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Analysis of cocaine and three of its metabolites in hair by gas chromatography-mass spectrometry using ion-trap detection for CI/MS/MS

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Abstract

A sensitive GC/CI/MS/MS method was developed for the simultaneous determination of cocaine (COC), anhydroecgonine methylester (cocaine pyrolysis product, AEME), ecgonine methylester (cocaine enzymatic hydrolysis product, EME) and cocaethylene (cocaine with ethanol trans-esterification product, COET) in human hair samples. After acid hydrolysis, hair samples were extracted with an automated solid phase extraction (SPE). The analysis of cocaine and its three metabolites was performed using an ion-trap spectrometer in positive chemical ionization with isobutane as gas reagent. The procedure was validated. Weighted linear regression was found appropriate in a concentration range of 0.10–5.00 ng/mg for AEME, 0.05–5.00 ng/mg for COC, EME and COET. The limit of detection was estimated at 0.005 ng/mg for COC and COET, at 0.025 ng/mg for EME, and at 0.050 ng/mg for AEME. Method performance was evaluated in terms of trueness and precision using quality control (QC) samples over the investigated ranges. Method selectivity and robustness were also demonstrated.

Keywords: Chemical ionization: CI; Cocaine; GC/MS/MS; Hair; Ion-trap; Validation

1. Introduction

Cocaine (COC), an alkaloid from the plant *Erythroxylum coca*, is one of the most widely used drugs of abuse. It is available in two forms: (a) COC hydrochloride, a white crystalline powder which can be snorted, swallowed or injected and (b) "crack", COC hydrochloride that has been processed into its freebase form through a reaction with either ammonia or bicarbonate and which is smoked.

Cocaine is rapidly metabolized to benzoylecgonine (BZE) by spontaneous chemical hydrolysis, to ecgonine methylester (EME) and ecgonine by esterase hydrolysis [1]. When COC is smoked, a pyrolysis product, anhydroecgonine methylester (AEME), is formed. COC is frequently taken together with ethanol because cocaethylene (COET), a psychoactive COC homologue of which properties are also searched, is formed arising through transesterification following concomitant intake of COC and alcohol (see Fig. 1).

The importance of hair analysis in drug testing has grown rapidly in recent years because this technique provides long-term information on drug use (depending on the hair length), gives complementary information to other biological matrixes (urine or blood) analyses and may offer crucial data in evaluating, interpreting and concluding with obtained results. Nowadays, hair analysis is routinely used as a powerful tool for the detection of drug use, not only in forensic science but also in clinical toxicology or in traffic medicine. The widespread use of this kind of analysis is due to the progress of separation techniques and the increased sensi-

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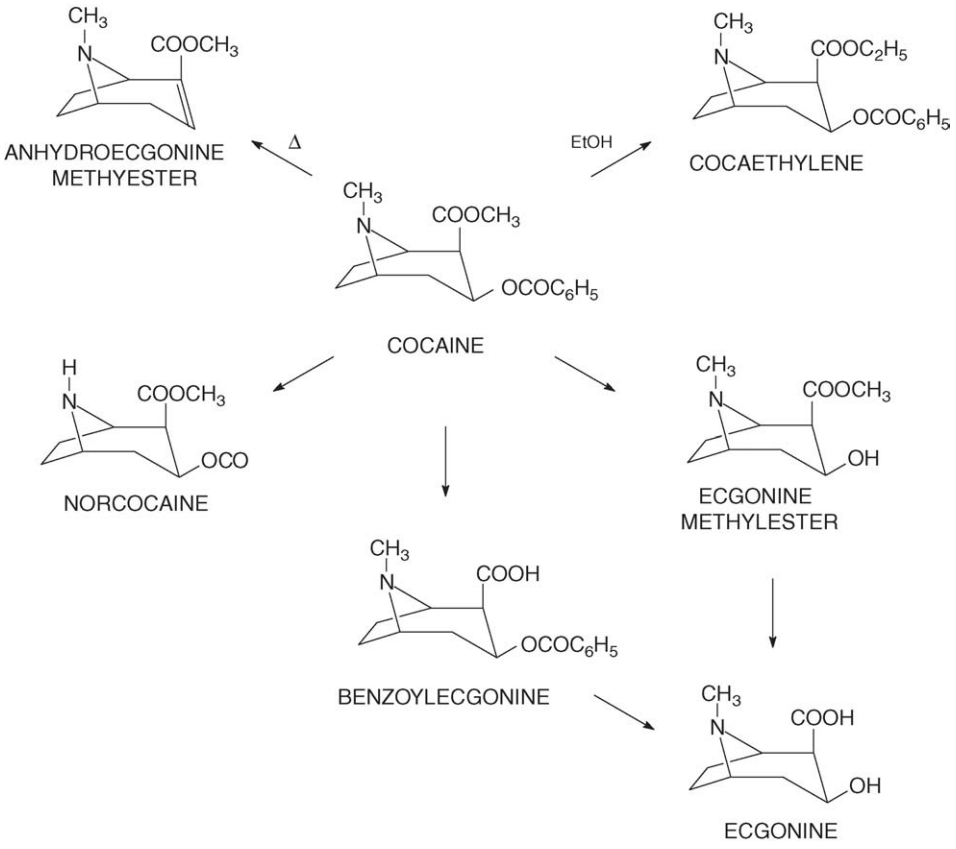

