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Hydrophilic interaction liquid chromatography–tandem mass spectrometry (HILIC-MS/MS) determination of cocaine and its metabolites benzoylecgonine, ecgonine methyl ester, and cocaethylene in hair samples

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Abstract This study reports the development and validation of a method using hydrophilic interaction liquid chromatography–tandem mass spectrometry (HILIC-MS/MS) for the analysis of cocaine and its metabolites benzoylecgonine (BE), ecgonine methyl ester (EME), and cocaethylene (CE) in hair samples. Decontamination was performed as follows: Firstly, the aliquot of hair was briefly rinsed with 2 mL dichloromethane, then was washed three times with 10 mL 0.01 M phosphate buffer, pH 6, for 15 min, followed by 2 mL 2-propanol for less than 2 min, and, finally, a last rinse with 2 mL dichloromethane was again done. Cocaine compounds were extracted from 10 mg of hair by incubation with 2 mL 0.1 M HCl at 50 °C for 12 h and purified by solid phase extraction with Oasis MCX cartridges. Analysis was performed by LC-MS/MS using an Atlantis HILIC silica chromatographic column. The method was fully validated. Linearity was established over the concentration range 0.020–10.0 ng/mg for cocaine (COC), 0.010–10.0 ng/mg for BE and CE, and 0.005–2.0 ng/mg for EME, and the correlation coefficients were all >0.99. Extraction efficiency was >70% for all analytes. Limits of detection were 0.0005 ng/mg for CE and 0.001 ng/mg for the other analytes (COC, BE, and EME). Lower limits of quantification were the lowest points of the calibration curves with acceptable accuracy and precision (coefficient of variation ≤20%). Intra- and inter-day imprecision ranged between 1.5% and 9.5% and 0.7% and 12.6%, respectively. Intra- and inter-day inaccuracy ranged from 0.5% to 12.3% and from 0.7% to 7.1%, respectively. With regard to matrix effects, suppression was <27.5% in all cases. The method was applied to the analysis of several samples derived from forensic cases.

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Introduction

Cocaine is a powerful addictive stimulant that directly affects the brain. Its abuse and addiction continues to be a problem that plagues the whole world. Cocaine remains the second most used illicit drug in Europe after cannabis, though levels of use vary greatly between countries. It is estimated that around 13 million Europeans have used it at least once in their lifetime, which represents 3.9% of adults aged 15–64 years. According to the last Annual Report (2009) from the European Monitoring Centre for Drugs and

Drug Addiction, Spain presents the highest prevalence of cocaine use among the European countries, with even higher values than in Australia or the USA [1].

Hair has become the third most fundamental biological matrix used for drug testing after blood and urine. One important reason is the wide diagnostic window of detection allowed by this specimen. Other characteristics and advantages of hair in comparison to blood and urine are the possibility of establishing both the chronological profile and the severity of drug consumption [2].

Reports on the analysis of cocaine compounds in hair samples, in the scientific literature, generally restrict analysis to the main compound (cocaine, COC) and one (benzoylecgonine, BE) [3, 4] or two (BE and cocaethylene, CE) [5–7] metabolites, while the number of publications including ecgonine methyl ester (EME) is considerably lower [8, 9]. This fact implies that the presence of EME in hair was seldom investigated. In the present method, we incorporate the analysis of EME as well as COC and BE in order to assess the usefulness of this important endogenous metabolite as a biomarker to establish powerful drug consumption discrimination criteria.

Although the concentrations of EME in blood and urine are significant, nevertheless, one of the main drawbacks for the analytical determination of EME in hair constitutes its low incorporation rate into the shaft, and consequently, highly selective and sensitive methods are required. For this purpose, the most adequate technique is tandem mass spectrometry (MS/MS) coupled to gas chromatography (GC-MS/MS) [10, 11] or to liquid chromatography (LC-MS/MS) [9, 12]. With respect to the latter, if we consider that EME has a higher polarity than COC, the analysis of this metabolite by standard reversed phase methods is particularly challenging since very short retention times are obtained, with a significant loss in sensitivity due to the co-elution with matrix interferences. In order to overcome these problems, hydrophilic interaction liquid chromatography (HILIC) seems to be a promising alternative since it allows adequate retention of highly polar metabolites as well as the corresponding parent compound. Moreover, the high concentrations of organic solvents in the eluting phase provide enhanced sensitivity when mass spectrometric methods are used as detection devices. In this context, several authors have previously reported the quantification of EME applying HILIC and mass spectrometric detection, most of them having been applied to the determination of EME in plasma, urine, or wastewaters [13–15]. To date, to the best of our knowledge, there are no reports dealing with the quantification of EME or the other cocaine-related compounds in hair matrix applying HILIC chromatography coupled to tandem mass spectrometry.

For the aforementioned reasons, the objective of this paper was the development and validation of a hydrophilic interaction liquid chromatography coupled to tandem mass spectrometry method for the analysis of cocaine and its metabolites benzoylecgonine, ecgonine methyl ester, and cocaethylene in hair samples.

