Journal of Chromatography B, 945–946 (2014) 84–91

Contents lists available at ScienceDirect

Journal of Chromatography B

journal homepage: www.elsevier.com/locate/chromb

Development of a method for the analysis of drugs of abuse in vitreous humor by capillary electrophoresis with diode array detection (CE–DAD)

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ARTICLE INFO

Article history:
Received 24 March 2013
Accepted 12 October 2013
Available online 21 October 2013

Keywords:
Capillary zone electrophoresis
Vitreous humor
Forensic toxicology
Cocaine
Liquid chromatography–mass spectrometry

ABSTRACT

This work presents the development of an analytical method based on capillary electrophoresis with diode array detection for the analysis of drugs of abuse and biotransformation products in vitreous humor. Composition of the background electrolyte, implementation of an online pre-concentration strategy and sample preparation procedures were objects of study. The complete electrophoretic separation of 12 analytes (amphetamine, methamphetamine, 3,4-methylenedioxyamphetamine (MDA), 3,4-methylenedioxymethamphetamine (MDMA), 3,4-methylenedioxyethylamphetamine (MDEA), ketamine, cocaine, cocaethylene, lidocaine, morphine, 6-monoacetylmorphine and heroin) and the internal standard *N*-methyl-1-(3,4-methylenedioxyphenyl)-2-butamine (MBDB) was obtained within 13 min of run. The method was validated presenting good linearity ($r^2 > 0.99$), recovery ≥90%, precision better than 12% RSD and acceptable accuracy in the range of 86–118% at three concentration levels (50, 100 and 500 ng/mL). LODs and LOQs in the order of 1–5 ng/mL and 5–10 ng/mL, respectively, were obtained. After validation, the method was applied to eighty-seven vitreous humor samples and the results were compared to those obtained by a liquid chromatography tandem mass spectrometry (LC-MS/MS) screening method, routinely used by the forensic toxicology laboratory of the Sao Paulo State Police, Brazil. Cocaine was detected in 7.1%, cocaethylene in 3.6%, lidocaine in 2.4% and ketamine in 1.2% of the total number of analyzed samples.

1. Introduction

In recent years, the use of non-conventional biological samples in replacement to the traditional ones – urine and blood – has been growing in forensic toxicology for verifying the intake of xenobiotics of several classes. Each biological matrix has its own peculiarities and presents advantages and disadvantages of usage. Knowledge of the stability of analytes in the biological material is essential for toxicological analyses, once various situations contribute to variable time intervals between the collection of the material, its transportation to the laboratory and the moment of analysis [1,2].

Vitreous humor constitutes a relatively simple matrix in analytical terms when compared to urine, blood or its derivatives (serum and plasma), and its usage has been recommended for the analysis of several xenobiotics of forensic interest, mainly in cases of analysis of polytraumatized bodies or bodies in advanced state of decomposition [1–5]. The fluid stays isolated in a compartment relatively protected from external contamination and invasion of microorganisms, constituting a privileged sample when putrefaction stage is of concern. The passage of xenobiotics to the vitreous humor occurs by simple diffusion through the lipidic barrier between the fluid compartment and the blood [1,6]. Drugs with low plasma protein binding and with adequate liposolubility to cross biological barriers, but still hydrosoluble, can diffuse directly from the bloodstream into the vitreous humor. Because vitreous humor contains a high percentage of water (90–98%), it enables the exchange of substances with the blood, and a good correlation between the levels of certain xenobiotics found simultaneously in both fluids, in a given moment, is usually obtained [4,6].

Chromatographic techniques, such as gas chromatography (GC) and high performance liquid chromatography (HPLC) are amply

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1570-0232/$ – see front matter © 2013 Published by Elsevier B.V.
http://dx.doi.org/10.1016/j.jchromb.2013.10.014

utilized in the determination of pharmaceuticals and drugs of abuse in vitreous humor and other biological fluids [3–5,7–9]. In the last decade, capillary electrophoresis (CE) has gained recognition as a separation technique of great utility in the analyses of xenobiotics in biological samples [10–18]. Some laboratories have replaced routine chromatographic methods with CE methods due to analysis time reduction and versatility: with CE it is possible to analyze compounds of varying chemical characteristics, such as mixtures of acidic, neutral and/or basic compounds, in a single run [12]. Other advantages of CE include high efficiency, low consumption of organic solvents and a plethora of separation modes, which allows the analysis of a large variety of solutes, including high-polarity, thermolabile and/or non-volatile compounds in the same capillary column [19–22].

This work describes the development and validation of an analytical procedure for determination of twelve drugs of abuse and a few of their metabolites (amphetamine, methamphetamine, 3,4-methylenedioxyamphetamine (MDA), 3,4-methylenedioxymethamphetamine (MDMA), 3,4-methylenedioxyethylamphetamine (MDEA), ketamine, cocaine, cocaethylene, lidocaine, morphine, 6-monoacetylmorphine and heroin) in vitreous humor by capillary electrophoresis with diode array detection (CE–DAD). The use of capillary electrophoresis for the analysis of vitreous humor samples has been described in the literature [23–26]. However, these articles focused mainly in potassium analysis to estimate post mortem intervals and nitrate levels in diabetic disorder. To our knowledge this is the first account for the analysis of drugs of abuse in human vitreous humor using CE–DAD. Eighty-seven humor vitreous samples were screened and the results were confirmed by a liquid chromatography tandem mass spectrometry (LC–MS/MS) routine methodology.

