have diminished activity relative to their dextrorotary counterparts (43).

Desomorphine is an amphoteric base, with a tertiary amine and phenolic group, which have disassociation constants of 9.69 and 10.62, respectively (42, 44). The isoelectric point (pI) of this zwitterionic drug is pH 10.16 (**Figure 1.2**). Analytically, zwitterions like desomorphine can be challenging to isolate from biological specimens. During extraction, the charge on the drug is manipulated by changing the pH. Using liquid-liquid extraction (LLE), drugs are preferentially partitioned into the organic phase in their uncharged state. For zwitterions, functional groups on the drug may be charged over a wider range of pH values. At the pI, although the net charge on the drug is zero, functional groups are still ionized. Although this can diminish extraction efficiency using LLE, it can be exploited using mixed-mode solid phase extraction if the stationary phase has anion or cation exchange capability.

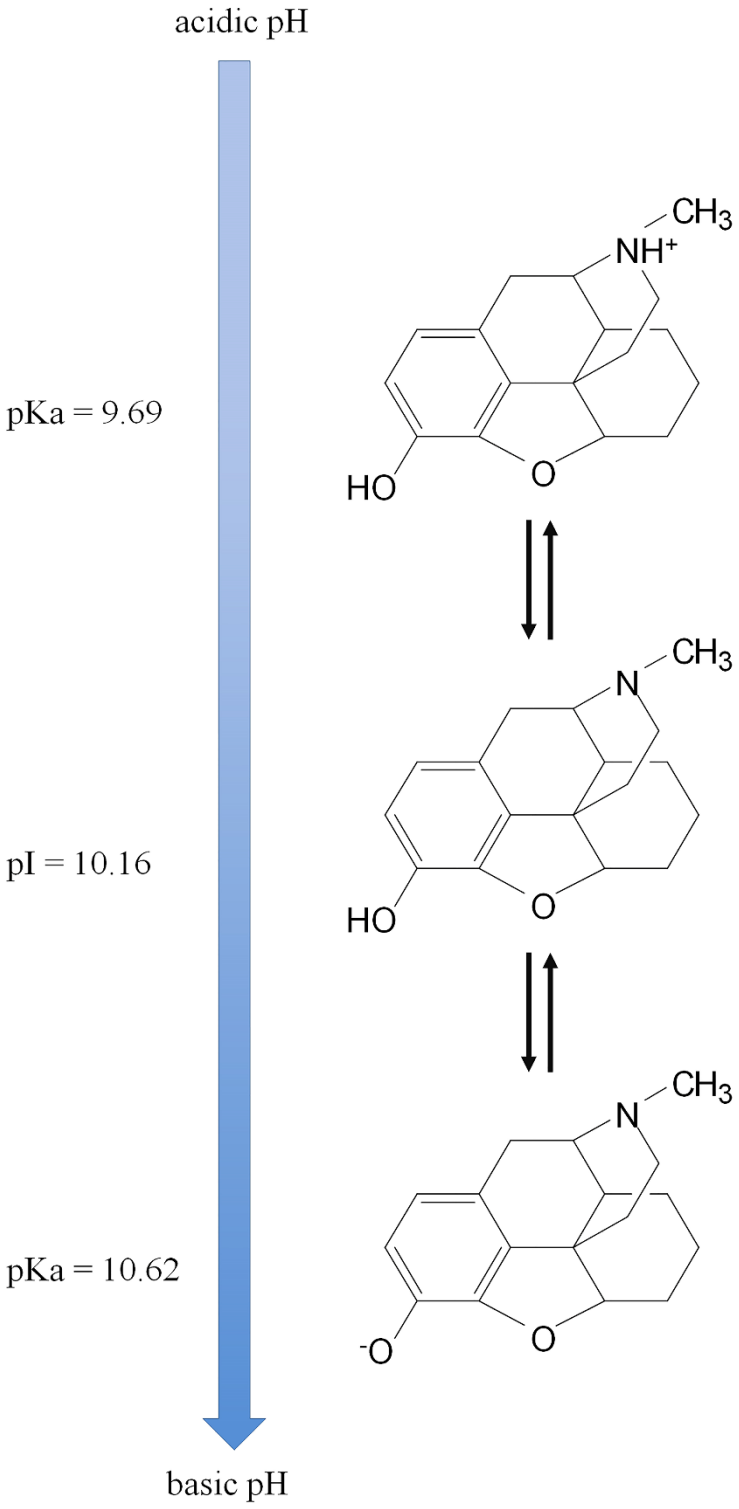

Figure 1.2. Charge states of desomorphine.

Synthesis

The structure of morphine was first published in 1925 by Gulland and Robinson (1). Shortly after, research emerged regarding chemical modifications to morphine's structure, in an effort to discover non-addictive analgesics that could be used in its place (40). In 1933 the first synthesis for desomorphine was published as part of a series on the catalytic hydrogenation of halogenocodides and halogenomorphides (45, 46). **Figure 1.3** depicts the structures of the different desoxymorphines and desoxycodeines produced by their research.

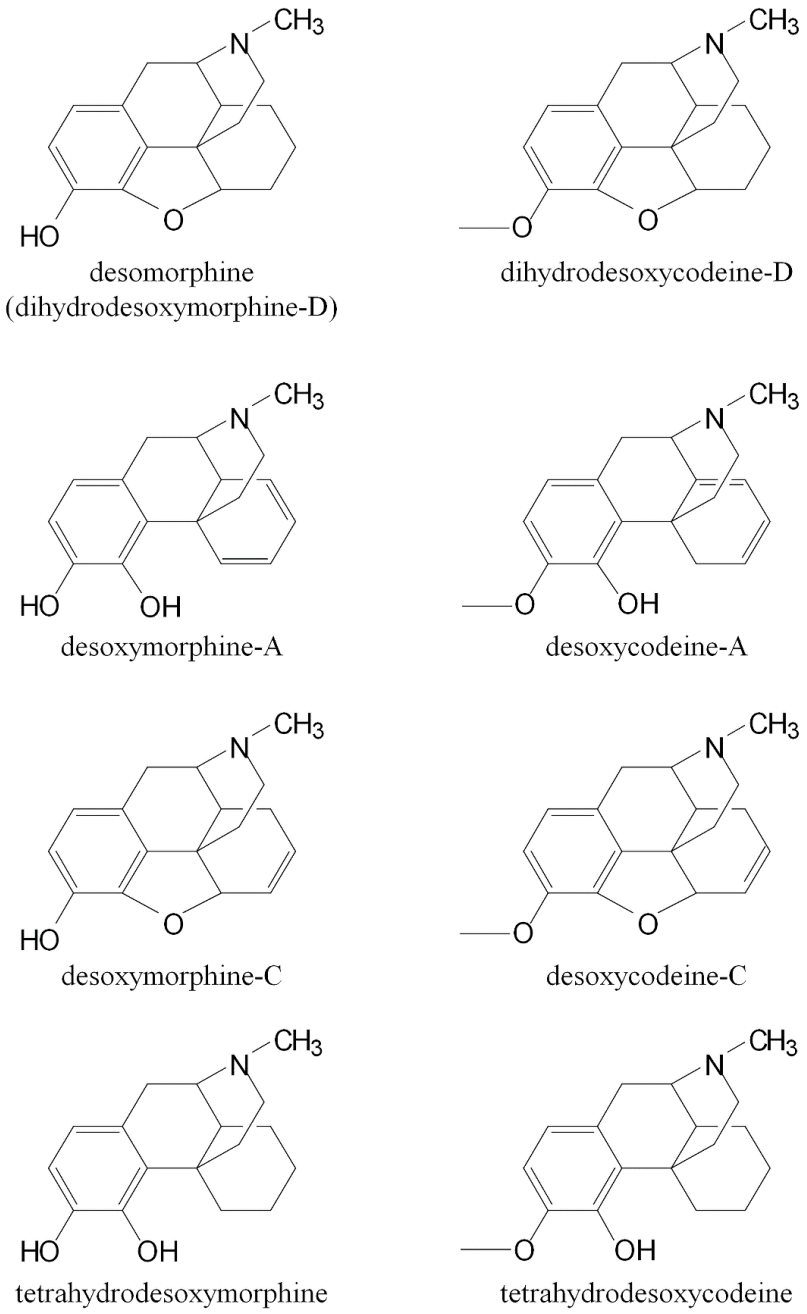

Figure 1.3. Desomorphine with examples of other desoxymorphines and desoxycodeines.

Beginning with chloromorphide and bromomorphide, palladium-barium sulfate was used to catalyze the reaction and produce dihyrodesoxymorphine-D ((5α,6α)-17-methyl-7,8-didehydro-4,5-epoxymorphinan-3-ol; desomorphine). They found that the yield of

desomorphine varied, depending on which compound was used as the precursor and what the reaction solvent was. **Figure 1.4** depicts the different reactions investigated and the yield of desomorphine associated with each precursor and solvent used. They noted that tetrahydrodesoxydesomorphine was also produced in smaller amounts from reactions starting with α-chloromorphide and an uncrystallizable oil was also produced from reactions with bromomorphide (2).

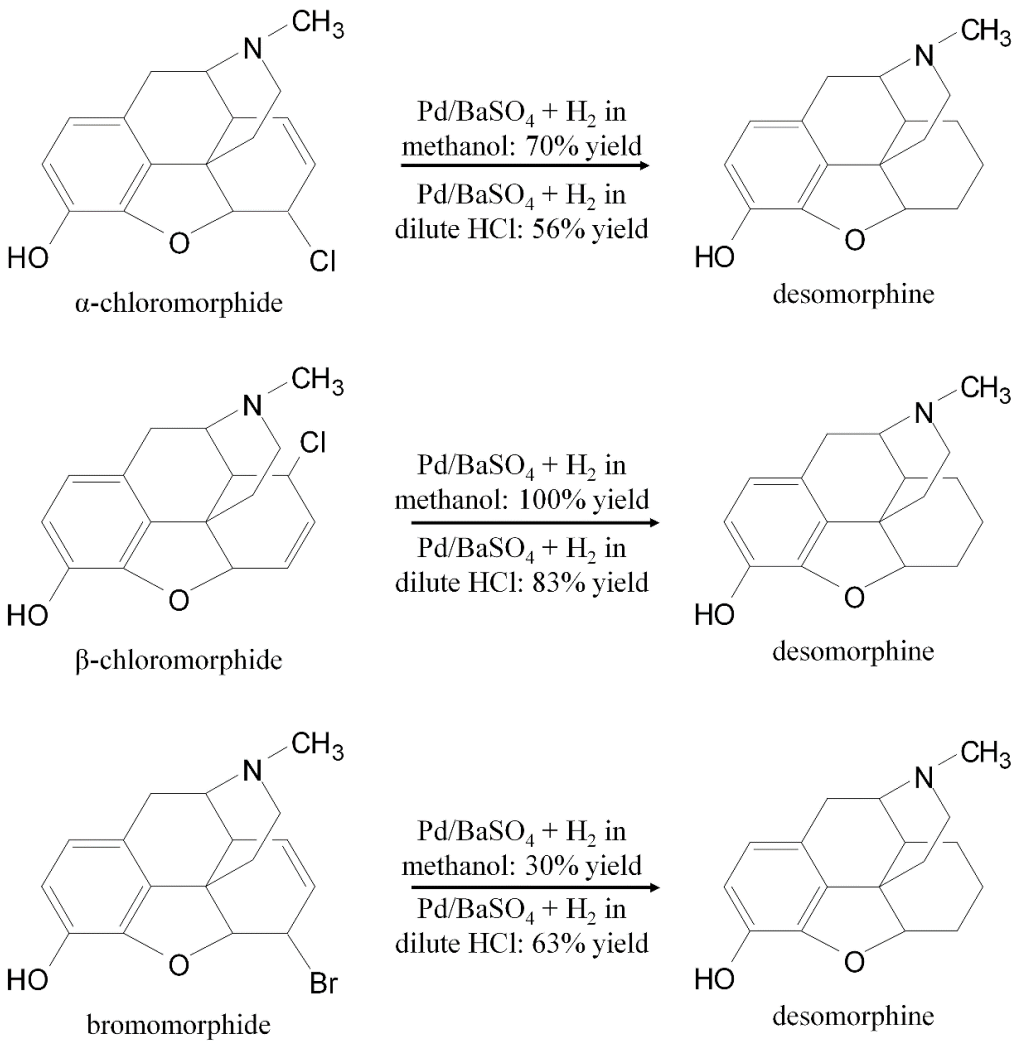

Figure 1.4. Desomorphine yield after dehalogenation of chloromorphide isomers and bromomorphide in either methanol or dilute hydrochloric acid (HCl).

Further research found that desomorphine could be obtained as a hydriodide from demethylating dihydrodesoxycodeine-D with a 93% yield (45). It was determined that this was the most practical pathway as dihydrodesoxycodeine-D could be prepared from α-chlorocodide, which was more easily accessible than the halogenomorphides (**Figure 1.5**) (2).

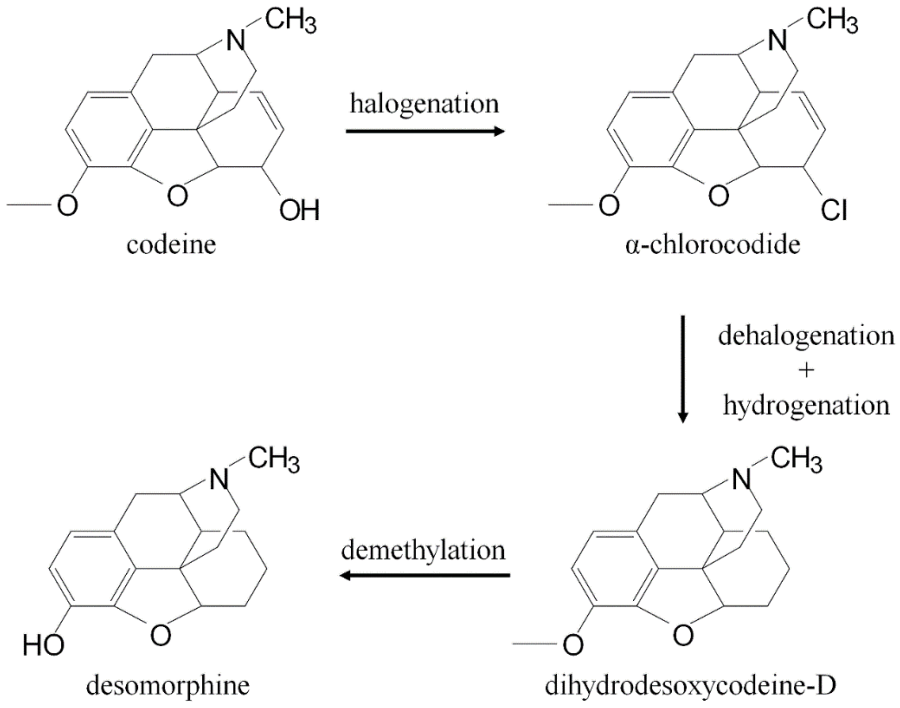

Figure 1.5. Synthesis of desomorphine using α-chlorocodide.

In 1952 Perrine and Small published their attempts to improve the synthesis of desomorphine (47). They first attempted to synthesize dihydrodesoxycodeine-D from dihydrocodeinone (hydrocodone) using hydrazine and ethyl mercaptan. These first attempts produced dihydrodesoxycodeine-C and dihydrothebainone but not desomorphine. They then adapted a synthesis published by Rapoport and Bonner (48). The original synthesis produced dihydrodesoxycodeine-D through detosylation of 6-toluenesulfonylcodeine. It had previously been shown that desomorphine could be

produced by demethylating dihydrodesoxycodeine-D (45) and Perrine and Small applied that to successfully derive desomorphine from 6-toluenesulfonylcodeine. The complete pathway is shown in **Figure 1.6**.

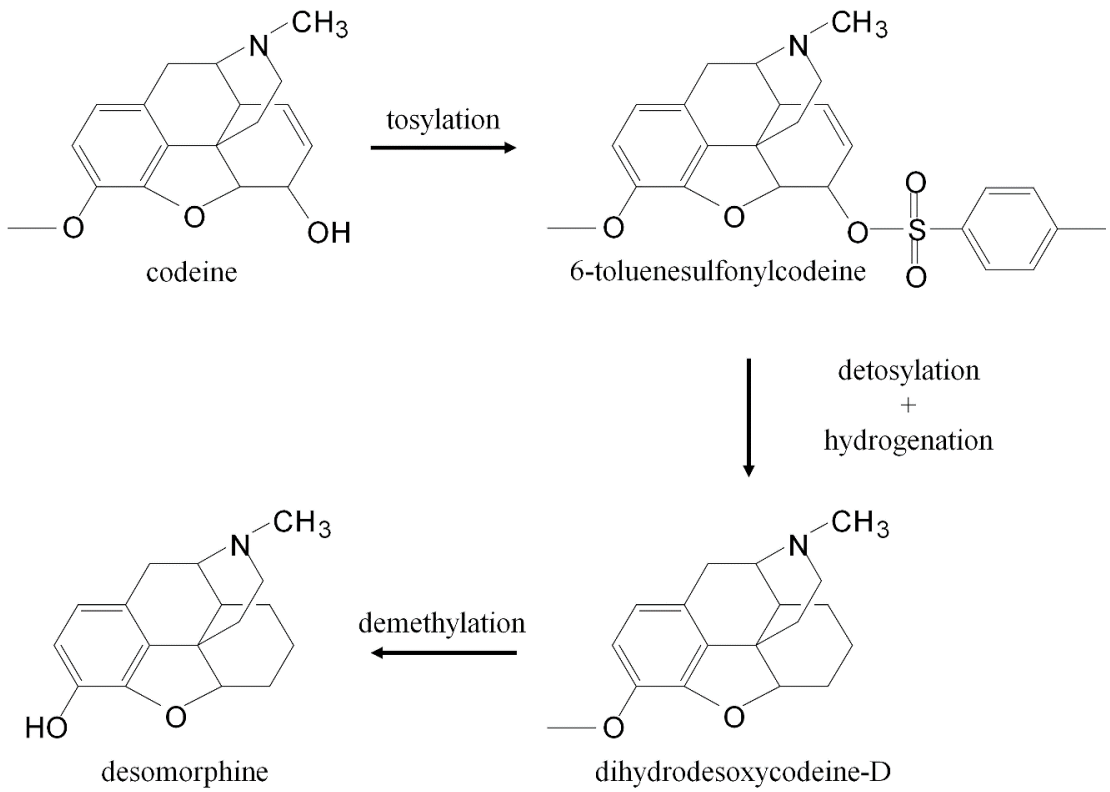

Figure 1.6. Synthesis of desomorphine using 6-toluenesulfonylcodeine.

After reports of Krokodil use began to surface, Srimurugan published a high yield synthesis for desomorphine and its deuterated analog (49). The reaction pathway is summarized in **Figure 1.7**. It proceeds in a similar fashion to that published by Perrine and Small (47) (**Figure 1.6)** however a mesyl group is added to codeine instead of a tosyl group due to a higher yield and shorter reaction time. The final product had a purity of 99% but the overall yield was 38%.

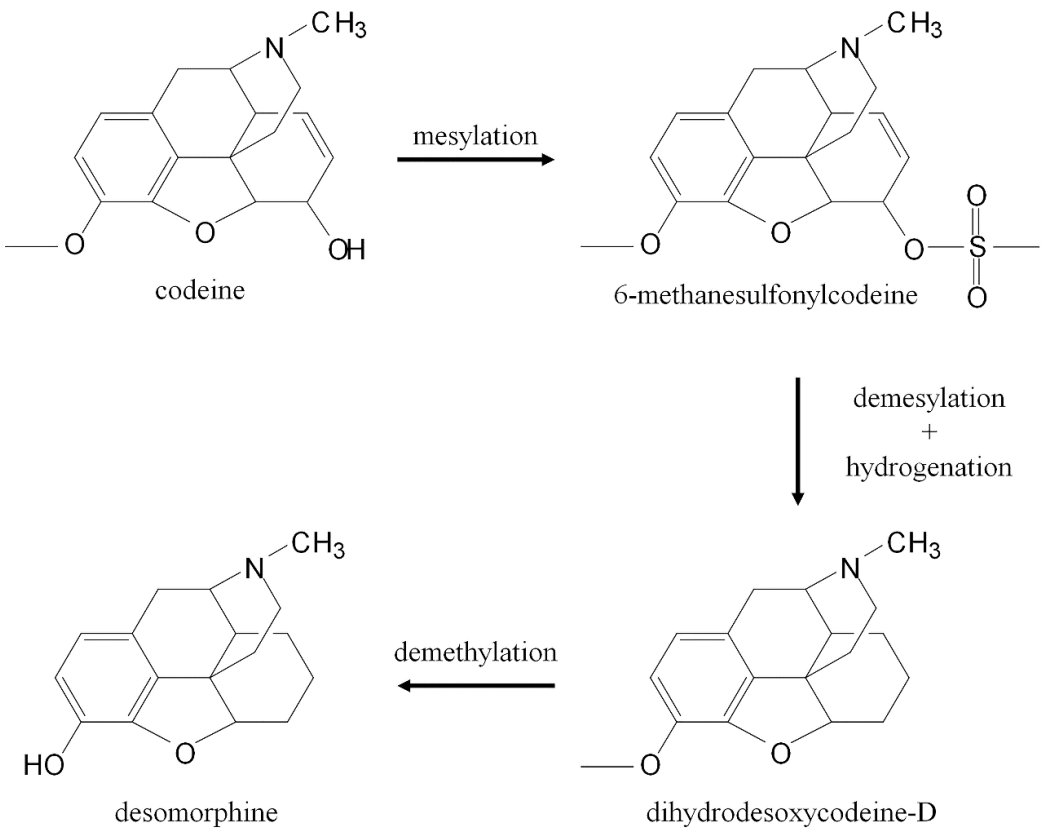

Figure 1.7. Synthesis of desomorphine using 6-methanesulfonylcodeine.

From the available information in published literature, the favored synthesis for the clandestine production of desomorphine resembles the Nagai method commonly used to synthesize methamphetamine (6, 50). A crude liquid-liquid extraction (LLE) with gasoline and alkali household cleaning products is used to extract codeine from over the counter (OTC) preparations. The isolated codeine is then mixed with iodine, hydrochloric acid and phosphorous. The mixture is heated and allowed to react for approximately forty-five minutes (50). The proposed reaction pathway is similar to that suggested by Small (2) but with α-chlorocodide replaced with α-iodocodide due to the presence of iodine (51) (**Figure 1.8**). Given that the reagents used for clandestine synthesis are not pure and that OTC

preparations of codeine can contain other drugs in addition to binders, it is unsurprising that recent studies have shown Krokodil to contain numerous side products in addition to desomorphine (51, 52). There is also evidence that variations in the source of reagents, for example different formulations between cleaning products, may also effect the composition of Krokodil (5).

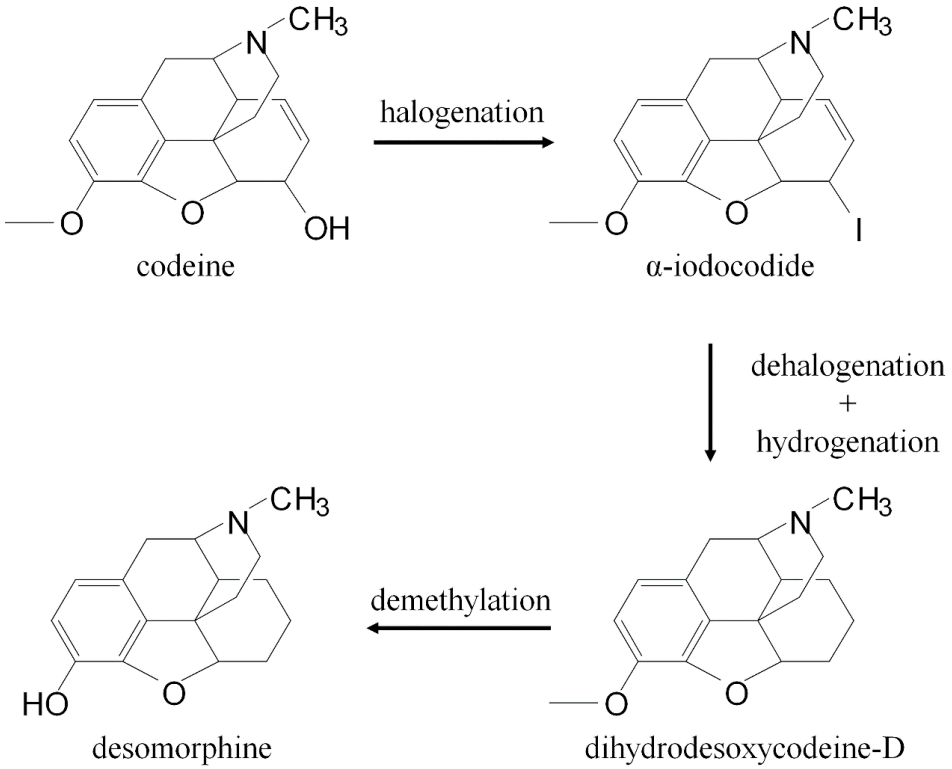

Figure 1.8. Clandestine synthesis of desomorphine.

Pharmacology

Pharmacodynamics

Desomorphine is a semi-synthetic opioid and as such its pharmacological effects are due to interactions with opioid-receptors which are present at several sites in the CNS.

A large cluster of opioid-receptors is located within the limbic system of the brain, which is involved with emotional response. Opioids, as narcotic analgesics, block pain responses by interacting with terminal nerve endings and blocking the release of neurotransmitters. This prevents the recognition of nociceptive signals which in turn inhibits a negative emotional response due to pain. In some instances, this interaction can also produce euphoria.

The three major receptor types involved with the pharmacological effects of opioids are the μ, κ and δ receptors. The μ receptors produce the largest number opioid effects including analgesia (supraspinal and spinal), euphoria, reduction of gastrointestinal motility, respiratory depression, hypothermia, bradycardia, tolerance and physical dependence. The κ receptors produce spinal analgesia, miosis, sedation, mild respiratory depression and diuresis. The analgesia produced by κ receptors can also be accompanied by psychomimetic effects. Spinal analgesia, respiratory stimulation, dysphoria, delusions, hallucinations and vasomotor stimulation are mediated via δ receptors. Opioids may also occasionally bind to σ receptors, which produce CNS excitation and can cause hypertension, tachycardia, tachypnea, mydriasis and hallucinations. Drug-receptor interactions can be agonistic or antagonistic in nature. Some opioids have agonistic effects at one receptor while having antagonistic effects at another. Opioids that are full antagonists tend to produce reduced analgesic effects and are often used to treat opioid intoxication, with naloxone and naltrexone being the most common examples. Opioids that are strong μ receptor agonists typically have the highest potential for abuse due to the production of euphoria and physical dependence.

The pharmacological response of an individual opioid is determined by its chemical structure. Structure-activity relationships of the opioids have been widely studied. Common structural alterations involve the C3 and C6 functional groups as well as the nitrogen ring (**Figure 1.9**). In general, changes to C3 decrease analgesic potency while changes to C6 increase it. A noted exception to this trend is diacetylmorphine (heroin) (43). Modifications to the nitrogen ring as a whole tend to significantly decrease potency, indicating that the tertiary amine plays a vital role.

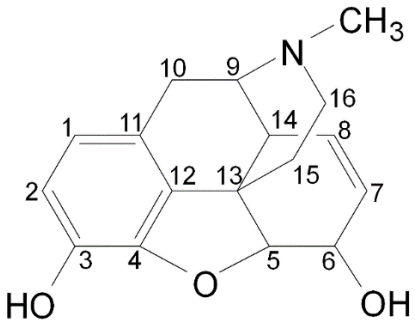

Figure 1.9. Morphine structure with carbon numbers labeled.

All *in vivo* animal and human studies involving desomorphine's pharmacology were performed in the early and mid-1900s (53). In study involving dogs, desomorphine produced a greater depressant effect compared to morphine. Desomorphine doses varied from 2-5 mg and morphine doses were 10-50 mg, with the effect of desomorphine noted as being greater than the 10 mg dose of morphine but less than the 50 mg dose. In cats, those dosed with desomorphine experienced less vomiting than those dosed with morphine and in a study using rhesus macaque monkeys, desomorphine showed ten times the depressant effects of morphine. Additionally, rapid tolerance was observed in the subjects, though the degree of tolerance was less than morphine. In rats, the LD_{50} of pure

desomorphine was found to be 27 mg/kg compared to 22 mg/kg for heroin, 300 mg/kg for codeine, and 226-318 mg/kg for morphine.

In humans, desomorphine was found to have a duration of effect of up to three hours (54). Increasing the dose of desomorphine did not noticeably extend the duration of effect but it was noted to be approximately ten times more potent than morphine. The reduced onset of action is linked to the elimination of the C6 hydroxyl group, rendering desomorphine more lipophilic than morphine and facilitating diffusion across the blood brain barrier. A decrease in respiration rate was noted with both desomorphine and morphine, but unlike morphine, the individuals who were treated with desomorphine did not develop tolerance with respect to respiratory depression with prolonged use. The increased potency relative to morphine and the reduced tolerance to respiratory depression could have serious consequences to individuals who abuse this drug. Patients who reported Krokodil at a drug treatment facility in the Republic of Georgia indicated that the short duration of action lead them to inject the drug anywhere from one to ten times daily (19).

The withdrawal effects experienced after ceasing desomorphine use appeared to be the typical abstinence syndrome experienced by opioid users (40). In 1943, Wright and Sabine published a study regarding the effects of morphine, hydromorphone, codeine and desomorphine on the activity of cholinesterase (55). It is the inactivation of cholinesterase that causes the respiratory depression characteristic of the opioids. Using multiple media, including human serum and brain, they found that desomorphine inactivated cholinesterase to a greater extent than morphine, hydromorphone and codeine. In summary, desomorphine is both more potent and more toxic than morphine and, based on its observed

pharmacological effects, a potent μ agonist with diminished affinities for both κ and δ receptors.

Due to the presence of multiple morphinans and traces of precursor reagents, the side effects of Krokodil are expected to exceed those of desomorphine but due to the limited number of published reports, the knowledge of those side effects is confined to anecdotal accounts from users and clinical observations from health care professionals. Reported side effects include abscesses, dermatological lesions and ulcers, thrombosis, necrotizing fasciitis, osteonecrosis, sepsis, damage to blood vessels, muscle, cartilage, and bone, multiple organ failure, endocarditis, hypotension, bradycardia, loss of cognitive functions, speech difficulty, changes in personality, loss of memory, hallucinations, and death (50, 56). Many of the severe effects, such as necrotic ulcers (**Figure 1.10**), are likely caused by impurities present from the synthesis.

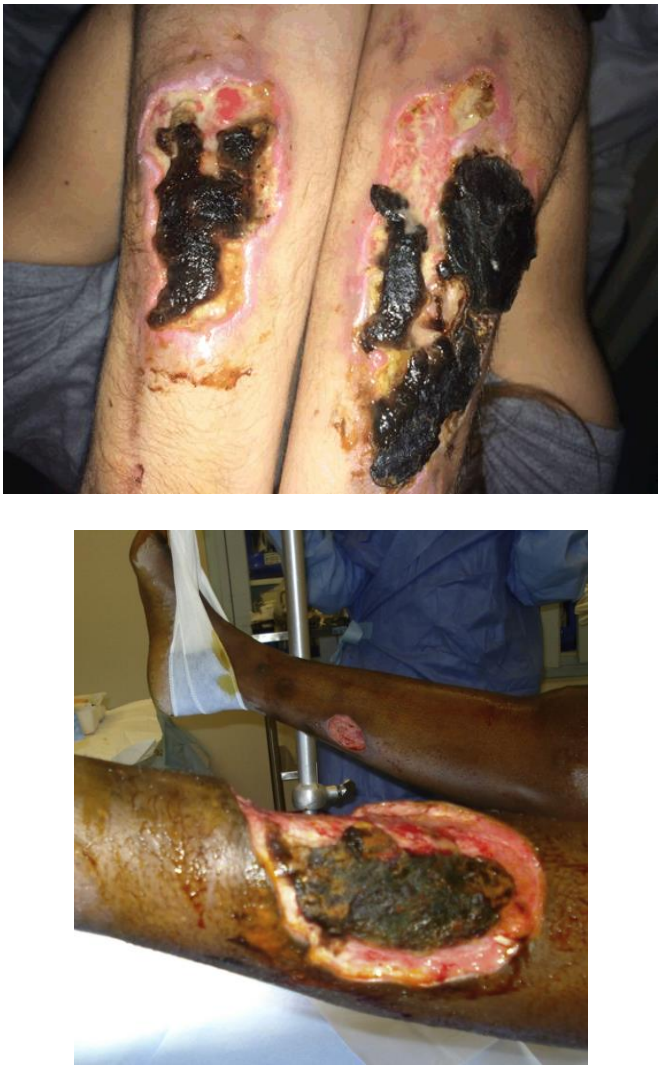

Figure 1.10. Necrotic ulcers associated with Krokodil use. Images originally published in (31, 57).

Pharmacokinetics

Knowledge of a drug's metabolism plays a key role in predicting drug-drug interactions and facilitating analytical detection. The rate of metabolism directly affects a drug's half-life and window of detection and known metabolites may serve as alternative biomarkers for analytical methods. When multiple drugs are metabolized by the same enzymes the duration of effect of one or both may be altered. Additionally, metabolites may also be pharmacologically active and may be more potent that the precursor, such as

morphine-6-glucuronide, which can create a secondary high (58). There are two types of reactions that take place during metabolism: the transformation of functional groups and the conjugation of the drug with endogenous substances. Phase I reactions, catalyzed by cytochrome P450 mono-oxygenases (CYPs), include demethylation, dealkylation, deamination, desulfuration, epoxide formation, hydrolysis, hydroxylation, *N*-oxide formation, sulfoxide formation and reduction. Phase II reactions may involve glucuronic acid (uridine diphosphate-glucuronosyltransferases, UGTs), sulfate (sulfotransferases) and glutathione (glutathione S-transferases). Phase I and II reactions generally occur synchronously, with many hydroxylated and carboxylated phase I species undergoing conjugation.

Opioids with free hydroxyl groups tend to be primarily conjugated during metabolism. Up to 75% of a morphine dose is conjugated to form morphine-3-glucuronide while approximately 5% converted to normorphine (59). Research into the enzyme isoforms (isozymes) involved with morphine metabolism has shown that the major phase II isozyme is UGT2B7 (60) which catalyzes the formation of both morphine-3-glucuronide as well as the minor metabolite morphine-6-glucuronide. Six additional isozymes (UGT1A1, UGT1A3, UGT1A6, UGT1A8, UGT1A9 and UGT1A10) have also been shown to catalyze the formation of morphine-3-glucuronide but not morphine-6-glucuronide (61). These additional biotransformation pathways likely contribute to morphine-3-glucuronide being morphine's major metabolite over morphine-6-glucuronide, which is significant as morphine-6-glucuronide is pharmacologically active while morphine-3-glucuronide is not. The CYP isozymes involved in morphine metabolism have also been explored. Both CYP2C8 and CYP3A4 have been shown to

catalyze the formation of normorphine (62), with CYP3A4 being the major contributor. CYP2D6 has also been shown to catalyze the phase I metabolism of levorphanol (63), which is structurally identical to desomorphine with the exception of the epoxide bridge. Approximately 31% of a levorphanol dose is conjugated and 2.5% is converted to norlevorphanol (64).

There is limited information regarding the pharmacokinetics of desomorphine. The primary route of administration reported is intravenous injection which means that it will not be subjected to first-pass metabolism (50). Its shorter duration of action likely corresponds to a shorter half-life but the only report of its window of detection, a few hours in blood and three days in urine, was not accompanied by any supporting data (11). The first study to investigate desomorphine's metabolism was published in 2016 (65). Using multiple models, including supersomes™, human liver microsomes and an *in vivo* rat study, seven phase I metabolites were identified (nordesomorphine, desomorphine-*N*-oxide and five hydroxydesomorphine isomers). Four phase II metabolites were also identified (desomorphine-glucuronide, desomorphine-sulfate, nordesomoprhine-glucuronide and desomorphine-*N*-oxide-glucuronide). CYP3A4 was found to catalyze the formation of most of the phase I metabolites but notably did not account for the formation of two of the hydroxydesomorphine isomers. Eight UGTs were found to catalyze the formation of desomorphine-glucuronide: UGT1A1, UGT1A8, UGT1A9, UGT1A10, UGT2B4, UGT2B7, UGT2B15 and UGT2B17. These results are largely consistent with what has been observed for morphine and levorphanol but there are notable differences. In this study, only CYP3A4 was shown to metabolize desomorphine in contrast to previous studies with morphine and levorphanol. Morphine has been shown to be metabolized by

CYP2C8 and CYP3A4, and levorphanol has been shown to be metabolized by CYP2D6 and CYP3A4. UGT2B7 was the major UGT isozyme involved in desomorphine metabolism, which is consistent with morphine. However, morphine is additionally metabolized by UGT1A3 and UGT1A6 which have not been shown to metabolize desomorphine. Conversely, morphine has not been observed to be metabolized by UGT2B4, UGT2B15 and UGT2B17, which were shown to metabolize desomorphine. These differences are likely due to the structural differences between morphine and desomorphine, which influences how each interacts with enzymatic binding sites.

Experimental approaches to drug metabolism

There are three categories of testing models that are used to study metabolism: *in vivo*, *in vitro*, and *in silico* (66). In brief, *in silico* models are predictions of drug metabolism based on existing knowledge of structurally similar drugs and enzyme crystal structures. *In vivo* models performed using living subjects (human or animal) provide information including but not limited to bioavailability, distribution, clearance rate, and duration of exposure. *In vitro* models work on a smaller scale than *in vivo*, but dispite their limitations, they can provide very reliable infomation (67, 68). *In vitro* models are widely used to investigate parameters including but not limited to metabolic stability, drug-drug interactions, and toxicity.

The ideal approach to determining the metabolism of desomorphine in humans would be to conduct an *in vivo* study, in which willing participants would be administered controlled doses. Urine and blood would be collected at set time intervals and analyzed for the parent drug and metabolites. However, the rate and efficiency of metabolism is highly variable between individuals. Age, disease, and genetic variation can all affect

metabolism and to draw any generalized conclusions from such a study would require a large population of subjects, which would be time consuming and costly (69-71). The use of an *in vitro* model has many advantages. In general terms hepatic *in vitro* models can be divided into the following categories: recombinant enzymes, subcellular fractions, whole cell cultures, and tissue cultures (72).

Recombinant enzymes

Recombinant enzymes are created by encoding the gene of an enzyme into a vector that can be expressed in a system other than its native environment. The gene is obtained by isolating messenger ribonucleic acid (mRNA) for an enzyme from tissue and using that mRNA to produce the complementary deoxyriboneucleic acid (cDNA) that encodes the enzyme (73). The cDNA can then be inserted into a vector for expression. Multiple enzymes have been expressed this way, the most common being CYPs, and more recently UGTs. Whole cell expression systems are commonly used, which includes mammalian cells, yeast cells, bacterial cells, and insect cells.

The species of mammalian cells used can vary based on availability and study needs. A virus or a plasmid is used to introduce the cDNA, and most mammalian cells offer the advantage of having an endogenous endoplasmic reticulum which helps support the activity of the expressed enzyme but may also have endogenous CYP enzymes. Using selection markers can amplify the cDNA copy number and increase the recombinant enzyme's expression beyond that of the endogenous enzymes (73).

The yeast species most commonly used for recombinant enzyme expression is baker's yeast, *Saccharomyces cerevisiae* though many others can be used (74). Insertion vectors are used, either replicating vectors or vectors that can be integrated with the host

genome, to insert the desired gene (73). Yeast also has an endoplasmic reticulum, however unlike mammalian cells the efficient expression of the recombinant enzyme often requires modification of the gene. This is accomplished by deleting a portion of the untranslated 5' region of the cDNA (75).

Escherichia coli is the most common bacterial species used for expressing recombinant enzymes. It is easily manipulated with numerous vectors but also requires extensive modification to the gene sequence to produce high expression of enzymes. Modification techniques used for inserted genes include modifications to the *N*-terminal and mutating the cDNA sequence to code for common *E. coli* codons rather than mammalian ones (76). Enzyme expression can also be enhanced by co-expressing cytochrome oxidoreductase and sometimes cytochrome b5 (77).