Fig. 1. Succinct and non exhaustive cocaine metabolism with only its principal metabolites.

tivity and selectivity of analytical instrumentation, which allows the detection of the small amount of drugs contained in hair.

During hair analysis, the parent substance is present predominantly. In case of COC, it is this, which is analysed concurrently with one or several of its metabolites allowing confirmation of whether consumption has really occurred. As COC decomposes spontaneously to BZE, the presence of the latter in hair is not a sufficient proof of COC use. Similarly, the unique occurrence of EME is not sufficient either. Moreover, determination of these two metabolites does not give any information about the way and the form of COC consumption. On the other hand, the presence of COET attests to the concurrent use of COC and ethanol, and the existence of AEME traces may demonstrate that crack was smoked. Therefore, it seems to be very worthwhile to analyse not only COC but also AEME, EME, COET to confirm COC consumption. Therefore, AEME and COET may be used as markers of different ways and forms of COC intake: AEME presence will reveal a crack consumption and COET presence will provide a concomitant intake of ethanol. So the monitoring of AEME can be useful in distinguishing intranasal and intravenous COC administration from smoking.

A large number of studies on hair analysis have dealt with drugs of abuse, especially cocaine. Different methodological approaches concerning the pre-treatment of hair samples, extraction of analytes from the matrix and isolation from incubation media have been proposed. Complete reviews of procedures for analysis of drugs of abuse and pharmaceuticals have been published [2–5]. Gas chromatography mass spectrometry in the electron impact mode is the most common procedure used, but in recent years positive and negative chemical ionization, GC/MS/MS or high-resolution MS were also adopted. An important application is sectional hair analysis, which could provide the drug history of a drug addict [6–8].

The present work was conducted to quantify simultaneously COC and its related metabolites AEME, EME and COET in hair of COC abusers by GC/CI/MS/MS. After acid hydrolysis, hair samples were extracted with an automated SPE and a Saturn 2000 Varian® ion-trap spectrometer used for detection in positive chemical ionisation with isobutane as gas reagent. The reported method was validated to provide evidence that the latter does what is it intended to do. All of the steps in the procedure have been considered, including sample preparation, chromatographic separation, detection and data evaluation. The validation process consisted of several parameters: trueness, precision, accuracy, limit of detection and limit of quantification. The validation procedure has been performed prior to the routine use of the analytical method.

2. Experimental

2.1. Chemicals and reagents

Acetonitrile solutions of COC, COET, EME, and AEME, 1000 μg/mL, were purchased from Cambridge Isotope Laboratories Inc. (Andover, MA, USA). Acetonitrile solutions of deuterated cocaine (COC-d_3) and deuterated ecgonine methylester (EME-d_3), 100 μg/mL (98% pure) were also purchased from Cambridge Isotope Laboratories Inc. Methanol, toluene, acetic acid (100%), hydrochloric acid concentrated solution (37%), ammonium hydroxide solution (25%), sodium hydroxide, potassium hydroxide, sodium hydrogenophosphate, and potassium dihydrogenophosphate were supplied by Merck (Darmstadt, Germany). Dichloromethane and isopropanol were obtained from Fluka (Buchs, Switzerland).

2.2. Instruments and GC/MS/MS conditions

Hair samples were pulverized in a ball mill MM 2000 provided by Retsch (Schieritz, Hauenstein, Switzerland). Automated extraction was performed on an ASPEC (Gilson Medical Electronics, Villiers-le-Bel, France) and with HCX Isolute (130 mg) cartridges, which were obtained from IST (Hengoed, UK).