2. Methods

2.1. Materials and reagents

Standard solutions at 1 mg/mL in methanol or acetonitrile of amphetamine (AMP), methamphetamine (MET), 3,4-methylenedioxyamphetamine (MDA), 3,4-methylenedioxymethamphetamine (MDMA), 3,4-methylenedioxyethylamphetamine (MDEA), ketamine (KET), cocaine (COC), cocaethylene (CET), lidocaine (LID), morphine (MOR), 6-monoacetylmorphine (6-MAM), heroin (HER) and *N*-methyl-1-(3,4-methylenedioxyphenyl)-2-butamine (MBDB, used as internal standard, (IS) were obtained from Cerilliant (Austin, TX, U.S.A.). The chemical structures of the compounds under investigation are depicted in Fig. 1. Phosphoric acid, triethylamine (TEA), methanol, acetonitrile and tris(hydroxymethyl)aminomethane (TRIS) of analytical grade were obtained from Merck (Darmstadt, Germany). Ultrapure water was obtained from a Milli-Q RG Millipore system (Bedford, MA, U.S.A.).

2.2. Instrumentation

2.2.1. Capillary electrophoresis with diode-array detection (CE–DAD)

The capillary electrophoresis experiments were carried out in a HP^{3D}CE instrument (Agilent Technologies, Palo Alto, CA, U.S.A.) equipped with on-column diode-array detector (set to acquire spectra from 190 to 400 nm). Analytes were monitored at 195 nm, except for MOR, 6-MAM and HER that were quantified at 208 nm. The separation was performed in the normal mode, by applying a 25 kV positive voltage across an uncoated fused-silica capillary (Polymicro Technologies, Phoenix, AZ, USA) with an inner diameter of 75 μm and a total length of 48.5 cm (40 cm effective length). The capillary was thermostatted at 20 °C. New capillaries were conditioned with 1 mol/L NaOH solution (30 min), followed by ultrapure water (20 min). At the beginning of the day, the capillary was conditioned with 1 mol/L NaOH for 5 min, followed by ultrapure water for 5 min and then the background electrolyte (BGE). Between runs, the capillary was rinsed with BGE for 2 min. The BGE was composed by a 95:5 v/v mixture of an aqueous solution of TRIS at 20 mmol/L concentration containing 0.4% TEA (pH adjusted to 2.5 with concentrated phosphoric acid) and methanol. Samples were introduced onto the capillary via electrokinetic injection (10 kV for 15 s).

2.2.2. Liquid chromatography tandem mass spectrometry (LC–MS/MS)

The LC–MS/MS analysis was based on the method proposed by Mueller et al. [27] with minor modifications. The liquid chromatography system was composed by an autosampler, column oven and a quaternary pump (Agilent 1200 Series, Agilent, Waldbronn, Germany). For the separation, a Synergi Polar RP analytical column (150 × 2 mm, 4 μm) with a 4 × 2 mm security guard cartridge (Phenomenex, Aschaffenburg, Germany) was used. Mobile phase A consisted of 0.1% formic acid/1 mmol/L ammonium formate solution. Mobile phase B consisted of a 95:5 v/v mixture of acetonitrile and 0.1% formic acid/1 mmol/L ammonium formate solution. A gradient starting with 10%B was increased linearly to 95%B in 20 min, kept constant at 95%B for 3 min, decreased to 10%B in 0.1 min and then the column was re-equilibrated for 10 min. The flow rate was 0.250 mL/min. For the MS/MS analysis, a 3200QTrap system with a Turbo V^{TM} ion source (electrospray ionization) (AB Sciex, Toronto, Canada) was used. The survey scan contained 298 multiple reaction monitoring (MRM) transitions for selected substances, listed by Mueller et al. [27]. The Information Dependent Acquisition (IDA) scan intensity threshold was set to 500 counts per second (cps). The dependent scan was an enhanced product ion (EPI) scan, which was carried out at three different collision energies (20, 35 and 50 eV). Three EPI scans were performed before switching back to MRM mode. The maximum cycle time (MRM and three EPI scans) was 3.8 s. Dynamic exclusion was set to 60 s to allow the detection of coeluting substances. The resulting EPI spectra were then compared to the mass spectral library.

2.3. Sample preparation

Eighty-seven vitreous humor samples were collected at the Institute of Legal Medicine of Sao Paulo, Brazil, without any distinction between right and left eye, by puncture, using a sterile and disposable syringe. The collected fluids were transferred to glass tubes closed by rubber caps, containing no preservatives, and stored at −20 °C until analysis. All samples were screened preliminarily by the LC–MS/MS method listed above (Section 2.2). Those with negative results for drugs of abuse were separated and constituted blank samples used in the method validation studies.

Sample preparation was conducted by liquid-liquid extraction (LLE). To separate conic polypropylene tubes with caps of the same material (capacity of 2 mL), 200 μL of vitreous humor samples and 50 μL of the methanolic solution of the internal standard (MBDB) at 1 μg/mL concentration were pipetted. Exactly 100 μL of 100 mmol/L sodium tetraborate buffer and 1 mL of ethyl acetate were then added, followed by vortexing (60 s) and centrifugation at 12,500 rpm for 5 min. After centrifugation, 900 μL of the supernatant were transferred to a polypropylene vial (proper for the automatic injector of the CE system). The extract was evaporated to dryness under nitrogen (at room temperature) and reconstituted with 100 μL of a hundred fold diluted BGE.

