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Capillary electrophoresis with laser-induced fluorescence and pre-column derivatization for the analysis of illicit drugs

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Abstract

In the current paper, we report the development of a new capillary electrophoresis method using pre-column derivatization and laser-induced fluorescence detection for the determination of ephedrine and amphetamine drugs. Our new method allows for the identification and quantification of six commonly used illicit drugs namely pseudoephedrine, ephedrine, amphetamine, methamphetamine, 3,4-methylenedioxyamphetamine, and 3,4-methylenedioxymethylamphetamine, respectively, as well as propafenone (internal standard). Following derivatization with fluorescein isothiocyanate, a total of six amphetamine drugs and the internal standard could readily be separated using a fused-silica 75 μm ID × 60 cm length (effective length: 50.2 cm) capillary column. The mobile phase consisted of buffer containing 20 mM borate (pH 12, adjusted with sodium hydroxide). Samples were injected in pressure mode with the capillary being operated at 25 kV/25 °C, and the detection of the derivatized compounds was sought using a laser-induced fluorescence (LIF) detector (λ_{ex} = 488 nm and λ_{em} = 520 nm), with a run-time of 20 min. The current method was validated with regard to precision (relative standard deviation, RSD), accuracy, sensitivity, linear range, limit of detection (LOD) and limit of quantification (LOQ). In human blood and urine samples, detection limits were 0.2 ng mL^{-1}, and the linear range of the calibration curves was 0.5–100 ng mL^{-1}. The intra-day and inter-day precisions were both less than 13.22%.

Keywords: Ephedrines; Amphetamines; Capillary electrophoresis; Laser-induced fluorescence; Pre-column derivatization; Fluorescein isothiocyanate; Solid-phase extraction

1. Introduction

The worldwide increase in the use of illicit drugs is having a serious effect on social problems around the world particularly with regards to drug dependence and increased drug-related crime. It is thus pertinent for forensic as well as clinical and pharmaceutical scientists to develop reliable, rapid and convenient methodologies for the identification and quantification of illicit drugs and their metabolites in human biological samples.

Amphetamines are stimulants that have a direct effect on the central nervous system. This family of drugs can induce anxiety, hallucination, violence and antisocial tropesis. Amphetamines are derived from the ephedrines that also have a stimulatory effect upon the central nervous system. Because amphetamines are easily synthesized, they have become the most popular recreational drugs in world [1,2]. Therefore, it is paramount that sensitive, rapid and specific methods for analyzing these drugs are available to the pharmacologist as well as forensic scientists. To date, several analytical techniques including high pressure liquid chromatography (HPLC)-UV [3,4], HPLC-fluorescence [5], HPLC-electrochemical [6], liquid chromatography–mass spectroscopy [7], gas chromatography–mass spectroscopy [8–13] detection methods have been developed to assay for amphetamines, the methodologies of which can be found in several recent reviews [14–17].

Capillary electrophoresis (CE) is a modern analytical technique that has attracted significant interest from the academic community due to its short analysis time, high separation efficiency, small sample size, and minimal solvent consumption. In the hands of forensic scientists, CE represents a powerful new analytical tool that could be readily used to determine the pres-

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ence of illicit drugs in seized preparations as well as in complex biological matrices [18,19]. Unfortunately, due to the relatively small sample sizes used in CE, sensitivity was often a limitation; however, new CE–MS techniques have overcome these shortcomings [20].

In the present study, we have developed a new CE method utilizing a pre-column derivatization step coupled with laser-induced fluorescence detection (LIF) for the separation of several commonly used illicit drugs. Sample treatment procedures and separation conditions were determined, with the new method exhibited excellent sensitivity (0.2 ng mL^{-1} in urine and blood) for all of six drugs analyzed.

2. Experimental

2.1. Chemicals

Pseudoephedrine hydrochloride, ephedrine hydrochloride, amphetamine sulfate, methamphetamine hydrochloride, 3,4-methylenedioxyamphetamine (MDA), 3,4-methylenedioxymethylamphetamine hydrochloride (MDMA) and propafenone (internal standard) were purchased from the National Institute for the Control of Pharmaceutical and Biological Products of China (NICPBP, Beijing, China). Fluorescein isothiocyanate (FITC) was obtained from Sigma–Aldrich (Switzerland). HPLC-reagent grade methanol was purchased from Tedia (USA). All other chemicals were of analytical grade and purchased from Shanghai Chemical Reagent Co., Ltd. High-purity water was prepared by a Direct-Q water purification system (Millipore USA). Standard stock solutions containing the amphetamine drugs used in the current study, as well as a control solution containing propafenone hydrochloride, were prepared in methanol to a final concentration of 1.0 mg mL^{-1} and stored in a refrigerator at −4 °C until required.

