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Quantification of drugs of abuse and some stimulants in hair samples by liquid chromatography–electrospray ionization ion trap mass spectrometry

Francesca Bucelli[a,*], Angela Fratini[b], Paolo Bavazzano[b], Nicola Comodo[a]

[a] Università di Firenze, Dipartimento di Sanità pubblica, Viale Morgagni 48, 50134 Firenze, Italy
[b] Azienda Sanitaria Firenze, Laboratorio di Sanità Pubblica, Via San Salvi 12, 50135 Firenze, Italy

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ABSTRACT

A qualitative and quantitative method for the analysis of drugs of abuse (cocaine and benzoylecgonine, opiates) and some stimulants in human hair was developed and validated. Hair samples were incubated with phosphate buffer (pH 5.0), chosen as the extraction medium, extracted with Bond Elut Certify cartridges and analyzed by LC–MS–MS and LC–MS^3 as confirmation for positive results. The method proved to be specific, accurate and precise across the calibration range (0.1–30 ng/mg) where good linearity was observed. Total extraction recovery, intra-assay accuracy and precision, limits of detection and limits of quantitation were estimated. The method was successfully applied to the analysis of hair samples collected from drug abusers and it was suitable for routine analytical applications in the Antidoping Laboratory of Public Health Laboratory.

1. Introduction

Analysis of drugs of abuse are important for the prediction of and protection from the risk to human health, especially for young people.

Data provided to the European Monitoring Centre for Drugs and Drug Addiction (EMCDDA) [1] estimate that amphetamines are the second most commonly used illicit substances in many European countries; 4.5 million Europeans have used cocaine in the last year and in many countries, opiates, mainly heroin, remain the principal drug the users seek treatment.

The use of drugs of abuse is increasing world wide and causing serious social problems. Depending on the actual compound, drug use may lead to health problems, social problems, physical dependence, or psychological addiction. Drug of abuse makes central nervous system effects, which produce changes in mood, levels of awareness or perceptions and sensations. Some drugs appear to be more likely to lead to uncontrolled use than others. The addiction is a disease that affects both brain and behaviour [2].

Several different tissues and fluids can be collected and used to detect and quantify abused drugs. Over more than 20 years hair analysis for drugs has been gaining increasing attention and recognition in various toxicological fields. Hair is a unique material for the retrospective investigation of chronic drug consumption; once incorporated, drugs remain very stable in hair, which allows for detection of substances up to several months after intake. In 2004 the Society of Hair Testing (SoHT) proposed confirmation cut-offs for amphetamines, cocaine and its metabolites, and opiates in hair [3].

Several methods have been reported for drugs determination in human hair. In particular, mass spectrometry is a powerful technique in terms of sensitivity and specificity for the detection of these drugs. The majority of developed methods involved gas chromatography–mass spectrometry (GC–MS) [4–9], using derivatization procedures. This generally involves excessive time consuming and sample loss. Other methods employed for the analysis of these drugs are based on the use of liquid chromatography–mass spectrometry (LC–MS) coupled to various ionization sources [10–12], but the analysis was generally limited to one or two drug groups. A qualitative LC–MS–MS method has been reported for the analysis of amphetamines, cocaine and its metabolites and opiates [13]. This method was not, however, developed for quantitative purposes but as an alternative to immunoassay screening.

The aim of this study was to develop and validate a liquid chromatography–electrospray ionization ion trap mass spectrometry method for the analysis of 16 drugs (cocaine and its metabolites, opiates and some stimulants) in human hair. In particular the advantages of ion trap use were greater sensitivity, good mass resolution and scan speed. The ion trap spectrometer afforded to work over the entire mass range in full scan mode, in MS/MS and MS^n mode.

* Corresponding author. Tel.: +39 0556263273.
E-mail address: francesca.bucelli@asf.toscana.it (F. Bucelli).