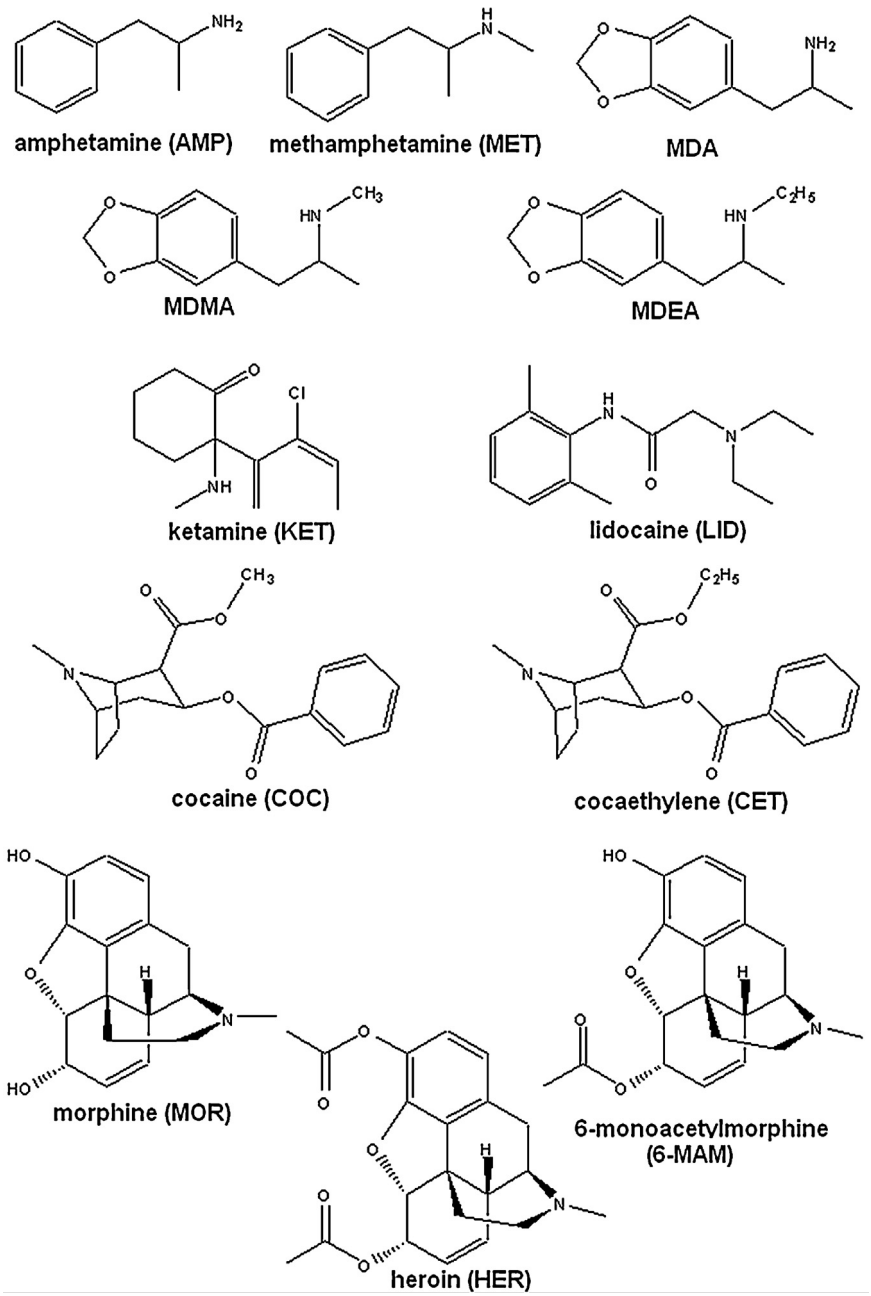

Fig. 1. Chemical structure analyzed of drugs of abuse and metabolites.

For confirmation purposes, the vitreous humor samples were diluted 10 fold in ultrapure water, filtered in 0.45 μm membrane, and injected in the LC–MS/MS system.

2.4. Method validation

The linearity study was conducted with blank vitreous humor samples enriched with analytes standards ranging from 5 to 500 ng/mL, with triplicate injection at each concentration level. Precision was established by analyzing vitreous humor samples at the following concentrations: 50, 100 and 500 ng/mL in three different days with six replicates for each concentration. Limits of detection (LOD) and quantification (LOQ) were evaluated by analyzing a series of vitreous humor blank samples fortified with decreasing amounts of all analytes until signal-to-noise ratios of 3 and 10 were reached, respectively. Accuracy was established at the same concentration levels used in the precision test by comparison of nominal values against values obtained in the analytical curve. Recovery tests were performed by spiking vitreous humor samples at 100 ng/mL of each drug (six replicates) and extracted by the LLE procedure described before (Section 2.3). The results were then compared to blank vitreous humor samples that where submitted to LLE, and spiked with drug standards only during the reconstitution of the dried material into the diluted BGE.