Materials and methods

Chemicals and reagents

Standards of COC, BE, EME, and CE, at a concentration of 1 mg/mL in methanol, and the corresponding deuterated internal standards COC-d3, BE-d3, EME-d3, and CE-d3 at a concentration of 100 µg/mL in methanol, were supplied by Cerilliant (Round Rock, TX, USA). LC-MS acetonitrile (99.9% pure), acetic acid (glacial), and hydrochloric acid 37% were purchased from Scharlau Chemie (Sentmenat, Spain). Methanol, dichloromethane, 2-propanol, sodium hydrogen phosphate, sodium dihydrogen phosphate, and ammonium hydroxide in a 25% solution were obtained from Merck (Darmstadt, Germany). Ammonium acetate was supplied by Panreac (Barcelona, Spain). Purified water was obtained in the laboratory using a Milli-Q water system (Le Mont-sur-Lausanne, Switzerland). Oasis MCX cartridges (3 cc 60 mg) were from Waters (Mildford, MA, USA).

Preparation of calibrators

A 10 µg/mL working solution containing all the standards was prepared by diluting the 1 mg/mL standard solutions with methanol. Appropriate dilutions of this solution were prepared to obtain 0.002, 0.02, 0.2, and 2 µg/mL working solutions for the generation of the calibration curves. A mixture of the deuterated internal standards (IS) was prepared at 0.02 µg/mL for EME-d3 and at 0.05 µg/mL for the other deuterated analogues. All working solutions were stored at −20 °C when not in use. For each batch, blank hair samples (10 mg) were fortified with the appropriate amount of standard solution to obtain the following drug concentrations in hair: 0, 0.005, 0.01, 0.02, 0.05, 0.1, 0.2, 0.5, 1, 2, 5, and 10 ng/mg of hair.

Hair samples

The collection of hair samples was made according to our local ethical committee. Three different types of samples have been used in this study: (a) Samples for method validation were blank samples obtained from laboratory staff volunteers. They were fortified with adequate concentrations of the different compounds to perform all the studies. The recommendations of the Society of Hair Testing (SoHT) for internal quality control were followed

[16]. (b) For the development and optimization of washing and extraction procedures, a pool of hair was prepared. The samples were obtained from a known cocaine consumer. (c) To demonstrate the applicability of the method, ten real hair samples were analyzed. These samples were provided by the National Institute of Toxicology and Forensic Sciences and sent to our lab with a reference not linked to the identity of the people, maintaining the anonymity of the donors. They were selected among those which previously tested positive for cocaine compounds.

Analytical method

Decontamination Hair samples were submitted to the following sequence of washes: Firstly, the aliquot of hair was briefly rinsed with 2 mL dichloromethane, then was washed three times with 10 mL 0.01 M phosphate buffer, pH 6, for 15 min, followed by 2 mL 2-propanol for less than 2 min, and, finally, a last rinse with 2 mL dichloromethane was again done. All wash solvents were kept for analysis if necessary.

Extraction of cocaine compounds from the hair samples Decontaminated hair was cut with scissors into small pieces (<1 mm), and 10 mg was weighed into a round glass tube. After the addition of 50 μL of IS mixture, the hair was incubated with 2 mL 0.1 M HCl at 50 °C for 12 h. The sample was then centrifuged and the supernatant subjected to solid phase extraction (SPE).

Solid phase extraction protocol (cleanup) The selected SPE column was OASIS MCX. This mixed mode, polymeric sorbent with strong cation exchange sulfonic acid groups located on the surface of a poly(divinylbenzene-*co-N*-vinylpyrrolidone) copolymer was employed successfully in a previous work involving determination of cocaine compounds in another biological matrix [17]. After conditioning the cartridges with 2 mL methanol and 2 mL water, the sample was applied directly into the SPE column. Cleanup was accomplished by sequentially washing with 2 mL each of water, 0.1 M HCl, and, finally, methanol/water (50:50). Cartridges were then dried for 15 min before performing the elution with 2 mL dichloromethane/2-propanol (75:25) with 5% NH_4OH. Eluates were evaporated to dryness with nitrogen, reconstituted in 100 μL acetonitrile/methanol (3:1), and 10 μL was injected into the LC-MS/MS.

Liquid chromatography–tandem mass spectrometry

The HPLC system was a Waters Alliance 2795 Separation Module with a Waters Alliance series column heater/cooler (Waters). For the chromatographic separation, an Atlantis®

HILIC Silica column (100 × 2.1 mm, 3 μm; Waters) was employed using 0.1 M ammonium acetate buffer, pH 4.5, and acetonitrile as mobile phase at a flow rate of 0.25 mL/min. The column temperature was maintained at 26 °C and the following gradient was applied: 82% acetonitrile until minute 1.5, then acetonitrile percentage was gradually decreased to 75% until minute 3 to continue decreasing until 40% at minute 4 and maintained for 2.5 min. From minute 6.5 to minute 7.5, it was returned to initial conditions and held for 6.5 min, yielding a total run time of 14 min. A divert valve was set to direct the LC flow to the mass spectrometer from 1.8 to 9 min and to waste the remaining time.