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2. Experimental

2.1. Chemical and reagents

Ecgonine methyl ester (EME), cocaine, benzoylecgonine, morphine, 6-monoacetylmorphine (6-MAM), codeine, amphetamine, methamphetamine, 3,4 methylenedioxyamphetamine (MDA), 3,4 methylenedioxyethylamphetamine (MDEA), 3,4 methylenedioxymethamphetamine (MDMA), N-methyl-1-(3,4 methylenedioxyphenyl)-2-butamine (MBDB), ephedrine HCl, phentermine, phendimetrazine, buprenorphine, amphetamine-d_5 and benzoylecgonine-d_8 were used as free bases and were obtained from Chemical Research S.r.l. 2000 (Rome, Italy). All solvents were analytical grade. Methanol, acetonitrile, dichloromethane and 2-propanol were purchased from Panreac Quimica Sau (Barcelona, Spain); formic acid, hydrochloric acid and potassium dihydrogen phosphate from Merck (Darmstadt, Germany); ammonium hydroxide was obtained from Sigma Aldrich (Milan, Italy). Bond Elut Certify SPE columns were obtained from Varian Corp. (Harbor City, CA, USA).

Stock solutions of the analytes and deuterated internal standards in methanol (1 mg/mL) were stored at −20 °C. Single analyte solution was prepared by diluting stock solution with methanol to obtain the desired concentration of each compound of interest.

2.2. Instrumentation

LC–MS–MS and LC–MS^3 analysis were performed on a Varian Pro Star 210 chromatography system consisting of vacuum degasser, two-piston gradient pump, autosampler Varian Pro Star 410, 5 μL sample-loop and connected to a Varian 500 MS ion trap mass spectrometer.

As confirmation for positive results MS^3 transitions were applied.

2.3. LC parameters

A Varian Polaris C18 column (100 mm × 2 mm, 3 μm), protected with a guard column Fusion-RP Phenomenex (4 mm × 2 mm) was used for chromatographic separations. For all the applications elution solvents were: ultra pure water–0.1% formic acid (solution A) and acetonitrile–0.1% formic acid (solution B). The following step-wise gradients, at constant flow rate of 0.2 mL/min, were used. For stimulants analysis: 90% A for 1 min, gradient to 20% A in 8 min, followed by 90% A with 5 min equilibration before the next injection. Total run time was 18 min. For opiates, cocaine and benzoylecgonine analysis: 90% A for 0.5 min, gradient to 20% A in 7.5 min, followed by 90% A with 5 min equilibration before the next injection. Total run time was 17 min. Injection volume was 5 μL.

2.4. MS parameters

The optimum tuning parameters, precursor, and product ions were identified for each analyte. To obtain mass spectral data of the different compounds, a solution of 5 μg/mL was directly infused into the mass spectrometer. Ionization of analytes was achieved using positive electrospray ionization (ESI). The drying gas temperature was maintained at 320 °C, the drying gas pressure was maintained at 20 psi and the needle voltage was 4800 V. The spray shield was set at 600 V. The nebulizer gas (nitrogen in the positive ionization mode and air in the negative ionization mode) was set at 30 psi for stimulants and at 50 psi for opiates, cocaine and benzoylecgonine. Helium was used as cooling gas for the ion trap.

2.5. Sample preparation

2.5.1. Hair samples

Authentic hair samples (20–50 mg each) were collected from drug abusers admitted to centers for detoxification treatment.

The hair was collected from the posterior vertex of the scalp and cut as close to the skin as possible and the proximal and distal ends were carefully identified. Blank hair samples were obtained in the same manner from volunteers.

Each hair sample was cut into small pieces, washed twice with dichloromethane (2 mL), mixed by vortex for 60 s, centrifuged for 5 min and dried under a nitrogen stream at room temperature.

2.5.2. Sample preparation and extraction

Phosphate buffer 0.1 N (pH 5.0) was chosen as the optimum hair incubation medium because of the high stability of cocaine and 6-monoacetylmorphine using this method [14]. To 20–50 mg of hair were added 200 ng of deuterated internal standard and 3 mL of phosphate buffer, the mixture was incubated at 45 °C for 18 h and then centrifuged for 5 min. Extraction was carried out with Bond Elut Certify cartridges. The cartridge was conditioned with methanol (2 mL) followed by 0.1 N phosphate buffer, pH 6 (3 mL). The sample was then passed slowly through the column. The cartridge was rinsed with 3 mL of water, followed by 1.5 mL of 0.1 M HCl, allowed to dry for 5 min under vacuum, then rinsed with 2 mL of methanol. Analytes were eluted with a mixture (3 mL) of dichloromethane/2-propanol/ammonia (80:20:2, v/v/v). The eluant was removed under a gentle nitrogen stream at 45 °C. The residue was reconstituted in 200 μL of mobile phase acetonitrile–water (10:90, v/v, 0.1% formic acid).