3. Results and discussion

3.1. Method development

The development of an analytical method by capillary electrophoresis must take into consideration the physicochemical characteristics of the analytes. All compounds under examination

in this work exhibit basic characteristics (pK_a values ranging from 7.5 to 10.1). Thus, they are all fully protonated in acidic medium, which allows their analysis by capillary zone electrophoresis as cations, and separation according to differences in electrophoretic effective mobility. Another important advantage the acidic medium offers is enhanced selectivity. At low pH electrolytes, with little or no electroosmotic flow (eof), possible interferents with acidic or neutral characteristics would bear negative or no charge at all and therefore would not pass the detector at measurable times [13]. Another parameter to consider in method development is the counter ion nature, which should be advantageously selected to promote adequate buffering capacity. For these reasons, the initially proposed BGE was an aqueous phosphate buffer solution with pH adjusted close to the system pK_a value (pH 2.50) for maximum buffer capacity.

In capillary zone electrophoresis it is routinary the use of additives indicated in four situations: to reduce the interaction of certain analytes with the capillary wall, to alter the analyte mobility, to modify the eof or to improve the analyte solubilization [28]. Adsorption of analytes of basic characteristics to the negatively ionized silanol groups at the capillary wall (of main concern here) leads to the formation of asymmetrical peaks and tailing, which may affect both qualitative and quantitative analyses. Due to peak broadening, resolution is compromised and peak integration is subjected to error. The deleterious effect of adsorption is often circumvented by incorporation of polyamines or alkylammonium salts to the BGE. Amines are known to block the silanol groups, making the undesirable interaction with the analytes less pronounced. The use of this kind of additive is recommended by several authors who have discussed the CE analysis of cationic substances [13,28–30]. In this work, 0.4% (v/v) TEA was selected as BGE additive.

Another important aspect of method development especially for small ionic analytes is to minimize the electrodispersive phenomenon. For this purpose, the electrolyte co-ion must have an electrophoretic mobility as similar as possible to the mobility of the analytes at the optimum pH. In this work, TRIS was selected as co-ion.

As a first attempt, a BGE composed of 20 mmol/L TRIS and 0.4% TEA (adjusted to pH 2.5 with phosphoric acid) was tested. With this BGE, a complete co-migration between cocaethylene (CET) and morphine (MOR) was observed. To improve resolution the use of organic solvents was further considered and the effective mobility of all analytes examined (Fig. 2A). When methanol was added to the BGE, selectivity was somehow enhanced, especially for the opiate compounds in the mixture. Resolution of COC and MOR improved considerably with increased methanol contents as Fig. 2B demonstrates. However, at higher methanol percentages the CET peak started to overlay its adjacent peak, lidocaine (LID), diminishing prohibitively the resolution between them. Therefore, methanol content was adjusted to modulate the position of CET right in between LID and MOR peaks in such way that the critical solute pairs COC/LID, LID/CET and CET/MOR present equivalent resolution. An optimal BGE composed by a 95:5 v/v mixture of aqueous solution of TRIS at 20 mmol/L containing 0.4% TEA (pH adjusted to 2.5 with phosphoric acid) and methanol was then selected.

Solute mobility is dependent on the solute volume and polar solute-solvent interactions. With methanol in the BGE, the cohesion forces of water decreases, and the solute's hydrophobicity differences are leveled despite intrinsic differences in volume. Therefore, polar solute-solvent interactions must be invoked to explain the separation selectivity. For protonated amines (tertiary amines COC, LID, CET and MOR), the electron pair in the nitrogen atom is no longer available, thus hydrogen bonding must occur via NH and the solvent oxygen electron pair. This interaction is somehow inhibited due to steric hindrance. What is left is dipole moment (identical for

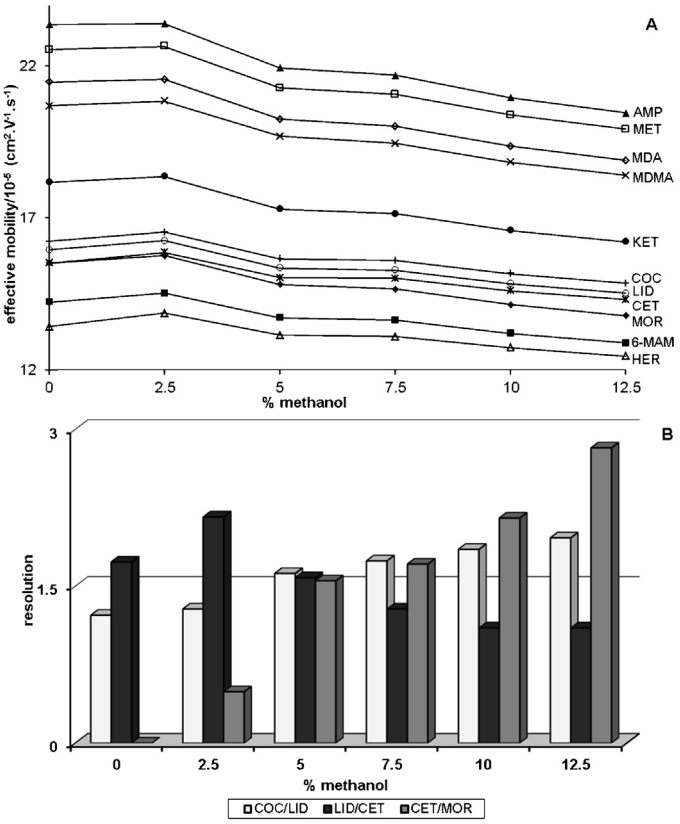

Fig. 2. Effect of the methanol content in the background electrolyte on the effective mobility of analytes (A) and resolution (B) of critical solute pairs. Peak legends as in Fig 1.

all solutes) and polarizability, which might be the discriminatory factor.

