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Chiral separation of amphetamines by high-performance capillary electrophoresis

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Abstract

The chiral separation of amphetamines is of great importance in clinical and forensic analysis, because it is well known that enantiomers do not have identical pharmacological activity; for example, *d*-amphetamine is 3–4 times more effective in central nervous system stimulation than the *l*-isomer, whereas the latter is slightly more potent in its cardiovascular action. This paper describes the method development for the chiral separation of a mixture of amphetamine analogues by cyclodextrin-modified capillary zone electrophoresis. The use of different cyclodextrin types as chiral selectors and the influence of experimental parameters such as the temperature, the voltage applied, the buffer concentration and the capillary length are investigated. The ability to determine the enantiomers in urine samples is also discussed.

1. Introduction

Amphetamine and its analogues are substances which have a potent central nervous system (CNS) stimulating effect. Some amphetamines have been accepted for their medical usage for years, whereas others have never been medically accepted and are purely drugs of abuse [1]. The amphetamine analogues most commonly consumed illegally in Europe are amphetamine (A) itself, methamphetamine (MA), methylenedioxyamphetamine (MDA), methylenedioxymethamphetamine (MDMA or Ecstasy) and more recently methylenedioxyethylamphetamine (MDE or Eve). All of these compounds possess a chiral centre and it is well known that the *d*- and *l*-enantiomers have different pharmacological activities. For example, *d*-methamphetamine is a more effective CNS stimulant than its *l*-enantiomer [2]. Hence it is necessary to develop an analytical procedure which can separate the enantiomers in biological matrices such as urine, serum, plasma or whole blood. Further, enantiomer separation can be useful for forensic analyses in order to identify the synthetic pathways of clandestine amphetamine samples [3].

The enantiomeric separation of amphetamine analogues can be carried out by numerous techniques. However, chromatographic methods such as gas chromatography (GC) [4–7] and high-performance liquid chromatography (HPLC) [8–12], in direct or indirect mode, are the most convenient. In the case of biological matrices, amphetamine concentrations have to be detected in the range 0.01–10 μg/ml [1] and often a prederivatization step is necessary in order to reach these limits of detection with both techniques. In the last few years, high-performance capillary electrophoresis (CE) has become

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a complementary analytical tool to the classical GC and HPLC techniques for the separation of drugs in biological matrices; the advantages of CE include small injection volumes, high separation efficiency, high resolution, low consumption of polluting solvents and rapid and inexpensive analyses.

Many recent publications have reviewed the applications of CE in enantiomeric separations [13–22] and numerous possibilities are available for the separation of drugs such as amphetamine analogues. As for chromatography, it is possible to separate enantiomers directly or after a pre-derivatization step. In the separation of amphetamine analogues, Lurie [23] used 2,3,4,6-tetra-O-acetyl-β-D-glucopyranosyl isothiocyanate (GITC) as a derivatization reagent and separated the diastereomers by micellar electrokinetic chromatography (MEKC). The chiral separation of amphetamine, methamphetamine, ephedrine, pseudoephedrine, norephedrine and norpseudoephedrine is carried out with UV detection. However, most applications are conducted by CE with β-cyclodextrin as chiral selector in the buffer mobile phase. Different β-cyclodextrin derivatives have been used with success in order to separate ephedrine and analogous compounds [24–31]. Nevertheless, few publications have dealt with the analysis of biological matrices and, to our knowledge, no enantiomeric separation of MDA, MDMA and MDE has been reported. This paper describes the enantiomeric separation of these compounds by capillary electrophoresis with (2-hydroxy)propyl-β-cyclodextrin as a chiral selector. Different parameters were optimized and the method was successfully applied to urine samples.

