DETECTION OF PRENATAL OPIATE EXPOSURES IN ALTERNATIVE MATRICES

by

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A thesis submitted in conformity with the requirements for the degree of Masters of Science

Collaborative program in Biomedical Toxicology

Graduate Department of Pharmacology and Toxicology

University of Toronto

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Detection of Prenatal Opiate Exposures in Alternative Matrices

Masters of Science (2010)

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Abstract

Identification of maternal opioid abuse in pregnancy is often difficult to ascertain in the absence of reliable self report. For this reason, physicians and child protection workers often turn to maternal and neonatal hair analysis for the detection of in utero opioid exposures. Since neonatal opiate hair analysis continues to prove difficult due to the scarcity of the hair sample and low drug concentrations, I developed a sensitive method utilizing headspace solid-phase microextraction (HS-SPME) coupled with gas chromatography-mass spectrometry (GC-MS) for the detection of three principal opiates (morphine, codeine, and 6-monoacetylmorphine) in human hair. Moreover, I characterized an at-risk neonatal population for in utero opiate exposures as well as for other drugs of abuse and alcohol. Equipped with a sensitive and specific method for the detection of opiate exposures and understanding the addiction profiles of pregnant women may lead to better clinical and social management and may benefit an at-risk population.
Acknowledgements

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I owe the greatest appreciation to my family and friends as their unconditional love and support has been the base upon which I have grown and flourished as an individual. My dedication to reaching my dreams is a result of their encouragement and dedication to me, and I will always be grateful to for their constancy.

Be true to your dreams.
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<th>Description</th>
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<tbody>
<tr>
<td>HSC</td>
<td>Hospital for Sick Children</td>
</tr>
<tr>
<td>NOUGG</td>
<td>National Opioid Use Guideline Group</td>
</tr>
<tr>
<td>LOPT</td>
<td>Long term opioid therapy</td>
</tr>
<tr>
<td>CNCP</td>
<td>Chronic, non-cancer pain</td>
</tr>
<tr>
<td>M3G</td>
<td>Morphone-3-glucuronide</td>
</tr>
<tr>
<td>M6G</td>
<td>Morphone-6-glucuronide</td>
</tr>
<tr>
<td>CYP2D6</td>
<td>Cytochrome P450 CYP2D6</td>
</tr>
<tr>
<td>AC</td>
<td>Adenylyl cyclase</td>
</tr>
<tr>
<td>CNS</td>
<td>Central nervous system</td>
</tr>
<tr>
<td>VTA</td>
<td>Ventral tegmental area</td>
</tr>
<tr>
<td>ADH</td>
<td>Antidiuretic hormone</td>
</tr>
<tr>
<td>NAS</td>
<td>Neonatal abstinence syndrome</td>
</tr>
<tr>
<td>6MAM</td>
<td>6-Monoacetylmorphine</td>
</tr>
<tr>
<td>SPME</td>
<td>Solid phase microextraction</td>
</tr>
<tr>
<td>HS-SPME</td>
<td>Headspace solid phase microextraction</td>
</tr>
<tr>
<td>GC-MS</td>
<td>Gas chromatography-mass spectrometry</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme linked immunosorbent assay</td>
</tr>
<tr>
<td>RIA</td>
<td>Radioimmunoassay</td>
</tr>
<tr>
<td>HPLC</td>
<td>High performance liquid chromatography</td>
</tr>
<tr>
<td>LC-MS/MS</td>
<td>Liquid chromatography-tandem mass spectrometry</td>
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1. INTRODUCTION

1.1 A Brief History of Opioids

The opium poppy, or *Papaver somniferum*, has been used for thousands of years for relief of pain. It is the main source of the crude opium substance which has been accounted to be used by ancient Egyptians, Greeks, and Romans throughout history, and has played a pivotal role in both medicine and trade. In fact, preserved opium pods and seeds have been discovered in various Swiss archeological sites of Neolithic dwellings dating back to the fourth millennium B.C. (Booth 1998). Moreover, primitive Sumerian ideograms from 2400 B.C. demonstrate their beliefs of the poppy to be the “joy plant”, and the ancient Egyptian city of Thebes was renowned for its extensive poppy fields from which opium trade began to flourish (Katz 2007).

The trade routes became quite pervasive and opium was soon carried into Europe where it enjoyed an extensive history of use and abuse for its euphoric and analgesic effects. The notion that opium possessed addictive qualities did not arise immediately; however controversy regarding opium dependency began to evolve in the medical literature as early as the 5th and 3rd centuries B.C (Katz 2007). Morphine was first isolated in 1805 from the crude opium extract by the German pharmacist Sertürner, who tested the compound on himself and subsequently named it “morphine” after Morpheus, the Greek god of dreams (Hamilton, Baskett 2000). Even Sir William Olser called morphine “God’s own medicine” after being treated with the analgesic for pain associated with renal colic (Osler 1910, Golden 2009). In 1895, Heinrich Dreser of The Bayer Company synthesized the potent opiate heroin which would find its way onto the market 3 years later where it was prescribed by physicians for the treatment of respiratory diseases and to attenuate morphine addiction (Hosztafi 2001, Berridge 2009).
At first, heroin was used to assist morphine addicts in attempting to abstain from the drug, but it was not long before physicians began to notice the severe effects of heroin withdrawal that were akin to that of morphine (Hughes et al. 1972). Opinions highlighting the dangers of opioids began to overshadow its potential benefits; as such the recent history of opioids has seen great peaks and troughs of availability and use. Morphine was extensively used throughout the American Civil War and into the early 1900’s until the Harrison Narcotics Act of 1914 imposed a dramatic restriction and tax on opioid products in the United States. In 1923 the U.S. treasury narcotics division banned all legal narcotic sales and imposed legal consequences for physicians prescribing the drug (Musto 1999). This prohibition forced addicted users to seek alternate sources of opium, morphine, and heroin, and thus the business of illegal street dealing began to thrive and evolve into the problematic system we deal with today.

In the late 20th century, in the face of improved cancer care initiatives, the patients’ rights movement, and the hospice movement among other social factors, opioids gradually returned to modern medicine (Katz 2007). The prevalence of opioid use and abuse has been substantially increasing since then (Havens, Oser & Leukefeld 2007, Havens et al. 2009, Peindl et al. 2007, Fischer et al. 2005). This is well exemplified by the increase in overall utilization of prescribed opioid analgesics in Canada from 2001 to 2005 which saw a 230%, 159%, and 28% increase in consumption for oxycodone, fentanyl, and morphine respectively (Fischer et al. 2008). Moreover, the types of opioids being consumed are changing rapidly; heroin users are becoming increasingly marginalized and prescription opioids are now predominantly abused (Fischer et al. 2006). For reference, the term ‘drug/opioid abuse’ refers to any intentional use of a medication
with intoxicating properties (i.e. opioids) outside of a physician’s prescription for a bona fide medical condition, excluding accidental misuse (Compton, Volkow 2006). It is difficult to determine whether this fundamental shift from illicit heroin use to non-medical opioid abuse is a product of supply or demand; however it should be noted that Canada is among the world’s top per capita consumers of opioids, creating an opioid-rich and ready environment (Fischer, Rehm 2006).

Hesitation among many physicians for the prescription of opioids, even to legitimately needing patients, stems from extensive misuse/abuse of these drugs. For this reason, the “Canadian guideline for safe and effective use of opioids for chronic non-cancer pain” was recently created by the National Opioid Use Guideline Group (NOUGG). NUOGG was formed in 2007 in order to oversee the development of these critical guidelines, which are aimed to assist physicians in their decision-making in prescribing to and managing patients requiring long term opioid therapy (LOPT) for chronic, non-cancer pain (CNCP) (Furlan, Reardon & Weppler 2010). It is hoped that equipped with these recommendations for clinical practice, the significant misuse of opioid containing preparations may be at least partially attenuated.

1.2 Opioid Pharmacology and Toxicology

There is some variation in the terminology of opioids but the following is most common: Opioid is the term used to refer to any compound acting at the opioid receptor that has morphine-like pharmacological properties, whether it is synthetic, semi-synthetic, or natural (i.e. morphine, methadone, or endogenous opioids such as β-endorphin). Opiate is the term used to describe the naturally occurring alkaloids found in opium, as well as their derivatives (i.e. morphine, codeine,
and heroin) (Brunton, Laurence L., John S. Lazo and Keith L. Parker 2006). The crude opium extract is obtained by incising the seedpod upon which a white latex substance oozes out and then turns brown and hardens. This sticky, gummy-like brown substance is opium, which contains approximately 20 different alkaloids, including morphine, codeine, thebaine, and papaverine. The former two alkaloids are clinically used analgesics, whereas the latter two are not themselves but are synthesized into compounds that act at opioid receptors, such as the opioid receptor antagonist naloxone (Booth 1998). Endogenous opioids, such as β-endorphin, are similar to opioid analgesics in their properties and mechanisms of action in the CNS.

Morphine is the principal alkaloid present in opium and constitutes approximately 10% of the crude extract. Codeine, a very commonly prescribed analgesic, constitutes less than 0.5% of the extract and therefore is synthesized commercially from morphine. Acetylation of both hydroxyl groups of morphine yields the semi-synthetic opiate diacetylmorphine, more commonly known as heroin. Figure 1 on the following two pages illustrates the chemical structure of a variety of opioids, including morphine, codeine, and diacetylmorphine (Coller, Christrup & Somogyi 2009).

Figure 1: (On next page) Diagram of various natural, synthetic, and semi-synthetic opioids. (Source: Coller, 2009)
1.2.1 Opioid Pharmacokinetics

Opiates are generally absorbed well from dermal, subcutaneous and intramuscular sites as well as mucosal surfaces such as the nose, mouth, or gastrointestinal tract. Distribution is heavily dependent on the physicochemical properties of the opioid, but all of these compounds bind to plasma proteins to some degree and rapidly leave to concentrate in highly profused tissues such as the kidneys, lungs, liver, and spleen (Trescot et al. 2008). Crossing the blood brain barrier is difficult for most opiates, except those with substituted aromatic hydroxyls at the C3 position, such as codeine and heroin. It is important to note that this essential barrier is not fully formed in neonates, and since opiates readily cross the placenta, their use in pregnancy may result in opiate-associated complications for the infant (van Lingen et al. 2002).

The metabolism of opiates generally consists of their conversion into more polar metabolites and excretion via the kidneys. Morphine and codeine are typically regarded as ‘short half-life opiates’ with values ranging from between 2-3.5 hours Methadone is an example of a ‘long half-life opioid’ and has a half-life of 22-24 hours if administered chronically (Verebely et al. 1975). Free hydroxyl containing opiates such as morphine are readily glucuronidated to morphine-3-glucuronide (M3G) and morphine-6-glucuronide (M6G), the latter of which has μ-receptor activity thought to be greater than even that of morphine in the central nervous system (Andersen, Christrup & Sjogren 2003). Esters such as heroin are rapidly hydrolyzed by common tissue esterases to 6-monoacetylmorphine, a more potent μ-agonist than morphine (Selley et al. 2001). Codeine itself is a weak μ-agonist but is a pro-drug and is converted during first pass metabolism by cytochrome P450 2D6 to the active analgesic morphine. Due to the polymorphic enzyme CYP2D6 involvement in opioid metabolism, inter-individual pharmacokinetic and
pharmacodynamic differences may be evident (Gasche et al. 2004). Figure 2 briefly demonstrates main metabolic pathways of the principal opiates, codeine, and morphine (Lotsch 2005). Heroin is converted to 6-monoacetylmorphine and further to morphine, then follows the same metabolic pathway as morphine.

**Figure 2:** Metabolic pathway of the principal opiates, morphine and codeine. (Source: Lotsch 2005).
Hepatic N-demethylation also contributes to opioid metabolism, however this pathway is considered minor. Polar metabolites and small amounts of unchanged parent compound are mainly excreted in the urine; enterohepatic circulation also represents a small portion of opioid excretion (Trescot et al. 2008).

