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Determination of cocaine, benzoylecgonine, cocaethylene and norcocaine in human hair using solid-phase extraction and liquid chromatography with tandem mass spectrometric detection

Christine Moore *, Cynthia Coulter, Katherine Crompton

Immunalysis Corporation, 829 Towne Center Drive, Pomona, CA 91767, United States

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Abstract

A quantitative analytical procedure for the determination of cocaine, benzoylecgonine and cocaethylene and norcocaine in hair has been developed and validated. The hair samples were washed, incubated, and any drugs present were quantified using mixed mode solid-phase extraction and liquid chromatography with tandem mass spectrometric detection in positive atmospheric pressure chemical ionization mode. For confirmation, two transitions were monitored and one ion ratio was determined, which was within 20% of that of the known calibration standards. The monitoring of the qualifying transition and requirement for its presence within a specific ratio to the primary ion limited the sensitivity of the assay, particularly for benzoylecgonine, however, the additional confidence in the final result as well as forensic defensibility were considered to be of greater importance. Even with simultaneous monitoring, the concentrations proposed by the United States Federal guidelines for hair analysis were achieved. The limits of quantitation were 50 pg/mg; the limit of detection was 25 pg/mg. The intra-day precision of the assays at 100 pg/mg ($n = 5$) was 1.3%, 8.1%, 0.8% and 0.4%; inter-day precision 4.8%, 9.2%, 15.7% and 12.6% ($n = 10$) for cocaine, benzoylecgonine, cocaethylene and norcocaine, respectively. The methods were applied to both proficiency specimens and to samples obtained during research studies in the USA.

Keywords: Hair; Cocaine; Benzoylecgonine; Cocaethylene; Norcocaine; LC/MS/MS

1. Introduction

Cocaine (COC) and its metabolites are included in the proposed United States Federal regulations for hair analysis. The suggested cut-off concentration for the metabolites is 50 pg/mg, which is difficult to achieve routinely using electron impact gas chromatography–mass spectrometry (GC/MS) [1,2]. This may be due to the inability to derivatize cocaethylene (CE) to improve its response; the co-elution of norcocaine (NC) and CE, or potentially similar ions for the derivatives of NC and benzoylecgonine (BZE). Procedures have been developed to approach the proposed detection requirements, including positive chemical ionization GC/MS [3], and gas chromatography with tandem mass spectrometry [4].

There are two publications describing the analysis of cocaine and its metabolites in hair using LC/MS/MS in APCI mode, in a similar manner to our approach [5,6]. The first of these analyzes only cocaine and benzoylecgonine, but more importantly, both procedures monitor only one transition in the multiple reaction-monitoring mode (MRM). Recently, several authors have focused on the need for the monitoring a second transition, allowing the ratio between the abundance of the primary and secondary ions to be calculated, and establishing more confidence in the final result. Maralikova and Weinmann noted that guidelines for confirmatory analysis using LC/MS/MS have not yet been established, and suggest that the monitoring of at least two transitions is required to provide sufficient identification of drugs [7]. Johansen and Bhatia [8] describe the analysis of cocaine and its metabolites in whole blood and urine using LC/MS/MS, focusing on the establishment of identification criteria based on two MRM transitions, their ratio, and retention time. This is particularly important in assays that include compounds with similar molecular weights

* Corresponding author. Tel.: +1 909 482 0840; fax: +1 909 482 0850.
E-mail address: cmoore@immunalysis.com (C. Moore).

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and chemical properties, since the same product ion is often present.

Using these suggestions for tandem mass spectrometry, we developed and validated a procedure using LC/MS/MS for the analysis of cocaine and its metabolites in hair, in order to provide additional confidence in the generated result. The method was applied to specimens received into our laboratory from proficiency programs and research studies.

2. Experimental

2.1. Standards and reagents

Deuterated internal standards (benzoylecgonine d3, cocaine-d3, norcocaine-d3 and cocaethylene-d8) as well as non-deuterated drug standards for each of the drugs were obtained from Cerilliant (Round Rock, TX). Solid-phase extraction columns (Clin II, 691-0353T) were obtained from SPEWare (San Pedro, CA). All solvents were HPLC grade or better, and all chemicals were ACS grade.

2.2. Calibrators

For the chromatographic calibration standards, a working solution containing deuterated internal standards was prepared in methanol at a concentration of 200 ng/mL. Unlabelled drug standards were prepared in methanol at the same concentration. All the working solutions were stored at −20 °C when not in use. For each batch, eight calibration standards were prepared in drug free hair (10 mg). Drug concentrations of 25, 50, 100, 200, 500, 1000, 2000 and 10,000 pg/mg of hair were prepared (internal standard concentration: 1000 pg/mg).