For the detection, a tandem mass spectrometer Quattro Micro™ API ESCI (Waters) with a triple quadrupole was employed. The instrument was operated in electrospray in the positive ionization mode (ESI+). Nitrogen, heated at 450 °C, was used as nebulization and desolvation gas at a flow rate of 500 L/h and as cone gas at a flow of 50 L/h. Capillary voltage and source block temperature were 3 kV and 150 °C, respectively.

To establish the optimal cone voltage value in order to obtain the most prominent pseudomolecular ion $[M+H]^+$, as well as the multiple reaction monitoring (MRM) transitions for the detection of each target analyte and IS, individual solutions of each were infused into the mass spectrometer (10 μg/mL in methanol at a flow rate of 10 μL/min) in "T" with the effluent of the chromatographic system (acetonitrile/ammonium acetate 0.1 M, pH 4.5, 50:50, at a flow rate of 0.25 mL/min). Collision-induced dissociation was performed using argon as collision gas, and optimal collision energy values to obtain the most abundant fragments were established. Data acquisition was controlled using MassLynx 4.0 software and processed with QuanLynx 4.0 software (Waters). In Table 1, MRM transition, cone voltage, collision energy, and retention time are indicated for each analyte.

Results and discussion

Optimization of the analytical method

Decontamination

One of the main challenges of cocaine analysis in hair samples is to avoid the risk of reporting false positive results due to external contamination of the hair. For this reason, the decontamination procedure is of paramount importance. For the optimization of this protocol, a pool of hair from a known cocaine abuser was subjected to different wash approaches. We followed the recommendations stated by the SoHT in 1997: a sequential washing

Table 1 Selected MRM transitions, cone voltage, collision energies, as well as retention times (R_t) for BE, CE, COC, EME, and their deuterated analogues

Analyte	MRM transition	Cone voltage (V)	Collision energy (eV)	Rt (min)
COC	*304.2≥182.3*	30	20	5.2
	304.2>82.0	30	30	
COC-d3	*307.2≥185.3*	30	20	5.2
BE	*290.2≥168.3*	30	20	4.0
	290.2>105.0	30	30	
BE-d3	*293.2≥171.3*	30	20	4.0
EME	*200.2≥182.2*	30	17	7.7
	200.2>81.9	30	23	
EME-d3	*203.2≥185.2*	30	17	7.7
CE	*318.2≥196.3*	30	19	5.1
	318.2>82.0	30	31	
CE-d3	*321.2≥199.3*	30	19	5.1

Quantifier MRM transitions are shown in italics

procedure with organic solvent, followed by water or aqueous buffer, and finally with an organic solvent once again [18]. Dichloromethane is an organic solvent frequently used to rinse the hair in a first attempt. This step removes sebum and hair treatments. Phosphate buffer washing time was optimized by comparing the amount of eliminated compound at different times. 2-Propanol washes were introduced to improve decontamination and transition between aqueous and organic solvent washing steps. After several times and a number of washes, hair samples were submitted to the sequence of washes previously described in "Analytical method."

Extraction of cocaine compounds from the hair samples

The most common treatments for the extraction of cocaine compounds involve the use of acidic solvents, methanolic solutions, or enzymatic digestions of the matrix with enzymes such as pronase, proteinase, etc. Cirimel et al. [19] studied the efficiencies of these three different extraction solutions and an additional alkaline incubation. Acidic conditions were, in general, the best option for COC and BE extraction, so finally, this was the condition selected in our method.

To assess the optimum incubation time, a similar experiment to that described by Clauwaert et al. [20] was performed. In our experiment, incubation times longer than 12 h did not result in a noticeable increase in recovery.

Application of HILIC chromatography

For the HILIC optimization, different mobile phase composition, gradients, injection volumes, flow rates, and column temperatures were investigated. Regarding the mobile phase composition, we followed the recommendations provided by the chromatographic column manufacturer about the most suitable reagents to use. The influence of acetonitrile and methanol as organic solvents and formic acid, ammonium formate (pH 3.0), and ammonium acetate (pH 4.5) as aqueous solvents were evaluated. Based on the chromatographic results, the chosen combination of 0.1 M ammonium acetate buffer, pH 4.5, and acetonitrile as mobile phase at a flow rate of 0.25 mL/min showed the optimum characteristics in terms of peak intensities, peak shape, selectivity, and elution time. HILIC chromatography allowed sufficient resolution of all analytes within 6 min, with a total chromatographic run time of 14 min. Identification was based on retention time as well as the two most prominent MRM transitions for each analyte. Deuterated analogues of all the compounds were employed as IS. Table 1 shows the quantification and qualification transitions selected for each analyte and IS as well as their retention times. It is noticeable that selectivity of the compounds are inverted when HILIC is performed instead of reversed phase liquid chromatography, moving EME to the last position in the chromatogram.

