Available online at www.sciencedirect.com
ScienceDirect
Journal of Chromatography A, 1159 (2007) 185–189
JOURNAL OF CHROMATOGRAPHY A
www.elsevier.com/locate/chroma

Hair analysis for illicit drugs by using capillary zone electrophoresis-electrospray ionization-ion trap mass spectrometry

Rossella Gottardo [a], Federica Bortolotti [a], Giorgia De Paoli [a], Jennifer Paola Pascali [a], Ivan Mikšík [b], Franco Tagliaro [a,*]

[a] *Department of Medicine and Public Health, Unit of Forensic Medicine, University of Verona, Verona, Italy*
[b] *Institute of Physiology, Academy of Sciences of the Czech Republic, Prague, Czech Republic*

Available online 10 January 2007

Abstract

In forensic toxicology, hair analysis has become a well established analytical strategy to investigate retrospectively drug abuse histories. In this field, gas chromatography–mass spectrometry and high-performance liquid chromatography–mass spectrometry are currently used, often after preliminary screening with immunoassays. However, on the basis of previous applications to pharmaceutical analysis, capillary zone electrophoresis coupled to ion trap mass spectrometry looks also highly promising. The purpose of the present work was the development of a simple and rapid CZE–MS method for sensitive and quantitative determination of the main drugs of abuse and their metabolites (namely, 6-monoacetylmorphine, morphine, amphetamine, methamphetamine, 3,4-methylenedioxyamphetamine (MDA), 3,4-methylenedioxymethampthetamine (MDMA), benzoylecgonine, ephedrine and cocaine) in human hair. Hair samples (100 mg) were washed, cut and incubated overnight in 0.1 M HCl at 45 °C, then neutralized with NaOH and extracted by a liquid–liquid extraction method. CZE separations were carried out in a 100 cm × 75 μm (I.D.) uncoated fused silica capillary. The separation buffer was composed of 25 mM ammonium formate, pH 9.5; the separation voltage was 15 kV. Electrokinetic injections were performed at 7 kV for 30 s under field amplified sample stacking conditions. ESI-ion trap MS detection was performed in the ESI positive ionization mode using the following conditions: capillary voltage 4 kV, nebulizer gas (nitrogen) pressure 3 psi, source temperature 150 °C and drying gas (nitrogen) flow rate 8 l/min. A sheath liquid, composed of isopropanol–water (50:50, v/v) with 0.5% formic acid, was delivered at a flow rate of 4 μl/min. The ion trap MS operated in a selected ion monitoring mode (SIM) of positive molecular ions for each drug/metabolite. Collision induced fragmentation was also possible. Nalorphine was used as internal standard. Under the described conditions, the separation of all compounds, except amphetamine/methamphetamine, MDA/MDMA and morphine/6-MAM was achieved in 20 min, with limits of detection lower than the most severe cut-offs adopted in hair analysis (i.e. 0.1 ng/mg). Linearity was assessed within drug concentration ranges from 0.025 to 5 ng of each analyte/mg of hair. Analytical precision was fairly acceptable with RSD's ≤3.06% for migration times and ≤22.47% for areas in real samples, in both intra-day and day-to-day experiments. On these grounds, the described method can be proposed for rapid, selective and accurate toxicological hair analysis for both clinical and forensic purposes.
© 2007 Elsevier B.V. All rights reserved.

Keywords: Capillary electrophoresis; Mass spectrometry; Drug analysis; Hair analysis

1. Introduction

The use of hair in forensic toxicology was introduced in the late 1970s by Baumgartner et al. [1], as this peculiar specimen proved to be suitable for investigating retrospectively drug abuse histories. In fact, drugs enter the hair stalk at the hair root together with the nutrients from the capillary blood and remain embedded in the hair matrix for all the life of this structure (from months to years). Since hair growth rate is about 1 cm per month, each centimeter of hair "keeps the record" of about 1 month of use or exposure of the individual to drugs. Current techniques for hair analysis include: immunoassays (mainly used for screening purposes), gas chromatography–mass spectrometry (GC–MS), high-performance liquid chromatography (HPLC) and HPLC–MS [2]. To the best of our knowledge, despite a wide acceptance in other areas of forensic interest, only few papers describe methods for hair analysis based on capillary electrophoresis (CE). Particularly, no application of CE–MS

* Corresponding author at: Department of Medicine and Public Health, Section of Forensic Medicine and Occupational Medicine, University of Verona, Policlinico, 37134 Verona, Italy. Tel.: +39 045 8124618; fax: +39 045 8027623.
E-mail address: franco.tagliaro@univr.it (F. Tagliaro).

