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Journal of Pharmaceutical and Biomedical Analysis 33 (2003) 463–473

JOURNAL OF PHARMACEUTICAL AND BIOMEDICAL ANALYSIS

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Determination of heroin metabolites in human urine using capillary zone electrophoresis with β-cyclodextrin and UV detection

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Received 8 January 2003; received in revised form 30 March 2003; accepted 8 May 2003

Abstract

A method has been developed for the detection of a mixture of morphine, codeine, 6-acetyl morphine (6-AM) and normorphine using capillary zone electrophoresis (CZE). The method utilized urinary 6-AM as a diagnostic indicator of heroin abuse because it is not a product of either morphine or codeine metabolism. The electrophoretic separation was achieved using an uncoated (50 μm I.D.) fused-silica capillary, 77 cm long, containing the detector window 10.0 cm from the outlet end. The running buffer (pH 6.0) contained 50 mM sodium phosphate and 0.015 M β-cyclodextrins (β-CD). The samples were first extracted using a mixed-mode solid-phase extraction procedure and then analyzed by CZE. The UV absorbance detection was monitored at 214 nm. It has been found that β-CDs can improve separation efficiency due to their hydrophobic cavity. The effect of the concentration of β-CD and pH was also evaluated. The application of electrokinetic injection with field amplified sample stacking results in low detection limits (40 ng/ml for each analyte) and the method has good reproducibility, precision, accuracy, and high recovery.
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Keywords: Heroin; 6-AM; β-Cyclodextrin; Electrokinetic injection, sample stacking, capillary zone electrophoresis

1. Introduction

Illicit opiate use remains a major problem around the world and still represents an analytical challenge for forensic analysts. Urine drug testing provides a tool for detecting users and for monitoring the compliance of subjects in recovery programs. However, the complex metabolic pathways of heroin, morphine, and codeine complicate the interpretation of test results [1,2].

Following administration, heroin undergoes rapid enzymatic hydrolysis to 6-acetyl morphine (6-AM) and then more slowly to morphine. The plasma half-life of heroin has been estimated at 2–8 min [3]. 6-AM is metabolically more stable than heroin (plasma half-life of 10–40 min) [4,5]. Morphine may be further metabolized in both the liver and intestine to normorphine and codeine, Fig. 1.

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doi:10.1016/S0731-7085(03)00298-X

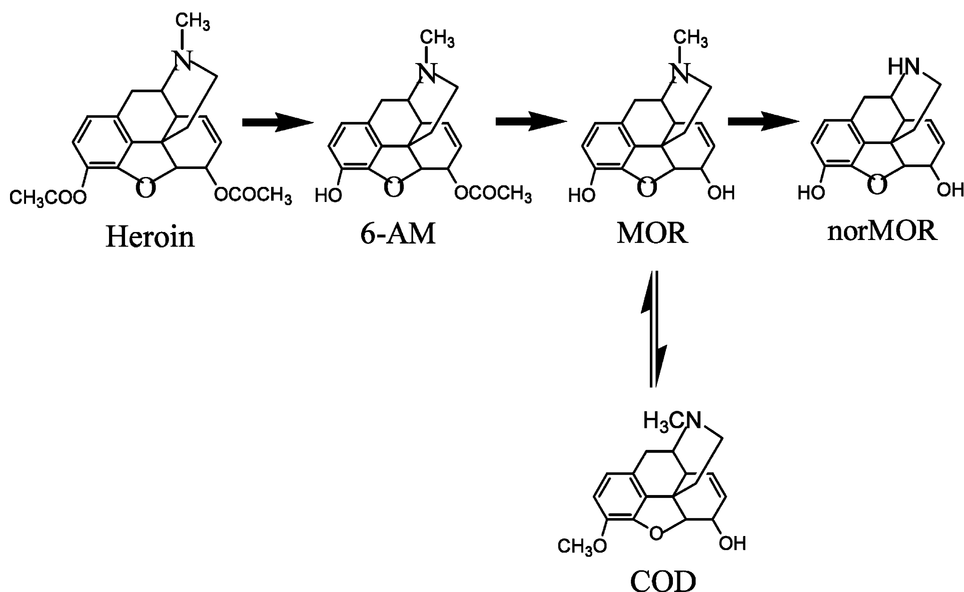

Fig. 1. Metabolic pathway of heroin [2].

The extreme labile nature of heroin limits its utility as a target analyte for routine abuse detection. 6-AM is considered the best marker for heroin use because there is no known natural source, it is not a codeine metabolite, and it is comparatively stable relative to heroin [2,6,7]. The window of detection for 6-AM in urine is 8-h [8,9].

Most laboratories adopt immunological assays such as enzyme immunoassay and radioimmunoassay for the determination of opiates in biological fluids. These assays are very sensitive, simple, and readily automated. However, immunoassays can be impaired by specific (cross-reaction of antiserums) and non-specific (pH, ion strength) interferences. Therefore, alternative techniques may be necessary to differentiate the opiates present and to quantitate their concentration.

