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November 2008

Research and Development in Forensic Toxicology

Analysis of Cocaine Analytes in Human Hair: Evaluation of Concentration Ratios in Different Hair Types, Cocaine Sources, Drug-User Populations, and Surface-Contaminated Specimens

FINAL REPORT

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ABSTRACT

Understanding the disposition of controlled substances in hair and the effects of environmental contamination on hair drug-testing results remains elusive. Current interpretation practices use a benzoylecgonine-to-cocaine (BE/COC) ratio of $\geq$0.05 to distinguish cocaine (COC) use from external contamination; however, ratios of other analytes, such as cocaethylene (CE) and norcocaine (NCOC), have not yet been fully investigated. This study's primary goal was to evaluate COC analyte concentrations and metabolite-to-parent drug concentration ratios in human hair to include hair type (e.g., color), COC source (e.g., pharmaceutical, street drug), and drug-environment conditions. This research evaluated the effects of COC composition, COC incorporation by ingestion and external contamination, and hair color (light and dark hair) on COC analytes and analyte-to-parent ratios found in hair. RTI International's[1] Center for Forensic Sciences evaluated hair obtained from commercial and professional sources and research facilities. Hair and refined illicit COC specimens were analyzed using liquid chromatography-tandem mass spectrometry. COC analyte concentrations and ratios for all drug-user populations agreed with concentrations previously reported by RTI and other researchers. Contamination studies with COC containing higher CE, BE, or NCOC concentrations resulted in significantly higher concentrations (p = 0.0001) of each of these drug compounds in the respective hair specimens. This indicates that the quantity of COC analyte found in illicit COC can affect the concentration of these compounds in the contaminated hair, although it may not be a linear relationship. BE/COC ratio increased significantly over time and could not be used reliably to identify COC-contaminated hair. Similar findings were observed when hair was contaminated with a COC hydrochloride (HCl) solution. Furthermore, criteria for distinguishing COC use from possible environmental exposure at realistic concentrations did not appear to be significantly improved by adding criteria that evaluated CE and NCOC to parent drug concentrations. In no instances did the cocaethylene-to-cocaine or the norcocaine-to-cocaine ratios result in confirmation rulings (e.g., COC positive or negative) different from that obtained by evaluating the BE concentration and the BE/COC ratio. The results also indicate that the COC cut-off concentrations and ratios currently used by many forensic drug-testing laboratories may not effectively discriminate between drug use and environmental exposure. Further research is needed to determine if using additional decision criteria, which may include a unique COC metabolite, wash criteria, or mathematical criteria that compare the presence of a drug in wash solutions to concentrations in hair, may be necessary to adequately and reliably identify external COC contamination.

[1] RTI is a trade name of Research Triangle Institute.

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EXECUTIVE SUMMARY

Statement of the Problem

Detecting the compound cocaine (COC) in hair is not sufficient to identify drug use because hair shafts may be contaminated by COC released into the air during smoking, and contaminated hands can transfer COC powder residue from surrounding surfaces where use occurred (Stout et al., 2006b; Kidwell and Smith, 2007). For these reasons, other COC analytes and even parent drug-to-metabolite concentration ratios are evaluated in hair drug-testing programs to ensure that the hair test only identifies the illicit use of COC; however, the efficacy of established cut-off concentrations for COC in hair is still debated.

There are up to four COC analytes routinely investigated for hair drug testing. These analytes include the parent compound (COC), as well as benzoylecgonine (BE), cocaethylene (CE), and norcocaine (NCOC). COC is the most abundant analyte, followed by BE (<10%–50% of COC), CE (<20% of COC), and NCOC (<10% of COC). In addition to analyte concentrations, a benzoylecgonine-to-cocaine (BE/COC) ratio can be monitored with the understanding that a ratio of <0.05 suggests that the hair is contaminated with COC and should not be reported as positive for drug use (SAMHSA, 2004a). Ratios for other COC analytes have also been monitored with less success (Bourland et al., 2000; Ropero-Miller et al., 2002; Cairns et al., 2004a; SAMHSA, 2004a; Scheidweiler et al., 2005).

Research evaluating COC analyte concentrations in hair following external contamination and fortification with drug standard solutions is limited. Reported contamination studies have focused on COC and BE after contaminating hair with COC, most likely because these are the primary analytes in hair and because the CE and NCOC were largely believed to be metabolites of COC and not by-products of the manufacturing process. Therefore, researchers did not expect to find CE or NCOC if they exposed the hair to high-purity COC. More recent studies (Scheidweiler et al., 2005; Stout et al., 2006b) have demonstrated that these COC analytes can occur at appreciable concentrations in studies where high-purity COC hydrochloride (HCl) powder was administered subcutaneously under controlled dosing and where it was applied to the hair during a short exposure time followed by 10 weeks of daily hygienic treatment (i.e., shampooing).

Project Purpose and Goals

The purpose of this study was to investigate COC analyte concentrations in hair and determine if concentration ratios could be established to distinguish between hair from a COC user and hair from a non-user. RTI International (RTI) evaluated the effects of COC source (e.g., pharmaceutical, street drug), and specimen type (e.g., drug user in the street environment, drug user in the controlled research environment, external drug fortification, and hair surface contamination) on analyte concentrations and metabolite-to-parent drug concentration ratios found in human hair. COC source and specimen type were evaluated separately in hair externally contaminated with a dry COC HCl powder (e.g., surface contamination model) and hair that was fortified with the same COC materials dissolved in a fortification solution (effectively used as an alternate contamination model). RTI contracted with established forensic hair-testing laboratories to analyze hair specimens by liquid chromatography-tandem mass spectrometry (LC-MS/MS). RTI used research designs and methods that are applicable to the current proposed Mandatory

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Guidelines and practices to determine the COC analyte concentrations in multiple drug-exposure situations. The research design and methods examined multiple potential effects in separate experiments, which included hair type, COC source, use pattern, laboratory fortification (i.e., spiking or drug incorporation methods), and environmental contamination. This study evaluated realistically expected situations to provide additional information about the potential differences in the COC concentration ratios observed. RTI has published the results of this study in the U.S. Drug Enforcement Administration's (DEA's) *Microgram Journal* and is currently in the process of submitting another manuscript to the *Journal of Analytical Toxicology* to achieve another project goal. RTI staff members have also presented the results at two annual Society of Forensic Toxicologists meetings.

Research Design and Methods

This study was designed to investigate COC analyte concentrations and their ratios in hair that was contaminated through drug ingestion, externally contaminated with a solid COC HCl powder (e.g., refined, illicit COC [street COC] and commercially available, pharmaceutical-grade COC), or externally fortified with a buffered solution of COC. The following are four stages of research grouped and discussed as sections in this report based on each experimental design, sample type and preparation, analysis procedures, findings, and conclusions:

- Stage I: Analysis of COC HCl powders
- Stage II: Analysis of hair from drug-user populations
- Stage III: Contamination of hair with COC HCl powders
- Stage IV: Fortification of hair specimens with COC HCl powders.

This experimental design was unique because some hair specimens were used in both Stages III and IV studies (e.g., externally incorporated COC protocols) and allowed for the direct comparison of analyte concentrations found after contaminating the hair with COC of varying purity (e.g., >99% COC, >1% CE and >10% BE, >5% NCOC). COC analyte concentration ratios were statistically evaluated to determine significant differences. All specimens were blinded prior to shipment to the testing laboratory, and drug-free blind control specimens were included with the study specimens. Sample types are listed in **Table ES-1** by the stage of the study, the number of specimens, and the type of analysis (e.g., singular, duplicate, or triplicate analysis).

Table ES-1. Number and Analysis of Specimens in the RTI Study

Stage of Study	Sample Description	Number of Specimens and Type of Analysis
Stage I	COC HCl powders	28 (singular and duplicate analysis)
Stage II	Drug-user hair with COC self-administered in a street environment (uncontrolled administration)	38 (singular and duplicate analysis)
Stage II	Drug-user hair from volunteers with COC administered in a clinical setting (controlled administration)	20 (singular analysis)

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Stage of Study	Sample Description	Number of Specimens and Type of Analysis
Stage III	Drug-free hair surface contaminated with high-purity pharmaceutical-grade COC (i.e., <1.1% CE or NCOC) collected at 13 time points	26 (triplicate analysis)
Stage III	Drug-free hair surface contaminated with low-purity street COC (i.e., 1.4% CE and 10.1% BE) collected at 13 time points	26 (triplicate analysis)
Stage III	Drug-free hair surface contaminated with low-purity street COC with higher amount of NCOC (i.e., 8.7% NCOC) collected at 13 time points	26 (triplicate analysis)
Stage IV	Drug-free hair fortified with COC of varying purity in a solvent solution (used same COC HCl as Stage III)	5 (triplicate analysis)
Stage II–IV	Drug-free and drug-user hair as controls in study	31 (singular and triplicate analysis)
Stage IV	Decontamination wash fractions	36 (singular)
TOTAL		251 (~400 with replicate analysis)

When possible, replicate analysis was performed, and the average of the results was used for data analysis. Approximately 400 specimens were tested for COC analytes with LC-MS/MS analysis. RTI submitted both drug-free and spiked blind control specimens as a quality assurance measure to ensure that specimens were handled correctly by the laboratory.

Results

Stage I: Analysis of Cocaine Hydrochloride Powders

COC HCl powders available illicitly (i.e., on the street) have a wide range of purity and manufacturing by-products that may affect the incorporation of drug into hair and its subsequent detection by hair tests. For this reason, RTI evaluated multiple COC sources that would realistically represent illicit COC available for ingestion and environmental contamination.

The U.S. Department of Justice's DEA Special Testing and Research Laboratory performed an in-depth COC signature analysis, and the Armed Forces Institute of Pathology (AFIP) Laboratory completed a limited gas chromatography-mass spectrometry (GC-MS) analysis to determine the composition of 28 COC HCl powders. At the completion of Stage I, RTI successfully identified three COC HCl powders at varying purities (DEA: 65%–85%) that met our study design criteria and could be used for the remainder of the project. The AFIP Laboratory's results corroborated DEA's NCOC results; therefore, RTI used DEA's results to make the final selection of COC HCl powders to use in the contamination and fortification studies of this project. COC materials included one illicit COC HCl with BE at 10%, and CE at 1.4%, one illicit COC HCl with NCOC at 8.7%, and a U.S. Pharmacopeia material that was 98% pure with 1.1% CE. No base preparations of COC were used in this study.

As an additional unplanned project result, RTI compared DEA's signature analysis to an analysis on the time-of-flight direct analysis real time (TOF-DART) system (AccuTOF-DART™ manufactured by JEOL USA, Inc.). Because there was no sample preparation, RTI investigated

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if the TOF-DART instrument could be used to complement traditional methods for determining compounds in seized materials. A total of nine COC analytes were identified by TOF-DART analysis. Anhydroecgonine methyl ester (AEME) and trans-cinnamoyl COC were easily detected in 23 of the 25 specimens. However, some COC analytes were difficult to identify (e.g., tropacocaine and truxilline isomers), whereas others (e.g., isomeric pair BE and NCOC) could not be distinctly determined because of their equal masses.

Stage II: Analysis of Hair from Drug-User Populations

Stage II compared COC analyte concentrations and ratios in the hair of various drug-user populations to the Division of Workplace Programs of the Substance Abuse and Mental Health Services Administration (SAMHSA) proposed Mandatory Guidelines criteria for federal workplace drug-testing programs and six other decision points of positive calls on confirmatory analytical results. The additional decision points were selected based on a review of the data and previously proposed criteria (Schaffer et al., 2007). Criteria 1 through 3 are the original criteria proposed in the Mandatory Guidelines. Criteria 4 and 5 evaluated the norcocaine-to-cocaine (NCOC/COC) ratios of ≥0.05 and ≥0.01, respectively. Criteria 6 through 9 evaluated the cocaethylene-to-cocaine (CE/COC) ratios of each of the following: ≥0.05, ≥0.02, ≥0.01, and ≥0.002. As a final evaluation, RTI compared the results of the drug-user populations in this study to the results of other drug-user populations using the same criteria (Cairns et al., 2004a; Bourland et al., 2000). These criteria are summarized in **Table ES-2**.

Table ES-2. Cocaine Analyte Concentrations (pg/mg) and Ratios Criteria for Determining Confirmation Results as Positive or Negative

Criteria 1 (BE criteria)	COC ≥500 and BE ≥50 and BE/COC ≥0.05
Criteria 2 (CE criteria)	COC ≥500 and CE ≥50
Criteria 3 (NCOC criteria)	COC ≥500 and NCOC ≥50
Criteria 4	COC ≥500 and NCOC ≥50 and NCOC/COC ≥0.05
Criteria 5	COC ≥500 and NCOC ≥50 and NCOC/COC ≥0.01
Criteria 6	COC ≥500 and CE ≥50 and CE/COC ≥0.05
Criteria 7	COC ≥500 and CE ≥50 and CE/COC ≥0.02
Criteria 8	COC ≥500 and CE ≥50 and CE/COC ≥0.01
Criteria 9	COC ≥500 and CE ≥50 and CE/COC ≥0.002

The federal criteria were selected for COC analyte concentrations and ratio criteria (Criteria 1 through 3) without the inclusion of any additional mathematical algorithms because these criteria are the only published criteria that, in the current state of hair testing, would be applicable to multiple laboratories.

For the drug-user population that self-administered COC in a street environment (i.e., street drug user [STREET], uncontrolled administration), only 50% of the population was positive for CE (i.e., 19 of 38 subjects). When the CE and NCOC criteria (Criteria 2 and 3) for each subject's results were compared to the BE criteria (Criteria 1), there was one subject who met these criteria, but did not meet the BE criteria. Conversely, there were four subjects who met the BE criteria (Criteria 1), but did not meet the NCOC criteria (Criteria 3). Therefore, the COC

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confirmatory rate did not increase when COC analytes other than COC and BE were evaluated with the proposed cut-off concentrations (e.g., change in confirmation ruling for a subject).