0021-9673/$ – see front matter © 2007 Elsevier B.V. All rights reserved.
doi:10.1016/j.chroma.2007.01.011

has so far been reported [3] in this specific field, although CE coupled with ion trap or quadrupole MS has been used for the analysis in urine of amphetamine and other drugs of forensic interest [4–6]. However, the features of minimal need of sample, easy operation, efficiency, rapidity and robustness typical of CE combined with the high sensitivity and selectivity offered by mass spectrometry look very attractive in hair analysis.

Taking advantage of the recent commercial availability of sound CE–MS coaxial interfaces based on electrospray ionization (ESI), the present work was aimed at developing and validating a rapid and simple, but unequivocal, method for the determination of the major drugs of abuse and their metabolites in human hair using an original capillary zone electrophoresis (CZE) method coupled to ESI-ion trap MS. Preliminary results from its application to real cases are also discussed.

2. Materials and methods

2.1. Standards and chemicals

Standards of morphine, cocaine, benzoylecgonine, 3,4-methylenedioxymethamphetamine (MDMA), 3,4-methylenedioxyamphetamine (MDA), 6-monoacethylmorphine (6-MAM), amphetamine, methamphetamine, ephedrine and nalorphine [used as internal standard (I.S.)], were obtained from Salars (Como, Italy). Stock solutions of each standard were prepared in methanol at an individual concentration of 2 mg/ml and stored at −20 °C. Water, methanol, isopropanol, ammonium formate, formic acid, ammonia and other chemicals used for the preparation of the CZE buffers and the ESI sheath liquid were of HPLC or "analytical" grade (Carlo Erba, Milan, Italy). Commercially available, ready-to-use liquid–liquid extraction tubes for basic compounds (Toxi-Tubes A) were supplied by Varian (Lake Forest, CA, USA).

2.2. Instrumentation and analytical conditions

A P/ACE 5500 automated capillary electropherograph (Beckman Coulter, Fullerton, CA, USA) equipped with a filter UV detector was used throughout the present study. In this instrument the UV detector was used only in the preliminary tests, because its location on the capillary too far from that of the MS (at the capillary outlet) precluded any usefulness of UV detection "in line" with the MS detector. Naked fused-silica capillaries (75 μm I.D., 100 cm total length) from Composite Metal Services (Worcester, UK) were used directly connected to the ESI needle at the cathodic end through a coaxial sheath liquid interface (Agilent Technologies, Palo Alto, CA, USA). For this purpose, an external detector adaptor provided by Beckman Coulter was used in combination with the standard capillary cartridge. In order to prevent siphoning through the separation capillary and to keep as short as possible the capillary length, the P/ACE 5500 was placed on a platform which was adjustable in height and position. The final CZE conditions were as follows. Buffer electrolyte, 25 mM ammonium formate adjusted to pH 9.5 with ammonia; separation voltage, 15 kV (forward polarity); room temperature (because of steric problems, only the first 20 cm of the capillary were thermostated, whereas the remaining part was exposed to the room environment without any cooling system). Electrokinetic injection was carried out as follows: the injection end of the capillary was preliminary dipped into doubly distilled water for 1 s for an external rinse, then a plug of water was hydrodynamically injected for 1 s at 0.5 psi and finally the sample was electrokinetically injected for 30 s at 7 kV. Doubly distilled water (500 μl) was used for reconstitution of the dried hair extracts. After each run, the capillary was rinsed with the separation buffer for 2 min at 20 psi. During injection, the spray needle voltage was set at 0 kV and the drying gas flow and the nebulizer pressure were also switched off.

