Effect of Four Laboratory Decontamination Procedures on the Quantitative Determination of Cocaine and Metabolites in Hair by HPLC–MS

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Abstract

The testing for drugs of abuse in hair is increasingly used to detect illicit substances. Laboratories have implemented various decontamination, or washing, procedures in order to eliminate concerns regarding potential contamination of the hair with drug from the environment. However, the effect of these decontamination procedures on drug incorporated into the hair shaft via systemic exposure is unknown. This study evaluated the effect of four simple laboratory wash procedures on the quantitative measurement of cocaine and its metabolites in hair from rats administered cocaine by intraperitoneal injection. Washes included (1) methanol only; (2) 0.1M phosphate buffer, pH 6.0; (3) 0.1M phosphate buffer, pH 8.0; and (4) isopropanol and phosphate buffer, pH 5.5. Cocaine and its major metabolites, benzoylecgonine, norcocaine, ecgonine methyl ester, and cocaethylene, were analyzed using high-performance liquid chromatography coupled to atmospheric pressure electrospray ionization mass spectrometry. All four washes resulted in significant differences from unwashed hair controls (p < 0.05) for some or all of the detectable analytes. Because different wash procedures lead to significant differences in the measured concentrations of analytes in hair known to contain drug, quantitative data must be interpreted cautiously based on the wash procedures employed.

Introduction

Methods for determination of drugs of abuse in hair are often hampered by their inability to distinguish systemically incorporated drug from drug contaminating the surface of the hair from the environment. Numerous laboratory decontamination procedures that attempt to remove potential contaminant drug from the surface of the hair have been described (1,2). Ideally, such procedures would also allow incorporated drug to remain in place and not be removed from the hair during the decontamination process. To permit comparison of quantitative hair measurements from laboratories, two aspects of the decontamination process must be evaluated: (1) the amount of incorporated drug removed from the hair by the process and (2) the amount of contaminant removed from the surface of the hair. The purpose of this study was to focus upon the first aspect of the decontamination process using an animal model. Specifically, we evaluated the effect of four simple laboratory wash procedures on quantitative measurement of cocaine and its metabolites in hair known to contain systemically incorporated drug and with minimal (undetectable) external contamination.

Materials and Methods

Chemicals and reagents

Cocaine (1 mg/mL in acetonitrile), benzoylecgonine (BE; 1 mg/mL in methanol), norcocaine (NORCOC) hydrochloride (1 mg/mL in acetonitrile), ecgonine methyl ester (EME; 1 mg/mL in acetonitrile), cocaethylene (CE; 1 mg/mL in acetonitrile), COC-d3 (100 ng/mL in acetonitrile), BE-d3 (100 ng/mL in methanol), NORCOC-d3 (100 ng/mL in acetonitrile), EME-d3 (100 ng/mL in acetonitrile), and CE-d3 (100 ng/mL in acetonitrile) were obtained from Radian Corp. (now Cerilliant, Austin, TX). Bond Elut Certify | solid-phase extraction (SPE) columns were obtained from Varian Corp. (Harbor City, CA). High-performance liquid chromatography (HPLC)-grade methanol, isopropanol, formic acid, and ammonium hydroxide were obtained from Fisher Scientific (Pittsburgh, PA). HPLC-grade dichloromethane was obtained from Burdick and Jackson (Muskegon, MI). All buffers and HPLC mobile phases were prepared using Milli-Q grade water (Millipore, Bedford, MA).

Standards and solutions

Stock solutions containing COC, BE, NORCOC, EME, and CE used for the preparation of calibration curves and quality control samples were prepared in HPLC-grade methanol and stored at −20°C. The stock solutions were used to make working solutions at 10, 1, 0.1, and 0.01 ng/μL. The working solutions were used to prepare quality control and calibration samples. Calibration curves were prepared by fortifying 20.0 ± 0.2 mg of drug-free rat hair samples in silanized glass tubes with drug prior to digestion at concentrations of 0.02, 0.05, 0.08, 0.1, 0.2, 0.3, 0.5, 1.0, 2.0, 3.0,
5.0, 10.0, 25.0, and 50.0 ng/mg. Quality control samples (0.5, 10.0, 25.0 ng/mg) were prepared similarly from solutions made with reference materials with lot numbers that were different from the reference materials used to prepare the calibration standards. A mixture of drug-free pigmented and unpigmented rat hair was used for the preparation of standard curves and quality control specimens.