Gas chromatographic analyses were performed on a Varian Star 3400 CX gas chromatograph (Walnut Creek, CA, USA) equipped with a Varian Saturn 2000 ion-trap detector (Walnut Creek, CA, USA). Ultra high purity helium was used as carrier gas with an inlet pressure of 0.069 Mpa (10 PSI). A J&W Scientifics (Folsom, CA, USA) fused silica capillary column of 15 m × 0.25 mm I.D. was used with a stationary phase of 5%-phenyl-methyl-polysiloxane (DB-5MS, film thickness 0.25 μm) and prior connected to an inert retention gap of 1–5 m × 0.53 mm I.D. The column oven temperature was programmed from an initial temperature of 75 °C held during 1 min, increased to 170 °C at 15 °C/min, then increased to 210 °C at 5 °C/min, and finally to 310 °C at 30 °C/min. The injection port temperature was programmed from an initial temperature of 75 °C held during 1 min, then increased to 280 °C at 50 °C/min and held during 1.40 min. Injections (3 μL) were made in cool on-column mode using the Varian 8200 CX autosampler (Walnut Creek, CA, USA). The ion-trap was operated in positive chemical ionisation (CI) with isobutane as gas reagent. The transfer line, manifold and trap temperatures were 290, 120 and 240 °C, respectively. Instrument control and data acquisition were carried out using the Saturn GC/MS Workstation Varian version 6.3. For the tandem mass spectrometry, the collision induced dissociation (CID) conditions used in non-resonant mode were reported in Table 1. For quantification, the following ions were used: COC *m*/*z* 182, AEME *m*/*z* 122, EME *m*/*z* 182, COET *m*/*z* 196, COC-d_3 *m*/*z* 185 and EME-d_3 *m*/*z* 185 (see Table 2). Typical GC/CI/MS/MS spectra of COC, AEME, COET and EME are shown in Fig. 1.

Table 1
CID conditions used for each compound

Compounds	Parent ions (*m*/*z*)	Excitation storage levels (*m*/*z*)	Excitation amplitudes (V)
COC	304.1	83.6	46
AEME	182.1	49.9	32
EME	200.1	54.9	34
COET	318.2	87.5	46
COC-d3	307.1	84.5	46
EME-d3	203.1	55.7	34

2.3. Hair sample collection

Hair samples were collected by cutting a tied tuft of about 5 mm in diameter from the vertex posterior region of the head, near the scalp, and by fixing it with scotch on a collection sheet of paper in order to identify the root and the tip of the lock. Real COC abusers hair samples were supplied by M. Markert from the Swiss Federal Office of Public Health and were stored at room temperature and sheltered from the light until its analysis.

Blank hairs collected from a young female volunteer, were tested for the presence of the compounds of interest, and were free from traces of COC and its metabolites. Prior to its utilisation, hair tufts were stored at room temperature and sheltered from the light.

2.4. Sample preparation

2.4.1. Hair decontamination

Before analysis, the totality of hair obtained from a person was washed successively with 10–50 mL of dichloromethane, 10–50 mL of water and finally 10–50 mL of methanol according to the size of hair tuft. This step is very important to eliminate possible external contamination. Then hair tufts were dried for 15 min at 60 °C. If possible, tufts were cut in three segments (root 3 cm for the first, 3–6 cm for the second and the third until tip extremity) and pulverized separately in a ball mill (maximum amplitude).

2.4.2. Digestion and extraction

Because drugs are fixed inside the hair matrix, a digestion procedure is required before extraction. About 50 mg of powdered hair samples were placed in a glass tube of 10 and 1 mL of hydrochloric acid 0.1 M was added. After an incubation overnight at 60 °C, the solution was neutralized with 1 mL

Table 2
Parent ions and principal product ions for each compound

Compounds	Parent ions → product ions[a]
COC	304 → <u>182</u>
AEME	182 → 105, <u>118</u>, 122, 150
EME	200 → 150, <u>182</u>
COET	318 → <u>196</u>

[a] Ions used for quantification are shown underlined.

of NaOH 0.1 M, buffered with 1 mL of phosphate buffer pH 7, and 25 μL of the internal standards (COC-d_3 and EME-d_3) solution at 1 μg/mL were added. After a centrifugation at 5000 × *g* for 10 min, the supernatant was transferred into a special glass tube for extraction. The ASPEC system was programmed to extract the hair samples in following steps: (1) the cartridges were conditioned successively with 2 mL of methanol and 2 mL of water; (2) 3 mL of the supernatant solutions were dispensing on the cartridges; (3) the latter were rinsed successively with 2 mL of water, 1 mL of acetate buffer pH 4 and 2 mL of methanol, and finally; and (4) after drying cartridges with hair, the compounds were eluted with 2 mL of (80:20:2) dichloromethane/isopropanol/ammonia hydroxide. The extracts were then evaporated to dryness under nitrogen at room temperature before being finally dissolved in 50 μl of toluene for the GC/CI/MS^2 analysis. This complete procedure has already been described in earlier articles [9,10].