The mass spectrometric instrumentation was composed of an ESI-ion source and an MSD-ion trap mass spectrometer, model SL, from Agilent Technologies. The coupling of the capillary electropherograph with the ESI interface was achieved with a commercial coaxial sheath liquid interface orthogonally positioned to the MS ion source. Nitrogen was used as both drying and nebulizing gas (drying gas flow rate, 8 l/min, drying gas temperature, 150 °C, nebulizer pressure, 3 psi). A mixture of isopropanol/water (50/50) added with 0.5% formic acid was delivered as sheath liquid at a flow rate of 4 μl/min through a 1:100 splitter by a HPLC pump (model 1100, Agilent Technologies). The spray voltage was set at 4 kV and the skimmer at 40 V. MS detection in the selected ion monitoring (SIM) mode was set at the individual m/z of the molecular ion $[M+H]^+$ of each drug [amphetamine 136.2, methamphetamine 150.2, MDA 180.2, MDMA 194.1, ephedrine 166.2, cocaine 304.1, benzoylecgonine 290.1, morphine 286.2, 6-MAM 328.2 and nalorphine (I.S.) 312.2]. For peak identification, the ion trap mass spectrometer was set to perform MS^2 on the molecular ion of the selected compounds, using helium as collision gas.

Quantification was carried out on the basis of molecular ion peak areas by using the internal standard method (I.S.: nalorphine).

2.3. Sample collection and preparation

Hair samples (∼100 mg) were cut from the scalp of the vertex posterior of the head and stored at room temperature until analysis, according to routine methods in use in our forensic toxicology laboratory. Blank hair was obtained from well-known subjects abstinent from any drugs for at least 6 months prior sample collection. When needed, blanks were spiked with suitable amounts of drugs to mimic concentrations usually found in real samples from drug users (0.025–5 ng/mg). "Positive" hair samples from drug abusers were preliminarily analyzed by routine methods based on immunometric screening and HPLC confirmation, as described in a previous article [7]. In short, hair samples were first washed twice with an aqueous solution of 0.3% Tween-20 (20 ml), in order to remove contaminants possibly present on the surface, then cut into small fragments and incubated overnight in 0.1 M HCl (1 ml) at 45 °C. The incubation mixture was then neutralized with equimolar amounts of NaOH and extracted into organic phase with Toxi-Tubes A. The organic layer was collected and evaporated under a stream of air. Finally,

the dried residue was reconstituted in 500 μl of bidistilled water.

Standard curves were prepared by spiking blank hair extracts with suitable amounts of analytes to mimic the following concentrations of drugs in hair: 0.025, 0.05, 0.10, 0.20, 0.50, 1.00, 2.00, 5.00 ng/mg; nalorphine (I.S.) was added to samples at fixed concentrations (1 ng/mg).

3. Results and discussion

3.1. CE–MS optimization

On the basis of the existing literature on CZE–ESI–MS analysis of basic drugs [8,9], an acidic background electrolyte composed of 100 mM formic acid at pH 2.5 was first tested. Unfortunately, the suppression of the electroosmotic flow and the unusual length of the capillary (1 m) required by steric hindrance of the electropherograph and the mass spectrometer, caused long residence times of the analytes in the capillary with consequent peak broadening. Even the application of 0.5 psi forward pressure in addition to voltage to speed up the run did not improve the separation, probably because of the formation of laminar flow inside the capillary. Thus, in order to reduce the analysis times, the electroosmotic flow was increased by raising the buffer pH to 9.5, by addition of ammonia to 25 mM formic acid. Under these conditions, a good separation of almost all analytes was achieved within 20 min.

In order to avoid siphoning between the two electrolyte jars, which could have affected analyte separation and sample stacking at injection [10,11], the relative level of the CE instrument in respect to the MS was carefully optimized, also taking into consideration the suction exerted by the ESI nebulizer. After optimization, a height difference of 9 cm was adopted between the two instruments (CE at a lower level than MS detector) with a nebulizer pressure of 3 psi.