1.2.2 Opioid Pharmacodynamics

Opioids provide their analgesic effects via opioid receptors of which there are 3 different isoforms, namely mu (μ), kappa (κ) and delta (δ) located in various locations in the central nervous system on nociceptive C and delta-A fibers. Receptors mediating analgesia are found in the dorsal horn of the spinal cord, as well as the periaqueductal gray matter and the thalamus. Receptors mediating the respiratory, antitussive, and pupillary constrictive effects of opioids are located in the ventral brain stem. Receptors affecting neuroendocrine secretions are located in the hypothalamus, and finally, those affecting mood and behaviour are located in the limbic structures of the brain, such as the hippocampus and the amygdala (Shook, Watkins & Camporesi 1990, Martin 1983, Fowler, Fraser 1994).

Seven subtypes of these opioid receptors have been characterized to date, including μ₁ and μ₂, κ₁, κ₂, and κ₃, and δ₁ and δ₂. They are G-protein coupled receptors, which act through G-proteins to inhibit adenylyl cyclase (AC) and thus attenuate cAMP production. Moreover, activation of inward rectifying K⁺ channels as well as inhibition of voltage gated Ca²⁺ channels occurs with opioid agonism and yields the overall effect of reduction in excitability and neurotransmitter release of neurons associated with pain pathways (Trescot et al. 2008, Martin 1983, Nestler 1996). Specifically, this indirectly inhibits the release of pain neurotransmitters
such as glutamate and substance P (McCleane, Smith 2007). Additionally, opioids cause disinhibition of dopaminergic neurons in the ventral tegmental area (or VTA), as well as increased concentrations of dopamine in the nucleus accumbens (NAcc) and thus an intense feeling of pleasure or euphoria ensues (Trescot et al. 2008). Therefore, opioids are widely used and typically abused for their outlined sedative, analgesic, and euphoric properties (Havens, Oser & Leukefeld 2007, Havens et al. 2009, Peindl et al. 2007, Fischer et al. 2005).

1.2.3 Clinical and Toxicological Effects

Although sedation and analgesia tend to be the predominant and desired clinical outcomes of opioid administration, this drug class elicits a variety of other effects on the body. Modulation of pain perception is mediated largely by µ opioid receptors which are highly concentrated in areas of the brain classically associated with analgesia, including the periaqueductal gray, nucleus raphe magnus, locus coeruleus, and medial thalamus (Pasternak 1988). Mediation of pain through δ and κ opioid receptors does play a small role in analgesia, but these receptors are located in the spinal cord and not the brain (spinal analgesia). Euphoria is produced by opioids via activation of the µ/δ receptor complex located in the ventral tegmental area which indirectly causes a release of dopamine into the mesolimbic system (Nestler 1996). The sedative effects of opioids are largely believed to be mediated by the µ receptors as well.

Although opioids are thought to be relatively safe and quite effective therapeutic agents (Barsan et al. 1993, Levy 1996), a wide array of toxicological events may occur as a result of their administration. These events may be predicted by and are extensions of opioid clinical pharmacology (i.e respiratory depression, sedation, and analgesia) however other symptoms may
present as well. Such clinical and toxicological effects of opioids are cardiovascular, pulmonary, neurologic, dermatologic, endocrinologic, gastrointestinal, and ophthalmologic in nature (Goldfrank's manual of toxicologic emergencies 2007).

Respiratory depression is a common and predominant adverse event caused by opioid analgesics via the $\mu_2$ receptor. Opioid agonists diminish the sensitivity of medullary chemoreceptors to hypercapnea as well as directly depressing ventilator responses to hypoxia, thus reducing ventilation overall and results in apnea (Weil et al. 1975). Pulmonary edema may occur with opioid administration as well, and has been documented to occur more commonly with heroin overdose and naloxone usage (Mell, Sztajnkrycer 2006, Johnson, Mayer & Grosz 1995). Opioids may elicit cardiovascular adverse events, which include peripheral vasodilation and orthostatic hypotension (Zelis et al. 1974, Ward, McGrath & Weil 1972). The administration of some opioids such as tramadol and fentanyl may also result in neurological sequelae including seizures and rigidity (Rehni, Singh & Kumar 2008, Rao, Mummaneni & El-Etr 1982, Viscomi, Bailey 1997).

Other important toxicological effects of opioids include pruritis and flushing (due to opioid induced histamine release), attenuation of ADH and gonadotropin release, and an array of gastrointestinal effects such as emesis, reduced motility and gastric acid secretion as well as increased biliary tract pressure and anal sphincter tone. Finally, a hallmark symptom associated with opioid ingestion is miosis, or “pinpoint pupils”, which is thought to be a result of either hyperpolarization of sympathetic nerves or hypopolarization of inhibitory neurons to the parasympathetic neurons. Overall, the constellation of symptoms produced by opioid ingestion is
described as the *Opioid Syndrome* and generally includes mental status depression, hypoventilation, miosis, and reduced bowel motility (Goldfrank's manual of toxicologic emergencies 2007).

### 1.2.4 Opioid Dependence and Addiction

The use of opioid analgesics for treatment of chronic pain is often hindered by the need for dose escalations due to the development of tolerance. Such dosage increases not only puts patients at risk for adverse events associated with opioid use, but also for the development of physical dependence and addiction (He, Kim & Whistler 2009).

The mechanisms behind the development of opioid tolerance and dependence has been linked to a complex interplay of a variety of cellular mechanisms, including but not limited to the activation of adenylyl cyclase (AC), and alterations and involvement of the N-methyl-D-aspartate receptor (NMDAR) and glucocorticoid receptor (GR). There is also speculation that trafficking and internalization of µ-opioid receptors plays a role in the development of tolerance; however failure to do so in various cell lines as well as adult neuronal tissue in the presence of high concentrations of morphine has instilled skepticism as to the contribution of this mechanism (Rodriguez-Munoz et al. 2007).

Substance dependence is of primary concern in the context of opioid abuse, according to ICD criteria (World Health Organization, 1993). Opioid dependence and addiction may lead users to seek the drug by alternative methods than that of legitimate prescription; it may also influence or provoke criminal and semi-legal activities (panhandling, prostitution, property
crimes) if the money required to maintain such dependence/addiction exceeds that of the individual’s income (Haydon et al. 2005). It is important to note that such drug seeking/obtaining behavior is likely exacerbated by the significant availability of such substances in the illicit market. According to the International Narcotics Control Board, Canada is among the world’s top per capita consumers of opioids: we are first in hydromorphone, second for morphine and oxycodone, and third for hydrocodone per capita consumption (International Narcotics Control Board, 2006). Moreover, the diversion and abuse of these drugs for non-medical purposes is becoming more apparent, coupled with increased levels of dependence and overdose (Compton, Volkow 2006, Haydon et al. 2005). Such diversion of prescribed opioids to the illicit drug market usually involves some form criminal activity, including robbery or fraudulent prescriptions, and sources for obtaining opioids have been reported to include dealers, physicians, partners/significant others, friends, and/or theft (Sajan, Corneil & Grzybowski 1998).

In the context of child protection, there are a variety of associated risks for the safety and well-being among children of parents facing opioid dependence that must be considered. Such risks include exposure to the buying and selling of drugs, contact with other drug users, as well as the drug-use itself (Hogan 2003). Moreover, these children tend to come from families of lower socioeconomic status, have significantly more academic, social, and family functioning difficulties, as well as significantly higher rates of psychopathology (Wilens et al. 2002). Therefore, identification of drug abusing and addicted parents is paramount for adequate social care for this vulnerable population of children.
1.2.5 Treatment of Opioid Toxicity

Clinical management of opioid toxicity may vary depending on the presentation of the patient as well as the type of patient. For instance, mild symptoms of toxicity may be treated with non-opioid pharmaceutical agents and supportive therapies, while life-threatening overdoses may require naloxone as an antidote. Naloxone is an opioid antagonist that competitively inhibits the binding of opioid agonists such as heroin. When such antagonism occurs by naloxone, a patient who experiences depressed ventilation as a result of opioid intoxication (or agonism) may abruptly resume spontaneous respirations (Gourlay, Coulthard 1983, Upadhyay et al. 2008, Yilmaz et al. 2003).

Neonates who are exposed *in utero* to opioids, especially close to delivery, may exhibit significant postnatal problems, most notably of which is the neonatal abstinence syndrome (NAS) or withdrawal (Finnegan et al. 1975, Finnegan 1985). It has been estimated that NAS occurs in approximately 55-94% of *in utero* opioid exposed neonates, and frequently requires supportive care (American Academy of Pediatrics on Drug Withdrawal 1998). Manifestation of NAS include a constellation of symptoms described by Finnegan in the mid 1970’s and include irritability, high-pitched cry, tremors, hypertonicity, poor feeding, vomiting, and diarrhea (Finnegan et al. 1975). Additionally, seizures have been described to occur between 2-11% of symptomatic infants (Herzlinger, Kandall & Vaughan 1977, Zelson, Rubio & Wasserman 1971, Kandall, Gartner 1974).

Naloxone is not used in the clinical management of NAS as it may precipitate enormous stress and a variety of undesirable sequelae in the infant; instead, neonates are administered
doses of opioids (predominantly morphine sulphate) and are slowly weaned off the drugs with other supportive methods (Johnson, Gerada & Greenough 2003b, American Academy of Pediatrics on Drug Withdrawal1998, Osborn, Jeffery & Cole 2005). If seizures do occur, phenobarbital is typically used to attenuate such CNS excitability, but benzodiazepines may also be used (Kandall et al. 1983, O'Grady, Hopewell & White 2009).
1.3 Confirmation of Opiate Use

In the past, self-reporting has been a widely used source of information pertaining to drug use in pregnancy, both for pre- and post-natal periods (Marques, Tippetts & Branch 1993). Due to the social stigma associated with drug abuse and addiction, especially in the circumstance of pregnancy and parenting, self-report is typically elusive and considered an inaccurate mode of substance abuse monitoring when compared to objective measures of drug consumption (Solbergsdottir et al. 2004, Katz et al. 2003). For this reason, social workers, researchers and physicians working in the realm of substance abuse monitoring as well as child protection have turned to toxicological analysis of body fluids/tissues to provide objective evidence of drug use and/or exposure.

1.3.1 Matrices for the Detection of Short-Term Opiate Exposures

1.3.1.1 Blood/Plasma Analysis

Obtaining blood levels for a drug and/or its metabolites may be applicable in acute toxicity cases (i.e. medical emergencies), primarily because blood drug levels are detectable for only a short period of time after intake. The detection of illicit substances in blood heavily relies on the time elapsed post-ingestion and the detection of drugs in blood generally does not confer quantitative information pertaining to dosage. Many drugs (including short-acting opioids) are eliminated quite rapidly (within minutes to hours) to undetectable levels after administration (Jenkins, Cone 1998), making blood analysis of drugs relatively useless in a substance abuse monitoring situation. Moreover, the invasive nature of blood sampling makes this method of testing undesirable. Testing umbilical cord blood to determine prenatal exposures possesses
similar drawbacks with respect to the narrow window of detection of most drugs, thereby limiting its use (Gray, Huestis 2007).

### 1.3.1.2 Sweat Analysis

Sweat analysis involves the adhesion of a sweat-patch to the skin of the monitored individual and has been employed to constantly monitor use. A critical drawback to sweat testing is the difficulty behind determining the volume of sweat produced in a given period of time. This volume can vary inter-individually as well as intra-individually due to environmental cues, as well as the mental and health status of the patient (Huestis et al. 1999). Moreover, issues with the site of patch placement as well as patient cooperation have been shown to compromise the reliability of sweat testing (Chawarski et al. 2007, Uemura et al. 2004).