2.3. Sample preparation for chromatographic analysis

An aliquot of hair (10 mg) was briefly rinsed with methylene chloride (1.5 mL) to remove hair treatments such as mousse, spray, gels, etc., and allowed to dry. The hair was cut into small pieces and internal standard was added (50 μL). 0.025 M phosphate buffer (pH 2.7; 1.5 mL) was added and the hair was sonicated at 75 °C for 3 h. The analytical recovery from authentic hair specimens was calculated following 1, 2 and 3 h incubation in the phosphate buffer. After 3 h, 82.7% of cocaine and 93.8% of benzoylecgonine were recovered from the hair matrix [9]. The buffer was decanted into clean glass tubes and 0.1 M sodium phosphate buffer (pH 6.0; 1 mL) was added to each calibrator, control or hair specimen. The mix was centrifuged for 10 min to ensure no hair strands were applied to the solid-phase extraction column. Solid-phase mixed mode extraction columns (Clin II, 691-0353T) were placed into a positive pressure manifold. Each column was conditioned with methylene chloride:methanol:ammonium hydroxide (78:20:2, v/v/v; 2 mL), ethyl acetate (2 mL), methanol (2 mL) and 0.1 M hydrochloric acid (1 mL). The samples were allowed to flow through the columns, and then the columns were washed with deionized water (2 mL), 0.1 M hydrochloric acid (2 mL), methanol (2 mL) and ethyl acetate (2 mL). The columns were allowed to dry between washes under nitrogen pressure (30 psi; 2 min). The drugs were finally eluted using freshly prepared methylene chloride:methanol:ammonium hydroxide (78:20:2, v/v/v; 3 mL). The extracts were evaporated to dryness under nitrogen at 40 °C and reconstituted in methanol (50 μL).

2.4. Liquid chromatography tandem mass spectrometry (LC/MS/MS)

An Agilent Technologies 1200 Series liquid chromatograph pump coupled to a 6410 triple quadrupole mass spectrometer (MS), operating in positive atmospheric pressure chemical ionization mode (APCI) mode was used for analysis. The liquid chromatographic column was a Zorbax Eclipse XDB C18 (4.6 × 50 mm × 1.8 μm), the column temperature was held at 40 °C and the injection volume was 2 μL. The mobile phase consisted of solvent A: 20 mM ammonium acetate (pH 6.4) and solvent B: methanol, and both the composition of the mobile phase throughout the run, and the flow rate were changed. The flow timetable is shown in Table 1. The post time necessary was 7 min to allow the system to re-equilibrate. The gas temperature was 350 °C, the gas flow was 5 L/min and the nebulizer pressure was 50 psi. Nitrogen was used as the collision gas and the capillary voltage was 4500 V.

Two transitions were selected and optimized for each drug using flow injection analysis. Table 2 shows the optimized fragment voltages for the parent ion (M + 1) as well as the collision energy for fragmentation of the product ions. Each subsequent analysis required the ratio between the quantitative ion and the qualifier ion to be within ±20% in order to meet the criterion for a positive result. The ion ratio for each drug was determined at a concentration of 100 pg/mg.

2.5. Data analysis

Calibration using deuterated internal standards was calculated using linear regression analysis over a concentration range of 25–10,000 pg/mg for all drugs. Peak area ratios of target analytes and their respective deuterated standards were calculated using Mass Hunter software (Agilent). The data were fit to a linear least-squares regression curve with a $1/x$ weighting and was not forced through the origin.

2.6. Selectivity

Drug free hair specimens were obtained from volunteers and extracted and analyzed according to the described

Table 1
Mobile phase program for LC/MS/MS analysis of cocaine and metabolites in hair

Time	Flowrate (mL/min)	Percentage of solvent B
0	0.9	25
1.5	0.9	30
4.5	1	55
5	1	60
7	1	75

Table 2
Transitions, optimized fragment voltage and collision energy for analytes

