National Institute on Drug Abuse

Hair Testing for Drugs of Abuse:

International Research on Standards and Technology

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Introduction and Overview: National Institute on Drug Abuse Special Publication on Hair Analysis for Drugs of Abuse

Edward J. Cone and Michael J. Welch

Abuse of controlled substances is a major factor in societal problems such as crime—particularly violent crime—and spread of diseases such as AIDS and hepatitis. In the workplace, substance abuse leads to higher rates of accidents and absenteeism and reduced job performance, resulting in serious losses of productivity, higher health costs, and increased liability claims. Consequently, there is considerable emphasis placed on testing individuals for evidence of use of controlled substances. Urine drug testing is widely applied for this purpose and has established a good record for accuracy. Unfortunately, urinalysis is effective for determining drug use only within the few days prior to collection of the urine sample. Long-term drug use may go undetected if the individual knows when his or her urine will be tested because he or she can refrain from drug use for the few days before the sample collection. Hair analysis is a promising alternative to urinalysis because it can provide a long-term record of drug abuse. However, several concerns have been raised about hair analysis that must be answered before this approach will be widely accepted as a valid determination of drug use.

In 1989 the National Institute of Standards and Technology (NIST), an agency of the U.S. Department of Commerce, began a study of

some issues regarding hair analysis for drugs of abuse. This work was supported through an interagency agreement, first with the National Institute of Justice and later with the National Institute on Drug Abuse (NIDA).[1] The work focused on four aspects: (1) development and evaluation of methods used to extract drugs from hair and to analyze the extracts; (2) investigation of environmental factors, specifically, the question of external contamination of hair with drugs of abuse and the question of the effect of hair treatments on levels of drugs incorporated into hair; (3) development of reference materials to support accurate measurement of drugs in hair; and (4) interlaboratory studies to determine how well laboratories could detect and quantify drugs in hair and to determine which approaches worked best. This last aspect was the focus of two workshops held at NIST, the more recent occurring in May 1993. Participants in the interlaboratory studies were invited to present their research on hair analysis, to discuss results from previous interlaboratory exercises, and to plan future activities. This document not only features papers submitted by participants in the workshop but also includes related papers submitted by other interested parties to give a more rounded picture of the status of hair analysis for drugs of abuse.

Seventeen papers were accepted for this publication. These papers provide an excellent overview of the state of the art and provide research results related to some of the more controversial aspects of hair analysis for drugs of abuse. The volume is divided into three sections: Drug Incorporation and Interpretation, Methods, and Applications. The following paragraphs briefly describe the subjects covered in this publication.

The subject of hair testing for drugs of abuse is introduced by Gropper, who provides a historical summary of this field and the research and development program sponsored by the National Institute of Justice and the National Institute on Drug Abuse.

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[1]NIDA agreement No. RA-ND-91-01, "Standards for Drug Testing and Analysis of Hair," between NIDA and the National Institute of Justice (NIJ) provided partial support for several grants of NIJ.

In the first section, Drug Incorporation and Interpretation, Kidwell and Blank discuss their investigation of the incorporation of drugs in hair and the effectiveness of various procedures to remove externally applied drugs. They used radioactive-labeled drugs to trace the location of the drugs after various procedures. Based on their results, they make strong recommendations about interpretations of the relationship between what is found in hair and whether drug use has occurred.

Henderson, Harkey, and Jones review the methods of determining the presence of cocaine and its metabolites in hair. This chapter includes discussions of controlled-dose studies, external contamination issues, appearance time of drugs in hair, and potential racial/hair type issues.

Cone and Wang describe their research on environmental drug exposure. They contaminated hair with cocaine, either as a vapor or an aqueous solution, and investigated how thoroughly the drug could be removed. They also investigated the potential of using the presence of certain cocaine metabolites in hair as a marker for drug use.

In the Methods section, Welch and Sniegoski report on the results of the first three interlaboratory studies on the analysis of hair for drugs of abuse conducted by NIST. Sixteen laboratories participated in a study to determine how well drugs could be detected and quantified in hair. Samples sent to the participating laboratories included hair from drug users, drug-free hair, and hair into which drugs had been soaked. Results described, both qualitative and quantitative, are associated with techniques used for extraction and analysis.

Baumgartner and Hill discuss the advantages of hair analysis for detecting use of illicit drugs and their procedures for differentiating between hair with external contamination and hair from drug users. They describe their wash kinetics approach and how they use these findings to decide whether drugs found are in the hair through drug use. They also describe how the presence of certain metabolites is used to confirm drug use.

Kintz describes a method for the simultaneous identification and quantitation of several opiates and cocaine-related compounds in hair. The method involves washing the hair with dichloromethane, powdering it in a ball mill, extracting with acid followed by solid-phase extraction, and derivatizing with silylation reagents, which promote a chemical reaction used to improve the chromatographic behavior of compounds subjected to gas chromatography analysis, for gas chromatography/mass spectrometry analysis.

Sachs and Moeller describe their investigations of various approaches for extracting drugs of abuse from hair. They powdered hair samples and compared how well acid, base, enzyme, and organic solvent extractions liberated drugs from hair.

Pichini, Pacifici, Altieri, Passa, Rosa, and Zuccaro describe the use of high-performance liquid chromatography (HPLC) and radioimmunoassay to measure nicotine and cotinine in the hair of both smokers and nonsmokers. Various extraction procedures were evaluated, both in terms of recoveries and in terms of interferences for the HPLC analyses.

Tagliaro, Deyl, and Marigo discuss the use of capillary electrophoresis for the analysis of hair for drugs of abuse using either conventional capillary zone electrophoresis or micellar electrokinetic capillary chromatography. The advantages of these two approaches are presented for a wide variety of drugs and metabolites.

Selavka and Riker discuss their experience in analyzing more than 1,000 hair samples from individuals in situations where there was reasonable suspicion that illegal drugs were being used. Percentages of positive findings for cocaine as well as levels of cocaine and metabolites are correlated with case categories. Procedures for removing external contamination and interpretation of findings are discussed.

In the Applications section, similar studies are reported by Cirimele, Kintz, and Mangin. They also treated powdered hair with acid, base, enzyme, and organic solvents and compared the efficiencies of the various extraction approaches.

Miller, Donnelly, and Martz describe research at the Federal Bureau of Investigation on the analysis of hair for drugs of abuse. They detail the procedures that they use to wash and extract hair and to differentiate environmental contamination from drug use. They also describe their use of chemical ionization tandem mass spectrometry on hair extracts to determine the presence of cocaine and benzoylecgonine. Related research and applications are mentioned briefly.

Moeller, Fey, and Sachs discuss the analysis of hair for opiates in forensic cases. They have analyzed more than 1,000 specimens from suspected opiate users for morphine, codeine, monoacetylmorphine, and dihydrocodeine. From these results, they propose guidelines for distinguishing true positive specimens from specimens where results are in doubt.

Uematsu, Mizuno, and Kosuge discuss their investigation of the incorporation of pharmaceuticals and other substances into hair. By combining carefully administered doses of easily detectable compounds with the segmental analysis of hair, they were able to measure hair growth rates and the distribution of the markers along the shaft. Studies of nicotine in the hair of smokers correlated well with these findings.

Klein and Koren describe their studies of cocaine in the hair of neonates exposed in utero. They discuss the advantages of using neonatal hair for determining cocaine exposure versus other approaches. They correlate nicotine and cotinine levels in maternal and neonatal hair with the mother's exposure to tobacco smoke and the incidence of low birth weight infants.

Katikaneni, Sallee, Ibrahim, and Hulsey describe their investigation of neonates to assess the effects of in utero exposure to cocaine. They analyze neonatal and maternal urine and hair for benzoylecgonine using a radioimmunoassay screening method to determine which newborns had been exposed. Infants' development is studied over the next several months. The investigators correlate cocaine exposure with several indices of fetal development using nonexposed newborns from the same socioeconomic background as controls.

It will be obvious in reading the chapters in this volume that there is international interest in hair testing. The potential for unique information that might be obtained from hair analysis has driven the rapid progress of the development of methods in this area. Hair analysis appears to offer a means of assessment of drug exposure that urine testing cannot provide. Consequently, it is expected that there will be multiple uses of hair analysis data. However, more work is needed to answer some basic questions of how drugs get into and are sequestered in hair. How long are drugs stable in hair, and what environmental factors are most important in the ultimate outcome of the test results? Many additional questions remain, but clearly, a critical mass of research has formed, and understanding of hair testing methods should grow quickly.

Historical Overview: Research and Development on Drug Testing by Hair Analysis

Bernard A. Gropper[1]

INTRODUCTION

This chapter summarizes the research and development program on hair analysis for drugs of abuse sponsored and supported by the National Institute of Justice (NIJ) and National Institute on Drug Abuse (NIDA). The program's goals were to develop enhanced methods for drug detection, diagnosis, and monitoring through hair analysis as a complement to other indicators. The program's long-range strategy supported systematic research in three complementary areas:

- *Scientific and interpretive* issues on the physiological and environmental factors affecting hair as a test medium

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[1]Bernard A. Gropper, Ph.D., former manager of the National Institute of Justice's research program on Drugs, Alcohol, and Crime, helped initiate and develop the NIJ research program on drug testing by hair analysis and served as program manager for the NIJ-NIDA interagency collaborative efforts on hair research described in this chapter.

- *Forensic and technological development* issues to evaluate alternative analytic techniques and to develop standards for hair analysis methods

- *Programmatic and evaluative* issues on detectable time-intensity usage profiles of major drugs of abuse and cost-benefit tradeoffs for individual and group applications

The chapter summarizes the major questions about the potential merits and limitations of hair testing for criminal justice and health-related applications and the program's approaches to addressing each of these issues.

In March 1991 NIJ, within the U.S. Department of Justice, and NIDA, within the U.S. Department of Health and Human Services (DHHS), initiated an interagency agreement to collaboratively support research and evaluation efforts toward developing scientifically sound and accurate procedures to analyze human hair for drugs of abuse (Gropper 1992*a*, pp. 463-467).[2]

The studies supported by these agencies were undertaken to explore and develop potential uses of hair analysis as a supplemental method of drug testing. Such applications include potential enhancements of existing capabilities to detect and characterize both individual and aggregate patterns of drug use, deter drug abuse, monitor

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[2]All Government-supported research is subject to review and potential change in light of many considerations. These include not only the results of the research efforts but also such exogenous factors as developments in related scientific, technical, programmatic, and policy areas and budgetary considerations. All information in this chapter on the activities, objectives, and long-term goals of these programs and studies reflects their rationale and progress during the periods described. They do not necessarily reflect their current status or imply any future commitments by the U.S. Departments of Commerce, Health and Human Services, or Justice or any other Government agency.

compliance, evaluate treatment effectiveness, and control drug-related crime.

BACKGROUND

Detection and deterrence of drug use are essential parts of the Nation's fight against drug abuse and drug-related crime. The President's National Drug Control Strategy (Office on National Drug Control Policy 1992, pp. 72, 127) called for wider use of drug testing in the workplace and at all levels of the criminal justice system.

However, no single testing medium or analytic method is optimal for all informational needs. The availability of alternative techniques with complementary characteristics can strengthen abilities to detect and control drug use and its consequences under conditions in which it might otherwise go undetected (U.S. General Accounting Office 1993, pp. 58-60).

NIJ and NIDA have mutual interests in research, evaluation, and development of enhanced and innovative drug testing capabilities. They have collaborated in many ways in meeting their respective agency missions and programmatic goals over the years. This partnership has included exchanges of data on factors affecting drug use and its consequences as well as joint support of studies to assess the nature and extent of these problems and to evaluate the impacts of prevention and control efforts in criminal justice and related drug service areas (National Institute on Drug Abuse 1991; National Institute of Justice 1992).

Since the mid-1980s NIJ and NIDA have supported the exploration and development of hair analysis as an approach to drug testing that may offer unique advantages compared with other currently used methods based on behavioral indicators or body fluids. Unlike such short-term indicators, hair retains drugs for much longer periods and thus may provide wider windows of detectability—weeks and months versus the 2 to 3 days that such rapidly eliminated drugs as cocaine or heroin can be detected in blood or urine. Therefore, drug users who are subject to hair testing may be less likely to evade detection,

and deterrence of use may be enhanced (Harkey and Henderson 1989, pp. 298-329; Baumgartner et al. 1989; Gropper 1989, 1992*a*, pp. 463-467). Another potential advantage of hair as a test medium is that specimens can be readily obtained by persons of either sex without the social invasiveness and privacy problems associated with obtaining urine samples or the physical invasiveness of drawing blood.

Although some current limitations of hair analysis can be resolved by systematic research and technical development, others cannot—because they are inherent to hair as a test medium. For example, hair cannot compete with urine or blood in assessing an individual's current clinical impairment or very recent drug use because of its relative lag in response time. Each approach monitors different effects or indicators of drugs on the body: Urinalysis and blood testing detect short-term reversible effects, whereas hair analysis detects longer term, more permanent effects. These media should be thought of as complementary rather than as competitive or alternative approaches to addressing such issues, with each offering unique potential advantages for answering specific questions (Gropper and Reardon 1993).

In addition, because hair, unlike urine or blood, is inherently exposed to the environment, other factors may need to be addressed in assessing the extent to which the detection of drug products in hair reflects a subject's use or environmental exposure. For some criminal justice evidentiary purposes, such as a client's compliance with a court order to remain drug-free and have no contact with drug materials, the distinction may be less important. Either ingestion or significant environmental exposure, such as that encountered in handling or dealing in drugs, would indicate a violation of the court conditions. However, for answering such health-related questions as assessment of drug use patterns and treatment need and for monitoring treatment effectiveness, distinctions between exposure and ingestion are much more significant (U.S. General Accounting Office 1993).

Initial priorities of these joint efforts focused on the basic and applied research issues of scientific validation and interpretation and technological standardization and quality assurance. When these

issues have been satisfactorily addressed, other more programmatic concerns, such as the reduction of current costs and testing times for deriving drug information from hair, can become the focus of possible further development and evaluation efforts.

GOALS AND OBJECTIVES

The program's overall goal has been to develop enhanced drug detection, diagnostic, and monitoring capabilities through hair analysis. Attaining this goal would permit hair analysis to serve as a method complementary to current techniques based on self-report, urinalysis, or other indicators to meet criminal justice and health and human service needs.

To achieve this goal, the NIJ-NIDA program's objectives were to:

- Support research, evaluation, and development of hair as a test medium and of analytic techniques for deriving drug-related information on major drugs of abuse
- Develop information needed to establish laboratory and field standards for sample acquisition and processing
- Evaluate alternative approaches to using hair analysis in conjunction with other forms of drug testing and address potential challenges to the accuracy of drug-related assessments in individual forensic evidentiary cases and in group applications for monitoring the nature and extent of drug usage (National Institute of Justice 1992)

PROGRAM STRATEGY

The program's strategy supported systematic research in three complementary priority areas: scientific and interpretive issues,

forensic and technological development issues, and programmatic and evaluative issues (Gropper 1992*b*).

Scientific and Interpretive Issues

- What are the characteristics of hair as a test medium, and how do analyses of hair compare with those based on other possible information sources? Differences in test results on samples from different body systems (e.g., breath, blood, urine, hair) may not imply error but rather differences in the characteristics of the systems involved.

- How rapidly are different drugs or drug-related products absorbed by various types of hair? How accurate are estimates of time intervals that correspond to various lengths of hair segments? Can hair analysis contribute to other data sources for developing more accurate prevalence estimates of total user populations for specific drugs?

- What is the best method to identify the reasons for differences in test results from samples that differ in the time it takes for ingested drugs and their metabolites to be deposited in or on the hair and in the rate of washout of drugs and their metabolites? Are there significant group differences in subpopulations that may be related to age, race, or sex that should be accounted for in the analytic techniques, specimen acquisition and handling procedures, or methods of interpreting test results?

Forensic and Technological Development Issues

Forensic and technological development priorities address analytic and testing techniques, standards, evaluative procedures, and possible challenges to drug test results from hair. They distinguish between two kinds of issues: those related to the characteristics and limits of hair as a test medium and those related to the techniques

for deriving drug-related information from hair. Related priority issues include:

- To what degree can the content or distributions of drug evidence within hair be increased, decreased, or contaminated by intentional efforts (e.g., hair treatments); environmental factors (passive exposure); physiological factors (sweating); or factors related to race, sex, or other group differences?

- To what extent are different procedures (such as washing or analyzing parent drug and metabolite ratios) able to compensate for these effects? Which drugs are most vulnerable? How readily can evasive attempts be detected?

- What are the relative merits of alternative extraction and analytic procedures in terms of sensitivity, specificity, and operational feasibility?

Programmatic and Evaluative Issues

Programs for monitoring drug offenders and detecting drug use in target populations may have to balance tradeoffs between frequency of testing, likelihood of detection (and associated deterrence), costs, and interference with jobs or other considerations in community-based settings. Optimal balances of these issues with any single type of test (e.g., breath, blood, urine, hair) or optimal combinations of them for screening or confirmation in applied settings require further evaluation.

- These considerations may include cost-benefit comparisons of alternative single method or multimethod programs for drug monitoring of offenders. What types of use patterns can be detected by different program schedules, tests, and cutoff criteria?

- How well can analysis of successive hair shaft segments be used to assess levels of drug use and periods of use or abstention to ensure conformity to requirements of nonuse in criminal justice and other operational settings?

- To what extent can hair tests complement other indicators (e.g., official records, clinical signs, urine and blood tests) in assessing the treatment needs of special populations (such as pregnant offenders and newborns in criminal justice and other settings) who might otherwise go undetected as drug abusers or as individuals who have drug-related problems?

PROGRAM DEVELOPMENTS

The missions and roles of NIJ and NIDA in these areas are complementary. NIJ directs its support primarily toward applied research and evaluation of testing methods in operational settings, and NIDA supports a broader research mandate encompassing many aspects from the basic physiological mechanisms and pharmacologic factors to the evaluation of analytic standards. Application and regulatory aspects, such as the accreditation of laboratories, which had been under NIDA's jurisdiction at the time of these initial studies and the interagency agreement, have been transferred to the new Substance Abuse and Mental Health Services Administration in the reorganization of DHHS. In addition to collaborating in planning, exchange of information, and support of studies in these areas (National Institute of Justice 1991*a*, 1991*b*, 1992, 1993; National Institute on Drug Abuse 1991), NIJ and NIDA have maintained close contact with other major organizations that conduct related research, such as the U.S. Navy, Federal Bureau of Investigation, American Academy of Forensic Sciences, Society of Forensic Toxicologists, and National Institute of Standards and Technology (NIST).

Initial studies supported under the collaborative NIJ-NIDA interagency agreement concentrated on research involving relatively rapidly excreted drugs, cocaine and heroin, which are of major

significance in the Nation's drug problem and should permit evaluation and demonstration of the potential advantages of hair analysis more readily than drugs with wider windows of detectability in urine.

The research included three major efforts:

- Studies by the University of California, Davis on the mechanisms by which cocaine is absorbed into hair and on other issues related to potential population characteristics and the interpretation of hair test results (Henderson et al. 1993).

- Laboratory standards development at NIST to evaluate alternative methods for washing, extracting, and analyzing hair samples with calibrated amounts of target drugs (cocaine, heroin) and environmental exposure of hair samples (Welch 1989).

- Comparisons of three drug detection methods among arrestees using (1) *hair analysis* (by radioimmunoassay), (2) *urinalysis* (by fluorescent polarization immunoassay and enzyme-multiplied immunoassay technique), and (3) *self-report* (Mieczkowski et al. 1991*a*). Assessments by hair analysis of drug use within the past 30 days revealed more cocaine use than by urinalysis, and self-report assessments revealed the least. Patterns of concordance between hair and urine results indicated that when cocaine use was frequent or at high levels, urine and hair testing tended to be equally effective, but for infrequent and low-level use, urinalysis was less effective than hair analysis (Mieczkowski et al. 1991*b*). Further details on each of these studies are reported elsewhere in this volume.

The NIJ-NIDA collaborative efforts under the initial interagency agreement on hair research have been concluded. The focus was on exploring the characteristics of hair as a potential medium for detecting drug use, assessing the relative merits of alternative

methods of hair analysis, and developing standardized guidelines and procedures for hair testing applications (Gropper and Reardon 1993).

Although these constitute major long-range systematic Federal efforts at basic research and development of hair testing, they are not the entire picture. Research on hair analysis issues is now worldwide, as evidenced by the ranges of medical and forensic questions and analytic approaches reported by the participants in the First International Conference on Hair Testing for Drugs of Abuse held in Genoa, Italy (Gropper 1992*b*). This expanded activity among growing numbers of independent investigators is encouraging. The exchange and integration of information gained from such multiple sources will stimulate and accelerate progress toward achieving these goals much more than would be possible without their respective contributions.

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Mechanisms of Incorporation of Drugs Into Hair and the Interpretation of Hair Analysis Data

David A. Kidwell and David L. Blank

INTRODUCTION

For more than a decade, the U.S. Department of Defense (DOD) has placed increased emphasis on detection and deterrence of drug use within the military. The employment of urinalysis to prevent drug use is one of the key components of this deterrence program. The U.S. Navy has been a strong proponent of urinalysis and is one of the world's largest drug-screening organizations. As the use of urinalysis has increased and become widely publicized, the self-reported use rate (past 30 days) in the U.S. Navy dropped from 33 percent in 1980 to less than 4 percent in 1992 (Bray et al. 1992). In the civilian sector also, as drug testing has expanded, the rate of drug use has declined (Newman 1994). The deterrent effect of urinalysis is suggested by the observation that 48 percent of a random sample of U.S. Navy personnel believe that urinalysis testing has contributed to reduced drug use in the military (Bray et al. 1992). Many of the procedures developed in the U.S. Navy and DOD laboratories have been adopted by the forensic community for application to the civilian sector.

Often the only evidence of drug use is a positive laboratory result. Therefore, laboratories must follow reliable, valid, and generally accepted procedures, and the procedures and results must be

subjected to internal and external review. Falsely accusing an individual of drug use could be devastating to the individual and have serious repercussions for the laboratory. To ensure program success, laboratories must use procedures that attach extreme importance to avoiding false accusation of drug use.

Notwithstanding the programmatic success of the U.S. Navy and DOD urinalysis programs, constant improvement is sought. For example, the short half-life of many drugs of abuse in urine makes detection of infrequent drug use difficult. Based on various reports (Baumgartner et al. 1981, 1982; Marigo et al. 1986; Smith and Liu 1986), the authors became interested in evaluating hair analysis as an adjunct to urinalysis. Military policy dictates that, when an individual is identified as positive for drug use by the urinalysis program, an administrative hearing or court martial may be held (U.S. Department of Defense 1994; U.S. Department of the Navy 1990). One application of hair analysis could be to support or refute an individual's testimony at such an administrative hearing or trial. Furthermore, hair analysis initially appeared to be capable of providing a long-term usage history so that informed decisions could be made on the accession or retention of an individual in military service (Baumgartner et al. 1979).

However, for hair analysis or any other type of analysis to be successful in furthering drug deterrence, it must enjoy the confidence of both the scientific community and the public. Thus, in 1986 several studies began that were designed to validate hair analysis as a useful forensic tool. The results of these initial studies were unexpected in that cocaine rather than benzoylecgonine (BZE) was present in cocaine users' hair, and phencyclidine (PCP), introduced from the external environment, was not readily removable (Kidwell 1988, pp. 1364-1365). As a result of these early studies, alternative explanations of the incorporation of drugs into hair were proposed (Blank and Kidwell 1989; Kidwell and Blank 1990; Kidwell 1992). These explanations hypothesized that drugs were incorporated into hair via sweat, and that if this were so, external contamination had to be considered in data interpretation. To our knowledge, sweat as a mechanism of transfer of drugs of abuse into hair was not considered before these reports. This concept has since polarized the hair-testing community. More

recent findings from the authors' laboratory, and findings of other investigators, have given support to this early contention (Blank and Kidwell 1993; Henderson et al. 1993).

Two theoretical positions have evolved to explain the incorporation of drugs into hair. The more prevalent model (figure 1a) proposes that drugs are incorporated into regions of hair from the bloodstream during the hair formation phase. These regions then become inaccessible after the hair matures. An alternative model (figure 1b) states that only minimal incorporation from the bloodstream occurs during the hair matrix formation process and that the major source of drugs in hair comes from drug-laden sweat or sebaceous excretions that bathe the hair shaft late in the hair formation process or after the hair matures. This latter model predicts that few or no regions in the hair are inaccessible to the external environment.

Other investigators have come to similar conclusions about the large role that environmental exposure can play in the incorporation of materials into hair. Chatt and Katz (1988, pp. 14-16, 77-81) discuss several fundamental assumptions concerning the incorporation of metal ions into hair and the sample preparation methods to reduce environmental contamination of metals. Testing hair for metal ions requires great care to exclude external contamination, and some authors believe that a substantial fraction of metal ions present in hair come from an external source such as sweat (Hopps 1977).

This chapter discusses the experimental evidence in light of the models cited above and the resultant consequences of the models for the interpretation of hair analysis data. It also addresses three major issues: (1) the mechanisms by which drugs are incorporated into and removed from hair, (2) considerations during the analysis of drugs in hair, and (3) interpretation of data within a theoretical framework.

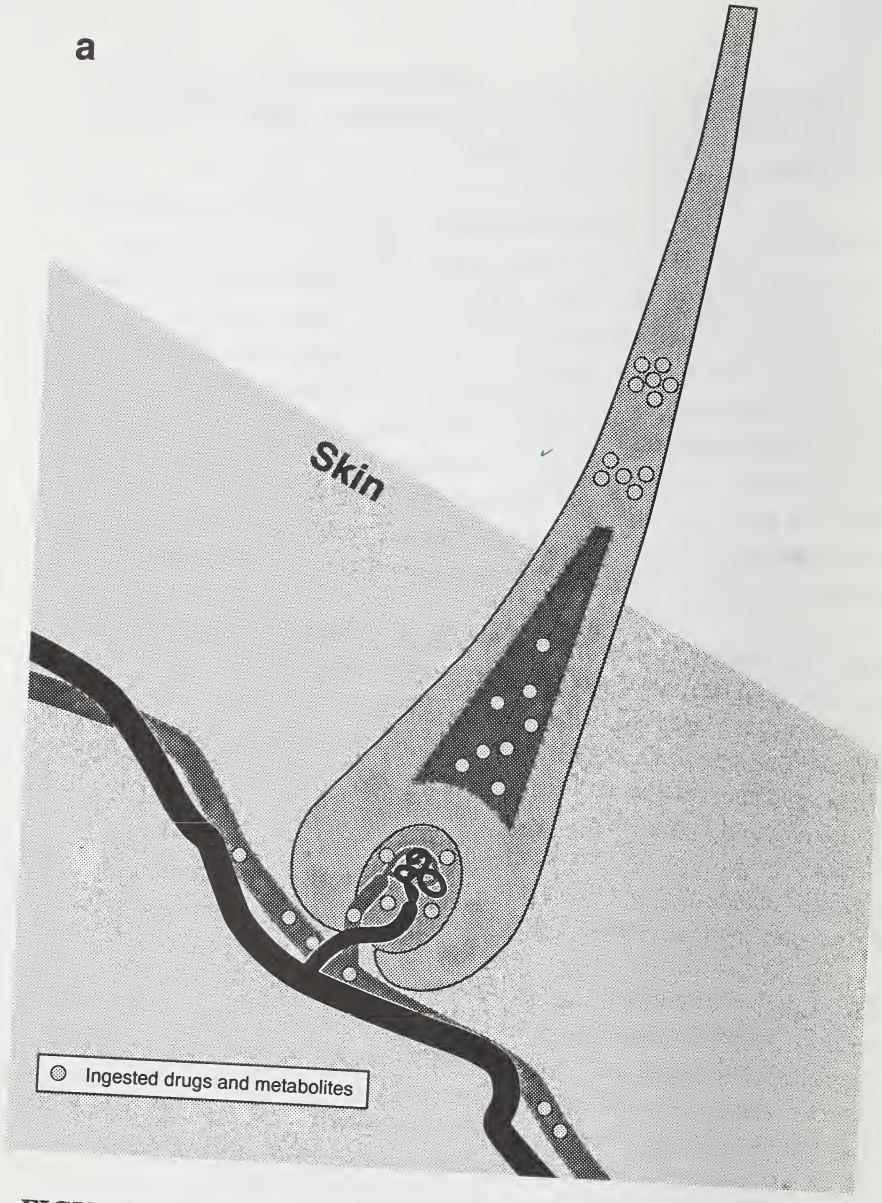

FIGURE 1a. *Prevalent model of bloodstream during drug incorporation*

SOURCE: Adapted from Holden, C., Hairy problems for a new drug testing method, *Science*, 249, 1099-1100, Sept. 7, 1990. Used with permission, ©AAAS.

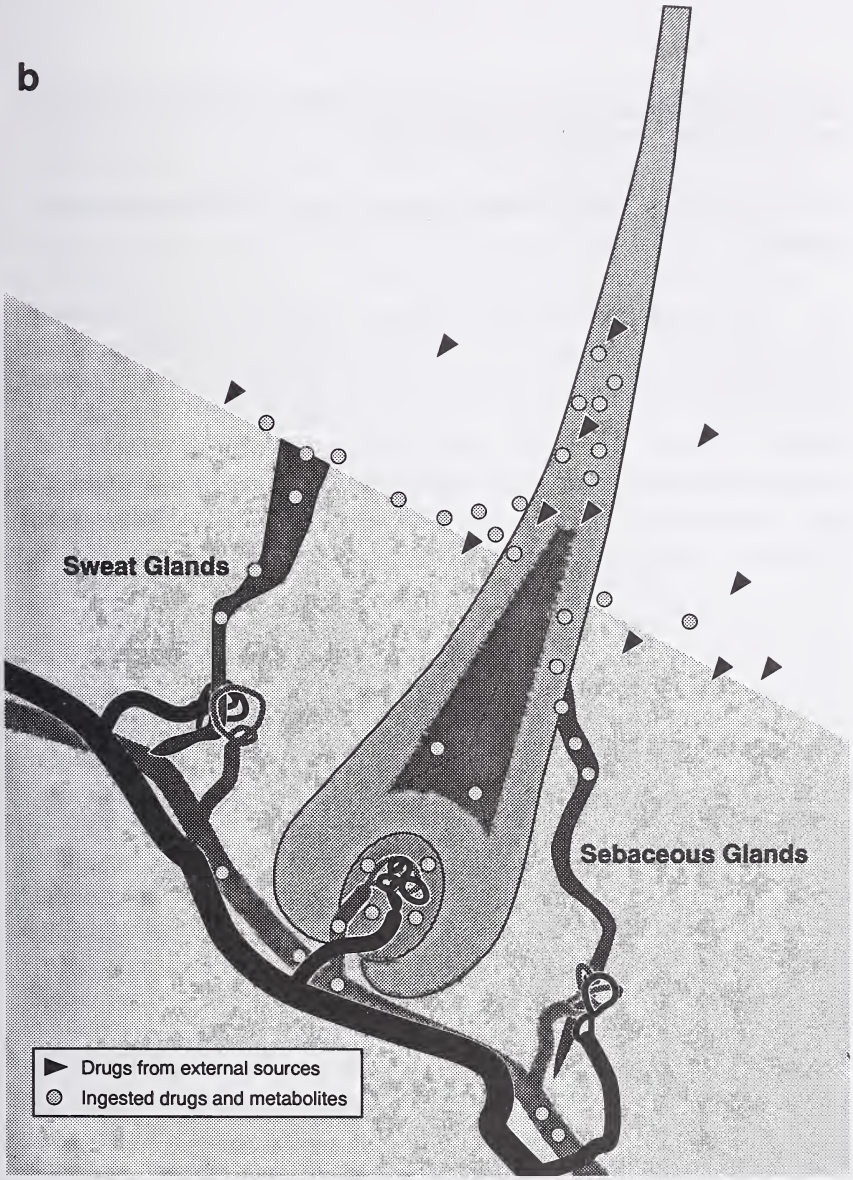

FIGURE 1b. *Alternative model of drug incorporation from multiple sources*

SOURCE: Adapted from Holden, C., Hairy problems for a new drug testing method, *Science*, 249, 1099-1100, Sept. 7, 1990. Used with permission, ©AAAS.

MECHANISMS BY WHICH DRUGS ARE INCORPORATED INTO AND REMOVED FROM HAIR

Can Exogenously Applied Drugs Be Incorporated Into the Hair Matrix?

Yes. Some evidence suggests that drugs deposited as a solid phase, such as crack cocaine smoke, can be successfully removed by certain decontamination procedures (Koren et al. 1992; Baumgartner and Hill 1992, pp. 577-597). In contrast, Welch and colleagues (1993) evaluated several decontamination procedures to remove cocaine placed on the hair in the solid form and found that none were successful in removing all external contamination. Also when Kidwell and Blank (1992, pp. 555-563) exposed hair to PCP vapors, large amounts of PCP could be removed by washing, but large amounts of PCP still remained in the hair when it was analyzed. Moreover, they observed a greater concentration of PCP in these passive exposure experiments than was found in analyses of users' hair. Similarly, Cone and colleagues (1991) found that substantial quantities of cocaine externally applied in an aqueous media could be removed if careful attention was paid to the experimental details of decontamination. Henderson and coworkers (1993, pp. 1-69) reported that a single exposure of hair to cocaine in vapor form (crack cocaine smoke) resulted in substantial amounts of cocaine in the hair extract, even after extensive washing. This study found that 149 to 526 ng cocaine/10 mg hair could be incorporated into the hair matrix by cocaine vapor exposure. These quantities compared with the upper range of amounts for cocaine users (Cone et al. 1993; Mieczkowski et al. 1991; Harkey et al. 1991; Fritch et al. 1992). Henderson and colleagues (1993) performed their experiments in two ways: (1) hair analysis after exposure but without decontamination and (2) hair analysis after exposure and decontamination using various washing procedures. In the unwashed hair, the amounts of cocaine were relatively constant between hair types. However, in the washed hair, substantial variations in amounts of cocaine occurred, suggesting that the effectiveness of incorporating drugs from the external environment may depend on hair type.

Drugs placed on the surface of hair must have some mechanism for entry into the hair matrix. If a solid is placed on the hair surface, most of it can be removed. However, after deposition of the solid drugs onto the hair surface, hair will be bathed at some point in an aqueous media—sweat, sebum, normal hygienic solutions, or the hair analysis procedure. An individual also could come in contact with a solution of a drug by transfer of sweat from another individual. For these reasons, the authors and other researchers have studied solution-phase transfer as the vector for admission and incorporation of drugs into hair.

When drugs are applied in the aqueous phase, they appear to be incorporated into the hair matrix and are difficult to remove by either normal hygienic washing or laboratory procedures. Kidwell (1988, pp. 1364-1365; 1992, pp. 98-120) and Kidwell and Blank (1990) have shown that hair readily adsorbs drugs from an aqueous solution. This work was extended using fluorescently labeled drugs (Kidwell and Blank 1992, pp. 555-563) and, more recently, radioactive tracers (Blank and Kidwell 1993). The concentration of drug in the applied aqueous media can be small. Henderson and coworkers (1993, pp. 1-69) have shown that hair soaked in a solution of cocaine at the 15-ng/mL level and repeatedly washed can produce a measurable amount of cocaine in hair. Other authors (Cone et al. 1991) have suggested that much of the cocaine applied externally can be removed. However, Cone and colleagues' (1991) work was based on experiments exposing the hair to a solution of cocaine at 1 mg/mL for 24 hours. Based on experience from the authors' laboratory, a solution of this concentration vastly exceeds that necessary to incorporate substantial quantities of cocaine into hair. In their experiments, Cone and colleagues (1991) may have reached a saturation point of the hair matrix for cocaine and thereby shifted the amount removed to much higher values.

In recent work with radioactive tracers, Blank and Kidwell (1993) developed a simple technique to monitor incorporation and removal of cocaine from hair, thus permitting them to perform many

experiments under a variety of conditions and with high precision.[1] The use of this technique allows the quantity of cocaine in various wash steps to be carefully monitored, and regardless of the decontamination technique, a substantial amount of drug could still be found in the final hair digest. These data were highly reproducible (figure 2).

The difference between figure 2 and data previously reported (Blank and Kidwell 1993) is that several hair prewashes were introduced prior to hair analysis. The contaminated hair was washed several times with distilled or tap water to mimic normal hygienic practices after hair contamination. Nevertheless, these preliminary decontamination procedures did not result in substantially different amounts of drug in the final hair digest from that previously reported for samples not subjected to the prewash steps.

Can Exogenously Applied Drugs Mimic Drug Usage?

Yes. To compare the extraction kinetics of drugs from external sources with those of endogenous drugs, hair from drug users was exposed to various drug analogs. A sample of hair from two cocaine users was exposed to an aqueous solution of p-bromobenzoylcocaine (a derivative of cocaine) at 10 μg/mL for 1 hour. It was then rinsed, air dried, and extracted according to procedures described in the literature available at that time for cocaine hair analysis (Baumgartner et al. 1982). Cocaine and p-bromobenzoylcocaine were quantitated in all solutions by gas chromatography/mass spectrometry (GC/MS), and the results are shown for an intravenous cocaine user (figure 3) and a crack cocaine smoker (figure 4). The washout kinetics for the cocaine and the

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[1]A possible criticism of the use of radiotracers for monitoring the uptake and release of cocaine could be the chemical reaction of the tracer with the hair matrix. A substantial chemical reaction is unlikely for two reasons. First, the radiotracer never exceeds 1 percent of the unlabeled drug. Therefore, *preferential* binding of the radiotracer is doubtful. Second, hair exposed to drugs without the radiotracer and analyzed by GC/MS shows the parent drug present.

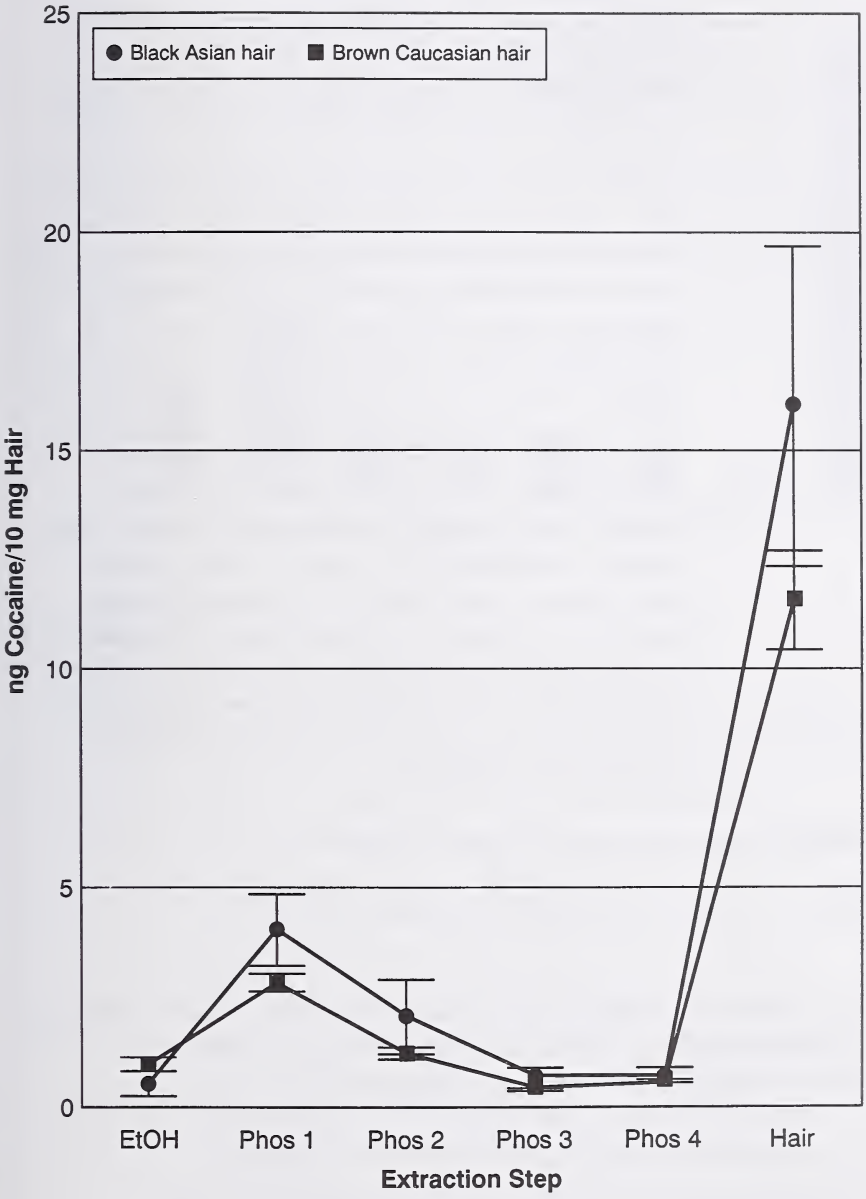

FIGURE 2. *Reproducibility of the authors' radiotracer technique for external drug exposure. Six samples each of two*

types of hair, black Asian and brown Caucasian, were exposed to 1 μg/mL cocaine in phosphate-buffered saline containing $1x10^6$ counts per minute (approximately 9 ng) 3,4-^{3}H-benzoylcocaine at pH 7 at 37 °C for 1 hour. The hair was rinsed three times with distilled water to remove most of the exposure solution and one time with ethanol to partially dry the hair. The hair was dried overnight under a vacuum. General decontamination and analysis procedure: The hair was extracted by shaking at 100 revolutions per minute in a water bath for 30 minutes at 37 °C in each solution shown in the figure. The hair then was digested with 0.25 M sodium hydroxide for 1 hour at 100 °C and was cooled; the digest was transferred to 20-mL scintillation vials; and a 15-mL Aquasol 2 universal liquid scintillation cocktail was added. Samples were counted in a scintillation counter using standard instrumental procedures. Blank samples were counted to determine the background counts, which were minimal for most analyses. This general decontamination and analysis procedure was followed for most of the radioactive experiments described in this chapter. In some cases, only three phosphate washes were employed.

NOTE: Error bars are ±1 standard deviation.

KEY: EtOH=ethanol; Phos=phosphate buffer

externally introduced p-bromobenzoylcocaine were similar, implying that external contamination could mimic drug use even with a contaminating solution as low as 10 μg/mL.[2]

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[2]Note that although the hair was not dissolved in the drug extraction step, significant amounts of both cocaine and p-bromobenzoylcocaine were found in the final hair extracts. The quantity of p-bromobenzoylcocaine was 5.4 times greater in the hair sample than in the last phosphate wash (Phos 3). A ratio

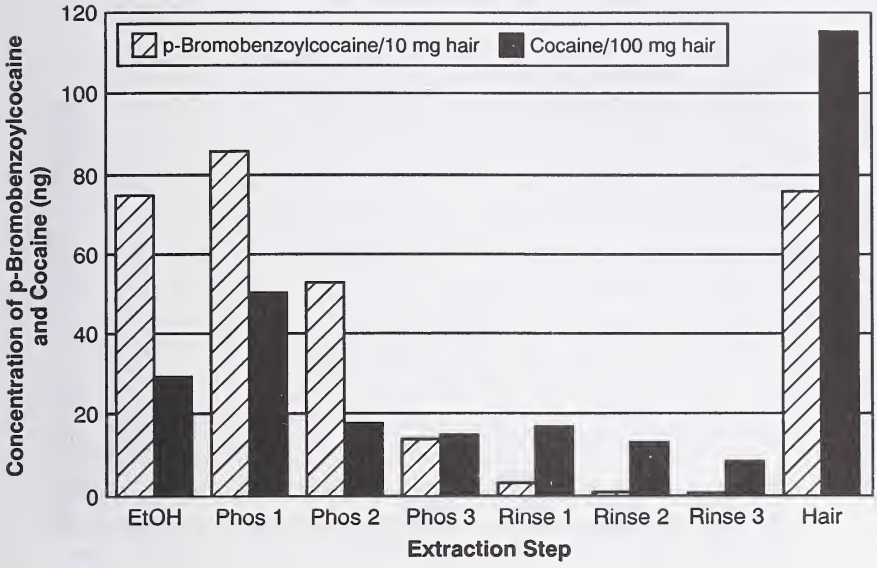

FIGURE 3. *Extraction profile of an intravenous cocaine user's hair exposed to a cocaine surrogate (p-bromobenzoylcocaine). After exposure to 10 μg/mL p-bromobenzoylcocaine, the hair was washed one time with ethanol, three times with phosphate buffer (pH 7), and three times with water; the cocaine remaining in the hair was extracted two times with 0.6* N *hydrochloric acid. The last two extracts were combined for the figure. The cocaine and p-bromobenzoylcocaine were quantitated in each solution by gas chromatography/mass spectrometry with selected ion monitoring. To display these data with a single ordinate scale, cocaine is expressed as ng/100 mg hair and p-bromobenzoylcocaine as ng/10 mg hair. Thus, the concentration of cocaine was approximately six times less than the cocaine surrogate.*

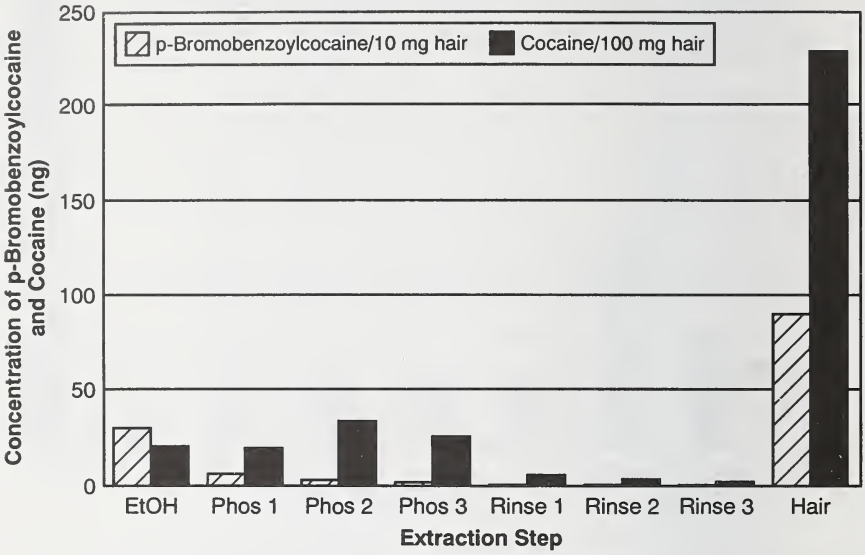

FIGURE 4. *Extraction profile of a crack cocaine smoker's hair exposed to a cocaine surrogate (p-bromobenzoylcocaine). The conditions and analysis procedure are the same as those in the figure 3 legend. In this figure, the scales are the same because of the higher concentrations of cocaine in this user's hair.*

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greater than 10 between the quantity extracted from hair and that in the last wash has been suggested (employing a different extraction procedure) to distinguish between active and passive exposure (Baumgartner et al. 1989). Using this factor, this cocaine user would not have been considered a user of p-bromobenzoylcocaine. Likewise, the ratio of cocaine in the hair to that of the third phosphate wash was 7.6:1; therefore, this individual also would not have been considered a cocaine user under this criterion.

The crack cocaine smoker's hair shown in figure 4 had substantially more cocaine at 229 ng/10 mg hair than did the hair of the individual depicted in figure 3, yet the quantities of p-bromobenzoylcocaine incorporated into the hair were similar (91 ng/10 mg hair). According to Baumgartner and coworkers (1989), an extract-to-last-wash ratio should be greater than 10 to ensure complete decontamination. The ratio of the drug surrogate (p-bromobenzoylcocaine) in the hair to the third phosphate wash was 54:1, greater than the suggested ratio of 10. This individual would have been considered a user of p-bromobenzoylcocaine under this suggested criterion. Interestingly, the ratio of cocaine in the user's hair extract to the third phosphate wash was 8.9:1, less than the suggested ratio of 10, and therefore, this individual would not have been considered a user of cocaine. However, although the hair was extracted twice with hydrochloric acid (HCl), it was not digested; therefore, some residual cocaine or p-bromobenzoylcocaine could have remained in the hair matrix.

Analogous experiments were performed with PCP. Figure 5 shows an analysis of a PCP user's hair that was exposed to a solution of p-methylphenylPCP at a concentration of 1 μg/mL for 1 hour. The final extract contained 23 ng PCP/10 mg hair, whereas p-methylphenylPCP was found at a concentration of 120 ng/10 mg hair. Clearly, more of the drug surrogate was found than was PCP incorporated in vivo.

Are Drugs Present in Sweat, and Can They Be Incorporated Into Hair?

It appears so. Drugs in sweat have been found by several researchers. For example, Balabanova and Schneider (1990) found that as much as 9.2 μg/cm^2/24 hours of cocaine could be deposited in the axillary region of a drug user. In a controlled-dose study using deuterated cocaine as tracers, Henderson and colleagues (1993, pp. 1-69) measured concentrations of the deuterated cocaine at levels as high as 50 μg/mL in sweat 1 hour after intranasal administration of cocaine at 0.6 mg/kg. Others have found varying amounts of amphetamine and dimethylamphetamine in sweat after low doses of the drugs were administered (Ishiyama et al. 1979). The concentrations of cocaine, BZE, and PCP analogs employed in

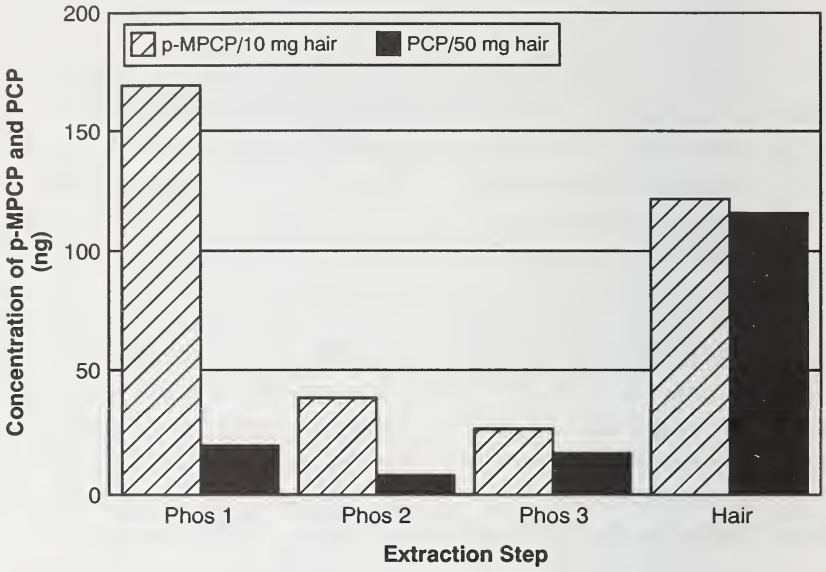

FIGURE 5. *Extraction profile of a PCP user's hair exposed to the PCP surrogate p-methylphenylPCP (p-MPCP). The conditions and analysis procedure are the same as those described in the figure 3 legend. Only the phosphate extractions are shown. To display these data with a single ordinate scale, PCP is expressed as ng/50 mg and p-MPCP as ng/10 mg. Thus, the concentration of PCP from the user's ingestion was approximately five times less than p-MPCP.*

the in vitro studies described in this chapter were well within the range found in sweat and were incorporated into the hair matrix after only short exposures.

The hypothesis that sweat was a vehicle for the transfer of drugs into hair became more apparent after examining hair from a male Korean who had used medicinally prescribed dihydrocodeine (Kidwell and Blank 1992, pp. 555-563). This individual had taken a total of 135 mg dihydrocodeine for 1 week after surgery. One week after the dihydrocodeine ingestion ceased, the hair was cut 2 to 4 cm

distal from the scalp to avoid collection of hair that could have grown during the period of administration of the dihydrocodeine. The subject did not undertake strenuous exercise or work during this 2-week period, and he washed his hair daily. Analysis by GC/MS showed dihydrocodeine at a level of approximately 0.4 ng/10 mg hair. Considering the small total dose, the amount of dihydrocodeine detected was high, particularly compared with such levels for most opiate abusers (1 to 20 ng opiates/10 mg hair).[3] Clearly, some mechanism other than deposition during hair growth contributed to drug incorporation in this hair sample.

A more conclusive experiment was performed by Henderson and coworkers (1993, pp. 1-69). Five volunteers were administered deuterated cocaine. Two hours later, these individuals held drug-free hair in their hands for 30 minutes. All the hair became contaminated with deuterated cocaine. The concentration of deuterated cocaine in these samples was as high as 478 ng/10 mg hair before decontamination of the hair samples and 109 ng/10 mg hair after decontamination. For one of the volunteers, Henderson and colleagues (1993, pp. 1-69) concluded, "Even after washing, the amount of deuterated cocaine in this externally contaminated hair sample was higher than that found in his hair when it was tested over the next few months."

Further evidence that sweat plays a role in the incorporation of drugs into hair can be gained from the timing of the administration and from the appearance of drugs in the hair. Cone (1990) and Püschel and colleagues (1983) reported that codeine could be

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[3]Püschel and coworkers (1983) reported levels of morphine of 15 to 18 ng/10 mg hair for medicinal morphine users and 0 to 32 ng/10 mg hair for opiate users. Offidani and colleagues (1989) reported morphine levels of 1 to 109 ng/10 mg hair for morphine users. Marigo and coworkers (1986) reported 1 to 150 ng/10 mg hair for 22 heroin addicts. Klug (1980) reported values ranging between 10 to 100 ng/10 mg hair for 10 drug-induced overdose cases. More recently, Kintz and colleagues (1993) reported levels of 6.2 to 270.1 ng/10 mg hair in 12 fatal heroin overdose cases.

detected in beard hair 24 hours after administration. A 5- to 7-day period would be necessary before the beard hair, which was forming during the period of maximum blood levels of codeine, emerged above the skin. Cone (1990) suggested that codeine secretion in sweat and its incorporation into hair could account for its initial appearance in beard hair.

The data suggest that, given the similarity between the in vitro studies reported here and those reported concerning sweat, drugs could be easily incorporated into hair from sweat prior to the hair's appearance above the surface of the skin.

Which Physicochemical Variables Affect Drug Incorporation Into Hair?

The amount of drugs incorporated into hair depends on a variety of factors: (1) The concentration of drugs in hair increases with concentration of the exposure solution and the time of exposure; (2) the concentration of drugs in hair decreases in the presence of competing cations such as sodium; and (3) the concentration of drugs in hair shows complex effects depending on the pH of the exposure solution and the chemical structure of the drug.

Concentration. The relationship between the concentration of cocaine in the exposure solution and the amount of cocaine in the final extract for two types of hair is shown in figure 6. Concentrations employed for this study were from 1 μg/mL cocaine plus radiotracer to 500 μg/mL cocaine plus radiotracer in phosphate-buffered saline (PBS), and all samples were soaked and dried as described in the legend for figure 2. Although figure 6 represents data obtained over a series of several experiments, the data are easily fitted to a linear plot. However, the amount of cocaine incorporated into hair varies depending on the hair type, reflecting a matrix bias that is explored in more detail below.

Time of Exposure. The association between the time of exposure to externally applied cocaine containing radiotracer solutions and the amount of cocaine incorporated in the hair is shown in figure 7. Increasing the duration of exposure increases the uptake of drug, although the relationship is not linear, especially for the black Asian

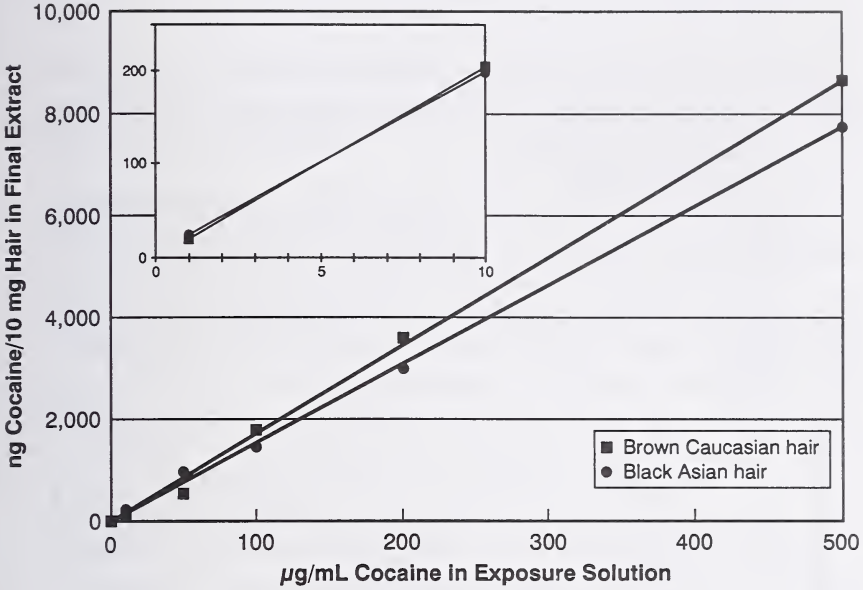

FIGURE 6. *Effect of changing the concentration of cocaine in the exposure solution on the incorporation of cocaine into hair. The inset expands the lower concentrations. Both types of hair were exposed to cocaine for 1 hour and were dried overnight before extraction as described in the figure 2 legend. No water rinses or ethanol predrying was used in this study.*

SOURCE: Blank and Kidwell 1993

hair sample. Even exposure times as short as 5 minutes produced specimens with detectable levels of drug. Exposure times greater than 1 day were attempted, but cocaine (and presumably the radiotracer) in the exposure solution decomposed substantially after 24 hours at 37 °C, affecting the concentration of cocaine in the soaking solution and therefore decreasing the amount of drug available for uptake. Furthermore, cocaine bound to the hair matrix

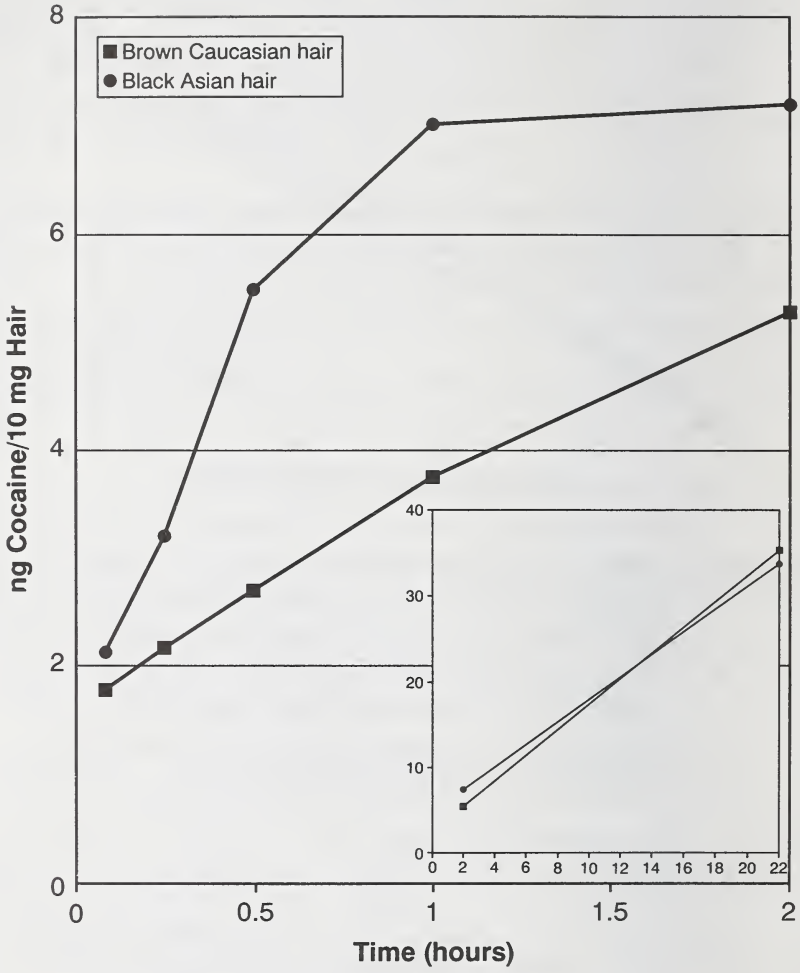

FIGURE 7. *Effect of increasing the exposure time on the incorporation of cocaine into hair. The inset displays the longer periods. Both types of hair were exposed to 1 μg/mL cocaine for varying lengths of time and were treated as described in the figure 2 legend. No water rinses or ethanol predrying was used in this study.*

during the early hours of exposure could be hydrolyzed,[4] resulting in conversion of the radioactive label to a material that does not bind well to the hair (see below).

Competing Cations. Figure 8 shows the effect of changing the concentration of competing cations in the externally applied cocaine solutions on the uptake of cocaine into hair. Cocaine plus radiotracer was added (final concentration 10 μg/mL) to each of four solutions with different sodium chloride concentrations ranging from zero to 500 mM. Two sets of samples were studied—each set containing either brown Caucasian or black Asian hair. The first set was dried after soaking and before decontamination/analysis for cocaine. The second set was carried directly through the decontamination procedure. As seen in figure 8, as the sodium ion concentration increases, the amount of drug incorporated into the hair decreases. What underlying mechanism could account for these data? If incorporation of drugs into hair is through ionic bonding, then increasing the concentration of cations in the soaking solution would increase the competition for available binding sites on the hair. Mass action would then dictate that the drug concentration would be lower.

The pH of the Exposure Solution and Charge State of Drug. The effect of changing pH on the uptake of tritiated cocaine and iodinated BZE from soaking solutions (1 μg/mL drug) is shown in figure 9. Raising the pH causes an increase in the incorporation of

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[4]The rate of degradation of cocaine depends on temperature and pH. Solutions of cocaine in PBS were incubated at 37 °C and pH 8.5. Samples were taken, concentrated to dryness, derivatized by silylation, and analyzed by GC/MS. The ratios after 1 hour were BZE to cocaine—40 percent; methyl ecgonine to cocaine—4.5 percent; and ecgonine to cocaine—1.9 percent. The ratios after 2 hours were BZE to cocaine—115 percent; methyl ecgonine to cocaine—9.1 percent; and ecgonine to cocaine—7.3 percent. If the cocaine incorporated into the hair were similarly affected, most of the radioactive label (cocaine, 3,4-^{3}H-benzoylcocaine) would have been converted to radioactive BZE or radioactive benzoic acid, which should bind poorly to hair.

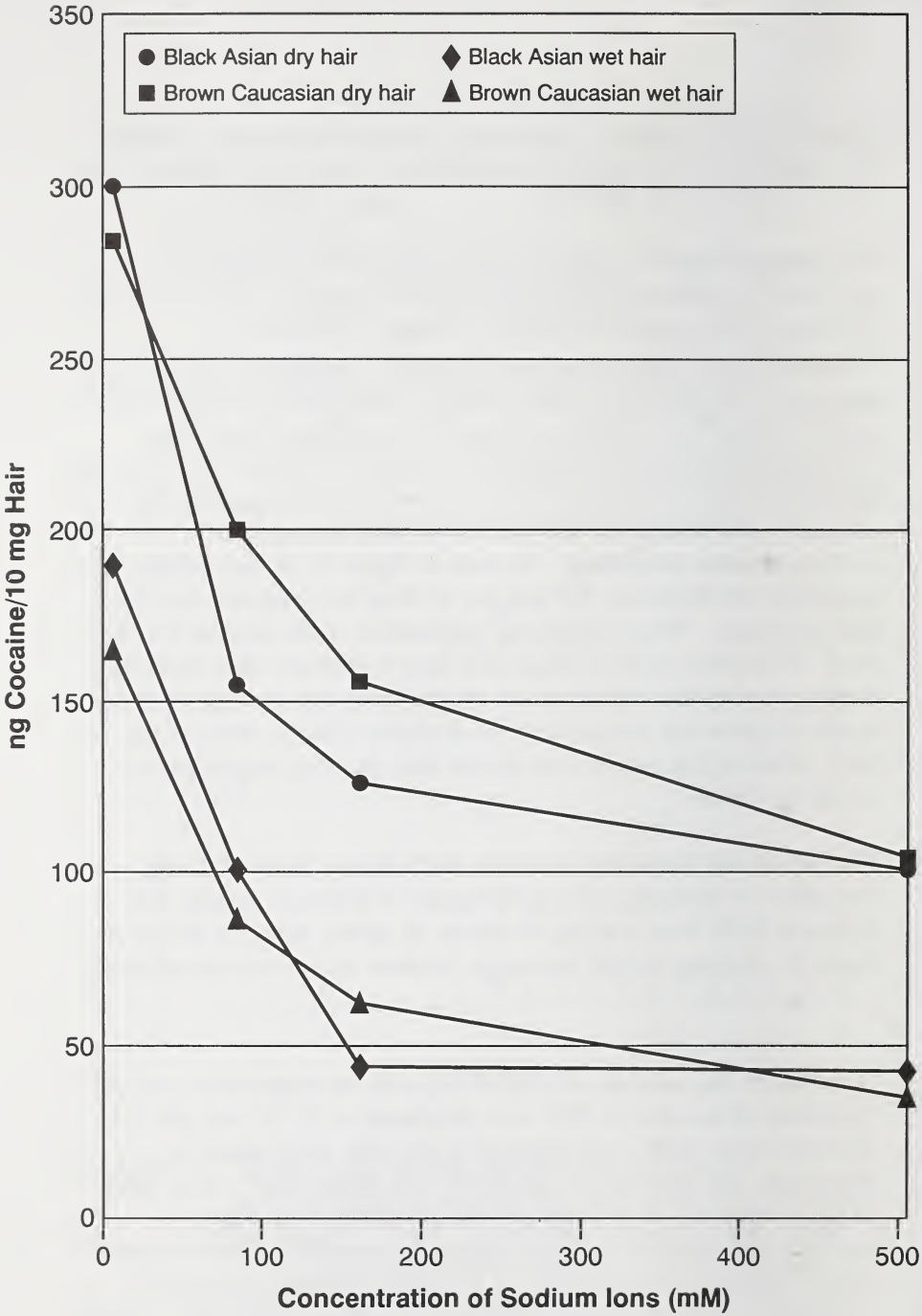


FIGURE 8. *Effect of changing the concentration of cations in the exposure solution on the incorporation of cocaine into*

hair. The hair was exposed to cocaine (10 μg/mL) and treated as described in the figure 2 legend, except that phosphate-buffered saline was replaced by phosphate buffer. The final concentration of sodium ions included the contribution from the buffer (10 mM phosphate, pH 5.6). Two sets of samples were employed for this study, each containing either brown Caucasian or black Asian hair. The first set was dried prior to decontamination and analysis for cocaine, and the second was carried directly through the decontamination procedure.

SOURCE: Blank and Kidwell 1993

cocaine into hair but a slight decrease in the incorporation of BZE, both followed by radiotracer analysis. The ratios of cocaine to BZE were as follows:

Concentration	Black Asian Hair	Brown Caucasian Hair
pH 5.3	2.3:1	1.6:1
pH 7.0	7.5:1	6.1:1
pH 8.5	15.8:1	21.5:1

No correction was made for the significant hydrolysis of cocaine in the exposure solution at pH 8.5 (see endnote 4). If this correction were made, the amount of cocaine incorporated would be increased. Cocaine (at pHs below 7) and BZE (at all pHs used) did not substantially degrade under these exposure conditions.

A model for incorporation of drugs into hair that is consistent with the effects of competing ions and pH is shown schematically in the three panels of figure 10. Hair contains three major functionalities whose characteristics can vary as a function of pH (i.e., side chains of aspartic acid, side chains of glutamic acid, and sulfate groups produced by oxygen degradation of the amino acid cysteine). The sulfate groups would be expected to be more prevalent in damaged or chemically treated hair, as supported by the observation of Pohl and coworkers (1985) that the isoelectric point of hair decreases

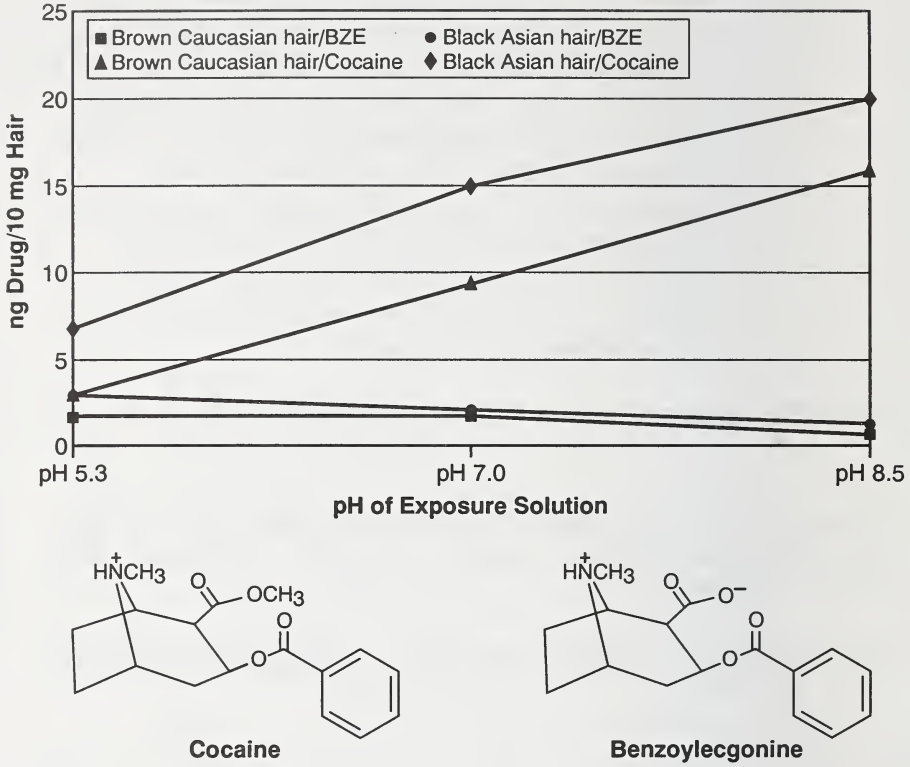
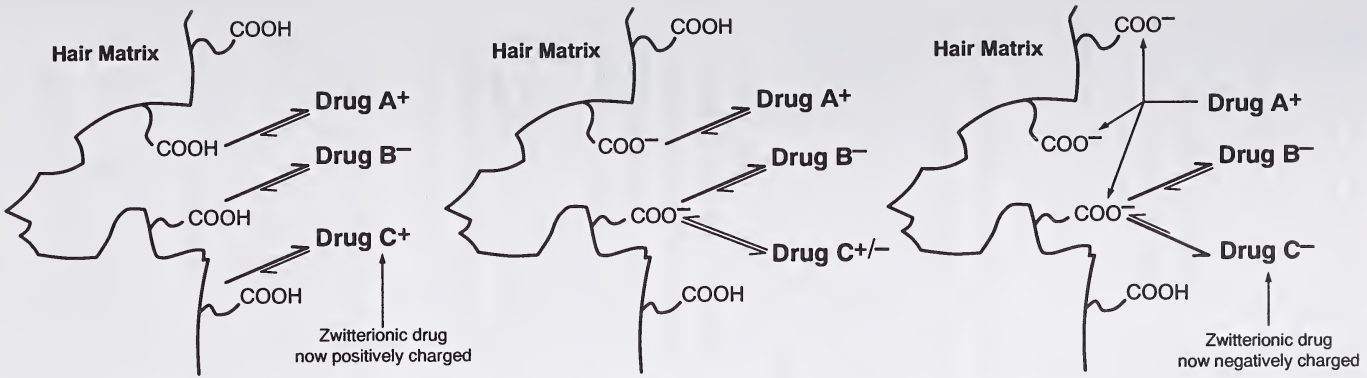


FIGURE 9. *Effect of changing pH on the uptake of tritiated cocaine and iodinated benzoylecgonine (BZE) from exposure solutions (1 μg/mL drug). The hair was exposed to cocaine and treated as described in the figure 2 legend, except that phosphate-buffered saline (PBS) was replaced by an equal mixture of 0.1 M phosphate buffer and PBS. On a nonradioactive control, the pH of the resultant solution was determined with a pH meter. The charge states of cocaine and BZE are shown over the pH range for these data.*

when the hair is damaged or when it ages. Hair has an isoelectric point of approximately pH 4. At this acidic pH, drugs could bind via weak hydrophobic interactions, as depicted in figure 10a. Thus,



a pH<4: Hair predominantly neutral; drugs bind via hydrophobic actions.

b pH 7: Hair negatively charged. Some drugs become zwitterionic; drugs bind or are repelled via ionic interactions.

c pH 9: Hair more negatively charged. Some drugs become negatively charged; drugs bind or are repelled via ionic interactions.

FIGURE 10. *A model of drug incorporation into hair consistent with the effects of competing ions and pH. Cocaine is represented by drug A. Biotin is represented by drug B. Benzoylecgonine is represented by the zwitterionic drug C, which would be positive, negative, or neutral depending on pH. Likewise, any of the compounds, drugs A through C, could represent a competing cation that could occupy a binding site on the hair and block a drug molecule from binding.*

drugs should bind equally well at low pHs provided they are similar in hydrophobicities. At pHs greater than the hair's isoelectric point, the side chains of aspartic and glutamic acids are deprotonated, and hair becomes negatively charged, as shown in figure 10b; therefore, the more positively charged the drug, the greater the likelihood of binding to hair. At still higher pHs, more side-chain carboxyl groups are deprotonated, and hair becomes increasing negatively charged, as shown in figure 10c. There are two effects that a change in pH can produce: (1) a change in charge state of the hair and (2) a change in charge state of the drug.

The exact charge state of the drug is complicated by zwitterionic species, such as BZE (see structure in figure 9), because their charge state changes from positive to negative depending on pH. Cocaine has a pK_b of 8.4 to 8.69 (Polášek et al. 1992) and should remain predominantly positively charged below pH 8.7. Cocaine is represented by drug A in figure 10. As modeled in figure 10, when the pH of the exposure solution is increased, more negatively charged sites are produced for binding of positively charged materials. Consequently, more cocaine should attach to the hair, because it would be attracted to a larger number of binding sites. On the other hand, BZE (pK_a about 3) would be zwitterionic within the pH range given in figure 9. As the pH of the soaking solution increases and the hair becomes more negatively charged, the zwitterionic BZE (represented by drug C in figure 10) would be repelled from the hair matrix, and less should bind. Also, because BZE is relatively hydrophilic, binding via hydrophobic interactions is not likely.

Negatively charged drugs (drug B in figure 10) should bind poorly to the hair matrix. In this model, a negatively charged drug, such as biotin (pK_a about 3.5), would be repelled from the hair matrix at all pHs studied and would bind poorly to hair compared with cocaine. The binding of biotin was compared with the binding of cocaine, both followed by radiotracer analysis. When hair was exposed to 1 μg/mL biotin at pH 7 in PBS for 1 hour, little was incorporated into hair—only 12 percent as much biotin as cocaine for black Asian hair and 4 percent for brown Caucasian hair. Likewise, the observations that hair from marijuana users contains

only trace quantities of negatively charged THC-carboxylic acid[5] and that aspirin (negatively charged, $pK_a=3.49$) also binds poorly to hair (Harrison et al. 1974*a*) would be consistent with the model presented here.

The preferential binding of drugs to the hair matrix varies with pH and therefore may be dependent on the pH of sweat, which varies considerably between individuals and increases with the flow rate (Lentner 1981). An average pH of 5.82 (Lentner 1981) has been reported for resting individuals and a range of 6.1 to 6.7 (Doran et al. 1993) for moderate exercise, but a pH of 4.5 also has been observed (Vree et al. 1972). The average ratio of cocaine to BZE in sweat is approximately 4.4:1 (D. Schoendofer, unpublished data). The ratio of cocaine to BZE in hair extracts varies among studies. By analyzing data provided from Cone and colleagues (1991), it is possible to calculate an average ratio of 6.7:1. Examination of the data of Henderson and coworkers (1992) yielded an average ratio of 7:1 before decontamination washing and 5.4:1 after washing. Assuming the sweat of these individuals was near the average pH of 5.8, one could calculate from the in vitro data given in figure 9 that the cocaine-to-BZE ratio in hair should be between 1.6:1 and 2.3:1. This ratio would depend on the hair type and assumes that the sweat contains equal concentrations of cocaine and BZE. Because sweat does not contain equal amounts of cocaine and BZE, the ratios must be adjusted by the drug ratios found in sweat.[6] Accordingly, when these adjustments are made, the ratio of cocaine to BZE in hair should be approximately 7 to 10:1, if sweat is the major contributor of drugs appearing in hair. Given the uncertainties of drug ratios in

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[5]Hayes and coworkers (1991) reported that in 200 samples of hair, the average value of THC-carboxylic acid was 4 pg/10 mg hair digest, which appeared to be substantially less than the average value of 6 pg/10 mg hair when the melanin fraction was examined.

[6]This calculation is as follows: (cocaine/BZE) in sweat from Schoendofer × (cocaine/BZE) incorporation ratio from our study = predicted cocaine/BZE ratio in hair extract. The average ratio of cocaine to BZE calculated from the data given by D. Schoendofer (unpublished data) was 4.43:1.

sweat, sweat pH, and natural biovariability in all the measurements, this calculated value of 7 to 10:1 comes remarkably close to the ratios observed by others of 6.7 to 7:1.

The model of ionic binding also was evaluated by comparing the binding to hair of fluorescein and rhodamine, two molecules of similar shape, size, and fluorescent quantum yield. Rhodamine, which is positively charged, is far more efficient at binding to hair than fluorescein;[7] this dramatic difference is seen in figure 11 for hair soaked in 10 μg/mL of each material. The disparity in binding of materials to hair is well known in the cosmetic industry. Goddard (1987) found that negatively charged hair care products bind poorly to hair, whereas positively charged materials bind tightly. These results should hold equally well for drugs of abuse as they do for hair care products.

Is There a Dose-Response Correlation for Drugs of Abuse?

Sometimes. In a data set from 1986, the authors observed poor correlation between self-reported drug use histories of military members in drug treatment and the amount of drugs found in hair (Kidwell and Blank 1992, pp. 555-563); selected data are shown in table 1. For example, one individual with black hair indicated use of approximately 250 mg cocaine on only one occasion, and yet 6.4 ng BZE equivalents/10 mg hair were found in the hair (as measured by radioimmunoassay [RIA]). In contrast, an individual with brown hair who also indicated a use of approximately 250 mg cocaine on five separate occasions had cocaine below the detection limit in his hair. The detection limit for cocaine in these studies was approximately 1 ng/10 mg hair (W. Baumgartner, personal communication, June 1983).

More recently, we reanalyzed data collected by Baumgartner and Hill (1991; in press). The selected data set contained a sufficient number of subjects (n=45) for statistical analyses. The correlation

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[7]Alternatively, merbromin (a mercury derivative of fluorescein) binds tightly to hair, presumably by covalent bonds with the sulfur residues rather than through ionic or cationic mechanisms.

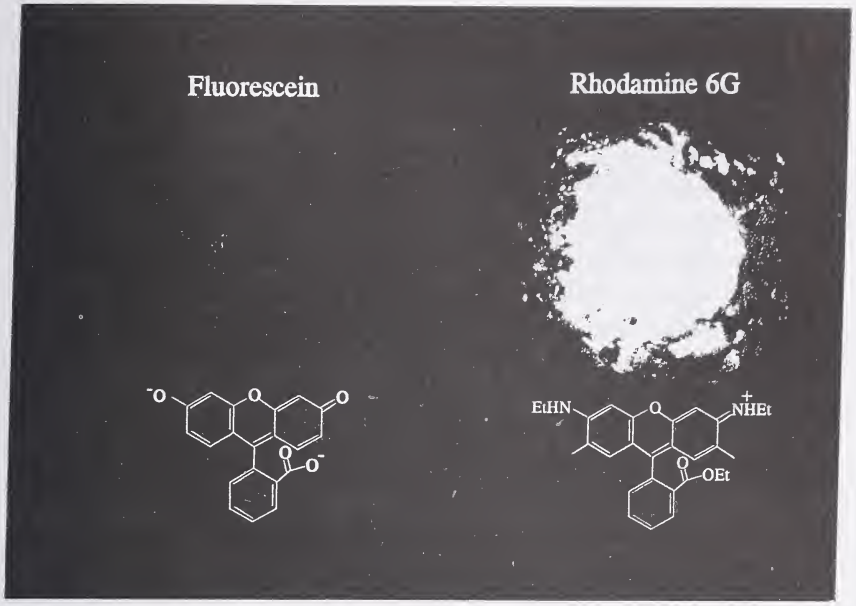

FIGURE 11. *The binding of fluorescein and rhodamine 6G to brown Caucasian hair. Brown Caucasian hair was exposed to either fluorescein or rhodamine (10 μg/mL) in 10 mM phosphate, pH 5.6 at 37 °C for 1 hour. The excess was rinsed off, and the hair was dried, mounted, and sectioned. The sections were photographed using a fluorescent microscope with appropriate excitation and barrier filters selected for each fluorescent compound. The exposure times were constant to allow comparison of fluorescent intensities. The photographs were scanned into a computer, converted to a gray scale, and the gamma curve adjusted to reflect the intensities of the image in the original photograph. The structures of fluorescein and rhodamine appear beneath the computer-generated illustrations of the respective hair specimens. The dyes are similar in shape and structure, although they vary in charge.*

coefficient between cocaine found in hair and self-reported use was 0.59 and was significant at $p < 0.01$. The regression line for this

TABLE 1. *Comparison of cocaine present in different hair types**

Black Hair		Brown Hair	
Use	Amount Detected BZE Equivalents (ng/10 mg hair)	Use	Amount Detected BZE Equivalents (ng/10 mg hair)
.25 g once	6.4	.25 g once	0
4-5 g total	3.5	.25 g 5 times	0
100 mg/week	2.2	2-4 g total	2.2
500 mg/week	18	5 g total	0
		250 mg/week	0
		250-500 mg/day	20

*Data were extracted from an unpublished U.S. Navy-sponsored study. Quantity of drug in hair was measured by radioimmunoassay and converted to BZE equivalents.