### 1.3.1.3 Saliva Analysis

Oral fluid has been regarded as more desirable than blood due to the less invasive collection procedure and somewhat prolonged window of detection. Saliva is a filtrate of blood: drugs present in the blood are filtered by the salivary glands and passed into the saliva. As a result, saliva portrays the toxicological profile of blood. Therefore, the detectable presence of many (but not all) drugs in this specimen is longer than that of plasma, with windows of detection approximated at around 5 days in chronic users (Cone, Weddington 1989, Kato et al. 1993, Thompson et al. 1987). However, if drug administration occurs via the oral cavity (the mouth), the concentrations of drug detected in the saliva may be unrepresentatively increased
(Huestis, Cone 1998). The transient nature of drugs present in saliva implies that this methodology is not optimal for long-term substance abuse monitoring due to the inconvenience and the high cost related to collecting samples every few days.

1.3.1.4 Urine Analysis

Urine toxicology is useful for detection of recent exposures to numerous illicit substances due to its rapidity and convenience. Like saliva, urine is also a filtrate of blood: drugs present in the blood are filtered by the kidneys and concentrated in the urine. Due to this concentration of drugs in a relatively small volume of urine, drugs that are undetectable in the blood several days after use will still be detectable in a urine sample. Moreover, the relatively short detection window for most drugs of abuse in urine (generally 1-5 days post-use) requires that twice weekly testing should be employed- with samples collected no more than three days apart- to be effectively reliable. Some drugs may even require sample collections up to three times weekly or even daily testing for adequate reliability (Halstead et al. 1988, Wolff et al. 1999).

1.3.2 Matrices for the Detection of Long-Term Opiate Exposures

There are a few matrices that retain drugs for long periods of time, including hair, nails, skin, and meconium (for in utero exposures). In particular, hair and meconium analyses have been significantly characterized in the literature to be effective methods for protracted substance abuse monitoring (Pragst, Balikova 2006, Gareri, Klein & Koren 2006, Koren, Hutson & Gareri 2008). Here, the science and applicability behind testing each of these matrices is reviewed.
1.3.2.1 Hair Analysis for the Detection of Opiate Exposure

Hair strand analysis has proven to be particularly effective and economical as a method of substance abuse monitoring in a child protection context. Drugs present in the bloodstream are incorporated into the growing hair shaft through the capillary blood supply to the follicle. Compounds trapped inside the hair shaft are protected from degradation by the external environment, and since hair grows at a semi-uniform rate across the population, hair analysis enables the determination of a retrospective timeline of drug exposure (Pragst, Balikova 2006). The degree to which a drug is incorporated into the hair shaft depends on the physiochemical properties of that drug, how it interacts with certain proteins in the hair, and the structural integrity of the hair strand itself.

Hair sample collection is particularly easy and non-invasive, and most importantly, it is nearly impossible for the patient to compromise sample integrity. Contrary to urine, which is generally sampled in private and given to the collector thereafter, hair samples are collected directly from the scalp of the patient by the sample collection agent (e.g. technician, nurse, physician, etc.). This feature of hair testing and the maintenance of sample integrity is a significant benefit in substance abuse monitoring. Hair samples are generally collected from the vertex posterior (i.e. “crown”) of the scalp, as this is the location of the most uniform hair growth in humans. If scalp hair is unavailable, body hair can be sampled such as arm, leg, armpit, chest, or pubic hair. It is important to note however, that body hair analysis provides much less interpretative value than head hair due to more inter individual variability in rates of growth, shedding, and drug incorporation (Pragst, Balikova 2006). Additionally, reference ranges
available from some laboratories for interpretation purposes are usually for scalp hair only and therefore would not be applicable to body hair. What body hair analysis can tell us is if someone has or has not used a particular drug in “the recent past”. Essentially, body hair provides a qualitative analysis indicating if the individual has a recent history of drug use.

The mean rate of human scalp hair growth is one centimetre per month (Society of Hair Testing 2004). By assessing the analysis in terms of length of hair versus the concentration of drugs found, the average pattern of drug use/exposure over relatively long periods of time can be determined. This is achieved by comparing sample results collected once every few months or by conducting segmental analysis of a single hair sample. Segmental analysis is carried out by cutting the hair into a series of sections of defined length, representing a chronological sequence of specific time-frames (e.g. 1cm = one month, 3cm = three months). Consequently, toxicological analysis of the segment closest to the scalp represents the most recent drug exposures, as diagrammed in Figure 3. Subsequent segments represent earlier time periods, thereby describing changes in the average intensity of drug use from one period to the next (counting backward). It should be noted that these time period are approximated based on a consensus standard hair growth rate: time frames cannot be considered exact to the day or week.
Although the primary route of drug deposition into the hair is through the blood, drugs can also be deposited via sweat and sebum. Additionally, drugs may also be deposited onto the external part of the hair shaft through environmental contaminants such as smoke and residues (Henderson 1993, Mieczkowski 1997); this is of particular importance for the determination of frequent second-hand drug exposure for both caregivers and young children. The degree to which a drug is incorporated into the hair shaft depends on the physiochemical properties of that drug, how it interacts with the hair, its primary route of deposition, and the structural integrity of the hair strand itself. For instance, cannabinoids have been found to incorporate poorly into the hair (relative to other substances) just by virtue of the chemical structures (more acidic) and the way they interact with the hair (can not be trapped in acidic environment like hair melanocytes).
(Pragst, Balikova 2006). Alternatively, once deposited into the hair, cannabinoids are harder to remove through aggressive chemical treatments such as bleaching (Jurado et al. 1997). Overall, cocaine is thought to have the highest incorporation rate into hair and cannabinoids have the lowest, while opioids are relatively in the middle (Nakahara, Takahashi & Kikura 1995).

Just as there are a variety of processes for drug deposition, there are also processes that contribute to the removal of drugs from hair. While regular shampooing is not a major contributor to the removal of drugs, aggressive cosmetic agents can variably decrease drug concentrations. Studies vary in their observations, but overall it is estimated approximately 30-60% of drug content can be removed through cosmetic hair treatment; cannabis is least affected followed by cocaine and then opiates (such as morphine) (Jurado et al. 1997, Cirimele, Kintz & Mangin 1995). The extent of such removal is thought to be highly dependent on original concentrations of drugs as well as conditions of the hair strand (i.e. severely damaged and porous vs. relatively healthy and structurally integral) (Skopp, Potsch & Moeller 1997). Figure 4 illustrates processes of deposition and removal of substances to the hair.
Overall, while the deposition, retention, and stability of drugs in hair is considered good, it is by no means perfect and is likely a large source of variation among hair test results. The type of drug tested, mechanism of incorporation and removal, and sources of environmental exposure should all be considered in devising the most accurate interpretation of such results. Ultimately, while cosmetic hair treatment can reduce the levels of drugs determined through hair analysis, false negative results are rare and regular drugs users are generally still identified.
1.3.2.2 Meconium for the Detection of *in utero* Opiate Exposure

Meconium is the contents of a neonate’s first few bowel movements and is characterized by a dark, shiny texture and lack of odor. Meconium begins to form during fetal life around the 12th week of pregnancy, corresponding to the time frame of initiation of fetal swallowing (Gareri, Klein & Koren 2006). Drugs and alcohol metabolites (Fatty Acid Ethyl Esters or “FAEE”) are incorporated into meconium through their presence in the shared maternal-fetal circulation and concentrated in meconium through fetal swallowing and digestion of amniotic fluid (Ostrea et al. 1994). The fetus may also release drug-containing urine into the amniotic fluid that is later swallowed and subject to metabolism in the gastrointestinal tract (and therefore deposited into the meconium) (Moore, Negrusz & Lewis 1998).

Evidence that formation of meconium begins around the 12th week of pregnancy translates to the notion that drugs and FAEEs accumulate in meconium throughout the remainder of the pregnancy (i.e. the second and third trimesters) until delivery, at which point the meconium is passed and can be sampled for analysis. Recent data has emerged indicating that third trimester exposure to drugs is more closely associated with positive meconium results than second trimester exposures. This suggests that while it is possible both the second and third trimester are represented in a meconium sample, the meconium drug-positive neonate is at a higher risk for late pregnancy drug exposure (Kacinko et al. 2008).
Meconium is optimally collected within twenty-four hours of birth; however some neonates are known to pass meconium for several days after birth thereby enabling later sample collection. After three days post-partum, it is highly unlikely that meconium will still be available: the newborn will likely have started to pass stool, which results from post-natal digestion and no longer reflects in utero exposures (Gourley, Kreamer & Arend 1990, Ostrea, Parks & Brady 1988, Verma, Dhanireddy 1993, Ostrea et al. 2001). Identification of in utero exposures to drugs, especially opioids, is of great importance since many neonatal complications may result.

1.3.2.3 Neonatal Hair for the Detection of in utero opiate Exposures

Neonatal hair is a unique and valuable matrix when evaluating in utero drug exposure. Akin to maternal hair, follicular incorporation of drugs into this specimen is an important mechanism of deposition; however deposition from the drug containing amniotic fluid plays a significant role as well (Bailey, Klein & Koren 1997). Neonatal hair begins to grow at approximately 28 weeks in utero, reflecting late pregnancy drug use at a time where the mother most likely knew she was pregnant (Bailey, Klein & Koren 1997). This highlights that use of illicit or non-prescribed drugs during the last trimester was occurring, which is a strong risk factor for possible maternal addiction to these substances. Moreover, neonatal exposure to drugs in pregnancy, especially late pregnancy, may precipitate undesirable symptoms in the neonate—both immediately following birth and long-term— and must be considered (Vinner et al. 2003a, Vinner et al. 2003b).
One study found that for cocaine, benzoylecgonine, and cannabis, meconium testing seemed to be more sensitive (95% and above) than neonatal hair testing for the detection of *in utero* exposures (Bar-Oz et al. 2003). This may be partly explained by the earlier formation of meconium compared with hair (roughly the second trimester compared with the third trimester). A significant advantage to neonatal hair testing, however, is that the prenatally grown hair can remain on the infants scalp up until 3-5 months post-partum. This allows for the determination of prenatal drug exposure history well after birth and after the short window for meconium collection has passed (Bar-Oz et al. 2003).
1.4 Detection Methods for Opiates in Hair

1.4.1 Immunologic Techniques

The determination of opioid exposures through hair analysis began in 1979 with immunological techniques, specifically through the employment of a radioimmunoassay (RIA) (Baumgartner et al. 1979). Since then, enzyme linked immunosorbent assay (ELISA) test kits have been developed for the detection of a variety of drugs of abuse, including opioids, and have been determined to be sufficiently sensitive for application to hair analysis (Spiehler 2000, Segura et al. 1999). For example, the Cozart® microplate ELISA kit for opiates was reported in the literature to demonstrate a sensitivity of 98% and 93% respectively for 106 hair specimens with a cut off of 0.2 ng/mg (Cooper et al. 2003). Typically though, immunoassays are used as rapid screening tools and are generally not confirmatory as these techniques have not gained general acceptance due to their lack of specificity. For instance, these tests may determine the class of drug but not the individual substance i.e. opioids vs. morphine, respectively. Therefore, positive test results should be confirmed with chromatographic techniques (i.e. with gas chromatography-mass spectrometry, or GC-MS) (Pragst, Balikova 2006).