2.2. Apparatus

The CE experiments were conducted using a Beckman P/ACE MDQ system (Beckman, USA) equipped with a diode array detector (DAD), a laser-induced fluorescence detector (LIF) and fused-silica capillary (Beckman, USA).

2.3. Reference solutions preparation

The six amphetamine drugs were dissolved in methanol to prepare stock solutions (1 mg mL^{-1}) and stored at −4 °C. All stock solutions were mixed and diluted with methanol to give suitable concentrations for CE–LIF analysis. The internal standard propafenone was added to each mixture to be analyzed to give a final concentration of 0.1 mg mL^{-1}.

2.4. Sample preparation

In this work, urine and blood samples were purified by a solid-phase extraction (SPE) clean-up procedure. An Oasis HLB column (1 mL/30 mg Waters, USA) was employed and conditioned with methanol and equilibrated with water. Aliquots of urine (1 mL) or blood (0.5 mL) were alkalized with phosphate, spiked with different drug concentration in the range of 0.5–100 ng mL^{-1} and mixed completely in 5 mL glass tubes containing the internal standards before introduction to the SPE column. Initially the SPE columns were washed with 1 mL 2% $NH_3 \cdot H_2O$/5% methanol in water followed by 1 mL 5% methanol for urine sample, and 1 mL 2% $NH_3 \cdot H_2O$–5% methanol in water followed by 1 mL *n*-hexane and followed by 5% methanol in water for blood sample, respectively. All the analytes were eluted using 1 mL ethyl acetate. The 20 μL 1% HCl was added to prevent the drugs from volatilizing when evaporating. The eluted solutions were evaporated at 50 °C under a stream of air and reconstituted with 50 μL water (urine samples) or 20 μL water (blood samples). Following the SPE extraction procedure, 10 mM borate buffer (pH 10), 0.1% pyridine/acetone, and FITC (n_{FITC}:n_{sample} = 10:1) were added to each biological sample for the derivatization reaction to proceed. Derivatization was conducted at room temperature in the dark for 12 h. The preparation procedures for urine and blood samples are shown in Table 1.

2.5. Separation procedures

Separation was performed using a fused-silica capillary of 75 μm ID × 60 cm length (effective length: 50.2 cm), using a method based on a CZE model. All buffer solutions were filtered through a 0.45 μm filter. The six amphetamine drugs were

Table 1
The sample procedure used for the analysis of amphetamines in blood and urine samples

Step	Urine (for basic drugs)	Whole blood (for basic drugs)
Condition	1 mL methanol	1 mL methanol
Equilibrate	1 mL water	1 mL water
Load	1 mL urine with 20 uL propafenone (I.S.)	0.5 mL spiked whole blood (1:3 dilute) with 20 uL phosphoric acid and 20 uL propafenone (I.S.)
Wash	1. 1 mL 2% $NH_3 \cdot H_2O$–5% methanol in water. 2. 1 mL 5% methanol in water (centrifuge: 3000 rpm, 3 min)	1. 1 mL 2% $NH_3 \cdot H_2O$–5% methanol in water 2. 1 mL *n*-hexane 3. 1 mL 5% methanol in water (centrifuge: 3000 rpm, 3 min)
Elute	1 mL ethyl acetate, 20 μL 1% HCl	1 mL ethyl acetate, 20 μL 1% HCl
Evaporate and reconstitute	50 °C under air stream, 50 μL H_2O reconstitute	50 °C under air stream, 20 μL H_2O reconstitute
Derivatization	10 mM, pH 10 borate buffer 430 μL n_{FITC}:n_{sample} = 10:1, 20 μL 0.1% pyridine/acetone, protected from light, room temperature for 12 h	n_{FITC}:n_{sample} = 10:1, 20 μL 0.1% pyridine/acetone, protected from light, room temperature for 12 h

Table 2
Calibration curves for the six amphetamines in urine

Drug	Linear range (ng mL^{-1})	Regression equation	r
MDMA	0.5–100	$Y = 0.05188x + 0.08393$	0.9997
Methamphetamine	0.5–100	$Y = 0.03874x + 0.53944$	0.9985
Ephedrine	0.5–100	$Y = 0.108748x + 0.34313$	0.9946
Pseudoephedrine	0.5–100	$Y = 0.28580x + 0.04185$	0.9974
MDA	0.5–100	$Y = 0.08174x + 0.17845$	0.9953
Amphetamine	0.5–100	$Y = 0.11522x + 0.03641$	0.9941

separated in a 20 mM borate buffer (pH 12) under an applied voltage of 25 kV and a capillary temperature of 25 °C. The detector was set to monitor wavelengths of 195 nm and 235 nm. A laser-induced fluorescence (LIF) detector was used at $\lambda_{ex} = 488$ nm and $\lambda_{em} = 520$ nm.