KEY: BZE=benzoylecgonine

SOURCE: Kidwell and Blank 1992, pp. 555-563

data set yielded a Y intercept of 49 ng/10 mg hair. Statistically, the Y intercept would predict the quantity of the drug in hair given a zero dose. Therefore, a positive Y intercept could be interpreted in two ways: Either (1) individuals who use drugs underreport their use—if they had accurately reported their use, the regression line would pass through the origin—or (2) environmental exposure and incorporation of drugs occur in individuals exposed to cocaine, which increases the apparent amount of cocaine in hair. Although statistically significant, the data reported by Baumgartner and Hill (1991; in press) had substantial scatter, with only 35 percent of the variance accounted for by an assumption of a linear dose-response.

In a controlled-dose study with cocaine, Henderson and colleagues (1993, pp. 1-69) did not find a predictable relationship between the amount of cocaine administered and the amount of drug eventually incorporated into hair. Overall, the correlation between the mean amount found in hair and the administered dose was 0.03. Using a

drug surrogate, methoxyphenamine, Nakahara and coworkers (1992) found that both the quantity of the drug appearing in the hair and its location along the hair shaft correlated with ingestion. However, they also found poor correlations between results for individuals with identical dosages. In contrast, for haloperidol, Matsuno and colleagues (1990) found a good correlation ($r=0.83$, $n=59$) between the amount of haloperidol administered and the amount of haloperidol and its metabolite in hair. These studies suggest that the concentrations of drugs found in hair are not related simply to the quantity of drugs ingested but may vary depending on the structure of the drug and certain other factors.

Do Different Physical Classes of Hair and Hair Care Habits Affect Drug Uptake?

Yes. As indicated in table 1, for a convenience sample of drug users, there appears to be a difference in the amount of drugs found in hair of differing types. Several variables, such as hair texture and hair care habits, could account for these differences. The variability of drug incorporation along racial lines also has been observed by several authors (Cone et al. 1993; Henderson et al. 1993, pp. 1-69) for a limited number of individuals.

In an attempt to examine this critical issue, the in vitro uptake of drugs for different hair types was extensively studied (Blank and Kidwell 1995). Frequently, black Asian hair incorporated more cocaine (as measured by radiotracer techniques) from the external environment than did brown Caucasian hair; the results (shown in figure 12) make this difference apparent. Six hair samples of varying lengths from female subjects were exposed simultaneously for 2 hours to a solution of cocaine containing a radiotracer at a concentration of 5 μg/mL. To address some concerns about drug uptake at the cut ends, the ends were sealed in wax prior to soaking. The hair samples were washed five times with distilled water to remove the relatively high concentration of cocaine in the soaking solution, and the samples then were dried. After drying, each hair type was sectioned into pieces 1 cm in length; the ends sealed in wax were discarded. Then each section was weighed and individually decontaminated (according to the method described in the figure 2 legend) and the cocaine tracer measured by liquid

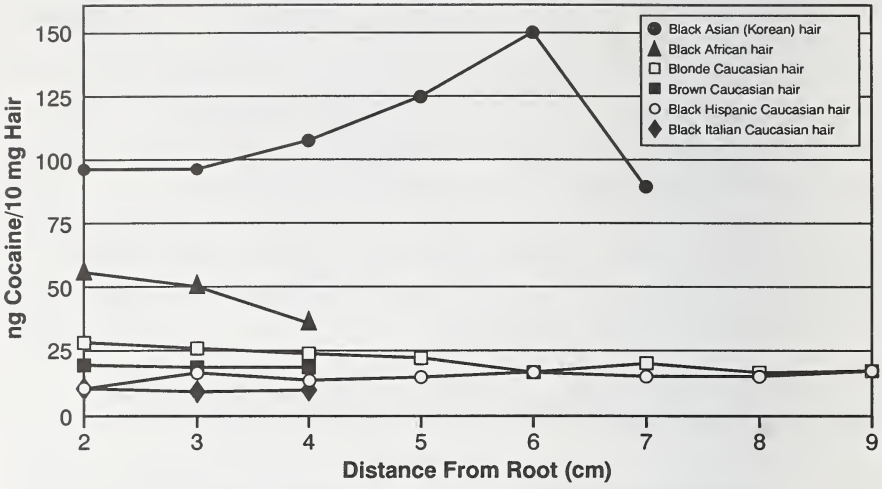

FIGURE 12. *The binding of cocaine to six different types of female hair*

SOURCE: Blank and Kidwell 1995

scintillation counting. The hair samples from the four Caucasian females showed relatively constant uptake of cocaine in all segments. The black African hair sample exhibited a decrease in uptake in the more distal segments, whereas the black Korean hair sample exhibited an increase in uptake in all but the last segment. The ratio of the average amount of cocaine incorporated into all segments of black African hair to all segments of Caucasian hair was 2.9:1, whereas the ratio of cocaine incorporated for all segments of black Korean hair to Caucasian hair was 6.8:1. There is no simple hypothesis that can account for these data. For example, the hair diameters of all segments were measured and were not related to cocaine uptake. Likewise, the Korean individual did not report any hair treatment other than normal hygiene. As suggested in the models outlined in figure 10, it might be expected that the charge state of the proteins of various hair types may be different. Studies are under way in the authors' laboratory to examine the effect.

The in vitro studies of Henderson and coworkers (1993, pp. 1-69) show a hair type bias similar to that depicted in figure 12. The rank order of cocaine incorporation into hair exposed to cocaine vapor parallels our data (i.e., the amount of cocaine incorporated into Asian hair > black African hair > Caucasian hair).

In real-life scenarios, both the uptake of drugs from the external environment and the loss of drugs incorporated into hair would be influenced by the individual's hair type and hair care habits. For example, drugs deposited into the hair matrix from external contamination or from sweat would be removed at rates influenced by normal hygiene and hair care. Both preliminary data collected from this laboratory and data reported in the literature (Purpura-Tavano 1992, p. 12) suggest that the frequency of hair washing is highly variable among individuals.

Although the in vivo studies cited above suggest bias among hair types (i.e., the differential incorporation or removal of drugs for different hair types at different doses), these studies either contained too few subjects to reach statistical significance or were based on self-reported use, which may be inaccurate. Mieczkowski and Newel (1993) have criticized such studies for these reasons. They compared self-reported use, urinalysis, and hair analysis data and found that hair analysis detected significantly more presumptive "drug users" than either urinalysis or self-report. They also compared the patterns of self-reported use with the patterns of urinalysis and hair analysis and concluded that there was no apparent bias among hair types. However, to observe bias, it is necessary to know whether an individual has used drugs and, if so, the dosage amounts. For example, if two individuals with different hair types consume the same amount of a drug and one is detected by hair analysis and the other is not, then a conclusion of bias could be drawn. This could occur if one type of hair incorporated the drug more readily, at higher levels, or released it more slowly.

Henderson and colleagues (1993, pp. 1-69), using a limited sample (n=14), calculated the half-life of drugs in hair and found that for Caucasians cocaine-d_5 (the administered tracer) had an apparent half-life of 2.1 months, whereas for non-Caucasians the half-life was 1.6 months. The observed difference in the half-life of cocaine may

not be accurate because of the limited number of subjects. The initial amounts of drugs must be considered with the half-life in determining the window of detection. Significantly, all the non-Caucasian hair samples originally had two to three times as much drug as the Caucasian hair samples, suggesting a longer window of detection for the small doses administered in this study.

Is Uptake of Drugs Different for Cosmetically Treated Hair?

Yes. As Baumgartner and colleagues (1989) noted, quantitation of drug levels in hair must be corrected for hair damage caused by cosmetic treatments. The authors studied the magnitude of the effect of damage for two types of hair. Hair was treated by exposure to Clairol Nice and Easy shampoo-in hair color (natural extra light beige blond) for varying lengths of time ranging from 20 minutes to 80 minutes. The manufacturer's instructions for this product noted an application time of 20 minutes for normal results. However, a customer may vary this application time or may perform several applications to achieve the color desired. Brown Caucasian hair was easier to color than was black Asian hair, as suggested by the manufacturer's literature and, therefore, was colored for the normal 20 minutes. Following cosmetic treatment, hair was exposed to a cocaine solution of 1 μg/mL plus radiotracer in PBS for 1 or 2 hours and carried through the normal prewashing and decontamination procedures noted in the figure 2 legend. These results are shown in figure 13. Two-hour exposure times resulted in greater incorporation of cocaine than did a 1-hour exposure for both hair types. The data reveal that for black Asian hair (13a), the longer the cosmetic treatment, the greater the incorporation of cocaine into the hair sample. In contrast, brown Caucasian hair that was cosmetically treated showed less uptake of cocaine than untreated hair (13b), as measured by radiotracer techniques.

Henderson and colleagues (1993, pp. 1-69) demonstrated an even more pronounced effect after a permanent wave solution was applied. In their studies, permed black Asian hair retained 82 ng/10 mg hair of cocaine after exposure to crack cocaine smoke compared with 22 ng/10 mg in brown Caucasian hair and 8 ng/10 mg in blonde Caucasian hair. These pronounced disparities among the quantities in the three types of hair must be considered with

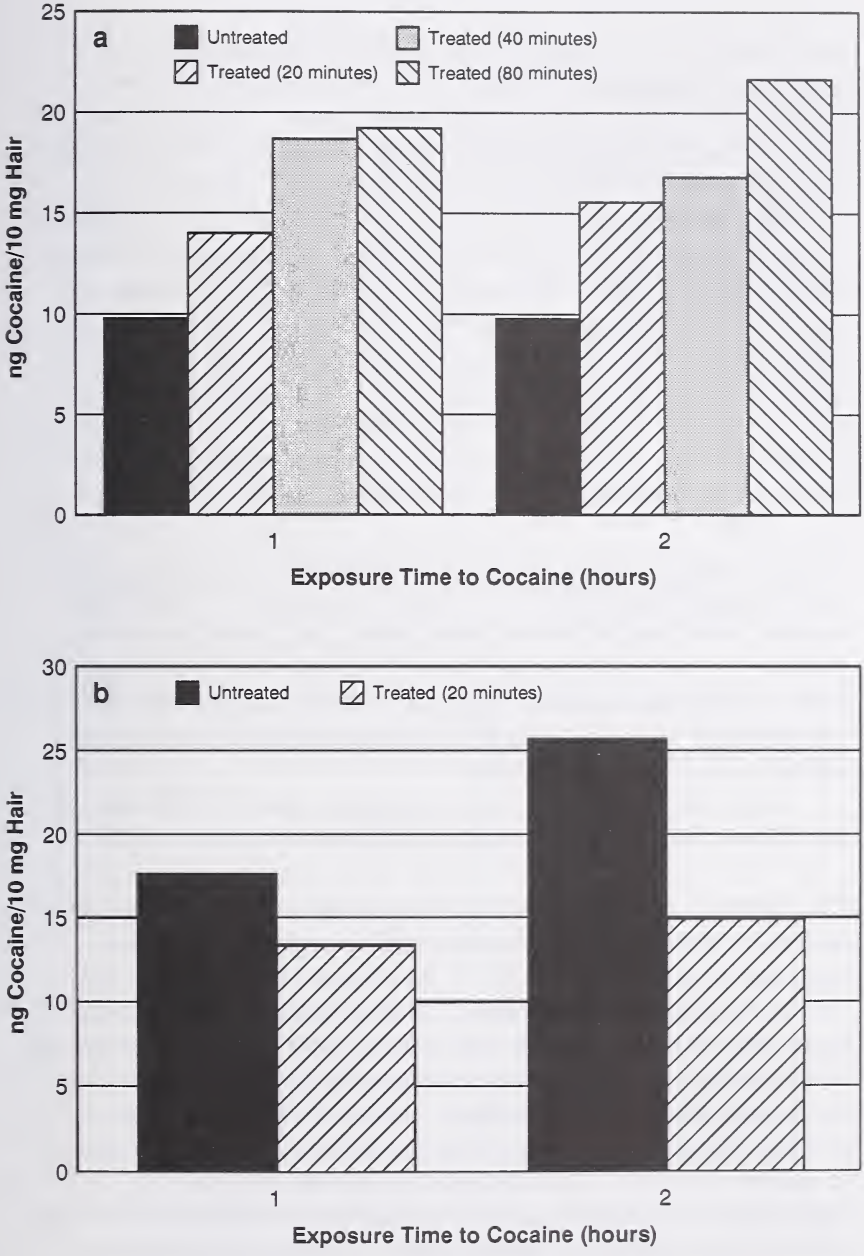

FIGURE 13. *Effect of cosmetic treatments on the incorporation of cocaine into two types of hair—(a) black Asian and (b) brown Caucasian*

caution because the different types of hair incorporated different amounts of cocaine before application of the permanent wave solution. The amounts of cocaine incorporated were as follows: black Asian, 526 ng/10 mg hair; brown Caucasian, 227 ng/10 mg hair; and blonde Caucasian, 149 ng/10 mg hair. However, the following percentage decreases from the untreated hair were similar: black Asian, 84 percent; brown Caucasian, 90 percent; and blonde Caucasian, 95 percent. Permanent wave solutions are among the most severe treatments to hair because they are basic and contain materials that break the disulfide bonds that give hair its texture. Consequently, perming solutions would be expected to cause more hair damage and loss of drugs than would various hygienic washes or hair coloring solutions. However, the percentage decrease in drugs removed is similar regardless of hair type.

Marques and coworkers (1993) also suggest the loss of drugs from the hair matrix as the result of cosmetic treatments. In studying cocaine in the hair of mother/infant pairs, they found that it was possible to account for an additional 21.6 percent of the variance in their data by eliminating "damaged hair" from the analysis. Unfortunately, by doing so, data for approximately one-half the sample pairs had to be dropped.

Are There Inaccessible Regions in Hair?

No. Several years ago, we began a series of experiments to determine whether there were inaccessible regions in hair that sequester drugs of abuse (Kidwell and Blank 1992, pp. 555-563). Two molecules were synthesized whose structures are shown in figure 14. PCP was labeled with a fluorescent compound to produce 5-dimethylamino-1-naphthalenesulfonic acid (DANSYL-PCP), and the control was DANSYL-aniline. DANSYL-PCP was synthesized to have only one potential cationic functionality, which was absent in DANSYL-aniline. The DANSYL group does contain a dimethylamino substituent, which if protonated when bound to hair would form a cationic ammonium salt. If this compound were retained in the hair, the DANSYL group would not be fluorescent.

Hair was exposed to solutions of these compounds, washed, embedded, and sectioned for microscopic observation. Although

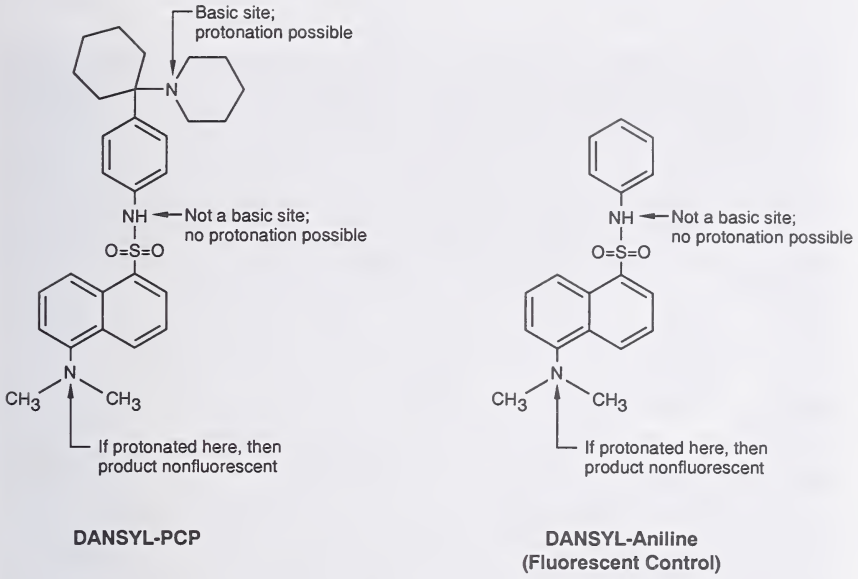

FIGURE 14. *Structures of fluorescently labeled DANSYL-PCP and DANSYL-aniline used in some exposure experiments*

KEY: DANSYL=5-dimethylamino-1-naphthalenesulfonic acid; PCP=phencyclidine

SOURCE: Kidwell and Blank 1992, pp. 555-563

hair soaked in a solution of DANSYL-aniline showed some fluorescence, its intensity was much less than that of the labeled PCP. This could indicate either that DANSYL-aniline did not bind as well as the labeled PCP or that, when it was bound, it was protonated and therefore not fluorescent. Because the source and history of the hair were known, it was clear that the hair was not damaged by bleaching or perming. Such damage could allow access of the fluorescent compounds to the interior. In many cross-sections of the hair shaft, the DANSYL-PCP was only on the surface; however, in a few cases the fluorescence was observed throughout the hair. In other sections, the fluorescence was both in the center and on the outside. These differential results were obtained on the

same strand of hair and did not indicate that the hair was resistant to penetration of the drug derivative (Kidwell and Blank 1992, pp. 555-563). Because a single strand of hair produced such varying results, even greater variability among hair samples from different individuals might be expected.

Further exploration of "inaccessible regions" by DeLauder and Kidwell (unpublished data) used fluorescent materials (rhodamine 6G and fluorescein) as drug surrogates. Eleven types of hair were exposed to these materials for varying times at 37 °C with varying concentrations of fluorescent compounds (1 to 10 μg/mL). The exposed hair was washed, mounted, and sectioned. Under a fluorescent microscope, cross-sections in almost every hair type showed incorporation of rhodamine throughout the hair. Some specimens required either longer exposure times or greater concentrations of fluorescent compound to incorporate it throughout the hair. A black Korean hair sample was the only one in which uniform rhodamine staining throughout the hair shaft was not achieved.

At low rhodamine concentrations, the rate of diffusion into the hair shaft was slow enough to be monitored. A sample of Caucasian hair exposed to rhodamine at 1 μg/mL is shown in figure 15. As exposure time was increased, more rhodamine reached the interior of the hair. At 120 minutes, although the surface was much brighter than the interior, substantial fluorescence was observed in the interior compared with unexposed hair or to hair exposed for 30 minutes.

These data suggest that, at least at the micron level (using × 20 or × 40 objectives), there are no inaccessible regions. Incorporation at the molecular level is not ruled out by these data, although stoichiometric considerations may suggest otherwise.[8] The issue

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[8]For example: Assume that one protein molecule can sequester one drug molecule. The molecular weight of most proteins is approximately 100,000 daltons and that of most drugs is approximately 300 daltons. Therefore, the molecular weight ratio of protein to drug is approximately 300:1. Further assume that

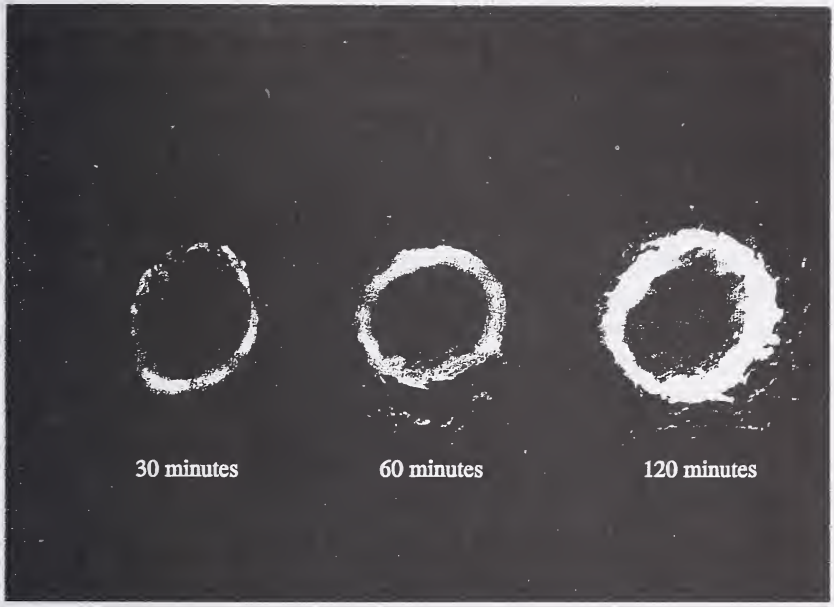

FIGURE 15. *Caucasian hair exposed to rhodamine 6G for varying lengths of time (30, 60, and 120 minutes). Following exposure to rhodamine (1 μg/mL), the hair was treated and the data processed as described in the figure 11 legend.*

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drugs are preferentially sequestered over other species in the blood at a 1,000:1 ratio. Cocaine has a maximum blood concentration of approximately 1 μg/mL blood or 1 ppm (parts per million). Thus, the maximum amount of cocaine that could be sequestered would be 30 ng/mg hair (1 mg hair[amount of protein] × 1,000[binding ratio]/(300[protein/drug ratio] × 1,000,000[1 ppm])), an amount substantially less than some drug users have in their hair. Furthermore, the authors believe that the preferential sequestration of drugs over other blood components is unrealistic, and dedication of that much protein for this purpose seems biologically unreasonable.

of inaccessible regions, which is critical for the theories of decontamination, continues to be studied in the authors' laboratory using different techniques and instrumentation.

Does the Hair Matrix Protect Previously Deposited Materials From Modification by the External Environment? Are Metabolites Adequate To Determine Usage?

No. In certain cases, such as absence of moisture, drugs have been shown to remain in hair for long periods (Springfield et al. 1993). However, this condition is unrealistic for living humans using normal hygienic practices. Nakahara and colleagues (1992) reported a loss of 50 percent of methoxyphenamine in five individuals after 5 months. Similarly, Henderson and coworkers (1993, pp. 1-69) showed a loss of 50 percent of deuterated cocaine in the hair after 6 months for individuals administered this drug and have suggested a half-life of approximately 2 months for drugs in hair.

For some drugs, such as amphetamines, PCP, and marijuana, the metabolites are either in low concentrations in the hair relative to the parent drug or nonexistent. For cocaine, the metabolites can be identical to hydrolysis products that occur in vitro. These degradation products are also present in street samples of cocaine. Thus, an individual could be exposed to these "pseudometabolites" without having ingested them, or they could be created in the person's hair by environmental degradation of passively incorporated cocaine. Five samples of street cocaine, furnished by the Drug Enforcement Administration and the U.S. Customs Service, were analyzed using GC/MS. The amounts of cocaine-related materials relative to cocaine for five samples are shown in table 2. Some samples, such as crack cocaine, have up to 13 percent BZE. Depending on a variety of circumstances, hair exposed to this impure cocaine could produce ratios of cocaine to BZE identical to those observed in specimens of cocaine user's hair (see discussion on sweat above).

TABLE 2. *Amounts of cocaine "metabolites" in street samples of cocaine**

Cocaine Source	Benzoylecgonine/ Cocaine	Methylecgonine/ Cocaine	Ecgonine/ Cocaine
Cocaine-HCl #1	0.86	0.57	1.76
Cocaine-HCl #2	2.39	3.41	17.30
Cocaine Free Base #1	4.96	0.07	5.41
Cocaine Free Base #2	3.63	0.10	5.45
Cocaine Free Base #3	12.70	0.09	29.70

*Values are relative to cocaine and were determined by gas chromatography/mass spectrometry analysis of the samples.

KEY: HCl=hydrochloric acid

Baumgartner and Hill (1993*a*) argue that the presence of drugs in archaeological artifacts demonstrates that drugs may remain in hair for long periods. Springfield and colleagues (1993) analyzed cocaine in the hair of 12 ancient coca chewers (Peruvian mummies dating from A.D. 1000). They found more cocaine metabolites than cocaine (ratios of cocaine to BZE of 0.04 to 0.19:1 compared with ratios of about 5.2:1 [Henderson et al. 1992] and 2.9:1 [Möller et al. 1992] in current day coca chewers). Springfield and coworkers (1993) suggested that this significant difference in ratios may be the result of degradation in situ.

Metabolites of cocaine could be produced in hair in vitro by exposing cocaine-contaminated hair to various conditions. The authors incorporated cocaine into two types of hair by exposure to cocaine solutions at 37 °C for 1 hour. After cocaine incorporation, the hair was exposed to either ultraviolet light to mimic sunlight or bicarbonate to mimic basic hair treatments such as shampoos. Table 3 shows the percentage of pseudometabolites relative to cocaine for these two types of hair as determined by GC/MS analysis. Only percentages that were at least two times the control values are presented. Clearly, mild bases, such as bicarbonate, can produce significant amounts of BZE and ecgonine from cocaine present in hair. Likewise, Nakahara and Kikura (1994) concluded

TABLE 3. *Percent degradation of cocaine in hair for metabolites relative to the cocaine remaining in the hair sample*

Metabolite:Parent Drug Ratio	Ultraviolet, 45 °C, 21 hours		Bicarbonate, Room Temperature, Overnight		Bicarbonate, 37 °C, Overnight	
	Brown	Black	Brown	Black	Brown	Black
Benzoylecgonine:cocaine	NS	NS	13%	8.4%	9.7%	24%
Ecgonine methyl ester:cocaine	3.7%	NS	NS	NS	NS	NS
Ecgonine:cocaine	NS	NS	NS	NS	11%	27%

NOTE: Analysis procedure: After exposure to the indicated environmental conditions, the "metabolites" were extracted from the hair with acid, the acid evaporated, the extract silylated, and the derivatives analyzed by gas chromatography/mass spectrometry. Controls were run to account for any degradation of cocaine during the incorporation and extraction stages.

KEY: NS=not significant if not at least two times larger than control values

from a study of rats injected with cocaine that some cocaine is hydrolyzed to BZE and methyl ecgonine. From these studies, it appears that the hair matrix does not present an insurmountable barrier to environmental degradation.

Alternatively, sweat contains nonspecific esterases (Herrmann and Habbig 1976*a*; Ryhanen 1983) and other enzymes (Loewenthal and Politzer 1962; Herrmann and Habbig 1976*b*), which may allow for degradation of the drug on the surface of the hair before incorporation into the hair matrix. These mechanisms for the production of metabolites in hair have not been considered in hair analysis.

An ongoing study at the University of Alabama at Birmingham is examining the children and spouses of cocaine users. The study assumes that children 3 to 10 years of age are unlikely to be self-administering cocaine. Preliminary analysis of the data from this study (Smith et al., in press) shows that several children of cocaine users have both cocaine and BZE in their hair in quantities greater than the drug-using parent. This may be the first study demonstrating that passive exposure occurs in household settings and that certain metabolites may not be an indicator of drug use.

Analysis of metabolites is complicated because drugs and metabolites are not incorporated at equal rates from the external environment. The plethora of hair treatments and environmental chemicals available that might be capable of influencing the production of pseudometabolites in hair would argue against automatically accepting the presence of metabolites in hair as the sine qua non of human drug ingestion.

Do Drugs Migrate Along the Hair Shaft?

No. Püschel and colleagues (1983) found that cut human hair placed in a solution containing codeine showed diffusion of the drug to the distal portions of the hair within 1 hour.[9] It is possible that the authors' in vitro contamination procedure would not mimic passive exposure in vivo if diffusion of drugs along the hair shaft were possible. To test the possibility of diffusion along the hair shaft, we examined two hair types (black Asian and brown Caucasian). Samples of both hair types were sealed at both ends by immersion of the hair in hot paraffin. Other samples of both hair types were cut into 1-cm sections and not sealed. The hair was exposed to cocaine plus radiotracer, washed, and analyzed as described in the figure 2 legend, except that the dry ethanol prewash was eliminated. As seen in figure 16, no differences were observed in the incorporation of cocaine. In another study, the possibility of diffusion of materials along the hair shaft was tested by placing the ends of 11 different hair types in 10 μg/mL rhodamine for 1 hour at 37 °C. The remainder of the hair was suspended above the liquid level. Following removal of the hair from the solution, the excess rhodamine was washed away in a flowing stream of water. The samples were cut near the liquid level, and both parts were mounted and sectioned so that the sections covered the part near the liquid level. Comparison of the two sections showed large amounts of rhodamine in the portion that was in contact with the solution and no

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[9]This is the only report in the literature that mentions diffusion along the hair shaft. It lacks mention of the amount of drug found in the distal ends, the concentration of the soaking solution, or the decontamination procedure. Furthermore, the assay for codeine consisted only of RIA.

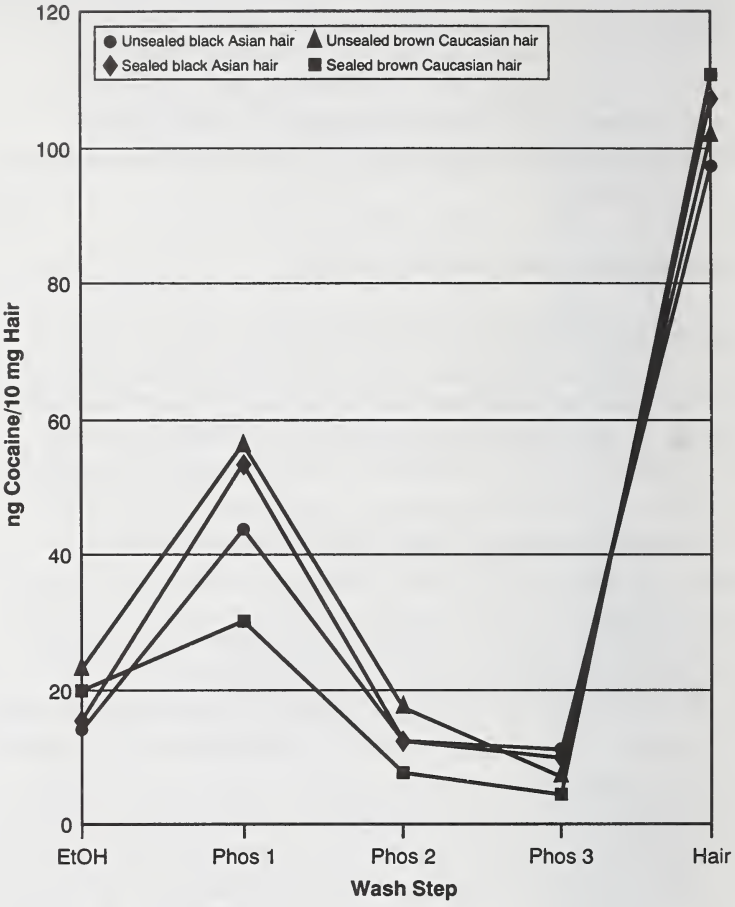

FIGURE 16. *Effect of sealing hair ends on uptake of cocaine*

KEY: EtOH = ethanol; Phos = phosphate buffer

rhodamine in the hair portion outside the solution. If migration of drugs occurs along the hair shaft, it would be over only a short distance.

In several controlled in vivo studies, Cone (1990) and Nakahara and coworkers (1992) showed that the thickness of the drug band in the hair shaft is greater than would be expected for the amount of hair

growth and time that the drugs are in the bloodstream. Whether this represents movement of the drug along the hair during the formation process or incorporation of the drugs from sweat has not been determined.

Does Melanin Play a Role in Drug Binding?

Perhaps. Harrison and colleagues (1974*a*, 1974*b*) suggest a specific structural requirement for a drug to be incorporated into melanin. Compounds structurally similar to the L-DOPA precursors of melanin (such as the amphetamines) may be incorporated into the synthesis of the melanin contained in the hair. On the other hand, Ishiyama and coworkers (1983) demonstrated that methamphetamine binds to the hair of albino animals in amounts similar to those found in pigmented animals, suggesting that drugs need not be incorporated into melanin. In contrast, Forrest and colleagues (1972) found differing rates of incorporation of chlorpromazine into the black and white areas of Dutch Belt rabbits but did not offer an explanation for this difference, such as varying fur growth rates. Forrest and coworkers (1972) monitored the incorporation of chlorpromazine only through radioactivity measurements. It is possible that the moiety with the radioactive label could have been metabolized to another compound that subsequently was used in the synthesis of fur pigments. Uematsu and colleagues (1990) also found substantial differences between the amount of haloperidol in white versus black hair in both rats and humans. In humans with some gray hair, the gray hair had about 10 percent as much haloperidol as black hair. In all Uematsu and colleagues' (1990) experiments, the haloperidol was extracted from the hair matrix after digestion of the hair with sodium hydroxide. Because no separation of the melanin fraction was performed, a clear demonstration of the association of haloperidol with melanin was never shown.

To examine the role of melanin for the incorporation of cocaine, the authors exposed two types of hair to cocaine plus radiotracer. The amount of cocaine present in the melanin fraction was not substantial (table 4). The sodium hydroxide digestion procedure was used to replicate previous digestion procedures. With this procedure, little cocaine was found in the melanin fraction alone. A potential criticism of this digestion methodology is that sodium hydroxide

TABLE 4. *Incorporation of cocaine into melanin as measured by digestion techniques*

	Sodium Hydroxide Digestion		Enzyme Digestion	
Matrix	Black Asian	Brown Caucasian	Black Asian	Brown Caucasian
Hair digest without melanin	9.98	5.97	7.49	6.09
Melanin fraction alone	0.69	10.27	0.15	10.20

NOTE: Numbers are in units of ng cocaine/10 mg hair. The hair was soaked in 2.5 μg/mL cocaine-containing tracer for 1 hour, rinsed two times with distilled water, and dried. The decontamination procedure was as described in the figure 2 legend. After centrifugation, all the digested hair in the supernatant could not be separated from the melanin pellet. Therefore, some radioactivity in the hair digest remained in the melanin fraction. The percentage of the supernatant remaining was estimated to be between 2 and 10 percent of the total fluid volume. Thus, the amount of cocaine associated with the melanin fraction is even less than that cited above.

would degrade the cocaine by converting the tritiated benzoyl group to tritiated benzoic acid, and because benzoic acid may not bind to melanin in the same manner as cocaine, the sodium hydroxide digestion may not reflect the true binding of cocaine to melanin. To address this concern, the enzyme digestion procedure of Baumgartner and Hill (1992, pp. 577-597) was adopted. Again, little cocaine was observed in the melanin fraction. We concluded that, regardless of hair type and digestion procedure, little cocaine was associated with melanin.

CONSIDERATIONS DURING THE ANALYSIS OF DRUGS IN HAIR

Can Externally Applied Drugs Be Removed?

Not with current procedures. In early work from the authors' laboratory, undamaged, unbleached Asian hair was soaked in a solution of PCP for 1 hour, removed, and allowed to dry. PCP was selected as a model drug because it is stable under almost all

extraction conditions. The hair was divided into several portions and extracted with several different solutions. The amount of residual drug in the hair was determined by thermal desorption tandem mass spectrometry (Kidwell 1993); results are shown in figure 17. The data are plotted relative to water. The efficiency of different solvents is plotted by rank order of efficacy for removal of externally applied PCP. Phosphate buffer, HIC, and sodium carbonate were more efficient in removing PCP than were surfactants, and some solutions were less effective than water in removing PCP.

Some external contamination can be washed away. In initial experiments, hair was exposed to vapors of PCP. Large amounts of PCP could be removed by washing (Kidwell and Blank 1992, pp. 555-563). However, large amounts of PCP still remained—amounts greater than that observed in the hair of PCP users—when the hair was extracted and the extracts analyzed by GC/MS. These experiments cannot be compared directly with the soaking experiments described above because hair may have a limited capacity to retain drugs. In many of these high-concentration experiments, large amounts of a drug are removed by washing, which may give the false impression that washing is effective in removing external contamination. It may be more appropriate to interpret these data in terms of the limited retention capacity of hair for drugs. If an individual's hair were exposed to an external source of drugs, a small percentage would be incorporated into the hair and the rest would remain on the surface. If a hair specimen were taken at that time, the decontamination kinetics would likely indicate that the hair was contaminated. However, after several normal hygienic washings, all the surface-bound drug would be removed, and only the drug in the interior would remain.[10] If a hair specimen were taken at that time, the decontamination kinetics would be substantially flatter, and the specimen would not likely be considered contaminated. Baumgartner and colleagues (1989) showed that even

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[10]For example, see figure 2 of Baumgartner and colleagues (1989). After two brief washings with shampoo, the kinetic wash data are essentially flat. However, 16.8 ng cocaine/10 mg hair (0.014 percent × 120,000) still remain in the subsequent analysis.

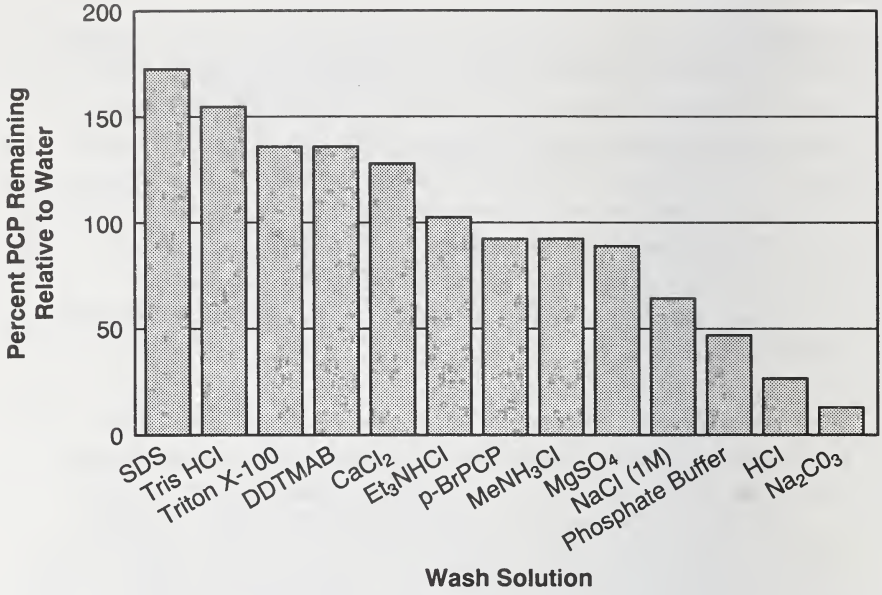

FIGURE 17. *PCP remaining in hair exposed to PCP after various wash solutions. The amount of PCP remaining in the hair is relative to water washing. All samples were washed with the respective 0.1 M solutions (1 M for sodium chloride [NaCl]) for 1 hour and the hair analyzed by thermal desorption tandem mass spectrometry. Amounts greater than 100 percent reflect that the solution was less effective than water at removing PCP. Surfactants included sodium dodecyl sulfate (SDS), Triton X-100, and dodecyltrimethylammonium bromide (DDTMAB). Salts included calcium chloride ($CaCl_2$), triethylammonium chloride (Et_3NHCl), methyl ammonium chloride ($MeNH_3Cl$), magnesium sulfate ($MgSO_4$), and NaCl. Buffers included tris(hydroxymethyl)aminomethane hydrochloride (tris HCl) and phosphate buffer at pH 7. HCl and sodium carbonate (Na_2CO_3) were an acid and a base, respectively. p-Bromophenylcyclohexyl-piperidine (p-BrPCP) was a PCP analog.*

SOURCE: Kidwell 1993

nine normal hygienic washings were unable to completely decontaminate hair exposed to crack cocaine smoke. Others have shown that hair samples soaked in solutions of drugs or exposed to the vapors are not completely decontaminated (Cone et al. 1991; Koren et al. 1992).

Several other investigators have proposed various means for decontaminating hair. For example, Martz (1988) substituted a methanol wash for the more widely employed anhydrous ethanol wash. He then examined the concentration of the drug in the methanol wash and compared it with the amount found in the hair to determine whether hair had been contaminated. The effectiveness of methanol and ethanol washes in decontaminating hair exposed to solutions of cocaine plus radiotracer is shown in figure 18 for brown Caucasian and black Asian hair. In this experiment, the hair samples were exposed to a cocaine solution plus radiotracer and washed with water four times prior to the 30-minute decontaminations with ethanol and methanol. Depending on hair type, methanol removed between 7 and 16 percent of cocaine compared with ethanol, which removed less than 1 percent. These hair samples continued to be washed with the normal series of phosphate washes, which removed only another 10 percent of the total cocaine. The amount of cocaine that remained was determined following digestion of the hair sample. Although methanol removed more cocaine than ethanol in these preliminary experiments, the amount of cocaine remaining in all these externally contaminated samples was still substantial. A false sense of security could be generated if analysis of the methanol extract were undertaken to determine the extent of external contamination. The quantity of cocaine extracted into the methanol, being only a fraction of that present, may be below the instrumental detection limit. Consequently, the methanol may be considered negative but the hair, with 10 times as much cocaine, could be positive.

The authors tested prewashing two types of hair with several different detergents. None was successful in removing all the cocaine from external contamination (see results in figure 19). A prewash with water was included as another control to correct for exposure of the hair to any aqueous solution. The difference

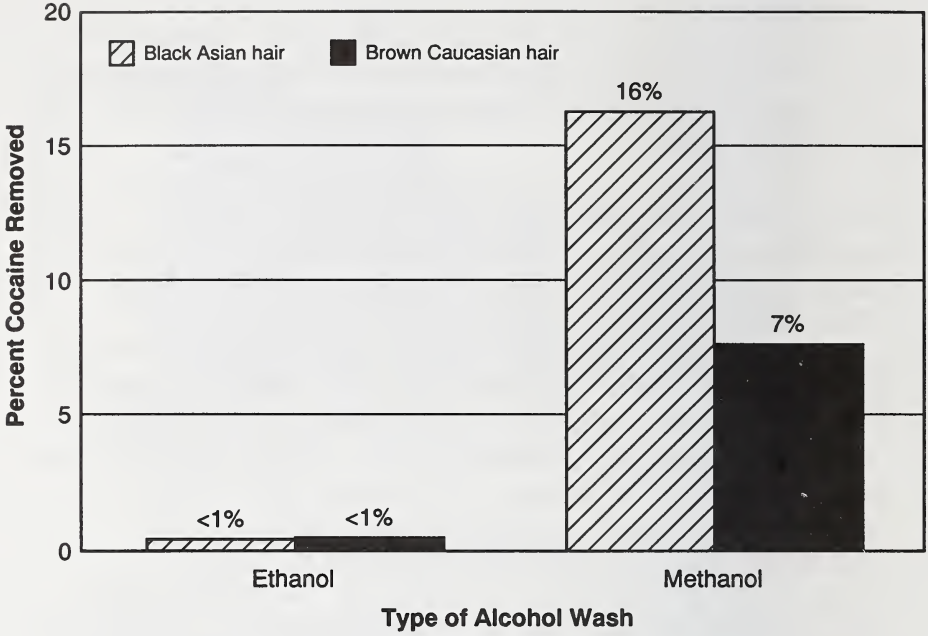

FIGURE 18. *Removal of cocaine with two types of alcohol wash. The hair was exposed to a solution of cocaine (10 μg/mL) and dried overnight before extraction. It was then extracted four times with water before the alcohol decontamination. The analysis outlined in the figure 2 legend was used to determine the amount of cocaine remaining in the hair. Percentages refer to the amounts removed from the hair by the alcohol relative to the total present in all the washes and the hair extract.*

SOURCE: Blank and Kidwell 1993

observed in figure 19 between a water prewash and no prewash is probably within experimental error.

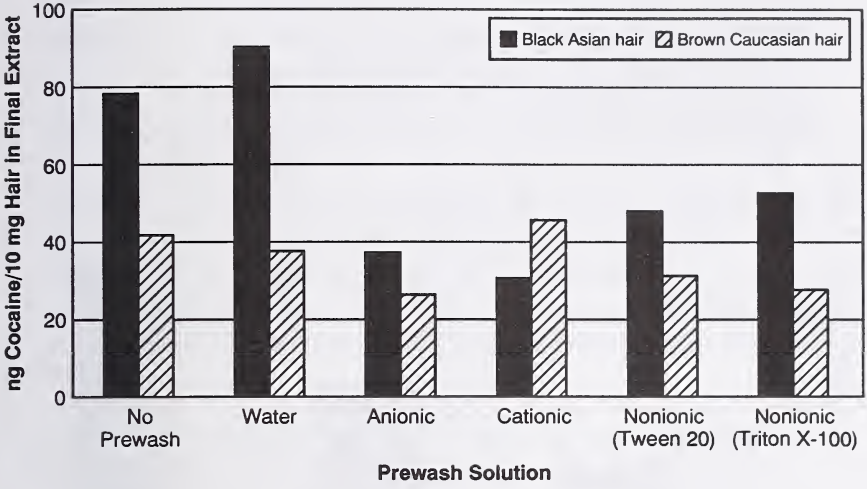

FIGURE 19. *Effect of prewashing the hair with different solutions on the amount of cocaine in the final extract. Two hair types (black Asian and brown Caucasian) were exposed to 1 μg/mL cocaine for 1 hour, prewashed for 30 minutes in the indicated solution, and then dried. The hair was decontaminated and analyzed as described in the legend for figure 2.*

Extraction Considerations

The two most prevalent types of extraction procedures are (1) digestion of the hair matrix and extraction of the drug from the digest with liquid-liquid or solid-phase extraction procedures and (2) extraction of the drug from the intact hair matrix. These techniques have been reviewed by Chiarotti (1993). Results of removing cocaine and radiotracer by acid extraction from *intact* hair are shown in figures 20a and b (Blank and Kidwell 1995). Cocaine was incorporated into hair by exposure to cocaine solutions containing tritiated cocaine as a tracer. After decontamination, the hair was extracted with 0.1 M HCl, and the radioactivity in the HCl extract was measured. The hair then was digested with NaOH, and the residual radioactivity was measured. The ratio of cocaine

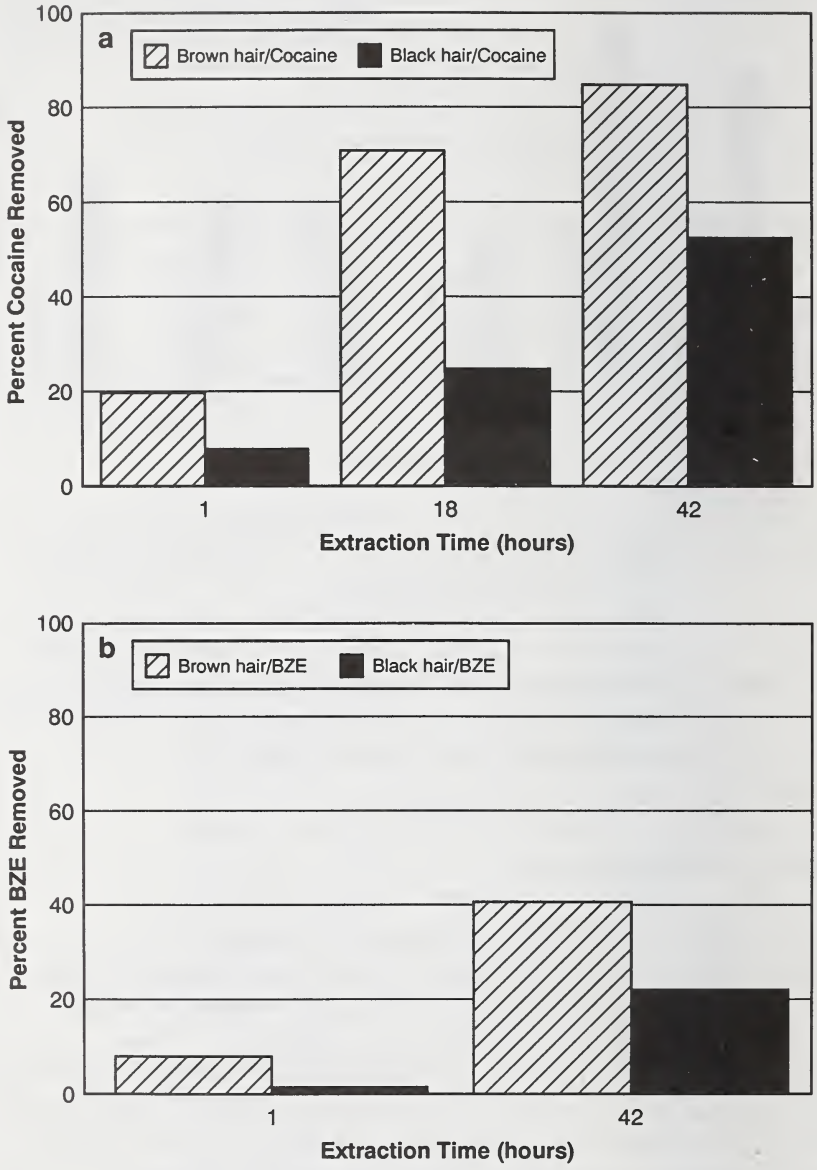

FIGURE 20. *Removal of cocaine and benzoylecgonine (BZE) by HCl extractions*

SOURCE: Blank and Kidwell 1995

extracted by HCl to the total amount of cocaine in the sample for different durations of extraction is shown in figure 20a. For the two types of hair studied, extraction efficiency varied by extraction time and ranged from 53 to 86 percent, even in the 42-hour extraction. This experiment was repeated for extraction of BZE (figure 20b), using a ^{125}I-labeled BZE tracer. The extraction efficiency never exceeded 41 percent. These data show that extreme caution must be exercised in the analysis of intact hair samples extracted by HCl. Data resulting from acid extraction can be biased in three ways: (1) incomplete extraction, (2) different extraction efficiencies across hair types, and (3) different extraction efficiencies across drug moieties.

Are There Quantitative Measures That Can Distinguish Between Endogenous and Exogenous Exposure?

No. All decontamination procedures are based on the assumption that drugs deposited in hair from the external environment are loosely bound to either the surface of the hair or the hair matrix and, therefore, can be removed by appropriate decontamination procedures. For example, Martz (1988) decontaminated the hair using successive brief methanol extracts and tested each extract until the last extract showed that no further drugs had been removed. Henderson and coworkers (1992) rinsed hair samples with a detergent followed by water followed by methanol prior to digestion and extraction. Cone and colleagues (1993) rinsed hair samples briefly with methanol, then extracted the drugs by incubating with methanol at 40 °C overnight. Mangin and Kintz (1993) decontaminated hair using a dichloromethane wash for 15 minutes at 37 °C. They found this method sufficient because a second wash was negative for drugs. Nakahara and coworkers (1993) washed hair samples with 0.1 percent sodium dodecyl sulfate and water. Moeller and colleagues (1993) attempted to eliminate external contamination with a 5-minute warm water wash followed by a 5-minute acetone wash. Several different decontamination procedures were tested to remove external contamination produced by solutions of drugs. These results are discussed above and shown in figures 17 through 19. None were sufficient to remove all drugs.

Baumgartner and Hill (1993*a*) have adopted a more rigorous and consistent approach to decontamination. First, the hair is evaluated for damage by measuring the uptake of methylene blue dye. The severity of the decontamination procedure varies depending on this evaluation and is made less severe when greater damage is observed in the hair sample. An undamaged hair sample is washed with anhydrous ethanol to remove surface contamination, oils, and dirt. Then a well-defined decontamination using three phosphate buffer washes is employed. The hair is then digested and analyzed. According to Baumgartner and Hill (1993*a*), drugs loosely adhering to the surface are removed with the initial alcohol wash. If the methylene blue staining indicates porous or damaged hair, the phosphate buffer washes are replaced by varying mixtures of ethanol and water, 5 to 15 percent. Unfortunately, the objective criteria for evaluating porosity are not reported. Each phosphate wash is analyzed for drug concentration, and the data are analyzed for external contamination by the criteria shown in table 5. The empirically determined cocaine cutoff values shown in table 5 may vary for other drugs (Baumgartner and Hill 1993*a*). If a specimen fails any of the three cutoff criteria by having a value less than those listed in table 5, it would be considered externally contaminated.

The authors tested Baumgartner and Hill's (1993*a*) decontamination procedure on more than 300 different hair samples that were then externally contaminated in the laboratory. In the initial phases of this study, we used four phosphate washes. In later phases, based on the comments of Baumgartner and Hill (1993*b*), our procedure was modified to use only three phosphate washes. However, in the radioactive tracer experiments that were typically performed, the final step of preparation was digestion. No radioactivity was lost by discarding solutions. Therefore, it is possible to calculate the effect of three phosphate washes on the decontamination of hair and on the various calculated criteria by adding the radioactivity found in the fourth wash to that found in the digest. No remarkable differences were found in the calculated criteria between our three-phosphate-extraction process and samples using four wash steps and then recalculated to reflect only three phosphate washes.

TABLE 5. *Definitions of kinetic wash criteria and values for cocaine**

$$R_{EW} = \frac{\text{amount of drug in digest}}{\text{amount of drug in last phosphate wash}}$$

$$R_{SZ} = \frac{\text{amount of drug in digest}}{\text{amount of drug in all three phosphate washes}}$$

$$R_{C} = \frac{\text{amount of drug in three phosphate washes}}{3 \times \text{amount of drug in last phosphate wash}}$$

Cutoff Values for Cocaine†

R_{EW}	10
R_{SZ}	0.33
R_{C}	1.3

*Definitions summarized from Baumgartner and Hill 1993*a*

†Specimens with values below these numbers are considered contaminated. Otherwise, the individuals are considered users of cocaine.

KEY: R_{EW}=extended wash ratio; R_{SZ}=truncated safety zone ratio; R_{C}=curvature ratio

SOURCE: Blank and Kidwell 1993

The purpose of any objective kinetic wash criterion is to assess how effective the decontamination procedure has been. Both the extended wash ratio (R_{EW}) and the truncated safety zone ratio (R_{SZ}) measure effectiveness of decontamination. However, the curvature ratio (R_{C}) does not appear to measure this variable. For example, the R_{C} value was examined for samples prepared by three different methods (Blank and Kidwell 1995). A sample of Asian hair was exposed to 50 μg/mL cocaine plus radiotracer for 1 hour and not prewashed with water prior to alcohol and phosphate decontamination (figure 21a). The second sample of Asian hair was exposed to 1 μg/mL cocaine plus radiotracer for 1 hour and

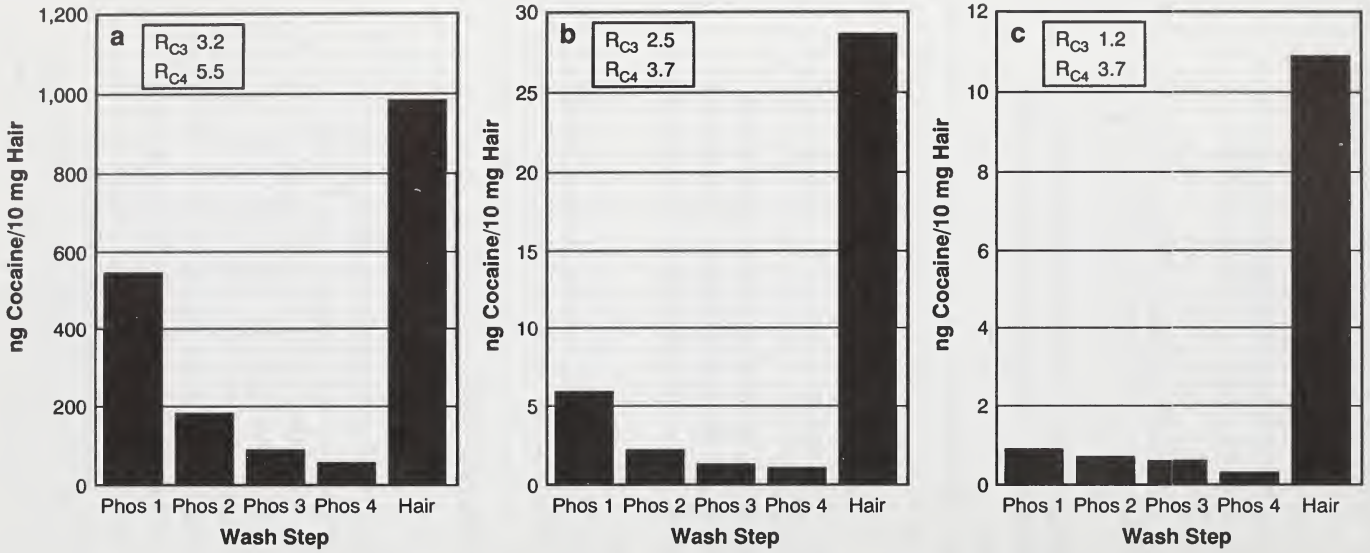

FIGURE 21. *Decontamination curves for three sets of Asian hair. The R_C values were taken as either R_{C3}=([Phos 1+Phos 2+Phos 3]/[3×Phos 3]) or R_{C4}=([Phos 1+Phos 2+Phos 3+Phos 4]/[3×Phos 4]).*

SOURCE: Blank and Kidwell 1995

also was not prewashed prior to decontamination (figure 21b). The third sample of Asian hair was exposed to 1 μg/mL cocaine plus radiotracer for 1 hour as well but also included three water prewashes before decontamination (figure 21c). As expected, samples that did not include a prewash exhibited a greater "curvature" (i.e., a greater decrease in the amount extracted by successive phosphate washes) than did the samples that included water prewashing. In other words, the greater the contamination, the greater the R_C value and the more easily it would exceed the cutoff criteria. Because the R_C value does not seem to have any special predictive value and the amount of drug in all phosphate washes and the amount of drug in the last phosphate wash appear in calculations of R_{EW} and R_{SZ}, it is not reported here. The calculated values for R_{EW} and R_{SZ} for 110 specimens are shown in figure 22. The data are plotted in order of increasing values for R_{EW}.

Of the 110 samples examined, only 3 fell below the cutoff values for these externally contaminated samples and then by only marginal amounts. Further criticisms of these data are that many samples pass the cutoff values by only trivial percentages and that these empirically determined cutoffs could be adjusted to take the data into account. A cutoff value of at least 214 for R_{EW} would exclude all the samples in figure 22 as contaminated, and a cutoff value of at least 137 would exclude 95 percent of the samples as contaminated. Cutoff values this high also would preclude analysis of many, if not all, user samples. For the data that pass the respective cutoffs, the frequency distributions for the samples shown in figure 22 are plotted in figure 23 along with data given in Baumgartner and Hill (1993*a*). Note the similarity in shape between these two frequency distributions.

The results of experiments in the authors' laboratory suggest that regardless of the decontamination procedure employed, external contamination cannot be fully removed.

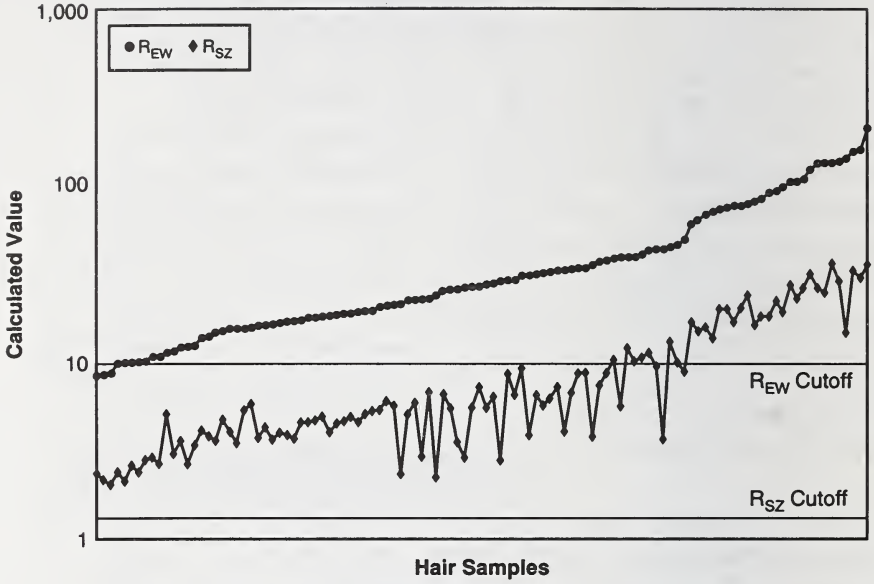

FIGURE 22. *Rank-ordered plot of R_{EW} and R_{SZ} values for 110 externally contaminated samples. Ten different types of hair were exposed to cocaine in phosphate-buffered saline at 5 μg/mL for varying lengths of time. The hair was then treated as described in the legend for figure 2. The R_{EW} and R_{SZ} values were calculated as if three phosphate washes were performed. (The concentration of cocaine in the fourth phosphate wash was added to the hair extract.) Any sample below the cutoff would be considered contaminated. Of the 110 samples, only 3 failed the R_{EW} cutoff, and none failed the R_{SZ} cutoff.*

SOURCE: Blank and Kidwell 1995

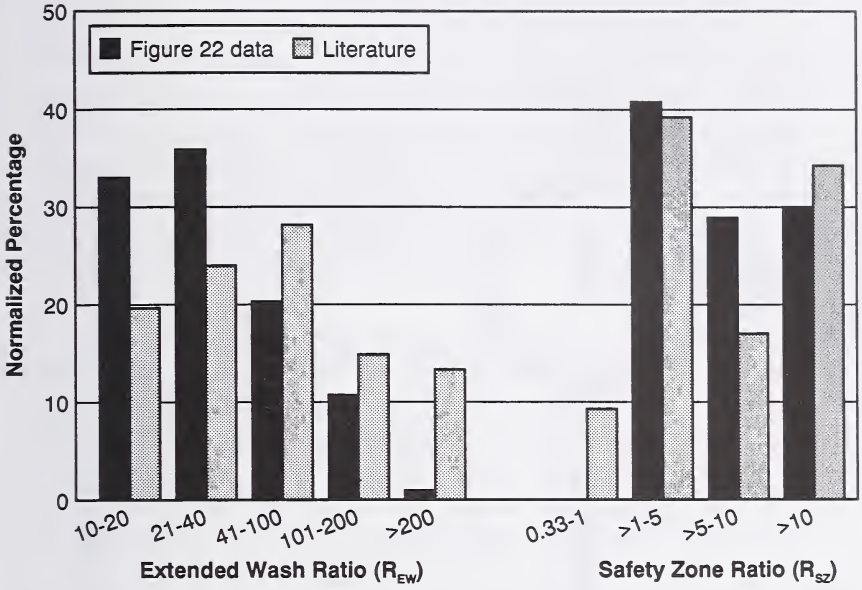

FIGURE 23. *Frequency distributions of R_{EW} (extended wash ratio) and R_{SZ} (truncated safety zone ratio) from the data in figure 22 and the literature. The three samples not meeting the R_{EW} cutoff were excluded.*

SOURCE: Literature values from Baumgartner and Hill 1993*a*

INTERPRETATION OF DATA WITHIN A THEORETICAL FRAMEWORK

A review of the data (collected in the authors' and other laboratories) cited above suggests that the mechanism for incorporation of drugs into the hair matrix is a complex process. A schematic diagram of possible routes of incorporation of drugs into hair is shown in figure 24. If an individual is a drug user, drugs may enter the hair by one or more of three different routes. The first is sweat. Ingested drugs and drug metabolites are excreted in sweat and sebum, bathe the hair shaft, and are incorporated into the hair matrix, possibly by an ion-exchange mechanism. The second is

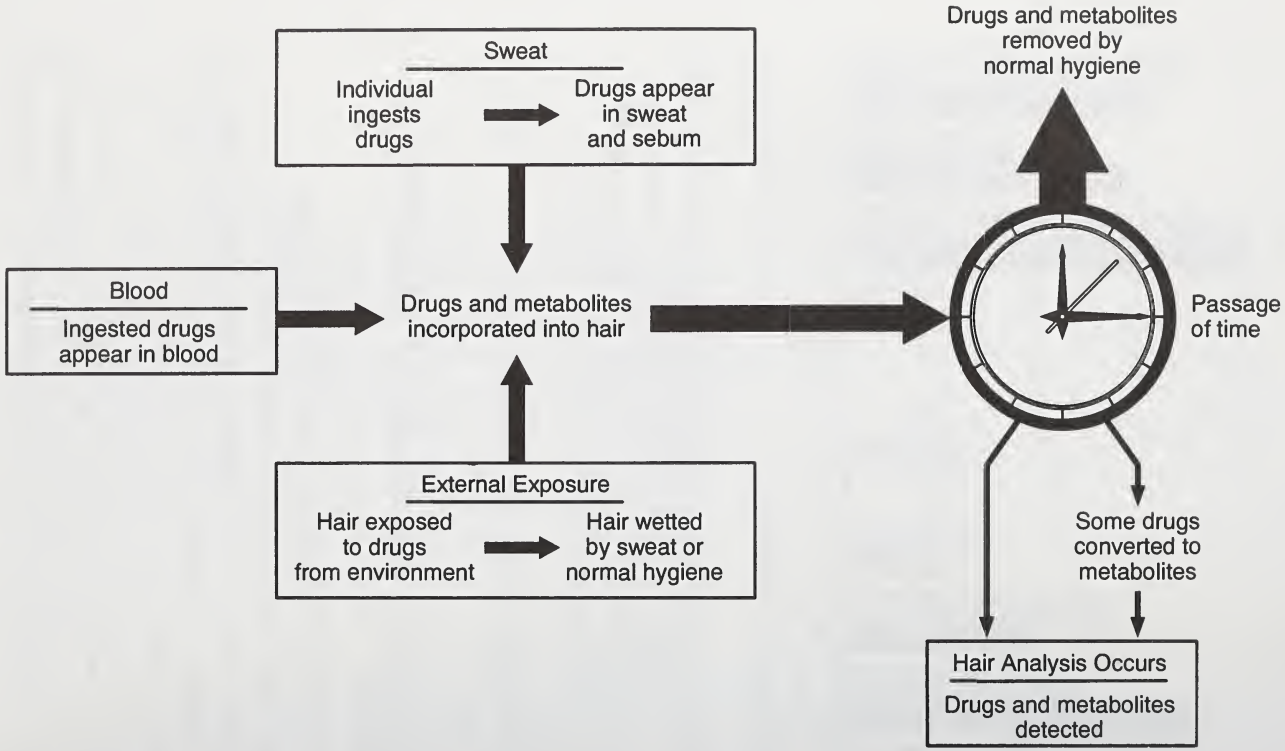

FIGURE 24. *Theoretical framework for the incorporation of drugs into and removal from hair*

SOURCE: Blank and Kidwell 1995

external exposure. It is likely that the environment of a drug user contains drugs of abuse. If the drugs come in contact with hair, they would be incorporated; perhaps incorporation would be mediated by solutions such as sweat or normal wetting of hair by hygienic practices. This route includes transfer of drug vapors or powders to the hair or physical transfer from drugs deposited on surfaces that could come in contact with the hair, such as hands, clothing, or money. The third route of transfer is blood, whereby drugs are transferred into the growing portion of the hair shaft from the capillary bed engulfing that structure.

As figure 24 illustrates, some time usually passes between ingestion and hair analysis. During that time, drugs loosely bound to the surface of the hair could be washed away by normal hygienic hair care. The removal of drugs depends on several variables, including the characteristics of the solutions used to wash or treat the hair. One may visualize these practices as an in vivo extraction of drugs of abuse and contrast them with laboratory extraction procedures.

The incorporation of drugs into the hair matrix of a nonuser would occur only via the mechanism of external exposure described above and shown in figure 24. Extremely minute quantities of drugs can be incorporated into hair over short periods, and the extraction kinetics of these externally deposited drugs mimic those from the hair of a drug user. The extent to which this would be observed in a nonuser would depend on the exposure to drugs from the environment in which that individual lives. For example, as described above, Smith and colleagues (in press) have shown that cocaine can be found in hair samples collected from spouses and children of cocaine users. In some of these cases (e.g., children 3 to 10 years of age), intentional ingestion of cocaine would be highly unlikely. It is well known that cocaine is found on circulating currency (Hudson 1989), but the extent to which that drug could be transferred from currency to hands to hair is not known. However, it must be emphasized that conditions on the head of an individual could favor the incorporation of drugs into the hair matrix. For example, if drugs are transferred to head sweat, their concentration would increase as the sweat evaporates, thus favoring incorporation into the hair matrix. In addition, infrequent

washing of hair or application of hair care products could also favor incorporation.

Drugs bound to hair are neither physically nor chemically protected from change. Thus, esterases, such as those found in sweat or highly basic hair care products, could degrade cocaine or other drugs to their metabolites over time. To use any metabolite as an analyte, it would be necessary to demonstrate that it could not be produced under in vitro conditions likely to exist in normal hair care or environmental degradation scenarios and in the presence of hair-bound enzymes.

The profound effects of inadvertent exposure to drugs are illustrated in figure 25. This hypothetical example assumes that two nonusing individuals are exposed to drugs. Individual A inadvertently ingests 80 to 100 mg cocaine, whereas individual B's head was inadvertently exposed to a few micrograms of cocaine. Figure 25 further assumes that both A and B are subjected to a random screening program (A, urinalysis; B, hair analysis) where either could be tested once on any day in a 120-day period. Individual B has a standard military hairstyle with hair 3 cm in length and obtains a haircut every 2 weeks. At the above-mentioned dose, assume that cocaine can be detected in A's urine for 3 days following ingestion (Ambre 1985) and the amount of cocaine that can be detected in B's hair decreases gradually over a 120-day period, the decrease in concentration accelerated by frequent haircuts. In this hypothetical example, it would be 37 times more likely for individual B to be positive on hair analysis than individual A on urinalysis.[11]

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[11]The following events are defined below: TP=testing positive; DP=drugs present in system (for individual A, this means ingestion of 80 to 100 mg cocaine into the body; for individual B this means hair is dusted with microgram quantities of cocaine); and EX=exposure to cocaine (i.e., physically in the presence of cocaine).

In general, the probability of a positive test can be expressed as the product: Probability of testing positive = (Probability of testing positive given drugs are present) × (probability of drugs

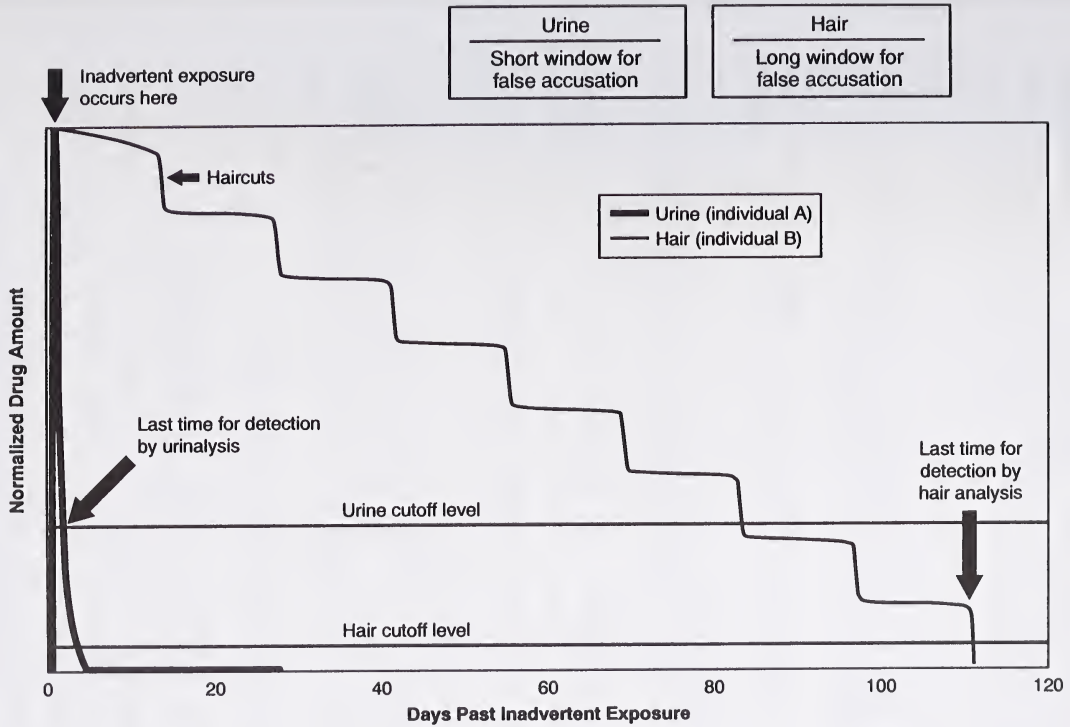

FIGURE 25. *Window of detection for urinalysis and hair analysis after inadvertent exposure to cocaine. Losses of cocaine from the hair come mainly from haircuts rather than the more gradual loss by normal hygiene.*

Clearly, given inadvertent exposure, the window for false accusation using urinalysis is far shorter than for hair analysis. This problem would be exacerbated by the fact that individual A could have noticed a physiological or psychological effect of inadvertent ingestion and could document that event in his or her defense, whereas individual B would have no knowledge of being passively exposed until that event was revealed by hair analysis. Given the longer window of detection for hair analysis, this would put the

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being present given a certain exposure) × (probability of the exposure). Mathematically this is represented by:

$$P(TP)=P(TP \mid DP) \times P(DP \mid EX) \times P(EX)$$

For individual A:
Probability of A testing positive = (Days that drugs are detectable in urine) × (probability of A being given a certain exposure) × (probability of exposure) or mathematically:

$$P_A(TP)=(3/120) \times P_A(DP \mid EX) \times P_A(EX)$$

and for individual B:
Probability of B testing positive = (Days that drugs are detectable in hair) × (probability of B being given a certain exposure) × (probability of exposure) or mathematically:

$$P_B(TP)=(110/120)\ P_B(DP \mid EX)\ P_B(EX)$$

If the probabilities of exposure are the same $[P_A(EX)=P_B(EX)]$, it would be 37 times more likely for individual B to be positive on a hair test than for individual A on urinalysis $(110/120 \div 3/120)$. However, inadvertent ingestion of 80 to 100 mg cocaine does not intuitively seem to be as likely as passive exposure of hair to 8 to 10 μg cocaine. The former would most likely require an intentional act of another individual, whereas the latter would require only that an individual be in an environment where cocaine is present.

burden of proof on the accused to document his or her exact whereabouts over a long period.

A more concrete example elucidates this issue. Suppose someone attends a party on Friday night and is given cocaine in a mixed drink, without his or her knowledge. On Monday the person is tested by urinalysis; by the end of the week, urinalysis results indicate that he or she tested positive for cocaine. Prior to a judicial proceeding, that individual recalls the inadvertent exposure event and, by so doing, presents data on that incident in his or her defense. Furthermore, witnesses may be produced to substantiate the claim that cocaine was used at the Friday night party and that the accused did not appear to participate in the use of cocaine. Contrast this situation to reconstructing these events 120 days later and not knowing when or where the inadvertent exposure had occurred. The credibility of any defense witnesses would be impaired by time and uncertainty when measured against the hard fact that cocaine exposure had occurred as determined by hair analysis.

At the current level of understanding, hair analysis data should be used with caution when the results are attributed to an individual and are used in an adverse manner. Conversely, hair analysis might be useful as an adjunct to paper-and-pencil population surveys where the prevalence of drug use is being measured. However, even in this case, the intended application of the data must be carefully considered. If trends in drug use in a large population are generated from a small cross-section of the population by hair analysis, the inclusion of a few false positives may seriously skew the data to an overly high use rate. If these data are used to make policy decisions, then a large or increasing use rate could prompt the Federal Government to divert resources or personnel or enact legislation in a wasteful manner.

CONCLUSIONS

Current findings suggest that cocaine and benzoylecgonine (and probably other drugs) may be readily incorporated into hair from environmental exposure and not removed by any one of several decontamination techniques. The procedures outlined in this chapter

using radiotracers allow many specimens to be analyzed under a variety of conditions with high precision. Any new decontamination procedure could be tested through the use of these techniques. Several authors have proposed wash ratios as one criterion to distinguish passive from active exposure. This chapter examined several hundred externally contaminated samples and found that the suggested criteria were inadequate to identify the samples as contaminated. Several decontamination scenarios employing detergents and solvents also were examined, and none decontaminated the hair. Metabolites of drugs in hair may distinguish active use from passive exposure; however, for cocaine, most of the metabolites may be produced by means other than drug ingestion.

Several variables besides the decontamination procedure must be considered in the analysis of hair. The most important is the method by which drugs are to be extracted from the hair matrix. Based on the discussion presented here, dissolution of the hair matrix before extraction of the drug appears to be necessary to achieve complete removal.

The evidence from the authors' laboratory and from others reviewed in this chapter reinforces and extends the serious concern that external contamination of hair by drugs of abuse can easily occur. Any interpretation of hair analysis data should be tempered by the prospect that the sample could have been externally contaminated. The pharmacokinetics of the incorporation of drugs into many tissues has been well elaborated (Hawks and Chiang 1987). However, the paucity of information on the incorporation of drugs into hair makes understanding the mechanisms indeterminate. Substantial additional information is needed on the mechanisms for incorporation of drugs into hair, the decontamination of hair, the differentiation between exposure to exogenous and endogenous drugs, and the meaning of the presence of metabolites in hair before hair analysis can be employed in many forensic applications. Given current knowledge, hair analysis could be useful as an adjunct to population surveys, as suggested by a recent U.S. General Accounting Office (1993) report. However, when the result of a hair analysis can be attributable to a single, identifiable individual, extreme care should be exercised in data interpretation.

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Analysis of Hair for Cocaine

Gary L. Henderson, Martha R. Harkey, and Reese T. Jones

INTRODUCTION

The use of hair as a specimen to detect cocaine use was first reported in 1981 (Arnold and Püschel 1981; Valente et al. 1981). In those studies, hair samples from suspected drug abusers were analyzed by radioimmunoassay (RIA) for the cocaine metabolite benzoylecgonine (BZE) in an attempt to verify a history of cocaine use. Additional studies using RIA followed shortly thereafter (Arnold and Püschel 1981; Valente et al. 1981; Baumgartner et al. 1982; Smith and Liu 1986; Michalodimitrakis 1987). The first gas chromatography/mass spectrometry (GC/MS) procedures for detecting cocaine in hair were not reported until 1987 (Balabanova and Homoki 1987). When this more specific technique was used, it was found that cocaine, not BZE, is the primary analyte in hair. The metabolites BZE and ecgonine methyl ester (EME), shown in figure 1, are present in such low and variable concentrations that they may result from environmental degradation of cocaine already present in hair. Because of the lack of specificity used in some of the early studies, it is difficult to evaluate much of this literature. For example, some investigators quantitated "cocaine" in hair using RIA antibodies highly specific for BZE, whereas others used RIAs more specific for cocaine. In addition, some digestion or extraction procedures could have caused degradation of cocaine.

The past few years have seen the development of highly specific and sensitive GC/MS methods for the simultaneous detection and quantitation of cocaine and several of its metabolites in hair. These methods have been applied to hair samples obtained from a variety of animal species and human subjects. It is now clear from these

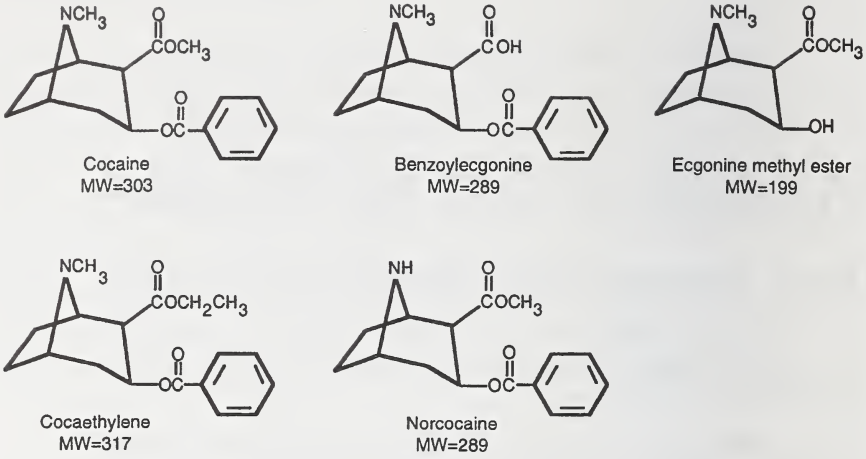

FIGURE 1. *Chemical structure and molecular weight (MW) of cocaine and the metabolites benzoylecgonine, ecgonine methyl ester, cocaethylene, and norcocaine*

more detailed studies that in all species studied and by all routes of administration, cocaine is incorporated into hair as the parent drug and can be detected in hair for months after administration. What has not been determined is the relationship between the dose and the amount of drug in hair or whether the position of the drug along the hair shaft can be used to determine the time of drug ingestion.

This chapter reviews results from the many laboratories throughout the world that are now engaged in hair analysis and compares them with the authors' controlled-dose studies involving human subjects.

HAIR ANALYSIS METHODS

Several analytical methods are used for detecting and quantitating cocaine and its metabolites in hair, but none has emerged as an accepted standard. Typically, each procedure includes the following steps: specimen collection, sample washing, digestion or extraction

of the hair sample, immunoassay screening, and confirmation or quantitation of the various analytes.

The greatest differences among the methods are in the sample preparation steps, that is, washing and digesting or extracting the hair sample. The authors used an elaborate method that incorporated "soft" digestion of the hair specimen to prevent any conversion of cocaine to BZE and solid-phase extraction of the digest so that relatively clean mass spectra were obtained for quantitating the low picogram amounts of drug found in their clinical studies (Harkey et al. 1991). The same methods have been used throughout so that results with various subjects studied during the past few years could be compared. Other laboratories have developed more rapid methods in which the samples were extracted with acid or organic solvent (Cone et al. 1991; Pirozhkov et al. 1992).

Hair Sampling Procedures

The site and technique for collecting the hair specimens are important because considerable differences in trace element and drug concentrations have been reported for hair samples collected from different sites (Chatt and Katz 1988, pp. 14-16). Morphine concentrations have been found to be highest in pubic hair, followed by axillary hair, then scalp hair (Kintz et al. 1993); methadone concentrations have been found to be highest in axillary hair, followed by pubic hair, then scalp hair (Balabanova and Wolf 1989).

Hair samples analyzed for cocaine are most typically obtained from the scalp. This site may be more susceptible to external contamination, but it also can be sampled less intrusively than the axillary or pubic regions. The posterior vertex region of the scalp is generally chosen as a sampling site because most hair in this area (85 percent) is in the active growing phase and thus more likely to incorporate drug. An adequate sample for testing, approximately 100 mg of hair, can be obtained by gripping a bundle of hair with the circumference of a pencil and then pulling gently to remove any loose strands of hair in the resting stage that are easily shed. The remaining strands are grasped firmly while the hair is cut as close to the scalp as possible. After cutting the hair, it is important to keep

the root ends of the bundle aligned so that the hair strands can be cut accurately for subsequent segmental analysis.

Segmental analysis often is used in hair analysis in an attempt to correlate time of ingestion with location of drug along the hair shaft. In this procedure, the root ends of the hair sample are aligned, and the sample is cut into segments that can be analyzed individually. Typically, the sample is cut into 1-cm segments with each segment corresponding to approximately 1 month's growth, assuming a hair growth rate of 1 cm/month. However, the length of the segments can be varied, and some laboratories have found that cutting the hair specimen into shorter segments (e.g., 2 mm) results in better resolution (Uematsu et al. 1993).