If additional criteria for the CE/COC and NCOC/COC ratios were considered (e.g., ≥0.05, ≥0.02, ≥0.01, ≥0.002) for this drug-user population, the following conclusions were drawn. Although 27 of 38 STREET drug users had NCOC concentrations ≥50 pg/mg at a NCOC/COC ratio requirement of ≥0.05 (Criteria 4), all subjects tested negative with the inclusion of this additional decision point. If the NCOC/COC ratio was lowered to ≥0.01, 19 subjects would have confirmed positive by Criteria 5 (≥0.01); however, an additional 9 subjects met the NCOC cut-off concentrations of ≥50 pg/mg, but did not meet the NCOC/COC ratio of ≥0.01 (Criteria 1). Inclusion of a ≥0.05 or ≥0.01 NCOC/COC ratio did not yield confirmation results similar to the current proposed Mandatory Guidelines for BE (Criteria 1: 36 of 38 subjects [94.7%]) or NCOC (Criteria 3: 33 of 38 subjects [86.8%]). Both decision points for the NCOC/COC ratio were too high for this drug-user population, and confirmed positive, at the most, 24 of 38 subjects (63.2%). This reduced the confirmed positive rate in this drug-user population by more than a 30%.

Criteria 6 through 9 had decision points using the CE/COC ratio and yielded the following positives for COC use: ≥0.05 (3 of 38 subjects [7.9%]); ≥0.02 (8 of 38 subjects, [21.1%]); ≥0.01 (12 of 38 subjects [31.6%]); and ≥0.002 (17 of 38 subjects [44.7%]). The number of STREET drug users that met any of the CE/COC ratio criteria was much lower then the BE concentration and the BE/COC ratio criteria of the proposed Mandatory Guidelines. For the most part, the CE concentration requirement of ≥50 pg/mg is largely the determining factor (19 of 38 subjects [50%]) for confirmation rulings evaluating CE. Even a CE/COC ratio of ≥0.002 would not be equivalent to the proposed BE and CE criteria for the STREET drug-user population.

Hair specimens collected from clinically administered drug users (CLINICAL) (controlled administration) during an in-patient clinical study were procured from the National Institute on Drug Abuse's Intramural Research Program (NIDA's IRP) for analysis and inclusion in RTI's study. NIDA protocols for this clinical study were reviewed and accepted by NIDA's Institutional Review Board.

For the CLINICAL group, a smaller percentage of the population tested positive for CE (i.e., 6 out of 20 subjects). When the CE criteria (Criteria 2) for each subject's results was compared to the BE criteria (Criteria 1), there were three CLINICAL subjects who met the CE criteria, but did not meet the BE criteria. These same subjects also met the NCOC criteria, but did not meet the BE criteria. Conversely, there were four CLINICAL subjects who met the BE criteria (Criteria 1), but did not meet the NCOC criteria (Criteria 3). Therefore, there was no increase in the overall COC confirmatory rate when additional COC analytes beyond COC and BE were evaluated with the proposed cut-off concentrations (e.g., change in confirmation ruling for a subject) for the CLINICAL subjects.

If additional criteria for NCOC/COC and CE/COC ratios were considered (e.g., ratios of ≥0.05, ≥0.02, ≥0.01, ≥0.002) for the CLINICAL population, the conclusions were as follows: 3 subjects (15%) were positive at ≥0.05 NCOC/COC; 14 subjects (70%) were positive at ≥0.01 NCOC/COC; 0 subjects were positive at ≥0.05 and ≥0.02 CE/COC; 2 subjects (10%) were positive at ≥0.01; and 6 subjects were positive at ≥0.002 (the same 6 subjects as Criteria 2).

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Overall, the NCOC criteria had equivalent positive calls to BE criteria, but there was a 40% reduction of positive calls using CE criteria for the CLINICAL drug-user group.

The CLINICAL drug-user population had a much smaller administered dose of COC compared to the other drug-user populations evaluated. As evidenced, the average BE concentration in hair was 7% of the COC concentration (434 pg/mg versus 6,171 pg/mg), the average CE concentration was 2% of the COC concentration (123 pg/mg versus 6,171 pg/mg), and the average NCOC concentration was 5% of the COC concentration (290 pg/mg versus 6,171 pg/mg).

Evaluation of the Cairns and colleagues (2004a) and Bourland and colleagues (2000) drug-user groups to the nine criteria yielded similar findings, so there was no significant advantage (<3% change in confirmation result) to evaluating additional COC analytes beyond COC and BE.

Stage III: Contamination of Hair with Cocaine Hydrochloride Powders

Stage III examined the ratios of analytes in hair after contaminating it with different source COC materials. The results of this study were consistent with what RTI has previously published for contaminating hair with pharmaceutical-grade COC (Stout et al., 2006b). All three COC sources resulted in significant quantities of COC on the hair and remaining on the hair over a 10-week period. As previously observed, there was a significant decline in the COC content over the course of the study.

In our study, the contamination with COC containing higher CE, BE, or NCOC concentrations resulted in significantly higher concentrations of each of these drug compounds in the respective hair specimens, indicating that the quantity of each of the drug compounds found in an illicit COC can affect the concentration of these compounds in the contaminated hair. As with COC, these compounds were resistant to removal by either hygienic treatment (i.e., shampooing) or laboratory decontamination. Additionally, exposure to the COC that contained CE at 1.4% of the COC material resulted in a maximum CE/COC ratio of 0.05, whereas the material containing 8% NCOC resulted in a maximum NCOC/COC ratio of 0.25. These results indicate that there may not be a direct relationship between the concentrations of CE and NCOC in hair and the concentration in the contaminating COC.

This study was also consistent with previous findings because the BE/COC ratio increased significantly over the course of the study period. In the recent study, the ratio exceeded the ≥0.05 point by Day 28 for those COC materials that contained less BE, instead of Day 21 as previously reported. For the illicit COC that had high CE and BE concentrations, this ratio increased after adding synthetic sweat and continued to rise over the course of the study. Hair specimens treated with the high NCOC illicit COC powder and the pharmaceutical-grade COC did not exhibit any decline in BE over the course of the study period.

A substantial number of analyzed specimens would have been determined as positive by most of the criteria applied. For the specimens exposed to COC that contained more CE, there were more specimens that would have resulted in positive calls. For these specimens, only the criteria, including a ≥0.05 CE/COC ratio, would have resulted in no positive results. At a ≥0.02 CE/COC ratio, there were 44% of the dark hair specimens and 33% of the light hair specimens that would have tested positive. For those specimens exposed to the high NCOC that contained COC, 33% of the light hair specimens and 92% of the dark hair specimens would have been

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determined as positive by all of the criteria using NCOC. A more complex pattern was observed with BE criteria because BE appeared in the hair from all sources; therefore, varied amounts of NCOC, CE, and BE in the contaminating COC can substantially confound the use of ratios to discriminate contaminated hair specimens, even after using a laboratory's decontamination protocol.

It is important to note that RTI applied these criteria to hair specimens because a reference laboratory would apply them under the proposed Mandatory Guidelines. In other words, the reference laboratory would have had to analyze the specimens and apply the cut-off concentrations directly to these results. As Schaffer and colleagues (2007) have noted in several publications (Cairns et al., 2004a and b), they have applied various ratios of compounds and have used various mathematical calculations using the amounts of a drug found in the last wash solution. As noted by Kidwell and Smith (2007), this wash criteria has evolved over the years. The proposed Mandatory Guidelines (SAMHSA, 2004a) do not have a provision for the use of such criteria; therefore, we did not use any wash criteria during this analysis. RTI has retained the last wash solutions of all hair specimens to potentially conduct this analysis at a later date.

Developing ratios to discriminate contaminated hair is problematic because of the potentially variable CE, NCOC, and BE contents in illicit COC and the high inter-individual variability in the way an external drug interacts with the hair. The ≥0.05 CE/COC ratio in high illicit COC may have eliminated positive results in contaminated hair using COC materials with higher purity and less CE composition; however, illicit COC may contain higher concentrations of CE, potentially confounding even this ratio.

Stage IV: Fortification of Hair Specimens with Cocaine Hydrochloride Powders

Stage IV examined the ratios and concentrations of the COC analytes following the introduction of COC to the hair by fortification procedures (i.e., effectively an alternate contamination model). Results indicated that COC analyte concentrations in hair following fortification with COC HCl solutions were >50 pg/mg for NCOC, but not for BE and CE. Evaluation of these specimens using current proposed Mandatory Guidelines suggest that fortified specimens (e.g., external contamination through COC HCl solution) may not be differentiated from the hair of COC users who actually ingested COC.

Hair drug-testing laboratories could use additional steps to help differentiate COC contamination from actual ingestion, but none of these steps is routinely practiced by most laboratories. For example, a decontamination wash calculation can be applied against the COC analyte concentration for a more conservative interpretation of a hair concentration. Cairns and colleagues (2004a) suggested that the COC concentration in the final decontamination wash should be measured, multiplied by a factor of five, and then subtracted from the final COC concentration in the hair sample to estimate the amount of COC that would be further removed with additional decontamination washes. The Cairns decontamination procedure takes 3.75 hours to perform. Alternatively, Tsanaclis and Wicks (2008) suggested that a drug detected in a dried-down methanolic wash that was obtained rapidly and analyzed could be used to calculate a wash-to-hair (W/H) ratio. Evaluation of the fortification specimens with either of these decontamination wash calculations indicates that the specimens were externally contaminated. Hair drug-testing laboratories could also consider analysis of another truly metabolic product that does have a pathway for its presence as a by-product from the manufacturing process, but the abundance of such an analyte may be small in comparison to COC.

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Overall Project Summary

After evaluating COC and COC analyte concentrations and ratios in user hair from various populations, contaminated hair with various sources of COC, and an alternate external application of COC to hair (fortification), the use of cut-off concentrations for any or all of the analytes would not be reliable to discriminate a user's hair from contaminated hair. The use of analyte ratios provides more information and some ability to discriminate user specimens from contaminated specimens; however, the use of CE and NCOC concentrations and ratios does not discriminate any more efficiently than does decision criteria using only BE and COC. All three analytes (i.e., CE, NCOC, and BE) can be present at varied concentrations in illicit COC as by-products of the manufacturing process, and as such, will confound the use of ratios to discriminate contamination from use. Contamination of hair with illicit COC materials that contain ≥1% to ≥10% (weight-to-volume) of CE, BE, and NCOC resulted in hair specimens that would not be discriminated from user hair by ratios or concentrations using the criteria applied. Even after decontaminating the hair, the application of concentration and ratio decision points does not adequately discriminate contamination from drug use.

In this study, RTI applied these decision criteria because laboratories would have to apply them under the current proposed federal Mandatory Guidelines. These guidelines do not have provisions for using additional decision criteria, which include wash criteria or those mathematical criteria that compare the presence of a drug in wash solutions to concentrations in hair. Published findings from Cairns and colleagues (2004a and b) and Tsanaclis and Wicks (2008), suggest some type of decision criteria is necessary to adequately and reliably identify contamination.

These results have implications for the proposed Mandatory Guidelines because the decision criteria, as proposed in this study, do not adequately discriminate contamination. This is of particular concern for individuals whose occupation (e.g., law enforcement) may put them in contact with large amounts of COC in their environment; therefore, a requirement for decontamination and further research are needed to determine the viability of comparative criteria using information from the decontamination.

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1. INTRODUCTION

1.1 Background

For more than 30 years, hair has been used as a biological matrix to detect controlled substances, such as cocaine (COC), and to indicate drug use. Although conventional matrices, such as blood, oral fluids, and urine, document an individual's drug exposure for a period ranging from minutes to days, hair can extend the detection period from months to years, depending on the hair sampled and the collection process. Moreover, conventional matrices can be both physically and socially invasive to collect, they can require preservation through refrigeration or freezing, and, as in the case of urine, they may be susceptible to adulteration or substitution. In contrast, hair is easy to collect, the drug is relatively stable in the hair, and hair is difficult to adulterate or substitute.

Although hair testing has many applications, including death investigations, workplace drug testing, drug-facilitated crimes, and violation of probation or parole, there are still many issues that limit its widespread use. These issues include the absence of standardized techniques between laboratories, consistent proficiency-testing materials, a laboratory certification program, consistent results within and between laboratories, and easily identifiable drug analytes that discriminate between environmental contamination and drug use, as well as the presence of a potential bias of drug incorporation into the hair (i.e., hair color or ethnic differences) (Ropero-Miller, 2007a).

The potential "color bias" in hair testing arises from the ability of drugs to associate predominantly with melanin, which is a pigment in hair that contributes to its color. Drugs may associate with other hair proteins as well, but they are not usually as abundant as melanin. There are two types of melanin in hair: eumelanin, which is present at much greater quantities, and pheomelanin. Eumelanin contributes brown and black pigments (darker hair), whereas pheomelanin contributes yellow-red pigments (lighter hair). Drugs associate with eumelanin with a strong affinity. After ingesting or being exposed to the same amount of a drug, individuals who have a darker hair will have a greater amount of drug incorporated into their hair than individuals who have lighter hair (Claffey et al., 2001; Joseph et al., 1996; Stout and Ruth, 1999; Kronstrand and Scott, 2007; Stout, 2006b).

