GAS CHROMATOGRAPHIC-MASS SPECTROMETRIC IDENTIFICATION OF COCAINE AND OPIATE ANALYTES IN KERATINIZED MATRICES OF HUMAN ORIGIN

By

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This dissertation is dedicated to the special people in my life:

  MY HUSBAND, Jeffrey
  MY FATHER, Sydney
  MY MOTHER, Kathy
  MY SIBLINGS, Kathy and Bill

Each has touched my heart as well as contributed, in some significant way, to my success in attaining my educational endeavors. For this, I give my endless love and gratitude.
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Chairperson: Bruce A. Goldberger, Ph.D.
Major Department: Pathology, Immunology and Laboratory Medicine

Previous research has demonstrated that analysis of keratinized matrices can be beneficial in detecting drug exposure. Hair and nails can identify long-term drug use while blood and urinalysis detect recent drug use.

Gas chromatography-mass spectrometry (GC/MS) was employed to investigate drug disposition of cocaine and codeine into keratinized matrices and to compare the sensitivity of hair and nail analysis with conventional techniques.

In project one, paired hair and nail specimens from eight volunteers enrolled in an inpatient study who were administered cocaine and codeine for a ten-week period were obtained for drug analysis. A significant dose-response relationship was observed for hair specimens. The mean peak concentrations after low dosing were half the concentration observed after high-dose administration.
Generally, no clear relationship was evident between nail drug concentrations and dose. Decontamination washes removed less than 20% of the total drug present in hair, but removed most of the drug concentrations (50-100%) in nail.

In project two, postmortem finger and toenail clippings of suspected cocaine users were obtained and subjected to drug analysis. Cocaine analytes were present in the nails of 14 out of 18 subjects (82%), whereas only 5 out of 18 (27%) subjects had positive results by conventional postmortem techniques. This study demonstrated a marked increase in the detection of cocaine use by nail analysis.

The final project utilized hair analysis to determine if detectability of drug exposure in SIDS cases could be improved. Head hair samples were obtained from 26 infants. Positive results were observed in 10 out of 17 SIDS cases (58%) and 1 out of 9 control cases (11%). Conventional postmortem analysis did not detect cocaine in the infants. Statistical analysis demonstrated a significant increase in drug detection with the use of hair analysis.

In conclusion, this dissertation demonstrated that hair and nail analyses are sensitive techniques for detecting drugs of abuse. In addition, drug incorporation into keratinized matrices may follow a predictable dose-response profile. However, further research investigating the mechanism of drug incorporation in keratinized matrices is needed.
CHAPTER 1
INTRODUCTION

Historical Overview

Since ancient times, man has consumed drugs in an effort to treat ailments, relieve pain, promote health, or induce pleasure and euphoria. As early as 4000 years ago, Sumerian and Assyrian/Babylonian cultures recorded opiate use for medicinal purposes (1). Similarly, cocaine was first used in the sixth century by Incan tribes of Peru and Bolivia (2-3). In modern times, a large population continues to abuse both licit and illicit drugs for a plethora of reasons and this has resulted in an epidemic in many global regions. In fact, the 1996 National Household Survey on Drug Abuse conducted by the Substance Abuse and Mental Health Services Administration reported that the number of current illicit drug users in the United States was approximately 13 million (4).

Drug abuse impacts society through decreased job productivity and earnings and increased crime, drug-related fatalities, health costs, prevention costs, and social welfare recipients. The Lewin Group for the National Institute of Drug Abuse and the National Institute on Alcohol Abuse and Alcoholism estimated that the total economic cost of alcohol and drug abuse in the United States was over 245 billion dollars in 1992. Of this
cost which are primarily absorbed by the government, almost 40% was attributed to drug abuse. These cost estimates increased nearly 50% from the 1985 data (4).

Rapidly increasing drug abuse among Americans has forced the Government to address the issue by creating agencies and new laws to regulate drugs in the United States. Agencies such as the Food and Drug Administration (1862), the National Institute of Drug Abuse (1974), and the Drug Enforcement Agency (1989) have emerged to address specific aspects of drug purity and control regulation. Likewise, many important legislative milestones concerning drugs have been established. In 1848, Congress passed the Drug Importation Act requiring U.S. Customs to prevent the passage of adulterated drugs from overseas into the States (5). In 1906, the Pure Food and Drug Act gave the Federal government more authority over the production, distribution, and marketing of food and drugs. This bill also deemed the U.S. Pharmacopeia "the legal standard of official preparations," requiring label disclosure of ingredients not found in this document (3). The Harrison Narcotic Act of 1914 imposed severe restriction on the sale and distribution of opiates and cocaine due to their adversities and addictive nature (1). New safety provisions were the focus of the 1938 Federal Food, Drug, and Cosmetic (FDC) Act. The FDC Act required manufacturers to demonstrate that newly marketed drugs were safe, including establishing safe tolerance doses. In 1965, the Drug Abuse Control Amendments were enacted to combat the growing abuse of depressants, stimulants, and hallucinogens (5).

The Schedule of Controlled Substances Act of 1972 placed many drugs on a schedule categorizing drugs based on their potential for abuse and their medicinal purposes. For example, certain narcotics, hallucinogens, and cannabinoids were classified
as Schedule I, restricting their legal use because of their high abuse potential and limited medicinal purposes. To minimize drug abuse in the workplace, the Drug-Free Workplace Act was enacted in 1988 by the National Institute of Drug Abuse (NIDA) requiring implementation of programs and policies for Federal employees, contractors, and grantees. Shortly afterwards, businesses within the private sector adopted similar workplace drug testing policies and procedures for pre-employment screens, random testing to promote safety, and potential drug-related accident investigations (6).

Drug testing has become a multi-billion dollar industry, whether its primary purpose is to investigate illegal behavior, to promote a drug-free workplace, to assist in clinical diagnosis and treatment, or to investigate accident and death scenes. In the past, many biological specimens have been investigated for their ability to demonstrate drug use by an individual. Biological tissues and fluids utilized for drugs of abuse testing include urine (7), blood (8-11), saliva (11-13), sweat (14-15), hair (16-18), nails (19-22), vitreous humor (7), cerebral spinal fluid (23-24), bile (7), meconium (25-28), amniotic fluid (29-30), breast milk (31-32), semen (33-34), and organs such as kidney, brain, and liver (7). In most instances, the conventional matrices chosen for drug testing are blood and/or urine. Use of these specimens provides sufficient quantities of material for testing, adequate concentration of the drug, and confirmation of recent drug use.

Although blood and urine are the most prevalent matrices for drug testing, there are several disadvantages associated with them. Both of these matrices require processing, and in some cases preservation and refrigeration to prevent degradation of the endogenous and exogenous chemical constituents. The collection of blood and urine can be invasive both physically and socially. Many individuals experience embarrassment
when a urine specimen is requested from them. The presence of a drug in blood or urine reflects use within a period determined by both metabolism and elimination. Drug in blood occurs within minutes to hours while drug in urine occurs within hours to days. Negative test results can occur depending on the elimination rate. For example, many cocaine and opiate abusers escape detection by conventional urine analysis since these analytes are eliminated rapidly, usually within 72 hours of use. All samples collected after elimination of drug is complete will have negative test results although drug was ingested. Conversely, false positive test results may result with the incorrect identification of an analyte due to interferences. For example, oxaprozin (Daypro®), a structurally unrelated nonsteroidal anti-inflammatory drug, produces false positive results with a number of benzodiazepine immunoassays (35). Due to these problems associated with blood and urinalysis, alternative matrices have been investigated as a complement to conventional matrices.

Hair and nails are two unconventional matrices recently investigated as complimentary specimens to blood and urine. As I will show, the complementary traits of these keratinized matrices make them a reasonable choice to increase detection of drug exposure. Numerous studies have shown that results of hair tests provide valuable information regarding relatively recent and past drug use (18, 36-41). Despite some controversy such as environmental contamination and color bias, results of hair tests have been utilized in clinical, forensic, and epidemiological studies, historical research, and have even been presented as evidence in civil, criminal, and military courts of law (42-44).

Hair analysis has entered the legal system with two specific purposes – as testimonial evidence and as an acceptable specimen for workplace drug testing. The
admissibility of hair analysis in American courts has made a gradual progression. At first, admissibility of hair analysis was based on the previously established Frye standard (1923) requiring that scientific principles be sufficiently established with general acceptance by the scientific community. In 1975, the Federal Rules of Evidence were adopted, allowing expert witnesses to give testimony "in the form of an opinion or otherwise" if it assisted "the trier of fact to understand the evidence or to determine a fact in issue" (45). However, these rules were not utilized until 1993 after a Supreme Court ruling in the Daubert v. Merrell Dow case. This new standard implemented a more liberal outlook allowing the judge to determine whether or not hair analysis evidence would lend understanding to the case, hence, superseding the "general acceptance" approach for admissibility (42).

Although hair analysis remains controversial, it has been deemed an "acceptable matrix" for drug testing in some States and agencies. The State of Florida Drug-Free Workplace Act was amended in 1996 to include hair as an acceptable specimen for drug detection. The U.S. Armed Forces have used hair testing since the late 1980s to investigate drug use and to serve as evidence in court-martial cases. The Federal Government is also considering hair as an alternative matrix to blood and urine for drug-free workplace testing.

Nail analysis for drugs of abuse has also been investigated since the early 1980s but research has been limited. The scientific community is just beginning to realize the potential of nail analysis. Due to its unfamiliarity, nail analysis has not entered the legal system like hair analysis. No matter the degree of general acceptability of these matrices,
their advantages and disadvantages should be carefully evaluated for each specified application in the forensic and clinical fields.

Unconventional Matrices in Drugs of Abuse Testing: Advantages and Disadvantages

Keratinized matrices offer drug testing laboratories many advantages which complement blood and urinalysis. One of the most complementary characteristics of keratinized matrices is their reflection of long-term drug use. Depending on the amount of growth available for collection, hair and/or nail analysis can potentially represent drug exposure from several months to years. Once incorporated into the final keratinized structure, the metabolic activities occurring within the body no longer influence internalized drug. Therefore, the hair or nail acts as a record of the metabolic events occurring at the time of its formation (46). Hair has also served as a “chronological ruler” using segmental analysis in which measured hair length was correlated with average hair growth to estimate the timing of drug exposure (47-49). The use of hair analysis to define the timing of drug exposure has proved beneficial in prenatal drug exposure, rehabilitation abstinence compliance, and therapeutic drug monitoring (23, 44, 50-51). Hair analysis has also been used to determine the pattern of drug use, differentiating between frequent/infrequent drug use (43, 52). Finally, temporary abstinence from drug use will not lead to a negative test result as seen with conventional matrices. Hence, several aspects of timing for drug exposure can be determined with hair analysis.

In addition to timing drug exposure events, hair analysis offers other advantages. Hair analysis can sometimes distinguish which form of the drug was actually ingested. For instance, the differentiation of opiate use as illicit heroin, prescribed morphine or codeine,
or consumed poppy seeds is possible in some cases. If codeine and morphine are detected, determination of licit or illicit drug use or poppy seed consumption can not be confirmed (53-54). However, if heroin or its major metabolite, 6-acetylmorphine, are detected, then illicit drug use is confirmed. Since heroin and 6-acetylmorphine are only detected in the blood and urine for minutes and hours after ingestion, respectively, finding these analytes within these specimens is rare. Nevertheless, these opiate analytes can be detected for a much longer period in hair and this increases the ability to detect illicit opiate use (55).

Another advantage of keratinized matrices is their chemical composition, which makes them very stable and less susceptible to environmental conditions. In postmortem cases, hair and nail are sometimes the only matrices present at the time of the body's discovery. In addition, the drug itself is more stable under normal conditions (i.e., no chemical treatment or physical stress) once it is incorporated into the interior regions of hair and nail. Drug analytes within the hair and nails are protected from further enzymatic degradation unlike blood and urine. The stability of drugs in hair has led to successful detection of drugs in the hair of Peruvian and Egyptian mummies dating 200-1500 AD and 1070 BC-395 AD, respectively (56-57). Stability also makes adulteration or manipulation of drug content in hair and nails more difficult to achieve (23). Therefore, the more stable nature of keratinized matrices provides many complementary advantages to conventional matrices.

Keratinized matrices also have advantages in specimen collection and analysis. The collection of hair and nails is relatively noninvasive and specimens are easy to obtain.
The ability to detect drugs in a small quantity of hair and nails is also beneficial. As little as 10 to 25 mg of keratinized tissue is required to detect many drugs.

Conversely, the inherent nature of hair and nails also leads to disadvantages of these matrices for drug testing. First, the mechanism of drug incorporation into hair and nails remains unknown. This, of course, complicates the interpretation of data for these matrices. Secondly, the lag period of drug incorporation into hair and nails makes them unacceptable for assessing current clinical impairment or very recent use (19, 48, 52). Several reports have estimated that it takes 3-7 days for a drug to enter these matrices and proceed to a point where collection of an appropriate specimen and identification of the drug are possible (58-60). Another potential problem of hair and nails is their inherent exposure to the environment and this subjects them to potential contamination leading to false positive test results. Decontamination washes are not always successful at removing the entire parent drug concentration. Researchers have demonstrated that 10 to 75% of the drug dose remained after standard wash procedures with organic solvents depending on the type of contamination (vapor or aqueous solution) and the type of drug investigated (cocaine, heroin, or 6-acetylmorphine) (48, 61). Some investigators have proposed "wash kinetics" to determine the contribution of environmental contamination while others do not believe these calculations are proven methods (48, 62). Finally, the chemical treatment of the hair including bleaching and shampooing can influence drug concentrations (48, 63-64).

Another controversy for keratinized matrices is a demonstrated color bias. Studies suggest that lighter-colored hair incorporates drug to a much lesser degree than darker-colored hair (62, 65). A possible mechanism for this color bias is the different
concentrations of pigment protein such as melanin present in hair (see following hair anatomy section). Since darker hair is more prevalent in specific ethnic groups compared to other groups, this color bias has been very detrimental for hair analysis (66-67). Since our judicial system is founded on equal treatment of all individuals, the legal system cannot risk any bias towards certain ethnic groups in accepted drug testing methodology.

Specimen preparation and laboratory handling of keratinized matrices also suffer from some disadvantages. Laboratory procedures to prepare hair and nails for analysis are often more tedious, time consuming, and costly than blood and urinalysis. These matrices usually require additional decontamination and solubilization/isolation steps that are not necessary for other matrices. Furthermore, technological standardization and quality assurance have not been established in this relatively new field of science (37, 52).

Anatomy and Physiology of Hair and Nail

Hair

A chemical analysis of hair shows the composition to consist of 65-95% proteins, 1-9% lipids, and 15-35% water. Hydration influences the percentage of each component (68).

The matrix proteins found in hair are referred to collectively as keratin. Hair and nail consist of hard keratins as opposed to the soft keratins found in skin. Keratins are fibrous in nature, insoluble in common protein solvents such as trypsin, and rich in sulfur content. These keratinized proteins are composed of several strands of highly oriented polypeptide chains wound into an α-helical structure known as microfibrils which combine
to form macrofibrils of the hair shaft (51, 46, 69). The amino acids cysteine, lysine, histidine, glutamic acid, and aspartic acid form the framework of the matrix proteins. Cysteine accounts for 11-18% of the keratin matrix (68, 70-71).

Strong covalent bonds are formed from cross-links between sulfylhydryl groups within the amino acid make-up (69). Electrostatic, hydrophobic, and ionic interactions can occur between small molecules and keratin’s functional groups such as carboxyl, amine, hydroxyl, and sulfylhydryl substituents. These interactions lead to the incorporation of exogenous components such as drug and metals into the hair matrix (65, 72-73). Trace elements and heavy metals partitioned into the hair matrix vary between 0.25% to 0.95%. The proteinacious bonds may be broken with such chemical treatments as permanent wave, bleaching, and enzymatic or acidic digest used in the laboratory. In addition, temperature extremes can denature matrix proteins (51).

The hair lipids include free and ecosinic fatty acids, phospholipids, cholesterol, and sulfates (73). The phospholipids may be chemically linked to keratin by their free fatty acid side chain (46). Alcohol and ester groups of these lipids may all contribute to both specific and nonspecific binding of drug to hair (65).

The anatomical structure of the hair consists primarily of the follicle, hair shaft, and surrounding glands. Figure 1-1 depicts the anatomy of the hair. The hair shaft, with an average diameter of 0.1 mm, is imbedded 3 to 4 mm below the surface of the epidermal epithelium of the skin. The hair shaft is a long cylindrical structure that protrudes from the hair follicle (46). The shaft is made up of tightly compacted cells found in three distinct regions – outer cuticle, cortex, and inner medulla. The elongated, overlapping cells of the cuticle protect the hair from the environment and anchor the shaft to the follicle. The
cuticle is not always present as it can be removed by chemical or physical stress to the shaft leaving only the inner regions (74). The shaft is composed of long, keratinized cells (100 μm) which form fibers through cross-linking bonds (51). The majority of the inner shaft is composed of bundles of macrofibrils that make up the cortex region. The innermost region is a hollow central area referred to as the medulla.

The hair follicle is a sac-like organ that houses the hair shaft, sebaceous gland, and in some regions the apocrine gland. There are approximately 100,000 follicles covering the human scalp (75). Apocrine glands secrete sweat in axillary, pubic, and perianal regions of the body. Apocrine glands secrete a specialized milky sweat that is rich in lipids (46). Eccrine glands are sweat glands within the epidermis, in close proximity to the hair follicle. Sweat of the eccrine glands is composed of a mixture of water, inorganic salts, amino acids, and waste products such as urea, uric acid, and lactic acid. Sebaceous glands secrete oily sebum composed of fats, cholesterol, proteins, and inorganic salts that keep the skin soft and pliable. Both secretions of the sebaceous and apocrine glands are released from ducts opening into the follicle. These secretions coat the skin and hair and act as a physical barrier to water, bacteria, and fungus.

Two capillary plexuses supply blood to the hair bulb (subcutaneous plexus) and to the epidermal area just beneath the stratum corneum surface (papillary plexus). Finally, a network of nerves surrounds the follicle at the level of the sebaceous gland (76).

Functionally, the follicle has three distinguishable growth zones. The zone of hair synthesis occurs at the base of the follicle where mitotically active matrix cells form the hair shaft. These cells change morphologically by elongating and increasing in size and volume. The cells move up the follicle to the keratogenous zone where the structural
protein, keratin, is synthesized. The final zone of permanent hair occurs after the cells lose their nucleus and dehydrate forming a condensed, cornified structure.

Hair color is attributed to pigment granules. The most predominant pigment is melanin, a copolymer composed of a protein matrix foundation with repeating 5,6-quinone units linked to form a polymer (77-78). Melanin contains many free carboxyl, phenolic and/or quinnonoid groups. Scatchard analysis of in vitro binding assays between melanin and several organic cations (i.e., chlorpromazine, chloroquine, and paraquat) indicate that more than one melanin binding site must exist for all investigated drugs. The binding of drugs to melanin is thought to occur through cation-exchange activity of the ionic drug with the carboxyl groups and phenolic groups of the melanin pigment granules. The protein moiety of the pigment granules may also bind to substances (79-80).

Melanin is synthesized in small organelles, melanosomes, located within specialized cells, melanocytes, of the hair bulb and other tissues such as skin. The melanosomes are subsequently transferred to keratinocytes within the medulla and cortex regions of the shaft. The average number of melanocytes on the scalp ranges from 1000-1200 melanocytes/mm². Melanin pigment granules originate from various substrates including tyrosine, L-dopa, dopamine, and catechol. Some melanin-synthesizing pathways occur in the presence of enzyme. For example, tyrosinase catalyzes the conversion of tyrosine to melanin. There are various melanin types; each vary in size, structure, and physiochemical properties (81).

Two types of melanin pigment human hair – eumelanin and pheomelanin. The types of melanin deposited, and the size, structure, distribution and density of the granules deposited within the cortex determine hair color (75). In a general, eumelanin produces
brown or black shades, pheomelanin produces blond, ginger and red shades, and lack of melanin leads to graying hair. In most hair, both melanin types exist and eumelanins are the most prevalent. The melanin content among ethnic groups is known to vary. For example, the melanin content of Chinese black hair (3%), European brown hair (1.2%), Irish red hair (0.3%), and Scandinavian blond hair (0.07%) varies as much as 40% (51).

Melanin content has become an issue for hair analysis because some researchers believe drugs vary in affinity for the different types of melanin. Researchers have proposed that drug binds to the functional chemical groups of melanin (48, 65, 69, 82-83). In vitro studies by Joseph et al. (65) demonstrated that more drug was detected in black or dark brown hair when compared to blond hair. These investigators suggested that the melanin fraction was the primary binding site, whereas the lipid fraction played a minor role. Animal studies with coexisting dark and light hair on individual rats showed that drug content was highest in black hair followed by brown then white (67, 84). Likewise, Reid et al. (86) demonstrated that hair analyzed from graying individuals had 1.3 to 6 times higher concentrations of cocaine analytes in pigmented hair when compared to nonpigmented (i.e., white) hair of the same individual. Nonetheless, the extent to which drug incorporation into hair is influenced by melanin content or the mechanism by which this relationship exist remains uncertain.

Nail

The chemical composition of the nail is very similar to the hair. Modified horny cells of the epidermis form the nail tissue. Nail is composed of a specialized keratin referred to as onychin. Like proteins of hair, the nail matrix cannot be dissolved by weak
acids/bases or pepsin digest, but stronger corrosives or enzymes can solubilize the nail. Essentially, growth occurs by the transformation of living cells of the nail matrix into layers of hardened, dead cells, forming a nail plate. Primates are the only mammals possessing true nails rather than claws. The tactile surface of nails is detached from the digit and this greatly increases sensitivity for exploration, scratching, grooming, and manipulation (87).

The basic structure of the perionychium, or nail and surrounding tissues, includes the nail root, cell matrix, lunula, nail bed, nail plate, eponychium, cuticle, and the distal free edge. Figure 1-2 illustrates the anatomy of a fingernail. The nail root constitutes the most proximal end of the germinal cell matrix located at the base of the nail plate just beneath the cuticle. The germinal matrix is the cell-producing component that contains lymph, blood vessels, and other nourishment required for nail formation. The lunula is the white, crescent-shaped nail area where the matrix connects with the nail bed. The lighter color persists either because the cells of the proximal nail plate are not entirely cornified and still contain keratohyalin granules, and/or due to variation in the nail plate’s adherence to the nail bed (87). The cuticle is the skin that overlaps the nail plate at its base and the eponychium is the point at which the nail enters the skin just beneath the cuticle. The nail fold guides the direction of the nail growth and it is divided into a dorsal roof and a ventral floor. A nail bed of highly vascularized and innervated tissue is located directly beneath the hardened nail plate. Two arterial arches run parallel to the lunula and free edge just above the distal phalanx. In addition, capillaries are abundant at the ventral floor, the major site of nail production (72, 87). The nail plate extends from the nail root area,
where it is anchored to the nail bed, to the distal free edge where it becomes detached from the soft tissue underneath it.

Nail growth occurs at the dorsal roof and the ventral floor of the nail fold as well as the nail bed located at the free margin of the nail. Utilizing silver-staining techniques with scanning electron micrographs, Zook et al. (87) demonstrated that the mature nail is composed of three layers formed by three separate processes. Nail layers and their respective formation process are as follows: 1) the dorsal nail arises from the proximal dorsal roof and ventral floor of the nail fold due to swelling of the cells, disappearance of nucleuses, and cell collapse; 2) the intermediate nail grows from the ventral floor and lateral walls of the distal nail fold to the lunula by parakeratosis (a gradual process where the epidermal cells broaden and flatten but keep the nuclei); and 3) the ventral nail layer originates from the distal lunula to the free edge of the nail. Though not always present, the ventral nail layer compensates for dorsal nail wear and leads to a thicker nail plate (72).

Nails also contain melanin housed in melanocytes, although the average melanocyte concentration is 5 to 10 times less than the amount present in hair of the scalp. In 1971, Hashimoto (88) was the first to demonstrate the presence of melanocytes in the normal nail matrix using histologically prepared tissue and high voltage electron microscopy. This study revealed that the development of melanosomes within melanocytes ranged from fully melanized in black subjects, to well developed in Japanese subjects, to poorly developed in Caucasian subjects. Melanosomes were rarely transferred to keratinocytes in Caucasian and the frequency of mature, dense melanosomes transferred to keratinocytes increased even more for Japanese and black nails (88). Another study
using L-dopa staining and immunochemistry with specific monoclonal antibodies to tyrosinase and proteins of the melanosomal vesicle, showed the proximal nail matrix contained unmelanized, immature melanocytes, whereas the distal nail matrix contained dormant and functionally differentiated and active melanocytes. The average count in the unmelanized compartment of the proximal epithelial sheets was $217 \pm 84/\text{mm}^2$, the distal matrix was $132 \pm 34/\text{mm}^2$, and the nail bed was $45 \pm 25/\text{mm}^2$. The active, melanized count was $103 \pm 17/\text{mm}^2$ for the distal matrix, relatively few in the proximal matrix, and none in the nail bed (89). Utilizing similar techniques, Higashi et al. (90) determined melanocyte count within the nail matrix to range between 208 to 576/\text{mm}^2. These investigators also determined that the maximum melanocyte count occurred in the distal portion of the nail matrix and the lower two to four layers of the matrix cells (90-91). Hence, depending on the region of the nail, melanocytes will have more or less melanin present. So if melanocytes having functional melanin do not occur until the distal portion of the nail, drug incorporation into nail may not be as strongly influenced by melanin content.

**Growth of Keratinized Matrices and Possible Routes of Drug Incorporation**

The growth of keratinized matrices is important to consider when evaluating drug exposure. How these matrices grow and the factors influencing their growth strongly affect the degree of drug incorporation. For example, drug content in plucked hair is different from shed hair because shed hair undergoes a resting stage before falling out (23).
Hair grows at an average rate of 0.35 mm/day, or about 1 cm/month and nail grows at an average rate one-third that of hair (48, 71, 92). On average, nail produced at the proximal nail fold takes between 70 to 160 days to reach the distal free margin (87). It takes approximately 10 to 15 days for newly incorporated matrix cells of the hair to move from the root to the surface of the scalp.

Growth rates for keratinized matrices are influenced by many biological and environmental factors. Factors influencing intra-individual growth rate of hair and nail include age, gender, ethnicity, heredity, climate, health, injury, and physical stress. In addition, location on the body influences growth (92-93). Average hair growth rates at different anatomical locations are as follows: scalp- 1.0 cm/month, axillary- 0.9 cm/month, facial 0.8 cm/month, pubic- 0.9 cm/month, and chest 1.3 cm/month (75, 94). Growth rate for the scalp displays regional variations ranging from the fastest growth occurring at the vertex (1.3 cm/month) to the temporal (0.87 cm/month) (75). Fingernails grow approximately four times faster than toenails. In addition, growth rate among digits varies slightly with longer digits having the fastest growth rate (72).

Hair undergoes a cyclic cycle; periods of growth are punctuated with periods of rest. The cycle includes a growth or anagen stage followed by resting (telogen) and degradation (catagen) stages lasting 2-5 years, 100-150 days, and 35 days, respectively. During anagen stage, matrix cells of the follicle undergo active mitotic division. Transition from the anagen phase to the catagen phase leads to rapid degeneration of the lower follicle matrix cells and subsequent shrinking of the follicle to only a small amount of undifferentiated cells. These undifferentiated cells form the new follicle. The portion of the follicle surrounding the hair base is lost and the hair develops a proximal swelling and
dies. Telogen hair is no longer secure and may fall out or is pushed out by the newly developing hair of the next anagen phase. The length of each cycle and the ratio of growth to rest vary depending on the body region, age, and gender (75). Chest hair has an equal or longer rest period lasting for two or more weeks, whereas the growth-to-rest ratio of scalp hair is 9:1. Scalp hair growth can last for years.

Data regarding the incorporation of drugs and drug metabolites into hair and nail is sparse and primarily hypothetical. However, based upon results from recent studies, several processes of internal and external incorporation of drug analyte in keratinized matrices have been proposed including

a) transport of the analyte directly into the hair follicle and hair cells through the blood supply to the hair follicle and nail bed;

b) diffusion of drug into secretions that can bath the hair follicles, maturing hair fibers, and nail perionchyium;

c) exposure of the hair fibers and nail surface to the external environment including drug residues, contaminated surfaces, or vaporized drug (48); and

d) exposure of the hair fibers and nail surface to outward transdermal diffusion of drug from the skin (95-96).

Many factors influence drug incorporation into keratinized matrices. First, the functional groups of the hair and nail matrix components enhance drug binding to intracellular components of hair cells. Acidic, basic, and peptide bonds will cause certain drugs to bind while other drugs will not. Because of this phenomenon, melanin and keratin content influences drug incorporation. Second, membrane permeability of keratinized matrices favors the incorporation of basic drugs such as cocaine and codeine.
(high pK_a) over neutral or acidic drugs. This is in agreement with the normal physiological ionization gradient between the follicular environment (pH 7.4) and the hair matrix (pI 4.0) allowing the unionized drug to cross the membranes (48, 51). Third, lipophilic drugs partition into hair and nail more readily than polar drugs. Previously published reports have demonstrated that cocaine is found in hair at a greater concentration than its metabolites, benzoylecgonine and ecgonine methyl ester (41, 97). This is contrary to cocaine analyte concentrations for urine, a more polar matrix. Finally, the metabolic profile of the drug determines the rate at which each analyte can potentially incorporate into these matrices (48).

Cocaine: A Brief Review

Use and Misuse

Cocaine is a naturally occurring alkaloid found in the leaves of *Erythroxylon coca*, a shrub indigenous to the warm climates of South America, Indonesia, and the West Indies (2). Radiolabeled carbon dating of tribal artifacts has documented the first known use of cocaine, by pre-Inca inhabitants, to be nearly 2000 years ago. The native tribes of Bolivia, Peru, and other South American countries used cocaine in religious rituals to treat ailments such as mountain sickness and fatigue (98). When the Spanish invaded and conquered these territories, they introduced cocaine to the European culture upon their return.

In 1857, Albert Neimann isolated cocaine from the coca leaf and found it to be the active alkaloid (99). For almost 20 years, anecdotal investigations from chemists and
physicians were reported within the literature but cocaine remained unnoticed by most. In the early 1870s, Charles Fauvel began experimenting with a tincture of alcohol and cocaine to relieve the painful sensations existing with surgical procedures of the throat and vocal chords. He introduced this concept to his cousin, Angelo Mariana, who brilliantly created a wine containing coca leaves. This wine, Vin Mariani, was successfully marketed to relieve many ailments including fatigue, insomnia, and despondency (3). In the mid-1880s, two well-known physicians, Sigmund Freud and Karl Koller published manuscripts promoting cocaine’s uses to treat morphine and alcohol addiction and as a local anesthetic in eye surgeries, respectively.

By the late 1890s as cocaine became a household product contained in the tinctures, wine, and syrup-based drinks (Coca-Cola®), America’s addiction to cocaine flourished. Adverse effects and addiction of cocaine and narcotics led to the enactment of the first American Drug laws of the early 1900s. These laws are the origin of drug regulation that continues today. In the mid-1980s, a highly addictive form of cocaine known as “crack” became available and it continues to dominate the Nation’s illicit drug problem.

In 1996, the estimated number of current cocaine users reported by SAMHSA’s National Household Survey on Drug Abuse was 1.75 million, which was slightly higher than the previous year (1.45 million). Although this statistic is lower than the peak incidence of 5.7 million reported in 1985, this survey estimated that over 650,000 Americans had tried cocaine for their first time in 1995 (4).

Cocaine is central nervous system (CNS) stimulant and sympathomimetic agent categorized as a Schedule II drug. Clinically, cocaine is used as a local anesthetic (4-10%
solution) and vasoconstrictor, especially for surgery of the nose, throat or cornea. Less common combination therapy includes tetracaine, epinephrine, and cocaine (TAC) used to treat scalp and facial lacerations in children and “Bromptom’s mixtures” prepared with cocaine and an analgesic-phentiazine solution used to treat oncology patients in European countries (2).

Unfortunately, cocaine has become a popular drug of abuse due to its highly desirable euphoric effects, availability, and low cost. The routes of administration vary depending on the form of cocaine ingested. The hydrochloride salt is administered intranasally (IN), orally (PO), or intravenously (IV), while free-base and cocaine base (“crack”) are smoked (SM). Coca paste is also smoked by applying it to the end of a tobacco or marijuana cigarette (98, 100-102).

The 85-90% purity of crack and free-base cocaine create a greater risk to the user. In addition, crack cocaine is readily absorbed, whereas absorption of insufflated cocaine hydrochloride is limited by its vasoconstrictive effects on the nasal mucosa (2). The normal illicit dose ranges between 10-120 mg by intranasal “snorting” or smoking (103). On the street, cocaine is often mixed with inert powders or other drugs to increase the amount for sale (lactose, talc, and sucrose) or to heighten the response (amphetamine, heroin, PCP) (98). Moreover, many cocaine users are drugs polydrug users, which can lead to a greater toxicity. For example, 30-60% of all cocaine users coingest alcohol; this can lead to more toxic metabolites and greater CNS effects (104).