1.4.2 Chromatographic Methods

Since a variety of licit and illicit substances comprise the opioid class, which can not specifically be determined using immunoassays, more definitive testing was required. This led to the progression to a variety of confirmatory chromatographic and spectrometric methods over the past 3 decades, including direct probe tandem mass spectrometry (Pelli et al. 1987), high performance liquid chromatography (HPLC) (Marigo et al. 1986), gas chromatography-mass
spectrometry (GC-MS) (Gambelunghe et al. 2005, Kintz, Ludes & Mangin 1992, Lachenmeier, Musshoff & Madea 2006, Romolo et al. 2003, Wu et al. 2008), and liquid chromatography-tandem mass spectrometry (LC-MS/MS) (Klys et al. 2007, Miller, Wylie & Oliver 2008, Moore et al. 2008, Xiang et al. 2006). While LC-MS/MS is typically more sensitive, the method is still restricted to a handful of laboratories due to expense; therefore GC-MS confirmation remains to be the standard method for evaluating opioid exposures in hair (Pragst, Balikova 2006).

There are numerous advantages to using GS-MS, but the highly specific and sensitive nature of this technique is most important; the procedures offer the ability for detection of a large variety of drugs and/or metabolites with excellent accuracy at very low concentrations. This is important for hair analysis since concentrations of drugs tend to be in the nanogram and even picogram range. The main prerequisite for substance detection using GC-MS is volatility and stability at high temperatures; however drugs with free amino (NH2), hydroxyl (OH) and carboxyl (COOH) groups do not possess these characteristics and must be derivitized first (Pragst, Balikova 2006). Overall, derivitization as well as differences and optimization of other sample preparation parameters allow for more precise detection of anylates using GS-MS procedures.

1.4.3 Sample Preparation: Solid Phase Microextraction

Evolution in hair analysis is in part due to advancements in sample preparation techniques. Typically, specimen pretreatment for GC-MS confirmation involves lengthy and cost-consuming methods; therefore the development of efficient, robust, and fast procedures are readily welcomed to the field. Solid phase microextraction (SPME) offers the aforementioned
advantages, in addition to being a solvent-free procedure, sensitive, and fully automated (Ulrich 2000). A method developed in 1989 by Pawliszyn and colleagues (Arthur, Pawliszyn 1990), SPME is now widely used, especially in toxicological laboratories.

SPME involves absorption of the analyte onto a specially coated fiber, which is then transferred to the GC injector port and subsequently desorbed for analysis by GC-MS. Headspace SPME (HS-SPME) allows for the extraction of semi volatile and volatile compounds from the headspace directly above the extract and is considered a cleaner sample preparation method as it can be performed without the involvement of organic solvents. The fiber is regenerated during sample desorption at 250 degrees, thereby avoiding sample carryover (Pragst, Balikova 2006). Figure 5 illustrates the principle of HS-SPME.
Figure 5: Basic illustration of HS-SPME. Semi-volatile drugs may be sampled/extracted directly from the headspace above the solution (extract), then desorbed into the GC-injection port for analysis. (Source: Pragst, 2006)
1.5 Statement of problems

1.5.1 Method Development for the Detection of Opiate Exposures in Hair

Hair analysis for drugs of abuse has evolved to become a powerful and widely used tool in forensic and clinical toxicology. This alternative matrix is often preferred over traditional matrices (urine and blood) due to its various practical advantages, including ease of collection, long window of detection, and stability of the specimen. Furthermore, segmental analysis can be very useful in the surveillance of drug abuse as it offers a detailed account of drug exposures over time (Pragst, Balikova 2006). Moreover, opiate hair analysis continues to prove difficult due to the scarcity of the hair sample and low drug concentration; knowledge of the specific type of opiate present in the specimen is often desired as well. Therefore, the development of sensitive and rapid techniques for the detection of drugs of abuse in hair is warranted to assist and improve the abilities of forensic and clinical toxicologists alike.

1.5.2 Maternal-Fetal Opiate Exposures

While it is largely known that opioids do cross the placenta and may inflict neonatal complications (Garland et al. 2008, Nanovskaya et al. 2002, Gerdin, Rane & Lindberg 1990), therapeutic use of these medications in pregnancy are not necessarily contraindicated. For example, the treatment of opioid addiction and use with methadone has been shown to decrease maternal and fetal morbidity and mortality and promote fetal stability and growth (Kandall et al. 1999). This is despite the fact that neonates exposed in utero to methadone can and may develop NAS, however. Moreover, maternal fluctuations of short-acting opioids may lead to fetal withdrawal or overdose (Bandstra et al. 2010). Furthermore, infants born to short-acting opioid-dependent mothers are frequently faced with significant postnatal problems, including jaundice,
aspiration pneumonia, transient tachypnea, infection (Finnegan et al. 1975, Finnegan 1985). Therefore, pharmacovigilence is warranted when treating the pregnant mother with opioid containing medications.

Since the prevalence of _in utero_ opioid exposed neonates may be high in some centres and frequently requires supportive care (Anonymous 1998b), identification of such at-risk infants is paramount. Maternal characteristics of the opioid abusing pregnant woman may also be cause for concern; substance abusing pregnant women are typically at high risk for malnourishment (Finnegan 1985), lack of adequate obstetric care, and these individuals typically reside in incompatible and often violent environments (Johnson, Gerada & Greenough 2003a). Therefore it is important to monitor opioid use in pregnancy, and identify potential drug abuse if possible; drug testing has played a considerable role in the evaluation of maternal and neonatal opioid exposures (Vinner et al. 2003b, Fendrich et al. 2004, Musshoff et al. 2006, Fishbain et al. 1999).
1.6 Specific Aims and Objectives

1.6.1 Method Development for the Detection of Opiate Exposures in Hair

This study aimed to develop a novel, optimized, and sensitive technique for the confirmation of 3 principal opiates, morphine, codeine, and the metabolite of heroin (6-monoacetylmorphine, or 6MAM) in human hair specimens using HS-SPME coupled with GC-MS. The detection of the parent compound heroin is nearly impossible since it is very rapidly metabolized once administered; therefore the detection of 6MAM, which persists in the body for much longer, serves to indicate heroin was the opioid used.

1.6.2 Estimation of Maternal-Fetal Opiate Exposures

This study aimed to isolate trends in neonatal opiate exposure and co-exposure by other drugs of abuse and alcohol through hair and meconium test results. In addition, the characterization of maternal-fetal passage of opiates by the placenta was assessed using hair test results of mother-infant dyads analyzed for opiates.
1.7 Rationale and Hypotheses

1.7.1 Method Development for the Detection of Opiate Exposures in Hair

HS-SPME methods have been developed and applied for the detection of environmental contaminants, chemicals, and a variety of drugs of abuse; however a method for the detection of opiates has yet to be reported. While opiates are not considerably volatile for the proposed HS-SPME method, the molecules can be derivatized (silylated) to achieve the necessary volatility. It was hypothesized that a sensitive HS-SPME method coupled with GC-MS could be developed and optimized for the detection of opiates (morphine, codeine, and 6MAM) in human hair.

1.7.2 Estimation of Maternal-Fetal Opiate Exposures

I hypothesized that 1) through the exploitation of maternal and neonatal hair and meconium specimens, maternal opiate use patterns in pregnancy and subsequent in utero fetal exposures may be determined. Moreover, since opioids readily cross the placenta and can precipitate undesirable sequelae in the neonate, it was hypothesized that 2) there would be good correlation between maternal-neonatal hair test results for opiates. Depending on the length of maternal hair tested, a correlation between maternal hair and neonatal meconium concentrations of opiates may also exist.
2. METHODOLOGY

2.1 Method Development for Detection of Opiate Exposure

2.1.1 Reagents

Codeine, morphine, and 6-acetylmorphine and the deuterated standards (codeine-d₃, morphine-d₃ and 6-acetylmorphine-d₃) were obtained in sealed ampoules from Cerilliant Corporation (Round Rock, TX) at concentrations of 1 mg/ml in methanol except 6-acetylmorphine and 6-acetylmorphine-d₃ that were in acetonitrile. BSTFA (N,O-bis(Trimethylsilyl)trifluoroacetamide), BSTFA+1% TMCS (Trimethylchlorosilane), and TFAA (Trifluoroacetic Acid Anhydride) were purchased from Pierce (Rockford, IL). Methanol and acetonitrile were of scientific grade and purchased from Sigma Aldrich (Oakville, ON).

2.1.2 Specimen Collection

Hair samples were submitted to the Motherisk Laboratory at the Hospital for Sick Children in Toronto for the analysis of opiates as well as other drugs of abuse/alcohol. Hair samples were obtained by cutting the strands as closely as possible to the scalp, then adhered to a clean piece of paper with the scalp end indicated accordingly. The samples were stored in dry conditions at ambient temperatures. The length of the clinical samples tested ranged from 1 cm to 4 cm on the proximal end. Drug free hair was obtained from laboratory personnel with no known opioid exposures. Blank hair from 6 volunteers was pooled and the absence of morphine, codeine, and 6-acetylmorphine was confirmed using GC-MS.
2.1.3 Preparation of Stock Solution and Standards

Individual stock solutions for codeine, morphine, and 6-acetylmorphine were prepared by adding 10 µL of each drug at an original concentration of 1 mg/ml to 990 µl of methanol (or acetonitrile for 6-acetylmorphine and its deuterated standard), and then diluted again to provide concentrations of 10 µg/ml and 100 ng/ml respectively. These were used to generate dilutions of 50, 20, 10, 5, 1, 0.5, and 0.1 ng/ml in methanol and/or methanol+hair combinations for calibration curves and determination of limits of detection and quantitation. Approximately 10 mg of hair was used in these experiments, therefore the concentrations of opiates/mg in hair were correspondingly 5, 2, 1, 0.5, 0.1, 0.05, and 0.01 ng/mg respectively. Deuterated codeine, morphine, and 6-acetylmorphine (D₃-opiates) were used as suitable internal standards at concentrations of 20 ng/ml or 2.0 ng/mg. Pure standards were injected directly on the GC-MS to ensure their reliability and reproducibility (linearity) at these concentrations.

2.1.4 Sample Preparation

Approximately 10 mg of clinical sample or pooled drug-free hair was aliquoted into 25 ml flat bottom glass vials. Deuterated opiate (-d₃) standards were added at concentrations previously described and the hair was chopped with scissors into a fine mulch. Methanol was added, the vials were capped, sealed with parafilm and incubated at 56°C for 18 hours (overnight) with agitation. The methanolic extract was decanted into 10 ml SPME vials using Pasteur pipettes and then dried under nitrogen (N₂). Finally, 10 µl of BSTFA+1%TMCS was added, and the vials were loaded onto the autosampler and subject to HS-SPME coupled with GC-MS.
2.1.5 Derivitizing Agents

A variety of derivitizing agents were investigated, including BSTFA, BSTFA+1% TMCS, and TFAA were all included in these experiments, and optimal volumes were deduced. Derivitization with these agents was attempted using the SPME method as well as direct injection into the GC/MS. Ethyl acetate (EA) was originally included in the derivitization mixture at a ratio of 3:2 EA to BSTFA. Unfortunately, repeated experiments demonstrated that EA caused the coating of the SPME fiber to swell which resulted in the assembly to crunch and break during auto-sampling. 20 ul of BSTFA+1% TMCS was added (BSTFA without TMCS and TFAA were tried as well). The vials were placed on the autosampler and subject to extraction and GC/MS analysis.

2.1.6 Direct Injection Sample Preparation Method

**BSTFA**

After drying under N₂, 30 ul BSTFA+1% TMCS and 20 ul of ethyl acetate were added to the clear glass conical vials, vortexed, capped immediately, and placed on a dry heating block at 70 degrees Celsius for 25 minutes. After heating, the solution was allowed to cool and then transferred to 50 ul spring glass inserts housed in brown glass autosampler injection vials (products!). Vials were capped immediately and placed on the autosampler for direct injection. One microlitre of the derivitized methanolic extract was injected into the GC injector port and subject to analysis by the same method as that used for the SPME preparation.