Animal protocol
In order to minimize external contamination of the hair from bedding or urine, male Long Evans (LE) rats (140–160g) were housed individually in bedding-free hanging wire-mesh cages. The rats were allowed food and water ad libitum. The rats were provided with a constant room temperature and a 12-h light/dark cycle. Prior to dosing, an approximately 1-in. square area of pigmented and non-pigmented hair was shaved from the dorsal region using an electric shaver. Cocaine hydrochloride dissolved in normal saline (5 mg/mL as free base) was administered by intraperitoneal injection once per day for five days with either 5, 10, or 20 mg/kg (n = 8 per dose). Fourteen days after the first dose, the same areas of pigmented and non-pigmented hair were again shaved to collect newly grown hair that contained cocaine and metabolites. All shaved hair was stored in aluminum foil at −20°C. A detailed description of animal protocols used has been previously described (3).

Sample preparation
A homogenous pool of combined pigmented and unpigmented rat hair known to contain drug was prepared from animals (n = 12) dosed with cocaine as described. To obtain a sufficient quantity of hair for use in all hair wash experiments, hair was pooled from several rats that had been administered either 5, 10, or 20 mg/kg cocaine. The rat hair was mixed thoroughly, cut finely into small pieces (approximately 1–2 mm length), mixed again, and weighed out in 20-mg aliquots. The mean concentrations of COC, NORCOC, and EME were 2.47 ± 0.16, 0.25 ± 0.02, and 0.68 ± 0.04 ng/mg, respectively, in the pool of hair as determined by liquid chromatography–mass spectrometry (LC–MS) (see Method).

Table I. Summary of Wash Procedures Performed on Hair from Dosed Rats

<table>
<thead>
<tr>
<th>Wash</th>
<th>Procedure</th>
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</table>
| A    | 1. Add 1 mL of methanol to hair.  
2. Vortex mix for 30 s.  
3. Centrifuge for 2 min at 2250 rpm.  
4. Remove liquid with glass Pasteur pipette and discard.  
5. Repeat steps 1–4 two more times.  |
| B    | 1. As wash A, but with 0.1M phosphate buffer (pH 6.0) instead of methanol.  |
| C    | 1. As wash A, but with 0.1M phosphate buffer (pH 8.0) instead of methanol.  |
| D    | 1. Add 1 mL of dry isopropanol.  
2. Place on shaking water bath for 25 min at 37°C and 100 rpm.  
3. Centrifuge for 2 min at 2250 rpm.  
4. Remove liquid with glass Pasteur pipette and discard.  
5. Add 1 mL 0.1M phosphate buffer (pH 5.5).  
6. Place on shaking water bath for 25 min at 37°C and 100 rpm.  
7. Centrifuge for 2 min at 2250 rpm.  
8. Remove liquid with glass Pasteur pipette and discard.  
9. Repeat steps 5–8 two more times.  |

A very small amount of BE (0.059 ± 0.015) was detected in the pool of hair; however this was likely a non-metabolic hydrolysis product of COC (see Results and Discussion). As expected, CE was not detected in the pooled hair.

Washing procedures
Twenty-milligram hair aliquots were washed in one of four ways as shown in Table I (Washes A, B, C, and D). Wash A consisted of three sequential 1-mL methanol washes. Wash B consisted of three sequential 1-mL 0.1M phosphate buffer (pH 6.0) washes. Wash C consisted of three sequential 1-mL 0.1M phosphate buffer (pH 8.0) washes. Wash D consisted of a 1-mL isopropanol wash followed by three sequential 1-mL 0.1M phosphate buffer (pH 5.5) washes. Sample vortex mixing was performed with a Fisher Scientific Vortex Genie 2®. Centrifugation between wash steps was performed with an International Centrifuges Size 2 Model K centrifuge.

After washing, the samples were allowed to completely dry in a fume hood at room temperature prior to extraction. At the time of extraction, five additional 20-mg aliquots of unwashed hair were co-analyzed and served as the unwashed control.

Sample preparation and extraction
Samples, calibrators, and quality control samples were fortified with 50 μL of 1.0 ng/μL COC-d₃, BE-d₃, NORCOC-d₃, EME-d₃, and CE-d₃ internal standard. All samples were then digested in 1.0 mL of 0.1N HCl overnight on a shaking water bath approximately 65 cycles/min at room temperature. After digestion, all samples were buffered with 400 μL of 0.1M phosphate buffer (pH 6) and subsequently divided into two 700-μL portions. One 700-μL aliquot was used to determine the cocaine and metabolite hair concentrations (the remaining extract was preserved for further experimentation not discussed here).