Reversed phase chromatography is one of the most commonly used separation techniques for the determination of cocaine and its main metabolites [21–23]. However, the polar cocaine metabolite EME is not easily analyzed using standard reversed phase chromatography due to poor retention profiles, which leads to very short elution times, even at the front solvent. This problem cannot be overcome by applying strategies such as normal phase chromatography or ion paring agents since they are not well suited to mass spectrometric detection [24]. Considering the interaction mode between analytes and stationary phase in HILIC [25], it seems a particularly well-suited technique to our needs since higher concentrations of organic solvents increase retention time of the analyzed compounds, while the addition of water (strong solvent) produces their elution. Therefore, good retention profiles for polar and less polar compounds are obtained when applying HILIC chromatography. The high concentrations of organic solvent in the eluting phase constitute an additional advantage of the

method since this feature has been reported to enhance the ionization efficiency in ESI [26], thus providing higher sensitivity in the overall quantification method.

Method validation

The method has been submitted to full validation which was performed according to the recommendations of the international organizations FDA [27] and ICH [28] and those of Shah et al. [29] and Peters and Maurer [30]. The following validation parameters have been evaluated: selectivity, linearity, limit of detection (LOD), lower limit of quantification (LLOQ), precision, accuracy, recovery, matrix effect, and cocaine hydrolysis.

Selectivity

Selectivity of the method was evaluated by the analysis of blank hair samples from ten different, well-known non-cocaine consumers.

The method proved to be selective as no interferences were observed at the retention times of any of the analytes in their MRM channels when blank hair specimens from ten different sources were analyzed. Figure 1 shows the MRM chromatograms of one of them.

In addition, we conducted an experiment to assess possible interferences of common drugs of abuse and/or medical drugs that could be present in the hair of a cocaine user and may interfere with the analysis. A single mixture of the following drugs was prepared at a concentration of 125 ng/mL in acetonitrile: 6-acetylmorphine, codeine, methadone, morphine, 2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine, amphetamine, 3,4-methylenedixyamphetamaine, 3,4-methylenedixyethylamphetamaine, 3, 4-methylenedixymethylamphetamaine, methamphetamine, tetrahydrocannabinol, d-lysergic acid diethylamide, ketamine, norketamine, gamma hydroxybutyrate, nicotine, cotinine, temazepam, alprazolam, 7-aminoflunitrazepam, clonazepam, diazepam, flunitrazepam, lorazepam, lormetazepam, nordiazepam, oxazepam, triazolam, nitrazepam, fentanyl, amitriptiline, paroxetine, zolpidem, zopiclone, ibuprofen, acetaminophen, omeprazole, and olanzapine. Fifty microliters of the solution was added into a clean round tube and evaporated to dryness. Compounds were redissolved in 100 µL of mobile phase, and a 20 µL aliquot was injected into the HPLC system. None of these compounds gave any peak or chromatographic interference in the MRM transitions investigated.

Linearity and sensitivity (LOD, LLOQ)

To establish the best calibration model fitted to our data, nine-to ten-point calibration curves from 0.020 to 10.0 ng/mg for

COC, from 0.010 to 10.0 ng/mg for BE and CE, and from 0.005 to 2.0 ng/mg for EME were analyzed on six different days. Acceptable criteria included correlation coefficients higher than 0.99.

Concentration to IS peak area ratio versus the theoretical concentration was fitted using least-squares linear regression ($y=\beta_0+\beta_1 x+\varepsilon$, ε being a zero mean error term) with a $1/x$ weighting factor for EME. However, for the other analytes, data were better fitted assuming a quadratic model ($y=\beta_0+\beta_1 x+\beta_2 x^2+\varepsilon$) because of the wider calibration range. According to some authors [29, 30], when the acceptance criteria are not satisfied with the linear model, the quadratic one should be employed instead, increasing the number of concentration levels to define the calibration range. After the model was visually evaluated, we analyzed the residual plots applying different regression models, choosing the one that minimized the residual value (maximum residual accepted is 15%, except 20% for LLOQ).

The LOD was defined as the lower concentration with a signal-to-noise ratio for all ions of at least 3 and was empirically determined by fortifying hair samples at decreasing analyte concentrations. The LLOQ was the lowest concentration that could be quantified with acceptable precision (coefficient of variation, %CV) ±20% and accuracy (mean relative error, MRE) <20%. LLOQ, in our method, was the lowest point of the calibration range. Table 2 shows LOD, LLOQ, calibration ranges, and correlation coefficients obtained for all analytes. The LLOQ values achieved with our method satisfy the requirements that two organizations, SoHT and Substance Abuse and Mental Health Services Administration, proposed for confirmation methods for the analysis of cocaine (0.5 ng/mg for cocaine and 0.05 ng/mg for metabolites) in forensic toxicology. In spite of the low LLOQ, we follow SoHT recommendations [16] before reporting a positive result for cocaine in hair; furthermore, the analysis must include cocaine and at least one of the following metabolites: BE, EME, CE, and norcocaine. Nevertheless, the high sensitivity of the method is crucial for the analysis of EME due to the low concentrations of this compound in this biological matrix.