3. Calibration and quality control samples

Calibration and quality control samples were prepared by adequately spiking hydrolysed hair solution after incubation (50 mg of hair added with 1 mL of HCl 0.1 M and placed at 60 °C during 12 h) with appropriate volumes of standard COC, AEME, EME and COET solutions.

4. Results and discussion

4.1. GC/CI/MS/MS development

The aim of this work was to develop an analytical method for the simultaneous analysis of COC and three of its principal metabolites, AEME, EME and COET, in human hair samples. During hair analysis, the parent substance is present in greater quantity than its metabolites. Therefore, the main difficulty was to obtain sufficient selectivity and sensitivity to quantify traces of metabolites in the possible presence of a relatively high concentration of COC. Because of the related structures of compounds of interest and their low molecular weight, a gas chromatography-tandem mass spectrometry using ion-trap detector in positive chemical ionisation (GC/CI/MS/MS) was considered for the simultaneous determination of COC, AEME, EME and COET in human hair samples.

At first, the GC/CI/MS/MS response of the four analytes was studied with methanol as liquid CI reagent and results obtained were conclusive and promising. According to the low concentration levels necessary to reach for the final method application, method development was mainly dedicated to achieve trace levels for the analytes of interest. Therefore, several parameters related to the detection conditions were investigated by a univariated approach. For this purpose, numerous investigations were achieved with standard solutions to determine optimal surroundings.

The inlet pressure of the carrier gas was initially studied and increased to 10 PSI (0.069 Mpa), as a compromise value between the nominal recommendations and peak quality. A better spectral peak reproducibility was enhanced by working at 10 PSI instead of five lower values without significant peak shape alteration.

The influence of the trap temperature on the peak shape was further studied as proposed by Libong et al. [11]. As previously observed, peak tailing decreased when the trap temperature was increased. This was probably due to an adsorption phenomenon at the electrodes surface, according to the polarity of the analytes and reduced when the trap temperature was enhanced. The trap temperature was therefore fixed at 240 °C for working and processing analyses instead of 200 °C.

As the four compounds of interest had a punted structure, the fragmentation remains a difficult process needing a higher energy. To avoid the ionic product lost with high excitation amplitude to fragment the molecules, the nominal q_z value was adjusted. Indeed, the latter was set at 0.25 in order to use higher excitation amplitude with a minimum lost of ionic signal when non-resonant mode was used for the CID fragmentation.

In order to achieve best sensitivity, the possibility to inject a sample volume higher than 1 μl (common used volume) was investigated. A retention gap was installed prior to the chromatographic column, allowing to inject higher sample volumes. An injection volume of 3 μl, which permitted to enhance the sensibility without adding significant interferences, was finally selected.

Because in chemical ionisation, the sensitivity is obviously dependant on the choice of the CI reagent, the latter was modified with reference to the results reported by Bouchonnet et al. [12]. So after having compared the sensitivity of our four compounds of interest by using methanol as liquid CI reagent or isobutane as gas CI reagent, we have noticed that the latter allowed an appreciable gain (factor 3–5) towards methanol and we have decided to work from then on with isobutane which has given a best sensitivity. As mentioned by Bouchonnet et al. [12], isobutane provides a greatest amount of MH^+ ions due to the combination of two factors: the formation of reagent ions is energetically low-costing and the storage of reagent ions is more efficient than with other "classical" reagents.

Other experiments were carried out to study different parameters related to CID. The concomitant influences of the maximum ionisation time (MIT) with the maximum reaction time (MRT) and the concomitant influences of the excitation amplitude (EXA) with the excitation time (EXT) were also studied. MIT corresponds to the time allowed for electroionization of the reagent (μs), MRT to the time allowed for the reaction of proton transfer (ms), EXA to the amplitude used to fragment parent ion and to form product ion (volts), and EXT to the time allowed for CID (ms). From all these