To understand the effect of methanol in the separation of the solutes under consideration, stepwise regressions of electrophoretic mobility versus a variety of solute properties (volume, polarizability, partial atomic charge of quaternary nitrogen, HOMO, LUMO, electronegativity, dipole moment, log *P*, etc) were attempted and the corresponding statistics calculated (not shown). Indeed, the results pointed to the polarizability of the protonated solutes as the most relevant variable. With the increase of methanol in the BGE, the ability of the solvent in neutralizing the solute charge decreases. As a result, the solute polarizability, which translates into the ability of the solute in spreading the charge over the molecule, becomes detrimental and governs the separation selectivity.

To improve method performance the use of an internal standard with physicochemical properties similar to the analytes under consideration is usually recommended. A few substances such as procaine, phemproporex, salbutamol and *N*-methyl-1-(3,4-methylenedioxyphenyl)-2-butamine (MDBD) were initially considered and MDBD was found adequate. Although classified as a designer drug, MBDB is rarely found as adulterating substance of Ecstasy pills [31,32].

Fig. 3 depicts an electropherogram extracted in 195 nm and the absorption spectra of the drugs of abuse in consideration in this work as well as a few of their biotransformation products at the optimum condition. Baseline separation of all substances (including the IS) was then achieved in less than 13 min, which is considerably shorter than most methods presented in the literature. Tagliaro and coworkers compared the use of capillary zone electrophoresis (CZE) and micellar electrokinetic capillary chromatography (MEKC) for the analysis of 20 drugs [33]. Most of them

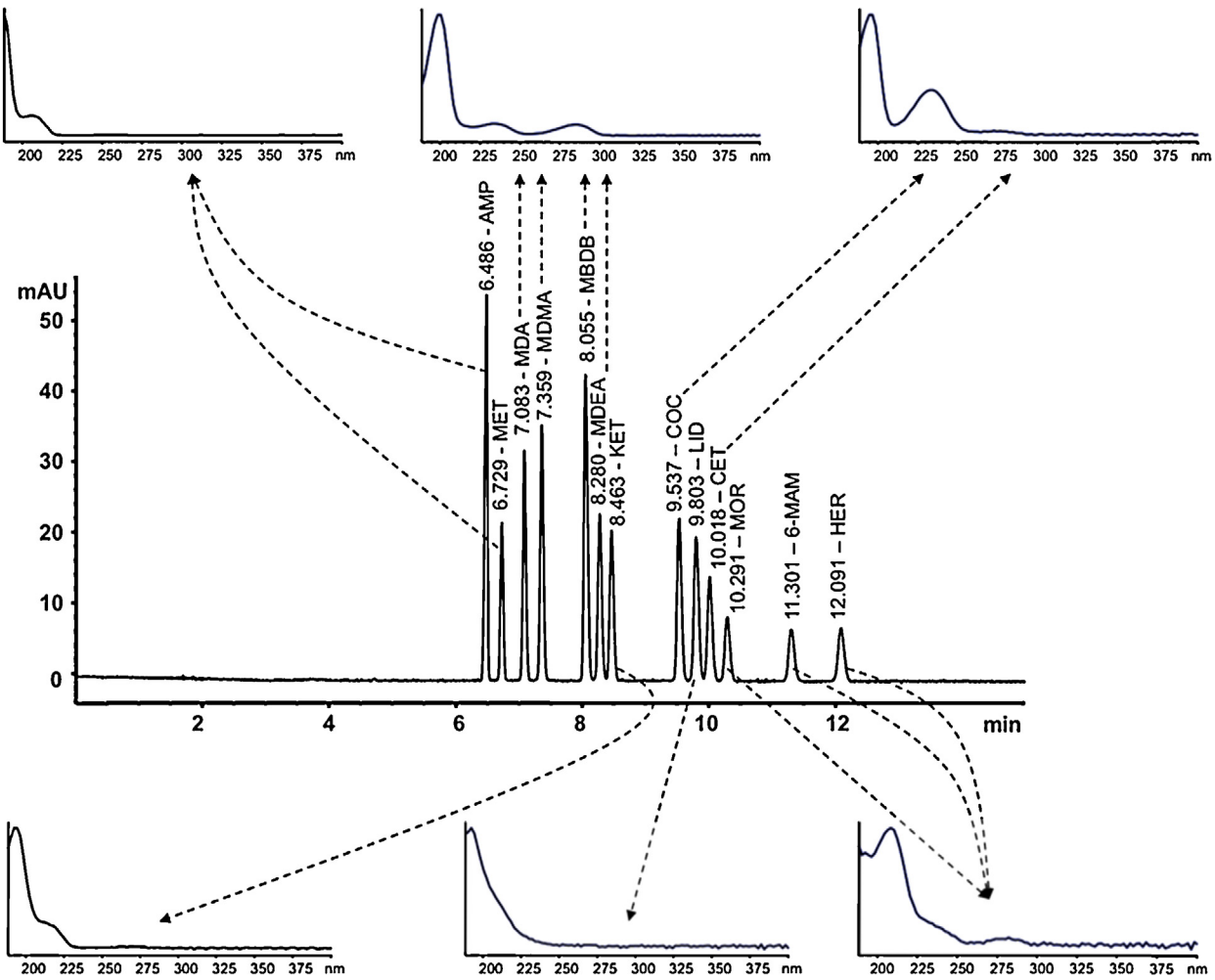

Fig. 3. Electropherogram of a mixture of drugs of abuse at 100 ng/mL obtained with the optimized electrolyte and corresponding on-line acquired UV absorption spectra. Electrophoretic conditions: fused silica capillary 75 μm i.d., 48.5 × 40 cm (effective length); BGE: 95 : 5 v/v mixture of TRIS at 20 mmol/L containing 0.4% TEA (pH adjusted to 2.5 with concentrated phosphoric acid) and methanol; 25 kV, 20 °C, 195 nm; electrokinetic injection: ultrapure water at 20 mbar/2 s, followed by sample at 15 kV/10 s. Peak legends as in Fig. 1.