Precision and accuracy

Precision and accuracy were evaluated at five concentrations for all analytes (LLOQ, three intermediate concentrations, and the upper limit of quantification). Intra-day precision and accuracy were assessed by analyzing five replicates at each concentration in the same run. Inter-day precision and accuracy were tested by analyzing the same previous concentration levels on six different days. Precision was expressed in terms of
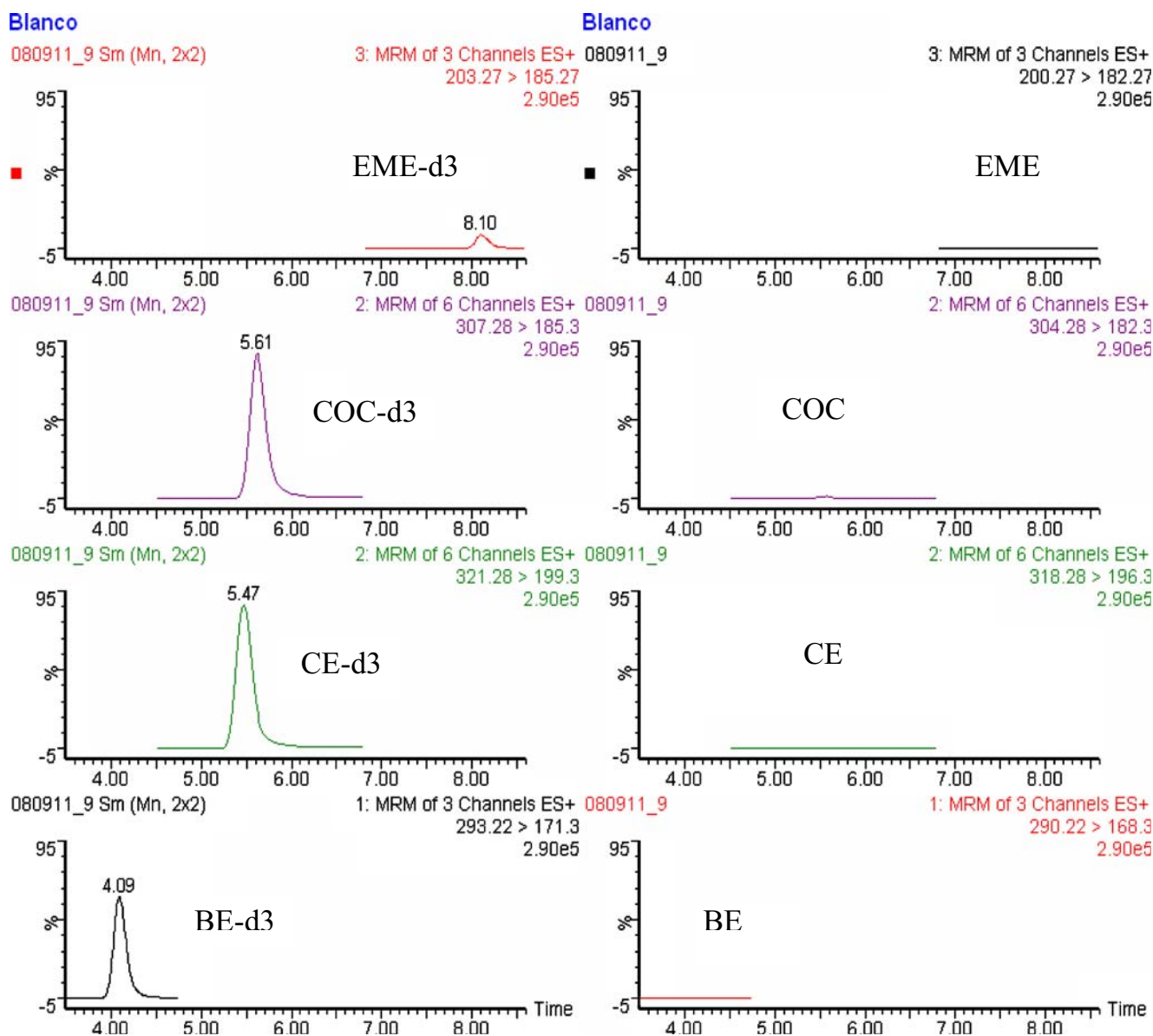
Fig. 1 Chromatograms of quantifier MRM transitions of EME, COC, CE and BE and its corresponding deuterated internal standards in a blank hair sample

imprecision by the calculation of the %CV of the measured values, which was required to be ±15%, except for the LLOQ for which values ±20% were accepted. Inaccuracy was evaluated by determining the MRE, which is the percentage deviation from the accepted reference value. MRE should be <20% of the target concentration for the LLOQ and <15% for the other calibrators.