Washing Procedures

Laboratory workers routinely wash the hair sample before analysis to remove lipids, oils, cosmetics, and any adhering drug. However, the efficacy of washing procedures in removing cocaine deposited on hair from external sources is controversial. Baumgartner and associates report that in most instances washing the hair specimen (5 to 10 mg) with 1 mL methanol for 15 minutes at 37 °C followed by three 30-minute washes with phosphate buffer (pH 6) at 37 °C removed any externally bound drug (Baumgartner 1989; Baumgartner and Hill 1992, pp. 577-597). In some cases they found that more extensive washing was required and proposed a "kinetic wash criteria"—in which the amount of drug found in the various washes was compared with the amount of drug in the washed, extracted hair specimen—to distinguish between passive exposure and active ingestion. Koren and colleagues (1992*a*) also found that washing removed all drug from a variety of hair samples that had been contaminated from externally applied cocaine. Their assay method, like that of Baumgartner, was RIA.

Other investigators have found that, despite extensive washing, enough residual cocaine remained on an externally contaminated hair sample to produce a positive test. Blank and Kidwell (1993) exposed control hair to radiolabeled cocaine in solution and found that washing removed most, but not all, the externally applied cocaine. They concluded that they were not able to distinguish

passively exposed samples from actively exposed samples using a kinetic wash criterion similar to the one proposed by Baumgartner and colleagues (1982). In addition, they found that washing removed cocaine metabolites as well and suggested that extensive washing with solvents might more aptly be called extraction (Blank and Kidwell 1993). In fact, methanol is the solvent used most often in washing procedures and is the solvent used by many for extracting cocaine from hair (see below). Kidwell (1993) recommended using pentane as a washing solvent because it removes surface oils from hair but does not extract drug from hair.

The authors also found that extensive washing removed most, but not all, of the cocaine deposited on hair either from "crack smoke" or from cocaine in an aqueous solution. For example, control drug-free hair was incubated for 72 hours in an aqueous solution of radiolabeled cocaine (4-^{3}H-cocaine, specific activity of 14.77 mCi/mg) at concentrations of 15 ng/mL and 1 mg/mL. The treated hair was washed extensively with various solutions (distilled water, acetone, chloroform, methanol), and significant amounts of drug remained in the hair even though no drug was detected in the final wash solutions. Figure 2 shows a typical uptake and washout curve for human hair incubated in a cocaine solution and washed exhaustively in distilled water (Henderson et al. 1993). Large amounts of cocaine were incorporated into hair, and repeated washings removed only about 80 percent of the drug. Similarly, when control hair was placed in an enclosed 4-cubic-foot acrylic chamber and exposed to 10 mg cocaine base that was vaporized by heating it to 200 °C, the hair was found to contain cocaine concentrations that were greater than 100 ng/mg before washing. Vigorous washing removed most (as much as 95 percent), but not all, of the externally applied drug. Washing also reduced the amount of BZE and EME in hair samples from chronic cocaine users (Henderson et al. 1992), which suggests that washing may remove internally incorporated drug as well as that present from external contamination.

Digestion and Extraction Techniques

A variety of methods are used for the digestion of hair and extraction of incorporated drug. The most efficient method of

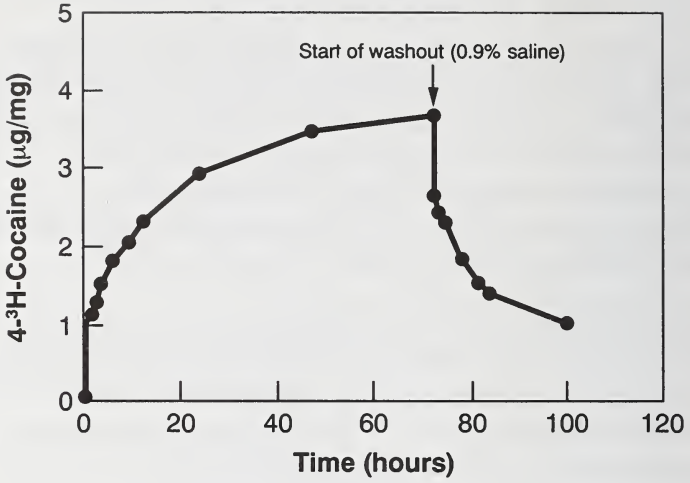

FIGURE 2. *Uptake of 4-^{3}H-cocaine into hair from a 1 mg/mL bathing solution followed by washout from a 0.9-percent saline solution. Each data point is the mean value of duplicate 10-mg samples.*

destroying the protein matrix is alkaline digestion (incubation in 1 *N* NaOH for 1 hour at 100 °C); however, this method cannot be used for cocaine analysis because it completely degrades cocaine. For the simultaneous quantitation of cocaine, BZE, and EME in hair, it is preferable to use either enzyme digestion or extraction with acid or solvent.

Acid Extraction. Extracting hair with mineral acid (hydrochloric acid [HCl] or H_2SO_4) is an efficient method for extracting cocaine and metabolites from hair, and only a small amount of cocaine is converted to BZE with this technique (Cone et al. 1991). A method used by Valente and colleagues (1981), Balabanova and coworkers (1987), and Balabanova and Homoki (1987) called for pulverizing approximately 50 mg hair, then incubating in 0.1 M HCl overnight at 45 °C. The acid extract was neutralized with 100 μL 1 M NaOH then diluted with phosphate buffer (pH 7.4) up to 2 mL. Martinez and colleagues (1993) used a similar procedure and incubated whole,

unpulverized hair samples with 1 mL 0.1 M HCl for 18 hours at 37 °C. Cone and colleagues (1991) used H_2SO_4 (1 mL, 0.05 M) in their extraction procedure and reported a recovery of 90 percent of the cocaine added to control hair with a 10-percent conversion to BZE.

Solvent Extraction. Methanol appears to be the solvent of choice for extracting cocaine and metabolites from hair. Pirozhkov and colleagues (1992) found that incubating washed hair samples (50 to 100 mg) in 3 mL methanol for only 2 hours at 60 °C yielded a recovery equivalent to acid extraction for 18 hours. Extending the incubation period to 4 hours did not appear to increase recovery. The extracts were purified by a differential pH liquid-liquid extraction procedure similar to that for the extraction of cocaine from plasma (Jatlow 1975, pp. 133-137). No recovery data were given; however, a limit of detection for cocaine of 0.2 ng/mg hair was reported.

Graham and colleagues (1989) described a similar extraction procedure for BZE in which washed hair samples (2 mg) were sonicated with 1 mL methanol for 30 minutes and then incubated overnight at 45 °C.

Enzyme Digestion. The authors used a soft digestion technique for hair samples similar to that reported by Gill and coworkers (1985) but modified it to improve the recovery of cocaine and reduce chemical background in the GC/MS analysis (Harkey et al. 1991). Approximately 10 mg hair was placed in a screw-capped silanized glass centrifuge tube (10 mm wide x 100 mm deep) with 2.6 mL digest buffer (1 mL 1 M Tris HCl buffer, 20 mL 10 percent sodium dodecyl sulfate, and 79 mL deionized water) and with 0.4 mL 0.4 M dithiothreitol in 10 mM sodium acetate buffer and then was vortexed and incubated for 2 hours at 40 °C. Then 55 μL proteinase K solution (10 mg/mL or 136 units/mL) was added; the sample was vortexed again and incubated overnight at 40 °C. Solid-phase extraction was used to isolate cocaine, BZE, and EME from the digested hair samples.

Möller and colleagues (1992) described a faster procedure in which 20 to 30 mg hair was pulverized and then digested for 2 hours at

40 °C in a solution containing 75 μL β-glucuronidase-aryl-sulfatase in 2 mL phosphate buffer (pH 7.6). They also used solid-phase extraction prior to derivatization and GC/MS analysis.

Cleanup Procedures

Both liquid-liquid extraction and solid-phase extraction have been used to purify hair extracts prior to analysis.

Liquid-Liquid Extraction. Pirozhkov and colleagues (1992) described a differential pH liquid-liquid extraction procedure for purifying hair extracts that is similar to a procedure for extracting cocaine from plasma (Jatlow 1975, pp. 133-137). Methanol hair extracts were evaporated under N_2 at 40 °C; the residue was dissolved in 2 mL 0.1 *N* HCl and then was extracted with 4 mL hexane containing 1 percent isoamylol. After the residue was centrifuged for 5 minutes at 2,500 rpm, the hexane layer was discarded, and the acid phase was made alkaline to pH 9.2 by adding 0.03 mL NH_4OH and 0.5 mL 10 percent K_2PO_4 and then was extracted with 4 mL hexane-isoamylol mixture. The organic phase was evaporated under N_2, and the residue was dissolved in methanol for subsequent analysis.

Solid-Phase Extraction. Möller and colleagues (1992) isolated cocaine and its metabolites from digested hair samples using Chromabond C_{18} extraction columns previously conditioned with 6 mL methanol and 3 mL water (H_2O). After the hair digest was added, the column was washed with 3 mL H_2O, 3 mL 0.25 *N* acetic acid, and then 3 mL H_2O. The column then was dried by passing air through it for 10 minutes and centrifuging at 4,000 rpm for 15 minutes. Absorbed drugs were eluted three times with 500 μL of three parts acetone to one part dichloromethane.

In the authors' laboratory, cocaine and metabolites were extracted from the digested hair samples using "double mechanism" (reversed phase and ion exchange) extraction columns (Bond Elut Certify). After the columns were conditioned with 2 mL methanol and 2 mL 0.1 M phosphate buffer, the hair digest was added to the columns and the columns were rinsed with 3 mL deionized water, 3 mL 0.1 M HCl, and 8 mL methanol. Cocaine, BZE, and EME then

were eluted with two additions of 2 mL methylene chloride:isopropyl alcohol (4:1) with 2 percent ammonium hydroxide. The extracts were evaporated under N_2 at 40 °C prior to derivatization and GC/MS analysis (Harkey et al. 1991).

Screening Methods

RIA is the most popular screening method for hair analysis, probably because of its sensitivity and the availability of commercial reagent kits. Because drugs and their metabolites are present in hair in ng/mg or pg/mg concentrations, it is likely that only immunoassays that use radiolabeled or chemiluminescence tracers have the requisite low limits of detection for hair analysis.

Radioimmunoassay. RIA has been used extensively both for screening and quantitating cocaine in hair. However, it was not appreciated by many of the earlier investigators that cocaine is the primary analyte in hair; therefore, some investigators used RIAs that were highly specific for BZE, whereas others used RIAs specific for cocaine. More recently, investigators have made their own modifications to commercially available reagent kits in an attempt to make their determinations more precise.

One of the earliest methods reported was a proprietary immunoassay procedure developed by Baumgartner and associates called RIAH (radioimmunoassay for hair). Unfortunately, the details of their proprietary methodology were not provided in any of their publications, which has prompted some editorial discussion in the literature (Sauls 1990; Needleman 1991).

Graham and Koren have used RIA reagents from two manufacturers for both screening and quantitating cocaine and BZE in hair (Graham et al. 1989; Koren et al. 1992*a*, 1992*b*). For the analysis of cocaine they used a reagent kit (Coat-A-Count, Diagnostic Products, Los Angeles, CA) with antibodies that have a much higher affinity for cocaine than for BZE (cross-reactivity with BZE is 0.5 percent). They further modified the manufacturer's procedure by using cocaine hydrochloride standards (1 to 500 ng/mL) instead of the BZE standards provided. They reported a sensitivity for the assay of 0.025 ng cocaine/mg hair. For the analysis of BZE, they

used a reagent kit containing antisera directed at the cocaine metabolite; however, there was a cross-reactivity with cocaine of 4 percent (Roche Abuscreen, Hoffman-La Roche). They reported a sensitivity of 0.25 ng BZE/mg hair.

Martinez and colleagues (1993) used the Coat-A-Count RIA kit with the BZE calibrators provided by the manufacturer. Hair extracts were centrifuged to separate particulate matter and then assayed directly. Their own cutoff concentrations were established by adding known amounts of BZE to 100-mg samples of drug-free hair and performing serial dilutions to give a final concentration in the ng/mL range. Their reported cutoff was 0.25 ng/mg hair expressed as combined cocaine and BZE, rather than BZE alone.

Fluorescence Polarization Immunoassay. Kintz and colleagues (1992) described the use of fluorescence polarization immunoassay (Abbott AD_x) in screening hair samples for a variety of drugs, including cocaine. Their alkaline hydrolysates of hair samples were neutralized with HCl, half diluted with AD_x buffer, and then analyzed directly for a positive or negative response according to the manufacturer's recommendations. They used the manufacturer's recommended cutoffs for plasma or urine, finding the method to be efficient, and because the antibody used can act directly on the hair hydrolysate, no prior purification was required.

Confirmation and Quantitation Methods

Gas Chromatography/Mass Spectrometry. A variety of GC/MS methods have been reported for confirming and quantitating cocaine and its metabolites in hair. The methods differ with regard to the reagents used to derivatize BZE and EME, the type of mass spectrometer used (mass selective detector [MSD], ion trap, or tandem mass spectrometer), or the ionization mode (electron impact or chemical ionization).

The authors' GC/MS procedure used ion-trap technology with chemical ionization and was developed specifically to quantitate the low levels of cocaine and metabolites likely to be found in hair following a single dose (Harkey et al. 1991). Hair digests were purified by bonded phase extraction, derivatized by adding

10 μL N-methyl-N-(tert-butyldimethylsilyl)-trifluoroacetamide (MTBSTFA), and incubated at 40 °C for 10 minutes. Aliquots (1 μL) were injected into the gas chromatograph/mass spectrometer for quantitation. Analysis was performed on a Finnigan model ITS-40 ion-trap mass spectrometer (San Jose, CA) equipped with a 15 m x 0.25 cm, 0.1 μm film thickness, DB-5 capillary column. The instrument was operated under chemical ionization conditions using isobutane as a reagent gas. Difluorococaine was used as an internal standard, rather than deuterated cocaine, because the studies required quantitation of both cocaine and pentadeuterated cocaine (d_5-cocaine). The limit of quantitation for this method was 100 pg/mg hair for cocaine and BZE and 500 pg/mg for EME. The coefficient of variation was approximately 15 percent for cocaine and 25 percent for BZE and EME at concentrations less than 1 ng/mg. Chemical ionization was particularly useful for quantitation when the concentrations of cocaine and metabolites were low. Welch and colleagues (1993) also found methane chemical ionization particularly useful in quantitating cocaethylene in hair. The abundant molecular ion at 318 m/z (mass-to-charge ratio) and the base peak at 196 m/z are essentially the only peaks in the spectra. Cone and coworkers (1991) derivatized their acid extracts of hair (after partial purification by solid-phase extraction) with N,O-bis(trimethylsilyl) trifluoroacetamide (BSTFA) and 1 percent trimethylchlorosilane; they then analyzed aliquots using a Hewlett Packard model 5890A gas chromatograph interfaced with a model 5970B MSD (Palo Alto, CA), which was fitted with a cross-linked fused-silica capillary column (0.20 mm × 12 m). They reported a limit of detection of 0.1 ng/mg for all analytes using a 50-mg hair sample.

Nakahara and colleagues (1992) used hexafluoroisopropanol (HFIP) to derivatize BZE and pentafluoropropionic anhydride (PFPA) to derivatize EME. Hair samples were digested with proteinase K, purified by solid-phase extraction, derivatized with HFIP and PFPA, and then injected into a Hewlett Packard model 5890A gas chromatograph (Palo Alto, CA) fitted with a capillary column (0.25 mm × 25 m, 100 percent dimethyl polysiloxane) coupled to a model 5970B MSD operated in the selected ion monitoring mode. They reported a limit of detection of 0.3 ng/mg hair for cocaine, BZE, and EME.

Möller and colleagues (1992) enzymatically digested hair samples and then used solid-phase extraction before derivatization with a mixture of PFPA and pentafluoropropanol. Quantitation was performed with a Hewlett Packard model 5890A gas chromatograph (Palo Alto, CA) fitted with a capillary column (cross-linked 5 percent phenyl methyl silicone, 0.33 μm film thickness, 0.20 mm ID $\times$ 12 m). The model 5971A MSD was operated in the electron impact mode, and the limit of detection (using 10 to 30 mg hair) was 0.1 ng/mg for cocaine and BZE and 1 ng/mg for EME.

Tandem Mass Spectrometry (MS/MS). The relatively high cost of purchasing and maintaining tandem mass spectrometers has limited their use to relatively few laboratories. MS/MS analysis for cocaine in hair has been conducted primarily by the U.S. Naval Research Laboratory (Kidwell 1993), the Federal Bureau of Investigation (Martz 1988; Martz et al. 1991), and the National Institute of Standards and Technology (Welch et al. 1993). With MS/MS, intact hair could be analyzed directly or, usually, an extracted residue of hair was analyzed. For the analysis of intact hair specimens, a few strands of hair were washed, dried, cut into small (0.5 cm) pieces, and then placed in a solid probe. Segmental analysis was performed using approximately five hair strands that had been cut into sequential 0.5-cm sections. The probe was inserted into the first MS stage and heated at a relatively low temperature (110 °C) to remove any moisture. Next, the probe was heated to vaporize volatile components in the sample. The volatile compounds were ionized and then transmitted out of the first mass spectrometer into the second mass spectrometer where they were detected and analyzed.

Tandem mass spectrometers are powerful instruments and can generate large amounts of mass spectral data about the specimen under analysis. On the other hand, they are more dependent on tuning conditions and type of hair than quadrupole or ion-trap mass spectrometers. Quantitation can be confounded by contaminants in hair that cause instrument detuning, and if the probe is heated too quickly, false-positive signals can be produced even with control drug-free hair. If the probe is heated too slowly, a bimodal desorption profile (e.g., two peaks for cocaine) can be observed. This is thought to result from the volatilization of loosely bound

cocaine (drug absorbed on the hair surface) followed by the volatilization of cocaine from the hair cortex (Kidwell 1993).

Thus, MS/MS analysis of hair, especially using thermal desorption, may be more sensitive to hair type and to instrument-tuning conditions than the more routinely used GC/MS. Welch and colleagues (1993) found that when intact hair samples were analyzed, it became increasingly difficult to distinguish between the hair of drug users and blank hair as the number of samples increased, probably because of contamination from the hair or various pyrolysis products. They achieved better results when cryogenically powdered hair samples rather than short hair segments were used. All laboratories reported much better results using extracts from hair rather than direct analysis of hair. Thus, MS/MS may provide greater sensitivity than GC/MS for the quantitation of cocaine, BZE, and EME in hair; however, MS/MS's high cost makes GC/MS the preferred method for most laboratories.

COCAINE AND METABOLITE CONCENTRATIONS IN HAIR

The range of cocaine, BZE, and EME concentrations found in human hair obtained from a variety of sources is summarized in table 1. Cocaine concentrations were low and typically ranged from 0.1 (the detection limit reported by most investigators) to approximately 50 ng/mg. Concentrations of BZE were invariably lower (0.2 to 6 ng/mg), and the concentrations of EME were even lower (trace to 4.4 ng/mg). The ratio of cocaine to BZE and EME was highly variable (e.g., 1.3:10 for C:BZE and 1.2:22 for C:EME). Table 2 shows representative values from human subjects and from rats.

The concentration of these analytes in the hair of experimental animals after dosing with cocaine was also low (table 3), and the ratio of cocaine to its metabolites generally paralleled that observed in humans. The drug concentration in hair of the various test groups is discussed below.

TABLE 1. *Range of cocaine, BZE, EME, and cocaethylene concentrations found in human hair*

Group	Self-Reported Dose	Number of Subjects	Cocaine (ng/mg)	BZE (ng/mg)	EME (ng/mg)	CE (ng/mg)	Reference
Drug treatment	Heavy user	10	6.4-19.2	0.3-2.5	0-1.9	0-2.6	Cone et al. 1991
Drug treatment	0.04-5 g/month	13	--	0.007-6.4	--	--	Baumgartner and Hill 1982
Drug treatment	Unknown	7	0.6-6.4	--	--	--	Balabanova and Homoki 1987
Drug treatment	1-3 times/week	6	0-5.7	0-1.1	--	--	Harkey et al. 1991
Coca chewers	100 mg/day	5	1.0-28.9	0.3-4.4	0-4.4	--	Henderson et al. 1992
Coca chewers	100 mg/day	20	1.4-50.6	0.4-17.6	Trace	--	Möller et al. 1992
Controlled-dose study	0.6-4.2 mg/kg (single dose)	25	0.1-5	0.1-0.36	Trace	--	Henderson et al. 1993
Arrestees	Unknown	22	--	0.26-18	--	--	Reuschel and Smith 1991
Mothers	Occasional	3	0.03-1.2	--	--	--	Graham et al. 1989
Mothers	Frequent	13	0.6-29.1	--	--	--	Graham et al. 1989
Neonates	Unknown	7	0.2-27.5	--	--	--	Graham et al. 1989
Infants	Unknown	2	4.3, 7.8	--	--	--	Graham et al. 1989

KEY: BZE=benzoylecgonine; EME=ecgonine methyl ester; CE=cocaethylene

TABLE 2. *Ratios of cocaine to metabolite concentrations reported in hair**

Group	Dose†	Number	Cocaine:BZE	Cocaine:EME	Reference
Coca chewers	About 100 mg/day	20	1.3-4.7	1.2-10.3	Möller et al. 1992
Drug treatment	Heavy users	10	5-10	5.2-22	Cone et al. 1991
Coca chewers	About 100 mg/day	5	2.1-8.6	6.6-15.5	Henderson et al. 1992
Controlled-dose study	0.6-4.2 mg/kg (single dose)	25	0.1-5	--	Henderson et al. 1993
Rats	5 mg/kg/day for 5 days	7	10‡	20.5‡	Nakahara et al. 1992

*Drug and metabolite concentrations were determined by gas chromatography/mass spectrometry.
†The amount of cocaine consumed was estimated or self-reported, except for the study involving the experimental animals (rats).
‡Mean value for seven animals.

KEY: BZE=benzoylecgonine; EME=ecgonine methyl ester

Arrestees

Hair samples weighing 5.3 to 61.2 mg were collected from a group of 48 jail detainees, extracted with ethanol, and analyzed by RIA specific for BZE. Of these samples, 22 were positive, and GC/MS analysis showed that BZE concentrations ranged from 0.26 to 18 ng/mg hair (Reuschel and Smith 1991). The samples were collected anonymously, and there was no attempt to elicit a drug use history.

Volunteers in Drug Treatment Programs

Baumgartner and colleagues (1982) used an RIA kit targeted at BZE to analyze hair samples from 13 admitted cocaine users in a drug rehabilitation program. Their self-reported cocaine use ranged from 0.04 to 5 g cocaine/month, and the drug concentrations found in

TABLE 3. *Range of cocaine, BZE, EME, and cocaethylene concentrations found in animal hair*

Species	Dose	Number	Cocaine (ng/mg)	BZE (ng/mg)	EME (ng/mg)	Reference
Rat	5 mg/kg/day (IP) for 5 days	9	16.4±4.8	1.7±0.4	0.8±0.3	Nakahara et al. 1992
Rat	3 mg/day (IM) for 10 days	7	--	0.02-0.05	--	Michalo-dimitrakis 1987
Mouse	40 mg/kg/day (IP) for 3 weeks	26	0.8-2.4	--	--	Pirozhkov et al. 1992
Mouse	20-50 mg/kg/day (IP) for 6 weeks	20	316-1,609	--	--	Poet et al. 1992
Sheep	2 mg/kg/day (IV) for 40 days	Not specified	1.5-3.0	--	--	Balabanova and Homoki 1987

KEY: BZE=benzoylecgonine; EME=ecgonine methyl ester; IP=intraperitoneally; IM=intramuscularly; IV=intravenously

their hair (reported as BZE equivalents) ranged from 0.007 to 6.4 ng/mg.

Cone and colleagues (1991) obtained hair specimens from 10 individuals who had completed a 180-day outpatient drug treatment program. The subjects identified themselves as heavy cocaine users; half the group identified themselves as intravenous users. The ranges of cocaine, BZE, and EME concentrations in their hair (quantitated by GC/MS) were 6.4 to 19.2 ng/mg, 0.3 to 2.5 ng/mg, and trace to 2.9 ng/mg, respectively. In addition, cocaethylene and norcocaine (chemical formulas shown in figure 1) were detected in low but quantifiable amounts in the hair of approximately half the subjects. The ratios of cocaine to metabolites varied among subjects and ranged from 5 to 10 for cocaine to BZE and from 5.2 to 22 for cocaine to EME. Balabanova and Homoki (1987), using an RIA for BZE, measured drug concentrations in the hair of seven cocaine users and their results were reported as the sum of cocaine and BZE. They reported drug concentrations in hair that ranged from 0.6 to 6.4 ng/mg. When these subjects were tested

again 3 months later, the drug concentrations in hair had decreased to between 0.3 and 0.5 ng/mg.

The authors analyzed the hair of five subjects who had applied for participation in the clinical studies. These subjects were self-identified as experienced cocaine users, but none exhibited clinical signs of cocaine dependency. The concentration of cocaine in the hair of these subjects (quantitated by GC/MS) ranged from 0 to 5.7 ng/mg hair. There was little correlation between the concentration of cocaine in hair and self-reported drug use. For example, the highest concentration of cocaine was found in a moderate user (i.e., self-reported drug use was 1 to 2 times a week), whereas no cocaine was detected in the hair of a subject who reported heavy (i.e., daily) use of cocaine (detection limit 0.1 ng/mg hair). BZE was found in the hair of only one subject and at a concentration of 0.1 ng/mg.

Pregnant Women and Neonates

There has been increasing interest in the use of hair samples from pregnant women, postpartum women, neonates, and infants as a method for measuring in utero exposure to cocaine. Graham and colleagues (1989) used RIA to measure the concentrations of BZE in the hair of pregnant women and neonates. The range of concentrations in those self-reporting as occasional users was 0.03 to 1.2 ng/mg and was 0.6 to 29.1 ng/mg in the hair of frequent users. Hair from seven neonates with a confirmed history of cocaine exposure had an average of 5.4 ng/mg BZE (range 0.2 to 27.5), whereas the hair of two infants (ages 2.5 and 3.5 months) had values of 4.3 and 7.8 ng/mg, respectively.

Coca Chewers

Hair obtained from South American native peoples provided interesting specimens for cocaine analysis. The native peoples of South America have chewed the leaves of the coca shrub for more than 3,000 years. Once prevalent throughout the continent, the custom today is restricted to Indians of Peru, Bolivia, Colombia, western Brazil, and northern Argentina (Carroll 1977). The concentration of cocaine in the plant material is variable, and there

is also considerable variation in the use of coca; however, if one assumes a typical quid (a cut or wad) of coca leaves (10 to 20 g) contains approximately 0.5 percent cocaine, dry weight, then a conservative estimate of the daily dose of cocaine ingested by coca chewers is 50 to 100 mg. Absorption of the drug likely takes place in the buccal cavity as well as in the intestine, and significant blood levels of cocaine (about 100 to 200 ng/mL at peak) following coca chewing have been reported (Holmstedt et al. 1979; Paly et al. 1980, pp. 86-89). This was a significant concentration but less than that reported for cocaine abusers (Jaffe 1989, pp. 535-584). Therefore, these hair samples might have been representative of someone who ingested approximately one line (approximately 25 mg) of cocaine per day.

The authors analyzed the hair from five South American Indians who acknowledged chewing coca leaves daily. Cocaine concentration in the hair of these subjects ranged from 1.0 to 28.9 ng/mg; BZE ranged from 0.3 to 4.4 ng/mg; and EME ranged from 0.5 to 4.4 ng/mg. The finding that cocaine was present at approximately 5 times higher concentration than BZE and approximately 10 times higher than EME was surprising considering these individuals likely had high steady-state plasma concentrations of BZE. It was also interesting that washing the hair before analysis not only reduced the concentration of cocaine but also reduced the concentration of BZE and EME as well. This finding, which has been reported by others (Cone et al. 1991), suggests that the washing procedures typically used in hair analysis probably extract analytes from the hair cortex as well.

Controlled-Dose Studies

The authors performed a series of controlled-dose experiments in which isotopically labeled cocaine (benzoyl-d_5-cocaine-HCl) was administered intravenously or intranasally to 25 human volunteers under controlled clinical conditions (Henderson et al. 1993). Sequential blood and sweat were collected for up to 3 days, and hair samples were collected for up to 10 months. All samples were analyzed by chemical ionization GC/MS for cocaine-d_5 and its metabolite benzoylecgonine-d_5 (BZE-d_5). The use of isotopically labeled cocaine distinguished between administered drug and any

cocaine used by the subjects either before or during the study. In both hair and sweat, the predominant analyte was the parent drug cocaine-d_5. In contrast, BZE-d_5 was the major analyte in blood, especially after approximately 2 hours. The sevenfold range of cocaine doses used in the study (0.6 to 4.2 mg/kg) resulted in 0.1 to 5 ng of cocaine-d_5 per hair sample and approximately one-sixth that amount of BZE-d_5. The minimal detectable dose by this GC/MS procedure was estimated as approximately 25 to 35 mg drug administered intravenously, which is approximately the amount found in a single line of cocaine. The authors found a poor correlation between the dose of drug administered and the amount of cocaine-d_5 incorporated into hair. Non-Caucasians in particular incorporated considerably more cocaine-d_5 into hair than did Caucasians (from 2 to 12 times, depending on how it was measured). These interindividual differences could not be explained by differences in the individuals' plasma pharmacokinetics. Also, there was little correlation between the time of drug administration and the position of drug along the hair shaft. Segmental analysis of the hair samples revealed that some subjects who received only a single dose had cocaine-d_5 distributed along most of the hair shaft, whereas some subjects who received multiple doses had the drug confined to a much smaller area. In addition, cocaine-d_5 was detected in hair as early as 8 hours after drug administration. Hair was obtained from four subjects 1 and 3 days after they received 0.6 mg/kg of cocaine-d_5 intranasally; cocaine-d_5 was found in three of the four subjects, which suggests that sweat or sebum may play a role in the incorporation of some drugs into hair.

Animal Studies

Cocaine and metabolites have been detected in the hair of laboratory animals following cocaine administration. The species studied to date include sheep (Balabanova et al. 1987), mice (Pirozhkov et al. 1992; Poet et al. 1992), guinea pigs (Koren et al. 1992*b*), and rats (Michalodimitrakis 1987). In general, the drug concentrations in their hair are similar to those found in human hair, and the ratios of cocaine to its metabolites appear to be similar as well.

Nakahara and colleagues (1992) analyzed hair from rats that had received 5 mg/kg/day intraperitoneally (IP) for 5 days and found the

average cocaine in hair to be 16.4±4.8 ng/mg; however, these researchers did not report the range of drug concentrations found. BZE and EME were present in approximately tenfold lower concentrations. Nakahara and colleagues (1992) also studied the plasma pharmacokinetics of cocaine in their test animals and found that the amounts of cocaine and metabolites in hair did not correlate well with the concentrations of drugs in the hair. They suggested that drug incorporation into hair must depend on the physicochemical properties of the drug.

Balabanova and Homoki (1987) administered cocaine to sheep for 12 days (dose was not indicated) and found cocaine concentrations in wool in the range of 2 to 3 ng/mg (measured by RIA); these concentrations remained relatively constant for the next 60 days. Pirozhkov and coworkers (1992) administered 20 mg/kg cocaine, cocaethylene, or cocaine and ethanol twice daily to mice for 3 weeks and (measuring with GC) found cocaine concentrations in hair that ranged from 0.9 to 2.4 ng/mg and cocaethylene concentrations that ranged from 2.4 to 2.8 ng/mg. In the animals that received cocaine plus ethanol, cocaethylene could not be detected. Pirozhkov's group reasoned that hair from cocaine and alcohol abusers contains much less cocaethylene than cocaine; thus, the expected concentration of cocaethylene in the hair of the mice would be below the limit of detection for their method.

In another study using mice as experimental animals, Poet and colleagues (1992) administered cocaine IP at 20 mg/kg/day for the first week, 30 mg/kg/day for the second week, and 50 mg/kg/day for the third week. They reported extremely high concentrations of cocaine in the hair of the mice. Hair samples were extracted with HCl, and cocaine concentrations (determined by an RIA specific for cocaine) ranged from 316 to 1,609 ng/mg. These concentrations are many orders of magnitude higher than have been reported in human or other animal hair and are difficult to explain. There was a significant variation in the amount of drug incorporated into hair of similarly treated and genetically identical animals.

Michalodimitrakis (1987) injected rats daily with 3 mg cocaine administered intramuscularly for 10 days and analyzed hair samples for BZE using RIA. No drug could be detected for the first 4 days;

thereafter, BZE concentrations in hair ranged from 0.024 to 0.048 ng/mg.

CONCLUSIONS

Analytical Methods

No single method has emerged as the best or only procedure; however, RIA and GC/MS are the most commonly used screening and confirmation techniques. Because of RIA's speed, sensitivity, and relatively low cost, most laboratories use this screening method. However, because cocaine is the primary analyte in hair, the antisera should be directed at cocaine rather than BZE, as is the case with many RIA kits used in urine drug screening. Analysts also should be aware that most commercially available RIA kits are designed for urine specimens and therefore have not been evaluated for possible matrix effects from different hair digestion techniques or for cross-reactivity to other possible components in hair, such as cosmetics.

Capillary column GC/MS using deuterated internal standards is becoming the preferred method for confirming and quantitating cocaine in hair. A variety of reagents have been used for derivatizing BZE and EME, including BSTFA, HFIP, MTBSTFA, and PFPA. No reagent appears superior, and the choice probably is determined by the laboratories' existing equipment and their prior experience with the reagent. The electron impact mode is more commonly employed (probably because it is more prevalent); however, chemical ionization may be more useful to measure analytes such as norcocaine and cocaethylene that are present in low levels.

MS/MS can be a powerful technique when great sensitivity is required and can work well using extracts of hair. However, when thermal desorption is used, its performance is more easily affected by hair type, sample size, tuning conditions, and the rate at which the solid probe is heated.

Dose-Response Relationships

The correlation between the dose of cocaine and the amounts of drug and metabolites detected in hair is unclear at the present time and remains controversial. Most studies have shown few correlations between dose of drug and concentration found in hair.

Self-Reported Drug Use. Data from studies comparing hair analysis results with self-reported drug use are conflicting; however, this might be expected because in many cases the minor metabolite BZE is quantitated, and it is fairly well accepted that self-reports of drug use may be inaccurate. Subjects may deliberately mislead, forget, or simply be unaware of the amount or purity of drug used.

Baumgartner and colleagues (1982) reported that cocaine concentrations in hair correlated approximately with the severity of drug use. Similarly, Graham and coworkers (1989) found that BZE levels in the hair of 3 women in their study who self-identified as occasional users were significantly lower than the BZE concentrations of the 13 women who self-identified as frequent users.

On the other hand, Martinez and colleagues (1993) analyzed hair samples from Hispanic males attending a community outreach program and concluded that hair analysis could identify drug use that was not detected by urinalysis; however, there was no particular relationship between the stated frequency of cocaine use and the levels of cocaine and BZE in the hair of their subjects. Möller and colleagues (1992) found no correlation between concentrations of cocaine or metabolites in hair and the reported use patterns of South American coca chewers.

The authors sampled five subjects who were experienced cocaine users and found little correlation between their reported use and the cocaine concentration in their hair (Harkey et al. 1991). In fact, one subject said he used cocaine more than three times a week, yet there was no cocaine or metabolite detected in his hair.

Controlled-Dose Studies. The authors administered precise doses of cocaine-d_5 to 25 subjects under controlled clinical conditions and

used GC/MS to quantitate the cocaine, BZE, and EME incorporated into hair (Henderson et al. 1993). A poor correlation was found between the dose administered and the amount of drug (cocaine-d_5) incorporated into hair. Under certain circumstances, increased doses did result in an increased amount of drug incorporated into hair; however, because of the considerable intersubject variability, it was impossible to infer the dose administered from the amount of drug in hair. However, the doses administered in the study were limited because of ethical and safety considerations and were no doubt considerably less than what might be used by compulsive cocaine users.

Hair as a "Calendar" of Drug Intake

The authors found that it was difficult to estimate precisely the time of drug administration from the position of cocaine along the hair shaft. In some subjects' hair, sectional analysis showed that cocaine was confined to a relatively small segment of the hair shaft and that it migrated with time along the axis at a rate of about 1 cm/month, the reported hair growth rate for humans (Saitoh et al. 1969, pp. 183-201; Montagna and Parakkal 1974, pp. 83-105). However, in many subjects little correlation was found between the position of the drug along the shaft and the time since drug administration. Variables that contribute to inaccuracies in correlating position of drug along the hair shaft with the time of drug ingestion include intersubject differences in hair growth rate, measuring hairs in different phases of their growth cycle, and variability in alignment of the hair strands prior to cutting. Hair growth rate can vary threefold among individuals (0.5 to 1.5 cm/month) (Saitoh et al. 1969; Montagna and Parakkal 1974, pp. 83-105).

Uematsu and colleagues (1993) used a single dose of a quinolone antimicrobial to measure hair growth rate in human volunteers and found the drug was distributed widely along the hair shafts (5 to 7 cm) when five strands of hair were measured. However, when only one strand of hair was measured, the drug was restricted to a 1- to 2-cm portion of the shaft. This suggests that there may have been some variability in aligning the hair prior to cutting or that not all hairs in the sample were in the growing phase when the drug was ingested. Finally, because cocaine is excreted in sweat, it is

possible that the drug may be incorporated directly into hair as the hair emerges from the scalp.

External Contamination as a Potential Source of False Positives

The possibility that a positive hair test for cocaine could result from external contamination is an important consideration when interpreting the results of a hair test. A few investigators have shown that washing can remove all externally deposited cocaine (Baumgartner and Hill 1992, pp. 577-597; Koren et al. 1992*b*). Others have found that hair exposed to cocaine in aqueous or other solution will accumulate large amounts of the drug; that washing will remove most, but not all, of the accumulated drug; and that the amount remaining will be consistent with very heavy drug use (Cone et al. 1991; Henderson et al. 1991; Welch et al. 1993; Blank and Kidwell 1993). The National Institute of Standards and Technology prepares reference materials by soaking hair in a solution of dimethylsulfoxide containing cocaine, BZE, morphine, and codeine. These materials are provided to laboratories as proficiency samples to evaluate the accuracy and precision of the laboratories' methods. Until this issue is resolved, external contamination should always be considered when interpreting hair analysis data, and both the collection site and the testing laboratory should have rigorous quality control measures to prevent contamination of any specimen. It has been proposed that unique metabolites such as cocaethylene or norcocaine be used as indicators of active exposure; that is, these metabolites, as well as cocaine and BZE, should be present in any hair sample declared positive (Cone et al. 1991). However, these metabolites are present in low, often undetectable, concentrations. In addition, cocaethylene or norcocaine has been identified in contraband cocaine samples, although this is likely a rare occurrence (Janzen 1992).

Hair Types and the Racial Issue

The possibility that drug incorporation into hair differs with hair type has been raised by several investigators and remains controversial. Kidwell (1993) found that thick, black hair takes up drugs more slowly from solution and releases them more slowly than fine, brown hair. Uematsu and coworkers (1993) have

suggested that certain drugs bind to melanin and found that in Japanese subjects the concentrations of haloperidol, chlorpromazine, nicotine, and quinolone anitimicrobials are higher in black hairs than in the "grizzled" (i.e., white) hairs of their subjects. In the authors' controlled-dose study with cocaine-d_5, all non-Caucasians (i.e., African-Americans, Hispanics, and East Indians) in the study incorporated significantly higher levels of cocaine-d_5 into their hair (Henderson et al. 1993). The plasma pharmacokinetics for cocaine in these subjects were not significantly different from the Caucasian subjects.

SUMMARY

Over the past decade, several methods for analyzing hair for cocaine have been developed, and laboratories throughout the world are conducting such tests. No single method or combination of methods has been accepted as a benchmark or standard. Because so many methods have been used, some specific and some nonspecific, it is difficult to compare many of the data, and serious questions still remain about how to interpret the results of a hair test for cocaine.

Nevertheless, the following is known about cocaine hair analysis: In humans (and experimental animals), cocaine ingestion can be detected by analyzing hair (or fur). Even a single dose of cocaine can be detected if sensitive methods are employed. Increasing doses usually result in increased levels of the drug and metabolites in hair; however, a clear dose-response relationship has not been established. This is not unexpected given the variety of methods that have been used and the paucity of controlled-dose studies. Also, it may be that the physicochemical characteristics of cocaine—for example, its high lipid solubility—allow this drug access to hair through alternate routes such as sweat and sebum. At the very least, it is known that the pharmacokinetics of cocaine incorporation into hair do not mirror its pharmacokinetics in plasma because cocaine is the major analyte in hair and the metabolites BZE and EME are present in low and variable amounts in hair.

Hair sampling procedures are becoming more standardized. Samples to be tested for cocaine are most often obtained from the

scalp, usually from the posterior vertex region that has a high population of hair follicles in the growing stage. However, some researchers have advocated using axillary or pubic hair because hair from these regions would less likely be contaminated from an external cocaine source. Unfortunately, little is known about the relative rates of cocaine incorporation into various types of hair or what role sweat may play. Even when a common sampling site is used, such as the posterior vertex region, there are indications that different hair types may preferentially incorporate cocaine. This so-called "racial bias" has not been established, but it should receive more attention.

Finally, the effectiveness of washing procedures in removing cocaine adsorbed or incorporated from the external environment remains controversial. This has important implications for the forensic application of cocaine hair testing results. At the very least, the lack of standardized washing procedures continues to make interlaboratory comparisons of data problematic.

In conclusion, there are several sensitive methods now available for the analysis of cocaine and metabolites in hair. However, until the testing technology is standardized, the mechanisms of cocaine incorporation into hair better understood, and unequivocal procedures for distinguishing between drug ingestion and external contamination developed, the results from hair tests for cocaine should be interpreted with caution.

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How Environmental Drug Exposure Can Affect Hair Testing for Drugs of Abuse

Edward J. Cone and Wen-Ling Wang

INTRODUCTION

Hair testing for drugs of abuse has received considerable attention because of its potential to serve as a long-term record of drug exposure. It is known that drugs can become sequestered in hair and can be detected for long periods after active drug use (Harkey and Henderson 1989, pp. 298-329). When compared with tests of accessible biological fluids, analysis of hair presents a clear time advantage. As illustrated in figure 1, blood and saliva drug testing provides information for approximately 24 hours after drug exposure. Urine testing may provide positive results for up to a week. Recent studies on sweat analysis suggest that this fluid can be collected in devices that can be worn up to 30 days (Cone et al. 1994). In contrast, hair testing can provide evidence for 3 months or longer, depending on the length of an individual's hair. Collection of hair samples also may be considered less invasive and less subject to tampering and adulteration than collection of other specimens. These considerations make hair an attractive matrix for use as evidentiary material to clinicians, treatment specialists, epidemiologists, forensic toxicologists, and law enforcement personnel.

Although hair offers certain advantages for testing over other biological tissues, there remain several unresolved, controversial issues that limit widespread acceptance of this technique (Holden

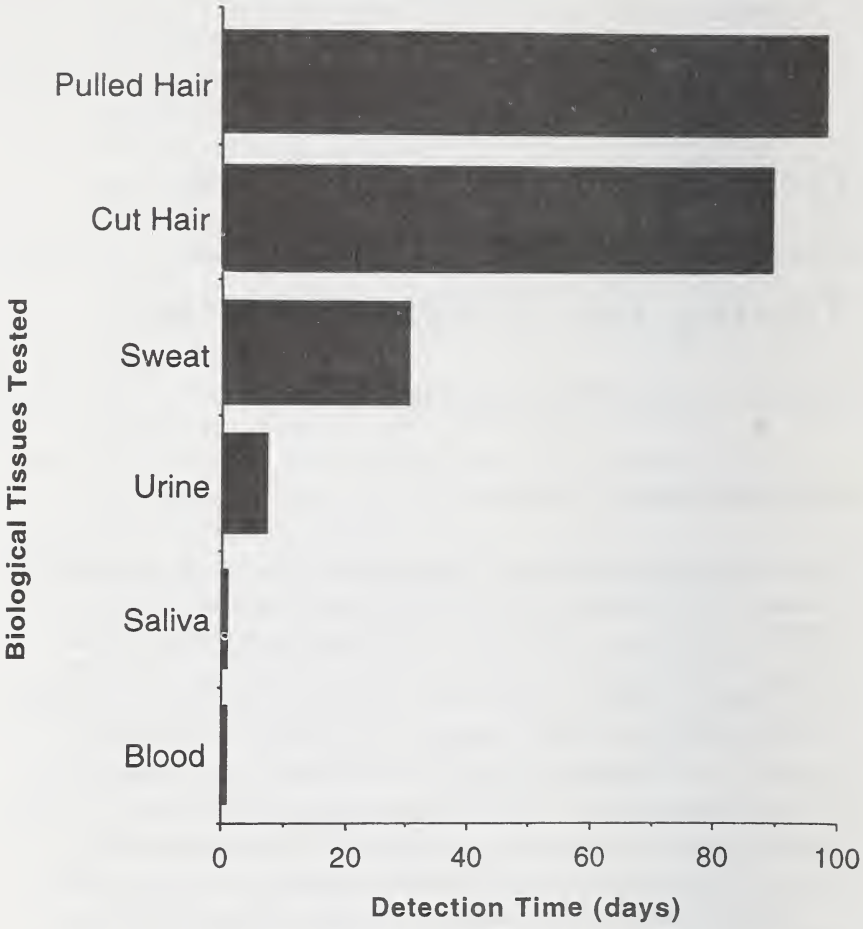

FIGURE 1. *Average detection times for drugs in hair, sweat, urine, saliva, and blood. Note that the longer detection time for "pulled" hair compared with "cut" hair results from inclusion of the root portion of hair. It is assumed that hair strands of approximately 3 cm are used for drug analysis.*

1990). One of the most serious issues that could affect the validity of hair testing is the potential generation of false-positive results

from environmental contamination. Hair serves several bodily functions, including that of being a barrier between living tissue and the external world. Clearly, the potential exists for contamination of hair from drugs in the environment. Hair, especially head hair, is exposed to and often collects substantial amounts of dust particles. Where large amounts of drugs are present, drug "dust" particles can form and settle on hair. These dust particles may consist of dry, powdered drug or vaporized drug (aerosols). Drugs also may be present in the environment in liquid form and may contaminate hair through physical contact with a drug solution or a residue from the solution (e.g., a drug-contaminated comb used for grooming).

MECHANISMS OF DRUG ENTRY INTO HAIR

Although it is not known how drugs enter hair, there are several possible mechanisms. Following a person's active drug use, diffusion from blood into the building blocks of hair could occur (figure 2). An intact, viable hair consists of a root section implanted

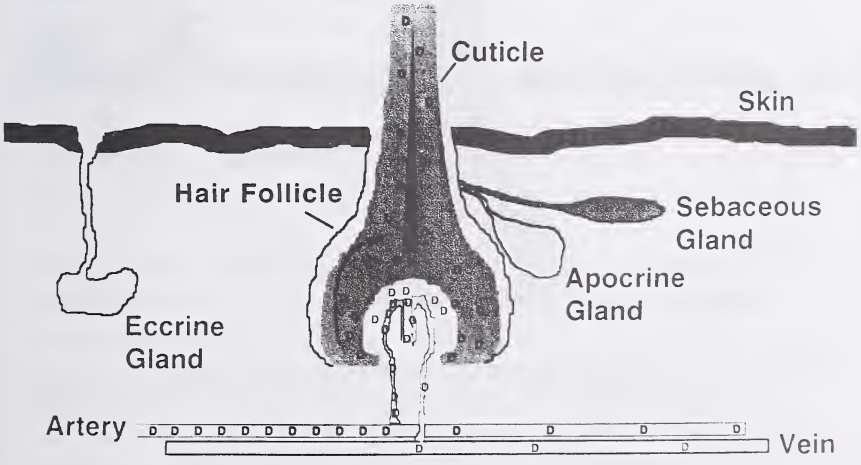

FIGURE 2. *Drug (D) entering hair by passive diffusion from arterial blood*

in the skin and a shaft above the skin surface. A hair root consists of an enlarged bulb encapsulated in a follicle that contains a dense network of capillaries. Arterioles supply nutrients to the follicle via the capillaries. It is likely that lipophilic drugs in blood traverse the thin capillary membranes and bind to or become sequestered in newly formed hair cells. As hair cells proliferate and migrate toward the skin surface, they die and become keratinized, forming the fiber-like material of hair. During this process, drugs and their metabolites become locally entrained in hair. It is assumed that drug sequestered in hair as a result of a single-drug episode will emerge at the skin surface as a narrow band without extensive diffusion in the hair matrix.

Several other possibilities exist for drug entry into hair. These mechanisms involve external contamination of the hair shaft (figure 3). The sebaceous gland secretes sebum, a wax-like substance, and the apocrine gland secretes an oily liquid. The eccrine (sweat) gland produces perspiration and functions primarily

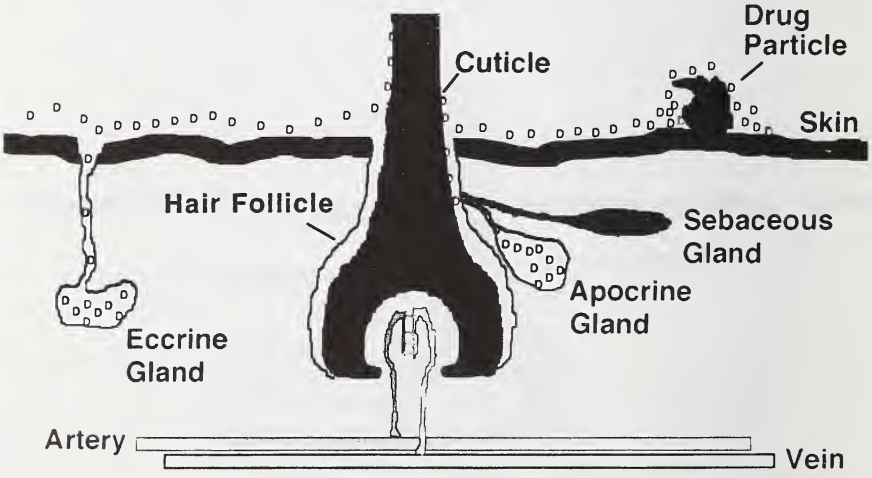

FIGURE 3. *Drug (D) entering hair by external contamination from the apocrine gland, sebaceous gland, eccrine gland, and the external environment*

to regulate body heat. Perspiration consists of approximately 99 percent water, with sodium chloride as the chief solute, but numerous other organic and inorganic trace constituents also are present. The secretions of all these glands coat the hair shaft. Consequently, when drugs are present in the organism, it is likely that they are deposited on the hair shaft via these processes. In a similar manner, environmental drugs, either in solid or liquid forms, also could be deposited on the hair shaft.

ENVIRONMENTAL DRUG CONTAMINATION

To address the issue of whether environmental drug contamination of hair could occur, the authors developed methods for artificially contaminating hair with drugs of abuse in solid and liquid forms. Because of the prevalence of cocaine use and its different modes of administration (intravenous, intranasal, and smoking) by drug users, cocaine was chosen as the model compound for contamination experiments. Cocaine is sold on the illicit market as a powder (hydrochloride salt) and as a base ("crack"). Cocaine hydrochloride is highly soluble in water (1 g dissolves in 0.4 mL of water), whereas cocaine base is nearly insoluble (1 g dissolves in 600 mL water). Heating or smoking cocaine hydrochloride leads primarily to its destruction. In contrast, cocaine base melts at 98 °C and can be vaporized or smoked efficiently at temperatures below 225 °C (Nakahara and Ishigami 1991). Because of these considerations, methods were developed for contaminating hair with cocaine-vapor (cocaine base) and cocaine hydrochloride solutions.

Cocaine-vapor contamination experiments were performed by placing drug-free hair in a chamber for 1 hour with vaporized cocaine base (10 mg, 200 °C). Samples were removed after exposure, washed three times with methanol for 15 minutes, and then extracted overnight with methanol. The wash and extract fractions were analyzed by gas chromatography/mass spectrometry (GC/MS). Cocaine hydrochloride solution experiments were performed by soaking drug-free hair overnight in an aqueous solution of cocaine hydrochloride (0.01 to 1.0 mg/mL). The contaminated hair was removed, blotted, and air dried. The samples

were analyzed in a manner similar to that employed for cocaine vapor-exposed hair.

Figure 4 illustrates GC/MS responses for cocaine (retention time=6.29 minutes) from (a) hair extracts, (b) control hair sample, (c) cocaine vapor-contaminated sample after a methanol wash, and (d) aqueous cocaine hydrochloride-contaminated sample after a methanol wash. It was obvious from this experiment that hair samples contaminated with both cocaine vapor and cocaine hydrochloride solutions remained contaminated with cocaine despite use of an extensive methanol wash procedure used to remove external contamination.

EFFECTIVENESS OF WASH PROCEDURES

When cocaine is deposited on hair as a result of environmental contamination, it is tempting to believe that cocaine is deposited only on the surface of hair (Koren et al. 1992). Although techniques have not been fully developed to visualize the location of cocaine in hair, the idea of cocaine contamination residing only on the surface of hair appears to be true for cocaine vapor contamination but not for aqueous cocaine contamination. When freshly contaminated hair (cocaine vapor contamination) is washed with an organic solvent like methanol or ethanol, the majority (>90 percent) of cocaine contamination is removed (Cone et al. 1991). After hair is washed, the residual cocaine that remains is no longer washable, suggesting penetration of the remaining cocaine into the hair matrix.

When hair comes in contact with aqueous solutions of cocaine hydrochloride, a somewhat different picture of contamination emerges. Washing freshly contaminated hair (aqueous cocaine contamination) with an organic solvent removes only about 25 percent of the cocaine contamination. The remainder is removed only with vigorous extraction conditions. Similar results have been obtained in contamination experiments with other drugs. When hair was contaminated with aqueous heroin hydrochloride (0.1 mg/mL) or with 6-acetylmorphine hydrochloride (0.1 mg/mL), the methanol wash procedure removed only 56.1 and 77.3 percent of the drugs, respectively, from the contaminated hair (Goldberger et al. 1991).

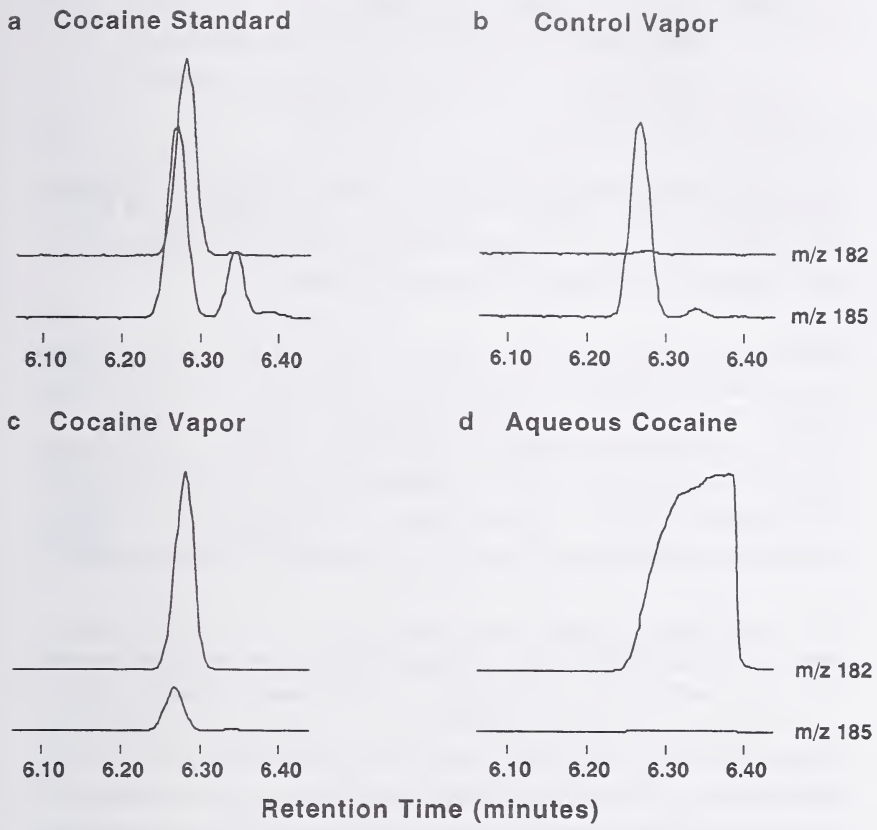

FIGURE 4. *Selected ion monitoring recordings of a standard cocaine hair extract and environmentally contaminated hair samples. Panel a represents the response for standard cocaine (50 ng) at mass-to-charge ratio (m/z) 182 (retention time [RT]=6.29 minutes) and deuterated cocaine (100 ng) at m/z 185 (RT=6.28 minutes). Panels b and c illustrate the response for the acid extract (after methanol wash) for cocaine-free control hair exposed to room air and cocaine vapor (10 mg in a 2×3×3 foot box), respectively. Panel d illustrates the response for the acid extract (after methanol wash) for cocaine-free control hair soaked for 48 hours in aqueous cocaine solution (1 mg/mL). Note the saturated response for cocaine in panel d.*

SOURCE: Cone et al. 1991. Reproduced from the *Journal of Analytical Toxicology* by permission of Preston Publications, A Division of Preston Industries, Inc.

These data suggest that the presence of water facilitates penetration of drug into the hair matrix. The greater water solubility of the salt form of the drug (e.g., cocaine hydrochloride versus cocaine base) also is likely to contribute to its entry into hair.

When hair is contaminated by cocaine vapor, penetrance is governed by the local conditions to which hair is subjected, especially during bathing. The authors evaluated the effects of shampoo soaking (overnight at room temperature) on cocaine vapor-contaminated hair. Shampoo soaking removed 93 to 98 percent of the initial cocaine contamination; however, the remaining cocaine was found primarily in the extract fraction, with much less present in the wash fraction.

These experiments suggest that contamination of hair with cocaine vapor differs substantially from contamination with aqueous cocaine only in the early stages immediately after cocaine exposure. On contact with water and pH modifiers (e.g., conditioners, shampoos, hair treatments), penetrance occurs, and the differences in contamination patterns fade. Thereafter, most cocaine resides in the extract fraction, similar to what was observed for hair samples contaminated with aqueous cocaine hydrochloride and with samples obtained from active cocaine users.

BIOLOGICAL MARKERS OF ACTIVE DRUG USE

Attempts have been made to identify a unique marker of active drug use that would clearly distinguish these samples from those tainted from environmental exposure. The requirements for such a marker include (1) the presence of a metabolite that cannot be produced as an artifact in hair over time or by the analytic procedure and (2) the marker's not being prevalent in illicit drugs because this prevalence would pose the risk of producing external contamination. Possible markers of active use for several illicit drugs are shown in table 1

TABLE 1. *Possible drug markers in hair to demonstrate active drug use and environmental contamination*

Drug	Possible Markers of Active Use	Possible Markers of Environmental Contamination
Cocaine	Cocaethylene Norcocaine Benzoylecgonine Ecgonine methyl ester	Anhydroecgonine methyl ester
Heroin	6-acetylmorphine	?
Codeine	Norcodeine	?
Marijuana	11-nor-9-carboxy-Δ-9-tetrahydrocannabinol	?
Phencyclidine	Hydroxy-metabolites	Phenylcyclohexene
Amphetamine	Hydroxy-metabolites	?
Diazepam	Oxazepam	?
Nicotine	Cotinine	?

along with markers that would indicate the likelihood of environmental contamination.

The usefulness of cocaethylene and norcocaine as possible markers of active cocaine use was evaluated (Cone et al. 1991). GC/MS analysis of hair samples from 10 active cocaine users provided evidence of cocaethylene in five samples and evidence of norcocaine in four samples. Ecgonine methyl ester was present in six samples, and benzoylecgonine was present in all samples. It was concluded from these data that when cocaethylene and norcocaine are present, they serve as useful markers of active cocaine use; however, their absence is not indicative of environmental contamination.

Although some investigators have advocated the usefulness of benzoylecgonine as a marker of active cocaine use (Koren et al. 1992), others have questioned its usefulness. Nakahara and Kikura (1994) indicated, on the basis of animal studies, that benzoylecgonine is a hydrolytic product of cocaine that is formed after cocaine is incorporated into hair. Little benzoylecgonine was

incorporated into hair in their study when benzoylecgonine was administered in the absence of cocaine. The authors evaluated benzoylecgonine as a marker of cocaine use in a series of studies with human hair. In hair samples contaminated with cocaine vapor, benzoylecgonine was not found in either wash or extract fractions, thus supporting its use as a possible marker of active cocaine use. Substantial amounts of anhydroecgonine methyl ester were detected in the wash fraction, suggesting that this analyte might serve as a marker of external contamination (table 1). In contamination studies with aqueous cocaine, trace amounts of benzoylecgonine were found; however, the ratio of benzoylecgonine to cocaine in these latter samples (<0.001) was much less than that found for active cocaine users (>0.01). Consequently, this ratio appeared to be a useful marker. A benzoylecgonine-to-cocaine ratio greater than 0.01 was assumed to represent active cocaine use and was a valid marker when applied to the analyses of 20 known cocaine users, all of whom exceeded this ratio.

Doubt about the general usefulness of the benzoylecgonine-to-cocaine ratio was raised in subsequent contamination experiments with aqueous solutions of cocaine hydrochloride and benzoylecgonine. When drug-free hair was contaminated with a 1:1 mixture of cocaine and benzoylecgonine (0.1 mg/mL), the benzoylecgonine-to-cocaine ratio (approximately 0.14) was similar to that found for active cocaine users (approximately 0.16). Because illicit samples of cocaine sometimes contain benzoylecgonine, the use of a benzoylecgonine-to-cocaine ratio to distinguish active cocaine use from contamination may not be valid in cases where exposure to benzoylecgonine is possible.

SUMMARY

A positive hair test resulting from environmental contamination would be an incorrect result (false positive) and could lead to accusing an innocent person of wrongdoing. Recent contamination studies with cocaine and other illicit drugs of abuse indicate that environmental contamination can be the cause of positive hair tests. Laboratory wash procedures designed to remove external contamination did not remove all the drug, and false-positive results

were obtained as a result of exposure of drug-free hair to cocaine vapor and to aqueous solutions of cocaine hydrochloride. A search for metabolic markers of active drug use was only partially successful. The presence of cocaethylene and norcocaine appeared to be reliable markers of active cocaine use. In addition, the finding of a BZE-to-cocaine ratio greater than 0.01 also was strongly suggestive of active use. Because of the risk of environmental contamination, caution is needed in the interpretation of positive hair test results.

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Interlaboratory Studies on the Analysis of Hair for Drugs of Abuse: Results From Three Exercises

Michael J. Welch and Lorna T. Sniegoski

INTRODUCTION

Laboratories around the world are investigating the potential of testing hair for drugs of abuse. Much research must be done before this approach is widely accepted. One important aspect is whether laboratories can reliably determine, both qualitatively and quantitatively, drugs of abuse in hair.

As part of a program to develop methods and standards to support hair testing for drugs of abuse, the National Institute of Standards and Technology (NIST) invited interested laboratories to participate in interlaboratory comparison studies to determine how well drugs in hair could be detected and quantified. Goals of the study include an evaluation of the state of the art and determinations of which extraction and measurement methodologies perform best, the relationship between drug concentration in hair and laboratory performance to provide data for possible cutoff levels, and how well laboratories can detect and correct for external contamination. Eleven laboratories participated in one or both of interlaboratory exercises 1 and 2 (for which cocaine, benzoylecgonine [BZE], and morphine were used) (Welch et al. 1993*a*). For exercise 3, 12 laboratories participated in testing hair for cocaine, BZE, morphine, and codeine. Hair samples included drug-free hair and

hair in which drugs had been fortified by soaking it in solutions of drugs. This chapter describes the results of the third exercise and summarizes the overall findings from all three exercises.

EXPERIMENTAL METHODS

The experimental methods used for exercises 1 and 2 are described elsewhere (Welch et al. 1993*a*). Drug-free hair was obtained from volunteers at NIST. Because drug-free hair was more readily available than drug users' hair, the goal was to determine whether drugs could be incorporated in drug-free hair well enough that such hair could act as a surrogate for drug users' hair. Hair was placed in dimethylsulfoxide (DMSO) solutions of cocaine, BZE, morphine, and codeine, and portions were withdrawn periodically over the course of a few weeks. Each portion was rinsed with methanol, air dried, and analyzed by gas chromatography/mass spectrometry (GC/MS) (as described below) to determine the drug content. Previous studies (Welch et al. 1993*a*) have shown that the concentrations of drugs in the hair initially rise rapidly, but increase slowly thereafter, and that increasing the concentration of the analytes in the solution increases the amount absorbed by the hair until, at higher concentrations, the amount absorbed reaches a plateau.

External Contamination

For exercise 3, sample 3, which had been soaked in DMSO solutions containing cocaine and BZE, also was treated with cocaine vapor for 2 hours to mimic passive exposure. The sample was prepared in this manner to determine whether laboratories could identify which samples had external contamination and how much of the total cocaine was attributable to the vapor deposition.

Cutting Hair Into Short Lengths

In exercise 3, all hair samples were in the form of short hair segments, primarily in the 2- to 5-mm range. Hair was cut manually with scissors. Despite repeated cuttings, some longer hair survived. All the cut hair for a particular sample was mixed

repeatedly, but because of static charges, the hair tended to lump together and stick to the walls of the glass containers. Thus, homogeneous distribution of drugs in the short hair segments could not be guaranteed.

For exercise 3, each of 12 laboratories was sent 8 samples of hair and asked to identify which samples contained cocaine, BZE, morphine, and codeine and to perform quantitative analysis for drugs in the hair. They also were asked to identify which samples had been externally contaminated with cocaine vapor.

Analytical Methods Used by Participating Laboratories

The participating laboratories provided general information about their methods. In many cases, details were sketchy, but it was still possible to group laboratories based on the approaches used. One laboratory reported results from two different methods, making a total of 13 methods from 12 laboratories. All the participating laboratories used some form of chemical treatment to liberate the drugs from the hair prior to analysis. Five laboratories used enzyme digestions, performed under neutral or slightly acidic conditions, to liberate the drug. Two of these laboratories used an overnight digestion with proteinase, and two others used a glucuronidase-arylsulfatase combination for 2 hours. The other laboratory using enzyme digestion did not specify its digestion procedure. Three laboratories used acid extractions, primarily with dilute hydrochloric acid (HCl) solutions and with gentle heating. Four laboratories used methanol extractions. For quantitation, one laboratory that determined opiates only used a basic hydrolysis to dissolve the hair. However, cocaine and BZE are unstable under such basic conditions. Most laboratories subjected the samples to further cleanup either with solid-phase or liquid-liquid extractions. For quantification, one laboratory used high-performance liquid chromatography (HPLC), and the remainder used GC/MS primarily with deuterated internal standards.

Analytical Method Used at NIST

Welch and colleagues (1993*b*) weighed (10 to 20 mg) samples of hair into screw-capped vials. They added 2 mL 0.1 *N* HCl to each

sample; appropriate amounts of deuterated internal standards also were added so that the unlabeled/labeled ratio was near 1; and the samples were heated at 45 °C for 20 to 24 hours. Solutions for the determination of cocaine and BZE were neutralized with 1 *M* potassium hydroxide (KOH) to pH 6, and then 2 mL 0.1 *M* phosphate buffer (pH 6) was added to each. Solutions for the determination of morphine and codeine were neutralized with 1 *M* KOH to pH 7 to 8. Each sample was put through a solid-phase extraction column designed for urine drug testing, according to the manufacturer's directions for urine samples. Cocaine and BZE were collected together under one set of conditions; morphine and codeine were collected together using another set. The eluents were dried under nitrogen and were dissolved in N,O-bis(trimethylsilyl)-acetamide (BSA). The trimethylsilyl (TMS) ester of BZE, the TMS ether of codeine, and the TMS diether of morphine are formed under these conditions. Cocaine is not derivatized, but the silylating reagent helps maintain the nonpolar character of the GC injector and column.

The samples were injected into a quadruple GC/MS system operated in the electron ionization mode and equipped with a 30-m fused silica capillary column with a nonpolar, bonded stationary phase. A splitless injection mode was used, with the injection port set at 270 °C and the column set at 150 °C for 3 minutes and then programmed at 25 °C/min to 260 °C, resulting in a retention time of approximately 7 minutes for cocaine. Selected ion monitoring was used to monitor a mass-to-charge ratio (m/z) of 182 and 185 for cocaine and cocaine-d_3, m/z 240 and 243 for BZE and BZE-d_3, m/z 371 and 374 for codeine and codeine-d_3, and m/z 429 and 432 for morphine and morphine-d_3. For the quantitative analyses, standard mixtures, consisting of known amounts of a crystalline drug standard of known purity and the same labeled material added to the samples, were analyzed to calibrate the system.

RESULTS AND DISCUSSION

Summary of Results From Exercises 1 and 2

For exercise 1, laboratories were sent eight samples of cryogenically ground hair (Welch et al. 1993*a*). Drug-users' hair, drug-free hair, and drug-soaked hair were included. Seven laboratories returned results. Qualitatively, the results were excellent and are summarized in table 1. No false positives were reported, and only three false negatives were reported (all from the same laboratory). Whether the hair was from drug users or was drug soaked had no appreciable effect on the success of the laboratories in determining the drugs present. The interlaboratory coefficients of variation (CVs) in the quantitative data ranged from 15 to 27 percent for the samples. Because all were powdered samples that could readily be mixed, inhomogeneous distribution of the drugs was unlikely, at least for samples of 10 mg or more. Because the powder has a large surface

TABLE 1. *Summary of three interlaboratory exercises for drugs of abuse in hair**

	Exercise 1	Exercise 2	Exercise 3
Hair type	Drug users' and drug-soaked	Drug users' and drug-soaked	Drug-soaked only
Hair form	Powder	Segments	Segments
Drug levels	High	High and low	High and low
% Positives correct	94	78	94
% Negatives correct	100	94	97
Number of laboratories (results/sent)	7/8	10/10	12†/13‡

*Drug-free samples were sent with each exercise.
†Number of laboratories.
‡Number of results sent.

area, extraction solvents perform more efficiently than for hair segments. Therefore, most of the imprecision is probably a reflection of interlaboratory variability in the measurements. The scatter appears to be random, with no obvious correlations between methods used and results. Nevertheless, the interlaboratory precision for the powdered samples used in exercise 1 was much better than was seen for exercises 2 and 3, where all samples were hair segments. The quantitative results for the drug-soaked samples were slightly more precise than those for the drug users' samples, but these differences may not be significant.

For exercise 2 (Welch et al. 1993*a*), the incidence of false positives and false negatives was higher than for exercise 1, where only powdered hair was used (see table 1). For exercise 2, the laboratories correctly identified all positive cocaine specimens with levels above 1 ng/mg hair, except for one missed by a laboratory using radioimmunoassay (RIA). There was only one instance of a laboratory that failed to detect BZE at levels above 1 ng/mg. There were no false positives reported for cocaine or BZE. For the two samples with levels below 1 ng/mg hair, 3 of the 10 laboratories found cocaine and/or BZE in both; 4 others found them in one of the samples; and 3 did not report them in either sample. All four false positives involved reports of opiates being detected when no morphine or codeine was present. There were five instances of not reporting morphine when it was present above 1 ng/mg, four of which were from one sample of drug user's hair and involved laboratories using GC/MS, RIA, and tandem mass spectrometry (MS/MS).

Quantitative results for exercise 2 were more scattered than they had been for exercise 1. For exercise 2 values that were more than a factor of 3 away from the means excluded, interlaboratory CVs ranged from 19 to 60 percent. Some of the difference may be that the drugs were less homogeneously distributed in the hair segments. The worst outliers were mostly from a few laboratories, suggesting methodological problems. One laboratory using MS/MS of intact hair without extraction reported concentrations that were generally an order of magnitude lower than those reported by the other laboratories. Another laboratory using RIA had two results that were more than an order of magnitude higher, suggesting some

nonspecificity in its analysis. There appears to be no correlation in the data between precision and type of drug.

Results From Exercise 3

Results were received from 12 laboratories, one of which analyzed the hair by two different methods and reported the results for each. Thus, when combined with NIST results, there were 14 sets of data. Five laboratories did not analyze for all the drugs, so some sets are not complete. The overall qualitative results for exercise 3 are shown in table 1 along with comparative results from exercises 1 and 2.

For exercise 3, 97 percent of the negative challenges were correctly reported as no drug detected. The samples included 16 negative challenges, if analyses were performed for all four drugs. Of the 14 data sets, 10 laboratories reported no positives for negative challenges, 3 reported one positive, and 1 laboratory reported two. In three of these positive reports, a trace level (detected but not quantifiable) was reported. In another instance, the level reported was near the limit of quantitation and might have been below any cutoff. Therefore, it is possible that only one of the positive results would be a true false positive under field measurement conditions. The positive responses by drug, in terms of number and percentage of negative challenges, were as follows: cocaine 0, 0 percent; BZE 1, 3.0 percent; morphine 3, 5.0 percent; and codeine 1, 1.8 percent. These data agree with those of exercise 2 in that morphine was more likely to be reported when not present than was cocaine.

For the positive challenges, 94 percent were correctly identified. The samples included 16 positive challenges, if analyses were performed for all four drugs. Nine of the fourteen results correctly identified all positive challenges, three missed one positive, one missed two, and another missed six. The negative responses by drug, in terms of number and percentage of positive challenges, were as follows: cocaine 5, 8.3 percent; BZE 6, 10.9 percent; morphine 0, 0 percent; and codeine 0, 0 percent. The morphine and codeine levels were relatively high compared with what is typically found in the hair of opiate users, whereas the cocaine and BZE levels were in the lower range of what is generally found in the hair

of cocaine users. Thus, cocaine levels were apparently more difficult to detect.

For the quantitative results, values that were more than a factor of 3 from the mean were considered outliers and were excluded for the purposes of calculating interlaboratory means and standard deviations. The resulting means, CVs (expressed as relative standard deviations), and excluded values are shown in table 2. Overall, there were 30 outliers, representing 16.3 percent of the positive challenges. Half the data sets had one or more outliers; four of these had six or more outliers. The breakdown by drug in terms of number of outliers and percentage of positives that were outliers, respectively, is as follows: cocaine 11, 18.3 percent; BZE 6, 10.9 percent; morphine 8, 22.2 percent; and codeine 5, 15.2 percent. With the outliers removed, interlaboratory CVs ranged from 31 to 66 percent. The variable effectiveness of various wash procedures prior to extraction for removal of drugs may be a significant contributor to the scatter observed, but it is not possible to quantify this effect.

Intralaboratory precision could not be evaluated for most laboratories because they analyzed each sample only once. Four laboratories provided data on the precision of their analyses for cocaine and BZE. These laboratories performed two to three analyses on each sample. Intralaboratory CVs ranged from zero to 53 percent for individual samples, with a mean of 15 percent. This mean did not vary much among the four laboratories, ranging from 13 to 17 percent, even though all three extraction and digestion approaches were represented among these four laboratories.

The laboratories were asked to determine whether any samples had external contamination. Nine laboratories correctly identified sample 3. Two other samples not treated with the vapor were identified once. For sample 3, which had been soaked in solutions of cocaine and BZE in addition to having been exposed to cocaine vapor, the quantitative results were particularly scattered. GC/MS measurements at NIST found 5.2 ng/mg of cocaine in this hair prior to the vapor deposition. The laboratories reported results ranging from a trace to nearly 20 ng/mg. The scatter is not entirely surprising because different approaches were used for removing the

TABLE 2. *Qualitative and quantitative results for each specimen in exercise 3*

			Interlaboratory Results					
Sample	Drug	NIST Mean (ng/mg)	No. of Positives	No. of Negatives	Interlaboratory Mean (ng/mg)	Coefficient of Variation (%)	Range (ng/mg)	Excluded Outliers (ng/mg)
1	Cocaine	0.9	9	3	0.7	37	0.5-1.1	0.2,0.2,5.1
	BZE	1.0	9	2	0.9	55	0.3-1.5	
	Morphine	0	2	10				
	Codeine	0	1	10				
2	Cocaine	0	0	12				
	BZE	0	1	10				
	Morphine	0	0	12				
	Codeine	0	0	11				
3	Cocaine	13.2	12	0	7.2	*	1.5-19.4	
	BZE	2.2	11	0	2.9	49	1.5-6.1	
	Morphine	0	0	12				
	Codeine	0	0	11				
4	Cocaine	0	0	12				
	BZE	0	0	11				
	Morphine	4.8	12	0	4.3	32	1.8-6.8	0.7
	Codeine	4.6	11	0	4.3	35	2.9-8.2	0.3

*This sample was contaminated with cocaine vapor. Different washing procedures produced highly variable results that could not be statistically evaluated.

TABLE 2. *(continued)*

Sample	Drug	NIST Mean (ng/mg)	Interlaboratory Results: No. of Positives	No. of Negatives	Interlaboratory Mean (ng/mg)	Coefficient of Variation (%)	Range (ng/mg)	Excluded Outliers (ng/mg)
5	Cocaine	2.0	11	1	1.0	55	0.4-2.0	Trace (2),0.1
	BZE	2.2	10	1	1.2	65	0.4-2.2	Trace (2),0.3
	Morphine	0	0	12				
	Codeine	0	0	11				
6	Cocaine	0	0	12				
	BZE	0	0	11				
	Morphine	0	1	11				
	Codeine	0	0	11				
7	Cocaine	2.7	11	1	2.4	31	1.0-2.9	0.4,0.6,0.7,13.2
	BZE	2.4	8	3	2.7	47	0.9-4.5	0.2,0.5
	Morphine	4.2	12	0	3.6	41	2.2-5.8	0.4,0.7,0.8,0.8,1.1
	Codeine	4.1	11	0	3.8	42	2.1-7.2	0.6,0.6,0.7
8	Cocaine	7.3	12	0	4.1	44	1.7-7.3	39.0
	BZE	1.6	11	0	2.6	66	1.1-5.4	0.7
	Morphine	11.9	12	0	7.8	51	4.8-15.1	0.5,2.5
	Codeine	6.7	11	0	5.7	42	3.2-9.9	0.8

KEY: NIST=National Institute of Standards and Technology; BZE=benzoylecgonine

external contamination. The laboratories were not asked explicitly about those procedures. However, a few laboratories provided some information about washing procedures. Solvents reported included water, methanol, ethanol, acetone, and phosphate buffer, but little is known about times and temperatures used. Some of these procedures probably removed more cocaine than others. The effectiveness of various wash procedures for removing external contamination is controversial, with many differences reported among the laboratories that have investigated this issue. Because the data for this sample were so scattered, no CV was calculated, and no results were discarded. However, vigorous washing procedures also can remove some of the drugs soaked into hair. It is possible that some of the low results reported for other samples may be at least partially due to strong washing procedures prior to extraction.

Relative Effectiveness of Extractions and Digestions

The qualitative results for exercise 3 in terms of the various approaches for removing drugs from the hair matrix are summarized in table 3. There was considerable scatter among the laboratories using either extraction or digestion. Comparable numbers of analyses were performed using enzyme digestions, acid extractions, and methanol extractions. Of the three approaches, the laboratories using acid extractions had the most instances of missing drugs that were present, whereas the laboratories using enzyme digestions had the most instances of false positives reported. Laboratories using methanol extractions had the most outliers. However, probably none of these differences are significant. For each of the three approaches, there were laboratories that provided accurate results, both qualitatively and quantitatively. When laboratories are allowed to choose what works best, results are generally better than when a detailed method is prescribed. Laboratories that use good quality assurance procedures, have well-trained staff, and use fundamentally sound methods and well-characterized materials for calibration and control generally produce high-quality results.

TABLE 3. *Comparison of various digestion and extraction approaches*

	Positive Challenges			
Results	Overall	Enzyme	Acid	Methanol
Positives	173	62	48	57
Negatives	11	1	8	2
Outliers	30	10	13	18

	Negative Challenges			
Results	Overall	Enzyme	Acid	Methanol
Positives	5	3	1	1
Negatives	179	54	55	60

OVERALL FINDINGS FROM THE THREE EXERCISES

The three exercises described above demonstrate that most laboratories can reliably detect the presence of the target compounds in hair, as seen in table 1. Most positives missed in exercises 2 and 3 were at low levels (≤ 1 ng/mg hair). Not surprisingly, laboratory performance improved with increasing concentration, as higher levels were seldom missed. Cocaine and BZE were the most frequently missed substances in the study.

The incidence of potential false positives has been low. Many of the positive levels reported on negative challenges have been low enough to be below likely cutoff levels. Almost all these positive reports have involved opiates. There have been no positives reported for cocaine when it was not present.

Quantitative results continue to be highly scattered. Interlaboratory CVs were much smaller for exercise 1, partially because the drugs were more homogeneously distributed in the powdered hair, but perhaps more important, because the drugs are much more easily extracted from the powder than from intact hair segments. With

most of the laboratories using GC/MS with isotope-labeled internal standards on exercises 2 and 3, the interlaboratory measurement precision should be considerably better than the overall precision observed in these studies. For exercise 2, laboratories were sent a hair extract to determine the contribution of measurements to the interlaboratory precision. Unfortunately, the analytes were not stable in the matrix. Consequently, the later the laboratories analyzed the material, the lower were the results. Nevertheless, it seems likely that the removal of the drug from the hair matrix is the major source of interlaboratory imprecision.

Extraction Versus Digestion

Unfortunately, these exercises have not demonstrated the superiority of any of the approaches used to date. Variability in hair pretreatment, extraction times, and extraction temperatures all contribute to considerable variability for a given approach. Results from exercise 2 suggest that there may be differences, depending on whether the sample is from a drug user or has been fortified with drugs through soaking. For exercise 2, both cocaine and BZE from drug users' hair yielded higher average results for the enzyme digestion than for the HCl extraction, whereas the opposite was true for fortified hair, suggesting that extracting cocaine and BZE from drug users' hair is more difficult than extracting from fortified hair. However, the 20- to 24-hour extractions with HCl performed at NIST produced results that were similar to the means of the enzyme results. Thus, the two approaches may produce equivalent results, providing that the extraction is sufficiently long and that the deuterated internal standard is present to equilibrate with the analyte in the hair.