SPE of the samples was performed using Bond Elut Certify® columns, a vacuum manifold device, and a vacuum source. The SPE columns were prepared by sequentially passing 2 mL methanol and 2 mL 0.1M phosphate buffer (pH 6) through the sorbent bed. Samples were each then transferred to a SPE column and allowed to pass through the column. The columns were then washed by sequentially passing 6 mL water, 3 mL 0.1M HCl, and 9 mL methanol through the sorbent bed. The columns were then thoroughly dried by applying a 10-mm Hg vacuum for approximately 3 min. Analytes were eluted from the column with 2 mL 78:20:2 (v/v/v) dichloromethane/isopropanol/ammonium hydroxide under gravity flow. Eluates were evaporated to dryness under a stream of air at 40°C. The residues were reconstituted in 50 μL of 90:10 0.1% formic acid/methanol and transferred to 300-μL conical bottom autosampler vials.

LC–MS analysis
Atmospheric pressure electrospray ionization (API-ES) analysis of sample extracts was performed using a Hewlett-Packard series 1100 LC–mass selective detector (MSD) (Hewlett-Packard Corp., Palo Alto, CA). The HPLC mobile
phase consisted of 75% water containing 0.1% formic acid and 25% methanol pumped isocratically at 0.25 mL/min at 30°C. Chromatographic separation of analytes was achieved using a Metasil basic 3-μm × 100-mm column (Metachem Technologies, Inc. Torrance, CA). An inline solvent filter (Upchurch Scientific, Oak Harbor, WA) and a Phenomenex SecurityGuard™ guard cartridge were employed to preserve column performance.

The spray chamber was operated with a drying gas flow of 10.0 L/min at a temperature of 300°C. The nebulizer pressure was 20 psig, and the capillary voltage was set to 1500 V. The fragmentor voltage was set to 70 V. The electron multiplier gain was set to 1.0.

The MSD was set to selective ion monitoring (SIM) mode to detect ions at m/z 290 (BE, NORCOC), 293 (BE-d₃, NORCOC-d₃), 304 (COC), 307 (COC-d₃), 300 (nMn), 303 (EME-d₃), 318 (Cₙ), and 321 (CE-d₃). The scan time was 0.6 s/cycle.

For the purposes of this experiment, a single ion was selected for analysis of the rat hair in order to maximize sensitivity, facilitating detection of subtle, yet reproducible changes in analyte concentration in the picogram-per-milligram range (4).

Quantitative analysis
Quantitation of cocaine and metabolites was accomplished by calculating the peak-height ratios for the molecular ions of each analyte and its respective deuterated internal standard. Linear curve fits for the analytes yielded the following linear ranges: 0.02–50 ng/mg for BE and CE, 0.05–50 ng/mg for EME and COC, and 0.05–10 ng/mg for NORCOC (r² > 0.99, all analytes). Hewlett-Packard ChemStation™ quantitation software was used to generate calibration curves and to calculate the concentrations of the five analytes.

Statistical analysis
Significance of drug concentration differences between washed rat hair and unwashed control was determined by a two-sample t-test (p ≤ 0.05) using DataDesk® version 4.0, Data Description, Inc.

Results and Discussion
Analytical method
Typical chromatograms obtained for a low hair calibrator (0.1 ng/mg) and a negative hair sample, respectively are shown in Figures 1 and 2. Chromatography was optimized to provide Gaussian peak shape and baseline chromatographic resolution of BE and NORCOC.

Precision and accuracy of the procedure was determined by analyzing drug-free rat hair fortified with known concentrations of COC, BE, NORCOC, EME, and CE. Results are summarized in Table II. The coefficients of variation for intra-assay precision was determined to be less than 4% for COC, BE, EME, and CE at concentrations of 0.5, 10, and 25 ng/mg, and less than 5% for NORCOC at concentrations of 0.5 and 10 ng/mg. The interassay precision was determined by comparing the quality control data from assays performed on six different days (n = 5 for each assay). The coefficients of variation for the interassay precision were less than 7% for COC, NORCOC, and EME. Precision data for incorporated BE and CE could not be determined as the dosed rat hair did not contain appreciable quantities of these two analytes.