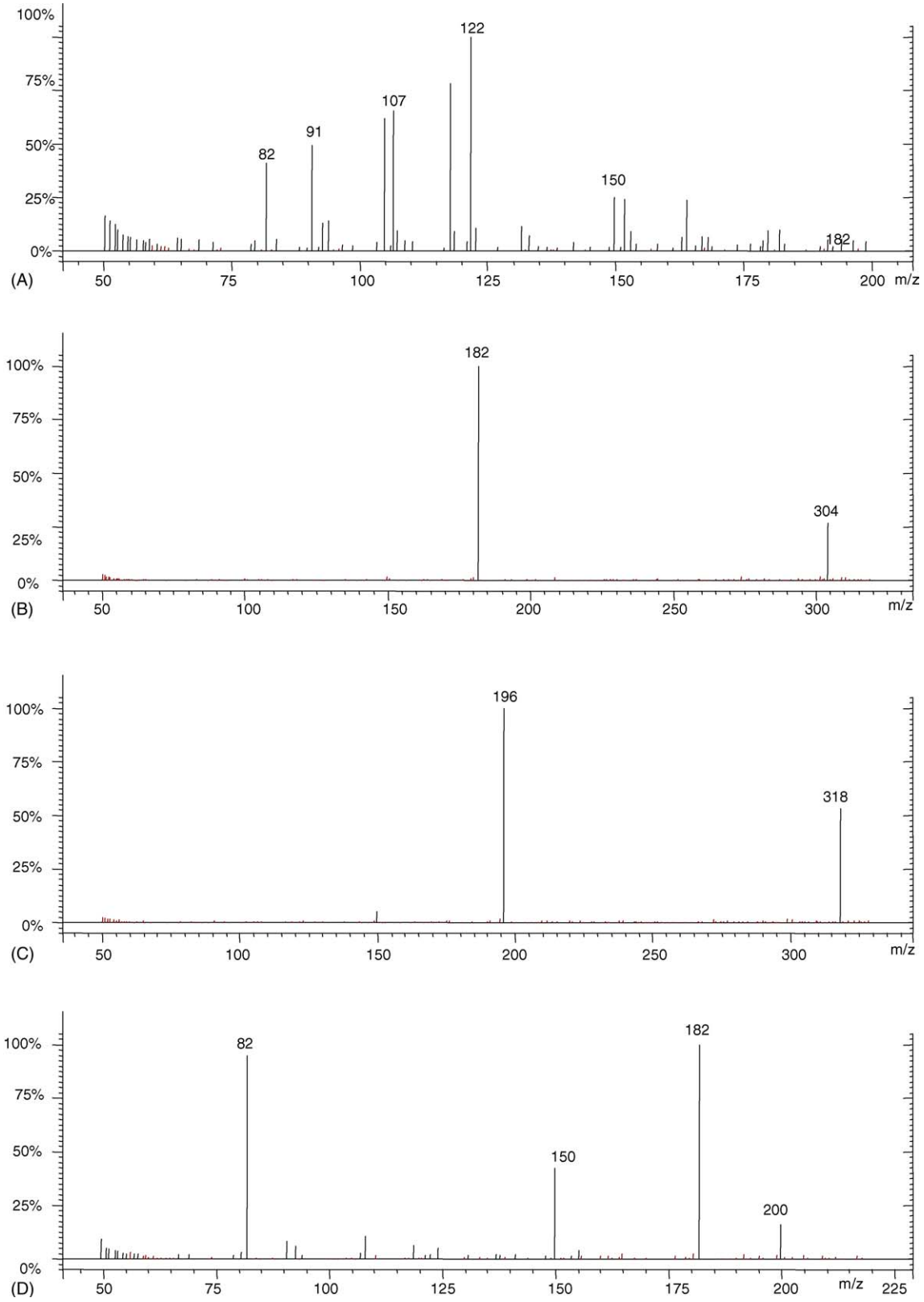

Fig. 2. Typical spectra of: AEME (A); COC (B); COET (C); and EME (D) obtained by GC/CI/MS/MS using ion-trap detection with isobutane as gas reagent.

Table 3
MIT, MRT, EXA and EXT optimized for each compound

Compounds	MIT (μs)	MRT (ms)	EXA (V)	EXT (ms)
COC	500	100	46	20
AEME	1000	80	32	40
EME	500	100	34	20
COET	500	100	46	20

studies, the best CID conditions for our four compounds of interest were selected (see Table 3).

A fragmentation difference for the analytes between standard solutions and matrix media was observed. Indeed, whereas during experiments with standard solutions a good fragmentation occurred, when the matrix extracts were analysed, less good spectra were obtained with an inferior fragmentation level of the parent ion. Unfortunately it was not always possible to compensate this phenomenon even by changing the CID conditions. Nevertheless, the developed method has shown correct performances.

In tandem mass spectrometry, it is generally recommended for the spectrum of the product ions to maintain a small proportion of the parent ion in order to keep a supplementary source of information. It is often advocated that the parent ion peak represent about 10% of the predominant product ion peak. In case of CI/MS/MS analysis of our four compounds of interest, it turned out that this was not always possible. The best example is COET (Fig. 2C) for which the peak corresponding to the parent ion remained always above about 50% even if a higher excitation amplitude was tested. In the same manner, the parent ion peak of COC (Fig. 2B) remained above about 25%. This phenomenon is probably due to the punted structure of the molecules, which accounts for their resistance.