2.6. Method validation

Calibration curves were developed for each amphetamine in samples of urine and blood. Samples were prepared as described above, and electrophoretic separations were performed under optimized conditions. Each curve was constructed from six concentration points with triplicate injections applied at each concentration. The linearity of each standard curve was confirmed by plotting the peak area ratio of the drug and I.S. as the ordinate versus the concentration of the drug (ng mL^{-1}) as the abscissa. The results are illustrated in Tables 2 and 3. The detection limits for the six amphetamine drugs were 0.2 ng mL^{-1} on the basis of a signal-to-noise ratio of 3.

Recovery efficiency was evaluated at three separate concentrations, each prepared in both urine and blood, by comparing the concentrations of the six amphetamines in the test solutions with the unextracted standard solution. The six amphetamines were added to urine or blood, and concentrations after SPE were compared to the standard solutions. The results of these comparisons are depicted in Tables 4 and 5.

The intra-day accuracy and precision were determined by analysis of five replicates at three typical assay concentrations (1, 20, 80 ng mL^{-1}, respectively) in urine and in blood. The inter-day accuracy and precision were evaluated by analyzing the same samples on five different days each in blood and in urine. Precision was characterized by calculating RSD, whereas accuracy was expressed as the comparison of the measured concentration with the added nominal concentration. This methodology shows that the accuracy was 95.48–107.74% with the RSD 1.01–12.54% in urine (Table 6), and 95.45–112.71% with the RSD 1.44–13.22% in blood (Table 7).

Table 3
Calibration curves for the six amphetamines in blood

Drug	Linear range (ng mL^{-1})	Regression equation	r
MDMA	0.5–100	$Y = 0.05344x + 0.07589$	0.9901
Methamphetamine	0.5–100	$Y = 0.04108x + 0.35895$	0.9911
Ephedrine	0.5–100	$Y = 0.12426x + 0.38055$	0.9984
Pseudoephedrine	0.5–100	$Y = 0.26258x + 0.074120$	0.9956
MDA	0.5–100	$Y = 0.08405x + 0.20148$	0.9936
Amphetamine	0.5–100	$Y = 0.12510x + 0.025674$	0.9970

Table 4
Extraction and recovery of amphetamines in urine ($n = 3$)

Drug	Added concentration (ng mL^{-1})	Mean absolute recovery (%)	RSD (%)
MDMA	1	78.56	2.26
	20	81.25	
	80	77.86	
Methamphetamine	1	74.56	3.34
	20	76.53	
	80	71.61	
Ephedrine	1	80.26	1.90
	20	82.46	
	80	79.51	
Pseudoephedrine	1	84.65	3.21
	20	82.63	
	80	79.41	
MDA	1	87.48	2.34
	20	84.32	
	80	83.79	
Amphetamine	1	86.96	3.16
	20	84.52	
	80	81.64	

3. Results and discussion

3.1. Optimization of the derivatization conditions

Several derivatization conditions were investigated including pH, buffer species, buffer concentration, ratio of drug with

Table 5
Extraction and recovery of amphetamines in blood ($n = 3$)

Drug	Added concentration (ng mL^{-1})	Mean absolute recovery (%)	RSD (%)
MDMA	1	75.64	3.78
	20	81.55	
	80	79.26	
Methamphetamine	1	70.25	5.15
	20	77.84	
	80	73.58	
Ephedrine	1	81.56	2.64
	20	82.56	
	80	78.46	
Pseudoephedrine	1	84.26	3.98
	20	80.54	
	80	77.85	
MDA	1	79.84	3.04
	20	81.26	
	80	76.56	
Amphetamine	1	81.25	2.95
	20	76.63	
	80	78.45	