Drug	Precursor ion	Fragment ion	Fragmentor voltage (V)	Collision energy (V)
Benzoylecgonine-d3	293.3	171.2	120	20
Benzoylecgonine	290.3	168.1	120	15
	290.3	105.1	100	15
Cocaine-d3	307.3	185.3	120	20
Cocaine	304.3	182.3	120	20
	304.3	82.3	120	25
Cocaethylene-d8	326.3	204.4	160	20
Cocaethylene	318.3	196.4	120	25
	318.3	82.2	120	25
Norcocaine-d3	293.3	171.4	120	15
Norcocaine	290.3	168.3	120	15
	290.2	136.3	120	25

procedures in order to assess interference from extraction or matrix, or potential ion suppression. Ion suppression is not as prevalent using APCI as it is in electrospray mode. In addition, interferences from commonly encountered drugs were added to the drug free hair specimens and subjected to the same extraction and analysis procedures. The following drugs were analyzed using the described procedures at a concentration of 20,000 pg/mg: morphine, 6-acetylmorphine, codeine, hydrocodone, hydromorphone, oxycodone, oxymorphone, tramadol, desmethyltramadol, fentanyl, gamma-hydroxybutyrate (GHB), tetrahydrocannabinol (THC), 9-carboxy-THC, amphetamine, methamphetamine, methylenedioxymethamphetamine (MDMA), methylenedioxyamphetamine (MDA), methylenedioxyethylamphetamine (MDEA), carisoprodol, methadone, phencyclidine, diazepam, nordiazepam, oxazepam, alprazolam, chlordiazepoxide, bromazepam, temazepam, lorazepam, flurazepam, 7-aminoflunitrazepam, α-hydroxyalprazolam, nitrazepam, triazolam, α-hydroxytriazolam, amitryptiline, nortriptyline, imipramine, protriptyline, doxepin, nordoxepin, trimipramine, secobarbital, pentobarbital, butalbital, and phenobarbital.

2.7. Linearity and sensitivity

The linearity of the assays was established with eight calibration points, excluding the drug free matrix. The sensitivity of the method was determined by establishing the limit of quantitation (LOQ) defined as the lowest concentration detectable with a signal to noise (S:N) ratio of at least 10 and retention time within 0.2 min of the calibration standard. The limit of detection (LOD) was determined from the lowest concentration detectable with a signal to noise (S:N) ratio of at least 3.

2.8. Accuracy and precision

The accuracy of the method at three concentrations: 50, 100 and 200 pg/mg was determined by the deviation of the measured concentration from the nominal concentration of the calibration standards. Inter- and intra-day precision of the assays was determined at one concentration, the calibration point of 100 pg/mg for all drugs. Intra-day data were obtained from 5 analyses performed on 1 day; inter-day data were obtained by analyzing a total of 10 specimens over 5 days (2 samples per day for 5 days; $n = 10$).

2.9. Stability

The stability of the drug extracts at a concentration of 50 pg/mg was determined by allowing the autosampler vials to remain in the liquid chromatographic chamber for 48 h after which time they were re-analyzed. The unit was maintained at 7 °C. The responses were compared to those achieved on the first day of analysis.

2.10. Application to authentic specimens

As part of various on-going research studies, our laboratory receives hair specimens for research purposes as well as proficiency specimens.

Table 3
Mean correlation, equation of the slope of the calibration curve and the qualifying ratio between the transitions monitored

Drug	Mean correlation ($n = 3$)	Equation for calibration curve	Allowable range of intensity for qualifying ion (%)
Benzoylecgonine	0.9989	$y = 0.00116x$	6.7–10
Cocaine	0.9995	$y = 0.00106x$	21.1–32.7
Cocaethylene	0.9987	$y = 0.00061x$	49.3–74
Norcocaine	0.9992	$y = 0.00096x$	65.9–98.9