2. Experimental

2.1. Instrumentation

The experiments were carried out on a HP^{3D}CE system (Hewlett-Packard, Waldbronn, Germany). This system consists of a capillary electrophoresis unit equipped with a diode-array detector, an autosampler and a high-velocity air-cooled capillary cartridge. The HP^{3D}CE ChemStation software was used for instrument control, data acquisition and data analysis. Hewlett-Packard capillaries of 50 μm I.D. (375 μm O.D.) and 64.5 cm total length (56 cm from inlet to the detector window) were used for all experiments unless stated otherwise. These capillaries were made of fused silica and equipped with an extended path-length detection window of 150 μm I.D. ("bubble cells"). New capillaries were flushed for 3 min with 1 *M* NaOH, then for 5 min with 0.1 *M* NaOH and finally for 10 min with water. Between each run, the capillaries were flushed for 5 min with phosphate buffer. When analysing biological samples, the capillaries were first flushed for 2 min with 0.1 *M* phosphoric acid and then for 5 min with phosphate buffer.

2.2. Chemicals

All the cyclodextrins were purchased from Cyclolab (Budapest, Hungary), except α- and β-cyclodextrin, which were from Sigma (St. Louis, MO, USA). Amphetamine, 3,4-methylenedioxyamphetamine (MDA) and 3,4-methylenedioxymethamphetamine (MDMA) were purchased as racemic mixtures from Sigma and the optically pure enantiomers (99%) of amphetamine and methamphetamine and racemic 3,4-methylenedioxyethylamphetamine (MDE) were purchased as standard solutions in methanol from Alltech (Deerfield, IL, USA). Phenylethylamine from Fluka (Buchs, Switzerland) was used as an internal standard. Individual stock solutions (100 μg/ml) of each racemic amphetamine were prepared in 0.1 *M* hydrochloric acid and were kept at 4°C. All the other reagents and solvents were of analytical-reagent grade and obtained from Fluka, except for methanol, which was supplied by SDS (Peypin, France). Ultrapure water was provided by a Milli-Q RG unit from Millipore (Bedford, MA, USA).

2.3. Electrophoretic conditions

Samples (5 nl) were injected by pressure (50 mbar for 4 s) and electrophoresis was performed

at a constant voltage of 25 kV (388 V/cm) after a 1-min ramp step. This ramp of voltage is used to ensure there is no loss of sample on injection, which could lead to some problems in quantification [32]. All runs were carried out at 15°C unless stated otherwise. Detection was performed using a diode-array detector scanning the wavelengths from 190 to 600 nm; the electropherograms were monitored at 200 nm with a band width of 10 nm and a reference signal at 350 nm (band width of 80 nm).

2.4. Buffer preparation

For 100 ml of 200 m*M* phosphate buffer (buffer A), we dissolved 1.153 g of 85% phosphoric acid and 1.212 g of 99% anhydrous NaH_2PO_4 in water in a 100-ml volumetric flask. The pH was adjusted to 2.5 with 1 *M* NaOH and the mixture was diluted to 100 ml with water. The buffer solution was filtered through a 0.45-μm nylon syringe filter.

In order to obtain a 20 m*M* (2-hydroxy)-propyl-β-cyclodextrin buffer solution, we dissolved 0.300 g of chiral selector in 10 ml of phosphate buffer. After the addition of the chiral selector, the buffer was filtered through a 0.2-μm nylon syringe filter prior to use.

The phosphate buffer used for solid-phase extraction experiments was 100 m*M* phosphate buffer prepared by dissolving 1.212 g of 99% anhydrous NaH_2PO_4 in water in a 100-ml volumetric flask. The pH was adjusted to 6.0 with 1 *M* sodium hydroxide and the mixture was diluted to 100 ml with water.

2.5. Preparation of urine samples

One part of a urine sample (blank) was spiked with 1 μg/ml each of racemic amphetamines (amphetamine, methamphetamine, MDA, MDMA, MDE and phenylethylamine as an internal standard), and was either pretreated using one of the following procedures, or not pretreated and directly injected. The same procedures were applied to the unspiked urine sample.