Among the chromatographic methods in use, thin-layer chromatography is the most simple and inexpensive. However, it suffers from a lack of sensitivity and specificity. Both sensitivity and specificity are available with gas chromatography mass spectrometry but this method requires derivatization of 6-AM. The application of a normal- or reversed-phase HPLC separation in this context has recently been demonstrated [10].

The advent of fully automated commercially available capillary electrophoresis (CE) systems has presented the forensic analyst with a new and viable analytical alternative to HPLC and TLC. The modern CE instruments are capable of the required sensitivity and precision, with similar characteristics to HPLC. In addition, CE offers several advantages, including highly efficient and fast separations, relatively inexpensive and long lasting capillary columns, very small sample size requirements, and low reagent consumption [11].

A variety of detectors have been used in CE, including: UV-visible absorbance [12,13], fluorescence [14], chemiluminescence [15], mass spectrometric [16], conductivity [17], radiometric [18] detection. Of these, the most widely used are UV-visible absorbance detectors.

The most widespread method for sample injection in CE is hydrodynamic injection, either achieved by applying pressure at the inlet or vacuum at the detector end of the capillary. Alternatively, ionized analytes, particularly those, which are cationic can be introduced into the capillary electrokinetically. It has been shown that, by using solid-phase extraction and electrokinetic injection, it is possible to get very low detection

limits to allow quantification of combinations of drugs in urine [11,19].

The unusual properties of cyclodextrins (CDs) originate in their unique structure. CDs consist of a family of oligosaccharides, composed of glucose units connected to each other through α-1,4-glucoside bonds. Despite a hydrophilic surface (the outside region due to the presence of hydroxyl groups—position 2, 3 and 6 of glucopyranose), CDs contain a hydrophobic cavity. It is the presence of this cavity that enables CDs to entrap analytes without formation of formal chemical bonds [20–22]. CDs are now widely used as run buffer additives for CE analyses. It has been shown that the selectivity of CE is enhanced by using CDs as chiral selectors due to their ability to include a wide variety of water-insoluble molecules into their hydrophobic cavity [23]. The effect of CDs on the spectral properties of guest molecules has led to their use as reagents in various spectrometric analyses, including UV-visible spectrophotometric analysis [24], fluorescence and chemiluminescence methods [15,25], and nuclear magnetic resonance spectroscopy [26].

Solid phase extraction (SPE) is a sample preparation technique that uses a small volume of a chromatographic stationary phase to selectively and efficiently extract specific compounds from complex mixtures. There are several advantages to using SPE over liquid–liquid extraction. These advantages include speed, reproducibility, selectivity, versatility, efficiency, and reduction of solvent usage. SPE is about 12-fold less time consuming and 5-fold less costly than liquid–liquid extractions [27]. Over the past 15 years the literature has catalogued a growing number of methods of separation using SPE [10,11].

It is the purpose of the present work to demonstrate that capillary zone electrophoresis (CZE) coupled with UV-visible absorption detection is capable of resolving a mixture of heroin metabolites and of quantifying these compounds in urine at levels comparable with those obtained using alternative techniques. Previous publications on opiate separation and quantification [24] using β-CD and hydrodynamic injection have been restricted to high sample concentrations. This method utilizes an electrokinetic injection, in which the ionic strength of the analyte is less than that of the buffer (producing a voltage divider) resulting in the concentration of the opiates in the capillary and increased sample loadings. In addition, this method utilizes a solid-phase extraction technique, which provides clean extracts with high recoveries for all heroin metabolites. The importance of β-CD concentration and pH adjustment in obtaining selectivity have been investigated and will be discussed, along with other factors found to be significant in obtaining high resolution and optimum sensitivity.

2. Experimental

2.1. Chemicals

6-AM·HCl, normorphine·HCl, noscapin·HCl, papaverine·HCl and nalorphine·HCl, used as the first internal standard (IS_1), were obtained from Lipomed Inc. (One Broaway, Cambridge, MA). Morphine-sulfate, codeine-sulfate, Thebaine, opium, and levallorphan, used as the second internal standard (IS_2) for SPE, were purchased from Sigma–Aldrich. Native β-cyclodextrins (β-CD) are commercially available from TIC America. Sodium phosphate and phosphoric acid were purchased from Spectrum Quality Products, Inc. (Gardena, CA). Bond Elut Certify SPE columns were obtained from Varian Sample Preparation Products (Harbor City, CA). All other solvents and reagents were of analytical grade.

2.2. Standard solutions

A mixture of stock solutions was prepared by adding 50 μL of codeine, morphine, normorphine, and 6-AM (1 mg/ml) to 10 μl (1% HCl in methanol). The resulting mixture was evaporated to dryness under a stream of N_2. The dried residues were reconstituted in 200 μl of slightly warm H_2O. The resulting mixture was diluted in water to prepare six different concentrations 500, 400, 300, 250, 150, and 100 ng/ml for calibration. Nalorphine and levallorphan were diluted in water to the desired concentration and were added to each sample. All standard solutions were stored at

4 °C. Calibration curves were constructed by plotting the ratios of peak areas of drugs to IS_1 against the known concentrations of drugs in the standards and fitted by linear regression analysis.