Finally, typical chromatograms of standard solutions of the four analytes with optimized GC/CI/MS/MS response are shown in Fig. 3.

4.2. Validation procedure

The strategy applied for the validation of the hair analysis of COC, AEME, EME and COET was based on the approach proposed by the "Société française des Sciences et Techniques Pharmaceutiques" (SFSTP) [13–17] and adapted for this specific case of forensic toxicology. The different experiments carried out have allowed the choice of the most appropriate model for the calibration curves, to evaluate the performances of the method (determination of trueness, precision-repeatability and intermediate precision-, accuracy and LOD/LOQ) over three days, and to estimate the robustness of the method in a given range of concentrations. To validate all these criteria, two kinds of samples were prepared: calibration (CAL) and validation samples corresponding to quality control (QC) samples used in routine analysis. These samples were prepared by adequately spiking hydrolysed hair solution after incubation (50 mg of hair added with 1 ml of HCl 0.1 M and placed during 12 h at 60 °C) with appropriate volumes of standard COC, AEME, EME and COET solutions.

COC-d_3 was used as internal standard (IS) for COC and COET, whereas EME-d_3 was used as IS for AEME and EME. Each CAL and QC samples contained the equivalent of 0.5 ng/mg of each IS.

In preliminary assays (data not shown), variance analysis indicated that the weighted linear regression was appropriate for establishing a relationship between the concentration and detection response of each compound. In order to establish the daily calibration curves for each substance of interest, six

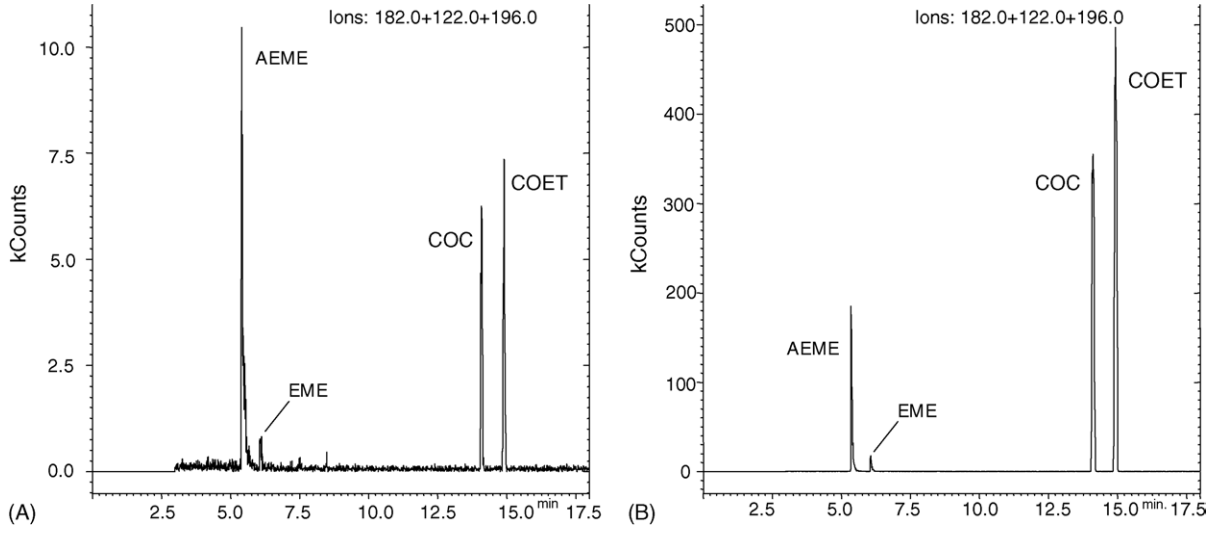

Fig. 3. Chromatogram of a standard solution at 25 ng/mL corresponding to 0.5 ng of drugs per mg of hair (A) and chromatogram of a standard solution at 250 ng/mL corresponding to 5 ng of drugs per mg of hair (B).

concentration levels (CAL = 0.05, 0.10, 0.20, 0.50, 1.00 and 5.00 ng/mg, $k = 6$) were prepared at each validation day and analysed three times ($n = 3$). The best weighting factor was chosen taking into account the relationship between natural variance logarithms and concentrations. For each analyte, the specially selected weighting factor was the inverse of the concentration raised to the λth power ($1/x^{\lambda}$), λ being the slope of the line fitted to the data on the logarithm scale round off the superior unit. Hence, the selected weighting factor was $1/x^2$ for each compound as described elsewhere [18].