were separated by either CZE or MEKC, however, with a running time of 50 min. Dahlén and Eckardstein developed a CZE method for the separation of amphetamine and 13 amphetamine analogues [34]. The method was applied to the analysis of street samples (seized drugs) and presented a running time of 23 min.

3.2. Sample preparation considerations

The small volume available of the biological fluid under consideration here (less than 2 mL) was determinant for the planning of the procedure adopted for extraction. The aliquots used in the analyses (200 μL) allowed replicate sampling for the proposed method as well as for the LC–MS/MS confirmatory method.

Initially sample preparation based on simple dilution of the vitreous humor sample and direct injection in the electrophoretic system was considered. This procedure was rejected because poor limits of detection were obtained. These inadequate results may be related to the composition of vitreous humor, which contains a large quantity of dissolved ions, mainly Na^+, K^+ and Cl^-, decreasing the efficiency of the electrokinetic injection.

Liquid–liquid extraction (LLE) is a simple and cost effective procedure, sometimes neglected due to high consumption of organic solvents (when compared to other techniques, such as solid-phase extraction, SPE). In the extraction method proposed here, a maximum of 2 mL of organic solvent per analyzed sample was considered. Since the entire extractive procedure was conducted in disposable polypropylene tubes using automatic pipettes with equally disposable tips, the possibility of accidental contamination during sample processing was drastically minimized, thus contributing to the reliability required for a method with forensic purpose.

The recovery of basic organic compounds in aqueous biological fluids during LLE with apolar solvents is augmented by sample alkalinization because it enhances the compound solubility in the organic phase favoring partition towards the solvent. Initially sample alkalinization with 100 μL of 1 mol/L NaOH was tested and ethyl acetate was used as organic extractor. An excellent extraction performance was observed for the amphetaminic derivatives (AMP, MET, MDA, MDMA, MDEA, KET) and for lidocaine (≥ 90%). However, recoveries for cocaine, cocaethylene and for the opioids (MOR, 6-MAM and HER) were poor, with 6-MAM and HER actually not being detected at all. This can be explained by the chemical instability of these substances, all presenting ester groups (see Fig. 1), which undergo hydrolysis with relative easy if the medium is too basic. The use of an aqueous solution of sodium tetraborate at 100 mmol/L concentration was next tested as sample alkalinizer. This solution was able to buffer the sample to approximately pH 9.2, as well as to promote analytes extraction with good recoveries (≥ 90% for all substances), without compromising sample stability.

There are innumerous organic solvents that can be used in LLE of drugs of abuse. Traditionally, it is required for the solvent to be immiscible with water to facilitate the separation of phases when in contact with the biological fluid. Recently, organic solvents of higher polarity, such as acetonitrile, have been used in LLE procedures, where the separation of phases is promoted by cooling the mixture followed by centrifugation [35,36]. Cold LLE seems to be very interesting when the subsequent analytical procedure is conducted in liquid phase, such as liquid chromatography or capillary electrophoresis. However, it must be emphasized that in cold LLE the extracting solvent is usually not evaporated at the end. Thus, an additional analyte preconcentration does not take place, which may be of concern if capillary electrophoresis is chosen as separation technique.

The extracting solvent, ethyl acetate, was chosen here based on procedures recommended by the International Program of Drugs Control of the United Nations [37]. It has relatively high volatility (which makes the evaporation step faster), lower toxicity when compared to other possible candidates, as *N*-hexane and toluene and it is not chlorinated, therefore not leaving persistent residues in the environment. In addition, the dry residues obtained after LLE were reconstituted in a 100 fold diluted BGE.

3.3. Online preconcentration

It is well recognized in the literature that capillary electrophoresis presents limitations regarding concentration sensitivity, in part due to the short optical pathlength represented by the capillary inner diameter, and the diminute amount of sample injected in the capillary, in the order of nanoliters [28,38]. Several on-line sensitivity enhancement strategies have been proposed throughout the years with varying performance being stacking, which relies on the velocity manipulation of analyte in relation to the BGE, one of the easiest to implement with fair enrichment gains [39,40]. In this work, a combination of injection of a water plug prior to electrokinetic sample injection was studied by a 2^3 factorial design. The design variables comprised injection time (2 and 4 s) for the water plug at 20 mBar, and applied voltage (5 and 10 kV) and injection time (5 and 15 s) for the sample plug. Considering the enhancement of signal-to-noise ratios of all analytes, a condition of water plug injection at 20 mBar for 2 s followed by electrokinetic sample injection of 10 kV for 15 s provided the best response (approximately 10 fold enhancement) and it was used for sample quantitation.

3.4. Method validation

The proposed method was validated in the parameters selectivity, linearity, limits of detection (LOD) and quantitation (LOQ), precision, accuracy, and recovery according to protocols commonly accepted in forensic analysis [41].