Ultrafiltration

We used a MicroSpin 24S centrifuge from Sorvall Instruments (DuPont, Wilmington, DE, USA) and MicroCon 10 filters units with a molecular mass cut-off 10 000 from Amicon (Beverly, MA, USA). For centrifugation, 500 μl of urine sample were transferred into the ultrafiltration device and were centrifuged at ca. 12 500 *g* for 20 min, then the filtrate was injected.

Solid-phase extraction (SPE)

Bond Elut Certify cartridges (Varian, San Fernando, CA, USA) were used on a Visiprep SPE vacuum manifold unit from Supelco (Bellefonte, PA, USA). The extraction procedure was carried out according to Gan et al. [33]. A 2-ml volume of urine was transferred into 15-ml plastic conical-bottomed tubes, to which were added 2 ml of 0.1 *M* phosphate buffer (pH 6.0). The tubes were vortexed mixed for 15 s. Initially, the Bond Elut Certify cartridges were conditioned with 2 ml of methanol and 2 ml of 0.1 *M* phosphate buffer (pH 6.0). Volumes of 2 ml of urine samples were introduced and slowly drawn through the columns under low vacuum. The columns were then rinsed with 1 ml of 1 *M* acetic acid, then dried under vacuum for 5 min. The columns were rinsed with 6 ml of methanol, and then dried again under vacuum for 2 min. The analytes were eluted with 5 ml of 2% ammonia solution in ethyl acetate (freshly prepared). The eluate was evaporated to dryness under a slow stream of nitrogen at room temperature. Two drops of acetic acid were added to the eluate to prevent losses of the volatile amphetamines during the evaporation step [34]. Finally, 1 ml of 0.1 *M* hydrochloric acid was added to solubilize the analytes before injection.

Liquid–liquid extraction

We followed the procedure described by Kinberger [35]. To 500 μl of urine were added 100 μl of 1 *M* sodium hydroxide. After vortex mixing for 30 s, 1 ml of diethyl ether was added and the tube was vortex mixed again for 1 min. After centrifugation (5 min at ca. 12 500 *g*), the organic layer was transferred into another tube, then 50 μl of 0.1 *M* hydrochloric acid were

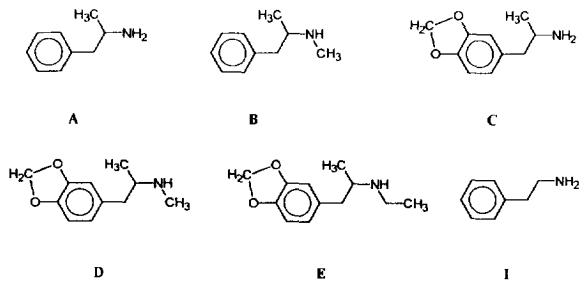

Fig. 1. Structures of the amphetamines tested. (A) Amphetamine; (B) methamphetamine; (C) 3,4-methylenedioxyamphetamine (MDA); (D) 3,4-methylenedioxymethamphetamine (MDMA, Adam or Ecstasy); (E) 3,4-methylenedioxyethylamphetamine (MDE or Eve); (I) phenylethylamine (internal standard).

added and the tube was vortex mixed for 1 min and centrifuged for 2 min. The diethyl ether layer was aspirated off and discarded. The acidic phase was injected without any further treatment.

3. Results and discussion

All the method development steps were carried out on a racemic amphetamine sample in order to determine the influence of some experimental factors on the resolution of the separation. Once the method had been optimized, we analysed a mixture of five racemic amphetamines (Fig. 1) with an internal standard for further quantification. Unless stated otherwise, all the samples used in this study were injected three times each.

3.1. Choice of the chiral selector and its concentration

In capillary zone electrophoresis (CZE), many types of chiral selectors, such as cyclodextrins, crown ethers, oligosaccharides and macrocyclic antibiotics, may be applied for enantiomeric separations. However, cyclodextrins (CDs) are by far the most commonly used. CDs are cyclic, non-reducing oligosaccharides with a truncated cylindrical molecular shape. They are produced from the decomposition of starch by the bacterial enzyme cyclodextrin glycosyltransferase. Many cyclodextrin derivatives have been developed in order to increase their solubility in water and to modify their cavity shape [36–38].