2.3. Apparatus and conditions

The P/ACE 5000 SERIES (Beckman Instruments, Inc. Fullerton, CA, USA) was used with a UV absorbance detector throughout the experiments. Control of the instrumentation, data acquisition and processing were performed with Chrom Perfect software version 3.5 (Justice Laboratory Software, Palo Alto, CA, USA).

The electrophoretic separation was achieved using an uncoated (50 μm I.D.) fused-silica capillary, 77 cm long, with the detector window 10.0 cm from the outlet end. The capillary was washed sequentially with distilled water (1 min), 0.1 M NaOH (1 min), and distilled water (2 min) at high pressure (20 p.s.i.), followed by reconditioning with running buffer (3 min of high pressure rinsing) between runs.

The UV absorbance detection was performed at 214 nm. The CZE system was operated using "normal" polarity (the cathode was located on the detector side). Samples were injected electrically. The electrokinetic injection is made by placing the capillary and the anode into the source vial and applying a voltage (5 kV) for 10 s. After the sample is introduced, the anode and capillary are placed back into the source vial, an electric field is applied, and electrophoresis proceeds. The system was run at 20 °C and at a constant voltage of 25 kV. Under optimized electrophoretic conditions, separations were carried out using a running buffer contained 50 mM sodium phosphate (adjusted to pH 6.0 with phosphoric acid) and 15 mM β-CD.

2.4. Samples preparation

2.4.1. Extraction of 6-AM

Levallorphan and 2 ml of 10 mM phosphate buffer (pH 6) were added to 5 ml urine samples spiked with heroin metabolites. The pH was then adjusted to 8.0–8.5 with 10 M KOH.

2.4.2. Extraction of normorphine, morphine and codeine

Levallorphan and 1 ml concentrated HCl were added to 5 ml urine samples spiked with heroin metabolites. The samples were immersed in a hot water bath for at least 30 min at 100 °C and cooled to room temperature before proceeding. 2 ml of 0.1 M phosphate buffer (pH 6) was added and the samples were vortexed. The pH was then adjusted to 8.0–8.5 with 10 M KOH.

2.5. Solid phase extraction

A 300 mg Bond Elut Certify SPE column was used for the extraction. The SPE columns were conditioned by the sequential passage of 2 ml of methanol and 2 ml of 10 mM phosphate buffer (adjusted to pH 8.0–9.0). The supernatant layers from the samples were applied to the SPE columns. The columns were washed with 2 ml of distilled water, 2 ml of 10 mM phosphate buffer adjusted to pH 4.0 with phosphoric acid in the case of extraction of 6-AM and/or 100 mM acetate buffer (pH 4) in the case of extraction of the other opiates, and 2 ml of methanol. The drugs were eluted with a solution consisting of a single phase mixture of dichloromethane/isopropanol/ammonium hydroxide (78/20/2) and collected in glass tubes. The elution solvent was evaporated to dryness under nitrogen stream. The dried residues were then reconstituted in slightly warm water, and nalorphine was added, before the electrical injection of the sample. The samples preparation and the SPE take approximately less than 40 min. In addition, as many as 12 samples can be simultaneously extracted using the Visiprep vacuum manifold (Supelco, Bellefonte, PA).

3. Results and discussion

In the first stage of our work, we used a buffer containing 50 mM sodium phosphate and 100 mM phosphoric acid at pH 6. A typical electropherogram from a mixture of heroin metabolites is shown in Fig. 2. Under these analytical conditions, analytes were only partially separated in the following order: normorphine + codeine, mor-

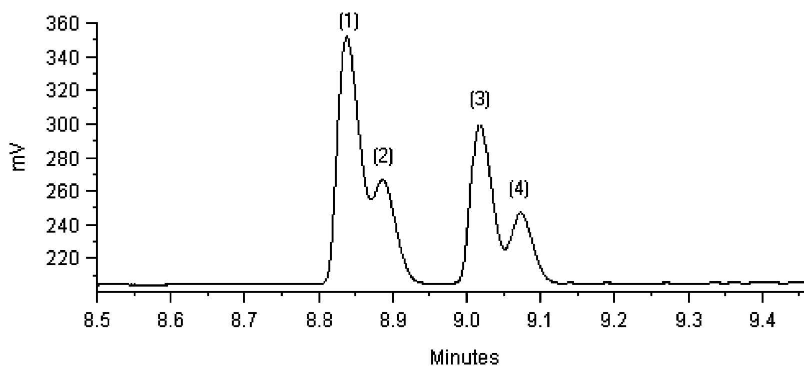

Fig. 2. Typical electropherogram of 500 ng/ml mixture of heroin metabolites. (1) Normorphine, codeine; (2) morphine; (3) 1000 ng/ml nalorphine and (4) 6-AM. Analytical conditions: injection, electrically (5 kV) for 10 s; capillary, uncoated (50 μm I.D.) fused-silica capillary, 77 cm long, and containing the detector window 10.0 cm from the outlet end; buffer, (pH 6.0) contained 50 mM sodium phosphate; potential 25 kV; detection, UV absorbance at 214 nm.

phine, nalorphine (IS_1), 6-AM. The lack of resolution may be attributed to the fact that all analytes migrate according to their mass to size ratio in CE, which in this case are very similar. Further experiments, performed by changing the pH and the buffer concentration, however, the results did not provide an acceptable separation (data not shown).