Table 6
Precision and accuracy of toxic alkaloids in urine

Drug	Intra-day ($n=3$)				Inter-day ($n=3$)		
	Added (ng mL^{-1})	Found (ng mL^{-1})	RSD (%)	Accuracy (%)	Found (ng mL^{-1})	RSD (%)	Accuracy (%)
MDMA	1	1.06	8.61	105.64	1.02	4.13	102.00
	20	19.86	7.46	99.28	19.63	9.65	98.15
	80	78.59	8.48	98.24	79.56	7.48	99.45
Methamphetamine	1	0.98	9.52	97.75	1.02	10.55	102.00
	20	21.55	9.48	107.74	20.58	4.56	102.90
	80	81.55	8.54	101.93	81.23	8.12	101.54
Ephedrine	1	0.98	10.25	97.56	1.02	9.44	102.00
	20	19.90	10.56	99.49	20.14	8.47	100.70
	80	84.16	7.84	105.20	79.35	6.54	99.19
Pseudoephedrine	1	1.04	5.65	103.65	1.03	6.63	103.00
	20	20.42	4.89	102.11	19.65	7.86	98.25
	80	81.56	4.94	101.96	83.14	8.54	103.93
MDA	1	0.98	10.25	97.58	0.96	9.58	96.00
	20	19.57	10.77	97.84	18.54	10.57	92.70
	80	82.79	1.24	103.48	81.12	2.89	101.40
Amphetamine	1	0.95	5.14	95.48	1.07	12.54	107.10
	20	21.06	1.01	105.29	21.24	2.87	106.20
	80	79.58	3.58	99.48	78.26	9.48	97.83

FITC, reaction temperature and reaction time. The peak area of amphetamine under differing conditions was used as an index to select for the optimum conditions for derivatization.

Because the FITC derivatizations were conducted following the conditions described [21], we primarily focused on the effects of pH and buffer concentrations used in the derivatization process. Three concentration of borate buffer solution were tested (5 mM, 10 mM and 20 mM) at a pH ranging from 8 to 11 (pH was adjusted using sodium hydroxide). These results are represented in Fig. 1. We found that when the pH was increased from pH 8 to 10, the peaks areas of our drugs of interest increased significantly. In contrast, when the pH was increased to pH 11, the peak areas of each drug were found to decrease particularly in the 5 mM and 10 mM buffer solutions. Therefore, we selected a buffer solution of 10 mM borate buffer at pH 10, as this gave the best stability and sensitivity, for all our future analyses.

We also determined the effects of different buffering species such as phosphate buffer, carbonate buffer and borate buffer

Table 7
Precision and accuracy of toxic alkaloids in blood

Drug	Intra-day ($n=3$)				Inter-day ($n=3$)		
	Added (ng mL^{-1})	Found (ng mL^{-1})	RSD (%)	Accuracy (%)	Found (ng mL^{-1})	RSD (%)	Accuracy (%)
MDMA	1	1.05	8.25	105.42	1.04	7.45	104.00
	20	19.60	7.99	97.99	20.60	8.46	103.00
	80	81.26	10.23	101.57	80.25	12.56	100.31
Methamphetamine	1	0.95	9.85	95.45	1.06	10.11	106.30
	20	22.54	8.47	112.71	21.65	4.78	108.25
	80	81.56	4.65	101.95	80.46	6.89	100.58
Ephedrine	1	0.96	2.89	96.48	0.99	6.44	98.70
	20	19.56	7.96	97.82	19.45	12.6	97.25
	80	81.56	4.13	101.96	80.26	9.58	100.33
Pseudoephedrine	1	1.01	7.85	100.52	1.03	5.47	103.20
	20	21.15	9.65	105.77	20.35	4.58	101.75
	80	80.56	1.44	100.70	79.25	5.46	99.06
MDA	1	1.05	5.64	104.78	0.99	9.54	98.70
	20	19.58	9.44	97.89	19.86	10.26	99.32
	80	82.79	2.65	103.48	80.24	9.54	100.30
Amphetamine	1	0.96	8.41	95.68	0.98	13.22	97.70
	20	21.56	9.65	107.82	20.64	10.57	103.20
	80	80.26	7.34	100.32	79.63	5.69	99.54

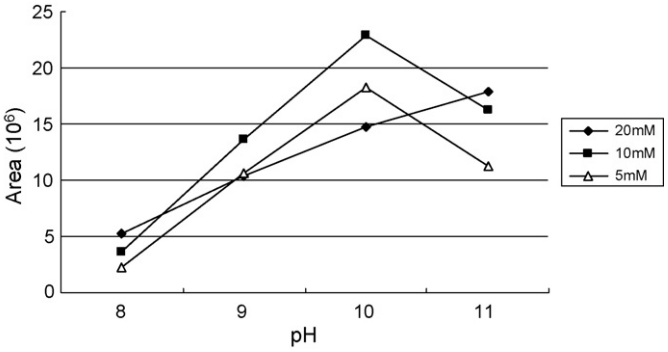

Fig. 1. The effects of buffer concentrations and pH on FITC derivatization of amphetamine drugs used in the current study.