The potential "ethnic bias" in hair testing is the concept that hair testing may produce results from the same exposure that are disproportionately more positive or negative for one ethnic group. This may be a result because of the demonstrated affinity of many drugs for melanin and because more drug is present in hair that contains more eumelanin. However, the ethnic bias is more complex because the distinction between ethnic groups is not straightforward, and it is unclear whether some ethnic groups have consistently and significantly more eumelanin than other ethnic groups. Additionally, cultural differences in hygienic treatments, cosmetic treatments, and environmental exposure could also produce differences in the interaction of drugs with hair between groups. Therefore, ethnic bias is a potential phenomenon that is more complex than simply color of the hair. Both dark and light hair specimens were used in this study as a preliminary investigation of potential hair color effects on the study design; however, the sample populations were too small for a statistical comparison.

The degree to which a drug analyte is incorporated into hair is dependent on hair growth patterns and biological and environmental factors influencing its growth. For example, the drug

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content in plucked hair is different from that found in shed hair because shed hair undergoes a resting period (no growth, or catagen phase) before falling out (Pichini et al., 1996). Factors that influence inter-individual growth rate of hair include age, gender, ethnicity, heredity, climate, health, injury and physical stress, as well as the anatomical site of hair growth (Hamilton et al., 1955; Hold, 1996; Robbins, 2002; Kronstrand and Scott, 2007).

1.2 Statement of the Problem

Detecting the parent compound, COC, in hair is not sufficient to identify drug use. COC is often smoked, and COC released into the air may coat the hair shaft, leading to environmental contamination. In addition, COC may be on the surfaces of areas where it was used and can be transferred to the hair by contaminated hands (Stout et al., 2006b; Kidwell and Smith, 2007). For this reason, other COC analytes, and even parent compound-to-metabolite ratios are evaluated in workplace drug-testing programs to ensure that the hair test only identifies illicit use of COC; however, the efficacy of established cut-off concentrations for COC in hair is still debated.

There are up to four COC analytes that are routinely investigated for workplace drug testing. These analytes include the parent compound (COC), as well as benzoylecgonine (BE), cocaethylene (CE), and norcocaine (NCOC). COC is the most abundant analyte followed by BE (10%–50% of COC), CE (<20% of COC), and NCOC (<10% of COC). Ratios for other COC analytes have also been monitored with less success (Bourland et al., 2000; Ropero-Miller et al., 2002; Cairns et al., 2004a; Scheidweiler et al., 2005; SAMHSA, 2004a).

Research that evaluates COC analyte concentrations in hair following external contamination and fortification with drug standard solutions has also been limited. Reported contamination studies have investigated COC and BE; however, the researchers did not report on other COC analytes, such as CE and NCOC. These investigations focused on COC and BE after contamination of hair with COC because these are the primary analytes in hair and because the CE and NCOC were considered to be metabolites of COC and not by-products of manufacture. Plus, the researchers did not expect to find these COC analytes when exposing the hair to high-purity COC. In this process, a benzoylecgonine-to-cocaine (BE/COC) ratio that is <0.05 suggests that the hair is contaminated with COC and should not be reported as positive for drug use (SAMHSA, 2004a).

More recent studies by Scheidweiler and colleagues (2005) and Stout and colleagues (2006b) have demonstrated that these COC analytes can occur at appreciable concentrations in studies where high-purity COC hydrochloride (HCl) was administered subcutaneously under controlled dosing and where it was applied to the hair during a short exposure time followed by 10 weeks of daily hygienic treatment, respectively.

More research is still needed to determine if current COC analyte concentrations and ratios that have been established to differentiate COC use from environmental exposure are adequate and if additional parent-to-metabolite ratio concentrations would assist in further discrimination.

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1.3 Review of the Literature

1.3.1 Use of Hair Drug Testing

Forensic laboratories use hair as a complementary and alternative matrix to blood and urine in testing for controlled substances. The disposition of many controlled substances in hair is cited in the literature, and hair test results have been used as evidence in civil, criminal, and military courts of law for more than 20 years (Huestis, 1996). Controlled substances reported in hair include amphetamines, COC, opiates, cannabinoids, barbiturates, phencyclidine, and benzodiazepines (Ropero-Miller et al., 2000 and 2005). Hair attributes include its durability, its ability to indicate long-term drug use (weeks to years depending on length of hair), and its ease of collection and storage. Applications of hair drug testing include postmortem analyses, human performance testing, parole and drug treatment programs, workplace drug testing, and crime scene investigations, including drug-facilitated crimes. However, interpreting hair test results for controlled substances has been complicated by many issues, including potential hair color bias, the need for more sensitive analytical techniques, external contamination, and high individual variability due to many factors such as age, gender, hygiene, drug biotransformation and excretion, and hair growth rate (Cone, 1990; Stout and Ruth, 1999; Bourland et al., 2000; Ropero-Miller et al., 2000; Claffey et al., 2001; Cairns et al., 2004a; Ruth and Stout, 2004). For hair testing to be accepted in forensic toxicology applications, it is crucial that an individual who is environmentally exposed to a drug can be differentiated from a drug user.

1.3.2 Cocaine in Hair

Detecting only the parent compound, COC, in hair does not prove COC use. COC is highly concentrated in the hair of demonstrated COC users, with BE, CE, and NCOC in amounts ranging from undetectable to >40% COC (Cairns et al., 2004a).

Historically, one way of differentiating a drug user from a person who may be occupationally exposed to the drug (e.g., narcotics officer, pharmaceutical researcher) or one who may have unknowingly come into contact with a drug-contaminated surface has been to identify a unique in vivo metabolite. Although BE, CE, and NCOC are readily accepted as COC metabolites, these analytes are not exclusively produced through biotransformation within the body. All of these COC analytes have been reported as impurities in pharmaceutical-grade and STREET COC in the literature, including work performed in RTI International's (RTI's) Center for Forensic Sciences (CFS) Laboratory (Casale and Klein, 1993; Casale and Moore, 1994a and b; Moore et al., 1994; Casale et al., 2005a and b). Furthermore, BE can be derived from COC by non-enzymatic hydrolysis under basic conditions and, consequently, cannot be conclusively used as a biological marker for COC ingestion because it is derived by hydrolysis in the environment, which means that the presence of BE in the hair is due to COC degradation (may only be environmental exposure) and not metabolism. Scheidweiler and colleagues (2005) detected CE (C_{max}: 71 pg/mg–143 pg/mg), which is a COC metabolite formed by trans-esterification with ethanol, in human hair after controlling COC administration (doses: 75 mg/70 kg and 150 mg/70 kg) in a closed residence-research facility where ethanol was unavailable. It was unclear where the CE came from during this study, and it is important to determine if these analytes were present as impurities or metabolites. Another study by Ropero-Miller and colleagues (2002) used the same clinical study protocol and dosing scheme as Scheidweiler and colleagues (2005) but only reported COC concentrations as maximum total drug concentrations by taking the sum of the concentrations found in the hair and the combined wash fractions. The results were reported

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this way to allow for the comparison of drug concentrations of nail scrapings collected at the same time to drug incorporation patterns of these keratinized matrices, which is the primary objective of this study. Ropero-Miller and colleagues (2002) said that other COC analytes were detected in initially collected specimens (anhydroecgonine methyl ester [AEME] and CE) and/or the first collection directly following drug administration (BE and NCOC). Generally, the combined concentration of all other COC analytes was <10% of the COC concentration for a given subject and collection, but in a few instances, the combined concentration of all other COC analytes was as much as 30% of the concentration of parent COC.

After extensive research, the U.S. Department of Health and Human Services (DHHS) proposed confirmatory test cut-off concentrations for COC analytes in hair, with the added stipulation that the parent drug compound must be present with at least one other COC analyte (Mangin, 1996). A BE/COC ratio of ≥0.05 was also specified. Due to limited data, ratios could not be determined for NCOC and CE. Other agencies, laboratories, and researchers have adopted similar practices (SOHT, 2004). **Table 1** summarizes established cut-off and threshold concentrations for COC in hair that have been published since 1998.

Table 1. Published Initial, Confirmatory, and Lower Limit Cut-Off Concentrations (pg/mg) for Cocaine in Hair

Agency or Organization	Testing Level	COC
Substance Abuse and Mental Health Services Administration (USA proposed 2004)	Initial	500
	Confirmatory	≥500 COC and ≥50 BE *and* BE/COC ≥0.05 **OR** ≥500 COC *and* ≥50 CE **OR** ≥500 COC *and* ≥50 NCOC
Society of Hair Testing	Initial	500
	Confirmatory	≥500 COC ≥50 metabolites
Gesellschaft fur Forensische und Toxikologische Chemie (Society of Toxicological and Forensic Chemistry) (Germany)	Initial	200
	Confirmatory	≥500 COC ≥100 metabolites
Societe Francaise de Toxicologie Analytique (French Society of Analytical Toxicology) (France)	Lower limit	500 COC, BE, and CE

Note: BE = benzoylecgonine; BE/COC = benzoylecgonine-to-cocaine ratio; CE = cocaethylene; COC = cocaine; NCOC = norcocaine; NCOC/COC = norcocaine-to-cocaine ratio

Table reprinted from Ropero-Miller, 2007a.

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1.3.3 Cocaine Analyte Concentration and Ratio Studies in Drug-User Populations

There are a limited number of reports in the literature that actually provide data in the form of COC analyte concentrations by subject. Similarly, specific data for COC analyte concentration ratios are equally sparse (Cassani and Spieher, 1993; Kidwell, 1993; SOHT, 1997). The Society of Hair Testing (SOHT) was one of the first organizations to recommend that the presence of metabolites should be used for interpretive purposes and that metabolite-to-parent drug ratios should be calculated. SOHT (1997) also suggested that a BE/COC ratio >0.05 might indicate drug use. **Table 2** summarizes concentration and ratios for BE and COC reported in the literature. Two more reports (Cairns et al., 2004a; Bourland et al., 2000) are discussed separately in the following paragraphs because more detail and subjects are included in these cases.

Table 2. Review of the Literature for Reporting of Cocaine Analyte Concentrations and Ratios

Author (Year)	COC (ng/mg)	BE (ng/mg)	BE/COC	Other Information
(Kidwell, 1993) *n* = 5	2	1.9	0.950	Solid-probe heating direct sample introduction; tandem mass spectrometry; also reported ecgonine and ratio
	98	8.9	0.091	
	40	2.9	0.073	
	12	2.8	0.233	
	6.85	5.1	0.745	
(Gaillard and Pepin, 1998)	5.5	1.5	0.273	
(Romolo et al., 2003) *n* = 17	3.4	0.7	0.206	Gas chromatography-mass spectrometry
	25.1	3.3	0.131	
	2.2	ND	Not calculated	
	18.3	4.5	0.246	
	0.5	ND	Not calculated	
	2.6	1.1	0.423	
	21.3	6.1	0.286	
	>100	24.7	0.247	
	0.9	ND	Not calculated	
	>100	>100	Approximately >1.0	
	30.5	8.1	0.266	
	3.3	3.7	1.121	
	6.3	3.5	0.556	
	8.6	4.2	0.488	
	29.8	11.2	0.376	
	16.4	3.3	0.201	
	0.7	ND	Not calculated	
	21.7	7.6	0.350	
	8.2	2.1	0.256	

Note: BE = benzoylecgonine; BE/COC = bezoylecgonine-to-cocaine ratio; COC = cocaine; ND = not detected

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One additional report from Cairns and colleagues (2004a) examined COC concentrations in two distinct populations: 75 confirmed drug users and more than 6,000 workplace drug-testing specimens. **Table 3** lists the distribution of COC concentrations in drug-user (confirmed positive urinalysis) and workplace populations (Cairns and colleagues [2004a] did not state total workplace population, they only confirmed positive hair-testing results at established limit of quantitation). Although 86.6% of the COC-containing hair from drug users had COC concentrations of 2,000 pg/mg and higher, 54.3% of the workplace population (individuals seeking employment or already gainfully employed) had similar concentrations. Additionally, the analyte profile for the workplace population showed the existence of BE levels >5% of the COC concentrations for most of the confirmed drug users, but in some individuals (4%–15% of those with COC ≥500 pg/mg), the BE was <5% of the COC values. Even in the drug-user population, 3%–5% of the COC-containing hairs did not contain BE at levels >5% of the COC concentration. For these individuals, their CE and/or NCOC concentrations would have tested positive using the proposed Mandatory Guidelines (SAMHSA, 2004a). Likewise, in the workplace population, more than half of the specimens contained CE >50 pg/mg hair, and approximately 78% of these specimens contained NCOC at >50 pg/mg hair.

Table 3. Distribution of Cocaine Concentrations in Drug-User and Workplace Populations Confirmed Positive by Hair Testing (Cairns et al., 2004a)

Cocaine in Hair (pg/mg)	Drug-User Population (%)	Workplace Population (%)
>20,000	39 (52)	725 (11.5)
10,000–20,000	9 (12)	633 (10.1)
5,000–9999	12 (16)	818 (13.0)
2,000–4,999	5 (6.6)	1,240 (19.7)
1,000–1,999	2 (2.7)	1,225 (19.5)
500–999	3 (4.0)	1,653 (26.3)
<500	5 (6.6)	Not reported
TOTAL	**75**	**6,294**

Table reprinted from Ropero-Miller, 2007a.

Cairns and colleagues (2004a) also reported all four COC analyte concentrations for each of the 75 confirmed drug users. These data are presented in **Table A-1** and are discussed in Sections 2.2.6 and 2.2.7. These data show the number of subjects with a detectable amount of COC analyte (i.e., above the limit of quantitation [LOQ]) and the pg/mg concentration range (mean and median) for each COC analyte as follows:

- 75 detectable results for COC at 30 to 227,000 pg/mg (mean: 43,038; median: 24,800)
- 73 detectable results for BE at not detected to 34,700 pg/mg (mean: 5,211; median: 2,380)
- 55 detectable results for CE at not detected to 12,790 pg/mg (mean: 1,218; median: 210)
- 70 detectable results for NCOC at not detected to 5,560 pg/mg (mean: 1,174; median: 810).