2.6. Method validation

The analytes were analyzed in two separate injections. We can program the mass spectrometer to acquire data differently by dividing the analysis into chromatographic time segments to minimize the number of scan events per time window. The first injection was for cocaine and its metabolites and opiates, the second injection was for stimulants. Fig. 1 shows an analysis of a blank hair

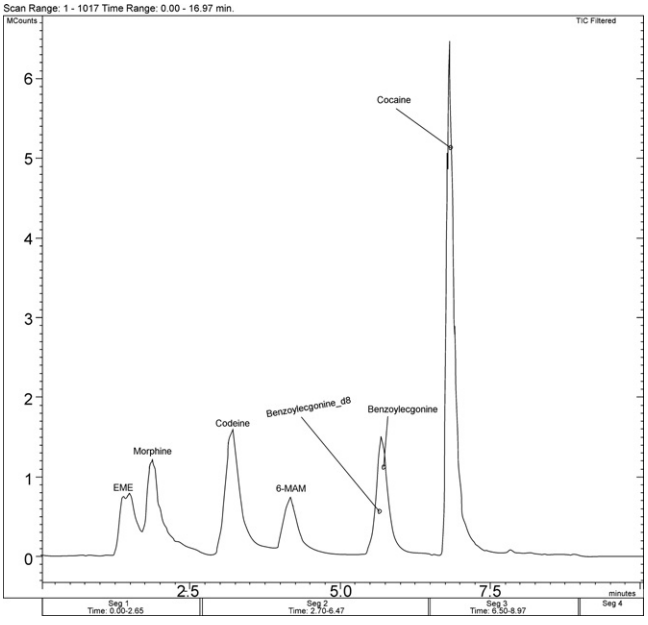

Fig. 1. Total ion chromatogram derived by LC–MS–MS analysis of a blank hair spiked with 5 ng/mg of cocaine and opiates.

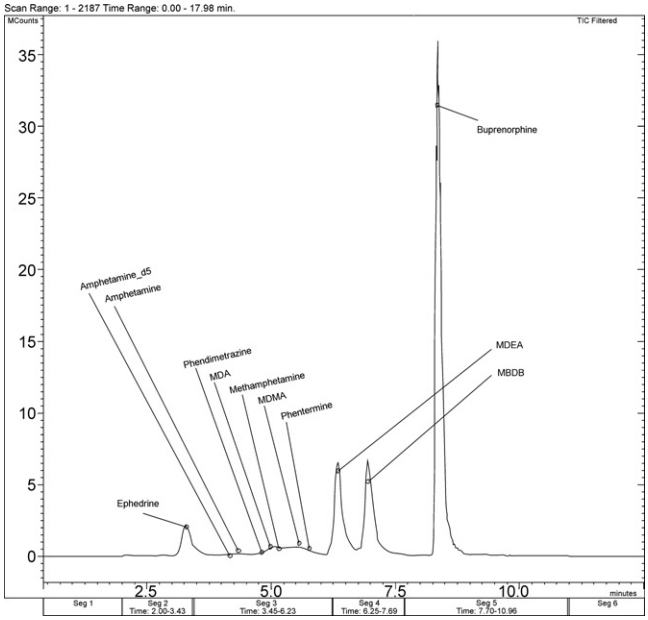

Fig. 2. Total ion chromatogram derived by LC–MS–MS analysis of a blank hair spiked with 5 ng/mg of all stimulants studied.

spiked with 5 ng/mg of cocaine and opiates. Fig. 2 shows an analysis of a blank hair spiked with 5 ng/mg of all stimulants studied.

Calibration was performed by addition of standard solutions to 50 mg of drug-free hair prior to incubation. Final concentrations were 0.1, 0.2, 0.5, 1, 5, 10 and 30 ng/mg.

The percent total extraction recovery was analyzed for each analyte at three different concentrations, 0.5, 1 and 5 ng/mg. This recovery takes into account a combination of the incubation step, SPE step and matrix effect because the reference was unextracted standards.

To determine intrabatch accuracy and precision six extracted 50 mg blank hair samples spiked at low, medium and high concentrations (0.5, 1, 5 ng/mg) were used. Precision is expressed as the relative standard deviation (RSD) of the concentrations calculated by the calibration graphs. Accuracy is expressed as the relative error of the estimated concentrations.