Each day, QC samples were prepared at four concentration levels (QC = 0.05, 0.10, 0.50 and 4 ng/mg) representing the entire calibration range. Each QC was treated four times ($n = 4$). The lowest concentrations were selected close to the preliminarily estimated LOQ (in preliminary assays, the latter was estimated with extracts spiked at different low concentration levels).

4.2.1. Linearity

The linearity of an analytical method is its ability within a definite range to obtain results directly proportional to the concentration (or amount) of the analytes in the sample [13,14,19]. The linearity was calculated by fitting the back-calculated concentrations of the QC versus theoretical concentrations by applying the linear regression model based on the least square method [20]. Good linearity (slopes close to 1 ± 0.005) and good closeness R^2 above 0.998 for all analytes were observed.

4.2.2. Trueness and precision

Trueness refers to the closeness of agreement between a conventionally accepted value and a mean experimental one [13,14,19]. The precision of a bioanalytical method is estimated by measuring repeatability and intermediate precision at different levels of concentration [13,14,19]. After fitting the calibration curves for each analyte and each day, concentrations of the four substances of interest in QC samples were computed from the analytical obtained responses. Trueness was expressed as the mean concentration found for all QC samples at each concentration level ($n = 12$). Variances of repeatability and intermediate precision were computed from the estimated concentrations and the precision was expressed by the relative standard deviation (RSD) at each level. Obtained trueness and precision results are presented in Table 4.

4.2.3. LOD and LOQ

The limits of quantification (LOQ) were determined by using the validation results for repeatability and precision. Each LOQ was determined at the concentration for which trueness was equal to $100 \pm 20\%$, the repeatability was inferior to 15% and RSD was inferior to 25% of intermediate precision. Hence, the LOQ were fixed at 0.10 ng/mg for AEME and at 0.05 ng/mg for COC, EME and COET.

The limit of detection (LOD) were estimated according to the signal to noise ratio (*S*/*N*), were fixed for a *S*/*N* equals 3 and corresponded to 0.050 ng/mg for AEME, 0.025 ng/mg for EME and 0.005 ng/mg for COC and COET.

4.3. Application to real cases

Real hair samples were collected from 80 male and female subjects, ranging from 18 to 50 years of age, who con-

Table 4
Trueness, repeatability and intermediate precision for QC samples

Compounds	Theoretical concentrations (ng/mg)	Found concentrations (ng/mg)	Trueness (%)	Repeatability (%)	Intermediate precision (%)
COC	0.05	0.05	100	11	23
	0.10	0.11	110	7	8
	0.50	0.49	98	3	3
	2.50	2.26	90	6	10
	4.00	3.64	91	9	14
AEME	0.05	0.02	40	58	96
	0.10	0.12	120	11	26
	0.50	0.44	88	13	14
	2.50	2.00	80	16	16
	4.00	3.45	86	17	23
EME	0.05	0.05	100	11	16
	0.10	0.11	110	15	15
	0.50	0.48	96	11	15
	2.50	2.28	91	7	12
	4.00	3.92	98	3	14
COET	0.05	0.05	100	7	12
	0.10	0.10	100	11	11
	0.50	0.50	100	5	5
	2.50	2.22	89	4	9
	4.00	3.71	93	6	8

sumed cocaine by inhalation, intravenous injection or by smoking. A tuft of hair for each of them was cut into two segments. The validated GC/CI/MS/MS method was applied to approximately 160 segments of cocaine consumer's hair. All samples were prepared as described in the experimental section and were injected in the GC/MS system. Several experiments (data not shown) have revealed that the linearity could be extended by extrapolation to 15 ng/mg for all the compounds by preserving the same method performances. Although more than 75% of the concentrations could be determined quantitatively and precisely for the three metabolites, we should recognise that the majority of the values determined for the COC are only good approximate considering the high retrieved concentrations. Nevertheless, the