Insect cell expression systems use baculoviruses as vectors (72), which are a group of viruses that exclusively infect insect cells (73). As with mammalian and yeast cells, insect cells contain endogenous endoplasmic reticulum. It is not necessary to modify cDNA for expression in insect cells but the co-expression of cytochrome oxidoreductase and sometimes cytochrome b5 for may still be necessary for efficient enzyme expression (78).

Recombinant enzymes are excellent tools for investigating individual enzyme pathways and the effect of enzyme polymorphism. Their high activity produces relatively large quantities of metabolites compared to other *in vitro* models, allowing for easier analysis of their structures. Interpretation of the results must be done with care however, for these enzymes are not functioning in their native environment and the strategies used to maximize their expression and activity mean that the metabolic products and reaction

rates might be shifted from *in vivo*. Studies comparing different expression systems also indicate varying levels of enzyme activity, with enzymes expressed in bactosomes being more active than those expresses in supersomes™ (79, 80), however bactosome expression requires more genetic modification, which could possibly account for the increase in activity but any additional modification can carry a risk of altering an enzymes function and potentially what metabolites are formed.

Subcellular fractions

Subcellular fractions are isolated from liver tissue. These fractions contain much of the infrastructure found in the whole cells, but they are not entirely self-supporting. It is necessary to add a source of elections, also referred to as a cofactor, for the reactions to proceed. There are three forms of subcellular fractions: microsomes, cytosol fractions, and S9 fractions.

Microsomes are vesicles of endoplasmic reticulum which contain CYPs and other membrane bound enzymes, such as UGTs (75). Enzymatic activity can vary between individuals so to overcome this, microsomes are 'pooled', meaning the final product contains microsomes from multiple individual donors to mitigate individual variation and allow for generalized conclusions to be drawn. Unpooled microsomes also have their uses as they can be used to investigate whether an enzyme's polymorphisms will affect the affinity towards a drug or change the final product formed. Additionally, pooled microsomes from donors of only one gender can be used to instigate the effect of gender on metabolism.

Cytosol fractions are portions of the cellular cytosol, which contains enzymes that are not bound to a membrane. This does not include CYPs but does include multiple phase

II enzymes, including glutathione S-transferase, sulfotransferase, and *N*-acetyl transferase (75). Cytosol fractions are not used as often as other subcellular fractions because these metabolic pathways are not commonly involved with drug metabolism but can prove useful in instances where extensive phase II metabolism occurs, such as the case with many opioids.

S9 fractions are a combination of microsomes and cytosol fractions. Like microsomes and cytosol fractions, S9 fractions still require cofactors for reactions to proceed and can be pooled from multiple donors. They give a more complete metabolic profile for a drug but overall have a lower enzyme activity compared to cytosol fractions and microsomes which can make detecting metabolites difficult (72).

Subcellular fractions are versatile tools for metabolism studies. They can be used to study drug interactions and individual enzyme pathways can be studied with the application of specific enzymatic inhibitors (66). S9 fractions can also be used to study the both phase I and phase II metabolic processes simultaneously. While more representative of *in vivo* than recombinant enzymes, subcellular fractions still lack extracellular factors and components that may influence metabolic pathways.

Whole cell culture and tissue fractions

As whole cell cultures consist of intact cells instead of enzymes or subcellular fractions, they are more representative of *in vivo* physiology, but must be cultured in specialized media to support their lifecycles. There are three types of whole cell cultures: liver cell lines, transgenic cell lines, and hepatocytes. Like subcellular fractions, cells from different donors can be pooled to reduce variation between individuals. However, in contrast to subcellular fractions, whole cell cultures contain not only all of the enzymes

involved in hepatic metabolism, but also the additional cellular structures present in eukaryotic cells, which may influence metabolism pathways.

Liver cell lines are isolated from primary tumors of liver parenchyma. As these are typically harvested from livers suffering from cirrhosis or hepatitis, they typically do not express all of the families of metabolic enzymes (75). There are multiple cell lines available, each of which expresses different families of enzymes. Enzyme expression can be increased through induction but it is still difficult to investigate individual enzyme pathways with cell lines (72). Their main advantage is that they are easier to cultivate than other cell cultures.

Transgenic cell lines can be considered both whole cell cultures and recombinant enzymes. They are liver cell lines that have been used to express recombinant enzymes in order to overexpress select enzymes (75). Transgenic cell lines share the advantages of both cell lines and recombinant enzymes. They are however limited in the number of enzymes that can be expressed at one time.

Hepatocytes are isolated from liver tissue obtained from liver transections (81). They are a popular *in vitro* model due to a strong resemblance to *in vivo* liver (82). Hepatocytes exist in three forms: primary, cultured, or cryopreserved. Primary hepatocytes refer to hepatocytes that are freshly isolated from tissue. They are generally only viable for a few hours unless cultured. Cultured hepatocytes are stable for up to four weeks but are subject to a decrease in CYP activity over time. Cryopreservation allows for hepatocytes to be stored for longer periods of time without a loss of activity, making them the most popular whole cell model (72).

Tissue cultures are the closest representative of *in vivo* interactions as they not only preserve the integrity of the cell but the extracellular environment as well, however they are the most difficult to prepare and work with. The two forms of liver tissue cultures are liver slices and isolated perfused liver. Like cell cultures they require specialized media to keep the tissue functioning (72). Slices of liver tissue can be powerful investigative tools, but their reproducibility is dependent on uniform thickness between slices (75). Long-term preservation of liver slices has not yet been developed so this model is only viable for a short period of time (83).

An isolated perfused liver is a whole liver that is maintained through specialized media. Human liver has never been used for this purpose due to the requirement of an intact liver (72). As animal liver is not always a good reflection of the human system this technique is rarely used in the realm of human drug discovery. It is typically only used when bile secretion is suspected to be important. Bile secretion is perhaps this model's only advantage over other models as it has a very short window of viability, generally about 3 hours (75).

Forensic toxicology

Forensic toxicology may include a variety of investigation types, including but not limited to fatalities (post mortem), impaired driving, and drug-facilitated sexual assault (ante mortem). The type of sample matrix encountered depends on the case type, although blood and urine are the most frequently encountered specimens. Postmortem toxicology may also involve tissues, stomach contents, bile, and vitreous humor. Additional sample matrices include hair, oral fluid and sweat.

In general, the analytical process consists of presumptive testing followed by confirmatory analysis. Presumptive testing, also called drug screening, is used to narrow down the class of drug that might be present and generally involves minimal sample preparation. Once that information has been obtained, the analyte is isolated from the matrix via sample preparation techniques that are tailored to the chemical characteristics of the drug(s) in question. After isolation the analyte, confirmatory testing takes place to specifically identify the analyte(s).

Any analytical methodology developed for use in forensic toxicology laboratories must be carefully documented and systematically assessed to ensure it will produce reliable results. The scope of validation is dependent on whether the method is intended to be presumptive, qualitative or quantitative. The validation of presumptive methods can include the limit of detection, potential interferences, the precision at a decision point, dilution integrity and stability. Validation of qualitative methods often includes the limit of detection, carryover, potential interferences, dilution integrity, stability, and matrix effects (if applicable). The validation of quantitative methods is the most rigorous and in addition to the parameters assessed with qualitative methods, should also assess bias, precision, the limit of quantitation and calibration model. Validation standards for forensic toxicology were published by the Scientific Working Group for Forensic Toxicology (SWGTOX) in 2013 (84). Those recommendations are currently undergoing a process to become an American National Standard.

Drug screening

Drug screening is intended to presumptively identify drugs or classes of drug that may be present in a specimen. Screening techniques used in forensic toxicology are

generally either immunoassay (IA) or mass spectrometry (MS) based. While IA techniques are most commonly encountered in forensic toxicology laboratories, MS-based screening is growing in popularity, likely due to the proliferation of designer drugs and new psychoactive substances (85).

IAs utilize antibody reagents, which are typically raised against an antigen in animal species. Antibodies isolated from serum can be immobilized onto a surface for immunoassay testing purposes. There are five immunoglobulin classes, IgG, IgM, IgA, IgE and IgD, with IgG being the most abundant. Polyclonal antibodies can be subject to considerable variation due to the nature of biological systems. Monoclonal antibodies are more reproducible, and are created by harvesting antibody cells, then fusing them to tumor cells to form hybridomas. These can be screened for specific antibody production and the desired cells cloned to increase antibody production.

Because most drugs are less than 2000 Da in mass, they are not naturally antigenic, necessitating linkage to a carrier protein to facilitate interaction. These carrier proteins are linked to the drugs using functional groups (amines, hydroxyls, etc) to attach the crosslinked 'bridge' between the drug, or hapten, and the carrier protein. For drugs with multiple functional groups, the site selected for covalent bonding may determine which part of the hapten can be recognized by the immune system. The nature of the attachment can significantly influence assay performance (86). Cross-reactivity arises in IAs when substances structurally related to the target drug bind to the antibody. The covalent bonding between the very large carrier protein and the small drug may occlude certain portions of the molecule, making them "invisible" to the immune system during antibody production. This can lead to decreased specificity at those sites, resulting in cross-

reactivity. However, this lack of specificity can also be exploited to create immunoassays capable of detecting multiple drugs within a class of structurally similar substances, such as the benzodiazepines or barbiturates.

The most commonly used IAs in forensic toxicology are enzyme-linked immunosorbent assays (ELISAs) and enzyme multiplied immunoassay technique (EMIT®) (85). All are competitive assays, meaning that any drug present in the sample will compete with labelled drug for antibody binding sites. In homogeneous assays it is not necessary to separate bound and unbound drug prior to detection. In a heterogeneous ELISA, unbound drug is removed prior to the addition of the enzyme substrate. In the case of ELISA, a colorimetric reaction is used to identify drug in the specimen. An established cut off concentration, is used to differentiate a positive from a negative result. While individual laboratories may establish cut off concentrations independently, recommendations for impaired driving casework have been published (87). In an ELISA, antibodies are frequently immobilized on the surface of microtiter wells. Drug in the sample and enzyme-labelled drug are incubated in the well, after which unbound components are discarded. Horseradish peroxidase-labelled drug and tetramethylbenzidine (TMB) are frequently utilized for colorimetric detection. Using this approach, there is an inverse relationship between color intensity (measured spectrophotometrically) and drug concentration. Heterogeneous ELISAs can be readily adapted to a wide variety of biological matrices. Other advantages include small sample volumes, high throughput, sensitivity, resistance to matrix effects, rapid analysis and potential for automation. Sodium azide, a common antimicrobial agent used to preserve urine, can interfere with horseradish peroxidase activity and should not be used.

Using EMIT, glucose-6-phosphate dehydrogenase oxidizes glucose-6-phosphate to gluconolacetone-6-phosphate and reduces nicotinamide adenine dinucleotide (NAD) to NAD^+. The absorbance of NAD^+ (340 nm) is measured as a function of time, with the amount of NAD^+ produced being directly proportional to the amount of free drug present in the sample. Advantages of EMIT include the wide concentration range and ease of automation. However, due to it homogeneous nature, EMIT is more susceptible to non-specific interferences and may require additional sample preparation for more complicated biological matrices (88).

IAs typically require minimal sample preparation for aqueous matrices, however solid matrices, such as tissues may require protein precipitation, centrifugation and/or dilution. The major limitation of all IAs is their reliance on structural similarity in order for a drug to be detected. Immunoassays for common opioids often utilize morphine as the target drug. As such they are incapable of detecting fentanyl, methadone or newer designer opioids that do not resemble morphine in structure. This may require the use of several IAs to detect a specific class of drug, which may be cost-prohibitive and inefficient. The development of a new commercial immunoassay is also a time-consuming process. In contrast, MS based screening techniques can be readily adapted for novel drugs, negating the time delay between the first appearance of a drug and the development of a suitable immunoassay. Due to the dynamic nature of the designer drug landscape, this adaptability is a significant advantage for forensic laboratories that already have MS instrumentation.

The MS is used to ionize molecules and separate them according to their mass-to-charge ratios. Accompanying mass spectral fragmentation of the molecule can provide valuable structural information. MS combined with separation techniques such as gas

chromatography (GC) and liquid chromatography (LC), are widely used for confirmatory analyses in forensic toxicology (85). While MS bases techniques may require more extensive sample preparation than ELISA, the existence of simultaneous extraction methods for acidic, basic and neutral drugs allows for broad spectrum drug screening (89, 90).

GC-MS has the advantage of being a well-established technique with large reference libraries available for comparison purposes (91). However, GC-MS is incompatible with large, nonvolatile compounds, as well as highly polar compounds, including many endogenous substances present in biological specimens. Derivatization is often needed for polar analytes (such as morphine), and conjugated metabolites must be hydrolyzed prior to extraction to facilitate their detection.

LC-MS is compatible with polar compounds, including conjugated species. Sample preparation is sometimes less extensive compared to GC-MS, but this approach can compromise sensitivity and matrix effects. High resolution mass spectrometry (HRMS) can be used to provide more detailed information in terms of structural elucidation. This is a significant advantage for novel drugs, for which reference materials or analytical standards may not be commercially available. One disadvantage of LC-MS based techniques include the potential for significant ionization suppression or enhancement. This can negatively impact analyte detection, particularly at low concentrations. Additionally, the fragmentation of molecules is not as reproducible as GC-MS, but careful control of ionization parameters allows for comparison of sample spectra with library databases (92).

Sample preparation

The goal of sample preparation is to isolate an analyte (drug) from the biological matrix with high recovery and minimal interferences from endogenous compounds. Regardless of the extraction technique being used, the sample should be homogeneous to ensure an equal distribution of the drug throughout the specimen. When necessary, this can be readily achieved for blood, urine and other body fluids using simple vortex mixing or sonication. However, solid samples including tissues typically require mechanical homogenization. Biological matrices rich in protein, such as blood and tissue, may require an additional step to precipitate the proteins and remove them from the matrix prior to extraction. This can be achieved through the use of organic solvents such as acetonitrile, acidic solutions such as trichloroacetic acid, or inorganic materials. After the proteins have been precipitated, the sample can be centrifuged or filtered to remove them from solution. Protein precipitation can be problematic with highly protein bound drugs as they may co-precipitate with the proteins (93, 94). In those instances, sample dilution, sonication, centrifugation or filtering may be preferable.

Opioids tend to undergo extensive phase II metabolism, much like cannabinoids and benzodiazepines. As such they are present in biological samples in both free and bound (conjugated) form. Often the ratio of free to bound drug in blood or tissue is calculated to provide insight regarding acute or chronic administration of the drug. The highly polar nature of conjugated metabolites increases the difficulty associated with their extraction from biological specimens and may also render them incompatible with gas chromatography-mass spectrometry (GC-MS). The hydrolysis of conjugated metabolites to yield free drug can make them more amenable to analysis, and as such samples are

typically analyzed twice, once to determine the amount of free drug present and again following hydrolysis to measure the total drug present. These two measurements are then used to calculate the proportions of bound and free drug. The two categories of hydrolysis reactions commonly used are chemical, using either strong acid or strong base, and enzymatic, using preparations of β-glucuronidase or sulfatase.

Chemical hydrolysis, which is commonly performed using acid at elevated temperatures, is less costly and requires less time than enzymatic options but has been shown to cause degradation in opioids (95-97), which can complicate interpretation of the results. As such, despite studies showing acid hydrolysis to be more efficient for hydrolyzing conjugated morphine metabolites, the more time-consuming enzymatic methods are often preferred to reduce loss of analyte and misinterpretation of the results. Enzymatic hydrolysis does not cause such degradation but some opioids, like morphine, are more resistant to enzymatic hydrolysis, resulting in extended incubation times to ensure complete hydrolysis (98-100). β-glucuronidase has been isolated from multiple species and each isoform varies in activity for different drugs (97, 101), necessitating the individual optimization of hydrolysis reactions for each.

Commonly used preparations of β-glucuronidase include *Escherichia coli*, *Helix pomatia* and *Patella vulgata* among others. When optimizing hydrolysis, enzyme concentration, temperature and pH are evaluated to determine which conditions will provide complete reproducible results in the least amount of time. It has been shown that the increasing the concentration of β-glucuronidase will not uniformly increase the extent of hydrolysis for all species (102). Increasing the temperature of the reaction may reduce reaction time, however each species has different limitations. In particular, *E. coli*, whose

optimal reaction temperature is 37°C, can tolerate temperatures up to 50°C without an appreciable decrease in efficiency while other species will be more efficient at 60°C (101). Manufacturers will recommend an optimal pH or pH range based on test substrates, and while in most cases that pH will be efficient for drug hydrolysis, a greater shift in pH may occasionally be necessary, as is the case for hydrolysis of morphine-glucuronides by *P. vulgata* (102). While assessing hydrolysis conditions, the stability of a glucuronide must also be considered as some glucuronides, particularly acyl- and *N*-glucuronides can be labile in neutral and alkaline solutions and may require hydrolysis with a β-glucuronidase that can tolerate more acidic conditions (103). The affinity for individual glucuronides may also vary between species. Comparison studies have shown *P. vulgata* to be more efficient for hydrolyzing morphine-glucuronides than *E. coli* and *H. pomatia* (98, 102), while the opposite is true for buprenorphine-glucuronides (97, 104).

Recently, recombinant β-glucuronidase preparations have become commercially available, which are genetically modified to increase both their activity and their affinity for analytes like the opioids. Two recent studies have been published evaluating one recombinant option, IMCSzyme™, with opioids. The first study compared hydrolysis with IMCSzyme™ with acid hydrolysis, showing that no degradation occurred when using the recombinant enzyme (95). The second study compared the recombinant enzyme with traditional preparations and found it to be either more efficient or comparable to the well-established enzymes for most of the substrates tested (105). Recombinant enzymes are purified preparations that do not contain additional endogenous proteins and compounds encountered in traditional β-glucronidase preparations. This may reduce the amount of

sample cleanup needed to remove impurities from the sample and reduce detector fouling and routine instrument maintenance.

Liquid-liquid extraction (LLE) is a commonly employed extraction technique for toxicological specimens. In brief, the aqueous sample matrix, after adjustment of the pH with buffer, is mixed with an immiscible organic solvent. After the drug partitions into the solvent it can be concentrated before analysis. Solvent polarity and hydrogen bonding are important considerations for LLE as most drugs that will be encountered have some degree of polarity. For example, a strongly polar drug will require a solvent with a high degree of polarity for extraction and a drug that is capable of accepting hydrogen bonds but not donating them will be more efficiently extracted with a solvent that can donate hydrogen bonds. The pKa of the drug is also an important point of consideration. Most drugs will more readily partition into the organic solvent when they are uncharged. While specific pH requirements can be calculated with the Henderson-Hasselbalch equation, acidic drugs are predominantly uncharged below their pKa, while basic drugs are uncharged above their pKa. Opioids require special consideration in this regard as many of them form zwitterions, therefore the pH of the sample is typically adjusted to their isoelectric point prior to LLE. For drugs with multiple pKa values that are similar in magnitude, relatively small variations in the sample pH may dramatically shift the ionization status of the drug. This may impact reproducibility of the assay and overall robustness.

One of the major disadvantages of LLE is the large quantity of solvent required for extraction. If the initial partition into the organic solvent has a poor yield, successive extractions or large solvent to sample ratios (e.g. 5:1 or more) may be needed to achieve sufficient analyte recovery (106). After the initial extraction, back-extraction techniques

are often required to remove additional impurities. This is readily achieved for basic compounds via the addition of an acidified buffer or reagent. For analysis with instruments incompatible with aqueous samples, a subsequent extraction with organic solvent is required. LLE is a relatively simple extraction with minimal steps, but while each additional step further purifies the sample, there is also a corresponding loss of drug, lowering the overall extraction efficiency. In addition to significant solvent volumes and the challenge of extracting zwitterions, the formation of an emulsion during the mixing of aqueous and organic phases is another potential complication.

Solid phase extraction (SPE) is an extraction technique that avoids some of the pitfalls of LLE. Silica or polymer-based sorbent in an SPE cartridge can facilitate the selective binding of drugs in an aqueous matrix, while allowing impurities to pass through unhindered. After washing with appropriate aqueous and organic solvents, the drug can be eluted with a minimal amount of solvent, resulting in a highly concentrated extract and creating minimal solvent waste. Functional groups bound to the stationary phase determine the chemical properties of the sorbent material and may consist of hydrocarbon chains, phenyl groups, polar groups, cation and anion exchange groups. Copolymeric sorbent phases, which contain multiple types of functional groups with different chemical properties, can be used to extract a wide range of drugs. Amphoteric bases, like the opioids, can be efficiently extracted using copolymeric or mixed-mode SPE. Using this approach, opioids can be readily extracted using SPE by adjusting the pH of the sample to ionize the basic nitrogen, introducing the sample to the SPE column, washing the column with aqueous, acidic and polar solvents to removed contaminants, drying the column and eluting

drugs with an alkaline organic solvent. While SPE requires more steps than LLE, it is typically faster, produces less solvent waste, and can be automated if necessary.

A final step that may be necessary is derivatization, particularly for analysis using GC-MS. Polar functional groups can result in poor chromatographic properties. Hydroxyl, carboxylic acid and amine functional groups are often derivatized, which reduces both their polarity and volatility, leading to improved chromatographic separation, peak shape and quantitative assay performance (107). As many opioids contain tertiary amines and hydroxyl groups, they can be readily derivatized using silylation and acylation. Silylation is often used to replace an active proton with an alkylsilyl group. A large number of silylating reagents are available for this purpose, including *N*,*O*-bis(trimethylsilyl)trifluoroacetamide and *N*-methyltrimethylsilyltrifluoroacetamide. Acylation usually involves the addition of a haloalkylacyl due to the associated increase in electron affinity. Although acylation is commonly used for drugs containing alcohols, amines, thiols and phenols, it is not effective for carboxylic acids (108). Examples of acetylation reagents include *N*-methyl-bis(trifluoroacetamide) and trifluoroacetic anhydride. Separation using LC instead of GC typically eliminates the need for derivatization because it is compatible with both aqueous samples and highly polar analytes.

Confirmatory analysis

Confirmatory testing is a vital step in forensic toxicology testing. Qualitative testing is used to specifically identify the drug(s) present, while quantitative testing is used to identify the quantity of the substance in the matrix. Instrumentation used for confirmatory testing requires a high degree of selectivity to provide sufficient confidence

in the results. Mass spectrometric (MS) techniques satisfy this requirement but cannot typically analyze multiple analytes simultaneously, necessitating some form of separation technique be applied.

Partition chromatography, invented by Martin and Synge in 1941 (109), is widely used in forensic toxicology laboratories for analytical separation. A sample is introduced to a chromatographic column and as it travels the length of the column, each component of the sample (drug or otherwise) will partition between a stationary phase and mobile phase. A component's affinity for the stationary phase will affect its rate of migration through the column and differences in migration rate result in separation.

The degree of separation is dependent on the chemical properties of the stationary phase. The main forms of interactions in partition chromatography are hydrogen bonding, dipole-dipole interactions and dispersion. A nonpolar stationary phase is limited to dispersion interactions with analytes, but a polar stationary phase is capable of hydrogen bonding and dipole-dipole interactions with polar analytes.

Chromatography columns may be either open tubular (capillary), with the stationary phase coating the wall of a capillary, or packed, with the stationary phase coating the packing material (typically silica particles). Capillary columns are commonly used with GC which uses an inert gas as the mobile phase. As such, samples analyzed using GC must be suitably volatile (boiling points typically <400°C). The order in which analytes elute from the column is affected by their boiling points. To vaporize samples, the inlet is maintained at an elevated temperature. Columns are housed in a thermostatically-controlled oven and the temperature can be increased over time to reduce the analysis time required for high boiling point compounds. Column efficiency is measured by the height

equivalent of a theoretical plate (HETP) and is in part a function of the choice of carrier gas for the mobile phase. It is desirable to produce the smallest HETP possible, while maintaining acceptable analysis time and reproducibility.

Hydrogen, helium and nitrogen are common choices for carrier gases as they are inert and will not react with the analyte. Nitrogen produces the highest efficiency in separations but small variations in flow rate can cause significant differences which may affect the chromatography. Hydrogen can provide the shortest analysis time and is less susceptible to differences from variations in flow rate, but its use at high temperatures is not favorable from a safety standpoint. Helium falls has intermediate properties, with a greater efficiency than hydrogen and a shorter analysis time than nitrogen. GC is most suitable for nonpolar analytes <1000 Da in mass and commonly utilizes a nonpolar column. Polar compounds such as desomorphine and other opioids may require derivatization prior to analysis to improve chromatographic behavior.

Packed chromatography columns are used with LC, which uses a liquid mobile phase. It is amenable to nonpolar, polar and high molecular weight compounds. Stationary phases can be polar (normal phase) or nonpolar (reverse phase). Nonpolar mobile phases are used with normal phase LC (NPLC) and polar mobile phases are used with reverse phase LC (RPLC). Analyte elution order will change depending the polarity of the stationary phase. With NPLC, nonpolar analytes will elute from the column first and with RPLC, polar analytes will elute first. As most drugs have some degree of polarity, RPLC is commonly used in forensic toxicology. In addition to polarity, elution order is also affected by analyte mass as the column packing material is generally composed of porous or superficially porous silica particles. Smaller analytes will migrate into small pores on

the particles while larger molecules will not, therefore drugs with larger mass will elute before smaller ones of similar polarity. The mobile phase consists one or more solvents, which may be aqueous or organic in nature, or a combination thereof. Isocratic elution is achieved by using the same composition of mobile phase throughout the separation. However, varying the ratio of aqueous and organic mobile phase solvents (gradient elution) adjusts the polarity of the mobile over time and can decrease the retention time of analytes that interact strongly with the stationary phase. While pure solvents can be used as mobile phase components, it is generally advisable to control the pH of the mobile phase during RPLC separation to achieve uniform movement and charge state of the analyte.

For forensic purposes, GCs and LCs are commonly coupled directly to an MS. Regardless of which separation technique is used, the ionized compounds must be in the gas phase to be detected by the MS. As such, MS instruments typically operate under high vacuum to facilitate the vaporization of high molecular weight compounds. There are multiple mass analyzers available, but the most common configurations encountered in forensic toxicology include quadrupole, ion trap and time-of-flight (TOF) mass analyzers.

Most mass analyzers are compatible with multiple ionization techniques, however not all ionization techniques are compatible with all separation techniques. The best example of this is electron impact (EI) ionization, which is widely used with GC-MS but is not capable of ionizing molecules in the liquid phase and is not used with LC-MS. During EI ionization molecules pass through a stream of electrons generated from a tungsten filament. The electrons have higher energy than the chemical bonds within the molecule, so as the molecule passes through the stream they not only ionize but fragment as well. The characteristic pattern associated with the fragmentation process are detected,

producing a mass spectrum that can be compared to reference libraries EI ionization is considered a "hard" ionization technique due to the high energy of the electrons and the significant fragmentation that ensues. In combination with GC, chemical ionization (CI) is a "softer" ionization technique, whereby high energy electrons are replaced with energized or charged gas molecules, such as methane or ammonia. Softer ionization techniques are also compatible with LC interfaces. These include electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI). Although these softer ionization techniques do not produce as many fragments as EI ionization, they can be used in tandem MS configurations to facilitate subsequent fragmentations of both precursor and product ions.

ESI ionizes analytes in the liquid phase, making it compatible with LC. Generally, ESI functions in either positive or negative ion mode, although some configurations can switch between the two during data acquisition, referred to as fast polarity switching. With positive mode ESI the column effluent is sprayed into the source through a nebulizer needle that is held at a high electric potential. This draws negative ions away, both isolating positive ions and repealing them out, breaking the surface tension and forming aerosol droplets. This process is often assisted by a flow of heated nitrogen. Once droplets are formed the internal coulombic repulsion caused by the excess charge causes the droplets to continue to separate which increases their charge. As this process continues, coulombic fission occurs, leaving only ions which pass into the mass analyzer.

APCI is a versatile ionization technique compatible with GC and LC as it is capable of ionizing analytes in both gas and liquid phases. The nebulizer needle used with APCI is a short tube maintained at a high temperature to vaporize the aerosol components as they

leave the needle. A corona discharge electrode near the end of the vaporization tube produces a cloud of electrons which initiates a cascade of reactions producing ions. APCI is a chemical ionization technique as the sample components are not directly ionized by the electron cloud, but by a series of reactions with reagent ions. APCI is particularly efficient at ionizing neutral compounds, such as steroids, that are not efficiently ionized with ESI, while ESI is often preferred for more polar compounds (110). However, because APCI uses high temperatures to vaporize eluent, it can thermally degrade labile analytes.

Both ESI and APCI are subject to matrix effects, which arise from reactions between an analyte and additional compounds that are present in the biological sample. These substances can either suppress or enhance ionization of the analyte. Although ion suppression is more prevalent, these matrix effects can greatly influence precision, bias and sensitivity of the assay. They are highly dependent on the matrix, analyte and sample preparation technique. Sources of ion suppression include both endogenous compounds, including ionic species, polar compounds, and organic molecules (carbohydrates, amines, urea, lipids, etc), and exogenous compound that may be introduced during sample preparation or analysis (polymer residues, phthalates, detergents, buffers, stationary phase particles, etc).

Mechanisms that have been proposed to explain ion suppression include: competition between the analyte and co-eluting compounds for available charge, matrix compounds binding to the analyte and causing co-precipitation, and analyte ions being neutralized through acid/base reactions in the gas phase (111). Choice of ionization technique can also have an effect, with ESI being more susceptible to matrix effects than APCI (112). Several strategies exist for reducing matrix effects. As only co-eluting

compounds can affect analyte ionization, the method can be optimized to achieve baseline separation between the analyte and all other sample components. Simplified sample preparations that do not separate analytes from sample components carry an increased risk of ion suppression. Extraction techniques like LLE and SPE remove more endogenous compounds but usually involve a pre-concentration step which may exaggerate ion suppression, in which case reducing the sample volume may be beneficial. Matrix matched calibrators and the use of internal standards (IS) mitigate the effect of ion suppression on quantitation. An isotopically labelled internal standard that coelutes with the analyte is ideally suited for this purpose because any decrease in ionization efficiency will influence the IS proportionally.

Quadrupole mass analyzers are frequently utilized with GC-MS and gain their name from four linear electrode rods arranged into two pairs, forming a diamond-like shape. Each pair generates an electrostatic field, one direct current and the other radiofrequency, which bisect each other perpendicularly. Manipulation of these fields create a resonate frequency for individual mass-to-charge ratios, allowing ions with specific *m/z* to fully pass through the quadrupole to the detector. Quadrupole analyzers are capable of varying the resonate frequency emitted to either scan the full mass range (scan mode) or allow only specific ions to pass through (selective ion monitoring, SIM).

Ion trap mass analyzers are variations of quadrupole analyzers whereupon the electrodes have been reconfigured to form a cylindrical shape with the electrodes at either end creating a direct current field and the electrode ring in between them creating a radiofrequency field. After ions enter the trap their energy is quenched using helium and they can then be selectively ejected from the trap by varying the potentials of the end

electrodes. Alternatively, the ions can be held within the trap and excited by varying the radiofrequency field, creating further fragmentation. As ion traps are compatible with multiple ionization techniques, they can provide a low-cost alternative to expensive tandem MS instrumentation.

Unlike quadrupole and ion trap analyzers, TOF mass analyzers are not capable of selecting specific masses. Instead, ions are pulsed from the source and accelerated through an electric field where they then pass through a flight tube to the detector (either a time-to-digital converter or an analog-to-digital converter). The detector is off-set from the ion source and a reflectron is used to focus the kinetic energies of the ions and repel them towards the detector once they reach the end of the flight tube. The principle of TOF mass analyzers is that larger ions take more time to reach the detector than smaller ones. Modern TOF instruments have high resolution, making them capable of accurate mass measurements which provide greater specificity than can be achieved with other mass analyzers.

Mass analyzers can be combined to create tandem MS (MS/MS), of which one of the most common configurations consists of three coupled quadrupoles. This is a powerful and sensitive analytical technique. Targeted acquisition can be achieved by either single reaction monitoring or multiple reaction monitoring, where specific precursor ions are selected for fragmentation in the second quadrupole (referred to as collision induced dissociation) and the third quadrupole operates in SIM mode monitoring specific product ions. Additional acquisition modes include product ion scans, where entire product spectra of selected precursors are recorded, precursor ion scans, where precursors ions of various masses are fragmented but only selected product fragments recorded, and neutral loss

scans, where precursor ions of various masses are fragments and all fragmentations leading to the loss of a specific neutral fragment are recorded. Coupled quadrupole/time-of-flight mass analyzers (Q/TOF-MS) take advantage of both the scanning capability of the quadrupole and the accurate mass capability of the TOF to create a highly specific instrument which can be particularly valuable for structural elucidation. Instruments can function in either full scan mode or in targeted mode, where specific precursors are selected for fragmentation and the entire spectrum is recorded.

Most types of mass analyzers detect charged species via an electron multiplier or photomultiplier and record the mass to charge ratio (*m/z*) of detected ions. No matter how efficient the ionization technique used, only a proportion of the total number of molecules that enter the MS are ionized and detected. Electron multipliers and photomultipliers amplify that signal to generate quantifiable data. For most drugs, only a single charge is produced during ionization such that the recorded *m/z* is analogous with the mass of the molecule, however complex molecules such as proteins can produce multiple charges during ionization. Despite the relatively low efficiency of ionization the proportion of molecule ionized to the whole remains sufficiently constant to allow for quantification based on the responses of known quantities of a drug. As the intensity of the signal produced is a function of the quantity of molecules present in the detector at any one time, it is vitally important for the calibrators used for quantitation to be analyzed under the same conditions as the unknown sample to ensure accurate and consistent results.

Analytical methodology for desomorphine

Desomorphine was reported to be detectable using one enzyme-linked immunosorbent assay, with a cross-reactivity of approximately 50% (113). However, there

has not been a thorough study of its cross-reactivity across multiple commercial ELISA platforms. Every commercially available ELISA has its own proprietary formulation and antibody reagent. As a result, the cross-reactivity of antibody reagents towards desomorphine could be highly variable, which could impact drug detection.

Richter applied metabolite-based screening approaches utilizing GC-MS and LC-HRMS, using an Orbitrap™ detector. Desomorphine and its metabolites were identified in urine using codeine-D_6 as the IS (65). For GC-MS, samples were hydrolyzed using acid hydrolysis, extracted with LLE and derivatized via acetylation before analysis. Using this approach, desomorphine and nordesomorphine were successfully identified. Desomorphine, nordesomorphine and desomorphine-glucuronide were successfully identified using LC-HRMS following protein precipitation and LLE. While originally described as screening approaches these methods could be adapted for confirmatory analysis.

There are very few published methods that describe the confirmatory analysis of desomorphine in biological matrices or even seized drug samples, and most are not validated for quantitative use. Savchuk was the first to describe analytical methodology to detect desomorphine using both GC-MS and LC coupled with an ultraviolet detector (LC-UV) to analyze urine and seized drug samples (5). Acid hydrolysis was used to hydrolyze conjugated metabolites and samples were extracted utilizing LLE. For GC-MS analysis both silylation and acetylation were successfully applied. While concentrations were reported, they were determined by comparison of absolute rather than relative peak area. The absence of an internal standard in this published study is problematic and it would not be considered appropriate for quantitative use in forensic toxicology.

Su was the first to describe a quantitative method, utilizing GC-MS to identify desomorphine in urine. However, the limit of quantitation was 250 ng/g which is not sufficiently sensitive for forensic purposes (114). Samples were extracted utilizing solid phase dynamic extraction and solid phase microextraction without derivatization. Su was the first to describe the use of desomorphine-D_3 as an IS however.

Alves used LC with diode array detection (LC-DAD) and GC-MS to analyze seized drugs with a limit of quantitation of 490 ng/mL (51) and later used GC-MS to analyze desomorphine in blood samples with a limit of quantitation of 103 ng/mL (115). Phenacetin, which bears no structural resemblance to desomorphine, was used as the IS. Drug samples were extracted using LLE, and silylated derivatives were analyzed using GC-MS. Blood samples were extracted using a QuEChERS (Quick, Easy, Cheap, Effective, Rugged and Safe) method before derivatization. This was the first method to describe the analysis of desomorphine in whole blood, however, given the lack of pharmacokinetic data for desomorphine, the reported LOQ of 103 ng/mL may not be sufficiently sensitive for forensic use.

Soares described a qualitative assay for Krokodil drug samples using both LC-DAD and LC-HRMS (Orbitrap™) (52). LLE was used to extract desomorphine but no IS was reported. The purpose of the study was to characterize Krokodil samples and in addition to desomorphine, a total of fifty-four morphinans were identified.

Using LC-MS/MS Eckart described a quantitative assay to identify desomorphine in serum, plasma and tissue (116). Samples were extracted utilizing SPE and codeine-D_6 was used as the IS. The reported limit of quantitation was 0.1 ng/mL, which provides sufficient sensitivity for forensic use. Urine, which is one of the most common biological

matrices encountered, was not evaluated in the study. The method was then applied to over two hundred authentic specimens, spanning clinical and forensic cases, though desomorphine was not identified in any of them. A summary of published analytical methods for desomorphine prior to this study is provided in **Table 1.2**.

Table 1.2

Summary of published analytical methodology for desomorphine.

Matrix	Extraction Method	Separation	Detector	Sample Volume	Derivatization	IS	Stationary Phase	LOQ	Reference
urine; drug samples	LLE	GC	MS	3 mL	acylation; silylation	NR	HP-SM5	NR	(5)
water; urine	SPME; SPDE	GC	MS	500 µL	None	desomorphine-D_3	DB-35MS	250 ng/g (SPDE); 500 ng/g (SPME)	(114)
drug samples	LLE	GC	MS	100 µL	Silylation	phenacetin	Rtx-5	490 ng/mL	(51)
blood	QuEChERS	GC	MS	300 µL	Silylation	phenacetin	Rtx-5	103 ng/mL	(115)
urine	LLE	GC	MS	10 µL	Acylation	codeine-D_6	TG-1MS	NR	(65)
urine; drug samples	LLE	LC	UV	3 mL	N/A	NR	C_{18}	NR	(5)
serum; plasma; tissue	SPE	LC	MS/MS	200 µL; 2 g	N/A	codeine-D_6	phenyl-hexyl	0.1 ng/mL	(116)
drug samples	LLE	LC	DAD	100 µL	N/A	phenacetin	silica	490 ng/mL	(51)

(continued)

urine	LLE	LC	Orbitrap™	2 mL	N/A	codeine-D_6	phenyl-hexyl	NR	(65)
drug samples	none	LC	DAD; Orbitrap™	10 µL	N/A	NR	C_{18}	NR	(52)

DAD, diode array detector; GC, gas chromatography; LC, liquid chromatrography; MS, mass spectrometry; N/A, not applicable; NR, not reported; QuEChERS, Quick, Easy, Cheap, Effective, Rugged and Safe; UV, ultraviolet detector

In addition to the described GC and LC methods, two recent reports described the qualitative analysis of desomorphine at trace levels utilizing a portable MS and direct sample analysis TOF-MS. The portable MS technique was developed for use in clandestine laboratory investigations (117). Items that may be encountered in clandestine laboratories (glassware, enameled cookware, Teflon-coated cookware, prescription bottles, etc) were swabbed and the swabs were directly introduced into the MS inlet. In the second method, samples were deposited directly on a mesh target screen for analysis (118). Sixty-four authentic seized drug casework samples were analyzed using this method but desomorphine was not detected in any. The method was also applied to urine samples however the complexity of the matrix produced inconsistent results.

Statement of the problem

Few case reports of Krokodil use have been published in scientific literature and to date only one case has been analytically confirmed. The lack of analytically confirmed cases makes estimating the true prevalence of Krokodil use difficult. Despite published clinical case reports, media reports and surveys of self-reported use, desomorphine abuse is not well understood.

While one study investigated its metabolism, the enzymes responsible for its biotransformation have not yet been investigated. Pharmacokinetic studies in humans are lacking and the only report of its window of detection was unsubstantiated by actual data. Despite this, it is reasonable to expect forensic analytical methods to have comparable sensitivity to that of morphine (i.e. low ng/mL). Existing analytical methodology may not be sufficiently sensitive for forensic use, particularly in light of reported delays between Krokodil use and specimen collection. Existing GC-MS methodologies do not have the

sensitivity that is required for forensic use and the only quantitative LC-MS/MS method was not developed for use with urine, which is one of the most common sample matrices in forensic toxicology.