The effect of the addition of CDs is shown in Fig. 3.When β-CD was added to the buffer, clear separation of the standard mixture of four opiates was achieved. In this case, base line separation was accomplished with a running buffer composed of 50 mM sodium phosphate (pH 6.0) and 0.015 M β-CD. Under these analytical conditions, analytes were separated in the following order: normorphine, morphine, 6-AM, codeine, nalorphine and Levallorphan. This order can be attributed to the influence of the hydrophobic cavity of the β-CDs. Solutes may partition into and out of the cavity, and the migration velocities of the solutes can be affected as well. When the solutes partition into the cavities their velocities are retarded. When present in the bulk phase or the interstitial space between β-CDs, the solutes run a normal electrophoresis. The differences of polarity, size and structure of the solute molecules cause the differences in their partitioning behavior, which then

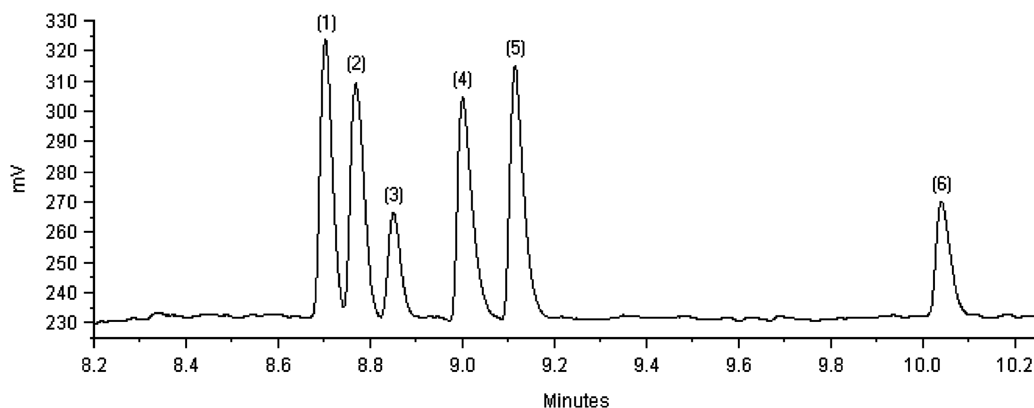

Fig. 3. Typical electropherogram of 500 ng/ml mixture of heroin metabolites. (1) Normorphine; (2) morphine; (3) 6-AM; (4) codeine; (5) 1000 ng/ml nalorphine and (6) 1000 ng/ml Levallorphan. Analytical conditions: buffer, (pH 6.0) contained 50 mM sodium phosphate and 0.015 M β-CDs, other conditions as Fig. 2.

results in differences in the migration velocities of the solutes and improvement of separation efficiency.

β-CD was also used in our lab to separate a standard mixture of morphine, codeine, thebiane, nalorphine (IS), papaverine and noscapin. The results were compared with a prepared opium sample Fig. 4.

To evaluate the linearity of the method, standard curves were prepared by analyzing six different concentrations of mixture of four opiates in the range of 100–500 ng/ml with a constant amount of nalorphine (1000 ng/ml). A linear regression analyses were performed using ratios of peak areas of drugs to internal standard against the respective drugs concentrations Fig. 5. The linear regression equation for normorphine, morphine, 6-AM, and codeine standard curves were $y = 0.0011x + 0.3511$ ($r = 0.996$), $y = 0.0007x + 0.2652$ ($r = 0.9977$), $y = 0.0005x + 0.1687$ ($r = 0.9985$) and $y = 0.001x + 0.3715$ ($r = 0.9971$), respectively.

The intra-day and overall accuracy and precision of the calibration curves were determined by analyzing three different concentrations of a mixture of pure standards of normorphine, morphine, 6-AM, and codeine and constant amount of nalorphine on three separate days. Three replicate