Reproducibility and accuracy of the assay for drug incorporated into hair could only be estimated from the analysis of the unwashed control hair. As shown in Table III, coefficients of variation for interassay precision were less than 10% for COC, NORCOC, and EME. Precision data for incorporated BE and CE could not be determined as the dosed rat hair did not contain appreciable quantities of these two analytes.

Analysis of COC and metabolites in rat hair subjected to laboratory wash procedures
Simple laboratory washes have been reported in published hair testing literature (1,2). Variants of
these procedures have been employed by many analytical laboratories to remove external environmental drug contaminant from the sample hair prior to digestion. However, it has been reported that such wash procedures do not always effectively remove surface contaminant (5-7). It has also been suggested that commonly employed wash procedures may also remove systemically incorporated drug from the hair shaft in addition to surface contaminants (8,9). Previously, we used an animal model to study the incorporation of COC and its metabolites into rat hair. These studies demonstrated that measured concentrations of COC were due to incorporated drug, rather than drug on the surface of the hair shaft from either sweat or saliva of rats (3). Therefore, this model was used in the present study to provide contaminant-free hair known to contain COC and metabolites incorporated into the hair shaft.

Four simple wash procedures were evaluated on two separate days of analysis. On each of the two days, each wash procedure was performed on five separate 20-mg aliquots of dosed rat hair. On each day of analysis, unwashed rat hair was concurrently extracted and analyzed to serve as the control value. The washed hair samples were digested and extracted as described in Analytical method. The quantitative wash results are summarized in Table III. Data are expressed as percentage of unwashed control hair and plotted in Figures 3-6. The measured COC concentrations in rat hair were decreased significantly from the control for washes B (82%), C (86%), and D (63%) (p < 0.05, 2-sample t-test, DataDesk). Wash A showed a sig-

![Figure 2. Reconstructed ion chromatograms for COC and metabolites in a contaminant-free (negative) hair specimen.](image-url)
**Table III. Concentrations of Detectable Analytes in Hair from Dosed Rats***

<table>
<thead>
<tr>
<th></th>
<th>Cocaine</th>
<th>Ergonine Methyl Ester</th>
<th>Norcocaine</th>
<th>Benzoylecgonine</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Concentration (ng/mg)</td>
<td>%CV</td>
<td>Concentration (ng/mg)</td>
<td>%CV</td>
</tr>
<tr>
<td>No Wash</td>
<td>2.47</td>
<td>6.5</td>
<td>0.68</td>
<td>5.9</td>
</tr>
<tr>
<td>Wash A</td>
<td>2.79†</td>
<td>8.8</td>
<td>0.65</td>
<td>6.2</td>
</tr>
<tr>
<td>Wash B</td>
<td>2.03†</td>
<td>7.4</td>
<td>0.50†</td>
<td>6.0</td>
</tr>
<tr>
<td>Wash C</td>
<td>2.12†</td>
<td>15.1</td>
<td>0.51†</td>
<td>9.8</td>
</tr>
<tr>
<td>Wash D</td>
<td>1.56†</td>
<td>8.3</td>
<td>0.33†</td>
<td>6.1</td>
</tr>
</tbody>
</table>

* n = 10 (2 separate batches, n = 5 each batch).
† Significantly different from unwashed control (p < 0.05).
‡ 6 of 10 values were negative (< LOQ). Calculation includes negative values as zero.

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**Figure 3.** Change in measured concentrations of COC, NORCOC, and EME after Wash Procedure A (methanol only). Data are expressed as percentage of unwashed control values (n = 10).

**Figure 4.** Change in measured concentrations of COC, NORCOC, and EME after Wash Procedure B (0.1M phosphate buffer, pH 6.0). Data are expressed as percentage of unwashed control values (n = 10).

Significant increase for COC (113%). Quantitative NORCOC concentrations were significantly decreased as compared to controls for Wash B (82%), C (86%), and D (64%). Measured NORCOC concentrations were significantly higher compared to controls for wash A (115%). EME concentrations were significantly different compared to controls in washes B (73%), C (74%), and D (48%). Wash A showed no significant difference in measured EME concentrations. It is possible that some of the COC was converted to EME through methanolation in wash A. However, it seems unlikely that this would contribute significantly to the EME concentration given that only small amounts of conversion (5%) have been detected under much more extreme conditions than those employed in wash A (10).