To facilitate identification in forensic toxicology investigations, a comprehensive study of desomorphine metabolism and analysis is required. Recombinant CYPs and UGTs were used to more thoroughly investigate the biotransformation pathways responsible for desomorphine's metabolism. Commercially available ELISAs were evaluated for cross-reactivity with desomorphine, and hydrolysis of the glucuronidated metabolite investigated. As instrumentation is variable between laboratories, analytical methodologies to identify and quantitate desomorphine in biological matrices were developed and validated using GC-MS, LC-MS/MS and LC-Q/TOF-MS.

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CHAPTER II

DESOMORPHINE SCREENING USING COMMERCIAL ENZYME-LINKED IMMUNOSORBENT ASSAYS[1]

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Abstract

Desomorphine (Krokodil) is a semi-synthetic opioid that has drawn attention as a recreational drug, particularly in Russia, neighboring former Soviet Republics, Eastern and Central Europe. It has no accepted medicinal uses and is currently a schedule I drug in the United States. In clandestine environments, desomorphine is synthesized from codeine using red phosphorous, hydroiodic acid and gasoline. Residual starting materials in illicit preparations have been associated with severe dermatological effects and extensive tissue necrosis. Desomorphine is not well studied, and there are limited reports concerning its pharmacology or detection in biological matrices. Immunoassays are widely relied upon for both antemortem and postmortem toxicology screening. Although desomorphine is an opioid of the phenanthrene-type, its ability to bind to conventional opioid antibodies has not been described. In this report we describe the cross-reactivity of desomorphine using six commercially available enzyme-linked immunosorbent assays (Immunalysis Opiates Direct ELISA, Immunalysis Oxycodone/Oxymorphone Direct ELISA, Randox Opiate ELISA, OraSure Technologies OTI Opiate Micro-plate EIA, Neogen Opiate Group ELISA and Neogen Oxycodone/Oxymorphone ELISA). Cross-reactivites were highly variable between assays, ranging from 77% to <2.5%. In general, assays directed towards morphine produced greater cross-reactivity with desomorphine than those directed towards oxycodone. The Immunalysis Opiates Direct ELISA produced the greatest cross-reactivity, although several of the assays evaluated produced cross-reactivity of a sufficient magnitude to be effective for desomorphine screening.

Keywords: Desomorphine, Cross-reactivity, ELISA

Desomorphine Screening Using Commercial Enzyme-Linked Immunosorbent Assays

Introduction

Desomorphine (dihydrodesoxymorphine) is a synthetic opioid that was briefly commercialized by Roche in the 1940s under the tradename Permonid. Structurally it differs from morphine in the absence of a hydroxyl group on C6 and the reduction of the C7-C8 double bond. The chemical structures of desomorphine, morphine and related opioids are shown in **Figure 2.1**. Desomorphine was first synthesized in the early 1900s as an alternative to morphine (3). Like other narcotic analgesics, it binds to the μ-opioid receptor and has been shown to have ten-times the analgesic potency of morphine (3, 4). Users experience euphoria and sedation as well as enhanced respiratory depression. Its shorter duration of action, no more than two hours, and greater toxicity compared to morphine caused it to be abandoned therapeutically (1). In the early 2000s it reappeared as an inexpensive alternative to heroin, most notably in parts of Eastern Europe and the Soviet Republic, where the cost of heroin is particularly high (2). The first published case study in the US was in the Midwest in 2014 (5) and although not widespread, an additional case involving Krokodil was reported in 2016 (6).

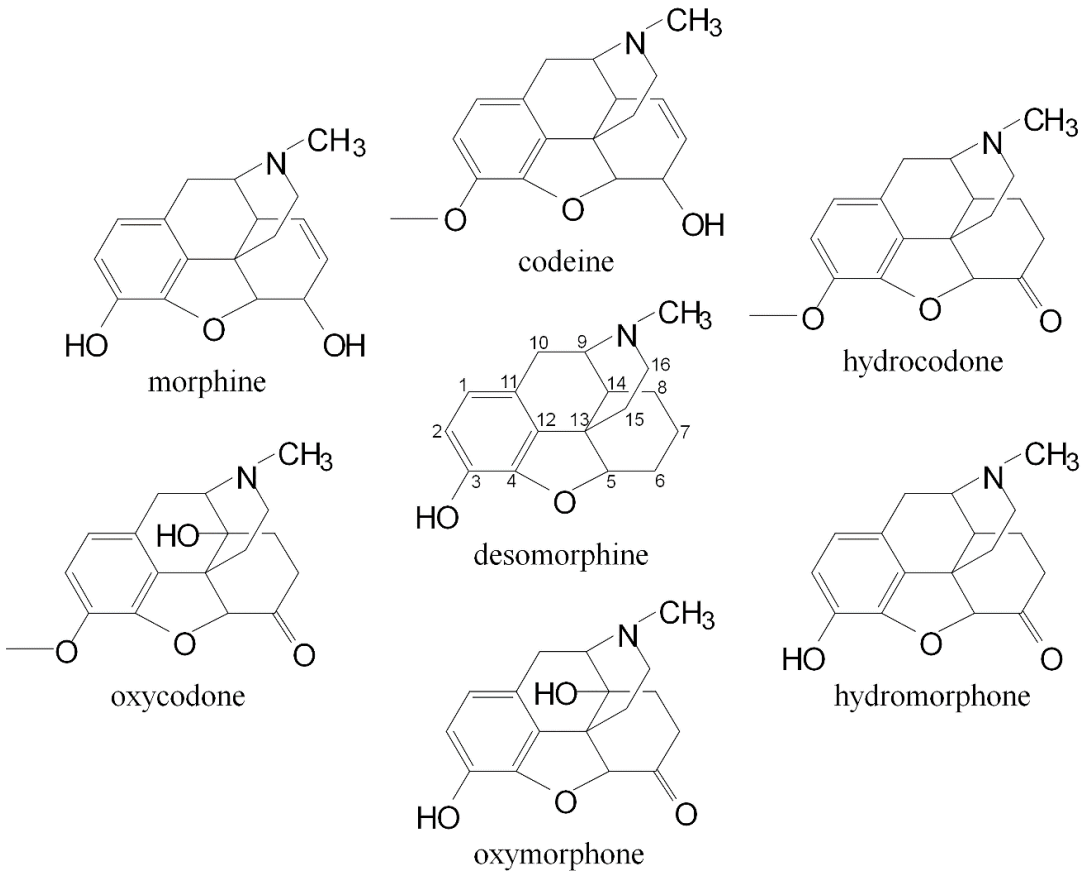

Figure 2.1. Structure of desomorphine and other structurally related opioids.

Desomorphine is readily synthesized via the reduction of codeine using hydroiodic acid and red phosphorus, which are commonly used during clandestine methamphetamine production. Household items, such as Drano® and gasoline, are often used for alkalinization and primitive solvent extraction. Following illicit manufacture, residual starting materials and impurities may remain posing significant harm (2, 7). The final product is typically a light brown liquid that is commonly administered via intravenous or subcutaneous injection. The reported adverse effects of desomorphine abuse include skin, tissue and vein lesions that may culminate in infection, gangrene or limb amputation (8). The tendency of the skin to appear rough and scaly around the site of injection is

responsible for desomorphine's street name of Krokodil (crocodile in Russian). In the United States the drug attracted sensational media attention in 2013 as a "flesh-eating drug" due to the severe dermatological sequale associated with its use. Nevertheless, domestic case reports have been somewhat limited, largely attributed to the widespread availability of other opioids, which hold much greater appeal to the US drug-abusing population.

In the United States desomorphine is a schedule I drug under the federal Controlled Substance Act (1). Codeine, the starting material for desomorphine, is controlled more strictly in the United States compared with many other parts of the world, where it is often available over-the-counter. Although there have been numerous reports of desomorphine use world-wide, there have been limited published reports involving analytical confirmation (9-12). In the absence of confirmed toxicological results, physicians rely heavily on patient history and admissions of Krokodil use (13). The absence of analytically confirmed cases is undoubtedly hampered by the scope of toxicological testing that is commonly employed. As a result, the true prevalence of desomorphine use in the United States is relatively unknown.

Immunoassay techniques are widely used for toxicological screening. However, the degree to which desomorphine cross-reacts with commercial opiate-based assays has not been systematically evaluated. Gas chromatography-mass spectrometry (GC-MS) and liquid chromatography-tandem mass spectrometry (LC-MS/MS) techniques are widely used to identify opiates in biological evidence. However, these confirmatory procedures typically target specific opioids, such as morphine, codeine, 6-monoacetylmorphine or keto-opioids (notably hydrocodone, hydromorphone, oxycodone and oxymorphone). As such, this targeted approach to identification (typically using selected ion monitoring or

multiple reaction monitoring) is unlikely to identify desomorphine. Although mass spectrometric screening techniques are readily adapted to emerging drugs of interest, immunoassays are commonly used. For this reason, cross-reactivity of desomorphine using commercial opiate assays could be highly beneficial and increase the likelihood of detection.

Enzyme-linked immunosorbent assays (ELISAs) are among the most widely used screening techniques in forensic toxicology laboratories. These heterogeneous immunoassays are amenable to blood, urine and tissue samples that are encountered in antemortem and postmortem toxicology investigations. Some assays are designed to be highly specific towards the target analyte, having little or no cross-reactivity towards other compounds in order to limit false positives. Conversely, there is often a need to develop assays with broad cross-reactivity in order to capture multiple drugs or metabolites within a specific class of drugs (e.g. benzodiazepines, barbiturates). The cross-reactivity of polyclonal antibodies that are used in commercial immunoassays is largely dependent on immunogen design and the site at which coupling of the hapten (drug) to the carrier protein takes place. Not surprisingly, crosslinkers that are commonly used to "bridge" the drug to the carrier protein make use of common functional groups such as amines, hydroxyls and others, to facilitate the coupling chemistry. The nature of the carrier protein and the length of the crosslinker also play an important role. The site at which coupling takes place on the drug molecule may be effectively hidden from view, resulting in antibodies that lack specificity for that site or region of the molecule (14). As such, bioconjugation techniques can play an important role in cross-reactivity, and ultimately assay utility and performance.

Structurally the opioids can be broadly classified a phenanthrenes (e.g. morphine, codeine, dihydrocodeine, 6-monoacetylmorphine, hydrocodone, hydromorphone, oxycodone, oxymorphone), phenylheptylamines (e.g., methadone, propoxyphene), phenylpiperidines (e.g., fentanyl, meperidine), morphinans (e.g., levorphanol, butorphanol), benzomorphans (e.g., pentazocine) and cyclohexanols (e.g., tramadol). The phenanthrenes (including desomorphine) are characterized by the morphine-like aromatic core. Although morphine is an effective target drug for many of the phenathrene-type opioids, characteristics of the antibody reagents varies considerably and can often elicit poor cross-reactivity towards some of the keto-opioids. For this reason, manufacturers may offer immunoassays specifically directed towards these popular opioids (namely, oxycodone and/or oxymorphone).

Despite the structural alterations at C6 and C7-8, desomorphine retains the morphine-like structure typical of the phenanthrenes, suggesting that it might lend itself to some degree of cross-reactivity with commercial opioid assays. In this study we evaluated a total of six commercial ELISAs directed towards morphine and oxycodone/oxymorphone. The latter were selected as possible candidates for desomorphine cross-reactivity because unlike morphine, these keto opioids are also saturated in the C7-C8 position (**Figure 2.1**).

Materials and methods

Morphine, desomorphine and oxycodone were obtained from Cerilliant (Round Rock, TX). Pooled human urine was collected from drug-free volunteers and preserved with 1% (w/v) sodium fluoride. Deionized water was generated from a Direct-Q 3 (UV) system (Millipore, Billerica, MA). Six immunoassay kits were obtained from four

commercial sources: Immunalysis Opiates Direct ELISA (catalog no. 207-0192) (IA-OPI) and Oxycodone/Oxymorphone Direct ELISA (catalog no. 221B-0096) (IA-OXY), (Pomona, CA); Randox Opiates ELISA (catalog no. OPI10014) (RX-OPI),(Kearneysville, WV); OraSure Technologies OTI Opiates Micro-plate EIA (catalog no.1150ET) (OS-OPI), (Bethlehem, PA); and Neogen Opiate Group ELISA (catalog no. 130419) (NG-OPI) and Oxycodone/Oxymorphone ELISA (catalog no. 130719) (NG-OXY) (Lexington, KY). Morphine and oxycodone were the target drugs in the opiate and oxycodone/oxymorphone assays, respectively.

Desomorphine cross-reactivity was measured relative to the target drug in urine. Calibrators were prepared by fortifying drug-free urine with drugs over a range of concentrations. Each assay was performed in accordance with the manufacturer's specifications, summarized in **Table 2.1**. Assays were performed manually using a multichannel pipette, a Biotek ELx50/8 Microplate Strip Washer (Winooski, TX) and a Dynex Technologies Opsys MR Plate Reader (Chantilly, VA).

Table 2.1

Summary of experimental conditions.

Assay	Recommended Matrix	Urine Dilution	Sample Volume	Enzyme Conjugate Volume/Incubation Time	Substrate Volume/ Incubation Time	Stop Reagent Volume
Immunalysis Opiates Direct (IA-OPI)	urine, whole blood, serum, plasma, oral fluid	1:20	10 µL	100 µL/60 min	100 µL/30 min	100 µL
Immunalysis Oxycodone/ Oxymorphone (IA-OXY)	whole blood, serum, plasma	1:10	10 µL	100 µL/60 min	100 µL/30 min	100 µL
Neogen Opiate Group (NG-OPI)	urine, whole blood, oral fluid	1:20	20 µL	180 µL/45 min	100 µL/30 min	100 µL
Neogen Oxycodone/ Oxymorphone (NG-OXY)	urine, whole blood, oral fluid	1:50	10 µL	100 µL/45 min	100 µL/30 min	100 µL
Orasure OTI Opiates (OS-OPI)	serum	1:10	25 µL	100 µL/30 min	100 µL/30 min	100 µL
Randox Toxicology Opiates (RX-OPI)	urine, whole blood	1:50	50 µL	75 µL/60 min	125 µL/20 min	100 µL

Briefly, drug-fortified urine at the appropriate dilution (in deionized water or supplied buffer) was added to microtiter wells, together with the appropriate volume of drug-enzyme conjugate. Manufacturer-recommended dilutions (provided in the assay specification sheet) were used unless none were specified. Following incubation for the specified time, excess (unbound) reagent and drug was removed using six washes with deionized water. The appropriate volume of tetramethylbenzidine substrate solution was added. Following incubation for the recommended time in the dark, the appropriate volume of acidic stop reagent was added, and the absorbance was measured (A 450nm – 630 nm). Dose-response curves were generated for each assay using replicate analyses (n=3) for both the target drug (morphine or oxycodone and desomorphine). Percent binding was calculated using Equation 1 where A0 was absorbance of the blank (drug-free urine). Unlike federally regulated workplace drug testing programs, the majority of laboratories that perform forensic toxicology investigations establish their own cutoffs, depending on the type of work they perform. For the purpose of this study a morphine cutoff of 200 ng/mL and an oxycodone cutoff of 100 ng/mL was selected to reflect recommended cutoffs for impaired driving casework (15). Although we recognize that cutoff concentrations throughout laboratories are not consistent, for the purposes of this study it was necessary to calculate cross-reactivities consistently between each assay. The cross-reactivity of desomorphine among the opiate assays was calculated using Equation 2 where C200 was the concentration of desomorphine required to give an absorbance reading equivalent to 200 ng/mL of morphine in urine. The cross-reactivity of desomorphine among the oxycodone/oxymorphone assays was calculated using Equation 3 where C100 was the

concentration of desomorphine required to produce an absorbance reading equivalent to 100 ng/mL of oxycodone.

$$\%\ \text{Binding} = \frac{A}{A_0}\ \text{X}\ 100 \qquad \textit{Equation 1}$$

$$\%\ \text{Cross} - \text{Reactivity} = \frac{200}{C_{200}}\ \text{X}\ 100 \qquad \textit{Equation 2}$$

$$\%\ \text{Cross} - \text{Reactivity} = \frac{200}{C_{100}}\ \text{X}\ 100 \qquad \textit{Equation 3}$$

Results and discussion

The cross-reactivity towards desomorphine and binding characteristics for each of the assays are summarized in **Table 2.2** and dose-response curves are depicted in **Figure 2.1**. Data represents the mean of triplicate measurements at each concentration in urine. Cross-reactivity towards desomorphine was widely variable between assays, ranging from 77% (**Figure 2.2**) to <2.5% (**Figure 2.4**). Error bars are omitted from the composite figure for clarity. Generally, the opiate immunoassays (directed towards morphine) produced greater cross-reactivity than those directed towards oxycodone. Although desomorphine and the keto opioids are saturated at C7 and C8 (unlike morphine), structural alterations at C3, C6 and C14 significantly diminish antibody binding, which is not unexpected.

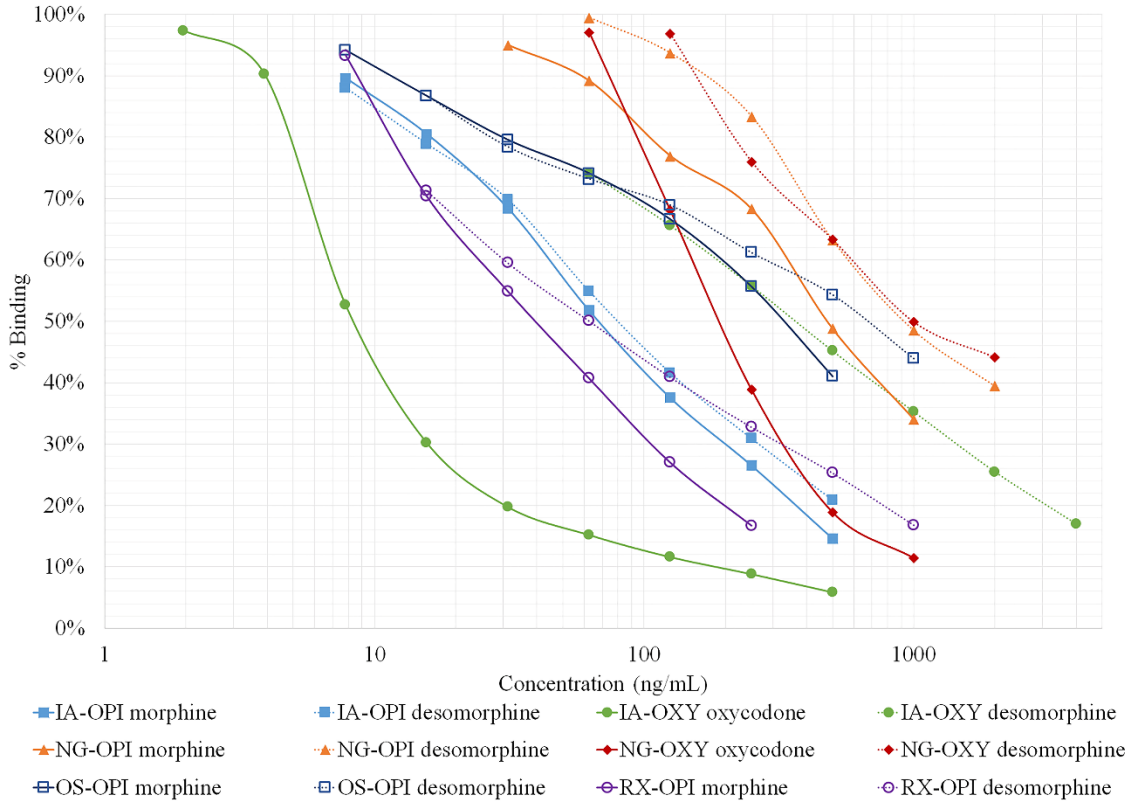

Figure 2.2. Dose-response curves for each assay using target drug (morphine or oxycodone) and desomorphine using Immunalysis Opiates Direct (IA-OPI) and Oxycodone/Oxymorphone Direct (IA-OXY), Neogen Opiates (NG-OPI) and Oxycodone/Oxymorphone (NG-OXY), OraSure Technologies OTI Opiates (OS-OPI) and Randox Toxicology Opiates (RX-OPI).

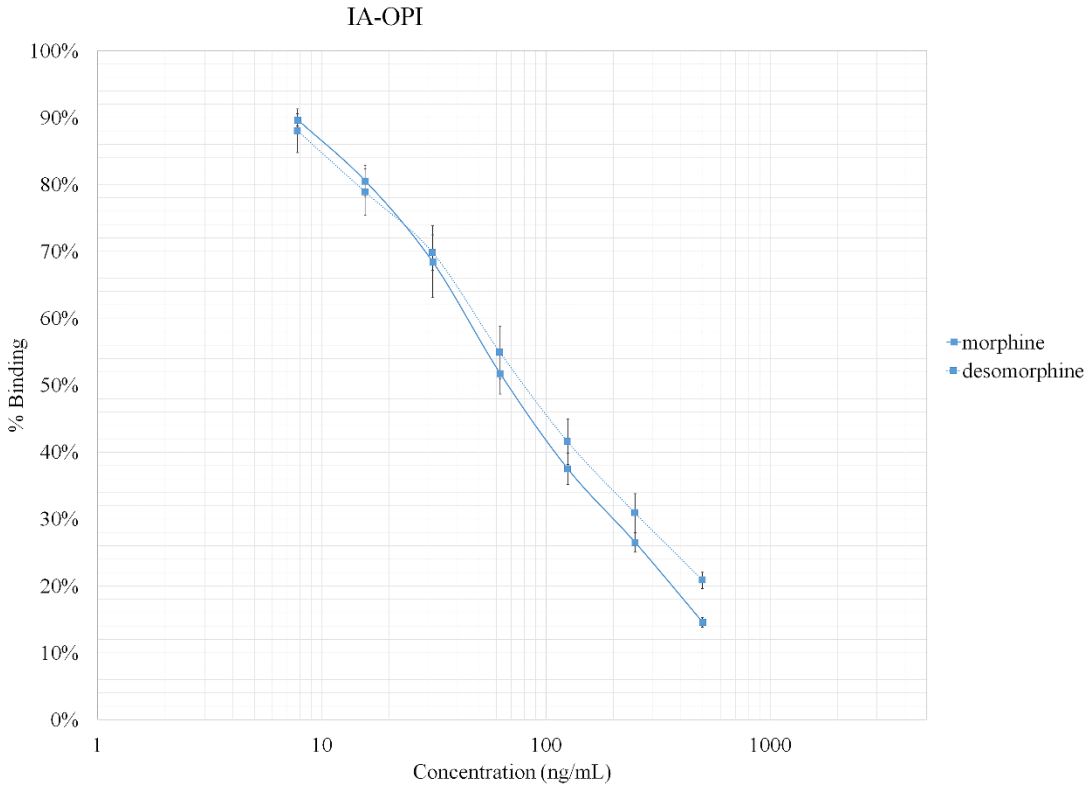

Figure 2.3. Immunalysis Opiates Direct (IA-OPI). Data are shown as the mean ± SD (n=3) at each concentration in urine.

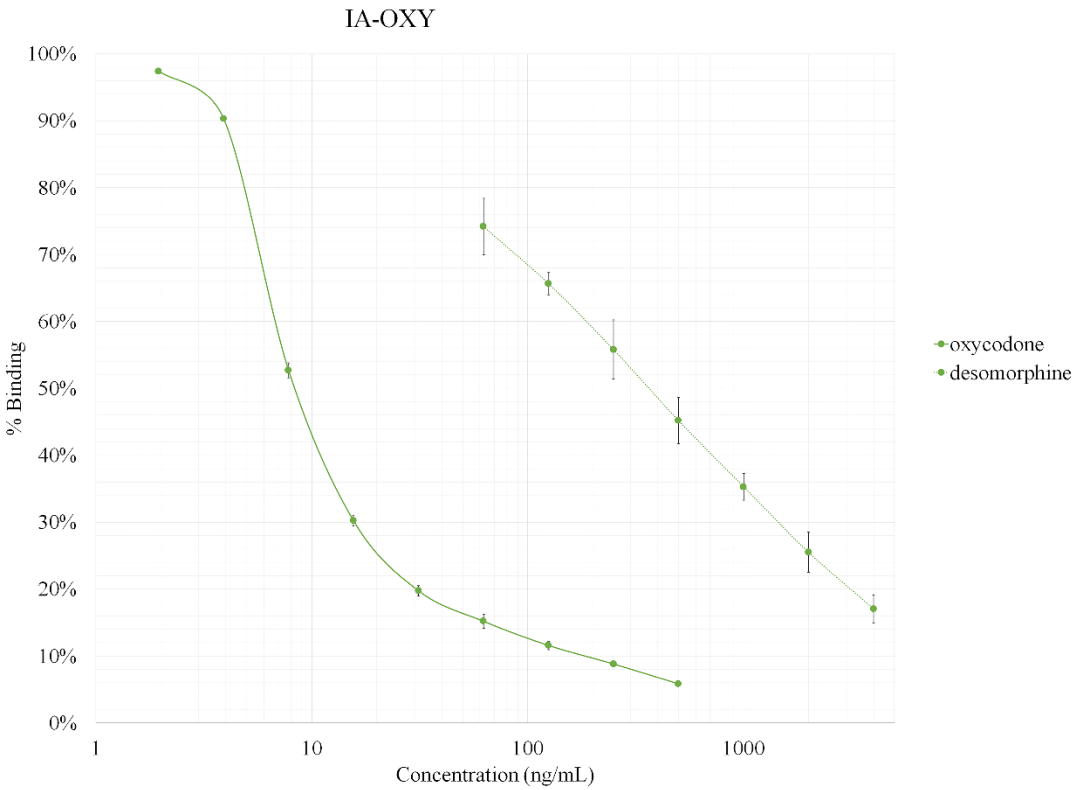

Figure 2.4. Immunalysis Oxycodone/Oxymorphone (IA-OXY). Data are shown as the mean ± SD (n=3) at each concentration in urine.

Table 2.2

Cross-reactivity and binding characteristics of commercial ELISAs towards desomorphine.

Assay	Target Drug	Cutoff (ng/mL)	Equivalent Desomorphine (ng/mL)	Cross-reactivity	EC50 Target Drug (ng/mL)	EC50 DESO (ng/mL)	EC50 Target/Desomorphine (%)
IA-OPI	morphine	200	260	77%	65	80	81%
OS-OPI	morphine	200	280	71%	340	700	49%
NG-OPI	morphine	200	390	51%	490	950	52%
NG-OXY	oxycodone	100	230	43%	190	1000	19%
RX-OPI	morphine	200	800	25%	40	65	62%
IA-OXY	oxycodone	100	> 4000	<2.5%	8	380	2%

Table 2.2 also summarizes antibody binding characteristics and the effective concentration for 50% binding (EC50) for both desomorphine and the target drug. Estimation of cross-reactivity at the EC50 is also possible but was not selected for two reasons. Doing so would effectively estimate the cross-reactivity for each assay at a different concentration; and second, the purpose was to determine the likelihood of identifying a desomorphine-positive sample among routine casework. For this reason, cross-reactivity was estimated at a uniform cutoff concentration for each assay. Nevertheless, the ratio of EC50 (Target Drug/Desomorphine) was generally well-correlated with the measured cross-reactivity. Numerical differences between these two approaches are expected and are caused by non-parallelism or binding cooperativity, which is inherent to antibody-antigen interactions.

The Immunalysis Opiate Direct assay produced the highest cross-reactivity towards desomorphine, with comparable binding to that of morphine (**Figure 2.2**). However, the Orasure Opiate (71%) and Neogen Opiate (51%) assays also proved effective for the purposes of desomorphine screening. Very little is understood concerning the metabolism of desomorphine and until recently there were no published pharmacokinetic studies. Preliminary data suggest that Cytochrome P450 (CYP) and uridine diphosphoglucuronosyl transferase (UGT)-mediated transformations take place, similar to other phenathrene-type opioids (16). Nevertheless, potential metabolites are not yet commercially available.

Cross-reactivity is greatly influenced by immunogen design and ultimately coupling chemistry between the drug and the carrier protein. The coupling has a tendency to obscure the site of action from “view”, resulting in immunoglobulins with reduced specificity for that region. Although antibody specificity is highly desirable in many

instances to reduce false positives, immunoassay screening for broad classes of drugs (e.g. benzodiazepines, barbiturates and opiates) can exploit this feature. Immunoassays exhibiting high cross-reactivity towards desomorphine (Immunalysis Opiates Direct) likely use an antibody reagent raised against an antigen or immunogen in which morphine was coupled in the C6 position (hydroxyl). The morphine-targeted assay with the lowest cross-reactivity towards desomorphine (Randox Toxicology Opiates) is more likely to have used an antigen or immunogen that obscured the C3 position (phenol) and/or nitrogen ring while leaving the C6 position free to interact with the antibody reagent. A similar immunogen design was likely used with the oxycodone-targeted assay that exhibited the lowest cross-reactivity overall, while the second oxycodone-targeted assay investigated more likely obscured the C3 hydroxy to some degree.

A limitation of the study was the use of different conditions for each of the assays (**Table 2.1**). Specimen dilutions, reagent volumes and incubation times varied between the assays. Recommended conditions are designed to optimize assay performance in the concentration range of interest. Use of uniform dilutions, reagent volumes or incubation times in all assays could have compromised the performance of some of the assays. For this reason, manufacturers' recommendations were followed.

Conclusions

Desomorphine (Krokodil) is a fast-acting and potent injectable opioid whose clandestine synthesis has drawn widespread attention due to extensive tissue necrosis. Despite reports of recreational use, analytically confirmed cases are rare. In the absence of commercially available metabolites or reference materials, identification of desomorphine in forensic toxicology casework relies upon the identification of the parent drug in

biological samples. Since targeted chromatographic-based assays may fail to identify desomorphine during routine confirmations, immunoassays can play an important role in terms of identifying its use. Although desomorphine cross-reactivity was highly variable, several existing commercial ELISAs produced sufficient cross-reactivity to be highly effective for this purpose.

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CHAPTER III

IN VITRO METABOLISM OF DESOMORPHINE[1]

This dissertation follows the style and format of *The Journal of Analytical Toxicology*.

[1]Winborn, J., Haines D., Kerrigan S. (2018). Forensic Science International, 289, 140-149.

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Abstract

Desomorphine is reported to be the principal pharmacologically active opioid in Krokodil, a homemade injectable drug that is perceived to be a cheaper alternative to heroin. There have been limited studies regarding its pharmacology or detection in biological matrices. The goal of this study was to contribute further knowledge regarding its metabolism. Recombinant human cytochrome P450 enzymes (rCYPs) and recombinant uridine 5'-diphospho-glucuronosyltransferases (rUGTs) were used to investigate the biotransformational pathways involved. Samples were analyzed by liquid chromatography/quadrupole-time of flight-mass spectrometry (LC-Q/TOF-MS). Seven rCYP (rCYP2B6, rCYP2C8, rCYP2C9, rCYP2C18, rCYP2C19, rCYP2D6 and rCYP3A4) enzymes were found to contribute to desomorphine metabolism and eight phase I metabolites were identified, including nordesomorphine, desomorphine-*N*-oxide, norhydroxydesomorphine, and five hydroxylated species. Inhibition assays were used to confirm individual rCYP isoenzyme activity. Nine rUGTs (rUGT1A1, rUGT1A3, rUGT1A8, rUGT1A9, rUGT1A10, rUGT2B4, rUGT2B7, rUGT2B15, and rUGT2B17) were found to contribute to the formation of desomorphine-glucuronide.

Keywords: Desomorphine, Krokodil, CYP450, Isozymes

In Vitro Metabolism of Desomorphine

Introduction

Desomorphine (dihydrodesoxymorphine, (5α)-17-methyl-4,5-epoxymorphinan-3-ol) is a synthetic opioid that is commonly derived from codeine. Its phenanthrene-type core is characterized by one aromatic and two saturated rings, in addition to a six-membered nitrogen-containing ring (morphinan). It is structurally similar to morphine but is reportedly more potent, and has a shorter onset and duration of action (1, 2). The absence of the 6-hydroxyl and saturation of the C7-8 bond differentiate the drug from morphine (**Figure 3.1**). Synthesis of illicit desomorphine is similar to the Nagai method for methamphetamine, involving red phosphorus and hydroiodic acid (1). When produced illicitly, Krokodil contains desomorphine and a significant number of other morphinans (3). Following intravenous use of the drug, severe dermatological effects have been reported, including abscesses, skin lesions, and necrosis (1, 4-6). This has been attributed to residual acid, reducing agent or solvent that may remain in Krokodil following its clandestine synthesis.

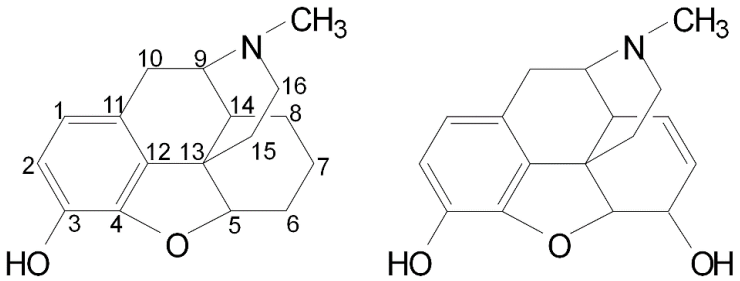

Figure 3.1. Structure of desomorphine and morphine.

Krokodil emerged as a drug of abuse in the 2000s in Russia and neighboring states (7-9). Reports of its use in European countries (including Romania, Germany, Poland, Czech Republic, France, Belgium, Spain and Italy) have been attributed to the migration of Krokodil users. To date there have been very few cases reported in the United States, although none have been analytically confirmed (5, 6, 10). Although the drug received widespread attention in the media as a "flesh eating drug", the U.S. Drug Enforcement Administration has reported a very limited number of drug seizures (11). However, detection of desomorphine in biological samples is a challenge because it is not within the normal scope of testing. Although some opioid immunoassays may have sufficient cross-reactivity to produce a positive screening result (12), mass spectral confirmation procedures may not target desomorphine. Additionally, published case reports involving desomorphine suggest that there is typically an extensive delay between use of the drug and seeking medical treatment for the severe dermatological side effects (5, 6, 13). By the time medical attention is sought, drug users have typically ceased using Krokodil due to the severity of the symptoms. As a consequence, the drug is no longer present and analytical confirmation is typically not possible. To date there has been only one published case report in the literature. This involved a fatal case of endomyocarditis where the presence of desomorphine was confirmed in the urine (13).

Ultra high pressure liquid chromatography (UPLC) coupled with high resolution time of flight (TOF) or quadrupole/time of flight-mass spectrometry (Q/TOF-MS) is a powerful tool for metabolite identification due to its mass resolving power, mass accuracy and sensitivity (14). Although Q/TOF-MS has lower resolution than other mass analyzers (e.g. Orbitrap™), it is compatible with the high data acquisition needs of UPLC and can

produce MS^2 spectra of diagnostic value. Furthermore, the use of superficially porous particles can improve chromatographic resolution and assist in the separation of challenging isobaric metabolites.

To date there has been only one published study describing desomorphine metabolism. In 2016, Richter investigated its biotransformation using pooled human liver microsomes (HLMs) and cytosol (15). Seven phase I metabolites were identified: nordesomorphine, desomorphine-*N*-oxide, and five hydroxydesomorphine isomers. Phase II metabolites included glucuronides of desomorphine, nordesomorphine and desomorphine-*N*-oxide, in addition to desomorphine sulfate. Cytochrome P450 (CYP) activity was investigated *in vitro* using recombinant isoenzymes (rCYP). Of the rCYPs investigated, only the CYP3A4 isoenzyme produced metabolic activity, but two of the hydroxydesomorphine isomers identified using HLMs were not identified using the rCYP microsomal incubations. Uridine 5'-diphospho-glucuronosyltransferase (UGT) activity was also assessed using recombinant enzymes (rUGTs). Metabolic activity was identified using UGT1A1, UGT1A8, UGT1A9, UGT1A10, UGT2B4, UGT2B7, UGT2B15, and UGT2B17.

Metabolite identification is important because it can help identify potential biomarkers, or compounds with pharmacological activity or unexpected toxicity. Identification of the enzymes responsible for metabolism can help predict the impact of genetic polymorphisms and the potential risk of drug-drug interactions. Recombinant isoforms are ideal for this task and can help identify the potential for induction or inhibition, whereby the pharmacokinetic profile of the drug can be significantly altered. Notwithstanding the limitations associated with *in vitro* techniques, this approach can

provide valuable information regarding potential metabolites and the involvement of CYP and UGT isoforms.

Previous *in vitro* studies have been reported for other structurally related opioids. In 2003 Projean found morphine metabolism to be catalyzed by numerous CYPs, including CYP2C8, CYP2C9, CYP2C19, CYP2D6, and CYP3A4 (16). Metabolism of the keto opioid hydromorphone was found to involve CYP2C9, CYP2D6, CYP3A4, and CYP3A5 (17). Levorphanol (which is structurally identical to desomorphine with the exception of the epoxy group) is reportedly mediated by CYP2D6 and CYP3A4 (16). In 1997, Coffman found UGT2B7 to be the major isoform involved in the formation of morphine-3-glucuronide and morphine-6-glucuronide (19). A subsequent study in 2003 by Stone suggested that numerous UGTs were also involved (UGT1A1, UGT1A3, UGT1A6, UGT1A8, UGT1A9 and UGT1A10) in the formation of morphine-3-glucuronide (20). In the study reported here, the CYP and UGT activity of desomorphine metabolism is further explored using rCYP and rUGT techniques and LC-Q/TOF-MS analysis. The terms rCYP and rUGT will be used when referring to the data collected during this study and the terms CYP and UGT used for broader discussion.

Materials and methods

Chemicals and reagents

Desomorphine, desomorphine-D3, ketoconazole and fluvoxamine were obtained from Cerilliant (Round Rock, Texas). Formic acid and ticlopidine were obtained from Sigma Aldrich (St. Louis, Missouri). Montelukast was obtained from Cayman Chemical (Ann Arbor, Michigan). Acetonitrile (LC/MS grade) was obtained from Thermo Fisher Scientific (Waltham, Massachusetts). Recombinant human cytochrome P450 (rCYP)

isoenzymes expressed in E. coli (bactosomes) were obtained from Xenotech, LLC (Kansas City, Kansas). Recombinant human uridine 5'-diphospho-glucuronosyltransferase (rUGT) isoenzymes expressed in baculovirus infected insect cells (supersomes™) were obtained from Corning (Glendale, Arizona). Reduced nicotinamide adenosine di-phosphate (NADPH) regenerating system solution A (40 U/mL glucose-6-phosphate dehydrogenase in 5 mM sodium citrate), solution B (26 mM NADP+, 66 mM glucose-6-phosphate and 66 mM magnesium chloride in aqueous solution), UGT reaction mix solution A (25 mM UDP-glucuronic acid), and UGT reaction mix solution B (250 mM Tris(hydroxymethyl)aminomethane-HCl (Tris-HCl), 40 mM magnesium chloride, and 0.125 mg/mL alamethicin) were obtained from Corning (Glendale, Arizona). All other chemicals and reagents (analytical grade) were obtained from VWR (Radnor, Pennsylvania). Deionized (DI) water was generated from a Direct-Q 3 (UV) system (Millipore, Billerica, Massachusetts).