Cocaine’s maximum safe adult intranasal dose ranges from 80-200 mg (1-3 mg/kg) and an adult fatal dose can be as high as 1.2 g. Solutions of the hydrochloride salt should not exceed 10%. The average therapeutic blood level reaches 0.2 mg/L one hour post-
administration (98). The toxic dose of cocaine averages greater than 200 mg resulting in detected blood levels approximating 4.6 mg/L for cocaine and 7.9 mg/L for benzoylecgonine, the major metabolite (103). A recently published report by Logan et al., (105) cautions that postmortem blood concentrations of cocaine analytes may not accurately reflect the perimortem concentrations due to “site-dependent differences and time-dependent changes believed to result from competing processes of tissue release and chemical and enzymatic degradation.” Hence, the presence of any postmortem concentration of cocaine could reflect toxicity and as such the investigators should evaluate the history, autopsy, and death scene of suspected cocaine-related deaths more thoroughly before assigning the cause of death.

Neurobiology

Pharmacologically, cocaine has three mechanisms of action on the nervous system. Cocaine acts on noradrenergic nerve terminals by inhibiting reuptake of catecholamines, such as dopamine and norepinephrine, following release from the presynaptic cells. Subsequently, these neurotransmitters pool at the postsynaptic receptors contributing to cocaine’s reinforcing and addictive effects (102). The increase in neurotransmitters also leads to cocaine’s sympathomimetic actions resulting in vasoconstriction, hyperthermia, hypertension, respiratory irregularities, and heart rate elevation (100, 106-107). Cocaine also influences the brain’s “stimulation-reward centers” consisting of dopaminergic, serotonergic, and opioid systems, playing an important role in psychotropically driven effects associated with feeding, drinking, male sexual motivation, and self-stimulation. Because cocaine can initiate this endogenous positive reward system, it encourages repeat
administration leading to high dependence rates (107). The quality and intensity of cocaine's psychotropic effects is dependent on the route of administration and the rate of rise of plasma levels (i.e., greater in the ascending portion of the plasma level vs. time curve plot) (106). Finally, cocaine's local anesthetic properties arise from its ability to block Na⁺ channel conductance thereby increasing the threshold required to generate an action potential (102).

Cocaethylene is a known CNS-active metabolite. In comparison to the parent drug, cocaine, CE binds with equal affinity to the dopaminergic receptor increasing the euphoric effects, but is 40 times less potent at the serotonin receptor. The serotonin receptor is believed to lessen the dysphoric effect associated with chronic COC use. CE persists longer and demonstrates a greater toxicity than its parent drug (108).

**Pharmacology**

The pharmacokinetic properties of cocaine originate from the body's ability to effectively metabolize and excrete this drug. The basic pharmacokinetic properties of cocaine in man are listed in Table 1-1. Cocaine, like many other basic drugs, binds to serum proteins such as albumin and α₁-acid glycoprotein (106). The onset of action for cocaine ranges from seconds (IV, SM) to minutes (IN, PO) (2). Cocaine's adsorption kinetics are route dependent with peak plasma levels achieved between 5 minutes (IV, SM) and 60 minutes (PO). Cocaine has a relatively short half-life of less than 1.5 hours. The cocaine effects of a given plasma level differ depending on whether the plasma concentration is rising or falling. For example, fewer psychological effects are manifested
during the downward slope of the dose-response curve (98). Biotransformation of cocaine produces predominantly inactivated, polar metabolites excreted in the urine.

**Metabolism**

Cocaine’s basic chemical structure includes a lipophilic benzoyl ring, an ionizable nitrogen-containing base (methylecgonine), and its ester substituents. The four primary metabolic pathways of cocaine include \( N \)-demethylation by P450 phase I monoamine oxidases, hydrolysis by liver esterases and plasma cholinesterases, spontaneous or chemical hydrolysis, and conjugation by P450 phase II enzymes. Other types of metabolism include hepatic transesterfication and hydroxylation, resulting in rapid hydrolysis of both ester linkages to inactivate cocaine (103).

Both chemical and enzymatic hydrolysis convert cocaine to its major metabolites, benzoylecgonine (BE) and ecgonine methyl ethyl (EME), respectively. Both metabolites can be further hydrolyzed to form ecgonine (ECG). Benzoylecgonine can be hydroxylated to form \( \text{m-hydroxybenzoylecgonine (MOHBE)} \) or \( N \)-demethylated to form norbenzoylecgonine (NBE). The \( N \)-demethylation of cocaine results in norcocaine (NCOC) which can further hydrolyze to form NBE. Liver carboxyesterases form a transesterfication product, cocaethylene (CE), with coingestion of cocaine and ethanol. Subsequent hydrolysis and \( N \)-demethylation of cocaethylene forms ecgonine ethyl ester (EEE) or BE, and norcocaethylene (NCE), respectively. Both CE and NCOC have been associated with hepatotoxicity (103-104, 109). Finally, crack cocaine forms a pyrolysis product, anhydroecgonine methyl ester (AEME). A schematic of cocaine metabolism is depicted in Figure 1-3.
Cocaine elimination normally follows first-order kinetics, but with saturation zero-order kinetics may occur. Cocaine’s rapid and complete metabolic disposition leads to low levels of unchanged drug in body fluids. As little as 1-9% (pH dependent) of unchanged drug can be readily detected in urine 4-6 h post-administration. The majority of a cocaine dose is excreted as urinary, polar metabolites. Cocaine’s major metabolites can be readily detected in the urine 24-72 h after administration. The combined data of twenty-four hour urine excretion studies show the percentage of each cocaine analyte to be as follows: 1-5% COC, 29-45% BE, 32-49% EME, 0.7% CE, 2.6-6.2% NCOC, 0% ECG, and trace amounts of other demethylated and hydroxylated products (98, 103, 106).

Adverse Effects and Treatment

Cocaine overdose can occur at relatively low doses resulting in a rapid death due to cardiovascular collapse, respiratory depression and arrest, dysrhythmias, and seizures.

The pathophysiology of acute toxicity originates from cardiovascular and neuropsychiatric complications associated with cocaine ingestion. The symptoms of acute toxicity include:

- vasoconstriction
- cerebrovascular accident
- hyperthermia
- sudden collapse
- hypertension
- pulmonary dysfunction
- myocardial infarction
- tachycardia
- intense paranoia
- bizarre and violent behavior
- stroke and grand-mal seizures
- coma
• rhabdomylosis
• atrial and ventricular fibrillation

One aspect of cocaine’s lethality is hyperthermia. Psychomotor hyperactivity, stimulation of hepatic calorigenic activity, and possibly stimulation of the hypothalamic thermoregulatory centers contribute to hyperthermia (98). Animal studies have demonstrated that major causes of death are linked to psychomotor agitation and hyperthermia (2). Cardiotoxicity associated with acute cocainism include myocardial ischemia and infarction, supraventricular and sinus tachycardia, systemic arterial hypertension, platelet aggregation, and in-situ thrombus formation. Both the left and right coronary arteries are subject to vasoconstriction leading to myocardial infarcts. These infarcts are commonly associated with delayed, atypical chest pain and can occur in subjects having no prior cardiac dysfunction (2). Pulmonary complications begin with repetitive short, deep breaths, progress to edema and depression, and ultimately to collapse (Cheyne-Stoke breathing). The vasoconstrictive nature of cocaine can also affect the vasculature of the skeletal muscle bed, resulting in severe rhabdomylosis. Individuals that conceal cocaine in body cavities, also known as body “packers” and “stuffers” may suffer acute gastrointestinal ischemia, colitis, and perforations.

Cocaine induces other adverse effects that are not acutely life threatening such as mydriasis, nausea and vomiting, aberrant psychological behavior, insomnia, and anorexia. Again, most of these symptoms are attributed to cocaine’s affect on the central nervous system.

Chronic exposure signs and symptoms include rhinitis, distorted perception, shortness of breath, increased and labored respiration, accelerated atherosclerosis,
tachycardia, cold sweats skin cellulitis/abscess, chronic cough and bronchitis, hypersensitive bronchial mucosa ("crack" lung), weight abnormalities, violent protective behavior, nasal septal perforation, psychiatric complications, and profound mood swings (2, 98, 110). Continual vasoconstrictive episodes and/or recurrent or diffuse ischemia can result in dilated cardiomyopathy. In rare cases, the aorta can actually dissect and rupture secondary to the mechanical stress of chronic hypertension and tachycardia. With continued IV use of cocaine, individuals are predisposed to bacterial endocarditis and deep vein thrombosis (98). Other behavioral effects mediated by stimulation of the "reward system" include self-medicating to control of euphoric response, anorexia, hyperactivity, and profound sexual excitement.

Cocaine toxicity requires initial treatment of the most life-threatening symptoms to stabilize the subject. Sedative-hypnotics like diazepam are administered to control the neurological complications (agitation, anxiety, seizures), sinus and supraventricular tachycardia, hypertension and other sympathomimetic symptoms. Calcium channel blockers (verapamil, nifedipine), oxygen, and cooling relieve hyperthermia and cardiotoxic effects (2). In addition, sodium carbonate is given to correct respiratory acidosis. If treatment occurs at the early excitatory phase during hypertensive effects, sodium nitroprusside or nitroglycerin drip may be administered. In addition, propanolol may reverse cardiovascular stimulation. During the late depressive phase of hypotension, administration of dopamine or norephedrine is more appropriate (98). Other treatment of cocaine toxicity mainly involves treatment of specific symptoms. Activated charcoal efficiently absorbs cocaine taken orally or in the cases of gastrointestinal drug smugglers (111). Psychotic reactions are treated with haloperidol or other antipsychotics. For
comatose or lethargic patients, naloxone should be considered in case of simultaneous ingestion of heroin (98).

**Opiates: A Brief Review**

**Use and Misuse**

“Opiates” are naturally occurring analgesic alkaloids derived from the opium poppy, *Papaver sominiferum*. Opium, derived from the Greek name for juice, is comprised of more than 25 distinct alkaloids, including morphine and codeine obtained from the milky exudate of the unripened, opium poppy seed (112). Morphine, the principle alkaloid of opium, was named after the Greek god of dreams, Morpheus, who was often depicted with poppy flowers (1,113). “Opioids” are natural and semisynthetic alkaloid derivatives prepared from opium, in addition to synthetic surrogates whose actions mimic those of morphine. Therefore, opioids include opiates, synthetic opioids, and opiopeptins (endogenous neuropeptides such as endorphins and enkephalins).

Opium alkaloids have been utilized as analgesics since the early 1800’s following the chemical isolation of pharmacologically active morphine (1803), codeine (1832), and papaverine (1848). These discoveries along with the invention of the hypodermic needle led to a predominant use of pure alkaloids rather than crude opium preparations. By World War II, opiate addiction became a problem prompting the clinical field to introduce opioids, like meperidine and methadone, which possess morphine-like analgesia but with less addiction potential. Then in the 1950s, opioid antagonists like nalorphine and naloxone were developed to reverse heroin and morphine toxicity (112-113).
Opiate analgesics are effective and common medications for treatment of mild to severe pain. Strong opiate analgesics are commonly referred to as narcotics, which is derived from the Greek word for stupor (113). Based on subjective patient reports, opioids are effective analgesics because they have the ability to change pain perception by raising the pain threshold. The Controlled Substance Abuse Act categorizes most opiate analgesics as Schedule II. They are also used to treat congestive heart failure (pulmonary edema) and anxiety. Opioids are commercially available as elixirs, pills, powders, suppositories, and solutions (114-116). Some commercial preparations are manufactured in combination with acetaminophen (Phenergan®, Tylenol® with codeine®) or aspirin (Empirin®). Common doses, given every four to six hours, are 15-60 mg (PO) for codeine, 2.5-10 mg (IM, SC, PO) for morphine, and 5-10 mg (PO, IM, SC) for heroin (112, 114).

Both the licit and illicit forms of opioids are often abused due to their desirable central nervous system (CNS) effects, especially euphoria. Heroin, first synthesized from morphine by Wright in 1874, is an illicit Schedule I drug that has no medicinal applications in the United States. Many opioids are highly addictive leading to physical and psychological dependence (116). Reports by the Drug Abuse Warning Network (DAWN) show a general trend towards an increased use of opioids during the period of 1992 to 1994 (117). Moreover, SAMHSA’s 1996 National Household Survey on Drug Abuse reported an increasing trend in new heroin users from 1992 to 1995, with estimated “past month” heroin users increasing from 68 to 216 thousand during this time period (4).
Chemical Structure

Phenanthrenes (4,5-epoxymorphinans), the most well known and characterized opioids, follow the basic morphine chemical structure illustrated in Figure 1-4. Drugs in this group possess various modifications including methylation or acetylation of the C3 and C6 hydroxy groups, oxidation of the C6 hydroxy group to a ketone functionality, saturation of the C7 - C8 double bond, and hydroxylation at the C14 position. These subtle molecular differences among opioids can change their pharmacological action (e.g., convert an agonist to an antagonist), receptor affinity, metabolic resistance to first-pass hepatic metabolism, and lipid solubility (103, 112, 114).

Neurobiology

The neurological mechanism of action for opioids is mediated through specific receptors located at various sites in the CNS and other peripheral organs. Opioids exert their pharmacological effects by mimicking endogenous neuropeptides (114). Opioid receptors are also located on several immune response cell types such as neutrophils, lymphocytes, and monocytes which are commonly associated with endogenous opioid peptides produced during a state of stress (116). Primary receptors include:

1) \( \mu \) (mu) receptors, responsible for euphoria, supraspinal analgesia, respiratory depression, miosis, reduced gastrointestinal motility, and physical tolerance and dependence

2) \( \kappa \) (kappa) receptors, mediate spinal analgesia, sedation, sleep, miosis, physical dependence, and limited respiratory depression
3) δ (delta) receptors, mediate dysphoria, delusions, hallucinations, 
respiratory stimulation, and vasomotor stimulation

4) σ (sigma), purported to have effects similar to the δ receptor.

These CNS receptors are part of the limbic system. The limbic system is involved in the arousal of emotion of man including and painful sensations and its negative emotional component and in some cases euphoria. Opioids bind to CNS receptors on terminal nerve endings and block the release of neurotransmitters involved in the transmission of pain stimuli (114).

On a cellular level, opioid receptors exert their effects through changes in Ca^{2+} and K^{+} flux associated with the cyclic AMP (cAMP) system of the nervous system (114). On a molecular level, opioid receptors are distinctly different. Through receptor binding and molecular cloning studies, it has been determined that these distinct opioid receptors are encoded by different genes expressed in discrete neuronal pathways or cell types. In addition, each opioid has differing selectivities for each receptor, which determine its pharmacological effect (113). For instance, heroin is a strong µ agonist while codeine is a weak µ and κ agonist, resulting in heroin's analgesic potency being 10 times that of codeine. Based upon their pharmacologic activity on the receptors, opioids are classified as full agonist, mixed agonist/antagonist, or full antagonist. Antagonist can preferentially displace agonist, which is why naloxone is so effective at reversing toxicity of opioid agonist. The ability of an opioid to bind to its receptor and the relative binding affinity are influenced by molecular structure and stereospecificity. For example, the levororatory isomer of morphine produces analgesia while the detrorotartory isomer of levorphanol (dextromethorphan) acts as an antitussive agent (116).
Prolonged use of opioids leads to tolerance. Tolerance begins with the first dose, but is usually clinically insignificant until about 2-3 weeks of chronic use. To minimize tolerance, opioids should be administered in small doses given at frequent intervals. Cross-tolerance among opioids is a prevailing characteristic (116).

Physical and psychological dependence makes opioid withdrawal and detoxification extremely painful and difficult. Typically, withdrawal signs from strong agonists appear within 6-8 hours after the last administration and peak at 36-72 hours. Psychological dependence produces strong craving that can lead the individual to pleas, demands, and manipulative behavior.

**Pharmacology**

Opioids share common pathways for their pharmacokinetic parameters including adsorption, distribution, metabolism, and elimination.

Since opioids are well absorbed by most tissues, numerous routes of administration are effective. Depending on the application, opioids can be ingested orally or injected intramuscular, intravenous, or subcutaneous. Some cautionary heroin users choose nasal insufflation (snorting) or inhalation of drug vapors ("chasing the dragon") for safety reasons. For instance, intravenous heroin use has a greater risk of overdose, transfer of diseases such as hepatitis and autoimmune deficiency syndrome (AIDS), and injection-site injuries. For analgesic and anesthetic purposes, alternate routes of administration include transdermal patches, epidural, and intrathecal injections (118-120).

The distribution of opioids depends upon specific drug properties, both chemical and physiological. The extent of first-pass metabolism influences opioid bioavailability
and pharmacological effects. Opioids can be as much as 95% bound by plasma proteins. Opioids concentrate in the tissues of highly perfused organs such as the lungs, brain, kidney, liver, and spleen. Opioids further accumulate in skeletal muscle and lipid reservoirs and cross-placental barriers to varying degrees (103).

**Metabolism**

Opioids are chiefly metabolized in the liver forming pharmacologically active and inactive metabolites. Some active metabolites have analgesic effects that are stronger than the parent drug itself. Metabolic pathways include reduction, oxidation, N- and O-dealkylation, hydroxylation, and conjugation. Both parent drug and metabolites are excreted through enterohepatic or renal circulation. The majority of opioids and their metabolites are excreted in the urine, with only a small amount of glucuronide conjugates eliminated in the feces (103). Figure 1-4 depicts the chemical structure and metabolism of common opioids.

**Adverse Effects and Treatment**

Individuals using opioids often experience adverse effects in addition to the desired therapeutic effects (Table 1-2). Common complaints include nausea, vomiting, constipation, and mood swings. The most life-threatening adversity, commonly observed following an overdose, is respiratory depression. Other risks associated with opioid toxicity include coma, hypothermia, seizures, and hypotension.

Treatment of opioid overdose consists mainly of supportive and antidotal measures such as:
1. Monitoring cardiovascular and respiratory status
2. Ventilation to reestablish respiratory exchange
3. Oxygen and anticonvulsants to combat seizure disorders
4. Intravenous fluids and vasopressors to regain normal blood pressure
5. Naloxone to counteract CNS and respiratory effects (115-116)

Generally, hemodialysis and forced diuresis are ineffective in treating opioid overdose patients.

Opioid abusers normally require a treatment program for successful detoxification. Hallmarks of withdrawal reflecting the physical dependence include irritability, insomnia, anorexia, violent yawning and sneezing, gastrointestinal abnormalities, elevated heart rate, profuse sweating, and piloeruction. Strong pains in the bones and muscles, and uncontrollable muscle spasms are consistent with withdrawal (98). The culmination of these symptoms is widely referred to as the abstinence or withdrawal syndrome. In most cases, these symptoms follow a characteristic chronology as shown in Table 1-3. The pharmacological activity of the opioid determines the severity of the withdrawal symptoms.

If the withdrawal symptoms are severe or prolonged, a methadone management protocol is followed to alleviate undesirable effects. Methadone is a slower acting opioid with a lower abuse liability that is frequently used in substitution pharmacotherapy. Patients are stabilized for 2-3 days before the methadone dosage is gradually decreased. Buprenorphine and propoxyphene have also been investigated as alternatives in the treatment of opioid dependence. Other treatments to alleviate withdrawal symptoms
include fluids and electrolytes, antispasmodics (propantheline), sedative-hypnotics (phenobarbital), and anti-adrenergics (clonidine) (116).

Scope of Dissertation

This dissertation embodies three projects, detailed below, that investigated the utility of hair and nail analysis for the identification of drug exposure. The current techniques for detection of drug exposure are flawed in many respects. Self-reports are notorious for underestimating drug use due to feared repercussions by the individual. Reported toxicological data derived from the analysis of conventional matrices, blood and urine, have many deficiencies which limit the sensitivity of the analytical methods. Therefore, the first objective of this dissertation was to develop and evaluate a more sensitive analytical technique using keratinized matrices to improve the detectability of drug exposure.

In the past two decades, hair analysis has undergone a metamorphosis from an unrefined research tool to a highly sensitive forensic technique. Despite the extensive research that has already been conducted, many questions and controversies remain. In addition, the potential utility of another keratinized matrix, nail, for identifying drug exposure is for the most part unknown.

The second objective of this dissertation was to investigate unknown aspects of hair and nail analysis that will contribute to a greater understanding of drug incorporation and detection of drugs in hair and nails. Examples of unanswered questions include: 1) Can hair and nail analyses improve the detection of drugs in comparison to conventional
matrices? 2) Is the current methodology utilized for hair appropriate for nails? 3) Do keratinized matrices demonstrate a dose-response relationship? 4) Is there a relationship between dose, time profile and concentration of drug in hair and nail? To accomplish this, highly sensitive and specific gas chromatography-mass spectrometry was employed to obtain analytical results for all three projects.

Chapter 1 is an introduction presenting a historical overview of the applications of drug testing, the laws addressing drug testing, and the matrices utilized in drug testing. It continues with a description of hair and nail anatomy, physiology, growth, possible routes of drug incorporation, and factors influencing drug incorporation. The advantages, disadvantages, and controversies associated with hair and nail analysis are also discussed. This introductory chapter also reviews the pharmacology, metabolism, and adverse effects of cocaine and opiates since these were the drug classes investigated during the dissertation.

The methodology employed for analysis of specimens within this dissertation are discussed in Chapter 2. It begins with a historical overview of analytical techniques used in the past and the present for identifying drugs in keratinized matrices. It focuses on each step of the analysis process including specimen collection and handling, decontamination wash procedures, isolation of analytes from keratinized matrices, and instrumental analysis. Performance data including accuracy, precision, recovery, and linearity are also presented.

Chapter 3 discusses the analysis of specimens collected and analyzed from an 11-week controlled clinical study in which eight black, male subjects were administered low and high doses of cocaine and codeine. Scalp hair from the posterior vertex region and
fingernail scrapings were collected weekly. The analytical results served to answer pharmacological questions about drugs in hair and nails. It also compared paired results of hair and nail to determine the potential utility of nails since much less is known about drug incorporation into this matrix. Lastly, it discusses the decontamination wash procedures and quantifies the amount of drug removed during each wash step.

Chapter 4 discusses a forensic application of nail analysis. The analytical results of conventional postmortem results using blood, urine, vitreous humor, and other tissue, were compared with the results of hair analysis. Nine cocaine analytes were investigated. Results of the cocaine analytes present in nails were compared to the subject’s history and the significance of certain unique analytes was discussed. The advantages, disadvantages, and potential applications of nail analysis were further presented.

Chapter 5 discusses a clinical application of hair analysis. Epidemiological studies have linked drug use, particularly cocaine, to some cases of sudden infant death syndrome (SIDS). Since the mechanism of SIDS is unknown and the risk factors are multifactorial and in many cases confounding, it is very important to identify all risk factors associated with an infant’s death. This project utilized a more sensitive toxicological technique, hair analysis, to identify the presence or absence of drug exposure in 26 deceased infants.

Chapter 6 summarizes the conclusions ascertained by these three projects and discusses the significance of this research.
Figure 1-1. The Anatomy of a Hair Follicle
Figure 1.2. The Anatomy of a Fingernail
Table 1-1. Pharmacokinetics of Cocaine in Humans (2, 103-104, 106)

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Details</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma Half-life (h):</td>
<td>0.7-1.5 (COC)</td>
</tr>
<tr>
<td></td>
<td>5-8 (BE)</td>
</tr>
<tr>
<td></td>
<td>3.5-8 (EME)</td>
</tr>
<tr>
<td></td>
<td>2.5 (CE)</td>
</tr>
<tr>
<td>Volume of distribution (L/kg):</td>
<td>1.6-2.7</td>
</tr>
<tr>
<td>Plasma protein binding (%):</td>
<td>96</td>
</tr>
<tr>
<td>Bioavailability (%):</td>
<td>20-60 (IN and PO)</td>
</tr>
<tr>
<td></td>
<td>90-100 (IV and SM)</td>
</tr>
<tr>
<td>Body clearance (L/min):</td>
<td>2.0</td>
</tr>
<tr>
<td>Peak plasma level (ng/mL):</td>
<td>IN 100-500 (1.5-2.0 mg/kg dose)</td>
</tr>
<tr>
<td></td>
<td>PO 50-90 (2.0 mg/kg dose)</td>
</tr>
<tr>
<td></td>
<td>IV and SM 500-1000 (32-50 mg dose)</td>
</tr>
<tr>
<td>Time of peak plasma level (min):</td>
<td>IN 15-60 (1.5-2.0 mg/kg dose)</td>
</tr>
<tr>
<td></td>
<td>PO 50-90 (2.0 mg/kg dose)</td>
</tr>
<tr>
<td></td>
<td>IV and SM 5 (32-50 mg dose)</td>
</tr>
<tr>
<td>pKa:</td>
<td>8.6</td>
</tr>
</tbody>
</table>

Abbreviations: cocaine (COC), benzoylecgonine (BE), ecgonine methyl ester (EME), cocaethylene (CE), intranasal (IN), oral (PO), smoked (SM), and intravenous (IV).
Figure 1-3. The Metabolic Pathway of Cocaine

Abbreviations: pyrolysis (Δ), cocaine (COC), anhydroecgonine methyl ester (AEME), benzoylecgonine (BE), cocaethylene (CE), ecgonine (ECG), ecgonine ethyl ester (EEE), ecgonine methyl ester (EEE), meta-hydroxybenzoylecgonine (MOHBE), norbenzoylecgonine (NBE), norcocaethylene (NCE), and norcocaine (NCOC).
Figure 1-4. Chemical Structure and Metabolic Pathway of Common Opioids (112)
Table 1-2. Therapeutic and Adverse Effects of Opioids (121)

**Central Nervous System Effects:**

*Nervous*  
- Euphoria  
- Analgesia  
- Sedation  
- Mental clouding and mood swings  
- Pulmonary  
- Respiratory depression  
- Decreased responsiveness

*Gastrointestinal*  
- Nausea  
- Vomiting

*Other*  
- Dizziness  
- Cough suppression  
- Miosis  
- Truncal rigidity  
- Flushing and warming of the skin  
- Sweating and itching

**Peripheral Effects:**

*Cardiac*  
- Bradycardia  
- Orthostatic hypotension when system stressed  
- Stroke

*Gastrointestinal*  
- Constipation  
- Decreased motility  
- Increased tone  
- Decreased gastric secretions  
- Biliary tract constriction of smooth muscle

*Genitourinary*  
- Decreased renal plasma flow  
- Increased urethral and bladder tone  
- Prolongation of labor  
- Menstrual abnormalities  
- Sexual dysfunction

*Neuroendocrine*  
- Increased antidiuretic hormone (ADH) release
<table>
<thead>
<tr>
<th>Time Interval</th>
<th>Symptoms</th>
</tr>
</thead>
<tbody>
<tr>
<td>8-12 Hours</td>
<td>Lacrimation, Yawning, Rhinorrhea, Perspiration</td>
</tr>
<tr>
<td>12-14 Hours</td>
<td>Irritability, Piloerection (&quot;Goose flesh&quot;), Restless sleep, Weakness, Mydriasis, Tremor, Anorexia, Muscle twitching</td>
</tr>
<tr>
<td>48-72 Hours</td>
<td>Increased irritability, Increased heart rate, Insomnia, Hypertension, Marked anorexia, Hot and cold flashes, Sneezing, Alternating sweating/flushing, Nausea and vomiting, Piloerection, Hyperthermia, Rapid or deep breathing, Abdominal cramps, Aching muscles</td>
</tr>
<tr>
<td>Syndrome duration:</td>
<td>7-10 days</td>
</tr>
</tbody>
</table>
CHAPTER 2
DEVELOPMENT OF A GAS CHROMATOGRAPHIC-MASS SPECTROMETRIC
ASSAY FOR THE MEASUREMENT OF COCAINE AND OPIATE ANALYTES IN
KERATINIZED MATRICES

Historical Overview of Methodology for Hair and Nail Analysis

The analysis of keratinized matrices to detect drugs of abuse is a relatively new field of study. The uniqueness of hair and nail requires specialized pretreatment steps to adequately measure drug incorporated into these matrices. In much the same way that plasma proteins are precipitated from blood to reduce interferences prior to measuring an analyte of interest, keratinized matrices require pretreatment steps to prepare them for analyte extraction. The chemical composition, structure, and exposure to the environment introduce new issues to the toxicologist interested in analysis of hair and nail.

The analysis of drug analytes in hair is a multi-step process involving the following: (1) decontamination of the surface of the hair fibers through washing; (2) sample preparation to facilitate ease of handling and release of drug analytes; (3) incubation or digestion to release drug analytes; (4) extraction and purification of drug analytes; and (5) analysis by an immunochemical or chromatographic technique. Conventional analysis of urine and blood usually only requires the last two steps. However, research has
demonstrated that the additional three pretreatment steps are necessary for the successful analysis of keratinized matrices (48, 62, 122).

First, keratinized matrices require decontamination procedures to remove unwanted interferents (i.e., lipids, oils, cosmetics) and exogenous analytes (e.g., drug) coating the surface from environmental exposure. A survey of the literature shows that researchers have investigated organic solvents, phosphate buffers, water, soaps, and various combinations of these for decontamination wash procedures. Table 2-1 summarizes representative decontamination wash procedures presented in the literature.

The extent to which keratinized matrices can be decontaminated from exogenous analytes depends on factors governing penetrance of the drug into the hair matrix such as route of drug administration, chemical treatment, and the water solubility of the drug. Drug entry into keratinized matrices is facilitated by water and the pH of the environment, both of which make decontamination procedures less effective (122).

Regardless of the procedure employed, the efficacy of decontamination washes has remained controversial. Some researchers are convinced that in most cases externally bound drug can be removed through multi-step decontamination processes (62, 123-124). If extensive washing does not remove residual drug, some investigators further propose that passive exposure and active ingestion are distinguishable through “wash kinetic criteria.” Wash kinetics compares the drug concentrations of the wash fractions and the extracted hair specimens (123). Others oppose complete efficacy of decontamination procedures, demonstrating that residual drug may remain after extensive washing procedures (18, 125-127).
Another required pretreatment step requires special preparation of hair and nails to optimize solubilization of these matrices during extraction or digest procedures. Researchers have found that by cutting hair and nails into small segments or pulverizing them into a fine powder, reagents can more readily enter the protein matrix, thereby releasing the analyte into solution. Moreover, measuring the segments of hair during this pretreatment step allows chronological analysis to estimate the timing of drug exposure.

Hair and nails are synthesized from hardened, structural proteins such as keratins that form a stable framework utilizing a variety of chemical bonds. Many of these bonds are strong and cannot be easily broken (e.g., covalent and electrostatic). Consequently, special reagents or environmental conditions must be employed to denature keratinized proteins. While some proteins can be denatured with reagents such as mineral acids, trypsin, or increased temperature (>37°C), complete solubilization of keratinized proteins requires harsher conditions.

Normally, to completely denature more resistant proteins, prolonged boiling or exposure to thiol-containing compounds and detergents (i.e., sodium dodecyl sulfate) or hydrogen bond-breaking reagents (i.e., urea or guanidine hydrochloride) are required (128). However, for drug testing of hair and nail, these treatments need to successfully solubilize the protein matrix to effectively remove the drug without destroying the drug itself. For example, extreme temperature or acidic environment should not be permitted in pretreatment steps for cocaine analysis because this would lead to degradation of the parent drug to its metabolite benzoylecgonine. In addition, chemical bonds between functional groups of drug analytes and other pigmentation proteins like melanin must be broken. Treatment with a strong acid or base depends on whether the drug is basic,
acidic, or neutral. Basic drugs are more readily extracted with strong bases while acidic/neutral drugs are normally extracted with strong acids.

It is not enough to subject keratinized matrices to simple liquid-liquid extraction (LLE) or solid-phase extraction (SPE) utilized for conventional liquefied matrices (i.e., blood and urine) to efficiently isolating the analyte. Rather, these matrices require degradation procedures prior to common extraction methods. The three primary degradation techniques employed by researchers include enzymatic digestion, caustic digestion, or solvent extraction. A myriad of these techniques exists in the literature and Table 2-1 summarizes a representative assay utilizing all of these. Many digest and extract solutions also contain chemicals like dithiothreitol and detergents that can break disulfide bonds of the protein matrix. These procedures are generally carried out under heated conditions and/or for prolonged periods to facilitate the degradation process. Once these degradation processes are completed, further extraction is necessary to improve analyte isolation and recovery.