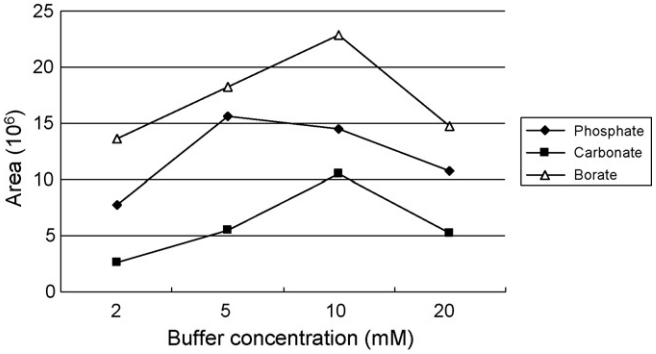

Fig. 2. The effects of phosphate, carbonate and borate buffer and their concentrations on the FITC derivatization reaction.

on the derivatization reaction of FITC with methamphetamine, as represented in Fig. 2. Under the same condition, the peak area in the borate buffer was higher than in those found for the phosphate and carbonate buffer solutions, with the 10 mM borate buffer showing the best peak area.

The effects of the derivatization regent, FITC, and the concentrations for optimum derivatization were next tested. Different ratios of sample amount (n_{sample}) to FITC amount (n_{FITC}) were mixed for derivatization. We found that the peak area was higher with a sample to FITC ratio of (n_{sample}:n_{FITC}) 1:10 (Fig. 3).

To determine the optimum time and temperature for the derivatization reaction, we selected three separate reaction temperatures, e.g. room temperature, as well as at 40 °C and 60 °C.

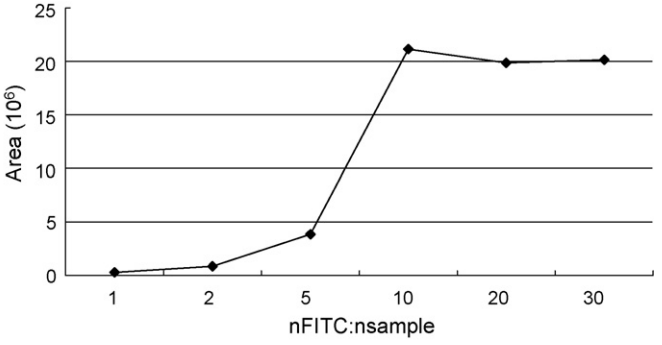

Fig. 3. The effects of the ratios of FITC amount (n_{FITC}) to sample amount (n_{sample}) on derivatization (10 mM, pH 10 borate, room temperature).

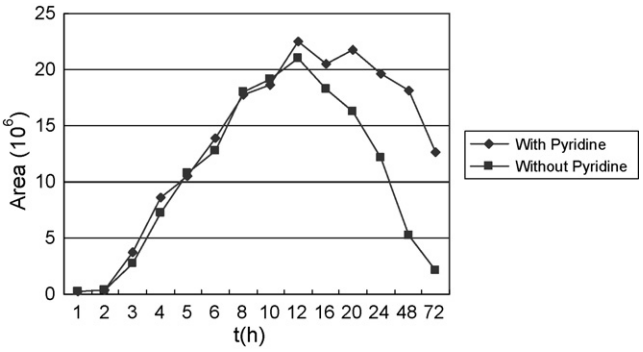

Fig. 4. The effect of adding pyridine to the reaction solutions to the FITC derivatization reaction conducted in 10 mM, pH 10 borate buffer at room temperature.

At each temperature the reaction was stopped at 2, 4, 6, 8, 12, 16 and 24 h, respectively, and the optimum temperature and time required for derivatization were determined. Our results show that the reaction achieved equilibrium after 12 h and the derivatization reaction was not significantly influenced by temperature. For further analyses, we selected room temperature and the 12 h reaction time for the derivatization reaction.

Two derivatization conditions were tested, plus or minus the addition of pyridine. We found that the addition of pyridine did not influence the derivatization reaction significantly before equilibrium was achieved. However, after 16 h the absence of pyridine in the reaction mixture demonstrated a marked decline in the derivatization process (Fig. 4). Therefore, in the current work 20 μL of 0.1% pyridine (in acetone) was added.