A crucial problem in hair analysis is analytical sensitivity, which is required by the small amounts of sample which for aesthetic reasons can be collected from individuals (typically 20–100 mg) and the low concentration of analytes in the hair matrix (typically 0.1–10 ng/mg). This is particularly relevant in CE analysis, which is typically characterized by a moderate concentration sensitivity. In order to overcome this problem, field amplified sample stacking (FASS) has been used with encouraging results in different biological matrices [11–15]. Consistently with the above assumption, the first experiments carried out with hydrodynamic injection (0.5 psi × ≤40 s) failed to achieve a sensitivity suitable for the determination of drug concentrations in real samples, whereas electrokinetic injection applied under FASS conditions was successful in achieving the needed sensitivity. In this injection procedure, the hair extracts were reconstituted with bidistilled water to keep their conductivity as low as possible. After rinsing the injection end of the capillary from the separation buffer salts, a plug of water was hydrodynamically loaded into the capillary; then the sample was electrokinetically injected at 7 kV for 30 s. During injection, the spray needle voltage was set at 0 kV and the drying gas flow and the nebulizer pressure were also off, to reduce to a minimum any disturbing effect on the stacking phenomenon [11]. Under these conditions the sensitivity was enhanced by approximately 20–50-fold in comparison with hydrodynamic injection without interference from matrix co-extractives.

Table 1
m/z of molecular ions and their main fragment

	$[M+H]^+$	Fragment
Amphetamine	136.2	119.2
Methamphetamine	150.2	119.2
MDA	180.2	163.2
MDMA	194.1	163.2
Ephedrine	166.2	148.2
Cocaine	304.1	182.0
Benzoylecgonine	290.1	168.0
Morphine	286.2	169.1
6-MAM	328.2	165.1
Nalorphine	312.2	270.2

The ESI–MS conditions were optimized in terms of spray voltage (3.5–6 kV), sheath liquid composition and flow rate (2–6 μl/min). As in most of CE–MS applications, the use of a sheath liquid flow was required to maintain the electric conductivity at the cathodic end of the capillary. To this aim, on the basis of literature data, a mixture of isopropanol/water (50/50) added with 0.5% formic acid was chosen. The best results were obtained with a sheath liquid flow of 4 μl/min. The use of a HPLC pump with a 1:100 flow splitter proved to cause a much smoother background signal in comparison with the standard syringe pump provided with the instrument. Temperature (150–350 °C) and flow rate of drying gas (3–10 l/min) were optimized on the basis of direct infusion experiments.

Because of the basic nature of the analytes, positive ion monitoring mode was chosen. Ion trap collision induced fragmentation provided fragment patterns similar to what is reported in the literature, as summarized in Table 1 [8].

3.2. CE–MS method validation

Under the described conditions, amphetamine, methamphetamine, MDA, MDMA, ephedrine, cocaine, benzoylecgonine, morphine, 6-MAM and nalorphine (I.S.) could be analyzed in less than 20 min. Overall, the peak shapes were symmetrical and the efficiency of the separation was excellent (about 400,000 plates/meter), being not inferior to that of the CZE methods with “in capillary” UV detection. The resolution of the different analytes was generally acceptable, with no interference from endogenous compounds, being the coelution of amphetamine and methamphetamine, MDA and MDMA and morphine and 6-MAM resolved on the MS side, on the basis of their different *m/z* (Fig. 1). No interferent peaks were observed at the selected *m/z* values in the electropherograms, from hair samples of subjects not exposed to drugs. Interferences from other major drugs of abuse (barbiturates, THC and its acid metabolite, benzodiazepines) were also excluded (Fig. 2).

The limit of detection (LOD) was calculated for each drug as the lowest analyte concentration in hair matrix giving a signal-