There are two methods used in the field of forensic toxicology to isolate drugs from biological matrices. The principle of LLE is based upon the partitioning coefficient of a drug. The coefficient is determined by the degree of affinity an isolate has for a chosen organic solvent and partitioning strength of the analyte between aqueous and organic phases. Given these criteria, an acidic drug is more soluble in an organic solvent than an aqueous solvent at an acidic pH and a basic drug is more soluble in an organic solvent than an aqueous solvent at a basic pH. A combination of extraction-back extraction-reextraction steps is employed to move the drug analyte from organic to aqueous to organic to obtain cleaner extracts using LLE (28).
Conversely, SPE is a sorbent phase bonded to a solid support such as silica contained within a column. There are many types of commercially available phases including hydrophobic (C18 octadecyl), hydrophilic (cyanopropyl), anion-exchange (quaternary amine), cation-exchange (benzene sulfonic acid), and copolymeric (hydrophobic and cation-exchange). SPE employs both physical and chemical properties to reproducibly interact with drug analytes to remove them from the liquid (i.e., serum, urine, amniotic fluid) or homogenate (i.e., liver, meconium) matrix in which they are contained. Multiple washings with various organic solvents can remove unwanted interferents prior to elution of the drug analyte from the SPE column (28).

Previously published methodology, describing the analysis of drugs in keratinized matrices, have utilized predominantly immunochemical or chromatographic technique. A description of each is outside the scope of this dissertation, however, Table 2-1 summarizes a cross-section of analytical techniques reported in the literature.

Developed Assays for the Detection and Measurement of Cocaine and Opiate Analytes in Keratinized Matrices by GC/MS Analysis

Collection and Preparation of Specimens

For this dissertation, several processes were employed to prepare the keratinized matrices. In all cases, hair was collected from the crown area of the scalp, or posterior vertex region. This region was chosen because it is purported to have the most follicles in active (anagen) growth at a given time. Growing at an approximate rate of 1.0 cm/month, this region also has a faster growing rate than other regions of the scalp (75). In addition, hair collection methods include cutting the hair as close to the scalp as possible or
plucking the hair from the scalp to include the root portion as well as the shaft. In all projects of this dissertation, hair was collected as close to the scalp as possible, knowing that the most recent drug exposure would not be detected.

In Chapter 3, hair was collected by the staff of the Intramural Research Program (IRP), NIDA. Grooming clippers were employed to remove the first collection of scalp hair from subjects, collecting different regions of the scalp (temporal, frontal, nape, posterior vertex, and anterior vertex) separately. For the study reported herein, only hair from the posterior vertex region was analyzed. The remaining stubble was removed and discarded with shaving cream and a straight edge razor. Hair from initial collection was stored in Ziplock™ plastic bags at room temperature until hair could be finely cut with scissors and transferred to separate glass vessels for storage at -30°C. For the remainder of the study, scalp hair (approximately 2-3 mm in length) was collected as close to the scalp as possible using a cleaned electrical shaver (Norelco®). Again, remaining stubble was removed and discarded using a straight edge razor. Since collected hair specimens only represented one week of growth, the hair was in small sections that could be weighed and used for analysis without further manipulation.

Infant hair analyzed in Chapter 5, was cut as close to the scalp as possible using cleaned scissors. Specimens were weighed into polyethylene vessels and 5 to 6 glass beads (0.5 mm) were introduced into the vessel to promote mechanical disruption of the hair. These specimens were pulverized into a fine powder by a Mini-beadbeater-8™ Cell Disrupter (Biospec Products, Bartlesville, OK) set at 80% power for 5 minutes. The hair powder was transferred to a disposable glass culture tube and the residual hair was removed from the polyethylene vessel using 8 to 10 methanolic rinses, which were
included in the subsequent overnight methanolic incubation step. Glass beads were removed after the overnight methanolic incubation period to prevent loss of analyte present in hair that coated the surface of the beads. It was believed that the glass beads were inert in the methanolic solution and would not interfere with noise to the assay.

Nail was collected from the digits of the hand and foot for the postmortem analysis study detailed in Chapter 4. Clean nail clippers were utilized to remove as much of the distal nail portion as possible. The nail was then finely cut into small pieces for analysis.

Fingernail specimens were also collected from subjects enrolled in the controlled clinical study (Chapter 3). The ventral surface of the nail was scraped with a sterile scalpel blade. Each digit was scraped 50-100 times and all scrapings were combined for analysis. These scrapings could be used for analysis without further manipulation. Nail scrapings were transferred to separate glass vessels for storage at -30°C until time of analysis.

Decontamination Wash Procedures

Two types of decontamination wash procedures were utilized in this dissertation. Both procedures have been previously employed by several research groups (18, 60, 123, 127, 129).

The first decontamination procedure (Chapter 3) used a multi-step process combining hydrating and non-hydrating solvents. First, a 15-min isopropanol wash (3.0 mL) was utilized to remove loosely bound lipids, soaps, and drug analyte present on the keratinized surface. Next, three successive 30-min washes in 3.0 mL of a 0.1 M phosphate buffer (pH 6.0) solution (1.36 g potassium phosphate monobasic in 1.0 L
deionized water) were employed to hydrate the keratinized specimen and remove analyte from its porous surface. All four steps were performed at room temperature and agitated by stirring or placing in an oscillating water bath. These wash fractions were collected for further analysis to determine the percentage of drug removed in each fraction.

Alternatively, Chapters 4 and 5 utilized a single-step decontamination wash procedure that was much less involved and less time-consuming. The prepared hair or nail specimens were transferred into a disposable culture tube and 3.0 mL of methanol was added to each. The specimen was then vortexed for 15 to 20 seconds and the methanol was immediately decanted and retained for further analysis. Specimens were not allowed to remain in the methanol longer than the time required for vortexing in order to minimize analyte extraction from the interior regions of the keratinized matrices.

Isolation of Drug Analytes from Keratinized Matrices

For the isolation of drug analytes from keratinized matrices, two different procedures were performed for this dissertation. Comparison studies of these two isolation procedures gave insight into the advantages and disadvantages of each.

A comparison study of enzymatic digestion and methanolic incubation demonstrated that both performed similarly and each had advantages over the other procedure. Replicate analyses (n=10) of an authentic drug-positive hair specimen were prepared using both isolation techniques. Specimens were otherwise subjected to the same sample preparation procedures and GC/MS analysis. Specimens were analyzed during the same run to minimize differences due to instrumental analysis. This comparison study demonstrated that the enzymatic digestion was able to isolate more drug analyte, but
it also resulted in more interference, and hence, a greater signal-to-noise ratio was observed. Mean concentration data for cocaine, cocaethylene, benzoylecgonine, and morphine are summarized in Table 2-2.

A comparison was also performed to determine which enzyme resulted in the best isolation of analyte. Two enzymes were chosen based on recommendations from the NIDA scientific group from which we obtained specimens. The enzymes, *Tritirachium album* and *Subtilisin A* were employed to isolate drug from the hair of a known drug abuser. Both *Tritirachium album* (proteinase K- Type XI) and *Subtilisin A* (protease - Type VIII) were purchased from Sigma Chemical Company, St. Louis. In addition to the respective enzyme, the digest solution also contained dithiothreitol (DTT-60mg/10 mL) and sodium dodecyl sulfate (SDS-20 mg/10 mL) to assist in breaking down the protein matrix of the hair. Mean concentration data for cocaine, cocaethylene, benzoylecgonine, and morphine are summarized in Table 2-2. Results were comparable for the two enzymes, however, *Tritirachium album* was ultimately chosen because it demonstrated greater recovery of minor analytes. In addition, these studies also demonstrated that SDS contributed background signal and did not improve recovery of analytes from the matrix. Therefore, SDS was not included in the digest solution during later specimen preparation.

A brief description of the enzymatic digest procedure used to degrade the protein matrix of hair and nail specimens collected from subject enrolled in the inpatient study (Chapter 3) follows. The enzymatic digest solution was prepared fresh daily by combining 60 mg of DTT, 0.5 mg of Protease XI (*Tritirachium album*), and 10 mL of 0.05M Tris buffer (preset pH 7.4).
Weighed samples were placed into 4.0 mL fritted filter (RFV02F4P-United Chemical Technologies; Bristol, PA) and 1.0 mL of digest solution was added to each. Trideuterated internal standards for major analytes (d$_3$-benzoylecgonine, d$_3$-cocaine, d$_3$-ecgonine methyl ester, d$_3$-codeine, and d$_3$-morphine) were added at a concentration of 100 ng/mg and minor analyte internal standards (d$_3$-cocaethylene, d$_3$-6-acetylmorphine) were added at concentration of 50 ng/mg.

Micro stir-bars were added to samples, fritted filters were capped and placed overnight (~16 hr) into a heated water bath (40°C) placed on top of stirring plates set at 80% of maximum speed. After completion of the digestion, the digestate was eluted from the fritted filters and collected into conical-shaped disposable culture tubes. The filters were rinsed with 2 x 2 mL volumes of 100 mM phosphate buffer (pH 6.0) which was collected into the same tube as the digestate. The filtered digestate was centrifuged at 4000 rpm for 10 minutes prior to SPE.

Specimens for studies presented in Chapters 4 and 5 employed an organic solvent incubation to isolate drug analytes from the keratinized matrices. Briefly, weighed and decontaminated specimens were placed in disposable culture tubes and 3.0 mL of methanol was added to each tube. Specimens were capped, vortexed, and placed overnight (~16 hr) into a heating manifold (40°C). Specimens were vortexed additional times during the incubation period. After the overnight methanolic incubation, samples were centrifuged at 3000 rpm for 5 to 10 min. The drug-containing supernatant was decanted from the hair pellet and evaporated at 40°C under a stream of nitrogen. Residues were reconstituted in 0.025 M phosphate buffer in preparation for SPE.
Assay Standardization

Calibration curves were prepared using working standards prepared in water or acetonitrile and stored at -30°C until needed. Calibrators and controls were prepared using drug-free hair or nail obtained from laboratory personnel. A minimum of six calibration points was included to construct a curve spanning a concentration range of 0.10-10.0 ng/mg. An unextracted standard was included in each run to assist with instrument setup and to help troubleshoot any potential problems with specimen preparation (i.e., to help determine if poor signal response was due to poor extraction of the specimen or instrumental problems). Negative controls containing drug-free hair or nail, with and without addition of internal standards, were included in each batch to assist in data interpretation to evaluate background noise and interference due to the matrix or reagents added during specimen preparation. Positive controls were included throughout the run (beginning, middle, and end) to measure curve stability. Positive controls were prepared fresh daily with standard materials prepared separately from calibrator materials and if possible, from different sources. Both high and low concentrations (0.5 ng/mg and 5.0 ng/mg), with respect to the calibration curve, were analyzed. Within-run and between-run variability for the positive controls are discussed later in the performance data section of this chapter.

Also included in each batch were hydrolysis controls for cocaine and 6-acetylmorphine ranging in concentration from 5 and 40 ng/mg depending on the project and the matrix being evaluated. Hydrolysis controls measured the spontaneous hydrolysis occurring during preparation and extraction methods. Table 2-3 summarizes the hydrolysis control data obtained for each project. Generally, hydrolysis was less than 5%
for cocaine and less than 10% for 6-acetylmorphine. Mean cocaine and 6-acetylmorphine concentrations were within 20% of the target concentration. Analytes detected due to hydrolysis included ecgonine methyl ester (EME), benzoylecgonine (BE), and morphine (MOR). Mean concentration ranges for detected analytes were as follows: EME- none detected (ND) to 1.0 ng/mg; BE- ND to 3.2 ng/mg; and MOR- 0.42 to 1.6 ng/mg.

**Extraction and Derivatization**

For this dissertation, SPE technology was chosen based on previous experience and the potential advantages offered. Since multiple analytes were being simultaneously analyzed, a copolymeric phase using hydrophobic and cation-exchange properties was chosen. Several commercially available SPE columns were compared prior to the selection of United Chemical Technologies CleanScreen® (ZSDAU020, 200mg/10 mL) column.

The same SPE procedure was employed during all projects of this dissertation. A step-by-step description of the procedure is presented. After completion of the appropriate isolation techniques (enzymatic extraction or methanolic reflux), specimens were reconstituted in phosphate buffer (0.01 M or 0.025 M; pH 6.0) prior to SPE using a vacuum manifold system. The extraction columns were conditioned with elution solvent (1 mL), methanol (3 mL), deionized water (3 mL), and phosphate buffer (pH 6.0, 3 mL). Specimens were added to SPE cartridges followed by deionized water (2 mL), 100 µM acetate buffer (pH 4.0, 2 mL), methanol (3 mL), and acetonitrile (1 mL). SPE columns were aspirated to dryness under a full vacuum. The analytes were collected into clean tubes inserted into the vacuum manifold system by addition of 4.0 mL of elution solvent.
consisting of methylene chloride-isopropanol-ammonium hydroxide (80:20:2 v/v/v).

During conditioning, sample introduction, and elution steps, the pressure was adjusted to maintain a flow rate of approximately 1-2 mL/min (20 mm Hg). Columns were not permitted to dry between addition of the phosphate buffer and sample introduction.

Samples were evaporated under a nitrogen purge using a heated water bath set at 40°C (Zymark® TurboVap®). Specimen tubes for Chapter 4 and 5 study received derivatizing agent, BSTFA [N,O bis(trimethylsilyl) trifluoroacetamide] with 1% TMCS (trimethylchlorosilane) at a volume of 40 µL, whereas specimens for Chapter 3 received 20 µL of BSTFA and 1% TMCS and 20 µL acetonitrile. Specimens were vortexed before derivatization at 65°C for 30 minutes. Derivatization was performed either directly in the autosampler vials (Chapter 3) or in a capped, disposable culture tube that was parafilmed around the seal. The later specimens were centrifuged for 5 min to allow adequate collection of the small volume. These specimens were then transferred to autosampler vials for analysis. In all cases, 1 µL of derivatized specimen was injected for GC/MS analysis.

The addition of a silyl derivative improves sensitivity in a number of ways. First, the trimethylsilyl (TMS) derivative increases molecular weight (MW) of the analyte of interest. Higher MW compounds demonstrate greater sensitivity and specificity due to fewer interferent signals associated with fragmentation products of other chemical products. Silyl derivatization also prevents interaction of the analyte functional groups with the GC column and this reduces analyte time on column and peak tailing due to column interactions.
Instrumentation

GC/MS analyses were performed using one of two Hewlett-Packard GC/MS mass selective detectors (Hewlett-Packard Company, Little Falls, DE).

Specimens in Chapter 4 and 5 were analyzed by a Hewlett-Packard (HP) 5890A Series II gas chromatograph and HP 7673 autoinjector interfaced with a HP 5972A series mass selective detector (MSD). The gas chromatograph was equipped with a cross-linked 95% dimethyl, 5% diphenylpolysiloxane capillary column (HP-5; 30 m x 0.10 mm i.d. x 0.10 μm film thickness). Automated injections were made in the splitless mode using a 2-mm i.d. silanized borosilicate liner in the injection port.

Similarly, a newer HP model was used during the Chapter 3 study. Analyses were performed with a HP 6890 Series II gas chromatograph and automatic liquid sampler interfaced with a Hewlett-Packard 5973 MSD. The gas chromatograph was equipped with a HP-1 crosslinked 1% diphenyl, 99% dimethylpolysiloxane fused-silica capillary column (12 m x 0.200 mm i.d. x 0.25 μm film thickness. Automated injections (1 μL) were made in the splitless mode, and a 4-mm i.d. silanized borosilicate liner with a glass wool plug was utilized.

Similar GC/MS parameters were employed for all techniques. The injection port and transfer line temperatures were maintained at temperatures of 250°C and 290°C, respectively. The oven temperature program was set at an initial temperature of 90°C (hold time- 0.5 min) and programmed to ramp at two intervals: 22.5°C/min up to 225°C and 17.5°C/min to a final temperature of 320°C (hold time- 4.0 min). The helium carrier gas was set at a constant flow rate of 1.0 mL/min. The septum purge flow was set at 2 to
3 mL/min. The MSD was operated in the selected ion monitoring mode at a dwell time of 20 ms.

A quantitating ion and at least two confirming ions were monitored for each analyte. Quantification of analytes was based upon the ratios of the integrated target ion areas to the corresponding deuterated internal standard analogs for each analyte. Those analytes which did not have commercially available internal standards were compared to the trideuterated analogue most similar in chemical structure. For example, norcocaine was quantitated based upon the ratio of its integrated ion peak-area to the integrated ion peak-area of trideuterated cocaine. The quantitating and confirming ions differed among projects due to observation of interferences for some ions. For instance, the 182 m/z was normally utilized to quantitate cocaine concentrations but specimens digested with the enzyme demonstrated a significant interferent for this ion. Consequently, the molecular ion, 303 m/z, was employed for Chapter 3. Table 2-4 summarizes all ions monitored for each analyte during this dissertation but does not differentiate between quantitating and confirming ions. Details of chosen ions are included in the respective chapters.

Analytes were identified based upon comparison of retention time and ion ratio with the corresponding values of calibrators assayed in the same run. Ion ratios were calculated by dividing the ion peak-area of the confirming ion by the ion peak-area of the quantitative ion. Quantification of analytes was based upon the ratios of the integrated ion peak-areas to the corresponding trideuterated standard analogues. Acceptance criteria were that ratios had to be within ±30% of the ratio value of a calibrator of similar concentration.
Figures 2-1-A through 2-1-M illustrate analyte abundances for monitored ions of a 25 ng/mg calibrator. Each figure depicts a representative selected ion chromatogram of the quantitation ion and a selective ion monitoring (SIM) spectrum showing the relative abundance for quantitating and confirming ions. In addition, Figures 2-2-A through 2-2-F illustrate representative selected ion chromatograms for the following specimens: unextracted 250 ng calibrator, extracted 250 ng cocaine and opiate calibrator in hair matrix, extracted negative control in hair matrix, and extracted posterior vertex hair and fingernail specimens from Subject M. The selected ion chromatograms depict signal due to SIM quantititating ions only and were normalized to the highest peak abundance.

**Performance Data**

Validation studies of new methodologies are necessary in order to verify performance, assess potential interferences, and compare hair analysis to established methods. Standard analytical procedures were employed to evaluate analytical parameters including recovery, precision, accuracy, linearity, and sensitivity.

The limits of detection (LOD) and quantitation (LOQ) were determined by replicate analysis of a series of standards decreasing in concentration. LOD and LOQ studies were performed during a 2 to 3 day period in separate batches in order to obtain more representative values. The LOD was defined as the concentration corresponding to a signal-to-noise ratio greater than or equal to 3.0. The LOQ was generally defined as the lowest standard that did not deviate from the target concentration by more than 20%. At very low concentrations (0.1 ng/mg) a less stringent criteria of ±30% was utilized. In addition, ion ratios and ±20% coefficient of variance (%CV) for the replicates were also
evaluated in determining the limit of quantitation. Tables 2-5, 2-7, and 2-10 summarize the LODs and LOQs for all studies.

The linearity of the standard curve was measured by two separate methods during the investigation. The initial assessment of linearity was determined by replicate analysis (n = 5) of standards prepared at a concentration of 10, 20, and 30 ng/mg. The calibration curve was considered linear if the mean concentration values did not deviate ±20% of the target concentration. All analytes were linear to at least to 10 ng/mg, which was the designated highest point of the calibration curve. Table 2-5 includes the limit of linearities demonstrated by this initial evaluation. Although some analytes demonstrated linearity above 10 ng/mg, results were reported as greater than 10 ng/mg for consistency.

Assessment of linearity during routine analysis was determined by established criteria that acceptable calibration points of the curve were within ±20% of their expected value. Along with visual observation of the standard curves, correlation coefficient ($r^2$) greater than or equal to 0.990 was considered acceptable.

Intra- and inter-assay variability were determined by analysis of controls prepared at low (0.5 ng/mg) and high (5.0, 7.5, and 10 ng/mg) concentrations. Seven to ten replicates were performed for both matrices and by both assay procedures. Within-run precision was determined by analyzing seven replicates. For between-run precision, data from positive controls included in each run were combined to calculate %CVs for each analyte. Tables 2-6, 2-8, and 2-11 summarize the precision studies for both assays and both matrices (hair and nail). Generally, the %CVs were acceptable at both the low and high concentrations. Analytes that demonstrated lower precision data included AEME, EEE, NBE, and NCOD.
Summary and Conclusions

Overall, the presented assays utilized for this dissertation performed favorably and reproducibly for the identification of cocaine and opiate analytes in hair and nails. These assays followed multiple-step procedures for specimen preparations that are generally accepted by forensic toxicologists for analyzing keratinized matrices. Specifically, specimens were decontaminated, subjected to an isolation procedure to remove analytes from the protein matrix, further purified by SPE, derivatized to improve specificity and sensitivity, and analyzed by GC/MS. Method validation studies for both assays presented in this dissertation, demonstrated acceptable efficiency, precision, and sensitivity for the analysis of keratinized matrices.
Table 2-1. Literature Survey: Assays to Detect Drugs in Keratinized Matrices

<table>
<thead>
<tr>
<th>Investigators (Year)</th>
<th>Reference Number</th>
<th>Analyte or Drug Class</th>
<th>Wash Procedure*</th>
<th>Isolation</th>
<th>Extraction*</th>
<th>Detection</th>
<th>Matrix</th>
</tr>
</thead>
<tbody>
<tr>
<td>Baumgartner et al. (1981)</td>
<td>(131)</td>
<td>PCP</td>
<td>3 x – Water</td>
<td>Methanol</td>
<td>None</td>
<td>RIA</td>
<td>Hair</td>
</tr>
<tr>
<td>Puschel et al. (1983)</td>
<td>(132)</td>
<td>Opiates</td>
<td>None</td>
<td>NaOH (1 min)</td>
<td>None</td>
<td>RIA</td>
<td>Hair</td>
</tr>
<tr>
<td>Offidani et al. (1989)</td>
<td>(133)</td>
<td>Morphine BE</td>
<td>2 x – Methylene chloride</td>
<td>1M NaOH (1 h) @ 80°C 1 mg/mL pronase/DTT</td>
<td>None</td>
<td>RIA</td>
<td>Hair</td>
</tr>
<tr>
<td>Suzuki et al. (1989)</td>
<td>(19)</td>
<td>Amphets</td>
<td>1) 5 x – Methanol 2) 5 x – Water</td>
<td>0.6N HCl</td>
<td>Chloroform/Isopropanol (3:1)</td>
<td>GC/MS</td>
<td>Hair</td>
</tr>
<tr>
<td>Nakahara et al. (1990)</td>
<td>(134)</td>
<td>Amphets</td>
<td>3 x – 0.1% SDS in water</td>
<td>Methanol/5M HCl (1 h) @ RT</td>
<td>None</td>
<td>GC/MS</td>
<td>Hair</td>
</tr>
<tr>
<td>Goldberger et al. (1991)</td>
<td>(18)</td>
<td>Heroin 6-AM</td>
<td>Methanol (20 s)</td>
<td>Methanol (18 h) @ 37°C</td>
<td>Tolulene/Heptane/Isoamyl alcohol (70:20:10)</td>
<td>GC/MS</td>
<td>Hair</td>
</tr>
<tr>
<td>Cone et al. (1993)</td>
<td>(17)</td>
<td>Cocaine Heroin</td>
<td>Methanol (20 s)</td>
<td>Methanol (overnight) @ 40°C</td>
<td>SPE</td>
<td>GC/MS</td>
<td>Hair</td>
</tr>
<tr>
<td>Traldi et al. (1993)</td>
<td>(135)</td>
<td>Cocaine</td>
<td>1) Diethyl ether 2) 0.01M HCl</td>
<td>0.1M HCl (16 h) @ 45°C</td>
<td>SPE</td>
<td>Ion trap MS/MS</td>
<td>Hair</td>
</tr>
<tr>
<td>Barrera et al. (1995)</td>
<td>(136)</td>
<td>Cocaine</td>
<td>Unspecified wash</td>
<td>1mg/mL Pronase/DTT</td>
<td>SPE</td>
<td>GC/MS</td>
<td>Hair</td>
</tr>
<tr>
<td>Cirimele et al. (1995)</td>
<td>(21)</td>
<td>Amphets</td>
<td>Unspecified wash</td>
<td>1M NaOH (10 min) @ 95°C</td>
<td>1) Ethyl acetate 2) 0.2 N HCl 3) Ethyl acetate</td>
<td>GC/MS</td>
<td>Nail</td>
</tr>
<tr>
<td>Authors</td>
<td>Year</td>
<td>Methodology</td>
<td>Solvents/Reagents</td>
<td>Temperature</td>
<td>Instrumentation</td>
<td>Detection</td>
<td>Sample Type</td>
</tr>
<tr>
<td>------------------</td>
<td>------</td>
<td>-------------</td>
<td>-------------------</td>
<td>-------------</td>
<td>----------------</td>
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<td>-------------</td>
</tr>
<tr>
<td>Jurado et al.</td>
<td>1995</td>
<td>Opiates</td>
<td>Dichloromethane</td>
<td>0.1N HCl (18 h) @ 50°C</td>
<td>Chloroform/Isopropanol/Heptane (50:17:33)</td>
<td>GC/MS</td>
<td>Hair</td>
</tr>
<tr>
<td>Kintz et al.</td>
<td>1995</td>
<td>Cannabinoids</td>
<td>Methylene chloride</td>
<td>1N NaOH (0.5 h) @ 95°C</td>
<td>n-Hexane/Ethyl acetate (9:1)</td>
<td>GC/MS</td>
<td>Hair</td>
</tr>
<tr>
<td>Henderson et al.</td>
<td>1996</td>
<td>Cocaine</td>
<td>1% SDS, Water, Methanol</td>
<td>Proteinase K/DDTT (overnight) @ 40°C</td>
<td>SPE</td>
<td>GC/MS</td>
<td>Hair</td>
</tr>
<tr>
<td>Smith et al.</td>
<td>1996</td>
<td>Cocaine</td>
<td>Dry ethanol, 0.01M Phosphate buffer</td>
<td>Dimethylformamide (1 h) @ 37°C</td>
<td>Diethyl Ether</td>
<td>GC/MS</td>
<td>Hair</td>
</tr>
<tr>
<td>Garside et al.</td>
<td>1998</td>
<td>Cocaine</td>
<td>Methanol (20 s)</td>
<td>Methanol (16 h) @ 40°C</td>
<td>SPE</td>
<td>GC/MS</td>
<td>Hair</td>
</tr>
<tr>
<td>Höld et al.</td>
<td>1998</td>
<td>Opiates</td>
<td>Unspecified wash</td>
<td>Proteinase K/DDTT (overnight) @ 40°C</td>
<td>SPE</td>
<td>GC/MS/PCI</td>
<td>Hair</td>
</tr>
</tbody>
</table>

Abbreviations: hydrochloric acid (HCl), sodium hydroxide (NaOH), dithiothreitol (DTT), sodium dodecyl sulfate (SDS), room temperature (RT), radioimmunoassay (RIA), tandem mass spectrometry (MS/MS), gas chromatography-mass spectrometry (GC/MS), gas chromatography-mass spectrometry-positive chemical ionization (GC/MS/PCI), benzoylecgonine (BE), 6-acetylmorphine (6-AM), and amphetamines (Amphets).

*Note: Numerical values represent multiple step procedures.*
Table 2-2. A Comparison Study of Enzymatic Digest versus Overnight Methanolic Incubation (n=3)

<table>
<thead>
<tr>
<th>Analyte Isolation Technique</th>
<th>COC ng/mg*</th>
<th>CE ng/mg*</th>
<th>BE ng/mg*</th>
<th>MOR ng/mg*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Enzymatic Digest using <em>Tritirachium Album</em> [16 h @ 40°C]</td>
<td>63 ± 8.1</td>
<td>0.62 ± 0.05</td>
<td>10 ± 0.78</td>
<td>1.40 ± 0.16</td>
</tr>
<tr>
<td>Enzymatic Digest using <em>Subtilisin A</em> [16 h @ 40°C]</td>
<td>47 ± 1.4</td>
<td>0.56 ± 0.08</td>
<td>12 ± 0.17</td>
<td>1.96 ± 0.01</td>
</tr>
<tr>
<td>Methanol Incubation [16 h @ 40°C]</td>
<td>29 ± 1.9</td>
<td>0.24 ± 0.01</td>
<td>5.6 ± 0.24</td>
<td>0.36 ± 0.06</td>
</tr>
</tbody>
</table>

Abbreviations: cocaine (COC), benzoylecgonine (BE), cocaethylene (CE), and morphine (MOR).

*Mean concentration results for replicate analyses of hair collected from a known drug user.
### Table 2-3. Mean Concentration Data for Cocaine and 6-Acetylmorphine Hydrolysis Controls (5 ng/mg) included in each Analytical Batch

<table>
<thead>
<tr>
<th>Hydrolysis Control Concentration (ng/mg)</th>
<th>COC (ng/mg)</th>
<th>EME (ng/mg)</th>
<th>BE (ng/mg)</th>
<th>6-AM (ng/mg)</th>
<th>MOR (ng/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>40 ng/mg (n=3)</td>
<td>38.4 ± 1.6</td>
<td>1.0 ± 1.3</td>
<td>0.16 ± 0.28</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>20 ng/mg (n=18)</td>
<td>17.9 ± 1.5</td>
<td>0.62 ± 0.24</td>
<td>3.2 ± 0.70</td>
<td>22.6 ± 3.5</td>
<td>1.6 ± 0.81</td>
</tr>
<tr>
<td>10 ng/mg (n=3)</td>
<td>8.6 ± 1.2</td>
<td>ND</td>
<td>ND</td>
<td>9.6 ± 0.13</td>
<td>0.49 ± 0.85</td>
</tr>
<tr>
<td>5 ng/mg (n=6)</td>
<td>5.9 ± 1.7</td>
<td>ND to &lt;0.20</td>
<td>0.49 ± 0.01</td>
<td>5.0 ± 0.13</td>
<td>0.42 ± 0.40</td>
</tr>
</tbody>
</table>

Abbreviations: cocaine (COC), benzoylecgonine (BE), ecgonine methyl ester (EME), 6-acetylmorphine (6-AM), morphine (MOR), not analyzed at this concentration (NA), and not detected (ND).
Table 2-4. Cocaine and Opiate Ions Monitored during GC/MS Analysis

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Ions (m/z)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anhydroecgonine methyl ester</td>
<td>152, 166, 181</td>
</tr>
<tr>
<td>d₃-Ecgonine methyl ester</td>
<td>85, 99, 274</td>
</tr>
<tr>
<td>Ecgonine methyl ester</td>
<td>82, 96, 271</td>
</tr>
<tr>
<td>Ecgonine ethyl ester</td>
<td>83, 96, 240, 285</td>
</tr>
<tr>
<td>d₃-Cocaine</td>
<td>85, 185, 275, 306</td>
</tr>
<tr>
<td>Cocaine</td>
<td>82, 182, 272, 303</td>
</tr>
<tr>
<td>d₃-Cocaethylene</td>
<td>85, 199, 275, 320</td>
</tr>
<tr>
<td>Cocaethylene</td>
<td>82, 196, 272, 317</td>
</tr>
<tr>
<td>d₃-Benzoyllecgonine</td>
<td>143, 243, 259, 364</td>
</tr>
<tr>
<td>Benzoylecgonine</td>
<td>140, 240, 256, 361</td>
</tr>
<tr>
<td>Norcocaine</td>
<td>140, 240, 346</td>
</tr>
<tr>
<td>Norbenzoylecgonine</td>
<td>140, 298, 404</td>
</tr>
<tr>
<td>m-Hydroxybenzoyllecgonine</td>
<td>82, 210, 240, 449</td>
</tr>
<tr>
<td>d₃-Codeine</td>
<td>181, 237, 374</td>
</tr>
<tr>
<td>Codeine</td>
<td>178, 196, 234, 371</td>
</tr>
<tr>
<td>Norcodeine</td>
<td>254, 292, 429</td>
</tr>
<tr>
<td>d₃-Morphine</td>
<td>236, 417, 432</td>
</tr>
<tr>
<td>Morphine</td>
<td>236, 414, 429</td>
</tr>
<tr>
<td>Normorphine</td>
<td>254, 308, 487</td>
</tr>
<tr>
<td>d₃-6-Acetylmorphine</td>
<td>290, 343, 402</td>
</tr>
<tr>
<td>6-Acetylmorphine</td>
<td>287, 340, 399</td>
</tr>
</tbody>
</table>
Figures 2-1-A through 2-1-M. 25 ng/mg Calibrator: Selected Ion Chromatogram of Quantitation Ion and Selective Ion Monitoring (SIM) Spectrum for Analytes Investigated
Figure 2-1-A. 25 ng/mg Cocaine Calibrator: Selected Ion Chromatogram of Quantitation Ion and Selective Ion Monitoring (SIM) Spectrum for Analytes Investigated Illustrating the Relative Abundance for Quantitating and Confirming ions
25 ng/g Calibrator: Cocaine (m/z 303)

Cocaine SIM Spectrum

m/z
Figure 2-1-B. 25 ng/mg Benzoylecgonine Calibrator: Selected Ion Chromatogram of Quantitation Ion and Selective Ion Monitoring (SIM) Spectrum for Analytes Investigated Illustrating the Relative Abundance for Quantitating and Confirming ions
25 ng/g Calibrator: Benzoylecgonine (m/z 240)

Benzoylcegonine SIM Spectrum
Figure 2-1-C. 25 ng/mg Anhydroecgonine Methyl Ester Calibrator: Selected Ion Chromatogram of Quantitation Ion and Selective Ion Monitoring (SIM) Spectrum for Analytes Investigated Illustrating the Relative Abundance for Quantitating and Confirming ions
25 ng/g Calibrator:
Anhydroecgonine Methyl Ester (m/z 152)

Anhydroecgonine Methyl Ester SIM Spectrum
Figure 2-1-D. 25 ng/mg Cocaethylene Calibrator: Selected Ion Chromatogram of Quantitation Ion and Selective Ion Monitoring (SIM) Spectrum for Analytes Investigated Illustrating the Relative Abundance for Quantitating and Confirming ions
25 ng/g Calibrator: Cocaethylene (m/z 196)

Cocaethylene SIM Spectrum
Figure 2-1-E. 25 ng/mg Eegonine Methyl Ester Calibrator: Selected Ion Chromatogram of Quantitation Ion and Selective Ion Monitoring (SIM) Spectrum for Analytes Investigated Illustrating the Relative Abundance for Quantitating and Confirming ions
25 ng/g Calibrator:
Ecgonine Methyl Ester (m/z 96)

Ecgonine Methyl Ester SIM Spectrum
Figure 2-1-F. 25 ng/mg Ecgonine Ethyl Ester Calibrator: Selected Ion Chromatogram of Quantitation Ion and Selective Ion Monitoring (SIM) Spectrum for Analytes Investigated Illustrating the Relative Abundance for Quantitating and Confirming ions
25 ng/g Calibrator: Ectonine Ethyl Ester (m/z 96)

Ectonine Ethyl Ester SIM Spectrum
Figure 2-1-G. 25 ng/mg Norcocaine Calibrator: Selected Ion Chromatogram of Quantitation Ion and Selective Ion Monitoring (SIM) Spectrum for Analytes Investigated Illustrating the Relative Abundance for Quantitating and Confirming ions
25 ng/g Calibrator: Norcocaine (m/z 140)

Norcocaine SIM Spectrum

m/z
Figure 2-1-H. 25 ng/mg Norbenzoylcoecgonine Calibrator: Selected Ion Chromatogram of Quantitation Ion and Selective Ion Monitoring (SIM) Spectrum for Analytes Investigated Illustrating the Relative Abundance for Quantitating and Confirming ions
25 ng/g Calibrator: Norbenzoylecgonine (m/z 298)

Norbenzoylecgonine SIM Spectrum

m/z
Figure 2-1-I. 25 ng/mg Codeine Calibrator: Selected Ion Chromatogram of Quantitation Ion and Selective Ion Monitoring (SIM) Spectrum for Analytes Investigated Illustrating the Relative Abundance for Quantitating and Confirming ions
25 ng/g Calibrator: Codeine (m/z 371)

Codeine SIM Spectrum

178 196 234

180 200 220 240 260 280 300 320 340 360 380

m/z
Figure 2-1-J. 25 ng/mg 6-Acetylmorphine Calibrator: Selected Ion Chromatogram of Quantitation Ion and Selective Ion Monitoring (SIM) Spectrum for Analytes Investigated Illustrating the Relative Abundance for Quantitating and Confirming ions
25 ng/g Calibrator: 6-Acetylmorphine (m/z 399)

6-Acetylmorphine SIM Spectrum

m/z
Figure 2-1-K. 25 ng/mg Morphine Calibrator: Selected Ion Chromatogram of Quantitation Ion and Selective Ion Monitoring (SIM) Spectrum for Analytes Investigated Illustrating the Relative Abundance for Quantitating and Confirming ions
25 ng/g Calibrator: Morphine (m/z 429)

Morphine SIM Spectrum

m/z

236 429

414
Figure 2-1-L. 25 ng/mg Norcodeine Calibrator: Selected Ion Chromatogram of Quantitation Ion and Selective Ion Monitoring (SIM) Spectrum for Analytes Investigated Illustrating the Relative Abundance for Quantitating and Confirming ions
25 ng/g Calibrator: Norcodeine (m/z 292)

Norcodeine SIM Spectrum

m/z
Figure 2-1-M. 25 ng/mg Normorphine Calibrator: Selected Ion Chromatogram of Quantitation Ion and Selective Ion Monitoring (SIM) Spectrum for Analytes Investigated Illustrating the Relative Abundance for Quantitating and Confirming ions
25 ng/g Calibrator: Normorphine (m/z 487)
Figure 2-2-A through 2-2-F. Selected Ion Chromatograms of Quantitation Ions for Simultaneously Detected Analytes by GC/MS
Figure 2-2-A. Selected Ion Chromatogram from an Unextracted 250 ng Calibrator*

Abbreviations:

Major Internal Standards (100 ng): trideuterated codeine (d₃-COD), and trideuterated morphine(d₃-MOR).
Minor Internal Standards (50 ng): trideuterated 6-acetylmorphine (d₃-6AM).