As mentioned by Terabe et al. [20], the enantiorecognition mechanism is based on the formation of an inclusion complex between the chiral selector and the analyte. The enantiomeric separation by cyclodextrin-modified CZE is based on the difference in the stability constants of a pair of enantiomers with the chiral selector.

In our study, we tested different kinds of α- and β-CDs as the γ-CDs are not suitable for the size of the tested amphetamines because their cavities are too large. As shown in Table 1, no resolution of the enantiomers occurs when using α-CDs. This is explained by the fact that, in

Table 1
Resolution obtained with different kinds of chiral selectors

Chiral selector	MT[a] (min)		Resolution
	l-Form	*d*-Form	
20 m*M* α-CD	11.9	11.9	0.0
17 m*M* (2-hydroxy)propyl-α-CD	12.3	12.3	0.0
20 m*M* dimethyl-β-CD	20.9	21.1	0.8
20 m*M* methyl-β-CD	16.8	17.0	0.9
22 m*M* β-CD in 1 *M* urea	19.6	19.9	2.1
20 m*M* (2-hydroxy)propyl-β-CD	15.7	16.0	2.2

Buffer A with a chiral selector. Other conditions: injection at 200 mbar s (pressure); $E = 388$ V/cm (1-min voltage ramp); $T = 15$°C; detection wavelength = 200 nm; bandwidth = 10 nm. Sample: *d,l*-amphetamine (15 μg/ml) in 0.1 *M* HCl.
[a] Migration time.

contrast to γ-CDs, the size of the cavity is too small for the inclusion of the amphetamine.

However, we obtained good resolution values with the β-CDs. This resolution seems to be dependent on the polarity and on the steric hindrance of the substituents. Good resolution was achieved with the native β-CD and also with (2-hydroxy)propyl-β-CD. It is important to note that urea has to be added to the native β-CD in order to increase its solubility in the buffer solution. Without urea or some other co-solvents such as acids or bases, the solubility of the β-CD is limited to 16 m*M* [39–41]. We chose to work further with (2-hydroxy)propyl-β-CD because it offers many advantages over native β-CD: a slightly better resolution, a shorter analysis time and a more stable baseline with UV detection at 200 nm. We also varied the concentration of the chiral selector from 10 to 40 m*M* in the phosphate buffer (Fig. 2). The resolution is improved with an increase in the chiral selector concentration, but at the expense of the analysis time. We chose a concentration of 20 m*M* of (2-hydroxy)propyl-β-CD in the buffer because the resolution was sufficient with a shorter analysis time. We also chose this concentration for economic reasons, because CD derivatives are fairly expensive.

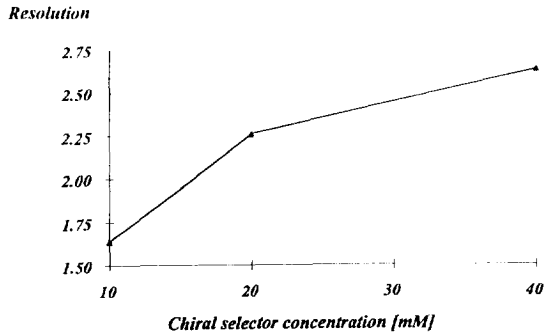

Fig. 2. Effect of chiral selector concentration on resolution. Buffer A with (2-hydroxy)propyl-β-CD as a chiral selector. Sample: *d,l*-amphetamine (15 μg/ml) in 0.1 *M* HCl. For other experimental conditions, see Table 1.

3.2. Effect of temperature

The temperature of the capillary has to be very carefully controlled as it can modify the viscosity of the buffer used for the separation and thus influence the electroosmotic flow velocity. This could lead to variations in the migration times (MT), and therefore to unsatisfactory run-to-run or day-to-day reproducibility.