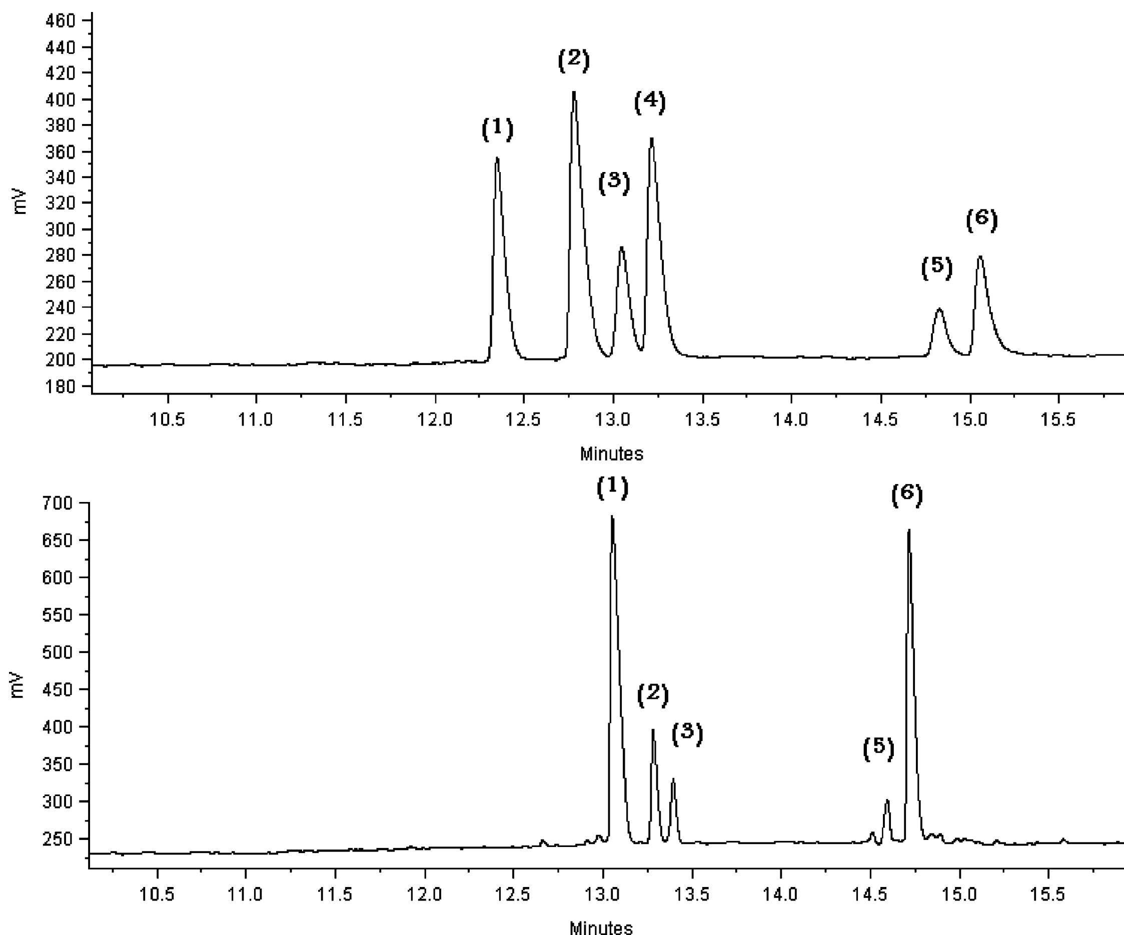

Fig. 4. Typical electropherogram of (a) 500 ng/ml mixture of (1) morphine; (2) codeine; (3) thebaine; (4) 1000 ng/ml nalorphine (IS); (5) papaverine and (6) noscapin; (b) prepared opium sample. Analytical conditions as in Fig. 3.

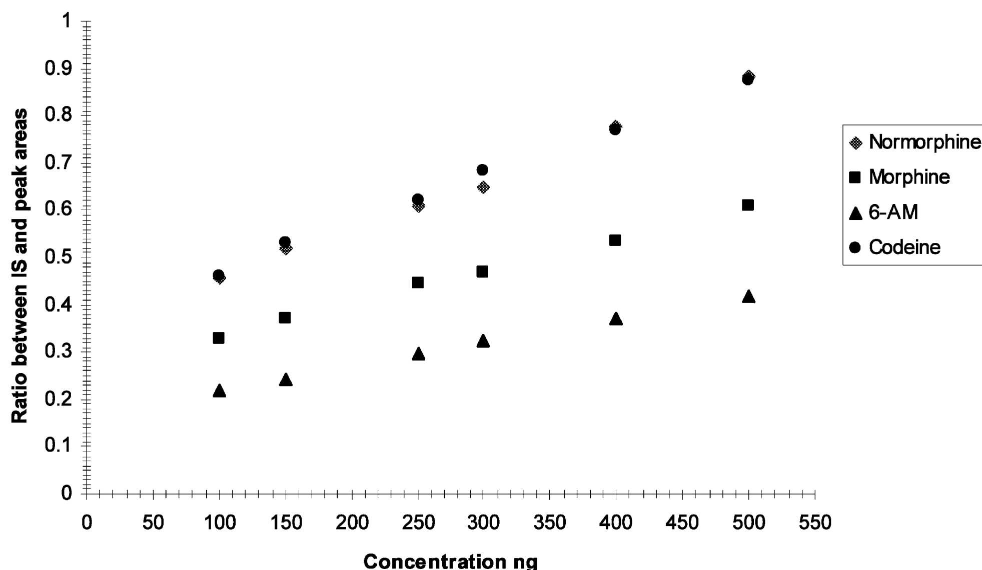

Fig. 5. Calibration curves of a mixture of normorphine, morphine, 6-AM and codeine with 1000 ng/ml nalorphine (IS_1) obtained from UV absorbance measurements at 214 nm.

determinations were made at each concentration level. The results of the reproducibility study are displayed in Table 1. The intra-day relative standard deviations (R.S.D.s) for migration times were $< 0.22\%$, and the overall precision was $< 0.14\%$. In case of peak area reproducibility,