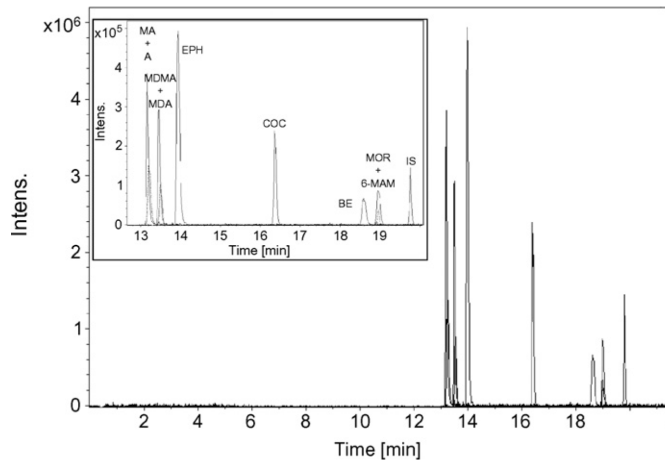

Fig. 1. Superimposed electropherograms of extracted ions from the analysis of a negative hair spiked with a standard mixture of: amphetamine (A), methamphetamine (MA), MDA, MDMA, ephedrine (EPH), cocaine (COC), benzoylecgonine (BE), morphine (MOR), 6-MAM and nalorphine (I.S.) mimicking a concentration of 2.0 ng/mg for each drug. (Insert: enlarged view). Analytical conditions: electrolyte buffer 25 mM ammonium formate, pH 9.5, separation +15 kV, electrokinetic injection 7 kV for 30 s, MS full scan detection in the positive mode. For details see text.

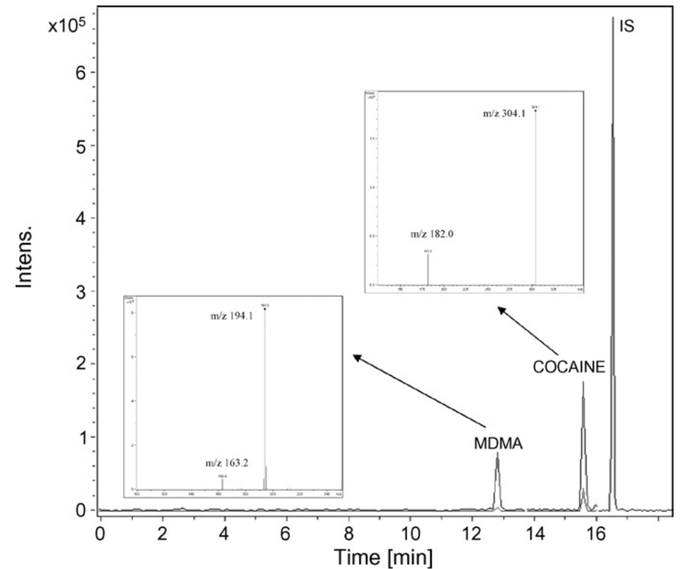

Fig. 3. Extract ion electropherogram of a hair sample from an occasional user of ecstasy and cocaine: presence of MDMA (m/z 194.1) at 0.033 ng/mg and cocaine (m/z 304.1) at 0.08 ng/mg. (Insert: mass spectra of MDMA and cocaine and their major fragment).

to-noise ratio ≥5 on the electropherogram corresponding to the molecular ion. Under the reported FASS conditions, LODs ranged from 0.005 ng/mg for ephedrine to 0.05 ng/mg for morphine, allowing in any case peak identification by fragmentation in the MS^2 mode. Because of its amphoteric nature, benzoylecgonine was less susceptible to FASS and could not be detected below 0.1 ng/mg. This represents a minor problem because benzoylecgonine is known to be incorporated into hair much less than its parent drug cocaine, which is the target analyte in hair for identifying cocaine abuse.

The limit of quantification (LOQ) was calculated for each drug as the lowest analyte concentration in hair matrix giving a signal-to-noise ratio ≥10. The results are shown in Table 2.

The method validation included the study of linearity of response carried out in blank hair extracts spiked with known amounts of each drug ranging from 0.025 to 5 ng/mg of hair. Analytical reproducibility was studied by repeated injections ($n = 5$) of hair extract spiked with the analytes at concentrations of 0.2, 1.0 and 5.0 ng/mg on the same day and on 5 different days. The results of intra-day experiments were characterized by RSD ≤3.3 and ≤1.0 for relative peak areas and relative migration times, respectively, for all the concentrations (0.2, 1.0 and 5.0 ng/mg). The results of day-to-day validation tests are shown in Table 2.