Drug Users' Hair Versus Fortified Hair

Exercise 3 did not use drug users' hair. For exercise 1, no significant differences were observed for results from drug users' hair versus results from fortified hair. For exercise 2, three false positives were reported from unfortified, drug-free samples; one was from a fortified sample. The absence of false positives from drug users' hair probably was related to the fact that in three of the four such samples all three drugs were present, and the fourth had two of

the drugs. Thus, there were few opportunities for laboratories to report drugs not present in these samples. The seven false negatives for drugs present at greater than 1 ng/mg hair were all from drug users' hair samples. This finding may relate to the apparent greater difficulty, discussed above, in extracting drugs from the hair of drug users compared with extracting from fortified hair. All the positive levels below 1 ng/mg hair were from drug users' hair samples. As mentioned above, quantitative results for these low-level samples were distinctly worse than for samples with higher levels of the substances and were worse for extraction versus digestion.

Accuracy by Measurement Method

For exercise 1, laboratories used GC/MS, MS/MS with extractions, and RIA. There were no false positives, and the positives that were missed were all from one laboratory, using GC/MS, that was making its initial attempt at hair analysis.

For exercise 2, laboratories using GC/MS, RIA, or MS/MS with extraction generally performed well qualitatively with only one false positive (RIA). Three of the four positives reported on negative challenges were obtained from direct probe MS/MS analyses of intact hair. Quantitative results were clearly best for GC/MS and MS/MS with extraction. The worst results were from the direct analysis of hair by MS/MS without extraction. Some RIA results were extremely high, indicating that cross-reactivity may be a problem for some RIA methods. For exercise 3, all but one laboratory used GC/MS. That one laboratory used HPLC and measured only cocaine and morphine. This laboratory produced results that were generally accurate qualitatively and did not produce any outliers quantitatively. These results suggest that although GC/MS is the preferred approach for measurements for most laboratories, other approaches, such as MS/MS with extraction and HPLC, may be satisfactory with proper care.

CONCLUSION

For the three exercises, most participating laboratories successfully identified most target compounds in most samples, with few false

positives. When compounds were present at lower levels, the incidence of not reporting the drugs was higher. Quantitatively, the results were more scattered for exercises 2 and 3, where the hair was in the form of short segments, compared with exercise 1, where the hair samples were powdered. Acid extraction, methanol extraction, and enzyme digestion generally performed about equally well. There was some evidence from exercise 2 that enzyme digestion may have performed slightly better for drug users' hair than did the acid extraction, except when long extractions (>20 hours) were used. Measurements using GC/MS, MS/MS with extraction, and HPLC provided generally consistent qualitative results. Measurements that involved RIA and MS/MS without extraction provided the least accurate results. Variations in the procedures for isolating the analytes from the hair matrix are probably responsible for most of the scatter observed in quantitative results.

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Hair Analysis for Drugs of Abuse: Probative Features of Hair Testing

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INTRODUCTION

Workplace drug testing programs in the private sector are commonplace and usually involve the analysis of urine specimens. Although not all such tests are federally regulated, they usually are performed according to regulations promulgated by the U.S. Department of Health and Human Services (DHHS) (U.S. Department of Health and Human Services 1994) or according to the guidelines issued for forensic urine drug testing by the College of American Pathologists (CAP) (College of American Pathologists 1992). Thanks to modern advances in analytical technology, low-cost, high-volume, reliable, and forensically acceptable urine drug testing has been achieved.

In developing drug testing of hair specimens, the authors benefited greatly from those developments (Baumgartner et al. 1979). Thus, DHHS and CAP policies and procedures developed for urinalysis were incorporated into hair analysis operations wherever possible (U.S. Department of Health and Human Services 1993). When such incorporation was precluded by differences in specimen or technologies, the authors followed the lead of laboratories performing forensic blood testing for drugs and developed appropriate forensic criteria of testing (Chen et al. 1990).

For example, hair testing uses the essential elements of DHHS policies relating to donor consent, chain-of-custody security, and confidential collection of specimen and reporting of test results (U.S. Department of Health and Human Services 1994). All initial positive radioimmunoassay (RIA) results are confirmed by gas chromatography/mass spectrometry (GC/MS).

Because only small amounts of hair can be obtained for workplace drug testing, it was also necessary to develop ultrasensitive and highly reliable chemical ionization or tandem mass spectrometry (MS/MS) procedures for identification and quantitation of drugs (Baumgartner et al. 1994, pp. 61-94). Accurate quantitation is achieved by adopting the DHHS practice of using deuterated internal standards, whereas technical false-positive mass spectrometer results (i.e., misidentification of a drug) are avoided by adhering to the mass spectrometric guidelines for analyte identification formulated by the U.S. Food and Drug Administration and various forensic organizations (Cairns et al. 1989*a*, 1989*b*; Chen et al. 1990). Testing and reporting of results are monitored by quality control procedures. These include an external quality control program with blind samples that are submitted through clients by an independent laboratory. In essence, this program serves in a capacity similar to that developed by Willette (1989; personal communications) for urinalysis prior to implementation of the DHHS and CAP laboratory certification programs.

PROBATIVE FEATURES OF HAIR ANALYSIS

The safety and effectiveness of drug testing by hair analysis are greatly enhanced by its unique probative features. For instance, because hair specimens can be collected under close supervision without embarrassment, hair testing is not subject to evasive maneuvers such as substitution of or tampering with the specimen. Hair, unlike urine, can be matched to an individual by its physical appearance or by microscopic examination. Hair has the advantage of providing an exceptionally stable matrix for the entrapped drugs that does not require refrigeration during shipment and storage. Exceptional analyte stability has been demonstrated with hair specimens whose age ranged from several hundred to more than a

thousand years and with hair on partially decomposed bodies (Baumgartner et al. 1989; Cartmell et al. 1991). Furthermore, the safety and accuracy of hair analysis are enhanced by the ability to collect a second specimen that, except for additional growth and the possibility of an intervening hair cut, is identical to the first collected specimen. This, in turn, enhances the confidence of test subjects and testers alike. Use of a second specimen also allows the rectification of problems in the chain of custody with the first specimen.

Hair analysis is complementary to urinalysis with respect to the timeframe of detecting drug use. In contrast to urinalysis, which detects only recent drug use, hair analysis has a wide window of detection that depends only on the length of available hair. For workplace testing, hair analysis typically covers the past 3 months if 3.9 cm of hair can be obtained. Thus, detection of drug use cannot be avoided by temporary abstention prior to a scheduled preemployment drug test. It also has been shown that various kinds of hair treatments (washing, getting a permanent [perming], dyeing) can reduce but not completely remove the drug content from hair (Marques et al. 1993; Welch et al. 1993). Drug use can even be effectively identified in the absence of head hair by analyzing body hair or the clippings or shavings of fingernails (Baumgartner et al. 1994, pp. 61-94). (Fingernail shavings have the advantage of readily accessing the same timeframe as head hair.) Thus, evidentiary false negatives are not a significant issue with hair analysis. Neither is the test encumbered by legal problems associated with unannounced and observed urine collections.

The advantages of hair analysis for the identification of drug users have been demonstrated by several blind field studies. For instance, these showed that hair testing was considerably more effective than unannounced and unexpected urine testing (e.g., in arrestee populations) (Mieczkowski et al. 1991) or than frequently applied, unannounced but expected urine testing (e.g., probationers and parolees) (Baer et al. 1991). With probationers and parolees, using only one-tenth the number of hair tests compared with urine tests, four times the number of positive cocaine results were obtained by hair analysis than by urinalysis (Baer et al. 1991). Only in cases of frequent drug use, as defined by the high drug concentrations in

hair, did the number of positive urinalysis results approach that of hair analysis as theoretically expected. The correlation between drug concentrations in hair and positive urine results provides independent support for the previously observed correlation between drug concentrations in hair and self-reports of the amount of consumed drug (Baumgartner et al. 1989). This important correlation permits distinguishing among heavy, intermediate, and light drug use—an important distinguishing feature for diagnosis, treatment, and custody referrals. Also, this dose correlation provides the theoretical basis for defining objective cutoff levels and guarding against evidentiary false positives caused by passive absorption of drugs (e.g., poppy seed ingestion) or passive inhalation of marijuana smoke from other people.

Another consequence of the dose correlation is the ability to establish the pattern of drug use from segmental hair analysis. The extent to which drug use remains constant, declines, or increases over a given time is an important clinical parameter. That this relative parameter can be measured with a higher degree of accuracy than the absolute dose correlation results from the test subject acting as his or her own control, thereby overcoming the vexing problem of biochemical individuality.

However, the greatest challenge to forensic quality drug testing—whether of urine, hair, blood, sweat, or saliva—is the avoidance of evidentiary false positives. Evidentiary false positives differ from technical false positives in that they are not caused by errors in the chemical analysis of the specimen but rather by the passive absorption of drugs present in the environment (endogenous false positives) or by contamination of the specimen (exogenous false positives). People specifically at risk for evidentiary false positives are narcotics agents, drug dealers, and those who cohabit with drug users.

Other causes of evidentiary false positives, particularly in the case of urinalysis (see below), are the ingestion of poppy seed-containing products or consumption of drug-spiked food or drink (the "brownie defense"). Only some of the evidentiary false-positive problems of urinalysis can be addressed by cutoff levels and by evaluating the clinical evidence of drug use; however, most of these problems can

be addressed effectively by hair analysis, as explained below, which suggests that hair analysis should be used as a followup test in cases of contested urinalysis results (Baumgartner and Hill 1993*a*; Harkey and Henderson 1989, pp. 298-329).

Both urinalysis and hair testing use cutoff levels to protect against endogenous evidentiary false positives. However, because of certain unique properties of hair as a test medium (see below), additional protective measures can be applied. These include cleansing of the hair surface by conventional hygienic practice, special cleansing procedures in the laboratory, evaluation of the effectiveness of these cleansing procedures by three-wash kinetic parameters, three-wash kinetic cutoff levels that have to be met simultaneously, metabolite-to-drug ratio criteria and their cutoff levels, reproducibility of results (including segmental analysis) with a newly collected specimen, and a tripartite reporting system: positive because of drug use, positive because of nontrivial contamination, and negative.

This chapter describes how these procedures confer on hair analysis unmatched safety against the occurrence of endogenous and exogenous evidentiary false positives. The review focuses specifically on these procedures and then addresses some concerns that have been expressed about hair analysis.

METHODS FOR AVOIDING ENDOGENOUS EVIDENTIARY FALSE POSITIVES

Urinalysis Safeguards

For urinalysis, the main method for avoiding evidentiary endogenous false positives caused by passive absorption of drugs in the environment is by setting appropriate cutoff levels (U.S. Department of Health and Human Services 1988). These are listed in table 1.

However, it is well known that these cutoff levels can be exceeded by several modes of passive drug exposure, for example, by the ingestion of small quantities of poppy seeds; however, the amount of morphine normally ingested via poppy seeds is well below the amount capable of producing psychotropic effects.

TABLE 1. *Cutoff values used in drug testing*

	Urine (ng/mL)		Hair (ng/g)	
Substance	Immunoassay	GC/MS	Immunoassay	GC/MS
Marijuana metabolites	100		1	
C-THC		15		0.05
Cocaine equivalents	300		500	
Cocaine/BZE		150		500*
Opiates	300		500	
Morphine		300		300
Codeine		300		300
PCP	25	25	300	300
Amphetamines	1,000		500	
Amphetamine		500		300
Methamphetamine		500		300

*Total of cocaine plus BZE

KEY: GC/MS=gas chromatography/mass spectrometry; C-THC=Δ-9-carboxytetrahydrocannabinol; BZE=benzoylecgonine; PCP=phencyclidine.

SOURCE: Baumgartner, W.A., and Hill, V.A., 1993*a*. Copyright 1993 by Elsevier Science Ireland Ltd. (Limerick).

With respect to environmental drug exposure, urinalysis has focused mostly on passive exposure to smoke (Cone et al. 1987). However, experience with poppy seeds (Selavka 1991), cocaine and benzoylecgonine (BZE) (Baselt and Chang 1987), and cocaine (Baselt et al. 1991) suggests that the absorption of small amounts of cocaine in the form of vapors or dust also may cause evidentiary false-positive urinalysis results. The probability of such an occurrence increases when individuals are chronically exposed to drug-containing environments.

Because of situations such as these, urinalysis is not a stand-alone test—that is, as an additional safety measure against endogenous evidentiary false positives (particularly those near the cutoff level), results must be evaluated by a medical review officer. Thus, in the case of low opiate positives and in the absence of the heroin metabolite monoacetylmorphine (MAM) or clinical evidence such as needle marks, medical review officers frequently opt for a passive

exposure interpretation. It has been reported that this is the interpretation in approximately 90 percent of positive opiate urinalysis results (Buskirk 1993). This dilemma could be solved if such opiate-positive urinalysis results were followed up with hair tests because MAM is effectively trapped by hair (Goldberger et al. 1991; Cone et al. 1993; Moeller et al. 1993).

The passive exposure problem of urinalysis with respect to drugs other than opiates is unclear. However, it is generally believed—based partly on hair analysis evidence (Mieczkowski et al. 1991)—that the incidence of evidentiary false-positive urinalysis results is low. It is generally agreed that the benefit of the doubt in preemployment testing goes to the employer and, in the case of safety-sensitive employees, to the public. With respect to preemployment situations, the point has been made (Baumgartner and Hill 1993*a*) that applicants are frequently denied employment on the basis of subjective criteria such as inappropriate appearance or demeanor. Consequently, employers are inclined to adopt the position of the criminal justice system of not tolerating in applicants (particularly in the case of safety-sensitive positions) the mere contact with drugs or drug users. However, with safety-sensitive employees, even though the public receives the benefit of the doubt, the authors (Baumgartner and Hill 1993*a*) and others (Harkey and Henderson 1989, pp. 298-329) have suggested that challenged positive urinalysis results be supported by a followup hair test.

The relatively rare situation where a negative hair test result is obtained on an individual with a positive urine (Mieczkowski et al. 1991) does not necessarily mean that the positive urinalysis result is an evidentiary false positive. For example, one can explain such disagreement between urinalysis and hair test by biochemical individuality, that is, by the unusual instance where a one-time deliberate or nondeliberate drug use is not detected by even "fine-grained" hair analysis—hair analysis performed on small hair segments corresponding to 1- to 2-week intervals. However, because of hair analysis' considerably higher efficiency of detecting drug use, particularly infrequent use (Mieczkowski et al. 1991), the authors maintain that an individual with negative hair test and positive urinalysis results should be given the benefit of the

doubt and be considered to have been passively exposed to nonpsychotropic amounts of drugs.

These considerations do not apply to marijuana because its use is currently identified by hair analysis with an effectiveness approximately equal to that of unannounced urinalysis (Mieczkowski and Newel 1993*a*). Furthermore, both tests are known to miss a certain fraction of drug users. This similarity in test performance relates to the fact that urinalysis for marijuana is the most effective of the urine tests for the "NIDA-5" drugs (marijuana, cocaine, opiates, PCP, and amphetamines [U.S. Department of Health and Human Services 1994]) because of the long excretion times of marijuana, whereas with hair analysis the marijuana test is the least effective test because of the low analyte concentration in hair.

Hair Analysis Safeguards

On the basis of correlations between self-reports of drug use and drug levels in hair, the authors also have developed endogenous cutoff levels as protection against endogenous evidentiary false positives. When these cutoff levels are expressed in units comparable with those used to test urine (i.e., per gram of hair versus per mL of urine), then except for marijuana, the cutoff levels for hair and urine have similar values (table 1). However, this similarity becomes significant only in combination with the amount of available specimen because specimen size and cutoff level define the required sensitivity of the analytical procedures that have to be used for hair analysis. In other words, the similarity in cutoff values bears no relationship to the relative effectiveness of urine and hair cutoff levels in guarding against endogenous evidentiary false positives.

An approach different from a simple comparison of the absolute value of cutoff levels is required for such an evaluation. The relevant parameter in this regard is the risk ratio (R_r), defined as:

$$R_r = \frac{\text{maximum endogenous drug concentration in hair or urine}}{\text{drug concentration cutoff level in hair or urine}}$$

If R_T approaches unity because of the adoption of high cutoff levels, then the probability of the occurrence of endogenous evidentiary false positives approaches zero. The effectiveness of identifying drug users also approaches zero under these conditions. As this ratio increases by lowering the cutoff level, the detection efficiency of the test increases, but the probability of endogenous evidentiary false positives also increases. However, the probability of the latter is not a linear but rather an S-shaped function of R_T. Thus, by the judicious choice of cutoff levels, the effectiveness of detecting drug users can be balanced against the effectiveness of avoiding false positives.

With hair, R_T is approximately 200 for cocaine and 20 for opiates, methamphetamine, marijuana, and PCP. The peak endogenous drug concentration of thoroughly washed hair for cocaine is approximately 1,000 ng/10 mg hair; for opiates, PCP, and methamphetamine, 100 ng/10 mg hair; and for marijuana, 10 pg C-THC/10 mg hair. Peak value estimates of these endogenous drugs (i.e., those in the inaccessible domain, defined below in the section on extended wash procedures) were obtained from field studies with heavy drug users in clinical and criminal justice facilities.

That urinalysis is faced with the poppy seed problem indicates that its R_T, at least for morphine, is much greater than the R_T for hair. This is further corroborated by a study in which test subjects chronically ingested poppy seed products for 1 month without yielding positive hair analysis results. On the other hand, randomly collected urine specimens reached levels of 500 ng/mL (Baumgartner et al. 1989).

However, in spite of its greater R_T values, unannounced urinalysis is still not as effective as hair analysis in identifying drug users. This apparent paradox can be explained by the pharmacokinetics of drug excretion that affects the outcome of urinalysis but not of hair analysis; that is, it is caused by the transient presence of drugs in urine. Consequently, the urine test at about the time of maximum drug excretion is too sensitive and, compared with hair testing, more prone to endogenous evidentiary false positives; on the other hand, during the terminal phase of drug excretion, the urine test

approaches insensitivity. During this latter phase, drug users escape detection (negative pharmacokinetic periods) by the urine test.

The effectiveness of the hair test in identifying drug users does not suffer from these difficulties because the test is time-insensitive; that is, hair carries within its structure a permanent record of both the severity as well as the chronicity of drug use. This feature gives hair analysis its greater detection efficiency in spite of lower R_r values. Only when drug use is frequent does unannounced urinalysis approach the detection efficiency of hair analysis (Mieczkowski et al. 1991). However, this occurs only under conditions of unexpected and unannounced urine testing, for example, with arrestees, but not with unannounced but expected testing, for example, with parolees (Baer et al. 1991), because the latter group apparently is still able to evade the urine test through various maneuvers.

Methods for Avoiding Exogenous Evidentiary False Positives

Hair analysis and urinalysis face entirely different problems with respect to the avoidance of exogenous evidentiary false positives. With urinalysis, exogenous false positives may result from contamination of the specimen in the laboratory or at the collection site through accidents or subversive activities. Because laboratory personnel can be a source of specimen contamination, it is important that they be monitored with an evasion-proof test such as hair analysis or frequently applied unannounced urine testing. Challenges to urine specimens on the basis of subversive activities at the collection site or in the laboratory are countered by safeguarding the urine specimen in secure locations and by strict chain-of-custody procedures. If any of these security measures is breached or not optimally documented, little can be done to correct this problem. Unlike the hair specimen, the urine specimen cannot be cleansed of exogenous contamination by washing, and a second essentially identical urine specimen cannot be collected at a later time.

With hair, exogenous evidentiary false positives caused by deposition of drugs from the environment on or in hair can be avoided with a high degree of certainty. It has been shown that drugs of abuse do not bind strongly to hair proteins. Furthermore,

the interior of hair (the inaccessible domain of hair, explained in the next section), unlike the lungs and gastrointestinal tract in the case of urinalysis, is highly resistant to penetration by exogenous drugs (Baumgartner and Hill 1992, pp. 577-597). Consequently, drugs can be removed readily from hair by conventional hygienic practices—the analog of the cleansing of the interior milieu by urine excretion.

However, of much greater importance is the ability to effectively decontaminate hair in the laboratory by specially developed wash procedures. These involve not only extensive washing with solvents especially chosen to match the condition of the hair (porous or nonporous) but also the use of specially developed wash kinetic procedures (Baumgartner and Hill 1993*a*; 1992, pp. 577-597).

Furthermore, the entry of exogenous drugs into hair—in contrast to urine, blood, and saliva—does not result in the formation of metabolites. Thus, the absence or presence of metabolites (in a certain ratio relative to the original drug and, once again, defined by cutoff values) provides powerful additional assurance against exogenous evidentiary false positives in the case of hair analysis, as explained below. The reproducibility of results with a second newly collected hair specimen is another effective means for distinguishing between external contamination and drug use, because contamination is generally a highly variable phenomenon.

Because the experimental procedures for these wash kinetic analysis and metabolite-to-drug ratio determinations have been extensively described in previous publications, they will be reviewed only briefly here.

Extended Wash Procedure

Experience with the decontamination of more than 20,000 positive hair specimens is summarized in figure 1. Essentially similar results are obtained with cocaine, opiates, amphetamines, and PCP. Only in the case of marijuana is a different situation encountered, as explained at the end of this section.

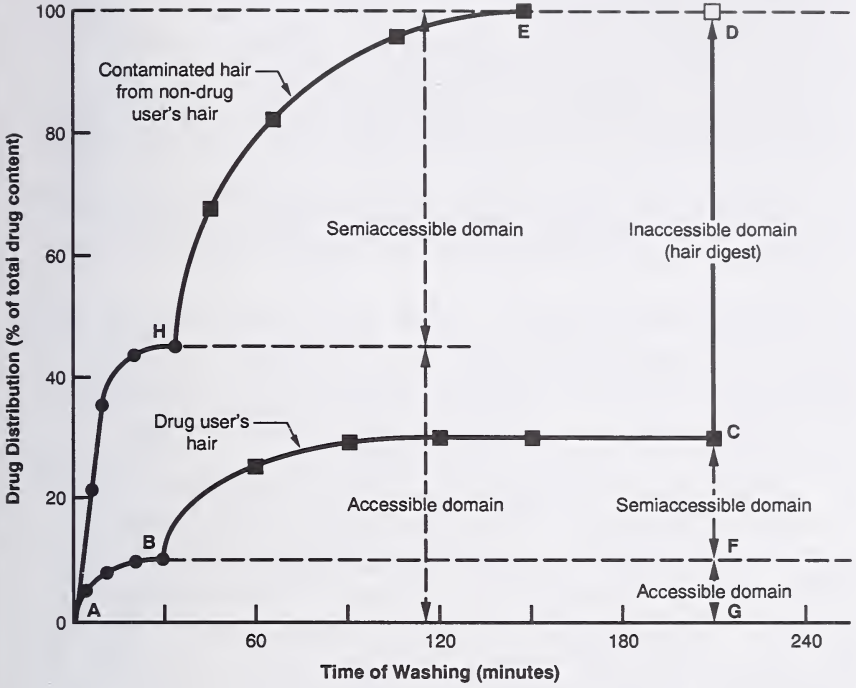

FIGURE 1. *Wash kinetics of contaminated hair and hair from a drug user. Hair treatments: ●, dry ethanol washes; ■, phosphate buffer washes; □, enzyme digestion.*

The results discussed are typical of strong (thick) nonporous hair from a drug user exhibiting a small amount of external contamination, for example, from smoke. The hair was nonporous (e.g., not permed or dyed) as determined by methylene blue staining (Baumgartner et al. 1989).

The first step in the wash procedure is to wash dry hair with a non-hair-swelling solvent such as dry ethanol. This initial wash is applied for two reasons: to remove substances (e.g., grease, dirt, oils) that can interfere with the subsequently applied aqueous wash procedure or the immunoassay and to remove any loose surface contamination of drugs—necessary for accurate application of the

wash kinetic parameters (see below). The amount of drug that can adhere loosely to the hair surface can range from none to a large quantity. The former would occur with hair freshly cleaned by normal hygienic practices and the latter with hair that has been freshly contaminated through contact with cocaine powder or smoke. Washing hair first with a non-hair-swelling solvent also drastically reduces the possibility of carrying surface contamination into interior structures with subsequently applied washes with hair-swelling solvents such as water (Baumgartner and Hill 1992, pp. 577-597).

The example shown in figure 1 reveals by the rapid attainment of a plateau in the wash kinetics (curve AB) that the four dry ethanol washes effectively remove readily accessible surface contamination. The contamination that can be removed by non-hair-swelling solvents such as dry ethanol, but not ethanol left open to the air that then becomes moist, is designated to have been removed from the accessible domain of hair. The relative absence of metabolites such as BZE suggests that the accessible domain contains predominantly exogenous drugs. The size of this domain is shown in figure 1 as line segment FG (accessible domain); as indicated above, its relative size can vary from zero percent of the total drugs present in hair to, theoretically, a large percentage with freshly contaminated hair.

After treatment with dry ethanol, hair (about 20 mg) is subjected to several washes (2 mL) with newly added phosphate buffer (.01 M, pH 5.5). The test tubes containing the hair are shaken at 100 cycles per minute. Washing is continued until a plateau is once again obtained in the wash kinetics. For optimum hair swelling, the contact time for each wash should be at least 30 minutes. With strong, nonporous hair, a plateau in the wash kinetics is generally reached after three to four 30-minute phosphate buffer washes. With more porous hair, particularly with hair that has been cosmetically treated, a plateau is more slowly attained, and a much greater percentage of the total drug present in hair is removed. However, these losses can be reduced by changing from a phosphate buffer wash to ethanol containing 5, 10, or 15 percent water. Ethanol with decreasing water content is used with increasingly porous hair specimens (Baumgartner and Hill 1992, pp. 577-597).

The drugs removed by buffer or water and ethanol washes are designated to have been removed from the semiaccessible domain of hair (figure 1, curve BC). This domain contains endogenous drugs and possibly exogenous drugs that may have been carried into the semiaccessible domain by perspiration or some other mechanism. The size of the semiaccessible domain is given by line segment CF.

Subsequent to the phosphate-buffer washes, hair is enzymatically digested by methods described previously (Baumgartner and Hill 1992, pp. 577-597; 1993*a*). This procedure releases the remaining drugs entrapped in the protein matrix of the hair in one large bolus (line segment CD in figure 1). However, this bolus of released drug does not include drugs that are entrapped in the melanin fraction, as was demonstrated by extracting drugs with solvents from the isolated melanin fraction and by noting how the extracted amount was affected by exposing melanin for varying periods (2 to 16 hours) to the digest (Nakahara 1988). Before the digest is chemically extracted for mass spectrometric analysis, the melanin fraction is removed by centrifugation. Thus, any biases in the analytical result caused by hair color can be effectively avoided (Baumgartner and Hill 1992, pp. 577-597; Mieczkowski and Newel 1993*b*). Such bias would occur only when a major fraction of the drug is contained in the melanin fraction. Melanin constitutes approximately 5 percent of the hair mass.

Hair digestion procedures suitable for mass spectrometric analysis have been described in several of the authors' previous publications (Baumgartner and Hill 1992, pp. 577-597; 1993*a*), whereas the drug content in the alcohol and phosphate washes can be readily determined by conventional immunoassay procedures. Therefore, all wash kinetic data can be checked by independent laboratories and are not influenced by any proprietary RIA screening technology of the digest.

Drugs released by hair digestion are designated to have been contained in the inaccessible domain (figure 1). This domain takes its name from the fact that exogenous drugs do not penetrate it to any significant extent even under severe conditions of contamination (Baumgartner and Hill 1992, pp. 577-597). Thus, under conditions

of realistic contamination, this domain contains only endogenous drugs.

Drug-binding studies in which the inaccessible domain has been destroyed suggest that the more effective sequestration of drugs in the inaccessible domain compared with that in the other two domains is not the result of stronger binding interactions with the protein structures of hair but rather of structural sequestration. The rope-like macroprotein structures as well as the organization of hair into cellular structures (figure 2) offer many possibilities for such a model. The semiaccessible domain may not necessarily involve only structures near the hair surface, that is, just below the accessible domain, but also pore-like structures, for example, those spaces accessed by water during the hair-swelling process. These are likely to extend all the way through the hair shaft; that is, they are envisaged to involve the interstitial spaces between the cells or macroprotein structures. Similar structural compartmentalization has been described by Robbins (1988, p. 59). Such microcompartmentalization of drugs cannot be expected to be identified by fluorescent staining techniques.

Figure 2 is not meant to imply that endogenous drugs can be deposited in the inaccessible domain only via the blood supply feeding the hair root. Any extracellular fluid containing endogenous drug that is in contact with the hair fiber-synthesizing apparatus in the follicle can bring this about. However, critical in this process is the contact secretions make with hair prior to the formation of the inaccessible domain. Once the inaccessible domain is formed, such entry cannot occur; that is, sweat and sebum are not mechanisms for the deposition of exogenous drugs into the inaccessible domain.

What is most important about the inaccessible domain of hair is its size relative to the semiaccessible domain. In nonporous hair, more than 90 percent of the endogenous drugs can be found in this domain. It is this distribution between the semiaccessible and inaccessible domains that provides the strongest wash kinetic criterion for distinguishing between external contamination and drug use—the extended and truncated safety zone ratios (see below).

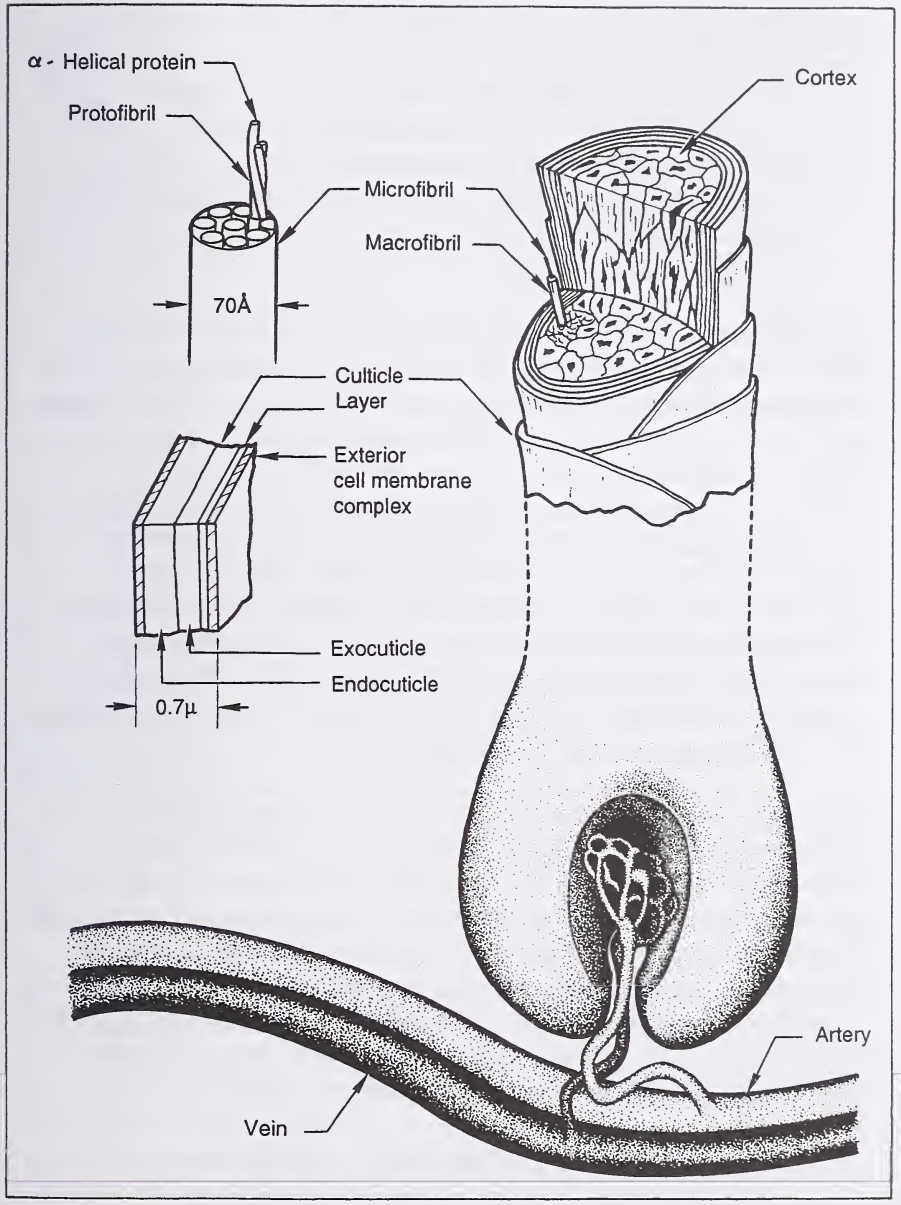

FIGURE 2. *Structure of human hair fiber and blood supply to hair follicle*

SOURCE: Baumgartner, W.A.; Cheng, C.C.; Donahue, T.D.; Hayes, G.F.; Hill, V.A.; and Scholtz, H. Forensic drug testing by mass spectrometric analysis of hair. In: Yinon, J., ed. *Modern Mass Spectrometry, Vol. 4, Forensic Applications of Mass Spectrometry*, 1994. p. 64. Reprinted by permission of CRC Press, Boca Raton, FL.

A typical example of a contaminated hair from a non-drug user is shown in figure 1 by curves AH and HE: AH represents the cleansing of drugs present in the accessible domain with dry ethanol and HE the cleansing of the semiaccessible domain by phosphate buffer. External contamination does not readily penetrate the semiaccessible domain; however, in contamination/decontamination studies (Baumgartner and Hill 1992, pp. 577-597), the authors purposely chose severe conditions for contamination to force exogenous drugs into the semiaccessible domain. For example, in contamination experiments hair was exposed for several hours to dense cocaine vapors in the confined space of a 1-L flask. Other conditions involved 3 1/2 hours of exposure to an aqueous solution of cocaine at a concentration of 5 μg/mL.

The most noteworthy fact gleaned from these contamination/ decontamination studies is that contamination is rapidly and effectively removed from the accessible and semiaccessible domains and that only a relatively small amount of drug remains in hair; this remaining amount is completely released on digestion of the hair. The relative drug content of the semiaccessible and inaccessible domains led to the definition "extended safety zone ratio" (R_{ESZ}) for the extended wash procedure for differentiation between exogenous and endogenous drugs. This ratio is defined as follows:

$$R_{ESZ} = \frac{\text{amount of drug per 10 mg hair in digest}}{\text{total amount of drug per 10 mg hair in all phosphate washes}}$$

For contaminated hair, R_{ESZ} is a small number that approaches zero. On the other hand, hair from drug users generally has values considerably greater than 1. The percentage distribution of R_{ESZ} values for different drugs is similar to those for the truncated safety

zone ratio (R_{SZ}). Based on the statistical distribution of R_{ESZ} values in a population of known drug users, a cutoff value of 0.25 has been chosen for R_{ESZ}.

The data in table 3 show that few hair samples exhibit R_{SZ} values in the vicinity of the cutoff level. The rare cases that have been encountered to date generally have come from individuals whose hair had been damaged beyond cosmetically acceptable levels by hair treatment (e.g., bleaching or perming). Such hair is readily recognized by its physical appearance and also by methylene blue staining. Hair with values below 0.25 is deemed to be contaminated, and hair with values above 0.25 is interpreted to be positive as a result of drug use.

Wash kinetic procedures are not used currently for the differentiation between contamination and drug use in the case of marijuana. Initially, it was more convenient to make this differentiation by measuring the marijuana metabolite C-THC in hair. This substance is not present in smoke. Although hair is readily cleansed of marijuana smoke contamination (Baumgartner and Hill 1992, pp. 577-597), it has not been possible to develop a convenient RIA procedure for the measurement of a specific marijuana compound in the wash solution that is also present in sufficiently high concentrations in hair to yield a favorable signal-to-noise ratio for effective R_{ESZ} measurements.

Truncated Wash Procedure

For forensic mass production testing, such as workplace testing, it is not practical to use wash procedures where every specimen is washed until a plateau is attained. Rather, except for special problem samples discussed later, a shortened version called the "truncated wash procedure" is used. This consists of one 15-minute dry ethanol wash and three 30-minute phosphate buffer washes.

In many cases the extended and the truncated methods give identical results, for example, with nonporous hair where plateau conditions are rapidly attained. However, when a plateau is reached slowly, as in the case of porous hair, then the wash kinetic parameters of the extended and truncated procedures differ significantly. There also is

the question of whether the residual bolus of presumed endogenous drug found in the hair digest after one dry ethanol and three phosphate buffer washes could have been depleted if the truncated wash procedure had been continued.

The authors have developed three truncated wash kinetic criteria for evaluating the probability of such an occurrence: prospective, retrospective, and contemporaneous approaches. In the prospective approach the question is: Given that the last wash (third phosphate buffer wash) contains X amount of drug, what is the probability that additional washes would have removed the residual drug found in the hair digest? This obviously depends on the amount of the residual drug relative to that in the last wash and on the degree of curvature of the wash kinetics at the time of the last wash, that is, on the extent to which a plateau had been approached. To evaluate the probability of the removal of X by additional washes, the authors defined the extended wash ratio (R_{EW}) and the curvature ratio (R_C):

$$R_{EW} = \frac{\text{amount of drug per 10 mg hair in digest}}{\text{amount of drug per 10 mg hair in last phosphate wash}}$$

$$R_C = \frac{\text{amount of drug per 10 mg hair in 3 phosphate washes}}{3 \times \text{number of phosphate buffer washes}}$$

The denominator of R_C is the number of phosphate buffer washes multiplied by 3 because this would make $R_C = 1.0$ in situations where perfectly linear wash kinetics are obtained.

In the retrospective approach, the question is: Having removed Y amount of drugs by the dry ethanol and three phosphate buffer washes, what is the probability that the residual drug found in the digest could have been removed by an extension of the truncated wash procedure? Here also the answer depends on the relationship of Y to the residual amount of drug in the hair digest and on the extent to which a plateau has been attained, that is, on R_C. The smaller Y is relative to the residue and the closer the approach to a plateau as measured by R_C, the smaller the probability of complete removal. The ratio evaluating this probability is termed the truncated safety zone ratio (R_{SZ}):

$$R_{SZ} = \frac{\text{amount of drug per 10 mg hair in digest}}{\text{total amount of drug per 10 mg hair in 3 phosphate washes}}$$

The drug contained in the dry ethanol fraction has to be excluded from all calculations because this drug could assume any high value as a result of loose surface contamination. In addition, the defined kinetic criteria (particularly the cutoff values discussed in the next paragraph) are valid only if the residual drug in the hair is measured by a method that guarantees the complete release of the entrapped drug (e.g., the authors' published digestion procedure for mass spectrometric analysis [Baumgartner and Hill 1992, pp. 577-597]). Strict adherence to other experimental details is also necessary. A recent attempt at an independent evaluation of these wash kinetic criteria developed in this laboratory was seriously flawed by major deviations from the published procedures.

The percentage distributions of values for the three truncated wash kinetic ratios for the four drugs for which the truncated wash procedures are used are given in tables 2, 3, and 4. In contrast to these values, those obtained for R_{EW} and R_C for the extended wash procedure are large numbers that approach infinity as the amount of drug found in the last wash approaches zero. The lowest value in each table—10, 0.33, or 1.3—is the cutoff value for the corresponding wash kinetic criterion. Samples producing values below these levels are interpreted as being contaminated unless overruled by metabolite criteria (see "Measurement of Metabolites" below). As with urinalysis, the certainty of a finding of drug use—as distinct from passive exposure—increases the farther the values of the wash kinetic ratios are from their cutoff values. For additional certainty, a diagnosis of drug use is made only when all three wash kinetic ratios exceed their cutoff values simultaneously.

Measurement of Metabolites

Another highly effective means of distinguishing drug use from external contamination is to identify and measure the presence of drug metabolites in hair. This approach is used in conjunction with other methods for avoiding evidentiary false positives. However, as shown below, wash procedures also play an important role in the measurement of metabolites because ratio measurements are

TABLE 2. *Percentage distribution of extended wash ratio (R_{EW}) values* using the truncated wash procedure*

Extended Wash Ratio	Percentage Distribution of Positive Samples			
	Cocaine	Opiates	PCP	Methamphetamine
10-20	19.6	0	20	42.6
21-40	24.0	0	25	32.7
41-100	28.2	23.7	20	14.8
101-200	14.9	29.3	15	8.3
>200	13.3	47.0	20	1.7

*R_{EW} = amount of drug per 10 mg hair in digest / amount of drug per 10 mg hair in last phosphate wash

KEY: PCP=phencyclidine

SOURCE: Baumgartner, W.A., and Hill, V.A., 1993*a*. Copyright 1993 by Elsevier Science Ireland Ltd. (Limerick).

TABLE 3. *Percentage distribution of safety zone ratio (R_{SZ}) values* using the truncated wash procedure*

Safety Zone Ratio	Percentage Distribution of Positive Samples			
	Cocaine	Opiates	PCP	Methamphetamine
0.33-1.0	9.3	1.7	4.4	22.7
>1.0-5.0	39.2	22.8	52.2	56.9
>5.0-10.0	17.1	24.6	17.3	11.5
>10.0	34.4	50.9	26.1	8.9

*R_{SZ} = amount of drug per 10 mg hair in digest / total amount of drug per 10 mg hair in 3 phosphate washes

KEY: PCP=phencyclidine

SOURCE: Baumgartner, W.A., and Hill, V.A., 1993*a*. Copyright 1993 by Elsevier Science Ireland Ltd. (Limerick).

TABLE 4. *Percentage distribution of curvature ratio (R_C) values* using the truncated wash procedure*

Curvature Ratio	Percentage Distribution of Positive Samples			
	Cocaine	Opiates	PCP	Methamphetamine
1.3-1.5	10.3	1.8	4.4	8.3
>1.5-2.0	14.6	7.0	30.4	30.2
>2.0-5.0	50.6	31.5	39.1	52.1
>5.0-10.0	17.4	19.3	4.4	7.3
>10.0	7.1	40.4	21.7	2.1

*$R_C = \frac{\text{amount of drug per 10 mg hair in 3 phosphate washes}}{3 \times \text{amount of drug per 10 mg hair in last phosphate wash}}$

KEY: PCP=phencyclidine

SOURCE: Baumgartner, W.A., and Hill, V.A., 1993*a*. Copyright 1993 by Elsevier Science Ireland Ltd. (Limerick).

involved here also, and these require the effective removal of drug contaminants.

Metabolites can sometimes be formed by nonmetabolic processes; the most notable example of this is the hydrolysis of cocaine to BZE. Therefore, it is preferable to measure metabolite-to-drug ratios rather than just metabolite concentration. The measurement of such ratios allows the monitoring of the contribution that a hydrolysis reaction may have made during the hair digestion process. Consequently, cocaine hydrolysis controls are included with every batch of digested hair samples. Also, the digestion is performed at pH 6.4, that is, under conditions where only a small fraction of cocaine hydrolyzes to BZE. A cutoff value of 4 percent BZE above the hydrolysis control is used to differentiate between cocaine use and cocaine contamination. However, the current best indicator of cocaine use is the presence of cocaethylene because this

metabolite (formed by the combined use of cocaine and alcohol) is not readily produced by nonmetabolic processes.

This laboratory has identified both methamphetamine and its metabolite amphetamine in hair (Baumgartner et al. 1994, pp. 61-94). The percentage of amphetamine in methamphetamine-containing hair samples from drug users ranges from 3 to 20 percent. Therefore, a cutoff value of 3 percent amphetamine has been chosen.

With opiates, several metabolic approaches can be used to distinguish between external contamination and drug use. Morphine is a metabolite of heroin. In addition, this laboratory and others have reported the presence of another heroin metabolite, MAM (Goldberger et al. 1991; Moeller et al. 1993; Cone et al. 1993). An indirect procedure developed in this laboratory for detecting the presence of morphine glucuronide in hair involves measuring the increase in free morphine in hair as a result of glucuronidase treatment (Baumgartner and Hill, in press). The percentage increase in morphine following hydrolysis ranges from 30 to 170. Thus, evidence for the presence of morphine glucuronide can be used to support wash kinetic data to differentiate between opiate use and opiate contamination. However, the presence of MAM can be used as an indicator of heroin exposure only. MAM does not necessarily indicate heroin use because MAM can be readily formed by hydrolysis from external heroin contamination. Therefore, wash kinetic data are required to distinguish heroin use from heroin contamination (Baumgartner and Hill 1994).

For marijuana, sensitive MS/MS procedures were developed to measure C-THC, which was found to be absent from marijuana smoke (Baumgartner et al. 1994, pp. 61-94). It has not been possible to define a suitable metabolite-to-drug ratio with this metabolite; drug use is indicated when C-THC values exceed 0.5 pg/10 mg hair.

The determination of accurate metabolite-to-drug ratios as well as endogenous cutoff levels depends critically on effective wash procedures—on the removal of metabolites and breakdown products present in external contamination. Thus, the measurement of

metabolites is not a substitute for effective washing of the hair specimen. Furthermore, good wash procedures are important for achieving reproducible results with a newly collected hair specimen. Reproducibility of results is an important criterion for excluding external contamination because contamination is generally characterized by highly variable results. A refinement of reproducibility includes a comparison of the segmental analysis of a first and second hair specimen; that is, the pattern of drug use must be reproducible.

DIAGNOSTIC ALGORITHM: AN OUTLINE

Several studies to evaluate the efficacy of the authors' methods for distinguishing between drug use and drug contamination have focused only on the use of the truncated wash kinetic procedures. This is an incorrect approach because the procedures used in the authors' laboratory also involve the use of metabolite criteria, evaluation of the porosity of the hair specimen by methylene blue staining, and the truncated and extended wash procedures according to the following algorithm, which is applied in the manner described below.

The rationale for such a combined diagnostic approach is rooted in the methods used for establishing the various kinetic and metabolite cutoff levels. After identifying the three domains in hair by extensive contamination (more severe than that likely to be encountered in everyday conditions) and decontamination experiments with hair from drug users and nonusers (as well as GC/MS measurements of drugs and metabolites in these hair specimens), the authors undertook a study of the statistical distribution of the numerical values of these parameters in hair from confirmed drug users. The criteria used for identifying drug users with certainty were self-reports, positive urinalysis results, and the presence of highly definitive metabolites in hair (e.g., cocaethylene, C-THC, indirect identification of morphine glucuronide). Once the statistical distribution of the various kinetic and metabolite parameters had been established for confirmed drug users, the authors investigated the distribution of these parameters in hair

specimens that had been contaminated under increasingly severe conditions.

The ability to discriminate between drug use and contamination is at an optimum when there is no overlap in the distribution curves of the various discriminating parameters in the population of drug users and nonusers. This ideal situation pertained with certain metabolites—cocaethylene and C-THC. With these substances, overlap in the distribution curves was purely hypothetical because their presence in contaminated samples could not be detected by even such ultrasensitive methods as MS/MS. However, in the case of BZE, a small amount of overlap was observed because of the inherent inaccuracies in measuring hydrolysis controls.

With the extended wash kinetic criteria, no overlap was observed between the contaminated and the user populations. However, this was not the case with the truncated wash kinetic criteria; here, a small but significant overlap was observed. Therefore, the cutoff levels indicated in tables 2, 3, and 4 were chosen at a point where the likelihood of misclassifying a realistically contaminated hair specimen was insignificant. This required that the cutoff level be set at a value where a small fraction of drug users are assigned to the contaminated category.

Although such assignments are dictated by benefit-of-doubt arguments, the matter does not rest here, because the truncated wash kinetic results constitute only the initial step in a multifactorial evaluation process. Multifactorial evaluations are particularly useful in situations where some diagnostic parameters fall into the gray zone near the cutoff level.

An example is the case where R_{SZ} and R_C indicate use and where R_{EW} has fallen just below the cutoff and indicates contamination. In addition to the usual metabolite evaluation, two additional actions are triggered by such a situation: an evaluation of the porosity of the hair and the rewashing of a second aliquot of the initial hair specimen by the extended wash procedure. The porosity evaluation is indicated because of the observation that the truncated wash kinetic parameters of porous hair from drug users cluster in the region of the truncated cutoff levels. Consequently, a finding of

nonporous hair strengthens an interpretation of contamination, whereas a porous hair result weakens such an interpretation.

However, the most important method for distinguishing between drug use and contamination is based on metabolite measurements. With respect to the example given above, if the metabolite percentage is marginal (near the cutoff), then the extended wash kinetic data serve as the tiebreaker. The identification of cocaethylene overcomes all marginal wash kinetic results in supporting a finding of drug use.

Conversely, samples initially judged as contaminated by the truncated criteria also are subjected to further evaluation. This involves washing by the extended wash procedure, evaluation of hair porosity, and metabolite measurements. The minimum criteria for overruling an initial determination of contamination are a positive metabolite finding and extended wash kinetic results indicating drug use.

Finally, a finding of contamination can be further subdivided into trivial and significant contamination. Results of trivial contaminations are scored as negatives. These are samples where, after application of the extended wash procedure, the drug content of the hair digest falls below the endogenous cutoff level (table 1). On the other hand, significant contamination is found when this cutoff level is exceeded after application of the extended wash procedure. Relatively few specimens fall into the significant contamination category. Such specimens often are obtained from individuals who are engaged in the manufacture or distribution of drugs.

The efficacy of the evaluation procedures outlined above has been extensively validated by trouble-free operations with more than 350,000 hair specimens involving applicants for employment as well as participants in numerous (mostly blind) field studies, which will be reviewed in the future. Applicants for employment are given every opportunity to appeal the finding of the first test by submitting a second hair specimen (the "safety net" specimen). The second specimens are carefully evaluated for any special circumstances and explanations given by the hair donor that could have influenced the validity of the first result. In addition to the complete battery of

tests outlined above, the identity of the two hair specimens is established by visual, microscopic, and staining procedures, and if required, the specimens are subjected to segmental analysis. Although several hundred of such repeat "safety net samples" confirming the analysis of the first specimen already have been performed, none of the safety net results have been further challenged.

In light of these considerations and this experience, it is highly inappropriate that individuals endeavoring to evaluate this laboratory's diagnostic procedures (see below) have focused exclusively on the truncated wash procedure, the first and most approximate step of the evaluations. Moreover, to date, all these endeavors have also been seriously flawed by major deviations from the authors' outlined methodology (see below). Anyone who wishes to apply the technique must do so by following the prescribed routine precisely as defined. Any deviation from the directions will not yield comparable results and will lead to incorrect conclusions about the value of the described routines.

FORENSIC STATUS OF HAIR ANALYSIS

The forensic status of hair analysis for drugs of abuse has been evaluated at various times. The first such review was that of Harkey and Henderson in 1988 for the State of California. Although this report was not released, a condensed version was subsequently published by Harkey and Henderson (1989, pp. 298-329), who concluded that hair analysis results should be interpreted with caution and suggested that the method could be used as a backup test for investigations of challenged urine results. Other scientific or legal reviews supporting the value of hair analysis have been published by various forensic or criminal justice experts (McBay 1991, pp. 29-41; Gropper and Reardon 1993; Mieczkowski 1992; Mieczkowski et al. 1993; Rob 1991; Zolla 1989; Zeese 1993, pp. 2-40—2-42).

In 1990 a critical report on hair analysis was issued by a group of urinalysis experts of the Society of Forensic Toxicologists (SOFT). Although this report (Bost 1990) emanated from a conference on

hair analysis, it did not reflect the opinion of hair analysis experts, that is, the legally relevant scientific community, who presented papers at the conference. The Honorable Judge Jack B. Weinstein, U.S. District Judge for the Eastern District of New York, the noted expert on scientific evidence and a participant of the conference, subsequently ruled in a precedent-setting case for the criminal justice system in favor of hair analysis (*United States* v. *Anthony Medina* 1990).

In 1992 SOFT updated its evaluation of hair analysis (SOFT-II) (Hearn 1992). SOFT-II recognized that the technology used for hair analysis (a preliminary immunoassay screen followed by GC/MS confirmation) was a valid and effective method for measuring drugs in hair. However, with respect to the interpretation or the forensic weight of a positive mass spectrometric result, the report raised several questions. It is not surprising that there should be questions with respect to the clinical interpretation of such data because such interpretations rely on the vagaries of the laws of biochemistry and biology. In contrast to these eccentricities, analytical data have as their basis the strict applications of the laws of physics and chemistry.

SOFT-II raised the question of whether hair color or race could bias the analytical results. This question was answered by the results of an independent blind field study of the technology described in this chapter with more than 1,000 specimens (Mieczkowski and Newel 1993*b*), which showed that such biases do not occur, at least not with hair digests from which the melanin fraction has been removed. However, at least in principle, such biases are possible when drugs are extracted from intact hair by solvent-based procedures. Biases resulting from melanin are possible only if a major fraction of the drug is present in the melanin fraction—a fraction that constitutes approximately 5 percent of the hair mass. There is also the possibility that the extraction efficiency of solvent methods is affected by the type of hair—fine, blonde Caucasian hair versus heavy, black Asiatic hair. Once again, problems of extraction efficiency do not occur with hair digests.

SOFT-II also advocated that hair analysis, at its present state of development, be used for forensic testing only when it is supported

by other competent evidence; that is, just as with urinalysis, a positive GC/MS-confirmed hair analysis result should not be used as a stand-alone test. The authors agree with this position, but only if this means that the positive result is to be interpreted by a medical or toxicological review officer in terms of the associated wash kinetic and metabolite data:

- Several unique drug metabolites and their metabolite/drug cutoff levels
- Three wash kinetic criteria and their cutoff levels
- Endogenous cutoff levels
- Procedures for identification of hair specimen
- Evaluation of condition of hair by methylene blue staining
- Drug dose/hair concentration correlation (heavy, intermediate, light drug use)
- Pattern of drug use by segmental analysis (constant, increasing, decreasing)
- A tripartite reporting scheme (no use, contamination, or drug use)
- Collection of a second specimen

A test regimen providing such a wealth of information to a medical or toxicological review officer along with its other forensic features of evasion-proof nonembarrassing collection, sample stability, and wide window of detection can hardly be called a stand-alone test.

Concerning any other supportive evidence for hair analysis, because of the timeframe difference, it is pointless to insist that positive hair data be backed by a positive urinalysis result. However, the converse is possible: A challenged urinalysis result can be supported by hair data. For example, a followup hair test promises

to be useful in cases of a positive opiate urinalysis result in which MAM is not found.

SOFT-II also questioned the value of measuring metabolites such as BZE that also can be formed by nonmetabolic processes. The authors agree with SOFT-II that considerable care must be taken in the evaluation of BZE levels in hair. However, in the authors' experience, meaningful metabolite measurements can be made by measuring the BZE-to-cocaine ratio (or percentage) in the protected inaccessible domain of hair. Most important is the application of appropriate experimental procedures: washing, digestion at pH 6.4, and inclusion of hydrolysis controls.

An investigation was performed of the BZE percentage in long hair as a function of time because it had been suggested that the alkaline condition of normal hygienic practices may cause changes in this percentage. The results with two types of hair, one porous and treated and the other nonporous and untreated, are shown in table 5. From the constant percentage values over many months, it is evident that the alkaline conditions of hygienic practices did not affect the

TABLE 5. *Stability of benzoylecgonine (BZE)-to-cocaine ratios in hair*

Hair Type	Percent Benzoylecgonine*				
Nonporous hair†	14.8	17.8	19.8	19.8	18.8
Time (months)‡	0-1	1-3	3-6	6-9	9-12
Porous hair	7.4	12.7	7.7	9.7	12.4
Time (months)	0-3	3-4	4-7	7-10	10-13

*BZE/(BZE+cocaine)×100. Hydrolysis in a cocaine hydrolysis control digested with the unknown samples was subtracted from the values shown.

†Porosity of the hair was determined by methylene blue staining.

‡Time represented by a segment is estimated by measuring the distance of the segment from the root of the hair and considering the growth rate of hair to be 1.3 cm/month.

relative BZE content in these hair specimens. These constant results can be attributed to the protected environment afforded by the inaccessible domain.

Work of numerous investigators further supports the value of BZE for establishing drug use (Harkey et al. 1991; Cone et al. 1991; Nakahara et al. 1992). Other cocaine metabolites such as cocaethylene and norcocaine are not always present in quantities measurable with conventional mass spectrometry. Approximately 50 percent of cocaine-positive hair specimens in this laboratory contain cocaethylene.

SOFT-II questioned the efficacy of the various wash procedures used by different investigators. The authors share this concern because it is clear from the published literature on hair analysis that many laboratories have not evaluated the effectiveness of their wash and extraction procedures. The procedures presented here have been validated with numerous field studies with confirmed drug users in terms of the three-domain model, including validation of the wash kinetic criteria for drug use with urinalysis, self-reports, and metabolite measurements. Therefore, it is not correct to imply that only freshly contaminated samples were used for this purpose (Hearn 1992). Freshly and excessively contaminated samples were used by the authors only in studies demonstrating the existence of the three domains for drug deposition (Baumgartner and Hill 1992, pp. 577-597).

Several investigators have reported the presence of residual cocaine contamination in hair after application of a limited number of washes (see below). Such residues are not surprising, for the cleansing of any item is, on the basis of dilution laws alone, an exponential process. The primary question is not whether contamination residues were found in hair but rather how much there was to start with and what fraction was removed—a relationship that is defined by the safety zone ratio.

The methods used for decontamination, particularly the first step, subsequent to massive contamination are also of great importance. With respect to this issue, it was reported recently (Welch et al. 1993) that hair after massive contamination was soaked for a half

hour in a phosphate buffer wash solution. Obviously, this is an inappropriate first step in decontamination because the high concentrations of drugs that quickly build up in the aqueous hair-swelling wash medium will cause the surface contamination to be carried into the more difficult-to-clean semiaccessible domain. That is, the first aqueous wash solution acted as a contamination rather than as a decontamination agent. As indicated previously, to guard against such problems, dry hair should have been exposed first to short washes with non-hair-swelling solvents such as dry ethanol.

Decontamination procedures used by other laboratories suffer from similar problems (Kidwell and Blank 1992, pp. 555-563). In several instances these also involved severe and highly unrealistic contamination conditions, for example, 24- or 48-hour soaking (Cone et al. 1991). A recent study under realistic contamination conditions (Wang and Cone 1995) applied only short decontamination procedures of unspecified duration. No attempt was made at an evaluation of wash kinetics. Therefore, it is once again not surprising that low levels of drugs, just above the endogenous cutoff level, were found to remain in hair.

Only one study to date has attempted to evaluate the efficacy of the chosen cutoff level for the truncated wash kinetic procedure (Blank and Kidwell 1993). Unfortunately, this study with hair samples soaked for 1 hour in concentrated drug solution was flawed by numerous and extensive deviations from the published procedures as well as by inappropriate pooling of data. In light of these procedural deviations, it was surprising that only some, and not all, of the obtained kinetic parameters fell outside the published cutoff criteria—and then only by a small margin. Even the most cursory corrections of the procedural deviations brought the truncated wash kinetic criteria into the appropriate range for contaminated specimens. This particular attempt at an evaluation of the wash procedures developed in the authors' laboratory underscores the fact that if laboratories introduce methodological changes, it is incumbent on the laboratories to develop their own in-house wash kinetic cutoff levels. A detailed criticism of this evaluation is given elsewhere (Baumgartner and Hill 1993*b*).

Laboratories are always free to adopt a theoretically simpler approach and, instead of using the truncated wash kinetic procedure, opt to use the extended wash procedure. Such an approach was apparently used in the contamination/decontamination studies of Koren and colleagues (1992). Here no significant contamination residues were reported after cleaning procedures.

The U.S. General Accounting Office (1993, pp. 58-60, 68-69) recently evaluated the status of hair analysis and recommended that it be tested as an objective measure of drug prevalence and to validate self-reports. A study of this kind has already been completed by the United Nations Interregional Crime and Justice Research Institute (Chiarotti and Tagliaro, in press).

The probative advantages and effectiveness of hair analysis for identifying drug users have been established by the publication of more than 160 scientific papers. Several were blind evaluations of the analytical procedures used in this laboratory. The authors, in collaboration with others (Siegel 1992, pp. 289-316), also have applied hair analysis in more than 300 forensic cases with only minor legal challenges. Currently, at least 12 forensic laboratories the world over, including those of the Federal Bureau of Investigation, have used hair analysis in hundreds of forensic investigations. The authors believe that these developments and their own experience with more than 350,000 hair specimens (i.e., 1.7 million analyses) have shown that hair analysis is an effective and safe method for establishing drug use.

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Testing Human Hair for Opiates and Cocaine by Gas Chromatography/Mass Spectrometry After Acid or Enzyme Hydrolysis

Pascal Kintz

INTRODUCTION

Morphological, serological, and chemical examination of human hair for forensic and medical investigation was initiated some years ago. A single human hair is sometimes the only remnant at the scene of a crime. In many cases, it serves to confirm or exclude a possible suspect. Because hair does not decompose like body fluids and soft tissues, it has been used by forensic pathologists to determine the cause of death. In the 1960s and 1970s, hair analysis was used to evaluate exposure to toxic heavy metals such as arsenic, lead, and mercury; the analytical method used in these analyses was atomic absorption, which detects amounts in the picogram range.

Later, analysis of hair by means of drugs labeled with radioactive isotopes established that these substances can move from blood to hair and are deposited there. Ten years after these first investigations for drugs in hair, simultaneously in the United States and the former West Germany, it became possible to measure various drugs by means of radioimmunoassay (RIA).

Using RIA, Baumgartner and colleagues (1979) published the first report on the detection of morphine in the hair of heroin abusers and found that differences in the concentration of morphine along the hair shaft correlated with the time of drug use. This was followed shortly thereafter by other studies on the analysis of drugs of abuse (e.g., Klug 1980; Arnold and Püschel 1981; Smith and Pomposini 1981; Valente et al. 1981). Initially, however, these methods, based on RIA, did not allow a differentiation among individual opiates.

Generally, the courts recognized chemical and toxicological analysis only in cases where results were confirmed by a second independent analytical method. This is the reason that gas chromatography/mass spectrometry (GC/MS) was used in hair analysis and is now the method of choice. At present, hair analysis is used as a tool of detection for drug use in forensic sciences, traffic medicine, occupational medicine, and clinical toxicology.

Various articles dealing with opiates (Sachs et al. 1993; Nakahara et al. 1992; Cone 1990; Goldberger et al. 1991) and cocaine (Fritch et al. 1992; Cone et al. 1991; Harkey et al. 1991; Henderson et al. 1992; Kidwell 1993) have been published, but only one (Moeller 1992) reported a simultaneous method for several compounds. This chapter describes a procedure that, in a single run, can identify and quantify opiates and cocaine and their major metabolites.

METHODS

Reagent and Standards

Methanol, dichloromethane, isopropanol, *N*-heptane, and chloroform were high-performance liquid chromatography grade. All other chemicals were analytical grade, including β-glucuronidase (12 IU/mL)/arylsulfatase (60 IU/mL).

The chemicals used were cocaine, benzoylecgonine (BZE), ecgonine methyl ester (EME) hydrochloride, morphine, codeine, 6-monoacetylmorphine (MAM), cocaethylene, cocaine-d_3, BZE-d_3, EME-d_3, morphine-d_3, codeine-d_3, MAM-d_3, cocaethylene-d_3, and N,O,-*bis*(trimethylsilyl)trifluoroacetamide (BSTFA)+1 percent

trimethylchlorosilane (TMCS). A ball mill was used to pulverize the hair samples.

MATERIALS FOR EXAMINATION

Positive hair samples were obtained from 78 male and 36 female subjects, ages 17 to 43 years, who either were deceased from fatal opiate (80 cases) or cocaine (24 cases) overdose or had been admitted to a detoxification center after heroin abuse (17 cases). Note that the 80 opiate cases overlap with the 24 cocaine cases, which explains the difference between the totals 114 (78 males + 36 females) and 121 (80 opiate cases+24 cocaine cases+17 heroin cases).

These positive samples were part of 450 specimens tested with the GC/MS method. To be considered positive, hair extracts must exhibit the presence of at least one compound.

Hair samples weighing at least 30 mg (50 mg is better) were cut from the posterior vertex as close as possible to the skin. For screening purposes, only the first 3 cm of hair from the root were tested. The hair was decontaminated by washing the specimen two times in 5 mL dichloromethane for 2 minutes at room temperature. This procedure was shown to remove external contamination because the GC/MS analysis of a third wash tested negative, although earlier washes were positive (Kintz and Mangin 1993).

SAMPLE EXTRACTION

The strands of hair were pulverized in a ball mill and then incubated at 56 °C overnight in 1 mL 0.1 M hydrochloric acid (HCl) in the presence of 100 ng each of the following standards: morphine-d_3, codeine-d_3, cocaine-d_3, MAM-d_3, EME-d_3, cocaethylene-d_3, and BZE-d_3. Samples were extracted with 10 mL chloroform isopropanol-*n*-heptane (50:17:33 v/v) under alkaline conditions (2 mL phosphate buffer at 1 mol/L and pH 8.4). After agitation and centrifugation, the organic phase was purified by an additional acid extraction (5 mL 0.2 M HCl), and the aqueous layer was reextracted

with 2 mL phosphate buffer, 1 mL 1 M sodium hydroxide (NaOH), and 5 mL chloroform. After further agitation and centrifugation, the organic phase was removed and evaporated to dryness at 45 °C in a centrifugal vacuum concentrator. BSTFA + 1 percent TMCS (30 μL) was added to the dry extract, which was sealed and heated at 70 °C for 20 minutes. A 2-μL portion of the derived extract was injected into the GC column.

For enzyme preparation, the sample was hydrolyzed with 75 μL β-glucuronidase/arylsulfatase for 2 hours at 40 °C in 2 mL phosphate buffer (pH 7.6) in the presence of deuterated internal standards (ISs) (Moeller 1992). After centrifugation, the supernatant was extracted as described previously.

GC/MS METHOD

The GC/MS system consisted of a chromatograph with a mass selective detector (MSD) operated at 70 eV with an ion source temperature of 180 to 190 °C. The electron multiplier voltage was set at +400 V above autotune voltage. The MSD was autotuned daily with perfluorotributylamine.

Carrier gas (helium, purity grade N55) flow through the capillary column (5 percent phenyl, 95 percent methyl siloxane; 30 m × 0.25 mm internal diameter) was 1.8 mL/min.

The column oven temperature was programmed from an initial temperature of 60 to 290 °C at 30 °C/min and was maintained at 290 °C for the final 5 minutes. Splitless injection with a split valve offtime of 0.75 minutes was used. Injector temperature was 260 °C.

Table 1 shows the ions monitored for the different drugs and the deuterated IS and retention time (RT) of each. Analytes were identified and quantified based on comparison of RTs and relative abundance of two confirming ions with the deuterated ISs. Ions used for quantitation are underlined.

TABLE 1. *Selected ions and retention times*

Drug	Ions	Internal Standard Ions	Retention Time (minutes)
Ecgonine methyl ester hydrochloride	96, 82	99, 85	7.13
Cocaine	182, 303	185, 306	9.72
Cocaethylene	196, 82	199, 85	9.93
Benzoylecgonine	240, 361	243, 364	9.97
Codeine	371, 282	374, 285	10.81
6-Monoacetylmorphine	429, 401	432, 404	11.03
Morphine	399, 340	402, 343	11.42

Standard calibration curves were obtained by adding 10 (0.2 ng/mg), 25 (0.5 ng/mg), 50 (1.0 ng/mg), 100 (2 ng/mg), 500 (10.0 ng/mg), and 2,500 (50 ng/mg) ng pure standards, prepared in methanol, to 50 mg pulverized blank control hair (obtained from laboratory personnel and previously tested to be drug-free).

Recovery and day-to-day precision were determined by adding 100 ng pure standards to 50 mg pulverized blank control, corresponding to 2 ng/mg hair.

RESULTS AND DISCUSSION

In all heroin cases, fatal overdose was clearly confirmed by the presence of MAM in the urine. Morphine blood concentrations were greater than 87 μg/L. In cases of cocaine overdose, blood BZE levels were greater than 1,200 μg/L. The concentrations determined are strongly influenced by the dose and the time of the last intake. For the medical examiner, it is sometimes useful to know whether the decedent was a chronic drug abuser. The absence of drug metabolism in hair may provide a historical account of drug use.

Before recognition by courts in France, the methodology must be fully validated. This was achieved by spiking control hair.

The correlation coefficients of the calibration curves were r=0.998 (cocaine), 0.995 (BZE), 0.991 (EME), 0.998 (morphine), 0.998 (codeine), 0.993 (MAM), and 0.996 (cocaethylene), showing linearity for all drugs between 0.2 and 50 ng/mg.

The interday coefficient of variation precision (n=6 persons/specimens), recovery, and limit of detection of each drug are presented in table 2.

Once the procedure was validated, it was possible to test real samples with the procedure. The hair samples were obtained from forensic and clinical cases and were tested with the presented method. Of the 450 specimens, 97 samples tested positive for opiates, whereas only 24 tested positive for cocaine. In some cases, only one analyte was identified. Particularly when cocaine concentrations were less than 2 ng/mg, no metabolites were found. The amounts of drugs found are shown in table 3.

TABLE 2. *Interday precision, recovery, and limit of detection of each drug; n=6 (number of persons/specimens)*

Drug	Interday Precision (%)	Recovery (%)	Limit of Detection (ng/mg)
Ecgonine methyl ester hydrochloride	17.7	68.6	0.50
Cocaine	8.4	83.2	0.05
Cocaethylene	9.6	81.6	0.05
Benzoylecgonine	14.3	72.1	0.15
Codeine	11.6	85.7	0.05
6-Monoacetylmorphine	15.7	69.4	0.15
Morphine	12.0	79.3	0.05

TABLE 3. *Amounts of drugs in human hair found using acid hydrolysis (n=450 samples)*

Drug	Number of Positives	Range (ng/mg)	Mean (ng/mg)
Ecgonine methyl ester hydrochloride	8	0.0 - 3.6	0.9
Cocaine	24	0.3 - 137.3	13.7
Cocaethylene	11	0.0 - 5.2	2.5
Benzoylecgonine	18	0.0 - 36.3	3.7
Codeine	97	0.1 - 19.6	2.9
6-Monoacetylmorphine	85	0.0 - 103.2	10.7
Morphine	97	0.2 - 27.1	5.2

Using alkali hydrolysis of hair samples, it was not possible to identify MAM or cocaine (Kintz and Mangin 1993). The present modifications were necessary to detect MAM as the confirmation marker for heroin exposure. Results revealed the presence of MAM as the major metabolite in hair of heroin abusers. This analyte has been reported previously by other investigators (Nakahara et al. 1992; Cone 1990; Goldberger et al. 1991).

Morphine was detected at 1/3 to 1/10 the levels of MAM. Morphine and codeine were present in all samples.

A particular problem in the detection of opiates is evaluating whether morphine has resulted from heroin or morphine consumption or from misuse of a medication containing codeine. Small amounts of morphine (approximately 5 to 10 percent) are produced from codeine by metabolic demethylation, but almost all illegally sold heroin contains acetylcodeine as an impurity of opium, which is quickly deacetylated to codeine after intake. The differentiation of heroin users from individuals exposed to other sources of opiate alkaloids can be achieved by identifying MAM in hair.

In the 12 cases (12 percent) where MAM was not detected, codeine levels were clearly higher than morphine levels, and it may be

assumed that only codeine had been ingested. In these cases, concentrations were 0.2 to 3.1 (mean 1.3) and 3.7 to 19.6 (mean 8.4) ng/mg for morphine and codeine, respectively. Only in 7 cases were opiates and cocaine simultaneously identified.

The major compound detected in the hair of cocaine abusers is the parent drug. BZE was found in 18 cases (75 percent), generally when cocaine concentration was greater than 2 ng/mg. Other researchers have found the same results (Fritch et al. 1992; Cone et al. 1991; Harkey et al. 1991; Henderson et al. 1992; Kidwell 1993). Cocaine was present at concentrations approximately 3 to 6 times higher than BZE and approximately 10 to 50 times higher than EME. Cocaethylene, which forms when cocaine and ethanol are used concurrently, was detected in 11 cases (46 percent). As proposed by Cone and coworkers (1991), cocaethylene's presence in hair could be considered a marker of cocaine exposure. All these findings illustrate that the stability and ratio of residual cocaine to that of its metabolites in hair differ from that in other commonly tested tissues in the human body.

Because the presence of cocaine and MAM as the primary analytes in hair cannot be explained by the usual pharmacokinetic considerations, some researchers have developed models of drug incorporation (Harkey et al. 1991; Henderson et al. 1992), including apocrine, sebaceous, or sweat gland secretions. Other factors, such as lipid solubility or degree of ionization of a drug, also may influence the incorporation of the drug in hair.

Stability experiments carried out in the laboratory showed both cocaine and MAM to be stable in 0.1 *n* HCl at 56 °C overnight and in β-glucuronidase/arylsulfatase pH 7.6 buffer. In both cases, the hair structures appeared to remain intact. Reextracting the same sample a second time showed negligible amounts of the analytes to be present, suggesting that the first extraction was efficient.

When acid and enzyme procedures were compared, both methods provided similar results in the range ± 28 percent, which were not statistically different. This can be attributed to analytical variations and demonstrates that neither approach consistently provides higher or more precise results. Based on these results, it is not possible to

determine which method performs better. Therefore, for forensic applications, positive samples obtained after acid hydrolysis are confirmed by a second GC/MS analysis after enzyme hydrolysis.

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Quantitative Results of Drugs in Hair Using Different Extraction Methods

Hans Sachs and Manfred R. Moeller

INTRODUCTION

The reason for comparison of extraction methods is not only to confirm the first gas chromatography/mass spectrometry (GC/MS) method by another independent procedure but also to get information—by modifying the method step by step—about the binding site of drugs in hair. Common extraction procedures are shown in figure 1. Since the first extraction method introduced by Klug (1980), several methods for extracting drugs from the keratin matrix have been published. Extractions by hydrochloric acid (HCl) were described by Valente and colleagues (1981), and improved versions have appeared in recent publications by Kintz and coworkers (1992). Enzymes were used to prepare the hair sample for solid-phase extraction (SPE) (Moeller and Fey 1990; Raff and Sachs 1990). The methanol extraction followed by SPE described by Cone (1990) that is used in many laboratories was simplified later by direct determination after methanol extraction by Kauert and colleagues (1992). Few methods disintegrate hair samples and extract drugs from the liquid phase. A comparison of extraction with buffer or methanol with the extraction from disintegrated hair can show whether drugs are completely extracted. In the present study, hair samples were analyzed in small batches. Different extraction methods were compared. The methods used in this comparison are described below.

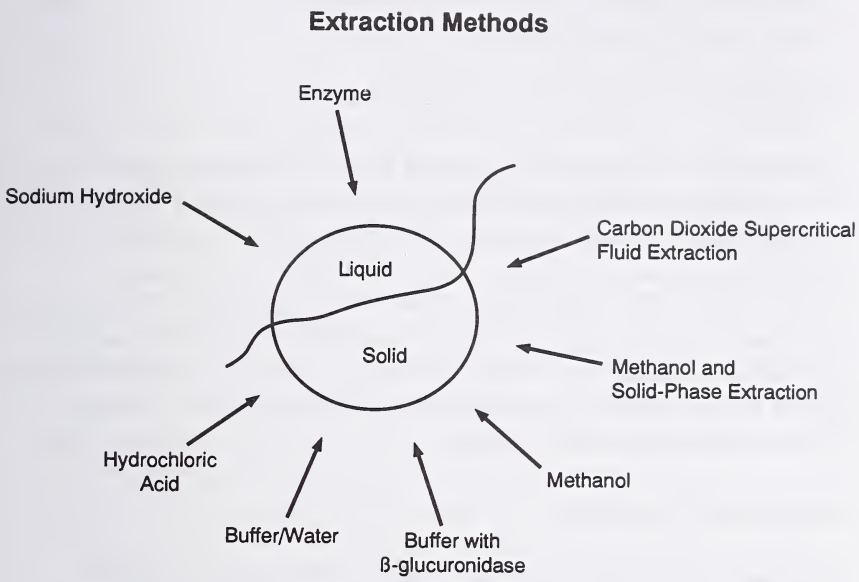

FIGURE 1. *Survey of known extraction methods for drugs in hair. Two methods disintegrate the hair to a liquid phase; the others leave hair matrix visibly unchanged.*

METHODS

Origin of Hair Samples

The hair samples were obtained from drug addicts by the police according to written instructions (Denk et al. 1992) or during autopsy in cases of fatal drug intoxication. Although different authors propose different washing procedures, to compare the extractions every hair bundle was washed with distilled water, acetone, and petroleum ether (10 mL each). Each washed hair bundle was cut into sections, when possible in 3-cm lengths, and each section was powdered in a ball mill. The pulverized hair was divided into fractions for the separate extraction procedures. Ethylmorphine was used as internal standard for determining the

opiates, and deuterated standards were used for the determination of cocaine and benzoylecgonine (BZE).

Materials

All reagents used were of analytical grade. ß-Glucuronidase (12 u/mL)/arylsulfatase (60 u/mL) was obtained from Merck (Darmstadt, Germany). Extractions were carried out on the following extraction columns: Extrelut (Merck, Darmstadt), Chromabond C18 ec (200 mg, 3 mL, Machery-Nagel, Dülmen, Germany), and Bond Elut Certify (ict Analytichem International, Frankfurt, Germany). Pentafluoropropionic anhydride (PFPA) and 2,2,3,3,3-pentafluoro-1-propanol were purchased from Aldrich-Chemie (Steinheim, Germany).

Extraction Procedures

Extrelut Extraction After Dissolution of the Hair by NaOH (Sachs and Brunner 1986; Sachs and Arnold 1989; Sachs et al. 1993). The individual hair fragments were heated in an evaporator with 3 mL 3 percent sodium hydroxide (NaOH) solution and 500 ng ethylmorphine as internal standard until the material was completely dissolved. Then the samples were hydrolyzed for 30 minutes together with 2 mL 25 percent HCl at 100 °C. After the sample was cool, solid sodium bicarbonate was added until a pH value of 8.5 to 9 was attained. The volume was adjusted to 20 mL with glycylglycine buffer, then extracted with a mixture of toluene/ *n*-butanol in an Extrelut-column, and reextracted in 10 mL 0.1 M sulfuric acid. The aqueous phase was adjusted to pH 8.5 to 9, and the solution was again extracted with dichloromethane-2-propanol by Extrelut. The organic phase was evaporated under a nitrogen stream at 50 °C.

Bond Elut Certify Extraction After Enzyme Digestion of the Hair (Raff and Sachs 1990). Approximately 50 mg pulverized hair sample was incubated in 1 mL 60 percent performic acid. After 30 minutes, 5 mL 5 percent ammonia and 10 mL water were added. The pH was adjusted to 7.5 with 5 percent ammonia or 2 percent performic acid as required. The surplus H_2O_2 was destroyed by adding 25 μL catalase and letting the sample stand for approximately

1 hour with occasional shaking. The solution then was incubated with 2 mg pronase (nonspecific enzyme produced by *Streptomyces griseus*) overnight at 40 °C. The extraction with Bond Elut Certify was carried out following the appropriate method for each substance according to the manufacturer's instructions. As opposed to other enzyme methods (Offidani et al. 1989), this dissolution leads to a residue of less than 5 percent, a result that is possible because the disulfide bonds in the keratin were broken with performic acid.

Solid-Phase Extraction After Treatment With Buffer and ß-Glucuronidase/Arylsulfatase (Moeller and Fey 1990). The authors added 2 mL Sorensen-buffer solution (pH 7.6) to about 40 mg pulverized hair together with the internal standards and 50 μL ß-glucuronidase/arylsulfatase. The solution was incubated for 2 hours at 40 °C and centrifuged. The buffer solution was decanted; then the residue shaken again with 2 mL new buffer and centrifuged. The combined aqueous layers were extracted on solid-phase columns, Chromabond C18 ec, and eluted three times with acetone dichloromethane at a ratio of 3:1.

Solid-Phase Extraction After Treatment With Buffer. The previous extraction method was changed as far as the enzyme was concerned. The extraction was performed with only 2 mL Sorensen-buffer (pH 7.6) under sonification for 2 hours.

HCl Extraction (Raff 1994). In this method, 50 mg pulverized hair was shaken in 1 mL 0.1 M HCl over a 1-day period. The liquid phase was neutralized with 0.1 mL 1 M NaOH and adjusted to pH 7.5 using phosphate buffer. The extraction was performed with Bond Elut Certify using the procedure for opiates written in the manual accompanying each product package.

Extraction With Methanol (Kauert et al. 1992). From 50 to 200 mg pulverized hair and the internal standard were extracted with 5 mL methanol in an ultrasound bath at 45 °C for 5 hours. After being centrifuged, the methanol was decanted and evaporated.

Quantification by Gas Chromatography/Mass Spectrometry

For GC/MS analysis the samples were derivatized with PFPA and 2,2,3,3,3-pentafluoro-1-propanol for 30 minutes at 65 °C. The samples were evaporated under a stream of nitrogen until odorless, then dissolved in ethyl acetate. One μL was injected without splitting into a GC/MS Hewlett Packard 5995A (with direct coupling) at 100 °C on an HP Ultra 2 column (cross-linked 5 percent phenylmethylsilicone, 12 m, 0.2 mm internal diameter, 0.33-μm film) and was analyzed from 250 to 280 °C programmed at 5 °C-increase per minute. For quantitative determinations, a mass-to-charge ratio of 414 was used for monoacetylmorphine (MAM) and morphine, 445 for codeine, 447 for dihydrocodeine, 182 for cocaine, and 300 for BZE. If deuterated standards were not available, ethylmorphine was used as internal standard if it could be determined that the drug addict had not used ethylmorphine. If the regression coefficients were not greater than 0.99, the assay was repeated.