Table 3 shows the data for imprecision and accuracy. Intra- and inter-day imprecision ranged between 1.5% and 9.5% and 0.7% and 12.6%, respectively. Intra- and inter-day inaccuracy ranged from 0.5% to 12.3% and from 0.7% to 7.1%, respectively.

SPE cleanup efficiency and matrix effect

These parameters were assessed following the recommendations of Matuszewski et al. [31]. Cleanup efficiency was determined at two concentrations (0.02 and 2 ng/mg) by comparing average peak areas of blank hair specimens fortified prior to extraction ($n=3$) with those obtained in specimens fortified after extraction ($n=3$). Cleanup efficiency was higher than 90% for COC, BE, and CE and 70% for EME (Table 4). These results are comparable for COC, BE, and CE with those achieved by Miller et al. [9] and Scheiweiler

Table 2 LOD, LLOQ, calibration range, correlation coefficient R^2 for COC, BE, EME, and CE

	LOD ng/mg	LLOQ ng/mg	Calibration Range ng/mg	R^2	β_0 (SE)	β_1 (SE)	β_2 (SE)
COC	0.001	0.02	0.02–10	0.9982	0.0291 (0.0098)	0.0035 (0.0001)	−8.5826E−08 (1.5246E−08)
BE	0.001	0.01	0.01–10	0.9986	0.0056 (0.0024)	0.0028 (0.0001)	−4.1687E−08 (7.9612E−09)
EME	0.001	0.005	0.005–2	0.9975	0.0311 (0.0124)	0.0101 (0.0002)	–
CE	0.0005	0.01	0.01–10	0.9986	0.0028 (0.0038)	0.0035 (0.0001)	−2.2127E−08 (1.6549E−08)

β_0, β_1, and β_2 are estimated coefficients of the calibration curves

SE standard error

and Huestis [8] using Clean Screen SPE columns, but slightly lower for EME. Previous experience, enough sensitivity for the application, and the availability in our lab were our reasons for choosing the selected OASIS MCX cartridges.

Matrix effect is defined as the effect of co-eluting residual matrix component on the ionization of the target analytes and is a limitation commonly associated with LC-MS analysis. Precision and/or accuracy of the method could be compromised by an enhancement or suppression of analyte response due to matrix effects. Matrix effect was calculated at 0.5 ng/mg by comparing average peak areas in five different blank hair eluates fortified after extraction to those obtained with analytes prepared in acetonitrile/methanol (3:1) at the same concentration ($n=5$). Suppression of the signal was observed for all analytes, being <27.5% in all cases (Table 4). These results are comparable in part to those achieved by Scheidweiler and Huestis [8] where COC and CE presented matrix suppression, while BE and EME showed matrix enhancement. Since ionization type, sample preparation, and chromatography were different, the different response by these two methods is not surprising. Miller et al. [9] performed matrix effect experiments for 0.5 ng/mg spiked hair extracts, achieving suppression of the signal for COC, CE, and BE, whereas EME presented signal enhancement. The use of deuterated internal standards for all compounds minimized the matrix effect since internal standards and analytes are equally affected by the matrix [8].

Table 3 Intra- and inter-day precision and accuracy for COC, BE, EME, and CE

	Concentration ng/mg	Intra- day precision and accuracy (n=6)		Inter-day precision and accuracy (n=5)	
		CV%	MRE%	CV%	MRE%
COC	0.02	2.2	−6.2	6.1	−1.9
	0.05	9.5	−8.1	7.7	−7.1
	0.2	4.5	−9.1	4.9	7.1
	2	3.1	8.2	4.1	2.0
	10	5.5	7.6	0.9	1.5
BE	0.01	3.5	−11.5	8.5	0.9
	0.02	7.0	6.1	4.4	4.1
	0.2	3.4	−8.8	5.6	2.3
	2	2.6	10.6	6.8	1.9
	10	6.2	5.3	0.7	0.7
EME	0.005	8.8	−2.1	12.6	−4.0
	0.01	6.4	−6.7	7.8	−2.9
	0.02	8.6	5.0	8.8	4.9
	0.05	2.4	−8.9	5.5	2.8
	2	2.3	0.5	3.2	−0.7
CE	0.01	8.7	−11.3	10.5	3.5
	0.02	3.9	9.2	3.4	1.2
	0.2	6.2	−12.3	7.1	1.3
	2	1.5	12.0	5.3	3.2
	10	4.9	3.4	0.7	0.7

Results are expressed as coefficient of variation percentage (CV%) and mean relative error percentage (MRE%)

Stability of cocaine

A study was designed to assess cocaine hydrolysis after performing all sample preparation steps. Blank hair samples were fortified with cocaine to achieve concentrations of 0.02, 2, 10, and 20 ng/mg of hair and submitted to the whole protocol. The stability of cocaine was established by calculating the concentrations of BE and EME formed during the process versus the concentration of added cocaine.