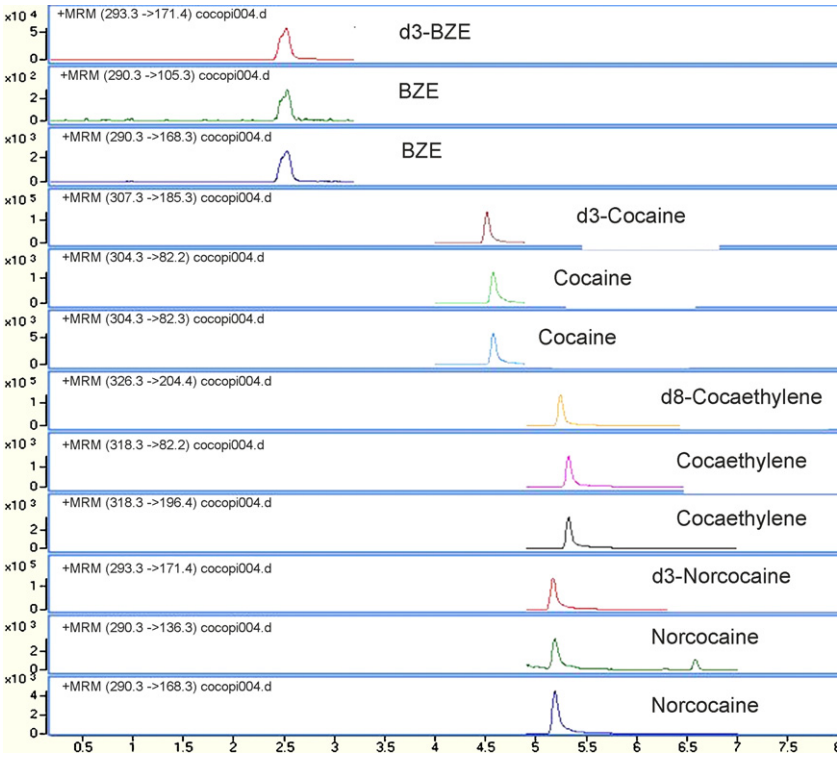

Fig. 1. Cocaine and metabolites extracted from hair at 50 pg/mg.

3. Results and discussion

3.1. Method development

The development of simple LC/MS/MS assays for the detection of cocaine and its metabolites in hair is reported. While these drugs have been detected in hair, the increasing utility of LC/MS/MS in laboratories makes development of confirmatory procedures necessary and timely. The monitoring of a second qualifying ion is reported for the first time for cocaine hair analysis, and is necessary for the improved confidence in the identification of the analyte.

3.2. Method validation

The chromatographic procedures developed for cocaine, benzoylecgonine, cocaethylene and norcocaine were validated according to accepted protocols. The limit of quantitation for each drug, and calibration curve data were determined as described in Section 2. Linearity was obtained with an average correlation coefficient for all the drugs of >0.99 over the dynamic range from 25 to 10,000 pg/mg of hair.

Table 3 shows the mean correlation, equation of the slope of the calibration curve and the qualifying ratio between the transitions monitored. The low intensity of the second transition for benzoylecgonine (6.7–10%), limited the sensitivity of the method for that particular drug, however, the importance of having a qualifying transition was considered to be of greater importance in forensic identification than sensitivity.

Hair specimens collected from drug free individuals showed no interference with any of the assays, which was not unexpected, since it is unlikely these drugs are similar to endogenous substances in hair. For exogenous interferences, commonly

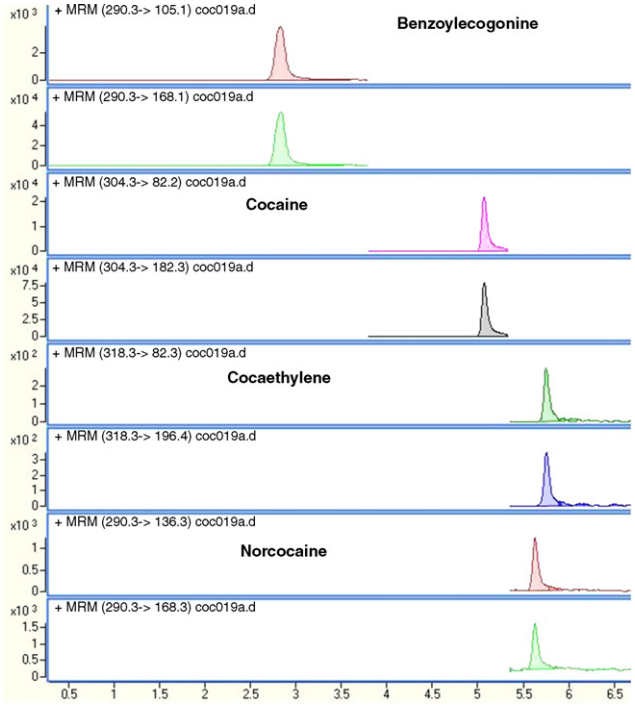

Fig. 2. Authentic drug user hair specimen: cocaine, benzoylecgonine, norcocaine and cocaethylene detected.