For the calculation of the limits of detection (LOD) and quantification (LOQ) six replicates at four different concentrations of the analytes (0.03, 0.05, 0.07 and 0.09 ng/mg) spiked in blank hair were used. The LOD and LOQ were calculated at a signal-to-noise ratio of 3 and 10, respectively. The analyte response was at least five times the response compared to blank response.

The calibration curves and quantitation data were obtained using ARPA software elaborated from Unichim manual [15].

Table 2
Absolute recovery (%) from spiked samples.

Analyte	Recovery (%) ($n = 3$)		
	0.5 ng/mg	1 ng/mg	5 ng/mg
EME	99.1	82.9	78.7
Cocaine	81.6	102.0	97.7
Benzoylecgonine	97.9	95.5	99.1
Morphine	122.0	130.0	107.0
6-MAM	89.8	83.5	77.9
Codeine	96.1	106.0	123.0
Amphetamine	92.4	84.3	85.6
Methamphetamine	94.5	87.5	79.8
MDA	87.4	74.4	74.0
MDEA	106.8	85.7	76.7
MDMA	93.7	97.1	73.1
MBDB	82.0	77.4	74.8
Ephedrine	77.2	73.0	74.0
Phentermine	95.1	86.7	78.4
Phendimetrazine	115.2	88.0	74.0
Buprenorphine	82.8	74.0	74.0

3. Results and discussion

3.1. MS tuning

Optimum tuning parameters, precursor ion, MS^2 product and quantitation ions and MS^3 product ions are shown in Table 1. The highest sensitivity was observed for ESI with the polarity in positive mode for all analytes. Figs. 3–4 show the results of some positive hair samples which were extracted using the developed method.

3.2. Validation of the quantification method

Standard curve plots for the analytes were linear in the range of tested concentrations with a coefficient of correlation (R^2) higher than 0.99.

Recovery was evaluated in triplicate samples at three different concentrations, by comparing the peak areas of analytes to the peak areas of corresponding compounds in samples prepared by spiking extracted blank hair with the same amount of compounds at the step immediately prior to injection. The recovery values for all the

Table 1
Optimum tuning parameters and precursor and product ions for each analyte.

Analyte	Capillary voltage (V)	RF loading (%)	Excitation amplitude (V)	Precursor ion (m/z)	Product ion MS^2 (m/z)	Product ion MS^3 (m/z)	Retention time (min)
EME	69.5	67	0.88	200.1	182[a]	150	1.35
Cocaine	80	83	0.77	304.2	182[a]	150	6.82
Benzoylecgonine	79.6	81	1.21	290.2	168[a]	150	5.72
Morphine	109	82	1.19	286.1	201[a], 229	183	1.88
6-MAM	105	84	1.35	328.1	211[a], 268	165	4.20
Codeine	105	81	1.24	300.1	215[a]	183	3.17
Amphetamine	32	55	0.68	136.0	119[a]	91	4.47
Methamphetamine	49	55	0.72	150.1	119[a]	91	5.17
MDA	44	61	0.81	180.2	163[a]	105, 135	5.08
MDEA	52	66	0.91	208.1	163[a]	105, 135	6.37
MDMA	45	63	0.86	194.1	163[a]	105, 135	5.57
MBDB	54	66	0.58	208.2	177[a]	135	6.96
Ephedrine	59	58	0.77	166.1	148[a]	117, 133	3.23
Phentermine	41	55	0.72	150.0	133[a]	91	5.89
Phendimetrazine	83	63	0.86	192.1	148[a]	133	5.07
Buprenorphine	150	64	1.42	468.3	414[a]	396	8.38

[a] Quantitation ion.

Table 3
Intraday accuracy and precision.