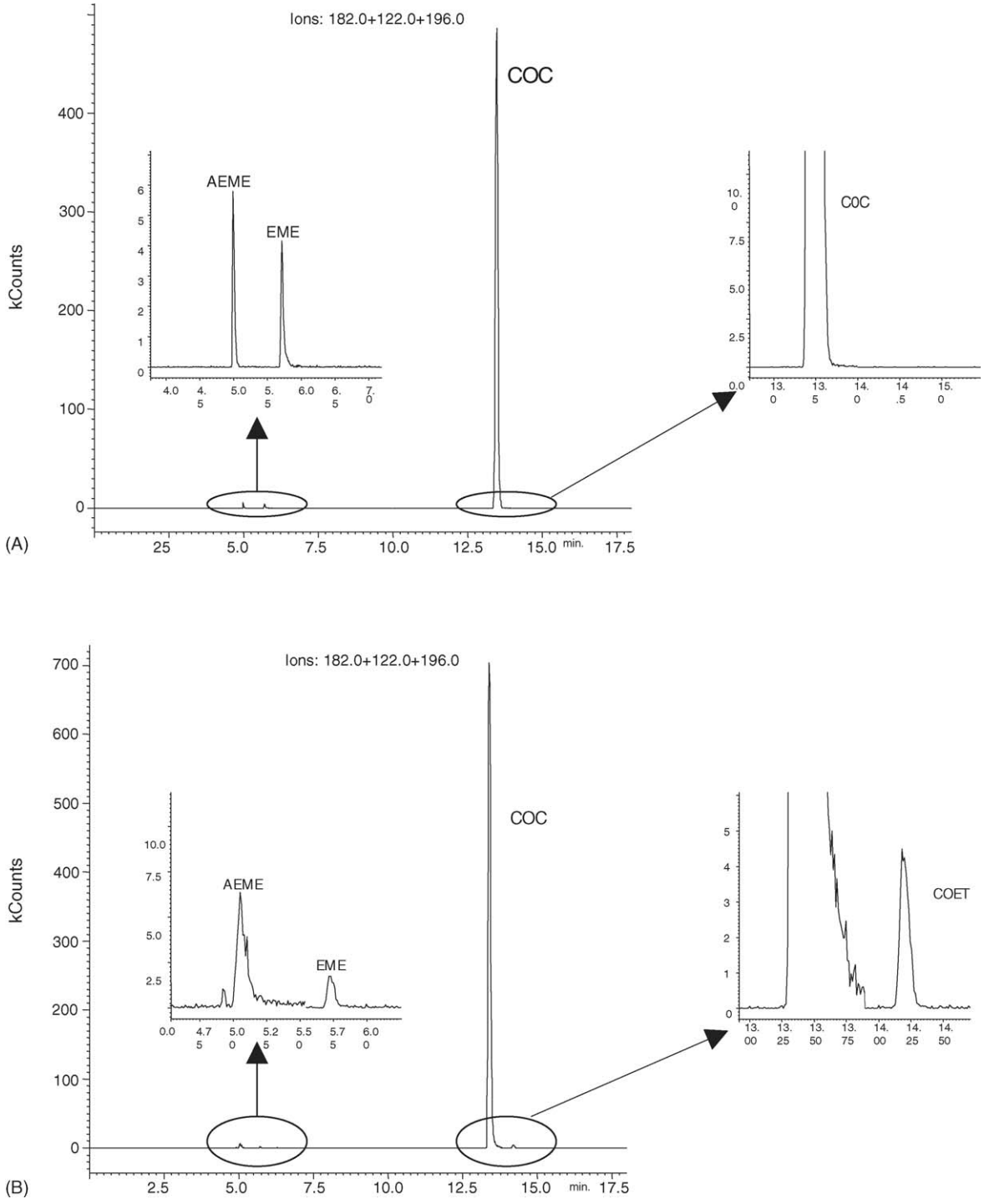

Fig. 4. Chromatogram of cocaine abuser (A) and chromatogram of cocaine abuser who had drunk concomitantly alcohol (B).

Table 5
Results obtained for the analysis of approximately 160 segments of cocaine abusers hair

	COC	AEME	EME	COET
Mean (ng/mg)	160	7.3	6.4	2.8
Min. (ng/mg)	0.2	0.0	0.0	0.0
Maximum (ng/mg)	3080	45	55	54
Median (ng/mg)	41	2.5	3.7	0.1
Percentile 25 (ng/mg)	15	0.9	0.7	0.0
Percentile 75 (ng/mg)	118	11.0	10.0	0.7

obtained results (see Table 5) showed that the method is suitable for the analysis of cocaine consumers' hair. Typical chromatograms of cocaine abusers' hair are shown in Fig. 4.

During this work, robustness itself was not studied, however the utilisation of the method for more than two months to analyse routinely about 100 real hair samples proved that this method is sufficiently robust towards current factors taking place in routine.

5. Conclusion

A semi automated and sensitive GC/CI/MS/MS method was developed for the simultaneous determination of cocaine (COC) and three of its metabolites by using ion-trap detection with isobutane as gas reagent. The method was fully validated in terms of robustness, linearity, trueness, precision and limits of quantification. The applicability of the validated method was demonstrated for the analysis of several real cases.

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