COMPARISON OF RESULTS

Different Extraction Methods in One Laboratory

Pronase Digestion and NaOH. Because heroin and MAM are hydrolysed to morphine in the NaOH procedure, the authors compared the morphine concentration of this procedure with the sum of MAM and morphine of the pronase digestion (figure 2). Each triangle represents a pair of results provided by the different examinations. In the majority of the cases there were higher concentrations after pronase digestion; the median of the deviation was 41 percent. It is necessary to take into account that both procedures dissolve the hair to a liquid phase and the extraction steps are comparatively complicated.

In five cases, dihydrocodeine concentrations were higher after pronase digestion than after treatment with NaOH (table 1). Especially at higher concentrations, pronase digestion showed higher concentrations.

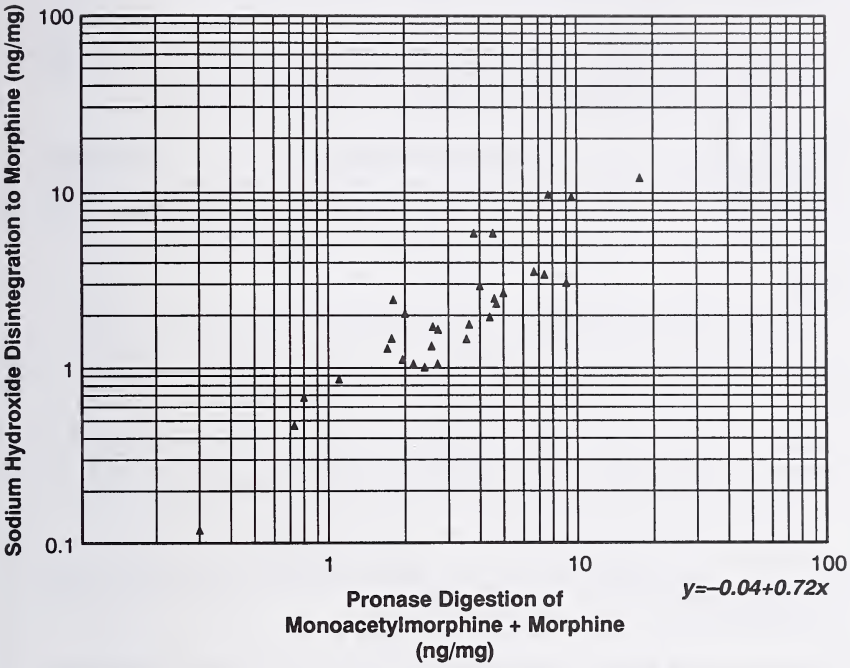

FIGURE 2. *The sum of concentrations of monoacetylmorphine and morphine after disintegration with sodium hydroxide compared with extraction after disintegration with performic acid and pronase*

Pronase Digestion and Buffer Extraction. Because the enzymes pronase and ß-glucuronidase cause destruction of MAM to morphine, the sums of MAM and morphine were compared (figure 3). The results demonstrated that pronase extraction on average produces higher results than the buffer extraction after ß-glucuronidase treatment.

Pronase Digestion and HCl Extraction. The results of the cocaine concentrations do not allow any conclusion as to whether the extraction rate is higher after extraction by pronase or HCl. However, it is obvious in the case of BZE that the disintegration of the hair leads to higher values than the extraction of the solid hair

TABLE 1. *Dihydrocodeine in hair of addicts determined after disintegration of the hair with performic acid and pronase compared with extraction after disintegration with sodium hydroxide*

Pronase (ng/mg)	Sodium Hydroxide (ng/mg)	Difference (ng/mg)
3.98	2.96	1.02
9.08	2.57	6.51
1.11	0.87	0.24
0.73	0.47	0.26
0.80	0.68	0.12
0.41	0.48	-0.07
0.22	0.56	-0.34

by HCl. The median values of cocaine and BZE were 8.8 for HCl extraction and 5.4 for pronase (table 2).

Methanol and Buffer Extraction. These two methods have been compared extensively in the authors' laboratory in normal routine cases because both methods allow screening for opiates, cocaine, cannabinoids, and amphetamines. As an example, cocaine and BZE results were compared. As shown in figures 4 and 5, the results do not coincide as well as for the opiates extracted by other methods, perhaps because the laboratory has more experience with opiates. In the majority of the compared cases, the cocaine concentrations were higher after methanol extraction than after the ß-glucuronidase and buffer extraction.

To exclude the influence of contamination when comparing two methods, substances can be examined only where the possibility of contamination is excluded. One substance that is abused to a certain extent is dihydrocodeine. Figure 6 shows a good correlation using the methanol and the ß-glucuronidase and buffer method for dihydrocodeine extraction. It is confirmed here that the extraction rate of the opiate is a little higher when the ß-glucuronidase and buffer method is used.

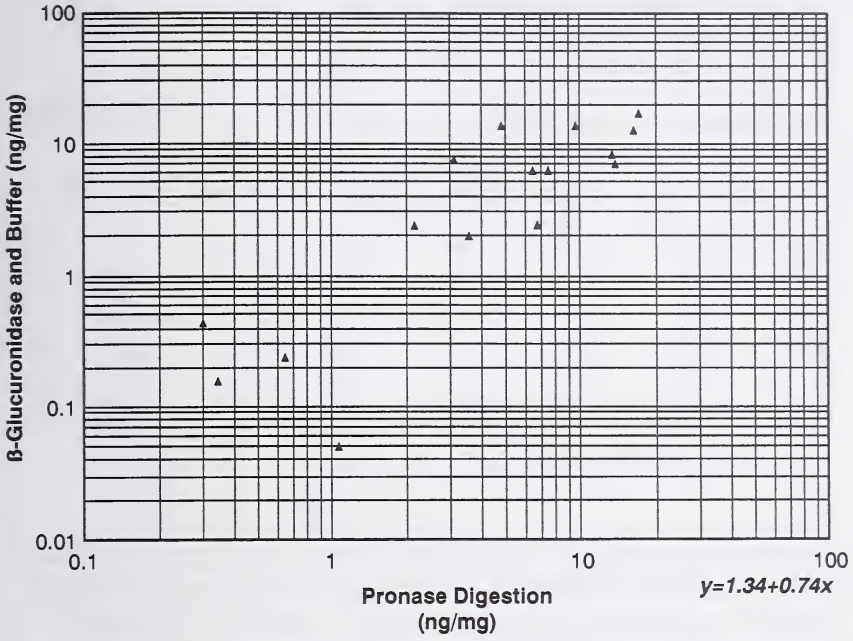

FIGURE 3. *The sum of concentrations of monoacetylmorphine and morphine after disintegration of the hair with performic acid and pronase compared with extraction after treatment with ß-glucuronidase and buffer followed by solid-phase extraction*

In all cases of drug addicts, there is a variety of sources for different results. MAM can be affected by enzymes. Different incubation times may lead to different MAM-to-morphine ratios.

The only way to observe morphine concentrations not affected by the extraction procedure is to examine the hair of patients who received morphine exclusively. The morphine concentrations in the hair of one of those patients are listed in table 3. The powdered hair was extracted with methanol, or buffer, or buffer with ß-glucuronidase.

TABLE 2. *Concentrations of BZE and cocaine after disintegration of the hair with performic acid and pronase compared with extraction after treatment with HCl followed by solid-phase extraction*

BZE		Cocaine		Sum		Cocaine/BZE	
HCl (ng/mg)	Pronase (ng/mg)	HCl (ng/mg)	Pronase (ng/mg)	HCl (ng/mg)	Pronase (ng/mg)	HCl	Pronase
6.40	13.30	23.80	27.80	30.20	41.10	3.72	2.09
14.03	23.11	68.96	67.00	82.99	90.11	4.92	2.90
18.63	30.13	104.60	71.19	123.23	101.32	5.61	2.36
0.40	0.82	3.52	7.66	3.92	8.48	8.80	9.34
0.39	1.47	4.99	10.60	5.38	12.07	12.79	7.21
0.59	1.39	6.01	7.52	6.60	8.91	10.19	5.41
0.29	0.99	4.81	7.08	5.10	8.07	16.59	7.15

KEY: BZE=benzoylecgonine; HCl=hydrochloric acid

The results show higher concentrations after using ß-glucuronidase, which leads to the assumption that at least a small part of morphine is bound to glucuronic acid as a conjugate. Buffer and methanol extractions provide similar results. It seems also that a dose/concentration relation exists, but this should be verified by further examinations.

INTERLABORATORY COMPARISON

The concentrations of MAM, morphine, codeine, and dihydrocodeine of 50 powdered hair segments of opiate addicts were determined in the laboratories of the universities in Ulm and Homburg/Saar. The determinations in Ulm were performed as the samples arrived in the laboratory during half a year, whereas the determinations in Homburg were performed in one series. These procedures may have caused an additional day-to-day error in Ulm but not in the laboratory in Homburg. Examples of the amounts of MAM and the sum of MAM and morphine determinations are shown in figures 7 and 8, respectively.

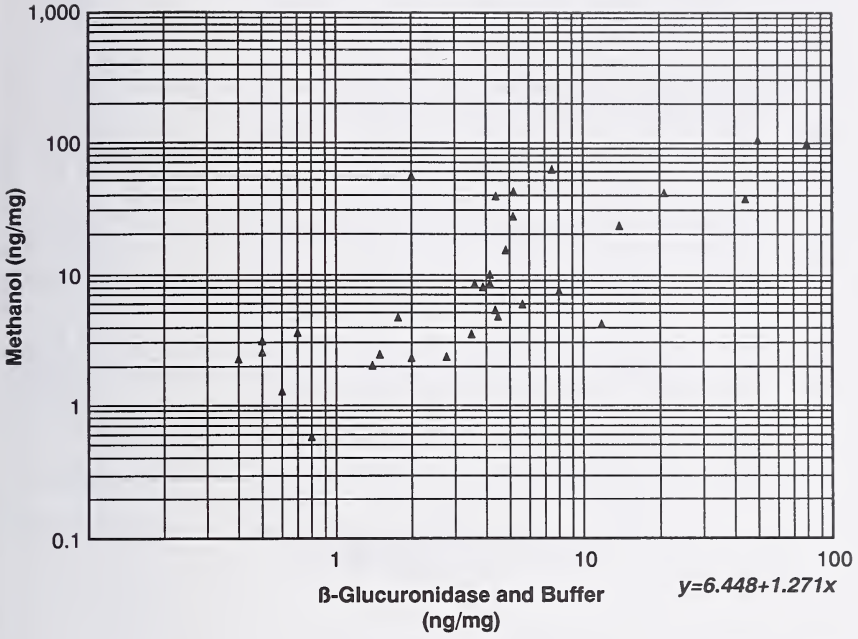

FIGURE 4. *Cocaine concentrations in hair of addicts after extraction with methanol and direct determination compared with extraction after treatment with ß-glucuronidase and buffer followed by solid-phase extraction*

DISCUSSION

For the different extraction methods, the treatment with performic acid and pronase showed the highest extraction rates. The comparisons also demonstrated that the disintegration of hair by NaOH or by enzymes is not necessary. In most cases only the qualitative results are evident. Because of irregular hair growth and unknown doses/concentration relations, the extractions by organic solvent, acids, or buffer are sufficient. ß-Glucuronidase may increase the extraction rate of morphine, but the deviation in single

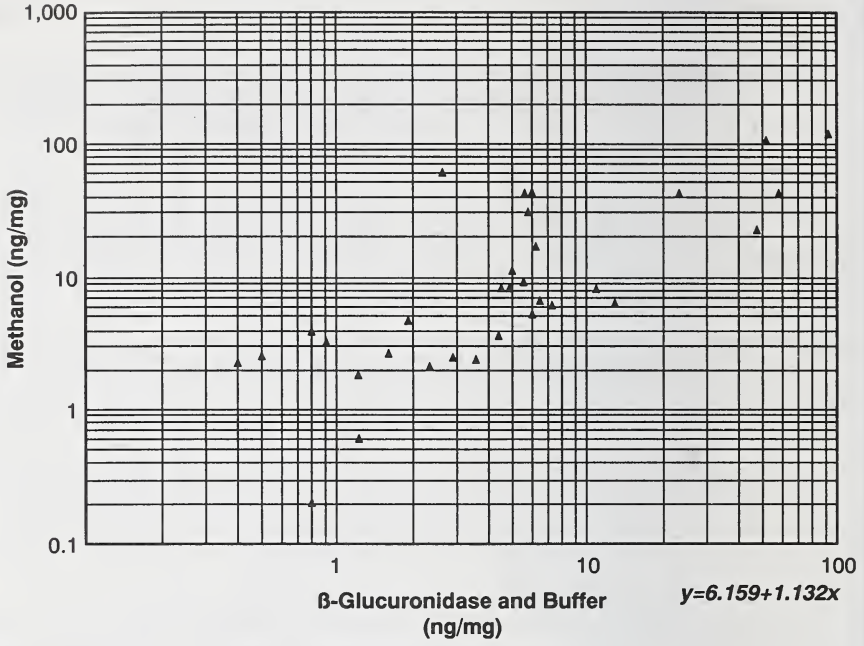

FIGURE 5. *The sum of concentrations of cocaine and benzoylecgonine in hair of addicts after extraction with methanol and direct determination compared with extraction after treatment with β-glucuronidase and buffer followed by solid-phase extraction*

cases corresponds to the different extraction effects on powdered hair.

Even if a high percentage of the morphine is metabolized initially when taken orally, the concentrations seem low compared with those of heroin addicts who probably cannot consume much more than 200 mg heroin daily. Therefore, these concentrations seem to confirm that lipophilic substances are more easily incorporated into hair than hydrophilic substances and that most opiates found in hair after heroin consumption are incorporated as MAM. A part of this metabolite may be metabolized or chemically converted to morphine during the analytical procedure.

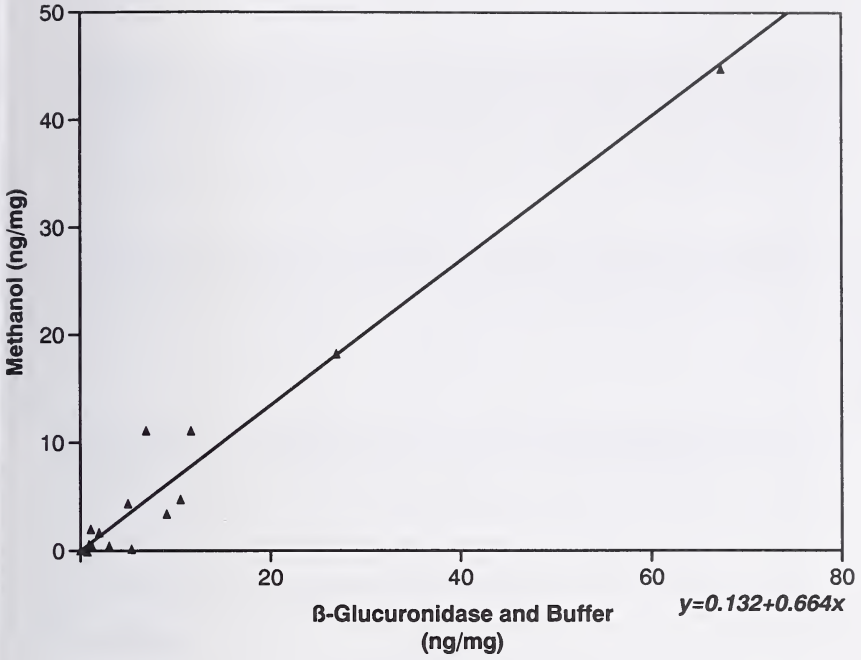

FIGURE 6. *Dihydrocodeine in hair of addicts determined after extraction with methanol and direct determination compared with extraction after treatment with β-glucuronidase and buffer followed by solid-phase extraction*

TABLE 3. *Morphine concentrations in the hair of a patient after controlled doses of morphine*

Daily Dose	0.5 g (ng/mg)	1.7 g (ng/mg)
Methanol	5.5	21.8
Buffer	4.0	19.9
Buffer with β-glucuronidase	6.5	24.4

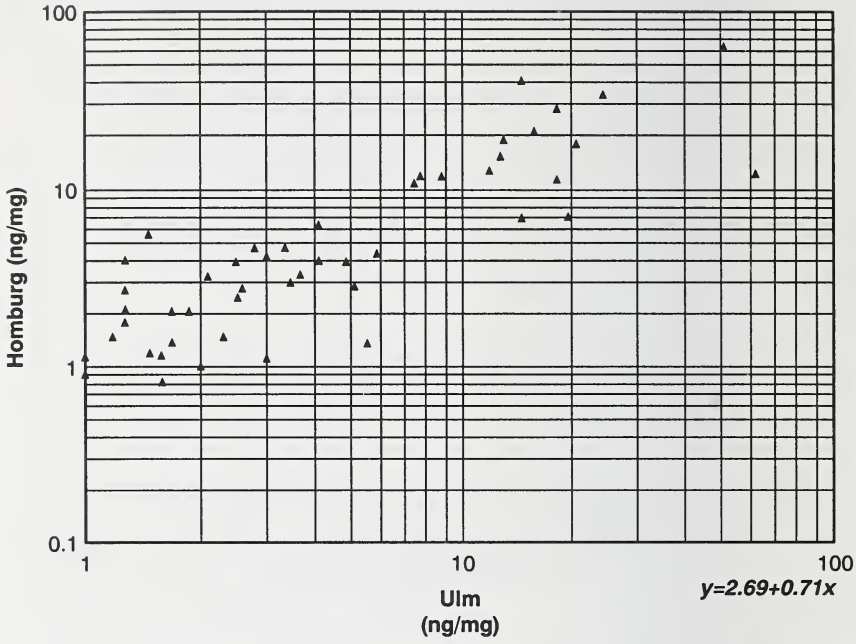

FIGURE 7. *Interlaboratory comparison of MAM concentrations in hair of addicts after treatment with β-glucuronidase and buffer followed by solid-phase extraction*

Buffer and methanol extractions allow screening for drugs of abuse like opiates, cocaine, cannabinoids, and amphetamines. In the laboratory in Ulm, screening for drugs is performed with several hair segments of 3-cm length using the buffer extraction. In positive cases the results of at least one segment are confirmed by determination after methanol extraction. In German courts the differentiation between regular use—more than one application a week—and irregular use is necessary. After this comparison and experience with more than 1,000 cases, it is assumed that a limit of 2 ng/mg for opiates and cocaine extracted by any of the described methods in at least 6 cm—2 segments of 3 cm—is suitable to prove that regular consumption has taken place.

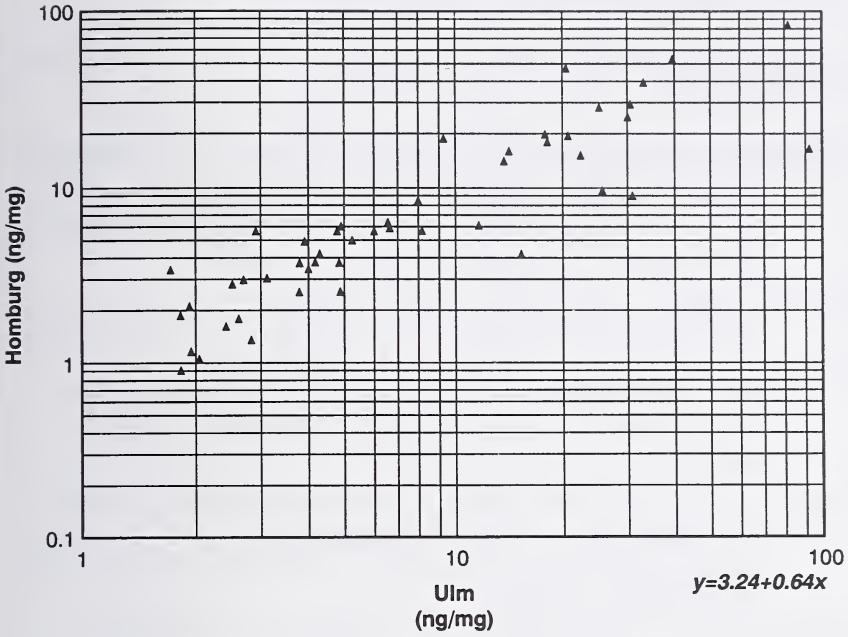

FIGURE 8. *Interlaboratory comparison of the sum of MAM and morphine concentrations in hair of addicts after treatment with ß-glucuronidase and buffer followed by solid-phase extraction*

SUMMARY

Comparisons of drug extraction methods can show the different qualities of these procedures when quantification is performed by the same GC/MS methods and the same derivatization (Sachs and Raff 1993). The authors' examinations were performed after dissolving the hair sample either with NaOH or pronase, after incubation of the hair in buffer, including ß-glucuronidase followed by solid-phase extraction, or after incubation with methanol and direct determination by GC/MS. Although the results do not match in every case, they show that drugs are easily extracted by water or

organic phases and that it is not necessary to dissolve the hair completely before extracting the drug from the liquid phase.

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Analysis of Nicotine and Cotinine in Human Hair by High-Performance Liquid Chromatography and Comparative Determination With Radioimmunoassay

Simona Pichini, Roberta Pacifici, Ilaria Altieri, AnnaRita Passa, Mirella Rosa, and Piergiorgio Zuccaro

INTRODUCTION

Inhalation of cigarette smoke is the leading cause of death from cancer in the world (U.S. Department of Health and Human Services 1988). More than 3,800 components of tobacco smoke have been identified, with nicotine being the most extensively studied (International Agency for Research on Cancer 1986, pp. 83-126; Dawson and Vestal 1982). Nicotine is a highly toxic alkaloid that causes stimulation of autonomic ganglia and the central nervous system (Benowitz 1988). Determination of the concentrations of nicotine and cotinine in blood (Hariharan et al. 1988; Zuccaro et al. 1993), saliva (Greenberg et al. 1984), urine (Parviainen et al. 1990; Hariharan and VanNoord 1991), and seminal fluid (Pacifici et al. 1993*a*) has been used to estimate uptake of nicotine during tobacco consumption and exposure to environmental tobacco smoke (ETS) and to validate abstinence after cessation of smoking (Widlox et al. 1979; Haley et al. 1983). Cotinine is considered to be the marker of choice, as its concentration in blood, urine, and saliva is higher

than that of the parent compound nicotine, and its half-life is approximately 10 times longer than that of nicotine (Benowitz et al. 1983). However, it is not always possible or desirable to sample the named body fluids from participants in a study. Furthermore, the above-mentioned biological matrices reflect nicotine intake and exposure to ETS only during the preceding 1 to 3 days. Information cannot be obtained on the frequency of smoking and on past exposure in subjects who might deliberately abstain for several days before sample collection.

Hair analysis is a noninvasive technique that has been used successfully to detect the presence of drugs and metabolites sequestered in the hair shaft; however, the mechanisms of drug incorporation are not fully understood (Welch et al. 1993). Hair samples are stable indefinitely, can be easily collected, and can provide a wide window of detection. However, it may be difficult to extract drugs from the hair matrix in a reproducible manner, and there is a lack of reference material with known quantities of analytes to assess various methodologies (Welch et al. 1993). Recently, results of several studies were published to determine nicotine and cotinine in hair samples. These studies used gas chromatography coupled with mass spectrometry (Kintz et al. 1992; Kintz and Mangin 1993) or nitrogen-phosphorus detection (Mizuno et al. 1993) and radioimmunoassay (RIA) (Haley and Hoffmann 1985; Eliopoulos et al. 1994).

This chapter describes high-performance liquid chromatography (HPLC) as used in analyzing the hair of smokers for the presence of nicotine and cotinine. HPLC results were compared with those of the RIA used by Haley and Hoffmann (1985). Moreover, different procedures for extraction of the analytes from hair were compared with those performed in the authors' laboratory.

MATERIALS AND METHODS

Hair was collected from 10 smokers among laboratory personnel. Smokers provided data on cigarette brand and the number of cigarettes smoked daily, which usually ranged from 5 to 40 cigarettes. Daily nicotine intake was calculated from the number

of cigarettes smoked per day multiplied by the nicotine yield. From each subject, about 500 mg hair was collected from the back of the head; the hair was cut at the scalp, and the full length was collected.

A portion of hair from all the smokers was cut and mixed to create a pool of samples to be used for different extraction procedures. Hair samples were washed twice with *N*-hexane by vortex mixing. The hexane solutions were evaporated under nitrogen, and the residues were assayed to determine whether this procedure removed external nicotine and cotinine.

The following extraction procedures were performed on 50 mg hair after a hexane wash:

- Hair samples were placed in a vial with 10 mL acetone and sonicated at room temperature for 30 minutes. The resulting acetone extract was dried under nitrogen (Haley and Hoffmann 1985) (extraction procedure 1).

- Hair samples were homogenized in 3 mL sodium hydroxide (NaOH) 1*N* for 1 hour at 100 °C; the analytes were extracted with 5 mL diethyl ether, which was evaporated to dryness before analysis (Kintz et al. 1992) (extraction procedure 2).

- The same procedure was repeated with NaOH 0.5*N* (extraction procedure 3).

- Hair samples were digested with 1 mL NaOH 0.6*N* overnight at 50 °C; the following day the solutions were neutralized with concentrated HCl and examined (Eliopoulos et al. 1994) (extraction procedure 4).

- Hair samples were digested with 3 mL NaOH 0.5*N* for 1 hour at 100 °C; the solution then was transferred to an Extrelut-3 glass column (Merck, Darmstadt, Germany), which was preconditioned with 10 mL dichloromethane the day before and left

to dry during the night. Fifteen minutes after the transfer, the analytes were eluted with 10 mL dichloromethane:isopropyl alcohol (9:1 v/v). The elute, with 300 μL methanolic HCl (25 mM) added to prevent nicotine loss, was evaporated to dryness before analysis (extraction procedure 5).

- The same procedure was repeated digesting the samples for 10 minutes, 4 hours, and 24 hours at room temperature (extraction procedures 6, 7, and 8) and for 30 minutes in a sonicating water bath (extraction procedure 9).

Table 1 shows the different extraction procedures used.

Residues from the hexane wash, samples extracted by different procedures, and hair extracted with procedure 7 (table 2) from each of the 10 smokers were dissolved in 1 mL RIA buffer or in 200 μL HPLC mobile phase and were analyzed. Nicotine and cotinine were quantified by RIA, using a double antibody RIA according to the technique described by Van Vunakis and colleagues (1993, pp. 293-299). The rabbit antiserum, H3 cotinine, and H3 nicotine were supplied by Dr. H. Van Vunakis from Brandeis University.

In addition, nicotine and its metabolites were determined by HPLC with a modification of the method described by Pacifici and colleagues (1993*b*). The HPLC system consisted of a Beckman solvent delivery system model #114 M, a Beckman 160 fixed absorbance detector (Beckman Analytical, Berkeley, CA) set at 254 nm, and a Varian 4290 integrator (Varian, Palo Alto, CA). The column was an LC8DB stainless steel column (5-μm particle size, 25 cm $\times$ 4.6 mm ID; Supelco, Bellefonte, PA). The mobile phase was water:acetonitrile (96.4:3.6 v/v) containing 2 mL/L triethylamine and 0.012 M each sodium heptanesulfonate, dipotassium hydrogen phosphate, and citric acid. The pH of the final solution was adjusted to 4.7 with citric acid. HPLC with diode array detection (Crystal 200 intelligent pump and Crystal 240 diode array detector, Unicam, Cambridge, U.K.) also was used to record the complete ultraviolet spectrum of the analytes in hair.

TABLE 1. *Nine different extraction procedures (50 mg hair)*

Procedure 1	Procedure 2	Procedure 3
10 mL acetone	3 mL NaOH (1*N*)	3 mL NaOH (0.5*N*)
30 min sonicating bath	1 hr at 100 °C	1 hr at 100 °C
	extraction with 5 mL diethyl ether	extraction with 5 mL diethyl ether
evaporation under nitrogen	evaporation under nitrogen of organic layer	evaporation under nitrogen of organic layer
Procedure 4	**Procedure 5**	**Procedure 6**
1 mL NaOH (0.6*N*)	3 mL NaOH (0.5*N*)	3 mL NaOH (0.5*N*)
overnight at 50 °C	1 hr at 100 °C	10 min at room temperature
neutralization with concentrated HCl	Extrelut-3 glass column	Extrelut-3 glass column
	10 mL dichloromethane: isopropyl alcohol (9:1)	10 mL dichloromethane: isopropyl alcohol (9:1)
	evaporation under nitrogen	evaporation under nitrogen
Procedure 7	**Procedure 8**	**Procedure 9**
3 mL NaOH (0.5*N*)	3 mL NaOH (0.5*N*)	3 mL NaOH (0.5*N*)
4 hr at room temperature	24 hr at room temperature	30 min sonicating bath
Extrelut-3 glass column	Extrelut-3 glass column	Extrelut-3 glass column
10 mL dichloromethane: isopropyl alcohol (9:1)	10 mL dichloromethane: isopropyl alcohol (9:1)	10 mL dichloromethane: isopropyl alcohol (9:1)
evaporation under nitrogen	evaporation under nitrogen	evaporation under nitrogen

KEY: HCl=hydrochloric acid

TABLE 2. *High-performance liquid chromatography determination of nicotine and cotinine in 50 mg smokers' hair with different extraction procedures*

Procedure	Nicotine (ng/mg hair)	Cotinine (ng/mg hair)
First hexane wash	3.0	ND
Second hexane wash	ND	ND
Extraction procedure 1	1.2	ND
Extraction procedure 2	IP	IP
Extraction procedure 3	43.0	IP
Extraction procedure 4	36.2	0.78
Extraction procedure 5	IP	ND
Extraction procedure 6	30.2	0.65
Extraction procedure 7	43.1	0.93
Extraction procedure 8	IP	IP
Extraction procedure 9	6.7	ND

KEY: ND=not detected; IP=interfering peak

RESULTS

Table 2 shows the results obtained with the different extraction procedures. Extraction 7 gave the best recoveries of nicotine and cotinine in the absence of interfering substances and caused no degradation of nicotine and cotinine when applied to aqueous standard solutions. Consequently, it was used to examine hair from each of 10 smokers.

The chromatographic separation of nicotine and cotinine in an aqueous standard and in a smoker's hair sample are presented in figure 1.

The assay showed good reproducibility, with intraday and interday variability coefficients of 2.2 and 3.4 percent, respectively. The detection limits (signal-to-noise ratio of 3:1) were 0.2 ng/mg hair for nicotine and 0.1 ng/mg hair for cotinine. Nicotine concentration in the hair of smokers ranged from 5.3 ng/mg to 158 ng/mg; cotinine

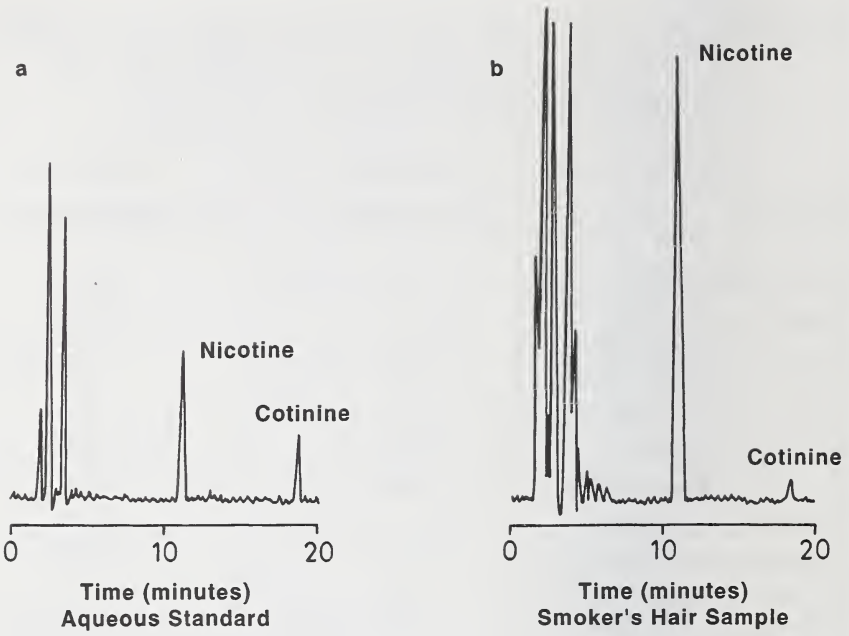

FIGURE 1. *Chromatograms of (a) an aqueous standard containing 500 ng/mL nicotine and 200 ng/mL cotinine and (b) a smoker's hair sample containing nicotine (38.7 ng/mg hair) and cotinine (0.48 ng/mg hair)*

concentration of smokers ranged between 0.48 ng/mg and 1.95 ng/mg and was detectable only for 7 smokers whose nicotine values exceeded 30 ng/mg hair. Hence, low cotinine levels were detectable only with RIA, in which the lower limits of sensitivity were 0.01 ng/mg hair for nicotine and 0.004 ng/mg hair for cotinine, when using 50 mg hair. The cross-reactivity of nicotine in the cotinine assay was 5.0 percent and that of cotinine in the nicotine assay was 2 percent. Figures 2 and 3 illustrate the relationship between HPLC and RIA values of nicotine and cotinine in active smokers' samples.

The correlation coefficients between nicotine and cotinine values determined by the two methods were 0.94 (significant at $p < 0.0001$) and 0.82 (significant at $p < 0.03$), respectively.

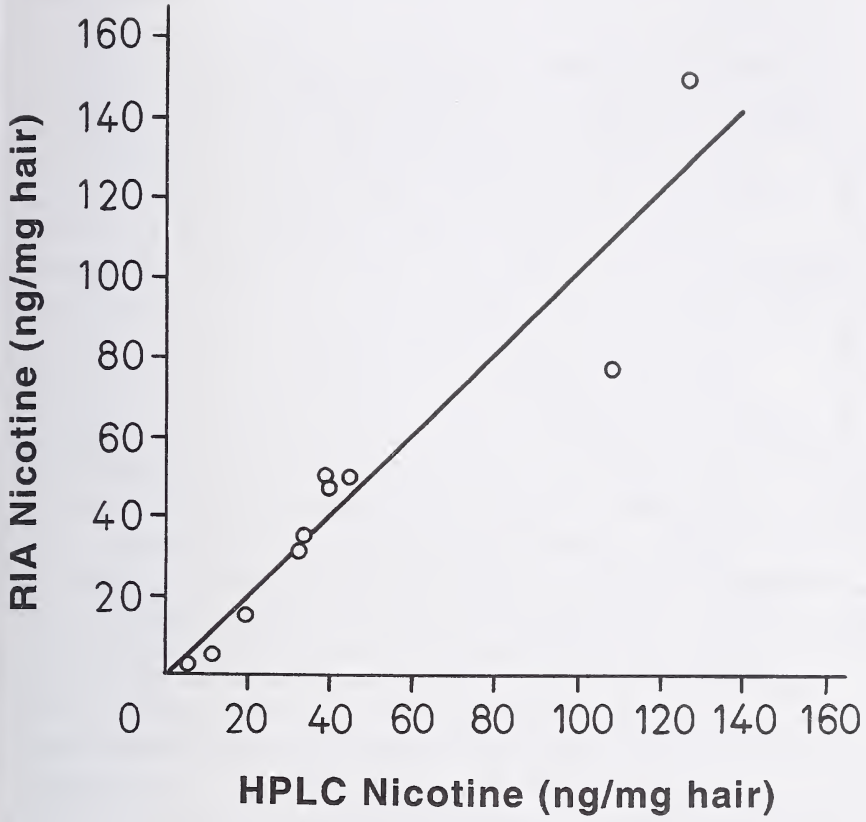

FIGURE 2. *Correlation between nicotine measured by HPLC and RIA in 10 smokers' hair samples ($r=0.94$, $p<0.0001$)*

KEY: RIA = radioimmunoassay; HPLC = high-performance liquid chromatography; r = correlation coefficient

The relationships between daily intake of nicotine and nicotine content in hair measured by HPLC and RIA exhibited correlation coefficients of $r=0.95$ (significant at $p<0.001$) and $r=0.97$ (significant at $p<0.001$), respectively.

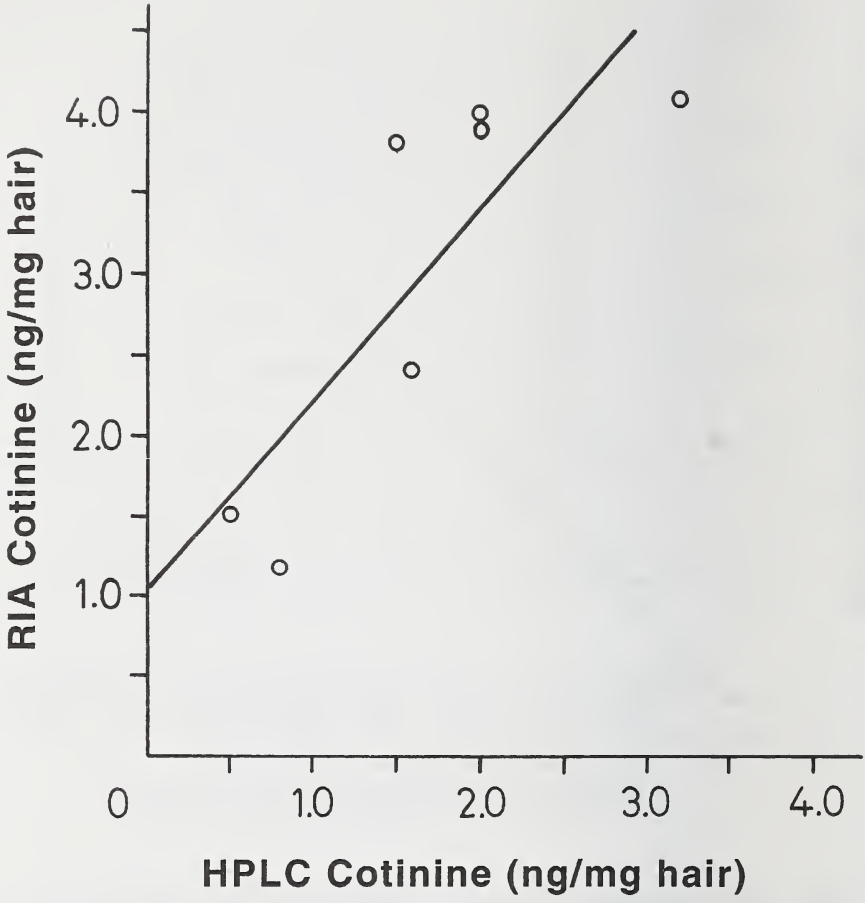

FIGURE 3. *Correlation between cotinine measured by HPLC and RIA in seven smokers' hair samples ($r = 0.82$, $p < 0.03$)*

KEY: RIA = radioimmunoassay; HPLC = high-performance liquid chromatography; r = correlation coefficient

DISCUSSION

The major problem when monitoring hair samples is the quantitative extraction of the drugs from the matrix. It is impossible to know whether all the nicotine and cotinine present in the hair is recovered. Spiked samples, commonly used for blood or urine to test for recovery, are less useful in this case because of the uncertainty of whether the added substances behave in the same manner as endogenous nicotine and cotinine. Complete dissolution of the hair is preferred to ensure that all the bound analytes are released from the hair. Extraction procedures that gave the lowest recoveries of nicotine included (1) extraction with acetone without NaOH treatment (extraction 1), (2) short digestion with 0.5 m NaOH (extraction 6), and (3) extraction without the addition of methanolic HCl addition to prevent the volatilization of nicotine. However, the severe conditions used for the total digestion of the hair (extractions 2, 3, 4, and 5) can destroy the analytes of interest and also extract interfering substances. Furthermore, hair washing prior to analysis was necessary to ensure the removal of contamination from the exterior of hair. The hexane wash was effective in the removal of external nicotine as evidenced from the results of the second wash (table 2). This step was especially important in the case of nicotine, whose concentration inside hair could be overestimated by external contamination. The measurement of hair levels of cotinine, a substance produced by human metabolism, in nonsmokers might be more indicative of absorption of nicotine. Interindividual variabilities (e.g., hair growth rate, growth uniformity, and alterations in scalp and hair) can influence the absolute concentration of these two substances.

In summary, the HPLC assay, coupled with a simple and reliable extraction, was easy to perform in routine analyses of active smokers. Nonetheless, the restrictions reported in the literature—for example, regarding white or dyed hair (Uematsu 1993; Mizuno et al. 1993)—should be taken into account prior to performing nicotine hair assays. When the amount of the sample was limited (e.g., in neonates and in smoking studies to distinguish between passively or externally exposed and nonexposed nonsmokers), low levels of cotinine could be measured only with RIA. Finally, the results demonstrated a high correlation between HPLC and RIA

determinations of nicotine and cotinine in hair samples. Moreover, the correlation between daily nicotine intake and nicotine content in hair appeared to confirm the usefulness of HPLC in assessing individual cigarette smoking behavior over a long period. However, the correlation between the amounts of these analytes in hair and active or passive exposure to nicotine remains uncertain.

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Capillary Electrophoresis: A Novel Tool for Toxicological Investigation: Its Potential in the Analysis of Body Fluids and Hair

Franco Tagliaro, Zdenek Deyl, and Mario Marigo

INTRODUCTION

Capillary electrophoresis (CE) evolved from traditional electrophoretic techniques, which for more than a century have been used, mainly in slab gels, in almost all laboratories that conduct biochemical, biomedical, and pharmaceutical research.

The industrial manufacture of tiny, but highly resistant, fused silica capillaries is a major technical achievement that is basic to the development of modern CE. The inherent anticonvective action, high heat dissipation (allowing the use of high electric fields), and high transparency of fused capillaries (allowing on-column detection) and the peculiar "plug-like" characteristic of the electro-osmotic flow in such capillaries permit the optimization of the main analytical parameters of electrophoresis (Mikkers et al. 1979; Jorgenson and Lukacs 1981*a*, 1981*b*) and the introduction of novel separation (chromatographic) modes in the electrophoretic environment (Terabe et al. 1984, 1985; Terabe 1989).

Overall, CE can be considered a new approach to the separation of ionic, hydrophilic, and lipophilic compounds, including inorganic

and organic ions, drugs, amino acids, biological amines, nucleic acids, and biopolymers such as peptides, proteins, oligonucleotides, and deoxyribonucleic acid (DNA) fragments.

A comprehensive presentation and discussion of the basic principles and modes of operation of CE are beyond the scope of this chapter. Several monographs on this topic have appeared in the past several years (Li 1992; Mosher et al. 1992) as well as symposium proceedings (Jorgenson 1991; Everaerts and Verheggen 1992; Karger 1993*a*, 1993*b*; Goodall 1993; Bocek and Fanali 1993; Thormann 1994; Krstulovic et al. 1994) and several reviews and overviews (Jorgenson and Lukacs 1983; Karger et al. 1989; Lauer and Ooms 1991; Perrett and Ross 1992; Pluym et al. 1992; Kuhr and Monning 1992; Campos and Simpson 1992; Krull and Mazzeo 1992).

The peculiarities of CE are the great versatility of application (ability to separate a broad spectrum of analytes ranging from inorganic ions to large DNA fragments), the extremely low demands on sample volumes, the relatively short running and equilibration times (usually $\leq$30 minutes), the simplicity of sample pretreatments, the minimum consumption of solvents (running buffer) and other consumables (e.g., capillaries), and the ruggedness and low cost of the basic instrumentation.

INSTRUMENTAL SETUP

The basic equipment (see figure 1) consists of an injection system, a capillary (in most instances made of fused silica, in general, 25 to 75 μm ID, 30 to 100 cm long), a high voltage (30 to 50 kV) source, electrodes and electrode jars, and detector. At the present stage of development, CE detection is accomplished mainly by ultraviolet (UV) visible absorption (and/or fluorescence) carried out directly on-column, which takes advantage of the high transparency of the silica wall of the capillary. Some commercial instruments recently have been fitted with diode array or fast-scanning UV detectors, allowing spectral analyses of the separated zones.

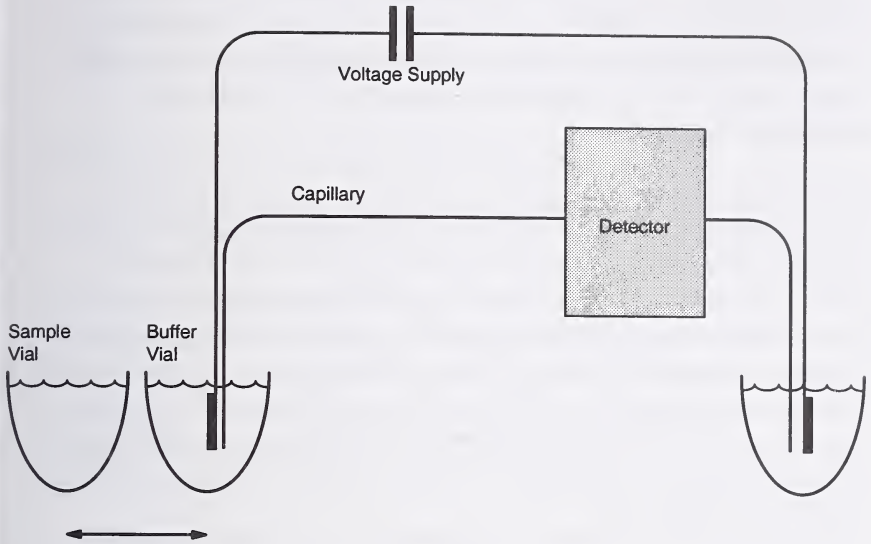

FIGURE 1. *General scheme of capillary electrophoresis instrumentation*

A variety of experimental detection methods currently in use, but not available commercially, include electrochemical (potentiometric, conductivity, and amperometric), laser-based thermooptical, refractive index, radioisotope, and notably, mass spectrometric.

SEPARATION PRINCIPLES AND MODES

CE technology can be applied according to different separation modes based on different physicochemical principles; thus, selectivity changes as a function of the chosen analytical conditions.

CE separations can be performed with plain capillary zone electrophoresis (CZE), micellar electrokinetic capillary chromatography (MEKC), capillary electrochromatography (CEC), capillary isoelectric focusing (CIEF), capillary gel electrophoresis (CGE), and capillary isotachophoresis (CITP).

For the analysis of drugs, CE primarily has been applied using CZE, MEKC, and CITP. For the sake of simplicity, CITP is not addressed in this overview; for a comprehensive presentation, the reader can refer to a fundamental volume by Everaerts and colleagues (1976).

In CZE and MEKC, in addition to the electrophoretic mobility, a factor originating from the negative charge of the silanols of the capillary wall, such as electro-osmosis, plays an important role. Electro-osmosis generates a flow of solvent and solutes, which in most cases is directed from the anodic to the cathodic end of the capillary. Considering that, in "normal" instrument setup, injection is at the anodic end and detection is close to the cathodic end and that electro-osmotic flow is greater than the electrophoretic velocity of most analytes, it is possible to detect cations, neutral species, and anions in the same run.

In CZE, the actual migration velocity of cations and anions results from the algebraic summation of the individual electrophoretic mobilities (in the same direction of electro-osmotic flow or in the opposite, respectively) and electro-osmosis. All neutral substances migrate at the same velocity as the electro-osmotic flow, which means that nonionic compounds are not separated.

To overcome this important limitation, Terabe and colleagues (1984, 1985) and Terabe (1989) introduced MEKC, in which a surfactant—in most cases sodium dodecyl sulfate (SDS)—is added to the buffer at supramicellar concentrations. The surfactant micelles, moving toward the electrode of opposite polarity (usually the anode) and in opposite direction to the electro-osmotic flow, affect the net mobility of other solutes, depending on the interactions they establish with them. Thus, a selectivity based on the partitioning of solutes in the lipophilic core of the micelles is introduced, which operates with nonionic substances in a "quasi-chromatographic" mode. In this mode, the micelles act as a peculiar form of "stationary phase" (also called pseudostationary), and the electro-osmotic flow of buffer (often added with organic solvents) is the corresponding "mobile phase."

MEKC, which somewhat resembles reversed-phase chromatography, has proved suitable for the separation of polar as well as nonpolar compounds, typically drugs, steroids, terpenes, and hydrocarbons.

APPLICATIONS IN FORENSIC TOXICOLOGY

Analysis of Illicit Preparations

CE applications recently have spread over areas typical of chromatography, for example, the analysis of drugs (biologically active compounds) and pharmaceuticals (preparations mixed with excipients, for example, and used for human and animal therapy) (Altria 1993) and compounds of forensic interest (Thormann et al. 1994).

To the best of the authors' knowledge, Weinberger and Lurie (1991) first applied CE, particularly MEKC, to the analysis of illicit substances. Weinberger and Lurie used 50 μm ID bare silica capillaries, 25 to 100 cm in length, and a hydroorganic buffer consisting of 85 mM SDS, 8.5 mM phosphate, 8.5 mM borate, and 15 percent acetonitrile at pH 8.5. The applied voltage was 25 to 30 kV; detection was by UV absorption at 210 nm. Under the described conditions, it was possible to separate acidic and neutral impurities in an illicit seizure sample of heroin with high efficiency. In general, relative standard deviation (RSD) for migration time was about 0.5 percent and for area and peak height, 4 to 8 percent. However, with the late peaks (migration time >40 minutes), analytical precision was less, a phenomenon ascribed to the inconsistent evaporation of the organic modifier from the buffer reservoirs. The same study demonstrated the separation of heroin, heroin impurities, degradation products, and adulterants. The drugs migrated in the following order: morphine, phenobarbital, 6-monoacetylmorphine (MAM), 3-MAM, methaqualone, heroin, acetylcodeine, papaverine, and noscapine. A sample of illicit cocaine also was analyzed, with excellent peak shapes and resolution of benzoylecgonine, cocaine, and *cis*- and *trans*-cinnamoylcocaine. MEKC was found to give greater efficiency, selectivity, peak symmetry, and speed than high-performance liquid chromatography

(HPLC) for the determination of illicit substances and for general drug screening (Weinberger and Lurie 1991).

The separation of enantiomers of amphetamine, methamphetamine, ephedrine, pseudoephedrine, norephedrine, and norpseudoephedrine with application to forensic samples was reported by Lurie (1992).

Trenerry and colleagues (1994*a*, 1994*b*) in two recent papers dedicated to illicit cocaine and illicit heroin, respectively, proposed the use of MEKC, with 50 mM cetyltrimethylammonium bromide as micellar agent, achieving better reproducibility of migration times than with SDS. Krogh and colleagues (1994) applied CE to the analysis of heroin and amphetamine.

Analysis of Biological Fluids

Concerning the toxicological investigation of biological fluids, Wernly and Thormann (1991) used MEKC with a borate-phosphate buffer (pH 9.1) containing 75 mM SDS to demonstrate the feasibility of analyzing many drugs of abuse and metabolites in urine, including benzoylecgonine, morphine, heroin, MAM, methamphetamine, codeine, amphetamine, cocaine, methadone, methaqualone, and benzodiazepines. Sample concentration, by means of solid-phase extraction of 5 mL urine followed by dissolution of the dried extract in 100 μL running buffer, allowed the determination of 100 ng/mL of drugs. Peak identification was obtained not only on the basis of retention times but also by obtaining absorbance spectra of the peaks and comparing them with computer-stored models. MEKC, with a sensitivity comparable to the common nonisotopic immunoassays, was proposed by the authors for confirmation testing following toxicological screening of urines by usual enzyme immunoassays (Wernly and Thormann 1991).

Also, MEKC with 50 mM SDS in borate-phosphate buffer (pH 7.8) allowed fast and high-resolution separations of seven barbiturates (barbital, allobarbital, phenobarbital, butalbital, thiopental, amobarbital, and pentobarbital), and the use of on-column multiwavelength detection allowed peak identification on the basis of their absorption spectra between 195 and 320 nm (Thormann et al.

1991). With urine, extraction of barbiturates prior to analysis was necessary, whereas with human serum, several barbiturates (including phenobarbital), eluted in an interference-free window of the electropherogram, allowed these substances to be determined by direct sample injection.

Wernly and Thormann (1992) reported the determination of 11-nor-Δ-9-tetrahydrocannabinol-9-carboxylic acid, the major metabolite of Δ-9-tetrahydrocannabinol, in urine. Wernly and colleagues (1993) recently reported the possibility of determining morphine-3-glucuronide in human urine with MEKC, omitting any hydrolysis. However, the sensitivity limit achieved—about 1 μg/mL after solid-phase extraction and concentration—was still unsatisfactory for confirmation of the results of the usual enzyme immunoassays.

A recent overview describes the strategies for monitoring drugs in body fluids (serum, urine, and saliva) by MEKC and focuses on buffer selection and sample preparation procedures, including direct injection, ultrafiltration, and solid-phase extraction (Thormann et al. 1993).

A CZE approach to drug screening was proposed by Chee and Wan (1993), who used 50 mM phosphate buffer (pH 2.35) and a 75-μm ID, 60 cm long, bare silica capillary to obtain the separation of 17 basic drugs of potential forensic interest, including amphetamine, methamphetamine, medazepam, lidocaine, diazepam, and methaqualone. Under these conditions, drugs with a lower pK_a, and consequently a less positive charge, showed higher migration times, but the influence of other factors (i.e., molecular size, tendency to interact with the column, and ability to form doubly charged species) prevented the observation of a clear correlation between pK_a and migration time. The migration time reproducibility was good, with RSD values in general of less than 1 percent; peak-area RSDs were between 1.5 and 4.3 percent. The described method also proved suitable for the analysis of plasma and urine, after simple chloroform-isopropanol (9:1) extraction from samples adjusted to pH 10.5. Clean electropherograms were obtained from blank plasma and urine, allowing the detection of amounts as low as 0.45 μg/mL of individual drugs.

The comparative use of MEKC, CZE, and CITP to determine the presence of drugs in body fluids was reported by Caslavska and coworkers (1993), who concluded that MEKC and CZE were easily applied, whereas CITP required careful selection of buffers and proved generally less sensitive.

Analysis of Hair

Based on this limited but recent and sound body of information and considering the potential advantages of CE in terms of the minimum amount of sample needed, high mass sensitivity, the wide spectrum of drugs susceptible to determination, high efficiency of separations, and costs of operation, the authors' research group first applied this technique to the investigation of illicit drugs in human hair as markers of chronic drug abuse.

Tagliaro and Marigo's first experiences focused on cocaine. They adopted CZE separations because this mode of operation was easier to use and replicate in different laboratories (Tagliaro et al. 1993*a*). The authors used a basic background buffer (i.e., 50 mM borate, pH 9.2) and a capillary (40 cm long, 50-μm ID) made of bare fused silica; the applied potential was 15 kV. Detection was by UV absorption at the wavelength of 238 nm instead of the generally adopted 195 to 200 nm, thus sacrificing sensitivity to increase selectivity.

The sample pretreatment was limited to washing hair (100 mg) with 30 mL 0.3 percent neutral surfactant and rinsing with water. The samples then were extracted by overnight incubation in 0.25 M hydrochloric acid, followed by liquid-liquid extraction. The organic phase was evaporated and the residue dissolved in 20 μL water. Before injection, tetracaine at the concentration of 25 μg/mL was added as the internal standard. Under these conditions, cocaine, behaving as a cation, gave a sharp and symmetric peak that migrated before tetracaine; separation efficiency was remarkable—about 350,000 theoretical plates.

After liquid-liquid extraction, the biological samples were still complex according to HPLC with UV detection (data not shown); however, the electropherograms from hair extracts were extremely

clean (figure 2). In practice, the electropherograms of blank samples showed only a single peak that could be ascribed to the matrix components comigrating with the neutral marker (i.e., at the same velocity as the electro-osmotic flow).

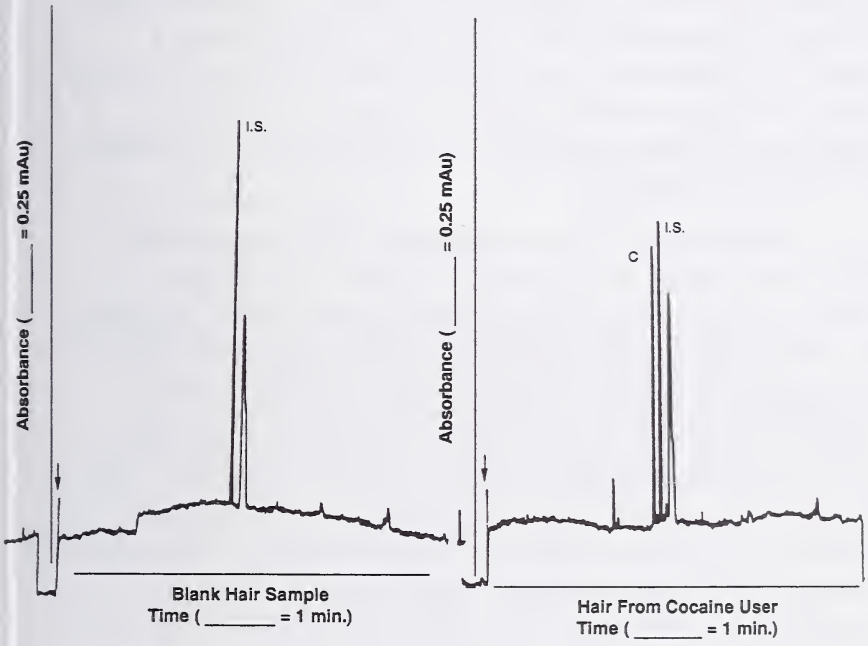

FIGURE 2. *Typical electropherograms of (left) a blank hair sample, with the addition of tetracaine (internal standard) (25 μg/mL), and (right) hair from a cocaine user, containing cocaine (C) at the level of 3.7 ng/mg. Arrows indicate injections. For conditions, see text.*

KEY: mAu=milliabsorbance unit; IS=internal standard

SOURCE: Tagliaro et al. 1993*a*. Copyright 1993 by Elsevier Science Ireland Ltd. (Limerick).

The linearity of cocaine determinations (in solutions) was satisfactory in the range of 0.78 to 100 μg/mL; accuracy (average recovery of 93 percent) and precision (intraday and interdays RSDs $\leq$7 percent) were comparable with those of chromatographic methods applied to the hair matrix.

The same CZE method also was applied with an automated instrument fitted with a photodiode array detector, recording online UV spectra of peaks from 200 to 400 nm. Notwithstanding a certain loss of sensitivity typical of diode array detectors, in cases of highly positive hair samples (e.g., 7.1 ng/mg), UV spectra of cocaine and tetracaine could be recorded, with obvious advantages for identification purposes.

A more extensive and formal investigation of the performances of CZE in hair analysis was reported by Tagliaro and colleagues (1993*b*). Using the analytical conditions reported above, but tuning the detection wavelength according to the absorbance maximum of each analyte (for cocaine 238 nm, for morphine 214 nm, and for both 200 nm), the authors achieved the detection of heroin and cocaine in human hair (figures 3 and 4, respectively). Nalorphine and tetracaine were chosen as internal standards, respectively. The separations were efficient (up to 350,000 theoretical plates) and repeatable (migration time RSDs $\leq$1 percent intraday, $\leq$3 percent interdays), and the determinations were accurate and precise (intraday RSD in the range of 3 to 5 percent). The sensitivity was $<$0.2 ng/mg for both analytes. Interferences from more than 90 therapeutic drugs and drugs of abuse were excluded.

However, two drawbacks of CZE appeared in these first tests. First, notwithstanding an excellent mass sensitivity, because of the limits in the injected volumes (few nanoliters), the sensitivity of the technique does not meet the requirements for hair analysis in terms of concentration. Second, CZE is inherently applicable only to ionized compounds, and the selectivity is based mostly on the charge differences. Thus, nonionic substances are not susceptible to analysis, and migration velocities of congeners having the same charged moieties could be the same.

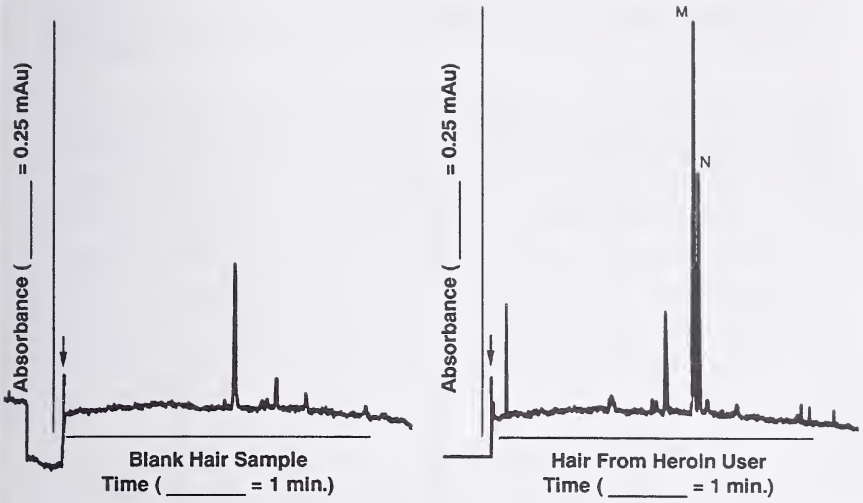

FIGURE 3. *Typical electropherograms of (left) a blank hair sample, and (right) hair from a heroin user, containing morphine (M) at the level of 3.0 ng/mg. Nalorphine (N) was the internal standard. Arrows indicate injections. For conditions, see text.*

KEY: mAu=milliabsorbance unit

SOURCE: Tagliaro et al. 1993*b*. Copyright 1993 by Elsevier Science (Amsterdam).

In further experiments, described below, the authors tested the different selectivity of MEKC in hair analysis.

EXPERIMENT

Capillary Electrophoresis Instrumentation and Methods

A manual capillary electropherograph equipped with an on-column UV detector and a splitted flow injector was used. Bare silica capillaries (60 cm total length, 40 cm to the detector, 50-μm ID)

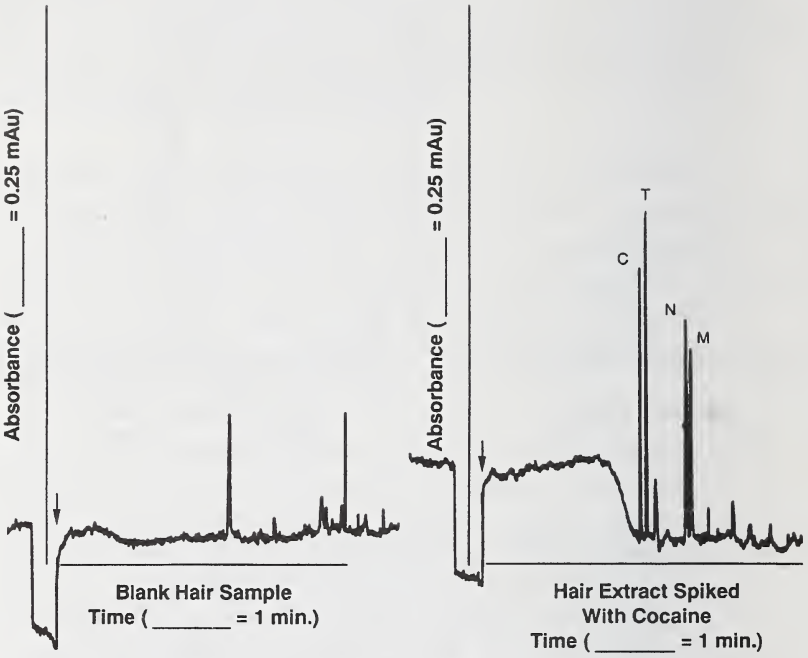

FIGURE 4. *Electropherograms of (left) a blank hair sample, and (right) hair extract spiked with cocaine (C), tetracaine (T), morphine (M), and nalorphine (N) to simulate a concentration of 4 ng/mg of each analyte in hair. The arrows indicate injections. For conditions, see text.*

KEY: mAu=milliabsorbance unit

SOURCE: Tagliaro et al. 1993*b*. Copyright 1993 by Elsevier Science (Amsterdam).

were adopted. Injections of hair extracts or standards (5 μL), reconstituted with the running buffer diluted half with water, were made manually with a syringe through the splitter, with a split ratio of about 1:800. Sample stacking experiments were carried out by

injecting as much as 25 μL of samples that had been reconstituted with plain distilled water.

The first MEKC separations were carried out using 0.050 M borate buffer as background electrolyte (pH 9.2) containing 0.050 M SDS. Further experiments were accomplished in a 0.050 M borate buffer (pH 9.2) containing 20 percent methanol and 0.050 M SDS. The adopted potentials were 15 to 20 kV to keep the current below 100 μA. Detection was by UV absorbance at the wavelength of 200 nm with full-scale range of 16 milliabsorbance units (mAu). After each injection, the capillary surface was flushed with the working buffer for 5 minutes.

All drug standards were from commercial sources. Sample pretreatment was as described in the authors' previous studies summarized above.

RESULTS AND DISCUSSION

Figure 5 shows an excellent MEKC separation of morphine, nalorphine, cocaine, and tetracaine, with a peculiar separation pattern clearly different from CZE. Under MEKC conditions, benzoylecgonine was also detectable, whereas with CZE, because of its high negative charge in basic media, this cocaine metabolite migrated very slowly, out of the time-window of reasonable detectability. Figure 5 also shows that the injection of real samples produced relatively clean electropherograms, allowing, in principle, the determination of all five analytes. However, Wernly and Thormann (1992) also observed that MEKC in plain aqueous buffer failed to resolve the most hydrophobic analytes (e.g., amphetamines and benzodiazepines).

The resolution of many drugs with a wide spectrum of hydrophobicity was achieved by adding low percentages of organic modifiers (i.e., 20 percent methanol) to the MEKC buffer, thus approaching the conditions proposed by Weinberger and Lurie (1991). A typical example is shown in figure 6, where as many as 20 therapeutic and illicit drugs, congeners, and naturally occurring impurities of heroin were separated in the same run. The

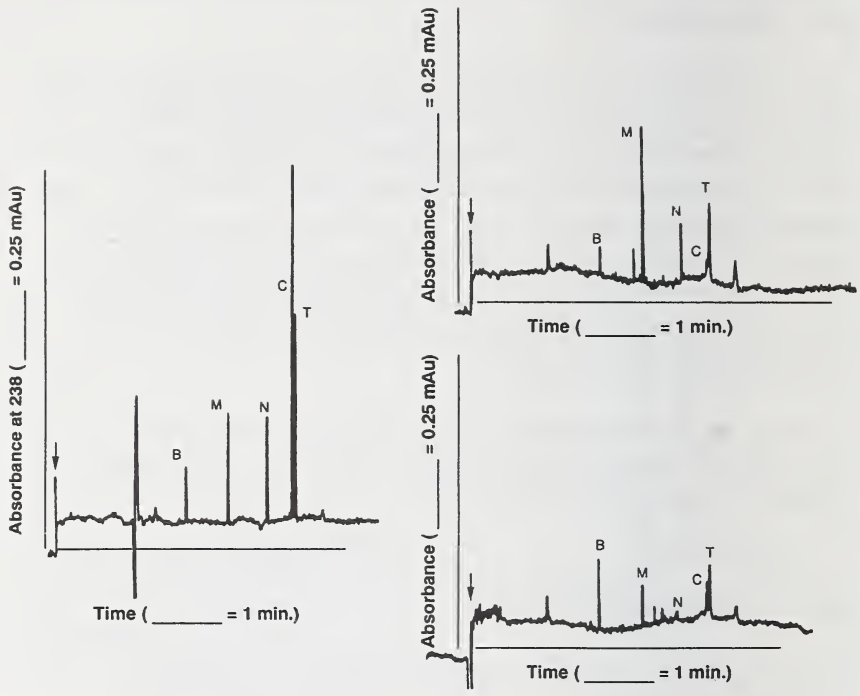

FIGURE 5. *Left: Micellar electrokinetic capillary chromatography (MEKC) separation of a standard mixture of benzoylecgonine (B), morphine (M), nalorphine (N), cocaine (C), and tetracaine (T); right: MEKC of blank hair extracts spiked with B, M, N, C, and T detected at 214 and 238 nm (top and bottom, respectively). In the spiking tests, the aim was merely qualitative; however, drugs were added to simulate concentrations in hair of about 1 to 2 μg/mg or less. Arrows indicate injections. Buffer: 50 mM borate (pH 9.2) containing 50 mM SDS; for other conditions, see text.*

KEY: nm=nanometer; mAu=milliabsorbance unit

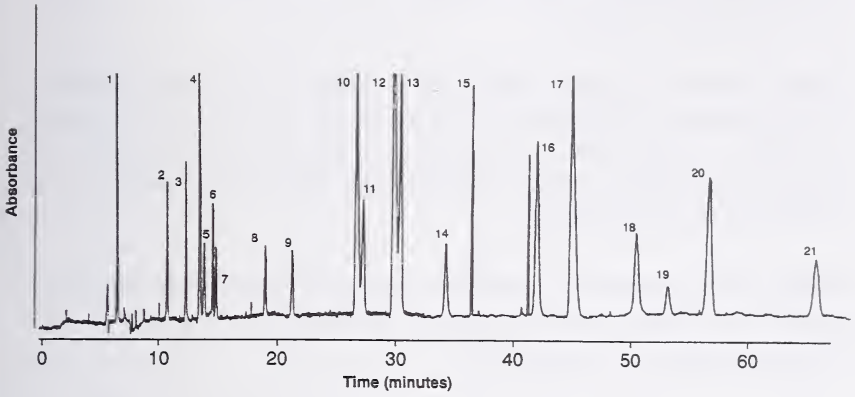

FIGURE 6. *Micellar electrokinetic capillary chromatography separation of a mixture of 20 drugs, congeners, and naturally occurring impurities (concentration range 10 to 40 μg/mL for each compound). Conditions were the same as in figure 5, with the addition of 20 percent methanol to the buffer. Separated compounds were caffeine (1), morphine (2), pentobarbital (3), barbital (4), narceine (5), monoacetylmorphine (quality/purity not determinable) (6), impurity in acetylcodeine (7), nalorphine (8), codeine (9), procaine (10), lidocaine (11), heroin (12), flunitrazepam (13), acetylcodeine (14), electric spike (15), hebaine (16), amphetamine (17), papaverine (18), cocaine (19), narcotine (20), and diazepam (21).*

reproducibility of absolute migration times was characterized by RSDs ranging from 2.0 to 8.4 percent; this relevant variability resulted mainly from variations in the electro-osmotic flow caused by nonreproducible conditioning of the capillary wall, which in the authors' instrument was made manually by flushing with buffer before each injection. After normalization on the basis of the flunitrazepam migration time, the reproducibility was improved, with RSDs of between 0.3 and 3.2 percent (n=5).

A remarkably clean electropherogram was obtained from negative hair, as shown in figure 7.

Figure 8 shows a typical result from a negative hair sample spiked with morphine, flunitrazepam, amphetamine, cocaine, and diazepam to mimic a concentration of 1 ng of each analyte per mg of hair (to check the sensitivity of the technique, the addition of standards was made after the extraction).

MEKC gave a pattern of separation completely different from CZE, which is consistent with the different separation mechanisms occurring in the two techniques.

MEKC, particularly with the addition of organic modifiers, proved able to resolve a wider spectrum of drugs, but the efficiency was lower than with CZE, with problems of peak dilution and consequently reduced sensitivity. However, using a technique of sample stacking (Chien and Burgi 1991), it was possible to inject much larger volumes of sample than in previous experiments (i.e., about 30 nL versus 6 nL) by injecting the sample in low conductivity solvent (distilled water) without observing overloading effects. This also allowed the reconstitution of the extracts with 100 μL solvent instead of 20 μL, with obvious advantages in the precision and practicability of the method.

Thus, notwithstanding a sensitivity still lower than with other more consolidated techniques, MEKC proved able to detect levels of drugs currently determined in hair (i.e., in the order of ng/mg), as demonstrated in figure 8.

The relatively few real samples until now analyzed were in good agreement with the MEKC pattern of the spiked hair. However, because no more than one drug was observed per sample, for the sake of presentation the more informative spiked hair was chosen.

CONCLUSION

CE represents a powerful new analytical tool in the hands of forensic toxicologists. It has proven suitable for investigating seized

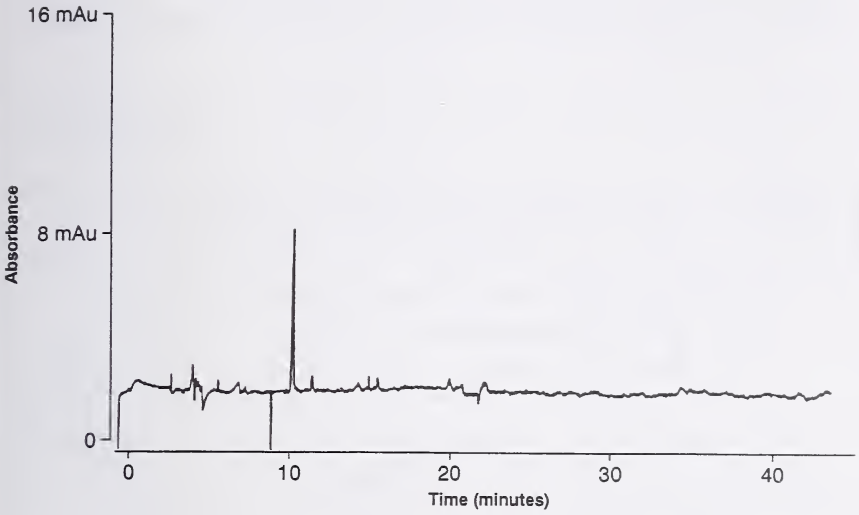

FIGURE 7. *Micellar electrokinetic capillary chromatography under sample stacking conditions from a 100-mg sample of blank hair, extracted and reconstituted with 100 μL water; volume injected: 25 μL (with 1:800 split ratio); other conditions as in figure 5.*

KEY: mAu=milliabsorbance unit

preparations of illicit drugs and biological samples, including hair. In its different modes, CE has several peculiar features, such as separation mechanisms, negligible consumption of sample, and multiple detection possibilities, including UV absorbance spectra acquisition and, still experimentally, mass spectrometry (Johansson et al. 1991), making it unique in modern analytical technology. Therefore, the use of CE in the forensic environment, as a method complementary to chromatography, seems extremely promising either "in parallel" or "in series" with more consolidated analytical techniques.

For use in the toxicological investigation of hair for drugs of abuse, CE is proving immensely effective, providing rapid, simultaneous determinations of different drugs without derivatization. The

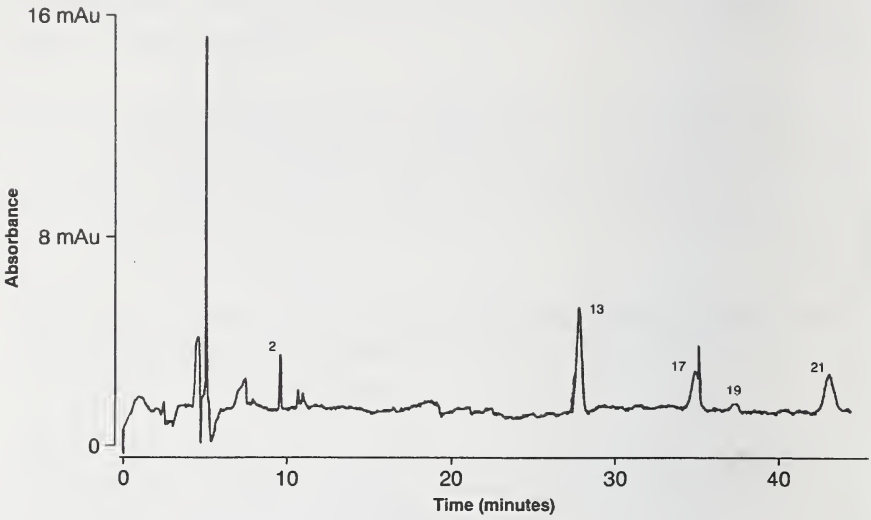

FIGURE 8. *Micellar electrokinetic capillary chromatography electropherogram from a 100 mg sample of blank hair extracted, reconstituted, and injected as in figure 7, after addition of morphine (2), flunitrazepam (13), amphetamine (17), cocaine (19), and diazepam (21) to mimic concentrations of 1 ng of each drug per mg of hair.*

KEY: mAu=milliabsorbance unit

continuing problems of sensitivity and selectivity toward matrix coextractives could be overcome by careful selection of sample purification and concentration procedures and with the adoption of more sophisticated detection techniques (e.g., fluorometric, electrochemical, mass spectrometric).

The possibility of carrying out separations based on different mechanisms (CZE, MEKC, CITP) with the same instrumentation provides an interesting additional possibility of "internal" confirmation of the results. Thus, the use of CE for toxicological

screening and confirmation of results is easily foreseeable because of the availability of reliable automated instrumentation.

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Casework Findings and a Practical Discussion of Rinsing Procedures in Forensic Hair Drug Testing

Carl M. Selavka and C. David Riker

INTRODUCTION

The convening of an international workshop on hair drug testing by the National Institute of Standards and Technology (NIST) was an occasion to explore the state of the science in this burgeoning field by those active in related research and casework. There is no doubt that hair drug testing holds significant potential (Inoue and Seta 1992; Mieczkowski et al. 1993; Welch et al. 1993).

For more than 20 years, the authors' laboratory has tested hair for metals, metalloids, drugs of abuse, and therapeutic agents for use in litigation, postmortem investigations, and clinical applications. However, only since late 1990 have historical data been routinely collected. Technically, testing hair for drugs is no more difficult or challenging than testing in many other alternative matrices (e.g., sweat, saliva, vitreous humor, seminal fluid, liver, bone, fingernails, fat, or skin). The application of analytical methods and instrumental approaches like immunoassays, chromatographic separations, and spectroscopic identifications is in most cases similar, regardless of the initial matrix.

The scientific community has demonstrated significant concern over the proper role that forensic hair drug testing should serve in

toxicological applications. In 1989, the Society of Forensic Toxicologists (SOFT) and the National Institute on Drug Abuse, in collaboration with the National Institute of Justice, convened a conference on hair analysis for drugs of abuse. As a result of this conference, a Consensus Opinion was published by SOFT (Bost 1990) that contained, among other points, the following opinions:

1. The use of hair analysis for employee and preemployment drug testing is premature and cannot be supported by the current information on hair analysis for drugs of abuse. Too many critical questions remain to be answered before the results can be accurately interpreted.

2. Hair may be a useful specimen in forensic investigations when supported by other evidence of drug use (e.g., analysis of blood, urine or other tissues) and when performed under generally accepted guidelines for forensic drug testing.

Because this opinion had a stifling effect on the application of hair drug testing in the workplace (gaming industry and other nonmandated or nonregulated testing environments), SOFT was asked to reevaluate the opinion. The recommended Revised Consensus Opinion on Applicability of Hair Analysis for Drugs of Abuse (Hearn 1992) contained a virtually identical opinion on workplace testing but softened the language in item 2: "Hair *is* a useful specimen in forensic investigations *when the data are supported by other competent evidence of drug use* and when assays are performed under generally accepted guidelines for forensic drug testing."

One interesting difference in the language of these two opinions on forensic testing was that "competent evidence of drug use" was now required (Hearn 1992), as opposed to more specific supporting information ("analysis of blood, urine or other tissues") as written in the 1989 version (Bost 1990). This suggests that reasonable suspicion or probable cause that an individual used a drug (the conventional standard of proof for obtaining arrest or search warrants in criminal investigations) could be used as the "other

competent evidence of drug use" when considering the application of hair drug testing in a forensic case.

The authors' involvement in forensic hair drug testing has primarily involved cases in which probable cause or reasonable suspicion exists that an individual has chronically or repetitively used or been exposed to drugs. Common testing forums involve requests from (1) attorneys and judges in child custody disputes and divorce proceedings; (2) administrators, investigators, and other officials in parole and probation revocation hearings; (3) physicians and nurses involved in the evaluation of neonates born to apparently drug-dependent mothers; (4) investigators and tribunals involved with violent crimes and violent criminals; (5) administrators and other officials involved in the review of workplace-related accidents or injuries ("for cause" testing); and (6) coroners, medical examiners, and forensic toxicologists involved in the investigation of unexplained deaths. There is also some call to perform hair drug testing on individuals who have applied for large insurance policies.

The following sections describe the tests performed and their results from May 1991 through November 1993, the distribution of findings among different tested populations, and the apparent ability of those ordering or performing specimen submissions to predict the drug-using behavior of tested subjects. Finally, a discussion of some practical aspects of rinsing protocols was developed to assist with the interpretation of cases and judgment of possible contributions of external contamination to case findings.

HISTORICAL CASEWORK SUMMARY

Testing has been performed for a wide range of individual therapeutic and controlled drugs in hair, with special emphasis on developing assays for drugs for which hair testing approaches have not been previously described (Selavka et al., in press). Table 1 lists commonly performed tests and summarizes the methods used for hair drug testing cases.

TABLE 1. *Description of commonly performed hair drug tests*

Test	Drug/Class	Analytes Included in Test
GC/MS*	Amphetamines	Amphetamine, methamphetamine, 3,4-methylenedioxyamphetamine, 3,4-methylenedioxyethylamphetamine, 3,4-methylenedioxymethamphetamine
GC/MS	Barbiturates	Amobarbital, butabarbital, butalbital, metharbital, barbital, pentobarbital, secobarbital, phenobarbital
GC/MS	Benzodiazepines	Alprazolam, chlordiazepoxide, desalkylflurazepam, diazepam, flurazepam, hydroxy-alprazolam, hydroxy-triazolam, lorazepam, midazolam, nordiazepam, oxazepam, temazepam, triazolam
GC/MS	Cocaine	Benzoylecgonine, cocaethylene, cocaine, ecgonine methyl ester
GC/MS	Opiates	Codeine, dihydrocodeine, 6-monoacetylmorphine, morphine, hydrocodone, hydromorphone, oxycodone, oxymorphone
GC/MS	PCP	Phencyclidine (PCP)
GC/NPD†	Fentanyls	Alfentanil, carfentanil, fentanyl, sufentanil
RIA‡	Cocaine	Cocaine/metabolites
EIA or RIA‡	Hair Screen I or III	Amphetamines, cocaine/metabolites, opiates, PCP
EIA or RIA‡	Hair Screen II or IV	Amphetamines, barbiturates, benzodiazepines, cocaine/metabolites, opiates, PCP
GC/NPD, EIA, and RIA§	Hair Screen V	GC screen for basic/neutral extractable therapeutic and controlled drugs, plus immunoassays for amphetamines, barbiturates, benzodiazepines, cocaine/metabolites, methadone, methaqualone, opiates, PCP, propoxyphene

*For the tests noted as "GC/MS," gas chromatography/mass spectrometry (GC/MS) is the first method performed on an extract of the submitted hair sample. If one or more of the analytes targeted in the analysis are detected by GC/MS, another aliquot of the submitted hair sample is analyzed using a complementary method (radioimmunoassay [RIA] for cocaine/metabolites; enzyme immunoassay [EIA] for other drug classes listed). Only if both methods are positive is a positive result reported, and quantitative findings from the GC/MS analysis are reported for each detected analyte.