Major Cocaine Analytes (250 ng): cocaine (COC), benzoylecgonine (BE), and ecgonine methyl ester (EME).
Major Opiate Analytes (250 ng): codeine (COD), and morphine (MOR).
Minor Cocaine Analytes (125 ng): anhydroecgonine methyl ester (AEME), cocaethylene (CE), ecgonine ethyl ester (EEE), norbenzoylecgonine (NBE), and norcocaine (NCOC).
Minor Opiate Analytes (125 ng): 6-acetylmorphine (6-AM), norcodeine (NCOD), and normorphine (NMOR).

*Note: All minor analytes are at one-half the concentration of major analytes. Data has been normalized to the highest peak abundance.
Figure 2-2-B. Selected Ion Chromatogram from a 250/125 ng/mg Cocaine and Opiate Hair Calibrator*

Abbreviations:

Major Internal Standards (100 ng): trideuterated cocaine (d₃-COC), trideuterated ecgonine methyl ester (d₃-EME), trideuterated benzoylecgonine (d₃-BE), trideuterated codeine (d₃-COD), and trideuterated morphine (d₃-MOR).
Minor Internal Standards (50 ng): trideuterated cocaethylene (d₃-CE) and trideuterated 6-acetylmorphine (d₃-6AM).

Major Cocaine Analytes (250 ng): cocaine (COC), benzoylecgonine (BE), and ecgonine methyl ester (EME).
Major Opiate Analytes (250 ng): codeine (COD), and morphine (MOR).
Minor Cocaine Analytes (125 ng): anhydroecgonine methyl ester (AEME), cocaethylene (CE), ecgonine ethyl ester (EEE), norbenzoylecgonine (NBE), and norcocaine (NCOC).
Minor Opiate Analytes (125 ng): 6-acetylmorphine (6-AM), norcodeine (NCOD), and normorphine (NMOR).

*Note: All minor analytes are at one-half the concentration of major analytes. Data has been normalized to the highest peak abundance.
Figure 2-2-C. Selected Ion Chromatogram from a Hair Negative Control*
Figure 2-2-D. Selected Ion Chromatogram of a Hair Negative Control with Cocaine Internal Standards Added

Abbreviations:

Major Internal Standards (100 ng): trideuterated cocaine (d$_3$-COC), trideuterated ecgonine methyl ester (d$_3$-EME), and trideuterated benzoylcegonine (d$_3$-BE).
Minor Internal Standards (50 ng): trideuterated cocaethylene (d$_3$-CE).
Hair Negative Control (with Internal Standards)

Minutes

3.5  4.0  4.5  5.0  5.5  6.0  6.5  7.0  7.5

d3-EME  d3-COC  d3-CE  d3-BE
Figure 2-2-E. Selected Ion Chromatogram of Posterior Vertex Hair from Subject M Taken at the Initial Collection (Day 0) Prior to Controlled Dosing

Abbreviations:

Major Internal Standards (100 ng): trideuterated cocaine (d\textsubscript{3}-COC), trideuterated eegonine methyl ester (d\textsubscript{3}-EME), trideuterated benzoylecgonine (d\textsubscript{3}-BE), trideuterated codeine (d\textsubscript{3}-COD), and trideuterated morphine (d\textsubscript{3}-MOR).
Minor Internal Standards (50 ng): trideuterated cocaethylene (d\textsubscript{3}-CE) and trideuterated 6-acetylmorphine (d\textsubscript{3}-6-AM).

Major Cocaine Analytes: cocaine and benzoylecgonine (BE).
Major Opiate Analytes: codeine (COD), and morphine (MOR).
Minor Cocaine Analytes: cocaethylene (CE) and norcocaine (NCOC).
Figure 2-2-F. Selected Ion Chromatogram of Fingernail Shavings from Subject M Taken at Initial Collection (Day 0) Prior to Controlled Dosing

Abbreviations:

Major Internal Standards (100 ng): trideuterated cocaine (d₃-COC), trideuterated ecdgonine methyl ester (d₃-EME), trideuterated benzoylecgonine (d₃-BE), trideuterated codeine (d₃-COD), and trideuterated morphine (d₃-MOR).

Minor Internal Standards (50 ng): trideuterated cocaethylene (d₃-CE) and trideuterated 6-acetylmorphine (d₃-6AM).

Major Cocaine Analytes: cocaine
Fingernail (Subject M - 268M-F001A)
Table 2-5. Limits of Detection (LOD), Quantitation (LOQ), and Linearity (LOL) for the Assay Utilized to Analyze Postmortem Specimens (Chapters 4 and 5)

<table>
<thead>
<tr>
<th>Analyte</th>
<th>LOD (ng/mg)</th>
<th>LOQ (ng/mg)</th>
<th>LOL (ng/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>COC</td>
<td>0.1</td>
<td>0.2</td>
<td>20</td>
</tr>
<tr>
<td>AEME</td>
<td>0.1</td>
<td>0.2</td>
<td>10</td>
</tr>
<tr>
<td>BE</td>
<td>0.1</td>
<td>0.3</td>
<td>20</td>
</tr>
<tr>
<td>CE</td>
<td>0.1</td>
<td>0.2</td>
<td>20</td>
</tr>
<tr>
<td>EME</td>
<td>0.1</td>
<td>0.2</td>
<td>10</td>
</tr>
<tr>
<td>EEE</td>
<td>0.4</td>
<td>0.5</td>
<td>20</td>
</tr>
<tr>
<td>MOHBE</td>
<td>0.2</td>
<td>0.5</td>
<td>10</td>
</tr>
<tr>
<td>NBE</td>
<td>0.4</td>
<td>0.4</td>
<td>10</td>
</tr>
<tr>
<td>NCOC</td>
<td>0.1</td>
<td>0.4</td>
<td>10</td>
</tr>
</tbody>
</table>

Abbreviations: cocaine (COC), anhydroecgonine methyl ester (AEME), benzoylecgonine (BE), cocaethylene (CE), ecgonine methyl ester (EME), ecgonine ethyl ester (EEE), \(m\)-hydroxybenzoylecgonine (MOHBE), norbenzoylecgonine (NBE), and norcocaine (NCOC).
Table 2-6. Precision Data for the Assay Utilized to Analyze Postmortem Specimens (Chapters 4 and 5)

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Within-run (0.5 ng/mg)</th>
<th>Within-run (10 ng/mg)</th>
<th>Between-run (0.5 ng/mg)</th>
<th>Between-run (7.5 ng/mg)</th>
<th>Between-run (10 ng/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>%CV n=5</td>
<td>%CV n=3</td>
<td>%CV n=13</td>
<td>%CV n=5</td>
<td>%CV n=9</td>
</tr>
<tr>
<td>COC</td>
<td>9.0</td>
<td>2.4</td>
<td>8.6</td>
<td>3.2</td>
<td>2.0</td>
</tr>
<tr>
<td>AEME</td>
<td>6.6</td>
<td>9.8</td>
<td>13.3</td>
<td>NA</td>
<td>8.7</td>
</tr>
<tr>
<td>BE</td>
<td>2.0</td>
<td>1.3</td>
<td>10.2</td>
<td>3.4</td>
<td>1.1</td>
</tr>
<tr>
<td>CE</td>
<td>1.8</td>
<td>1.5</td>
<td>8.4</td>
<td>2.9</td>
<td>1.4</td>
</tr>
<tr>
<td>EME</td>
<td>4.5</td>
<td>4.2</td>
<td>16.1</td>
<td>19.4</td>
<td>8.3</td>
</tr>
<tr>
<td>EEE</td>
<td>8.1</td>
<td>5.7</td>
<td>22.7** (n=11)</td>
<td>29.5**</td>
<td>6.1</td>
</tr>
<tr>
<td>MOHBE</td>
<td>14.5</td>
<td>5.9</td>
<td>18.9 (n=12)</td>
<td>11.6</td>
<td>7.8</td>
</tr>
<tr>
<td>NBE</td>
<td>12.2</td>
<td>4.5</td>
<td>32.8** (n=11)</td>
<td>55.2** (n=4)</td>
<td>12.9</td>
</tr>
<tr>
<td>NCOC</td>
<td>14.6</td>
<td>5.3</td>
<td>11</td>
<td>40**</td>
<td>8.8</td>
</tr>
</tbody>
</table>

Abbreviations: coefficient of variation (CV), analyte not analyzed at this concentration (NA), cocaine (COC), anhydroecgonine methyl ester (AEME), benzoyllecgonine (BE), cocaethylene (CE), cecgonine methyl ester (EME), cecgonine ethyl ester (EEE), m-hydroxybenzoyllecgonine (MOHBE), norbenzoyllecgonine (NBE), and norcocaine (NCOC).

**Note: Concentration was greater than ±20% of the target CV value.
Table 2-7. Limits of Detection (LOD) and Quantitation (LOQ) for the Assay Utilized to Analyze Hair of Inpatient Study Subjects (Chapter 3)

<table>
<thead>
<tr>
<th>Analyte</th>
<th>LOD (ng/mg)</th>
<th>LOQ (ng/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>COC</td>
<td>0.1</td>
<td>0.2</td>
</tr>
<tr>
<td>AEME</td>
<td>0.1</td>
<td>0.1</td>
</tr>
<tr>
<td>BE</td>
<td>0.1</td>
<td>0.2</td>
</tr>
<tr>
<td>CE</td>
<td>0.1</td>
<td>0.3</td>
</tr>
<tr>
<td>EME</td>
<td>0.1</td>
<td>0.3</td>
</tr>
<tr>
<td>EEE</td>
<td>0.1</td>
<td>0.3</td>
</tr>
<tr>
<td>NBE</td>
<td>0.2</td>
<td>0.2</td>
</tr>
<tr>
<td>NCOC</td>
<td>0.1</td>
<td>0.2</td>
</tr>
<tr>
<td>COD</td>
<td>0.1</td>
<td>0.1</td>
</tr>
<tr>
<td>6-AM</td>
<td>0.1</td>
<td>0.1</td>
</tr>
<tr>
<td>MOR</td>
<td>0.1</td>
<td>0.1</td>
</tr>
<tr>
<td>NCOD</td>
<td>0.2</td>
<td>0.2</td>
</tr>
<tr>
<td>NMOR</td>
<td>0.1</td>
<td>0.2</td>
</tr>
</tbody>
</table>

Abbreviations: cocaine (COC), anhydroecgonine methyl ester (AEME), benzoylecgonine (BE), cocaethylene (CE), ecgonine methyl ester (EME), ecgonine ethyl ester (EEE), norbenzoylecgonine (NBE), norcocaine (NCOC), codeine (COD), 6-acetylmorphine (6-AM), morphine (MOR), norcodeine (NCOD), and normorphine (NMOR).
Table 2-8. Precision Data for the Assay Utilized to Analyze Hair of Inpatient Study Subjects (Chapter 3)

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Within-run (0.5 ng/mg)</th>
<th>Within-run (5 ng/mg)</th>
<th>Between-run (0.5 ng/mg)</th>
<th>Between-run (5.0 ng/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>%CV n=10</td>
<td>%CV n=14</td>
<td>%CV n=22</td>
<td>%CV n=22</td>
</tr>
<tr>
<td>COC</td>
<td>10.8</td>
<td>2.7</td>
<td>10.7</td>
<td>4.6</td>
</tr>
<tr>
<td>AEME</td>
<td>12.4</td>
<td>10.5</td>
<td>13.0 (n=17)</td>
<td>18.6</td>
</tr>
<tr>
<td>BE</td>
<td>9.6</td>
<td>10.1</td>
<td>15.8 (n=17)</td>
<td>10.7</td>
</tr>
<tr>
<td>CE</td>
<td>13.4</td>
<td>2.1</td>
<td>14.8</td>
<td>7.2</td>
</tr>
<tr>
<td>EME</td>
<td>18.8</td>
<td>9.0</td>
<td>16.8</td>
<td>8.1</td>
</tr>
<tr>
<td>EEE</td>
<td>15.6</td>
<td>5.8</td>
<td>12.0 (n=17)</td>
<td>7.3</td>
</tr>
<tr>
<td>NBE</td>
<td>36.3** (n=9)</td>
<td>17.3 (n=8)</td>
<td>22.8 (n=16)</td>
<td>24.1 (n=13)</td>
</tr>
<tr>
<td>NCOC</td>
<td>45.6**</td>
<td>5.8</td>
<td>15.3 (n=17)</td>
<td>6.6</td>
</tr>
<tr>
<td>COD</td>
<td>8.9</td>
<td>8.3</td>
<td>5.8</td>
<td>5.4</td>
</tr>
<tr>
<td>6-AM</td>
<td>7.9</td>
<td>5.2</td>
<td>13.7</td>
<td>3.8</td>
</tr>
<tr>
<td>MOR</td>
<td>14.5</td>
<td>2.1</td>
<td>11.7</td>
<td>4.8</td>
</tr>
<tr>
<td>NCOD</td>
<td>7.9</td>
<td>6.8 (n=8)</td>
<td>20.0</td>
<td>8.1 (n=19)</td>
</tr>
<tr>
<td>NMOR</td>
<td>7.9</td>
<td>12.4</td>
<td>10.2</td>
<td>7.9</td>
</tr>
</tbody>
</table>

Abbreviations: coefficient of variation (CV), cocaine (COC), anhydroecgonine methyl ester (AEME), benzoylecgonine (BE), cocaethylene (CE), ecgonine methyl ester (EME), ecgonine ethyl ester (EEE), norbenzoylecgonine (NBE), norcocaine (NCOC), codeine (COD), 6-acetylmorphine (6-AM), morphine (MOR), norcodeine (NCOD), and normorphine (NMOR).

**Note: Concentration greater than ±20% of target CV value.
Table 2-9. Recovery Data for the Assay Utilized to Analyze Hair and Nails of Inpatient Study Subjects (Chapter 3)

<table>
<thead>
<tr>
<th>Analyte</th>
<th>% Recovery (1.0 ng/mg) n=5</th>
<th>% Recovery (8 ng/mg) n=5</th>
</tr>
</thead>
<tbody>
<tr>
<td>COC</td>
<td>68.1</td>
<td>74.7</td>
</tr>
<tr>
<td>AEME</td>
<td>60.3</td>
<td>60.5</td>
</tr>
<tr>
<td>BE</td>
<td>85.8</td>
<td>88.5</td>
</tr>
<tr>
<td>CE</td>
<td>83.2</td>
<td>80.2</td>
</tr>
<tr>
<td>EME</td>
<td>71.5</td>
<td>73.4</td>
</tr>
<tr>
<td>EEE</td>
<td>71.8</td>
<td>66.6</td>
</tr>
<tr>
<td>NBE</td>
<td>127</td>
<td>123</td>
</tr>
<tr>
<td>NCOC</td>
<td>113</td>
<td>75.7</td>
</tr>
<tr>
<td>COD</td>
<td>82.2</td>
<td>83.8</td>
</tr>
<tr>
<td>MOR</td>
<td>84.3</td>
<td>85.8</td>
</tr>
<tr>
<td>NCOD</td>
<td>83.9</td>
<td>82.7</td>
</tr>
<tr>
<td>NMOR</td>
<td>90</td>
<td>79.5</td>
</tr>
</tbody>
</table>

Abbreviations: cocaine (COC), anhydroecgonine methyl ester (AEME), benzoylecgonine (BE), cocaethylene (CE), ecgonine methyl ester (EME), ecgonine ethyl ester (EEE), norbenzoylecgonine (NBE), norcocaine (NCOC), codeine (COD), morphine (MOR), norcodeine (NCOD), and normorphine (NMOR).

**Note: Concentration greater than ±20% of target value.**
Table 2-10. Limits of Detection (LOD) and Quantitation (LOQ) for the Assay Utilized to Analyze Fingernail Scrapings of Inpatient Study Subjects (Chapter 3)

<table>
<thead>
<tr>
<th>Analyte</th>
<th>LOD (ng/mg)</th>
<th>LOQ (ng/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>COC</td>
<td>0.20</td>
<td>0.26</td>
</tr>
<tr>
<td>AEME</td>
<td>0.26</td>
<td>1.0</td>
</tr>
<tr>
<td>BE</td>
<td>0.13</td>
<td>0.26</td>
</tr>
<tr>
<td>CE</td>
<td>0.13</td>
<td>0.26</td>
</tr>
<tr>
<td>EME</td>
<td>0.13</td>
<td>0.40</td>
</tr>
<tr>
<td>EEE</td>
<td>0.13</td>
<td>0.50</td>
</tr>
<tr>
<td>NBE</td>
<td>0.13</td>
<td>1.0</td>
</tr>
<tr>
<td>NCOC</td>
<td>0.13</td>
<td>0.26</td>
</tr>
<tr>
<td>COD</td>
<td>0.13</td>
<td>0.20</td>
</tr>
<tr>
<td>6-AM</td>
<td>0.13</td>
<td>0.20</td>
</tr>
<tr>
<td>MOR</td>
<td>0.13</td>
<td>0.13</td>
</tr>
<tr>
<td>NCOD</td>
<td>0.13</td>
<td>0.40</td>
</tr>
<tr>
<td>NMOR</td>
<td>0.13</td>
<td>0.40</td>
</tr>
</tbody>
</table>

Abbreviations: cocaine (COC), anhydroecgonine methyl ester (AEME), benzoylecgonine (BE), cocaethylene (CE), ecgonine methyl ester (EME), ecgonine ethyl ester (EEE), norbenzoylecgonine (NBE), norcocaine (NCOC), codeine (COD), 6-acetylmorphine (6-AM), morphine (MOR), norcodeine (NCOD), and normorphine (NMOR).
Table 2-11. Precision Data for Assay Utilized to Analyze Fingernail Scrapings of Inpatient Study Subjects (Chapter 3)

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Within-run (1.0 ng/mg)</th>
<th>Within-run (4.0 ng/mg)</th>
<th>Between-run (1.0 ng/mg)</th>
<th>Between-run (10 ng/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>%CV n=4</td>
<td>%CV n=3</td>
<td>%CV n=14</td>
<td>%CV n=14</td>
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<td>5.7</td>
<td>9.5</td>
<td>5.2</td>
</tr>
<tr>
<td>AEME</td>
<td>32.7**</td>
<td>22.4**</td>
<td>12.5</td>
<td>13.7</td>
</tr>
<tr>
<td>BE</td>
<td>13.4</td>
<td>2.9</td>
<td>17.4</td>
<td>6.5</td>
</tr>
<tr>
<td>CE</td>
<td>7.8</td>
<td>4.9</td>
<td>13.1</td>
<td>5.8</td>
</tr>
<tr>
<td>EME</td>
<td>3.6</td>
<td>4.2</td>
<td>13.9</td>
<td>7.9</td>
</tr>
<tr>
<td>EEE</td>
<td>2.1</td>
<td>2.3</td>
<td>10.9</td>
<td>6.8</td>
</tr>
<tr>
<td>NBE</td>
<td>2.3</td>
<td>7.1</td>
<td>6.8</td>
<td>24.0**</td>
</tr>
<tr>
<td>NCOC</td>
<td>31.7**</td>
<td>8.7</td>
<td>21.1**</td>
<td>15.8</td>
</tr>
<tr>
<td>COD</td>
<td>15.1</td>
<td>14.1</td>
<td>19.6</td>
<td>18.6</td>
</tr>
<tr>
<td>6-AM</td>
<td>8.7</td>
<td>19.3</td>
<td>12.7</td>
<td>8.2</td>
</tr>
<tr>
<td>MOR</td>
<td>18.8</td>
<td>18.7</td>
<td>25.4**</td>
<td>9.7</td>
</tr>
<tr>
<td>NCOD</td>
<td>6.3</td>
<td>21.6**</td>
<td>25.7**</td>
<td>24.3**</td>
</tr>
<tr>
<td>NMOR</td>
<td>33.1**</td>
<td>17.3</td>
<td>13.7</td>
<td>17.8</td>
</tr>
</tbody>
</table>

Abbreviations: coefficient of variation (CV), cocaine (COC), anhydroecgonine methyl ester (AEME), benzoylecgonine (BE), cocaethyle (CE), ecgonine methyl ester (EME), ecgonine ethyl ester (EEE), norbenzoylecgonine (NBE), norcocaine (NCOC), codeine (COD), 6-acetylmorphine (6-AM), morphine (MOR), norcodeine (NCOD), and normorphine (NMOR).

**Note: Concentration greater than ±20% of target CV value.
CHAPTER 3
THE DISPOSITION OF COCAINE AND OPIATE ANALYTES IN KERATINIZED MATRICES OF VOLUNTEERS IN A CONTROLLED CLINICAL STUDY

Introduction

Hair analysis for drugs of abuse testing has emerged as a complementary, sensitive and specific technique to conventional blood and urinalysis. Its ability to identify long-term drug exposure has made it very useful in drug testing applications. Despite its controversies such as environmental contamination and color bias, hair analysis is being introduced into more and more laboratories worldwide. A large consensus of toxicologists believe hair analysis has transgressed the boundaries of being solely a research tool. There is still a critical need for research to investigate the disposition of drugs in hair. Furthermore, investigation of less studied alternative matrices like nail is also warranted since these matrices can potentially offer advantages similar to hair.

Numerous research groups have investigated various aspects of drug disposition into hair. Dose-concentration relationships reported for therapeutic drugs and drugs of abuse range, from no apparent correlation to a significant correlation. Drug analytes investigated in animal studies reporting a linear correlation between dose and analyte concentration in hair include cocaine (139-140), cocaine metabolites (benzoylecgonine and ecgonine methyl ester) (41, 140), amphetamines (134, 139), phencyclidine (41), phenobarbital (67), ofloxacin (84), methaqualone (41), codeine (67), morphine (41, 139),
6-acetylmorphine (139), and methoxyphenamine (139). Human studies investigating drug disposition in hair have focused on various analytes including cocaine (41, 48, 97), digoxin (41), cannabinoids, (41), haloperidol (47), and methamphetamine (134).

Human studies performed by Uematsu et al. (47) demonstrated a significant correlation of haloperidol in hair with trough plasma concentrations \( r=0.772 \) and daily dose concentrations \( r=0.555 \). Another study reported that morphine and codeine concentrations in beard hair of two subjects after single dose administration appeared to be dose-related when screened by radioimmunoassay and confirmed by GC/MS (141). Likewise, Baumgartner et al. (41) observed varying linear relationships between dose and digoxin, benzoylecgonine, phencyclidine (PCP), heroin, and marijuana concentrations in human scalp hair utilizing radioimmunological techniques. A previous report by Baumgartner and colleagues (131) even categorized subjects as low, medium, or high PCP users based on the total drug content in whole hair. Blank and Kidwell (48) reported two studies of cocaine concentrations in hair and self-reported drug use. In one study, these investigators found statistically significant correlation coefficients between self-reported drug use and cocaine found in hair \( r = 0.59, p<0.01 \), but in the other study investigating benzoylecgonine a metabolite of cocaine, they observed a poor correlation.

Other investigators report no correlation between administered doses of drug and concentrations in hair. Püshel et al. (132) used a combination of study designs including controlled codeine dosing of guinea pigs and voluntary probationers, carcinoma patients receiving morphine hydrochloride, and admitted heroin users to conclude that no correlation exists between administered doses of opiates and their concentrations found in hair. Kintz et al. (16) investigated the dose-concentration relationships in hair of subjects
in a controlled heroin-maintenance program. Twenty subjects were administered heroin hydrochloride salt at total doses ranging from 14.1 to 71.5 g during an undisclosed period. Heroin analytes were detected at the following concentrations: heroin (0-4.53 ng/mg), 6-acetylmorphine (0.38-10.11 ng/mg), and morphine (0.71-5.20 ng/mg). No correlation between doses of administered heroin and total opiates detected in hair could be determined ($r = 0.346$). However, correlation coefficients did increase ($r = 0.12$ to $r = 0.64$) with analytes having a longer plasma-half life. In a study conducted by Henderson et al. (66), incorporation of isotopically-labeled cocaine analytes administered intravenously and/or intranasally in doses of 0.6 to 4.2 mg/kg demonstrated that hair analysis by GC/MS does not accurately predict amount, time, or duration of drug use.

Drugs of abuse and their metabolites including cocaine analytes (22, 130, 142), amphetamines (19-21), and opiates (142) have also been successfully detected in nails. Although investigators have identified drugs of abuse in fingernails and toenails, dose-response studies in nails have been limited to the investigation of therapeutic drugs.

Uematsu et al. (47) found a significant correlation of haloperidol concentrations in nails and daily administered dose ($r = 0.525$, $p > 0.05$) in 20 human subjects. Although haloperidol was always present in the fingernails and toenails of all subjects at concentrations ranging from 0.67 to 16.89 ng/mg, the concentrations of drugs in nail were always less than that found in hair ($4.32 \pm 3.17\%$, mean $\% \pm SD$).

Several studies have found a positive correlation for dose-response relationships of the antifungal drugs, terbinafine and itraconazole, in nails. Pharmacokinetic studies by Faergermann et al. (143) showed that toenails had measurable amounts of terbinafine (0.1 ng/mg) seven days after beginning a daily oral dose regime (250 mg). Terbinafine levels
continued to increase in nails until one day after cessation of treatment when a peak level of 0.4 ng/mg was observed. Schatz et al. (58) also investigated oral terbinafine concentrations (250 mg daily) in toenails of two study groups: one received treatment for six weeks (Group A) and the other received treatment for 12 weeks (Group B). Combined nail analysis demonstrated that the area-under-the-curve values for a 48-week period were consistently higher (50-60%) in Group B (finger-18.1 µg x weeks/g; toe- 23.0 µg x weeks/g) than Group A (finger-15.5 µg x weeks/g; toe- 11.2 µg x weeks/g). Median terbinafine concentrations were consistently higher in the nails of group B subjects throughout the study period. Similar results were observed by Willemsen et al. (144) when a therapeutic dose (100 mg) and a high dose (200 mg) of itraconazole were administered for three months. Levels of itraconazole in the finger- and toenails were constant during the six-month follow-up and mean values for the 100 mg and 200 mg dose were 0.15 and 0.67 µg/g, respectively. Hence, several research groups have observed a dose-response relationship for therapeutic drugs in nails.

Presently, there is no agreement whether the time of drug use can be reliably inferred from analysis of keratinized matrices, or if the drug concentrations detected in these matrices accurately reflect the extent of drug use. Discrepancies among dose-response relationship studies suggest that drug disposition into hair varies from drug to drug. Some drugs display a dose-relationship, while others may not. Moreover, nonstandardized analytical methodology may affect intra-laboratory results of dose-response studies. Therefore, if the concentration of drug incorporated into hair does not correlate with the dose administered, the extent of drug use cannot be estimated using these matrices.
To our knowledge, there have been no reported investigations analyzing paired hair and nails from the same subject in a controlled inpatient study. This study utilized a controlled clinical environment to investigate the similarities and differences in the disposition of cocaine and opiate analytes into two keratinized matrices, hair and nail.

**Materials and Methods**

**Standards and Reagents**

All organic solvents were HPLC grade and chemicals were reagent grade. Anhydroecgonine methyl ester, benzoylcegonine, d₃-benzoylcegonine, cocaine, d₃-cocaine, cocaethylene, d₃-cocaethylene, ecgonine ethyl ester, ecgonine methyl ester, d₃-ecgonine methyl ester, norbenzoylcegonine, norcocaine, 6-acetylmorphine, d₃-6-acetylmorphine, codeine, d₃-codeine, morphine, d₃-morphine, norcodeine, and normorphine standards were purchased from Radian International LLC (Austin, TX). N,O-Bis(trimethylsilyl)trifluoroacetamide (BSTFA) with 1% trimethylchlorosilane (TMCS) was obtained from Pierce Company (Rockford, IL). Dithiothreitol (DTT) and micro stir-bars were purchased from Fisher-Scientific (Atlanta, GA). Protease Type XI (*Tritirachium album*) and Tris (hydroxymethyl) aminomethane (Tris reagent) were purchased from Sigma Chemical Company (St. Louis, MO). Fritted filter reservoirs (4 mL, RFV02F4P) and solid-phase extraction (SPE) cartridges (Clean Screen®, ZSDAU020) were purchased from United Chemical Technologies, Horsham, PA.