Analyte	Intrabatch precision (RSD%, $n = 6$)			Accuracy (error%, $n = 6$)		
	0.5 ng/mg	1 ng/mg	5 ng/mg	0.5 ng/mg	1 ng/mg	5 ng/mg
EME	15.7	17.7	6.8	26.0	3.3	5.0
Cocaine	6.4	6.5	2.9	0.5	6.5	2.9
Benzoylecgonine	7.2	6.6	3.9	6.2	1.7	2.9
Morphine	7.7	7.8	10.5	30.0	30.0	7.2
6-MAM	8.8	9.8	6.6	5.5	14.2	5.7
Codeine	7.3	17.5	14.0	6.0	11.4	3.1
Amphetamine	5.8	13.9	3.50	0.4	6.2	3.6
Methamphetamine	13.5	14.6	6.6	3.5	14.6	3.5
MDA	10.9	13.7	5.8	15.2	7.8	12.8
MDEA	5.8	14.6	17.4	22.8	28.9	9.7
MDMA	19.2	20.8	6.2	26.3	21.4	0.5
MBDB	19.7	13.1	19.7	5.7	13.9	13.8
Ephedrine	11.6	11.9	9.1	2.2	18.3	9.3
Phentermine	19.3	20.4	10.1	30.0	26.5	3.6
Phendimetrazine	22.6	26.9	7.7	18.1	15.4	2.6
Buprenorphine	8.9	16.8	17.1	15.9	1.2	13.1