LC-Q/TOF-MS analysis

Samples were analyzed using an Agilent Technologies (Santa Clara, California) 1290 Infinity LC system equipped with a 6530 Accurate Mass Q/TOF-MS. Gradient elution was performed using a Poroshell 120 EC-C18 column (2.1 x 100 mm, 2.7 µm) with a Poroshell 120 EC-C18 guard column (2.1 x 5 mm, 2.7 µm). LC separation of metabolites was achieved at 35°C in a thermostatically controlled column compartment. Mobile phase A consisted of 0.1% formic acid in DI water and mobile phase B consisted of 0.1% formic acid in acetonitrile. Optimal separation of metabolites was achieved using a flow rate of 0.3 mL/min with the following gradient: 10% B 0-2 mins, increasing to 37% B by 6 mins and 90% B by 10 mins. Metabolites were identified using electrospray ionization (ESI) in

the positive mode. MS conditions for rCYP incubations were as follows: gas temperature 150°C, gas flow 13 L/min, nebulizer 45 psi, sheath gas temperature 200°C, sheath gas flow 12 L/min, VCap voltage 4000 V, nozzle voltage 1000 V, fragmentor 150 V. MS conditions for rUGT incubations were as follows: gas temperature 350°C, gas flow 10 L/min, nebulizer 20 psi, sheath gas temperature 400°C, sheath gas flow 12 L/min, VCap voltage 2500 V, nozzle voltage 0 V, fragmentor 150 V. MS^2 spectra were generated using collision induced dissociation (CID) energies of 30, 40 and 50 eV. Scan speeds were 8 spectra/sec for MS and 3 spectra/sec for MS^2. The mass range was 100 - 1000 *m/z*. Data was acquired in full scan mode using a preferred list of suspected metabolites. Data files were processed using MassHunter software (Agilent Technologies).

rCYP incubations

Each rCYP isoenzyme (rCYP1A2, rCYP2B6, rCYP2C8, rCYP2C9, rCYP2C18, rCYP2C19, rCYP2D6 and rCYP3A4) was incubated separately to evaluate individual metabolic activity for desomorphine. Once microsomal conditions had been fully optimized, incubations were performed using replicate measurements (n=4). Incubations were carried out at 37°C in the presence of 200 µM desomorphine and 50 pmol/mL rCYP isoenzyme. The incubation mixture also contained 100 mM potassium phosphate buffer (pH 7.4), 1.3 mM NADP+, 3.3 mM glucose-6-phosphate, 3.3 mM magnesium citrate, and 0.4 U/mL glucose-6-phosphate dehydrogenase. Incubation mixtures were assayed at t = 0, 30, 60, 120, 180 and 240 minutes. Each 25 µL aliquot was quenched using an equal volume of ice-cold acetonitrile containing 0.1% formic acid and 5 µM internal standard (desomorphine-D3). The solution was centrifuged at 10,000 x g at 4°C for 3 minutes. The supernatant was mixed with an equal volume of a 50/50 mixture of mobile phase A/B and

2 µL was injected onto the LC-Q/TOF-MS. Control and blank samples were included in each assay. In controls, rCYP isoenzymes were replaced with Xenotech control bactosomes and in the blank samples, desomorphine was replaced with phosphate buffer (no drug).

Inhibition studies

For the inhibition study, each rCYP isoenzyme was incubated side by side, in the presence and absence of inhibitor (n=3). Conditions were identical to those described earlier, with the exception of the inhibitor. Ketoconazole (20 µM) was used for CYP2C9, CYP2C18, CYP2C19 and CYP3A4; fluvoxamine (20 µM) was used for CYP1A2 and CYP2D6; ticlopidine (10 µM) was used for CYP2C8 and montelukast (10 µM) was used for CYP2B6. For the inhibition studies, aliquots were removed at t = 0 and 240 minutes and samples were analyzed using LC-Q/TOF-MS as described above.

rUGT incubations

Each rUGT isoenzyme (rUGT1A1, rUGT1A3, rUGT1A6, rUGT1A7, rUGT1A8, rUGT1A9, rUGT1A10, rUGT2B4, rUGT2B7, rUGT2B15, and rUGT2B17) was incubated separately to evaluate individual metabolic activity for desomorphine. Once microsomal conditions had been fully optimized, incubations were performed using replicate measurements (n=3). Incubations were carried out at 37°C in the presence of 200 µM desomorphine and 0.25 mg/mL rUGT isoenzyme. The incubation mixture also contained 90 mM Tris-HCl (pH 7.5), 8 mM magnesium chloride, 25 µg/mL alamethicin, and 2 mM uridine 5'-diphospho-glucuronic acid (UDPGA). Incubation mixtures were assayed at t = 0, 30 and 120 minutes. Each 25 µL aliquot was quenched using an equal volume of ice cold acetonitrile containing 0.1% formic acid and 5 µM internal standard (desomorphine-D3). The solution was centrifuged at 10,000 x g at 4°C for 3 minutes. The supernatant

was mixed with an equal volume of a 50/50 mixture of mobile phase A/B and 2 µL was injected onto the LC-Q/TOF-MS. Control and blank samples were included in each assay. In controls, rUGT isoenzymes were replaced with Corning control supersomes™ and in the blank samples, desomorphine was replaced with Tris-HCl buffer (no drug).

Metabolite identification

Potential metabolites were first identified by observing changes in each rCYP and rUGT incubation mixtures over time. Using the optimized LC separation, the abundance of each potential metabolite was normalized to the internal standard (desomorphine-D3). Measurement of the relative peak area (RPA) minimized random errors associated with volumetric steps, injection volume and ionization efficiency. The exact masses of predicted metabolites were monitored using a preferred list and MS^2 spectra of compounds that appeared to increase over time were further investigated over a range of CID voltages.

Results and discussion

Identification of phase I metabolites

A total of eight potential metabolites were identified including nordesomorphine (*m/z* 285, 3.04 mins), desomorphine-*N*-oxide (*m/z* 288, 4.11 mins), five hydroxylated species (*m/z* 288, 0.99, 1.35, 1.79, 2.41 and 3.66 mins) and norhydroxydesomorphine (*m/z* 274, 2.50 mins). The chromatographic separation of desomorphine and its metabolites is shown in **Figure 3.2** and MS^2 spectra are shown in **Figure 3.3**. Each metabolite's chemical formula, exact mass, accurate mass, mass error, and product ions are detailed in **Table 3.1**. Mass errors for all assignments were within 5 ppm for all metabolites except norhydroxydesomorphine (5.2 ppm), a newly identified metabolite which was produced at low intensity.

Table 3.1

Retention time, chemical formula, exact mass, accurate mass and mass error for desomorphine and its metabolites.

Compound Name	Retention Time	Chemical Formula	Exact Mass (M+1)	Accurate Mass (M+1)	Mass Error (ppm)
desomorphine	3.22	$C_{17}H_{21}NO_2$	272.1645	272.1643	0.91
nordesomorphine	3.04	$C_{16}H_{19}NO_2$	258.1489	258.1482	2.54
desomorphine-*N*-oxide	4.11	$C_{17}H_{21}NO_3$	288.1594	288.1597	1.11
hydroxydesomorphine isomer 1	0.99	$C_{17}H_{21}NO_3$	288.1594	288.1593	0.25
hydroxydesomorphine isomer 2	1.35	$C_{17}H_{21}NO_3$	288.1594	288.1591	1.07
hydroxydesomorphine isomer 3	1.79	$C_{17}H_{21}NO_3$	288.1594	288.1595	0.38
hydroxydesomorphine isomer 4	2.41	$C_{17}H_{21}NO_3$	288.1594	288.1591	1.20
hydroxydesomorphine isomer 5	3.75	$C_{17}H_{21}NO_3$	288.1594	288.1594	0.03
norhydroxydesomorphine	2.50	$C_{16}H_{19}NO_3$	274.1438	274.1423	5.21

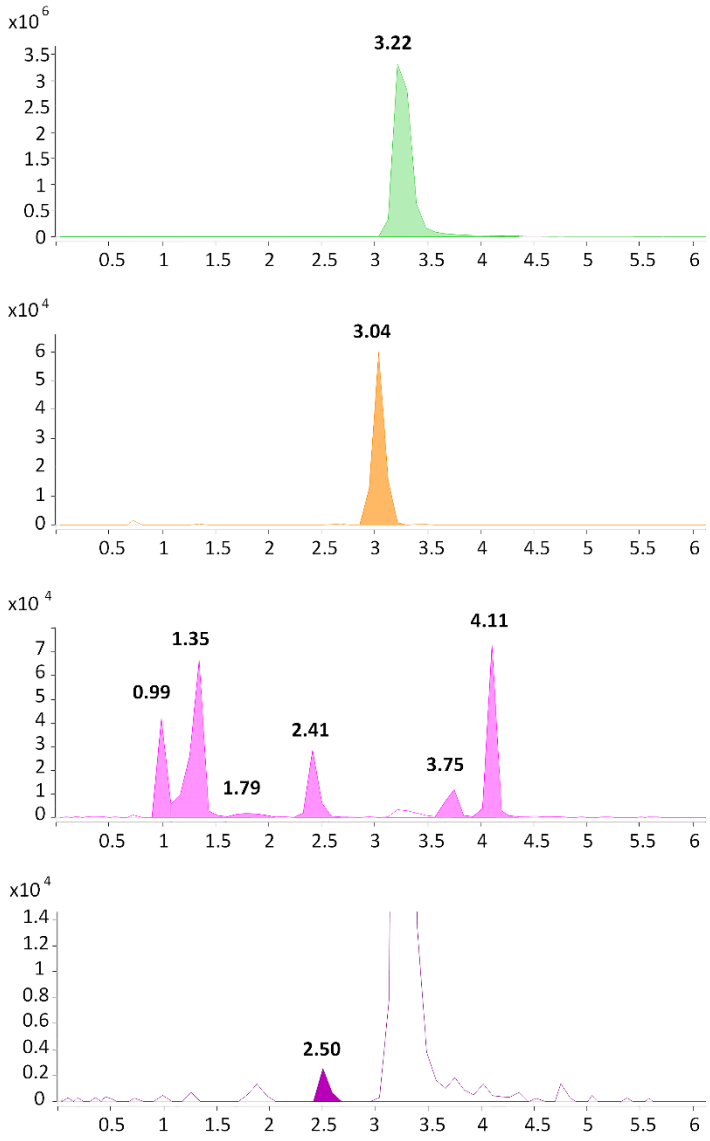

Figure 3.2. Extracted ion chromatograms for desomorphine, 3.22 min (*m/z* 272); nordesomorphine, 3.04 min (*m/z* 258); desomorphine-*N*-oxide, 4.11 min and hydroxydesomorphine, 0.99, 1.35, 1.79, 2.41 and 3.75 min (*m/z* 288) and norhydroxydesomorphine, 2.50 min (*m/z* 274).

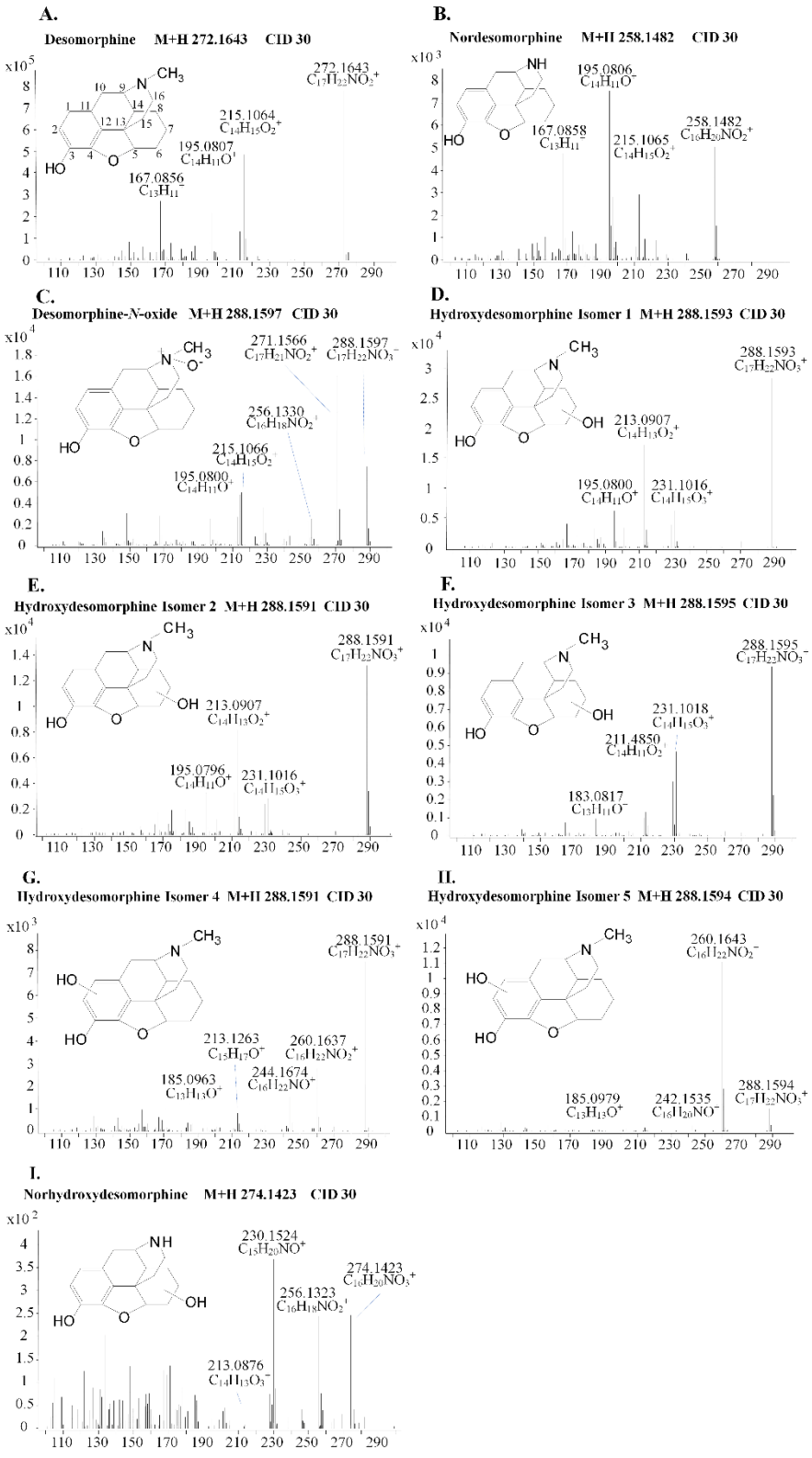

Figure 3.3. MS^2 spectra of desomorphine (A), nordesomorphine (B), desomorphine-*N*-oxide (C), hydroxydesomorphine (D-H), and norhydroxydesomorphine (I).

The fragmentation of desomorphine (*m/z* 272, M+1) is dominated by the loss of the nitrogen ring (*m/z* 215, M-57), which is characteristic for phenanthrene-type opioids (21). Subsequent loss of hydrogen, water and CO produce ions at *m/z* 213, 195 and 167 (**Figure 3.3**). In combination, HRMS and these characteristic losses were used to identify metabolites and their associated ions. Structural assignments and associated mass errors for desomorphine and its metabolites are presented in **Table 3.2**. Not surprisingly, the most significant mass shifts were observed with some of the least abundant ions, or metabolites produced at low intensity. All of the metabolites of desomorphine undergo fragmentation consistent with the loss of the nitrogen ring, resembling the parent drug. Product ion spectra for the *N*-demethylated species (nordesomophine) and *N*-oxide bear the closest resemblance to the parent drug. Desomorphine-*N*-oxide shows an immediate oxygen loss, which is highly characteristic of *N*-oxides (22). Five of the metabolites identified in this study have an additional oxygen, consistent with hydroxylation or isobaric internal heteroatom dealkylation products. In the absence of analytical standards, specific assignments were not attempted, although mass spectra did provide some distinguishable information.

Product ion spectra (**Figure 3.3**) depict water losses (*m/z* 231/229 to 213/211 and *m/z* 213 to 195) in hydroxydesomorphine isomers 1, 2 and 3, which are absent in the product ion spectra of isomers 4 and 5. Protonated ions of aliphatic hydroxylated metabolites are reported to undergo more facile water loss than aromatic hydroxylation (18, 22). As a result, isomers 1-3 were tentatively identified as aliphatic hydroxylated species, in contrast to isomers 4 and 5. While isomers 1-3 showed significant dehydration consistent with stable aliphatic hydroxylation, isomers 4 and 5 did not. This might be

attributed to aromatic hydroxylation, or products of aliphatic hydroxylation adjacent to heteroatoms followed by spontaneous ring-opening to isobaric ketones. The mass spectrum for norhydroxydesomorphine also shows significant dehydration, suggestive of aliphatic hydroxylation. This is also consistent with the equivalent norhydroxylated product observed by Bonn for oxidation of levorphanol, which is identical in structure to desomorphine, with the exception of the epoxy group (oxygen bridge) (18).

Table 3.2

Chemical formula, exact mass, accurate mass and mass error for product ions of desomorphine, nordesomorphine, desomorphine-*N*-oxide, hydroxydesomorphine isomers and norhydroxydesomorphine.

Compound	Chemical Formula	Exact Mass (M+1)	Accurate Mass (M+1)	Mass Error (ppm)
desomorphine	$C_{14}H_{15}O_2^+$	215.1067	215.1064	1.00
	$C_{14}H_{11}O^+$	195.0804	195.0807	1.08
	$C_{13}H_{11}^+$	167.0855	167.0856	0.53
nordesomorphine	$C_{14}H_{15}O_2^+$	215.1067	215.1065	0.74
	$C_{14}H_{11}O^+$	195.0804	195.0806	1.04
	$C_{13}H_{11}^+$	167.0855	167.0855	0.19
desomorphine-*N*-oxide	$C_{17}H_{21}NO_2^+$	271.1567	271.1566	0.27
	$C_{16}H_{18}NO_2^+$	256.1332	256.1330	0.77
	$C_{14}H_{15}O_2^+$	215.1067	215.1066	0.42
	$C_{14}H_{11}O^+$	195.0804	195.0800	2.40
hydroxydesomorphine isomer 1	$C_{14}H_{15}O_3^+$	231.1016	231.1016	0.06
	$C_{14}H_{13}O_2^+$	213.0910	213.0907	1.40
	$C_{14}H_{11}O^+$	195.0804	195.0796	4.33
hydroxydesomorphine isomer 2	$C_{14}H_{15}O_3^+$	231.1016	231.1010	2.33
	$C_{14}H_{13}O_2^+$	213.0910	213.0915	2.53
	$C_{14}H_{11}O^+$	195.0804	195.0800	0.10
hydroxydesomorphine isomer 3	$C_{14}H_{15}O_3^+$	231.1016	231.1018	0.92
	$C_{14}H_{11}O_2^+$	211.0754	211.0748	2.67
	$C_{13}H_{11}O^+$	183.0804	183.0817	7.09
hydroxydesomorphine isomer 4	$C_{16}H_{22}NO_2^+$	260.1645	260.1637	2.92
	$C_{16}H_{22}NO^+$	244.1696	244.1674	8.92
	$C_{15}H_{17}O^+$	213.1274	213.1263	5.19
	$C_{13}H_{13}O^+$	185.0961	185.0963	1.19

(continued)

hydroxydesomorphine isomer 5	$C_{16}H_{22}NO_2^+$	260.1645	260.1643	0.62
	$C_{16}H_{20}NO^+$	242.1539	242.1535	1.95
	$C_{13}H_{13}O^+$	185.0961	185.0979	9.85
	$C_{12}H_{13}^+$	157.1012	157.1013	0.98
norhydroxydesomorphine	$C_{16}H_{18}NO_2^+$	256.1332	256.1323	3.45
	$C_{15}H_{20}NO^+$	230.1539	230.1524	6.83
	$C_{14}H_{13}O_2^+$	213.0910	213.0876	15.82

rCYP activity

Of the eight rCYP isoenzymes tested, metabolic activity was observed using rCYP2B6, rCYP2C8, rCYP2C9, rCYP2C18, rCYP2C19, rCYP2D6 and rCYP3A4. No detectable metabolism was observed with rCYP1A2. **Figure 3.4** shows metabolite formation (30-240 mins) for nordesomorphine and **Figure 3.5** shows metabolite formation (30-240 mins) for hydroxydesomorphine isomers 1-5, desomorphine-*N*-oxide and norhydroxydesomorphine. As these metabolites cannot be quantified without an analytical standard, their abundance was compared relative to the internal standard. Comparing the relative peak areas (RPAs) of all of the metabolites, nordesomorphine was the major metabolite using all rCYPs, except for rCYP2B6 where hydroxydesomorphine isomer 3 was the major metabolite produced.

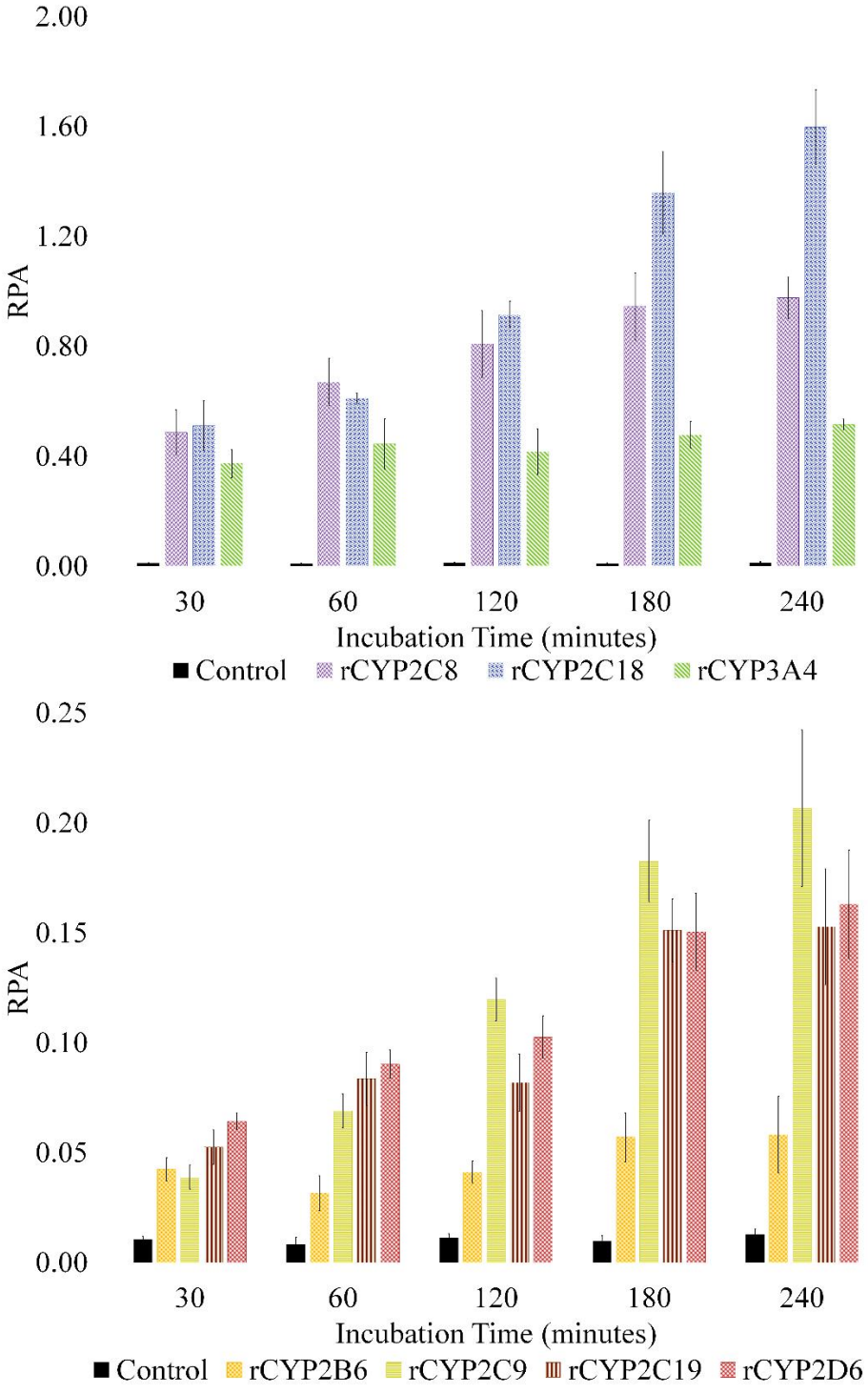

Figure 3.4. Production of nordesomorphine over time using rCYP2C8, rCYP2C18, and rCYP3A4 (top) and rCYP2B6, rCYP2C9, rCYP2C19, and rCYP2D6 (bottom). The quantity of nordesomorphine is expressed using relative to the internal standard. Data is shown separately due to scale. Error bars represent mean ± 1 SD (n=4).

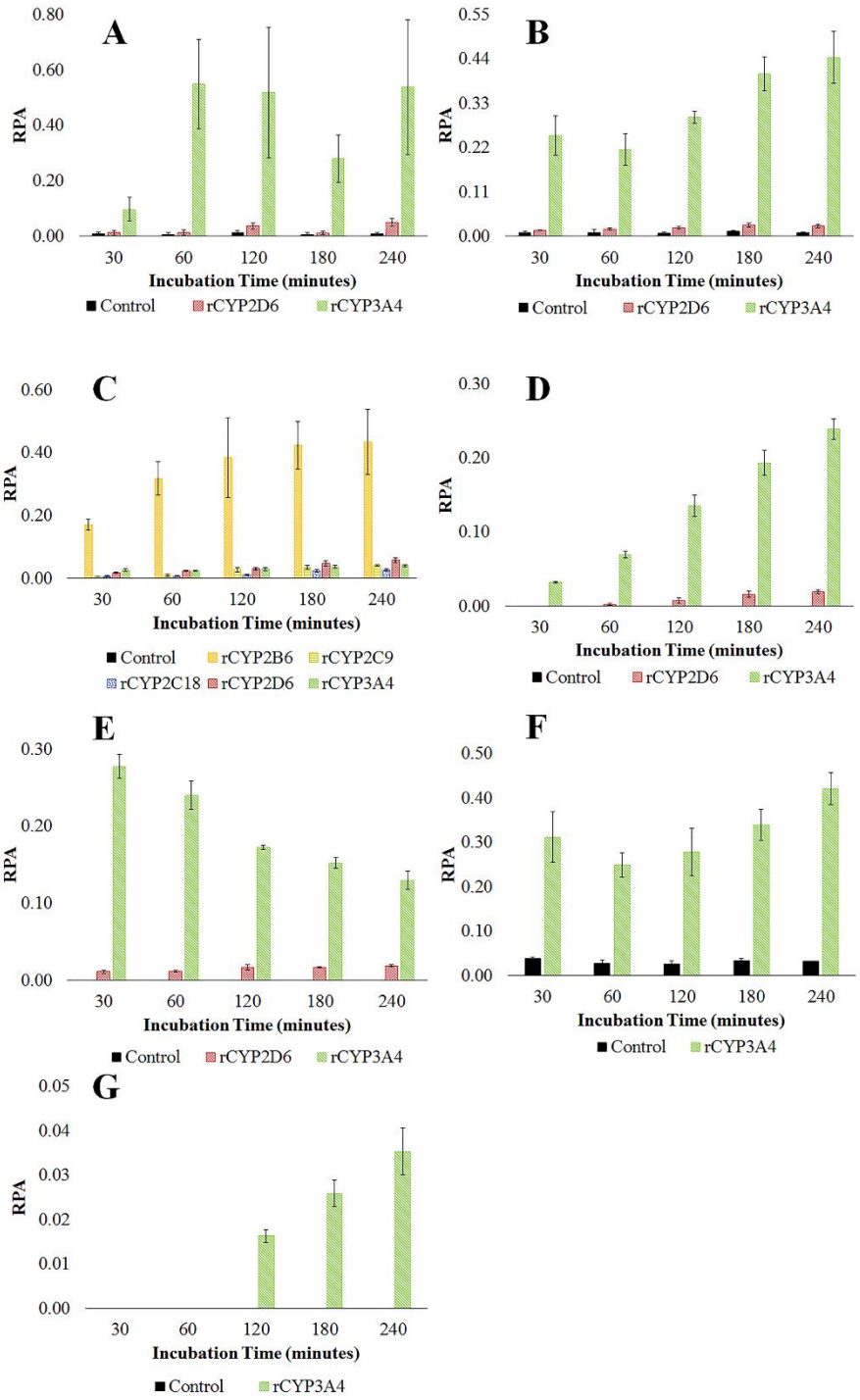

Figure 3.5. Metabolite production over time for hydroxydesomorphine isomer 1 (A), hydroxydesomorphine isomer 2 (B), hydroxydesomorphine isomer 3 (C), hydroxydesomorphine isomer 4 (D), hydroxydesomorphine isomer 5 (E), desomorphine-*N*-oxide (F), and norhydroxydesomorphine (G). The quantity of each metabolite is expressed relative to the internal standard. Error bars represent mean ± 1 SD (n=4).

No detectable metabolism was observed in any of the controls or blanks. The rate of metabolite formation was not estimated because *in vitro* rates of reaction using rCYPs may not be reflective of *in vivo* kinetics. While the lack of analytical standards or certified reference materials means that the concentrations of metabolites in each rCYP reaction cannot be determined, a comparison of the relative abundance between rCYPs does provide useful information (**Figure 3.6)**. This demonstrates that although rCYP3A4 was the only isoform to produce desomorphine-*N*-oxide and norhydroxydesomorphine, other isoforms were involved for six of the eight metabolites. Moreover, rCYP2C18 was the major isoform responsible for the formation of nordesomorphine. While the results here confirm the activity of CYP3A4 first reported by Richter (15), our results provide supplemental information regarding the role of other isoforms, in addition to a novel metabolite (norhydroxydesomorphine).

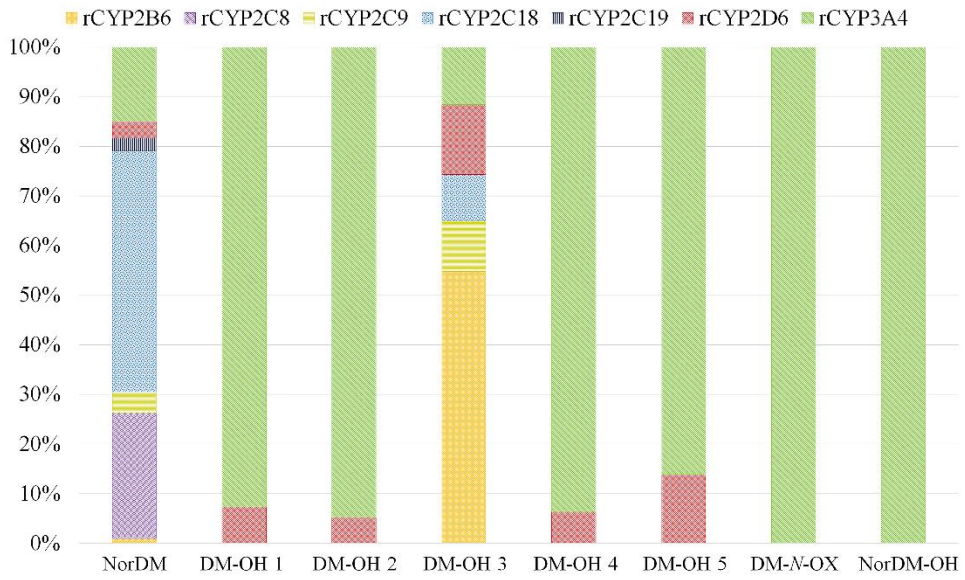

Figure 3.6. Relative contribution of rCYPs during in vitro metabolism studies. Abbreviations: NorDM, nordesomorphine; DM-OH, hydroxydesomorphine; DM-*N*-OX, desomorphine-*N*-oxide; NorDM-OH, norhydroxydesomorphine.

Inhibition studies were performed to verify observed isoenzyme activity. Uninhibited and inhibited reactions were in parallel, and the RPAs of the metabolites produced in each were compared and used to calculate the decrease in production that occurred (% inhibition). The RPAs of uninhibited and inhibited reactions of rCYPs capable of metabolizing desomorphine to nordesomorphine are shown in **Figure 3.7**. Significant inhibition (>20%) was observed for all rCYPs for all phase I metabolites identified (**Table 3.3**). Isoenzymes capable of phase I metabolism are summarized in **Figure 3.8**. Although the formation of the *N*-oxide was solely attributed to CYP3A4, several other isoforms were capable of *N*-demethylation and hydroxylation. Although *in vitro* assays using recombinant enzymes can identify possible pathways, they do not always mimic *in vivo* biotransformations. These pathways, and the extent to which each isoform may contribute, are variable. Despite the convenience of *in vitro* studies, this is an important limitation of this approach.

Table 3.3

Percent inhibition for phase I metabolites (n=3).

Metabolite	% Inhibition ± Standard Deviation
nordesomorphine	rCYP2C18 (89% ± 2%), rCYP2C8 (93% ± 1%), rCYP3A4 (91% ± 1%), rCYP2C9 (64% ± 3%), rCYP2D6 (41% ± 12%), rCYP2C19 (48% ± 8%), rCYP2B6 (57% ± 11%)
desomorphine-*N*-oxide	rCYP3A4 (55% ± 7%)
hydroxydesomorphine isomer 1	rCYP3A4 (91% ± 3%), rCYP2D6 (69% ± 10%)
hydroxydesomorphine isomer 2	rCYP3A4 (90% ± 2%), rCYP2D6 (46% ± 11%)
hydroxydesomorphine isomer 3	rCYP2B6 (88% ± 5%), rCYP2D6 (58% ± 6%), rCYP2C9 (69% ± 5%), rCYP3A4 (86% ± 1%), rCYP2C18 (86% ± 3%)
hydroxydesomorphine isomer 4	rCYP3A4 (97% ± 1%), rCYP2D6 (68% ± 2%)
hydroxydesomorphine isomer 5	rCYP3A4 (90% ± 2%), rCYP2D6 (65% ± 7%)
norhydroxydesomorphine	rCYP3A4 (100% ± 0%)

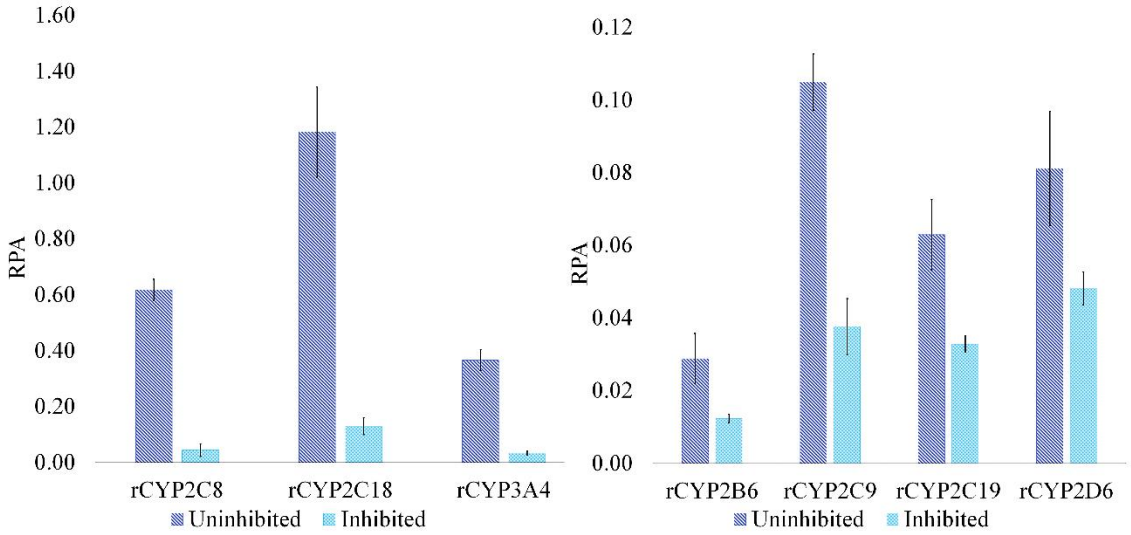

Figure 3.7. Inhibition of nordesomorphine production using rCYP2C8, rCYP2C18, rCYP3A4 (left) and rCYP2B6, rCYP2C9, rCYP2C19, rCYP2D6 (right) at 240 minutes.The quantity of nordesomorphine is expressed relative to the internal standard. Error bars represent mean ± 1 SD (n=3).

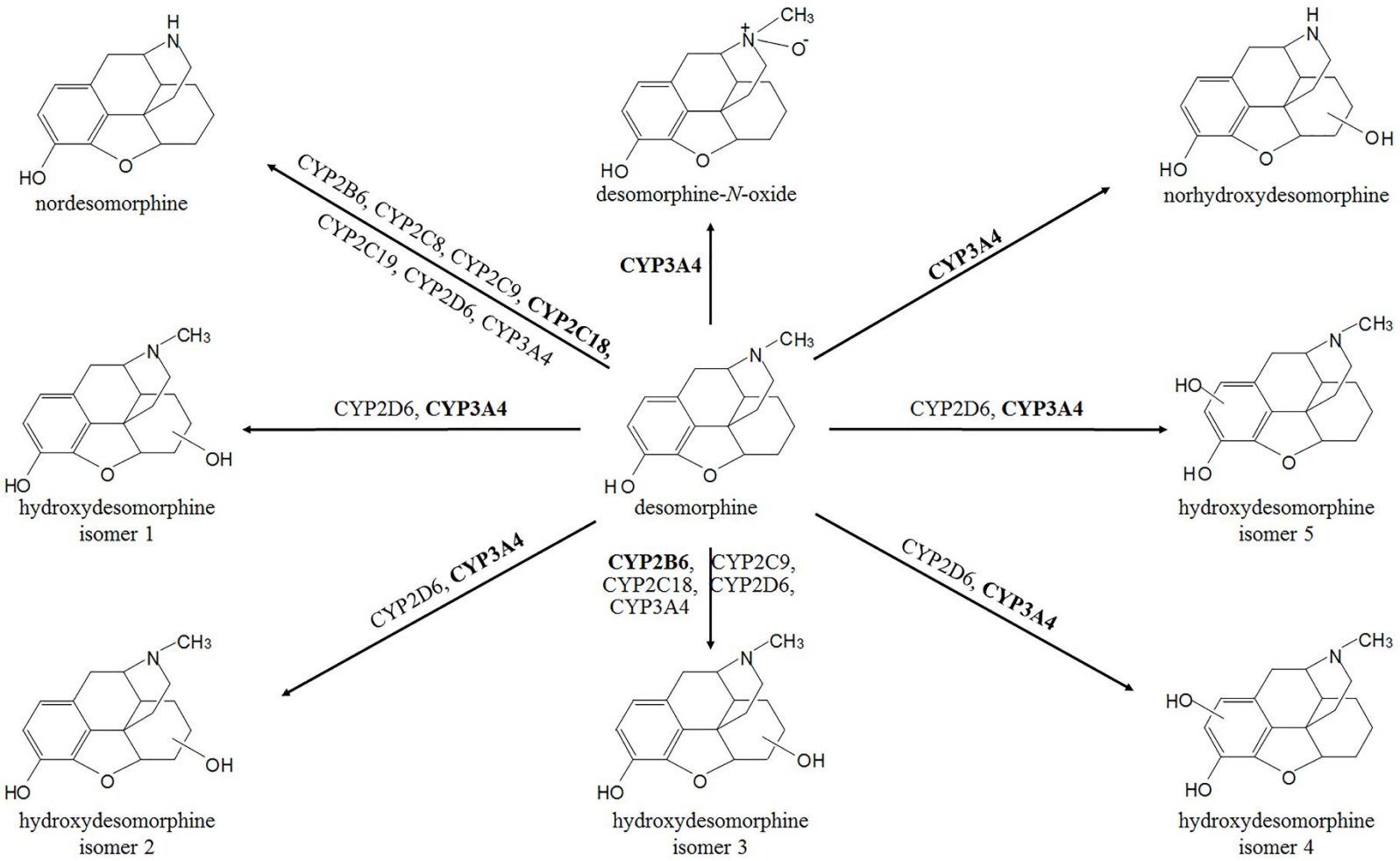

Figure 3.8. Formation of Phase I metabolites *in vitro*. The major contributing rCYP isozyme is shown in bold.

rUGT activity

No detectable metabolism was observed in any of the controls or blanks. Of the ten rUGTs evaluated, metabolic activity was observed in rUGT1A1, rUGT1A3, rUGT1A8, rUGT1A9, rUGT1A10, rUGT2B4, rUGT2B7, rUGT2B15, and rUGT2B17. No detectable metabolism was observed in rUGT1A6. The rate of metabolite formation was not estimated because *in vitro* rates of reaction using rUGTs may not be reflective of *in vivo* kinetics. Desomorphine-glucuronide was identified. Its MS^2 spectrum and mass assignments are summarized in **Figure 3.9** and **Table 3.4**, respectively.