On increasing the temperature, higher diffusion occurs which enhances the band broadening of the peaks and decreases the efficiency. As can be seen in Fig. 3, the resolution between the enantiomers decreases with increase in temperature. We chose 15°C, which is the lowest temperature for the instrument when working at ambient temperature (25°C).

Wren and Rowe [29,42] proposed a mathematical model to explain the chiral separation of a pair of enantiomers in capillary zone electrophoresis. In their model, the resolution between two enantiomers depends on several parameters, including the equilibrium constants of the inclusion complexes [42,43]. Varying the temperature affects the thermodynamics of complex formation and therefore modifies the resolution between enantiomers.

3.3. Effect of applied voltage

The applied voltage influences mainly the electroosmotic flow velocity, the Joule heating and also the peak efficiency. We found that on increasing the voltage, the migration time de-

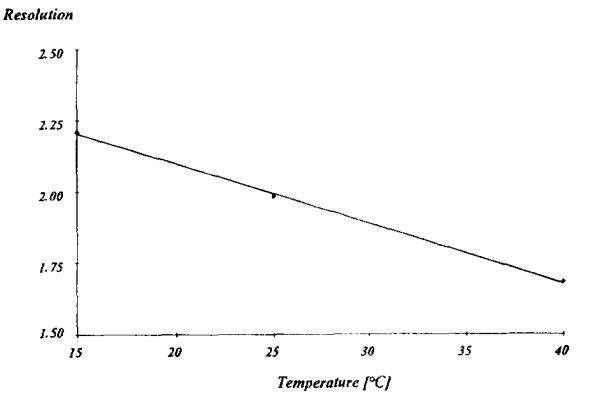

Fig. 3. Effect of temperature on resolution. Buffer A with 20 m*M* (2-hydroxy)propyl-β-CD. Sample: *d,l*-amphetamine (30 μg/ml) in 0.1 *M* HCl. For other experimental conditions, see Table 1.

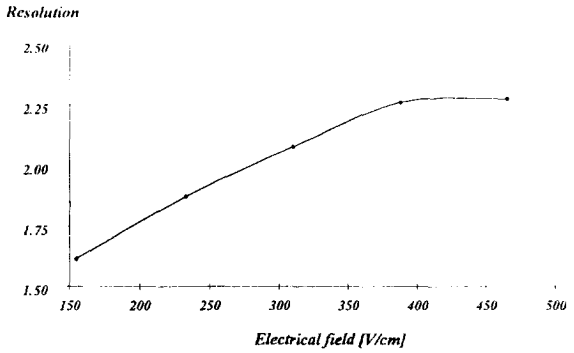

Fig. 4. Effect of applied voltage on resolution. Buffer A with 20 m*M* (2-hydroxy)propyl-β-CD. Sample: *d,l*-amphetamine (30 μg/ml) in 0.1 *M* HCl. For other experimental conditions, see Table 1.

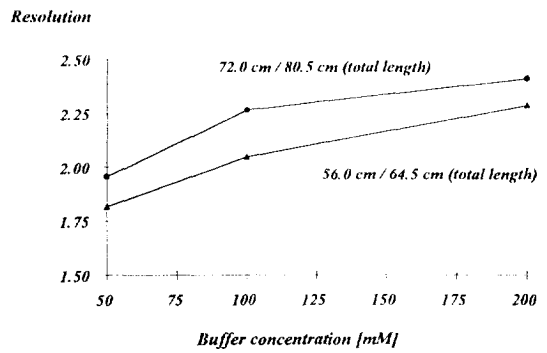

Fig. 5. Effects of buffer concentration and capillary length on resolution. Buffer A at different concentrations with 20 m*M* (2-hydroxy)propyl-β-CD. Sample: *d,l*-amphetamine (30 μg/ml) in 0.1 *M* HCl. For other experimental conditions, see Table 1.

creases and the resolution increases (Fig. 4). We chose to work at 25 kV (388 V/cm), as the gain in resolution at 30 kV (465 V/cm) is too low in comparison with the amount of heat generated by the Joule effect. We also noticed that at 25 kV (388 V/cm) we were no longer in the linear range of Ohm's law. Working at a high voltage leads to higher efficiencies and a faster analysis time as long as there are no bubbles in the capillary to cause disturbances in the analysis.