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Many times, when the parent drug COC was detected, the other COC analytes were not detected in this drug-user population. For all COC analytes, the mean concentration was higher than the median, which indicates that the population distribution curves were skewed toward the left, or lower, concentrations. If the results from each subject were compared to the Substance Abuse and Mental Health Services Administration (SAMHSA) confirmatory cut-off concentrations (Table A-1), the number of subjects and the percentages of positive test results for this drug-user population were: 70 subjects positive for COC (93%), 69 subjects positive for BE (92%), 37 subjects positive for CE (49%), and 64 subjects positive for NCOC (85%). CE was the only analyte that appeared in two subjects at detectable concentrations but the concentrations were not high enough to confirm positive results by the proposed Mandatory Guidelines (SAMHSA, 2004a) (COC: 70 of 75, BE: 69 of 73, CE: 37of 55, and NCOC: 64 of 70).

In a second population study, Bourland and colleagues (2000) reported COC concentrations in 30 human head hair specimens. These specimens were randomly chosen production specimens that had previously been reported as positive by an enzyme-linked immunosorbent assay (ELISA) and gas chromatography-mass spectrometry (GC-MS); the data provided in this report were from re-analyzing these specimens. The COC analyte concentration ranges and means are listed in **Table 4**. Based on the proposed Mandatory Guidelines and SOHT Guidelines listed in Table 1, at least one subject (i.e., Subject X: COC 420 and BE 100) would have been reported as negative by SOHT Guidelines and two subjects (i.e., Subject E: COC 21,260 and BE 620; Subject X: COC 420 and BE 100) would have been reported negative for COC by the proposed Mandatory Guidelines. COC concentrations reported by Cairns and colleagues (2004a) and Bourland and colleagues (2000) are consistent (Tables A-1 and **A-2**); however, the smaller population studied by Bourland and colleagues had a lower percentage of subjects with concentrations <2,000 pg/mg (20% versus 45%).

Table 4. Cocaine Analyte Concentration Ranges and Means for 30 Human Head Hair Specimens Submitted for a Workplace Drug-Testing Program, with Method Limit of Detection and Limit of Quantitation (Bourland et al., 2000)

Analyte	Concentration Range (pg/mg)	Mean	LOD	LOQ	Percentage
COC	420 to 2,000 (*n* = 6) 2,001 to 35,500 (*n* = 24)	10,350	10	50	100
BE	70 to 4,710	1330	10	50	12.8
CE	<LOD to 10,870	1590	10	50	15.4
NCOC	<LOD to 1,580	260	10	50	2.5

Note: BE = benzoylecgonine; CE = cocaethylene; COC = cocaine; NCOC = norcocaine; LOD = limit of detection; LOQ = limit of quantitation

Table reprinted from Ropero-Miller, 2007a.

The actual subject data for all four COC analyte concentrations of the 30 drug users was reported by Bourland and colleagues (2000) and are presented in Table A-2. The number of subjects with a detectable amount of COC analyte (i.e., greater than or equal to the LOQ), the pg/mg concentration range, and the mean and median for each COC analyte could be determined for the subject data and are summarized as the following:

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- 30 detectable results for COC (97%) at 420 to 35,500 (mean: 10,348; median: 6,320) pg/mg
- 30 detectable results for BE (100%) at 70 to 4,710 (mean: 1,327; median: 705) pg/mg
- 19 detectable results for CE (60%) at not detected to 10,870 (mean: 1,590; median: 610) pg/mg
- 23 detectable results for NCOC (77%) at not detected to 1,580 (mean: 325; median: 220) pg/mg.

Although COC and BE were detected in all subjects, the other two COC analytes were not detected in 23% to 37% of this drug-user population. Again, the mean concentration was higher than the median for all COC analytes. For BE, 5 subjects who met the confirmatory cut-off concentration of 50 pg/mg did not meet the criteria of a BE/COC ratio of ≥0.05.

1.3.4 Preliminary RTI Studies

Preliminary RTI studies suggest that BE, CE, and NCOC may be deposited during in vitro surface contamination of the hair and can be detected at the current proposed DHHS cut-off concentrations for up to 10 weeks after applying COC. This recently published research by RTI's CFS Laboratory found that it was difficult to remove COC in hair after applying COC HCl powder to the hair surface, and, in some cases, it was difficult to discern contaminated hair from drug-user hair by the detection of metabolites (Stout et al., 2006b). Our work to date has corresponded with that of Romano and colleagues (2001). **Figure 1** shows data that RTI has produced for COC in contaminated hair that was subjected to different decontamination strategies.

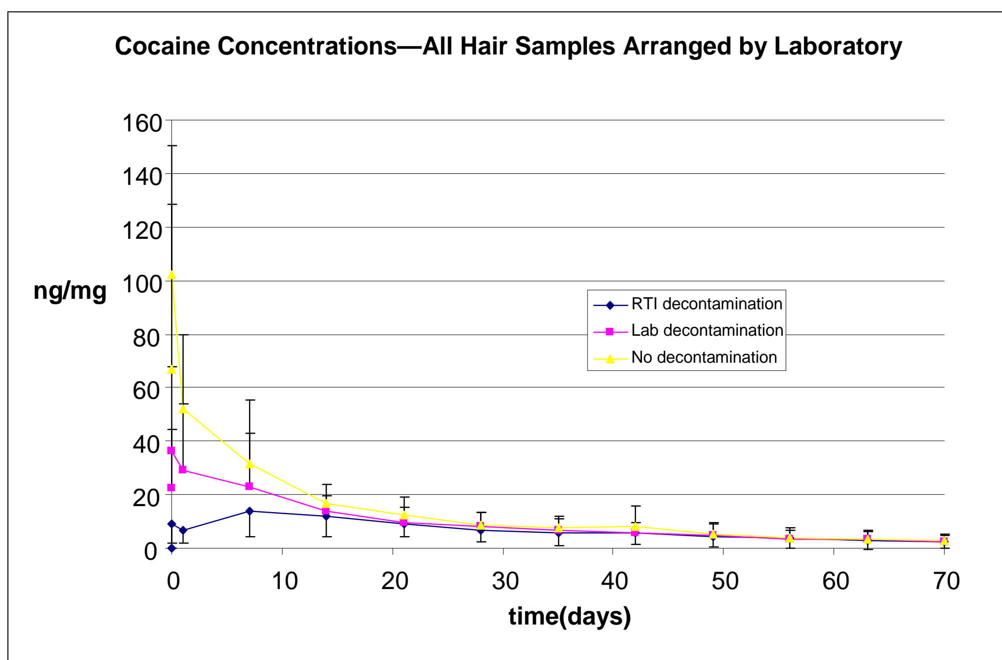

Figure 1. Data from RTI's investigations show COC concentrations in hair after contamination with 15 mg of COC hydrochloride powder distributed on hands. Hair specimens were decontaminated by RTI, decontaminated by testing laboratories, or not decontaminated. Error bars are 1 standard deviation, and each point represents the mean of 15 observations. The three lines are significantly different (p <.00001).

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Significant COC concentrations were observed in hair specimens over the entire study period from a single contamination event.

Figure 2 presents the BE/COC ratio over the study period from hair decontaminated with an extensive buffer wash performed by RTI. The BE/COC ratio exhibited a significant increase over the study period (p <0.0001) and increased above the ratio of 0.05 proposed for hair testing in federal workplace drug-testing programs (SAMHSA, 2004b). There was no apparent relationship between the rate and the extent of ratio increase and hair color.

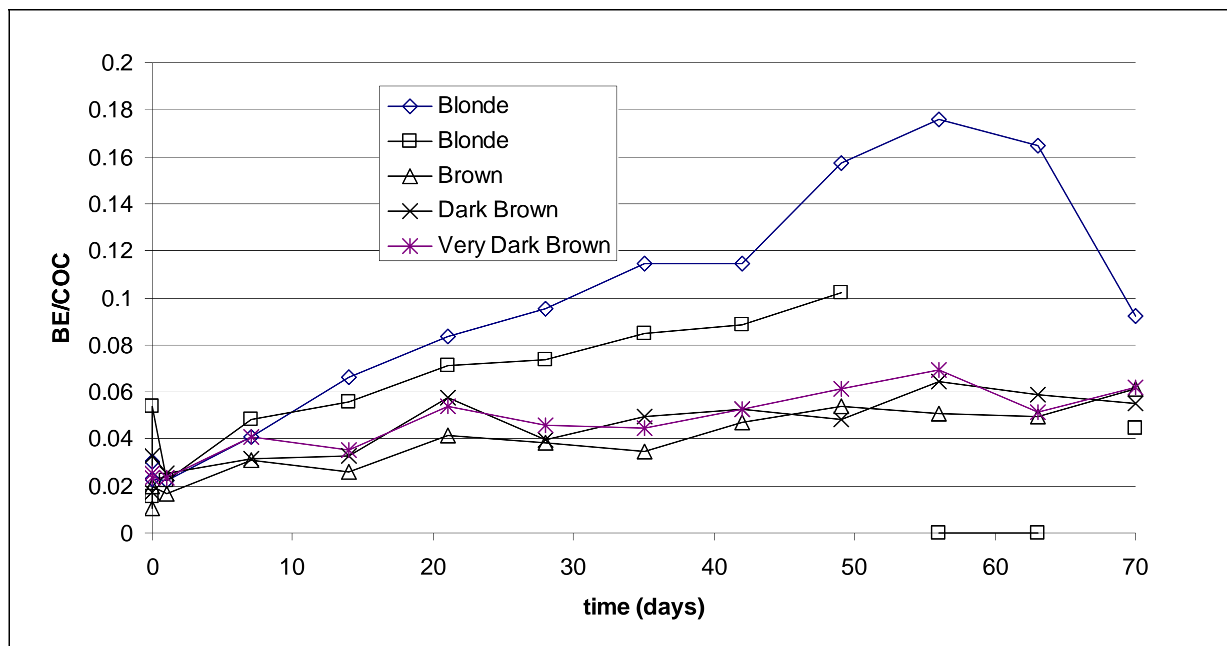

Figure 2. Benzoylecgonine-to-cocaine (BE/COC) ratio in hair over time in hair decontaminated with an extended aqueous buffer wash. Significant (p <0.0001) increases in the BE/COC ratio were observed during the 10-week period, with wide variability between hair types without any apparent relationship to hair color.

External contamination of hair with COC HCl powder resulted in the presence of COC, BE, CE, and, to a lesser extent, NCOC, which was resistant to removal over 10 weeks of model hygienic treatment and laboratory decontamination. The presence of trace quantities of CE and NCOC (<1%) in the COC used in the study confounded the use of ratios, cutoffs, and other mathematical criteria to distinguish a contaminated sample. This is likely to be a greater issue with illicit COC, which is reported to have up to 20 times the NCOC and 3 times the CE as the COC used in the study (Casale and Klein, 1993; Casale and Moore, 1994a and b; Moore et al., 1994; Casale et al., 2005a). BE also appeared to increase in comparison to COC, as evidenced by a significant linear increase in BE/COC ratios over the study period.

Within the small sampling of hair types used, there did not appear to be any simple relationships between concentrations of COC, BE, CE, or NCOC with total melanin. RTI used the Schwarzkopf color scale (Schwarzkopf, 2001), which is employed by professional cosmetologists, to characterize the hair in this study and measured total melanin content using a digestion procedure and a spectrophotometric method modified from those developed by Kronstrand and colleagues (1999). Several researchers have associated higher melanin content with a proportional increase in drug incorporation (Joseph et al., 1996; Slawson et al., 1998).

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Table 5 indicates that a range of melanin concentrations was used. These data suggest that the binding and retention of COC is a complex function of melanin and other hair characteristics.

Table 5. Hair Specimens—Hair Color (Based on the Schwarzkopf Color Scale), Subject Demographics, Texture Description of Hair, and Total Melanin in Hair (Kronstrand et al., 2001)

Schwarzkopf Color Scale (Modified by RTI)	Subject Demographics and Texture Description	Mean Total Melanin μg/mg (Standard Deviation)
Blonde 9.0	Caucasian female, thin strands	6.6 (5.4)[a]
Light brown 7.5	Caucasian female, thin strands, easily tangled	7.0 (4.5)[a]
Brown 6.5	Caucasian female, slight wave, smooth thick strands	31.1 (6.6)[b]
Dark brown 5.5	Caucasian female, slight wave, smooth thick strands	60.7 (10.5)[c]
Very dark brown 4.0	Asian female, thick fibers, straight and smooth strands	57.4 (6.2)[c]

[a, b, c] Indicates groups of specimens that were significantly different from one another when measured by a single factor analysis of variation (p <0.0001).

Contamination of the hair's surface may result in the incorporation of analytes into the hair without the addition of sweat. In RTI's study with COC, we observed that specimens decontaminated with an aggressive phosphate buffer procedure 1 hour after contamination and prior to contact with any moisture were completely decontaminated (no detectable COC or metabolites). Significantly more COC and metabolites were detected in specimens taken 1 hour after contamination and prior to contact with any moisture, and they were packaged for shipment to testing laboratories for decontamination. These results indicate that contaminating COC could only be removed from the hair for a short period of time by any means. The laboratories decontaminated the hair at least 5 days after the contamination event, and the drug was not removed by any of the decontamination procedures used in the study.

Adding moisture to the hair as artificial sweat markedly increased the concentrations of drug analytes in the hair. Wetting the hair only once resulted in significant COC and metabolites detectable in the hair after all decontamination procedures. After the drug analytes were absorbed into the hair, they were resistant to removal by shampooing and/or by current laboratory decontamination wash procedures used by other researchers and reported in the literature.