3.2. Optimization of the separation conditions

We experimented with a number of modifications to the running buffer including pH, species, concentration, and choice of organic solvent before selecting the buffer that offered the best resolution for all of the amphetamine drugs.

As the derivatization conditions were performed in borate solution, the borate buffer was used for the running buffer. We tested the running buffer at pH 8, 9, 10 and 11, respectively. We found that in a running buffer of pH < 10, all of six amphetamine drugs could not be separated. Similarly, in a running buffer of pH > 13, we found that the electric current increased greatly resulting in increased Joule heating. Therefore, we investigated the pH range between pH 10 and 12.5. The results are represented in Fig. 5, from which it can be seen that a pH range between pH 11.5 and 12.5 was the optimal condition. All future analyses in the current study were conducted using a running buffer of pH 12. The studies on buffer conditions demonstrated that the borate buffer concentration did not significantly influence the resolution, but high concentrations resulted in increased Joule heating; therefore, the 20 mM borate buffer was chosen as the running buffer.

Under the optimum conditions determined in the current work, six amphetamine drugs and an internal standard can be separated in less than 20 min. Typical electropherograms for standard mixtures in urine and blood are represented in Fig. 6A and B, respectively.

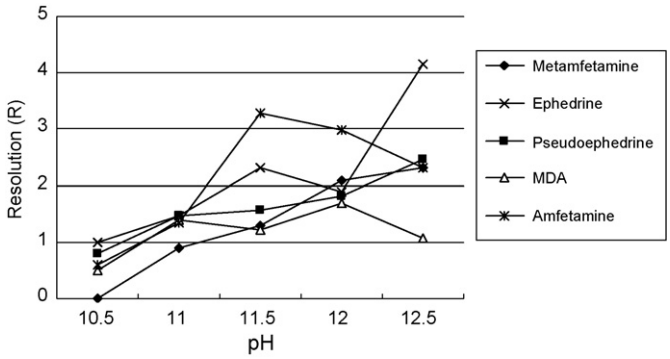

Fig. 5. The effect of borate buffer pH (20 mM borate, room temperature) on the derivitization reaction.

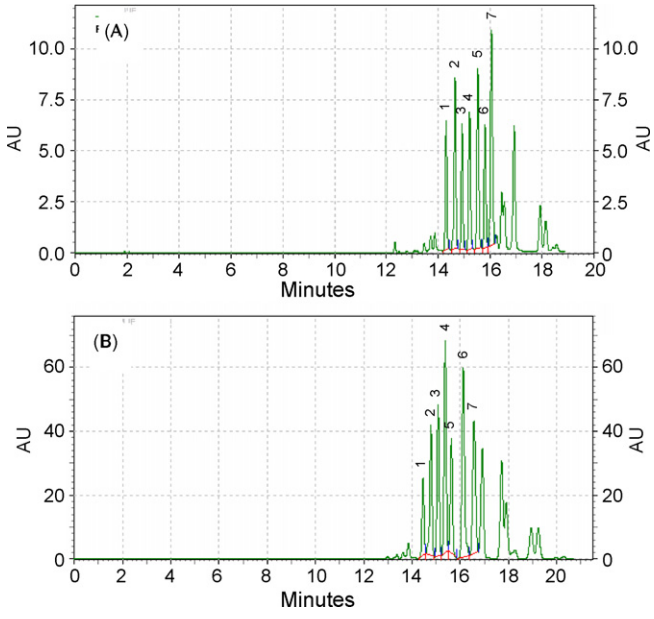

Fig. 6. Typical electropherograms for standard mixture solution of amphetamines and the internal standard (15 ng mL^{-1}) in urine (A), and 50 ng mL^{-1} in blood (B). 1, MDMA; 2, methamphetamine; 3, ephedrine; 4, pseudoephedrine; 5, MDA; 6, propafenone; 7, amphetamine.

4. Conclusion

A CE–LIF method based on pre-column derivatization was established to determine the presence of amphetamine drugs in biological samples. Our new method shows excellent sensitivity when compared with the more commonly used analytical methods such as GC/MS [11]. The LOD both in blood and plasma was 0.2 ng mL^{-1}. We also validated the method in precision, accuracy, sensitivity and linearity. All the results of these parameters indicated the high selectivity and favorable accuracy of the current method. Given the rapid and simple nature of the reported new analytical methodology, we propose that it would be suitable for first-aid treatment in clinical cases and for forensic investigations.

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