3.4. Effect of buffer concentration and capillary length

When working at a high buffer concentration, which means at high ionic strength, the zeta potential of the double layer is decreased, and this results in a decrease in the electroosmotic flow [44–46]. A buffer of high ionic strength gives significantly higher efficiencies and enhances the resolution, since the analytes remain longer in the capillary (Fig. 5). Buffers of high ionic strength also prevent analyte–analyte or analyte–wall adsorption in the capillary, and thus lead to better quantification and reproducibility [45]. A major drawback to working at high buffer concentrations is the generation of a high current through the capillary, owing to an enhanced conductivity of the buffer. This phenomenon therefore limits the usable buffer concentration, since a higher temperature could cause disturbances in the separation, as seen before.

We also noted that the resolution is better with an 80.5-cm capillary (total length) than a 64.5-cm capillary, but leads to a longer analysis time. By using the shorter capillary, we were able to analyse five racemic amphetamines in less than 30 min.

3.5. Injection volume

As mentioned by Shihabi and Garcia [47], a rule of thumb restricts the sample volume to less than 1% of the total capillary volume in order to avoid overloading of the capillary and distortion of the peaks. We varied the injection volume of a racemic amphetamine sample in order to optimize this parameter. We injected 2.4–35.7 nl, which represents 0.2–2.8% of the total capillary volume (1.27 μl). As shown in Fig. 6, the relationship between the injection volume and the height of the peaks is linear for the whole range tested. Nevertheless, we chose to use a sample volume of 14.3 nl (600 mbar s), because the resolution between the peaks decreases with larger sample volumes injected.

3.6. Electrophoretic analysis

Once the method had been optimized, we injected a mixture of five racemic amphetamines (amphetamine, methamphetamine, MDA, MDMA and MDE) (Fig. 7). We achieved their

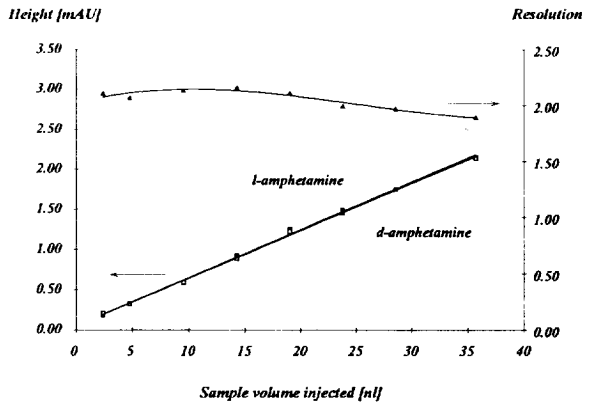

Fig. 6. Linearity of the injection volume. Buffer A with 20 m*M* (2-hydroxy)propyl-β-CD. Sample: *d,l*-amphetamine (1 μg/ml) in 0.1 *M* HCl. For other experimental conditions, see Table 1.

enantiomeric separation in less than 30 min and always with baseline resolution. We then tested the repeatability of the method (12 injections intra-day) with a standard mixture containing 1 μg/ml of each racemic amphetamine and 1 μg/ml of the internal standard (phenylethylamine) (Table 2). For the migration times we obtained R.S.D. values between 0.3 and 0.4%. However, for the peak areas the R.S.D. values were between 4.3 and 9.1%, as shown in Table 2. These poor values are due to the low concentration of the amphetamine sample, as mentioned by Silverman and Shaw [49]. It should be mentioned that the peak areas of the enantiomers were corrected by their respective migration times in order to take into account the difference in mobilities between the analytes [48].