**TFAA**

50 ul of TFAA was added to the vials and vortexed. The vials were then heated at 80 degrees Celsius for 30 minutes. The solution was dried down again under B2, and the extract was
reconstituted in 50 ul of ethyl acetate. The solution was passed into 50 ul spring glass inserts housed in brown autosampler injection vials and capped immediately. One microliter was injected onto the GC/MS.

2.1.7 HS-SPME Sample Preparation Method

BSTFA
After drying under N₂, 30 ul BSTFA+1% TMCS (± 20 ul of ethyl acetate) were added to SPME autosampler vials containing standards or samples. Vials were capped immediately and placed on the autosampler for HS-SPME and GC/MS analysis.

TFAA
50 ul of TFAA (± 50 ul ethyl acetate) was added to the vials, capped, and placed on the autosampler for HS-SPME and GC/MS analysis.

2.1.8 Autosampler Conditions for SPME

Conditions were optimized to produce the highest area counts and sharpest peaks as computed by GC/MS analysis. Agitation time, temperature, needle penetration, and fiber coatings were among the altered parameters and tested in triplicate. Agitation times tested were 10, 15, 20, 25, and 30 minutes. Temperatures ranged from 70 to 130 degrees Celsius. Needle penetration into the GC injector varied from 54 to 45 um. Lastly, multiple SPME fiber assemblies were used for the experimentation of different fiber coatings. 100 um and 30 um PDMS (Polydimethylsiloxane) non-bonded coatings as well as a 65 um PDMS/DVB (Divinylbenzene) bonded coating were obtained from Supelco (Belfonte, PA) and used. These fibers had similar practical guidelines of utilization which was useful for consistency between
experiments, including recommended maximum, operating, and conditioning temperatures, as well as conditioning times. Other SPME autosampler parameters included 300 seconds (5 minutes) pre-incubation time, agitation for one minute with 15 second intervals, 1200 second (20 minute) extraction time, and 600 second (10 minute) desorption time.

2.1.9 Gas Chromatography-Mass Spectrometry

All experiments were completed on a QP2010 GC/MS coupled with a AOC-5000 Autosampler (Shimadzu, Columbia, MD, USA). The separation was achieved on a FactorFour Capillary Column (30 m, 0.25 mm, i.d. 0.25 film thickness; Varian, Inc, Palo Alto, CA, USA). Peak integration and analysis was performed using Shimadzu GCMSsolution Version 2.50 software. Fibers composed of 100 µm and 30 µm Polydimethylsiloxane (PDMS) non-bonded coatings, as well as a 65 um PDMS/DVB (Divinylbenzene) bonded coating were obtained from Supelco (Bellfonte, PA). Each fiber was used for no more than 100 injections. SPME vials (10 mL) were obtained from Supelco (Belfonte, PA). High purity helium was used as the carrier gas.

The instrument parameters for the GC were as follows: column oven temperature of 70 degrees Celsius, injection temperature of 260 degrees in splitless injection mode; 1.30 ml/min column flow and 3 ml/min purge flow; pressure was set as 61.5 kPa, total flow at 46.1 ml/min, and linear velocity at 36.7 cm/sec, and; the column oven temperature program began at 70 degrees held for 2 minutes, then increased at a rate of 15 degrees per minute until reaching 220 degrees and held for another 2 minutes, then increased at a rate of 5 degrees per minute until 255 degrees, at which point the temperature ramp increased quickly to 30 degrees per minute until reaching 300 degrees and was held here for 3 minutes. The instrument parameters for the MS
were as follows: ion source and interface temperatures were 230 and 310 degrees respectively; the detector gain was operating relative to tune (between 0.65 and 0.7 kV above tune); the solvent cut time was 6.50 minutes, the detector start time was 10 minutes, and the end time was 25.50 minutes, and the MS was operating in scan mode for m/z between 85 and 500 amu at an interval of 0.5 seconds.

The retention times for the BSTFA-derivitized opiates were 21.581, 21.354, and 22.381 minutes; for the deuterated standards the retention times were 21.553, 21.323, and 22.381 minutes. Quantification was performed by taking the ratio between the peak areas for the molecular ions (m/z) of the opiate TMS-derivatives morphine, codeine, and 6-acetylmorphine (429, 371, and 399 respectively) to their TMS-derivative deuterated standards (432, 374, and 402 respectively).

2.1.10 Calculation of Method Limits

Limits of detection (LOD) and limits of quantification (LOQ) were calculated using Shimadzu GCMSsolution Version 2.50 software; the signal to noise calculation function was used. The LODs were defined as those concentrations of each opiate found to exhibit a signal to noise ratio of greater than 3. Limits of quantification were defined as those concentrations of each opiate found to exhibit a signal to noise ratio of 10. Precision and accuracy data were obtained using two different concentrations- one high (10 ng/ml) and one low (and 1 ng/ml).
2.1.11 Method Validation Using Clinical Samples

Hair samples from nine different sources (donors) tested positive for opiates by ELISA in our laboratory were tested using the optimized HS-SPME GC/MS method described. If sufficient quantity allowed, duplicates of these samples as well as multiple segments were tested. Additionally, in one case the specimen had also been sent to a US drug testing facility for the LC/MS/MS confirmation of morphine, codeine, and 6-acetylmorphine and compared to our results.

Approximately 10 mg of hair from these clinical samples was chopped in methanol already containing the deuterated internal standards of the three opiates (at a concentration of 20 ng/ml or 2.0 ng/mg). Hair was incubated and agitated overnight as previously described (section 2.2.3); the methanolic extract was then decanted, dried down, dervitized with BSTFA+TMCS, and subject to the optimized HS-SPME GC/MS method for analysis. A five point standard curve created by spiking pooled blank hair with the pure standards and their deuterated counterparts was also included, as well as appropriate quality control samples.
2.2 Estimation of Maternal-Fetal Exposures to Opiates

2.2.1 Study Population

The Motherisk Program in the Division of Clinical Pharmacology and Toxicology at the Hospital for Sick Children receives thousands of hair and meconium specimens from across Canada testing of licit and illicit drugs each year. Requisitions of specimen analysis usually come from child protection authorities (Children’s Aid Societies) or health care providers and follows suspicion of parental substance abuse. The tested population primarily includes women and their children, although many paternal samples are also tested and depends on the requirements of the authorities. Between June 2007 and January 2009, nearly 9000 specimens were received for analysis, and of these, over 1500 neonates were assessed for opiate drug exposure via hair and/or meconium (396 neonates were tested using both hair and meconium).

2.2.2 Hair and Meconium Analysis for Drugs of Abuse and Alcohol

The analyses for alcohol and drugs of abuse are routinely performed in the Motherisk Laboratory by established methods (Klein J, Bar-Oz, Hutson). Briefly, for hair analysis of drugs of abuse, hair was finely cut in 1 ml methanol and incubated overnight on agitation at 56 degrees Celsius. On the next day, the methanol extract was decanted and dried under N2 at 40 degrees Celsius. Next, 400 ul of PBS (phosphate buffer saline, pH7.4) was added and the individual drugs were analyzed by ELISA (enzyme linked immunosorbent assay) using their respective kits manufactured by Immunalysis (San Diego, CA). For meconium testing, approximately 0.5-1.0 g was extracted with methanol, centrifuged, and the supernatant diluted 1:5 with PBS. The PBS extract was analyzed using the ELISA kits previously described. FAEE analysis in meconium first required 50 mg of the specimen to be mixed with 1 ml PBS, transferred to HS-SPME
autosampler vials, and subject to GC/MS analysis for four principle FAEE, namely ethyl palmitate, ethyl linolate, ethyl oleate, and ethyl stearate. Results for FAEE concentration are recorded as a sum of these FAEE.

The opiate ELISA kit primarily detects, morphine, codeine, hydrocodone, hydromorphone, 6-acetylmorphine, 6-acetylcodeine, has limited cross-reactivity to oxycodone, oxymorphone, normorphine, norcodeine, noroxycodone, noroxymorphone, and nalorphine, and no cross-reactivity to other synthetic opioids such as methadone, meperidine, fentanyl and buprenorphine. See www.immunalysis.com for ELISA kit product information for specific drugs of abuse.

2.2.3 Identification of Neonatal Hair and Meconium Results Tested for Opiates

Hair and meconium drug test results are available in electronic (database) and hardcopy form in the laboratory. Ethics approval was obtained from the Research Ethics Board at the Hospital for Sick Children for the study of these results in order to identify trends of in utero drug exposure. Neonatal hair and meconium sample results (for all drugs/alcohol) were recorded/included if the specimen was tested for opiates. If the sample was not tested for opiates, the data were excluded. Results were recorded on a spreadsheet with only the corresponding sample number of the specimen and no other potentially identifying information. Throughout the study, all spreadsheets containing test result information were password protected and maintained on the secure Hospital server.
2.2.4 Identification of Mother-Infant Dyads Tested for Opiates

Hardcopy records of tests conducted between June 2007 and January 2009 were examined for potential mother-infant pairs. Hardcopy records were used because potentially identifying maternal information may have been included on requisition forms for neonatal specimens; such information may not have been available in the electronic database. In addition, the last name of any woman tested for opiates was used for an electronic database search for a potential infant pair.

2.2.5 Analysis of Neonatal Hair and Meconium Test Results

Descriptive statistics were used to characterize the trends of in utero exposure to drugs of abuse and alcohol. Logistic regression analysis was used to determine the likelihood of the presence of other drugs of abuse in conjunction with opiates in neonatal hair and meconium specimens. Odds ratios and their 95% Confidence Intervals (CI 95%) were calculated using SigmaStat Software. For some drugs of abuse that did not coexist with opiates in any samples, the odds ratios could not be calculated (eg. Benzodiazepines in neonatal hair).
3. RESULTS

3.1 Method Development for the Detection of Opiate Exposures

3.1.1 Optimization of Experimental Parameters

Optimization of this method was first carried out using pure standards and then progressed to using blank hair samples spiked with each of the three opiates and their deuterated standards at concentrations mentioned in methods section 2.1.2. Preliminary work was performed on an older, less sensitive GC/MS machine; therefore higher concentrations were used in these experiments (100 ng/ml (10 ng/mg) and 50 ng/ml (5 ng/mg)). Each experiment was performed with n=3 at each parameter. All experiments were performed on separate occasions, using the same source of pooled blank hair for consistency.

The choice of fiber was first assessed between the 100 um PDMS, 30 um PDMS, and 50 um PDMS-DVB fibers. All three fibers were capable of adsorbing the opiates, however the 100 um PDMS fiber produced the highest peak area and was therefore used throughout these experiments. Of importance, originally this method began with the use of ethyl acetate in conjunction with BSTFA for derivitization and sample preparation as we simply extrapolated conditions from the direct injection method. The ethyl acetate was found to swell the aforementioned fibers to the point where they would not be able to retract into the SPME fiber assembly and would subsequently break; therefore ethyl acetate was no longer used in the HS-SPME method.
The retention times for the BSTFA-derivitized opiates, or TMS-opiates, is summarized in Table 6. The ions monitored after silylation have been described in the literature (Kintz, Mangin 1995). Quantitation was performed by taking the ratio between the peak areas for the molecular ions (m/z) of the opiate TMS-derivatives morphine, codeine, and 6-acetylmorphine (429, 371, and 399 respectively) to their TMS-derivative deuterated standards (432, 374, and 402 respectively).