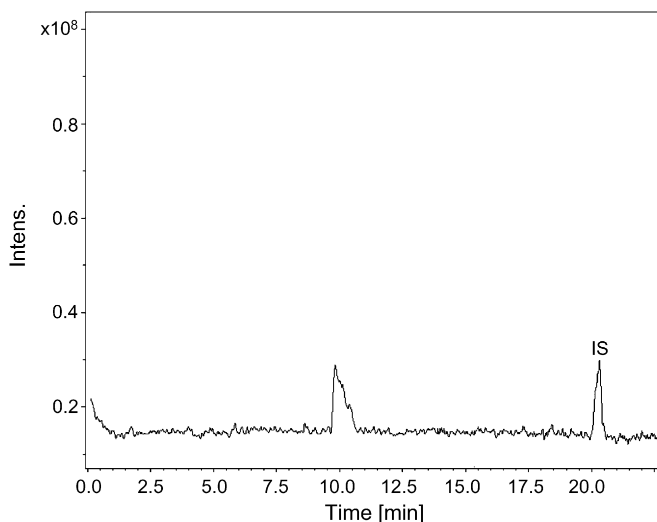

Fig. 2. Total ion electropherogram of a “blank” hair sample (100 mg) spiked with nalorphine (I.S.). For analytical conditions, see text.

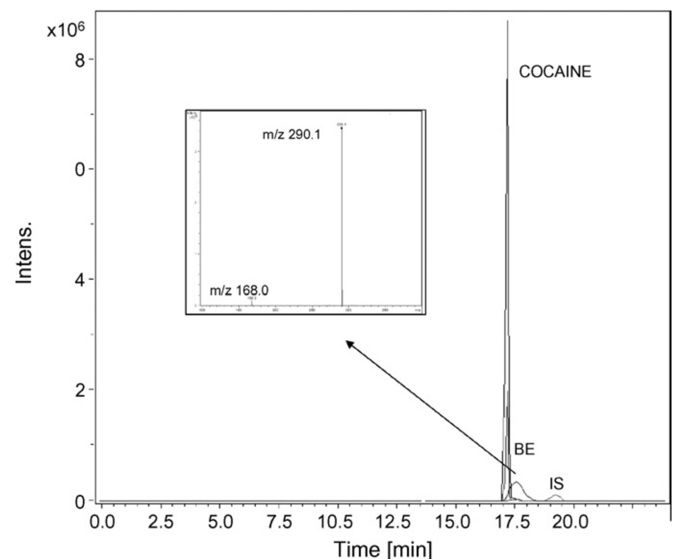

Fig. 4. Extract ion electropherogram of a hair sample from heavy user of cocaine. Determination of cocaine (m/z 304.1) at 4.0 ng/mg and benzoylecgonine (BE) (m/z 290.1) at 0.72 ng/mg. (Insert: mass spectrum of benzoylecgonine, cocaine metabolite, and its major fragment).

Table 2
Validation figures: linearity, analytical sensitivity and precision

Analyte	Linearity	R^2	LOD (s/n = 5) (ng/mg)	LOQ (s/n = 10) (ng/mg)	Repeatability (RSD) day-to-day ($n = 5$)[a]					
					0.2 ng/mg		1 ng/mg		5 ng/mg	
					Area	Time	Area	Time	Area	Time
Amphetamine	$y = 0.9893x - 0.0547$	0.9940	0.040	0.080	22.47	2.72	12.46	3.06	7.86	2.45
MA	$y = 2.2287x - 0.0239$	0.9683	0.025	0.050	16.78	2.09	13.20	3.01	10.20	2.21
MDA	$y = 1.1889x - 0.1411$	0.9892	0.030	0.060	17.67	2.26	15.79	2.84	13.81	2.07
MDMA	$y = 3.3385x + 0.1142$	0.9944	0.020	0.040	18.55	2.08	10.24	2.76	7.22	1.87
Cocaine	$y = 3.5598x + 0.1819$	0.9758	0.015	0.030	14.00	2.52	9.52	2.11	6.51	1.54
Ephedrine	$y = 3.7964x - 0.0064$	0.9725	0.005	0.010	21.25	2.61	20.97	2.08	20.01	1.88
Morphine	$y = 1.0455x - 0.0346$	0.9867	0.050	0.100	6.81	0.93	6.62	0.64	5.53	0.52
6-MAM	$y = 1.112x + 0.0390$	0.9776	0.020	0.040	4.70	0.46	4.26	2.01	4.15	1.22

[a] Data relative to the internal standard.