Phosphate buffer (10 mM and 100 mM; pH 6.0 ± 0.1) was prepared from a mixture of potassium phosphate monobasic and deionized water. Acetate buffer (10 mM;
pH 4.0 ± 0.1) was prepared from a mixture of glacial acetic acid, sodium acetate, and deionized water. Tris buffer (50 mM; pH 7.4 preset) was prepared by adding 7.6 g Tris reagent to 1.0 L of deionized water. All buffers were stored refrigerated (0-5°C) for up to six months while not in use. The pH of the buffers was checked periodically prior to use. The enzymatic digest solution was prepared fresh daily by combining 60 mg of DTT, 0.5 mg of Protease XI, and 10 mL of 50 mM Tris buffer. The SPE elution solvent, methylene chloride–2-propanol–ammonium hydroxide (80:20:2, v/v/v) was prepared daily.

**Study Design**

Detailed information about the subjects and study design was described in a previous publication by Joseph *et al.* (145). Briefly, eight healthy, black males were voluntarily enrolled in a ten-week inpatient study conducted by the Intramural Research Program (IRP), National Institute of Drug Abuse (NIDA), National Institutes of Health (Baltimore, MD). All subjects had a history of drug use, which was confirmed by drug screening prior to admittance to the study. Medical and psychological evaluations were performed to verify each subject’s health prior to study participation. All subjects provided informed consent and were compensated for their participation in the study.

The study timeline for dosing and specimen collection is illustrated in Figure 3-1. Both hair and nail specimens were collected the first day of the study (day 0) and weekly collection continued for the remainder of the study. Drugs were not administered during the first three weeks to allow time for all previously administered drug to be removed from the hair and nails (i.e., washout period). Beginning in week four, subjects were administered a low dose of cocaine hydrochloride (75 mg/70 kg) by subcutaneous
injections on Monday, Wednesday, and Friday. During the same period, codeine sulfate (60 mg/70 kg) was administered orally on Tuesday, Thursday, and the following Monday. Following low dosing, subjects were administered placebo doses subcutaneously and orally during weeks 6 and 7, observing the same daily schedule. Beginning in week eight, subjects were administered a high dose of cocaine hydrochloride (150 mg/70 kg) and codeine sulfate (120 mg/70 kg) following the same schedule. Specimen collection was continued for one week after final dosing for follow-up of drug elimination after high dose administration.

Collection of Specimens

Head hair was collected by the staff of the IRP, NIDA. Grooming clippers were employed to remove the first collection of scalp hair from subjects, collecting different regions of the scalp (temporal, frontal, nape, posterior vertex, and anterior vertex) separately. For the study reported herein, only hair from the posterior vertex region was analyzed. The remaining stubble was removed and discarded with shaving cream and a straight edge razor. Hair from the initial collection was stored in Ziplock™ plastic bags at room temperature until hair could be finely cut with scissors and transferred to separate glass vessels for storage at -30°C. After initial collection, scalp hair (approximately 2-3 mm in length) was collected as close to the scalp as possible using a cleaned electrical shaver (Norelco®). Again, remaining stubble was removed and discarded using a straight edge razor.
Fingernail was collected from each digit of both hands of the subject and combined for analysis. The ventral surface of the nail was scraped 50-100 times with a sterile scalpel blade.

If adequate specimen was available, hair and nail scrapings were split into two portions. One portion was analyzed after decontamination wash procedures while the other portion was analyzed without first being subjected to decontamination wash procedures. In cases of limited specimen amounts, only a washed specimen was prepared for analysis. In most cases, there were only ample nail scrapings for a washed specimen analysis. Approximately 25 mg of hair and 15 mg of nail scrapings were weighed into fritted filtered reservoirs in preparation for analysis.

Decontamination Washes

A multi-step procedure was employed to decontaminate the hair and nail surfaces. Briefly, a 15-min isopropanol wash (3 mL) was followed by three successive 30-min washes in 3.0 mL of a 100 mM phosphate buffer (pH 6.0). All steps were performed at room temperature and specimens were agitated by stirring or placing in an oscillating water bath. Wash fractions were collected for further analysis. All four wash fractions for initial specimen collections (day 0) were analyzed separately, whereas the phosphate buffer wash fractions for the following weeks were combined prior to analysis.

Enzymatic Digestion

Specimens were placed in 1.0 mL of digest solution containing 0.05 mg/mL Protease XI and 60 mg DTT dissolved in 50 mM Tris buffer (pH 7.4). The final pH of the
digest solution was approximately 7.2. Specimens were spiked with trideuterated internal standards (d₃-benzoylecgonine, d₃-cocaine, d₃-cocaethylene, d₃-ecgonine methyl ester, d₃-6-acetylmorphine, d₃-codeine, and d₃-morphine) at a concentration of 50 or 100 ng. Micro stir-bars (2-3 mm) were added and the fritted filters were capped and placed in a 40°C water bath overnight (~16 hr). The digestates were eluted from the fritted filters. The filters were rinsed with 2x2 mL volumes of 100 mM phosphate buffer (pH 6.0) which was collected into the same tube as their respective digestate. The filtered digestates were centrifuged at 4000 rpm for 10 minutes prior to SPE.

Solid-Phase Extraction

The SPE cartridges were conditioned sequentially with elution solvent (1 mL), methanol (3 mL), deionized water (3 mL), and 100 mM phosphate buffer (2 mL). Centrifuged digestates were loaded onto the cartridges, and the cartridges were washed sequentially with deionized water (2 mL), 10 mM acetate buffer (2 mL), methanol (3 mL), and acetonitrile (1 mL) before aspirating to dryness under full-vacuum conditions. Analytes were recovered from the columns with elution solvent (4 mL), and the eluents were evaporated to dryness at 40°C under a stream of nitrogen. The residues were reconstituted in 20 µL of acetonitrile, vortexed, and transferred to autosampler vials. BSTFA with 1% TMCS (20 µL) was added to each of the vials. Autosampler vials were capped, vortexed, and heated at 65°C for 30 min.
GC/MS Analysis

Analyses were performed with a Hewlett-Packard 6890 Series II gas chromatograph and automatic liquid sampler interfaced with a Hewlett-Packard 5973 Mass Selective Detector (MSD, Hewlett-Packard Company, Little Falls, DE). The gas chromatograph was equipped with a HP-1 crosslinked 1% diphenyl, 99% dimethylpolysiloxane fused-silica capillary column (12 m x 0.2 mm i.d. x 0.25 µm film thickness, Hewlett-Packard Company). Automated injections (1 µL) were made in the splitless mode, and a 4-mm i.d. silanized borosilicate liner with a glass wool plug was utilized. Parameters for gas chromatograph were previously detailed in Chapter 2 of this dissertation.

The MSD was operated in the selected ion monitoring mode. The following ions were monitored at a dwell time of 20 ms (quantitation ions are italicized):

- anhydroecgonine methyl ester: \( m/z \) 152, 166, and 181; \( \text{d}_3 \)-ecgonine methyl ester: \( m/z \) 85, 99, and 274; ecgonine methyl ester: \( m/z \) 82, 96, and 271; ecgonine ethyl ester: \( m/z \) 83, 96, and 240; \( \text{d}_3 \)-cocaine: \( m/z \) 185, 275, and 306; cocaine: \( m/z \) 182, 272, and 303; \( \text{d}_3 \)-cocaethylene: \( m/z \) 199, 275, and 320; cocaethylene: \( m/z \) 196, 272, and 317; \( \text{d}_3 \)-benzoylecgonine: \( m/z \) 243, 259, and 364; benzoylecgonine: \( m/z \) 240, 256, and 361; norcocaine: \( m/z \) 140, 240, and 346; norbenzoylecgonine: \( m/z \) 140, 298, and 404; \( \text{d}_3 \)-6-acetylmorphine: 290, 343, and 402; 6-acetylmorphine: 287, 340, and 399; \( \text{d}_3 \)-codeine: 181, 237, and 374; codeine: 178, 234, and 371; \( \text{d}_3 \)-morphine: 239, 417, and 432; morphine: 236, 414, and 429; norcodeine: 254, 292, and 429; and normorphine: 254, 308, and 487. Analytes were identified based upon comparison of retention time and ion ratios with the corresponding values of calibrators assayed in the same run. Ion ratios were
calculated by dividing the ion peak-area of the confirming ion by the ion peak-area of the quantitative ion. Quantification of analytes was based upon the ratios of the integrated ion peak-areas to the corresponding trideuterated standard analogues.

Calibration curves were constructed with a minimum of six calibrators prepared at a concentration range of 0.1-10.0 ng/mg utilizing either 25 mg of pre-washed drug-free hair or 15 mg of pre-washed drug-free nails. The calibrators were fortified with standard solutions of anhydroecgonine methyl ester, ecgonine methyl ester, ecgonine ethyl ester, cocaine, cocaethylene, benzoylecgonine, norcocaine, norbenzoylecgonine, 6-acetylmorphine, codeine, morphine, norcodeine, normorphine, and their respective trideuterated analogues. Cocaine and 6-acetylmorphine analytical controls (5 or 10 ng/mg) were included in all batches in order to monitor the hydrolysis during sample preparation and GC/MS analysis. Negative and positive controls (0.5 to 10 ng/mg), prepared in drug-free matrix, were also included in each run.

**Pharmacokinetic Measurements and Statistics**

Maximum concentration values (Cmax) were determined by visual inspection of a time vs. concentration graph (Figures 3-2, 3-3 and Tables 3-1, 3-2). In most cases after high dose administration, the last collected specimen was treated as an "apparent" maximum concentration since no collection followed to confirm if drug concentrations began to decrease after this time due to completion of the study.

Cocaine and codeine concentrations in unwashed hair were analyzed by nonlinear regression analysis using WinNonlin™ (Scientific Consulting Inc., Apex, NC) software. Model-independent methods were used to obtain area-under-the-curve (AUC) values in
hair for cocaine and codeine. AUC measures were only performed for low dosing intervals. In instances where the drug concentration did not return to baseline following low dose administration and prior to high dose administration (cocaine for subjects A, C, G, K, M, and N), the AUC was estimated by extrapolation of the line to baseline. AUC high dose measures could not be accurately determined since drug concentrations generally demonstrated an ascending phase of terminal drug elimination during the two week collection period prior to completion of the study. \( AUC_{0-\infty} \) was determined by the following formula:

\[
AUC_{0-\infty} = AUC_{0-t} + C_t/\lambda_e
\]

Estimates for the elimination constant \( (\lambda_e) \) were obtained by linear regression analysis of drug concentrations in hair and nail during weeks seven and eight prior to the high dose week. AUC measurements were not performed for nail analysis due to lack of analyte response.

The Wilcoxon signed rank test was used for statistical comparison of: 1) \( C_{max} \) measurements in washed hair and nail following low and high doses of cocaine and codeine; 2) \( C_{max} \) measurements in washed and unwashed hair analysis following low and high doses of cocaine and codeine; and 3) total drug removed \( C_{max} \) (TDR-Cmax) measurements in hair and nails following low and high doses of cocaine and codeine. The appropriateness of this test was based on the study's small sample size \( (N=8) \). The test evaluated the null hypothesis of no significant difference between variables with a chosen alpha level of 0.05.
Results

Hair and nail specimens were prepared for GC/MS analysis by subjecting weighed hair and nail scrapings to a decontamination wash, overnight enzymatic digest, solid-phase extraction, and derivatization. Performance data for this assay including sensitivity, extraction efficiency, accuracy, and precision, are summarized in Table 2-7 through 2-11.

The limit of detection (LOD) of the assay was generally 0.1 ng/mg for hair and 0.13 ng/mg for nail scrapings (signal-to-noise ratio ≥ 3 for the quantitative ion). Norbenzoylecgonine and norcodeine demonstrated a slightly higher LOD of 0.2 ng/mg for hair analysis. Cocaine and anhydroecgonine methyl ester demonstrated a slightly higher LOD of 0.20 ng/mg and 0.26 ng/mg for nail analysis, respectively. The limit of quantitation (LOQ) for cocaine and codeine analytes in hair ranged from 0.1 to 0.3 ng/mg with cocaethylene, ecgonine methyl ester, and ecgonine ethyl ester having the highest LOQs. The LOQs for the analysis of nail scrapings ranged from 0.13 to 1.0 ng/mg with anhydroecgonine methyl ester and norbenzoylecgonine having the highest LOQs. The calibration curves were linear over a concentration range of 0.1 to 10 ng/mg and the correlation coefficients of all standard curves were ≥ 0.990.

Hydrolysis controls were included in all batches. Observed hydrolysis was less than 5% for cocaine and less than 10% for 6-acetylmorphine. Negative controls of drug-free hair and nail were monitored for contribution of matrix interference. Codeine was the only analyte demonstrating consistent interference for both qualitative ions (m/z 178 and 234). Consequently, ion ratios for another ion (m/z 196) were monitored to verify all codeine signal responses. Positive controls ranging from 0.5 to 10 ng/mg were analyzed
at the beginning, middle, and end of each run. Quantitative results for each analyte were only reported if positive controls were within 20% of the expected value.

**Analysis of Washed and Unwashed Specimens**

By the end of the washout period (week 3), drug was eliminated from both hair and nail scrapings in a majority of the subject specimens. Cocaine was the primary cocaine analyte in washed and unwashed hair and was detected in the first hair specimen collected within 3 days after completion of drug administration. Peak cocaine concentrations generally occurred in hair collected 2-3 weeks after dosing and concentration began to decrease in hair collected approximately 4 weeks after dosing. Codeine was the primary opiate analyte in washed and unwashed hair and the timing profile followed the same pattern as cocaine.

Other analytes present in hair included anhydroecgonine methyl ester, ecgonine methyl ester, cocaethylene, norcocaine, benzoylecgonine, morphine, and 6-acetylmorphine. These analytes were normally present in initially collected specimens (day 0) and/or the first collection directly following drug administration. The combined concentration of all other analytes present in a hair specimen was as much as 30% of the primary analyte concentration (i.e., cocaine or codeine) but was generally less than 10% of the primary analyte concentration.

Dose-response relationships were determined by the evaluation of AUC and Cmax measurements. AUC measurements for unwashed hair following the low dose week ranged from 23.6 to 218 ng-min/mg for cocaine and 20.8 to 110 ng-min/mg for codeine. The mean ± SD for unwashed hair AUC measurements were COC: 92.6 ± 64.6 ng-
min/mg and COD: 53.7 ± 30.2. Since the last day prior to high dose was at peak drug concentrations following low dose administration, subject G required an extra time point to determine the terminal elimination phase.

Cocaine Cmax values for washed hair ranged from 1.7 to 15 ng/mg after low-dose administration and from 4.0 to 27 ng/mg after high-dose administration. Table 3-1 summarizes the maximum time and concentration measurements for cocaine and codeine following low- and high-dose administration in washed hair. Unwashed Cmax values for hair were 5 to 50% higher. In one instance (subject M), the washed cocaine Cmax value (27 ng/mg) for hair was higher than the unwashed specimen (24 ng/mg) following high dose administration. Given the high concentration, the small difference could be explained by the nonhomogeneity of the sample matrix or analytical error. Peak codeine concentrations in washed hair followed similar timing profiles ranging from 0.71 to 5.7 ng/mg after the low dose week and from 1.8 to 8.7 ng/mg after the high dose week. Again, the unwashed codeine Cmax values were 8 to 55% higher. Figures 3-2 A through D illustrate the time-concentration relationship of cocaine and codeine in washed versus unwashed hair specimens.

Wilcoxon sign rank sum test demonstrated a significantly higher maximum drug concentration in unwashed hair analysis based on low dose administration of cocaine (p=0.0078) and low and high dose administration of codeine (p=0.0078 and p=0.0156, respectively). However, this test showed no significant difference in Cmax between unwashed and washed data after high dose administration of cocaine (p=0.1484). The nonsignificant difference between these two analyses can be explained with subject M data discussed previously. Disregarding subject M data, the observed significance level
decreased (P=0.0156) and demonstrated a significant difference for unwashed and washed analyses.

Generally, cocaine was only detected in nail specimens during the washout period prior to clinical drug administration (0 to 1.6 ng/mg). Codeine was not present in nail scrapings. No clear relationship was evident between nail-drug concentrations and dose. Presence of analytes other than the primary analytes in nail scrapings was rare. Other analytes present in nail scrapings included ecygonine methyl ester, cocaethylene, norcocaine, and benzoylecgonine. These analytes were present in initially collected specimens (day 0) and/or the second collection directly following high dose drug administration. The combined concentration of all other analytes present in a nail specimen ranged from more than the primary analyte concentration (i.e., cocaine or codeine) to approximately 40% of the primary analyte concentration.

The observed drug disposition profiles were different for these two keratinized matrices. Higher drug concentrations were observed in the hair itself rather than the decontamination wash fractions, whereas nail specimens had lower drug concentrations than their decontamination washes. Less than 20% of drug in hair specimens was removed by solvent decontamination washes while most of the drug concentrations (≥ 60%) were removed by washes of in nail specimens.

**Analysis of Decontamination Washes**

All four decontamination wash fractions for the initially collected hair and nail specimens were analyzed separately. Generally, the first phosphate buffer wash fraction had the greatest concentration of drug analyte. The second phosphate buffer wash
fraction or the isopropanol wash fraction had the second highest concentration and the last phosphate buffer wash fraction had the lowest concentration of drug analytes. In most instances, cocaine and codeine were the only analytes detected in the wash fractions. Occasionally, benzoylecgonine and 6-acetylmorphine were detected at concentrations near the LOQ.

The total amount of drug removed by washing was calculated for hair using the following formula:

\[
\text{Total drug removed by washing (\%) = } \frac{[\text{combined wash fractions (ng/mg)}] - [\text{washed specimen (ng/mg)}]}{[\text{unwashed specimen (ng/mg)}]} \times 100
\]

Generally, the total drug removed by washing the hair was 30-50% of the total drug removed. In a few specimens, the percentage of drug removed by washing was greater than the drug concentration found in the hair itself. This observation usually occurred directly following drug administration. The combined wash fraction concentration was normally 5-30% of the washed hair specimen concentration.

The previously mentioned calculation could not be performed for nail specimens since unwashed specimens were not analyzed due to a limited quantity of specimen. However, drug concentrations in the wash fractions were greater than concentrations observed in the nail scrapings themselves. In many specimens, cocaine and opiate analytes were not detected in the nail scrapings but they were detected in the wash fractions. When cocaine and opiate analytes were detected in both nail scrapings and in wash fractions, the nail scraping concentrations were 5-40% of the combined concentration of the wash fractions.
Comparison of Total Drug Removed from Hair and Nails from the Same Subject

Drug concentrations in hair and nails collected from the same subject at the same time point were compared. Results for paired analysis were reported as “total drug removed” during analysis to allow an overall evaluation of the drug disposition into keratinized matrices. This calculation was necessary since disposition into these matrices behaved differently. The calculation for determining the total drug removed was as follows:

\[
\text{Total drug removed from hair/nails} = \text{washed specimen result (ng/mg)} + \text{combined wash fractions results (ng/mg)}
\]

Figures 3-3 A through D illustrate the time-concentration graphs of total drug removed (TDR) by hair and nail analysis following low and high dose administration of cocaine and codeine. To compare hair and fingernail analysis, maximum concentration of total drug removed following high and low dose administration of cocaine and codeine were obtained and statistically evaluated. Table 3-2 summarizes maximum time and concentration results (TDR-Cmax and TDR-Tmax) for hair and nail analysis. The mean ± SD calculation for the TDR-Cmax of cocaine and codeine present in hair specimens after low dose (COC: 75 mg/70 kg; COD: 60 mg/70 kg) administration was 6.1 ± 4.0 ng/mg and 2.9 ± 1.6 ng/mg, respectively. Mean TDR-Cmax values for nails after low dose administration were COC- 0.85 ± 0.47 ng/mg and COD- 0.18 ± 0.09 ng/mg. The mean TDR-Cmax values for cocaine and codeine present in hair specimens after high dose (COC: 150 mg/70 kg; COD: 120 mg/70 kg) administration were 12 ± 7.3 ng/mg and 5.1 ± 2.7 ng/mg, respectively. Mean TDR-Cmax values for nail analysis were COC- 1.2 ± 0.79
ng/mg and COD- 0.17 ± 0.04 ng/mg. A dose-response relationship based on the mean maximum drug concentration of total drug removed after low and high dose administration was demonstrated for cocaine by hair and nail analyses. A dose-response relationship for codeine was demonstrated for hair analysis at low and high dosing intervals and by nail analysis after low dose administration. A dose-response relationship was not observed for codeine in nails at high dose administration. This observation may be attributed to the low concentration of codeine detected in nails. At high and low dose concentrations, the TDR-Cmax values for hair analysis were generally 5- to 20-times higher than those observed for nail analysis. Figure 3-4 illustrates the mean Cmax measurements for TDR from hair and nails after low and high dose administration.

Wilcoxon Sign Rank sum test was used to compare the difference of the mean maximum concentration of total drug removed from hair and nail after high and low dose administration. This test determined that hair analysis showed a significantly higher amount of total drug removed than nail analysis. This analysis showed significance for both drug analytes at both dosing intervals. The p-values were as follows: 1) COC low dose p<0.0078; 2) COC high dose p<0.0156; 3) COD low dose p<0.0078; 4) COD high dose p<0.0156.

Discussion

Previous research has demonstrated that analysis of hair and nails can be beneficial in detecting drug exposure. The sensitivity of hair and nail analysis to detect drug
exposure cannot be fully appreciated without further investigation of dose-response relationships and the mechanisms existing for drug disposition into these matrices.

This clinical inpatient study conducted with eight black male subjects evaluated the disposition of cocaine, codeine, and their metabolites in hair and nail. It describes the time course for appearance, elimination, and peak drug concentrations following low and high dose administration. This study also investigated the dose-response relationship for these drugs in keratinized matrices. The primary objectives of this study were to determine if drug disposition into these matrices occurred in a similar fashion and if either of these matrices demonstrated a predictable dose-response relationship for cocaine and codeine. Moreover, results were evaluated to determine if any mechanism of drug disposition could be supported by this study.

In order to evaluate drug disposition into the hair and nails of the same subject combined results of the washed specimens and their respective wash fractions were reported as “total drug removed” from hair or nails during analysis. This method of calculation was necessary because of insufficient quantities of nail specimens available for analysis (nails were only analyzed unwashed) and the different drug disposition profiles exhibited by hair and nails. Both hair and nail analyses demonstrated a dose-response relationship for cocaine based on the mean maximum drug concentration of total drug removed after low and high dosing levels. A dose-response relationship for codeine was also demonstrated by hair analysis at both dosing levels but only the low dosing level showed a relationship by nail analysis. This observation may be due to the low concentration of codeine observed in nail specimens. However, cocaine and codeine concentrations in hair were 5 to 20 times higher than nail-drug concentrations.
Information derived from AUC measurements for this study was limited. AUC measurements for high dose administration could not be reliably determined due to inadequate collection time following drug administration. Collection of specimen only continued for 2 weeks after high dose administration and terminal drug elimination was still in its ascending phase. However, AUC measurements after low dose administration did demonstrate high intra-subject variability for cocaine and codeine disposition into hair. This variability shows the degree of difficulty in interpreting data for hair analysis since these measurements were obtained from subjects of the same race and gender and under strictly controlled conditions.

This project also demonstrated that the observed drug disposition profiles were different for hair and nails. Less than 20% of drug in hair specimens was removed by decontamination wash procedures while most of the drug concentrations were removed by washes of nail specimens.

There are two possible explanations for this observation. One explanation may be that the location of drug disposition into the hair and nail matrix might be somewhat different. Based on findings of this research, it appears that drug may incorporate more deeply into the inner matrix of hair in comparison to nails. If drug incorporates into the inner region of the hair shaft this would prevent easy removal by decontamination washes. Conversely, drug incorporation into fingernails may be more surface-oriented, which would allow decontamination wash procedures to more readily remove drug. Another explanation for this discrepancy may involve the method of specimen collection. Fingernail specimens were collected by scraping the dorsal surface of the nail plate to obtain shavings. The proposed mechanism of nail growth and incorporation of epidermal
cells (i.e., keratinocytes) includes growth beginning at the proximal end of the nail plate beginning at the nail fold and from the ventral side of the nail plate directly above the highly vascularized nail bed. Therefore, the nail specimen collected for this study may represent the most inaccessible region of drug-positive keratinocytes recently incorporated into the nail plate.

Nail grows at an average rate less than 0.1 cm/week. By completion of this study, nails would have grown a little over one-half a centimeter which would barely transfer the new growth at a position where the scraping for specimen collection occurred. Moreover, research has demonstrated that more mature melanocytes possessing melanin are mainly located towards the distal edge of the nail plate. Melanin has been proposed as a primary drug-binding site, this drug compartment was underrepresented by the method of specimen collection for this study. The latter explanation is more plausible if one considers the data obtained from another project within this dissertation (Chapter 4). This study demonstrated that reasonably high cocaine concentrations were easily detected in distal, free-edge nail specimens using the same analytical technique of GC/MS analysis. Although drug was removed by the methanolic wash procedure used during specimen preparation, the drug concentrations in the washes were always less than or equal to drug concentrations observed in the nail specimens themselves.

Although this study did not fully investigate the mechanism by which drugs are deposited into keratinized matrices in real-life situations, it did give evidence of some mechanistic contribution. This study does support drug diffusion from the vasculature into matrix cells of the hair and nail. However, results of this study suggest that drugs may absorb closer to the nail surface and can be easily removed by wash procedures,
whereas drugs may penetrate further into the inner matrix of the hair preventing easy removal. This study also supports transfer of drug from sweat, sebum, and skin.

The study design employed in this investigation limited the degree of contribution from environmental contamination and bodily secretions that are proposed mechanisms of drug disposition. Because drug was administered by oral ingestion and subcutaneous injections, environmental contamination resulting from drug aerosols and vapors were not represented in this study. In addition, collection of hair by shaving the entire scalp each week limited the transfer of drug from sweat, sebum, and skin. Instead of a hair shaft of several inches available for deposition of drug-containing secretions, the hair collected for this study had one week’s worth of drug secretions coating its surface. The role of this study limitation is not apparent since it is unknown to what extent bodily secretions coat the hair near the surface of the scalp in comparison to hair of the distal shaft.

Reports investigating drug concentrations in hair and nail of the same subject have been limited. All of these reports are of small sample size. Table 3-3 summarizes results from these reports and the current study. Most of these investigations only report detection of a drug in these keratinized matrices and they do not discuss dose-response relationships. Those investigating dose-response relationships only discuss therapeutic drugs. This is the first investigation to report dose-response relationships for drugs of abuse in paired hair and nail analysis. These reports differ in their observations as to whether drug concentrations are higher in hair or in nail. Reports investigating the same drug agree in their findings. These investigations demonstrate that both hair and nails may show a dose-response relationship, but the relationship appears to be stronger for hair than for nails.
Because the data from paired hair and nail analyses are limited for dose-response relationship investigations, it is necessary to rely on investigations of hair alone to discuss the limitations and problems of these types of kinetic studies. Conclusions surmised from investigations into dose-response relationships in hair analysis remain contradictory due to differences associated with study design, analyte of interest, and analytical methodology.

Differences in study designs to determine dose-response relationships include use of experimental animals versus human subjects and determination of dose based on self-reports or clinical studies with known administered concentrations. Analytes of interest in disposition studies are influenced differently by physiochemical properties such as lipophilicity, melanin affinity, plasma protein binding, molecular weight, and membrane permeability. Finally, analytical methodology can influence drug disposition studies due to differences in efficiency of analyte isolation and instrumental sensitivity and specificity.

Human studies among investigators vary in their method of determining dose-response relationships. Some investigators depend on self-reporting of drug use while others control the actual dose administered. Moreover, conclusions made by investigators based on results from similar study designs vary in their degree of conservativeness. Although controlled studies are more valid for determining dose-response relationships, they are also more cost-prohibitive and dosing schemes are limited by safety considerations. The human studies performed by Baumgartner et al. (41) were largely based on self-reported drug use. Digoxin was the only drug administered under controlled conditions. For these studies, a poor correlation between dose and self-reported cannabinoid concentrations in hair was observed, but the investigators attributed this to technical difficulties associated with the monoclonal antibody used by their
radioimmunoassay. Nakahara et al. (134) also employed self-reports of patients admitted to a psychiatric hospital for methamphetamine abuse to determine the relationship of sectional analysis of hair by GC/MS analysis and drug histories. In 9 of 11 subjects, a positive correlation was demonstrated between methamphetamine concentrations in hair and self-reported drug histories. These authors did not discuss dose and concentration of drug in hair due to the uncertainty of the dose, unknown purity of the illicit drug, and the intra-individual variability of drug uptake from blood to hair. As demonstrated from just these two studies, there is wide disparity with conclusions made by investigators using similar study designs.

Not only are drug disposition studies influenced by methodology and study design, different physiochemical properties of drug analytes can influence results of drug disposition and dose-response studies. For example, Gygi et al. (67) performed an animal study investigating the differences in acid-base nature and lipophilicity of phenobarbital (weak acid, highly lipophilic) and codeine (weak base, less lipophilic) demonstrated different incorporation patterns for these two drugs. This study showed that codeine was incorporated at higher concentrations in pigmented versus nonpigmented hair of the same animal but phenobarbital concentrations were equal in both hair types. This study suggests that higher concentrations of drug in hair for drug incorporation studies could be influenced by physicochemical properties and may not be due to greater ingestion of drug alone.

In summary, this study demonstrated that drug can be detected in hair and nail by GC/MS analysis. The time profiles for drug disposition into hair and nails are similar with drug concentrations appearing within three days following last drug administration,
peaking within two to three weeks of drug administration, and decreasing approximately four weeks post-administration. The difference in drug disposition profiles for hair and nails was demonstrated by the greater drug removal of drug by decontamination wash procedures for nail than hair. This difference may be attributed to different growth rates or to the difference in drug disposition pattern (surface-oriented versus confined to the inner matrix). By evaluating the maximum concentration of total drug removed from the matrix (i.e., combined concentrations of washed hair/nail plus wash fraction results), this study demonstrated that a dose-response relationship was observed for cocaine and codeine in hair and nails. However, nail analysis failed to demonstrate a dose-response relationship for codeine at high dose administration. Drug concentrations in hair were generally 5- to 20-times higher than nail concentrations.

Future study is needed to further determine similarities and differences of drug disposition into hair and nails. In addition, studies are needed to delineate the mechanisms that contribute to observed differences in timing profiles for drug disposition into these keratinized matrices of similar composition, vascularization, and inherent exposure to the environment.
Study Timeline

Wash-out period

PLACEBO

0 1 2 3 4 5 6 7 8 9 10

LOW DOSE WEEK
Mon, Wed, Fri-75 mg/70 kg coc HCl
Tue, Thur, Mon-60 mg/70 kg cod sulfate

HIGH DOSE WEEK
Mon, Wed, Fri-150 mg/70 kg coc HCl
Tue, Thur, Mon-120 mg/70 kg cod sulfate

Biological specimen collections
Hair - once each week
Fingernail Shaving - once each week

Figure 3-1. Time Line for Inpatient Study

Abbreviations: cocaine (coc), codeine (cod), subcutaneous (SC), oral (PO), and hydrochloride salt (HCl).
Table 3-1. Maximum Time and Concentration Measurements for Cocaine and Codeine Following Low and High Dose Administration in Washed Hair

<table>
<thead>
<tr>
<th>Subject</th>
<th>HAIR COC Cmax (75 mg/70 kg)</th>
<th>HAIR Tmax (Wks. after dose)</th>
<th>HAIR COC Cmax* (150 mg/70 kg)</th>
<th>HAIR Tmax (Wks. after dose)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>6.6</td>
<td>2</td>
<td>9.9</td>
<td>3</td>
</tr>
<tr>
<td>C</td>
<td>5.7</td>
<td>3</td>
<td>8.6</td>
<td>3</td>
</tr>
<tr>
<td>F</td>
<td>2.6</td>
<td>3</td>
<td>5.1</td>
<td>3</td>
</tr>
<tr>
<td>G</td>
<td>5.0</td>
<td>4</td>
<td>10</td>
<td>3</td>
</tr>
<tr>
<td>H</td>
<td>1.7</td>
<td>3</td>
<td>10</td>
<td>3</td>
</tr>
<tr>
<td>K</td>
<td>7.5</td>
<td>3</td>
<td>15</td>
<td>3</td>
</tr>
<tr>
<td>M</td>
<td>15</td>
<td>3</td>
<td>27</td>
<td>3</td>
</tr>
<tr>
<td>N</td>
<td>2.2</td>
<td>2</td>
<td>4.0</td>
<td>3</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Subject</th>
<th>HAIR COD Cmax (60 mg/70 kg)</th>
<th>HAIR Tmax (Wks. after dose)</th>
<th>HAIR COD Cmax* (120 mg/70 kg)</th>
<th>HAIR Tmax (Wks. after dose)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>1.7</td>
<td>2</td>
<td>2.4</td>
<td>3</td>
</tr>
<tr>
<td>C</td>
<td>3.3</td>
<td>3</td>
<td>4.2</td>
<td>3</td>
</tr>
<tr>
<td>F</td>
<td>1.7</td>
<td>3</td>
<td>2.0</td>
<td>3</td>
</tr>
<tr>
<td>G</td>
<td>3.3</td>
<td>4</td>
<td>5.5</td>
<td>3</td>
</tr>
<tr>
<td>H</td>
<td>1.3</td>
<td>3</td>
<td>5.9</td>
<td>3</td>
</tr>
<tr>
<td>K</td>
<td>5.7</td>
<td>3</td>
<td>7.7</td>
<td>3</td>
</tr>
<tr>
<td>M</td>
<td>4.3</td>
<td>3</td>
<td>8.7</td>
<td>3</td>
</tr>
<tr>
<td>N</td>
<td>0.71</td>
<td>2</td>
<td>1.8</td>
<td>3</td>
</tr>
</tbody>
</table>

Abbreviations: cocaine (COC), codeine (COD), total drug removed (TDR), maximum concentration (Cmax), maximum time (Tmax), and weeks (Wks.)