<table>
<thead>
<tr>
<th>Opiate</th>
<th>Ions Monitored</th>
<th>Deuterated (d₃) Ions</th>
<th>Retention Times</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Morphine</strong></td>
<td>*429, 414</td>
<td>*432, 417</td>
<td>21.36 / d₃ - 21.33</td>
</tr>
<tr>
<td><strong>Codeine</strong></td>
<td>*371, 343</td>
<td>*374, 436</td>
<td>21.10 / d₃ - 21.06</td>
</tr>
<tr>
<td><strong>6-Acetylmorphine</strong></td>
<td>*399, 340</td>
<td>*402, 343</td>
<td>22.22 / d₃ - 22.20</td>
</tr>
</tbody>
</table>

*Quantitation ion

### 3.1.1.1 Assessment of Optimal Incubation Temperatures

The effect of incubation temperature was assessed during two separate experiments. The first began with two incubation temperatures, one high (125 degrees Celsius) and one low (80 degrees Celsius). The second experiment assessed temperature over a more broad range, from 70 degrees to 130 degrees in 20 degree increments in order to support the findings of the first experiment. Overall, it was determined that 125 degrees Celsius conferred the highest peak area
counts for most opiates tested (figure 6), especially 6-acetylmorphine. In addition, this incubation temperature showed the greatest reproducibility and the least variability at two separate concentrations (100 ng/ml and 50 ng/ml). Therefore, 125 degrees Celsius was used as the incubation temperature throughout the HS-SPME method development.

Figure 6: The effect of incubation temperature on mean peak area counts for 100 ng/ml of morphine, codeine, and 6MAM. Data is expressed as the mean (n=3) ± S.D.
3.1.1.2 Assessment of Optimal Extraction Time

Next, the effect of extraction time on peak area using the HS-SPME method was measured from 10 to 30 minutes in 5 minute intervals. All three opiates, including their deuterated standards, achieved extraction equilibrium between the liquid phase and the fiber coating at 25 minutes, as demonstrated by figure 7. Therefore, 25 minutes was used.

![Figure 7: The effect of extraction time on peak area counts for 100 ng/ml morphine, codeine, and 6MAM. Data is expressed as the mean (n=3) ± S.D.](image-url)
3.1.2 Assessment of Linearity, Limits of Detection and Quantification

Finally, linearity was assessed using these optimized conditions using pure standards and then blank hair spiked with standards. When using pure standards, the concentrations ranged between 0.01 ng/ml – 10 ng/ml for morphine, codeine, and 6-acetylmorphine, and an $R^2 > 0.999$ was obtained for each opiate compound as seen in figure 8.

![Pure Standard Opiate Curve](image)

**Figure 8:** Standard curve of morphine, codeine, and 6-acetylmorphine using optimized methods for HS-SPME GC/MS analysis. Concentrations ranged from 0.1 ng/ml – 10 ng/ml.

When blank hair was spiked with pure standards and linearity was assessed, concentrations ranged from (0.005 for codeine) 0.01 ng/mg – 5 ng/mg. Correlation coefficients ($R^2$) all resided about 0.992, as seen in figure 9 and summarized in Table 2.
Table 2: Linear Regression*, Limits of Detection and Quantification

<table>
<thead>
<tr>
<th></th>
<th>Linearity (ng/mg)</th>
<th>m</th>
<th>b</th>
<th>R²</th>
<th>LOD (ng/mg)</th>
<th>LOQ (ng/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Codeine</td>
<td>0.005 – 5.0</td>
<td>0.5272</td>
<td>0.0543</td>
<td>0.9975</td>
<td>0.002</td>
<td>0.005</td>
</tr>
<tr>
<td>Morphine</td>
<td>0.01 – 5.0</td>
<td>0.6342</td>
<td>0.117</td>
<td>0.9976</td>
<td>0.005</td>
<td>0.01</td>
</tr>
<tr>
<td>6MAM</td>
<td>0.01 – 5.0</td>
<td>0.2525</td>
<td>0.3743</td>
<td>0.9921</td>
<td>0.005</td>
<td>0.01</td>
</tr>
</tbody>
</table>

*m=gradient; b=axis intercept; R²= correlation coefficient
Figure 9: Standard curve of morphine, codeine, and 6-monoacetylmorphine spiked in pooled blank hair using the optimized HS-SPME GC/MS method.

Limits of detection (LOD) were defined as those concentrations of each opiate found to exhibit a signal to noise ratio of greater than 3; the LOD was 0.005 ng/mg for morphine and 6-acetylmorphine and 0.002 ng/mg for codeine. Limits of quantification (LOQ) were defined as those concentrations of each opiate found to exhibit a signal to noise ratio of 10; the LOQ for morphine and 6MAM was 0.01 ng/mg and 0.005 ng/mg for codeine. Precision and accuracy data were obtained using two different concentrations with n=5 at each concentration: 5 ng/mg and 0.5 ng/mg. Intra-day and inter-day precision data is summarized in Table 3.
Table 3: Intraday and Interday Precision at Concentrations of 0.5 ng/mg and 5.0 ng/mg

<table>
<thead>
<tr>
<th></th>
<th>0.5 ng/mg</th>
<th></th>
<th>5.0 ng/mg</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Intraday P (%)</td>
<td>Interday P (%)</td>
<td>Intraday P (%)</td>
<td>Interday P (%)</td>
</tr>
<tr>
<td>Codeine</td>
<td>3.44</td>
<td>11.46</td>
<td>3.05</td>
<td>4.17</td>
</tr>
<tr>
<td>Morphine</td>
<td>5.11</td>
<td>5.26</td>
<td>5.04</td>
<td>14.34</td>
</tr>
<tr>
<td>6MAM</td>
<td>5.14</td>
<td>14.77</td>
<td>4.07</td>
<td>5.51</td>
</tr>
</tbody>
</table>

Figures 10 and 11 represent typical chromatograms seen in blank hair without the opiates (but with internal standard) and in blank hair spiked with morphine, codeine, and 6-acetylmorphine and their deuterated internal standards.

**Figure 10:** Typical chromatogram of blank hair sample (i.e. not spiked with any opiate but including the deuterated internal standards at a concentration of 1 ng/mg).
3.1.3 Application of HS-SPME Method to Clinical Samples

As previously described, we assessed clinical hair samples for opiates using our optimized HS-SPME method. In total, 12 hair samples were tested using our described method. Figure 12 below shows a typical chromatogram obtained from an opiate positive specimen (Sample 1A).

Figure 11: Typical Chromatogram of opiates in hair at a concentration of 1 ng/mg.
Figure 12: Typical chromatogram obtained from a clinical sample testing positive for codeine, morphine, and 6MAM. Peaks for each drug and their deuterated standards are indicated as well as their retention times.

Ten of the samples were positive as determined by ELISA; of these, nine were positive using our method indicating one ELISA positive test result may have been either false or due to consumption of other opioids such as hydromorphone or hydrocodone (which our assay did not detect). Two samples were determined by ELISA to be below limit of detection and these results were confirmed by this HS-SPME method as well. One positive and one negative sample were confirmed by the US Drug Testing Laboratory using LC-MS/MS; our results were consistent with this. Furthermore, the confirmed positive opiate test results for sample 1 (A and B)
indicated the presence of morphine, codeine, and 6MAM by our newly developed HS-SPME method as well as the US facility. A summary of opiate concentrations obtained using our newly developed GC/MS method as compared to the ELISA method (and LC-MS/MS confirmations when available) is summarized in Table 4.

<table>
<thead>
<tr>
<th>SAMPLE ID</th>
<th>Morphine</th>
<th>Codeine</th>
<th>6MAM</th>
<th>Total</th>
<th>LC-MS/MS* or ELISA</th>
</tr>
</thead>
<tbody>
<tr>
<td>1A*</td>
<td>0.503</td>
<td>0.334</td>
<td>1.333</td>
<td>2.17</td>
<td>&gt; 4*</td>
</tr>
<tr>
<td>1B*</td>
<td>0.484</td>
<td>0.341</td>
<td>1.301</td>
<td>2.126</td>
<td>&gt; 4*</td>
</tr>
<tr>
<td>2</td>
<td>Below LOD</td>
<td>0.536</td>
<td>Below LOD</td>
<td>0.536</td>
<td>6.99</td>
</tr>
<tr>
<td>3 (0-2 cm)</td>
<td>Below LOD</td>
<td>0.582</td>
<td>Below LOD</td>
<td>0.582</td>
<td>4.06</td>
</tr>
<tr>
<td>3 (2-4 cm)</td>
<td>Below LOD</td>
<td>0.377</td>
<td>Below LOD</td>
<td>0.377</td>
<td>3.7</td>
</tr>
<tr>
<td>4A</td>
<td>Below LOD</td>
<td>0.457</td>
<td>Below LOD</td>
<td>0.457</td>
<td>5.36</td>
</tr>
<tr>
<td>4B</td>
<td>Below LOD</td>
<td>0.396</td>
<td>Below LOD</td>
<td>0.396</td>
<td>5.36</td>
</tr>
<tr>
<td>5</td>
<td>Below LOD</td>
<td>0.947</td>
<td>Below LOD</td>
<td>0.947</td>
<td>5.72</td>
</tr>
<tr>
<td>6 (0-3)A</td>
<td>Below LOD</td>
<td>0.977</td>
<td>Below LOD</td>
<td>0.977</td>
<td>5.08</td>
</tr>
<tr>
<td>6 (0-3)B</td>
<td>Below LOD</td>
<td>1.036</td>
<td>Below LOD</td>
<td>1.036</td>
<td>5.08</td>
</tr>
<tr>
<td>6 (3-6)A</td>
<td>Below LOD</td>
<td>0.243</td>
<td>Below LOD</td>
<td>0.243</td>
<td>4.41</td>
</tr>
<tr>
<td>6 (3-6)B</td>
<td>Below LOD</td>
<td>0.212</td>
<td>Below LOD</td>
<td>0.212</td>
<td>4.41</td>
</tr>
<tr>
<td>7</td>
<td>Below LOD</td>
<td>0.571</td>
<td>Below LOD</td>
<td>0.571</td>
<td>2.16</td>
</tr>
<tr>
<td>8A</td>
<td>Below LOD</td>
<td>0.425</td>
<td>Below LOD</td>
<td>0.425</td>
<td>5.01</td>
</tr>
<tr>
<td>8B</td>
<td>Below LOD</td>
<td>0.496</td>
<td>Below LOD</td>
<td>0.496</td>
<td>5.01</td>
</tr>
<tr>
<td>9</td>
<td>Below LOD</td>
<td>Below LOD</td>
<td>Below LOD</td>
<td>Below LOD</td>
<td>3.14</td>
</tr>
<tr>
<td>10</td>
<td>Below LOD</td>
<td>2.896</td>
<td>Below LOD</td>
<td>2.896</td>
<td>4.98</td>
</tr>
<tr>
<td>11*</td>
<td>Below LOD</td>
<td>Below LOD</td>
<td>Below LOD</td>
<td>Below LOD</td>
<td>Below LOD*</td>
</tr>
<tr>
<td>12</td>
<td>Below LOD</td>
<td>Below LOD</td>
<td>Below LOD</td>
<td>Below LOD</td>
<td>Below LOD</td>
</tr>
</tbody>
</table>

*LC-MS/MS confirmed
Highlighted specimens are duplicates from the same sample.
3.2 Estimation of Maternal-Fetal Opiate Exposures

3.2.1 Neonatal Hair and Meconium Results for Opiates

1515 neonates were tested for opiates in hair and meconium from June 2007 to January 2009 from which 563 hair and 1318 meconium specimens were assessed. Of these, 64/563 (11.4%) and 224/1318 (17.0%) tested positive for opiates in hair and meconium respectively. Additionally, 396 neonates were tested for opiates using both hair and meconium specimens; 37/396 (9.3%) infants tested positive in both hair and meconium, 36/396 (9.1%) tested positive only in the meconium but not in hair, and 10 (2.5%) tested positive in the hair and not meconium. From these values, neonates testing positive for opiates in meconium had an odds ratio of 32.17 (95% CI 14.76–70.11) for also testing positive in hair.