Table 1
Analytical precision expressed as intra-day ($n = 3$) and overall ($n = 9$) R.S.D.% of relative migration times and peak areas

Concentration (ng/ml)	R.S.D. (%), migration time				R.S.D. (%), peak area			
	Day 1	Day 2	Day 3	Overall	Day 1	Day 2	Day 3	Overall
Normorphine								
400	0.010	0.009	0.220	0.142	1.4	1.3	1.6	1.5
250	0.008	0.005	0.044	0.126	1.2	1.5	1.1	1.2
100	0.005	0.043	0.015	0.105	0.1	0.5	1.2	1.0
Morphine								
400	0.011	0.047	0.197	0.114	0.4	2.2	0.7	4.7
250	0.009	0.002	0.037	0.109	1.4	1.2	2.1	1.4
100	0.008	0.048	0.009	0.089	1.8	1.5	3.0	2.0
6-AM								
400	0.007	0.014	0.171	0.096	2.1	0.8	1.8	1.5
250	0.018	0.004	0.041	0.077	1.0	1.2	1.6	2.8
100	0.013	0.041	0.026	0.058	0.9	1.6	0.8	1.3
Codeine								
400	0.012	0.017	0.078	0.049	1.6	0.6	0.6	1.4
250	0.006	0.005	0.015	0.009	0.4	0.2	0.6	0.8
100	0.011	0.006	0.005	0.009	1.2	2.1	0.5	1.3

Table 2
Detection limits for a mixture of pure standards of normorphine, morphine, 6-AM, and codeine using electrokinetic injection and UV-visible absorbance detector

Analyte	LOD (ng/ml)
Normorphine	30
Morphine	30
6-AM	40
Codeine	40

R.S.D.s were $<4.7\%$. The IS_1 was introduced to minimize the variation result from the fluctuation of the electroosmotic flow.

The detection limits (signal-to-noise ratio of 3) shown in Table 2 are in the region of 30–40 ng/ml using the conditions specified in the optimized assay method. These low detection limits are more than adequate for the usual analytical requirements for controlled drugs analysis in forensic laboratories (the total 6-AM results from heroin metabolites is greater than 350 ng/ml and the ratio of 6-AM/morphine is greater than 100% [2]).

Fig. 6 shows the plots of the mobility of heroin metabolites versus β-CD concentration and it is apparent that, mobility decreases as β-CD concentration increases, until the limit of 0.015 M, above which the solubility of the CD decreases resulting in a reduction in the reproducibility of the method. On this basis a concentration of 0.015 M was chosen as optimum.

Fig. 7 shows the importance of pH adjustment in obtaining selectivity. The pH of the buffer affects the degree of ionization of the drug under study. At low values of pH, drugs may become protonated, causing decreases in their charge-to-size ratios with subsequent decreases in their mobilities. As the pH is raised, their mobilities increase and their migration times get shorter. All four opiates are resolved between 5.5 and 6.5 and separations were found to be most reproducible in this pH range. On this basis a pH of 6.0 was chosen as optimum.

Fig. 8a shows a representative electropherogram of urine, spiked with heroin metabolites and

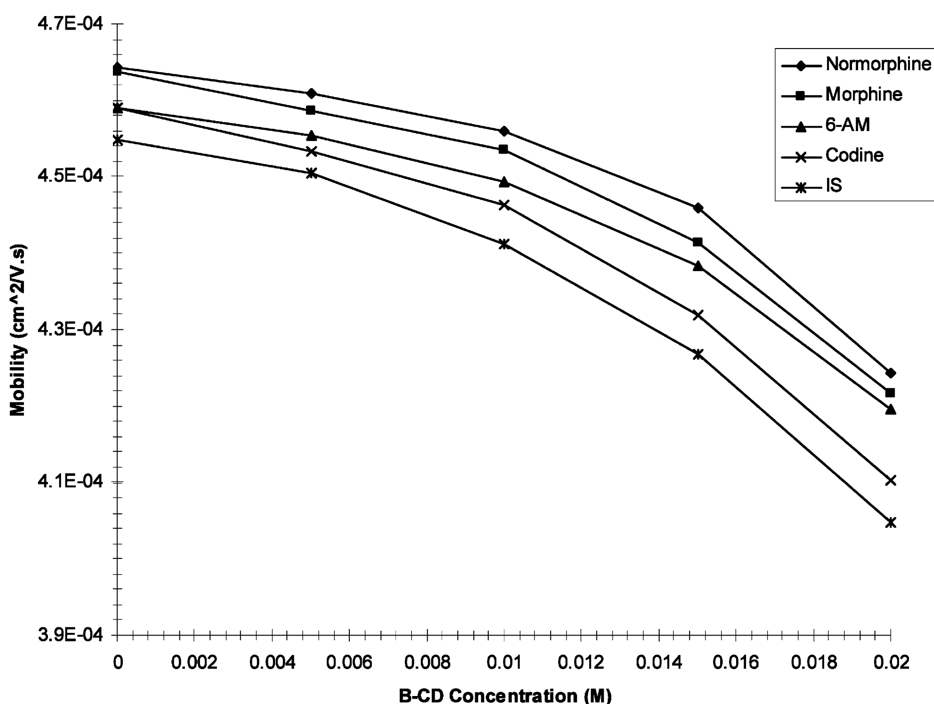

Fig. 6. Plots of β-CD concentration vs. the mobility of mixture of heroin metabolites; analytical conditions as in Fig. 3.