Washes B, C, and D all employed 0.1M phosphate buffer, yet there was wide variability in quantitative results between wash D and those of washes B and C. The results of washes B and C were similar to each other.

The pH differences between the buffer solutions do not explain the pattern of drug removal. The results of washes B and C were similar even though the pH of the B wash buffer (pH 6.0) was closer to that of wash D (pH 5.8) than to that of wash C (pH 8.0). Given that the $pK_a$ of cocaine is approximately 8.6, the average charge state of cocaine molecules in contact with buffer is positive for all three washes. If the mechanism of drug binding to hair and drug removal depends on the charge state of the drug, then there should not be substantial differences in removal patterns between these three buffers. The difference between the results of wash D and those of washes B and C may be due to the greater duration, the heating of the wash solution, and the isopropanol pre-treatment in wash D.

BE and CE were not detected as metabolites in rat hair after administration of COC. This is consistent with previously published data for COC and metabolites in rat hair (3,11). Nakahara and Kikura have suggested that BE found in hair is the result of hydrolysis of incorporated COC. Our previous study with COC incorporated into rat hair determined that the measured BE concentrations were approximately 2% of the COC concentration after either a 5-, 10-, or 20-mg/kg dose. In this present study, we again determined that a very small amount of BE (0.059 ng/mg ± 0.015; approximately 2.4% of COC) could be detected in the unwashed rat hair (control) after digestion and extraction. To determine whether the BE was produced as an analytical artifact, we co-analyzed five quality-control specimens fortified with 10 ng/mg COC only. After digestion, extraction and analysis of the quality control specimens, the
mean measured BE concentration was 0.08 ng/mg ± 0.034, or approximately 0.8% of the fortified COC concentration. Thus, the BE concentrations (2.4% of COC) measured in the pooled rat hair could be attributed to both analytical artifact and hydrolysis of incorporated COC. Regardless of the source of the BE, none of the wash procedures completely removed the BE from the hair (Table III).

CE was undetectable in the rat hair samples because the rats were not administered ethanol during the dosing procedure. This analyte was, therefore, not expected to be present. It was nevertheless analyzed for as part of an analytical method originally designed for broader applications.

The methanol wash (Wash A) resulted in a statistically significant increase in the measured concentrations of COC and NORCOC. We believe that this effect may be due to a relative increase in the extraction efficiency of the acid digestion of hair previously treated with methanol.

The results of this study demonstrate that wash procedures vary in the degree to which they remove incorporated drug from rat hair. This study was not intended to develop, optimize, or otherwise recommend a particular wash procedure for hair, but rather to simply demonstrate that different wash procedures can produce different quantitative hair results and that this may contribute to variability in quantitative data between laboratories. For this reason, comprehensive analyses of the individual wash fractions were not conducted.

The variability in the degree to which wash procedures remove incorporated drug demonstrated in this study clearly indicate that evaluation of the removal of incorporated drug should be included in the validation of analytical procedures for hair.

It is possible that there are differences in hair structure between rats and humans that might result in different relative percentages of drug-removal upon washing. For example, human and rodent hair differ in the ultra-structure of the medulla region of the hair shaft. Rats possess a discontinuous medulla with wide or cubic aerial vesicles. Humans have an intermediate or fragmented medulla with small aerial vesicles (12). If, for example, binding of drugs in hair was localized to the region of the medulla, structural differences might greatly impact the pattern of results observed for wash data. There may also be differences in the permeability of the two hair types that would affect the accessibility of some regions of the hair to the wash solutions. Our studies cannot address these issues directly. Similar wash studies need to be performed using human hair; however, sources of contaminant-free human hair containing known quantities of incorporated COC are not readily available. Despite differences in human and animal hair, it is probable that laboratory wash procedures contribute to variability in quantitative drug measurements between laboratories for human hair.

Conclusions

This paper describes a sensitive and specific method for the determination of low concentrations of cocaine and its four major metabolites in hair using API-ES LC-MSD. The procedure was then used to evaluate the effect of four simple wash procedures on the quantitative measurement of COC and metabolites in rat hair. The quantitative data obtained after the four wash procedures demonstrate that systemically incorporated drug and/or metabolite can be removed...
from the hair shaft. This would result in significantly different quantitative results in comparison to unwashed hair. The data clearly demonstrate that the effect of wash procedures on quantitative analysis should be determined during validation of analytical methods. The specific wash procedure(s) employed by laboratories should be considered when interpreting quantitative hair data.

Acknowledgments

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References