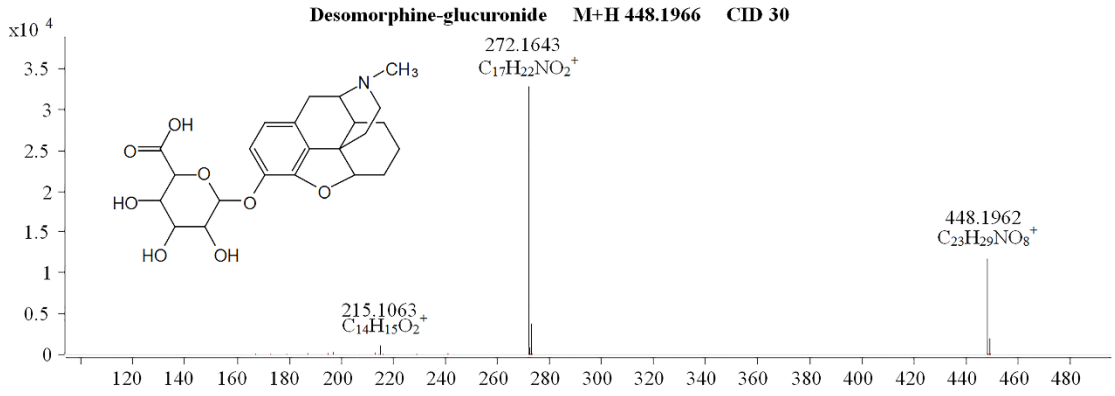

Figure 3.9. MS^2 spectra of desomorphine-glucuronide.

Table 3.4

Chemical formula, exact mass, accurate mass and mass error for precursor and product ions of desomorphine-glucuronide.

m/z	**Chemical Formula**	**Exact Mass (M+1)**	**Accurate Mass (M+1)**	**Mass Error (ppm)**
448	$C_{23}H_{29}NO_8^+$	448.1966	448.1962	0.80
272	$C_{17}H_{22}NO_2^+$	272.1645	272.1643	0.66
215	$C_{14}H_{15}O_2^+$	215.1067	215.1063	1.67
167	$C_{13}H_{11}^+$	167.0855	167.0847	4.92

A shorter timeline was used for the rUGT incubations compared to the rCYP incubations due to the fact that production of desomorphine-glucuronide appeared to plateau at 120 minutes. Because of this, the source parameters for analysis were further optimized in order to improve sensitivity. **Figure 3.10** depicts the relative abundance of desomorphine-glucuronide produced by each rUGT at 120 minutes. The greatest activity was observed using rUGT1A9, rUGT2B4 and rUGT2B7.

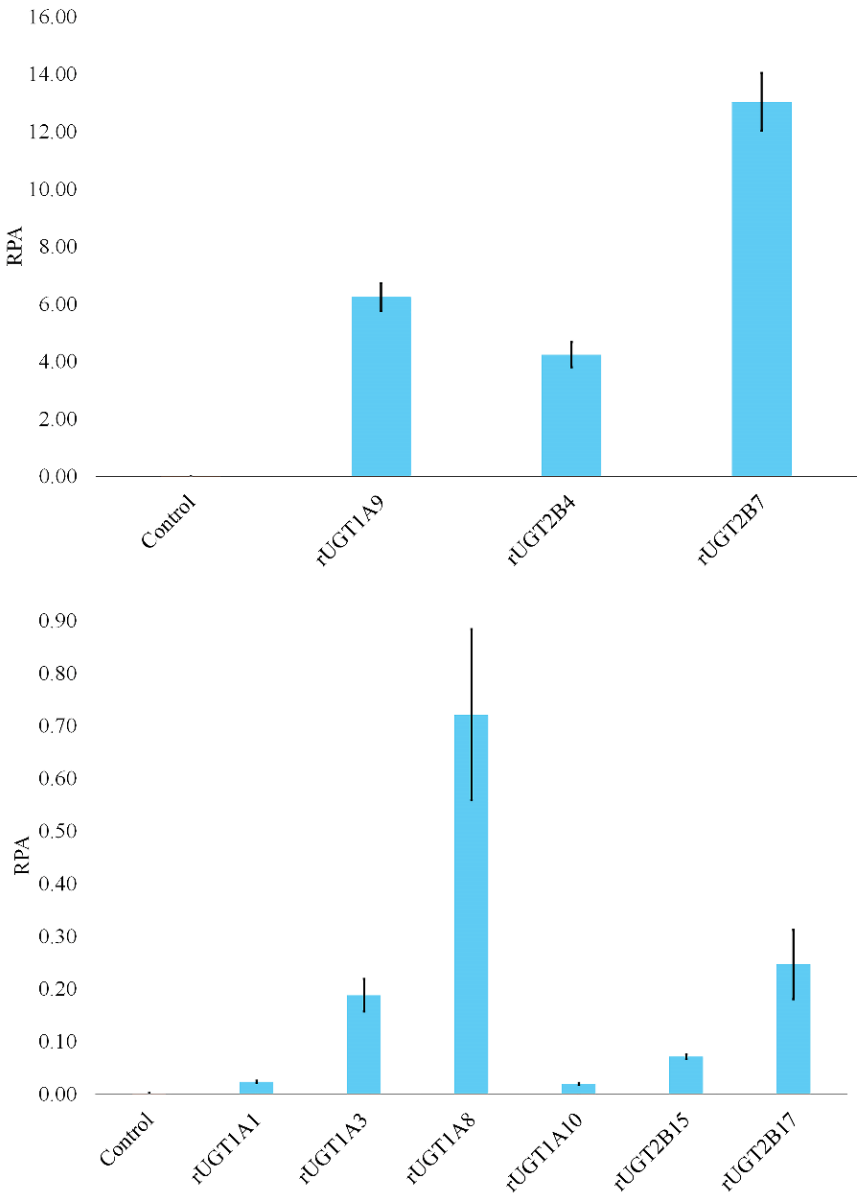

Figure 3.10. Desomorphine-glucuronide production by rUGT1A9, rUGT2B4, rUGT2B7 (top) and rUGT1A1, rUGT1A3, rUGT1A8, rUGT1A10, rUGT2B15 and rUGT2B17 (bottom) at 240 minutes. The amount of desomorphine-glucuronide is expressed using the relative peak area (RPA). Error bars represent mean ± SD (n=3).

Comparison with previous studies

The results of this study regarding the individual CYPs involved in desomorphine metabolism complement previous work (15). Richter's study investigated rCYP1A2,

rCYP2A6, rCYP2B6, rCYP2C8, rCYP2C9, rCYP2C19, rCYP2D6, rCYP2E1, rCYP3A4 and rCYP3A5. Metabolic activity was only observed with rCYP3A4 and norhydroxydesomorphine was not identified in any of the *in vitro* or *in vivo* experiments. These differences may be attributed to differences in microsomal incubation conditions and the use of rCYPs expressed in bactosomes, rather than supersomes™. There is evidence to suggest that bactosomes have greater activity and turnover compared to supersomes™ (23). One of the unique metabolites identified in our study (the doubly oxidized norhydroxydesomorphine) was not present in sufficiently high yield until two hours, which may explain why is was not detected previously using a 30 minute incubation (15).

The results of this study are in good agreement with previously published metabolism studies using phenanthrene-type opioids. Metabolism of morphine involves CYP2C8, CYP2C9, CYP2C19, CYP2D6 and CYP3A4 isoforms (16). CYP3A4 is known to preferentially catalyze the oxidative *N*-demethylation of drugs within this class. Like desomorphine, levorphanol is also saturated at the C7-8 bond and is fully reduced at carbon 6. Consistent with our results, Bonn observed that CYP3A4 was solely responsible for the formation of levorphanol-*N*-oxide, while *N*-demethylation involved both CYP3A4 and CYP2D6 (18). Accordingly, the role of other isoforms involved in hydroxylation was also identified in that same study. Hydroxylation of levorphanol was found to involve CYP3A4, and to a lesser extent CYP2D6, also consistent with our findings (**Figure 17**). As in the levorphanol study, a product was identified that was both hydroxylated (appearing to be an aliphatic hydroxylation in both cases) and *N*-demethylated.

In this study, rCYP2C18, rCYP2C8 and rCYP3A4 were the major cytochrome P450 enzymes responsible for *N*-demethylation. Although hydroxylation was largely mediated by rCYP3A4 (and to a lesser extent rCYP2D6), hydroxydesomorphine isomer 3 was produced predominantly by rCYP2B6. Although *in vitro* assays do not mimic *in vivo* transformations, the abundance of nordesomorphine in the microsomal incubations, relative to other metabolites, is consistent with this being the major metabolite *in vivo.* As opioids are known to be preferentially metabolized by glucuronidation, it is highly probable that nordesomorphine is the most likely phase I metabolite to be identified in forensic toxicology investigations.

The results for the UGTs in this study also complement previous work. Richter previously reported that rUGT1A1, rUGT1A8, rUGT1A9, rUGT1A10, rUGT2B4, rUGT2B7, rUGT2B15, and rUGT2B17 metabolized desomorphine (13). The study reported here was the first to investigate rUGT1A3 using desomorphine. Previous studies have shown UGT1A1, UGT1A3, UGT1A6, UGT1A8, UGT1A9, UGT1A10, and UGT2B7 to have metabolic activity for morphine with UGT2B7 being the major contributor (19). All are also involved in the metabolism of desomorphine. The main contributor is UGT2B7, with UGT1A9 and UGT2B4 being the next most active. Stone also tested UGT1A6 and UGT2B15 for morphine metabolism and UGT1A6 metabolized morphine, while UGT2B15 did not (20). The opposite is true for desomorphine.

Conclusions

This study identified a new phase I metabolite, additional CYP450 isoenzyme activity, and an additional UGT isoenzyme that may be involved in the biotransformation of desomorphine. The results presented here complement existing work and are consistent

with metabolic pathways of other phenanthrene-type opioids. The identification of potential metabolites is important in forensic toxicology so that appropriate compounds can be targeted in biological fluids. Furthermore, a comprehensive understanding of isoenzymes involved in drug metabolism is also important in terms of drug-drug interactions and the potential for adverse drug reactions.

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CHAPTER IV

STABILITY AND HYDROLYSIS OF DESOMORPHINE-GLUCURONIDE[1]

This dissertation follows the style and format of *The Journal of Analytical Toxicology*.

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Submitted for publication in the Journal of Analytical Toxicology

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Abstract

Desomorphine, the principal opioid in Krokodil, has an analgesic potency approximately ten-times that of morphine. Similar to other opioids, during phase II metabolism it undergoes conjugation with glucuronic acid to form desomorphine-glucuronide. Although hydrolysis of conjugated species is sometimes required prior to analysis, desomorphine-glucuronide has not been fully investigated. In this study, six hydrolysis procedures were optimized and evaluated. Deconjugation efficiencies using chemical and enzymatic hydrolysis were evaluated and stability in aqueous solution was assessed. Acid hydrolysis was compared with five β-glucuronidase sources (BGTurbo™, IMCSzyme™, *Escherichia coli*, *Helix pomatia* and *Patella vulgata*). At optimal conditions, each hydrolysis method produced complete hydrolysis (≥96%). However, under simulated challenging conditions, *P. vulgata* was the most efficient β-glucuronidase for the hydrolysis of desomorphine-glucuronide. Both BGTurbo™ and IMCSzyme™ offered fast hydrolysis with no need for sample cleanup prior to liquid chromatography-quadrupole time of flight mass spectrometry (LC-Q/TOF MS) analysis. Hydrolysates using *E. coli*, *H. pomatia* and *P. vulgata* underwent additional sample treatment using β-Gone™ cartridges. Additionally, the stability of free and conjugated drug was evaluated at elevated temperature (60°C) in aqueous solutions between pH 4-10. No degradation was observed for either desomorphine or desomorphine-glucuronide under any of the conditions tested.

Keywords: Desomorphine, Krokodil, Hydrolysis, Beta-glucuronidase, Acid hydrolysis

Stability and Hydrolysis of Desomorphine-Glucuronide

Introduction

Krokodil is a homemade heroin substitute that has been used in Russia and surrounding countries since the early 2000s (1). In recent years, reports of its use have also emerged from the Republics of Armenia (2-4) and Georgia (5), Germany (6), Italy (7), Poland (8), Russia (9, 10), Spain (11), the United Kingdom (12) and the United States (13-17). To date, only one analytically confirmed case has been published, where desomorphine was identified in the post-mortem urine of a 39 year old male at 270 ng/mL (7). Krokodil is clandestinely produced from codeine using hydroiodic acid and red phosphorus, producing a mixture of morphinans, of which desomorphine is a major component (18, 19). Desomorphine is structurally similar to morphine, with differences at C6 (absence of the hydroxyl) and saturation of the C7-C8 double bond (**Figure 4.1**). These structural differences make desomorphine approximately ten times more potent than morphine, with a faster onset of action but a shorter duration of effect (20). Krokodil is predominately used intravenously, which bypasses many of the human body's natural safeguards. Residual precursor chemicals used during synthesis may remain in the final product, which may contribute to the severe dermatological sequela associated with its use, including necrotic ulcers and osteonecrosis.

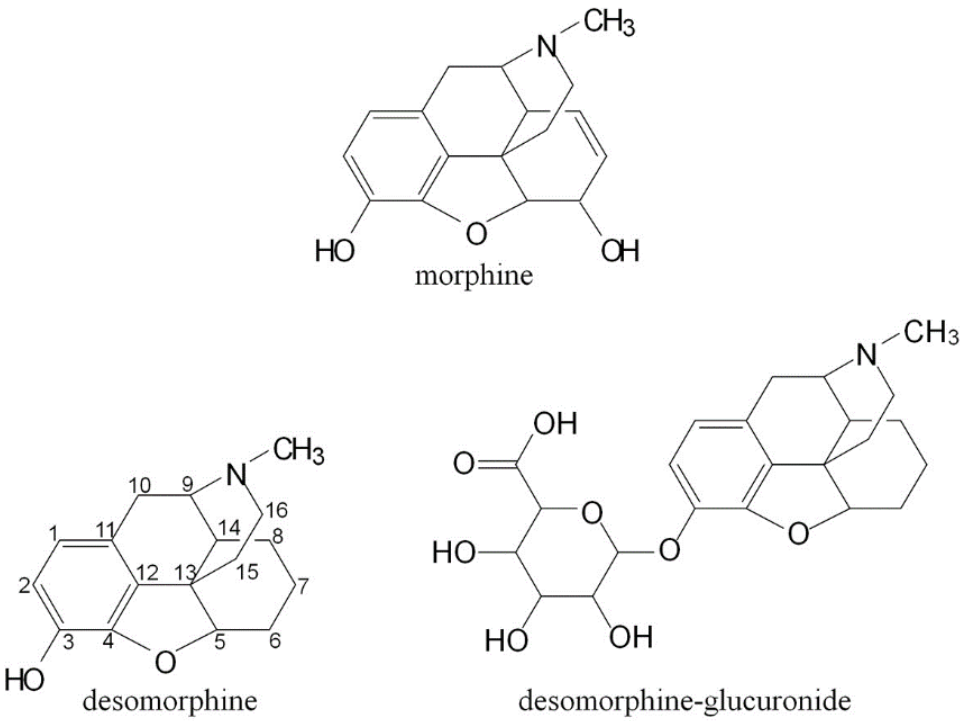

Figure 4.1. Chemical structures of morphine, desomorphine and desomorphine-glucuronide.

Recent studies indicate that desomorphine undergoes conjugation with glucuronic acid and sulfate during phase II metabolism, as well as *N*-demethylation, hydroxylation and *N*-oxide formation during phase I (21, 22). Data from both studies indicate desomorphine-glucuronide to be the major phase II metabolite formed. This is consistent with morphine, of which approximately 75% of a dose is excreted as morphine-3-glucuronide (23). Polar glucuronidated species are not amenable to gas chromatographic analysis. If gas chromatography-mass spectrometry (GC-MS) based methods are used, glucuronides must be hydrolyzed prior to analysis. Liquid chromatography (LC) based techniques are advantageous from this standpoint, because both free and conjugated drug can be identified simultaneously. However, if both free and conjugated species are to be isolated from a biological matrix using either liquid-liquid extraction (LLE) or solid phase

extraction (SPE), careful selection of solvents and potential sorbent phase is necessary (24). Further limitations associated with the direct analysis of glucuronides may include their increased cost, availability, and for some conjugates, the absence of a stable isotope internal standard (IS).

Conjugated metabolites are frequently hydrolyzed prior to extraction and instrumental analysis. In doing so, the amount of free and bound drug is determined indirectly from the free (untreated) and total (hydrolyzed) fractions. Although this approach requires the sample to be analyzed twice, the quantity of free and bound drug can provide valuable information from an interpretive standpoint.

The hydrolysis of glucuronide species is typically achieved either chemically or enzymatically. Strong acids or bases (sometimes at elevated temperatures) or β-glucuronidase enzymes can be used, both of which have advantages and disadvantages. Chemical hydrolysis is often less costly, faster and sometimes more efficient that enzyme hydrolysis but can cause degradation, particularly with opioids (25, 26). Conditions for enzyme hydrolysis are typically less harsh and do not cause degradation, but the efficiency of β-glucuronidase enzymes varies significantly between individual drugs and drug classes, typically requiring much longer incubation times (24, 27, 28). Additionally, there are commercially available β-glucuronidases isolated from multiple species, each of which can have varying activities towards different substrates. As a result, optimum enzymatic systems and conditions must be explored during method development (29-31).

Previous studies involving opioids have shown that acid hydrolysis is often the most efficient method for hydrolysis, particularly with morphine (27, 30). However, under some conditions degradation may occur with 6-acetylmorphine, buprenorphine and keto

opioids (25, 26, 29), complicating the interpretation of the results. Comparisons of different sources of β-glucuronidases have shown *Patella vulgata* to have a greater affinity for morphine compared with Escherichia *coli* and *Helix pomatia* (27), but conversely it has the lowest affinity of the three for buprenorphine (29). Although appropriate glucuronidated controls should always be used to monitor the efficiency of the hydrolysis step, incomplete hydrolysis may reduce the overall sensitivity of the analytical method. Longer enzymatic incubations may increase the extent of hydrolysis, but the increased overall analysis time may be undesirable for high throughput laboratories or those with large caseloads.

To date, only two studies have described the hydrolysis of desomorphine metabolites. Savchuk used chemical hydrolysis using hydrochloric acid on human urine samples prior to GC-MS analysis (32). Richter used β-glucuronidase from *Helix pomatia* to facilitate the identification of phase I metabolites of desomorphine in rat urine, and later acid hydrolysis was used during the development of a screening assay to detect desomorphine (21). However, no comparison between different hydrolysis methods has been performed for desomorphine-glucuronide to date.

In order to gain additional insight into the efficiency of desomorphine-glucuronide hydrolysis, six deconjugation processes were evaluated. Chemical hydrolysis (acid) and five enzymatic methods (BGTurbo™, IMCSzyme™, *Escherichia coli*, *Helix pomatia* and *Patella vulgata*) were compared side-by-side using desomorphine-glucuronide generated *in vitro* using recombinant human uridine 5'-diphospho-glucuronosyltransferase (rUGT) isoenzyme. Given that some glucuronides are known to be labile (33), the stability of

desomorphine and desomorphine-glucuronide was briefly investigated over a range of pH values (4-10).

Materials and methods

Reagents

Recombinant human uridine 5'-diphospho-glucuronosyltransferase isoenzyme 2B7 (rUGT2B7) expressed in baculovirus infected insect cells (supersomes™), UGT reaction mix solution A (25 mM UDP-glucuronic acid), and UGT reaction mix solution B (250 mM Tris(hydroxymethyl)aminomethane-HCl (Tris-HCl), 40 mM magnesium chloride, and 0.125 mg/mL alamethicin) were obtained from Corning (Glendale, Arizona, USA). Desomorphine, desomorphine-D_3 (IS) and morphine-3-glucuronide were obtained from Cerilliant (Round Rock, Texas, USA). Acetonitrile (LC/MS grade) was obtained from Thermo Fisher Scientific (Waltham, Massachusetts, USA) and acetic acid was obtained from Mallinckrodt Chemicals (St. Louis, Missouri, USA). Concentrated ammonium hydroxide was purchased from Macron Fine Chemicals (Center Valley, Massachusetts, USA) and concentrated hydrochloric acid was from J.T. Baker (Center Valley, California, USA). Formic acid (>95%), potassium phosphate (monobasic and dibasic), ammonium bicarbonate, ammonium acetate, TRIS-base, TRIS-HCL, β-glucuronidase from *Escherichia coli* (Type IX-A, lyophilized powder, 1,000,000-5,000,000 units/g), β-glucuronidase from *Helix pomatia* (Type H-1, partially purified powder, ≥300,000 units/g) and β-glucuronidase from *Patella vulgata* (Type L-II, lyophilized powder, 1,000,000-3,000,000 units/g) were obtained from Sigma Aldrich (St. Louis, Missouri, USA). IMCSzyme™ (genetically modified β-glucuronidase, purified solution, >50,000 units/mL) and its proprietary Rapid Hydrolysis Buffer were obtained from Integrated Micro-

Chromatography Systems, LLC (Irma, South Carolina, USA). BGTurbo™ (genetically enhanced β-glucuronidase solution for 10 min flash-hydrolysis, ~1 mg/mL, >90% purity, >200,000 units/mL) was obtained from Kura Biotec® (La Piedra Biotecnología, Puerto-Varas, Chile). β-Gone™ hydrolysis sample clean-up tubes were from Phenomenex (Torrance, California, USA) and deionized (DI) water was generated from a Direct-Q 3 (UV) system (Millipore, Billerica, Massachusetts, USA). Unless otherwise stated, all inorganic salts, acids and bases were ACS grade.

LC-Q/TOF-MS

An Agilent Technologies (Santa Clara, California) 1290 Infinity LC system equipped with a 6530 Accurate Mass Q/TOF-MS was used for analysis. An Agilent Poroshell 120 EC-C18 column (2.1 x 100 mm, 2.7 µm) with a Poroshell 120 EC-C18 guard column (2.1 x 5 mm, 2.7 µm particle size) was used to separate compounds in a thermostatically controlled column compartment (35°C). Mobile phase A and B consisted of 0.1% formic acid in DI water and 0.1% formic acid in acetonitrile, respectively. The flow rate was 0.3 mL/min with the following gradient: 10% B 0-2 mins, increasing to 37% B by 6 mins and 90% B by 10 mins. Ionization was achieved using electrospray ionization (ESI) in the positive mode. MS conditions were as follows: gas temperature 350°C (10 L/min), nebulizer 20 psi, sheath gas temperature 400°C (12 L/min), fragmentor 150 V, VCap voltage 2500 V, nozzle voltage 0 V. Data was acquired in full scan mode using a preferred list of analytes (desomorphine, internal standard and desomorphine-glucuronide). Collision induced dissociation (CID) energies of 30, 40 and 50 eV were used to generate product MS^2 spectra. Scan speeds for MS and MS^2 were 8 spectra/sec and 3 spectra/sec,

respectively and the mass range was 100 - 1000 *m/z*. Data files were processed using MassHunter software (Agilent Technologies).

Glucuronide production

In the absence of a commercially available standard or authentic urine specimens from Krokodil users, desomorphine-glucuronide was generated *in vitro* using rUGTs as previously described (22). Although as many as nine UGTs may be involved in the glucuronidation of desomorphine, rUGT2B7 was selected for the *in situ* production of the conjugate, due to its increased activity relative to the other isoforms (22). Incubations were carried out at 37°C in the presence of 25 µM desomorphine and 0.25 mg/mL rUGT2B7 isoenzyme. The incubation mixture also contained 90 mM Tris-HCl (pH 7.5), 8 mM magnesium chloride, 25 µg/mL alamethicin, and 2 mM uridine 5'-diphospho-glucuronic acid (UDPGA). The reaction was stopped by heat shocking the mixture at 80°C for 15 minutes. The solution was centrifuged at 14,000 x g at 4°C for 10 minutes to remove proteins. An aliquot of supernatant was diluted 1:1 with 90:10 A:B mobile phase containing IS (5 µM) and 2 µL was injected onto the LC-Q/TOF-MS for analysis. The remaining supernatant containing desomorphine-glucuronide was hydrolyzed as described below prior to analysis.

Acid hydrolysis

An equal volume of supernatant containing desomorphine-glucuronide (500 µL) and concentrated hydrochloric acid (500 µL) was combined and incubated at 95°C for 60 minutes (n=2). The sample was allowed to come to room temperature then neutralized (pH 7) with ammonium hydroxide solution. The sample was diluted 1:1 with 90:10 A:B mobile phase containing the internal standard (2.5 µM) and 2 µL was injected onto the LC-Q/TOF-

MS for analysis. The concentration of the IS was adjusted to give the same drug:IS ratio in all samples to facilitate comparison between methods.

Enzyme hydrolysis

A 50 µL aliquot of supernatant containing desomorphine-glucuronide was used for each enzymatic hydrolysis reaction described below. Each reaction was performed in duplicate, and where possible the recommendations of the manufacturer were followed. For IMCSzyme™, the reaction mixture contained sample, 30 µL of IMCSzyme™, 90 µL of proprietary buffer, 30 µL of IS (prepared at 9.96 µM in 50:50 water:methanol) and sufficient DI water to bring the reaction volume to 300 µL. The reaction mixture was incubated at 55°C for 30 minutes. Following dilution (1:1) with 90:10 A:B mobile phase, 2 µL was injected onto the LC-Q/TOF-MS for analysis. For BGTurbo™, the reaction mixture contained sample, 15 µL of BGTurbo™, 15 µL of IS (prepared at 16.7 µM in 50:50 water:methanol) and sufficient 50 mM phosphate buffer (pH 6.8) to achieve a final reaction volume of 150 µL. The reaction mix was incubated at 55°C for 30 minutes. The sample was then diluted 1:1 with 90:10 A:B mobile phase and 2 µL was injected onto the instrument for analysis.

For hydrolysis with *E. coli* β-glucuronidase, the reaction mixture contained sample, 150 µL of enzyme (prepared at 10,000 units/mL in 100 µM phosphate buffer pH 6.8), 15 µL of IS (prepared at 9.96 µM in DI water) and sufficient 100 mM phosphate buffer (pH 6.8) to achieve a final volume of 300 µL. The reaction mixture was incubated at 37°C for 180 minutes before being subjected to sample cleanup prior to analysis. For hydrolysis with *H. pomatia* β-glucuronidase, the reaction contained sample, 150 µL of enzyme (prepared at 10,000 units/mL in 100 µM acetate buffer pH 5.0), 15 µL of IS (prepared at

9.96 µM in DI water) and sufficient 100 mM acetate buffer (pH 5.0) to reach a final volume of 300 µL. The reaction mixture was incubated at 65°C for 180 minutes prior to sample cleanup. For hydrolysis with *P. vulgata* β-glucuronidase, the reaction mixture contained sample, 150 µL of enzyme (prepared at 10,000 units/mL in 100 µM acetate buffer pH 5.0), 15 µL of IS (prepared at 9.96 µM in DI water) and sufficient 100 mM acetate buffer (pH 5.0) to achieve a final volume of 300 µL. The reaction mixture was incubated at 65°C for 180 minutes, then subjected to sample cleanup prior analysis. In accordance with manufacturer recommendations, sample cleanup was achieved by adding 200 µL of the reaction mixture to 133 µL of 0.1% formic acid in methanol before allowing the sample to flow through a β-gone™ column under gravity. The eluent was diluted 1:1 with 90:10 A:B mobile phase and 2 µL injected onto the LC-Q/TOF-MS for analysis.

Short-term stability

Acetate (pH 4-6), Tris-HCl (pH 7) and bicarbonate buffers (pH 8-10) (50 mM) were prepared at pHs ranging from 4 to 10. Desomorphine-glucuronide generated *in situ* (100 µL) was diluted 1:3 with each buffer. An aliquot was immediately removed for t=0 and diluted 1:1 in 90:10 A:B mobile phase. The buffered solutions were then incubated at 60°C. Aliquots were removed at 5, 10, 15, 30, 60, 120 and 180 minutes and diluted 1:1 in 90:10 A:B mobile phase. Samples were refrigerated prior to analysis using LC-Q/TOF-MS.

Results and discussion

In Situ production of desomorphine-glucuronide

Due to the absence of reference material or authentic specimens, deconjugation efficiencies were determined using desomorphine-glucuronide generated *in situ* using

rUGT enzyme. As a result, the total concentration of conjugated drug may be lower than expected in an actual biological specimen. As a consequence, the efficiency of each deconjugation procedure was evaluated by varying the amount of enzyme, rather than increasing the quantity of drug. This approach was necessary to provide challenging conditions that would allow the deconjugation efficiency of each approach to be compared.

During initial method development the quantity of desomorphine-glucuronide generated *in situ* was optimized. The activity of the rUGT supersomes™ diminished beyond a two-hour incubation (22), so a large excess of desomorphine (25 µM) was utilized. In the absence of a reference standard for desomorphine-glucuronide it was not possible to determine the exact concentration of metabolite produced *in situ*. However, using the optimized conditions, the absolute peak area of desomorphine-glucuronide produced a signal equivalent to 1,000 ng/mL of morphine-3-glucuronide. The extracted ion chromatogram (EIC) and MS^2 spectrum of desomorphine-glucuronide (M+H, *m/z* 448) in the diluted supernatant are depicted in **Figure 4.2**. Not surprisingly, the MS^2 spectrum indicates the major loss of the glucuronide moiety (M-176) with the fragmentation of the parent (*m/z* 448) to *m/z* 272.

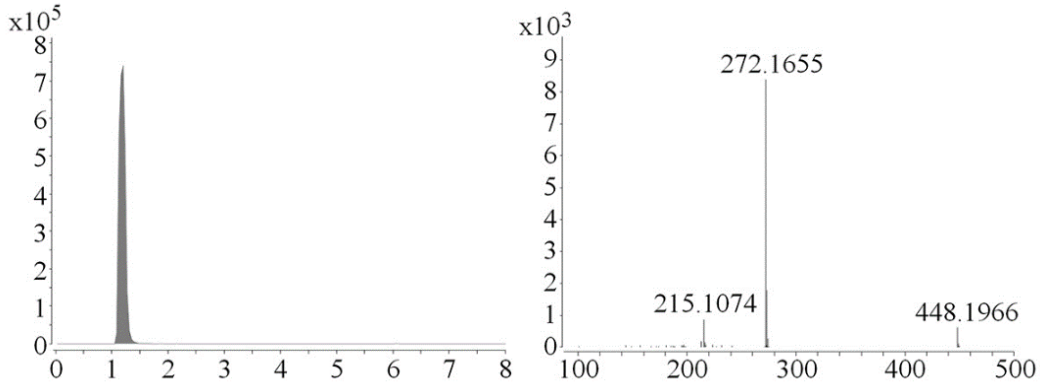

Figure 4.2. Extracted ion chromatogram of desomorphine-glucuronide (RT, 1.21 min) and associated MS^2 spectrum (CID 40V).

Stability

Chemical hydrolysis of desomorphine-glucuronide produced complete hydrolysis, which is consistent with what has been observed with other opioids (27, 30). No degradation of desomorphine was observed using the conditions tested, despite reports otherwise for other opioids (25). As such, the stability of desomorphine-glucuronide was further investigated in the form of a short-term stability study conducted at 60°C and different pH values ranging from 4-10. The temperature was selected based on the incubation temperatures of the enzymes used for hydrolysis. The time was limited to 3 hours as that was the longest incubation period used for hydrolysis. The pH range was expanded to further explore pH-dependent effects (pH 4, 5, 6, 7, 8, 9 and 10). The pH of each sample was adjusted and a t=0 aliquot was taken prior to placing samples into the heating block. To account for changes in signal that might occur between samples, the relative peak area (RPA) of desomorphine-glucuronide/IS (desomorphine-D_3) was used for comparison, and the coefficient of variation (%CV) between time stops was calculated for each pH. Degradation was present if the RPA decreased by 20% or more. Additionally, the appearance of potential degradation products was monitored. For desomorphine-glucuronide the %CV was less than 20% for each pH evaluated and changes in RPAs over time were not significant, indicating that the conjugate was stable. The RPAs of desomorphine were also compared to assess potential degradation. For desomorphine the %CV was less than 5% for each pH and no potential degradation products were found. These results are summarized in **Tables 4.1** and **4.2**. Although no degradation was observed for either desomorphine or desomorphine-glucuronide, the stability was evaluated in aqueous buffer rather than biological matrix. Additionally, because the main

purpose was to evaluate the potential for pH dependent instability during deconjugation, only short-term stability was evaluated (over hours). Therefore, inferences regarding the stability of these compounds in authentic biological matrices, which may be stored for extended periods, should not be made.

Table 4.1

Results of short-term stability of desomorphine in aqueous solution. Mean relative peak area (RPA) ± SD (n=8) for all time stops.

pH	Average RPA	%CV
4	4.28 ± 0.05	1%
5	4.42 ± 0.05	1%
6	4.39 ± 0.06	1%
7	4.43 ± 0.06	1%
8	4.44 ± 0.02	1%
9	4.43 ± 0.16	4%
10	4.49 ± 0.01	2%

Table 4.2

Results of short-term stability of desomorphine-glucuronide in aqueous solution. Mean relative peak area (RPA) ± SD (n=8) for all time stops.

pH	Average RPA	%CV
4	0.10 ± 0.02	15%
5	0.11 ± 0.02	17%
6	0.11 ± 0.01	8%
7	0.10 ± 0.01	9%
8	0.11 ± 0.01	10%
9	0.12 ± 0.02	16%
10	0.12 ± 0.01	9%

Deconjugation efficiency

H. pomatia and *E. coli* are widely used to hydrolyze opioids, although studies have shown *P. vulgata* to have a comparatively higher affinity for morphine, though less so for other opioids (29, 31). IMCSzyme™ is a genetically modified β-glucuronidase that is purported to be more efficient that traditional preparations. BGTurbo™ is another recombinant β-glucuronidase that has been modified to increase the reaction efficiency. Both recombinant enzymes have been purified to reduce the need for post-hydrolysis cleanup.

First, the conditions for each hydrolysis reaction were optimized to maximize deconjugation of the drug. The concentration of the IS used for each reaction was adjusted based on the total dilution factor for each protocol so that the RPAs for each reaction mixture could be directly compared. Each enzyme evaluated produced complete, or near complete hydrolysis (≥96%) of desomorphine-glucuronide using optimized conditions. **Table 4.3** summarizes the optimum conditions for each system in terms of reaction time and percent hydrolysis. Lacking commercially available reference material for desomorphine-glucuronide, the ratio of drug to enzyme was increased by diluting the enzyme in appropriate buffer, in order to test the limits of the enzymatic systems. Hydrolysis reactions were repeated (n=2) for each enzyme under more challenging conditions using successive 1:5 dilutions. Differences in hydrolysis efficiency were first discernable at dilutions of 1:25 and above. Under these conditions, *P. vulgata* was the optimum deconjugation enzyme for desomorphine-glucuronide, hydrolyzing 92% of the drug. This trend is consistent with what has been observed for morphine (27, 31), though it should be noted that newer recombinant β-glucuronidase enzymes have not been

investigated until recently. Only two published studies to date have evaluated IMCSzyme™ (25, 34), only one of which compared it to other enzymes, and no published studies have evaluated BGTurbo™. The extent of hydrolysis observed for all enzymes at all dilutions tested is shown in **Figure 4.3**. One-way Analysis of Variance (ANOVA) was used for statistical comparisons. At undiluted and 1:5 dilutions, no significant differences were observed. Under the most challenging conditions tested (1:125 dilution of enzyme), results were highly significant ($F(4,9)=18.5$, $p=0.003$).

Table 4.3

Incubation time and extent of hydrolysis at optimized conditions for each β-glucuronidase evaluated (n=2).

Enzyme	**Incubation Time (Minutes)**	**% Hydrolysis (Mean ± SD)**
BGTurbo™	15	100 ± 0
IMCSzyme™	30	96 ± 1
E. coli	180	100 ± 0
H. pomatia	180	97 ± 1
P. vulgata	180	100 ± 0

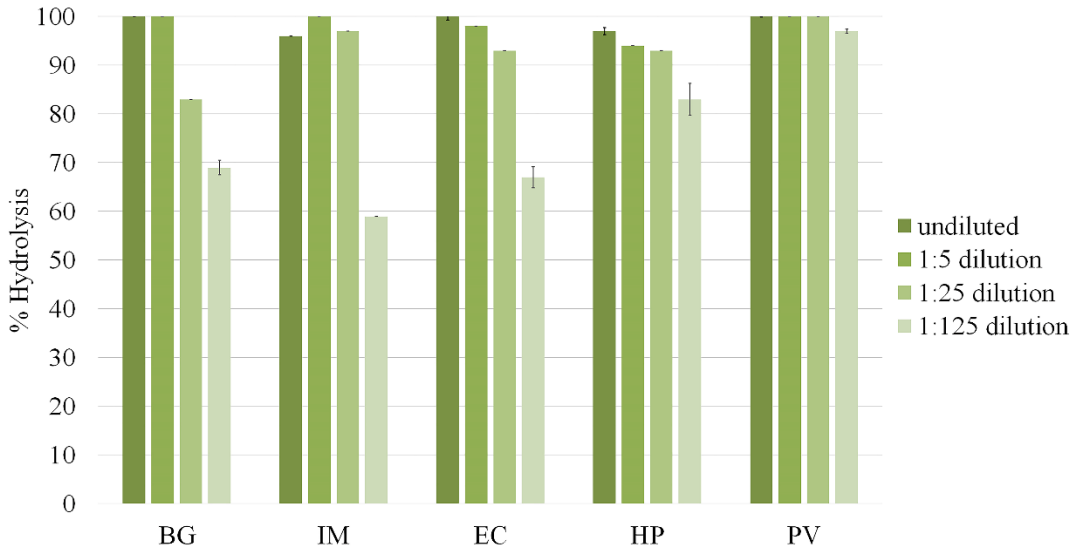

Figure 4.3. Extent of hydrolysis for IMCSzyme™ (IM), BGTurbo™ (BG), *E. coli* (EC), *H. pomatia* (HP) and *P. vulgata* (PV) at undiluted, 1:5 diluted, 1:25 diluted and 1:125 diluted enzyme levels. Error bars represent the standard deviation (n=2).

While more prone to loss of activity with dilution, in terms of reaction time and sample cleanup both recombinant systems offer key advantages over traditional enzymes. Optimum reaction times for both IMCSzyme™ and BGTurbo™ (**Table 4.3**) were much shorter than traditional enzymes. Additionally, the increased purity of both meant that no sample cleanup was necessary. This was also evident from the total ion chromatograms (TICs) of each preparation, which are shown in **Figure 4.4**. Samples hydrolyzed with IMCSzyme™ and BGTurbo™ were analyzed without sample cleanup while samples hydrolyzed with *E. coli*, *H. pomatia* and *P. vulgata* were further purified using β-gone™ columns prior to analysis. These proprietary columns remove excess enzyme which can cause detector fouling. Prior to use, β-gone™ columns were evaluated to ensure that analyte (desomorphine and desomorphine-glucuronide) was not lost during the cleanup step. No analyte loss was observed. Examination of the TICs showed that even after

sample cleanup there were many extraneous compounds in the sample which could potentially cause interference as well shorten the life of the column.