The percentage of degradation to BE and EME was lower than 0.75% and 0.6%, respectively. In a previous paper, Clauwaert et al. [20] performed a similar study with a cocaine concentration of 1000 ng/mg. They found degradation <0.2% to BE. Degradation to EME was not determined in that study.

Table 4 Cleanup efficiency and matrix effect from fortified hair

	Concentration (ng/mg)	Recovery (%)	Matrix effect (n=5)	
			%	CV
COC	0.02	92.5		
	0.5		16.9	6.7
	2	90.6		
BE	0.02	96.1		
	0.5		−1.4	5.5
	2	91.4		
EME	0.02	81.8		
	0.5		27.5	4.3
	2	73.0		
CE	0.02	91.3		
	0.5		10.5	4.8
	2	93.2		

Quantification of samples above the ULOQ

When applying this procedure in the routine of the laboratory, it was noticeable that COC and metabolite concentrations in forensic specimens were sometimes above the upper limit of quantification (ULOQ) of the present analytical method (Table 5). To calculate drug concentrations in these cases, we attempted to extend the validated calibration range; however, this would lead to a lack of linearity because of the saturation of the mass spectrometer when increasing the amount of drug and the extremely large calibration range. Another possibility was to weigh less amount of sample for the analysis, but this was also not appropriate as it led to an inherent weighting error because of the low amount of hair used in this method (10 mg). For this reason, a sort of "dilution procedure" was designed consisting of adding an IS solution ten times more concentrated and diluting the reconstituted eluent 1:10 with the mobile phase. To validate this procedure, blank hair samples were fortified with the analytes to obtain concentrations of 20 and 80 ng/mg (n=5 each). After adding 50 μL of IS (0.20 μg/mL for EME-d3 and at 0.5 μg/mL for the other deuterated analogues), they were submitted to the previously described protocol. Then, an aliquot of 20 μL of the final reconstituted eluent was diluted to 200 μL with the mobile phase (acetonitrile/methanol, 3:1), and 10 μL was injected into the LC/MS-MS. Diluted samples were quantified with a calibration curve prepared according to the method described in this manuscript. For all analytes, precision and accuracy in the diluted samples were <7.0% and ±12% of the target concentrations, respectively.

Concentrations above the ULOQ in real hair samples were determined by reanalyzing the specimens after applying the described dilution procedure (Table 5).

Application to the analysis of real hair samples

In order to demonstrate the applicability of the method, ten real hair specimens from forensic cases were analyzed according to the previously described and validated method. The samples were provided by the National Institute of Toxicology and Forensic Sciences (INT-CF). They were selected among the samples which tested positive for cocaine compounds. Table 5 shows the results obtained for all the analytes. MRM chromatograms from case number 3 are shown in Fig. 2.

In addition, the results for COC and BE obtained in our lab applying the present method were compared with those obtained in the INT-CF, applying a method based on decontamination with dichloromethane, followed by 0.1 M HCl extraction and analysis by GC-MS [32]. The analyses, performed in both laboratories, gave very homogeneous results since the correlation coefficients were 0.9393 and 0.8926 for COC and BE, respectively.

Few papers have been published that include the analysis of EME in hair. However, EME concentrations achieved with our method are in accordance with those obtained by other authors [8, 9]. Miller et al. [9] presented hair results from nine cases, five of which tested positive to cocaine. Among these, only three were positive for EME. This could be due to their high LLOQ for EME (0.08 ng/mg), which is not sensitive enough for the detection of EME when a low dose of cocaine was taken. In our method, with an LLOQ

Table 5 Concentrations (ng/mg) of COC, BE, EME, and CE in ten real hair specimens

Specimen	COC	BE	EME	CE
1	6.07	1.46	0.059	0.11
2	3.64	1.12	0.041	0.45
3	2.23	0.66	0.036	0.17
4	9.93	2.50	0.213	0.10
5	8.32	1.87	0.01	0.08
6	1.07	0.484	0.017	<0.005
7	5.49	2.59	0.067	0.71
8[a]	53.90	36.70	1.401	0.34
9[a]	23.21	8.40	0.221	0.11
10[a]	38.50	13.11	0.321	0.31