†Gas chromatography/nitrogen-phosphorus detection (GC/NPD) analysis of an extract of the submitted hair is performed to screen for fentanyl (with or without its analogs). If this test detects one or more of these analytes, another aliquot of the submitted hair sample is extracted and analyzed using GC/MS. Only if both methods are positive is a positive result reported, and quantitative findings from the GC/NPD analysis are reported for each detected analyte.

‡EIA or RIA of an extract of the submitted hair is performed to screen for the noted analytes. If this test is positive, another aliquot of the submitted hair sample is extracted and analyzed using GC/MS. Only if both the preliminary and confirmation methods are positive is a positive result reported, and quantitative findings from the GC/MS analysis are reported for each detected analyte.

§GC/NPD, EIA, and RIA of an extract of the submitted hair are performed to screen for the noted analytes. If this test is positive, another aliquot of the submitted hair sample is extracted and analyzed using GC/MS. Only if both the preliminary and confirmation methods are positive is a positive result reported, and quantitative findings from the GC/NPD analysis (for basic or neutral therapeutic drugs) or GC/MS analysis (all other analytes) are reported for each detected analyte.

The authors' approach to testing involved first the use of either a presumptive test—radioimmunoassay (RIA), enzyme immunoassay (EIA), or gas chromatography/nitrogen-phosphorus detection (GC/NPD)—for a group of drugs or gas chromatography/mass spectrometry (GC/MS) for a group of analytes within the drug class (table 1). If the first test detected one or more analytes in the hair extract, another portion (aliquot) of the hair sample was prepared and tested using the appropriate alternative technique. Regardless of the order in which the tests were performed, the GC/MS result was referred to as the "identifying" *confirmation* and the RIA or EIA as the *verification*. In the case of a positive finding, the quantitative results were taken either from the GC/NPD analysis (for basic or neutral extractable drugs) or from the GC/MS analysis (for all other compounds).

The analytical preparation steps involved in the testing have been previously published (Fritch et al. 1992), and the rinsing protocols and interpretive guidelines used are discussed in significant detail later in this chapter. Unless otherwise requested, testing was performed using 50 mg hair, without segmentation. The rinsing procedure is described below. Drugs were liberated from the hair matrix by overnight incubation with 0.1 *N* hydrochloric acid. The data presented here include results on hair collected from the head or (in a few cases) the pubic area of subjects. No specific data for the race of subjects were made available to the laboratory, and the age of subjects was provided in only about half the cases.

During the 2 1/2 years of this research, more than 1,000 hair specimens were analyzed. As shown in figure 1, most of the testing was performed for cocaine. More than half the submitted specimens were tested for cocaine first by GC/MS. Nearly 100 other specimens were tested for cocaine first by RIA. The largest number of screen ("panel") tests performed targeted amphetamines, barbiturates, benzodiazepines, cocaine, opiates, and phencyclidine (PCP). Figure 1 also demonstrates that the more focused the testing, the more likely the specimen resulted in a positive finding. The targeted analyses for cocaine (RIA and GC/MS) demonstrated a positive rate of nearly 50 percent, and similarly high rates were evidenced for amphetamines and fentanyl and its analogs.

The overall positive rate (any drug or class) was 34 percent for hair specimens tested from May 1991 through November 1993. This rate is higher than the 6.2-percent positive rate reported in a preemployment workforce (Baumgartner and Hill 1990) and suggests that the probable cause or reasonable suspicion used to justify the collection of hair from subjects was generally reliable. Although it could be argued that "reasonable" suspicion should give rise to even higher positive rates, it is important to remember that several factors related to hair testing may not be subject to adequate control:

- There may be an indication of impairment or other drug-related behavior that allows an observer to form a reasonable suspicion, but if the hair test requested does not target the drug being abused by the donor, the result will be negative.

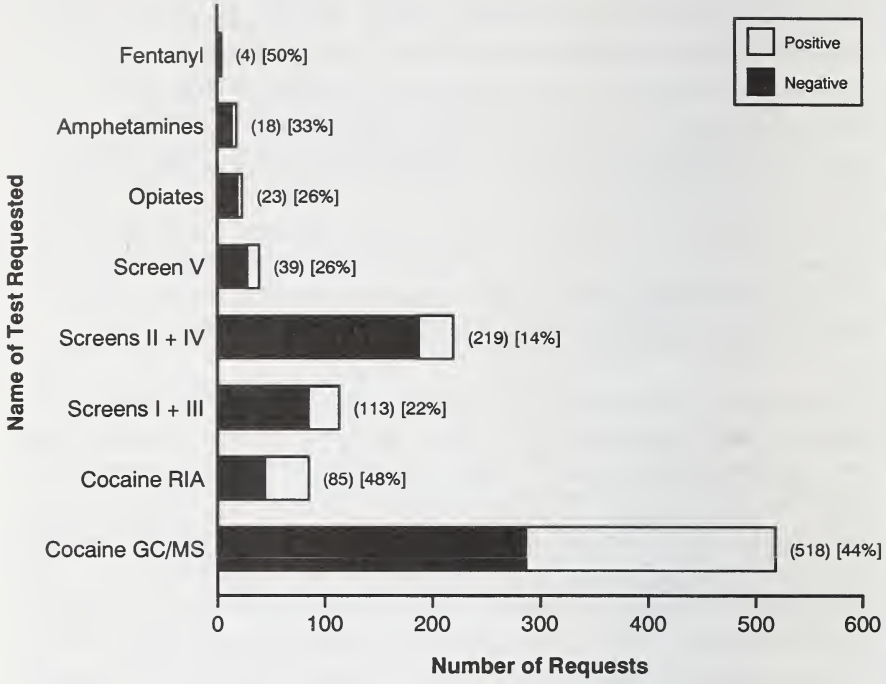

FIGURE 1. *Distribution of testing requests for specimens tested from May 1991 through November 1993—1,024 hair specimens were tested (5 are not shown). "Fentanyl," "Amphetamines," "Opiates," and "Cocaine GC/MS" refer to specimens for which GC/MS was requested for this drug or class of drugs. See table 1 for specific analytes included in each of these tests. Numbers of cases are shown in parentheses; positive rates are noted in brackets.*

KEY: RIA = radioimmunoassay; GC/MS = gas chromatography/ mass spectrometry

- If the number of uses by the donor is not sufficient to deposit detectable quantities of drug or metabolite in the hair, the result will be negative.

- If the period of suspected use is not known or relayed to the laboratory, it is possible that the time period represented by the hair length does not contain the period of suspected use.

- The stability of drugs in hair can be expected to be somewhat variable: It has been demonstrated that the apparent level of drug in a portion of hair may decrease with the increasing age of the hair as a function of the time since drug deposition or as a result of repetitive hair treatments (Henderson et al. 1992; Nakahara et al. 1992).

The reason for hair collection or the specific issues under contention in a case are not always provided. However, when this information is known, the data offer some insight into the "reasonableness" of the "reasonable suspicion" surrounding the collection of hair for drug testing. For example, the high positive rate for neonates suggests that the clinical signs of infant drug influence or dependency, and the objective measurement techniques used by neonatal intensive care nurses and physicians to judge these signs, are being used effectively in the evaluation of newborns. Similarly, the relatively high positive rates for hair specimens collected pursuant to child custody proceedings or in criminal or civil investigations suggest that the investigative process is providing relatively reliable information about the effect that drug use may be having on the individuals under scrutiny (e.g., parents, guardians, suspects). Data that demonstrate higher positive rates in individuals tested in workplace-related cases than previously reported in preemployment cases (Baumgartner and Hill 1990) support the working hypothesis that most cases represented in the data are related to "for cause" investigations of workplace injuries or disciplinary matters.

Two interesting results of the analysis in figure 2 involve the positive rates in divorce and insurance cases. The low positive rates in divorce cases may mean that the accusations are often inaccurate because they clearly are not commonly substantiated. Such accusations may be part of a litigation strategy: An accuser may make allegations of someone's drug use with knowledge that

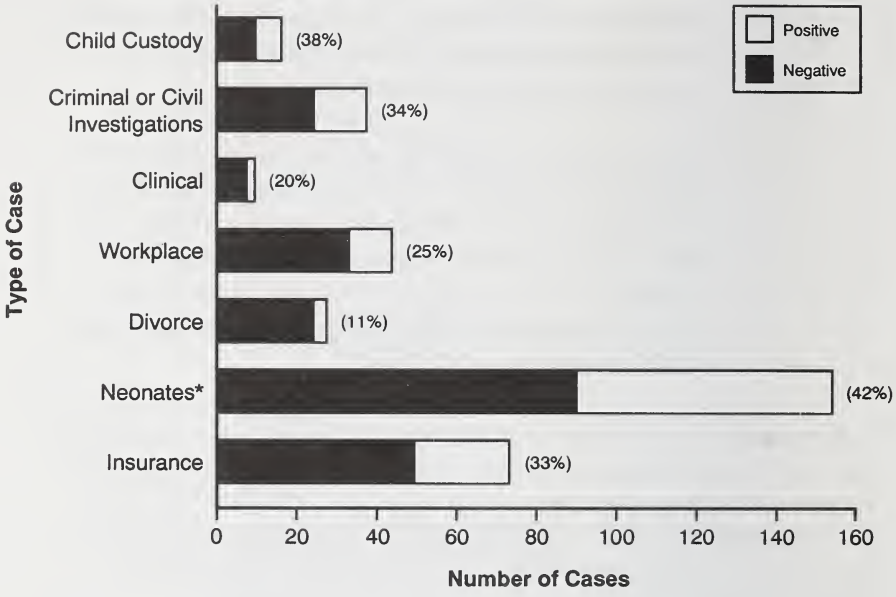

FIGURE 2. *Distribution and comparison of findings for hair drug tests performed from May 1991 through November 1993. Data are provided only for those specimens for which the type of case was communicated to the laboratory. Positive rates for each type of case are noted in parentheses.*

*This category includes hair samples from newborns or their mothers.

negative urine or blood tests cannot completely remove the stigma of reputed drug use because of the relatively short window of detection of drug use in these two matrices. It is also possible that the testing does not target the drug or class used by the accused or that the accused does not use drugs often enough to lead to detectable levels in hair. On the other hand, hair submitted for testing pursuant to an insurance investigation is often triggered by a previous positive urinalysis result or by lifestyle or behavior issues communicated to insurers by investigator(s).

In addition to detecting cocaine, morphine, codeine, and methamphetamine relatively routinely in positive hair test findings, hair testing has identified many unusual drugs. In most cases, these drugs or drug classes were the targets of the analyses, although some findings also began with a presumptive identification in the basic or neutral extract with GC/NPD analysis. Less common positive findings include the following drugs or metabolites:

6-monoacetylmorphine
fentanyl
propoxyphene
fluoxetine
diphenhydramine
hydrocodone
oxycodone
phentermine
meperidine
butalbital
dihydrocodeine

Although these substances were not routinely detected, it should be noted that the most commonly requested testing regimens (targeted cocaine analysis, followed by screens for six common controlled drugs or classes) would not detect most of these more unusual analytes. Therefore, the positive rates for these drugs among the historical data cannot be used to estimate the prevalence of use or abuse within the tested population.

The large number of cocaine-positive cases allows for review of the empirical profile of cocaine and its analytes in hair. The findings for 319 cocaine-positive hair specimens are presented in figure 3. One of the more striking findings of this analysis is that, in nearly half the positive hair samples, cocaine was detected in the absence of any of its major metabolites (at or above a reporting limit of 0.20 μg/g hair). This finding is consistent with much more limited studies by Harkey and colleagues (1991) on patients with self-reported cocaine use histories. This summary finding suggests that the use of one or more metabolites as the only "true marker" of blood-borne cocaine incorporation in hair does not seem to be a viable approach to differentiating "endogenous" cocaine from

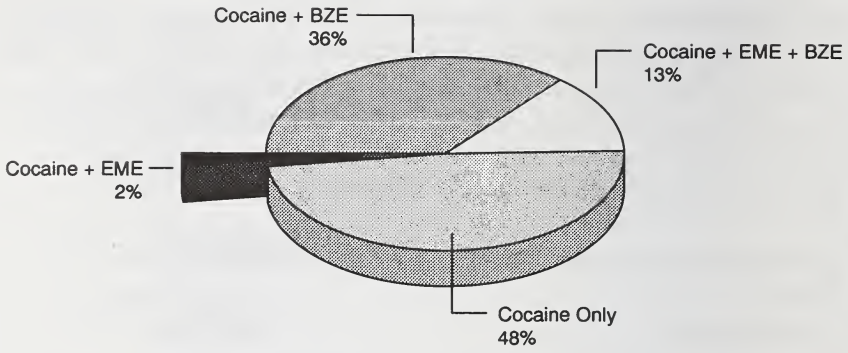

FIGURE 3. *Summary of analyte mixtures found for 319 cocaine-positive hairs tested from May 1991 through November 1993. The reporting limits for analytes are 0.2 μg/g (cocaine, benzoylecgonine [BZE]) or 0.5 μg/g (ecgonine methyl ester [EME]).*

NOTE: Cocaethylene was identified in 61 of 319 hair specimens (19 percent).

possible cocaine contamination on the exterior of the hair, an approach that was previously advanced by Koren and coworkers (1992).

The results displayed in figure 3 for a presumably "normal" cross-section of drug users and others chronically exposed to cocaine (e.g., infants) are different from those reported by Möller and colleagues (1992) for 20 Bolivian coca chewers, who displayed levels not only of cocaine but also of benzoylecgonine (BZE) and ecgonine methyl ester (EME). Figure 3 also demonstrates that cocaethylene is detected in 19 percent of the cocaine-positive hair specimens. There is no particular context into which this finding can be placed because there have not been any other reports that describe the presence or absence of cocaethylene in a large number of cocaine-positive hair specimens. However, as noted by Cone and coworkers (1991), cocaethylene findings often carry substantial weight in litigations and administrative proceedings (e.g., military

separation boards, wrongful termination hearings, union arbitrations). Such a finding quickly defuses discussion of the possible contribution of external cocaine contamination to positive hair test findings, although there has been one anecdotal report of "nonphysiological" formation of cocaethylene (Janzen 1992).

A common interpretive question posed when cocaine results are positive is whether a person used a lot of cocaine or just a little. A standard answer to this inquiry is that a positive hair test result can be interpreted as meaning that the donor has chronically or repetitively used or been exposed to the drugs identified in the hair, although "chronic" and "repetitive" are not defined in the same way for all individuals. To say much more about the meaning of a quantitative drug finding in hair puts a laboratory at great risk of overstating the information content of the test results. However, summary data have been collected that may be of assistance by providing contextual meaning for a given type of case.

Such contextual meaning can be gleaned from figure 4, which demonstrates the distribution of findings for cocaine in positive hair specimens. Grouping results into various quantitative categories, although similar to the format used by Baumgartner and Hill (1990), does not label the results for the apparent "severity of use." However, one way to use these categories is to rank a new positive cocaine value in terms of its percentile. For example, if a new hair submission produces a positive cocaine finding at 7.4 μg/g, this finding would fall into the 74th percentile of all positive hair cocaine historical findings reviewed. Although it cannot be concluded that the individual used 24 percent more drugs or used drugs 24 percent more often than a person whose quantitative value for cocaine was in the 50th percentile, there are cases where such a system of order ranking might assist those who make the ultimate judgments in legal disputes.

There seem to be higher findings in particular kinds of some categories of cases than in others. However, only after amassing sufficient numbers of positive findings has it been possible to assess the accuracy of this suspicion. Figure 5 demonstrates the relative percentages of quantitative findings in insurance cases and neonatal testing (containing results only for the neonates, not combined with

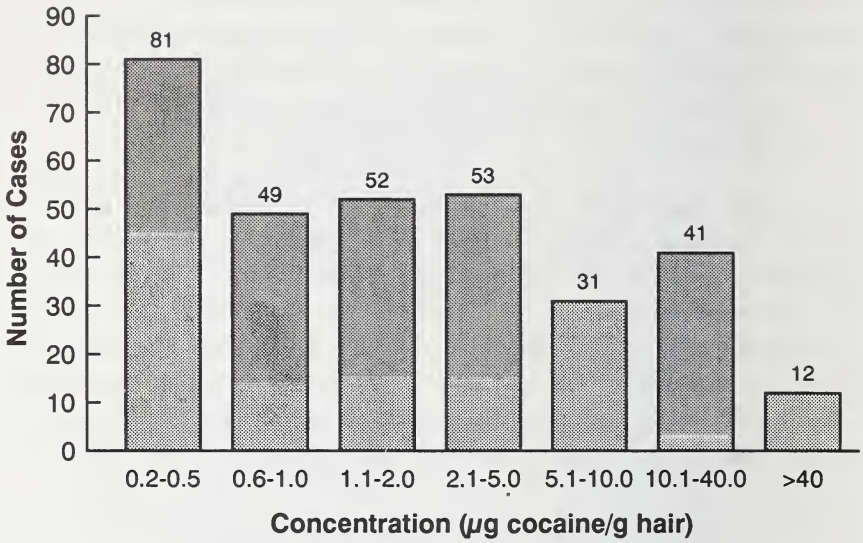

FIGURE 4. *Distribution of quantitative findings for hair speciemns in which cocaine was detected from May 1991 through November 1993 (n=319 cases)*

mothers as in figure 2). The distribution in "all other identified" cases (n=116) (figure 5, white columns) is provided as a potential control or at least to summarily demonstrate the rest of the cases. Other categories are not segregated for display because there were far fewer numbers of these cases identified for which positive cocaine findings were obtained. Although the data are not conclusive, they suggest that the quantitative findings for neonates are biased toward higher values than those related to insurance testing and other testing motivations.

This finding duplicates the general findings previously reported by Kintz and Mangin (1993*a*), who commented that the elevation of results in neonatal hair over findings in other cases suggested that the constant "bathing" of fetal hair in drug-containing amniotic fluid in utero could cause unique hair drug incorporation. In addition, nothing is known about differences to be expected in the incorporation mechanisms of drugs into lanugo (fine hair on fetus'

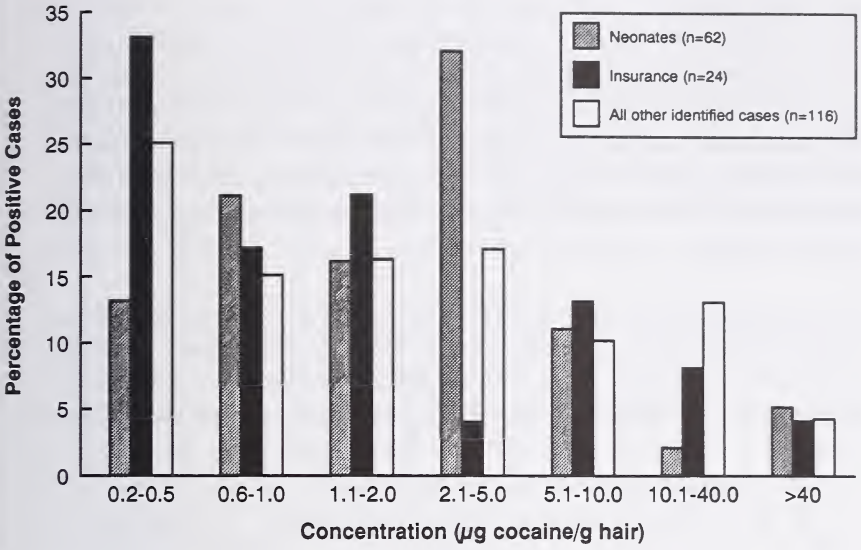

FIGURE 5. *Distribution of positive hair cocaine quantitative findings for samples tested from May 1991 through November 1993 and for which the type of case was identified. For neonates, n=62 positive findings; for insurance cases, n=24 positive findings; and for all other identified cases, n=30 positive findings. There were many positive cases for which the reason for testing was not identified.*

body) and vellus (fine hair that succeeds the lanugo) hair nor about the relative ease with which these drugs can be extracted from different types of hair. Without controlled-dose studies and information on the incorporation and release mechanisms for drugs in hair, the exact interpretation of this elevated positive neonate population is not clear.

Just as the distribution of cocaine into ranges may be useful in the interpretation of findings, it is also possible to similarly plot the distribution of BZE, EME, and cocaethylene. It appears that the likelihood of detecting metabolites of cocaine in positive cocaine hair

specimens increases as the cocaine concentration increases. As shown in figure 6, when the parent drug cocaine was the only analyte identified in a hair sample, the typical quantitative findings were relatively low (compare with figure 4). Figure 6 also suggests that there was a difference between the levels of cocaine determined for neonates and other types of cases. Once again, the reason for this apparent difference in drug incorporation in neonate hair is not easily explained.

When metabolites of cocaine were detected, the relative metabolite concentrations also were somewhat predictable. In general, cocaine tended to predominate over BZE by a factor of between 2 and 10. In addition, consistent with the findings of Möller et al. (1992), the

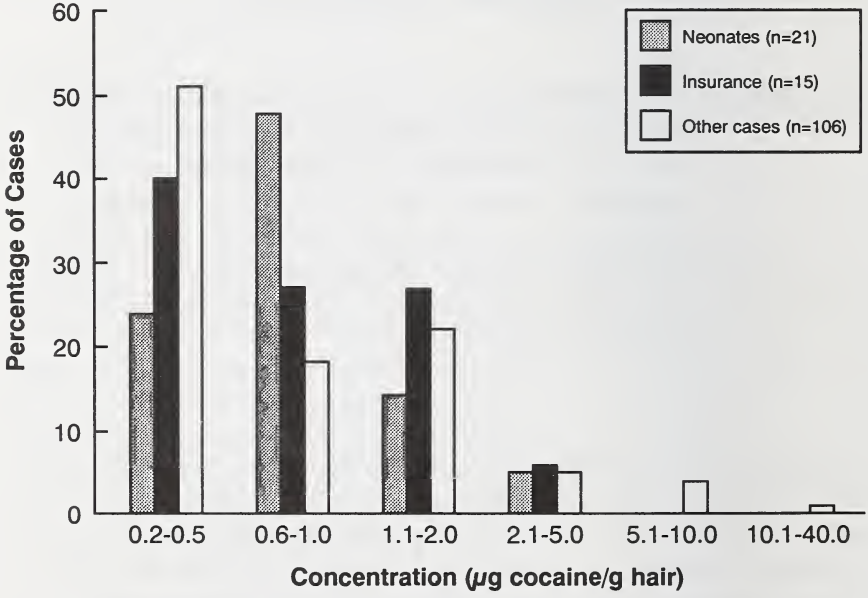

FIGURE 6. *Plot of the distribution of cocaine quantitative findings by category of case, when cocaine was the only analyte detected, for samples received for testing from May 1991 through November 1993*

relationship between the analyte concentrations was generally cocaine > BZE > EME ≈ cocaethylene. The distribution of findings for these three metabolites of cocaine in positive hair specimens is shown in figure 7. In addition, in no cases were detectable levels of metabolites detected in hair in the absence of the parent drug cocaine. Although there may be other interpretations of this finding, it suggests that the incorporation mechanism for cocaine and its metabolites into hair is relatively consistent.

As mentioned above, cocaethylene findings can carry significant weight in administrative and judicial processes. One question related to cocaethylene that arises with some frequency is whether the likelihood of detecting cocaethylene in a cocaine-positive hair is related to the type of case involved. The legend for figure 3 notes that cocaethylene was detected in 19 percent of all cocaine-positive hair specimens. As demonstrated in figure 8, the prevalence of

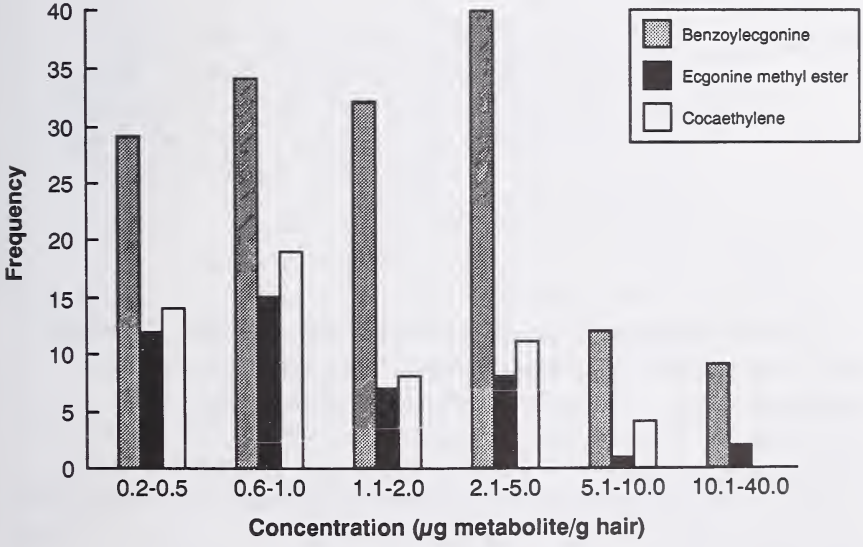

FIGURE 7. *Distribution of findings for cocaine metabolites in positive cocaine hair specimens tested from May 1991 through November 1993*

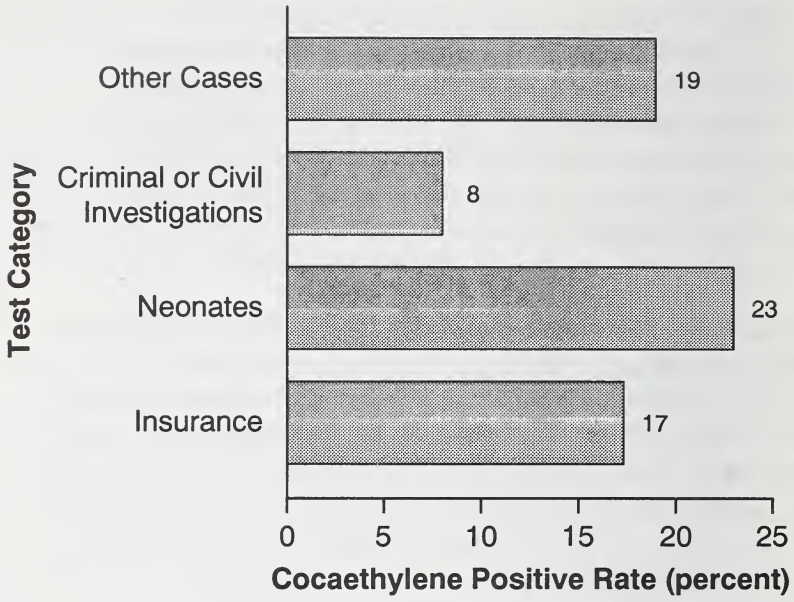

FIGURE 8. *Prevalnce of positive findings for cocaethylene in samples from several groups of hair donors. These data include only those samples and groups for which the testing category was identified and for which there were more than 10 positive cases during the period May 1991 through November 1993.*

cocaethylene is higher in positive neonate specimens than in positive specimens collected pursuant to criminal or civil investigations or insurance cases. A recent related study by DiGregorio and colleagues (1993) discussed the determination of cocaethylene in hair from pregnant women who admitted to the use of alcohol, cocaine, or both. The findings from this related study tended to confirm the potential problem for unborn children posed by the pharmacologically active cocaethylene. However, because the DiGregorio study used preselection of subjects from whom the hair specimens were collected, the results reported in this chapter cannot be interpreted to unequivocally support or refute the suggestion that the prevalence of cocaethylene in mothers and their neonates is

disproportionately higher than in the general population of cocaine users.

General experiences noted to this point can be summarized as follows:

- Cocaine is the drug for which the majority of "single drug" testing requests are targeted.
- The positive rates are highest when specific analytes are targeted.
- Those hair specimens collected pursuant to neonatal testing, insurance policy investigations, child custody proceedings, and criminal or civil investigations are more likely to be positive than those collected in a workplace setting, clinical studies, or divorce cases.
- Nearly half the cocaine-positive hair specimens did not contain any levels of BZE, EME, or cocaethylene at or above their reporting limits. When present, the quantitative order of analytes tended to follow the relationship cocaine > BZE > EME $\approx$ cocaethylene.
- A quantitative bias toward higher cocaine values was detected in neonates compared with other cocaine-positive hair donors. Cocaine-positive hair findings were more likely to include the detection of cocaethylene for neonates than such findings for other tested populations.

PRACTICAL CONSIDERATIONS FOR HAIR RINSING PROTOCOLS

The previous discussion of the SOFT Revised Consensus Opinion on Applicability of Hair Analysis for Drugs of Abuse (Hearn 1992) focused primarily on language regarding the use of hair drug testing in cases in which supportive, competent evidence of drug use exists.

Another primary concern of this advisory committee was the potential problem of evidentiary false positives, or those positive findings that might be caused by (among other reasons) external deposition of drug on the hair. To address this concern, the committee posed the following questions (Hearn 1992):

> To what extent is an externally applied drug from environmental exposure retained in hair, and what is the effect of hygienic washing practices and cosmetic treatments on externally applied drugs?
>
> What is the effect of various laboratory hair pretreatment procedures in removing externally applied drug or internally incorporated/bound drug? Are washing procedures effective in distinguishing between environmental contamination and internal incorporation of drugs?

These questions have engendered much activity by researchers, probably because the matter of evidentiary false positives is easy to study without (1) direct involvement in testing in actual cases, (2) access to hair from bona fide drug users, or (3) controlled-dose studies (and the attendant requirement for approval by human use committees). It could be argued that involvement in actual testing and having access to hair from bona fide drug users are helpful in performing and interpreting the findings for research about this concern. In this way, the research can better place findings in a human context and weigh the importance of findings for distinguishing evidentiary false-positive results from those obtained from actual drug users.

Several, often conflicting models have been used to describe the manner in which a drug is incorporated into hair (Kalasinsky et al. 1993; Spiehler 1993). Because of the complexity of this issue and because no new mechanistic information is provided herein, no indepth discussion is included. However, synthesis of the various current models for the microenvironment of hair and the suspected manner in which drugs are incorporated lead to the following working hypotheses:

- *Hair porosity* is not a fixed feature. It varies among ages, races, types of hair, and periods of growth and is affected by hygienic treatments, environment, nutrition, and perhaps many other factors.

- *A drug incorporated from active drug use* may be found in hair resulting from a circulating drug being incorporated into undifferentiated hair matrix cells in the highly mitotic, vascular region of the dermal papilla by absorption of the drug carried by sebaceous excretions in the keratinization zone of the hair root; and/or through absorption of the drug carried by sweat exposed to keratinized hair.

- *An externally applied drug* is incorporated in hair differently from a drug incorporated from active drug use. When subjected to hair rinsing procedures, an externally applied drug demonstrates different kinetics of removal from hair.

Current reports on rinsing in the literature on hair drug testing are diverse. They describe studies that used no rinsing (Nagai et al. 1989; Kintz et al. 1992*a*; Kintz and Mangin 1992); rinsing with methanol or ethanol alone (Suzuki et al. 1984; Cone et al. 1991; Harkey et al. 1991; Martz et al. 1991; Forman et al. 1992; Kintz et al. 1992*b*; Koren et al. 1992); rinsing with alcohol followed by rinses with aqueous buffers (Baumgartner et al. 1989; Cone et al. 1991; Baumgartner and Hill 1992, pp. 577-597; 1993; Fritch et al. 1992; Selavka et al., in press); rinsing with methylene chloride alone (Kintz and Mangin 1993*b*, 1993*c*; Kintz 1993*a*); rinsing with surfactants alone (Graham et al. 1989; Nakahara et al. 1991, 1992; Uematsu et al. 1992, 1993; Martinez et al. 1993); rinsing with water (Uematsu et al. 1994); rinsing with water and acetone (Möller et al. 1992); rinsing with pentane (Kidwell 1993); and rinsing with ethyl ether and hydrochloric acid (Gamaleya et al. 1993; Tagliaro et al. 1993). Recently, several methods of rinsing were summarized and compared by Kintz (1993*b*, pp. 37D-3—37D-32) and Welch and colleagues (1993). Sometimes these rinses were tested, but many times they were not. The procedure described in this chapter called for rinsing with reagent alcohol (which was discarded), then

replicating the rinse with pH 7 phosphate buffer. Both the first and last replicates of these buffer rinses were tested using the appropriate immunoassay.

For purposes of interpretation, rinse protocols and review guidelines similar to those most recently described by Baumgartner and Hill (1993) were adopted in the authors' laboratory (Fritch et al. 1992). However, because of differences in procedures, research of historical findings were used to guide the creation of unique interpretive rules for cocaine cases. This process employed retrospective evaluation of 100 cases in which rinsing test data were available and the hair extract was positive for cocaine by either RIA, GC/MS, or both. As shown in figure 9, these 100 cases were categorized using the results from their first buffer rinse (positive [+] or negative [-] by RIA), their last buffer rinse (+ or - by RIA), RIA of the hair extract (+ or -), and GC/MS of the hair extract (+ or -). A shorthand notation was created to denote the findings for each of these four tests, in the order of first rinse / last rinse / RIA / GC/MS. Using this model, a sample having a positive first rinse, a negative last rinse, a positive RIA, and positive GC/MS would be noted as "+/-/+/+."

Figure 9 demonstrates that, within the paradigm of the working hypotheses stated above, the issue of the contribution of external contamination to a positive hair drug test result does not apply to 89 percent of the samples captured in this retrospective analysis. This 89 percent comprises those samples for which the last rinse was negative, yet the hair extract was positive by both RIA and GC/MS (+/-/+/+ [12 percent of cases] and -/-/+/+ [77 percent]). Three percent of the cases demonstrated -/-/+/- findings, wherein the GC/MS result did not confirm the positive RIA findings. This phenomenon has been studied further, but because there were few specimens in this group, it cannot be stated conclusively whether this type of finding is an artifact of the quantitative thresholds used (0.20 μg/g by GC/MS and 0.10 μg/g "apparent cross-reactants" by RIA) or has some other cause.

The raw data for +/+/+/+ cases in figure 9 are provided in table 2. This group of samples was found by GC/MS to contain relatively high levels of cocaine. (Using figure 4, all these findings

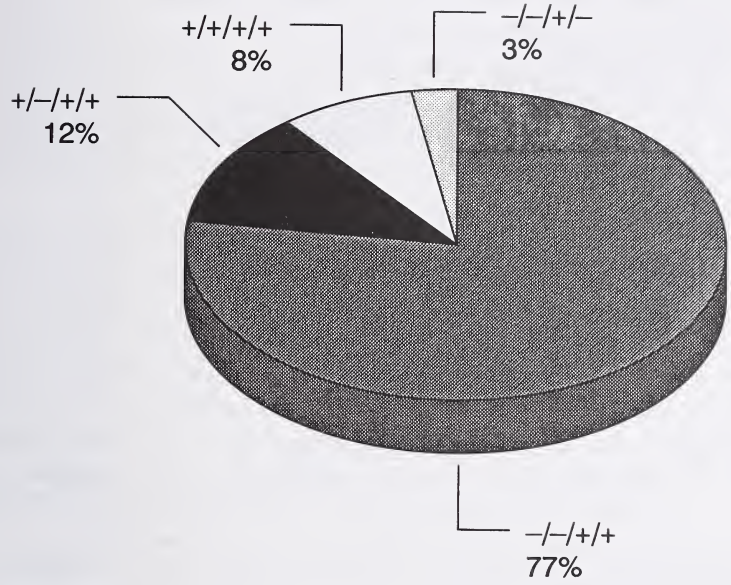

FIGURE 9. *Summary of findings for the first aqueous buffer rinse, last aqueous buffer rinse, extract cocaine RIA result, and GC/MS cocaine result for 100 samples tested from August 1992 through April 1993 for which either RIA or GC/MS returned positive findings for the hair extract (see text for more detail on the notations used)*

fall within or above the 74th percentile of positive cocaine cases.) In the review of cases, a hair extract result was reported as positive as long as the RIA value for the last rinse was less than 10 percent of that for the hair extract. If this criterion was not met, then the sample had to be aliquoted again and a greater number of rinses used. All the +/+/+/+ hair samples met this criterion with either the fourth or eighth rinse. It has been suggested that rinses that contain large amounts of drug may indicate contamination caused by environmental (external) sources (Kidwell 1993). Baumgartner and Hill (this volume) suggest that the rinse kinetics for an externally

TABLE 2. *Data for +/+/+/+* cases*

Semiquantitative Cocaine RIA Results† (Apparent ng/mL Cross-Reacting Substances)				GC/MS Result for Hair	
Rinse 1	Rinse 4	Rinse 8	RIA Hair	Cocaine (μg/g)	Last Rinse/Cocaine‡
7,715		722	22,494	8.8	82.1
22,737		802	121,027	8.9	90.1
3,761	509		55,963	16.0	31.8
9,390		1,916	105,922	21.2	90.4
9,573	697		67,973	22.8	30.6
10,061		513	59,593	24.3	21.1
3,330		769	86,237	62.7	12.3
18,646		1,779	225,552	93.1	19.1
Average values				32.2	47.2
Ranges				8.8-93.1	12.3-90.4

*See text discussion of figure 9 for a description of "+/+/+/+" nomenclature.

†The radioimmunoassay was performed using Diagnostic Products Corp. Coat-A-Count RIA kits for cocaine.

‡The semiquantitative finding for the last "positive" rinse [value >500 ng/mL cross-reacting substances] was divided by the GC/MS quantitative value for cocaine.

KEY: RIA=radioimmunoassay; GC/MS=gas chromatography/mass spectrometry

applied drug are significantly different from those for a drug deposited from active drug use.

To create a relatively sensitive measurement technique for identifying possibly contaminated hair specimens, the ratio of the apparent concentration of cross-reacting substances in the last rinse (RIA data) to the cocaine quantitation by GC/MS can be used as an interpretive guideline in +/+/+/+ cases. For those hair specimens depicted in table 2, this ratio was found to range between 12.3 and 90.4. To test the utility of this measurement as a sensitive indicator of contaminated hair specimens, the same measurements were made for the eight cocaine-positive hair specimens supplied within the last NIST round-robin set. The NIST round-robin set consists of hair specimens submitted as proficiency/challenge samples to voluntarily

participating laboratories that are active in hair drug analysis or research. This batch was known to have been created using negative hair specimens soaked in dimethylsulfoxide solutions containing drugs and could therefore be considered to represent externally contaminated hair specimens (i.e., those not containing drug resulting from natural incorporation mechanisms through active drug use).

Table 3 demonstrates results for the NIST round-robin set. It is clear that, although the last rinse was positive by RIA for these hairs, the cocaine in the hair extract exhibited low quantitation by GC/MS, giving rise to rinse/cocaine ratios for all eight cut hair specimens that were far above the range described by the +/+/+/+ hair set shown in table 2. Although this one set of contaminated samples from NIST is not representative of all possible contaminated hair specimens, the findings allow for creation of interpretive criteria within the general list for cocaine-positive cases:

- If the last rinse is positive, but cocaine in the extract by GC/MS is less than 5 μg/g, the sample is suspect.
- If the "last rinse (RIA)/cocaine by GC/MS" ratio is greater than 125, the sample is suspect.
- If the last rinse (RIA) is greater than the first rinse (RIA), the sample is suspect.
- If the first rinse is negative, the sample is presumptively uncontaminated.
- If the sample contains cocaethylene, it is presumptively uncontaminated.

It should be emphasized that these are general interpretive guidelines and may be rejected, or other criteria may be employed, if the reviewing official in a case believes it is necessary for proper case interpretation. Ongoing research with hair contaminated by vaporization deposition of cocaine and deposition through soaking with aqueous solutions may lead to new criteria or validate continued use of the current guidelines without modification.

TABLE 3. *Data for NIST round-robin specimens (n=8) containing cocaine*

Sample	Semiquantitative Cocaine RIA Results† (Apparent ng/mL Cross-Reacting Substances) Rinse 1	Rinse 4	Rinse 8	GC/MS Result for Hair Cocaine (μg/g)	Last Rinse/Cocaine†
1	1,693	268	70	0.45	596
3	9,822	1,870	390	1.47	1,272
5	3,476	623	96	0.57	1,093
7	3,896	2,074	650	0.36	1,806
8	4,885	2,300	1,362	2.41	565
Average values				1.05	1,066
Ranges				0.36-2.41	565-1,806

*The radioimmunoassay was performed using Diagnostic Products Corp. Coat-A-Count RIA kits for cocaine.

†The value represents the semiquantitative finding for the last "positive" rinse (value >500 ng/mL cross-reacting substances) or rinse 4 (if neither rinse 4 nor rinse 8 is >500 ng/mL) divided by the GC/MS quantitative value for cocaine.

NOTE: See text discussion of figure 9 for a description of "+/+/+/+" nomenclature.

KEY: RIA-radioimmunoassay; GC/MS=gas chromatography/mass spectrometry

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Comparison of Different Extraction Procedures for Drugs in Hair of Drug Addicts

Vincent Cirimele, Pascal Kintz, and Patrice Mangin

INTRODUCTION

A critical element in the acceptance of hair analysis for the detection of drugs of abuse is the validity of the methodology. Each technique must accurately determine which drugs are present and at what levels.

Cocaine and its metabolites—cocaethylene, benzoylecgonine (BZE), and ecgonine methyl ester (EME)—and opiates—including morphine, codeine, and 6-monoacetylmorphine (MAM)—can be extracted after hydrolysis by acid (Kintz et al. 1993), alkali (Sachs et al. 1993), or enzyme (Möller et al. 1992) or directly by methanol (Kauert et al. 1992). Although many reports have been published about these extraction procedures, only a few compare these techniques (Sachs 1993; Welch et al. 1993).

Therefore, to complete previous studies, the authors used 19 hair samples obtained from drug abusers to test the various analytical approaches to compare extraction efficiencies for cocaine, heroin, and their metabolites.

MATERIALS AND METHODS

Chemical Reagents

All chemicals were provided by Merck (Darmstadt, Germany): Methylene chloride (CH_2Cl_2), chloroform, isopropanol, *n*-heptane, and methanol were high-performance liquid chromatography grade; all others were analytical grade.

Deuterated internal standards, cocaine-d_3, cocaethylene-d_3, BZE-d_3, EME-d_3, morphine-d_3, codeine-d_3, and MAM-d_3, were purchased from Radian Corp. (Austin, TX). Stock solution was prepared at 10 mg/L in methanol.

N,O,-bis (trimethylsilyl) trifluoroacetamide (BSTFA)+1 percent trimethylchlorosilane (TMCS) was purchased from Interchim (Montluçon, France).

Sodium dihydrogen phosphate (NaH_2PO_4) buffer (10 mM, pH 7.6) was prepared as follows:

- 137.99 mg NaH_2PO_4 were mixed with 70 mL H_2O.
- pH was adjusted at 7.6 with 1 M sodium hydroxide (NaOH), and H_20 was added to complete to 100 mL.

Diammonium hydrogen phosphate [$(NH_4)_2HPO_4$] saturated buffer pH 8.4:

- 70 mL H_2O were saturated with $(NH_4)_2HPO_4$, adjusted at pH 8.4 with 1 M hydrochloric acid (HCl), and mixed with H_2O to complete to 100 mL.

Materials for Examination

Hair samples were obtained from 19 drug abusers ages 18 to 37, including 10 fatal cases. Strands of hair in the vertex region were cut as close as possible to the scalp.

All samples were decontaminated twice in 5 mL CH_2Cl_2 for 2 minutes at room temperature. This was the most efficient of the five wash solutions tested (water, phosphate buffer, acetone, sodium dodecyl sulfate, and CH_2Cl_2) (Kintz and Mangin 1993). Samples of 500 mg each were pulverized in a Retsch MM2 type ball mill (Haan, Germany) and homogenized to eliminate drug concentration fluctuations along the hair shaft.

Sample Extraction

Pulverized samples were divided into 4 parts, and 50 mg each were incubated under the following conditions and in the presence of 200 ng deuterated internal standards:

- Acid hydrolysis: 1 mL 0.1 *N* HCl 16 hours at 56 °C
- Alkali hydrolysis: 1 mL 1 *N* NaOH 10 minutes at 100 °C
- Enzyme hydrolysis: 2 mL phosphate buffer 10 mM pH 7.6 containing β-glucuronidase and arylsulfatase at 12 units/mL and 60 units/mL, respectively, 2 hours at 40 °C
- Methanol direct extraction: 5 mL methanol, sonication 5 hours at 45 °C

After incubation, all samples were extracted according to the following procedure (Kintz and Mangin 1993). Samples were buffered at pH 8.4 with 2 mL saturated $(NH_4)_2HPO_4$ solution and extracted with 10 mL chloroform/isopropanol/*n*-heptane (50:17:33, volume of solute per volume of solvent [v/v]). After the samples were agitated (95 cycles/minute) and centrifuged (3,000 revolutions per minute [RPM]), the organic phase was purified by an additional acid extraction (5 mL 0.2 *N* HCl). After neutralization by 1 mL 1 *N* NaOH and the addition of 2 mL pH 8.4 phosphate buffer, the aqueous layer was reextracted with 5 mL chloroform. After agitation and centrifugation of the samples under these conditions, the organic phase was evaporated to dryness in a

Varian Speed Vac Concentrator. For methanol direct extraction, it was necessary to filtrate the incubation solution on 0.45-μm Millex-HV filters (Millipore, Bedford, MA) and to evaporate the methanol to dryness before the three-step extraction. Drugs were silylated using 40 μL BSTFA+1-percent TMCS added to the dry extract and stored in a sealed tube at 70 °C for 20 minutes.

Gas Chromatography/Mass Spectrometry Method

A 2-μL portion of the derivatized extract was injected through the column (HP-5MS capillary column, 5-percent phenyl to 95-percent methyl siloxane, 30 m $\times$ 0.25 mm) of a Perkin Elmer 8500 chromatograph. The injector temperature was 250 °C, and splitless injection was used with a split-valve off-time of 1 minute.

Gas head pressure (helium, purity grade N 55) was 15 pounds per square inch. The temperature column was programmed to rise from an initial temperature of 60 °C (kept for 1 minute) to 295 °C (kept for 6 minutes) at 30 °C/minute.

The Perkin Elmer 8500 gas chromatograph was coupled with an ion-trap detector (ITD) operating in electronic impact mode at 70 eV and with an ion source temperature of 230 °C. The electron multiplier voltage was between 1,600 and 2,000 volts. The ITD was autotuned daily.

Qualitative and quantitative analyses were obtained in the selected ion monitoring mode by the comparison of retention times and relative abundance of two confirming ions with the deuterated internal standards.

RESULTS AND DISCUSSION

In this study, hair samples were obtained from 19 drug abusers. It was possible to identify MAM in all the samples, clearly indicating heroin abuse. Moreover, in nine cases, cocaine and its metabolites also were detected. Ranges of drug concentrations obtained by the four methods were in accordance with those

reported in previous papers (Möller et al. 1992; Kauert et al. 1992; Welch et al. 1993; Baumgartner et al. 1989; Cone 1990).

Alkali hydrolysis, which completely dissolved the hair matrix, allowed solubilization of all the drugs. Unfortunately, under these conditions, MAM, a unique proof of heroin abuse, was hydrolyzed to morphine. It was therefore not possible to differentiate between medical intake of codeine or morphine and heroin abuse. Consequently, the alkaline preparation could be retained as the method of choice.

Figures 1 through 5 show that extraction recoveries were dependent on the method.

- For codeine (figure 1), the acid method gave higher concentrations eight times, and in six cases, extraction recoveries were more than twice those of methanol extraction recoveries. Higher concentration findings were obtained by enzyme hydrolysis five times.

- For morphine (figure 2), the acid method produced higher results five times, with extraction recoveries two to three times higher than those obtained with the methanol method.

- Results were more controversial for MAM (figure 3) because the histogram did not indicate a preponderant method.

- For cocaine (figure 4), acid hydrolysis obtained higher concentrations five times.

- For BZE (figure 5), the three methods were equivalent and gave similar results.

The maximal differences in extraction recoveries were observed when drug concentrations were elevated. In these cases, the acid and enzyme preparations always showed higher drug concentrations compared with methanol extraction. However, methanol direct

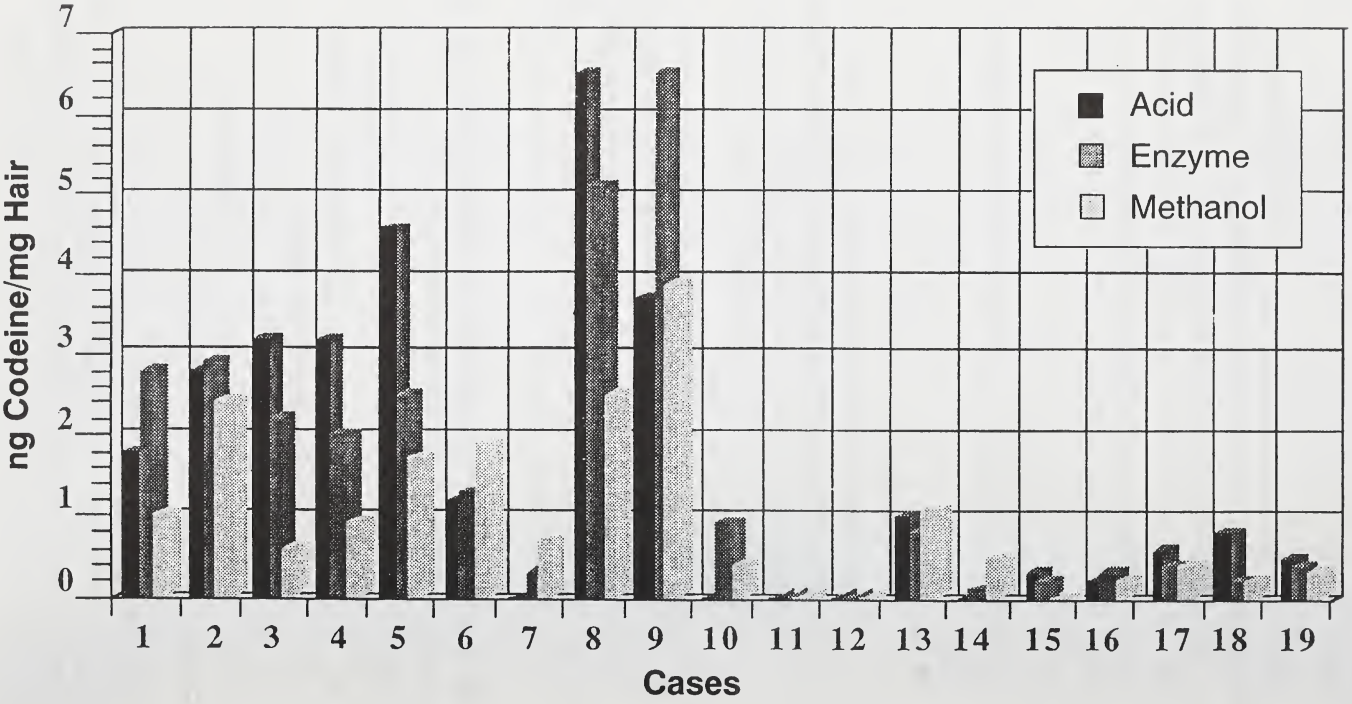

FIGURE 1. *Distribution of codeine concentrations in 19 hair samples after 3 different extraction procedures*

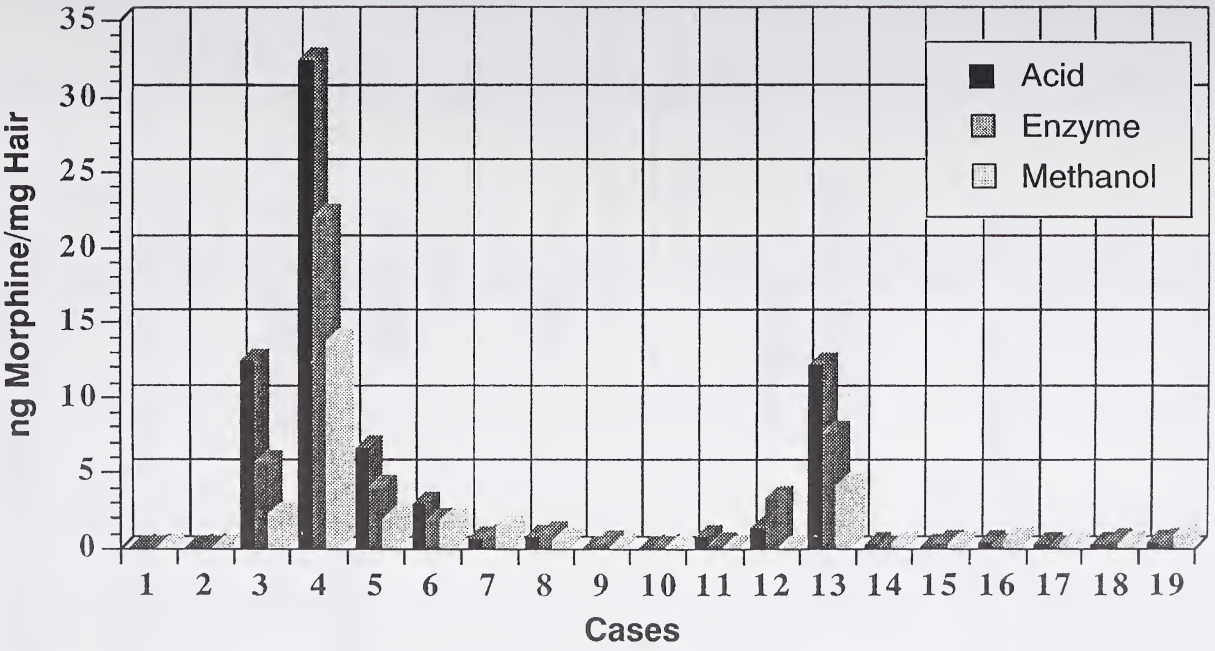

FIGURE 2. *Distribution of morphine concentrations in 19 hair samples after 3 different extraction procedures*

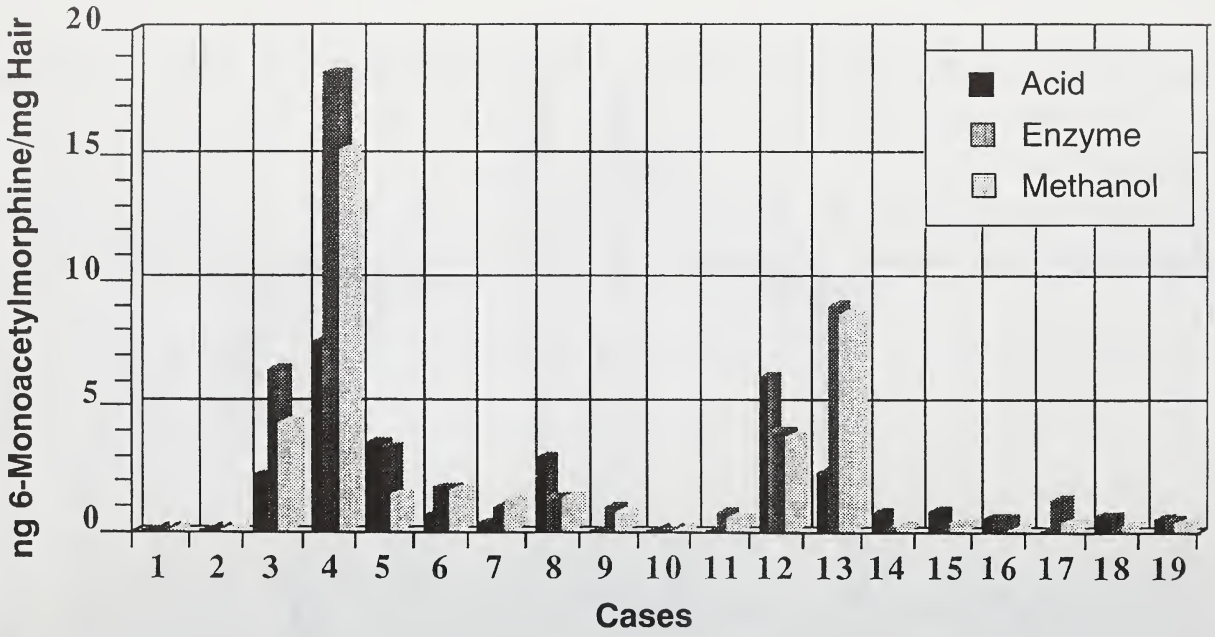

FIGURE 3. *Distribution of 6-monoacetylmorphine concentrations in 19 hair samples after 3 different extraction procedures*

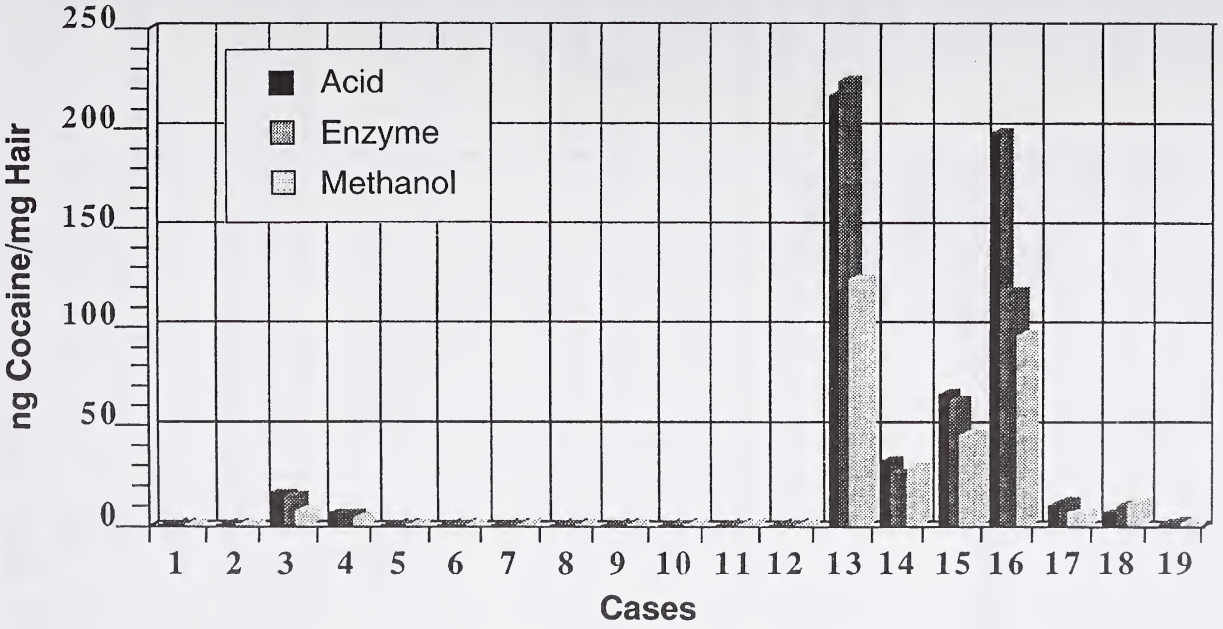

FIGURE 4. *Distribution of cocaine concentrations in 19 hair samples after 3 different extraction procedures*

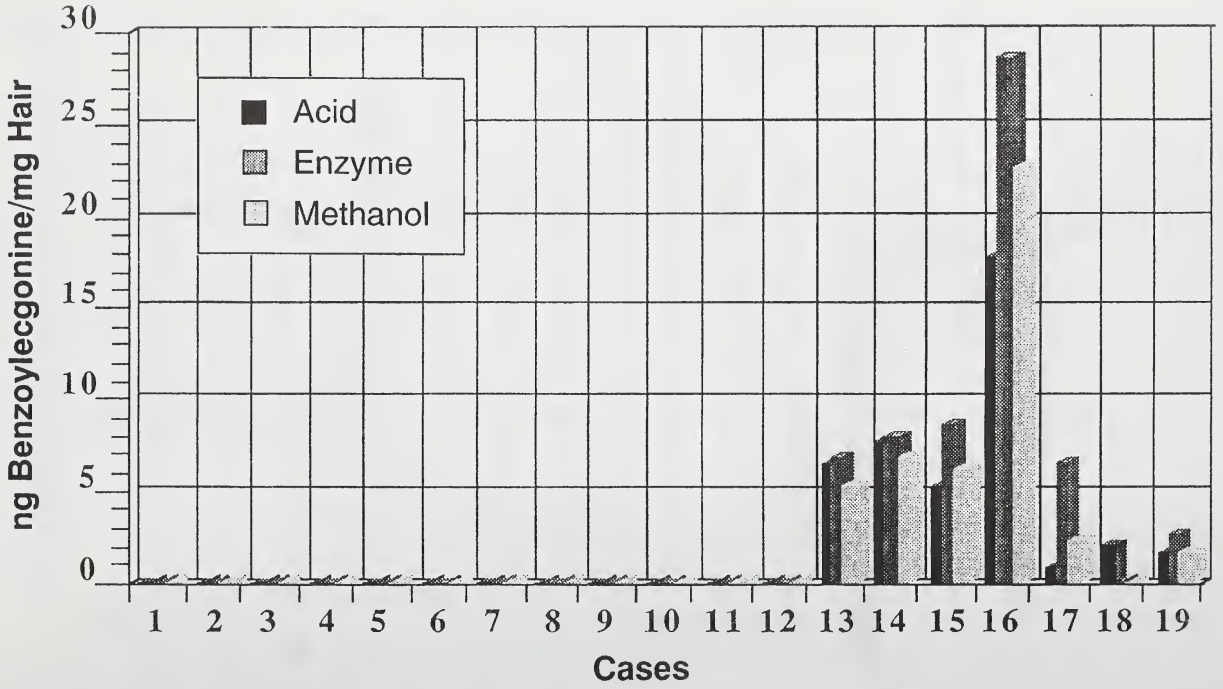

FIGURE 5. *Distribution of benzoylecgonine concentrations in 19 hair samples after 3 different extraction procedures*

extraction seemed to give results similar to the two other methods when drug concentrations in hair samples were low. These results are in accordance with those of Sachs and colleagues (1993) who reported that enzyme hydrolysis gave better extraction recoveries for opioids than did methanol direct extraction. In contrast, the authors reported better results for cocaine by methanol extraction. In their study, smaller drug concentrations often were found after methanol extraction, and close results were seen between the other two methods. Welch and colleagues (1993) also observed small differences of drug concentrations between acid and enzyme hydrolyses. This study revealed that each of the target drugs was extracted by each of the extraction conditions.

Other analytical parameters must be considered. Acid preparation takes more time (18 hours) compared with enzyme preparation (4 hours). Nevertheless, the latter is more expensive. Methanol direct extraction is less time consuming (7 hours) compared with acid preparation. It is less expensive; and extracts can be injected directly into a gas chromatography/mass spectrometry column after evaporation. Unfortunately, because the extracts were of poor quality and there was a background noise on the chromatograms, it was difficult to determine drugs in low concentrations. Therefore, it was necessary to purify the dry extracts by the same three-step extraction procedure (used for the three other preparations) before injection.

With one exception, all the methods presented have the capacity to extract opiates, cocaine, and their metabolites. Alkali hydrolysis did not detect the presence of MAM, a unique proof of heroin abuse.

Except for methanol extraction, which identified lower drug concentrations compared with acid and enzyme hydrolyses, it is not possible to determine which method performs best.

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Research at the Federal Bureau of Investigation in Hair Analysis for Drugs of Abuse

Mark L. Miller, Brian Donnelly, and Roger M. Martz

INTRODUCTION

The analysis of hair for drugs of abuse has been a visible but small portion of the casework at the Federal Bureau of Investigation (FBI) laboratory. The strengths of hair analysis are its ability to give some indication of chemical exposure on a historical basis (Martz et al. 1991) and its noninvasive nature. Hair analysis is generally conducted in cases where there is prior evidence of drug use, such as a positive urine test. In these instances, a defendant's innocent plea usually can be either substantiated or refuted based on the results of hair analysis. Currently, the only requested drug analysis performed on hair in the FBI laboratory is for cocaine because of its popularity in U.S. culture and the amount present in hair. Methods are being researched for drugs other than cocaine, with particular emphasis on opiates. Screening and confirmation analyses currently performed with mass spectrometry (MS) and future procedures are anticipated to continue to rely on MS for some time because of its sensitivity and its acceptability in court.

The FBI's first use of hair analysis for drugs of abuse was in 1987 (Martz 1988) in the case of a drug smuggler who claimed to have no knowledge of how several kilograms of cocaine came to be in his car trunk. He also claimed never to have used cocaine. The credibility of the alleged smuggler was in question because a lock of his hair had been found in one of the car trunk cocaine packages,

which linked him directly to the drugs. A sample of his head hair was collected and analyzed for cocaine by the Chemistry-Toxicology Unit of the FBI laboratory. The results were positive for cocaine, demonstrating untruthfulness in his statements and thus further reducing the possibility of the smuggler's innocence. Hair testing was crucial to the suspect's pleading guilty. The conviction of the smuggler demonstrated the power of hair analysis in forensic science and encouraged further developments in methodology.

There has been tremendous growth in the use of hair analysis for drugs of abuse at the FBI since 1987 (figure 1). Beginning with the first case in 1987, the number of cases increased to 35 in 1992. As many as 40 cases were projected for 1993. The future annual growth in cases will depend on court proceedings and testimonies as legal precedence becomes more established.

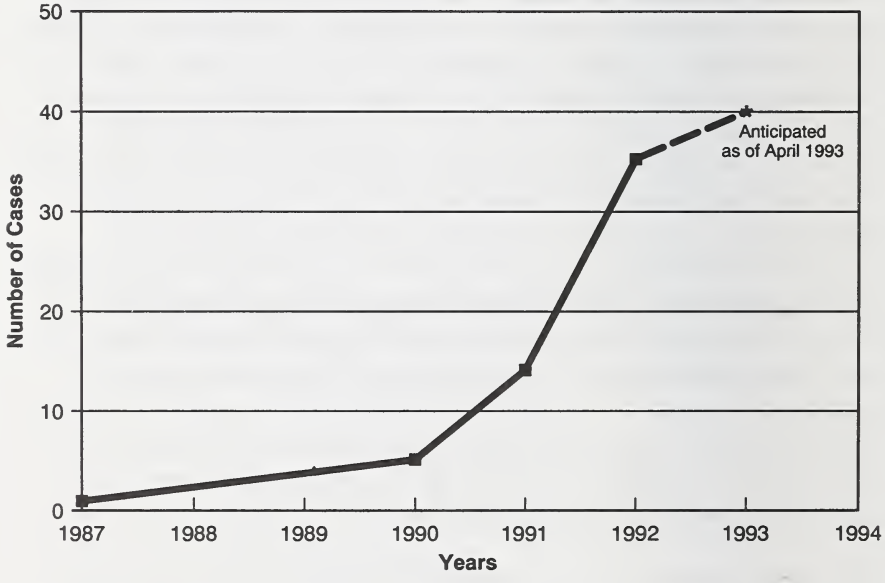

FIGURE 1. *Annual number of hair cases (1987-1993) tested by the Federal Bureau of Investigation's Chemistry-Toxicology Unit*

Diverse uses have been made of hair analysis at the FBI for the benefit of both the defense and the prosecution. More than half the requests thus far have been from military investigators for consideration in sentencing; drug urinalysis can corroborate previous test results or establish whether long-term drug abuse has occurred. Illegal drug use by military personnel is grounds for court-martial and expulsion. Although precise dose/concentration relationships have not been determined for cocaine in hair, findings of tens of nanograms per milligram of hair are consistent with long-term or at least heavy, short-term drug use as has been shown in hair sectioning studies (Martz et al. 1991; Möller et al. 1992).

Nonmilitary uses of hair analysis have involved cases such as parole hearings where freedom is conditional on an individual's being drug-free, prison inmate use, allegations of drug use by Federal employees with high-level security clearances, and allegations of drug use by court personnel such as a prosecutor or a defense attorney. In one instance, a police shooting victim who was in an apparent drug-induced state was confirmed to be a drug user by hair analysis. In another case, two police officers were tested because they were on trial for stealing drugs and money from the shakedown of arrestees. In one well-publicized case, a mayor from a large metropolitan city was tested to substantiate long-term use of cocaine.

Few cases have required in-person testimony (military or civilian) from the FBI for work done by the Chemistry-Toxicology Unit. Frequently, defendants have pleaded guilty when results of the hair analysis have been positive. Most military cases have resulted in courts-martial. In cases where testimony was given, hair analysis results have helped defend or convict in all but two cases. In one exception, a military person was found innocent of drug abuse in spite of positive hair results because his wife stated that more than once she had slipped cocaine into his drink without his knowledge.

DETERMINATION OF COCAINE IN HAIR

The collection of evidential hair is performed by medical and law enforcement personnel. Approximately 100 hairs are gathered by cutting them from the vertex of the scalp, and approximately 5 mg

hair are used in each analysis. Although this is a small sample size, it is sufficient to establish whether a person is a cocaine user.

The first requirement before extracting the hair is to wash it twice with 1 mL methanol by vortexing for 30 seconds. Methanol has broad solvating powers and can remove drugs or oils from the surface of the hair. Also, 100 ng trideuterated cocaine (cocaine-d_3) are added to the wash as a quantitative standard. The methanol wash is evaporated under nitrogen, and the residue is taken up in 20 μL methanol. The wash is quantitated by loading 5 μL of the sample into a glass crucible for tandem mass spectrometry (MS/MS) analysis (see below). Wash values are usually much lower than extract concentrations (<1 ng/mg hair). Higher values are indications of possible environmental contamination. When contamination of scalp hair is suspected, a new specimen is requested from the subject from an area other than the head. In one case, a high level of cocaine was observed in the wash (5 ng/mg) of hair treated with petroleum jelly and coloring agents. In another case, analyzing hair from a hairbrush showed a high level of drug in the wash; it was likely to have been contaminated by the user in daily handling. The pubic region is a preferred alternative sampling area because it is less likely to be the subject of styling treatments and colorants.

Once the hair has been washed, it is ready for extraction. To the specimen are added 2 mL 1 *N* HCl and the internal standards 100 ng cocaine-d_3 and 100 ng pentadeuterated benzoylecgonine (BZE) (BZE-d_5). The sample is heated 45 minutes at 60 °C in a covered test tube; then a few drops of concentrated ammonium hydroxide are added to neutralize the acid before the sample is extracted in the alkali-buffered Toxi-tube A for 20 minutes on a laboratory shaker. The organic supernatant is removed from the Toxi-tube and evaporated under nitrogen for reconstitution in 20 μL methanol.

If the hair extract tests positive, a BZE sample is prepared by adding methylene chloride to the fill line on the cocaine-extracted Toxi-tube A. The tube is then shaken for 20 minutes and spun in a centrifuge at 2,500 rpm for 10 minutes. The organic subnatant is removed and dried with a small amount of sodium sulfate and evaporated to

dryness under nitrogen. This residue is reconstituted with 15 μL methanol.

Both the cocaine and BZE reconstituted extracts are analyzed by solid probe chemical ionization MS/MS using a fast heating rate to 400 °C. For the cocaine analysis, the instrument is set up to monitor neutral losses of 122 daltons for the transition of the (M+H) ion at a mass-to-charge ratio (m/z) of 304 to the fragment ion at 182 (figure 2). In this example, the 304 ion is the parent ion, and any ions that arise from its fragmentation (e.g., 182) are known as daughter ions. A fragment-ion spectrum of a particular parent ion is called a daughter spectrum in MS/MS. A parent-ion spectrum is obtained by looking only for ions that precede a specific daughter ion. The cocaine-d_3 standard is observed at m/z 307 and its fragment at m/z 185 (figure 3). The BZE analysis is performed by scanning for parents of 168 at m/z 290 for BZE and 295 for the quantitation standard BZE-d_5 (figure 4). Argon is used as the collision gas for the MS/MS experiments, and methane serves as the chemical ionization reagent. Quantitation is accomplished by the

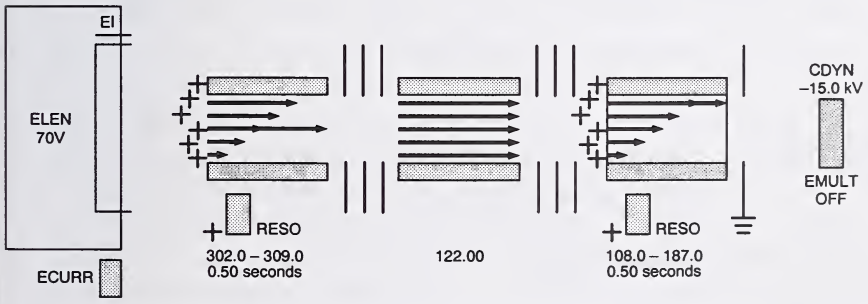

FIGURE 2. *Chemical ionization tandem mass spectrometry analysis for cocaine*

KEY: EI=electron impact; ELEN=electron energy; ECURR=electron current; RESO=resolution; CDYN=conversion dynode; EMULT=electron multiplier

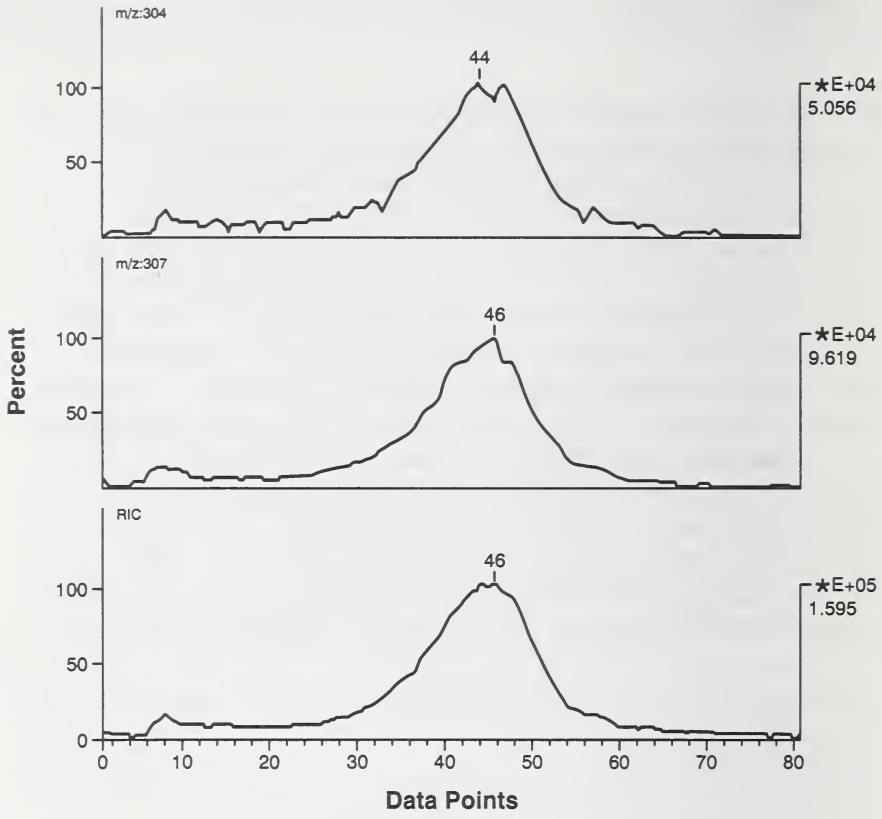

FIGURE 3. *Tandem mass spectrometry chromatograms of hair extract containing cocaine (ion trace 304), internal standard (ion trace 307), and reconstructed ion chromatogram*

KEY: m/z:304 = ion monitored for detection of cocaine; m/z:307 = ion monitored for detection of deuterated cocaine; RIC = reconstructed ion chromatograph; * = full-scale intensity of signal for the chart; E+ = notation for 10 to the power of the number following (e.g., 04, 05) times the number below (e.g., 5.056, 9.619)

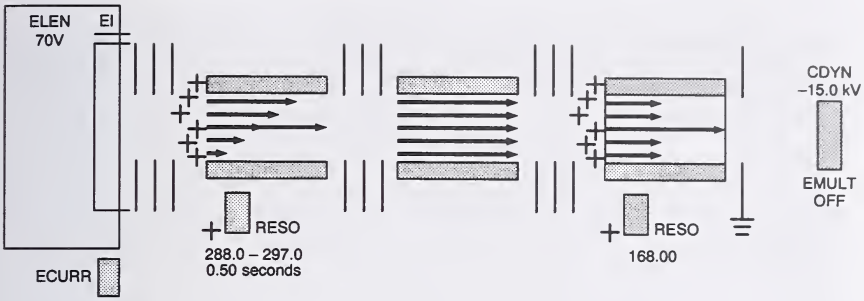

FIGURE 4. *Tandem mass spectrometry analysis for benzoylecgonine*

KEY: EI=electron impact; ELEN=electron energy; ECURR=electron current; RESO=resolution; CDYN=conversion dynode; EMULT=electron multiplier

ratio of the unknown intensity to the internal standard. The cutoff for cocaine positives is approximately 1 ng/mg hair because confirmational MS/MS daughter spectra are difficult to obtain below this level for a 5-mg specimen under these experimental conditions. The ratio of cocaine to BZE normally encountered is 5:1. Confirmation of cocaine can be made by acquiring an MS/MS daughter spectrum of the (M+H) ion at m/z 304.

Solid-probe MS/MS analysis using acid extraction with Toxi-tube A cleanup is accurate and reproducible. The method has been tested on National Institute of Standards and Technology (NIST) reference material code number 8448 hair segments and found to give results within two standard deviations of the best estimate values. In addition, analysis of the methylene chloride extract after the extraction of cocaine in a mild alkali Toxi-tube A has not shown any degradation of cocaine to BZE in the short time required to prepare the sample. The methanol wash step is important in the elimination of possible false positives resulting from what is considered surface contamination. True surface contamination is external to the scales of the hair and should be easily removed because cocaine base and

cocaine hydrochloride are soluble in methanol at these levels. The short contact time of the wash does not extract the hair to any significant extent as evidenced by previous in-house studies that did not show the presence of BZE metabolite in the wash for cocaine-positive samples.

EFFECT OF EXTRACTION METHODOLOGY ON HAIR MORPHOLOGY

Practitioners in the field of hair analysis for drugs currently use one of four extraction methods to remove these substances from hair: base, enzyme, acid, or solvent extraction. To investigate the effect these methods have on the morphology of hair, a microscopic study was made using a scanning electron microscope (SEM). Solvent extraction was not included in the study because it is unlikely to result in any visible effects, and a quicker method was desired. The goal of a clean extraction (an extract solution that contains only the analyte of interest) is to remove the analyte from the matrix without also generating extraneous substances, (e.g., breakdown products from hair) that can be potential interferants. Destruction or changes in the morphology of the hair are a possible indication of the presence of interfering compounds in the extract. These background compounds include protein fragments and other components from degradation of the hair matrix. An increase in the amount of material (other than the analyte) in the final glass crucible for solid-probe MS/MS analysis may decrease the sensitivity for the analyte by ionization interference or detuning (when voltages on the ion source are not optimized for the analyte detection) of the ion source. Methods such as solid-probe MS/MS that do not employ chromatographic separation before MS are prone to losses in ion production as the ion source becomes dirty (contains chemicals other than the analyte of interest) (Kidwell 1993). Therefore, the ideal extraction method for solid-probe MS/MS should not lead to the destruction of the hair.

The first sample set involved base extraction of hair using 0.1 *N* sodium hydroxide. Figure 5 shows a hair with clearly visible scales on the cuticle before any treatment. Examination of the same hair after only 30 minutes in the base extraction media (figure 6)

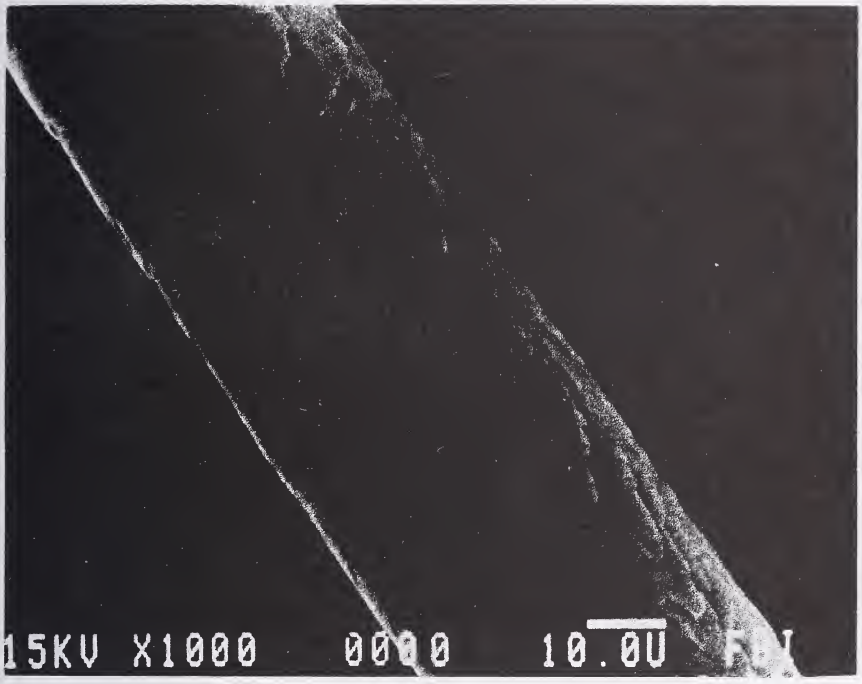

FIGURE 5. *Hair before treatment with sodium hydroxide (see figure 6 for posttreatment effects)*

KEY: 15kV=electron beam energy; × 1,000=magnification; 0000=picture counter reset to zero; 10.0μ=bar giving dimensions; FBI=Federal Bureau of Investigation

indicates extensive damage because the scales were removed by the caustic solution. In addition, an inherent disadvantage of using a strong base for the extraction is the decomposition of cocaine to BZE.