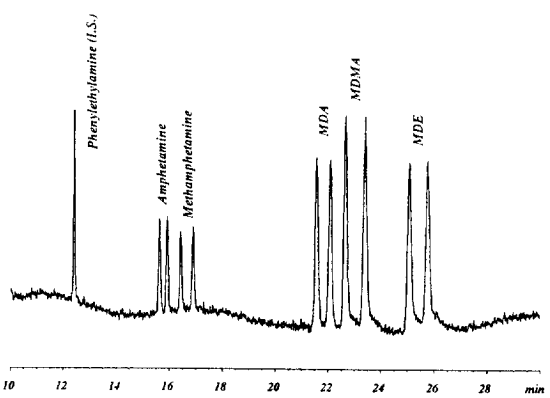

Fig. 7. Separation of a sample containing the five racemic amphetamines (1 μg/ml). Buffer A with 20 m*M* (2-hydroxy)-propyl-β-CD each. Other conditions: injection at 600 mbar s (pressure); $E = 388$ V/cm (1-min voltage ramp); $T = 15$°C; detection wavelength = 200 nm; bandwidth = 10 nm.

Preliminary results showed good linearity of the method in the concentration range 500–10 000 ng/ml with standard aqueous samples ($r > 0.999$ for all enantiomers tested). Nevertheless, the linearity, the repeatability and the limits of detection and quantification of the method will be determined further directly for the determination of the amphetamines in real urine samples and illicit tablets.

3.7. Application of the method to urine samples

We tested four modes of pretreatment for the urine samples before the capillary electrophoretic analysis. A blank urine sample was spiked with 1 μg/ml of each racemic amphetamine (amphetamine, methamphetamine, MDA, MDMA, MDE) and with 1 μg/ml of phenylethylamine as internal standard. A fraction of this sample was injected directly without any pretreatment, a fraction was purified by ultrafiltration and the two last fractions were purified by liquid–liquid extraction (LLE) and by solid-phase extraction (SPE). The same blank urine sample, but this time unspiked, was treated as described above.

All the analyses were carried out under the conditions described in Fig. 7 and each sample was injected three times. As shown in Fig. 7, all the amphetamines (racemic mixtures) and the phenylethylamine were resolved in less than 30 min for a standard aqueous solution. Without any pretreatment, the unspiked and the spiked samples gave a complex electropherogram (results not shown). Neither quantitative nor qualitative results were obtained even if it was possible to assign some amphetamines. The samples purified by ultrafiltration also gave very complex electropherograms which cannot be used for analytical purposes. In view of these results, it appears that, in order to purify the sample

Table 2
Repeatability of the method

Compound	MT[a] (min)	R.S.D. (MT) (%)	Area[b]/MT (mAU)	R.S.D. (area/MT) (%)	Relative area[c]	R.S.D. (rel. area) (%)
Internal standard	12.43	0.3	0.735	5.5	–	–
l-Amphetamine	15.71	0.4	0.369	6.8	0.50	6.2
d-Amphetamine	16.00	0.4	0.385	7.0	0.52	7.0
l-MA	16.53	0.3	0.326	7.6	0.45	9.1
d-MA	17.00	0.4	0.309	5.5	0.42	6.8
MDA-1	21.80	0.4	0.762	2.8	1.04	4.3
MDA-2	22.34	0.4	0.763	2.9	1.04	4.6
MDMA-1	22.94	0.4	0.950	5.3	1.30	6.1
MDMA-2	23.70	0.4	0.900	4.9	1.23	4.9
MDE-1	25.46	0.4	0.722	4.8	0.98	4.7
MDE-2	26.17	0.4	0.696	4.5	0.95	4.7

For experimental conditions, see Fig. 7. Sample: mixture of five racemic amphetamines (1 μg/ml each) in 0.1 *M* HCl.
[a] Migration time.
[b] The corrected areas (area divided by migration time) are preferably used for the calculations rather than the simple areas, as has been shown by Altria [48].
[c] The relative area is the corrected area of one enantiomer divided by the corrected area of the internal standard.

before the analysis, an extraction step is necessary.