*Note: These values are apparent Cmax values.
Table 3-2. Total Drug Removed-Maximum Time and Concentration Measurements for Cocaine/Codeine Following Low/High Dose Administration in Hair and Nails

<table>
<thead>
<tr>
<th>Subject</th>
<th>HAIR COC TDR-Cmax (Wks. after dose)</th>
<th>HAIR TDR-Tmax</th>
<th>HAIR COC TDR-Cmax (Wks. after dose)</th>
<th>HAIR TDR-Tmax</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>75 mg/70 kg</td>
<td>150 mg/70 kg</td>
<td>150 mg/70 kg</td>
<td>150 mg/70 kg</td>
</tr>
<tr>
<td>A</td>
<td>6.8</td>
<td>11</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>C</td>
<td>5.8</td>
<td>9.1</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>F</td>
<td>2.9</td>
<td>5.1**</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>G</td>
<td>5.4</td>
<td>11</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>H</td>
<td>2.2</td>
<td>11</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>K</td>
<td>7.9</td>
<td>16</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>M</td>
<td>15</td>
<td>28</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>N</td>
<td>3.3</td>
<td>5.5</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Mean ± SD</td>
<td>6.1 ± 4.0</td>
<td>12 ± 7.3</td>
<td>12 ± 7.3</td>
<td>12 ± 7.3</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Subject</th>
<th>NAIL COC TDR-Cmax (Wks. after dose)</th>
<th>NAIL TDR-Tmax</th>
<th>NAIL COC TDR-Cmax (Wks. after dose)</th>
<th>NAIL TDR-Tmax</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>75 mg/70 kg</td>
<td>150 mg/70 kg</td>
<td>150 mg/70 kg</td>
<td>150 mg/70 kg</td>
</tr>
<tr>
<td>A</td>
<td>0.82</td>
<td>1.6</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>C</td>
<td>0.25</td>
<td>0.73</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>F</td>
<td>1.6</td>
<td>1.5</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>G</td>
<td>0.64</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>H</td>
<td>0.7</td>
<td>0.68</td>
<td>3</td>
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<tr>
<td>K</td>
<td>0.75</td>
<td>0.62</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>M</td>
<td>0.54</td>
<td>0.57</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>N</td>
<td>1.5</td>
<td>2.7</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Mean ± SD</td>
<td>0.85 ± 0.47</td>
<td>1.2 ± 0.79</td>
<td>1.2 ± 0.79</td>
<td>1.2 ± 0.79</td>
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</table>
Table 3-2. continued.

<table>
<thead>
<tr>
<th>Subject</th>
<th>HAIR COD TDR-Cmax 60 mg/70 kg</th>
<th>HAIR TDR-Tmax (Wks. after dose)</th>
<th>HAIR COD TDR-Cmax* 120 kg/mg/70</th>
<th>HAIR TDR-Tmax (Wks. after dose)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>1.9</td>
<td>2</td>
<td>3.1</td>
<td>3</td>
</tr>
<tr>
<td>C</td>
<td>3.3</td>
<td>3</td>
<td>4.2</td>
<td>3</td>
</tr>
<tr>
<td>F</td>
<td>1.7</td>
<td>3</td>
<td>2.0**</td>
<td>3</td>
</tr>
<tr>
<td>G</td>
<td>3.3</td>
<td>4</td>
<td>5.5</td>
<td>3</td>
</tr>
<tr>
<td>H</td>
<td>1.8</td>
<td>3</td>
<td>6.5</td>
<td>3</td>
</tr>
<tr>
<td>K</td>
<td>5.9</td>
<td>3</td>
<td>8.2</td>
<td>3</td>
</tr>
<tr>
<td>M</td>
<td>4.6</td>
<td>3</td>
<td>9.3</td>
<td>3</td>
</tr>
<tr>
<td>N</td>
<td>1.0</td>
<td>2</td>
<td>2.3</td>
<td>3</td>
</tr>
<tr>
<td>Mean ± SD</td>
<td>2.9 ± 1.6</td>
<td></td>
<td>5.1 ± 2.7</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Subject</th>
<th>NAIL COD TDR-Cmax 60 mg/70 kg</th>
<th>NAIL TDR-Tmax (Wks. after dose)</th>
<th>NAIL COD TDR-Cmax* 120 kg/mg/70</th>
<th>NAIL TDR-Tmax (Wks. after dose)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>0.12</td>
<td>1</td>
<td>0.2</td>
<td>2</td>
</tr>
<tr>
<td>C</td>
<td>0.12</td>
<td>1</td>
<td>0.19</td>
<td>2</td>
</tr>
<tr>
<td>F</td>
<td>n.c.o.</td>
<td>n.t.o.</td>
<td>0.12</td>
<td>2</td>
</tr>
<tr>
<td>G</td>
<td>n.c.o.</td>
<td>n.t.o.</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>H</td>
<td>0.17</td>
<td>3</td>
<td>0.2</td>
<td>1</td>
</tr>
<tr>
<td>K</td>
<td>0.31</td>
<td>3</td>
<td>0.19</td>
<td>1</td>
</tr>
<tr>
<td>M</td>
<td>n.c.o.</td>
<td>n.t.o.</td>
<td>n.c.o.</td>
<td>n.t.o.</td>
</tr>
<tr>
<td>N</td>
<td>n.c.o.</td>
<td>n.t.o.</td>
<td>0.12</td>
<td>2</td>
</tr>
<tr>
<td>Mean ± SD</td>
<td>0.18 ± 0.09</td>
<td></td>
<td>0.17 ± 0.04</td>
<td></td>
</tr>
</tbody>
</table>

Abbreviations: cocaine (COC), codeine (COD), total drug removed (TDR), maximum concentration (Cmax), maximum time (Tmax), no Cmax observed (n.c.o.), no Tmax observed (n.t.o.), no specimen available for analysis (NS), standard deviation (SD), and weeks (Wks.)

*Note: These values are apparent Cmax values.

**Note: Wash fractions lost during specimen preparation. This value is for washed specimen measurement only.
Figure 3-2 (A-D). Concentration of Drug Analytes in Washed and Unwashed Portions of Hair from Subjects Enrolled in 10-week Inpatient Clinical Study. Subjects were given low and high doses of cocaine and codeine and hair was collected weekly during study time. (A) Cocaine concentrations for Subjects A, C, F, G; (B) Cocaine concentrations for Subjects H, K, M, N; (C) Codeine concentrations for Subjects A, C, F, G; and (D) Codeine concentrations for Subjects H, K, M, N.
Figure 3-2 A

Subject A
Washed / Unwashed Hair

Subject C
Washed / Unwashed Hair

Subject F
Washed / Unwashed Hair

Subject G
Washed / Unwashed Hair
Figure 3-2B

Subject K
Washed / Unwashed Hair

Washed - Unwashed

Subject M
Washed / Unwashed Hair

Washed - Unwashed

Subject N
Washed / Unwashed Hair

Washed - Unwashed
Figure 3-2 C

Subject A
Washed / Unwashed Hair

Subject C
Washed / Unwashed Hair

Subject F
Washed / Unwashed Hair

Subject G
Washed / Unwashed Hair
Figure 3-2 D

Subject H
Washed / Unwashed Hair

Subject K
Washed / Unwashed Hair

Subject M
Washed / Unwashed Hair

Subject N
Washed / Unwashed Hair
Figure 3-3 (A-D). Total Drug Removed ("Combined Washed + Wash Fractions") from Hair vs. Fingernail Scrapings after Low and High Dose Administration of Cocaine and Codeine over a 10-week period. (A) Cocaine concentrations for Subjects A, C, F, G; (B) Cocaine concentrations for Subjects H, K, M, N; (C) Codeine concentrations for Subjects A, C, F, G; and (D) Codeine concentrations for Subjects H, K, M, N.
Figure 3-3 A

**Subject A - Total Drug Removed**

Week of Study

**Subject C - Total Drug Removed**

Week of Study

**Subject F - Total Drug Removed**

Week of Study

**Subject G - Total Drug Removed**

Week of Study
Figure 3-3 B

Subject H - Total Drug Removed

Subject K - Total Drug Removed

Subject M - Total Drug Removed

Subject N - Total Drug Removed
Figure 3-3-D

Subject H- Total Drug Removed

![Graph showing the removal of drug from nails and hair over weeks of study.]

Subject K- Total Drug Removed

![Graph showing the removal of drug from nails and hair over weeks of study.]

Subject M- Total Drug Removed

![Graph showing the removal of drug from nails and hair over weeks of study.]

Subject N- Total Drug Removed

![Graph showing the removal of drug from nails and hair over weeks of study.]

**MISSING SAMPLE**
Figure 3-4. Mean Cmax Measurements for Total Drug Removed from Hair and Nails after Low and High Dose Administration

Abbreviations: cocaine (COC), codeine (COD), maximum concentration (Cmax), low dose (LD), and high dose (HD).
### Table 3-4. Investigations of Drugs in Paired Hair and Nail Samples

<table>
<thead>
<tr>
<th>Investigators (Year)</th>
<th>Analyte</th>
<th>Drug Concentrations</th>
<th>Method of Analysis</th>
<th>Dose-Response Results</th>
<th>Basis of Dose-Response</th>
</tr>
</thead>
<tbody>
<tr>
<td>Suzuki et.al. (1984) (20)</td>
<td>Amphetamines (A, MA)</td>
<td>Nail&gt;Hair (5 out of 7)</td>
<td>GC-MS-CI</td>
<td>Not Investigated</td>
<td>NA</td>
</tr>
<tr>
<td>Suzuki et.al. (1989) (19)</td>
<td>Amphetamines (A, MA)</td>
<td>Nail&gt;Hair (6 out of 10)</td>
<td>GC-MS-EI</td>
<td>Not Investigated</td>
<td>NA</td>
</tr>
</tbody>
</table>
| Uematsu et.al. (1989) (47) | Haloperidol                  | Hair>Nail (n=20)     | RIA                | Yes: Hair b/o trough plasma conc. (SS) \(r = 0.772\)  
Yes: Hair b/o daily dose \(r = 0.555\)  
No: Nail b/o trough plasma conc. (SS)  
Yes: Nail b/o daily dose \(r = 0.525\) | Clinical Study |
| Faergemann et.al. (1993) (143) | Terbinafine                  | Hair>Nail (n=12)     | HPLC               | Yes: Hair-followed typical time-concentration pharmacokinetic profile;  
[Hair]<[Plasma]  
Yes: Nail-followed typical time-concentration pharmacokinetic profile;  
[Nail]<[Plasma] | Clinical Study |
| Miller (1994) (22)     | Cocaine BE                   | Hair>Nail            | MS-MS              | Not Investigated                                                                    | NA                      |
| This Study            | Cocaine (Opiates)            | Hair>Nail (n=8)      | GC-MS-EI          | Yes: Hair- b/o TDR-Cmax. COC and COD at low and high dosing intervals  
Yes: Nail- b/o TDR-Cmax. COC (low/high) and COD (low)  
No: Nail- b/o TDR-Cmax. COD and at high dosing interval | Clinical Study |
Abbreviations: amphetamine (A), methamphetamine (MA), 3,4-methylenedioxyamphetamine (MDA), 3,4-methylenedioxymethamphetamine (MDMA), cocaine (COC), benzoylecgonine (BE), codeine (COD), gas chromatography-mass spectrometry-electron impact (GC-MS-EI), gas chromatography-mass spectrometry-chemical ionization (GC-MS-CI), mass spectrometry-mass spectrometry (MS-MS), radioimmunoassay (RIA), high-pressure liquid chromatography (HPLC), total drug removed (TDR), maximum concentration (Cmax), steady-state (SS), based on (b/o), and not applicable to this study (NA).
CHAPTER 4
IDENTIFICATION OF COCAINE ANALYTES IN POSTMORTEM FINGERNAILS AND TOENAILS

Introduction

Since the 1800s, nails have been used in the investigations of death associated with poisons. Historically, nails have been subjected to elemental analysis in a variety of applications including arsenic in forensic investigations (146-149) and the transition metals for occupational (150) and environmental exposure (151-153). In addition, nail analysis has been applied to the clinical diagnosis of various disease states (154-156), and more recently, to therapeutic drug monitoring (47, 58, 157).

In 1984, Suzuki et al. (20) were the first to identify drugs of abuse in nails by successfully detecting amphetamine and methamphetamine in the nail clippings of methamphetamine abusers. Since then, Suzuki et al. (19) and Cirimele et al. (21) have pursued the analysis of nails for amphetamines, the latter group also detecting 3,4-methylenedioxyamphetamine and 3,4-methylenedioxymethamphetamine. In 1994, preliminary findings by the Federal Bureau of Investigation’s (FBI) Toxicology Laboratory demonstrated the presence of cocaine and benzoylecgonine in nails obtained from cadavers (22).
The literature, however, remains scant in the area of nail analysis for drugs of abuse. This report describes a sensitive and specific gas chromatographic/mass spectrometric (GC/MS) method for the isolation and detection of cocaine analytes in fingernail and toenail clippings. In addition, we discuss the possible mechanism of drug entry and incorporation, and describe the advantages and disadvantages of nail analysis for drugs of abuse.

**Materials and Methods**

**Standards and Reagents**

All organic solvents were HPLC grade, and chemicals were reagent grade. Anhydroecgonine methyl ester, benzoylecgonine, d$_3$-benzoylecgonine, cocaine, d$_3$-cocaine, cocaethylene, d$_3$-cocaethylene, ecgonine ethyl ester, ecgonine methyl ester, d$_3$-ecgonine methyl ester, m-hydroxybenzoylecgonine, norbenzoylecgonine, and norcocaine were purchased from Radian International LLC (Austin, TX). $N,O$-Bis(trimethylsilyl)trifluoroacetamide (BSTFA) with 1% trimethylchlorosilane (TMCS) was obtained from Pierce Company (Rockford, IL).

Phosphate buffer (25 mM; pH 4.0 ± 0.1) was prepared from a mixture of potassium phosphate monobasic and deionized water. Acetate buffer (100 mM; pH 4.0 ± 0.1) was prepared from a mixture of glacial acetic acid, sodium acetate, and deionized water. The solid-phase extraction (SPE) elution solvent, methylene chloride–2-propanol–ammonium hydroxide (80:20:2, v/v/v) was prepared daily.
Subjects

Fingernail (N=17) and toenail (N=15) clippings were obtained using cosmetic nail clippers from subjects examined at the 8th District Medical Examiner’s Office (Gainesville, FL). Nail clippings from all digits were combined for each subject (approximately 7-50 mg). The selection of subjects was based upon the likelihood of drug use indicated by information gathered by the Medical Examiner’s Office. Upon receipt in the laboratory, the nail specimens were stored at room temperature in grip-lock plastic bags.

Epidemiologic data, and the cause and manner of death for each subject are shown in Table 4-1. In addition, 12 fingernail specimens were obtained as controls from individuals who were unlikely to be cocaine users (i.e., laboratory personnel, law enforcement officers, children).

Conventional postmortem drug analyses using immunoassay, gas chromatography, and GC/MS were performed on blood and/or urine specimens by a commercial laboratory (SmithKline Beecham Clinical Laboratory, Leesburg, FL) or a university laboratory (University of Florida College of Medicine, Gainesville, FL). Testing of antemortem blood was performed by a hospital clinical laboratory. The results of these analyses are also shown in Table 4-1.

Solid-Phase Extraction

The solid-phase extraction of cocaine analytes from nails and subsequent analysis by GC/MS was adapted from the procedure previously reported by Cone et al. (11).

Nail clippings were cut into small pieces and approximately 7-25 mg aliquots were placed into disposable culture tubes. The samples were washed with methanol (3 mL) by
vortexing for 10 s. The methanolic wash was decanted into a fresh tube and dried down at 50°C under a stream of nitrogen (TurboVap®, Zymark, Hopkinton, MA). The washes were retained for further analysis. Control nail specimens and their washes were treated in the same manner as suspected cocaine user specimens. Methanol (3 mL) and trideuterated internal standards (5 ng/mg) were added to the washed nails, the tubes capped, and the mixture heated under reflux at 40°C for 16 h. After cooling, the methanolic extracts were decanted into fresh tubes and evaporated to dryness at 50°C under a stream of nitrogen.

After adding trideuterated internal standard (5 ng/mg) to the wash residues, both the nail extracts and wash residues were reconstituted in phosphate buffer (3 mL) prior to further extraction. The SPE cartridges (Clean Screen®, ZSDAU020, United Chemical Technologies, Horsham, PA) were conditioned sequentially with elution solvent (1 mL), methanol (3 mL), deionized water (3 mL), and phosphate buffer (2 mL). Vortexed samples were loaded onto the cartridges, and the cartridges were washed sequentially with deionized water (2 mL), acetate buffer (2 mL), methanol (3 mL), and acetonitrile (1 mL) before aspirating to dryness. Analytes were recovered from the columns with elution solvent (4 mL), and the eluents were evaporated to dryness at 50°C under a stream of nitrogen. The extracts were derivatized with 30 µL of BSTFA with 1% TMCS at 60°C for 30 m and transferred to autosampler vials.

**GC/MS Analysis**

Analyses were performed with a Hewlett-Packard 5890A Series II gas chromatograph and 7673B automatic liquid sampler interfaced with a Hewlett-Packard 5972A Mass Selective Detector (MSD, Hewlett-Packard Company, Little Falls, DE). The
gas chromatograph was equipped with an HP-5MS crosslinked 5% diphenyl, 95%
dimethylpolysiloxane fused-silica capillary column (30 m x 0.25 mm i.d. x 0.25 µm film
thickness, Hewlett-Packard Company). Automated injections (1 µL) were made in the
splitless mode, and a 2-mm i.d. silanized borosilicate liner was utilized.

The MSD was operated in the selected ion monitoring mode. The following ions
were monitored at a dwell time of 20 ms (ions in italics were used for quantitation):
anhydroecgonine methyl ester: m/z 152, 166, and 181; d₃-ecgonine methyl ester: m/z 85, 99, and 274; ecgonine methyl ester: m/z 82, 96, and 271; ecgonine ethyl ester: m/z 83, 96, and 285; d₃-cocaine: m/z 185, 275, and 306; cocaine: m/z 182, 272, and 303; d₃-
cocaethylene: m/z 85, 199, and 320; cocaethylene: m/z 82, 196, and 317; d₃-
benzoylecgonine: m/z 85, 243, and 364; benzoylecgonine: m/z 82, 240, and 361; 
norcocaine: m/z 140, 240, and 346; norbenzoylecgonine: m/z 140, 298, and 404; and m-
hydroxybenzoyllecgonine: m/z 82, 210, and 240. Analytes were identified based upon
comparison of retention time and ion ratio with the corresponding values of calibrators
assayed in the same run. Ion ratios were calculated by dividing the ion peak-area of the
confirming ion by the ion peak-area of the quantitative ion. Quantification of analytes was
based upon the ratios of the integrated ion peak-areas to the corresponding trideuterated
standard analogues.

Calibration curves were constructed with a minimum of six calibrators prepared at
a concentration range of 0.1-10.0 ng/mg utilizing 25 mg of pre-washed drug-free nails.
The calibrators were fortified with standard solutions of anhydroecgonine methyl ester,
ecgonine methyl ester, ecgonine ethyl ester, cocaine, cocaethylene, benzoylecgonine,
norcocaine, norbenzoylecgonine, m-hydroxybenzoyllecgonine, and their respective
trideuterated cocaine analogues. Cocaine and ecgonine methyl ester analytical controls (10 ng/mg) were included in all batches in order to monitor the hydrolysis of cocaine during extraction and GC/MS analysis, and the formation of anhydroecgonine methyl ester during GC injection, respectively.

Statistics

McNemar’s test was utilized to compare the rate of cocaine positive results for nail analysis and conventional postmortem toxicological methodology. This test was chosen based due to small sample size (n = 18) and the categorical nature of the data (i.e., same subjects analyzed by both methods). The test evaluated the null hypothesis of no significant difference between variables at a chosen 95% confidence level (α = 0.05).

Results

Methanolic extracts of fingernail and toenail clippings were purified by SPE and assayed by GC/MS for the presence of cocaine analytes. The results of the nail analyses are listed in Table 4-2 and summarized in Table 4-3. The limit of detection (LOD) of the assay was approximately 0.10 ng/mg (signal-to-noise ratio ≥ 5 for the quantitative ion) for all cocaine analytes, except for ecgonine ethyl ester and norbenzoylecgonine, which had an LOD of approximately 0.40 ng/mg. The limits of quantitation (LOQ) ranged from 0.20 to 0.5 ng/mg. Ecgonine ethyl ester, m-hydroxybenzoylecgonine, and norbenzoylecgonine and norcocaine had LOQs 2.0 to 2.5 times higher than those of the lowest analytes. Table 2-5 summarizes the limits of detection, quantitation, and linearity for this assay. Intra- and inter-assay data are summarized in Table 2-6 in Chapter 2 of this dissertation. The
calibration curves were linear over a concentration range of 0.1 ng/mg to 10 ng/mg; the correlation coefficients of all standard curves were $\geq 0.993$.

Hydrolysis of cocaine was monitored in all batches and was less than 5%. All analytical controls fortified with either cocaine or ecgonine methyl ester alone were negative for anhydroecgonine methyl ester. These controls verified that the degradation of these analytes during the analytical process was insignificant.

Cocaine and benzoylecgonine were the predominant cocaine analytes in all postmortem nail specimens. Cocaine analytes were present in 14 (82.3%) subjects utilizing nail analysis. Out of those 14 subjects, only 5 (27.7%) were positive by conventional postmortem drug analysis. Anhydroecgonine methyl ester, ecgonine methyl ester, ecgonine ethyl ester, cocaethylene, norcocaine, and norbenzoylecgonine were found in a limited number of specimens. Results of the 12 control nail specimens were negative for all cocaine analytes. McNemar's test showed that nail analysis significantly improved the detection of cocaine exposure relative to conventional postmortem toxicological methods ($p = 0.003$).

Analysis of the wash fractions revealed the presence of cocaine analytes in 5 subjects (no. 3, 5, 7, 13, and 17). Cocaine analytes present in the washes included cocaine, benzoylecgonine, anhydroecgonine methyl ester, ecgonine methyl ester, and cocaethylene. The wash concentrations ranged from less than 0.10 ng/mg to greater than 10.0 ng/mg. Analysis of the wash fractions for the control nails was negative for all cocaine analytes.
Discussion

Description of the Nail and Mechanism of Drug Incorporation

Nails are derived from the same cells as the epidermis and hair and consist of hard, dead keratinous cells. Nails are colorless, but appear pink due to the blood supply in the underlying dermis. The anatomy of the nail, with the relevant anatomic features indicated, is shown in Figure 1-2 of the Chapter 1.

The mechanism of drug entry and incorporation into the nail matrix is unknown. However, it is assumed that the dividing cells responsible for nail formation also incorporate drug. During formation, drug may be incorporated continuously or as a single event. It has been reported that drugs gain rapid access to the distal nail plate during nail production by incorporation into cornified cells of the nail bed. Incorporation of drug by diffusion from the nail bed to the ventral portion of the nail plate is thought to be minimal (158-159). Studies have also shown that nail production and drug incorporation occurs in the lunular germinal matrix as the nail plate grows distally from the base of the nail (160-161).

Another potential mechanism of drug entry is through exposure to environmental contamination and biological fluids including sweat, sebum, saliva, and urine. In addition, processes previously identified in hair may also influence incorporation (95). These include the chemical nature of the drug analyte (e.g., lipophilicity and state of ionization), the metabolic profile of the drug, and the composition of the matrix.

The degree to which each of these proposed routes contributes to the overall drug content of nails is unknown.
Cocaine Analysis of Nails

In the present study, fingernail and toenail analysis greatly increased the detection of drug use. While conventional postmortem drug analysis of blood and urine identified recent cocaine use in 27.7% of the subjects, nail analysis detected cocaine exposure in 82.3% of the cases. In addition, positive test results were observed in 5 of 6 subjects known to authorities as cocaine users based upon conventional postmortem analysis, whereas all 6 subjects were positive by nail analysis. Moreover, the nails of these subjects contained minor cocaine analytes not seen in other subjects.

The concentration of cocaine analytes in fingernails was generally greater than toenails. In addition, cocaine analytes were not detected in the toenails of several subjects (no. 4, 8, 12, and 15) with positive fingernail analysis. Several factors may contribute to this finding including potential differences in the degree of environmental contamination, personal hygiene, rate of nail growth, and blood supply to the nail bed.

Cocaine and benzoylecgonine were the predominant cocaine analytes detected in nails, with cocaine present at a concentration approximately 2-10 times greater than benzoylecgonine. This is similar to the well-established cocaine-to-benzoylecgonine ratio observed in hair (162). However, the ratio is contrary to that previously observed by the FBI Toxicology Laboratory (22) which reported the presence of cocaine and benzoylecgonine in nails to be approximately in equal amounts.

The detection of anhydroecgonine methyl ester, a pyrolysis product of cocaine, in nails indicates exposure to “crack” cocaine (163). Furthermore, the presence of anhydroecgonine methyl ester in toenails suggests that the individual actively inhaled
“crack” smoke since toenails are much less likely to be externally contaminated than fingernails. While cocaine, benzoylecgonine, and anhydroecgonine methyl ester can be incorporated into the nail through environmental contamination, the presence of metabolic markers such as norcocaine and norbenzoylecgonine strongly support cocaine ingestion followed by metabolism and incorporation into nails.

Our study demonstrated that several cocaine analytes are less frequently detected in nails compared to cocaine and benzoylecgonine. Egonine methyl ester, ecgonine ethyl ester, and cocaethylene were detected in the nails of one subject at low to moderately low concentrations. Cocaethylene, a cocaine metabolite commonly observed following the coingestion of cocaine and ethanol, was not detected in the nails of the subjects with other reliable evidence indicating concomitant use of these drugs. For example, cocaethylene was surprisingly absent in the nails of subject no. 7 who was a known alcohol and cocaine user, and whose conventional postmortem toxicology tests indicated high concentrations of both ethanol and benzoylecgonine. Finally, m-hydroxybenzoylecgonine was the only cocaine analyte not found in the nails analyzed.

Cocaine analytes were present in the fingernail washes of subjects found to contain high quantities of cocaine analytes in their nails, but were found to a lesser extent in the corresponding toenail washes. Generally, the pattern of cocaine analytes in the nail washes was similar to that observed in the nail extracts. As expected, cocaine analytes that are present in the environment including cocaine, benzoylecgonine, and anhydroecgonine methyl ester, were the predominant analytes found in the washes. Although ecgonine ethyl ester and cocaethylene were detected in the nails, these analytes were not found in the corresponding washes.
Advantages and Disadvantages of Nail Analysis

The principal advantage to nail analysis may be its ability to provide a long-term measure of drug use representing several months to years. Conventional matrices, such as blood and urine, can only provide recent accounts of drug exposure. In addition, nails are a relatively noninvasive specimen to collect, probably even more so than hair. Although not evaluated in this study, we believe the likelihood for racial bias is minimal since melanin pigmentation within the nails is limited in comparison to hair, which has been attributed as a source of bias in hair (61). The concentration of cocaine analytes found in nails has proven to be high enough to readily detect drug use, even when small quantities (<25 mg) of nail are available for analysis.

Like hair, nail specimens are easy to store and the drug analytes are presumably stable within the nail matrix. The stability of drugs in nails makes their analysis a valuable tool for postmortem investigations. For example, in cases having decomposed remains, conventional toxicological analysis could potentially produce negative test results due to the instability of the drug in the body fluids, or alternatively, cannot be performed due to lack of body fluids.

Clearly, the most significant disadvantage of nail analysis is the limited work that has been conducted in this area, particularly with drugs of abuse. With nail analysis, recent drug use may not be readily detectable and the potential for environmental contamination leading to positive test results exists. If drugs are incorporated through the nail bed and along the entire nail following drug use, then nail analysis cannot determine time and magnitude of drug exposure through sectional analysis such as that previously
reported for hair analysis. Given the numerous factors that may affect nail growth such as age, sex, heredity, environment, and the difference in nail growth for individual digits, the interpretation of nail analysis is currently speculative (87, 93). In addition, the unknown mechanism of drug incorporation further complicates the interpretation of nail analysis data.

Conclusions

Nail analysis can successfully detect drug exposure in situations where conventional postmortem analysis has proved unsuccessful, corroborating prior drug-use history. The presence of several unique cocaine analytes in nails contributes additional information regarding the subject’s drug exposure. For example, the presence of unique cocaine analytes reveal information regarding concomitant cocaine and ethanol use (cocaethylene), route of cocaine administration (anhydroecgonine methyl ester), and the likelihood of drug ingestion and subsequent metabolism (norcocaine and norbenzoylecgonine).

The potential use of nail analysis in drug testing applications including workplace, clinical, forensic, epidemiological, and historical, necessitates further investigation. Currently, interpretation of nail analysis is challenging and should be considered semi-quantitative at best. Future study, including paired hair and nail analysis, may prove the utility of nail analysis as an alternative biological matrix for the testing of drugs of abuse.
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Abbreviations: gun shot wound (GSW), motor vehicle accident (MVA), multiple blunt traumatic injury (MBTI), multiple massive blunt traumatic injury (MMBTI), and blunt traumatic head injury (BTHI).

† Note: These specimens were obtained antemortem. A complete toxicological screen was not performed.
### TABLE 4-2. Results of Nail Analysis

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<td>13.1</td>
<td>ND</td>
<td>0.91</td>
</tr>
<tr>
<td>18</td>
<td>FN</td>
<td>TN</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

Abbreviations:

- FN = fingernail
- TN = toenail
- AEME = anhydroecgonine methyl ester
- BE = benzoylecgonine
- CE = cocaethylene
- COC = cocaine
- EME = ecgonine methyl ester
- NCO = norcocaine
- NBE = norbenzoylecgonine
- ND = none detected

† Note: Trace refers to a concentration less than the limit of detection. Concentrations have been weight-corrected based upon a sample weight of 25 mg.
### TABLE 4-3. Comparison of Postmortem Drug Analysis and Nail Analysis

<table>
<thead>
<tr>
<th>Subject Number</th>
<th>Conventional Postmortem Drug Analysis</th>
<th>Fingernail Analysis</th>
<th>Toenail Analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>-</td>
<td>+</td>
<td>NS*</td>
</tr>
<tr>
<td>2</td>
<td>-</td>
<td>+</td>
<td>NS</td>
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<tr>
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<td>+</td>
<td>+</td>
</tr>
<tr>
<td>18</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

**Total Positive (%)**  
5 (27.7) 14 (82.3) 8 (53.3)

Abbreviation: no specimen was available for analysis (NS).
CHAPTER 5
THE UTILIZATION OF HAIR ANALYSIS IN THE IDENTIFICATION OF DRUG INVOLVEMENT IN SIDS-RELATED DEATHS

Introduction

Sudden Infant Death Syndrome

Sudden infant death syndrome (SIDS) is the most common cause of death in infants between the age of 1 week and 1 year, accounting for approximately 40-50% of all deaths in this age group. The peak incidence of SIDS occurs 2 to 4 months post partum (164). National SIDS rates for the general population range from 1-2 per 1000 live births (1.3/1000 in U.S.) (165). SIDS is a “diagnosis of exclusion” whereby all other causes of death are ruled out.

The original definition for SIDS, adopted in 1969 at the Second International Conference on the Causes of Sudden Death in Infants, reads as follows, “the sudden death of any infant or young child, which is unexpected by history, and in which a thorough postmortem examination fails to demonstrate an adequate cause for death” (166). Since the original definition, clarifications have been proposed for clinical and research purposes. The following features have been suggested for an update of the SIDS definition:

1. Age: limit the age to 8-12 months;
2. Prone prevalence: temporally associated with sleep;
Thorough investigation: should include autopsy, examination of the death scene, and review of the clinical history (167);

In addition, categorization of SIDS is proposed by assigning cases into groups following descriptive standards; category I- for “classical” SIDS meeting clinical criteria after thorough investigation, category II- “apparent SIDS” which slightly deviates clinically from classical pathology, and category III- “presumed SIDS” appears to meet clinical criteria, however, all required studies are not performed (168).