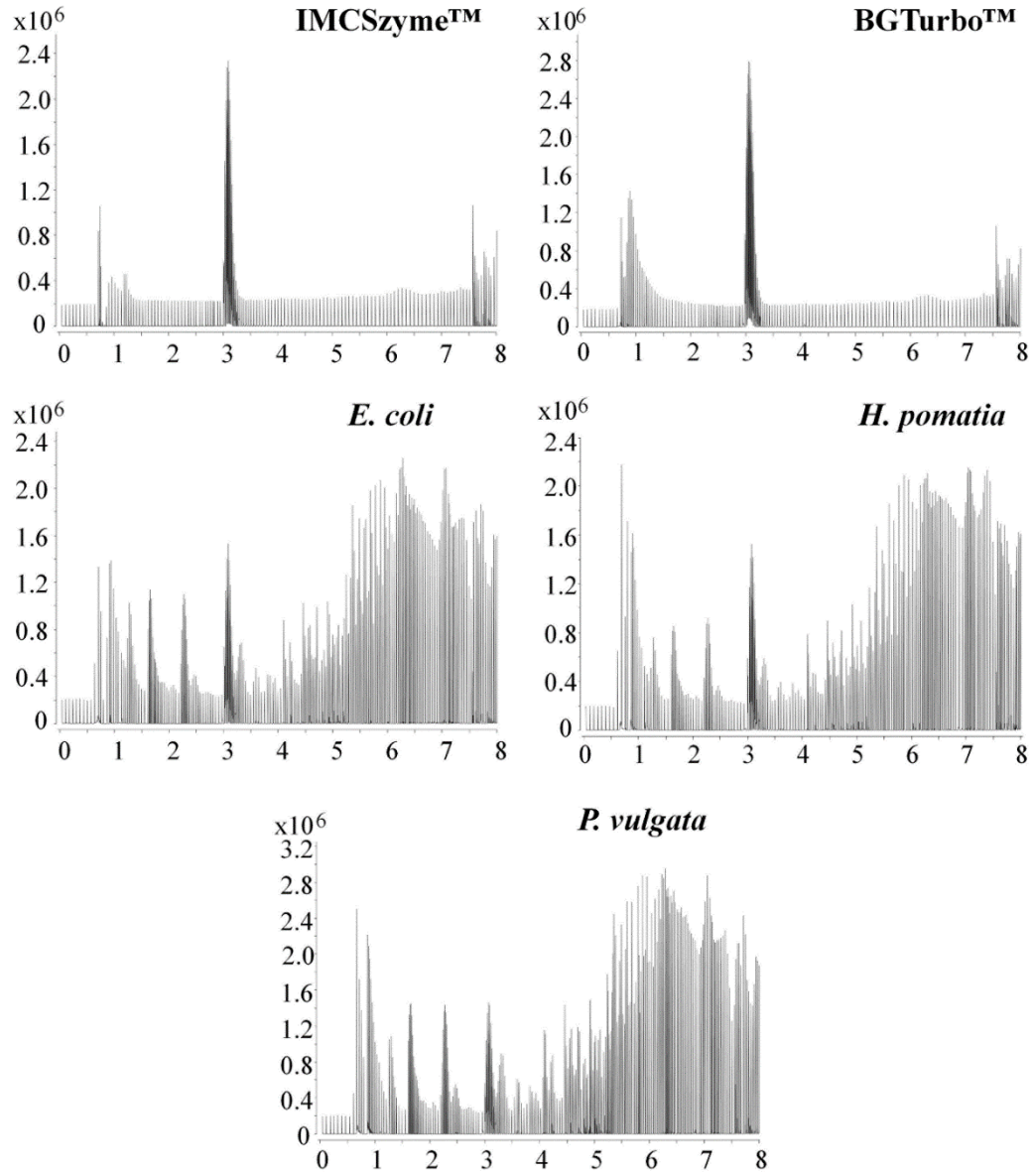

Figure 4.4. Total ion chromatograms (TICs) of samples hydrolyzed with IMCSzyme™, BGTurbo™, E. coli, H. pomatia and P. vulgata. No sample cleanup was used with IMCSzyme™ and BGTurbo™. Sample cleanup was used with E. coli, H. pomatia and P. vulgata. Desomorphine retention time is 3.2 minutes.

Conclusions

The short-term degradation of desomorphine and desomorphine-glucuronide at elevated temperature and various pH values was investigated and no degradation of either desomorphine or desomorphine-glucuronide was observed. Using optimum conditions for chemical and enzymatic hydrolysis, deconjugation rates of 96-100% were achieved for all systems tested. Although all five enzymes were as effective as chemical hydrolysis under optimum conditions, *P. vulgata* was the most efficient enzyme under challenging conditions. In terms of incubation time and sample cleanliness, the recombinant enzymes IMCSzyme™ and BGTurbo™ were superior compared to the traditional β-glucuronidase enzymes. However, all of the enzymatic approaches are considerably more expensive than chemical hydrolysis.

In this first report to evaluate the stability and deconjugation efficiency of desomorphine-glucuronide, the following limitations should be considered. Desomorphine-glucuronide was generated *in situ* due to the absence of commercial reference material or authentic urine specimens from Krokodil users. As such, the concentration of desomorphine-glucuronide used might be considerably less than the concentration found in authentic blood or urine specimens from desomorphine users. In an effort to differentiate deconjugation efficiencies between the different enzymatic systems, sub-optimal conditions were employed. Deconjugation efficiencies were evaluated in aqueous solution rather than biological matrix which may influence overall performance (24). Finally, although both desomorphine and desomorphine-glucuronide were stable for up to three hours at incubation temperatures up to 60^{o}C over a range of pHs (4-10), drug stability in biological matrix should not be inferred from these results.

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CHAPTER V

QUANTITATIVE ANALYSIS OF DESOMORPHINE IN BLOOD AND URINE USING SOLID PHASE EXTRACTION AND GAS CHROMATOGRAPHY-MASS SPECTROMETRY[1]

This dissertation follows the style and format of *The Journal of Analytical Toxicology*.

[1]Winborn, J., Kerrigan, S. (2018) Quantitative Analysis of Desomorphine in Blood and Urine Using Solid Phase Extraction and Gas Chromatography-Mass Spectrometry. *Journal of Chromatography B*, in press.

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Abstract

Desomorphine, a semi-synthetic opioid, is a component of the street drug Krokodil. Despite continued reports of Krokodil use, confirmation via toxicological testing remains scarce. The lack of confirmed desomorphine reports may be in part due to the limited published analytical methodology capable of detecting desomorphine at forensically relevant concentrations. In an effort to assist with identification efforts, a robust analytical method was developed and validated. Solid phase extraction (SPE) and gas chromatography-mass spectrometry (GC-MS) were used to determine desomorphine in blood and urine using a deuterated analog as the internal standard. Data was acquired using selected ion monitoring (SIM) mode. Extraction efficiencies in blood and urine were 69% and 90%, respectively. The limits of quantitation in blood and urine were 5 ng/mL and 8 ng/mL, ten-fold lower than previously published methods. Intra- and inter-assay CVs were 2-4% (n=3) and 3-7% (n=15), respectively. The method was fully validated in accordance with published guidelines for forensic use. Furthermore, it provides a means by which desomorphine can be identified in toxicology specimens at forensically relevant concentrations, without the need for derivatization.

Keywords: Desomorphine, Krokodil, Blood, Urine, GC-MS

Quantitative Analysis of Desomorphine in Blood and Urine Using Solid Phase Extraction and Gas Chromatography-Mass Spectrometry

Introduction

Desomorphine ((5α)-17-methyl-4,5-epoxymorphinan-3-ol) is a semi-synthetic opioid that gained notoriety in the early 2000s when reports of its abuse began to surface. Although the drug originated in Siberia, its popularity in Russia and the former Soviet Republics (as a less expensive alternative to heroin) increased significantly (1). By the mid-2010s, its use had spread to central European countries. Desomorphine is currently listed as a Schedule I drug in the United States, where reports of use have been somewhat limited (2). It is reported to have approximately ten-times the analgesic potency of morphine, with a faster onset but shorter duration of action (3). As its name suggests, dihydrodesoxymorphine is structurally differentiated from morphine by the absence of the C6 hydroxyl group and saturation of the C7-C8 double bond (**Figure 5.1**).

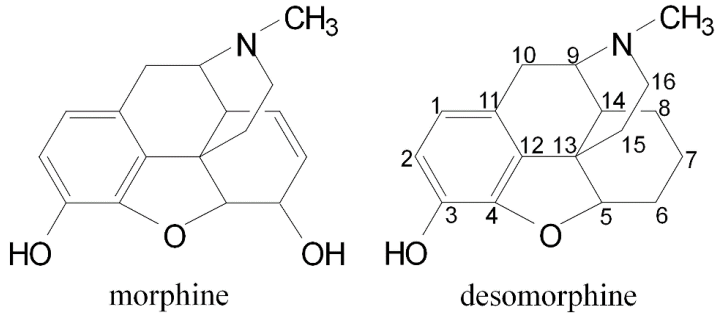

Figure 5.1. Chemical structures of morphine and desomorphine.

Krokodil is synthesized from codeine using harsh reagents such as hydroiodic acid and red phosphorous, also used in the clandestine manufacture of methamphetamine (4). Although Krokodil contains a number of morphinans, desomorphine is the principal component (5,6). The clandestine synthesis often leaves behind byproducts and traces of

the precursor chemicals, contributing to the severe dermatological side effects. The name of the street drug comes from the crocodile-like appearance of the skin following intravenous or subcutaneous injection. The sequela of abuse, including necrotic ulcers, gangrene and even osteonecrosis, may necessitate the amputation of limbs (7). At least one fatality involving lethal endomyocarditis has been reported following chronic use of the drug (8). Most published case reports originate from emergency departments and clinical settings, with cases originating from the Republics of Armenia (9-11) and Georgia (12), Germany (13), Italy (8), Poland (14), Russia (15. 16), Spain (17), the United Kingdom (18) and the United States (19-23). However, to date only one published case report included positive toxicology findings for desomorphine (8). This might be attributed to the time difference between actual drug use, and the manifestation of severe dermatological effects. However, analytical factors must also be considered.

Immunoassays are widely used for both clinical and forensic screening purposes. Enzyme linked immunosorbent assays are commonly used in forensic toxicology settings because these assays are amenable to a variety of biological matrices including blood, urine, tissue, hair, etc). Non-specific binding of compounds structurally similar to the target drug can be exploited to create immunoassays capable of detecting multiple drugs within a particular class of drug (e.g. benzodiazepines). A recent evaluation of commercially available ELISAs indicated highly variable cross-reactivity towards desomorphine (24). Cross-reactivities using commercial opiate assays ranged from <2.5% to 77%. Reliance on immunoassay-based screening alone may not be sufficient to detect the drug if the cross-reactivity is not sufficiently high. For forensic purposes, immunoassay-based screening

results also require analytical confirmation. GC-MS is widely used for this purpose and has long been considered the gold standard for confirmatory drug testing (25).

Krokodil is typically administered intravenously. Although pharmacokinetic studies in humans have not been reported, desomorphine is more lipophilic than morphine, hence its faster onset of action. While desomorphine's half-life is unknown, it is reported to have a duration of action approximately half that of morphine (3). Although one published case report suggested detection windows of a few hours in blood and three days in urine, no supporting data was provided (13). Recent studies identified several desomorphine metabolites using human liver microsomes and recombinant cytochrome P450 isoforms (rCYPs) (26, 27). Phase I metabolism includes *N*-demethylation, hydroxylation and *N*-oxidation. However, reference standards for these metabolites are not commercially available, precluding them from analysis. Therefore, forensic laboratories must rely upon analytical methods with sufficient sensitivity to detect the parent drug in both blood and urine.

There have been very few published methods for desomorphine analysis. Five reports describe the identification of the drug in either water, blood, urine or seized drug material using GC-MS. These are summarized in **Table 5.1**. Of these, only one was fully validated for quantitative analysis in a biological matrix (28). In this method, Alves utilized a QuEChERS (quick, easy, cheap, effective, rugged and safe) extraction to identify desomorphine in silylated blood extracts with a limit of quantitation of 103 ng/mL. Phenacetin, which bears no structural similarity to desomorphine was used as the internal standard. Although liquid-liquid extraction was reported most frequently, it is known to have lower extraction efficiencies for zwitterionic drugs like desomorphine. Su used solid

phase dynamic extraction (SPDE) and solid phase microextraction (SPME) to isolate desomorphine from water and urine (29). Although a deuterated internal standard was employed, the method also lacked requisite sensitivity for forensic purposes, with limits of quantitation ranging from 250-500 ng/g. In addition to GC-MS methodology, a liquid chromatography-tandem mass spectrometry (LC-MS/MS) published by Eckart, was capable of identifying desomorphine in plasma, serum and tissue at concentrations as low as 0.1 ng/mL [30]. While this method is significantly more sensitive than available GC-MS methods, not all forensic toxicology laboratories have access to LC-MS/MS instrumentation.

Table 5.1

GC-MS analysis of desomorphine.

Matrix	Extraction Method	Derivatization	Limit of Quantitation	Internal Standard	Reference
urine; drug samples	LLE	acylation; silylation	NR	NR	(34)
water; urine	SPME; SPDE	none	250 ng/g (SPDE); 500 ng/g (SPME)[1]	desomorphine-D_3	(30)
drug samples	LLE	silylation	490 ng/mL[2]	phenacetin	(6)
blood	QuEChERS	silylation	103 ng/mL[2]	phenacetin	(28)
urine	LLE	acylation	NR	codeine-D_6	(26)

[1]LOQ criteria: signal to noise ratio >10:1.
[2]LOQ criteria: bias/precision ±20% and signal 5x that of a blank sample.
LLE, liquid-liquid extraction; NR, not reported; QuEChERS, quick, easy, cheap, effective, rugged and safe; SPDE, solid phase dynamic extraction; SPME, solid phase microextraction.

Derivatization of polar compounds is sometimes necessary to improve their gas chromatographic properties and detectability. Desomorphine is a zwitterion like morphine, bearing both amine (pKa 9.69) and phenol (pKa 10.62) functional groups (27, 28). It is therefore no surprise that the majority of the GC-MS methods described (**Table 5.1**) utilized derivatizaton (silylation and acylation). Although derivatization reduces polarity, imparts volatility and can improve detectability, it can result in detector fouling and introduces an additional step into the analytical scheme.

Forensically relevant concentrations in blood and urine are dose-dependent and greatly influenced by the tolerance of the user. In the absence of human pharmacokinetic studies, an assay range comparable to that of morphine is desirable. Ideally, methods should be capable of identifying low ng/mL concentrations of desomorphine. The purpose of this study was to develop a highly sensitive GC-MS method for desomorphine in blood and urine for forensic use, and to fully validate the method in accordance with published recommendations (33).

Materials and methods

Chemicals and reagents

Desomorphine, and desomorphine-D3 were obtained from Cerilliant (Round Rock, TX, USA). Pooled drug free urine obtained from Utak Laboratories (Valencia, CA, USA) was preserved with 1% sodium fluoride prior to use. Bovine blood preserved with 1% sodium fluoride and 0.2% potassium oxalate was obtained from Quad Five (Ryegate, MT, USA). Methanol (LCMS grade) and concentrated hydrochloric acid were obtained from J.T. Baker (Center Valley, MA, USA). Ethyl acetate (HPLC grade) was obtained from Fisher Scientific (Fair Lawn, NJ, USA) and concentrated ammonium hydroxide was

obtained from Macron Fine Chemicals (Center Valley, MA, USA). Dichloromethane and isopropyl alcohol were obtained from Mallinckrodt Chemicals (St. Louis, MO, USA). Acetonitrile (LCMS grade) was obtained from Thermo Fisher Scientific (Radenor, PA, USA). *N*-methyl-*N*-(trimethylsilyl)trifluoroacetamide (MSTFA) was obtained from Sigma Aldrich (St. Louis, MO, USA). Deionized (DI) water was generated from a Direct-Q 3 (UV) system (Millipore, Billerica, MA, USA). PolyChrom Clin II 3cc (35 mg) solid phase extraction (SPE) columns were obtained from SPEware (Baldwin, CA, USA).

Working standards of desomorphine were prepared in acetonitrile at 50, 5 and 0.5 µg/mL for fortification in urine, and 25, 2.5 and 0.25 µg/mL for fortification in blood. The internal standard solution was prepared in acetonitrile at 0.5 µg/mL for urine and 0.25 µg/mL for blood. Elution solvents containing concentrated ammonium hydroxide were prepared daily.

Instrumentation

SPE was performed using a Supelco Visiprep vacuum manifold (Supelco, Bellefonte, PA, USA) and extracts were evaporated to dryness under nitrogen using a Turbovap LV concentration workstation (Biotage, Charlotte, NC, USA). An Agilent 5975B VL MSD was coupled to an Agilent 6890N GC oven equipped with an Agilent 7683B autosampler (Agilent Technologies, Santa Clara, CA, USA). Separation was achieved using an Agilent DB-5 capillary column (30 m x 0.25 mm, 0.5 µm film thickness). The optimized GC-MS conditions were as follows: injection mode: split (2:1); injector port temperature: 250 °C; carrier gas: helium with flow rate 1.3 mL/min; oven program: 160 °C with initial hold time 0.5 min, 30 °C/min to 290 °C with a final hold time of 7.67 min; ion source temperature: 230 °C. Preliminary mass spectral data were obtained in full scan

mode from *m/z* 50 to 600 amu. Selective ion monitoring (SIM) mode was used for quantitation. The monitored ions for desomorphine were **271.1**, 256.1, and 228.1 and for desomorphine-D3 were **274.1**, 259.1, and 231.1. Quantitation ions are shown in bold. The dwell time for each ion was 25 ms and the total run time was 12.5 minutes. All data was analyzed using Agilent ChemStation software.

Urine extraction

Internal standard solution (20 µL) was added to 1 mL of urine to achieve a final concentration of 10 ng/mL. Urine was diluted with 1 mL of 0.1 M hydrochloric acid and transferred to PolyChrom Clin II SPE columns (3 cc, 35 mg). Samples were allowed to flow through under gravity or sufficient vacuum to maintain constant flow (approximately 1 mL/min). Columns were successively rinsed with 1 mL of deionized water, 0.1 M hydrochloric acid, methanol and ethyl acetate. After drying columns for five minutes at full vacuum, desomorphine was eluted using two 0.5 mL aliquots of elution solvent (4% ammonium hydroxide in ethyl acetate). Extracts were evaporated to dryness under nitrogen at 50 °C then reconstituted in 30 µL of ethyl acetate. An aliquot of 1 µL was injected onto the GC-MS for analysis.

Blood extraction

Internal standard solution (20 µg/mL) was added to 0.5 mL of whole blood to achieve a final concentration of 10 ng/mL. A protein precipitation was performed with the addition of 2 mL of ice-cold acetonitrile while vortex mixing, followed by centrifugation at 4000 rpm for 10 minutes. The supernatant was decanted and diluted with 1 mL of 0.1 M hydrochloric acid. Samples were transferred to SPE columns (3 cc, 35 mg) and extracted

as described for urine. Extracts were reconstituted in 30 µL of ethyl acetate and 1 µL was injected onto the GC-MS.

Method development

To evaluate the need for derivatization, desomorphine was silylated with MSTFA. On-column derivatization was achieved by adding 10 µL of 0.1 mg/mL desomorphine standard to 10 µL of MSTFA. Data was acquired in full scan using a modified oven temperature programming: 80 °C (0.5 min), 30 °C/min to 290 °C. All other conditions were as described earlier. Ions were selected for SIM acquisition based on abundance and specificity for desomorphine.

To optimize the extraction efficiency, samples of urine (1mL) were fortified with 250 ng/mL of desomorphine and extracted as described earlier. The solvents evaluated were 2% ammonium hydroxide in 80:20 dichloromethane/isopropanol, 4% ammonium hydroxide in 80:20 dichloromethane/isopropanol, 2% ammonium hydroxide in ethyl acetate and 4% ammonium hydroxide in ethyl acetate. Additionally, samples of whole blood (0.5 and 1 mL) were fortified with 250 ng/mL of desomorphine and extracted as described earlier.

Method validation

The method was validated in accordance with the Scientific Working Group for Forensic Toxicology recommendations (33). Performance was evaluated in terms of extraction efficiency, limit of detection (LOD), limit of quantification (LOQ), bias, precision, calibration model, interferences, and carryover. The extraction efficiency in blood and urine (250 ng/mL) was calculated by comparing extracted and non-extracted samples. Matrix containing internal standard (10 ng/mL) was extracted with and without

desomorphine. Samples extracted without desomorphine were fortified with an equivalent amount post-extraction, but prior to evaporation and reconstitution. Recovery of the analyte was calculated by comparing the relative peak area (drug/IS) of samples extracted in the presence of desomorphine (n=3) with those fortified post-extraction (n=3).

The limits of detection and quantitation were determined empirically using drug-free matrix fortified with desomorphine. Three sources of drug-free matrix were analyzed in duplicate over three independent runs. The LOD was defined as the lowest concentration of drug that produced a reportable result (relative retention time ± 2% of the standard, ion ratios ±5% and a signal-to-noise ratio of 3:1 or greater). The LOQ was defined as the lowest concentration to produce a reportable result (as described above) with a signal-to-noise ratio of 10:1 or more, and a quantitative result within 20% of the expected value. Calibration models were evaluated statistically using the coefficient of determination (R^2), f-tests and standardized residual plots. A total of nine non-zero calibrators were used in blood (5, 10, 25, 50, 100, 250, 500, 750 and 1000 ng/mL) and urine (8, 10, 25, 50, 100, 250, 500, 750 and 1000 ng/mL).

Bias and precision were evaluated at three concentrations (low, medium and high) in urine (30, 400 and 800 ng/mL) and blood (15, 400 and 800 ng/mL) in triplicate over five runs. Within-run precision was calculated for each run at each concentration (n=3). Between-run precision was calculated over all five runs for all three concentrations (n=15). Bias was calculated from the following equation: $\frac{calculated\ concentration - expected\ concentration}{expected\ concentration} \times 100$. The threshold for acceptable bias and precision was ± 20%.

Interferences from the matrix, isotopically labeled internal standard, common drugs and structurally related compounds were also evaluated. Ion contributions from the stable isotope internal standard were evaluated by fortifying drug-free matrix with internal standard (10 ng/mL) and monitoring the signal of desomorphine. Matrix interferences were examined using ten drug-free matrix samples from independent sources without internal standard (n=2). Forty-two common drugs and twenty-four opioids were selected for drug interference, giving a total of sixty-six compounds. These are summarized in **Table 5.2**. Interferences were evaluated using negative and positive controls in blood and urine. A 1:1 ratio, 10-fold excess and 100-fold excess of interferent (relative to desomorphine) was used for interference testing. The negative control contained internal standard (10 ng/mL) and 1000 ng/mL of interferent. Positive controls were prepared with internal standard (10 ng/mL), interferent (1000 ng/mL) and desomorphine at 1000, 100 and 10 ng/mL, respectively.

Carryover was assessed using by injecting a blank matrix (no drug or IS) or negative control (IS only) immediately following the high control (1000 ng/mL). Carryover was deemed to be present if the blank matrix or negative control produced a reportable result (S/N ratio of 3:1 or greater, relative retention time ± 2% and acceptable ion ratios).

Table 5.2

Summary of compounds included in interference study. Compounds were separated into two groups: common drugs (n=42) and opioids (n=24).

Common Drugs	Opioids
7-aminoclonazepam, 7-aminoflunitrazepam, acetaminophen, alprazolam, amitriptyline, amobarbital, amphetamine, butalbital, caffeine, carbamazepine, carisoprodol, clonazepam, cocaine, cotinine, cyclobenzaprine, dextromethorphan, diazepam, fluoxetine, flurazepam, gabapentin, ketamine, MDMA, meprobamate, methaqualone, 11-nor-9-carboxy-Δ9-THC, nordiazepam, oxazepam, pentobarbital, phencyclidine, phenobarbital, phenytoin, pseudoephedrine, salicylic acid, secobarbital, sertraline, temazepam, Δ9-THC , trazodone, valproic acid, zaleplon, zolpidem, zopiclone	6-acetylcodeine, 6-acetylmorphine, buprenorphine, codeine, dihydrocodeine, fentanyl heroin, hydrocodone, hydromorphone, levorphanol, meperidine methadone, morphine nalorphine, norcodeine, norhydrocodone, normeperidine, normorphine, noroxycodone, oxycodone, oxymorphone, propoxyphene, thebaine, tramadol

MDMA, methylenedioxymethamphetamine; THC, tetrahydrocannabinol.

Results and discussion

Method development

Total ion chromatograms and mass spectra for desomorphine and its trimethylsilyl derivative are depicted in **Figure 5.2**. The mass spectrum of the underivatized drug is characterized by an intense molecular ion (*m/z* 271) with subsequent loss of a methyl group $[M-15]^+$ (*m/z* 256), hydroxyl $[M-17]^+$ (*m/z* 254), CH_3N $[M-29]^+$ (*m/z* 242), C_2H_5N $[M-43]^+$ (*m/z* 228) and C_3H_7N $[M-57]^+$ (*m/z* 214) representing complete cleavage of the nitrogen ring. Since the phenanthrene-type opioids fragment in a predictable fashion, the silylated derivative also produced a mass spectrum with prominent M-15, 29, 43 and 57 losses, in addition to the loss of the trimethylsilyl (TMS) group $[M-72]^+$. Full scan acquisition confirmed that the derivatization of desomorphine was complete (100% yield). Although the abundance of fragment ions improved with derivatization, chromatographic and mass

spectral properties were sufficient to proceed with method development without MSTFA. This approach decreases analysis time, detector fouling and routine maintenance.

Several ions (*m/z* 271, 256, 242, 228, 214) were evaluated during method development of underivatized desomorphine using SIM acquisition. Three ions were selected based upon their specificity. The quantification ion was the molecular ion, *m/z* 271 (also the base peak) and the two qualifier ions selected were *m/z* 228 and 256. Due to the ion ratios for *m/z* 228 and 256 being 20% and 18% respectively, a 5% absolute abundance threshold was established, instead of ± 20% relative abundance, which is often used [33].

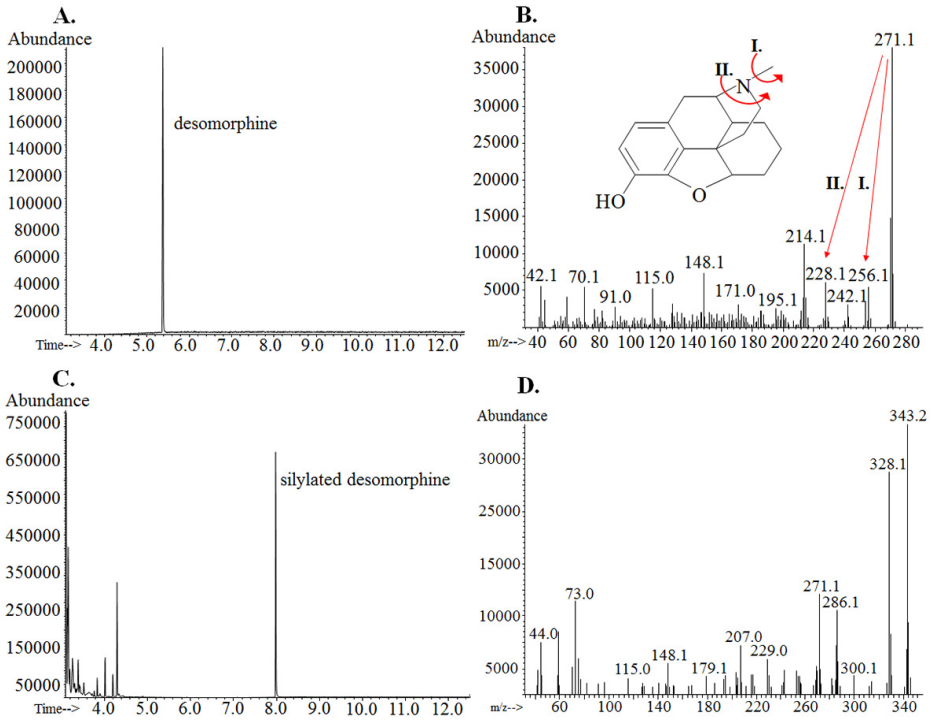

Figure 5.2. Total ion chromatogram (TIC) and mass spectrum of desomorphine (A and B), and of silylated desomorphine (C and D). The retention time of desomorphine was 5.4 minutes and of desomorphine-TMS was 7.9 minutes. Fragmentation of qualifier ions are shown with arrows I (*m/z* 256) and II (*m/z* 228).

Optimum extraction efficiencies in blood and urine were achieved using ethyl acetate containing 4% concentrated ammonium hydroxide. **Figure 5.3** depicts a box plot

of desomorphine peak areas obtained using all four elution solvents (measured in triplicate) and **Figure 5.4** shows representative extracted ion chromatograms (EICs) for each elution solvent and the average S/N ratios in urine. The improved extraction efficiency using highly basified ethyl acetate is not surprising given the high pKa of the tertiary amine (9.69). Ethyl acetate was a far superior elution solvent to dichloromethane/isopropyl alcohol (80/20), and increasing the ammonium hydroxide concentration from 2 to 4% more than doubled the desomorphine peak area. Although absolute recovery was improved using ethyl acetate, all elution solvents evaluated were free from coextractive interferences (**Figure 5.4**). Sufficient sensitivity was achieved using a reduced volume of blood, so method validation was performed using 1 mL urine and 0.5 mL blood.

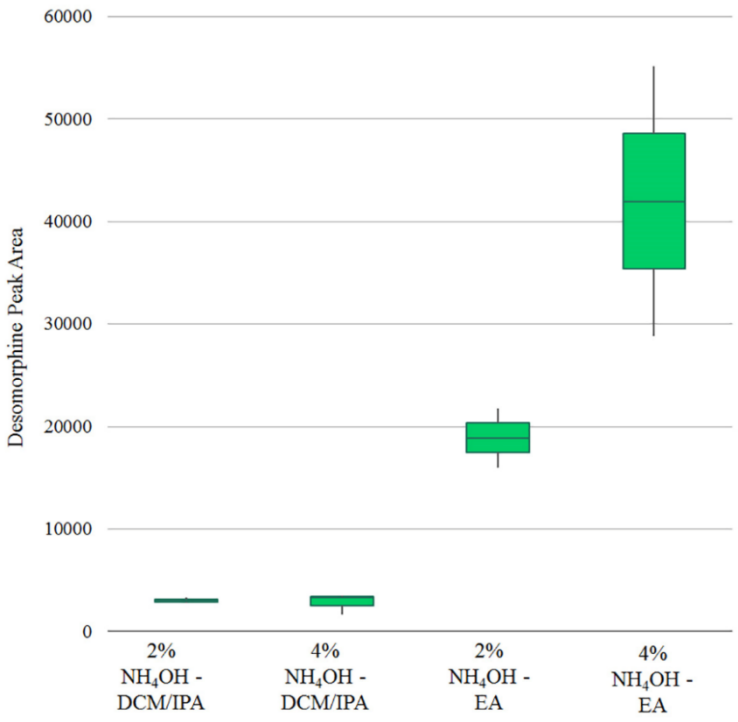

Figure 5.3. Box plots comparing the desomorphine peak area from urine (250 ng/mL) extracted with different elution solvents (n=3). Elution solvents: 2% ammonium hydroxide in 80:20 dichloromethane/isopropanol (2% DCM/IPA), 4% ammonium hydroxide in 80:20 dichloromethane/isopropanol (4% DCM/IPA), 2% ammonium hydroxide in ethyl acetate (2% EA) and 4% ammonium hydroxide in ethyl acetate (4% EA).

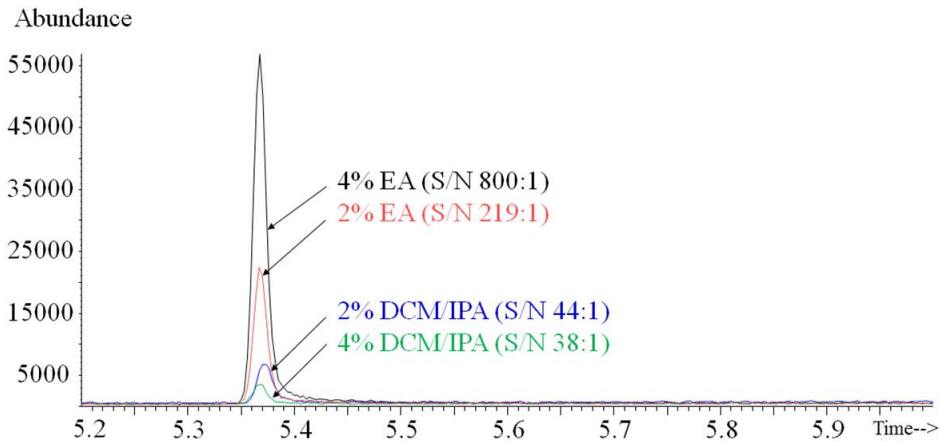

Figure 5.4. Representative EICs (*m/z* 271) of desomorphine from urine using different elution solvents. The average signal-to-noise (S/N) for each solvent (n=3) is shown in parentheses. Elution solvents: 2% ammonium hydroxide in 80:20 dichloromethane/isopropanol (2% DCM/IPA), 4% ammonium hydroxide in 80:20 dichloromethane/isopropanol (4% DCM/IPA), 2% ammonium hydroxide in ethyl acetate (2% EA) and 4% ammonium hydroxide in ethyl acetate (4% EA).

Method validation

Extraction efficiencies were 90 ± 1% in urine (n=3) and 69 ± 2% in blood (n=3). The limits of detection and quantitation were 5 ng/mL in blood (n=18) and 8 ng/mL in urine (n=18). Concentrations as low as 1 ng/mL were evaluated, and while the S/N ratios exceeded 3 at all concentrations, the secondary acceptance criteria (ion ratios ±5% absolute) was not met at any concentration less than 5 ng/mL for blood or 8 ng/mL for urine. Residual plot analysis identified a weighted (1/x) quadratic as the preferred calibration model. However, using the *f*-test to determine the value of the addition of the quadratic term showed no significant difference from a linear model. Additionally, in practice a weighted (1/x) linear calibration model gave the best quantitative performance at low concentration, while maintaining acceptable bias and precision at the high end of the calibration range. The R^2 values were above 0.99 for both matrices.

Bias, precision and S/N ratios are summarized in **Table 5.3** and EICs at the LOQ are shown in **Figure 5.5**. Bias and precision were evaluated at three concentrations (low, medium and high) in triplicate over five days. Intra-assay CVs were 2-3% in blood and 2-4% in urine. Inter-assay CVs were 3-5% in blood and 3-7% in urine. Bias and precision were within acceptable ranges (± 20%) at all concentrations tested and are summarized in **Tables 5.4** and **5.5**.

Table 5.3

Limits of detection and quantitation for desomorphine in blood and urine using GC-MS. The mean, standard deviation (SD), signal-to-noise ratio (S/N), bias and covariance (CV) at the LOQ are shown.

Sample Matrix	LOD (ng/mL)	LOQ (ng/mL)	Mean ± SD (ng/mL) n=18	S/N	Bias	CV
blood	5	5	4.42 ± 0.25	70:1	-12%	6%
urine	8	8	7.96 ± 0.20	261:1	-1%	3%

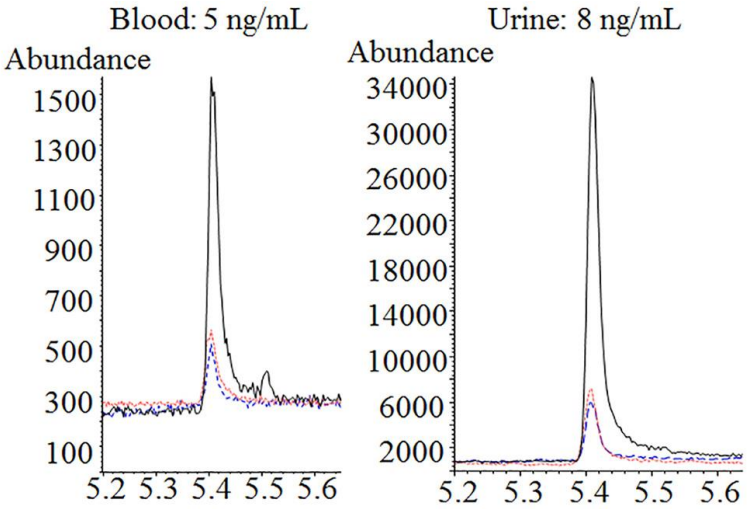

Figure 5.5. EICs of desomorphine in blood and urine at the limit of quantitation.
Table 5.4

Precision and bias (n=15) for desomorphine in blood at low (15 ng/mL), medium (400 ng/mL), and high (800 ng/mL) concentrations.

Concentration (ng/mL)	**Intra-assay Precision (n=3, CV)**	**Inter-assay Precision (n=15, CV)**	**Bias (n=15)**
15	2%	5%	1%
400	3%	3%	5%
800	3%	3%	-3%

Table 5.5

Precision and bias (n=15) for desomorphine in urine at low (30 ng/mL), medium (400 ng/mL), and high (800 ng/mL) concentrations.

Concentration (ng/mL)	**Intra-assay Precision (n=3, %CV)**	**Inter-assay Precision (n=15, %CV)**	**%Bias (n=15)**
30	4%	7%	1%
400	2%	3%	9%
800	2%	3%	0%

Interferences (matrix, isotopically labeled internal standard and other drugs) were systematically evaluated. For both blood and urine, ten independent drug-free sources were analyzed. No quantitative or qualitative interferences were observed using either matrix, and no qualitative or quantitative inferences were observed from the deuterated analog. Sixty-six drugs, including twenty-four opioids were evaluated for potential interferences (**Table 5.2**). No quantitative inferences were observed for any of the compounds. However, when present at a 100-fold higher concentration than desomorphine

in blood, levorphanol produced a qualitative interference due to the presence of *m/z* 228 and 256 ions in the mass spectrum. Although alternative ions (*m/z* 242 and 214) were also evaluated for desomorphine during method development, it is noteworthy that these ions are also present for levorphanol and as such, would not have mitigated the qualitative interference at excess concentrations. The same interference was observed for levorphanol at 10- and 100-fold concentrations in urine. Although bias and precision were still within acceptable ranges, this does highlight the potential for levorphanol to interfere qualitatively due to unacceptable ion ratios. Although levorphanol is not widely used, its *d*-isomer (dextrorphan) is a metabolite of dextromethorphan. GC-MS cannot differentiate between these *d* and *l* isomers. Therefore, desomorphine might not be reportable in the presence of large excesses of either of these substances. Carryover was evaluated in both blank matrix and negative control samples. No carryover was observed using methanol as a wash solvent with fifteen post-injection washes.

Conclusions

Opioid abuse in the United States and elsewhere has reached unprecedented levels. Forensic toxicology laboratories must have at their disposal analytical methods to detect a wide-range of substances. The prevalence of Krokodil use is difficult to estimate but it continues to be self-reported by drug users seeking medical treatment. The absence of sufficiently sensitive analytical methodology may in part explain why desomorphine is typically not reported in published case reports.

In this report, a validated method for the quantitative analysis of desomorphine in blood and urine is described. Using small sample volumes, solid phase extraction and GC-MS was used to identify desomorphine at low ng/mL concentrations without the need for

derivatization. Analytical performance greatly exceeded those of earlier studies and the method was validated in accordance with published recommendations for forensic use.

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CHAPTER VI

ANALYSIS OF DESOMORPHINE IN URINE USING LIQUID CHROMATOGRAPHY-TANDEM MASS SPECTROMETRY[1]

This dissertation follows the style and format of *The Journal of Analytical Toxicology*.

[1]Winborn, J., Kerrigan, S. (2018) Analysis of Desomorphine in Urine Using Liquid Chromatography-Tandem Mass Spectrometry. *Journal of Analytical Toxicology*, in press. Submitted for publication in the Journal of Analytical Toxicology

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Abstract

Desomorphine is a primary component of the drug Krokodil. While reports of Krokodil use continue to appear in the literature, analytically confirmed cases remain quite scarce. This might be attributed to trends in geographical use and limited published analytical methodology to detect its use. A sensitive analytical method to detect desomorphine was developed and validated to assist with identification efforts. Solid phase extraction (SPE) and liquid chromatography-tandem mass spectrometry (LC-MS/MS) were used to quantitatively identify desomorphine in urine. An isotopically labelled analog was used as the internal standard. Assay performance was evaluated in accordance with published guidelines. The extraction efficiency for desomorphine in urine was 90% and limits of detection and quantitation were 0.5 ng/mL. The calibration range of the assay was 0.5 to 500 ng/mL. Bias ranged from -1-2% (n=15) and the intra- and inter-assay CVs were 2-3% (n=3) and 3-6% (n=15), respectively. Ion suppression was -20% and -10% at low and high concentrations, respectively. Interferences were assessed using common drugs, including twenty-four opioids and structurally related compounds. Using this approach, the quantitative analysis of desomorphine in urine is described at forensically relevant concentrations.

Keywords: Desomorphine, Krokodil, Urine, LC-MS/MS

Analysis of Desomorphine in Urine Using Liquid Chromatography-Tandem Mass Spectrometry

Introduction

Desomorphine ((5α,6α)-17-methyl-4,5-epoxymorphinan-3-ol) is a semi-synthetic opioid that was first synthesized in the early 1930s (1). As its name suggests, this 4,5-epoxymorphinan can be structurally differentiated from morphine by the absence of a hydroxyl group (C6) and a saturated bond (C7-C8) (**Figure 6.1**). Desomorphine has a faster onset of action compared to morphine due to its increased lipophilicity, but its duration of action is observed to be approximately half that of morphine (2). From 1940 to 1951 desomorphine was used therapeutically in Switzerland under the trade name Permonid (3). Notably, small scale production continued until 1981 for use by a single patient. At the 1961 United Nations Single Convention on Narcotic Drugs, attended by over seventy countries, desomorphine was placed into the most restrictive category (4). At present, the drug is controlled in many countries including Australia, Brazil, the United Kingdom, Austria and Germany (5). In the United States, desomorphine is listed under Schedule I of the Federal Controlled Substances Act (6).