As shown in Figs. 8A and 9A, the SPE procedure gives a cleaner extract than LLE for the same blank urine sample. With the former method, no or few interference peaks appear in the time window expected for the amphetamines. Figs. 8B and 9B also show the electropherograms of the spiked urine samples after the SPE and LLE pretreatments and indicate that,

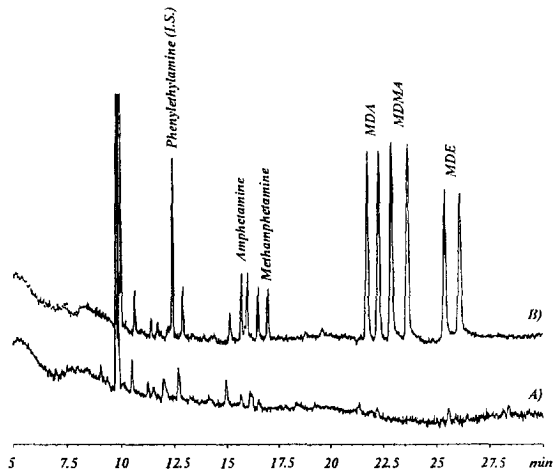

Fig. 8. Separation of a sample containing the five racemic amphetamines after SPE pretreatment. (A) Blank urine sample (unspiked); (B) spiked urine sample (1 μg/ml of each racemic amphetamine and 1 μg/ml of internal standard). For experimental conditions, see Fig. 7. Sensitivity: 2 mAUFS.

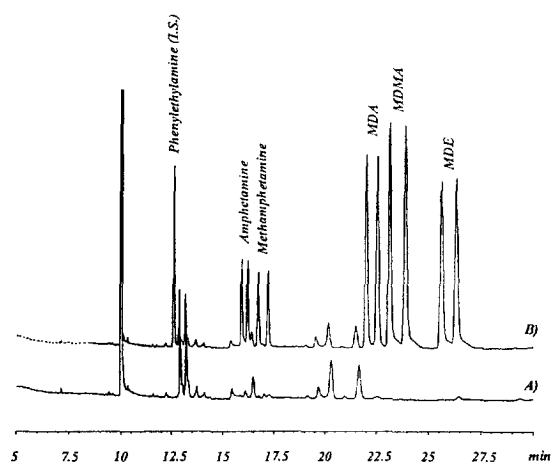

Fig. 9. Separation of a sample containing the five racemic amphetamines after LLE pretreatment. (A) Blank urine sample (unspiked); (B) spiked urine sample (1 μg/ml of each racemic amphetamines and 1 μg/ml of internal standard). For experimental conditions, see Fig. 7. Sensitivity: 20 mAUFS.

with the SPE procedure, qualitative and quantitative analyses can be easily carried out. The LLE procedure can also be used, but with more interfering peaks which might induce some errors during quantitative analysis; nevertheless, this procedure is faster than SPE.

These preliminary results show that, before undertaking a capillary electrophoretic analysis of urine samples, an extraction procedure is necessary. Work is in progress in order to determine statistically the recoveries of the SPE and LLE procedures for all the enantiomers and to validate the entire analytical method.

4. Conclusions

Because of its high efficiency, high-performance capillary electrophoresis has been shown to be a useful additional tool to the analytical techniques available for the resolution of enantiomers. We have demonstrated that five racemic amphetamines can be separated in less than 30 min with a very good resolution.

After having optimized certain parameters, we concluded that the most significant one is the nature of the chiral selector. The mechanisms of enantiorecognition with the substituted CDs are not yet well defined, and their study is under investigation in order to determine what kind of selector has to be used for the separation of a class of pharmaceuticals. Some experimental work is in progress for complete validation of the method for the determination of amphetamines in biological fluids such as urine, serum and saliva.

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