3.2.2 Neonatal Hair Test Results for Opiates and Other Drugs of Abuse

Of the available 563 neonatal hair specimens tested for opiates, 550 were also tested for other drugs of abuse. 32/550 (5.8%) tested positive for opiates and one or more other substances, 30/550 (5.4%) tested positive for opiates but negative for other substances, and 185 (33.6%) tested negative for opiates but positive for other substances. The breakdown of frequency of co-exposures to other drugs of abuse as determined by hair testing is outlined in Table 5. The odds ratio calculated for testing positive for other drugs of abuse if testing positive for opiates was significant at 1.75 (95% CI 1.03–2.97). Hence, infants were more likely to be co-exposed to other drugs of abuse if testing positive for opiates, as determined by hair testing.
Table 5: Drug co-exposures among positive and negative opiate hair tests

<table>
<thead>
<tr>
<th>Drug Co-exposure Frequency in Hair</th>
<th>Opiate Positive</th>
<th>Opiate Negative</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of different drugs</td>
<td>n</td>
<td>%</td>
</tr>
<tr>
<td>1</td>
<td>30</td>
<td>48.4</td>
</tr>
<tr>
<td>2</td>
<td>30</td>
<td>48.4</td>
</tr>
<tr>
<td>3</td>
<td>2</td>
<td>3.2</td>
</tr>
<tr>
<td>Total</td>
<td>62</td>
<td>100</td>
</tr>
</tbody>
</table>

Evidently, many other drugs of abuse are frequently tested for alongside opiates, the most common being cocaine (558/563 or 99.1%) and cannabinoids (504 or 89.5%), followed by barbiturates (163 or 29%), amphetamine and methamphetamine (161 and 148, or 28.6% and 26.3% respectively), benzodiazepines (94 or 16.7%), oxycodone (50 or 8.9%) and methadone (45 or 8.0%). Among neonates tested positive for opiates, the odds ratios for testing positive for cocaine/BE were calculated to be 1.58 and 1.61 (95% CI\textsubscript{cocaine} 0.90-2.76, CI\textsubscript{BE} 0.92-2.82). For marijuana, the odds ratio was 0.97 (95% CI 0.44-2.16), for methadone the odds ratio was 5.5 (95% CI 0.89-33.99), and for oxycodone the odds ratio was 66 (95% CI 4.57-953.28). Positive benzodiazepine, barbiturate, amphetamine, methamphetamine and nicotine hair tests did not coincide with positive opiate hair tests, precluding the calculation of odds ratios for these drugs. Additionally, there were no positive results for phencyclidine, and meperidine in this neonatal study population. FAEE cannot currently be detected in neonatal hair in any laboratory, but effort is underway to develop methods suitable for this analysis. The statistics described above are summarized in Table 6.
Table 6: Itemized drug co-exposures as determined by neonatal hair testing and the corresponding odds ratios

<table>
<thead>
<tr>
<th>Drugs</th>
<th>Test (opiate tested population)</th>
<th>Positive‡</th>
<th>Simultaneous Positive†</th>
<th>Odds Ratio (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n&lt;sup&gt;a&lt;/sup&gt;</td>
<td>%</td>
<td>n&lt;sup&gt;b&lt;/sup&gt;</td>
<td>%</td>
</tr>
<tr>
<td>Cocaine</td>
<td>558</td>
<td>99.1</td>
<td>150</td>
<td>26.9</td>
</tr>
<tr>
<td>Cannabinoids</td>
<td>504</td>
<td>89.5</td>
<td>76</td>
<td>15.1</td>
</tr>
<tr>
<td>Barbiturates</td>
<td>163</td>
<td>29</td>
<td>1</td>
<td>0.6</td>
</tr>
<tr>
<td>Amphetamine</td>
<td>161</td>
<td>28.6</td>
<td>1</td>
<td>0.6</td>
</tr>
<tr>
<td>Methamphetamine</td>
<td>148</td>
<td>26.3</td>
<td>3</td>
<td>2.0</td>
</tr>
<tr>
<td>Benzodiazepines</td>
<td>94</td>
<td>16.7</td>
<td>1</td>
<td>1.1</td>
</tr>
<tr>
<td>Oxycodone</td>
<td>50</td>
<td>8.9</td>
<td>4</td>
<td>8.0</td>
</tr>
<tr>
<td>Methadone</td>
<td>45</td>
<td>8.0</td>
<td>9</td>
<td>20.0</td>
</tr>
<tr>
<td>Phencyclidine</td>
<td>45</td>
<td>8.0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Meperidine</td>
<td>23</td>
<td>4.1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Nicotine</td>
<td>8</td>
<td>1.4</td>
<td>3</td>
<td>37.5</td>
</tr>
</tbody>
</table>

*Odds Ratio is significant
‡Percent positive describes those tested for individual drug (i.e. n<sup>b</sup>/n<sup>a</sup>)
†Drug tested positive simultaneously with positive opiate result; of percentage of tests conducted for individual drug (i.e. n<sup>c</sup>/n<sup>a</sup>)

3.2.3 Neonatal Meconium Results for Opiates and Other Drugs of Abuse and Alcohol

Out of the 1318 meconium specimens analyzed for opiates, 1304 were tested for other drugs of abuse or alcohol (FAEEs) as well. Among these tests, 62 (4.8%) tested positive for opiates only, 129 (9.9%) tested positive for opiates and one or more other substances, and 628 (48.2%) tested negative for opiates but positive for another substance in meconium. The frequency of co-exposures to other drugs of abuse as determined by meconium testing is outlined in Table 7. If testing positive for opiates in meconium, the odds ratio for testing positive for other drugs was calculated to be 1.61 (95% CI 1.16-2.22). Hence, infants testing positive for opiates were more likely to be co-exposed to other substances, as determined by meconium test results and coinciding with odds ratios determined using neonatal hair specimens as well (section 3.1.2).
Table 7: Drug co-exposures among positive and negative opiate meconium tests

<table>
<thead>
<tr>
<th>Drug Co-exposure Frequency in Meconium</th>
<th>Opiate Positive</th>
<th>Opiate Negative</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of different drugs</td>
<td>n</td>
<td>%</td>
</tr>
<tr>
<td>1</td>
<td>62</td>
<td>32.5</td>
</tr>
<tr>
<td>2</td>
<td>81</td>
<td>42.5</td>
</tr>
<tr>
<td>3</td>
<td>36</td>
<td>18.8</td>
</tr>
<tr>
<td>4</td>
<td>10</td>
<td>5.2</td>
</tr>
<tr>
<td>5</td>
<td>1</td>
<td>0.5</td>
</tr>
<tr>
<td>6</td>
<td>1</td>
<td>0.5</td>
</tr>
<tr>
<td>Total</td>
<td>191</td>
<td>100</td>
</tr>
</tbody>
</table>

Akin to the neonatal hair data, when testing for opiates, the most frequently co-tested drugs were cocaine (1283/1304 or 98.4%) and marijuana (1250 or 95.9%). These substances were followed by, in descending order, amphetamine (344 or 26.4%), methamphetamine (340 or 26.1%), FAEE (325 or 24.9%), oxycodone (228 or 17.5%), benzodiazepines (227 or 17.4%), and lastly methadone (149 or 11.4%).

Among neonates tested positive for opiates in meconium, the odds ratios calculated for testing positive for cocaine/BRE were 1.51 and 1.69 respectively (95% CI<sub>cocaine</sub> 1.11-2.07, 95% CI<sub>BE</sub> 1.25-2.28). For cannabinoids, the odds ratio was 0.77 (95% CI 0.56-1.06), for benzodiazepines the odds ratio was 1.72 (95% CI 1.17-20.56), and for amphetamine and methamphetamine the odds were 2.54 and 1.72 respectively (95% CI<sub>amph</sub> 0.62-10.49, 95% CI<sub>meth</sub> 0.34-8.73). Lastly, the odds ratios calculated for methadone, oxycodone, and FAEE were 3.9 (95% CI 1.63-9.35), 8.05 (95% CI 3.01-21.5), and 2.35 (95% CI 1.13-4.9) respectively. There were no positive meconium test results for barbiturates, phencyclidine, or meperidine in this study population. Collectively, this data suggests that neonates testing positive for opiates in
meconium are significantly more likely to test positive for cocaine, benzodiazepines, methadone, oxycodone, and FAEE. This is summarized in Table 8.

### Table 8: Itemized drug co-exposures as determined by meconium testing and the corresponding odds ratios

<table>
<thead>
<tr>
<th>Drugs</th>
<th>Tested§</th>
<th>Positive</th>
<th>Simultaneous Positive†</th>
<th>Odds Ratio (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>nᵃ</td>
<td>%</td>
<td>nᵇ</td>
<td>%</td>
</tr>
<tr>
<td>Cocaine</td>
<td>1283</td>
<td>98.4</td>
<td>356</td>
<td>27.7</td>
</tr>
<tr>
<td>Cannabinoids</td>
<td>1250</td>
<td>95.9</td>
<td>467</td>
<td>37.4</td>
</tr>
<tr>
<td>Amphetamine</td>
<td>344</td>
<td>26.4</td>
<td>9</td>
<td>2.6</td>
</tr>
<tr>
<td>Methamphetamine</td>
<td>340</td>
<td>26.1</td>
<td>8</td>
<td>2.3</td>
</tr>
<tr>
<td>FAEE</td>
<td>325</td>
<td>24.9</td>
<td>65</td>
<td>20.0</td>
</tr>
<tr>
<td>Oxycodone</td>
<td>228</td>
<td>17.5</td>
<td>20</td>
<td>8.8</td>
</tr>
<tr>
<td>Benzodiazepines</td>
<td>227</td>
<td>17.4</td>
<td>8</td>
<td>3.5</td>
</tr>
<tr>
<td>Barbiturates</td>
<td>224</td>
<td>17.2</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Methadone</td>
<td>149</td>
<td>11.4</td>
<td>35</td>
<td>23.5</td>
</tr>
<tr>
<td>Phencyclidine</td>
<td>84</td>
<td>6.4</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Meperidine</td>
<td>52</td>
<td>4.0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

*Odds Ratio is significant

§ n value and respective percentage tested for indicated drug among opiate tested population

‡Percent positive describes those tested for individual drug (i.e. nᵇ/nᵃ)

†Drug tested positive simultaneously with positive opiate result; of percentage of tests conducted for individual drug (i.e. nᶜ/nᵃ)

#### 3.2.4 Opiate Levels in Mother-Infant Dyads

In total, 18 mother-infant dyads tested for opiates were identified from our database. Ten pairs were tested for opiates in maternal and neonatal hair and fourteen pairs consisted of maternal hair and neonatal meconium; of these, six mother-infant dyads had both neonatal hair and meconium test results (Table 9). Among these six mother-infant pairs, four tested positive in all three specimens (maternal hair, neonatal hair, and meconium), one pair tested positive in
maternal and neonatal hair only, and one pair tested positive in maternal hair and meconium only.

Results for the ten maternal-infant hair tests for opiates showed positive maternal hair tests in 100% of the pairs. Two neonatal hair tests (2/10 or 20%) were negative for opiates despite a positive maternal result, thereby having 80% concordance between the tests. Alternatively, out of the fourteen maternal hair test and neonatal meconium test pairs, only 9/14 (64%) demonstrated concordance, as 2 maternal hair specimens and 3 meconium specimens were negative for opiates (mutually exclusive).

<table>
<thead>
<tr>
<th>Table 9- Mother-infant dyad test results for opiates</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mother-Infant Pair Number</td>
</tr>
<tr>
<td>---------------------------</td>
</tr>
<tr>
<td>1</td>
</tr>
<tr>
<td>2</td>
</tr>
<tr>
<td>3</td>
</tr>
<tr>
<td>4</td>
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<tr>
<td>5</td>
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<tr>
<td>16</td>
</tr>
<tr>
<td>17</td>
</tr>
<tr>
<td>18</td>
</tr>
</tbody>
</table>
Because the data were not normally distributed, we used a Spearman Rank Test to determine if there was any significant correlation between mother-infant concentrations of opiates in these specimens. There was a significant correlation between maternal-neonatal hair test results for opiates as seen in figure 13 (Spearman’s $r=0.657$, $p=0.03$); maternal hair test results and neonatal meconium results were not significantly correlated.