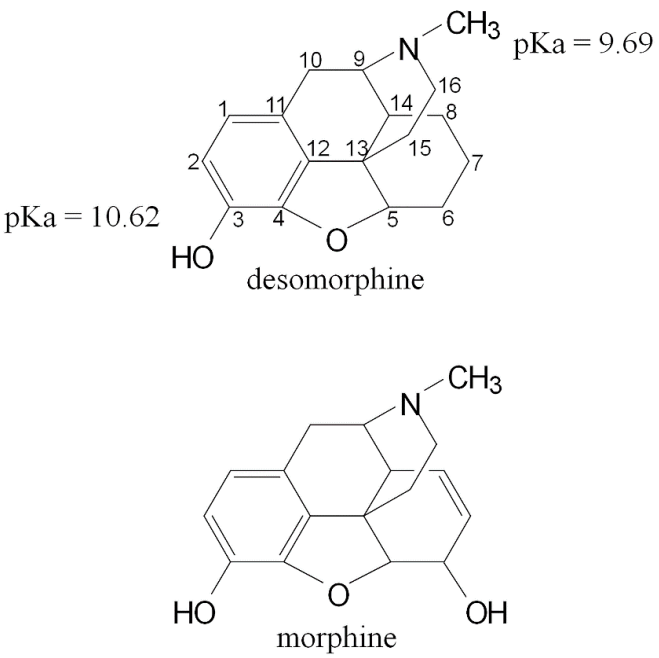

Figure 6.1. Chemical structures of desomorphine (dihydrodesoxymorphine) and morphine.

In 2003, desomorphine emerged as a drug of abuse in Siberia and Russia (3). Using harsh chemicals, such as red phosphorous and hydroiodic acid, codeine is converted to desomorphine along with other morphinans to produce Krokodil (7-9). The drug is typically administered intravenously as a substitute for heroin. Precursor chemicals that remain following clandestine synthesis can cause severe dermatological side effects. Complications associated with Krokodil use include necrotic ulcers, gangrene and osteonecrosis, which may necessitate invasive surgeries or even amputation of limbs (10).

In the years following its first appearance, reports of Krokodil use spread from Siberia and Russia through neighboring countries to central Europe and the United States. Despite anecdotal evidence suggesting that at one time there were approximately 100,000 people using Krokodil in Russia alone (7), analytically confirmed cases were not being reported in the literature. In contrast, numerous clinical case reports were described from

multiple countries, including Russia (11, 12), Republics of Georgia (13) and Armenia (14-16), Germany (17), Italy (18), Spain (19), Poland (20) and the United States (21-25). Most of these reports originate from drug users seeking medical attention for the complications of Krokodil use, although three involved fatalities (17, 18, 22). To date only one case has been analytically confirmed, with desomorphine identified by gas chromatography-mass spectrometry (GC-MS) in the urine of a decedent at a concentration of 270 ng/mL (18).

The lack of analytically confirmed cases makes it difficult to assess the true prevalence of Krokodil use. There is no published half-life for desomorphine and the only report of its window of detection (a few hours in blood and three days in urine) was not accompanied by any supporting data (17). Pharmacokinetic studies in humans are generally lacking. Richter was the first to investigate desomorphine metabolism using human liver microsomes (26, 27). Phase I metabolism pathways include *N*-demethylation, hydroxylation and *N*-oxidation, while Phase II metabolism includes conjugation with glucuronic acid and sulfates. Studies using recombinant cytochrome P450 isoenzymes (rCYPs) identified eight phase I metabolites (nordesomorphine, desomorphine-*N*-oxide, five hydroxydesomorphine isomers and norhydroxydesomorphine) that were produced by seven rCYPs (CYP2B6, CYP2C8, CYP2C9, CYP2C18, CYP2C19, CYP2D6 and CYP3A4) (27). An investigation using recombinant uridine 5'-diphospho-glucuronosyltransferase isoenzymes (rUGTs) suggested that desomorphine-glucuronide was formed by nine UGTs, including UGT1A1, UGT1A3, UGT1A8, UGT1A9, UGT1A10, UGT2B4, UGT2B7, UGT2B15 and UGT2B17 (27). Unfortunately, reference materials for desomorphine metabolites are not commercially available, precluding quantitative identification in forensic investigations. Despite the absence of

pharmacokinetic studies, it is reasonable to expect that analytical methods for forensic toxicology purposes should have comparable sensitivity to those of morphine.

Enzyme-linked immunosorbent assays (ELISAs) are commonly used for toxicological screening purposes because they are compatible with multiple biological matrices. However, immunoassay-based screening is typically directed towards one target analyte. Although antibody reagents with poor to moderate specificity can be exploited for the purposes of identifying a class of related compounds, the extent to which this is possible depends on the cross-reactivity. An evaluation of commercially available opioid ELISAs showed that cross-reactivities towards desomorphine ranged from <2.5% to 77% (28). Should an immunoassay with low cross-reactivity be used, there is an elevated risk that the drug will go undetected.

There are very few published methods that describe the quantitative analysis of desomorphine in biological matrices, and some lack the sensitivity necessary to be effective in toxicological investigations. Su (29) and Alves (30) utilized GC-MS to identify desomorphine in urine and blood with limits of quantitation of 250 ng/g and 103 ng/mL, respectively. We recently reported a more sensitive GC-MS assay, with limits of quantitation of 5 and 8 ng/mL in blood and urine (31). Liquid chromatography-mass spectrometry-based techniques are becoming increasingly widespread in toxicology laboratories for confirmatory testing. Improved sensitivity and versatility with respect to polar compounds and metabolites is a distinct advantage over traditional GC-MS approaches. Savchuck described LC with ultraviolet (UV) detection for the qualitative identification of desomorphine in urine and seized drug samples (32). Alves used LC with diode array detection (DAD) in seized drugs with a limit of quantitation of 490 ng/mL (8)

and Soares described a qualitative assay using both DAD and high-resolution mass spectrometry (HRMS) (9). Richter also used HRMS (Orbitrap™) to identify desomorphine and metabolites qualitatively in urine (26). Using LC-MS/MS Eckart described a quantitative assay to identify desomorphine in serum, plasma and tissue (33). These methods, including the internal standard (IS) and limits of quantitation (LOQ) are summarized in **Table 6.1**. Validated methods capable of detecting desomorphine in urine at forensically relevant concentrations have not been described to date. The purpose of this study was to isolate desomorphine from urine with high extraction efficiency, quantify the drug using LC-MS/MS, and to validate the method in accordance with published recommendations (34).

Table 6.1

Summary of LC-based methods to identify desomorphine.

Matrix	Sample Volume	Extraction Method	Internal Standard	Stationary Phase	LOQ	Detector	Reference
urine; drug samples	3 mL	LLE	NR	C_{18}	NR	UV	(32)
serum; plasma; tissue	200 µL or 2 g	SPE	codeine-D_6	phenyl-hexyl	0.1 ng/mL	MS-MS	(33)
drug samples	100 µL	LLE	phenacetin	silica	490 ng/mL	DAD	(8)
urine	2 mL	LLE	codeine-D_6	phenyl-hexyl	NR	Orbitrap™	(26)
drug samples	10 µL	None	NR	C_{18}	NR	DAD; Orbitrap™	(9)

DAD, Diode array detector; LLE, Liquid-liquid extraction; NR, Not reported; SPE, Solid phase extraction; UV, Ultraviolet detector.

Materials and methods

Reagents and equipment

Desomorphine and desomorphine-D_3 (IS) were purchased from Cerilliant (Round Rock, TX, USA). Pooled drug free urine from Utak Laboratories (Valencia, CA, USA) was preserved with 1% sodium fluoride before use. Deionized (DI) water was generated using a Direct-Q 3 (UV) system (Millipore, Billerica, MA, USA) and PolyChrom Clin II 3cc (35 mg) solid phase extraction (SPE) columns were purchased from SPEware (Baldwin, CA, USA). Methanol (LCMS grade) and concentrated hydrochloric acid were from J.T. Baker (Center Valley, MA, USA). Ethyl acetate (HPLC grade) was purchased from Fisher Scientific (Fair Lawn, NJ, USA), concentrated ammonium hydroxide was from Macron Fine Chemicals (Center Valley, MA, USA), acetonitrile (LCMS grade) was from Thermo Fisher Scientific (Waltham, MA, USA) and formic acid (>95%) was from Sigma Aldrich (St. Louis, MO, USA). Desomorphine working standards were prepared in acetonitrile at 25, 2.5 and 0.25 µg/mL for fortification in urine and the internal standard solution was prepared at 0.25 µg/mL in acetonitrile. The elution solvent (prepared daily) consisted of 4% concentrated ammonium hydroxide in ethyl acetate. Samples were extracted using SPE using a Supelco Visiprep vacuum manifold (Supelco, Bellefonte, PA, USA) and extracts were evaporated under nitrogen using a Turbovap LV concentration workstation (Biotage, Charlotte, NC, USA).

Solid phase extraction

Urine (0.5 mL) was fortified with 50 µL of IS solution (0.25 µg/mL). An aliquot of 1 mL of 0.1 M hydrochloric acid was added to the sample before transferring it to the SPE column. Samples were allowed to flow under gravity, or when necessary, sufficient

vacuum to keep a constant flow of approximately 1 mL/min. Columns were washed (successively) with 1 mL of DI water, 0.1 M hydrochloric acid, methanol and ethyl acetate. After drying under full vacuum for 5 minutes, desomorphine was eluted using two 0.5 mL aliquots of elution solvent. Extracts were evaporated under nitrogen at 50°C before being reconstituted in 30 µL of 92:8 (A:B) mobile phase (described below). An aliquot of 2 µL was injected onto the LC-MS/MS for analysis.

LC-MS/MS analysis

An Agilent Technologies 6470 triple quadrupole LC-MS/MS equipped with an Agilent 1290 Infinity II autosampler was used to analyze samples (Agilent Technologies, Santa Clara, CA, USA). Agilent MassHunter software was used for acquisition and data analysis. Nitrogen was generated using a Genius 3040 nitrogen generator (Peak Scientific, Billerica, MA, USA). Separation was achieved using an Agilent Poroshell 120 EC-C18 column (2.1 x 100 mm, 2.7 µm particle size) with a matching guard column kept in a thermostatically controlled column compartment (35°C). The mobile phase consisted of 0.1% formic acid in deionized water (A) and 0.1% formic acid in acetonitrile (B). The gradient elution profile was as follows: 8% B, flow rate 0.3 mL/min (0-2 min); 20% B, 0.3 mL/min (2-6 min); 90% B, 0.4 mL/min (6-6.5 min); 8% B, 0.4 mL/min (6.5-7 min). Data was acquired between 2 and 6 minutes. The LC-MS/MS was equipped with an electrospray ionization (ESI) source that was operated in positive mode. The optimized source conditions were as follows: drying gas temperature, 350°C; drying gas flow rate, 8 L/min; nebulizer, 20 psi; sheath gas temperature, 400°C; sheath gas flow rate, 10 L/min; capillary voltage, 4000 V. Data was acquired using multiple reaction monitoring (MRM). Precursor and product ions, collision energies, and fragmentor voltages for desomorphine and

desomorphine-D_3 are detailed in **Table 6.2**. The dwell time was 100 ms and the cell accelerator voltage was 7 V.

Table 6.2

Transition ions (*m/z*), fragmentor voltage and collision energies for desomorphine. Relative abundance is shown in parentheses.

Compound	Precursor Ion (*m/z*)	Product Ion (*m/z*)	Fragmentor (V)	Collision Energy (V)
desomorphine	272.2	195 (76%)	138	33
		167 (100%)	138	41
		152 (86%)	148	60
desomorphine-D_3 (IS)	275.2	195 (75%)	148	33
		167 (100%)	148	41
		152 (87%)	148	60

Assay performance

The method was validated in accordance with published guidelines from the Scientific Working Group on Toxicology (SWGTOX) (34). Extraction efficiency in urine was determined by comparison of extracted and non-extracted samples at 250 ng/mL as described earlier (31). The limits of detection and quantitation (LOD and LOQ) were established using drug-free urine fortified with desomorphine and IS. Three independent sources of drug-free matrix were analyzed in duplicate over three days. The LOD was the lowest concentration of drug to produce a signal-to-noise ratio (S/N) of 3:1 or greater, retention time ± 2% of the control and relative ion ratios ± 20%. The LOQ was defined as the lowest concentration of drug to produce a quantitative value within 20% of the expected value, a signal-to-noise (S/N) ratio of 10:1 or greater, retention time ± 2% of the control, ion ratios within ±20%, and precision and bias within 20%.

Bias and precision were evaluated in urine (2, 250 and 450 ng/mL) using three samples of pooled fortified matrix over five runs. Intra- and inter- assay precision was determined for each concentration using the one-way analysis of variation (ANOVA) approach (34). Bias was evaluated simultaneously with precision and tolerance for both was 20%. The calibration model was established using five independent runs using ten non-zero calibrators (0.5, 1, 2, 5, 25, 75, 150, 250, 350 and 500 ng/mL). The optimum calibration model was established using residual plot analysis. Significance testing and the coefficient of determination (R^2) were also considered.

Interferences from the isotopically labeled IS, the biological matrix and other drugs were also evaluated. Ion contributions from the IS were evaluated by fortifying drug-free urine with internal standard (25 ng/mL) and matrix interferences were evaluated in duplicate using ten independently sourced drug-free urine samples extracted in the absence of IS. Drug interferences were evaluated using a total of sixty-six analytes (**Table 6.3**). Both negative and positive controls were used. The negative control consisted of urine fortified with internal standard (25 ng/mL) and interferent (500 ng/mL). Positive controls contained internal standard (25 ng/mL) and desomorphine (5, 50 or 500 ng/mL) in the presence of interferent at equivalent, ten-fold and 100-fold concentration (relative to desomorphine).

Table 6.3

Summary of compounds used in interference study (n=66).

Group	Compounds
Common Drugs	7-aminoclonazepam, 7-aminoflunitrazepam, acetaminophen, alprazolam, amitriptyline, amphetamine, amobarbital, butalbital, caffeine, carbamazepine, carisoprodol, clonazepam, cocaine, cotinine, cyclobenzaprine, dextromethorphan, diazepam, fluoxetine, flurazepam, gabapentin, ketamine, MDMA, meprobamate, methaqualone, 11-nor-9-carboxy-Δ9-THC, nordiazepam, oxazepam, pentobarbital, phencyclidine, phenobarbital, phenytoin, pseudoephedrine, salicylic acid, secobarbital, sertraline, temazepam, Δ9-THC, trazodone, valproic acid, zaleplon, zolpidem, zopiclone
Opioids	6-acetylcodeine, 6-acetylmorphine, buprenorphine, codeine, dihydrocodeine, fentanyl, heroin, hydrocodone, hydromorphone, levorphanol, meperidine, methadone, morphine, nalorphine, norcodeine, norhydrocodone, normeperidine, normorphine, noroxycodone, oxycodone, oxymorphone, propoxyphene, thebaine, tramadol

MDMA, methylenedioxymethamphetamine; THC, tetrahydrocannabinol.

Matrix effects were examined at low and high concentrations (20 and 400 ng/mL). Ten drug-free urine samples from independent sources were extracted in the absence of desomorphine and were fortified with drug post-extraction. Ion suppression or enhancement was calculated by comparing the mean peak areas of desomorphine in urine with the equivalent concentration in mobile phase. Carryover was assessed using both blank matrix and negative controls analyzed immediately following the highest calibrator (500 ng/mL). Carryover was present if a blank or negative control produced a detectable result (above the LOD). Processed sample stability was evaluated at 24 hours after storage in the autosampler (4°C) at three concentrations (2, 250 and 400 ng/mL) in triplicate. Quantitative results and absolute response (peak areas) at 0 and 24 hours were compared.

Results and discussion

The SPE was previously optimized to maximize the extraction efficiency (31). An elevated concentration of ammonium hydroxide was necessary due to the high pKa of the basic nitrogen on desomorphine (pKa 9.69) (35). Using this approach, extraction efficiencies of 90 ± 1% (n=3) were achieved for this zwitterionic drug (31). The limits of detection and quantitation were 0.5 ng/mL (n=18), the lowest concentration tested. The associated bias, precision and S/N ratios are summarized in **Table 6.4** and an extracted ion chromatogram at the LOQ is shown in **Figure 6.2**. Ions for MRM were selected based upon abundance and specificity. The quantification ion for desomorphine was *m/z* 167 and qualifier ions were *m/z* 195 (79%) and *m/z* 152 (86%). Relative ion abundances are shown in parentheses. Bias and precision were calculated at three concentrations (2, 250 and 400 ng/mL) extracted in triplicate over five days using one-way ANOVA. Intra-assay CVs were 2-3% and inter-assay CVs were 3-6%. The bias ranged from -1-2% (**Table 6.5)**.

Table 6.4

LC-MS/MS assay performance at the LOD and LOQ.

LOD (ng/mL)	**LOQ (ng/mL)**	**Mean ± SD (ng/mL) (n=18)**	**S/N Range (Mean) (n=18)**	**Bias Range (Mean) (n=18)**	**CV (n=18)**
0.5	0.5	0.52 ± 0.06	58-594:1 (238:1)	-19%-19% (11%)	2%

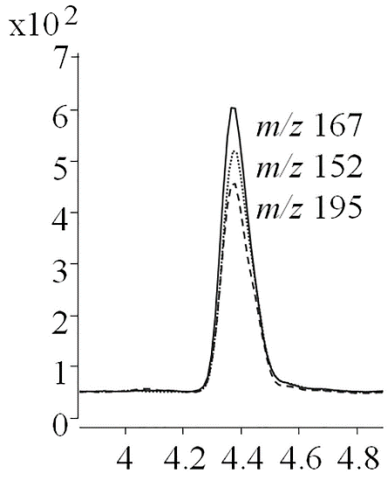

Figure 6.2. Extracted ion chromatograms of desomorphine in urine at the LOQ (0.5 ng/mL).

Table 6.5

Precision and bias (n=15) for desomorphine in urine at low (2 ng/mL), medium (250 ng/mL), and high (400 ng/mL) concentrations.

Concentration (ng/mL)	**Intra-assay Precision (n=3, CV)**	**Inter-assay Precision (n=15, CV)**	**Bias (n=15)**
2	2%	6%	-1%
250	3%	3%	1%
400	2%	3%	2%

A weighted (1/x) quadratic calibration model was selected as the calibration model for quantitative analyses (0.5 – 500 ng/mL). Although R^2 values were greater than 0.99 for all calibration models evaluated, standardized residual plots were optimal using a quadratic model. Although an *F*-test indicated there was a significant difference between unweighted and weighted (1/x) quadratic models, the weighted (1/x) quadratic calibration model produced the highest R^2 values and minimized bias at both the low and high ends of

the calibration. Although the only published report involving desomorphine was within the calibration range of the assay, urinary drug concentrations in authentic case samples could exceed this range. In those circumstances dilution integrity would need to be established.

Interferences from matrix, isotopically labeled IS and other drugs were examined. Ten independent drug-free urine samples were analyzed (n=2) and no inferences were observed. The deuterated analog also caused no inference. Sixty-six drugs, including twenty-four opioids were evaluated for potential interferences (**Table 6.3**). Qualitative and quantitative interferences were evaluated in the presence of 1-, 10- and 100-fold excess of the interfering substance. No qualitative or quantitative inference were observed for any of the drugs tested. Extracted ion chromatograms (EICs) of desomorphine and the closest eluting opioids are shown in **Figure 6.3**. While baseline separation was not achieved between desomorphine (*m/z* 272, MH^+) and norhydrocodone (*m/z* 286, MH^+), mass selectivity and the use of isotopically labelled internal standard prevented any interference, even in the presence of a 100-fold excess of the interferent.

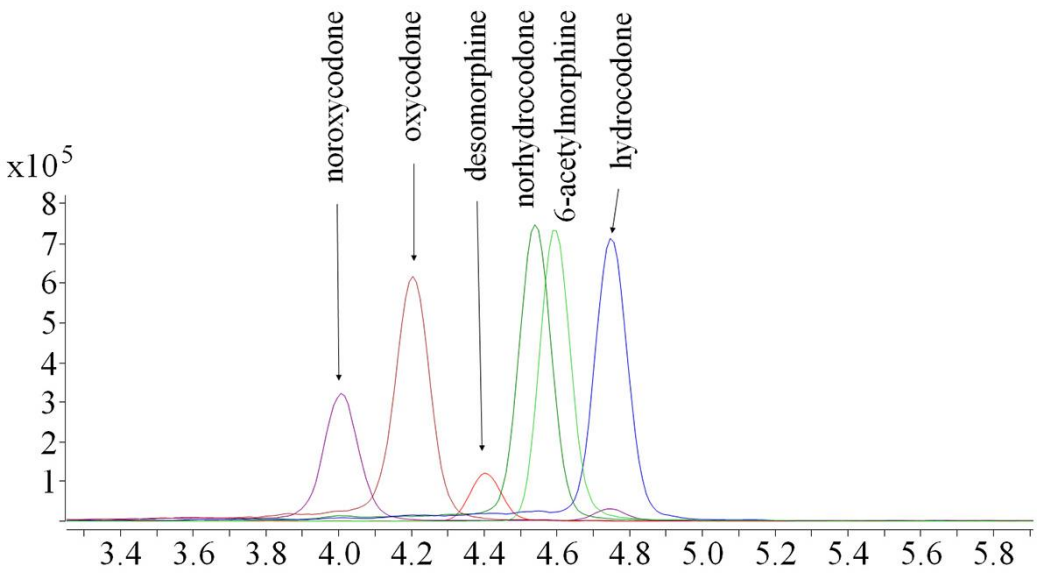

Figure 6.3. Extracted ion chromatogram depicting desomorphine (*m/z* 272) and closely eluting opioids (noroxycodone, *m/z* 302; oxycodone, *m/z* 316; norhydrocodone, *m/z* 286; 6-acetylmorphine, *m/z* 328; hydrocodone, *m/z* 300) at a 10-fold excess concentration (relative to desomorphine). Data was acquired in full scan in order to identify the elution order.

The potential for ion suppression or enhancement was investigated using ten drug-free sources of urine with desomorphine added post-extraction at low and high concentrations (20 and 400 ng/mL). The average ion suppression was -20% at 20 ng/mL (n=10) and -10% at 400 ng/mL (n=10), with CVs of 16% and 7% respectively (**Table 6.6**). In addition to matrix intereferences, the use of an isotopically labelled internal standard also minimizes the possibility of decreased ionization efficiency caused by coeluting drugs.

Table 6.6

Matrix effect and associated bias and precision of desomorphine in urine (20 and 400 ng/mL; n=10).

Concentration (ng/mL)	Matrix Effect Mean ± SD	Matrix Effect CV (n=10)	Calculated Concentration Mean ± SD (ng/mL)	Bias (n=10)	CV (n=10)
20	-20 ± 13%	16%	22.16 ± 0.54	11%	2%
400	-10 ± 7%	7%	402.29 ± 3.97	1%	1%

Carryover was evaluated using blank matrix and negative control samples after injection of the highest calibrator (500 ng/mL). No carryover was observed. Processed sample stability was evaluated following 24 hours storage in the autosampler tray (4°C). Three concentrations (2, 250 and 400 ng/mL) were analyzed in triplicate at 0 and 24 hours and the average response for each concentration compared. At 24 hours, absolute peak areas for desomorphine and the IS were within 2-3% of the original response, and bias was within 0.5-1.2% for all concentrations tested.

Conclusions

Drug users seeking medical treatment continue to self-report Krokodil use, but its prevalence is difficult to estimate due to a lack of analytically confirmed cases. No authentic specimens from Krokodil users were available for this study. Although abuse of the drug appears to be highly geographical, its analgesic potency is approximately ten-fold greater than morphine and intravenous use has been associated with severe and life-threatening consequences. We describe the use of solid phase extraction to isolate desomorphine from urine with high analytical recovery (90%). Using LC-MS/MS,

desomorphine was identified at sub-ng/mL concentrations, facilitating identification of the drug within the range of forensic interest.

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CHAPTER VII

QUANTITATIVE ANALYSIS OF DESOMORPHINE IN URINE USING SOLID PHASE EXTRACTION AND LIQUID CHROMATOGRAPHY-QUADRUPOLE/TIME OF FLIGHT-MASS SPECTROMETRY[1]

This dissertation follows the style and format of *The Journal of Analytical Toxicology*.

[1]Winborn, J., Basiliere, S., Kerrigan, S. (2018) Quantitative Analysis of Desomorphine in Urine Using Solid Phase Extraction and Liquid Chromatography-Quadrupole/Time of Flight-Mass Spectrometry. *Forensic Toxicology*, in review
Submitted for publication in Forensic Toxicology

Abstract

The semi-synthetic opioid desomorphine is a primary component of Krokodil, the use of which has been associated with severe dermatological side effects. Confirmatory toxicological test results for desomorphine are scarce despite reports associated with its use, which may in part be attributed to delays seeking medical treatment, geographical trends and limited published methodology to detect its presence in biological matrices. To assist with identification efforts, a sensitive method to detect desomorphine in urine was developed using solid phase extraction (SPE) and liquid chromatography-quadrupole/time of flight-mass spectrometry (LC-Q/TOF-MS). A deuterated analog was used as the internal standard and assay performance was assessed in accordance with published guidelines. This is the first report to describe the use of LC-Q/TOF-MS for the analysis of desomorphine in a biological matrix. SPE yielded an extraction efficiency of 90% for urine. The limits of detection and quantitation were 0.5 ng/mL and the associated calibration range was 0.5-500 ng/mL. Ion suppression averaged -1% at 20 ng/mL and -7% at 400 ng/mL, with associated CVs of 11% and 2%, respectively. No qualitative or quantitative interferences were observed for common drugs, including twenty-four opioids and structurally related compounds. Intra- and inter-assay CVs were 3-5% (n=3) and 4-7% (n=15) respectively, and bias ranged from -4-0% (n=15) at 2, 200 and 400 ng/mL. The metabolism of desomorphine has been reported but reference materials are not yet available, preventing the inclusion of metabolites in quantitative assays at this time. In the absence of authentic samples from Krokodil users, recombinant enzymes were used to generate desomorphine metabolites for analysis. Ten phase I and phase II metabolites were identified, including a second norhydroxydesomorphine isomer not previously reported.

The LC-Q/TOF-MS method is capable of detecting both desomorphine and its phase I and phase II metabolites which should improve identification efforts in forensic and clinical toxicology laboratories.

Keywords: Desomorphine, Krokodil, Urine, LC-Q/TOF-MS

Quantitative Analysis of Desomorphine in Urine Using Solid Phase Extraction and Liquid Chromatography-Quadrupole/Time of Flight-Mass Spectrometry

Introduction

The semi-synthetic opioid desomorphine ((5α,6α)-17-methyl-4,5-epoxymorphinan-3-ol) is a constituent of the drug Krokodil which has been abused, primarily in Russia and surrounding countries since the early 2000s (1). First synthesized in the 1930s, desomorphine is an amphoteric base and zwitterionic species. Like morphine, it contains a basic nitrogen in addition to the phenol. The key structural differences between desomorphine and morphine are the absence of the C6 hydroxyl and the saturated bond between C7 and C8 (**Figure 7.1**). Desomorphine was briefly sold under the trade name Permonid in Switzerland from 1940 to 1951 (1). While desomorphine is up to ten times more potent than morphine with a faster onset of action, its duration of effect is shorter (2). This coupled with concerns regarding its abuse liability led to discontinuation of its therapeutic use. In the United States, desomorphine is a Schedule I drug in the Federal Controlled Substances Act (3). Internationally, desomorphine is restricted under the 1961 United Nations Single Convention on Narcotics Drugs, which was attended by over seventy countries (4). Several countries have legislation restricting desomorphine use, including the Republic of Armenia, Australia, Brazil, Germany, Russia and the United Kingdom (5-7).

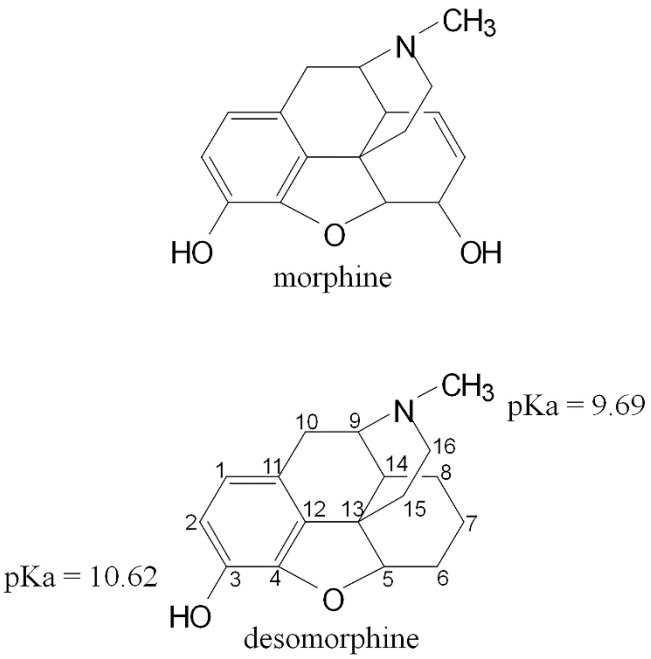

Figure 7.1. Chemical structures of morphine and desomorphine.

Krokodil is commonly synthesized from pharmaceuticals containing codeine, using harsh chemicals such as hydroiodic acid and red phosphorous (8). The final product is typically a liquid, of which desomorphine is a major component, with other morphinans also produced as side products (9, 10). The first reports of Krokodil use appeared in 2003 in Russia. Since then its use has been reported through neighboring countries into central Europe and the Unites States. Some reports suggest that at one point as many as 100,000 people were using Krokodil in Russia and 20,000 in Ukraine (8). Its popularity was attributed to a reduction in heroin availability due to a disease among poppy crops (11). Similar to heroin, Krokodil is commonly injected intravenously. As such, remnants of the harsh precursor chemicals can enter the blood stream, bypassing the body's natural safeguards. Necrotic ulcers, gangrene and osteonecrosis have all been associated with Krokodil use, which may necessitate amputations or other invasive surgeries (12).

Despite reports of Krokodil use in the media, published case reports are rare, with the first originating from Germany in 2013 (13). The case involved a fatality of a Krokodil user, but post-mortem toxicological analysis for desomorphine was negative. Clinical case reports have since emerged from the Republic of Armenia (14-16), the Republic of Georgia (17), Italy (18), Poland (19), Spain (20), Russia (21, 22), the United Kingdom (23) and the United States (24-28). Three of these involve fatalities (13, 18, 27) but the vast majority describe clinical and emergency department related reports. To date only one case has been analytically confirmed, involving a fatality of a 39 year old male with desomorphine detected in post-mortem urine (270 ng/mL) (18). Published case reports suggest that there is often a delay between time of drug use and seeking medical treatment, which may in part explain the lack of analytically confirmed reports. Limitations in methodology may also contribute to the lack of analytically confirmed forensic cases.

Forensic toxicology screening is commonly accomplished using enzyme-linked immunosorbent assays (ELISAs) because they are compatible with multiple biological matrices. Immunoassay-based screening techniques are often directed towards one target analyte but may be used to identify a class of compounds due to non-specific binding with structurally similar compounds. A recent study evaluated commercially available opioid ELISAs for cross-reactivity with desomorphine, which ranged from <2.5% to 77% (29). As a consequence, some ELISAs are unlikely to identify desomorphine in forensic toxicology specimens.

Pharmcokinetic studies of desomorphine in humans are limited to date. The first study on desomorphine metabolism was published by Richter in 2016 (30). Utilizing rat models, human liver microsomes, recombinant cytochrome P450 isoenzymes (rCYPs) and

recombinant uridine 5'-diphospho-glucuronosyltranferase isoenzymes (rUGTs), phase I biotransformations included *N*-demethylation, hydroxylation and *N*-oxidation, while phase II metabolism involved glucuronidation and sulfation. A subsequent investigation utilizing recombinant CYPs found that seven isoenzymes (CYP2B6, CYP2C8, CYP2C9, CYP2C18, CYP2C19, CYP2D6 and CYP3A4) may contribute to the formation of phase I metabolites (nordesomorphine, desomorphine-*N*-oxide, five hydroxydesomorphine isomers and norhydroxydesomorphine) (31). The same study also investigated recombinant UGTs and found that nine rUGTs (UGT1A1, UGT1A3, UGT1A8, UGT1A9, UGT1A10, UGT2B4, UGT2B7, UGT2B15 and UGT2B17) may contribute to the formation of desomorphine-glucuronide. While metabolites can be valid markers for drug use, the lack of commercially available reference materials precludes quantitative analysis at this time.

Published methods describing the analysis of desomorphine in biological matrices are limited, and some lack the requisite sensitivity to be effective in toxicological investigations. While gas chromatography-mass spectrometry (GC-MS) has traditionally been the most widespread technique for confirmatory testing in toxicology laboratories, liquid chromatography (LC) based approaches are becoming increasingly widespread due to an improved sensitivity and versatility with respect to polar compounds and metabolites (32). GC-MS was used by Su to identify desomorphine in urine with a limit of quantitation of 250 ng/g (33) and by Alves to identify desomorphine in blood with a limit of quantitation of 103 ng/mL (34). More recently, the authors published a more sensitive GC-MS assay for blood and urine, with limits of quantitation of 5 and 8 ng/mL, respectively (35).

The qualitative identification of desomorphine in urine and seized drug samples utilizing LC with ultraviolet detection was described by Savchuk (7) and Richter described a qualitative assay utilizing high resolution mass spectrometry (HRMS) using an Orbitrap™ detector to identify desomorphine and its metabolites in urine (30). Desomorphine was qualitatively identified in drug samples by Soares, utilizing both LC coupled to a diode array detector (DAD) and LC-HRMS (10). Quantitatively, desomorphine has been identified in drug samples with a limit of quantitation of 490 ng/mL using LC-DAD (9). Eckart described a quantitative LC method utilizing tandem mass spectrometry (MS/MS) to identify desomorphine in serum, plasma and tissue with a limit of quantitation of 0.1 ng/mL (36). Previously published methods have used phenacetin and deuterated codeine as internal standards. We previously described a validated quantitative LC-MS/MS assay to detect desomorphine in urine with a limit of quantitation of 0.5 ng/mL, which was the first LC-MS method to utilize a deuterated desomorphine analogue as the internal standard. (37). LC-based methods for both seized drugs and biological matrices are summarized in **Table 7.1**. The purpose of this study was to develop a method to quantitate desomorphine in urine using LC-Q/TOF-MS, validate the method in accordance with published recommendations (38) and utilize that method to analyze desomorphine metabolites generated *in vitro*, in the absence of authentic specimens.

Table 7.1

Summary of LC-based analytical methods targeting desomorphine in seized drugs and biological matrices.

Matrix	LOQ	Sample Volume	Extraction Method	Internal Standard	Detector	Reference
urine	0.5 ng/mL	500 µL	SPE	desomorphine-D_3	MS/MS	(37)
drug samples	490 ng/mL	100 µL	LLE	phenacetin	DAD	(9)
serum; plasma; tissue	0.1 ng/mL	200 µL or 2 g	SPE	codeine-D_6	MS/MS	(36)
drug samples	NR	10 µL	None	NR	DAD; Orbitrap™	(10)
urine	NR	2 mL	LLE	codeine-D_6	Orbitrap™	(30)
urine; drug samples	NR	3 mL	LLE	NR	UV	(7)

DAD, Diode array detector; LLE, Liquid-liquid extraction; NR, Not reported; SPE, Solid phase extraction; UV, Ultraviolet detector.

Materials and methods

Chemicals

Desomorphine, desomorphine-D_3 and compounds used to assess interferences (**Table 7.2**) were obtained from Cerilliant (Round Rock, TX, USA). Ethyl acetate (HPLC grade), dibasic and monobasic potassium phosphate were obtained from Fisher Scientific (Fair Lawn, NJ, USA). Acetonitrile (LCMS grade) was purchased from Thermo Fisher Scientific (Radenor, PA, USA) and formic acid (>95%) was from Sigma Aldrich (St. Louis, MO, USA). Concentrated ammonium hydroxide was obtained from Macron Fine Chemicals (Center Valley, MA, USA). Dichloromethane and isopropyl alcohol were procured from Mallinckrodt Chemicals (St. Louis, MO, USA). Concentrated hydrochloric acid and methanol (LCMS grade) were from J.T. Baker (Center Valley, MA, USA). Pooled drug free urine obtained from Utak Laboratories (Valencia, CA, USA) was preserved with 1% sodium fluoride prior to use. PolyChrom Clin II 3cc (35 mg) solid phase extraction (SPE) columns were obtained from SPEware (Baldwin, CA, USA). Deionized (DI) water was generated from a Direct-Q 3 (UV) system (Millipore, Billerica, MA, USA). Recombinant human cytochrome P450 isoenzyme 3A4 (rCYP3A4) expressed in E. coli (bactosomes) were purchased from Xenotech, LLC (Kansas City, Kansas). Recombinant human uridine 5'-diphospho-glucuronosyltransferase isoenzyme 2B7 (rUGT2B7) isoenzymes expressed in baculovirus infected insect cells (supersomes™) were purchased from Corning (Glendale, Arizona). UGT reaction mix solution A (25 mM UDP-glucuronic acid), UGT reaction mix solution B (250 mM Tris(hydroxymethyl)aminomethane-HCl (Tris-HCl), 40 mM magnesium chloride, and 0.125 mg/mL alamethicin), reduced nicotinamide adenosine di-phosphate (NADPH) regenerating system solution A (40 U/mL

glucose-6-phosphate dehydrogenase in 5 mM sodium citrate), and NADPH regenerating system solution B (26 mM NADP+, 66 mM glucose-6-phosphate and 66 mM magnesium chloride in aqueous solution) were also obtained from Corning.

Table 7.2

Drugs used to assess interference, divided into opioids (n=24) and common drugs (n=42).

Opioids	Common Drugs
6-acetylcodeine, 6-acetylmorphine, buprenorphine, codeine, dihydrocodeine, fentanyl, heroin, hydrocodone, hydromorphone, levorphanol, meperidine, methadone, morphine, nalorphine, norcodeine, norhydrocodone, normeperidine, normorphine, noroxycodone, oxycodone, oxymorphone, propoxyphene, thebaine, tramadol	7-aminoclonazepam, 7-aminoflunitrazepam, acetaminophen, alprazolam, amitriptyline, amobarbital, amphetamine, butalbital, caffeine, carbamazepine, carisoprodol, clonazepam, cocaine, cotinine, cyclobenzaprine, dextromethorphan, diazepam, fluoxetine, flurazepam, gabapentin, ketamine, MDMA, meprobamate, methaqualone, 11-nor-9-carboxy-Δ9-THC, nordiazepam, oxazepam, pentobarbital, phencyclidine, phenobarbital, phenytoin, pseudoephedrine, salicylic acid, secobarbital, sertraline, temazepam, Δ9-THC , trazadone, valproic acid, zaleplon, zolpidem, zopiclone

MDMA, methylenedioxymethamphetamine; THC, tetrahydrocannabinol.

Sample preparation

Working standards of desomorphine were prepared at three concentrations (25, 2.5 and 0.25 µg/mL) in acetonitrile and the internal standard was prepared at 0.5 µg/mL in acetonitrile. The elution solvent was prepared daily and consisted of 4% ammonium hydroxide in ethyl acetate. A Supelco Visiprep vacuum manifold (Supelco, Bellefonte, PA, USA) and a Turbovap LV concentration workstation (Biotage, Charlotte, NC, USA) were used for extraction and evaporation.

Aliquots of urine (0.5 mL) were fortified with 50 µL of IS solution to achieve a final concentration of 25 ng/mL. Samples were diluted with 1 mL of 0.1 M hydrochloric

acid then transferred to PolyChrom Clin II SPE columns (3 cc, 35 mg) and allowed to flow through under gravity (approximately 1 mL/min). When necessary, vacuum was applied to maintain the flow rate. Samples were successively washed with 1 mL of deionized water, 0.1 M hydrochloric acid, methanol, and ethyl acetate. After the final wash, samples were dried for five minutes on full vacuum. Desomorphine was eluted using two 0.5 mL volumes of elution solvent. Extracts were evaporated to dryness under nitrogen (50°C) before being reconstituted in 30 µL of mobile phase (92:8 A:B). Samples were injected (2 µL) onto the LC-Q/TOF-MS for analysis.