Enzyme digestion was studied using proteinase K (pronase) for the release of analytes from the matrix. Figure 7 shows a hair prior to digestion. The photomicrograph in figure 8 reveals that most scales were destroyed by the enzyme in 30 minutes. However, compared

FIGURE 6. *Hair after 30 minutes of treatment with sodium hydroxide*

KEY: 15kV=electron beam energy; × 1,000=magnification; 0000=picture counter reset to zero; 10.0μ=bar giving dimensions; FBI=Federal Bureau of Investigation

with the base digestion, the enzyme does not decompose the keratin as quickly.

The final experiment was the 1 *N* HCI extraction of hair. The scales are clearly visible on the same hair before (figure 9) and after (figure 10) treatment with acid for 30 minutes. Apparently, hydrochloric acid (HCl) selectively removes analytes from the protein matrix (without destruction) after swelling of the hair

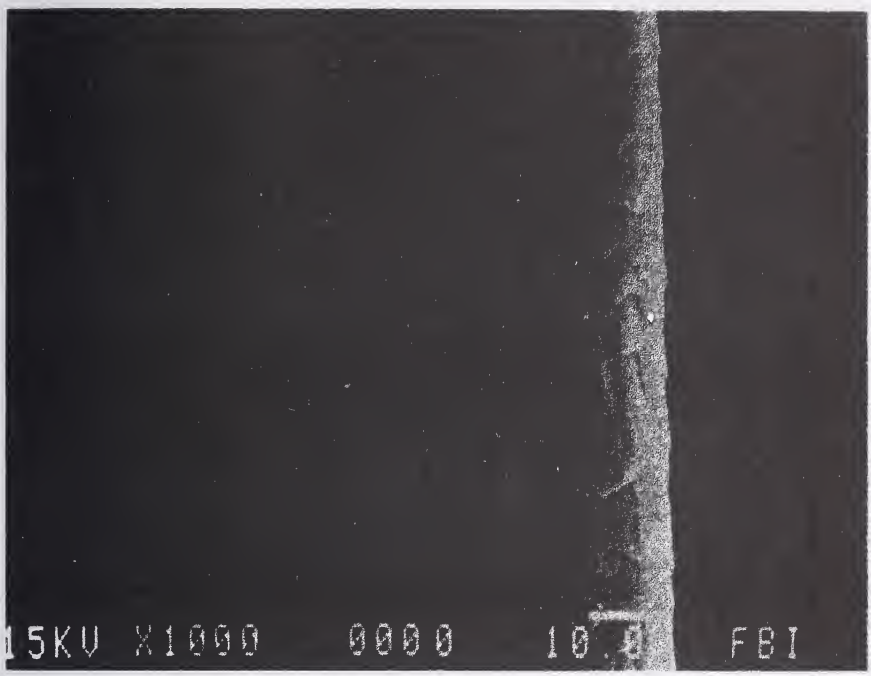

FIGURE 7. *Hair before treatment with proteinase K (pronase) (see figure 8 for posttreatment effects)*

KEY: 15kV=electron beam energy; × 1,000=magnification; 0000=picture counter reset to zero; $10.0\overline{\mu}$=bar giving dimensions; FBI=Federal Bureau of Investigation

structure. Once the solution makes contact with the hair matrix, the cocaine is highly soluble in HCl.

Swelling of the hair is obvious after 30 minutes of treatment by base, enzyme, or acid in the three sets of photomicrographs. SEM images of the hair after longer periods of treatment illustrate the complete destruction of the keratin by base and enzyme, but not by acid. Therefore, cleaner extracts are more probable by acid treatment than by base or enzyme treatment because protein

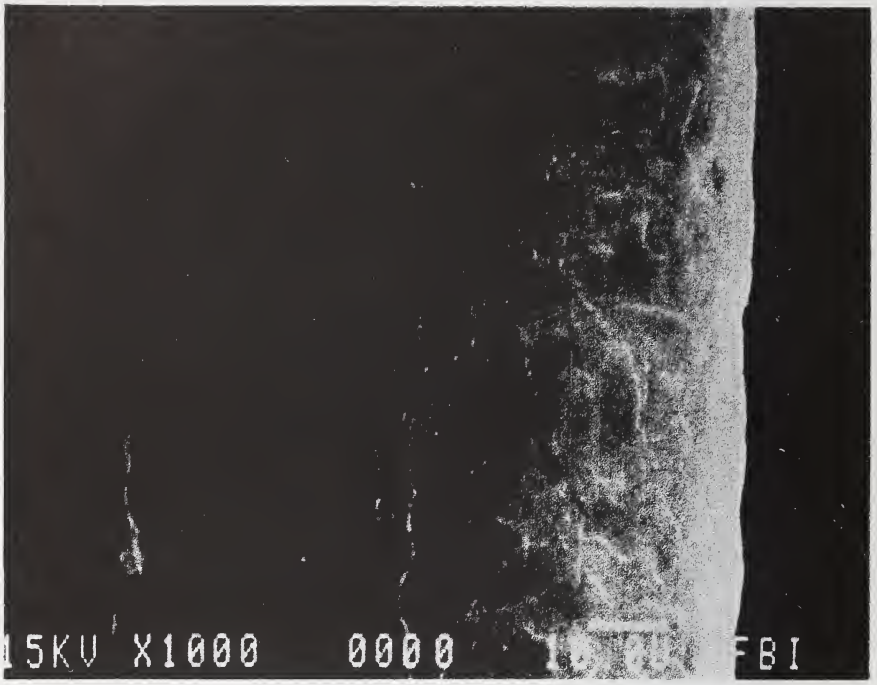

FIGURE 8. *Hair after treatment for 30 minutes with proteinase K (pronase)*

KEY: 15kV=electron beam energy; × 1,000=magnification; 0000=picture counter reset to zero; 10.0μ=bar giving dimensions; FBI=Federal Bureau of Investigation

fragments and other extraneous compounds from the degradation of the hair matrix are not generated in quantities as large by acid treatment as would result from complete disintegration (i.e., base or enzyme digestion).

OPIATE ANALYSIS IN HAIR

Current research at the FBI for drugs of abuse in hair focuses on opiates because, after marijuana, they are the most common type

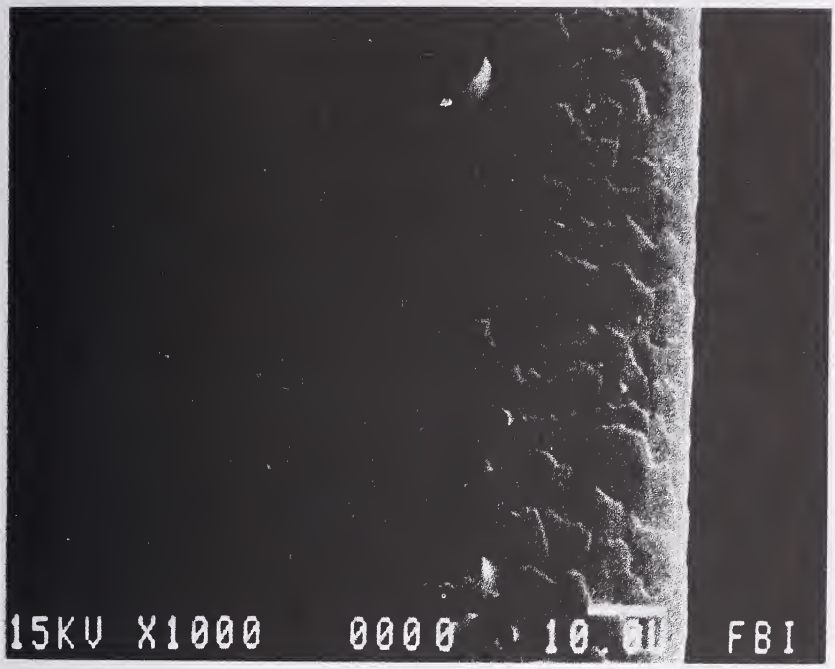

FIGURE 9. *Hair before treatment with hydrochloric acid (see figure 10 for posttreatment effects)*

KEY: 15kV=electron beam energy; × 1,000=magnification; 0000=picture counter reset to zero; $10.0\overline{\mu}$=bar giving dimensions; FBI=Federal Bureau of Investigation

of drug casework. Indicators for marijuana in hair are not being pursued because of their low concentration and because tetrahydrocannabinol (THC) metabolites can still be detected in urine up to a month after marijuana use. The analysis of opiates in hair is different from that of cocaine because the finding of morphine alone in a subject's hair does not prove heroin use. Morphine is not only a heroin metabolite but also a metabolite of the legal drug codeine and an alkaloid in poppy seeds. Therefore, a defendant could claim a "poppy seed defense" to escape conviction (Selavka 1991). To

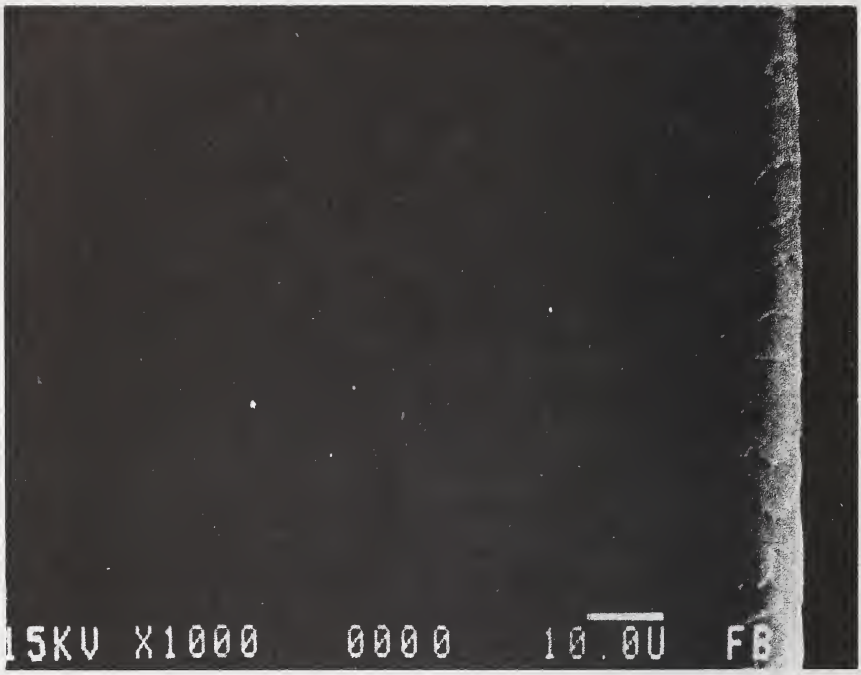

FIGURE 10. *Hair after 30 minutes treatment with hydrochloric acid*

KEY: 15kV=electron beam energy; × 1,000=magnification; 0000=picture counter reset to zero; $10.0\underline{\mu}$=bar giving dimensions; FBI=Federal Bureau of Investigation

conclusively prove heroin use from hair, it is necessary to determine heroin's major metabolite, 6-monoacetylmorphine (MAM), or heroin itself.

Morphine has been detected at the FBI in a manner similar to the cocaine-detecting technique using solid-probe electron impact MS/MS. However, the concentration of morphine in the hair of heroin users tends to be lower than the concentration of cocaine (Möller et al. 1992; Cone et al. 1991; Goldberger et al. 1991). For

the morphine analysis, a Toxi-tube A extract after HCl extraction was used, and the MS/MS was set to scan for neutral loss of 123 daltons over ions 285 (morphine) and 288 (morphine-d_3 internal standard) (figure 11). The analysis is more difficult with this technique because the morphine evolves gradually from the solid probe and at a lower concentration. The result is a broad and diffuse peak of morphine that is not easy to detect and quantitate in the background (figure 12). Another weakness of this method is the lack of a confirming metabolite for morphine compared with the use of BZE for cocaine because MAM and heroin are destroyed by acid extraction.

An alternative to the determination of opiates by solid-probe MS/MS is the more common use of gas chromatography/mass spectrometry (GC/MS). This method was studied using opiate-soaked hair that was prepared using procedures established by NIST (M. Welch, personal communication, November 16, 1992). Opiate-soaked hair was prepared by placing hair in dimethyl sulfoxide (DMSO) solutions of morphine or heroin at a concentration of 0.5 mg/mL for 5 months.

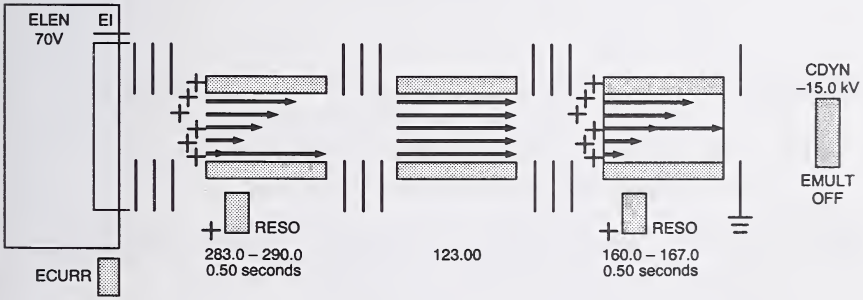

FIGURE 11. *Tandem mass spectrometry analysis for morphine*

KEY: EI=electron impact; ELEN=electron energy; ECURR=electron current; RESO=resolution; CDYN=conversion dynode; EMULT=electron multiplier

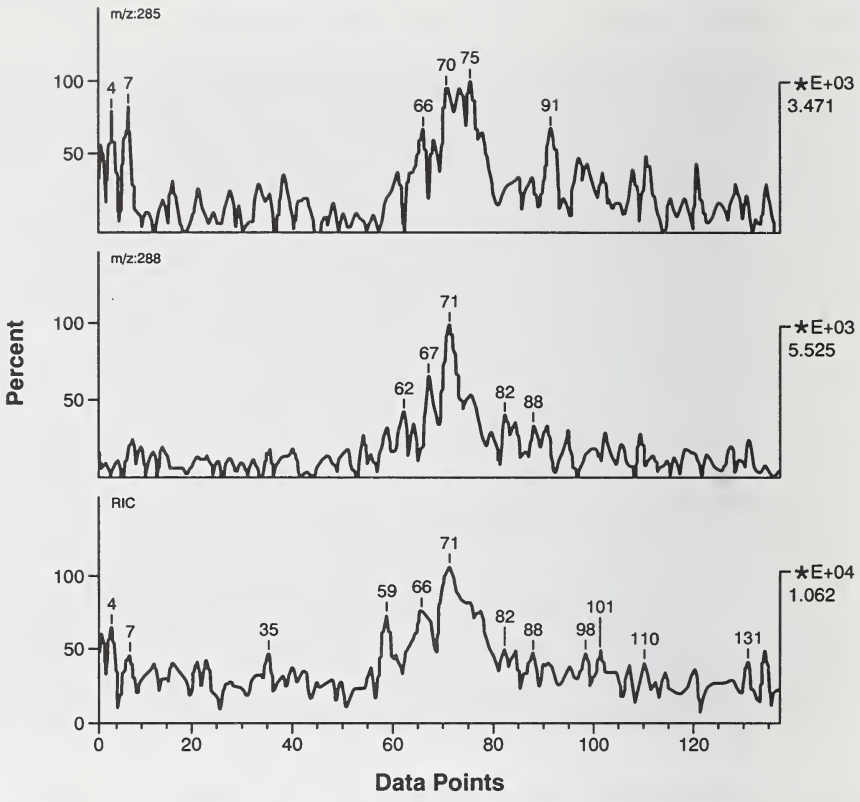

FIGURE 12. *Tandem mass spectrometry chromatograms for hair extract containing morphine (ion trace 285), internal standard (ion trace 288), and reconstructed ion chromatogram*

KEY: m/z:304 = ion monitored for detection of cocaine; m/z:307 = ion monitored for detection of deuterated cocaine; RIC = reconstructed ion chromatograph; * = full-scale intensity of signal for the chart; E+ = notation for 10 to the power of the number following (e.g., 03, 04) times the number below (e.g., 3.471, 1.062)

Following the method of Goldberger and colleagues (1991), two sets of opiate hair (one of heroin and one of morphine) and a blank hair

were extracted overnight with methanol containing 100 ng morphine-d_3 internal standard. After the liquid-liquid solvent extraction, the extract residue was derivatized with N-methylbis-(trifluoroacetamide) (MBTFA) for GC/MS analysis. A visual comparison of the total ion chromatograms of derivatized opiate standards and blank hair indicated that most peaks were from the MBTFA reagent and that there were coeluting peaks interfering with the determination of morphine (figure 13). Combined ion traces (364+369 daltons) for the opiate hair samples revealed the presence of morphine in both samples and MAM and heroin in the heroin hair sample (figure 14). Quantitation of the morphine hair indicated a high level of the drug at 237 ng/mg hair. Results of the heroin hair analysis revealed that most heroin ended up via decomposition as MAM at a concentration of 160 ng/mg hair. A considerable amount of morphine also was found in the hair at 11.1 ng/mg, whereas only a small fraction of the total opiate was the parent heroin at 2.4 ng/mg hair. The opiate levels in both synthetic hair samples were much higher than actual concentrations reported in the literature, which are about 1 ng/mg hair or less (Goldberger et al. 1991). The high-percentage conversion of heroin to MAM and morphine in the result for the heroin hair brought the analysis procedure into question. For example, the MBTFA-treated morphine-d_3 and heroin standards chromatogram (figure 14) exhibited a peak for MBTFA-derivatized MAM at a level approximately one-fourth the size of the heroin peak. High-performance liquid chromatography (HPLC) analysis of the heroin standard before derivatization did not indicate the presence of MAM. Therefore, the MBTFA-derivatized MAM peak must have been a heroin degradation product of the MBTFA treatment. It is desirable to use an analysis procedure that is not a major contributor to degradation of a drug species present in the hair.

ELECTROSPRAY IONIZATION AND ATMOSPHERIC PRESSURE CHEMICAL IONIZATION FOR HAIR ANALYSIS

In the search for new or alternative instrumentation and techniques that are more sensitive or less dependent on additional time-consuming derivatization steps, electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI) are being

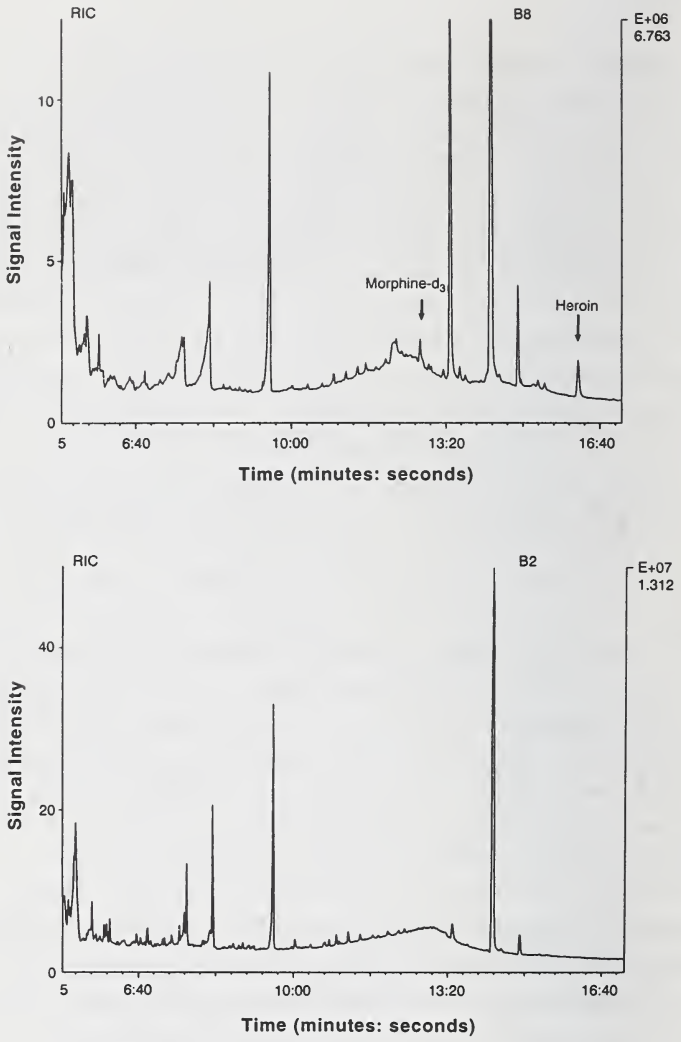

FIGURE 13. *Reconstructed ion chromatograms of N-methylbis-(trifluoroacetamide)-derivatized opiate standards and blank hair extracts*

KEY: MBTFA = N-methylbis(trifluoroacetamide); morphine-d_3 = trideuterated morphine; RIC = reconstructed ion chromatogram; B8 = magnification power of 8; E+ = 10 to the power of the number following (e.g., 06, 07) times the number below (e.g., 6.763, 1.312); B2 = magnification power of 2

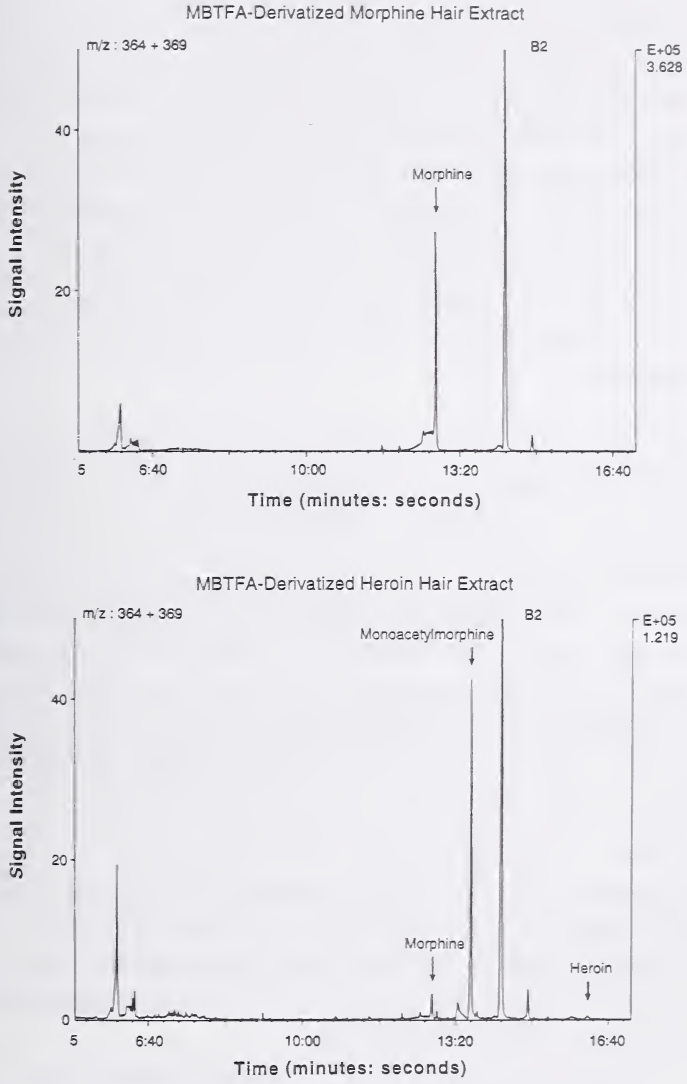

FIGURE 14. *Ion chromatograms of N-methylbis-(trifluoroacetamide)-derivatized morphine and heroin hair extracts*

KEY: MBTFA = N-methylbis(trifluoroacetamide); B2 = magnification power of 2; m/z = mass to charge ratio; E+05 = 10^5 times the number below (e.g., 3.628, 1.219)

investigated as mass spectral techniques for the analysis of hair. Both MS-sample-introduction techniques permit ionization of aqueous or organic effluents at atmospheric pressure and are thus suitable for HPLC/MS or flow injection analysis (FIA) of biological extracts. FIA/MS is similar to HPLC/MS except that no chromatographic separation column is used with FIA/MS. ESI generates analyte ions under the influence of a high electric field between a spray nozzle and a heated capillary sampler inlet. In APCI, sample molecules are ionized by reagent ions in a plasma that is formed by a corona discharge needle. The predominant product species of both techniques are protonated molecular ions with little decomposition.

Several drugs of abuse were analyzed by FIA ESI/MS (figure 15) and APCI/MS (figure 16) at a level of 100 ng or less without instrumental or mobile-phase composition (the liquid effluent) optimization. The ESI and APCI interfaces were recently acquired by the FBI laboratory. As experience is developed with these new techniques, it is expected that gains will be made in sensitivity. Using data acquired by scanning a wide range of masses instead of looking for an ion in one mass, each of the drugs exhibited a high signal intensity for the monoprotonated molecular ion by each technique. Therefore, each drug has the potential for MS/MS and selected reaction monitoring to increase detection specificity. In addition, the intensities were reproducible and should be quantifiable. With optimization of the instrumental conditions, mobile phase, and scan range (or selected ion monitoring) for specific drugs, the detection limits should reach into the subnanogram range with limited sample preparation. These techniques are bright future prospects for the direct analysis of hair extracts without derivatization.

FUTURE DIRECTIONS

The direction of research in keratin analysis at the FBI is a broad topic and encompasses samples other than hair. For example, hair and chitin are both keratin biopolymers and can be examined for drugs of abuse by similar procedures. This has been demonstrated by the analysis of beetles and fly puparial cases for amitriptyline and

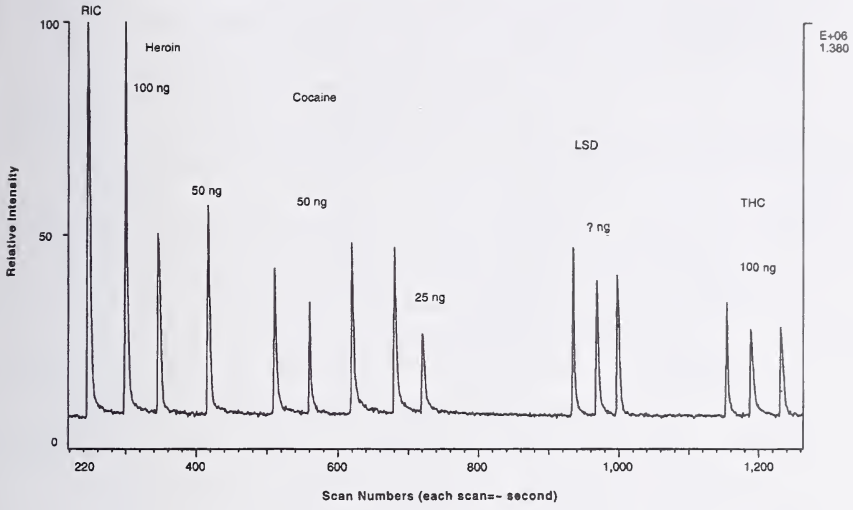

FIGURE 15. *Electrospray ionization/mass spectrometry chromatogram of drugs of abuse*

KEY: RIC=reconstructed ion chromatogram; LSD=lysergic acid diethylamide; THC=tetrahydrocannabinol; E+06=10^6 times the number below (1.380)

nortriptyline using acid or base extraction and GC/MS (Miller et al. 1993, pp. 859a-859b). Drugs were detected at the ng/mg-of-chitin level in insect parts found on human remains and in controlled laboratory studies.

Another area of keratin analysis for drugs that has not been well explored is research on fingernails and toenails. Nails offer an alternative site for sampling a subject. To date, one of the few drugs to have been detected in nails is methamphetamine (Suzuki et al. 1989). The FBI is in the process of collecting hair and nail samples for correlating drug analysis results from these types of specimens.

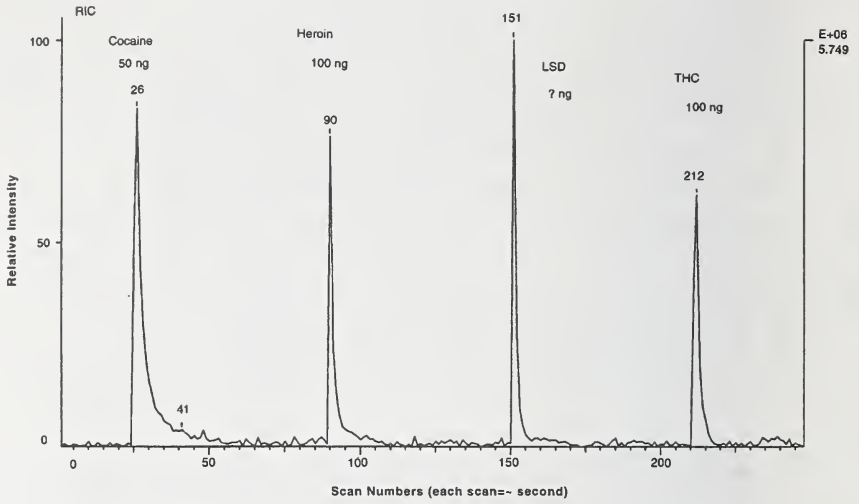

FIGURE 16. *Atmospheric pressure chemical ionization/mass spectrometry chromatogram of drugs of abuse*

KEY: RIC=reconstructed ion chromatogram; LSD=lysergic acid diethylamide; THC=tetrahydrocannabinol; E+06=10^6 times the number below (5.749)

A more immediate goal for research at the FBI is to develop and validate a method for the analysis of opiates in hair. The critical point of this procedure will be to distinguish the abuse of heroin from the ingestion or use of other morphine-related substances.

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Hair Analysis for Opiates in Forensic Cases

Manfred R. Moeller, Peter Fey, and Hans Sachs

INTRODUCTION

Hair analysis for drugs of abuse is an ideal addendum to blood testing and urinalysis to prove or disprove drug use. Hair analysis cannot pinpoint a date of drug consumption, but it can reveal information in addition to that generally available through analysis of either urine or blood.

This chapter focuses on opiates because heroin abuse has been the most significant drug problem in most European countries, including Germany.

In 1986 the first procedure for the detection of morphine and codeine in hair specimens by gas chromatography/mass spectrometry (GC/MS) was described by Sachs and Brunner (1986). Earlier radioimmunological methods were not specific enough to differentiate between heroin or morphine consumption and codeine use. Both codeine and morphine can be found in most positive cases. In cases of heroin or morphine use, higher concentrations of morphine than codeine can be expected because morphine is the main metabolite of heroin. However, codeine is also detectable because it is present as a by-product in street heroin samples. In cases of codeine use, higher concentrations of codeine than morphine can be found because only a small amount of codeine (5 to 13 percent) is metabolized to morphine (Adler et al. 1955).

A significant advancement in proving heroin use was the detection of 6-monoacetylmorphine (MAM) in hair (Tagliaro et al. 1987, pp. 115-127; Raff et al. 1991; Goldberger et al. 1991). Other semisynthetic opiates, like dihydrocodeine (DHC) and pholcodine, have been found in hair, and procedures for analysis have been developed (Sachs et al. 1993; Maurer and Fritz 1990). DHC abuse is a serious problem in Germany because many addicts treat their heroin withdrawal symptoms with dihydrocodeine therapy. This substitute drug can be obtained on the illicit drug scene or by physician prescription, mostly from general practitioners who regard DHC as an alternative to methadone.

GC/MS is the method of choice for analyzing hair specimens for drugs of abuse because of its sensitivity and specificity (Moeller 1992). Immunological methods are not satisfactory because they are able to detect only classes of drugs as opposed to single substances. Furthermore, with regard to immunological techniques, each class of drugs requires a special procedure, and specimens must be divided into several fractions, which diminishes the amount of material available for each immunoassay. Because a positive immunological result must be confirmed by GC/MS, depending on the number of positive cases, starting with GC/MS may be the more practical solution.

Several GC/MS methods have been described for the detection of various drugs (Moeller 1992). One procedure, which has already been used for routine determinations (Möller et al. 1992; Moeller et al. 1993), is presented here.

All hair samples were quantitatively analyzed for MAM, codeine, DHC, and morphine. The additional analysis for amphetamine, benzoylecgonine (BZE), cocaine, and methadone and its metabolite EDDP (dl-2-ethyl-1,5-dimethyl-3,3-diphenylpyrrolinium) is also possible using the same procedure (Moeller et al. 1993).

MATERIALS

Chemicals and Equipment

All reagents were analytical grade. The substances used were MAM, codeine, DHC, morphine, MAM-d_3, codeine-d_3, morphine-d_3, pentafluoropropionic anhydride (PFPA), pentafluoropropanol (PFPOH), and ß-glucuronidase (30 u/mL)/arylsulfatase (20 u/mL).

The solid-phase extraction was carried out with extraction columns (200 mg, 3 mL) and a Vac-Elut SPS 24 system. A ball mill (type MM2) also was used.

Instrumentation

The following Hewlett Packard instruments were used: 7673A automatic sampler, 5890 series II gas chromatograph, 5971A mass selective detector, Vectra QS/16S Workstation, and G1034B software.

The gas chromatograph was equipped with a capillary column (cross-linked 5 percent phenylmethyl silicone, 12 m × 0.2 mm × 0.33-μm film thickness, temperature programmed from 70 °C [1 minute hold] to 155 °C at 30 °C/minute, to 240 °C at 10 °C/minute, and to 300 °C at 30 °C [5 minutes hold]), directly inserted into the ion source. The injector temperature was 260 °C; the GC/MS interface temperature was 280 °C; and the helium carrier gas flow rate was 2 mL/minute. The mass spectrometer was operated in the electron impact (EI) mode with electron energy of 70 eV.

Sample Collection

The hair samples were obtained on orders of police, courts, driver's license authorities, and drug treatment centers. The specimens were taken by cutting the hair as close as possible to the scalp and were fixed with adhesive tape with the root directions and tip directions marked. A band of hair about half the diameter of a pencil was taken with each sampling. Blank control hair samples were obtained

from five coworkers of the authors' institutes, which were analyzed for the absence of opiates by GC/MS prior to use.

METHODS

Extraction and Derivatization for Gas Chromatography/Mass Spectrometry Analysis

The locks of hair were fixed with strings so that the roots (cut ends) of the single strands remained aligned. The hair was washed with warm water (5 minutes) and acetone (1 minute) to eliminate external contaminants and then dried in a stream of warm air. The locks of hair were cut into segments of 2 cm, and the segments were pulverized separately in a ball mill.

To 10 to 30 mg pulverized hair were added 2 mL phosphate buffer (pH 7.6) and 100 ng trideuterated standards (MAM-d_3, codeine-d_3, and morphine-d_3). The sample was hydrolyzed with 75 μL ß-glucuronidase/arylsulfatase for 2 hours at 40 °C. After centrifugation, the supernatant was removed to a clean vessel, and 2 mL phosphate buffer were added to the residue, shaken, and centrifuged and the supernatant removed. A solid-phase extraction column was conditioned by sucking through 6 mL methanol and then 3 mL distilled water. The two buffer fractions were combined, transferred to the column, and drawn through. The column then was washed consecutively with 3 mL distilled water, 3 mL 0.6 M sodium bicarbonate, and 3 mL distilled water and was dried by passing air through for 10 minutes and centrifuging at 4,000 u/minute for 15 minutes. The adsorbed drugs were eluted with 3×500 μL acetone/dichloromethane (3:1).

The eluent was evaporated to dryness under a stream of nitrogen at 60 °C. The residue obtained was derivatized with 100 μL PFPA and 70 μL PFPOH for 30 minutes at 60 °C. The mixture was dried again under nitrogen at 60 °C and reconstituted in 30 μL ethyl acetate.

Gas Chromatography/Mass Spectrometry Analysis

Aliquots (2 μL) of the derivatized sample were injected into the GC/MS system. The mass spectrometer was operated in the selected ion monitoring (SIM). Table 1 shows the retention indices that were determined according to a published method (Deutsche Forschungsgemeinschaft Commission for Clinical-Toxicological Analysis 1992) and the ion masses monitored for the pentafluoropropionic (PFP) derivatives of MAM, morphine 2, codeine, DHC, and the corresponding trideuterated compounds. Because a useful separation of all analyzed substances in one GC/MS run was not possible with any of the columns (cross-linked methyl silicone and 5 percent phenylmethyl silicone gum phases; column lengths 12 and 25 m, respectively), each sample was analyzed twice. With the selected column, the mass spectrometer was adjusted in the first run to the masses of morphine, codeine, and MAM. DHC was measured in the second run because it was eluted close to morphine. The mass 448 of deuterated codeine was used for quantification of DHC because deuterated DHC was not commercially available.

TABLE 1. *Selected ions and retention indices used for identification of monoacetylmorphine (MAM) pentafluoropropionic (PFP), morphine 2 PFP, codeine PFP, and dihydrocodeine (DHC) PFP (underlined ions used for quantification)*

		Ion	
Substance	Retention Index	Undeuterated	Deuterated
MAM PFP	2,433	<u>414</u> 473	<u>417</u> 476
Morphine 2 PFP	2,334	<u>414</u> 577	<u>417</u> 580
Codeine PFP	2,366	<u>445</u> 282	<u>448</u> 285
DHC PFP	2,355	<u>447</u> 284 390	<u>448</u>*

*The mass 448 of deuterated codeine was used to quantitate DHC because deuterated DHC is not commercially available.

Calibration curves were obtained in double runs through the described analysis using 10, 50, 100, 250, and 500 ng of the standards (MAM, morphine, codeine, and DHC); 100 ng corresponding trideuterated compounds as internal standards; and 20 mg pulverized blank control hair (corresponding to 0.5, 2.5, 5.0, 12.5, and 25.0 ng/mg hair).

The limit of detection (LOD) and limit of linearity (LOL) were determined (n=6) from methods given by the International Union of Pure and Applied Chemistry (Long and Wineforder 1983). To 20 mg pulverized blank control hair, 100 ng deuterated standards were added; extraction was followed by derivatization. For each substance, the standard deviation of the blank value ($\bar{x}_B$) was determined. The LOD was defined as $\bar{x}_B + 3s_B$ and the LOL as $\bar{x}_B + 10s_B$.

RESULTS

Identification

The EI mass spectra of the PFP derivatives are shown in figures 1a (MAM), 1b (morphine 2 PFP), 1c (codeine), and 1d (dihydrocodeine). Figures 2a through 2d show the SIM chromatograms of the PFP derivatives of MAM, morphine 2, codeine, and dihydrocodeine, respectively. The extracts are from drug users' hair samples.

Quantitation

Table 2 shows the correlation coefficients (*r*) of the calibration curves and LOD and LOL for each substance.

The possible conversion of MAM to morphine during the extraction procedure was studied by adding 100 ng MAM to 20 mg pulverized blank control hair followed by extraction. Before derivatization, 100 ng internal standards were added, and the derivatized mixture was analyzed by GC/MS. Two percent of MAM was converted to morphine. The determinations were performed twice.

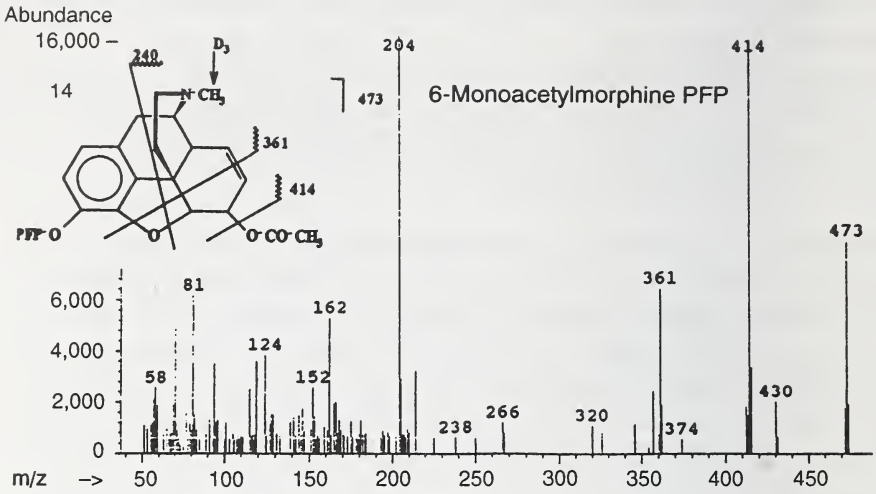

FIGURE 1a. *Electron mass spectrum of the pentafluoropropionic derivative of 6-monoacetylmorphine*

TABLE 2. *Correlation coefficient (r), x_B, standard deviation $\bar{x}_B$, limit of detection, and limit of linearity for codeine pentafluoropropionic PFP, dihydrocodeine (DHC) PFP, monoacetylmorphine (MAM) PFP, and morphine 2 PFP*

Substance	Correlation Coefficient (*r*)	x_B (ng/mg)	Standard Deviation $\bar{x}_B$ (ng/mg)	Limit of Detection (ng/mg)	Limit of Linearity (ng/mg)
Codeine PFP	0.9995	0.02	0.006	0.04	0.06
DHC PFP	0.9995	0.02	0.006	0.04	0.06
MAM PFP	0.9998	0.1	0.02	0.16	0.3
Morphine 2 PFP	0.9999	0.02	0.006	0.04	0.08

Recovery

To 20 mg control hair, 100 ng internal standards were added and the sample treated as described above. Prior to the derivatization, 100 ng internal standards were added, and the specimens were

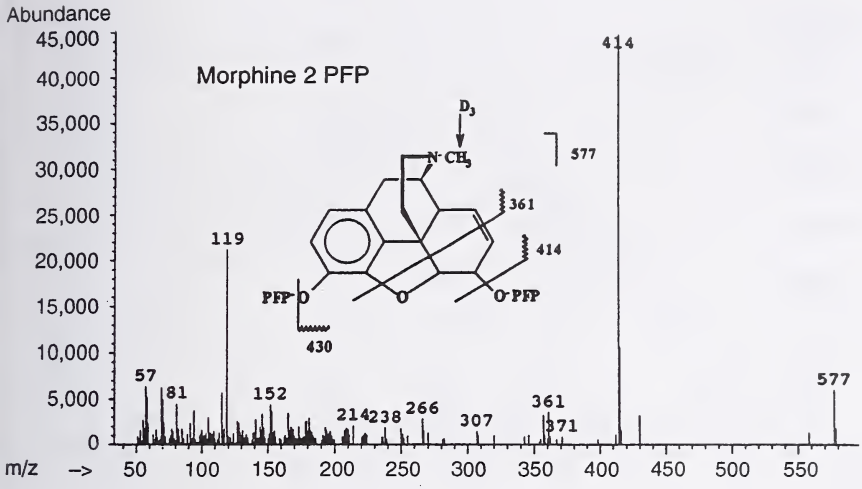

FIGURE 1b. *Electron mass spectrum of the pentafluoropropionic derivative of morphine 2*

analyzed by GC/MS. The peak areas of the ions used for quantitation were compared. The analyses were performed five times. The mean recoveries and the corresponding coefficients of variation were as follows: MAM PFP, 70 percent (6 percent); morphine 2 PFP, 70 percent (5 percent); and codeine PFP, 80 percent (3 percent). Deuterated DHC was not commercially available; therefore, the analytical data of codeine were used for the quantitation of DHC.

Reproducibility

A mixture was made of pulverized hair samples from several individuals, and various amounts (7.6 to 50.6 mg) of the mixture were analyzed (n=5) for codeine PFP and morphine 2 PFP. The standard deviations and the coefficients of variation are shown in table 3.

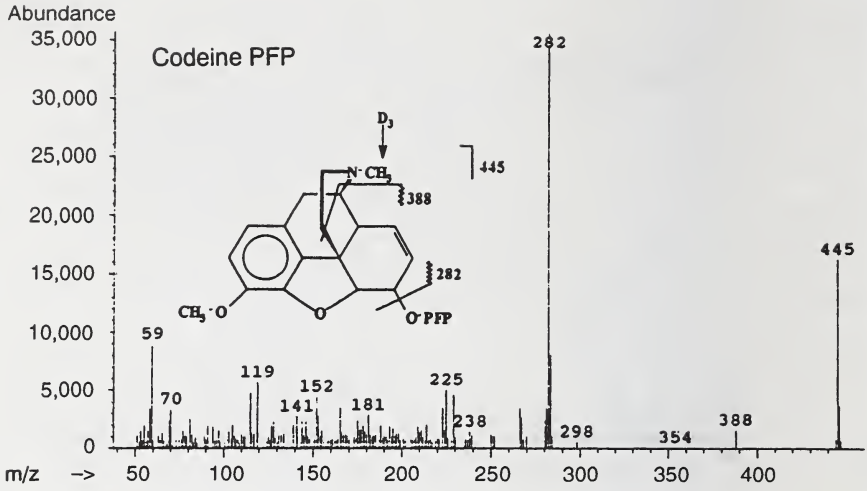

FIGURE 1c. *Electron mass spectrum of the pentafluoropropionic derivative of codeine*

TABLE 3. *Standard deviations and coefficients of variation*

Substance	Analyzed Amount (ng/mg)	Standard Deviation (ng/mg)	Coefficient of Variation (%)
Codeine PFP	1.7	±0.2	±12%
Morphine	7.7	±0.3	±4%

KEY: PFP=pentafluoropropionic

Washing Procedure

To investigate the amount of MAM/morphine that can be washed out during the described procedure, several unpulverized hair samples from drug users were divided into two parts. One part was extracted unwashed, and the other was washed three times alternately with warm water and acetone. The three aqueous

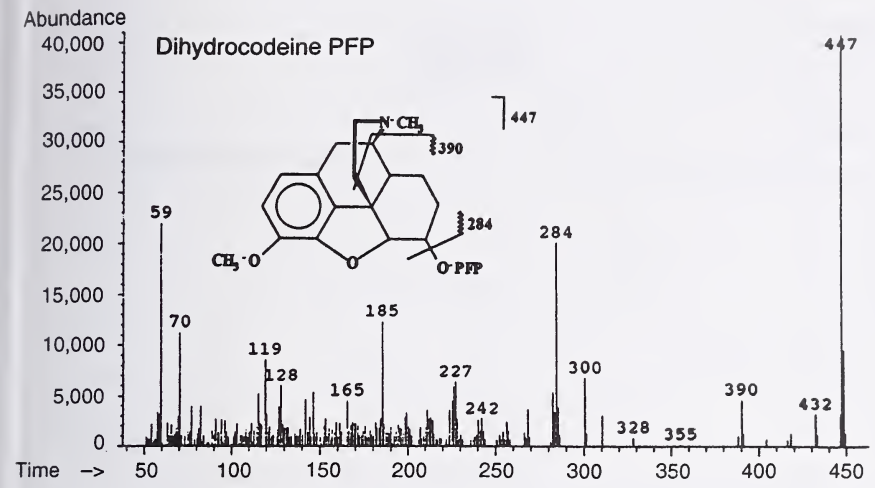

FIGURE 1d. *Electron impact mass spectrum of the PFP derivative of dihydrocodeine*

SOURCE: Moeller, M.R., Fey, P.J., and Wennig, R., 1993. Copyright 1993 by Elsevier Science Ireland Ltd. (Limerick).

solutions and the unwashed and washed hair samples were extracted and analyzed according to the described procedure. The acetone phases were evaporated to dryness. After addition of the internal standards, the residues were derivatized and analyzed as described. The three washings extracted about 5 percent morphine and 15 percent MAM. Between 70 and 95 percent of the extracted material was in the first aqueous solution. Only traces of the substances could be found in the acetone phases.

CASE EXAMPLES

In most cases arising from police or court orders, the subjects were prosecuted for selling drugs. The suspects may have confessed to buying drugs only to satisfy their own addictions. In these cases,

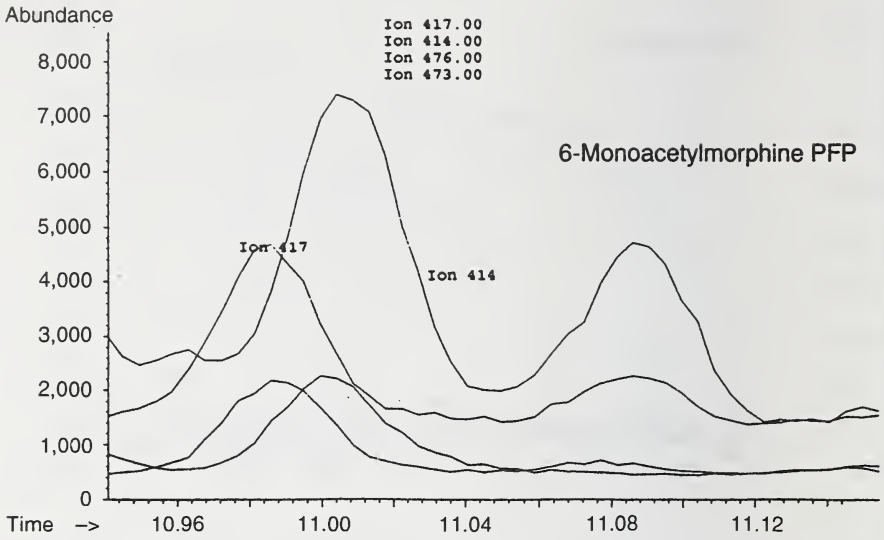

FIGURE 2a. *Selected ion monitoring chromatogram of the pentafluoropropionic derivative of 6-monoacetylmorphine and its trideuterated analog*

they normally reported high consumption rates. Such statements cannot be scientifically validated in cases where only low morphine concentration in hair had been found, that is, below the mean value of 6.7 ng/mg (table 4).

In a 1991 court case, a suspect accused of possessing heroin asserted that the heroin was for her own use (figure 3). The sectional hair analysis showed a high MAM value for the time in question (section 12 to 14 cm)—the time before she was imprisoned. The sections closer to the root showed decreasing concentrations due to the dormant hair effect. Her statement was in accordance with the analytical results and could therefore be scientifically confirmed.

A different result can be seen in a case (figure 4) where a man accused of robbery claimed that he was addicted to heroin at the

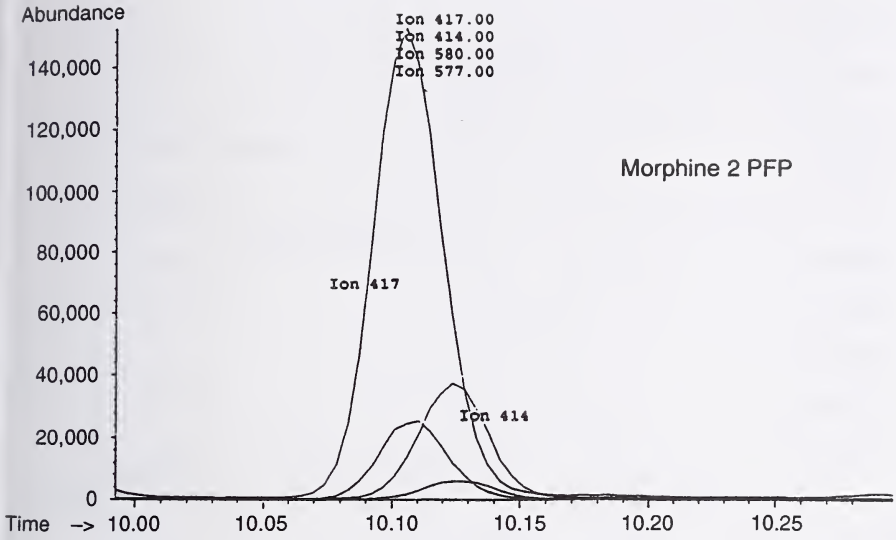

FIGURE 2b. *Selected ion monitoring chromatogram of the pentafluoropropionic derivative of morphine 2 and its trideuterated analog*

TABLE 4. *Results of hair analysis for opiates from January 1989 until December 1991. Evaluated as heroin abuse were cases with a morphine-to-codeine ratio of 2:1 for morphine levels greater than 1 ng/mg and 5:1 for levels between 0.1 and 1 ng/mg hair.*

Number of analyzed segments	1,184
Number of morphine-positive segments (> 100 pg/mg)	762
Number of segments evaluated as heroin consumption	499

Morphine Concentration	(ng/mg)
Mean	6.7
Median	2.7
Maximum	86.5

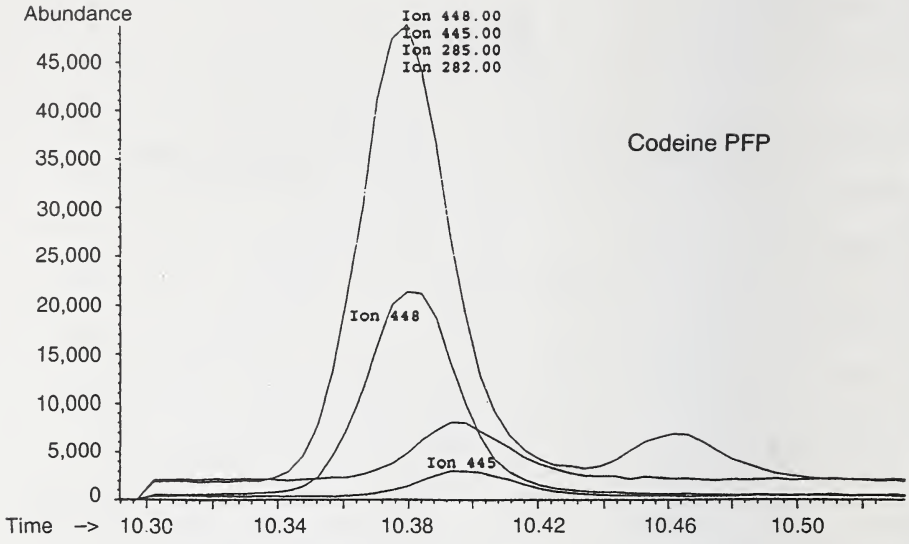

FIGURE 2c. *Selected ion monitoring chromatogram of the pentafluoropropionic derivative of codeine and its trideuterated analog*

time he committed the crime. A hair sample was taken 6 months after the crime was committed, cut into segments, and analyzed for opiates. In the segments from 4 to 9 cm from the root, no opiates could be detected. The concentration in the other segments supported a low, but not more than moderate, use during the time. During the trial, the man was confronted with the result of the hair analysis and subsequently confessed that he had not used heroin at the time of the robbery.

The sectional hair analysis of a heroin addict who was treated with dihydrocodeine therapy by a general practitioner is shown in figure 5.

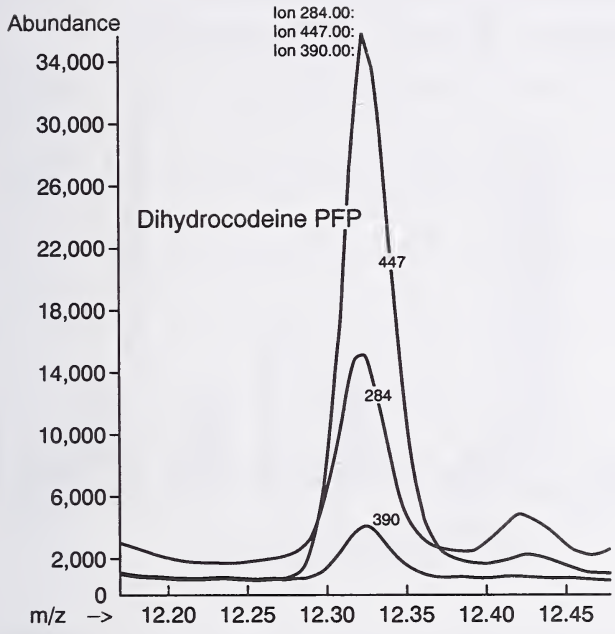

FIGURE 2d. *Selected ion monitoring chromatogram of the pentafluoropropionic derivative of dihydrocodeine (no trideuterated analog was available for dihydrocodeine)*

SOURCE: Moeller, M.R., Fey, P.J., and Wennig, R., 1993. Copyright 1993 by Elsevier Science Ireland Ltd. (Limerick).

DISCUSSION

The data and case examples presented here demonstrate the usefulness of hair analysis as evidence in forensic cases. In cases of opiate use, some general recommendations for interpretation can be made. These recommendations are based on the authors' experiences and results after analyzing more than 1,000 hair segments. It is important to note that other procedures may give different results and different limits (Sachs and Raff 1993).

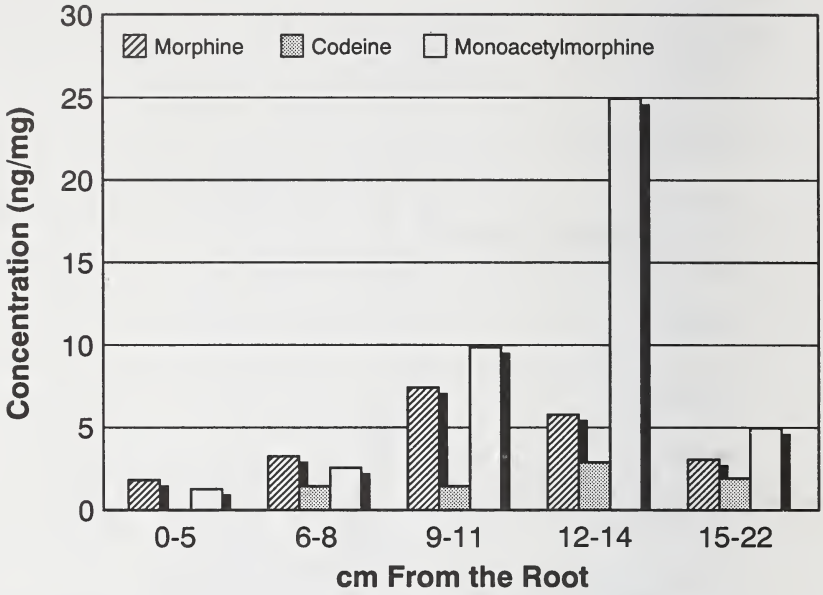

FIGURE 3. *Sectional analysis of hair sample from heroin dealer who confessed to personal heroin use before imprisonment (hair sample taken about 10 months after dealer was imprisoned)*

SOURCE: Moeller, M.R., Fey, P.J., and Wennig, R., 1993. Copyright 1993 by Elsevier Science Ireland Ltd. (Limerick).

In several cases, there was uncertainty as to whether the presence of morphine or codeine was from heroin use. Such cases were eliminated from discussion, and strict guidelines were set to interpret opiate concentrations found in hair (table 4). In cases where the total morphine in hair was less than 1 ng/mg, it was postulated that the morphine-to-codeine ratio must be at least 5:1 to indicate heroin or morphine use, whereas with more than 1 ng/mg morphine in hair, that a morphine-to-codeine ratio of 2:1 was necessary for heroin consumption. On the other hand, long-term or chronic

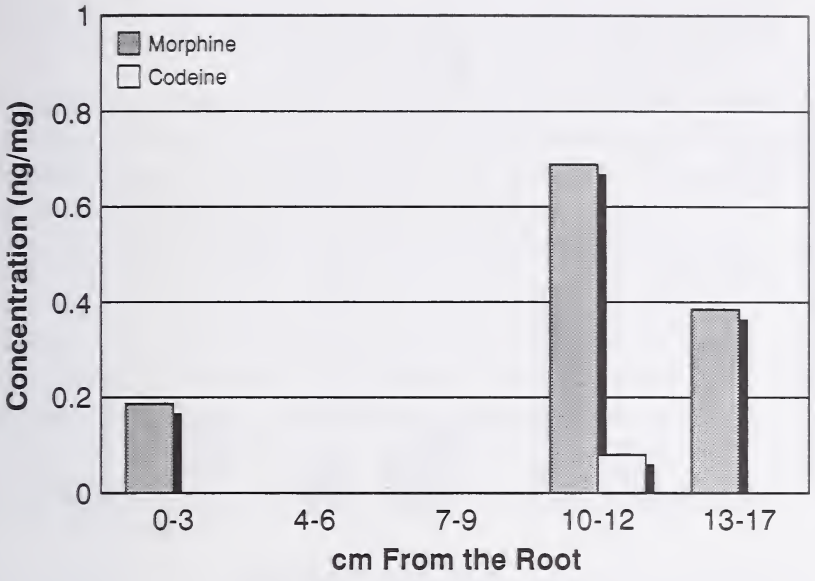

FIGURE 4. *Sectional analysis of hair sample from subject accused of robbery who was imprisoned several months later*

SOURCE: Moeller, M.R., Fey, P.J., and Wennig, R., 1993. Copyright 1993 by Elsevier Science Ireland Ltd. (Limerick).

heroin use was indicated when the total morphine concentration in hair exceeded the median value of 2.7 ng/mg.

Measurements of MAM in hair have simplified the problem of interpretation. Table 5 shows the relationship of MAM, morphine, and codeine and the ratios of MAM to morphine and MAM + morphine to codeine in 95 selected cases. Only hair segments with concentrations greater than 1 ng morphine/mg hair and where heroin use was confirmed by additional evidence (self-reports, urinalysis, needle marks, witness statements) are considered here. Heroin consumption is in accordance with a morphine concentration of at

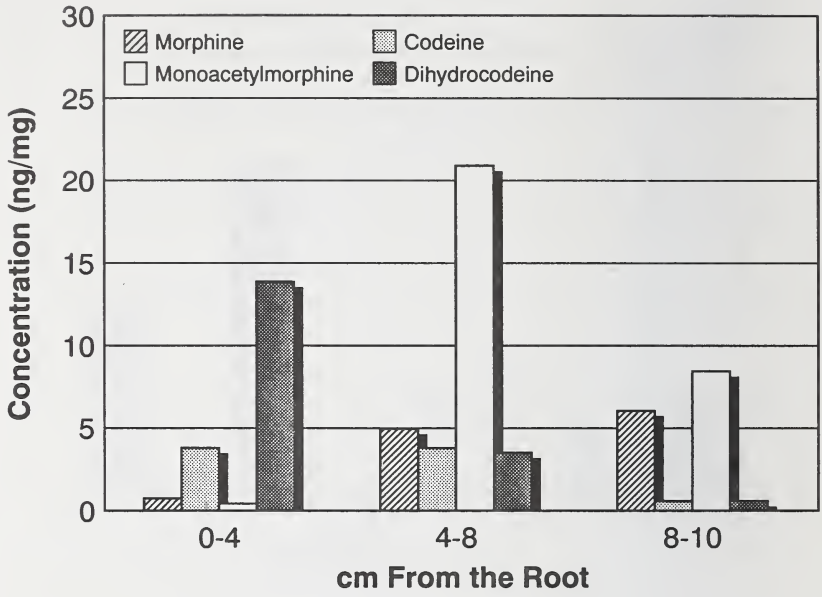

FIGURE 5. *Sectional analysis of hair sample from heroin addict treated with dihydrocodeine therapy*

SOURCE: Moeller, M.R., Fey, P.J., and Wennig, R., 1993. Copyright 1993 by Elsevier Science Ireland Ltd. (Limerick).

TABLE 5. *Ratios of monoacetylmorphine (MAM), morphine, and codeine in cases of heroin abuse (number of analyzed segments = 95)*

				Ratios	
Drug Concentration	MAM (ng/mg)	Morphine (ng/mg)	Codeine (ng/mg)	MAM: Morphine	MAM+Morphine: Codeine
Range	0-121.0	1.0-28.5	0-6.7	0-10.5	1.4-72.7
Median	7.0	3.5	0.9	2.3	12.8
Mean	14.6	6.5	1.6	2.4	14.7

least 1 ng/mg hair, together with at least 1 ng/mg MAM, and not more than 50 percent codeine in relation to the morphine value.

The ratio of MAM + morphine to codeine proved to be a better indicator of heroin use. Of the 95 cases, 69.5 percent had a ratio of between 6 and 25 for MAM + morphine to codeine. Twenty-five percent had ratios above 25 (< 147), and in only 5 percent of the cases, the ratio of MAM + morphine to codeine was less than 2 (1:1.7), which could be explained because of additional codeine use. In all 46 cases where concentrations of both morphine and codeine were greater than 1 ng/mg hair, the ratio of MAM + morphine to codeine was greater than 8:1 in 82.6 percent of cases. In the remaining cases, the ratio was between 2:1 and 6:1. Additional investigation is necessary before this relationship can be precisely established; however, heroin use cannot be concluded if MAM is not detectable.

MAM must be detectable in all cases where heroin use is suspected. Similar observations can be made with other drugs and their metabolites (e.g., cocaine, methadone, tetrahydrocannabinol [THC]). In these examples the parent drug or, in the case of heroin use, the first metabolite, is always found in the highest concentration.

Kidwell and Blank (1992, pp. 555-563) characterize hair as being an ion-exchange membrane that takes up cationic compounds (cocaine, amphetamine, opiates, phencyclidine [PCP]) better than anionic species, such as THC-carbocyclic acid or aspirin. However, the incorporation of drugs and their metabolites seems to be multifaceted where bioavailability, plasma elimination half-lives of the drugs, pK_a values, volume of distribution, and/or lipid solubility as well as the possible inhibition of the transport of the more polar compounds (metabolites) through cell membranes (Ellenhorn and Barceloux 1988) also must be taken into consideration. The binding of drugs in hair also seems to depend strongly on the drug structure. Enzyme hydrolysis gives a high yield for morphine (70 percent) and codeine (50 percent) but not for cocaine and BZE (Fey 1993).

Concentrations of morphine less than 1 ng/mg hair, which indicate infrequent use, are near the LOD (0.1 to 0.5 ng/mg hair) and should be interpreted carefully, even if the 5:1 morphine-to-codeine ratio is

met. In cases with low concentrations (< 1 ng/mg), often only the metabolite morphine is found, and in cocaine cases, only its metabolite BZE is found. One possible cause is the degradation of the parent compound to the corresponding metabolites; a second is that the less polar parent compounds do not bind strongly and are washed out more easily than their metabolites.

The highest value of morphine found was 86.5 ng/mg hair. About 100 ng/mg can be related to a maximal daily dose of 200 mg (Baselt and Cravey 1989). Thus, a range of approximately one use per week (with concentrations near the LOD) to several times a day can be seen. In forensic cases, the concentration of a drug found in hair can be located in this scale. Although the concentration found cannot relate precisely to the frequency of consumption, it can provide additional or useful information for the court.

More research is needed for more accurate interpretations, especially when sectional analysis is done and when low concentrations are found in hair, possibly stemming from passive contamination. Further work is in progress to answer some of these questions as well as including other drugs of abuse, with the goal of developing more precise guidelines.

SUMMARY

Because hair analysis can be used for the determination of drug use months after drug consumption, hair analysis data often can provide important and even decisive evidence in the courtroom. Recently developed GC/MS methods offer excellent sensitivity and specificity that were not possible by earlier radioimmunoassay techniques. This chapter describes a method for the simultaneous detection and quantitation of MAM, codeine, DHC, and morphine in hair. The hair samples were washed, cut into 2-cm segments, pulverized, and incubated with phosphate buffer and ß-glucuronidase/arylsulfatase. After solid-phase extraction and derivatization with PFPA and PFPOH, the drugs were identified and measured by GC/MS using their trideuterated analogs as internal standards. The method is reproducible with detection limits < 0.1 ng/mg hair for most tested substances.

The authors' experience with more than 1,000 hair samples has shown that morphine-to-codeine ratios necessary to determine heroin use are 5:1 for low morphine concentrations (< 1 ng/mg hair) and 2:1 for concentrations > 1 ng/mg hair. Further proof of heroin use can be obtained by the analysis of the metabolite MAM. As can be seen from several case examples, hair analysis cannot pinpoint an exact date of opiate use, but it can be used to validate or invalidate a subject's statement about drug consumption. However, great caution must be used in interpreting the test results.

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Human Scalp Hair as Biopsy Material Suitable for Quantitative Analysis in Therapeutic Drug Monitoring

Toshihiko Uematsu, Atsuhiro Mizuno, and Kazuhiro Kosuge

INTRODUCTION

When drugs of abuse are analyzed in hair samples, it is difficult to verify the results in a quantitative manner. This is because the absolute amount of ingested drugs and the duration and frequency of drug exposure might be—in the absence of validation—other than self-report, and the circumstances might dictate that the subject could be legally accused or lose his or her job immediately on identification of drug addiction. Therefore, the results of hair testing are considered to be only qualitative or, at best, semiquantitative. In addition, there is strong criticism against using hair analysis for identifying drug abusers (Holden 1990), and it is appropriate to assess whether hair is a biopsy material intrinsically suitable for quantitative analysis.

Hair testing is a subject of growing interest not only in the field of forensic science (Baumgartner et al. 1979, 1981, 1982; Suzuki et al. 1984) but also in clinical pharmacology because it has been shown that the clinically applied antipsychotic agents, haloperidol and chlorpromazine, are excreted into hair in proportion to the given doses (Uematsu et al. 1989; Matsuno et al. 1990; Sato et al. 1993). Moreover, the centimeter-by-centimeter analysis of axial distribution of a drug (e.g., haloperidol, chlorpromazine, ofloxacin) along the

hair shaft represents the month-by-month dosage history, assuming a mean hair growth rate of about 1 cm per month (Sato et al. 1989; Uematsu and Sato 1990). These observations are from studies conducted with inpatients in psychiatric hospitals, where medications were administered under strict supervision by doctors or nursing staff—in other words, where patient compliance was considered to be good. Thus, hair has been shown to possess desirable characteristics as a biopsy material that functions like a "tape recorder," storing along its length information on individual drug therapy over the past several months and possibly up to years in a quantitative manner.

FACTORS AFFECTING DRUG CONCENTRATION IN HAIR

Hair Color

Almost all Japanese people have black hair, although some subjects, especially older ones, have gray hair. When the concentration of haloperidol in a white hair was compared with that in a black hair, both of which had been collected simultaneously from the same subject with gray hair, haloperidol was always much lower in white hair (less than 10 percent) than in black hair (Uematsu et al. 1990). Haloperidol concentration in nails, the other keratinized tissue containing less melanin than hair, was also much lower than that in black hair and comparable to that in white hair (Uematsu et al. 1989). These findings suggest that the transfer of haloperidol from blood into hair bulb cells is highly dependent on the presence of melanin. In the case of chlorpromazine (Sato et al. 1993), as well as fluoroquinolones (Uematsu and Nakashima 1992, pp. 39-55) and nicotine (Mizuno et al. 1993) (see below), similar results have been obtained. This phenomenon has been confirmed experimentally by comparisons of drug concentrations in hair between albino and pigmented rats (Uematsu et al. 1990, 1992; Sato et al. 1989; Mizuno et al. 1993). Such observations might suggest that hair analysis is applicable only to people with black hair. However, the concentration of haloperidol in blond hair sampled from Caucasian psychiatric patients (n=5) was almost the same as that in black hair on the basis of daily dose (unpublished observation). Therefore, the role of other components, such as melanosomes and precursors of

melanin, should be clarified in the context of incorporation of a drug into hair.

Physicochemical Properties of Drugs

In the case of haloperidol, the incorporation of a major metabolite (reduced haloperidol) into hair was proportional to the area under the plasma concentration-time curve and comparable with that of the parent compound (Uematsu et al. 1993*a*). However, for the few other drugs investigated, the concentration of metabolites in hair was much less than that of the parent compound or was even below the detection limit, although the plasma concentration of metabolite was higher than that of parent drug (unpublished observation). For example, hydroxylated metabolites of chlorpromazine and their conjugates were undetectable in hair. Indoxyl sulfate, a putative uremic toxin and hydrophilic substance, has been tested as an index of renal insufficiency, although it is not a drug but an endogenous substance. Hair samples were obtained from anuric patients receiving hemodialysis maintenance therapy. Although indoxyl sulfate was always present in the blood of anuric patients in high concentrations (up to 10 μg/mL) and the detection limit of the analytical method was approximately 10 ng per tube, this substance was undetectable in hair (n=4) (unpublished observation). These findings suggest that the more hydrophilic a substance, the more difficult its transfer from blood into hair bulb cells.

Sampling Location

Head hair has been used for therapeutic drug monitoring because quantitative aspects of drug incorporation into other types of hair, such as axillary and pubic hairs, remain unclarified. The authors applied the technique for analyzing ofloxacin in head hair to the other types of hair as well. With head hair, growing-stage hairs are usually identified and collected by cutting hair strands of the frontal region close to the scalp and pulling them tightly to exclude loose stands that may be in the resting stage and easily shed. This sampling technique also was applied to axillary and pubic hairs, and it was found that ofloxacin was detected in some hairs but not in others. Therefore, five strands of axillary or pubic hairs were used together, cut simultaneously into 5-mm lengths serially from the skin

ends, and analyzed for ofloxacin to determine the axial distribution along the bundle of five axillary or pubic hair shafts. Comparison of the axial distribution of ofloxacin along the hairs collected 1 month after administration versus those collected 2 or 3 months later gave the following results: Axillary hairs grow at a rather constant rate for the first few months but then enter a long resting stage over a few months, whereas in pubic hairs, the proportion of resting-stage to growing-stage hair and the growth rate are variable among subjects, with two or three peaks in the axial distribution of ofloxacin along the pubic hair shafts. Although, in the case of drugs of abuse, interest has focused on which type of hairs accumulate drugs to the highest degree, the differences in growth rate, proportion of resting-stage hair, and duration of the growing stage among the three types of hair appear to have a great influence on drug concentration in hair.

Problems Related to Variable Growth Rate and Cycle Stage of Hair

Hair growth rate varies both within and among subjects, although it is limited within a rather narrow range. According to dermatological studies (Saitoh et al. 1967, pp. 21-34), the growth rate of head hair ranges from about 0.5 to 2 cm per month. Therefore, at the extremes of the range, a 1-cm length of hair may represent information on drug use over a period of a half-month or 2 months. Moreover, each strand of hair has its own growth cycle of 2 to 8 years or more, consisting of growing, intermediate, and resting stages (Saitoh et al. 1970). Consequently, the "tape speed" of this tape recorder is not necessarily uniform. Therefore, uncertainties inherent in the growth rate and cycle stage of each hair segment may negate the results obtained from hair testing. To solve this problem, identification has been tried with a drug that could be detected in a single hair even after a brief exposure and that moves outward along the hair shaft as the hair grows. By using this drug as a time marker within hair, the growth rate and cycle stage of each single hair can be determined.

After various drugs were screened, ofloxacin was selected. It is one of the most widely and frequently prescribed antimicrobial fluoroquinolones worldwide (Uematsu et al. 1991; Miyazawa et al.

1991). Ofloxacin could be detected in a single hair when a daily dose (300 mg/day in three divided doses) was taken by the subject for only 1 day. A single hair was cut into 1-cm lengths serially from the scalp end, and each hair portion was dissolved in 1 M sodium hydroxide (NaOH) by heating at 80 °C for 30 minutes. High-performance liquid chromatography was used together with a fluorescence detector, with a detection limit of less than 0.5 ng per tube (Miyazawa et al. 1991). Hair samples were collected at 1-month intervals for 3 months from a subject who had taken 300 mg per day of ofloxacin for 2 or 3 days. The axial distribution of ofloxacin along a single hair shaft was analyzed on a centimeter-by-centimeter basis. The hair portion containing ofloxacin was clearly shown to grow outward along the hair shaft month by month at the rate of about 1 cm per month. The drug was not detectable from a shed hair (figure 1, panel b), which is easily separated from the head with a simple tug; the axial distribution along a shed hair shaft was different from that along a hair shaft with a visible hair bulb plucked from the same subject (figure 1, panel a). These phenomena could be explained by assuming that the shed hair either had been in the resting stage for a few months prior to testing or entered the resting stage immediately after the administration period. Thus, ofloxacin was not incorporated in a shed hair (figure 1, panel b).

The more detailed distribution of ofloxacin along a single hair shaft was analyzed by cutting a single hair into 2-mm lengths serially from the scalp end. Ofloxacin was distributed only in two or three consecutive 2-mm sections of the hair (figure 2). Therefore, the hair growth rate could be calculated for each hair strand as the length of the hair strand from the scalp end to the position of peak ofloxacin concentration divided by the time interval between the dates of drug intake and hair sampling. For the three different hair strands depicted in panels b, c, and d of figure 2, the growth rates thus calculated were panel b, 0.89 (2.4/2.7); panel c, 1.22 (3.3/2.7); and panel d, 1.22 (3.3/2.7) cm per month. The growth rate thus obtained for the frontal hair of young adults was 1.12 ± 0.11 cm per month (mean $\pm$ standard deviation, $n=7$), with intraindividual variability of 4.8 to 18.1 percent as the coefficient of variation (Miyazawa and Uematsu 1992). This value is similar to published values for growth rate (Saitoh et al. 1967, pp. 21-34).

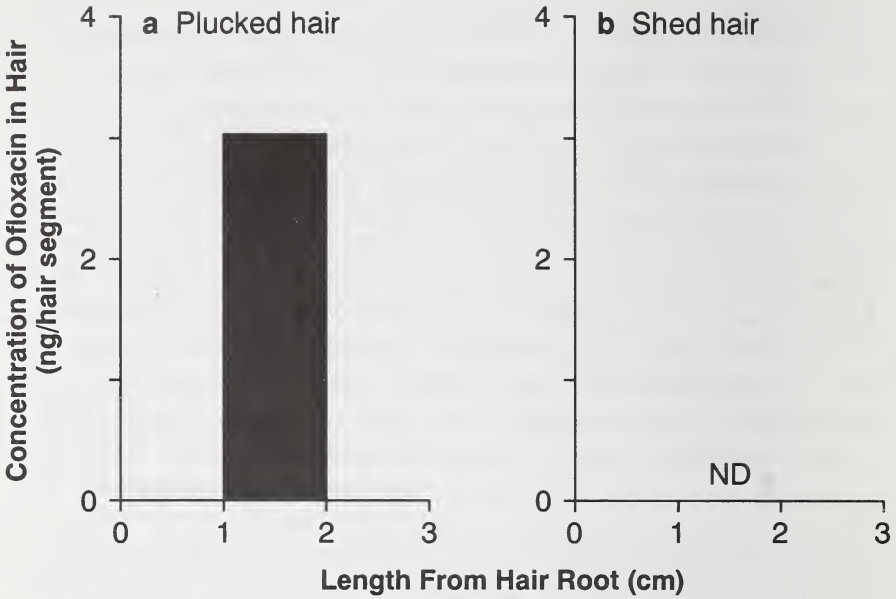

FIGURE 1. *Representative axial distribution of ofloxacin along a plucked hair shaft (panel a) and a shed hair shaft (panel b). Both types of hair were collected simultaneously 2 months after drug intake from a subject who had taken 300 mg/day of ofloxacin for 3 days.*

KEY: ND = not detected

The same desirable drug characteristics have also been observed with other fluoroquinolone derivatives, including therapeutic agents such as norfloxacin and ciprofloxacin (Mizuno et al. 1994) and newly developed compounds such as OPC-17116 (Uematsu et al. 1993*b*), AM-1155 (Uematsu et al. 1993*c*), temafloxacin (Uematsu et al. 1994*a*), and Q-35 (Uematsu et al. 1994*b*). These fluoroquinolones may be used in patients with infective diseases concurrent with other illnesses or in otherwise healthy subjects with only minor upper respiratory or urinary infections. There should be many candidate drugs that could serve as time markers in hair other

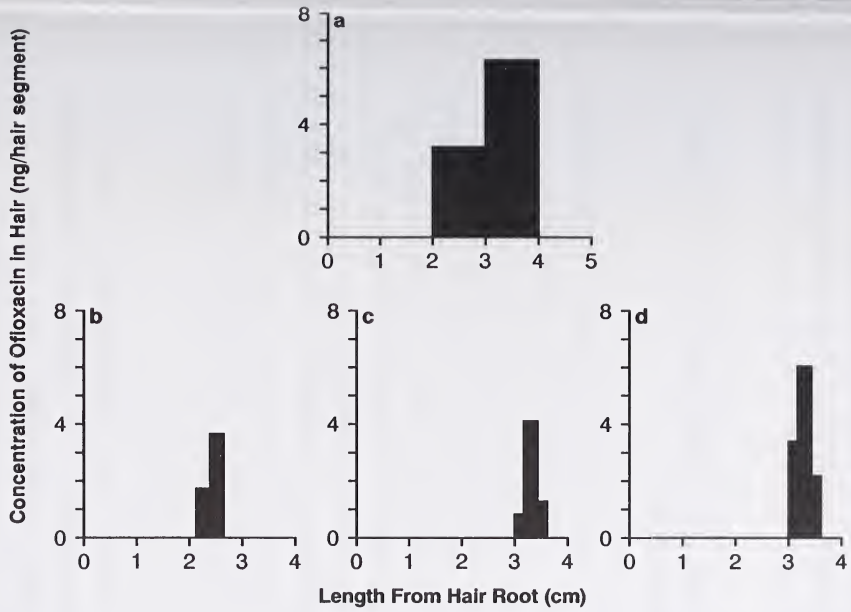

FIGURE 2. *Representative detailed distribution of ofloxacin along a single hair shaft. Four strands of hair were obtained simultaneously 2.7 months after drug intake from a subject who had taken 300 mg/day of ofloxacin for 3 days. The distribution of ofloxacin was roughly determined on a centimeter-by-centimeter basis by using the first single hair strand shown in panel a. Then the distribution of ofloxacin was analyzed in detail along each of the other three hair shafts. Panels b, c, and d show the detailed distribution of ofloxacin along the second to fourth single hair. The mean rate of hair growth was calculated in this subject as 1.1 cm/month and, in a total of 7 subjects, tested as 1.12±0.11 cm/month.*

than fluoroquinolones, which are detectable in hair after a short exposure. For example, antibiotic aminoglycosides have a generally high affinity to melanin and are used at doses ranging from 100 to 1,000 mg, although they have not been tested yet.

Detailed analysis of the distribution of a drug such as ofloxacin or other fluoroquinolones along a single hair shaft also may be useful in the field of forensic science. For example, this method may be used to identify a subject by comparing the results of hair analysis with past medical records.

ESTIMATION OF CIGARETTE SMOKING BEHAVIOR BY HAIR ANALYSIS OF NICOTINE

Nicotine associated with cigarette smoking is one of the most widely abused chemical substances in the world. It has been well established that cigarette smoking is a major cause of disease, disability, and death (U.S. Department of Health and Human Services 1990). Cigarette smoking is now regarded as the foremost preventable cause of death in the United States (Tsevat 1992). Research concerning cigarette smoking behavior and smoking prevention and cessation programs necessitates accurate measurement of smoking behavior. In addition, cigarette smoking is well known to influence the pharmacokinetics of various drugs by enhancing or inhibiting diverse hepatic drug-metabolizing enzymes. Therefore, noninvasive validation of individual smoking behavior is desirable for therapeutic drug monitoring.