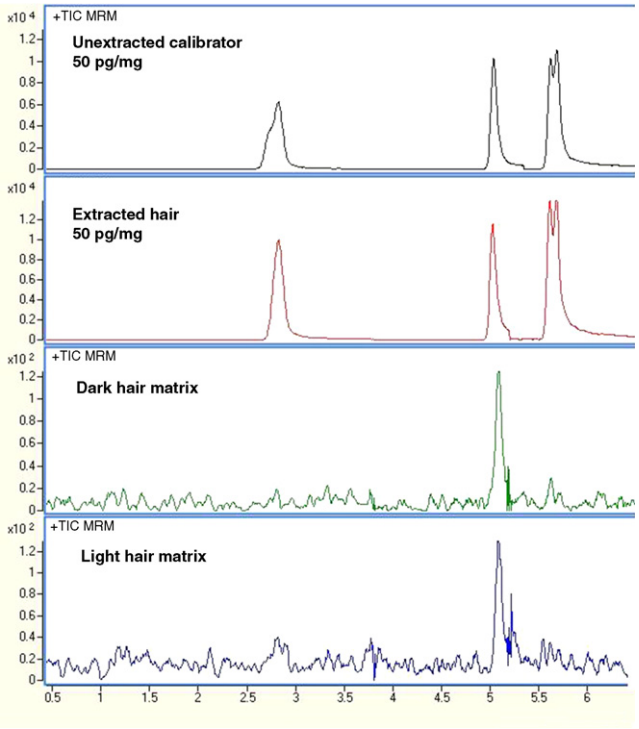

Fig. 3. Total ion chromatograms: (a) unextracted calibration standard (50 pg/mg); (b) extracted hair specimen fortified at 50 pg/mg; (c) dark hair extracted according to described procedure; (d) light/blonde hair extracted according to described procedure.

encountered drugs of abuse were studied as described in Section 2. No chromatographic interference was observed in the channels of these transitions.

An example of an extracted hair specimen at a concentration of 50 pg/mg is shown in Fig. 1. Monitored transitions from the hair of an authentic cocaine user are shown in Fig. 2. The specimen quantitation was cocaine (6181 pg/mg), benzoylecgonine (4568 pg/mg), cocaethylene (58 pg/mg) and norcocaine (112 pg/mg).

Ion suppression was assessed by analysis of both light and dark colored hair, drug free and fortified with the analytes at 50 pg/mg. Apparently, the color of the hair did not affect the background, which is to be expected, since all hairs are subjected to exhaustive solid-phase extraction before analysis. Some response was observed in the drug free matrix around the retention time of cocaine, but comparison with fortified hair indicates the degree of suppression is minimal. The mass spectral response between unextracted and extracted hair calibration standards was similar (Fig. 3).

At nominal concentrations of 50, 100 and 200 pg/mg for all compounds, the inter-assay accuracy was 99.9%, 101.4% and 94.5% for benzoylecgonine; 101.7%, 93.7% and 94.3% for cocaine; 99.3%, 92.5% and 88.4% for cocaethylene; and finally 108%, 88.4% and 86.1% for norcocaine, respectively. The inter-day (between day) and intra-day (same day) precision of the assays was determined using replicate analyses as described. For benzoylecgonine, cocaine, cocaethylene and norcocaine, the inter-day precision was 9.2%, 4.8%, 15.7% and 12.6%, respectively ($n = 10$). For same day precision ($n = 5$), the values were 8.1%, 1.3%, 0.8% and 0.4%, respectively. Finally, the stability of the drugs in the collection system and the stability of the extracts were assessed. The extracts were stable for at least 2 days when kept in the instrument rack inside the autosampler, which was maintained at 7 °C. There was less than a 5% difference in the quantitation of the extracts after 48 h.

3.3. Authentic specimens

The procedures were applied to authentic specimens as well as proficiency specimens received into the laboratory. In the proficiency program managed by the International Society of Hair Testing (SoHT), the specimens are also screened using enzyme linked immunosorbent assay (ELISA) techniques to provide additional information regarding the validity of the results. The proficiency program managed by Research Triangle Institute (RTI), which includes both hair from drug users and fortified specimens, currently asks laboratories not to wash hair specimens. This is because wash procedures between laboratories are not standardized, and any fortified specimens could be subject to variance in drug removal depending upon the length and type of washing protocol. The laboratory performance was excellent, with all quantitation being within 10% of the group mean identified by the program administrators.

4. Conclusions

The determination of cocaine, benzoylecgonine, cocaethylene and norcocaine in hair is described. The LC/MS/MS procedure is reproducible, robust and precise. The assay includes the monitoring of a qualifying transition and calculation of a ratio, required to be within 20% of that of a known calibration standard in order for definitive identification to be made. The method is easily incorporated into routine laboratory testing.

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