LC-Q/TOF-MS analysis

An Agilent Technologies 6530 LC-Q/TOF-MS equipped with an Agilent 1290 Infinity autosampler was used to analyze samples (Agilent Technologies, Santa Clara, CA, USA). Data was acquired and analyzed using Agilent MassHunter software. An Agilent Poroshell 120 EC-C18 column (2.1 x 100 mm, 2.7 µm particle size) equipped with a matching Poroshell 120 EC-C18 guard column was for separation. The autosampler chamber and column compartment were maintained at 4°C and 35°C, respectively. Mobile phase A consisted of 0.1% formic acid in deionized water and mobile phase B consisted of 0.1% formic acid in acetonitrile. The following elution profile was utilized: 8% B, 0-2 min (0.3 mL/min); 8-20% B, 2-6 min (0.3 mL/min); 20-90% B, 6-6.5 min (0.4 mL/min); 90-8% B, 6.5-7 min (0.4 mL/min), followed by a re-equilibration period of 2 min. Nitrogen was generated using a Genius 3040 nitrogen generator (Peak Scientific, Billerica, MA, USA). An electrospray ionization source with Jet Stream technology was used in positive mode and Q/TOF-MS conditions were as follows: drying gas temperature, 350°C (10 L/min); sheath gas temperature, 400°C (10 L/min); nebulizer, 20 psi; capillary voltage,

2500 V; nozzle voltage, 0 V; fragmentor, 150 V; skimmer, 65 V. Data was acquired using targeted MS/MS acquisition with a mass range of 100-1000 Da, with MS and MS/MS scan rates of 5 spectra/s. Precursor and product ions, collision energies and retention time for desomorphine and desomorphine-D_3 are summarized in **Table 7.3**.

Table 7.3

Transition ions, collision energy (CE) and retention time for desomorphine and desomorphine-D_3. Ion ratios are shown in parentheses and quantitation ions are shown in bold.

Compound	Precursor Ion (Exact Mass)	Product Ions (Exact Mass)	CE (V)	Retention Time (min)
desomorphine	272.1645	**167.0855 (100%)** 195.0804 (35%) 152.0621 (43%)	45	4.4
desomorphine-D_3	275.1833	**167.0855 (100%)** 195.0804 (36%) 152.0621 (39%)	45	4.4

Validation procedure

Assay performance was evaluated in terms of extraction efficiency, calibration model, bias, precision, limit of detection (LOD), limit of quantification (LOQ), interferences, matrix effects, carryover and processed sample stability.

The extraction efficiency in urine was determined previously by direct comparison of the relative peak area (drug/IS) for extracted samples (n=3) with the mean relative peak area for non-extracted samples (n=3) (35). Extracted samples contained IS (25 ng/mL) and desomorphine (250 ng/mL) and non-extracted samples contained IS only. Non-extracted samples were fortified post-extraction with an equivalent amount of desomorphine. The calibration model was determined with five independent runs using ten non-zero calibrators

(0.5, 1, 2, 5, 25, 75, 150, 250, 350 and 500 ng/mL). Calibration models were evaluated based on performance, the coefficient of determination (R^2) and standardized residual plots. Carryover was evaluated using negative controls (IS only) and blank matrix (no IS) immediately following injection of the highest calibrator (500 ng/mL, n=3). Carryover was present when a reportable result was produced: S/N ratio of 3:1 or greater, retention time ± 2% of the standard and ion ratios within ±20% of the established value).

Bias and precision were determined simultaneously over five runs in triplicate using pooled fortified matrix at three concentrations (2, 250 and 450 ng/mL). The tolerance was ±20% for both bias and precision. Within-run precision was calculated for each concentration (n=3) over each run and between-run precision was calculated for each concentration over all five runs (n=15). Limits of detection and quantitation (LOD and LOQ) were determined using three sources of drug-free matrix, analyzed in duplicate over three days. The LOD was the lowest concentration to produce a reportable result, with a signal-to-noise ratio(S/N) ≥3:1, ion ratios ± 20%, and retention time ± 2% of the standard. The LOQ was the lowest concentration to produce a reportable result with quantitative values within 20% of the expected concentration, a signal-to-noise (S/N) ≥10:1 and acceptable bias and precision.

The biological matrix and isotopically labeled IS were evaluated for potential interferences. Ten drug-free urine samples from independent sources were extracted, in duplicate, in the absence of IS to evaluate matrix interference. Ion contribution from the stable isotope IS was evaluated by fortifying drug-free urine with IS (25 ng/mL) and monitoring the signal of desomorphine. Drug interferences were evaluated for sixty-six drugs including twenty-four opioids (**Table 7.2**). Interferences were assessed qualitatively

and quantitatively using negative and positive controls. The positive controls contained desomorphine at one of three concentrations (5, 50 or 500 ng/mL) and were fortified with IS (25 ng/mL) and interferent (500 ng/mL). The negative control was fortified with internal standard (25 ng/mL) and interferent (500 ng/mL).

Processed sample stability was evaluated at three concentrations (2, 250 and 400 ng/mL) at 0 and 24 hours. Samples were stored in the autosampler (4°C) and analyzed in triplicate. The average quantitative response and average peak area of desomorphine at 0 and 24 hours were compared for all three concentrations. Matrix effects were quantitatively assessed using post-extraction addition at two concentrations (20 and 400 ng/mL) using ten drug free matrixes. Ion suppression or enhancement was calculated by comparing the mean peak areas of desomorphine in matrix with the equivalent concentration of the drug in mobile phase (no matrix).

Analysis of desomorphine metabolites

Due to the absence of metabolite standards or authentic urine samples, desomorphine metabolites were generated *in vitro* using recombinant enzymes (rCYP3A4 and rUGT2B7) as described previously (31). Reactions were performed in duplicate at 37°C in the presence of 200 µM desomorphine. The rCYP3A4 reaction contained 50 pmol/mL rCYP isoenzyme, 100 mM potassium phosphate buffer (pH 7.4), 1.3 mM $NADP^+$, 3.3 mM glucose-6-phosphate, 3.3 mM magnesium citrate, and 0.4 U/mL glucose-6-phosphate dehydrogenase. The rUGT2B7 reaction contained 0.25 mg/mL rUGT isoenzyme, 90 mM Tris-HCl (pH 7.5), 8 mM magnesium chloride, 25 µg/mL alamethicin, and 2 mM uridine 5'-diphospho-glucuronic acid (UDPGA). Both reactions were terminated after 2 hours using an equal volume of ice cold acetonitrile containing 0.1%

formic acid and 5 µM internal standard. The samples were diluted 1:1 in 92:8 (A:B) mobile phase and 2 µL was injected onto the LC-Q/TOF-MS and analyzed using the parameters and elution gradient described earlier for LC-Q/TOF-MS analysis.

Results and discussion

Method validation

The SPE extraction was previously optimized to minimize the loss of desomorphine during the extraction process (35). Extraction efficiencies of 90% were achieved using 4% ammonium hydroxide in ethyl acetate. The increased concentration of ammonium hydroxide may in part be due to the high pKa of the tertiary amine (9.69) (39). Both LOD and LOQ were 0.5 ng/mL (n=18). **Table 7.4** summarizes the associated bias, precision and S/N ratios at the LOQ and **Figure 7.2** depicts the extracted ion chromatogram (EIC) of desomorphine at 0.5 ng/mL in urine. Precision at 2, 250 and 400 ng/mL produced intra- and inter-assay CVs of 3-5% (n=3) and 4-7% (n-15) respectively. Bias ranged from -4-0% (n=15) (**Table 7.5**). **Figure 7.3** shows the MS^2 spectrum of desomorphine with the quantitative and qualifier ions identified. Quantitate and qualitative ions were selected based upon abundance and specificity. Optimum CID voltages produced qualifier ion ratios of 35% and 43% for *m/z* 195.0855 and *m/z* 152.0621.

Table 7.4

Limits of detection and quantitation of desomorphine in urine.

LOD (ng/mL)	LOQ (ng/mL)	Mean ± SD (ng/mL; n=18)	S/N Range (Mean; n=18)	Bias Range (Mean; n=18)	CV (n=18)
0.5	0.5	0.53 ± 0.05	10:1 - 21:1 (16:1)	-11 - 20% (5.6%)	9.1%

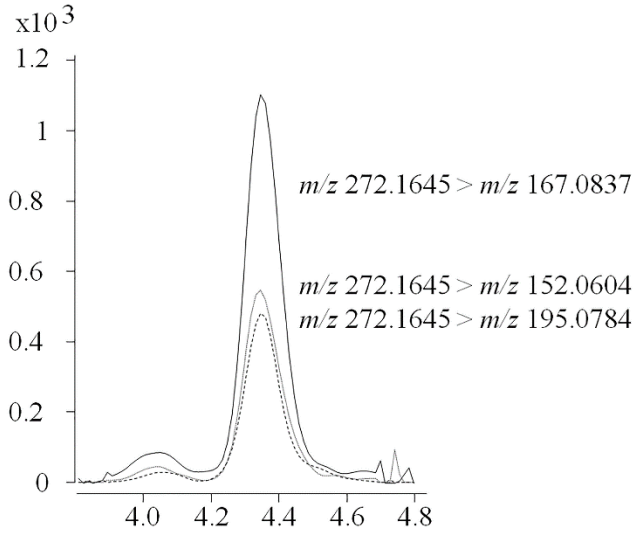

Figure 7.2. Extracted ion chromatogram of desomorphine in urine at the LOQ (0.5 ng/mL).

Table 7.5

Bias and precision (n=15) for desomorphine in urine at 2, 250 and 400 ng/mL.

Concentration (ng/mL)	Intra-assay Precision (CV; n=3)	Inter-assay Precision (CV; n=15)	%Bias (n=15)
2	3%	4%	-4%
250	4%	4%	0%
400	5%	7%	-1%

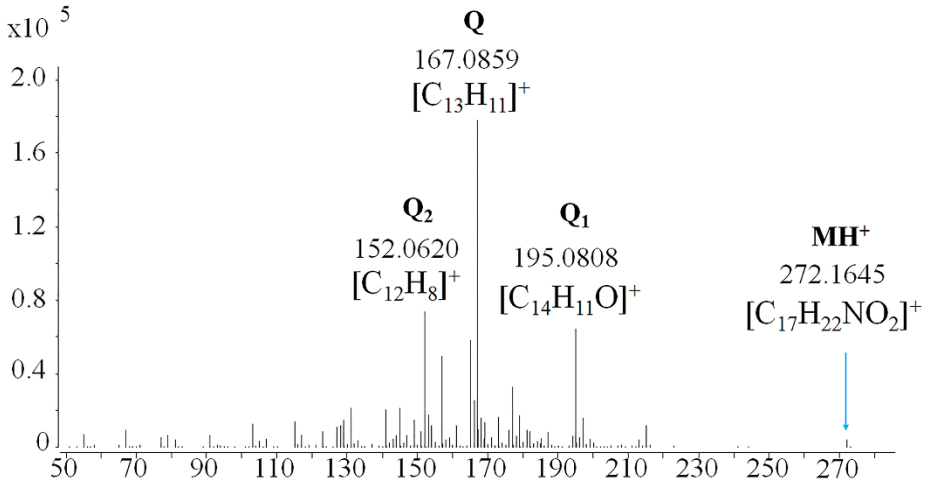

Figure 7.3. MS^2 spectrum with chemical formulas and accurate masses of the precursor (MH^+), quantitation (Q) and qualifier ions (Q_1 and Q_2).

No carryover was observed under the conditions tested and processed samples were stable for 24 hours, with absolute peak areas within 0-5% of the original result (t=0) and the bias of the quantitative results ranging from -3 to 2% (n=3). The calibration model selected for quantitative analysis (0.5 – 500 ng/mL) was a weighted (1/x) quadratic calibration model, which produced the highest R^2 values and minimized bias at the low and high ends of the calibration. While an *F*-test indicated no significant difference between unweighted and weighted (1/x) models, nor between linear and quadratic models, a weighted (1/x) quadratic model was selected based upon the residual plot analysis (**Figure 7.4**). No interferences from either the matrix or isotopically labeled internal standard were present. No qualitative or quantitative interference were observed with any of the sixty-six drugs tested at 1-, 10- and 100-fold excess (relative to desomorphine) (**Table 7.2**). Ion suppression and enhancement were assessed using ten drug-free sources of urine fortified with desomorphine post-extraction at two concentrations (20 and 400 ng/mL). The mean

ion suppression was -1% and -7% at 20 and 400 ng/mL, with associated CVs of 11 and 2%, respectively (**Table 7.6).**

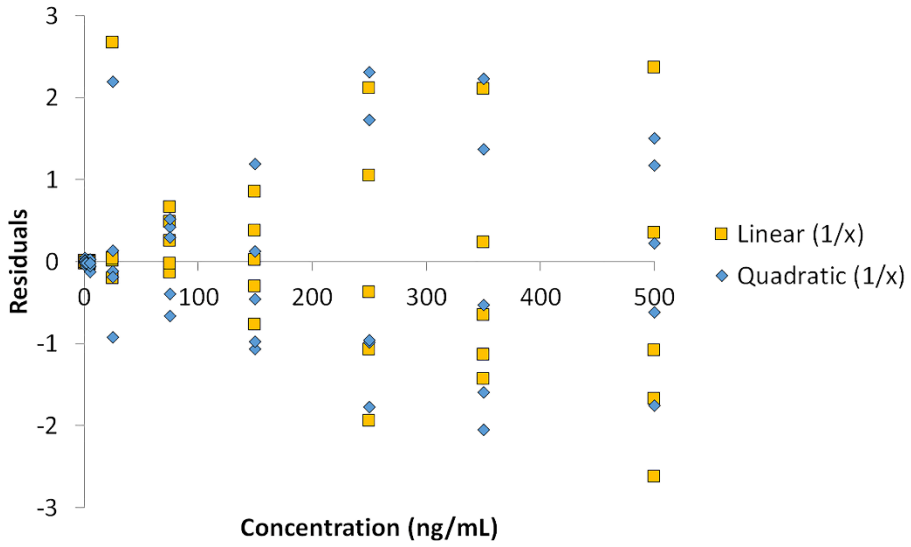

Figure 7.4. Standardized residual plot for linear and quadratic weighted (1/x) calibration models. Calibrator concentrations: 0.5, 1, 2, 5, 25, 75, 150, 250, 350 and 500 ng/mL.

Table 7.6

Matrix effect and associated bias for desomorphine in urine.

Concentration (ng/mL)	**Matrix Effect Range (Mean; n=10)**	**Matrix Effect CV (n=10)**	**Calculated Concentration Mean ± SD (ng/mL; n=10)**	**Bias Range (Mean; n=10)**	**CV (n=10)**
20	-22 - 11% (-1%)	11%	18.51 ± 0.54	-12 - -4% (-8%)	3%
400	-22 - 2% (-7%)	2%	362.10 ± 15.30	-14 - -3% (-10%)	4%

Analysis of desomorphine metabolites

Although previous studies have identified potential desomorphine metabolites, reference materials are not yet commercially available (30, 31). In the absence of authentic urine samples, desomorphine metabolites generated in vitro were also identified using the targeted LC-Q/TOF-MS method described here. EICs of the metabolites are shown in **Figure 7.5**, where in addition to the nine metabolites identified in our previous study (31), a second norhydroxydesomorphine isomer (RT 2.49 min) was also identified. The identification of the second norhydroxydesomorphine isomer may be attributed to the different mobile phase gradient utilized in the validated method, which was further optimized to provide baseline separation between desomorphine and closely eluting opioids (37). The samples containing phase I metabolites were reanalyzed in full scan mode and MS^2 spectra were generated. Mass spectra for both norhydroxydesomorphine metabolites (CID voltage 35) are depicted in **Figure 7.6**. Structural assignments and associated mass accuracy for all identified desomorphine metabolites are shown in **Table 7.7.**

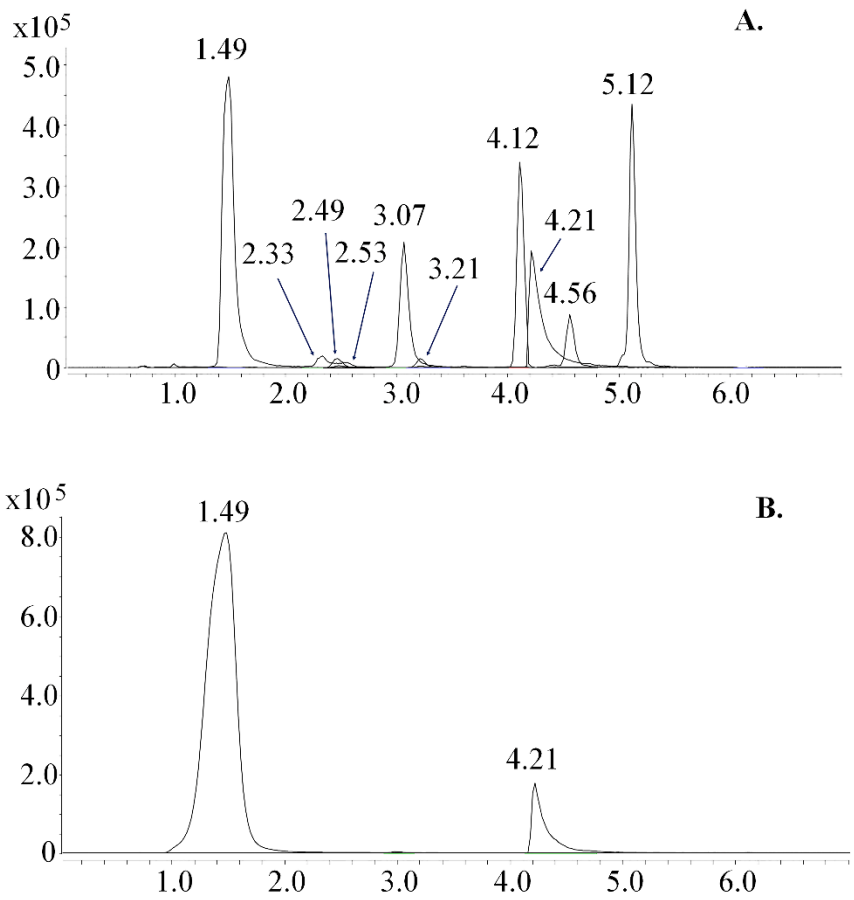

Figure 7.5. Extracted ion chromatograms for phase I metabolites (A): nordesomorphine, 4.12 min (*m/z* 258); desomorphine-*N*-oxide, 5.12 min, and hydroxydesomorphine, 1.49, 2.33, 2.53, 3.07 and 4.56 min (*m/z* 288); norhydroxydesomorphine, 2.49 and 3.21 min (*m/z* 274), and the phase II metabolite (B) desomorphine-glucuronide, 1.49 min (*m/z* 448). Desomorphine-D_3 (4.21 min, *m/z* 275) is shown for comparison.

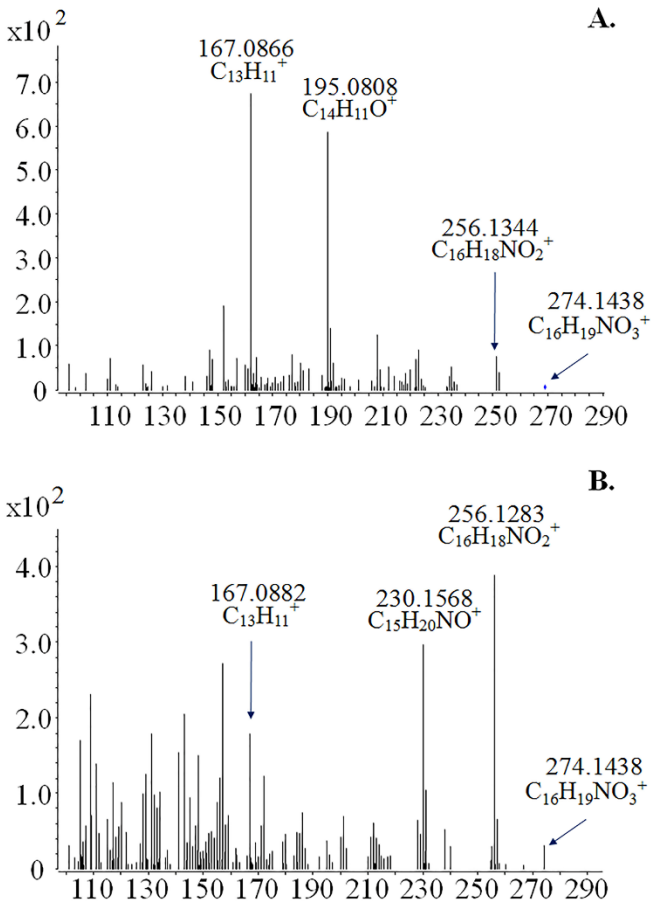

Figure 7.6. MS^2 spectra of (A) norhydroxydesomorphine isomer 1 and (B) norhydroxydesomorphine isomer 2. Data acquired in full scan.

Table 7.7

Retention time (RT), chemical formula, exact mass, accurate mass and mass error (PPM) for desomorphine and its metabolites. Data acquired in full scan. Structural assignments for desomorphine-glucuronide, nordesomorphine, hydroxydesomorphine isomers 1-5, desomorphine-*N*-oxide and norhydroxydesomorphine isomer 2 were reported in a previous study (31).

Metabolite	RT	Chemical Formula	Exact Mass	Accurate Mass	PPM
desomorphine-glucuronide	1.49	$C_{23}H_{29}NO_8^+$	448.1966	448.1962	0.80
		$C_{17}H_{22}NO_2^+$	272.1645	272.1643	0.66
		$C_{14}H_{15}O_2^+$	215.1067	215.1063	1.67
nordesomorphine	4.12	$C_{16}H_{20}NO_2^+$	258.1489	258.1482	1.75
		$C_{14}H_{11}O^+$	195.0804	195.0807	1.08
		$C_{13}H_{11}^+$	167.0855	167.0856	0.53
hydroxydesomorphine isomer 1 (aliphatic)	1.49	$C_{17}H_{22}NO_3^+$	288.1594	288.1593	0.25
		$C_{14}H_{13}O_2^+$	213.0910	213.0907	1.40
		$C_{14}H_{11}O^+$	195.0804	195.0796	4.33
hydroxydesomorphine isomer 2 (aliphatic)	2.33	$C_{17}H_{22}NO_3^+$	288.1594	288.1595	0.38
		$C_{14}H_{15}O_3^+$	231.1016	231.1018	0.92
		$C_{14}H_{11}O_2^+$	211.0754	211.0748	2.67
hydroxydesomorphine isomer 3 (aliphatic)	2.53	$C_{17}H_{22}NO_3^+$	288.1594	288.1591	1.07
		$C_{14}H_{15}O_3^+$	231.1016	231.1010	2.33
		$C_{14}H_{13}O_2^+$	213.0910	213.0915	2.53
hydroxydesomorphine isomer 4 (aromatic)	3.07	$C_{17}H_{22}NO_3^+$	288.1594	288.1591	1.20
		$C_{16}H_{22}NO_2^+$	260.1645	260.1638	2.64
		$C_{16}H_{22}NO^+$	244.1696	244.1681	6.18
hydroxydesomorphine isomer 5 (aromatic)	4.56	$C_{17}H_{22}NO_3^+$	288.1594	288.1594	0.03
		$C_{16}H_{22}NO_2^+$	260.1645	260.1643	0.62
		$C_{16}H_{20}NO^+$	242.1539	242.1535	1.95

(continued)

desomorphine-*N*-oxide	5.12	$C_{17}H_{22}NO_3^+$	288.1594	288.1597	1.11
		$C_{17}H_{21}NO_2^+$	271.1567	271.1566	0.27
		$C_{14}H_{15}O_2^+$	215.1067	215.1066	0.42
norhydroxydesomorphine isomer 1 (aliphatic)	2.49	$C_{16}H_{20}NO_3^+$	274.1438	274.1443	2.09
		$C_{14}H_{11}O^+$	195.0804	195.0808	1.69
		$C_{13}H_{11}^+$	167.0855	167.0866	6.63
norhydroxydesomorphine isomer 2 (aliphatic)	3.21	$C_{16}H_{20}NO_3^+$	274.1438	274.1423	5.21
		$C_{16}H_{18}NO_2^+$	256.1332	256.1323	3.45
		$C_{15}H_{20}NO^+$	230.1539	230.1524	6.83

Conclusions

In the absence of widespread screening and testing, the prevalence of Krokodil use is difficult to estimate. Despite numerous published case reports, only one analytically confirmed case has been reported to date. We describe a procedure to identify desomorphine in urine at sub ng/mL concentrations using LC-Q/TOF-MS. This method was fully validated in accordance with published guidelines.

In the absence of authentic urine samples from Krokodil users, recombinant enzymes were used to generate desomorphine metabolites *in vitro*. Ten phase I and phase II metabolites were identified using this method, which in addition to previously published work (31), included a second norhydroxydesomorphine isomer. This new procedure to identify desomorphine in urine should assist with identification efforts in both clinical and forensic toxicology.

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CHAPTER VIII

CONCLUSIONS

Desomorphine is a semi-synthetic opioid that is a major constituent of Krokodil, a clandestinely produced heroin substitute. Although Krokodil abuse was first reported in Russia and its surrounding countries, its true prevalence is unknown. Published case reports have originated from the Republics of Armenia and Georgia, Germany, Italy, Poland, Russia, Spain, the United Kingdom and the United States. To date only one reported case has been analytically confirmed in a biological matrix. Although not widespread, its use in the United States is supported by data from the National Forensic Laboratory Information System in the early 2000s. The delay between Krokodil use and the development of severe dermatological symptoms that require medical intervention may explain why few clinical case reports are analytically confirmed.

To facilitate the identification of desomorphine, a comprehensive study of its metabolism and analysis was conducted. As a commonly used screening technique, commercially available ELISAs targeting morphine and oxycodone (Immunalysis Opiates Direct ELISA, Immunalysis Oxycodone/Oxymorphone Direct ELISA, Randox Opiate ELISA, OraSure Technologies OTI Opiate Micro-plate EIA, Neogen Opiate Group ELISA and Neogen Oxycodone/Oxymorphone ELISA) were evaluated for cross-reactivity with desomorphine, which was found to be highly variable (<2.5% to 77%). Depending on the choice of ELISA, desomorphine may go undetected during immunoassay screening. The Immunalysis Opiates Direct ELISA produced the greatest cross-reactivity and several other assays also produced cross-reactivity of sufficient magnitude to be effective for screening purposes.

The metabolism of desomorphine was investigated using recombinant CYPs and UGTs. Eight phase I metabolites were identified in the initial investigation: nordesomorphine, desomorphine-*N*-oxide, five hydroxydesomophine isomers and norhydroxydesomorphine. Norhydroxydesomorphine was a novel metabolite that had not previously been reported in the literature. Later analysis of desomorphine metabolites using a validated method for the quantitative analysis of desomorphine identified an additional norhydroxydesomorphine isomer that was not previously observed. Seven CYPs were found to contribute to desomorphine's metabolism (CYP2B6, CYP2C8, CYP2C9, CYP2C18, CYP2C19, CYP2D6 and CYP3A4), with CYP3A4 mediating the production of all eight metabolites but CYP2C18 being the major contributor to the production of the major phase I metabolite, nordesomorphine. Desomorphine-glucuronide was identified during phase II metabolism, with nine UGTs contributing to its production (UGT1A1, UGT1A3, UGT1A8, UGT1A9, UGT1A10, UGT2B4, UGT2B7, UGT2B15, and UGT2B17). UGT2B7 showed the greatest activity and the involvement of UGT1A3 was a novel pathway not previously reported in the literature.

Hydrolysis of conjugated metabolites to determine the relative proportions of free to total drug is sometime required. The hydrolysis of desomorphine-glucuronide was investigated using acid hydrolysis and enzymatic hydrolysis with β-glucuronidase from BGTurbo™, IMCSzyme™, *E. coli*, *H. pomatia* and *P. vulgata*. Acid hydrolysis with hydrochloric acid produced complete hydrolysis, and at optimal conditions all five enzymes also produced near complete hydrolysis. At simulated challenging conditions *P. vulgata* maintained the highest activity. BGTurbo™ and IMCSzyme™ offered the shortest incubation time necessary to produce complete hydrolysis and their purified preparations

required minimal sample cleanup compared to *E. coli*, *H. pomatia* and *P. vulgata*. A short-term stability study of desomorphine and desomorphine-glucuronide at 60°C indicated that both species were stable for three hours over a range of pH values (4-10).

SPE and GC-MS were used to quantify desomorphine in blood and urine using desomorphine-D_3 as the IS, without the need for derivatization. The extraction efficiencies were 69% for blood and 90% for urine and the LOQs were 5 ng/mL and 8 ng/mL, respectively, which was ten-fold lower than previously published methods. The intra- and inter-assay CVs were 2-4% (n=3) and 3-7% (n=15), respectively. A weighted (1/x) linear calibration model was used with a calibration range of 5-1000 ng/mL for blood and 8-1000 ng/mL for urine. No carry over was observed. A qualitative interference was observed with levorphanol at 10- and 100-fold concentrations (relative to desomorphine). As levorphanol is a stereoisomer of dextrorphan (a metabolite of dextromethorphan), an interference from this substance is also expected.

LC-MS/MS and LC-Q/TOF-MS were used to quantitate desomorphine in urine after SPE extraction. For LC-MS/MS the LOD and LOQ were 0.5 ng/mL, with a calibration range of 0.5-500 ng/mL using a weighted (1/x) quadratic calibration model. The bias ranged from -1-2% (n=15), and the intra- and inter-assay CVs were 2-3% (n=3) and 3-6% (n=15), respectively. Ion suppression ranged from -44-0% at low concentrations and -23-4% at high concentrations. LC-Q/TOF-MS also had a LOD and LOQ of 0.5 ng/mL. The bias ranged from -4-0% (n=15), with intra- and inter-assay precision CVs of 3-5% (n=3) and 4-7% (n=15), respectively. The calibration model used was a weighted (1/x) quadratic model with a range of 0.5-500 ng/mL. The ion suppression was -22-11% at low concentrations and -22-2% at high concentrations.

All three analytical methods were capable of detecting desomorphine at forensically relative concentrations, although some differences in performance were observed. The greatest calibration range was achieved using GC-MS, but this was accompanied by reduced sensitivity compared with LC-MS/MS and LC-Q/TOF-MS. Overall, improved S/N ratios were observed using LC-MS/MS compared to LC-Q/TOF-MS; however less ion suppression was observed with LC-Q/TOF-MS.

In the absence of authentic urine samples from Krokodil users, recombinant enzymes were used to generate desomorphine metabolites for analysis using the validated LC-Q/TOF-MS method. Ten phase I and phase II metabolites were identified using retrospective data analysis, which included a second norhydroxydesomorphine isomer that was not previously identified in the initial investigation of desomorphine's metabolism.

As drug users continue to self-report Krokodil use, it is important for laboratories to have sensitive and robust analytical methodology to detect desomorphine in biological specimens. This research provides the forensic toxicology community with comprehensive information regarding desomophine's metabolism and its analysis utilizing multiple techniques, which will aid in its identification. This study was the first to describe the analysis of desomorphine in urine using LC-MS/MS and the first to describe the use of LC-Q/TOF-MS to analyze desomorphine in biological specimens.

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APPENDIX

Abbreviations

AL	Alprazolam
AMP	Amphetamines
BAR	Barbiturates
BZO	Benzodiazepines
CAN	Cannabis
cDNA	Complementary deoxyribonucleic acid
CEDIA	Cloned enzyme donor immunoassays
CNS	Central nervous system
COC	Cocaine
CSA	Controlled Substances Act
CYP	Cytochrome P450 mono-oxygenase
DAD	Diode array detector
ELISA	Enzyme linked immunosorbent assay
EMIT	Enzyme multiplied immunoassay technique assays
GC	Gas chromatography
HER	Heroin
HLM	Human liver microsomes
HRMS	High resolution mass spectrometry
IA	Immunoassay
Ig	Immunoglobulin
IS	Internal standard
LC	Liquid chromatography
LLE	Liquid-liquid extraction
MDMA	Methylenedioxymethamphetamine
MET	Methamphetamine
MRM	Multiple reaction monitoring
mRNA	Messenger ribonucleic acid
MS	Mass spectrometry
MS/MS	Tandem mass spectrometry
OTC	Over the counter
PNS	Peripheral nervous system

(continued)

Q/TOF-MS	Quadrupole/time of flight-mass spectrometry
QuEChERS	Quick, Easy, Cheap, Efficient, Rugged and Safe
SIM	Selected ion monitoring
SPE	Solid phase extraction
THC	Tetrahydrocannabinol
TOF	Time of flight
UGT	Uridine diphosphate-glucuronosyltransferase
UV	Ultraviolet detector

VITA

Jessica Winborn

Relevant Professional Experience

Sam Houston State University - August 2013 to Present

- Graduate Research Assistant
- Laboratory Assistant: Aided in laboratory preparation, inventory, administrative duties, and troubleshooting instruments
    - Gas chromatography-mass spectrometry (GC-MS), liquid chromatography-tandem mass spectrometry (LC-MS/MS) and liquid chromatography-quadrupole/time of flight-mass spectrometry (LC-Q/TOF-MS)

US Customs and Border Protection Southwest Regional Laboratory - June 2014 to September 2015

- Student Trainee (controlled substances and latent print units)
- Experience using Gas Chromatography-Mass Spectrometry/Flame Ionization Detector (GC/MS/FID) and Fourier Transform Infrared Spectroscopy (FTIR)
- Collaborative research project analyzing seized marijuana samples using headspace solid phase microextraction (HS-SPME) (Award Number 2014-R2-CX-K005)

Education

Sam Houston State University, Huntsville, TX August 2013 – Present

- Pending Doctor of Philosophy in Forensic Science
- GPA: 4.0
- Graduation: December 2018 (Anticipated)

Sam Houston State University, Huntsville, TX August 2008 – May 2013

- B.S. in Forensic Chemistry (ACS certified) with minors in Criminal Justice and Biology
- Graduated Magna Cum Laude May 2013

Relevant Education Experience

Sam Houston State University

- Forensic Instrumental Analysis, Advanced Instrumental Analysis, Advanced Forensic Chemistry, Neuropsychopharmacology, Drug and Toxin Biochemistry, Advanced Biochemistry, Chromatographic Separations, Forensic Statistics and Interpretation, Statistical Genetics, Controlled Substances, Pattern and Physical Evidence Concepts, Crime Scene Investigation, Trace Evidence and Microscopic Analysis, Biochemistry, Quantitative Analysis, Instrumental Analytical Chemistry, Forensic Chemistry

Skills and Qualifications

Screening and Sample Preparation

- Enzyme-linked immunosorbent assays (ELISA)
- Liquid-liquid extraction (LLE)
- Solid phase extraction (SPE)
- Solid phase microextration (SPME)

Instruments

- Agilent Technologies gas chromatography-mass spectrometer
  - 5975B VL MSD
- Agilent liquid chromatography-quadrupole/time of flight-mass spectrometer
  - 6530 Q/TOF-MS
- Agilent liquid chromatography-tandem mass spectrometry
  - 6470 triple quadrupole MS/MS

Software

- Agilent Technologies ChemStation and MassHunter, R Statistical Software, ACD/ChemSketch

Research Grant Funding

- National Institute of Justice – Graduate Research Fellowship (2015-R2-CX-0031)
  - *Metabolism and Analysis of Desomorphine*
  - PI: Jessica Winborn, CO-PI: Sarah Kerrigan

Publications in Peer Reviewed Journals

1. Winborn, J., Kerrigan, S. (2017). Desomorphine Screening Using Commercial Enzyme-Linked Immunosorbent Assays. Journal of Analytical Toxicology, 41(5), 455-460.

2. Winborn, J., Haines, D., Kerrigan, S. (2018). *In vitro* Metabolism of Desomorphine. Forensic Science International, 289, 140-149.

3. Winborn, J., Kerrigan, S. (2018) Quantitative Analysis of Desomorphine in Blood and Urine Using Solid Phase Extraction and Gas Chromatography-Mass Spectrometry. Journal of Chromatography B (In press).

4. Winborn, J., Kerrigan, S. (2018) Analysis of Desomorphine in Urine Using Liquid Chromatography-Tandem Mass Spectrometry. Journal of Analytical Toxicology (In press).

5. Winborn, J., Basiliere, S., Kerrigan, S. (2018) Quantitative Analysis of Desomorphine in Urine Using Solid Phase Extraction and Liquid Chromatography-Quadrupole/Time of Flight-Mass Spectrometry. Forensic Science International (In revision).

6. Winborn, J., Kerrigan, S. (2018) Stability and Hydrolysis of Desomorphine-Glucuronide. Journal of Analytical Toxicology (In revision).

Peer-Reviewed Presentations/Posters

1. Winborn, J., Haines, D., Kerrigan, S. Identification of Desomorphine in Urine. Proceedings of the Society of Forensic Toxicologists (2018), Minneapolis, MN. (Poster Presentation)

2. Winborn, J., Haines, D., Kerrigan, S. Phase I Metabolism of Desomorphine, Proceedings of the Society of Forensic Toxicologists/International Association of Forensic Toxicologists (2017), Boca Raton, FL. (Oral Presentation)

3. Winborn, J., Sweet, J., Yu, J.C.C. Differentiation of Seized Marijuana Samples using Automated Headspace Solid-Phase Microextraction Coupled to Gas Chromatograph – Mass spectrometer/ Flame Ionization Detector and Principal

Component Analysis, Proceedings of the American Academy of Forensic Sciences (2016), Las Vegas, NV. (Poster Presentation)

4. Winborn, J., Hanson, M., Figueroa, L., Konarik, A., James, D. Chen, K. Dassau, T., Sweet, J. Yu, J.C.C. Analysis of Cannabinoids Found in Seized Marijuana Using Automated Headspace Solid-Phase Microextraction Coupled with Gas Chromatography/Mass Spectrometry, Proceeding of the American Academy of Forensic Sciences (2015), Orlando, FL. (Poster Presentation)

Webinars

- Winborn, J., Haines, D., Kerrigan, S. Phase I Metabolism of Desomorphine, Proceedings of webinar "Novel Forensic Chemistry Research from Early-Career Scientists" presented by the Forensic Technology Center of Excellence, National Forensic Science Week 2018.

Professional Affiliations

- American Academy of Forensic Sciences (AAFS) – General Member (2013 - current)
- Society of Forensic Toxicologists (SOFT) – Student Member (2017 – current)

Continuing Education

- Bloodborne and Airborne Pathogens
- OSHA Certification in Blood Bourne Pathogens and Laboratory Standard
- RTI International Forensic Science Education
  - Answering the NAS: The Ethics of Leadership and the Leadership of Ethics
  - Introduction to Uncertainty in Forensic Chemistry and Toxicology
  - Standard Operating Procedure (SOP) Writing for ISO 17025 Accreditation
  - To Hell and Back: The Ethics of Stewardship and the Stewardship of Ethics
  - Applications of Higher Resolution Mass Spectrometry in Drug Testing
  - Fundamentals of Chromatography used in Toxicology
  - Best Practices: Synthetic Drugs Online
  - Exploiting the Power of LC-TOF Data Mining
- Forensic Technology Center of Excellence
  - Opioids and Death Investigations: A Perfect Storm

  - Novel Psychoactive Substances in Forensic Casework Session I: The Synthetic Drug Crisis-Identifying NPS in Forensic Casework
  - Novel Psychoactive Substances in Forensic Casework Session I: Analysis of NPS – Practical Considerations and Analytical Approaches
- Attended Workshop “Risky Business: The Dance Between ISO/IEC 17025:2017’s Risk Based Requirements and Forensic Toxicology Laboratories” at the Society for Forensic Toxicologists Annual Meeting in Minneapolis, MN, October 2018.
- Attended Short Course “High Resolution Mass Spectrometry for Qualitative and Quantitative Analysis: An Introduction” at the American Society for Mass Spectrometry Annual Meeting in San Antonio, TX, June 2015.
- Attended LC/MS Master Class offered by Agilent Technologies in Austin, TX, January 2014.