The authors have applied the technique of hair testing to measure a subject's smoking behavior. Black hair samples were obtained from many people who had smoked cigarettes daily for more than 3 years. The concentration of nicotine in hair was measured by a capillary gas chromatograph with a nitrogen-phosphorus detector after each hair was dissolved in 2.5 M NaOH and nicotine was extracted using diethyl ether (see Mizuno et al. 1993). The nicotine concentration in hair was approximately proportional to the number of cigarettes consumed daily.

In a separate experiment, hair samples were collected from subjects who had participated in a 6-month smoking cessation program and had succeeded in stopping smoking or in reducing the number of cigarettes smoked. The axial distribution of nicotine along the hair shafts was analyzed by cutting several hairs simultaneously into 1-cm lengths serially from the scalp ends and measuring nicotine concentration in each length. When hair growth was assumed to be 1 cm per month, the centimeter-by-centimeter distribution of nicotine approximated self-reporting regarding the month-by-month change in mean number of cigarettes smoked per day (figure 3).

In the case of nicotine analysis in hair, the sample washing technique was critical because hair is inherently an oily tissue that may easily adsorb nicotine from environmental dust. Hair samples were washed successively with a 1 percent solution of sodium dodecyl sulfate and distilled water for 30 minutes. This procedure was repeated twice. White as well as black hair samples were collected simultaneously from subjects with gray hair, and the nicotine concentrations in both types of washed hair were measured and compared. In the same subject, the concentration of nicotine in white hair was always much lower than that in black hair (Mizuno et al. 1993). This finding was also confirmed in an animal experiment in which nicotine (3 mg/kg/day) was continuously administered to pigmented rats for 4 weeks via an osmotic pump implanted subcutaneously; the nicotine concentration was compared between the whitish hair on the thorax and brown hair on the back (1.5 vs. 38.5 ng/mg). If the two types of human hair are assumed to equally adsorb nicotine from environmental dust, the idea is supported that most of the nicotine measured in black hair was derived from nicotine incorporated into hair through the blood. Therefore, the washing technique adopted in this study was considered to be practically sufficient for removing adsorbed nicotine, which may interfere with tracing the changes of individual smoking behavior.

CONCLUSIONS

Human scalp hair is a useful biopsy material for therapeutic drug monitoring and for forensic and toxicological investigations because information about drug exposure is stored along the hair shaft in a

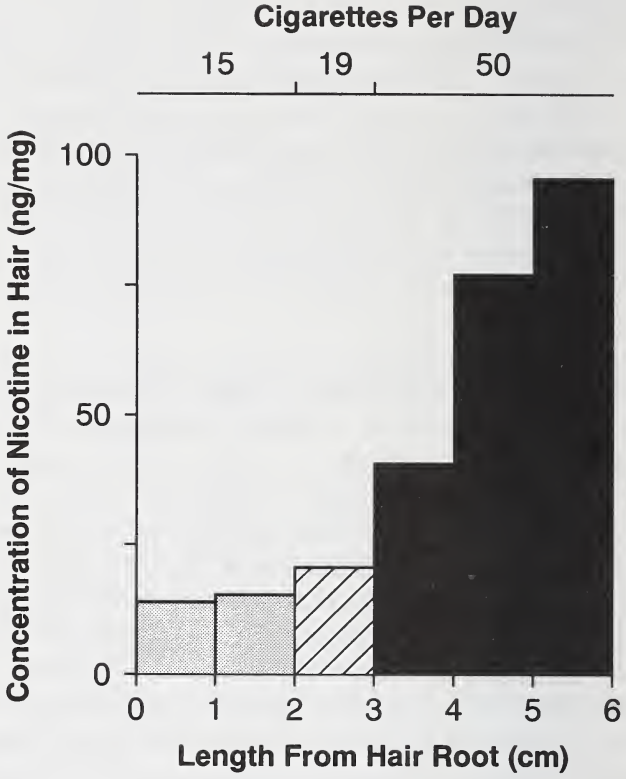

FIGURE 3. *Representative axial distribution of nicotine along hair shafts collected from a subject who participated in a smoking cessation program for 6 months and succeeded in reducing the number of cigarettes smoked per day. Five hair strands collected at the end of the program were cut together into 1-cm lengths serially from the scalp ends. The mean number of cigarettes smoked daily, which was reported by the subject every month before and during the program, is depicted above the graph as number of cigarettes per day. This top horizontal axis shows that the subject consumed, on average, 50 cigarettes per day from 6 months until 3 months before the hair sampling, 19 cigarettes per day from 3 months until 2 months, and 15 cigarettes per day in the final 2 months of the*

cessation program, assuming the hair growth rate of 1 cm/month, depicted in the bottom horizontal axis. Each column shows the nicotine concentration. In a total of seven subjects tested, the rise and fall in nicotine concentration along the hair shafts approximated that in the self-reported number of cigarettes smoked per day.

quantitative manner. It can be said that hair acts as a tape recorder that continuously stores all data about the drug use history of a subject. The duration of data storage ranges from several months to years depending on the length of hair. However, because growth rate may differ both within and among subjects according to each hair's growth cycle, it is useful to find a marker drug, such as one of the fluoroquinolones, along the hair shaft. The growth rate of each single hair can be obtained by analyzing in detail the axial distribution of a fluoroquinolone along the hair shaft because the hair segment containing the drug, which is formed by hair bulb cells at the time of dosing, has been shown to grow outward, thereby keeping pace with hair growth.

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Neonatal Hair Analysis: A Tool for the Assessment of In Utero Exposure to Drugs

Julia Klein and Gideon Koren

INTRODUCTION

Maternal exposure to drugs and chemicals is increasingly being recognized as adversely affecting the developing fetus (MacGregor et al. 1987; Chasnoff 1989; Chasnoff et al. 1990; James 1990). A major issue in studying the adverse effects of illicit drugs is the lack of standardized techniques to ascertain intrauterine exposure. Traditionally, the evidence of drug exposure is based on maternal self-report; however, it has been demonstrated by scores of studies that these reports are by no means accurate. The inaccuracy ranges from total denial to downplaying the amount and frequency of drug use. This phenomenon is not surprising considering the potential legal consequences of maternal illicit drug use (Frank et al. 1988; Zuckerman et al. 1989; Forman et al. 1994). Pregnant women are known to downplay even the number of cigarettes they smoke, probably out of guilt, shame, and so forth (Feldman et al. 1989; Eliopoulos et al. 1994). Blood and urine testing have proven unreliable as well, mainly because of the short elimination half-life of many commonly used drugs and their metabolites (Saxon et al. 1988).

The illicit drug most commonly used by the Canadian and U.S. populations is cocaine. Because women of reproductive age constitute a sizable percentage of the population and many women in this age group use cocaine, many fetuses are exposed in utero to

cocaine (Smart 1988; Abelson and Miller 1987, pp. 35-49). The most common adverse perinatal effects associated with cocaine use are higher rates of miscarriage; placental abruption; stillbirth; intrauterine growth retardation; and in the neonatal period, prematurity, small head circumference, intracranial bleeding, sudden infant death syndrome, congenital malformations, and developmental delay (Madden et al. 1986; MacGregor et al. 1987; Bauchner et al. 1988; Chasnoff et al. 1988).

During the past few years neonatal hair has been shown to be a sensitive biological marker that not only may yield a dichotomous answer to exposure (yes or no) but also may project a cumulative estimate of cocaine exposure (Graham et al. 1989; Forman et al. 1992).

Neonatal hair starts growing during the last 3 to 4 months of gestation, reflects intrauterine drug exposure for the last part of gestation, and is available for as long as 4 to 5 months of postnatal life (Smith and Gong 1974). This is a marked advantage for hair analysis over meconium analysis. Meconium starts to form in the second trimester and reflects in utero drug exposure from that point until 2 days after birth (Ostrea and Romero 1993).

The difficulty of neonatal hair analysis begins with obtaining a sufficient sample. Most newborn babies have little hair, and mothers are often reluctant to give permission to cut even a few strands. Because of the sparsity of hair, sensitive methods are needed for analysis.

METHODOLOGICAL ISSUES

Washing or Decontamination Procedures

Although distinguishing between systemic exposure and external contamination is an extremely important issue, with legal implications in the adult population, in the neonatal age group this issue is substantially less important. The source of contamination in this case is the amniotic fluid. Forman and colleagues (1992) showed that soaking the cocaine-free hair of a guinea pig in a

solution containing cocaine and benzoylecgonine (BZE) at concentrations similar to those measured in amniotic fluid (50 ng/mL cocaine and 350 ng/mL BZE) caused low measurable levels to be observed. After hair is washed with ethanol using a procedure described previously (Baumgartner et al. 1982), levels of cocaine and BZE in hair become undetectable.

However, the important question in this case is the following: Is contamination of neonatal hair by the amniotic fluid a "true" external contamination? After all, the fetus swallows the amniotic fluid at a rate of 0.5 L per day (Seeds 1981); moreover, while the fetus bathes in it, toxins circulating in the amniotic fluid reach the fetus via the transdermal route (Baselt et al. 1990). Fetal skin, because of low keratinization, is readily permeable by exogenous substances (Luck et al. 1985); therefore, the distinction between external contamination and systemic exposure to drugs in utero becomes less important.

Extraction Procedures

There are numerous procedures described in the literature (Valente et al. 1981; Ishiyama et al. 1983; Baumgartner et al. 1982; Arnold 1987) for hair extraction of drugs in general and cocaine in particular. These procedures can be grouped into four categories:

- Organic solvent extraction
- Alkali digestion
- Acid digestion
- Enzyme digestion

Although organic solvent extraction might be slower than other methods, it has the advantage of leaving the hair intact. Alkali digestion cannot be used with cocaine because of cocaine decomposition to BZE. Both acid digestion (Cone et al. 1991) and enzyme digestion (Harkey et al. 1991) are widely used methods. The digestion step is usually followed by a liquid-liquid or solid-phase extraction into an organic solvent followed by a concentration step.

Although the liquid-liquid extraction is the older, more traditional method of extraction, solid-phase extraction has gained in popularity because of the dramatic timesaving it offers. Unlike liquid-liquid extraction, which often results in variable sample recoveries, solid-phase extraction is based on specific molecular interactions for more reproducible results. Extraction columns are packed with a variety of sorbents that selectively retain specific types of chemical compounds from a surrounding matrix.

QUANTITATION OF DRUGS (COCAINE) IN THE EXTRACT

There are many techniques available for the quantitation of drugs of abuse in general and cocaine in particular following extraction (Baumgartner et al. 1982; Franceschin et al. 1987; Balabanova and Homoki 1987; Sandberg and Olsen 1990; Kidwell 1993). The accepted standard method of analysis is gas chromatography/mass spectrometry. When deuterated internal standards are used, quantitation is accomplished by the ratio of the unknown intensity to the internal standard. Gas chromatography/mass spectrometry is undoubtedly an accurate and sensitive method, but in the case of hair analysis, the limiting factor is the efficiency of extraction of the drug from the hair matrix.

A technique that is not dependent on extraction is tandem mass spectrometry (Kidwell 1993). The instrumentation for this procedure is costly, and only a few clinical pediatric units are equipped with or have access to such instruments. A less expensive alternative is high-performance liquid chromatography, which, with a good detection system, is satisfactory for medical diagnosis and clinical investigation (Sandberg and Olsen 1990). For mass screening, radioimmunoassay (RIA) has emerged as a simple and cost-effective alternative (Koren et al. 1992). Antibody specificity is the limiting factor in this case. When BZE in hair extract is measured, cross-reactivity of the antibody with cocaine and other metabolites must be considered. Consequently, results (positive and negative) should be confirmed with a procedure involving mass spectrometry.

ASSESSMENT OF FETAL EXPOSURE TO COCAINE IN A TORONTO COHORT

Although there is a general perception of Toronto, Ontario, Canada, as being safer and "cleaner" than other cities of similar size, there is increasing evidence from the police and social services providers that the use of cocaine is on the rise. Using hair as a biological marker, the prevalence of in utero exposure to cocaine in Toronto in 1990 and 1991 was studied.

Three hospital nurseries contributed 200 cases each, for a total of 600 neonatal hair and urine samples that were randomly collected. The hair samples were obtained by clipping the hair as close to the scalp as possible using fine scissors. The urine samples were collected in pediatric collection bags. Maternal and neonatal charts were reviewed. The hair and urine samples were analyzed for cocaine and BZE by RIA. The hair samples (2 to 5 mg) were sonicated in 1 mL methanol for 30 minutes and subsequently incubated overnight at 45 °C. Following incubation, the methanol was removed and evaporated at 40 °C under a stream of nitrogen; the samples were reconstituted with 0.1 mL phosphate-buffered saline (pH 7.5). To measure cocaine, Coat-A-Count (Diagnostic Products, Los Angeles, California) for cocaine metabolite in urine was used, but instead of the standards provided with the kit, cocaine hydrochloride standards (1 to 500 ng/mL) were prepared. The antiserum in this particular kit has a much higher affinity for cocaine than for BZE; therefore, the cross-reactivity with BZE is only 0.5 percent. The sensitivity of the assay is 1 ng/mL, which corresponds to 0.05 ng cocaine per mg hair. For the analysis of BZE in the extracts, the Roche Abuscreen (Hoffman-La Roche, Nutley, New Jersey) for cocaine metabolite in urine was used. The cross-reactivity with cocaine was determined to be 4 percent, and the sensitivity of the assay is 5 ng/mL, which corresponds to 0.25 ng BZE per mg hair. For urine samples, only BZE was measured with the same RIA kit as for the hair analysis, but the cutoff value of 300 ng BZE per mL suggested in the kit was lowered to 30 ng BZE per mL.

From the 34 hair samples that tested positive for BZE, 20 samples (59 percent) were confirmed by thermal desorption/mass

spectrometry. The hair sample is heated, and the volatile compounds are vaporized. The parent and daughter ions are monitored using two mass spectrometers. The rest of the positive samples were not confirmed because of a shortage of specimens. No false positives were observed. From the 20 confirmed positive hair samples, 3 were positive by all three methods considered, 3 were positive by RIA and had positive maternal history but negative urine, and 14 had neonatal hair positive for BZE but negative urine and negative maternal history. From the negative samples, 5 percent were reanalyzed by thermal desorption/mass spectrometry.

A total of 37 infants were determined to be exposed to cocaine, corresponding to an average rate of 6.25 percent in utero exposure. In one hospital the rate was 12.5 percent. Hair samples were positive in 34 of the 37 cases; however, urine was positive for only 9 of the 37 cases, and maternal histories indicated cocaine exposure in only 7 of the 37 cases.

It is likely that the true rates of fetal exposure were even higher than indicated by hair analysis because even the hair test failed to detect 10 percent of cases that were positive by urinalysis. The data suggest that exposure below a certain level may not be detectable in fetal hair. Although maternal urine was not tested in this particular study, it is possible that the women whose babies' urine tested positive while the hair was negative restarted using cocaine in the last few days of pregnancy and that the exposure did not have time to be reflected in fetal hair. There is evidence that addictive women often use cocaine to induce labor (Volpe 1992).

In comparing maternal self-report with urinalysis, it has been hypothesized that the combination of history and urine testing would still miss some cases of fetal exposure. In this study, the combination of maternal history and urinalysis would miss 76 percent of the cases identified by hair analysis (table 1). The data indicate that women whose history reveals voluntary report of cocaine use are likely to be heavier users, as evidenced by hair and urine concentrations of BZE higher by an order of magnitude.

TABLE 1. *Characteristics of methods of detection of fetal exposure to cocaine (n=37)*

Category	Number of Positive Cases	Percent of All Positive Cases
Positive history	7	19
Positive urine for BZE	9	24
Positive hair test	34	90

KEY: BZE=benzoylecgonine

SOURCE: Forman, R.; Klein, J.; Barks, J.; Mehta, D.; Greenwald, M.; Einarson, T.; and Koren, G. 1994. Copyright 1994 by *Clinical and Investigative Medicine*, University of Toronto Press (Toronto, Ontario).

Probably the most accurate way to identify in utero cocaine exposure is to combine as many screening methods as possible: maternal self-report and hair and neonatal hair, urine, and meconium. Taking into consideration cost-effectiveness versus efficacy of the screening method, neonatal hair analysis by RIA emerges as the single most useful method in identifying in utero cocaine exposure in the last trimester of pregnancy. Although it has been shown previously that cocaine crosses the placenta, because of interindividual variability, some placentas have the ability to bind large quantities of cocaine arriving at the placental vessels, subsequently protecting the fetus from cocaine exposure (Potter et al. 1994). In such cases maternal hair analysis would not be useful in indicating in utero cocaine exposure. Urinalysis, although less expensive, reflects only recent exposure, whereas meconium analysis—at the same cost as hair analysis—has been documented as being less sensitive than hair testing (Callahan et al. 1992).

Infants exposed to cocaine were of smaller birth weight than those not exposed (table 2); however, when the cocaine-exposed group was stratified according to maternal tobacco-smoking status, the results revealed that babies of smoking and cocaine-using mothers had significantly lower birth weight (2,899±750 g) than those of

TABLE 2. *Comparison between infants exposed versus infants not exposed to cocaine in utero*

Parameter	Exposed (n=37)	Not Exposed (n=563)	Significance
Birth weight (g)	3,162±645*	3,391±573*	$p<0.05$
Birth length (cm)	49.9±2.8*	51.1±3.1*	$p<0.05$
Need for medical support and resuscitation	51%	30%	$p<0.05$
Unresponsiveness at birth	8%	3%	NS
Grunting	7%	1.5%	NS
Sepsis	7%	1.5%	NS
Congenital malformations	5(0.9%)	0%	NS

*Mean±standard deviation

KEY: NS=not significant

SOURCE: Reprinted from *Reproductive Toxicology*, vol. 7, Forman, R.; Klein, J.; Barks, J.; Mehta, D.; Greenwald, M.; Einarson, T.; and Koren, G. Maternal and neonatal characteristics following exposure to cocaine in Toronto, pp. 619-622, Copyright 1993, with kind permission from Elsevier Science Ltd, The Boulevard, Langford Lane, Kidlington 0X5 1GB, UK.

mothers who did not smoke but used cocaine (3,423±612 g, $p<0.05$). In fact, the birth weight of babies from mothers who used cocaine but did not smoke was not different from the birth weight of nonexposed babies.

These results highlight the urgent need for finding a biochemical marker for nicotine exposure in utero, as a confounder for cocaine exposure and possibly for other illicit drugs and also as a way to better understand the effects of maternal smoking on the fetus and the neonate.

In another study, after consent was obtained from mothers, hair samples were collected from 94 mother-infant pairs in two Toronto nurseries (Langone et al. 1973). The mothers were stratified into

three categories: active smokers, passive smokers, and nonsmokers. Passive smokers were defined as women who were exposed during their pregnancies on a regular and steady basis to other persons' cigarette smoke, either at home (e.g., smoking by husband or partner) or in the workplace. Hair analysis was performed by RIA after digesting the samples in alkaline solutions.

Briefly, 2 to 5 mg hair samples were washed with a detergent, mixed with water, and dried at 37 °C overnight. The following day the samples were digested with 1 mL 0.6 *N* sodium hydroxide at 50 °C overnight; after the solutions were neutralized, an aliquot was used to measure nicotine and cotinine concentration by RIA. Ten percent of the samples in each of the three categories were reanalyzed and confirmed by thermal desorption/mass spectrometry.

The sensitivity of the assay was 0.25 ng/mg hair for nicotine and 0.1 ng/mg hair for cotinine. The cross-reactivity of nicotine in the cotinine assay was 5 percent and 2 percent for cotinine in the nicotine assay. The other metabolites that retained only one of the two-ring systems (the pyridine or *n*-methylpyrolidine rings) did not exhibit cross-reactivity with either assay. The 3-hydroxycotinine metabolite of cotinine exhibited less than 10 percent cross-reactivity in the cotinine RIA. This is a minor metabolite that is not measurable in plasma and has not yet been reported in hair.

The study revealed significant differences in the cotinine concentration in the three groups in both maternal and neonatal hair (figure 1) and a good correlation between maternal and neonatal cotinine concentration (figure 2).

FUTURE DIRECTIONS

Since their introduction, neonatal hair tests for drugs of abuse have raised hopes that they will become biological markers of fetal exposure to these toxins.

There are still many unanswered questions. Because maternal history has proven inaccurate, the correlation between maternal dose of cocaine and infant hair concentration is not known. Ultimately,

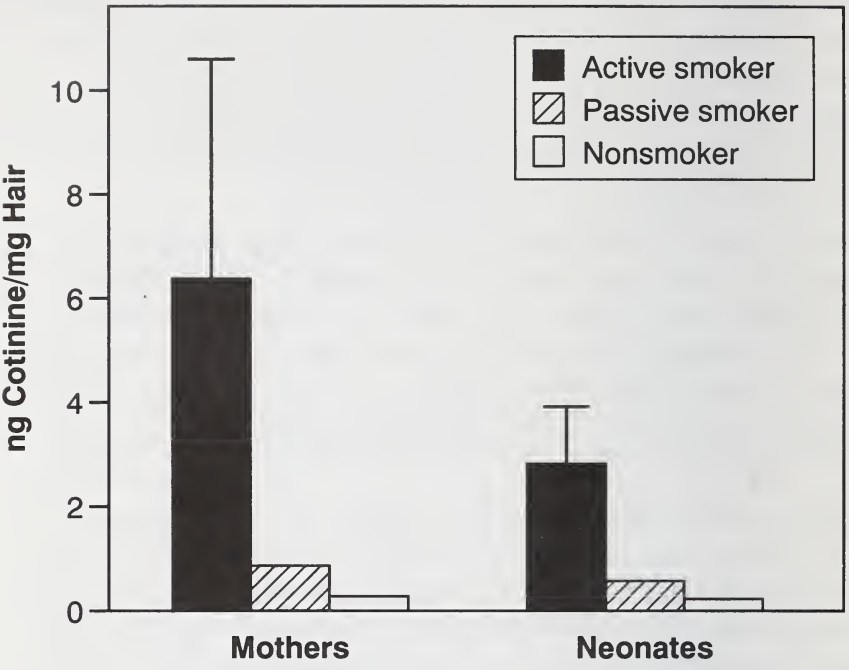

FIGURE 1. *Maternal and neonatal concentrations of cotinine in hair for active smoking, passive smoking, and nonsmoking mothers*

SOURCE: Eliopoulos, C.; Klein, J.; Phan, M.K.; Knie, B.; Greenwald, M.; Chitayat, D.; and Koren, G. *Journal of the American Medical Association* 271:621-623, 1994. Copyright 1994, American Medical Association.

more data are needed to assess the ability of neonatal hair concentrations of drugs to predict rates and the extent of perinatal complications.

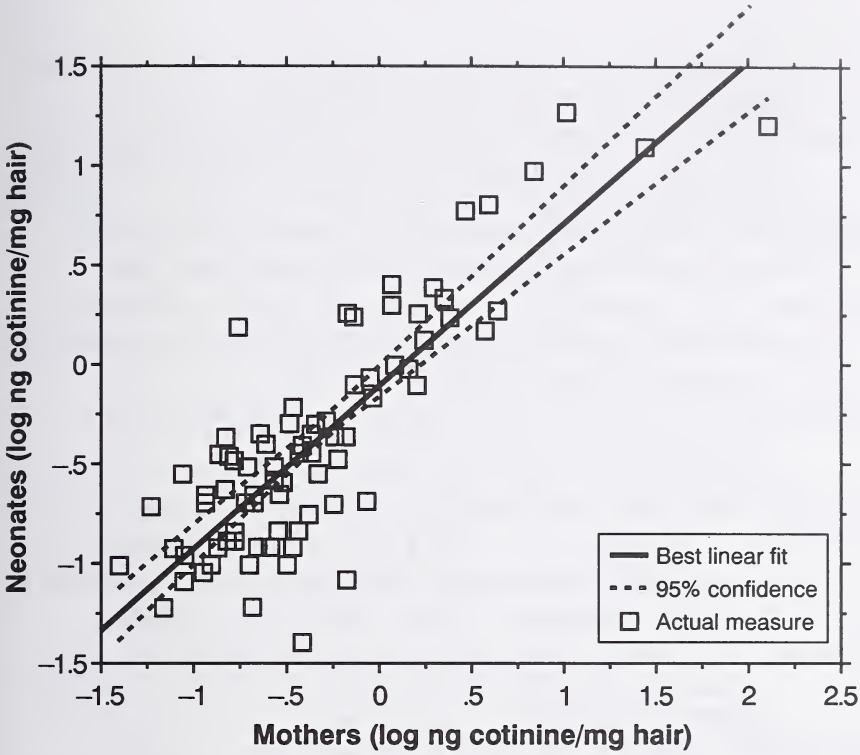

FIGURE 2. *Correlation between maternal and neonatal hair concentrations of cotinine*

SOURCE: Eliopoulos, C.; Klein, J.; Phan, M.K.; Knie, B.; Greenwald, M.; Chitayat, D.; and Koren, G. *Journal of the American Medical Association* 271:621-623, 1994. Copyright 1994, American Medical Association.

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Neonatal Hair Analysis for Benzoylecgonine: A Marker for Gestational Cocaine Exposure, Growth at Birth, and Neurodevelopment Based on Short-Term Followup (4 to 8 Months)

Lakshmi D. Katikaneni, Floyd R. Sallee, Hoda O. Ibrahim, and Thomas C. Hulsey

INTRODUCTION

Cocaine use during pregnancy may have serious implications for both maternal and fetal well-being. Maternal health may be affected by predisposition to hypertension and placental abruption. The consequences of fetal exposure to cocaine during gestation may be related to the extent of maternal drug use (dose, route, duration, frequency of drug intake) and the time, duration, and degree of fetal exposure. Drug metabolism (in the mother and fetus) and amount of placental transfer may determine the degree of fetal exposure (Van Dyke and Byck 1982; Farrar and Kearns 1989; Stewart et al. 1979). Identification of the prevalence of drug use by pregnant women and the impact of regional differences, racial distribution, and socioeconomic status (SES) is the initial step in assessing fetal exposure. Furthermore, the chronicity and degree of exposure and delineation of the impact of confounding variables—such as maternal malnutrition, polydrug habit, and risk from sexually transmitted

diseases (STDs), including syphilis and those diseases resulting from cytomegalovirus and human immunodeficiency virus (HIV)—are important issues in determining fetal and long-term growth and neurodevelopmental effects.

Maternal admission of drug use is helpful but not always reliable in diagnosis, and the degree of fetal exposure is difficult to assess. Urinalysis for cocaine using enzyme-multiplied immunoassay technique (EMIT), if positive, indicates only acute maternal intake. Availability of a biological marker that is stable, easily accessible, noninvasive, quantitative, sensitive, specific, technically sound and consistent, and cost-effective; that requires only a small sample for analysis; and that serves as an indicator for prolonged exposure would be the ideal tool to assess chronic gestational cocaine exposure (Evans 1992). Hair analysis for benzoylecgonine (BZE), a major metabolite of cocaine, appears to be a promising biological maker that satisfies some of these criteria.

THESIS

Because of the nature of the pathological effects of cocaine (vascular compromise and neurotransmitter interference), it is conceivable that central nervous system (CNS) disability is possible and variable depending on the degree and duration of exposure in utero. Moreover, depending on the CNS area involved and its functional maturation during postnatal development, neurodevelopmental problems may surface on followup. Children of drug-abusing mothers may also be exposed to other detrimental factors (e.g., in utero infections and nutritional deprivation) that individually can hamper brain growth and development. In the absence of intrauterine infections and detriment of socioeconomic conditions, maternal cocaine use during pregnancy may cause harmful effects on fetal brain and body growth depending on circulating fetal blood and brain drug levels. The latter may be reflected in neonatal hair BZE levels. Because neurodevelopmental disability (NDD) in these children is not an all-or-nothing phenomenon, establishing a range of exposure and relating it to the degree of disability is an initial step for future intervention strategies.

Thus, the aim of the current prospective study was threefold: (1) to establish a range of hair BZE levels in a large sample of predominantly black neonates exposed to cocaine in utero as a marker for the degree of exposure (Study 1), (2) to assess the impact of hair BZE levels on growth status at birth (birthweight [BWT] and birth head circumference [BHC]) (Study 2), and (3) to evaluate the relationship between the degree of exposure at birth and the severity of NDD (Study 3). A total of 151 gestationally cocaine-exposed newborns with appropriate controls of 100 newborns not exposed to cocaine formed the basis for Study 1. A cohort of 64 newborns from the Study 1 group and 63 from the control group who were hair-positive or -negative for BZE formed the Study 2 group. In addition, 41 hair-positive newborns from Study 2 and 14 hair-negative controls were followed for 4 to 8 months and formed the Study 3 group (figure 1).

Although it is known that cocaine crosses the placenta to reach the fetus, the extent of transfer is not well understood in human beings because of methodological difficulties. Maternal cocaine use during pregnancy predisposes for preterm deliveries, placental abruption, maternal hypertension, intrauterine growth retardation, and CNS abnormalities in the fetus (Bingol et al. 1987; Burkett et al. 1990; Chasnoff and Griffith 1989; Chasnoff et al. 1989*a*; Dombrowski et al. 1991; Oro and Dixon 1987; Neerhof et al. 1989; Zuckerman et al. 1989; Gillogley et al. 1990; Cherukuri et al. 1988; Volpe 1992; Kliegman et al. 1994). Furthermore, fetal morbidity may be enhanced by poor maternal SES, inadequate prenatal care and nutritional support, increased risk for STDs, and polydrug use such as alcohol and tobacco. In addition, when the mother and child are discharged from the hospital, a deficient nurturing environment and parenting skills interrupt infant caregiver "attachment," a fundamental building block needed for learning, cognitive, and behavioral development.

In evaluating the effects of gestational cocaine exposure on fetuses and NDD in children, the following methodological issues must be addressed (Volpe 1992; Neuspiel and Hamel 1991; Hansen and Ulrey 1993, pp. 115-125; Zuckerman and Frank 1994):
(1) Accurate determination of gestational cocaine exposure necessitates the development of tests that are simple, sensitive, and

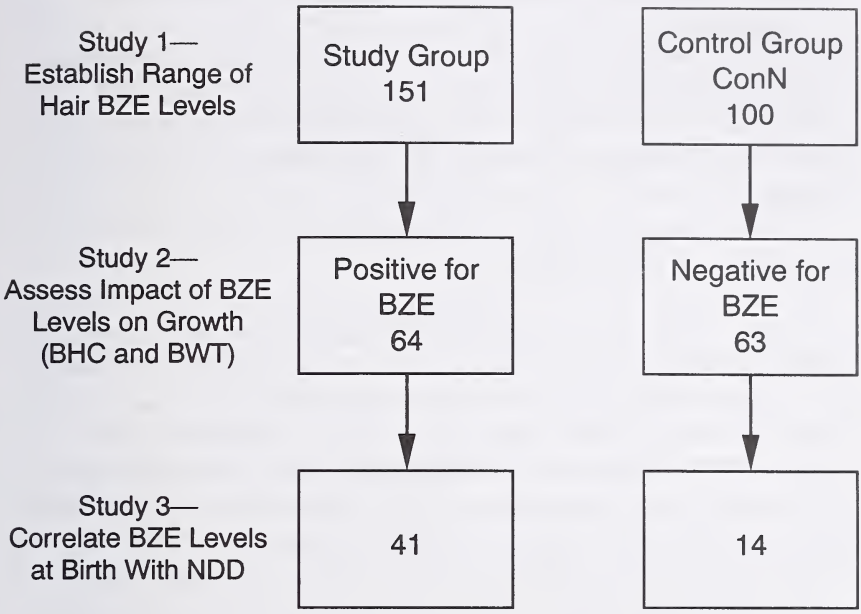

FIGURE 1. *Algorithm of study groups*

KEY: BZE=benzoylecgonine; BHC=birth head circumference; BWT=birth weight; NDD=neurodevelopmental disability

specific. (2) The degree and timing of gestational cocaine exposure may determine the major pathological effects such as growth retardation and CNS abnormalities as indicated by well-controlled animal studies. (3) Confounding variables need to be eliminated. These variables include intrauterine infections, congenital abnormalities, heavy alcohol or tobacco use, and low SES, each of which individually is associated with NDD. (4) The cocaine-specific toxic effects on a developing CNS, with special attention to areas of potential compromise, need to be understood. (5) The assessment of infant functioning should be carried out serially. Human infant assessment needs to be evaluated chronologically because different functions mature at different ages. In all developing organisms, exposure to drugs at different times during prenatal development may have different effects. (6) Finally, developmental tests should

be included that identify all the areas of potential damage by cocaine such as cognitive, motor, and behavior skills. The tests also should include assessment of the home environment, caregivers' parenting skills, and emotional attachments of the caregivers to the child. Appropriate control subjects with confirmed maternal negative drug history are essential to identifying developmental deficits specific to cocaine.

Pathological Cocaine Effects

In well-controlled animal studies involving maternal/infant pairs, the damaging effects of cocaine on the fetus are well defined, especially the CNS pathology (Dow-Edwards 1989, 1991; Dow-Edwards et al. 1990; Raum et al. 1990; Segal et al. 1989, p. 519; Spear et al. 1989*a*, 1989*b*; Sobrian et al. 1990; Volpe 1992). This pathology includes structural and functional deficits demonstrated in a series of neurobehavioral, neuropharmacological, neurochemical, and physiological studies, primarily in rats. Structural defects may be noted in the hippocampus (crucial for memory and learning), the nigrostriatal (important in regulation of movement), the mesolimbic dopaminergic system (needed for reinforcing effects of drugs like cocaine and control of interactive neurobehavior with the environment), and the hypothalamus (important for reproductive function and osmotic balance) (Dow-Edwards et al. 1990; Raum et al. 1990). Furthermore, definite dose-dependent effects from cocaine, including low BWT, slow postnatal weight gain, delay in physical maturation at birth, sensory neuronal hearing deficit, and diminished conduction potentials and behavior responses, were shown in prenatally exposed rats (Church et al. 1990). Volpe (1992) emphasized that CNS pathology related to cocaine ingestion may be attributed to a variety of cocaine's actions, namely, activation of the adrenergic system by interfering with epinephrine and norepinephrine reuptake by presynaptic nerve endings resulting in hypertension and vasoconstriction; influence on dopamine, serotonin, and sodium permeability interfering with several CNS functions; and vasoconstricting effects on fetal/placental circulation (causing fetal hypoxia and nutritional depletion) and cerebral vasculature (Farrar and Kearns 1989; Van Dyke and Byck 1982; Woods et al. 1987). Thus, the circulating blood and brain levels of cocaine and its metabolites in the fetus during rapid brain growth

and functional organization of the brain may be critical factors that determine the extent of damage. In mice, the concentrations of cocaine detected in the brain were up to five times higher compared with concentrations detected in plasma (Patrick et al. 1993). The degree of transfer across the placenta is difficult to assess in humans for ethical reasons. In monkeys, using a dose equivalent to an average intake by a chronic drug-abusing adult, a 30-percent transfer to fetal blood vessels was noted by Slikker and colleagues (1991).

Currently, in utero exposure is ascertained by testing maternal urine for cocaine and its metabolites, interviewing the mother, or using both methods. The importance of using both approaches was shown by Zuckerman and coworkers (1989) in a prospective study of 1,226 mothers in whom cocaine use was determined by both interview and urinalysis. On the basis of the interview alone, 24 percent of exposed infants would have been missed; urinalysis alone would have missed 47 percent of exposed infants. The detection of cocaine and its metabolites in both blood and urine is limited by a short elimination time. Hence, urine testing in neonates or their mothers provides evidence only of recent exposure to cocaine.

Recently, several studies have documented that cocaine and its metabolite BZE are incorporated into hair during the growth of the hair shaft and remain there for the whole life of the hair (Baumgartner et al. 1982; Graham et al. 1989; Koren et al. 1992; Callahan et al. 1992; Forman et al. 1992; Magura et al. 1992). Because the rate of hair growth is about 1 cm per month (Saitoh et al. 1967), the time and duration of exposure can be obtained by cutting the hair into segments. Three to four cm of hair growth may suggest drug ingestion for at least 3 to 4 months prior to testing. Similar to adults, the newborns of mothers who used cocaine late in pregnancy have BZE in their hair (Graham et al. 1989; Callahan et al. 1992). Cocaine is rapidly metabolized either spontaneously or by serum and hepatic cholinesterase to active compounds (such as norcocaine) and inactive compounds (such as BZE and ecgonine methyl ester) that are renally excreted (Stewart et al. 1979; Roe et al. 1990). Cocaine is less polar; therefore, it accumulates more rapidly in the hair than BZE. However, over time BZE incorporation in the hair increases because of its longer elimination

half-life. The pharmacokinetics of cocaine follow first-order elimination (Jatlow 1988). Similar to nutrients and other small molecules, drugs are transferred into the growing hair shaft through the capillaries that nourish the shaft. Although the mechanics of drug transport into hair are not well understood, it is pharmacokinetically conceivable that systemic exposure to the drug, measured as the area under the concentration curve, will dictate the amount of drug in the hair. Drugs become detectable in the hair in about 3 to 4 days. Furthermore, Koren and colleagues (1992) have shown that crack cocaine smoke may result in cocaine, but not BZE, deposition in the hair and that such a deposition is washable, differentiating contamination versus true systemic exposure. They also showed that the presence of cocaine in amniotic fluid is unlikely to cause false-positive hair BZE levels by contamination in guinea pig neonatal fur. BZE levels in neonatal hair thus appear to be from systemic exposure. The rate of accumulation depends on the degree and duration of exposure, which in turn may be dictated by the degree of placental transfer, deficiency of cholinesterase, and placental and fetal clearances of cocaine. Because brain, spleen, kidney, and urine are known to concentrate cocaine at levels higher than blood (Baselt 1982; Mittleman and Wetli 1984), we hypothesize that higher BZE levels in the hair may indicate increased levels in the brain.

Cocaine and its metabolites may be detected by radioimmunoassay (RIA) and gas chromatography/mass spectrometry (GC/MS) (Reuschel and Smith 1991; Harkey et al. 1991; Baumgartner et al. 1982). GC/MS can be used to analyze cocaine, BZE, ecgonine methyl ester, cocaethylene (a byproduct of cocaine and alcohol in vivo), and norcocaine (an oxidized product of cocaine in vivo) (Stewart et al. 1979). Although GC/MS methodology is the accepted standard for the confirmation of positive drug screens in urine, meconium, and hair, it is technically difficult and time consuming and not cost-effective for routinely analyzing large numbers of hair samples. On the other hand, the modified RIA screening for BZE (Abuscreen, Roche Diagnostic Systems, Nutley, NJ) method used in the current study not only analyzes the BZE present in the sample but also measures parent compound cocaine that is converted to BZE during extraction procedures. Although the modified Abuscreen RIA test is not within the technical reach of the

average laboratory, it still can be used for testing multiple hair samples for a reasonable price. Furthermore, in the authors' experience, GC/MS confirmation of RIA for cocaine metabolites has not revealed any false positives due to testing errors.

STUDY 1: NEONATAL HAIR ANALYSIS FOR BENZOYLECGONINE—A QUANTITATIVE MARKER FOR GESTATIONAL COCAINE EXPOSURE

Materials

A total of 151 newborn subjects (born at the Medical University Hospital in Charleston, South Carolina) were enrolled prospectively for a period of 32 months. The study comprised newborns of mothers with a positive drug history during pregnancy and a positive urine drug screen for cocaine metabolites at delivery (study group = 151). Drugs screened in the urine were opioids, amphetamines, cocaine metabolites, benzodiazepine, barbiturates, and cannabinoids. Although the extent of maternal drug use behavior is difficult to assess by urine drug screen, a cocaine-positive urine screen at the time of delivery at least confirms that the neonate was exposed to cocaine late in pregnancy. The control newborns (control group = 100) were born during the same period and were selected to match the study subjects in sex distribution, gestational age (GA) (range 27 to 42 weeks), race ($\geq$75 percent black), and maternal demographic variables such as marital status, race, educational level, insurance status, and age distribution. The mothers of control newborns had confirmed negative histories, and the neonates had negative urine screens for cocaine metabolites. The neonates had been closely monitored prenatally with many prenatal clinic visits compared with the study population. The study was approved by the Institutional Review Board for Human Research of the Medical University of South Carolina.

Methodology

Maternal information was gathered from computerized data collection systems, chart review, and the central toxicological laboratory. Maternal data included sociodemographic data (marital

status, race, educational level, insurance status, age distribution); height; gravidity and parity; pregnancy and intrapartum complications (including mode of delivery, instrumentation at delivery, history or presence of STDs); and prenatal care information as shown in table 1. Newborn data included GA, BWT, BHC, growth status as measured by Babson's fetal infant growth curve (Babson and Benda 1976), level of care, and urine and hair analysis for cocaine metabolites. GAs of the infants were estimated using the Ballard Modification of the Dubowitz Assessment Scale (Ballard et al. 1979), whereas the weight and head growth percentiles for GA were obtained from Babson's fetal infant growth curve.

Hair Sampling and Hair Analysis

During the first 48 hours after birth, hair samples were taken from infants by clipping hairs as close to the scalp as possible in the occipital region. From 10 to 25 mg (50 to 100 strands) were collected by the principal investigator (PI) or by someone under the PI's supervision. Shaven hair samples were used for some newborns who were undergoing intravenous therapy for fluid management and needed scalp vein use. Because the sample size was small, no disfigurement of the scalp was noted. The length of hair of newborns varied between 0.5 and 2.5 cm. Maternal hair samples also were obtained close to the scalp in 18 mothers; the length of hair clippings varied from 3 to 6 cm.

Hair Benzoylecgonine Analysis

Before RIA, the procedure employed for extracting BZE hydrolyzed the cocaine that was present to the major metabolite BZE. Therefore, the BZE concentrations reported here are composites of both parent compound cocaine and major metabolite BZE.

Extraction

A 25-mg sample of hair was washed in mild detergent, immersed in 1 mL 0.1 mol/L hydrochloric acid (HCl), and incubated in a water bath at 45 °C for 48 hours. The pH of the liquid was then adjusted

TABLE 1. *Maternal variables retrieved from the perinatal information system*

Sociodemographic Data
Marital status—married/single
Race—black/white
Educational level
Insurance status—private/self-payment/medicaid/indigent
Age distribution
Height
Gravidity and parity
Pregnancy and Intrapartum Complications
Hypertension/preeclampsia/eclampsia
Poor weight gain
Drug dependence (alcohol, tobacco, cocaine, etc.)
Placental abruption
Preterm labor
History or presence of sexually transmitted diseases (syphilis, gonorrhea, herpes, acquired immunodeficiency syndrome)
Instrumentation at delivery/mode of delivery
Fetal distress/meconium stain
Prenatal Care
Number of prenatal visits
Date care began
No prenatal care

to 8.5 using 0.5 percent sodium hydroxide (NaOH). Cocaine metabolite was then extracted with a 10-mL mixture of isopropanol and chloroform (1:9). After the mixture was shaken for 1 hour, the organic layer was separated and the aqueous layer discarded. The organic layer was dried under vacuum and the residue dissolved in 0.1 mL phosphate-buffered saline. The extraction procedure, modified from Valente and colleagues (1981), indicates that the

48-hour hot HCl method gives improved (100 percent) recovery compared with methanol extraction from hair. Confirmation of cocaine extraction was made by spiking control hair samples with known concentrations of cocaine from 0.1 to 10 ng/mg hair and subjecting them to our extraction and assay procedure. Recoveries of 95 to 98 percent were obtained, with all cocaine converted to BZE.

Benzoylecgonine Assay

A modification of RIA provided by Roche (modified Abuscreen) and based on that of Graham and coworkers (1989) was performed in triplicate. A calibration curve from 32 to 1,000 ng BZE was constructed using a BZE standard obtained from SIGMA Chemical Company (St. Louis, MO), with little cross-reactivity with parent compound cocaine or other cocaine metabolites. The assay was linear in the concentration range of 32 to 1,000 ng/mL with a limit of sensitivity of 5 ng/mL, an intra-assay variability of 1 percent, and an interassay variability of 5 percent as determined from standards included in each run. Typically, an assay was performed with as little as 25 μL analyzate. Confirmation of initial hair BZE samples (n=30) for BZE also was performed using GC/MS.

Maternal (at delivery) and infant (within 24 hours of birth) urine drug screens were performed using EMIT, and confirmation of positive tests was by thin-layer chromatography or GC/MS.

Statistical Analysis

Using the hair analysis data of 251 newborns and confirmed or negative maternal drug use, positive predictive value, negative predictive value, sensitivity, and specificity of hair analysis for defining gestational cocaine exposure were calculated. The similarities or differences between the control and study mother/infant pairs were analyzed using a chi-squared test for categorical variables, median tests for ordinally scaled variables, and Student's *t*-test for continuous variables. A p value of less than 0.05 was considered statistically significant (Armitage and Berry 1988).

Results

Study mothers were significantly older (25+ years), had fewer prenatal visits or less late prenatal care, and had increased incidence of STDs and number of pregnancies compared with control mothers (table 2). The percentage of black mothers in the study group was significantly higher. The urine drug screen for cocaine was negative in all control mothers and positive in all study mothers. Eighteen study group mothers also had hair analysis findings positive for BZE, with mean $\pm$ standard deviation (SD) 9,051 $\pm$ 5,904 (ng/g hair) (range 2,389 to 23,710 ng/g hair).

No significant differences were noted between study and control groups in GA distribution ($<$33 weeks, 34 to 37 weeks, and $>$37 weeks) or BWT (very low birth weight [$<$1,500 g], weight between 1,500 and 2,499 g, and $>$2,500 g). No significant difference was noted in the admissions of the neonates to either normal, intermediate, or intensive care nurseries. The sex distribution of neonates was similar.

Significant Differences Between the Study and Control Groups. Table 3 shows that the mean BHC and BWT were significantly decreased in the study group compared with the control group (BHC 31.9 cm versus 32.6 cm; BWT 2,552 g versus 2,756 g; $p<0.03$). The study group also had significantly decreased head growth percentile measurements (26.2 percent, $p<0.05$) based on the fetal infant growth curve (Babson and Benda 1976) compared with the control group (33.5 percent, $p<0.05$). Urine cocaine and hair BZE level findings were all negative (100 percent) in the control group. In the study group, 88.1 percent (n=133) had hair positive for BZE, and 11.9 percent had negative hair analysis findings. Urine EMIT screen results for cocaine were positive in 83.5 percent and negative in 16.5 percent of the study group. Neonatal hair analysis for BZE, as an indication for gestational cocaine exposure, had a sensitivity of 88 percent, a specificity of 100 percent, a positive predictive value of 100 percent, and a negative predictive value of 93 percent. Distribution of BZE levels in the hair-positive newborns (n=133) is shown in figure 2. The range of BZE was between 99 and 23,300 ng/g hair. Of the hair-positive newborns, 79 percent had BZE levels less than 5,000 ng/g hair; 96 percent had less than

TABLE 2. *Differences in maternal profiles of study and control mothers for Study 1*

Variable	Study Newborns (%)	Control Newborns (%)	*P* Value
Age (years)			
<20	4.8	29.6	<0.001
20-24	24.7	30.6	--
25+	70.5	39.8	--
Race			
Black	93.0	76.3	<0.01
White	7.0	23.7	--
Prenatal care			
No prenatal care	31.1	19.0	<0.002
First trimester care	18.9	40.5	--
Late care	50.0	40.5	--
Incomplete care	--	--	--
Number of prenatal visits			
0-4	56.3	43.0	<0.03
5-10	28.9	32.3	--
11+	14.8	24.7	<0.001
Gravidity			
Gravida I	2.8	23.7	--
Gravida II-IV	62.7	57.0	--
Gravida V+	34.5	19.3	--
Parity			
Para I	7.0	30.1	<0.001
Para II-III	75.4	57.0	--
Para IV+	17.6	12.9	--
Pregnancy and/or intrapartum complications			
STDs	33.5	22.5	<0.04
Fetal distress/ meconium stain	31.7	21.3	<0.04

KEY: STDs=sexually transmitted diseases

TABLE 3. *Differences between findings for newborns in the study and control groups for Study 1*

Variable	Study Group	Control Group	*P* Value
1-minute Apgar	32.2%	20.4%	<0.04
1-minute Apgar 8+	67.8%	79.6%	--
Mean head circumference	31.9 cm	32.6 cm	<0.03
Mean birth weight	2,552.0 g	2,756.0 g	<0.03
Birth weight*	31.0%	38.4%	<0.07
Birth head circumference*	26.2%	33.5%	<0.05
Urine EMIT for cocaine			
Negative	16.5%	100.0%	<0.001†
Positive	83.5%	0.0%	--
Newborn hair			
Negative BZE	11.9%	100.0%	<0.001†
Positive BZE	88.1%	0.0%	--

*Measurements are based on Babson's fetal infant growth curve (Babson and Benda 1976).
†Fischer's exact test (Fleiss 1981).

KEY: EMIT=enzyme-multiplied immunoassay technique; BZE=benzoylecgonine

10,000 ng/g hair; and only 3.9 percent had levels greater than 10,000 ng/g hair. No significant difference was noted in hair BZE levels between male and female neonates and blacks and whites. The ratio of maternal to infant hair BZE levels varied between 0.5 and 8.

STUDY 2: HAIR BENZOYLECGONINE LEVELS AT BIRTH VERSUS GROWTH-STATUS (BIRTH WEIGHT, BIRTH HEAD CIRCUMFERENCE, AND GESTATIONAL AGE)

A subgroup of 64 hair-positive newborns formed the Study 2 study group, and 63 hair-negative newborns formed the Study 2 control group from the original cohort (figure 1). The subgroups were

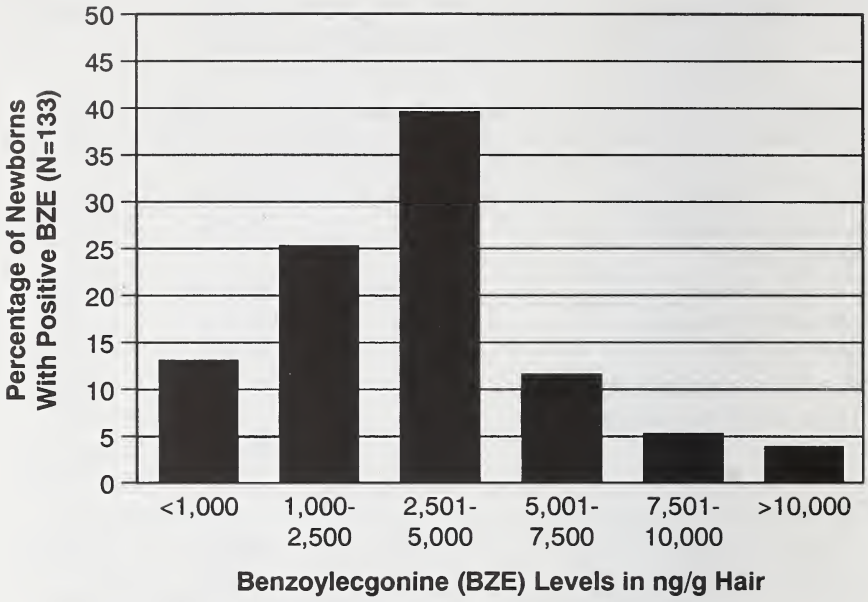

FIGURE 2. *Hair-positive newborns grouped according to benzoylecgonine levels*

selected after matching maternal profiles of both groups for age and SES and deleting mothers with STDs that could affect growth as well as fetal congenital anomalies. Sexual distribution and rates of prematurity incidence were similar in both groups.

The toxicities of cocaine may be related to the degree of exposure, which in turn may be related to the size of the organism or surface area exposed. Based on the hypothesis that similar exposure may have more deleterious effects on a smaller compared with a larger baby, the authors computed the amount of cocaine exposure in ng/g hair per square meter of body surface area (BZS [benzoylecgonine in ng/g hair/m^2 of body surface area]). BZS is also helpful in normalizing the data. The study group had both BZE and BZS levels available.

Data available on the neonates included sex, race, BWT, GA, BHC, 1- and 5-minute Apgar scores (given at birth to assess cardiorespiratory and neurological status), Babson's fetal infant growth curve (Babson and Benda 1976), and clinical problems (respiratory, CNS, feeding).

Statistical Analysis

The means of neonatal variables of the study and control groups were compared using Student's *t*-test. The association of BZS and BHC and BWT was evaluated by Pearson's correlation coefficient (Snedecor and Cochran 1980, pp. 175-193). Analysis of variance was used to compare the means of BHC, BWT, and GA of the subgroups with BZS values greater or less than 15,000.

Results

The study group had a significantly lower BHC compared with the control group (31.9 versus 33.0 cm, $p=0.01$) (table 4). The percentage of infants with head circumference (HC) less than the 10th percentile was significantly higher in the study versus the control group (50.0 versus 38.7 percent). The reduction in BWT of the study group (2,658 g) approached significance compared with the control group (2,887 g, $p=0.06$). The mean $\pm$ standard deviations (SDs) of BZE and BZS of the study group were $2{,}901 \pm 1{,}988$ ng/g hair (range 99 to 10,667 ng/g hair), and $15{,}953 \pm 11{,}440$ (range 521 to 53,335), respectively. No correlation was noted among BZE, BZS, and GA. However, a significant negative correlation was observed between BZS and BHC ($p<0.03$) and BWT ($p<0.001$). Only 47 of 64 of the study group (73.4 percent) had both hair and urine test positive, and 17 (26.6 percent) had only hair test positive for BZE. The latter group had significantly less BZE (1,567 versus 3,384 ng/g hair) and BZS (8,378 versus 18,693) compared with those in the study group who had both positive hair and urine.

Further review of the data revealed that the study group newborns who were urine-positive and had BZS levels greater than 15,000 appeared to be the most severely affected group with respect to BWT (2,233 g) and BHC (30.9 cm) and were more likely to be born

TABLE 4. *Clinical data for newborns in the study and control groups for Study 2*

Variable	Study Group (N=64)	Control Group (N=63)
Gestational age	37.4±3.5 weeks	38.2±2.9 weeks
Birth weight	2,658±717 g	2,887±644 g
Birth head circumference	31.9±2.5 cm	33.0±2.0 cm
1-minute Apgar	6.7±2.2	7.7±1.8
5-minute Apgar	8.2±1.5	8.7±0.9
BZE	2,901±1,988 ng/g hair	--
BZS	15,953±11,440	--
<10th percentile HC*	50.0%	38.7%
<25th percentile HC*	64.0%	50.8%
Males	52.0%	49.0%
Females	48.0%	51.0%
Neonatal problems	--	--
Prematurity	25.0%	28.6%
Respiratory distress syndrome	54.6%	19.0%
Withdrawal	4.7%	0.0%
Rule out sepsis	1.6%	6.3%
Central nervous system	14.0%	4.8%
Digestive	4.7%	2.2%

*Measurements are based on Babson's fetal infant growth curve (Babson and Benda 1976).

KEY: BZE=benzoylecgonine; BZS=benzoylecgonine in ng/g hair/m^2 of body surface area; HC=head circumference

prematurely (36.2 weeks) compared with urine-positive study group newborns with hair BZS levels less than 15,000. Least affected were the study group newborns who were urine-negative and had BZS levels less than 15,000. The latter group had a mean BWT of 3,056 g, BHC of 33.3 cm, and GA of 39.1 weeks (figure 3). Thus, it appears that simultaneous hair and urine analysis for BZE is a

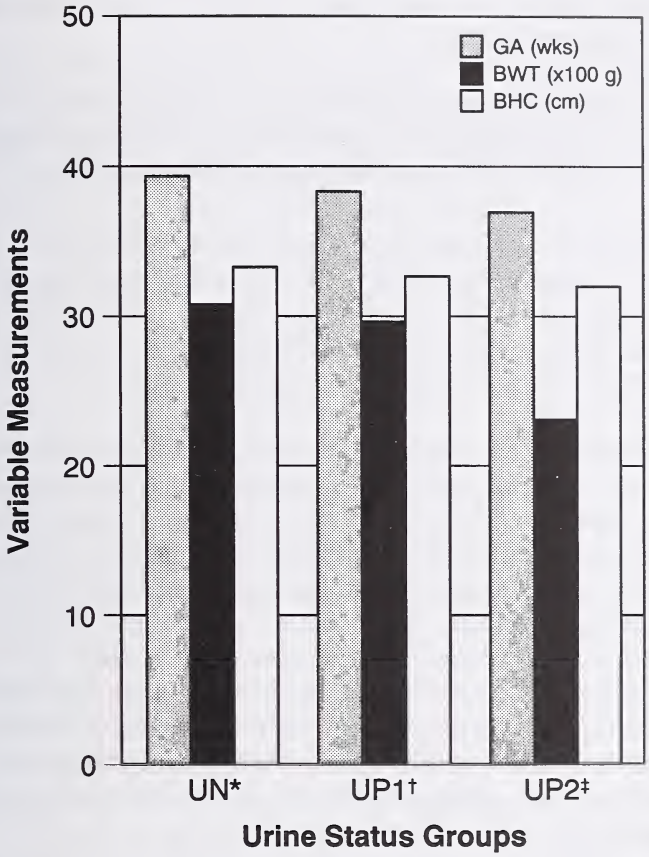

FIGURE 3. *Enzyme immunoassay screen for cocaine in urine and benzoylecgonine in hair of neonates for Study 2*

*UN = Urine-negative group with BZS < 15,000 (14 newborns)
†UP1 = Urine-positive group with BZS < 15,000 (25 newborns)
‡UP2 = Urine-positive group with BZS > 15,000 (22 newborns)

KEY: GA = gestational age; BWT = birth weight; BHC = birth head circumference; BZS = benzoylecgonine in ng/g hair/m^2 of body surface area

superior prognosticator of the degree of growth retardation and the risk for prematurity at birth.

STUDY 3: HAIR BENZOYLECGONINE LEVELS AT BIRTH AND NEURODEVELOPMENTAL DISABILITY AT 4 TO 8 MONTHS AND 19 MONTHS OF FOLLOWUP

From Study 2, 41 hair-positive newborns made up the study group for Study 3. These were matched with 14 control newborns from Study 2 to make up the control group for Study 3. Newborns currently in the developmental followup clinic were subjects for Study 3. None had birth asphyxia (5-minute Apgar ≤ 3).

During the followup clinic visits at 4 to 8 months and 19 months, the following data were collected: (1) BWT, BHC, and growth percentiles (Babson's fetal infant growth curve [Babson and Benda 1976] and the National Center for Health Statistics [Hammill et al. 1979]) and (2) neurodevelopmental assessments performed by developmental pediatricians and physical therapists specifically evaluating four areas of development—gross motor, fine motor/cognitive, behavior, and feeding problems—per our standard protocol (Cattell 1980; Illingsworth 1990). Furthermore, depending on the developmental area affected, an NDD scoring system was devised with scores ranging from zero to 4 to describe infants with no problems through severe problems such that zero=none, 1=motor problems only (minimum), 2=1+fine motor/cognitive (mild), 3=2+behavioral/sensory (moderate), and 4=3+feeding problems (severe). At birth, 56 percent were placed under foster care, and 44 percent of biological mothers undergoing therapy for drug addiction were caregivers. The developmental assessments were performed blinded to hair BZE and BZS levels.

The relationship between BZE and BZS and birth variables BWT, BHC, GA, 1-minute Apgar, 5-minute Apgar, and NDD profile was analyzed with SAS using a one-way analysis of variance, Pearson's and Spearman's correlation coefficients (Snedecor and Cochran 1980, pp. 175-193), and Student's *t*-test.

Study Infants

All study infants had hair analysis findings for BZE that were positive at birth with a mean±SD 2,903±1,950 ng/g hair and mean BZS 16,417±11,288 ng/g hair/m^2 of body surface area. There were 21 males and 20 females with mean±SD for BWT (2,510±747 g), GA (36.7±3.9 weeks), BHC (31.37±2.7 cm), and 1- and 5-minute Apgars of 6.5±2.1 and 8.0±1.6, respectively, as shown in table 5. Mean BWT and BHC percentages were 26.5±23.6 and 32.8±24.0, respectively. Of the 41 study infants, 58.5 percent had respiratory problems, and 12.2 percent had CNS pathology (irritability, seizures, abnormal tone) (table 5). No correlation was found between BZS and GA. However, 17 percent had spent fewer than 33 weeks in the womb. There was a significant negative correlation between BZS and BWT (r -0.36, $p<0.02$). Infants with BZS greater than 15,000 (17/41, 41.5 percent) had a significantly lower BWT (2,092 versus 2,806 g, $p<0.002$), BHC (30.2 versus 32.2 cm, $p<0.02$), with a tendency for prematurity (35.2 versus 37.8 weeks, $p<0.06$), and significant length of hospital stay (30 versus 8 days, $p<0.03$) compared with those with BZS levels less than 15,000 (figure 4). On followup at 4 to 8 months, 88 percent of these infants had a catchup growth pattern (as defined by increment-in-growth percentiles postnatally for BHC and weight of between 10 and 25 percent when compared with those at birth, who were in the <10th or <25th percentiles for gestational age) for BHC, and 85 percent had a catchup pattern for weight. However, the NDD profile (zero to 4) correlated significantly with BZS levels ($r=0.34$, $p<0.03$) after controlling for BWT. BZE also correlated significantly with the NDD score. However, it did not correlate with BWT and BHC.

All but two of the study infants had some degree of NDD (95 percent). Nineteen (46.3 percent) had NDD score 1, and the other 20 infants had NDD scores between 2 and 4—NDD 2=7, NDD 3=4, and NDD 4=9. The latter group (NDD 2 to 4) had similar BWT, BHC, and GA distributions compared with NDD zero to 1 (21) and differed in BZE (2,408 versus 3,232 ng/g hair) and BZS (13,425 versus 19,558) (figure 5). Those study group infants with NDD 3 to 4 (n=13) had significantly increased BZE and BZS

TABLE 5. *Clinical and drug analysis (hair and urine) data of 41 infants exposed to cocaine in utero reported for Study 3*

Variable	Mean±Standard Deviation	Range
Sex		
Male	21	
Female	20	
Race		
Black	39	
White	2	
Gestational age	36.7±3.9 weeks	25-42 weeks
Birth weight	2,510±747 g	866-3,940 g
Birth head circumference	31.37±2.7 cm	5-36 cm
1-minute Apgar	6.5±2.1	1-9
5-minute Apgar	8.0±1.6	3-9
Mean birth weight percentage*	26.5±23.6%	<10-75%†
Mean birth head circumference percentage*	32.8±24.0%	<10-90%‡
Respiratory problems	24 (58.5%)	
Central nervous system	5 (12.2%)	
Urine drug screen	33 (80.5%)	
Hair for BZE	41 (100%)	
BZE	2,903±1,950 ng/g hair	99-10,667 ng/g hair
BZS	16,417±11,288	521-53,335
BZS		
Female	14,758±12,149	
Male	18,160±10,327	

*Measurements are based on Babson's fetal infant growth curve (Babson and Benda 1976).

†Of 41 infants, 76 percent were below the 25th percentile for birth head circumference; of these, 65 percent were below the 10th percentile.

‡Of 41 infants, 54 percent were below the 25th percentile for birth weight; of these, 55 percent were below the 10th percentile.

KEY: BZE=benzoylecgonine; BZS=benzoylecgonine in ng/g hair/m^2 of body surface area

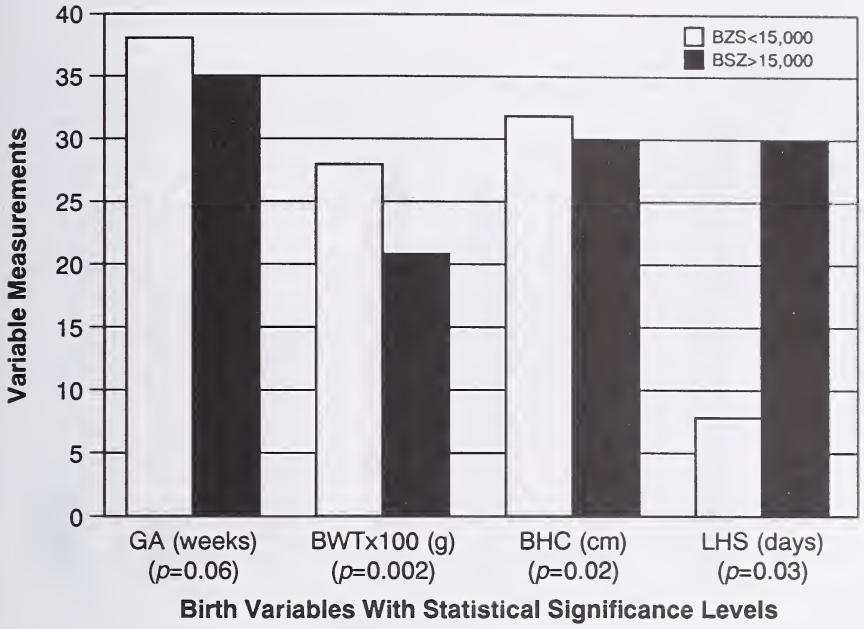

FIGURE 4. *Birth time variables in neonates with BZS less than 15,000 and those with BZS greater than 15,000*

KEY: BZS=benzoylecgonine in ng/g hair/m^2 of body surface area; GA=gestational age; BWT=birth weight; BHC=birth head circumference; LHS=length of hospital stay

compared with the study group infants with NDD zero to 2, $p < 0.05$ (figure 6).

At a subsequent followup of 27 study group infants at 19 months of age, those with persistent NDD had significantly higher BZS (16,700) and a lower mental developmental index (MDI) finding (82.2) compared with those with transient NDD at 4 to 8 months (9,700, $p < 0.05$; 96.4, $p < 0.05$) (figure 7).

Study infants had significant reduction in BWT (2,510 versus 2,909 g) and BHC (31.4 versus 33.6 cm) compared with control infants ($p < 0.05$). On followup, growth was greater than 25 percent for control infants, and only 7 percent (1/14) had NDD compared with 95 percent for the study infants.

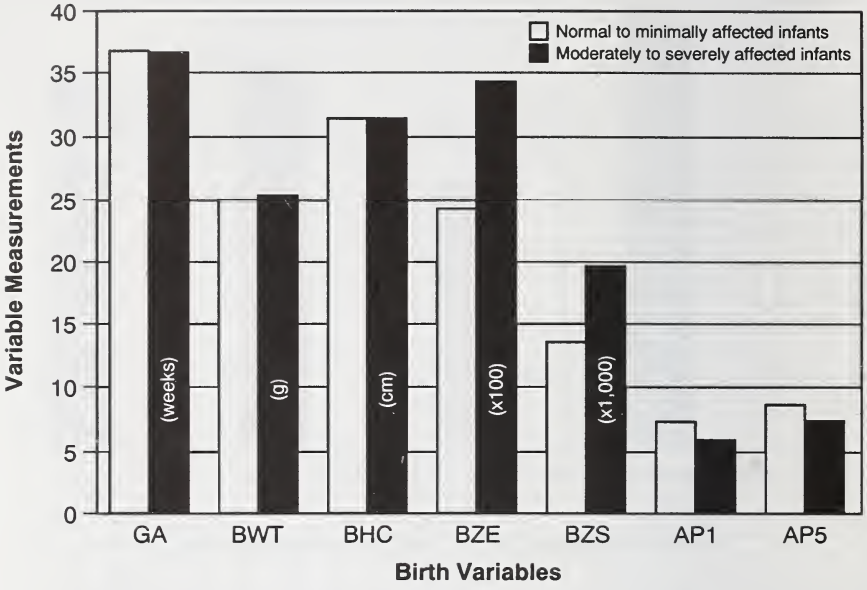

FIGURE 5. *Birth variables and degree of neurodevelopmental disability in normal to minimally affected infants and moderately to severely affected infants*

KEY: GA=gestational age; BWT=birth weight; BHC=birth head circumference; BZE=benzoylecgonine; BZS=benzoylecgonine in ng/g hair/m^2 of body surface area; AP1=Apgar score at 1 minute; AP5=Apgar score at 5 minutes

DISCUSSION

Cocaine metabolites have been detected in the hair of newborn infants of women who used cocaine during pregnancy and are detectable in the hair until 2 to 3 months of age (Graham et al. 1989). Recently, Callahan and colleagues (1992) evaluated the sensitivity of infant's hair, meconium, and urine in detecting gestational cocaine exposure in 59 predominantly white

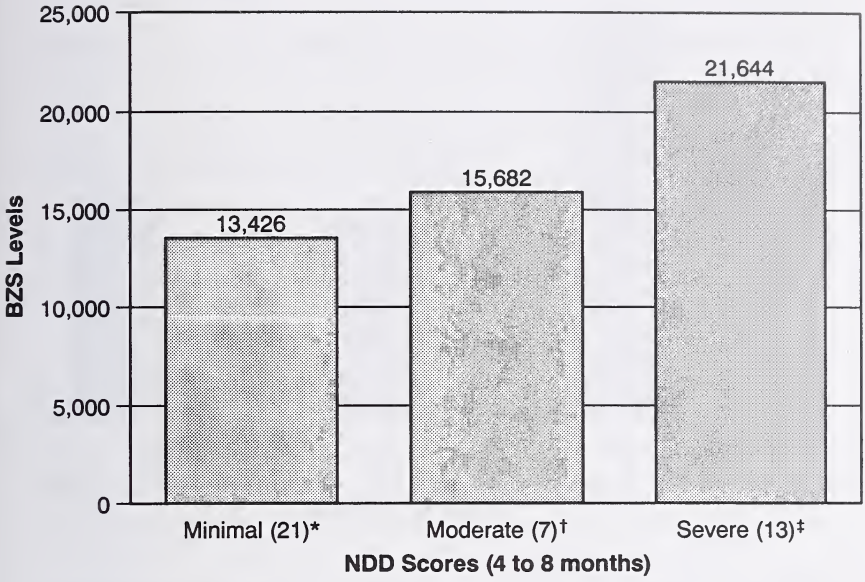

FIGURE 6. *Correlation of neurodevelopmental disability at 4 to 8 months with mean BZS at birth*

*NDD zero to 1 (includes two normal neonates with positive BZE)
†NDD 2
‡NDD 3 to 4

KEY: BZS=benzoylecgonine in ng/g hair/m_2 of body surface area; NDD=neurodevelopmental disability

mother/infant pairs. Maternal hair also was analyzed for BZE in three sections corresponding to the first, second, and third trimesters of pregnancy. Of the biological specimens, hair had the highest sensitivity (78 percent), followed by meconium testing by GC/MS (74 percent), and urine immunoassay (38 percent). The quantity of BZE in the newborns' hair correlated best with a proximal segment of mother's hair representing the last 9 to 12 weeks of antepartum hair growth ($r<0.83$).

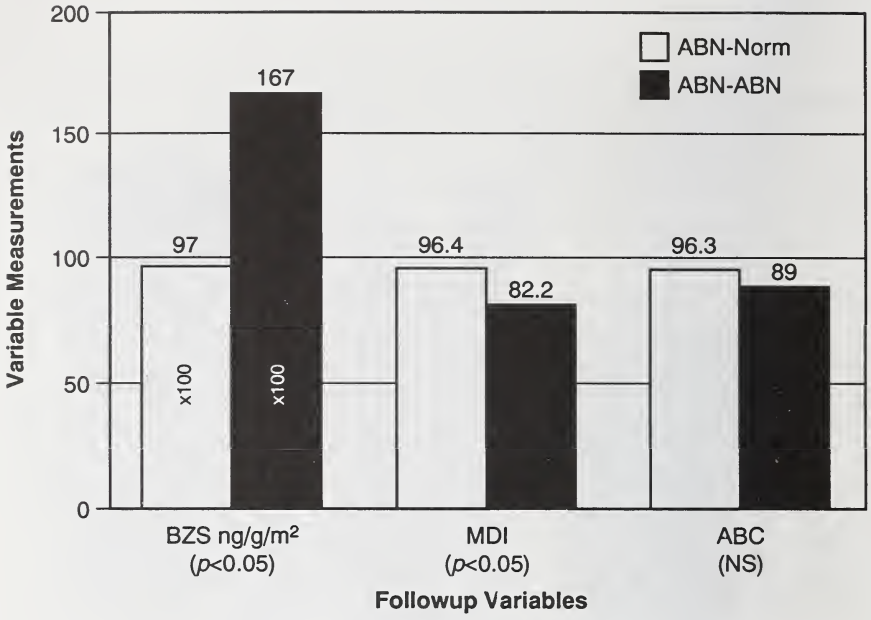

FIGURE 7. *Neurodevelopmental disability versus BZS at 19-month followup*

KEY: BZS=benzoylecgonine in ng/g hair/m^2 of body surface area; ABN-Norm=NDD present at 4- to 8-month evaluation that became normal at 19 months; ABN-ABN=NDD present at 4- to 8-month evaluation that persisted at 19 months; MDI=mental development index; ABC=adaptive behavior composite; NS=not significant

The data from hair analysis for BZE collected prospectively from a sample size of 151 gestational cocaine-exposed newborns and 100 negative control newborns satisfy several factors necessary for testing a biological marker for drug ingestion passively using the fetus via the placenta (Evans 1992). Hair analysis for BZE is noninvasive and needs a small sample size (15 to 25 mg). Neonatal hair analysis for BZE, as an indication for gestational cocaine exposure, had a sensitivity of 88 percent, a specificity of 100 percent, a positive predictive value of 100 percent, and a negative predictive value of 93 percent. No false positives were noted; however, 12 percent of the newborns had negative hair

analysis. The mothers of these neonates may have used the drug inconsistently. The intent of neonatal hair BZE analysis is to identify the degree of in utero cocaine exposure, not to detect maternal cocaine use during pregnancy. Furthermore, the hair analysis for BZE was quantitative, with 96 percent of the newborns having BZE levels less than 10,000 ng/g hair (range 99 to 23,700 ng/g hair). BZE hair levels of study mothers (18 tested) were similar to those obtained by Graham and coworkers (1989) in adult heavy cocaine users (9,315 versus 8,775 ng/g hair). In their study, Graham and colleagues found that neonates exposed to cocaine in utero had hair BZE levels between 200 and 27,500 ng/g hair, which is similar to the distribution found in the present study. The extraction and the assay used in the current hair BZE analysis identify total cocaine exposure as determined by BZE that is already present plus BZE from the parent cocaine compound. Because hair growth is constant at about 1 cm a month, the presence of BZE in the newborn's hair may indicate chronic gestational exposure beginning at least 6 to 12 weeks before birth (Graham et al. 1989). The ratio of mother-to-infant hair BZE levels was 0.5:8, which may be indirect evidence of placental transfer. Drug uptake and retention seem to be greater in black hair compared with brown hair (Kidwell and Blank 1990), with similar differences noted in experimental animals (Harrison et al. 1974; Forrest et al. 1972). In the current study, the population was predominantly black, and no significant differences in hair BZE levels were noted in black and white newborns. The disadvantages of RIA are that its radioactive waste needs appropriate disposal and the equipment is not routinely available in smaller hospitals.

Although meconium analysis appears to be more sensitive than urine analysis (Ostrea et al. 1992), it has several limitations as a biological marker of intrauterine exposure. Meconium is available only during the first few days of life, whereas hair will stay positive for at least 3 to 4 months (Graham et al. 1989). It is not clear how much cocaine in the meconium stems from the newborn swallowing amniotic fluid versus intrahepatic circulation of the drug. The dose-response curve for cocaine in meconium has not yet been established.

The most accepted teratogenic consequence of cocaine exposure is intrauterine growth retardation (IUGR) (Oro and Dixon 1987; Neerhof et al. 1989; Gillogley et al. 1990; Cherukuri et al. 1988; Doberczak et al. 1988; Chouteau et al. 1988). Zuckerman and colleagues (1989) and Zuckerman and Frank (1994) found independent contributions of cocaine and marijuana with regard to decreased growth pattern of both BWT and length and an independent contribution of cocaine with regard to decreased BHC. Several studies to date also confirm decreased BHC in cocaine-exposed newborns (Hadeed and Siegel 1989; Zuckerman et al. 1989; Handler et al. 1991; Fulroth et al. 1989; Gillogley et al. 1990; Chasnoff and Griffith 1989; Little and Snell 1991; Bingol et al. 1987; Frank et al. 1990). The degree of cocaine exposure, nutrition, and intrauterine infections may play a role in IUGR.

A comparison of 64 infants exposed to cocaine in utero as confirmed by quantitative positive hair analysis for BZE at birth with appropriately matched SES in 63 controls with negative hair analysis for BZE indicated that the in utero cocaine-exposed infants had significantly smaller BHC compared with the control group; BWT differences approached significance. In the hair-positive group, there was a significant negative correlation between BZS and BWT and BHC, although no correlation was found between BZS and GA. Furthermore, 47 infants whose hair and urine tested positive for cocaine metabolite had a significantly higher amount of BZE ng/g hair and BZS compared with infants who were positive only in hair. In addition, babies who had a positive urine test and BZS levels greater than 15,000 had significant growth reduction for BWT and BHC; these babies also had a tendency for prematurity compared with infants with less than 15,000 BZS and a positive urine test. Babies' BWT and BHC were less affected if only their hair tested positive (while urine tested negative) and if BZS was less than 15,000 (figure 3). A simultaneous urine and hair analysis appears to be a better predictor of growth at birth (Katikaneni et al. 1991, 1992; Sallee et al. 1995).

Neurodevelopmental Deficits Following Gestational Cocaine Exposure

Numerous studies have reported neurobehavioral and developmental deficits in infants exposed to cocaine in utero. Neurological findings in neonates include increased jitteriness, tremors, irritability, high-pitched and excessive crying, hypertonia and hypotonia, vigorous sucking, hyperactivity, and movements (Cherukuri et al. 1988; Dixon and Bejar 1989; Doberczak et al. 1988; Fulroth et al. 1989; Chasnoff et al. 1989*b*; Parker et al. 1990). Other investigators have reported abnormal electroencephalograph findings and visual- and auditory-evoked responses (Dixon and Bejar 1989; Doberczak et al. 1988). In studies using the Brazelton Neonatal Behavioral Assessment Scale (NBAS) (Brazelton 1984, pp. 1-125), an omnibus assessment of infant neurobehavioral organization, cocaine-exposed infants have been observed to have poorer control of sleep and awake states and impaired interactive abilities relative to control infants. Alterations in neurobehavioral functioning noted by NBAS have been reported for other drugs such as alcohol and marijuana (Richardson and Day 1991; Coles et al. 1992).

Cognitive and Behavior Outcomes

Several high-risk variables that could affect both the biological vulnerability and the postnatal nurturing environment that are present in gestationally cocaine-exposed infants could also cause developmental deficit. Controversies exist as to the contribution to the degree of NDD of cocaine plus polydrug use, lack of a nurturing environment, and maternal SES characteristics. In addition, the presence of prematurity, IUGR, and chronic infections increases NDD independent of cocaine exposure (Villar et al. 1984). Because of the nature of damaging effects of cocaine (vascular insults and neurotransmitter perturbations), outcome measures at later ages should include not only evaluation of vascular insults but also potential effects of neurotransmitter interference, particularly frontal lobes, with increased dopaminergic projections (Welsh and Pennington 1988). The neurobehavioral risks include social competence and regulation of arousal and attention. The latter risk becomes important in preschool and elementary school years.

Cocaine-exposed very low birth weight infants had a higher incidence of Grade I to II intraventricular hemorrhage (IVH) and cognitive deficits even after correcting for the effects of IVH and chronological age in a followup study at 16.5 ± 8 months (Singer et al. 1994). The only well-controlled and blind clinical studies published to date on infants at 2 years and older (Chasnoff et al. 1992; Azuma and Chasnoff 1993; Griffith et al. 1994) come from the National Association for Perinatal Drug Addiction Research and Education. In those three studies, the cognitive abilities were similar in children exposed to cocaine/polydrug use in utero compared with controls as measured by MDI at 2 years and Stanford-Binet test at 3 years. However, approximately one-third of drug-exposed children displayed delays in normal language development and/or problems in attention and self-regulation. In the absence of intrauterine infection, similar SES compared with the non-drug-exposed newborns, and absence of heavy maternal alcohol or tobacco use, it is conceivable that degree and duration of exposure (reflected by the hair BZE level) may determine the extent of NDD.

A total of 41 infants exposed to cocaine in utero had mean BZE 2,909 ng/g hair and BZS 16,417 levels at birth. Growth reduction (BWT and BHC) was significantly associated with BZS greater than 15,000 (BWT 2,092 versus 2,806, $p < 0.002$; BHC 30.2 versus 32.2, $p < 0.02$) compared with those infants with BZS less than 15,000. On 4- to 8-month followup, 88 percent of these infants had a catchup growth pattern for HC, and 85 percent had a catchup growth pattern for weight. However, NDD correlated significantly with BZS ($p < 0.03$) after controlling for BWT. Moreover, infants with NDD greater than 2 areas (3 and 4) had the highest mean BZS levels (21,645) compared with those mildly affected by NDD (NDD=2, 15,682) and minimally affected by NDD (NDD zero to 1, 13,425), $p < 0.05$ to BZS levels (21,645, $p < 0.05$). At a subsequent followup of 27 infants at 19 months of age, those children with persistent NDD had significantly higher BZS (16,734) and lower MDI (82) compared with those having transient NDD (9,746, $p < 0.05$; 96.4, $p < 0.05$) (Katikaneni and Sallee 1993; Katikaneni et al. 1993). Targeting this population with early intervention and environmental support may be cost-effective.

SUMMARY

Neonates with positive hair and urine drug tests for cocaine metabolites at birth appeared to be much more severely affected with regard to growth (BWT and BHC), with a tendency for prematurity and need for prolonged hospital stay if BZE ng/g hair per square meter of body surface area was greater than 15,000. However, on followup at 4 to 8 months, 88 percent of these infants showed a catchup growth pattern for BHC, and 85 percent had a catchup growth pattern for weight, although NDD persisted and significantly correlated with BZS levels at birth. Thus, this study shows that quantitative hair BZE levels at birth may be an important indicator for gestational cocaine exposure, degree of cocaine exposure, degree of growth reduction at birth (BWT and BHC), and degree of NDD on 4- to 8-month and 19-month followups. Further studies are in progress.

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