[a] Concentrations of some analytes in samples 8, 9, and 10 were higher than the ULOQ. They have been quantified following the procedure described in section Quantification of samples above the ULOQ.
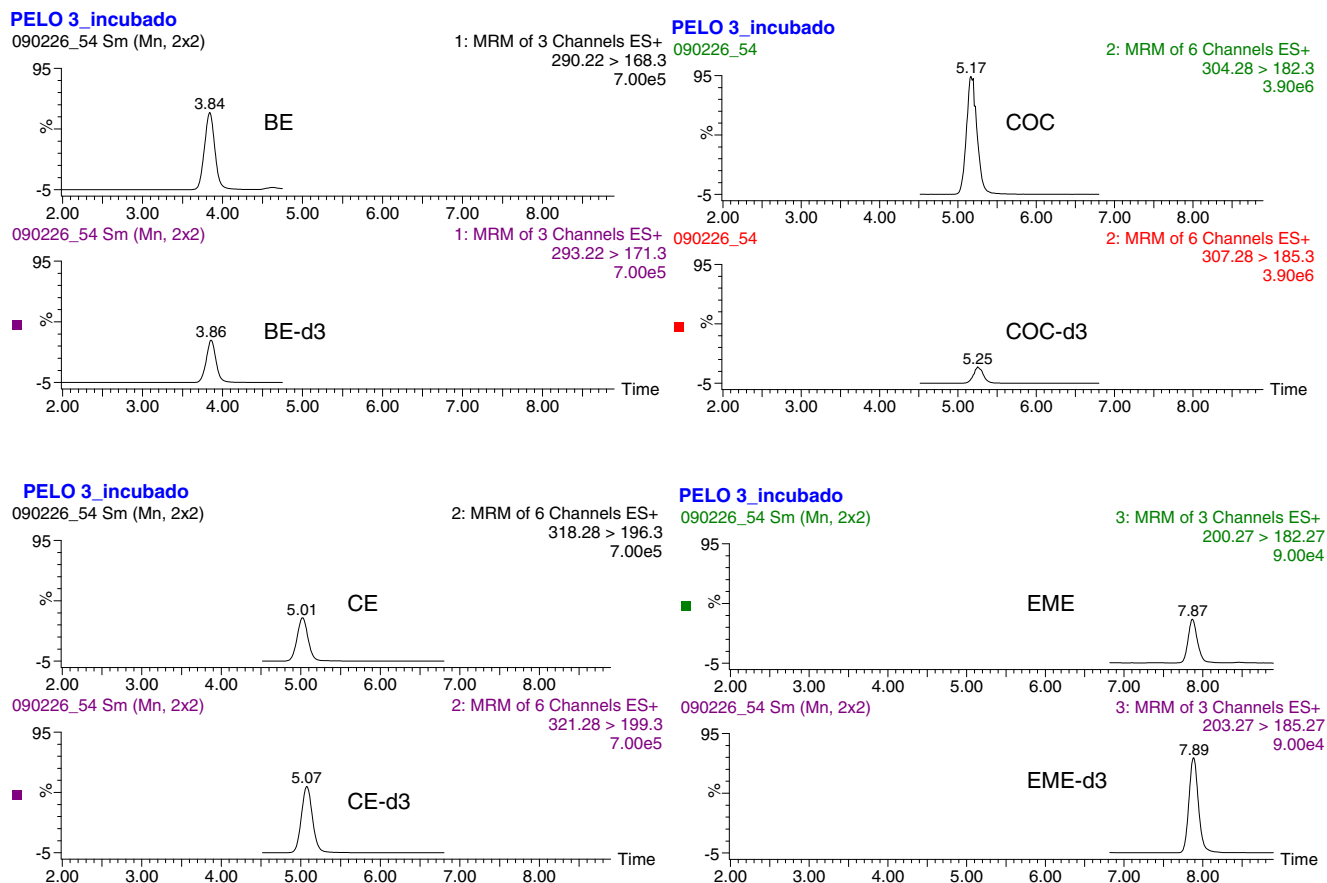
Fig. 2 Chromatograms of quantifier MRM transition from sample 3. The concentrations determined were: COC, 2.23 ng/mg; BE, 0.66 ng/mg; EME, 0.036 ng/mg; and CE, 0.17 ng/mg

of 0.005 ng/mg, we are able to detect EME in all the analyzed specimens.

Conclusion

A new HILIC-MS/MS method for the analysis of cocaine and its metabolites benzoylecgonine, ecgonine methyl ester, and cocaethylene in hair has been developed. The method has been fully validated in terms of selectivity, linearity, sensitivity, precision, accuracy, recovery, matrix effect, and cocaine hydrolysis. Since the LC/MS/MS method is robust, reproducible, and precise, it was incorporated into the routine of our laboratory. The applicability of the method has been demonstrated by analyzing several hair samples from forensic cases. HILIC showed to be a suitable option for the determination of cocaine compounds, especially if the very polar metabolite EME had to be included in the analysis of cocaine-related compounds. The analytical procedure allowed the determination of target compounds in the low pictograms per milligram of hair, which seemed to be crucial for detecting EME. From a preliminary study presented at the last SoHT Meeting (14th Scientific Meeting of the Society of Hair Testing, Rome, Italy, June 14–16, 2009), EME could be an interesting biomarker for the consumption of cocaine in hair. The presented method will be applied to the analysis of a significant number of specimens in order to obtain a strong conclusion on the usefulness of EME to differentiate passive incorporation from active consumption of cocaine.

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