However, the majority of researches agree that the definition should remain in its simplest form so that each SIDS case can be determined independently without unwarranted restraints. Others feel that without clarification, the broad SIDS definition can become a “catch-all” for unexplained deaths among infants as a combined consequence of its broad definition, varying knowledge of SIDS etiology among physicians, and inconsistent death scene investigation protocols.

SIDS is not a new phenomenon of this century. Transcribed references dating as far back as 500 BC indicate that sudden, unexpected infant death resulted from a mother or wetnurse “overlaying” upon the child (169). Subsequently, many other hypotheses including status thymico-lymphaticus, viremia, hypoparathyroidism, and hypersensitivity to cow’s milk have been proposed. These theories, like others, were eventually disproved.

Today, proposed causes of SIDS include spontaneous idiopathic apnea, progressive bradycardia, and central nervous system (CNS) abnormalities including damage to critical control centers in the brain and alterations of monoaminergic pathways (norepinephrine, dopamine and serotonin) (170-171). These hypotheses may, in fact, be
linked with the aberrant autonomic control being the primary mechanism and the first
two being subsequent sequela (172). The worldwide interest in this perplexing
phenomenon continues to grow exponentially. With continued interest in the scientific
and social communities, one or a number of hypotheses may be proven in the future.

The cardiorespiratory control hypothesis is the most comprehensive and accepted
theory as to the cause of SIDS. Figure 5-1 gives a flow diagram schematic of the
cardiorespiratory control hypothesis (173). This hypothesis purports a brainstem
developmental abnormality or maturational delay related to neurodevelopment of
regulatory centers for cardiorespiration, sleep/wake status, and circadian rhythm.
Dysregulation contributes to impaired arousal responsiveness characterized by prolonged
apnea and bradycardia, ultimately, resulting in SIDS (174). Components of this
hypothesis, such as chronic asphyxia and abnormal brainstem development, are supported
by directly related postmortem pathological findings. Over 66% of all SIDS cases show
presence of tissue markers for chronic asphyxia such as elevated cerebrospinal fluid
levels of hypoxanthine, a purine base intermediate in oxidative process of uric acid
synthesis (165). Another tissue marker associated with SIDS studies was reported
decreased levels of homovanillic acid, a dopamine metabolite associated with various
disease states such as hypotension (170). Brain stem abnormalities found in SIDS cases
include focal astrogliosis, persistent dendritic spines, hypomyelination, increased
medullary reactive astrocytes, and immature medullary catecholaminergic neurons (165,
175).

Many investigators believe that a deficit in arousal responsiveness is insufficient
in itself to cause SIDS and that the presence of other biologic and/or epidemiologic risk
factors is required (165). These risk factors act as "promoters" inducing the events ultimately resulting in an unexplainable infant death. Presently, scientific evidence has not fully explained how these risk factors interact with physiological homeostasis to increase the risk of death.

Based on combined clinical, pathological, and epidemiologic data, many risk factors for SIDS have been proposed. Table 5-1 lists an abbreviated, categorized list of potential SIDS risk factors (176-178). Most of these factors are confounding, high-risk variables associated with SIDS and cannot be easily delineated for research and diagnostic purposes. Presently, there is no proven strategy for intervention nor an accurate method for identification of SIDS infants. However, certain factors, such as alternatives to prone sleeping position and home monitoring of SIDS siblings and premature infants, represent risk markers that have been successfully adopted in intervention programs in several countries in efforts to reduce the frequency of SIDS (179).

In 1992, the American Academy of Pediatrics adopted a philosophy of supine or side-sleeping position and, by 1995, a reduction of SIDS incidence was reported in some populations (164). One case-control study showed a reduction in prone prevalence to be directly proportional to a reduction in SIDS rates, demonstrating a 50% or more decrease in SIDS incidence and a drop in SIDS rates to 0.4-0.5/1000 livebirths (176).

**Risk Factors And Adverse Effects Associated With Cocaine Use And Pregnancy**

About 50% of women who use illicit drugs are in the childbearing age group of 15 to 44 years of age and the prevalence of drug use among pregnant woman continues to grow. During 1992 to 1993, NIDA conducted a survey of hospitals in the United States
known as the National Pregnancy and Health Survey. This survey reported that 221,000 women used illegal drugs during their pregnancy and marijuana and cocaine were the most prevalent at 53% and 20%, respectively. This survey also found that illegal drug use was higher in unwed women who were less educated, unemployed, and were welfare recipients (4). In the past decade, it has been estimated through self-reports and toxicological screening of newborns that 10-45% of woman admitted to large inner city teaching hospitals used cocaine during their pregnancy (173-180).

The full implications of fetal exposure to drugs of abuse are unknown. Cocaine use during pregnancy has many deleterious, perinatal and neonatal effects on the mother and the fetus including premature labor, spontaneous abortion, abruptio placentae, stillbirth, decreased head circumference, low birth weight, neonatal cerebral infarction, cystic cortical lesions, and altered CNS maturation (102, 181-186). Sometimes infants of cocaine-abusing mothers experience transplacental myocardial depression manifested at birth as a lower cardiac output. This complication usually resolves within a day. Although infants exposed to cocaine \textit{in utero} can suffer devastating effects at parturition, the long-term morbidity may not be as high as first estimated (187).

Infants of cocaine-addicted mothers experience withdrawal after birth, however, the symptoms are less pronounced than opiate-addicted infants. Symptoms of neonatal cocaine withdrawal usually begin within 24 to 48 hours after birth (2). Common withdrawal signs include irritability, poor eye contact, vigorous sucking, and restlessness. The withdrawal lasts approximately 2-3 days.

The prolonged effects of cocaine exposed infants have not been investigated thoroughly. Reports indicate that cognitive, neurological, and behavioral effects can
result from prenatal cocaine exposure (170, 188-190). Animal studies have demonstrated a prolonged effect of cocaine on the developing rat brain resulting in neurochemical changes in the dopaminergic system and associated early- and late-onset behavioral abnormalities (170). A study of three groups of 2-year-olds, categorized as polydrug with cocaine use, polydrug without cocaine use, and no drug exposure, showed that both groups exposed to multiple drugs had a significantly lower mean developmental scores in comparison to unexposed infants (190). In a study of the same subjects at age three, Azuma et al. (189) observed both direct (i.e., and indirect effects (i.e., head size, perseverance at a task) of drug use on cognitive function. One prospective study investigating neurological effects in cocaine-exposed infants demonstrated that hypertonia of the lower and upper extremities was significantly higher at 6- and 12-months of age and resolved by 2 years when compared to unexposed infants (188). Finally anecdotal behavioral effects including attention deficit disorder, visual memory, attention span, and distractibility have been reported in the literature (170).

Many researchers believe that cocaine affects the respiratory and sleep arousal centers of the hind brain, and that immaturity of these centers may inevitably lead to sudden infant death (191-194). In fact, a preliminary animal study investigating the mechanistic effects of cocaine on the norepinephrine system at the level of the locus coeruleus, the area of the brain associated with arousal from sleep-related apnea, reported that cocaine did alter neurotransmitters important in respiratory control (195).

In addition to this theory, there are several pharmacological explanations as to why cocaine adversely affects the fetus and the newborn. Cocaine is highly lipophillic and readily crosses the placenta and blood-brain barrier. In adults, cocaine
concentrations in the brain and plasma have a 4:1 ratio, indicating cocaine’s ability to reach and adversely affect the CNS. The acute effects of cocaine’s effect on the adrenergic system include hypertension, tachycardia, and vasoconstriction (173). Vasoconstriction essentially occurs in all organ systems contributing to multi-system abnormalities. The increase in maternal spontaneous abortion, hemorrhage, and placental abruption can be attributed to increased systolic blood pressure, decreased uterine and placental perfusion, and increased uterine contractility associated with cocaine’s vasoconstrictive action (183). Moreover, plasma and liver cholinesterase activity is reduced in pregnant women, fetuses, and newborns, which may result in a decrease in drug clearance and potential toxicity (183, 186). Finally, the fetal human liver has a glucuronidation capacity approximately one-sixth that of an adult liver, which can further reduce drug elimination (196). Therefore, cocaine accumulates in the body leading to greater toxicity.

Background and Significance of Drug Testing

Many researchers have investigated the relationship between maternal drug use during pregnancy and subsequent SIDS. Currently, the mechanism(s) involved in SIDS and the role that drugs play in this mechanism remains undetermined.

In the late 1970s, several studies linking opiate use to SIDS in drug-abusing mothers were reported (197-199). Similarly, the emergence of “crack” cocaine use in the mid-1980s, led to several epidemiological studies that associated cocaine use with an increased risk of SIDS (200-203). However, risk ratios reported in other epidemiological studies showed no increase in cocaine-related SIDS deaths when compared to sociodemographically matched controls (204). Table 5-2 summarizes a survey of the
literature. In 1997, Fares et al. (205) completed a meta-analysis of 10 published studies on SIDS incidence and found an increased risk for SIDS in cocaine-exposed infants (5.8 per 1000) compared with drug-free infants (1.35 per 1000). However, when the incidence for SIDS in cocaine-exposed infants was compared to polydrug users who used illicit substances other than cocaine (i.e., methadone, heroin, and alcohol there was no increased risk. The results of the later subset analysis suggest that confounding variables associated with a drug user’s lifestyle contribute to an increased incidence of SIDS and may have independent effects that cannot be easily differentiated.

Most of these retrospective epidemiological studies included limited toxicological analysis, instead depending primarily on unreliable self-reports. Toxicological analyses was usually performed on blood and/or urine obtained from the mother or child. These screening methods had many deficiencies that may have contributed to a high false negative rate. Examples of how these methods were insensitive and inaccurate measures of drug exposure include the following:

1) Conventional methods of drug detection are inadequate. Urinalysis, the most common technique, will reveal recent and/or heavy drug use only. Recent abstinence will result in negative maternal and neonatal urine test results.

2) Parental self-reports are inaccurate. Reluctance of parents to disclose their drug use stems from fear of the potential consequences including loss of child custody.

3) Lack of comparison of subjects with accurate controls, considering age, race, parity, socioeconomic status, duration and frequency of drug administration, and polydrug use.
Some or all of the above points may account for the discrepancies found in the literature to date. Unfortunately, finding a sample population large enough that eliminates all these limitations would be extremely difficult, if not impossible. For instance, the confounding variables of a drug user’s lifestyle and low socioeconomic status would be difficult to separate from one another.

It is certain that not all SIDS cases are linked to drug use. Moreover, the extent to which SIDS can be attributed directly to drug use or to confounding variables associated with a drug-user's lifestyle such as lack of prenatal care and other socioeconomic factors has not been determined. Although epidemiological data seems to suggest an association, sufficient and direct analytical links between sudden infant death syndrome and exposure to an illicit drug are lacking. If it is unequivocally proven with continued research that drug use contributes significantly to the incidence of SIDS, then similar intervention programs including postnatal care, supportive counseling, parenting skills, and public health nurse visits could be implemented (206).

Hair Analysis as a Measure of Gestational and Prenatal Cocaine Exposure

Historically, gestational cocaine exposure is routinely identified through maternal self-report, and analysis of maternal and infant urine. Diagnosing intrauterine cocaine exposure is paramount in explaining perinatal/neonatal complications and in identifying cocaine-addicted mothers who need drug counseling and assistance in caring for their infant.

To improve the sensitivity of detection of cocaine-exposed infants, many researchers and clinicians have began analyzing meconium and amniotic fluid (26-27, 29-
Meconium and amniotic fluid may increase the detection period of drug exposure but the timing of collection is critical.

Meconium represents the intestinal content of the fetus from the 12th week of gestation until birth. Proposed mechanisms of drug incorporation include: 1) infant swallowing of amniotic fluid containing drug analytes excreted in the urine which becomes a portion of the amniotic fluid; and 2) secretion of drug-containing bile that is deposited into the intestines (210). Meconium can only be collected from the neonate during the first 2-3 days of life and the concentration of drug significantly diminishes with subsequent stool specimens (26). Therefore, the sensitivity of meconium analysis varies depending on whether the first stool, second stool, or subsequent stools are collected for analysis. Other studies comparing meconium and urinalysis show the sensitivity of these matrices to be equivalent or meconium slightly better when the same analytical method was employed (211-213). Problems associated with meconium collection include non-homogeneity of the specimen, lack of control and proficiency specimens, and inability to collect specimen prior to hospital discharge (209).

The concentration of drug in amniotic fluid directly affects the degree to which the fetus is exposed to drug. The fetus is bathed in amniotic fluid resulting in transdermal diffusion of drug into the fetus. After keratinization of the skin at about six months gestation, transdermal transfer is reduced (30). Also, the fetus swallows amniotic fluid at a rate of 0.5 liters per day leading to direct ingestion of the drug (180). Cocaine and its metabolites have been detected in amniotic fluid by several research groups (29-30, 210, 214). Ripple et al. (29) detected higher concentrations of cocaine analyte in the amniotic fluid of 5 subjects (COC range: trace to 24 ng/mL; BE range: 51 to 836 ng/mL) in
comparison to maternal serum (COC range: none detected; BE range: 0 to 205 ng/mL).
These results are consistent with the hydrophilic nature benzoylecggonine and its
increased ability to partition into polar compartments such as urine.

Maternal and/or infant hair analysis provides another alternate source to
determine gestational drug exposure (215-220). The theoretical mechanism of
incorporation of cocaine analytes into fetal hair is two-fold — internal incorporation
through placental circulation and external incorporation through exposure to amniotic
fluid. Prenatal hair develops during the third trimester of pregnancy. The fine hair first
generated is referred to as lanugo. Replacement with a more coarse hair begins around
the eighth month of pregnancy and continues six months post partum (46). The presence
of cocaine analytes in hair obtained immediately following parturition indicates in utero
exposure. But as time elapses following parturition, it becomes more difficult to
differentiate gestational and postnatal drug exposure. After the first three to four months
of life, hair analysis can no longer determine in utero cocaine exposure due to the
common loss of neonatal hair (180).

A number of studies utilizing maternal and infant hair have been conducted to
assess in utero cocaine exposure. Iwersen et al. (196) recently reported an autopsy case
of a heroin-related death of a woman in her 32\textsuperscript{nd} week of gestation, compared hair
analysis with other tissues and fluids analyzed from the mother and fetus. This report
showed that the fetal opiate concentration in all tissues and fluids, including hair, were
less than the maternal concentrations, however, toxic concentrations were found in both.
Segmental analysis also revealed that the opiate concentrations within the maternal and
fetal hair increased during pregnancy and suggested a dose-dependent transplacental
diffusion for opiates. Several investigators have reported that hair analysis is a more sensitive technique for detecting gestational and neonatal drug exposure.

Callahan and colleagues (221) demonstrated that hair analysis by using RIA was more sensitive ($p<0.02$) than analysis of meconium (FPIA, GC/MS) and urine (EMIT) in detecting gestational cocaine exposure. The sensitivity of the tests was 78%, 74%, 52%, and 38%, respectively. However, this study did not demonstrate completely the sensitivity of hair analysis since different analytical techniques were employed for the different matrices. Another study employing RIA to compare hair and urine of drug users demonstrated a 55% positivity rate in the hair specimens while only 4.3% of the urine specimens tested positive, a greater than 10-fold increase utilizing hair analysis (38). Ursitti et al. (180) conducted a four-year study on neonatal hair of infants who were clinically suspected to have drug exposure. In 182 cases that were negative by urinalysis and maternal self report, 55 (30%) of the neonatal hair specimens tested positive demonstrating a statistically significant increase ($p<0.001$) in cocaine exposure when compared to a 5.5% positivity rate found in a previous population-based study performed by the same researchers. This study also found that 8 of 13 (67%) mothers suspected of drug use tested positive by hair analysis. Furthermore, all four of the collected mother-infant paired hair specimens were in agreement for mother and infant (3 pairs positive in both maternal and neonatal hair and 1 pair negative in both) (180).

As seen in the studies discussed above, sampling times relative to drug exposure for both meconium and urinalysis are critical in comparison to hair. Maternal abstinence to drug use a few weeks prior to analysis can result in a false negative by analysis of both matrices, falsely classifying drug exposed babies into a control group. Given these
reports, hair analysis may be a more sensitive analytical technique for determining drug exposure in infants, particularly those who die of SIDS.

The objective of this study was to develop a sensitive analytical technique utilizing hair analysis by gas chromatographic/mass spectrometric methods to improve the detectability of cocaine exposure in infant deaths, either SIDS or other assigned causes of death (i.e., controls). The results of hair analysis were compared to postmortem toxicological methods to determine the reliability and sensitivity of hair analysis. With improved analytical techniques, a better interpretation of the true incidence of drug exposed infants and the frequency of SIDS-related deaths can be more appropriately determined.

Materials and Methods

Reagents and Materials

All organic solvents were HPLC grade, and chemicals were reagent grade. Anhydroecgonine methyl ester, benzoylecgonine, d3-benzoylecgonine, cocaine, d3-cocaine, cocaethylene, d3-cocaethylene, ecgonine ethyl ester, ecgonine methyl ester, d3-ecgonine methyl ester, m-hydroxybenzoylecgonine, norbenzoylecgonine, and norcocaine were purchased from Radian International LLC (Austin, TX). N,O-Bis(trimethylsilyl)trifluoroacetamide (BSTFA) with 1% trimethylchlorosilane (TMCS) was obtained from Pierce Company (Rockford, IL).

Phosphate buffer (25 mM; pH 6.0 ± 0.1) was prepared from a mixture of potassium phosphate monobasic and deionized water. Acetate buffer (100 mM; pH 4.0 ±
0.1) was prepared from a mixture of glacial acetic acid, sodium acetate, and deionized water. The solid-phase extraction (SPE) elution solvent, methylene chloride–2-propanol–ammonium hydroxide (80:20:2, v/v/v) was prepared daily.

Subjects, Specimen Collection, and Data Handling

Hair specimens were obtained by the University of Maryland Brain and Tissue Banks for Developmental Disorders through a collaborative relationship with the Office of the Chief Medical Examiner (OCME) in Baltimore, Maryland. All specimens were obtained with informed consent and submitted to our laboratory without identifiers as to the donor and cause of death. Once the Brain and Tissue Bank received the hair analysis results, epidemiological, pathological and conventional toxicology data were transmitted to our laboratory for comparison.

The procurement staff collected approximately 25-50 strands of hair from the crown area (posterior vertex) of the head by cutting as close to the scalp as possible. Maintaining orientation of the hair strands, the hair was placed into a secured container (i.e., aluminum foil and envelope or paper towel and test tube). All samples were labeled with a unique subject number. To maintain the integrity of the hair, the hair was stored at room temperature prior to analysis.

Blinded “analytical” control samples obtained from drug-free individuals and known drug users were also submitted to the laboratory for analysis with the intent to challenge the laboratory’s analytical capabilities.
Specimen Preparation

Hair samples were first decontaminated by a 20-second, methanolic wash in which samples were vortexed. The methanolic wash was decanted and collected for further analysis. Specimens were dried at 40°C under a nitrogen stream (Zymark Turbovap® LV). Specimens were weighed (~ 25 mg) into polyethylene vials, 5 to 6 glass beads (0.5 mm) were added, and the specimens were pulverized into a fine powder by a Mini-beadbeater-8™ Cell Disrupter (Biospec Products, Bartlesville, OK). The pulverized hair was transferred to disposable culture tubes and the polyethylene vials were rinsed 8 to 10 times with a small aliquot of methanol (ca. 0.5 mL) to remove all residual hair. The methanolic rinses were collected into the respective tube containing the powdered hair.

A 3.0 mL volume of methanol and 125 ng of trideuterated internal standard [d3-ecgonine methyl ester (d3-EME), d3-cocaine (d3-COC), d3-cocaethylene (d3-CE), and d3-benzoylcegonine (d3-BE)] were added to each specimen tube. Specimens were capped, vortexed, and heated for approximately 16 hours at 40°C in a heated manifold. The specimens were vortexed during the last half of the incubation period. After completion of the incubation, specimens were vortexed and centrifuged at 3000 rpm for 10 min. The supernatant was collected and dried at 40°C under a nitrogen stream.

Calibration curves were prepared using working calibrator solutions for all cocaine analytes added to drug-free control hair obtained from laboratory personnel in a concentration range of 0.10-10.0 ng/mg (a minimum of 5 concentration levels). To evaluate background interference, instrument stability, and analyte degradation, appropriate controls were included for analysis. Negative controls and positive controls (0.5 ng/mg and 5 ng/mg) were analyzed after every 15 specimens. Cocaine controls (10
ng/mg) were analyzed to measure spontaneous hydrolysis of cocaine to benzoylecgonine during specimen preparation and introduction to the GC/MS.

A modified method of Cone et al. (11) was employed for the solid-phase extraction of cocaine analytes from hair and subsequent analysis by GC/MS. The salient features are summarized below.

After adding the internal standard to the decontamination washes, both the hair extracts and wash residues were reconstituted in phosphate buffer (3 mL) prior to further extraction. The SPE cartridges (Clean Screen®, ZSDAU020, United Chemical Technologies, Horsham, PA) were conditioned sequentially with elution solvent (1 mL), methanol (3 mL), deionized water (3 mL), and phosphate buffer (2 mL). Vortexed samples were loaded onto the cartridges, and the cartridges were washed sequentially with deionized water (2 mL), acetate buffer (2 mL), methanol (3 mL), and acetonitrile (1 mL) before aspirating to dryness. Analytes were recovered from the columns with elution solvent (4 mL), and the eluents were evaporated to dryness at 40°C under a stream of nitrogen. The extracts were derivatized with 30 μL of BSTFA with 1% TMCS at 65°C for 30 min and transferred to autosampler vials for GC/MS analysis.

**GC/MS Analysis**

Analyses were performed with a Hewlett-Packard 5890A Series II gas chromatograph and 7673B automatic liquid sampler interfaced with a Hewlett-Packard 5972A Mass Selective Detector (MSD, Hewlett-Packard Company, Little Falls, DE). The gas chromatograph was equipped with an HP-5MS crosslinked 5% diphenyl, 95% dimethylpolysiloxane fused-silica capillary column (30 m x 0.25 mm i.d. x 0.25 μm film
thickness, Hewlett-Packard Company). Automated injections (1 μL) were made in the splitless mode, and a 2-mm i.d. silanized borosilicate liner was utilized.

The MSD was operated in the selected ion-monitoring mode utilizing a 20 ms dwell time. Table 5-3 summarizes the ions monitored for each analyte. Analytes were identified based upon comparison of retention time and ion ratio with the corresponding values of calibrators assayed in the same run. Ion ratios were calculated by dividing the ion peak-area of the confirming ion by the ion peak-area of the quantitative ion. Ion ratios were compared to the ion ratio for a mid-range calibrator with acceptance based on ±20% criteria. Quantification of analytes was based upon the ratios of the integrated ion peak-areas to the corresponding trideuterated standard analogues.

Statistics

The Fisher’s Exact Test was used for statistical comparison of cocaine concentrations in hair of SIDS and control infants. The appropriateness of this test was based on the study’s small sample size (N=26) and lack of five subjects in each category which is required for Chi-Square analysis. A two-sided comparison was employed. The test evaluated the null hypothesis of no significant difference in the incidence of SIDS between cocaine-exposed and unexposed infants at a chosen 95% confidence interval (α=0.05).
Results

The limit of detection (LOD) was determined to be 0.1 ng/mg for all analytes (S/N>3.0), except EEE, which had a slightly higher LOD of 0.3 ng/mg. Most of the analytes had a limit of quantitation (LOQ) of 0.2 ng/mg, whereas the LOQs for EEE, NBE, and MOHBE were 2-2.5 times higher (S/N>3.0, observed value ± 20% of expected value). Most analytes had a limit of linearity greater than 20 ng/mg.

Forty-seven subjects were analyzed for this preliminary study. Originally, 29 infant specimens were submitted for analysis but three infants did not meet the age-match requirements and were subsequently moved into the analytical control group. Twenty-six infants (17 SIDS and 9 age-matched controls) ranging in age from 1 day to 14 months were included in the study. The epidemiology, cause of death, and conventional postmortem data for each subject is reported in Table 5-4. In addition, 21 blinded analytical control specimens were submitted for analysis and these subjects ranged in age from 3 to 55 years.

GC/MS analysis revealed the presence of cocaine and its analytes in 11 out of 26 infants (Figure 5-2). The results of the hair analysis, summarized in Table 5-4, show that 10 out of 11 specimens testing positive for cocaine were obtained from infants whose death were attributed to SIDS. Analyte ranges for the cocaine positive cases (mean ± SD) for each analyte were: COC 5.87 ± 10.08 ng/mg (n=11); BE 0.47 ± 0.60 ng/mg (n=11); NCOC 0.22 ± 0.26 ng/mg (n=6); and EME 0.10 ± 0.0 ng/mg (n=3). Trace amounts of CE, an active metabolite of cocaine and ethanol, was observed in one infant
(SIDS No. 584). The incidence of cocaine positive SIDS cases was 58.8% while cocaine positive control-infant was 11.1%. Table 5-5 compares conventional postmortem results with hair results for SIDS and control infants.

Generally, a cocaine-to-benzoylecgonine ratio ranging from 5 to 10:1 was observed in the infants, this compared favorably to ratios previously reported for adult populations. As depicted Ugher in most subjects. Subject 1002 was the only cases in which COC and BE were both at equivalent concentrations approaching the limit of quantitation.

All washes from infants were negative for cocaine analytes. However, 5 of the decontamination washes of analytical controls were positive at low concentrations (<10% of determined concentration for respective hair) for COC and/or BE.

Conventional postmortem toxicological tests were performed by the Toxicology Laboratory at the Office of the Chief Medical Examiner (OCME) using a combination of techniques including immunoassay, gas chromatography, and gas chromatography/mass spectrometry. Matrices analyzed included blood, urine, liver, bile, and vitreous humor. Results for all infants were negative for cocaine analytes. In several infants, drug analysis revealed the presence of therapeutic drugs such as trimethoprim, atropine, ketamine, dextromethorphan, and phenylpropanolamine. All therapeutic drugs were consistent with resuscitative measures or infant’s history.

Analysis of hair revealed the presence of cocaine analytes in 8 out of 21 of the analytical controls. Results for analytical controls are summarized in Table 5-6. In most cases, hair analysis corroborated subject history and toxicological results performed by the OCME. In addition, hair analysis identified cocaine analytes in four cases in which
conventional postmortem analysis failed to detect cocaine concentrations. The cocaine-to-benzoylcegonine ratio for analytical controls ranged from 2 to 10:1. Cocaine analyte concentrations ranged from trace amounts to the following maximum concentrations: COC- 25.8 ng/mg (n=8); BE- 16.1 ng/mg (n=6); NCOC- 1.15 ng/mg (n=6); EME- 1.65 ng/mg (n=3); and NBE-1.82 ng/mg (n=2). In addition, one subject (No. 1023) had trace levels of AEME, a crack pyrolysis product. Another subject (No. 645) had 1.92 ng/mg CE present, which was consistent this subject’s cause of death (cocaine intoxication) and postmortem toxicological results (positive cocaine and ethanol results).

Discussion

The diagnosis of SIDS is made following the exclusion of all other known causes. Currently, no evidence exists that indicates that the mechanism of SIDS is different in infants with or without indications of past exposure to cocaine. Neither is there evidence that exposure to cocaine in SIDS cases contributes directly to infant mortality.

Due to the complex nature of hair analysis and the number of variables which effect drug disposition, both internally and externally, distinguishing gestational exposure from postnatal exposure by hair analysis is, at best, an extrapolation of the data and infant history to obtain an estimation of exposure time. The only way in utero exposure can be determined unequivocally is to collect the hair immediately following parturition. Positive hair analysis findings may indicate: internal incorporation during the gestational period; exposure to cocaine analytes in amniotic fluid; active ingestion of cocaine
through breast-feeding; exposure to contaminated surfaces; and/or environmental contamination.

The ability of conventional postmortem analysis to detect cocaine exposure in infants is limited. Unless there is chronic and/or acute ingestion, detection of cocaine in blood and other postmortem tissues, such as the liver, is uncommon. Many investigators recognize the sensitivity of hair analysis in determining drug exposure (18, 37-38, 43-44, 222). As such, hair analysis can be utilized as a sensitive complementary technique to conventional analyses, including immunoassays and GC. The sensitivity of hair analysis makes it a useful tool to identify drug-exposed infants during postmortem investigation. Currently, most hair analysis studies focus on COC and BE as analytes for determining cocaine exposure. By including additional cocaine analytes such as AEME, NCOC, EEE, CE, NBE, and MOHBE, a more comprehensive evaluation of cocaine exposure can be determined. For example, active metabolism of cocaine through disposition of liver-metabolized analytes (i.e., norcocaine and cocaethylene) into the hair can serve as a postmortem indicator of drug exposure prior to infant death. The presence of these hepatotoxic metabolites can be even more detrimental to the fetus, since the fetus already has limited hepatic function.

Since little is known about drug biotransformation and disposition into body fluids and tissues in infants, this study attempted to evaluate if differences such as cocaine-to-benzoylecgonine ratios, concentrations of active metabolites, and body compartmentalization exist for infants when compared to adult profiles. In each case, the infant profiles for hair analysis seemed consistent with adult data. No conclusions could be reached concerning toxicity and adverse effects since conventional postmortem
analysis did not detect the presence of cocaine analytes; toxic concentrations of cocaine analytes at the time of death did not exist.

While cocaine and its major metabolite, benzoylecgonine were the predominant analytes present in hair, other analytes present in infant specimens included EME, NCOC, CE, and NBE. AEME and MOHBE were not detected in any of the infant hair specimens. In most instances, minor metabolites (NCOC, NBE, EME) were present at trace amounts, however, in cases that could be quantified, concentrations were 5 to 30 times lower. These findings are also consistent with data obtained from adults studies (17, 60, 97, 136, 138).

The presence of cocaine and its analytes in hair, and not blood and urine, suggests that the infants were not exposed to cocaine immediately prior to death. Since the majority of the hair specimens were obtained from infants less than 3 months old, the data further suggests in utero drug exposure. An alternate theory is that the cocaine analytes were incorporated into the hair postnatally through parental administration of drugs and subsequent consumption of breast milk by the infant and/or accidental environmental exposure, and/or parental administration of drugs. Our results could not delineate which route(s) of drug exposure was the predominate route.

The incidence of cocaine exposure was high in the SIDS infants (10 out of 17) when compared to control infants (1 out of 9). Hair analysis of this small-based population of infants demonstrated that the relative risk of SIDS infants being exposed to cocaine was 4.7 times higher than the age-matched controls (Table 5-2). However, statistical analysis utilizing the Fisher’s Exact Test demonstrated that the incidence of
cocaine-exposed SIDS cases was significantly higher than age-matched controls (p<0.024).

In this study, the cocaine-to-benzoylecgonine ratio approximated 5-10:1 in most cases (5 of 8); this agrees with ratios for adult populations (51, 140). In the remaining positive cases, an equal ratio was observed at concentrations close to the detection limit. This may be attributed to systematic error that is associated with concentrations at or near detection limits. Alternatively, Ursitti et al. (180) suggested a much lower cocaine-to-benzoylecgonine ratio in newborns in comparison to adults may be attributed to lack of medullated hair in newborns. Data suggest that BE partitions into nonmedullated hair regions while cocaine is located deeper within the hair matrix. Hence, benzoylecgonine would preferentially incorporate into nonmedullated hair shaft of neonates and cocaine would be less likely to incorporate in neonatal hair.

Decontamination washes were performed and analyzed to remove unnecessary interferents (i.e., lipids, soaps, and dirt) and to assist in determining what type(s) of exposure the infant experienced. Analysis of methanolic decontamination washes demonstrated an absence of cocaine analytes was detected in the washes of infant subjects. These results suggest that the contribution due to environmental drug exposure was minimal or nonexistent. Regardless, in this study external contamination was not considered an issue since it also contributes to the infant’s exposure to drugs and subsequent adverse effects and potential increased risk to SIDS. Low concentrations of cocaine and benzoylecgonine were identified in a few decontamination washes of the analytical control cases in which high concentrations of cocaine were usually found in the
hair extract itself. Moreover, review of the history for these cases revealed that all died of either drug intoxication or homicide.

There were several limitations to this study. The small sample size analyzed within this preliminary study is limited and may not accurately reflect the true incidence of cocaine exposure in the total SIDS population. The incidence of confounding variables was unknown to us. We did not receive information pertaining to other risk factors for SIDS such as the socioeconomic background, duration and frequency of drug administration by the parents, polydrug use, and smoking habits. The National Pregnancy and Health Survey estimated that nearly one-third of pregnant woman reporting illegal drug use also smoked cigarettes and drank alcohol, which may also contribute to the results of this study (4). Moreover, the occurrence of two other risk factors – black ethnicity (57.6%) and male gender (73%) – were unusually high within our study population. The relative risk of SIDS in black infants is between 1.7 and 5.2 when compared to white infants (165). However, the rate of illegal substance abuse is higher in black women when compared to white women. It is uncertain to what degree these risk factors may have affected the incidence of SIDS among study infants, but these risk factors were also proportionately high in the SIDS cases (black-72% and male-90%).