In summary, further investigation of COC analyte concentrations and metabolite-to-parent drug ratios is needed to determine if additional federal or laboratory-based guidelines can be established to correctly distinguish between a COC user and an individual who may have been unknowingly exposed to COC in the environment. Although several researchers have investigated COC analyte concentration ratios in a limited specimen type or sample size, no one group has simultaneously investigated COC analytes under varying factors, including hair type, COC source, use pattern, laboratory fortification, and environmental contamination, using the same sample preparation and instrumental analysis.

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1.4 Rationale for the Research (Statement of Hypothesis)

The purpose of this study was to investigate COC analyte concentrations with respect to each other to determine if appropriate concentration ratios could be established to distinguish between COC user and non-user hair. RTI sought to evaluate metabolite-to-parent drug concentration ratios in human hair as affected by COC source to include pharmaceutical and street drugs, specimen type to include drug users in street environment, drug users in a controlled research environment, external drug fortification, and surface contaminated. RTI separately evaluated COC source and specimen type in hair externally contaminated with a dry COC HCl powder (e.g., surface contamination model) and also evaluated ratios obtained from fortifying hair with the same COC materials as used in external contamination. Although variables such as hair type (e.g., color, texture) were a part of the hair selection process because both light hair and dark hair were included in all stages of the study, statistical evaluation of the hair type differences could not be performed due to the small sample populations. We contracted with established forensic hair-testing laboratories to analyze hair specimens by using liquid chromatography-tandem mass spectrometry (LC-MS/MS). RTI used research designs and methods that are applicable to the current proposed Mandatory Guidelines and practices to determine concentrations of four COC analytes (i.e., COC, BE, CE, NCOC) in multiple situations. The research design and methods evaluated as many situations as can be realistically expected to occur and provided additional information about the potential differences in the concentration ratios observed. In addition, RTI has published, and will continue to publish, the results in prominent forensic journals and has presented some findings at national meetings of leading forensic organizations.

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2. RESEARCH DESIGN AND METHODS

This study was designed to investigate COC analyte concentrations and their ratios in hair that was contaminated through drug ingestion, externally contaminated with a solid COC HCl powder (e.g., refined, illicit COC and commercially available, pharmaceutical-grade COC), or externally fortified with a buffered solution of COC. The following are four stages of research that are grouped and discussed as sections in this report based on each experimental design, sample type and preparation, analysis procedures, findings, and conclusions:

- Stage I: Analysis of COC HCl powders
- Stage II: Analysis of hair from drug-user populations
- Stage III: Contamination of hair with COC HCl powders
- Stage IV: Fortification of hair specimens with COC HCl powders.

This experimental design was unique because some of these hairs (e.g., externally incorporated COC protocols of Stages III and IV) were introduced to the same COC of varying purities (e.g., >99% COC, >1% CE, >5% NCOC) at similar concentrations to investigate COC analyte concentration ratios obtained by different routes of externally introduced COC exposure. All analyses included the quantitation of COC, BE, CE, and NCOC by using LC-MS/MS hair-testing procedures. COC analyte concentration ratios were statistically evaluated to determine significant differences. All specimens were blinded prior to shipment to the laboratory, and drug-free blind control specimens were included with the study specimens.

More than 200 hair specimens were analyzed during this study. When possible, specimens were analyzed by replicate analysis and were averaged for data analysis. For example, for the contamination experiments, there were 257 total hair specimens (replicate analyses for some samples) analyzed plus 23 blind specimens. Therefore, approximately 400 hair specimens were tested for COC, BE, CE, and NCOC concentrations using 10-mg aliquots for LC-MS/MS analysis. RTI submitted both drug-free and spiked blind control specimens as a quality assurance measure to ensure that specimens were handled correctly by the laboratory.

The hair sample types that RTI analyzed and the approximate number for each are the following:

- Drug-user hair from the street environment (n = 38)
- Drug-user hair from subjects who were administered COC in a clinical setting (n = 20)
- Drug-free hair surface-contaminated with high-purity pharmaceutical-grade COC (i.e., insignificant amount [<1%] of CE or NCOC) collected at 13 time points (n = 26)
- Drug-free hair surface-contaminated with low-purity street COC with a higher amount of CE collected at 13 time points (n = 26)
- Drug-free hair surface-contaminated with low-purity street COC with a higher amount of NCOC collected at 13 time points (n = 26)
- Drug-free hair fortified with COC for proficiency testing at various concentrations similar to proposed cut-off concentration in Mandatory Guidelines for federal workplace drug testing (n = 20)

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- Drug-free and drug-user hair as controls in study (n = 31)
- Decontamination wash fractions from the Stage IV fortification study (n = 36).

2.1 Stage I: Analysis of Cocaine Hydrochloride Powders

2.1.1 Experimental Design

Stage I of this research project focused on analyzing COC analytes in COC HCl powders, both refined illicit COC sources and commercially available pharmaceutical sources, to select an appropriate COC source to use in the COC surface-contamination study (Stage III) and the COC fortification study (Stage IV). RTI obtained street COC as 1-g sample of COC bricks seized by the U.S. Drug Enforcement Administration (DEA) and sent to RTI under contract to purify the COC for use by the National Institute on Drug Abuse (NIDA). RTI also obtained a 1-g sample from each seized COC HCl powder for this research.

Twenty-eight COC HCl powders were submitted to laboratories to analyze the powders' purity by using procedures commonly used for signature analysis (e.g., GC-MS) and other methods. This testing determined the concentrations of COC analytes, including CE, BE, CE, and NCOC. Other COC analytes, such as AEME, trans-cinnamoyl COC, tropacocaine, and truxilline isomers; manufacturing by-products, including solvents, such as methyl ethyl ketone (MEK), methyl isobutyl ketone (MIBK), and petroleum ether; and adulterants, such as lactose, mannitol, and caffeine, were also detected in the signature analysis process performed by DEA laboratories. Determining these cutting agents is routinely performed by DEA laboratories and can help law enforcement track high-level dealers of illicit substances and identify new local or national illicit manufacturing trends. **Figure 3** shows the chemical structures of all COC analytes determined during this stage of the study. (It is important to note that some analytes were determined in other stages as well, but this section determined all COC analytes to determine purity.)

After these specimens were correctly identified as COC specimens and their purity was determined by using traditional analytical procedures, most of these specimens were also analyzed by using a novel screening instrument as an additional study design. The purpose of the analysis was to determine if this novel time-of-flight direct analysis real time (TOF-DART) mass spectrometer using exact mass determination had the potential to greatly improve controlled substances screening in forensic laboratories (Laks et al., 2004; Song et al., 2004; Cody et al., 2005; Ojanpera et al., 2005). Although COC signature analyses have been routinely performed in many forensic laboratories, these laboratories could benefit from a rapid screening method to identify controlled substances (Ehleringer et al., 2000). Using the TOF-DART instrument in a procedure that would require minimal to no sample preparation was investigated to determine if this instrument could be developed and used to complement traditional methods for determining drug compounds in seized materials. If so, it would facilitate the tracking of drugs and other substances that are added to dilute or cut scheduled drugs to increase bulk and profit margins. So, as an adjunct project that was not originally proposed as part of this research, RTI directly compared COC signature analyses of crude illicit COC specimens with a new mass spectral technology that shows promise for identifying the controlled substances.

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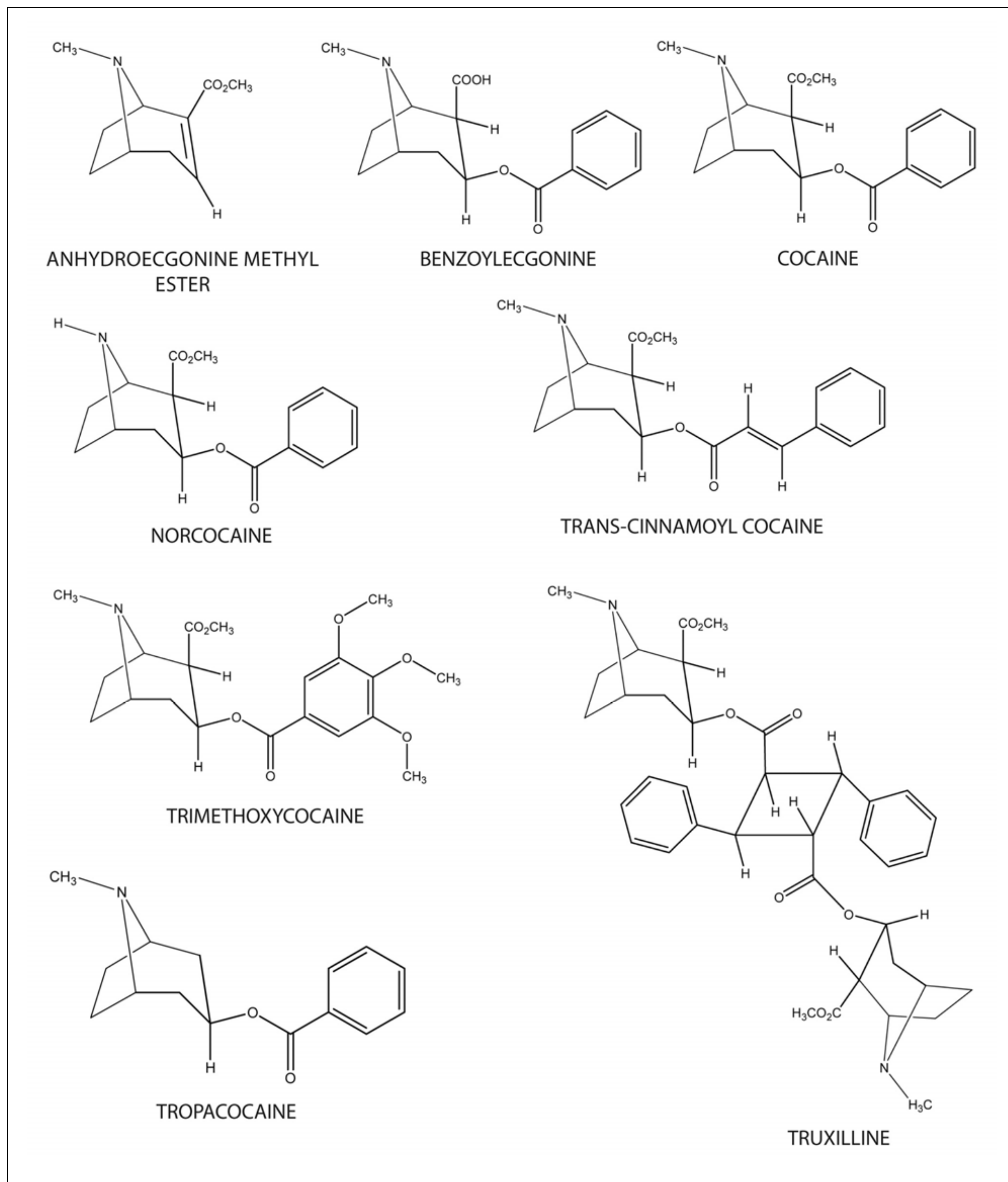

Figure 3. Chemical structures of cocaine analytes.

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2.1.1.1 Powdered Cocaine Hydrochloride Specimens

There were two sources of powdered COC HCl specimens obtained for Stage I of this project. First, 25 illicit COC HCl powder specimens were obtained from NIDA's Division of Neuroscience and Behavioral Research. RTI obtained additional refined illicit COC powder from DEA laboratories when testing found that the specimens from NIDA did not have a CE concentration >1% as proposed in RTI's study design. DEA provided RTI with a COC sample from its inventory of illicit COC seizures after determining that the sample contained the appropriate CE concentration.

RTI also obtained a commercially available, pharmaceutical-grade COC HCl powder from the U.S. Pharmacopeia (USP). A certificate of analysis and further analytical testing by DEA laboratories confirmed that this COC source was of high purity (<1% of impurities).

2.1.2 Materials

COC analyte standards (i.e., COC, AEME, BE, CE, NCOC) were purchased as HCl salt (1 mg/mL) or base solutions of methanol (BE) or acetonitrile by Cerilliant Corp. Polyethylene glycol, which was used as the calibrating reagent for the TOF-DART instrument, was of reagent-grade quality and was obtained from Sigma-Aldrich Chemical Co. All other solvents and standards used by laboratories were of analytical grade and high purity.

2.1.3 Methods

For Stage I of this study, we used three protocols at three separate research facilities to identify components contained in the examined COC HCl powders. First, an in-depth COC signature analysis was performed by the U.S. Department of Justice's DEA Special Testing and Research Laboratory (henceforth referred to as the DEA Laboratory). Second, a limited GC-MS analysis was performed by the Armed Forces Institute of Pathology (henceforth referred to as the AFIP Laboratory). Third, a novel screening technique for controlled substances, such as COC, was performed by RTI in Research Triangle Park, NC. The results of Stage I of this study were published in DEA's *Microgram Journal* (Ropero-Miller, 2007b).

Cocaine Signature Analyses by DEA Laboratories

COC signature analyses were conducted by using GC-MS, capillary gas chromatography with electron capture, and flame ionization detection as reported by many researchers (Casale, 1991; Casale and Moore, 1994a and b; Morello and Meyers, 1995; Moore et al., 1996; Ehleringer et al., 2000). The GC-MS analysis was performed using an Agilent Technologies Model 5973 quadrupole mass-selective detector (MSD) interfaced with an Agilent Technologies Model 6890 gas chromatograph. The MSD was operated in the electron ionization mode with an ionization potential of 70 eV, a scan range of 34–700 mass units, and at 1.34 scans per second. The gas chromatograph was fitted with a 30 m x 0.25 mm I.D. fused-silica capillary column coated with 0.25 μm DB-1 (J&W Scientific). The oven temperature was programmed as follows: initial temperature = 100°C; no hold, program rate, and 60°C per minute; final temperature = 300°C; with a final hold time of 5.67 minutes. The gas chromatograph injector was operated in the split mode (21.5:1) and at a temperature of 280°C. The auxiliary transfer line to the MSD was operated at 280°C.