A final test of the validation procedure was a comparison of the CE–MS results with those from a well established HPLC methods currently used for routine hair analysis [7]. Fig. 3 shows the electropherogram of a hair sample from a real cocaine and ecstasy user. Under the described conditions it was possible to detect MDMA (m/z 194.1) and cocaine (m/z 304.1) at concentrations (0.033 and 0.08 ng/mg, respectively) below the cut-off value of 0.1 ng/mg currently adopted in hair testing. These results were in agreement with the HPLC data (0.045 and 0.09 ng/mg, respectively). Fig. 4 shows a second example from a cocaine addict, "positive" at a HPLC analysis for both cocaine (4.5 ng/mg) and benzoylecgonine (0.78 ng/mg). CE–MS confirmed the HPLC determination, giving concentrations of 4.0 and 0.72 ng/mg for cocaine and benzoylecgonine, respectively.

4. Conclusions

On the basis of the data reported and discussed in the present work, the hyphenation of CZE with MS has proved suitable for rapid, sensitive and unequivocal confirmation of the presence of the major drugs of abuse and their metabolites in hair, thus offering an analytical tool simpler and more versatile than the traditional methods based on GC–MS, requiring solid-phase extraction and derivatization. The present method offers a multi drug screening opportunity by using SIM, with the possibility of confirmation using MS^2, with LODs always better than the traditional cut-off adopted in hair analysis (~0.1 ng/mg) [16]. Quantification has also proved fairly acceptable, even without deuterated I.S., thus offering a much cheaper alternative to GC–MS for quantitative analysis.

Because of a superior versatility of CE in comparison to either gas or liquid chromatography and its demonstrated easy, efficient and robust coupling to MS, the introduction of CZE-ion trap MS in the forensic toxicology laboratories can be seen as a new important achievement to increase both productivity and reliability of results.

Acknowledgements

This work was co-funded by research grants awarded by Ministry of University and Scientific Research (PRIN #2005064329) and by Donazione "Loro-Cherubini".

References

[1] A.M. Baumgartner, P.F. Jones, W.A. Baumgartner, C.T. Black, J. Nucl. Med. 20 (1979) 748.
[2] P. Kintz, Drug Testing in Human Hair, CRC Press, 1996.
[3] F. Tagliaro, F. Bortolotti, Electrophoresis 27 (2006) 231.
[4] E. Varesio, S. Cherkaoui, J.-L. Veuthey, J. High Resol. Chrom. 21 (1998) 653.
[5] A. Ramseier, J. Siethoff, J. Caslavska, W. Thormann, Electrophoresis 21 (2000) 380.
[6] A.B. Wey, J. Caslavka, W. Thormann, J. Chromatogr. A 895 (2000) 133.
[7] F. Tagliaro, R. Valentini, G. Manetto, F. Crivellente, G. Carli, M. Marigo, Forensic Sci. Int. 107 (2000) 121.
[8] W.F. Smyth, P. Brooks, Electrophoresis 25 (2004) 1413.
[9] W.F. Smyth, Electrophoresis 27 (2006) 2051.
[10] P. Schmitt-Kopplin, M. Frommberger, Electrophoresis 24 (2003) 3837.
[11] A.B. Wey, W. Thormann, J. Chromatogr. A 924 (2001) 507.
[12] C.-X. Zhang, W. Thormann, Anal. Chem. 68 (1996) 2523.
[13] C.-X. Zhang, W. Thormann, Anal. Chem. 70 (1998) 540.
[14] F. Tagliaro, G. Manetto, F. Crivellente, D. Scarcella, M. Marigo, Forensic Sci. Int. 92 (1998) 201.
[15] G. Manetto, F. Tagliaro, F. Crivellente, V.L. Pascali, M. Marigo, Electrophoresis 21 (2000) 2891.
[16] O. Drummer (Ed.), Guidelines for Testing Drugs Under International Control in Hair, Sweat and Saliva, United Nations International Drug Control Programme, New York, 1998.