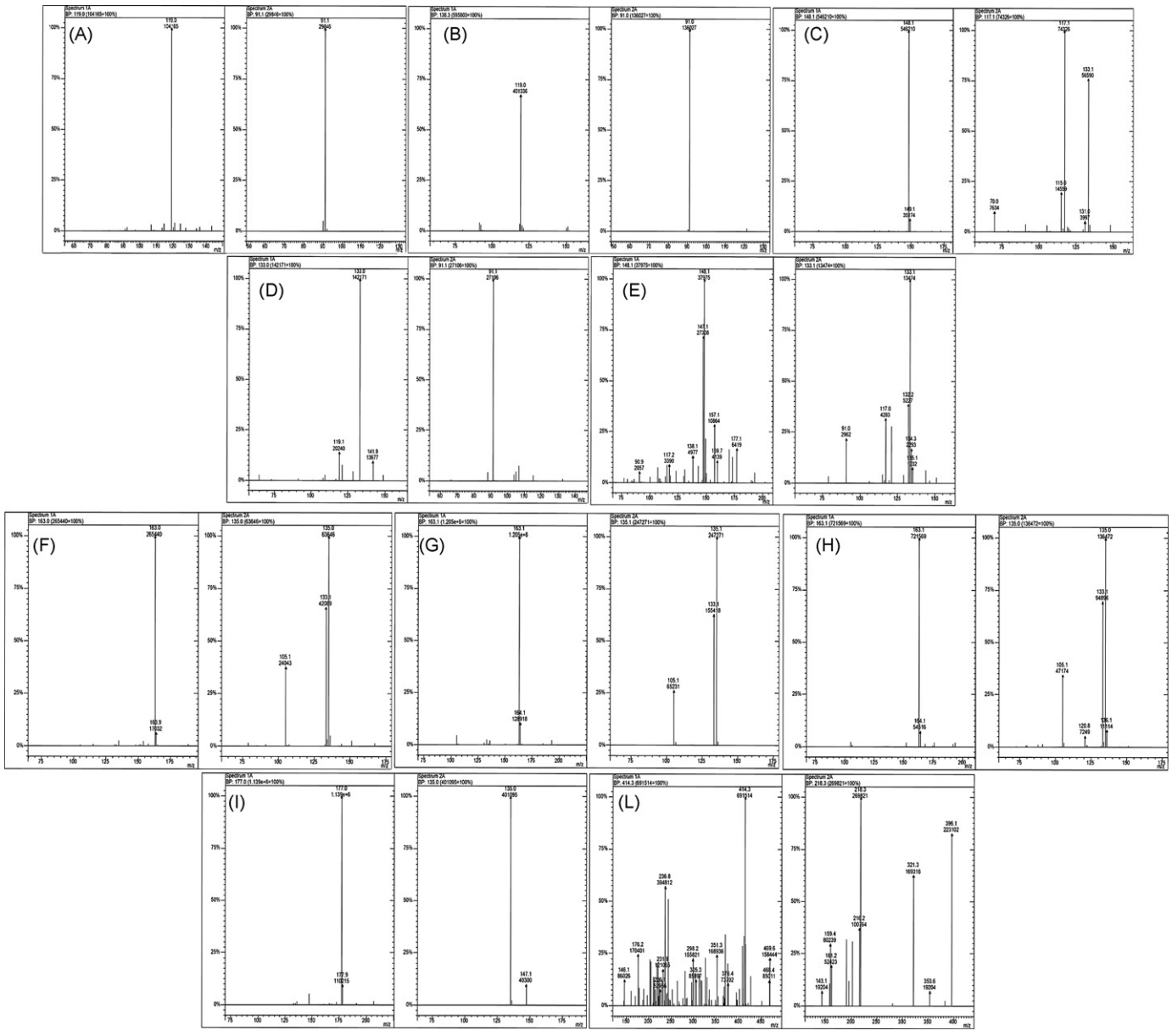

Fig. 3. MS^2 (Spectrum 1A) and MS^3 (Spectrum 2A) of amphetamine (A), methamphetamine (B), ephedrine (C), phentermine (D), phendimetrazine (E), MDA (F), MDEA (G), MDMA (H), MBDB (I), buprenorphine (L).

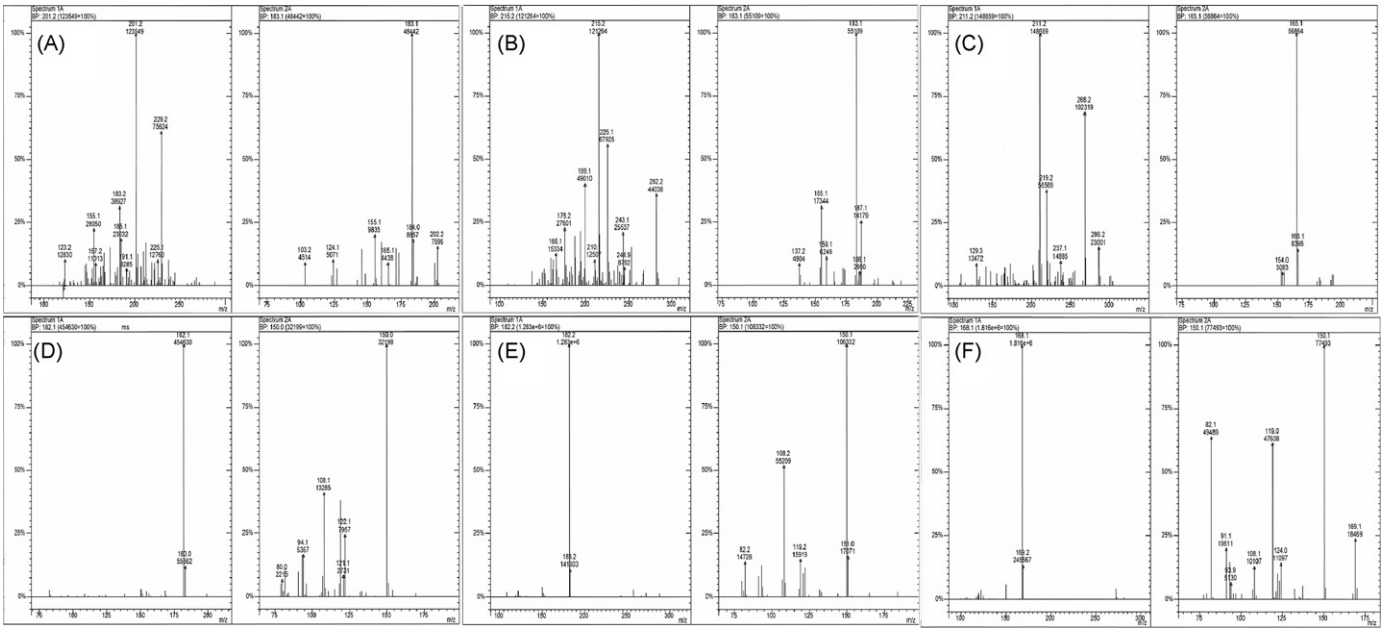

Fig. 4. MS^2 (Spectrum 1A) and MS^3 (Spectrum 2A) of morphine (A), codeine (B), 6-MAM (C), EME (D), cocaine (E), benzoylecgonine (F).

analytes in spiked hair are given in Table 2. All gave high recoveries (>73%) at all three concentrations.