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Gas Chromatography-Mass Spectrometry by the AFIP Laboratory

COC concentrations in illicit COC specimens (>50%) are relatively high compared to other analytes (generally <5%); therefore, the specimens were tested separately using standard sample preparations and GC-MS analysis. AFIP used three separating GC-MS methods to analyze all four COC analytes.

TOF-DART Analysis by RTI Laboratories

RTI used the AccuTOF-DART (JEOL USA, Inc.) system to perform analyses, which were conducted using the positive mode on the DART ion source. COC HCl powders were introduced to the ion source as solid specimens (manual and autosampler introduction) and as methanolic solutions (autosampler introduction). The specimens were introduced into the ion source by dipping a glass probe into the sample, and then passing this through the stream. The AccuTOF-DART system was calibrated with polyethylene glycol before each sample run. When available, the monoisotopic ion plus the hydrogen ion (M + H) values of the COC analytes were verified using certified drug standard solutions.

The source was operated with a ring lens voltage of 5V, an orifice 1 voltage of 20V, and an orifice 2 voltage of 5V. The mass range was initially set from 60 to 1,000 daltons, but it was later reduced to a range of 60 to 700 daltons so more difficult compounds, such as alcohols, could be further evaluated. Electrodes 1 and 2 of the TOF-DART source were set to 150V and 350V, respectively, and the TOF-DART temperature was set to 300°C. The detector was optimized at 2,200V.

2.1.3.1 Modifications to the Research Design and Rationale

After discussions, NIDA's Division of Neuroscience and Behavioral Research and collaborating laboratories mutually agreed to release 25 1-g specimens of COC HCl powders for this project. This number was larger than the originally proposed number of 20, but even with these representative COC HCl powder specimens, RTI was unable to find a COC HCl powder that contained a higher amount of CE (>1%), which was proposed. The DEA Special Testing and Research Laboratory (Dulles, VA) determined (through database and inventory searches) that they possessed a representative high CE sample and could release it to RTI for inclusion in this project.

Because the STREET COC was more realistic to use for this study, RTI decided that inclusion of one commercially available, pharmaceutical-grade COC was sufficient for meeting the study design objective. We obtained certified COC analyte reference materials from USP instead of Cerilliant Corp. as originally proposed. Furthermore, RTI was awarded a National Institute of Justice (NIJ) grant in 2006 (award number 2006-91774-NC-IJ), which is evaluating a novel analytical instrument for another forensic application. The AccuTOF-DART system has also recently shown applicability for identifying controlled substances. Because RTI possesses this instrumentation, we were able to include an additional study within Stage I to further evaluate these COC HCl specimens by using a novel screening technique for controlled substances identification.

Overall, RTI made minor modifications to the original study design proposed. With the current study design, RTI analyzed slightly more COC HCl powders than was originally proposed and still maintained and achieved the goals of this research.

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2.1.4 Findings

The DEA Laboratory provided the primary results for the identification of the COC HCl powder specimens. Besides determining the relative concentration of all analytes investigated in this study to the parent COC concentration ratio, the comprehensive COC signature analysis performed by DEA Laboratory provides additional information that helps to characterize the purity, geographical origin, and manufacturing processes used to transform the COC plant materials into the COC HCl that is available illicitly on the street. For this study, the results of the COC signature analyses are presented as the COC analytes' characterization and purity and the presence of solvents and adulterants from the manufacturing process. A complete description of this process is outside the scope of this study but was described by Ehleringer and colleagues (2000).

The DEA COC signature analysis determines the relative amounts of the following COC analytes with respect to the parent COC: NCOC; CE; BE; AEME; trans-cinnamoyl COC; 3', 4', 5'-trimethoxycocaine; tropacocaines; and the truxilline isomers, among others. All specimens were positively identified as COC. The COC HCl powders had purity ranges from 65%–88.5%. BE concentrations ranged from 0.14%–1.1% (mean: 0.37%), and the NCOC concentrations ranged from trace up to 8.7% (mean: 0.9%). All specimens that contained BE also contained NCOC. All specimens contained only trace or non-detectable quantities of CE. Ethanol was in 14 of the 25 specimens (not reported for 3 specimens) with concentrations and ranged from 0.003%–0.12% (mean: 0.06%). **Table 6** summarizes the COC purity and the presence of each of the COC analytes that was detected in at least one of the submitted COC HCl powders.

Diluents and adulterants that were selected to be included in COC manufacturing processes and determined by the DEA Special Testing Laboratory's COC signature analysis were sodium chloride (NaCl), mannitol, caffeine, dimethylterephthlate, and lactose. Solvents determined to be included were isopropyl acetate, n-propyl acetate, petroleum ether, ethyl acetate, MEK, and MIBK. The base origin and HCl process for each COC HCl powder was determined as a Peruvian or Columbian manufacturing process. Table 6 shows the presence of each of these COC constituents in the COC HCl powders evaluated for this project.

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Table 6. Details of the Content of the 25 NIDA Cocaine Specimens and the Three Cocaine Specimens Used in the Contamination and Fortification Experiments (Highlighted Yellow)

Count	Source	Purity	BE	NCOC	CE	Total cinnamoyls	Tropa-cocaine	Trimethoxy-cocaine	Truxillines	EtOH %	cis-cinnamoyl EEE %	trans-cinnamoyl EEE %
COC_HCl_1	NIDA	87.5	0.37	0.33	ND	2.3	0.10	0.18	6.6	0.08	ND	ND
COC_HCl_2	NIDA	86.9	0.42	0.28	ND	2.4	0.08	0.18	6.7	0.09	ND	ND
COC_HCl_3	NIDA	87.5	0.41	0.32	ND	2.3	0.12	0.18	7.1	0.09	ND	ND
COC_HCl_4	NIDA	85.1	0.31	0.06	0.0018	1.0	0.21	0.15	9.0	0.12	ND	ND
COC_HCl_5	NIDA	84.0	0.22	0.06	0.0017	1.0	0.33	0.14	9.6	0.11	ND	ND
COC_HCl_6	NIDA	79.6	0.24	0.05	ND	1.0	0.39	0.07	9.2	0.02	ND	ND
COC_HCl_7	NIDA	78.9	0.16	0.04	ND	1.1	0.38	0.08	9.4	0.01	ND	ND
COC_HCl_8	NIDA	80.9	0.27	0.04	ND	1.0	0.38	0.06	8.5	0.02	ND	ND
COC_HCl_9	NIDA	78.1	0.31	0.05	ND	0.9	0.44	0.07	9.1	0.02	ND	ND
COC_HCl_10	NIDA	82.4	0.24	Trace	ND	1.0	0.34	0.00	8.2	0.02	ND	ND
COC_HCl_11	NIDA	83.8	0.47	0.07	ND	8.2	0.06	0.18	5.8	ND	ND	ND
COC_HCl_12	NIDA	84.9	0.69	0.08	ND	5.6	0.06	0.20	5.6	ND	ND	ND
COC_HCl_13	NIDA	85.5	0.43	Trace	ND	6.4	0.04	0.12	5.9	ND	ND	ND
COC_HCl_14	NIDA	74.8	ND	ND	ND	6.5	0.19	0.22	9.9	ND	ND	ND
COC_HCl_15	NIDA	65.1	0.62	Trace	ND	5.4	0.10	0.10	10.1	0.04	ND	ND
COC_HCl_16	NIDA	74.6	ND	ND	ND	5.3	0.22	0.22	11.5	ND	ND	ND
COC_HCl_17	NIDA	87.3	ND	ND	0.0015	8.5	0.08	0.12	2.1	ND	0.0039	ND
COC_HCl_18	NIDA	71.2	ND	ND	ND	1.3	0.28	0.20	13.2	ND	ND	ND
COC_HCl_19	NIDA	79.6	1.1	0.04	ND	6.0	0.21	0.15	11.5	0.003	ND	ND
COC_HCl_20	NIDA	88.5	0.16	8.7	0.0031	0.2	0.08	0.34	3.3	0.06		
COC_HCl_21	NIDA	82.3	0.65	Trace	0.0019	6.0	0.33	0.10	7.7	0.09	ND	ND

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Count	Source	Purity	BE	NCOC	CE	Total cinnamoyls	Tropa-cocaine	Trimethoxy-cocaine	Truxillines	EtOH %	cis-cinnamoyl EEE %	trans-cinnamoyl EEE %
COC_HCl_22	NIDA	85.2	0.29	2.0	0.0027	6.0	0.51	0.00	6.4	ND	ND	ND
COC_HCl_23	NIDA	84.8	0.14	1.4	0.012	6.6	0.25	0.08	7.3	ND	0.003	0.0027
COC_HCl_24	NIDA	87.8	0.19	0.65	ND	5.3	0.15	0.25	4.5	ND	ND	ND
COC_HCl_25	NIDA	79.0	0.17	1.8	ND	4.1	0.82	0.00	15.0	ND	ND	ND
COC_HCl_26	DEA[a]	93.3	NR	NR	2.30	NR	NR	NR	NR	NR	NR	NR
COC_HCl_27	DEA[b]	82.2	10.10	0.8	1.43	2.1	0.08	NR	NR	NR	NR	NR
COC_HCl_28	USP	98.9	Trace	NR	1.10	NR	NR	NR	NR	NR	NR	NR
	COUNT	25	21	21	10	25	25	25	25	14	2	1
	MIN Range	65.1	0.14	0.0	0.0015	0.2	0.04	0.00	2.1	0.00	0.0030	0.0027
	MAX Range	88.5	1.10	8.7	2.30	8.5	0.82	0.34	15.0	0.12	0.0039	0.0027
	Mean	81.8	0.37	0.9	2.06	3.8	0.25	0.14	8.1	0.06	0.0035	0.0027

Note: BE = benzoylecgonine; CE = cocaethylene; COC = cocaine; DEA = U.S. Drug Enforcement Administration; EEE = ecgonine ethyl ester; EtOH = ethanol; NCOC= norcocaine; ND = not detected; NIDA = National Institute on Drug Abuse; NR = not reported; USP = U.S. Pharmacopeia

[a] The sample was enriched with CE prior to conversion to HCl, this was not used for the study.

[b] The sample contained CE at this amount when seized and was then converted to HCl by the Peruvian process by DEA.

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The AFIP Laboratory performed a limited GC-MS analysis for 25 of the 28 COC HCl powders. The AFIP Laboratory procedure evaluated a limited number of COC analytes (did not evaluate BE); therefore, this procedure was only used to corroborate the primary analysis of the DEA Laboratory. Again, the AFIP Laboratory identified all submitted specimens as having COC and its analytes present at varying concentrations. The AFIP Laboratory identified 10 COC HCl powders that contained NCOC concentrations ranging from 0.1%–6.9% (mean: 1.3%). The AFIP Laboratory did not detect CE in any of the specimens, and although they detected AEME in concentrations ranging from 0.3%–0.8%, the results were not ultimately considered positive. The amounts of AEME determined at concentrations ranging from 0.5%–0.8% were lower than the 2% decision point for AEME to be considered as part of the illicit manufacturing artifact from the COC processing (as an acid hydrolysis product) and not an analytical artifact of the GC-MS analysis.

The results from the TOF-DART analyses were qualitatively compared to the results obtained by the AFIP and DEA laboratories. A total of nine COC analytes could be identified by AccuTOF-DART analysis (see **Table 7**). AEME and trans-cinnamoyl COC were easily detected in 23 out of the 25 specimens; however, some COC analytes were difficult to identify (e.g., tropacocaine and truxilline isomers), and others, such as isomeric pair BE and NCOC, could not be distinctly determined because of their equal masses.

In addition, some adulterants and solvents (e.g., dimethylterephthalate, MIBK, MEK) were minimally detected. **Table 8** summarizes these results.

Table 7. Comparison of TOF-DART Results with DEA Signature Analysis and AFIP Analysis of COC Materials[a]

Analytes	AccuTOF-DART (RTI Laboratory)	Cocaine Signature Analyses (DEA Laboratory)	GC/MS Analysis (AFIP Laboratory)
COC	25	25	25
BE[b]	25	21	NR
CE	ND	3	ND
NCOC[b]	25	21	7
AEME	23	ND	23
Trans-cinnamoyl COC	23	25	NR
3',4',5'-trimethoxy COC	ND	25	NR
Tropacocaine[c]	5	25	NR
Truxilline isomers[c]	7	25	NR

Note: AccuTOF-DART = time-of-flight direct analysis real time; AFIP = Armed Forces Institute of Pathology; AEME = anhydroecgonine methyl ester; BE = benzoylecgonine; CE = cocaethylene; COC = cocaine; DEA = U.S. Drug Enforcement Administration; GC-MS = gas chromatography-mass spectrometry; NCOC= norcocaine; ND = not detected; NR = not reported

[a] The first 25 COC HCl samples appear in Table 6; the other 3 samples were analyzed only by the DEA Laboratory.

[b] Ions of these isomers (i.e., BE and NCOC) were indistinguishable by using the AccuTOF-DART system.

[c] These analytes were analyzed multiple times to verify the presence or absence of the analytes.