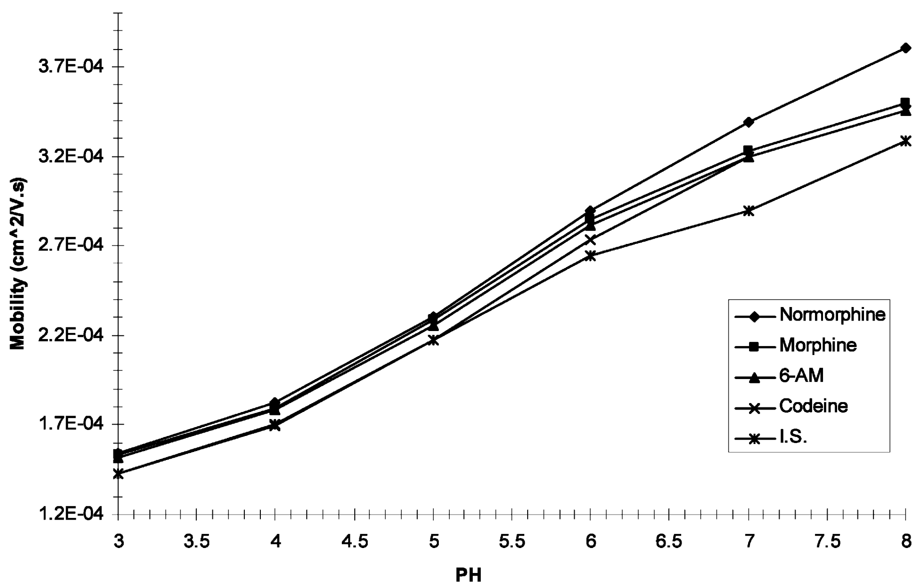

Fig. 7. Plots of pH vs. mobility of mixture of heroin metabolites; analytical conditions as in Fig. 3.

Levallorphan (IS_2) and including nalorphine (IS_1) after SPE. In the same figure, the electropherogram obtained by subjecting blank urine to the same treatment is shown Fig. 8b. It is evident that very few endogenous compounds in the urine are being extracted and applied to the capillary under the conditions used. The endogenous species detected have migration times shorter than 6-AM.

The other major metabolites of heroin are normorphine, morphine and codeine. These meta-

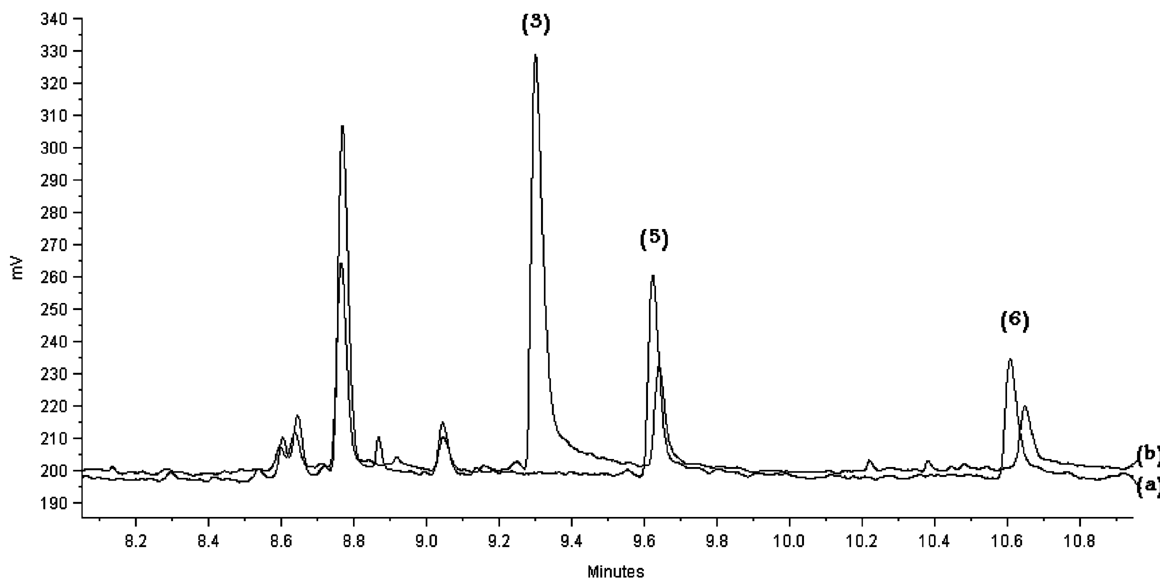

Fig. 8. Typical electropherogram of (a) urine spiked with 500 ng/ml heroin metabolites and 1000 ng/ml levallorphan (IS_2); (b) blank urine sample spiked with IS_2. The nalorphine (IS_1) was added after extraction. Peaks identification: (3) 6-AM; (5) nalorphine and (6) Levallorphan, other conditions as in Fig. 3.