Table 3 shows the intrabatch accuracy and precision values, these were generally acceptable by SOFT guidelines [16] of ±20% for the three concentrations tested; however, the guidelines also declare that ±25–30% may be acceptable for some analytes. Precision (RSD%) ranged from 2.9% to 26.9% for all analytes; accuracy (error%) ranged from 0.4% to 28.9%.

Calculated LOD and LOQ were in the range 0.005–0.08 ng/mg and 0.02–0.25 ng/mg, respectively. The analyte response at the lowest concentration detectable for each analyte was reproducible with a precision of 20% and accuracy of 80–120%. Results are shown in Table 4.

These results were in agreement with the results reported in other studies which used different mass spectrometers for drugs determination in human hair [6–13].

3.3. Analysis of real samples

The qualitative and quantitative analytical procedure was applied routinely for the analysis of hair samples in the Anti-doping Laboratory. In particular, the developed method was used to analyze hair samples collected from 18 voluntary and anonymous drug abusers between 19 and 50 years old. When possible, sectional analysis of hair samples was performed. As reference samples we used a blank hair spiked with the analytes studied and a Hair Control sample obtained by Medichem (Medidrug DHF 1/08-A H-plus) with reference values for the analytes contained. Eight of the 18 hair samples tested positive with the LC–MS^2 analysis for at least one drug and LC–MS^3 analysis were carried out for these samples. The hair samples were reported as positive for a particular drug group according to the SoHT guidelines, in particular the Society of Hair Testing recommended cut-off values of 0.2 ng/mg for opiates, 0.5 ng/mg for cocaine (0.05 ng/mg for benzoylecgonine) and 0.2 ng/mg for amphetamines. One sample tested positive for amphetamine (0.25 ng/mg) and one for MDMA (0.30 ng/mg). In Table 5 shows the LC–MS^2 results of positive hair samples for cocaine and opiates: seven samples tested positive for cocaine, three for morphine. The ratio benzoylecgonine on cocaine resulted in the range 0.1–0.7 whereas the ratio of 6-MAM on morphine in the range 2–3; the ratio values found were within the ranges suggested by the Society of Hair Testing.

Table 4
LOD, LOQ, precision and accuracy values.

Analyte	LOD (ng/mg)	LOQ (ng/mg)	Precision (%)	Accuracy (%)
EME	0.02	0.06	17	113
Cocaine	0.02	0.07	13	92
Benzoylecgonine	0.005	0.02	7	87
Morphine	0.04	0.15	20	120
6-MAM	0.03	0.09	19	92
Codeine	0.02	0.06	12	102
Amphetamine	0.04	0.13	19	106
Methamphetamine	0.03	0.08	17	94
MDA	0.06	0.19	12	76
MDEA	0.04	0.13	20	110
MDMA	0.04	0.12	19	120
MBDB	0.02	0.06	13	100
Ephedrine	0.03	0.09	15	118
Phentermine	0.08	0.25	21	110
Phendimetrazine	0.05	0.17	21	108
Buprenorphine	0.02	0.08	20	80

Table 5
Concentration (ng/mg) of cocaine and opiates in the hair of drug abusers; I is the proximal segment; II is the distal segment; – is value inferior to cut-off.

Sample	EME	COCA	BEG	MOR	6-MAM	COD
1	–	1.12	0.37	1.42	4.39	0.56
2	–	–	–	1.03	3.86	–
3	–	1.10	0.18	–	–	–
4_I	–	3.36	2.20	–	–	–
4_II	–	5.99	4.06	–	–	–
5	–	1.60	0.66	–	–	–
6	–	4.09	1.35	–	–	–
7	–	17.08	2.09	0.82	1.70	–
8	–	–	–	–	–	–

Abbreviations: COCA, cocaine; BEG, benzoylecgonine; MOR, morphine; COD, codeine.

4. Conclusions

The liquid chromatography–electrospray ionization mass spectrometry method reported in this paper to analyze cocaine and benzoylecgonine, opiates and some stimulants in hair was validated according to guidelines proposed by the Society of Hair Testing.

The qualitative and quantitative method proved suitable for routine use in the Antidoping Laboratory, resulting specific, accurate and precise across the calibration range. In this way the possibility to use the ion trap mass spectrometer for drugs determination in human hair was demonstrated. The results achieved were in agreement with the results reported in other studies where different mass spectrometers were used.

Additional experiments are currently in progress to include other drugs and metabolites into the method.

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