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Table 8. Summary of Additional Solvent Characteristics from DEA's Cocaine Signature Analysis

Cocaine	Number of Specimens	Comments
Major diluents/adulterants	10	NaCl (5), mannitol (3), dimethylterephthlate (1), lactose (1)
Trace diluents	1	Caffeine
Solvent A	20	Isopropyl acetate (4), n-propyl acetate (9), petroleum ether (4), ethyl acetate (3)
Solvent B	11	MIBK (3), MEK (8)
Base origin	1 Peruvian and 25 Columbian	
HCl process	25 Columbian	

Note: HCl = hydrochloride; MIBK = methyl isobutyl ketone; MEK = methyl ethyl ketone; NaCl = sodium chloride

2.1.5 Conclusions

COC signature analyses performed by DEA laboratory and GC-MS performed by the AFIP Laboratory identified that 3 of the 16 COC HCl powder specimens sent for analysis met RTI's study design criteria for use in our contamination and fortification studies. For these studies, RTI required one highly pure COC with <1% impurities and two less pure COC HCl powder specimens with higher concentrations of other COC analytes (>1 CE and >2% NCOC and BE). These findings allowed the proposed studies to proceed with only minor modifications to the research design. The modified study design, which included three COC sources, was adequate for the evaluation of COC analyte concentrations and metabolite-to-COC ratios.

The TOF-DART instrument allowed for the rapid analysis of the 25 illicit COC specimens without the need for sample preparation. Although this direct analysis resulted in the rapid production of data, it also caused inconsistent results. The TOF-DART is a novel approach to forensic analysis; however, it could not detect many drug compounds that are used to trace a COC sample to its geographic origin. Forensic laboratories may want to use the TOF-DART as a rapid screening test for preliminary sample-to-sample comparison of the analytes that can be detected with this technology.

At the completion of Stage I, RTI successfully identified three COC HCl powders at varying purities (DEA: 65%–85%) that met our study design criteria and could be used in the remainder of the study. The AFIP Laboratory's results corroborated DEA's NCOC results; therefore, RTI used DEA's results to determine which COC HCl powders to use in the contamination and fortification studies of this project. COC HCl powders that are available on the street have a wide range of purity and manufacturing by-products that may affect the incorporation of the drug into hair and its subsequent detection by hair drug testing using the proposed Mandatory Guidelines. For this reason, RTI evaluated multiple COC sources that would represent realistic COC available for ingestion and environmental contamination for use in this project.

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2.2 Stage II: Analysis of Hair from Drug-User Populations

2.2.1 Experimental Design

Stage II of this research project focused on analyzing COC analytes in hair from multiple drug-user populations to determine biomarker concentrations and the metabolite-to-parent concentration ratios. These data were statistically evaluated to determine the mean, median, and concentration range for each sample. In addition, drug-user populations' data were compared to confirmatory cut-off criteria of the proposed Mandatory Guidelines (SAMHSA, 2004a). When available, demographics and hair characterization were reported as part of this stage of the study. Before proceeding to other stages of this research, RTI evaluated COC analyte concentrations in hair by comparing the metabolic disposition of COC in head hair from two study populations. The study followed self-reported drug users (e.g., native environment) after ingesting the street drug and drug users who voluntarily participated in a 10-week in-patient NIDA study during which they were clinically administered COC in a double blind, placebo-controlled design. Participants provided written informed consent, and the protocol was approved by NIDA's Institutional Review Board.

It was important to evaluate drug-user populations as part of this research due to the limited availability of data reporting on all the COC analytes of interest. Furthermore, this parallel study design allowed for the evaluation of COC analyte disposition into the hair under controlled and non-controlled environments. COC analyte concentrations in the hair of drug users in a native environment are highly variable, and the dose is largely unknown, whereas COC analyte concentrations in hair following controlled drug administration allow researchers to investigate drug analyte distribution into human hair following a known dose. Therefore, a direct comparison of these populations by the same researchers who use the exact or similar analytical procedures was essential to the overall study design because this provided a foundation for understanding COC analyte concentrations and ratios in hair.

Tables 9 and **10** show the study design for Stage II based on dose setting (i.e., street environment versus clinical research). Table 9 shows hair samples from 38 drug users in a street environment (STREET drug user population) collection process that were obtained from a commercial source (27 subjects; Robert Fassio, Boston, MA) and hair collection from 11 subjects upon admission into an inpatient clinical research facility because these samples represented street use. Table 10 shows hair collected during controlled COC dosing from the same 11 subjects who were voluntarily enrolled in one of the two controlled dosage studies at an inpatient clinical research facility.

2.2.1.1 Street Drug-User Population

Human hair was voluntarily collected from self-reported drug users in their native environment; these specimens were purchased from a commercial source. All subjects provided informed consent and were compensated for their participation in the study. If known, demographic information (e.g., gender, age, and race) was obtained and reported to RTI with each hair specimen submission. Hair specimens were collected and analyzed from 38 drug users in this street environment. In addition, for purposes of this study, the head hair specimens collected from NIDA's Intramural Research Program (IRP) at the time of admittance and prior to controlled drug administration, which represented street drug use, were grouped and evaluated with this population. Head hair specimens from an additional 7 subjects were included in this

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street drug-user population and all are referred to as STREET specimens, for a total of 38 subjects. Subject demographics, hair characterization, and the type of collection for the STREET population are listed in **Table 11.**

Table 9. Experimental Study Design and Hair Specimen Collection and Analysis Scheme for the STREET Population

Hair Specimen Collection																				
Subject Number		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
Rep	1																			
	2																NA	NA	NA	NA
Subject Number		20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38
Rep	1																			
	2	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
												TOTAL ANALYSES								53

Note: NA = specimen unavailable; STREET = street drug user

Table 10. Experimental Study Design and Hair Specimen Collection and Analysis Scheme for Subjects Enrolled in NIDA's IRP Controlled Drug Administration Study in Which Cocaine Administration at Low and High Doses Occurred During a 9- to 10-Week Study Period (i.e., CLINICAL)

Hair Specimen Collection											
Subject Number	B	C	E	F	I	K	L	M	P	R	W
Low Dose (75 mg/70 kg)											
High Dose (150 mg/70 kg)					NA				NA		
						TOTAL ANALYSES					20

Note: CLINICAL = clinically administered drug users; IRP = Intramural Research Program; NA = specimen unavailable; NIDA = National Institute on Drug Abuse

Table 11. Summary of Demographic Information for the STREET Drug-User Population

Subject	Race	Age	Sex	Hair Type	Schwarzkopf Scale (RTI Modified)	Location and Type of Hair Collection
STREET 1	C	43	F	Medium blonde/ dyed	8	Unspecified; street/native environment collection
STREET 2	C	20	F	Medium blonde	9	Unspecified; street/native environment collection
STREET 3	C	24	F	Medium blonde	7	Unspecified; street/native environment collection
STREET 4	C	42	M	Dark brown/ some gray	4	Unspecified; street/native environment collection
STREET 5	C	41	F	Reddish brown	6	Unspecified; street/native environment collection

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Subject	Race	Age	Sex	Hair Type	Schwarzkopf Scale (RTI Modified)	Location and Type of Hair Collection
STREET 6	AA	36	F	Dark brown	3	Unspecified; street/native environment collection
STREET 7	C	24	F	Reddish brown/ dyed	6	Unspecified; street/native environment collection
STREET 8	C	52	M	Gray	10	Unspecified; street/native environment collection
STREET 9	C	44	M	Light brown	6	Unspecified; street/native environment collection
STREET 10	C	29	M	Dark brown	5	Unspecified; street/native environment collection
STREET 11	C	46	F	Medium brown	6	Unspecified; street/native environment collection
STREET 12	C	41	M	Dark brown	3	Unspecified; street/native environment collection
STREET 13	C	26	F	Blonde	8	Unspecified; street/native environment collection
STREET 14	C	48	M	Dark brown	5	Unspecified; street/native environment collection
STREET 15	UNK	UNK	UNK	Dark brown/ possibly colored	UNK	Unspecified; street/native environment collection
STREET 16	UNK	UNK	UNK	Light brown	5	Unspecified; street/native environment collection
STREET 17	UNK	UNK	UNK	Dark blonde	6	Unspecified; street/native environment collection
STREET 18	UNK	UNK	UNK	Dark blonde	6	Unspecified; street/native environment collection
STREET 19	UNK	UNK	UNK	Reddish brown	6.5	Unspecified; street/native environment collection
STREET 20	UNK	UNK	UNK	Dark blonde	6	Unspecified; street/native environment collection
STREET 21	UNK	UNK	UNK	Medium brown	4	Unspecified; street/native environment collection
STREET 22	UNK	UNK	UNK	Dark blonde	6	Unspecified; street/native environment collection
STREET 23	UNK	UNK	UNK	Ultra-light blonde	10	Unspecified; street/native environment collection
STREET 24	UNK	UNK	UNK	Medium blonde	7	Unspecified; street/native environment collection
STREET 25	UNK	UNK	UNK	Dark blonde	6	Unspecified; street/native environment collection
STREET 26	UNK	UNK	UNK	Light brown	5	Unspecified; street/native environment collection
STREET 27	UNK	UNK	UNK	Light brown	5	Unspecified; street/native environment collection
STREET 28	UNK	UNK	UNK	Light brown	5	Unspecified; street/native environment collection

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Subject	Race	Age	Sex	Hair Type	Schwarzkopf Scale (RTI Modified)	Location and Type of Hair Collection
STREET 29	UNK	UNK	UNK	Dark brown	3	Unspecified; street/native environment collection
STREET 30	UNK	UNK	UNK	Light brown	5	Unspecified; street/native environment collection
STREET 31	UNK	UNK	UNK	Medium brown	4	Unspecified; street/native environment collection
STREET 32	C	34	M	UNK	UNK	AVH; admission to the clinical study
STREET 33	AA	39	M	Dark brown	3	Unspecified; admission to the clinical study
STREET 34	C	28	M	UNK	UNK	Unspecified; admission to the clinical study
STREET 35	AA	32	F	Dark brown	3	Unspecified; admission to the clinical study
STREET 36	AA	40	M	Dark brown	3	Unspecified; admission to the clinical study
STREET 37	AA	29	F	Dark brown	3	Unspecified; admission to the clinical study
STREET 38	C	26	M	UNK	UNK	Unspecified; admission to the clinical study

Note: STREET = street drug user; C = Caucasian; AA = African American; M = male; F = female; UNK = unknown; AVH = anterior vertex head hair

2.2.1.2 User Population—Clinically Administered Cocaine

Human hair collected during inpatient clinical studies was procured through collaboration with NIDA investigators. Participants in this study provided written informed consent, and the protocol was approved by NIDA's Institutional Review Board.

Detailed information about the participants and the study design was described in previous publications (Joseph et al., 1998; Huestis et al., 1999; Ropero-Miller et al., 2000; Kolbrich et al., 2003; Kacinko et al., 2005; Scheidweiler et al., 2005). Briefly, 11 healthy subjects voluntarily enrolled in a 9- to 10-week inpatient study conducted by the Chemistry and Drug Metabolism Section of the IRP, NIDA, and the National Institutes of Health. All subjects had a self-reported history of COC use and tested positive by urine drug tests. Medical and psychological evaluations were performed to verify each subject's health before participating in the study. All subjects provided informed consent and were compensated for their participation. Details of the subjects' demographics, head hair specimens, and dosages are provided in **Table 12.**

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Table 12. Subjects Demographics and Hair Specimen Types in NIDA's IRP Controlled Drug Administration Study (i.e., Clinically Administered Drug-User Population)

Subject	Ethnicity	Age	Gender	Location Hair Collection	Time (Week)	Cocaine Dose (mg/70 kg)
B	C	34	M	AVH	Admission	UNK
				PVH	6	75 (low)
				FH	10	150 (high)
C	AA	39	M	Non-specific	Admission	UNK
C	AA	39	M	FH	6	75 (low)
C	AA	39	M	FH	10	150 (high)
E	C	28	M	Non-specific	Admission	UNK
E	C	28	M	PVH	6	75 (low)
E	C	28	M	PVH	10	150 (high)
F	AA	23	M	TH	6	75 (low)
F	AA	23	M	FH	10	150 (high)
I	AA	34	M	FH	6	75 (low)
K	AA	36	M	TH	6	75 (low)
K	AA	36	M	TH	10	150 (high)
L	AA	32	F	Non-specific	Admission	UNK
L	AA	32	F	PVH	6	75 (low)
L	AA	32	F	FH	10	150 (high)
M	AA	40	M	Non-specific	Admission	UNK
M	AA	40	M	TH	6	75 (low)
M	AA	40	M	TH	10	150 (high)
P	AA	29	F	Non-specific	Admission	UNK
P	AA	29	F	PVH	6	75 (low)
R	AA	35	F	AVH	6	75 (low)
R	AA	35	F	AVH	10	150 (high)
W	C	26	M	Non-specific	Admission	UNK
W	C	26	M	AVH	6	70
W	C	26	M	AVH	10	150 (high)

Note: AA = African American; AVH = anterior vertex head; C= Caucasian; F = female; FH = frontal head; IRP = Intramural Research Program; M = male; NIDA = National Institute on Drug Abuse; PVH = posterior vertex head; TH = temporal head; UNK = unknown

The time line for dosing and specimen collection is shown in **Figure 4**. Head hair was completely shaved the first day of the study (i.e., during admittance or Day 0), and weekly collection continued during the remainder of the study. Drugs were not administered during the first 3 weeks to allow time for all previously administered drugs, including COC, to be removed from the hair (i.e., the washout period). Hair was collected and analyzed during this period and