**Figure 13:** Correlation between maternal and neonatal opiate concentrations in hair. Spearman’s $r=0.657$; $p=0.03$. 
4. DISCUSSION

Hair and meconium analysis are increasingly being used by child welfare authorities and legal services for the accurate and objective determination of drug use (Lewis et al. 1997). This is not only beneficial for children potentially at risk, but also for parents mislabeled as drug abusers (Klein, Karaskov & Koren 2000). Thus, characterization of drug use and exposures by these susceptible populations is warranted for improved clinical and social care.

Neonates who are exposed in utero to opioids, especially close to delivery, may exhibit significant postnatal problems. Predominantly, a significant portion of opioid exposed neonates may experience NAS (American Academy of Pediatrics1998) of which symptoms include irritability, high-pitched cry, tremors, hypertonicity, poor feeding, vomiting, and diarrhea (Finnegan et al. 1975). It’s thought that NAS occurs due to the fact that opioids are readily able to cross the placenta as well as the blood brain barrier as the latter is not fully formed in neonates (van Lingen et al. 2002).

Neonatal withdrawal syndrome may occur due to opioid tolerance or physical dependence, primarily a result of opioid receptor desensitization and upregulation of cyclic AMP (cAMP) (Suresh, Anand 2001). The former process may occur by either downregulation or internalization of opioid receptors, or uncoupling of the receptors from their G-proteins. cAMP upregulation might result from supersensitization of adenylyl cyclase as well as enhanced coupling of opioid receptors with the Gs-protein (Suresh, Anand 2001).
Opioid exposed neonates may also experience a constellation of other pathologies such as jaundice, aspiration pneumonia, transient tachypnea, infection (Finnegan et al. 1975, Finnegan 1985), and seizures may also occur in a small percentage of symptomatic infants (Herzlinger, Kandall & Vaughan 1977, Zelson, Rubio & Wasserman 1971, Kandall, Gartner 1974). Therefore, identification of these opioid exposed infants is of chief importance to ensure they receive adequate and supportive medical care in the first few days of life.

It is important to note that opioids are widely prescribed to and used by women of reproductive age and are not contraindicated in pregnancy; therefore the assessment of fetal exposures to such compounds and other drugs of abuse using these alternative matrices deserves consideration. It has been estimated that between 5-10% of pregnancies are affected by drug abuse in the general population (Koren et al. 2002, Huestis, Choo 2002). Specifically, opiate exposures in pregnancy vary from 1.2 – 8.7%, as estimated by meconium analyses (Yawn et al. 1994, Pichini et al. 2005). Although general population figures exist, estimates for at-risk populations are still lacking. For this reason, I aimed to develop a sensitive and specific method to evaluate such exposures for maternal and neonatal hair analysis. Moreover, I aimed to assess the opioid exposure profile of neonates in an at-risk population.

4.1 Method Development for the Detection of Opiate Exposures in Hair

I developed and reported in the literature (Moller et. al. 2010) a novel, and optimized technique for the confirmation of codeine, morphine, and 6MAM in hair specimens using HS-SPME coupled with GC-MS. Compared with conventional methods, this method for detection of
opiates is fast, simple, and accurate, with sensitivity and specificity required in forensic toxicology.

Upon comparison of our results and those previously obtained results from the ELISA and LC-MS/MS methods, minor discrepancies became evident. Among some clinical hair samples, nearly ten-fold variations were evident between the concentrations of opiates found with the ELISA method and our newly developed HS-SPME GC-MS method. Moreover, although similar, the opiate concentrations were not identical between the LC-MS/MS method used to examine Sample 1 (A and B) and our newly developed HS-SPME GC-MS method. Here we propose valid explanations for these discrepancies.

The first consideration that must be made is the age of the sample, or the time between sampling and analysis. Upon receipt of any hair sample to the Motherisk Laboratory, it is analyzed by ELISA for the requested drugs of abuse immediately, leaving little time for loss of drug via degradation, as described in the literature to be possible (Balabanova, Albert 1994). Additionally, the policy of our facility is that after testing, remaining hair specimens are to be kept for 12 months and not to be used for research in case additional testing is required. With the exception of samples 1 and 11, which had already been confirmed using LC-MS/MS and therefore would unlikely be re-tested, all other specimens were at least one year old. While we make great effort in maintaining ambient temperatures and humidity, conditions do change within the hospital and therefore may play a role in the potential degradation of drug molecules over this extended period.
Secondly, in some cases there was insufficient specimen quantity to re-analyze for opiates using our method (as outlined above for sample 1). Using more distal segments than originally tested would likely result in decreased concentrations (due to environmental degradation) however this would also depend on drug consumption habits of the donor. Therefore, degradation of drug molecules over time may have played a significant role in the differences of results.

Another possible explanation for the discrepancies is cross reactivity. The ELISA kit used by our laboratory will cross react with other opiates such as hydromorphone (Dilaudid®) and hydrocodone (Vicodin®). These opioids may have been taken by our substance abusing population. This would result in a positive ELISA test but since our method is specific for the 3 mentioned opiates, it would be negative for morphine, codeine, and 6MAM upon GC-MS confirmation. Therefore, discrepancies in total concentrations, as well as the ELISA positive, GC-MS negative test result of one specimen may be attributed to this very basic and fundamental aspect of differences in drug consumption by the donor as well.

Differences in opiate concentrations obtained between our HS-SPME method and the LC-MS/MS method are likely due to a unique situation. Since there was not a sufficient amount of hair specimen remaining for samples 1A and 1B, we were required to examine the 3-6 cm segment rather than the 0-3 segment originally tested (and reported). The client’s opioid intake may have fluctuated [on average] during those two 3 month time periods, and this may afford the differences in opiate concentrations detected. Our laboratory does not receive drug intake information, therefore we have no way of knowing whether this particular client’s use changed at
all during the respective time periods tested. Moreover, the condition of the hair segments may have also been different: if the client had chemically treated his/her hair in the previous three months (3-6 cm segment) but not in the most recent 3 months (0-3 cm segment), the former segment would be more subject to drug removal/degradation.

Finally, while samples that were tested in duplicate did not result in identical concentrations for each of the three opiates tested, the values were comparable. The fact that individual hair strands are completely independent of each other in terms of blood supply, and therefore drug incorporation (Pragst, Balikova 2006), may also afford the minor differences observed.

Therefore, while our results are comparable but not identical to those previously obtained using different immunologic/chromatographic methods, the inherent mechanistic variability introduced by these methods as well as the theoretical variability introduced by the hair and hair shaft condition itself all likely play a role in such differences.

4.2 Estimates of Maternal-Fetal Opiate Exposures

Here, I aimed to characterize and report in the literature (Moller, Karaskov & Koren 2010) an at-risk neonatal population for opiate exposures as well as other drugs of abuse and alcohol. Our results indicate a higher than previously estimated rate of opiate exposures: we detected 17% opiate exposures by meconium analysis and 11.4% by neonatal hair analysis. This elevation in exposures was intuitively expected since the population tested was that of high risk and sent to us upon suspicion of drug abuse by child welfare authorities. Additionally, the higher
rate of detected exposures through meconium versus hair is in agreement with previous literature investigating prenatal cocaine exposures; the authors found that meconium had nearly a 10% greater diagnostic sensitivity than neonatal hair (Garcia-Bournissen et al. 2007a). Therefore, opiate use in this population appears to be greater than that of the general population by approximately 10%, and should be flagged as a potential public health concern.

In assessing poly-drug exposures among the infants tested for opiates, we found that if the infant tested positive for opiates in both hair and meconium, they were more likely to test positive for other drugs of abuse as well. Specifically, in neonatal hair, the synthetic opioid oxycodone was found more likely to test positive with a positive opiate result; in meconium, cocaine, benzodiazepines, methadone, oxycodone, and FAEE were more likely to be positive with a positive opiate result. With larger sample sizes, it is possible that the neonatal hair statistics would match that of meconium; however in our laboratory the latter matrix is much more common for toxicological testing simply because it is a discarded material. Moreover, there is a usually sufficient quantity of meconium available for testing while neonatal hair is often very scarce.

The fact that our results indicate maternal polydrug abuse in this at-risk population is not unanticipated either- we previously determined maternal cocaine or methamphetamine abuse was associated with such behavior in the same population (Garcia-Bournissen et al. 2007a, Garcia-Bournissen et al. 2007b). Moreover, the drugs found to be significantly correlated with positive opiate exposures fit the scenario quite well. Cocaine is the leading choice of stimulant in North America (Huestis, Choo 2002) and is commonly used by high risk populations. Oxycodone,
methadone, and benzodiazepines are all prescribed medications and may be prescribed or abused concurrently with opiates.

Finally, positive alcohol (FAEE) testing was significantly correlated with a positive opiate meconium test. A positive alcohol test in our laboratory indicates chronic/excessive alcohol consumption which may have significant consequences to the development of the neonate (Kulaga et al. 2009). Alcohol is widely consumed by the general public, especially among those of high risk (i.e. low socioeconomic status and/or of minority) (Vilamovska, Brown Taylor & Bluthenthal 2009, Gomberg 2003, Curtis, McCullough 1993, Freisthler, Weiss 2008). Since the opiate class may include legitimately prescribed substances that are not contraindicated in pregnancy, our findings of increased neonatal exposure to alcohol among those also positive for opiates may have implications on the social, medical, and legal systems alike (Stade et al. 2007, Stade et al. 2009).

Lastly, we assessed the transplacental transfer of opiates as quantified by maternal and neonatal levels in hair. We found that these concentrations were significantly correlated while meconium concentrations did not correlate with maternal hair concentrations. This is not surprising since maternal hair was generally segmented to represent only the final trimester of pregnancy (0-3 cm segment); meconium opiate concentrations represent in utero exposures after approximately 12 weeks gestation and would include second trimester exposures. These would not be accounted for in the maternal hair segment. Concentrations found in neonatal hair represent in utero exposures after approximately the 20th week of pregnancy (i.e. beyond the 2nd trimester) and this corresponds well with the time period that maternal hair segment represents.
A stronger correlation between maternal and neonatal hair concentrations may not have been evident due to variation in pharmacokinetic parameters of the maternal-fetal unit, including differences in transplacental transfer/distribution, maternal and fetal opiate metabolism, and maternal/fetal clearance (Garland et al. 2005, Mucklow 1986). Thus, these results should stimulate further placental research, perhaps in the form of profusion or uptake studies to assist in the mechanistic determination of fetal opiate exposure.
5. CONCLUSION

I report an optimized, reliable, and fast HS-SPME coupled with GC-MS technique for the simultaneous quantification of morphine, codeine, and 6MAM in human hair. This method offers numerous advantages including speed and environmental and cost conservatism. To my knowledge, this method boasts limits of detection and quantification lower than previous reported in the literature. This is paramount when assessing in utero opiate exposures through neonatal hair analysis as concentrations of drug and specimen amounts tend to be low. Through the epidemiological portion of this work, I have found that this ability to assess in utero opiate exposures to neonates is a chief incentive for method development; I have highlighted an increased exposure rate to opiates in this at-risk population as compared with the general population. Additionally, I have described other substances that were more likely to have been co-consumed with opiates, including benzodiazepines, cocaine, alcohol, and the synthetic opioids methadone and oxycodone. Since in utero opioid exposures may predict NAS, and maternal abuse of other drugs and alcohol may precipitate poor neonatal prognoses, these objective methods for assessing such exposures may lead to improved pediatric clinical care.
6. REFERENCES


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7. APPENDIX

List of Publications


**Directly relevant to thesis; full article included in appendix.**