Selectivity was established by inspection of blank vitreous humor samples spiked with drugs of abuse likely to be found in intoxication episodes, overdose situations or in cases that denote regular use. Within this context, as Fig. 3 confirms, the method can be considered selective.

Table 1 depicts the results of method validation regarding linearity and limits of detection and quantification. Plots of peak area ratios (analyte to IS) versus concentration exhibited adequate linearity for the studied analytes with acceptable statistical parameters, coefficient of determination >0.98 and small regression error.

LOD and LOQ values were estimated by serial dilutions of stock standard solutions until concentrations that provided signal-to-noise ratios of 3 and 10, respectively. In Table 1, LODs and LOQs were smaller than 5 ng/mL and 10 ng/mL, respectively. In overdose situations the concentration of xenobiotics in blood and probably in vitreous humor is significantly high (over 500 ng/mL). The method presented here is capable of detecting the presence of these drugs in concentrations much lower than this threshold value, and quantifying samples with concentrations a hundred fold lower. It was decided to work here in a lower concentration range than what is expected in overdose situations because it would be possible to identify and to quantify drugs not only in acute intoxications, but also in recreational uses. Positive samples that presented higher concentration than the upper limit of the analytical curve were diluted with blank vitreous humor and reprocessed for reliable quantitation.

Table 1
Method validation regarding linearity and limits of detection (LOD) and quantification (LOQ).

Analyte	r^2	Regression error	LOD (ng/mL)	LOQ (ng/mL)
AMP	0.988	0.062	1	5
MET	0.997	0.022	2	5
MDA	0.997	0.047	2	5
MDMA	0.993	0.072	2	5
MDEA	0.999	0.022	2	5
KET	0.999	0.017	2	5
COC	0.999	0.018	2	5
LID	0.999	0.011	2	5
CET	0.986	0.046	2	5
MOR	0.998	0.0075	5	10
6-MAM	0.975	0.025	5	10
HER	0.976	0.024	5	10

Table 2 compiles the results of the method precision and accuracy reported for three concentration levels. Relative standard deviations better than 12% and accuracy between 86 and 118% were found.

3.5. Sample analysis

All the analytes studied here possess chromophoric groups in their chemical structure, with significant absorption of the electromagnetic radiation in the ultraviolet region (190–350 nm) and regions of maximum absorptivity between 195 and 210 nm as depicted in the inserts of Fig. 3. In order to obtain maximum sensitivity, quantitative analyses were conducted with electropherograms extracted in the wavelength of 195 nm (except for MOR, 6-MAM and HER, where the quantification was done in 208 nm). Relative migration times were used as an initial identification criterion and relative peak areas were considered in the sample quantitation. Even though an UV spectrum is not as informative as a mass spectrum of a given substance, it provides another substance-dependent information and, combined with migration time, it is usually more reliable for peak assignment than migration time alone. Thus, the combination of relative migration time with a comprehensive UV spectra database (made in house by injection of authentic standard solutions of each analyte) was used here as analyte identification criterion.

Fig. 4 depicts electropherograms of two real vitreous humor samples submitted to the proposed analytical method with positive results for cocaine (Fig. 4A) and for cocaine, lidocaine and cocaethylene (Fig. 4B).

Table 3 compiles the CE–DAD quantitative results of the examined samples that presented at least one of the substances investigated here, i.e. 9.5% of the total number of analyzed samples (eighty seven). As observed, cocaine was detected in 7.1%, cocaethylene in 3.6%, lidocaine in 2.4% and ketamine in 1.2% of the total number of analyzed samples. The highest incidence of positive results for cocaine was expected, once this substance is recognizably related to criminal occurrences, and conflicts or disputes related to the narcotraffic due to increased feeling of alertness, supremacy, restlessness, irritability and aggressiveness usually associated to cocaine consumption. Similarly, the absence

Table 2
Parameters of analytical reliability of the validated CE–DAD method for determination of drugs of abuse and biotransformation products in vitreous humor.

	AMP	MET	MDA	MDMA	MDEA	CET	COC	LID	CET	MOR	6-MAM	HER
Precision (%RSD)*												
50 ng/mL	6.8	7.5	3.4	2.9	4.2	4.2	5.7	6.8	4.5	10.3	9.0	12.3
100 ng/mL	4.5	3.8	2.7	3.3	3.1	3.4	2.7	4.7	3.5	8.8	7.5	8.4
500 ng/mL	5.3	4.7	4.9	5.3	8.1	8.6	3.2	7.9	8.4	7.3	5.6	5.1
Accuracy (%)												
50 ng/mL	89	86	101	96	86	101	100	95	111	89	101	115
100 ng/mL	92	100	100	94	104	100	97	104	99	105	118	115
500 ng/mL	98	99	100	99	99	100	99	100	97	101	103	104

* RSD, relative standard deviation (%).