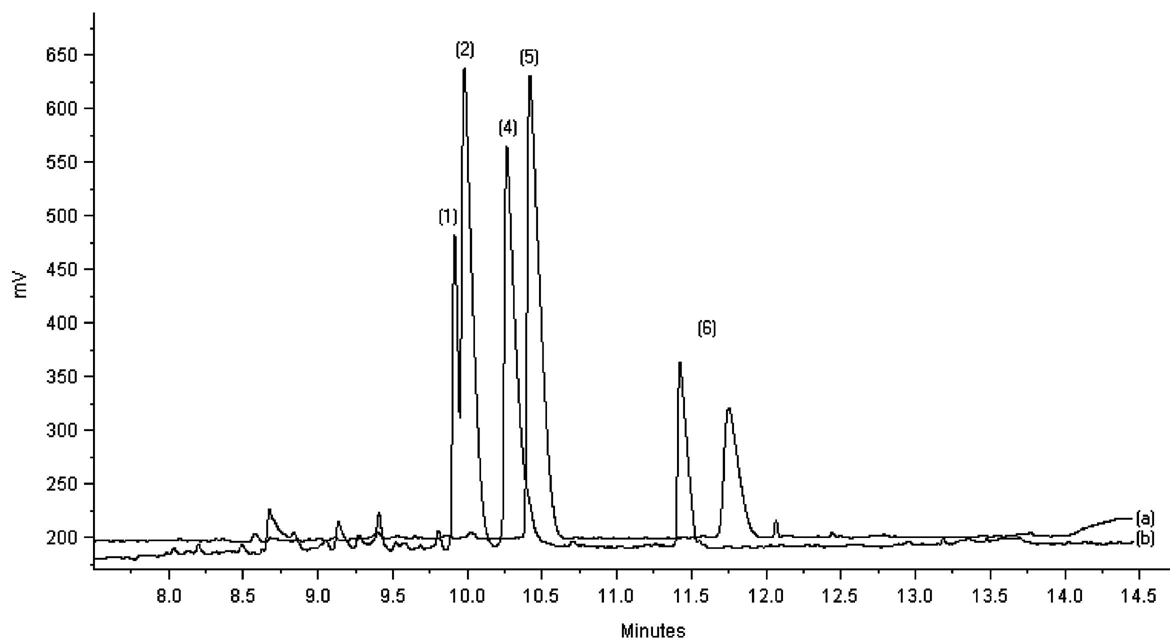

Fig. 9. Typical electropherogram of (a) urine spiked with 500 ng/ml heroin metabolites and 1000 ng/ml IS_2; (b) blank urine sample spiked with IS_2. Normorphine (IS_1) was added after extraction. Peaks identification: (1) normorphine; (2) morphine; (4) codeine; (5) IS_1 and (6) IS_2, other conditions as in Fig. 3.

bolites were assayed for any possible interferences and the chromatograms are shown in Fig. 9. There are no interferences present at any of the respective retention times. Moreover, results from urine samples were also extremely reproducible (Table 3).

Table 3
Reproducibility of analysis for urine samples spiked with normorphine, morphine, 6-AM, and codeine, ($n = 3$)

Actual concentration (ng/ml)	Calculated concentration (ng/ml) mean ± S.D.	R.S.D. (%)	Recovery (%)
Normorphine			
500	490 ± 5.23	1.20	98
400	380 ± 3.21	2.09	95
250	240 ± 2.08	1.82	96
Morphine			
500	512 ± 8.59	1.08	102
400	394 ± 6.42	2.09	99
250	240 ± 2.36	2.98	96
6-AM			
500	480 ± 10.88	2.79	96
400	394 ± 8.54	2.08	99
250	235 ± 5.55	3.63	94
Codeine			
500	456 ± 9.65	2.73	91
400	393 ± 5.33	3.60	98
250	220 ± 4.43	3.60	88

4. Conclusions

The analysis of heroin metabolites in human urine using CZE with β-CD and UV detection is a sensitive, precise, reproducible and simple screening technique for forensic analysis. This analytical method has a low detection limit when compared with other studies (below 30–40 ng/ml). This low detection limit may be attributed to the combination of electrokinetic injection and the stacking phenomenon.

The use of the Bond Elut Certify SPE column resulted in an excellent extraction and separation of heroin metabolites as well as good peak shape. There were no interferences with components from extracted endogenous compounds. In addition, the experimental work discussed in the present paper was directed towards investigating the effect of β-CD as a complexing agent for improving the separation selectivity of heroin metabolites. It has been found that β-CDs can improve the separation efficiency due to their hydrophobic cavity. This work further demonstrates that pH adjustment is very important for obtaining high resolution. All four opiates were resolved between pH 5.5–6.5 and separations were found to be most reproducible in this pH range.

Acknowledgements

The authors would like to thank Ira Lurie for useful discussions. This work was supported in part by a grant from the National Institute of Justice.

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