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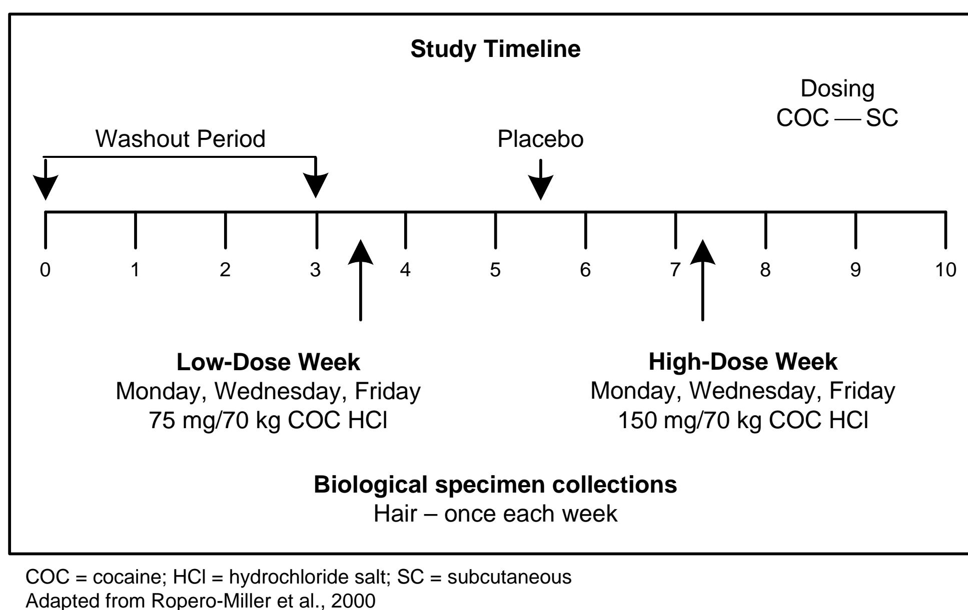
was determined to be mostly drug-free by the end of the washout period. Beginning in Week 4, subjects were administered a low dose of COC HCl powder (75 mg/70 kg) through subcutaneous injections on Monday, Wednesday, and Friday. Following low dosing, subjects were administered placebo doses subcutaneously during Weeks 7 and 8, observing the same daily schedule, with weekly head shaving. Beginning in Week 8, subjects were administered high doses of COC HCl (150 mg/70 kg) subcutaneously according to a similar dosing scheme. Specimen collection continued for 1 week after final dosing for follow up of drug elimination after high-dose administration. A total of 27 head hair specimens from 11 subjects were obtained from the specimen inventory of this NIDA IRP controlled drug administration study for inclusion in this research. Seven specimens that were collected at the time of admission were included in the STREET population because these were not collected under controlled drug administration and represented self-administration of street drugs (i.e., unknown COC dose). The remaining 20 head hair specimens from subjects were included as part of the controlled drug administration (clinically administered drug users [CLINICAL]) population.



COC = cocaine; HCl = hydrochloride salt; SC = subcutaneous
Adapted from Ropero-Miller et al., 2000

Figure 4. NIDA's IRP controlled drug administration study (i.e., clinically administered drug-user population) used the time line above for dosing and specimen collection.

2.2.2 Materials

Collection of Drug-User Hair Specimens by a Commercial Source—A commercial source pre-cleaned scissors with methanol, and then collected approximately 1 g of hair that was cut as close to the scalp as possible. After the specimens were collected, they were placed into sealed plastic bags at room temperature; any personally identifiable information was removed and shipped to RTI with limited demographic data when available.

Collection of Hair Specimens at NIDA's IRP During a Clinical Study—Hair was collected weekly by NIDA staff. For the first collection, cleaned grooming clippers were used to remove hair from different regions of the scalp (e.g., temporal, frontal, nape, posterior vertex, anterior vertex). For this study, hair was analyzed from the temporal, posterior vertex, and anterior vertex regions. Shaving cream and a straight-edge razor were used to remove remaining stubble, which was discarded. Hair from the first collection was stored in sealed plastic bags at

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room temperature until it was finely cut with scissors and transferred to separate glass vessels for storage at −30ºC. For the remainder of the study, a cleaned electrical shaver was used to collect hair (approximately 2–3 mm in length) as close to the scalp as possible. Remaining stubble was removed and discarded with shaving cream and a straight-edge razor. Hair specimens only represented 1 week of growth; these pieces were weighed and analyzed for COC analytes. A portion of each specimen was sent to RTI for analysis.

Collection of Hair Specimens by RTI for Stage II of This Study—Human hair specimens were collected from three sources to meet all requirements for different variables and hair types. All specimens collected were human head hair that was cut with scissors from various regions of the scalp (e.g., frontal, temporal, posterior vertex, anterior vertex) and shaved with a cleaned electric razor close to the scalp (i.e., drug-user hair, clinical hair specimens after Week 1) or was not noted what was root end (drug-free hair, STREET population, and some CLINICAL population hair specimens before Week 1).

Drug-free hair was collected for use as blind negative analytical controls for Stage II of this research. Professional cosmetologists collected drug-free dark and light hair samples, which were not chemically treated (e.g., straightened, permanent waved, colored), during the normal process of a hair cut. If available, cosmetologists also collected demographic information (e.g., gender, age, ethnicity) to submit to RTI with hair specimens. The sources of these data are not publicly available. RTI collected information and recorded it in such a manner that human subjects cannot be identified, directly or through identifiers linked to the subjects. All drug-free hair specimens were analyzed for analytes of interest before inclusion in this study. Drug-free hair was used as a negative control specimen and was shipped in a blinded manner, appearing as actual study specimens, to the Immunalysis Corp. for quality assurance purposes. This drug-free hair was also used as the precursor for the contaminated hair specimens and the fortified proficiency specimens detailed in Stages III and IV of this research.

Preparation of Drug-User Hair Specimens for Shipment to the Reference Hair-Testing Laboratory—Before shipping hair specimens to the reference laboratory, all drug-user hair specimens were subjected to a decontamination procedure as described in Section 2.2.3.1. All specimens were labeled with unique identifiers that could not be linked to the type of hair specimen being submitted for analysis. Approximately 100 specimens were analyzed for Stage II of this study.

2.2.3 Methods

2.2.3.1 Hair Characterization and Decontamination Procedures

Characterization of Hair Specimens—The Schwarzkopf color scale, which is used by professional cosmetologists to categorize hair color, was modified by RTI (**Table 13**) to determine hair color (1 = black, up to 10 = light blonde and gray) samples during this study. One person visually and physically determined all hair specimens by hand, and another individual confirmed these determinations separately (Schwarzkopf, 2001). Prior to fortification, the hair was received into the inventory, weighed, and visually evaluated for color using the Schwarzkopf scale modified by RTI.

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Table 13. RTI's Color Scale Based on the Schwarzkopf Scale

Hair Color	Schwarzkopf Scale
Black	1
Dark brown	2 through 4
Brown	5 and 6
Light brown	7
Medium blonde	8 and 9
Light blonde and gray	10

Decontamination of Hair Specimens—Prior to sending the specimens to reference hair-testing laboratories, RTI decontaminated the hair by using a protocol published by Cairns and colleagues (2004b), which employed the use of an extended aqueous buffer wash. Following the receipt procedure, including characterization, each 50 mg–100 mg hair specimen was shaken vigorously at 120 rpm at 37°C for 15 minutes in 20 mL of isopropanol. Hair specimens were then shaken at 120 rpm in sufficient 0.01 M phosphate buffer with 0.01% bovine serum albumin (pH 6) for 30 minutes at 37°C for three 30-minute intervals and two 60-minute intervals, and the phosphate buffer was replaced after each wash step. The shaker was configured so that the sample tubes traveled a short distance and experienced an abrupt change in direction at the ends of the shake cycle (bumped at the ends). This was repeated two more times, followed by two 60-minute buffer washes using the same conditions. Specimens were allowed to dry overnight before aliquoting and preparing them for shipment to the reference laboratories.

2.2.3.2 Data Collection and Statistical Analysis

All statistical analyses were performed using SAS (version 9.1.3) or Microsoft Excel. For Stage II comparisons, a one-way analysis of variation or student t-test was used. Specimens were analyzed in singular or in replicate as presented in Tables 9 and 10.

2.2.4 Analytical Procedures

All specimens were submitted to Immunalysis Corp. for Stage II analyses by established standard operating procedures for performing hair drug testing. This laboratory quantified COC analytes in the hair specimens and was compensated for performing the analytical work. Appropriate digestion methods for hair were selected to maintain all COC analyte concentrations with minimal COC hydrolysis to BE, which can be more labile under many hair-digestion methods. Matrix-matched quality controls were included in the analysis to monitor for hydrolysis, with ≤5% considered acceptable.

Liquid Chromatography-Tandem Mass Spectrometry—LC-MS/MS was used for hair testing according to previously published and peer-reviewed methods (Moore et al., 2007). Quantitative analytical procedures for determining COC, BE, CE, and NCOC in hair were performed on an Agilent Technologies 1200 Series liquid chromatograph pump coupled to a 6410 triple quadrupole mass spectrometer that was operated in positive APCI mode. For confirmation, two transitions were monitored, and, in some cases, one ion ratio was determined and found to be acceptable if it was within 20% of the ratio performance of known calibration standards. **Figure 5** and **Table 14** show representative LC-MS/MS chromatography and validation statistics for the primary reference laboratory's (Immunalysis Corp.) method used for hair analysis for this study.

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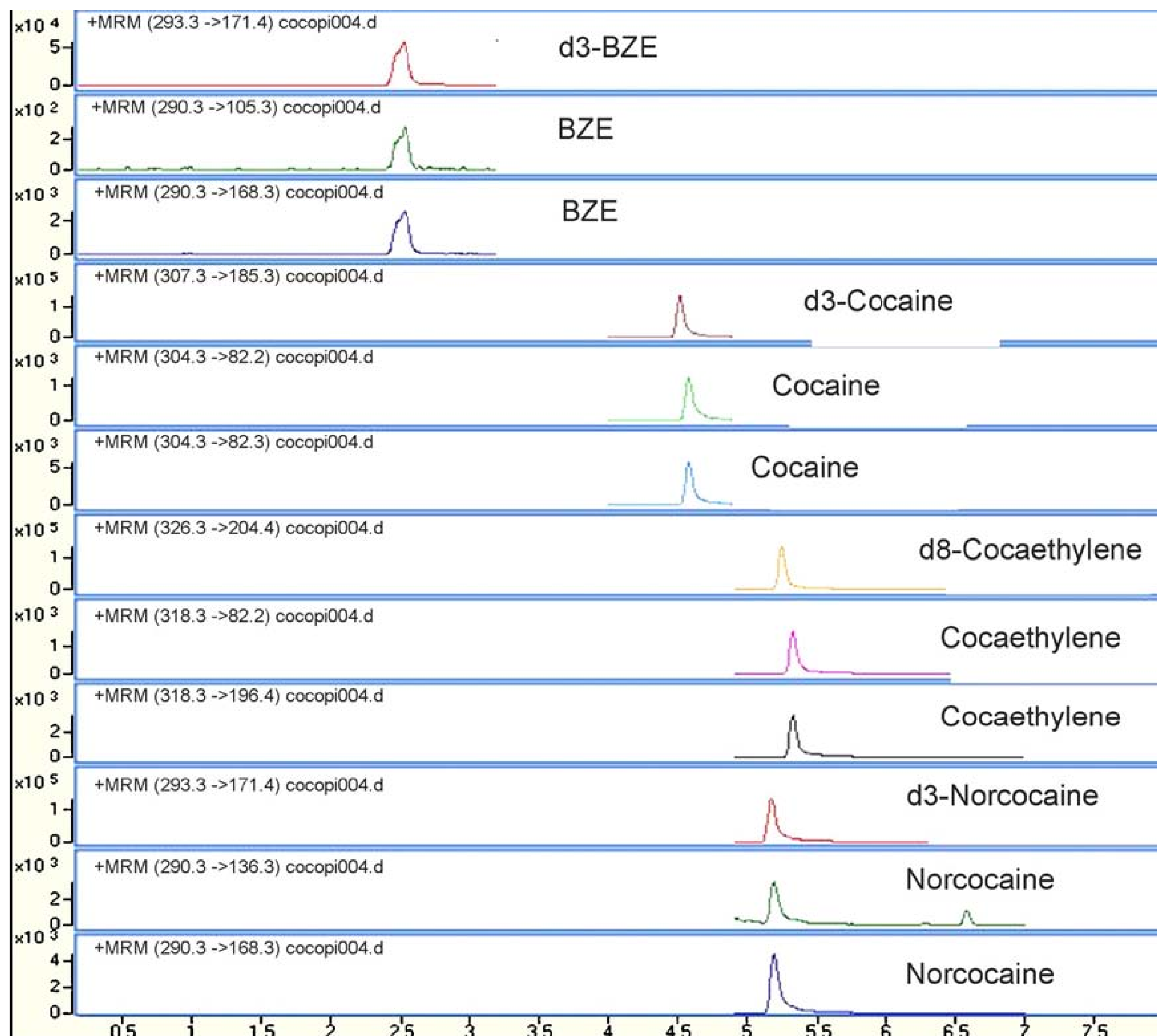

Figure 5. LC-MS/MS chromatography performance from Immunalysis Corp. for a standard material at 50 pg/mg.

Table 14. Validation Statistics for the Primary Reference Laboratory's (Immunalysis Corp.) Method Used for Hair Analysis and Reported to RTI for This Research

LC-MS/MS Operating in APCI Mode	COC	BE	CE	NCOC
Limit of detection (pg/mg)	25	25	25	25
Limit of quantification (pg/mg)	50	50	50	50
Limit of linearity (pg/mg)	10,000	10,000	10,000	10,000
Accuracy; *n* value	5	5	5	5
50 pg/mg target; % accuracy	99.9	101.7	99.3	108.0