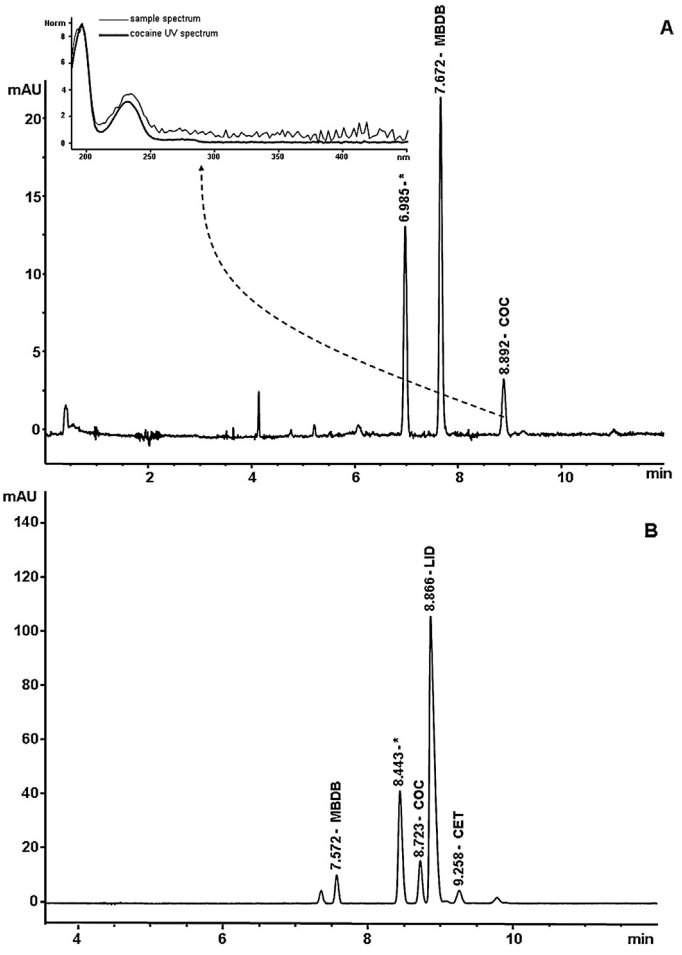

Fig. 4. Electropherograms of two real vitreous humor samples submitted to the proposed analytical CE–DAD method with positive results for cocaine (A, sample #1, Table 3) and for cocaine, lidocaine and cocaethylene (B, sample 5, Table 3). Electrophoretic conditions as in Fig. 3 (*unidentified peak).

of positive results for the amphetaminic derivates (MDMA, MDA and MDEA) can be explained by the effects they cause and by the circumstances related to their use, as these substances produce euphoria and empathy of the users, without increasing aggressiveness. Amphetamine, methamphetamine and heroin (consequently 6-monoacethylmorphine and morphine) were not detected probably due to the pattern of use of these substances, which represent a serious public health problem in the United States of America and in some countries of European Union, but still little used in Brazil.

All positive results found with the CE–DAD method were confirmed by a screening method currently under routine use in the forensic toxicology laboratory of the Sao Paulo State Police based on LC-MS/MS. The results were also compiled in Table 3. In the case of positive samples for the presence of cocaine, this confirmation comes from the similarity among mass spectra, as well as from the presence of other biotransformation products, benzoylecgonine (BEC) and ecgonine methyl ester (EME). It is important to note that benzoylecgonine and ecgonine methyl ester were not detected by the CE–DAD method. In fact ecgonine does not absorb in the UV wavelength used for detection but benzoylecgonine with an aromatic moiety is expected to. Nevertheless BEC was not detected within an allowed timeframe of 1 h run. One possible explanation relies on BEC physical chemical properties: it presents a pK_a of 3.15 related to the dissociation of the carboxylic acid moiety and a pK_a of 9.54 related to the protonation of the amine group. Therefore, at the optimal BGE pH (2.5), the molecule exhibits only a partial positive charge, translated into a marginal electrophoretic mobility.

The use of liquid chromatography with diode array detection (HPLC–DAD) in toxicological analysis is well established in the literature [42–47]. It has been shown in a systematic study with more than 2500 toxicologically relevant substances that UV spectra have a fairly high specificity with respect to substance structure [29]. In this way the proposed CE–DAD method could work as a second technique based on a different chemical principle to forensic toxicology analyses. Moreover, in accordance to the principles of forensic analysis the quantitation of an analyte by CE–DAD may serve as acceptable confirmation of its identity if it was initially detected by a LC–MS/MS.

Table 3
Results obtained in the analyses of drugs of abuse and biotransformation products in vitreous humor by the proposed CE–DAD and confirmatory LC–MS/MS methods.

Sample no.	Criminal occurrence	Results	
		CE–DAD	LC–MS/MS
1	N/A	COC (28 ng/mL)	COC, BEC, EME
2	Car accident	KET (630 ng/mL)	KET
3	Car accident	COC (52 ng/mL), CET (23 ng/mL)	COC, BEC, EME, CET
4	Homicide	COC (6 ng/mL), CET (15 ng/mL)	COC, BEC, EME, CET
5	Homicide	COC (119 ng/mL), LID (1420 ng/mL), CET (21 ng/mL)	COC, BEC, EME, CET, LID
6	Homicide	COC (15 ng/mL)	COC, BEC, EME
7	Homicide	COC (68 ng/mL)	COC, BEC, EME
8	Fall	LID (985 ng/mL)	LID, tramadol, bupivacaine,

Legend: COC: cocaine; lido: lidocaine; CET: cocaethylene; BEC: benzoilecgonine; EME: ecgonine methyl ester; KET: ketamine; LID: lidocaine.

4. Conclusions

In this work, a fast and reliable CE–DAD method for the screening and quantitation of drugs of abuse and a few of their transformation products in vitreous humor samples has been proposed. Because the method is based on a distinct separation mechanism it can be considered complementary to well established liquid chromatography methods in the forensic scenario. Moreover, it provided acceptable performance characteristics and identity confirmation for toxicologically relevant substances.

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