Epidemiological risk factor studies, both retrospective and prospective, have continued to show contradictory data regarding the incidence of SIDS and its association with infant drug exposure (Table 5-1). However, the current data suggest that an animal study in which the fetus is exposed to cocaine could provide insights into SIDS. If distinct characteristics are associated with cocaine exposure in animal models, and the same characteristics are subsequently observed in human infants exposed to cocaine, then
an isolated subpopulation of infants diagnosed with SIDS could be identified. Improved detection by more reliable techniques such as hair analysis could be utilized to better identify these populations.

Conclusions

In conclusion, hair analysis is a valuable analytical technique that complements conventional techniques in determining drug exposure. As such, hair analysis can be utilized as a sensitive technique to identify an infant’s exposure to cocaine through various routes. Thus far, this preliminary study demonstrates a significant positive trend for the association of cocaine exposure with an increased risk of SIDS.

It should be stressed that this study evaluated a small sample population and, therefore, does not represent the total SIDS population. This study is currently being expanded to include a larger number of infants from different geographical locations.
Regulation of sleep/wake status
Brainstem Dysfunction/Immaturity
Regulation of cardiorespiration
thermoregulation
arousal response
Regulation of circadian rhythm
Prolonged apnea and Bradycardia
SIDS

BIOLOGICAL RISKS

Epidemiological Risks

Figure 5-1. Cardiorespiratory Control Hypothesis for SIDS (173)
Table 5-1. Potential Risk Factors Contributing to the Incidence of SIDS

**Biological and Clinical Factors:**

<table>
<thead>
<tr>
<th>Risk Factor</th>
<th>Risk Factor</th>
</tr>
</thead>
<tbody>
<tr>
<td>genetic disorders</td>
<td>intrauterine hypoxia</td>
</tr>
<tr>
<td>apnea</td>
<td>fetal growth retardation</td>
</tr>
<tr>
<td>hyperthermia</td>
<td>maternal anemia</td>
</tr>
<tr>
<td>prematurity</td>
<td>nutritional deficiency</td>
</tr>
<tr>
<td>family history of SIDS</td>
<td>low birth weight</td>
</tr>
<tr>
<td>age</td>
<td>maternal urinary tract infection</td>
</tr>
<tr>
<td>gender</td>
<td>recent (febrile) illness</td>
</tr>
<tr>
<td>ethnicity</td>
<td>upper respiratory tract infection</td>
</tr>
<tr>
<td>thermal stress</td>
<td>deficient brain stem control</td>
</tr>
</tbody>
</table>

**Epidemiological and Environmental Risk Factors:**

<table>
<thead>
<tr>
<th>Risk Factor</th>
<th>Risk Factor</th>
</tr>
</thead>
<tbody>
<tr>
<td>lack of prenatal care</td>
<td>soft sleeping surface</td>
</tr>
<tr>
<td>prone sleeping position</td>
<td>low socioeconomic status</td>
</tr>
<tr>
<td>geographical</td>
<td>cigarette smoking</td>
</tr>
<tr>
<td>cold climate</td>
<td>illicit drug exposure</td>
</tr>
<tr>
<td>change in climate</td>
<td></td>
</tr>
<tr>
<td>INVESTIGATOR (YEAR)</td>
<td>NUMBER OF CASES (SID'S INCIDENCE)</td>
</tr>
<tr>
<td>---------------------</td>
<td>----------------------------------</td>
</tr>
<tr>
<td>Bachman et al. (1990)</td>
<td>E=23 (0)</td>
</tr>
<tr>
<td>Bauchner et al. (1988)</td>
<td>E=175 (1)</td>
</tr>
<tr>
<td>Chasnoff et al. (1987)</td>
<td>E=66 (10)</td>
</tr>
<tr>
<td>Chasnoff et al. (1989)</td>
<td>E=32 (0)</td>
</tr>
<tr>
<td>Durand et al. (1990)</td>
<td>E=966 (9)</td>
</tr>
<tr>
<td>Fulroth et al. (1989)</td>
<td>E=368 (0)</td>
</tr>
<tr>
<td>Kandall et al. (1993)</td>
<td>E=8868 (41)</td>
</tr>
<tr>
<td>Ostrea et al. (1997)</td>
<td>E=903 (5)</td>
</tr>
<tr>
<td>Rajegowda et al. (1978)</td>
<td>E=383 (8)</td>
</tr>
<tr>
<td>Rosen et al. (1988)</td>
<td>E=29 (2)</td>
</tr>
<tr>
<td>Silvestri et al. (1991)</td>
<td>E=61 (2)</td>
</tr>
<tr>
<td>Ward et al. (1990)</td>
<td>E=1674 (14)</td>
</tr>
<tr>
<td>Fares et al. (1997)</td>
<td>E=11,587 (68)</td>
</tr>
<tr>
<td>Fares et al. (1997)</td>
<td>E=365 (12)</td>
</tr>
</tbody>
</table>
Table 5-2—continued

<table>
<thead>
<tr>
<th>INVESTIGATOR</th>
<th>NUMBER OF CASES (SIDS INCIDENCE)</th>
<th>RELATIVE RISK †</th>
<th>DRUG(S) INVESTIGATED*</th>
<th>RISK ASSESSMENT</th>
</tr>
</thead>
<tbody>
<tr>
<td>Current study (1998)</td>
<td>E= 17 (10) NE = 9 (1)</td>
<td>4.7</td>
<td>cocaine</td>
<td>increased risk</td>
</tr>
</tbody>
</table>

Abbreviations: cocaine exposed infants (E), comparison group of infants not exposed to cocaine (NE), infants of methadone-maintained mothers (NARC), and polydrug exposure (P).

* Laboratory-dependent panel of illicit drugs: heroin, methadone, barbiturates, cocaine, benzodiazepines, alcohol, marijuana, and phencyclidine.

† In studies having no comparison group or no SIDS incidence rate for the comparison group, the U.S. incidence rate of 1.3/1000 was used to calculate the relative risk.

‡ These studies were included in the Fares (1997) study.
### Table 5-3 Cocaine Analytes and Ions Monitored for GC/MS Analysis

<table>
<thead>
<tr>
<th>Analyte†</th>
<th>Ions (m/z)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anhydroecgonine methyl ester</td>
<td>152, 166, 181</td>
</tr>
<tr>
<td>d&lt;sub&gt;3&lt;/sub&gt;-Ecgonine methyl ester</td>
<td>99, 85, 274</td>
</tr>
<tr>
<td>Ecgonine methyl ester</td>
<td>96, 82, 271</td>
</tr>
<tr>
<td>Ecgonine ethyl ester</td>
<td>96, 83, 285</td>
</tr>
<tr>
<td>d&lt;sub&gt;3&lt;/sub&gt;-Cocaine</td>
<td>185, 306, 275</td>
</tr>
<tr>
<td>Cocaine</td>
<td>182, 303, 272</td>
</tr>
<tr>
<td>d&lt;sub&gt;3&lt;/sub&gt;-Cocaethylene</td>
<td>199, 85, 320</td>
</tr>
<tr>
<td>Cocaethylene</td>
<td>196, 82, 317</td>
</tr>
<tr>
<td>d&lt;sub&gt;3&lt;/sub&gt;-Benzoylecgonine</td>
<td>243, 364, 259</td>
</tr>
<tr>
<td>Benzoylecgonine</td>
<td>240, 361, 256</td>
</tr>
<tr>
<td>Norcocaine</td>
<td>140, 240, 346</td>
</tr>
<tr>
<td>Norbenzoylecgonine</td>
<td>404, 140, 298</td>
</tr>
<tr>
<td><em>m</em>-Hydroxybenzoylecgonine</td>
<td>240, 82, 210, 449</td>
</tr>
</tbody>
</table>

† d<sub>3</sub>-Analogues are internal standards used for quantitation.

* Quantitating ions appear first and in Italics.
<table>
<thead>
<tr>
<th>Sample Number</th>
<th>Case Type</th>
<th>Hair Results (ng/mg)</th>
<th>Age</th>
<th>Race</th>
<th>Gender</th>
<th>Cause of Death</th>
<th>PM Toxicology Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>461</td>
<td>Control</td>
<td>negative</td>
<td>1.1 y</td>
<td>C</td>
<td>F</td>
<td>Asphyxia</td>
<td>trimethoprim</td>
</tr>
<tr>
<td>469</td>
<td>SIDS</td>
<td>negative</td>
<td>3 m</td>
<td>C</td>
<td>M</td>
<td>SIDS</td>
<td>negative</td>
</tr>
<tr>
<td>524</td>
<td>Control</td>
<td>COC 1.12</td>
<td>4 m</td>
<td>B</td>
<td>M</td>
<td>Myocarditis</td>
<td>negative</td>
</tr>
<tr>
<td>528</td>
<td>SIDS</td>
<td>negative</td>
<td>3 m</td>
<td>C</td>
<td>M</td>
<td>SIDS</td>
<td>negative</td>
</tr>
<tr>
<td>536</td>
<td>Control</td>
<td>negative</td>
<td>8 m</td>
<td>C</td>
<td>F</td>
<td>Cardiomyopathy</td>
<td>atropine</td>
</tr>
<tr>
<td>540</td>
<td>SIDS</td>
<td>negative</td>
<td>2.5 m</td>
<td>B</td>
<td>F</td>
<td>SIDS</td>
<td>negative</td>
</tr>
<tr>
<td>543</td>
<td>SIDS</td>
<td>negative</td>
<td>4.5 m</td>
<td>B</td>
<td>F</td>
<td>SIDS</td>
<td>negative</td>
</tr>
<tr>
<td>572</td>
<td>SIDS</td>
<td>negative</td>
<td>3 m</td>
<td>C</td>
<td>M</td>
<td>SIDS</td>
<td>negative</td>
</tr>
<tr>
<td>583</td>
<td>SIDS</td>
<td>negative</td>
<td>2.5 m</td>
<td>B</td>
<td>M</td>
<td>SIDS</td>
<td>dextromethorphan</td>
</tr>
<tr>
<td>584</td>
<td>SIDS</td>
<td>COC 6.82</td>
<td>5 m, 10 d</td>
<td>B</td>
<td>M</td>
<td>SIDS</td>
<td>phenylpropanolamine</td>
</tr>
<tr>
<td>678</td>
<td>SIDS</td>
<td>negative</td>
<td>3 m</td>
<td>C</td>
<td>M</td>
<td>SIDS</td>
<td>negative</td>
</tr>
<tr>
<td>710</td>
<td>SIDS</td>
<td>COC 44.8</td>
<td>2 m</td>
<td>C</td>
<td>M</td>
<td>SIDS</td>
<td>negative</td>
</tr>
<tr>
<td>Case</td>
<td>Diagnosis</td>
<td>BE</td>
<td>COC</td>
<td>Age</td>
<td>Gender</td>
<td>Cause of Death</td>
<td>Additional Information</td>
</tr>
<tr>
<td>------</td>
<td>-----------</td>
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<td>-----</td>
<td>--------</td>
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</tr>
<tr>
<td>716</td>
<td>SIDS</td>
<td></td>
<td>0.39</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>718</td>
<td>Control</td>
<td>negative</td>
<td></td>
<td></td>
<td>1 y</td>
<td>B</td>
<td>F</td>
</tr>
<tr>
<td>719</td>
<td>Control</td>
<td>negative</td>
<td></td>
<td></td>
<td>1.1 y</td>
<td>C</td>
<td>F</td>
</tr>
<tr>
<td>780</td>
<td>Control</td>
<td>negative</td>
<td></td>
<td></td>
<td>1 d</td>
<td>B</td>
<td>M</td>
</tr>
<tr>
<td>874</td>
<td>Control</td>
<td>negative</td>
<td></td>
<td></td>
<td>10 m</td>
<td>B</td>
<td>M</td>
</tr>
<tr>
<td>1001</td>
<td>SIDS</td>
<td>COC BE</td>
<td>0.30</td>
<td></td>
<td></td>
<td>3 m</td>
<td>B</td>
</tr>
<tr>
<td>1002</td>
<td>SIDS</td>
<td>COC BE</td>
<td>0.19</td>
<td>0.22</td>
<td></td>
<td>2 m</td>
<td>B</td>
</tr>
<tr>
<td>1003</td>
<td>Control</td>
<td>negative</td>
<td></td>
<td></td>
<td>4 m</td>
<td>C</td>
<td>M</td>
</tr>
<tr>
<td>1004</td>
<td>SIDS</td>
<td>COC BE EME NCOC</td>
<td>3.17</td>
<td>0.88</td>
<td>trace</td>
<td>4 m</td>
<td>B</td>
</tr>
<tr>
<td>1005</td>
<td>SIDS</td>
<td>COC BE NCOC</td>
<td>2.04</td>
<td>0.24</td>
<td>trace</td>
<td>2 m</td>
<td>B</td>
</tr>
<tr>
<td>1006</td>
<td>SIDS</td>
<td>COC BE</td>
<td>0.29</td>
<td></td>
<td>trace</td>
<td>5 m</td>
<td>B</td>
</tr>
<tr>
<td>Sample Number</td>
<td>Case Type</td>
<td>Hair Results (ng/mg)</td>
<td>Age</td>
<td>Race</td>
<td>Gender</td>
<td>Cause of Death</td>
<td>PM Toxicology Results</td>
</tr>
<tr>
<td>---------------</td>
<td>-----------</td>
<td>----------------------</td>
<td>-----</td>
<td>------</td>
<td>--------</td>
<td>----------------</td>
<td>----------------------</td>
</tr>
<tr>
<td>1022</td>
<td>SIDS</td>
<td>COC 4.55  BE 0.93 NCOC 0.77</td>
<td>2 m</td>
<td>H</td>
<td>M</td>
<td>SIDS</td>
<td>negative</td>
</tr>
<tr>
<td>1024</td>
<td>SIDS</td>
<td>COC 0.96 BE trace</td>
<td>2 m</td>
<td>B</td>
<td>M</td>
<td>SIDS</td>
<td>negative</td>
</tr>
</tbody>
</table>

Abbreviations: ecgonine methyl ester (EME), cocaine (COC), benzoylecgonine (BE), norcocaine (NCOC), analyte concentration is less than the limit of quantitation (trace), day (d), month (m), year (y), black (B), Caucasian (C) and Hispanic (H) and postmortem (PM).
Table 5-5. Comparison of Hair Analysis Results and Conventional Results

<table>
<thead>
<tr>
<th>Case Classification</th>
<th>Cocaine Positive (%)</th>
<th>Cocaine Negative (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SIDS</td>
<td>10 (58.8)</td>
<td>7 (41.1)</td>
</tr>
<tr>
<td>Control</td>
<td>1 (11.1)</td>
<td>8 (88.8)</td>
</tr>
</tbody>
</table>
Table 5-6. Results of Blinded Analytical Controls

<table>
<thead>
<tr>
<th>Sample Number</th>
<th>Case Type</th>
<th>Hair Results (ng/mg)</th>
<th>Age</th>
<th>Race</th>
<th>Gender</th>
<th>Cause of Death</th>
<th>PM Toxicology Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>585</td>
<td>Cerebral ocular facial syndrome</td>
<td>Negative</td>
<td>14 y</td>
<td>B</td>
<td>M</td>
<td>Pending</td>
<td>L = negative</td>
</tr>
<tr>
<td>590</td>
<td>Depression</td>
<td>COC 0.36, BE 0.17</td>
<td>46 y</td>
<td>B</td>
<td>M</td>
<td>Drug overdose</td>
<td>BL = chlor Diazepoxide, chlorpheniramine, cocaine, dextromethorphan, diphenhydramine, fluoxetine, norfluoxetine, pseudoephedrine U = same with addition of cocaine metabolites</td>
</tr>
<tr>
<td>614</td>
<td>Psychiatric case, on Serzone</td>
<td>negative</td>
<td>55 y</td>
<td>W</td>
<td>M</td>
<td>Undetermined</td>
<td>negative</td>
</tr>
<tr>
<td>618</td>
<td>Remote drug use</td>
<td>COC 1.81, BE 0.55, NCOC 0.40</td>
<td>28 y</td>
<td>C</td>
<td>M</td>
<td>Drug overdose</td>
<td>BL = cocaine, free morphine, quinine U = cocaine &amp; metabolites, free morphine, 6-acetylmorphine, procaine, quinine</td>
</tr>
<tr>
<td>629</td>
<td>MVA</td>
<td>negative</td>
<td>7 y</td>
<td>B</td>
<td>M</td>
<td>Multiple injuries</td>
<td>L = negative</td>
</tr>
<tr>
<td>630</td>
<td>MVA</td>
<td>negative</td>
<td>19 y</td>
<td>C</td>
<td>M</td>
<td>Multiple injuries</td>
<td>BL = ethanol, lidocaine, VH = ethanol</td>
</tr>
<tr>
<td>635</td>
<td>Control</td>
<td>COC 0.89, BE 0.10</td>
<td>33 y</td>
<td>C</td>
<td>M</td>
<td>Narcotic intoxication</td>
<td>BL = free morphine, phencyclidine, quinine Bile = free morphine, phencyclidine, quinine</td>
</tr>
<tr>
<td>Case</td>
<td>Control</td>
<td>COC</td>
<td>BE</td>
<td>EME</td>
<td>CE</td>
<td>NCOC</td>
<td>NBE</td>
</tr>
<tr>
<td>-------</td>
<td>------------</td>
<td>------</td>
<td>-----</td>
<td>-----</td>
<td>-----</td>
<td>------</td>
<td>-----</td>
</tr>
<tr>
<td>645</td>
<td>Control</td>
<td>25.8</td>
<td>16.1</td>
<td>1.65</td>
<td>1.92</td>
<td>1.15</td>
<td>1.82</td>
</tr>
<tr>
<td>650</td>
<td>Control</td>
<td>negative</td>
<td>26 y</td>
<td>C</td>
<td>F</td>
<td>Multiple injuries</td>
<td>BL= ethanol, cocaine, lidocaine, quinine</td>
</tr>
<tr>
<td>679</td>
<td>Control</td>
<td>negative</td>
<td>3 y 3.5 m</td>
<td>C</td>
<td>M</td>
<td>Trisomy 7</td>
<td>negative</td>
</tr>
<tr>
<td>690</td>
<td>Control</td>
<td>negative</td>
<td>4 y 11 m</td>
<td>C</td>
<td>M</td>
<td>Kawasaki's disease</td>
<td>negative</td>
</tr>
<tr>
<td>1007</td>
<td>Control</td>
<td>negative</td>
<td>23 m</td>
<td>NA</td>
<td>F</td>
<td>Pneumonia</td>
<td>negative</td>
</tr>
<tr>
<td>1014</td>
<td>Negative control</td>
<td>negative</td>
<td>Adult</td>
<td>NA</td>
<td>F</td>
<td>NA</td>
<td>no tests performed</td>
</tr>
<tr>
<td>1015</td>
<td>Negative control</td>
<td>negative</td>
<td>Adult</td>
<td>NA</td>
<td>F</td>
<td>NA</td>
<td>no tests performed</td>
</tr>
<tr>
<td>1016</td>
<td>Negative control</td>
<td>negative</td>
<td>Adult</td>
<td>NA</td>
<td>F</td>
<td>NA</td>
<td>no tests performed</td>
</tr>
<tr>
<td>1017</td>
<td>Negative control</td>
<td>COC</td>
<td>trace</td>
<td>Adult</td>
<td>NA</td>
<td>F</td>
<td>NA</td>
</tr>
<tr>
<td>1018</td>
<td>Negative control</td>
<td>negative</td>
<td>Adult</td>
<td>NA</td>
<td>F</td>
<td>NA</td>
<td>no tests performed</td>
</tr>
<tr>
<td>1019</td>
<td>Negative control</td>
<td>negative</td>
<td>Adult</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>no tests performed</td>
</tr>
<tr>
<td>Sample Number</td>
<td>Case Type</td>
<td>Hair Results (ng/mg)</td>
<td>Age</td>
<td>Race</td>
<td>Gender</td>
<td>Cause of Death</td>
<td>PM Toxicology Results</td>
</tr>
<tr>
<td>---------------</td>
<td>-----------</td>
<td>----------------------</td>
<td>-----</td>
<td>------</td>
<td>--------</td>
<td>----------------</td>
<td>-----------------------</td>
</tr>
<tr>
<td>1020</td>
<td>Control</td>
<td>COC 0.14</td>
<td>29 y</td>
<td>B</td>
<td>M</td>
<td>Drug overdose</td>
<td>BL= morphine, lidocaine, quinine U= lidocaine, 6-acetylmorphine, quinine</td>
</tr>
<tr>
<td>1021</td>
<td>Control</td>
<td>COC 11.1 BE 2.39 EME trace NCOC 0.33 [Wash COC ]</td>
<td>20 y</td>
<td>B</td>
<td>M</td>
<td>Homicide</td>
<td>BL= negative U= negative</td>
</tr>
<tr>
<td>1023</td>
<td>Control</td>
<td>AEME trace COC 9.57 BE 4.41 NCOC 0.28 [wash COC ]</td>
<td>49 y</td>
<td>C</td>
<td>M</td>
<td>Combined drug intoxication</td>
<td>Morphine, sertraline, tramadol</td>
</tr>
</tbody>
</table>

Abbreviations: anhydroecgonine methyl ester (AEME), ecgonine methyl ester (EME), cocaine (COC), benzoylecgonine (BE), norcocaine (NCOC), norbenzoylecgonine (NBE), analyte concentration is less than the limit of quantitation (trace), motor vehicular accident (MVA), postmortem (PM), qualitative summary of decontamination washes [wash], month (m), year (y), black (B), Caucasian (C), information not available (NA), blood (BL), liver (L), urine (U), and vitreous humor (VH).
Figure 5-2. Results of Hair Analysis for SIDS and Control Infants
Figure 5-3. Comparison of Cocaine and Benzoylecgonine Concentrations in SIDS Infants

**Note: Actual cocaine concentration was 44.8 ng/mg for Subject 710.
CHAPTER 6
CONCLUSIONS AND FUTURE WORK

Within the last two decades in the United States, hair analysis has transcended from a research tool that could successfully detect drugs of abuse to its acceptance within our court system as a legally defensible matrix. On the other hand, investigations focusing on the utility of nail analysis are at a stage of infancy. Forensic applications of both keratinized matrices have remained an analytical quandary. Researchers have been unable to fully explain the mechanism of drug incorporation into keratinized matrices or extent of the contribution of environmental contamination and color bias to hair analysis results. Consequently, the full implications of data interpretation are complex and incomplete. The dilemmas of hair and nail analysis can not be eliminated without studies focusing on drug disposition into these matrices. Moreover, overall acceptance of hair and nail analysis by the scientific community is dependent upon proof of its utility to enhance detection of drug exposure in special clinical and forensic applications.

The projects within this dissertation investigated pharmacokinetic and drug disposition properties of hair and nails, as well as, explored the utility of analyzing keratinized matrices in specific applications to determine if identification of drug exposure could be improved. Goals of this research concentrated on answering several fundamental questions concerning hair and nail analysis including: 1) Do drugs of abuse such as cocaine and codeine enter into keratinized matrices in a predictable dose-response fashion? 2) Are
the pharmacokinetic profiles similar for hair and nails? 3) Can analysis of keratinized matrices offer a complementary and sensitive technique to detect drug exposure and in some cases identify exposure that may, otherwise, go undetected? 4) Can techniques introduced for hair analysis work equally well for nail analysis? 5) Can new methodology identify unique analytes, in addition to parent drug, that would offer supplementary information as to the history of the individual's drug use?

Chapter 3 of this dissertation presented data suggesting that both cocaine and codeine, given at multiple dosing levels (COC: 75 and 150 mg/70 kg; COD: 60 and 120 mg/70 kg) in a controlled inpatient study, incorporate into hair in a predictable dose-response manner. Cocaine and codeine were detected in washed hair in the first hair specimen collected within 3 days after the last drug administration and peaked within 2-3 weeks after dosing. For a majority of the subjects, these data demonstrate a two-fold increase in the measured dose-response parameters when the dose was doubled. Table 6-1 summarizes the peak concentrations (Cmax) for cocaine and codeine after low and high dose administration.

| Analyte          | Low Dose (Cocaine: 75 mg/70 kg  
|                 | Codeine: 60 mg/70 kg)          | High Dose (Cocaine: 150 mg/70 kg  
<table>
<thead>
<tr>
<th></th>
<th></th>
<th>Codeine: 120 mg/70 kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cocaine Cmax (ng/mg)</td>
<td>1.7 to 15</td>
<td>4.5 to 27</td>
</tr>
<tr>
<td>Codeine Cmax (ng/mg)</td>
<td>0.71 to 5.7</td>
<td>1.8 to 8.7</td>
</tr>
</tbody>
</table>
In contrast, nail analysis of the same subjects did not demonstrate a dose-response relationship for either cocaine or codeine.

This project also demonstrated that the observed drug disposition profiles were different for hair and nails. Less than 20% of drug in hair specimens was removed by decontamination wash procedures while most (50-100%) of the drug concentrations were removed by washes of nail specimens.

This observation was unexpected given the following reasons: 1) these matrices are of similar chemical composition and vascularization which would suggest similar drug disposition into these matrices; and 2) cocaine concentrations (sometimes greater than 10 ng/mg) were successfully detected in postmortem distal nail clippings also analyzed during the course of this dissertation (Chapter 4) and the decontamination wash procedures for this method only removed 20-50% of the total amount of drug detected.

In the future, a study designed to investigate whether different collection techniques for nails can influence the results obtained by nail analysis. The present study may not have allowed enough time for the nails to grow out to a point where drug was incorporated and, hence, collected for analysis. On the other hand, it is perplexing that the initial nail scrapings did not have higher concentrations of drug since these individuals were known drug abusers. Therefore, a longer dose-response study design could help to determine whether observations of the present study were a result of the slower growth rate for nails.

The chosen study design did not allow critical evaluation of all routes of drug disposition into hair. Since subjects were administered drug either by oral ingestion or subcutaneous injections, environmental contamination due to vapors or contaminated
surfaces present in the “real-world” environment did not contribute to drug disposition into hair during this study. Further study is needed to determine if drugs administered by smoking also demonstrate a dose-response relationship in hair. The method of collecting hair specimens introduced another limitation of study design. By shaving the entire scalp each week, the transfer of drugs from other matrices (i.e., sweat and sebum) to hair was limited to hair grown only in the week during drug administrations. Hence, a study is needed to determine whether drugs in biological secretions are deposited only in hair near the surface of scalp or if drugs in secretions can be transferred to sections of hair distal to the scalp.

In Chapter 4, postmortem fingernail (N=17) and toenail (N=15) clippings from 18 suspected cocaine were analyzed for cocaine analytes. Methodology currently utilized for hair analysis was employed for nail analysis. Specimens were decontaminated, cut into small pieces, incubated overnight in methanol, subjected to solid phase extraction and derivatization, and analyzed by GC/MS. Nails results were compared to conventional postmortem testing results and are summarized in Table 6-2.

Table 6-2. Comparison of Nail Analysis Results and Conventional Results

<table>
<thead>
<tr>
<th>Analysis</th>
<th>Cocaine Positive (%)</th>
<th>Cocaine Negative (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nails</td>
<td>14 (77.7)</td>
<td>4 (22.2)</td>
</tr>
<tr>
<td>Conventional</td>
<td>5 (27.7)</td>
<td>13 (72.2)</td>
</tr>
</tbody>
</table>

Comparison of nail analysis to blood and urine analysis revealed a marked increase in the detection of cocaine use (82% versus 27%). In addition, this assay detected several unique cocaine analytes such as anhydroecgonine methyl ester, cocaethylene, norcocaine, and norbenzoylecgonine. The presence of these analytes in nail specimens revealed
additional information about the subjects drug use including confirmation of drug ingestion and subsequent metabolism (norcocaine), route of administration (anhydroecgonine methyl ester), and concomitant cocaine and ethanol use (cocaethylene).

Chapter 5 investigated the potential use of hair analysis to determine if detectability of drug exposure in SIDS cases could be improved. Although epidemiological data seems to suggest an association between drug use and SIDS, sufficient and direct analytical links between sudden infant death syndrome and exposure to an illicit drug are lacking.

Head hair samples were obtained from 26 infants and analyzed in a blinded-fashion by GC/MS. Conventional postmortem results were subsequently obtained and compared to hair analysis. The results of both analyses are summarized in Table 6-3.

Table 6-3. Comparison of Hair Analysis Results and Conventional Results

<table>
<thead>
<tr>
<th>Case Classification</th>
<th>Cocaine Positive (%)</th>
<th>Cocaine Negative (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SIDS</td>
<td>10 (58.8)</td>
<td>7 (41.1)</td>
</tr>
<tr>
<td>Controls</td>
<td>1 (11.1)</td>
<td>8 (88.8)</td>
</tr>
</tbody>
</table>

Statistical analysis demonstrated a significant increase in drug detection with the use of hair analysis. Since conventional analysis reflects more recent drug in comparison to hair analysis, the results of these analyses suggest that the infants were not exposed to cocaine immediately prior to death.

Although these infants were exposed to cocaine, there is no evidence that this exposure contributed directly to the infant’s death. Animal studies exposing fetuses and/or young may help in the understanding of the mechanism by which drug exposure and the extent the drug’s effect have on the fetus and/or neonate. If specific traits are
observed in animal populations, this information could be useful in identifying a subpopulation of infants at risk for SIDS.

The current study looked at an isolated population of infants from the same geographical location and was not representative of the total SIDS population. Therefore, a nationwide study including a much larger population would provide more comprehensive and complete information.

In conclusion, answers to the previously stated questions summarize the results obtained by the projects of this dissertation. A dose-response relationship was observed for cocaine and codeine incorporation into hair but not fingernails. Hair and nails demonstrated different pharmacokinetic profiles by this study design. Analysis of keratinized matrices to detect drugs of abuse in special applications in the fields of toxicology and clinical medicine can offer complementary information to conventional testing techniques. Techniques employed for hair analysis can be utilized for nail analysis. Finally, assay inclusion of unique analytes can offer additional historical information of the subject’s drug use if these analytes are present.

A final cautionary note about analysis of keratinized matrices is warranted for conclusions of this research. Although this dissertation demonstrated a dose-response relationship of drug disposition into keratinized matrices in some instances, the mechanism of drug incorporation was not elucidated. Until the mechanism of drug incorporation into keratinized matrices is more fully understood, data interpretation will be difficult. Moreover, other unresolved controversies associated with analysis of keratinized matrices that further complicate interpretation of data. External contamination, color bias, and nonstandardized analytical procedures will continue to hinder the advancement of hair and
nail analysis until research can provide answers to these debilitating, but important, issues. Until this time, analysis of these keratinized matrices should be executed with caution to avoid overstating the meaning of the results.
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BIOGRAPHICAL SKETCH

The author, Jeri Diane Ropero-Miller, was born the youngest daughter of Sydney Anthony and Helen Kathleen Ropero on December 4, 1967. Her birthplace, and the town, in which she lived for the first eighteen years of life, was Orlando, Florida. She has one older sister, Kathleen Parker, and one younger brother, Bill Strickland.

The author graduated eighth in her class from Maynard Evans High School in June of 1985. She continued her education at Wesleyan College in Macon, Georgia, where she obtained liberal arts degree in chemistry with a strong background in biology and math. She graduated with cum laude honors with a Bachelor of Arts degree in May 1989. During her last semester of college, she met and fell in love with her future husband, Jeffrey Joiner Miller.

Upon completion of her undergraduate degree, the author accepted an analytical chemistry position as a Research Scientist with Conoco, Inc, a refinery and subsidiary of DuPont. This required her to relocate to Ponca City, Oklahoma, where she resided for the next three and a half years. This position allowed her to learn a myriad of spectroscopic techniques. It also enabled her to determine that, while she enjoyed an analytically based career, she would like to pursue a career in the forensic sciences. Consequently, she decided to return to Florida and prepare to enter graduate school.

The author relocated to Gainesville, Florida, in December 1992. While awaiting the re-establishment of her Florida residency, she accepted a supervisory position in the
Inorganic Atomic Spectroscopy Group with Environmental Science & Engineering, Inc. She remained in this position until the fall of 1994, when she was accepted into the University of Florida College of Medicine Graduate Program in the Department of Pathology. After a year of course work and several laboratory rotations, the author decided to begin her Ph.D. research in forensic toxicology under the guidance and mentoring of Dr. Bruce A. Goldberger.

On February 1, 1997, the author married her long-term companion in Orlando, Florida. After graduation, the author has accepted a position at the Office of Chief Medical Examiners in Chapel Hill, North Carolina in the field of postmortem forensic toxicology.
I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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Roger L. Bertholf
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Ian R. Tebbett
Professor of Medicinal Chemistry
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December, 1998

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Dean, College of Medicine

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Dean, Graduate School