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Determination of morphine and codeine in urine using poly(dimethylsiloxane) microchip electrophoresis with electrochemical detection

Qian-Li Zhang, Jing-Juan Xu, Xiang-Yun Li, Hong-Zhen Lian, Hong-Yuan Chen *

The Key Laboratory of Analytical Chemistry for Life Science, Department of Chemistry, Nanjing University, Nanjing 210093, China

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Abstract

In this paper, a poly(dimethylsiloxane) (PDMS) microchip with electrochemical (EC) detection was developed for rapid separation and detection of morphine and codeine. It was found that morphine and codeine were well separated within 140 s in phosphate buffer solution (PBS) (pH 6.6, 40 mM)–β-cyclodextrin (β-CD) (20 mM)–acetonitrile (30%, v/v). The detection limit was 0.2 μM for morphine and 1 μM for codeine. The protocol was successfully applied to monitoring the amount of morphine and codeine in human urine. Compared with the conventional methods, the presented method had many advantages such as lower instrument cost, less reagent consumption and shorter analysis time.

Keywords: Poly(dimethylsiloxane) (PDMS) microchip; Electrochemical (EC) detection; Morphine; Codeine; β-Cyclodextrin (β-CD); Acetonitrile

1. Introduction

Morphine and codeine were the main alkaloids in poppy seeds having pharmacological and toxicological activity. As drugs, codeine is a common cough suppressant and both of them were usually used for treating acute and chronic pain [1,2]. But excessive or habitual use of morphine and codeine causes toxic symptom. Additionally, morphine and codeine were reported to be important indicators of poppy drug abuse, which lead to a lot of society problems [3,4]. For instance, when 6-monoacetylmorphine, the definitive metabolite of heroin, could not be detected for the short half-life of approximately 30 min [5], a detectable amount of morphine and codeine and the morphine-to-codeine ratio of higher than 2 were important criteria for judging recent heroin use [3]. Thus, the rapid separation and determination of morphine and codeine in biological fluids was of vital interest in clinical toxicology, control of drug abuse and forensic cases.

Chromatographic separation techniques, such as high performance liquid chromatography (HPLC) with UV detection [6] and MS detection [7], gas chromatography (GC) with MS detection [2,8–11] had been reported for the simultaneous quantification of morphine and codeine. However, the analysis process was time-consuming and the expensive devices and maintenance also limited their applications. Therefore, the development of sensitive, rapid, cheap and simple technique for the determination of morphine and codeine was very important. For this purpose, CE had been employed for the separation and detection of the two analytes [1,12]. Over conventional CE, microchip CE had attracted a lot of attention recently because it had outstanding advantages in separation speed, the cost of reagents and related instruments, and integration and miniaturization of the analytical instrument. It was reported that microchip CE would be the attractive technology for the next generation of CE instruments [13].

Miniaturized CE devices were initially fabricated in glass [14]. The fabrication procedure was complicated and expensive, and the fabricated device was fragile. Recently, polymer PDMS had become an important alternative for the fabrication of microchip CE device due to its low price and simple operation [15–18]. Similar to conventional CE, different detections including ultraviolet (UV), EC and laser induced fluorescence (LIF) detection can be coupled with PDMS microchip CE. Generally, EC detection was the most suitable detection mode for the micro-system because EC detection was sensitive, cheap, simple and easily miniaturized without sensitivity lost [19]. PDMS

* Corresponding author. Tel.: +86 25 83594862; fax: +86 25 83594862.
E-mail address: hychen@nju.edu.cn (H.-Y. Chen).

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microchip with EC detection had been successfully used for the separation of amino acids, proteins and some small biomolecules [20–24].

Here, the system was employed to develop a rapid, cheap, and sensitive method for the separation and detection of morphine and codeine. Though they had been separated in conventional CE, the separation of them was still a challenge work in PDMS microchip because the separation channel length was much shorter than that of conventional CE (usually 4–70 cm). In order to improve the resolution, the combination usage of β-CD and acetonitrile as buffer additives was developed for the separation of morphine and codeine. The factors that influence the separation and detection were studied in detail.

2. Materials and methods

2.1. Reagents and materials

All reagents were of analytical grade and used as received. Morphine and codeine were provided by China Doping Control Center. Disodium hydrogen phosphate dodecahydrate, sodium dihydrogen phosphate dihydrate, hydrochloric acid, β-cyclodextrin and acetonitrile were purchased from Shanghai Chemical Reagents Factory (Shanghai, China). Sylgard 184 (PDMS) was from Dow Corning (Midland, MI, USA). SPE cartridge columns (C_{18}, 250 mg) used for extracting the analytes from human urine were obtained from Hanbang Science and Technology Co. Ltd. (Huai'an, China). All solutions were prepared with doubly distilled water and passed through a 0.22-μm cellulose acetate filter (Xinya Purification Factory, Shanghai, China). In addition, all experiments were performed at ambient temperature.

2.2. Apparatus

The PDMS chip was horizontally fixed on a laboratory-made Plexi glass microchip holder as we reported previously [22]. Briefly, the position of working electrode was adjusted by a 3D adjustor (Shanghai Lianyi Instrument Factory of Optical Fiber and Laser, Shanghai, China) and inserted into the channel about 40 μm with the help of a XTB-1 microscope (Jiangnan Optical Instrument Factory, Nanjing, China). EC detection was performed with a CHI 832 electrochemical workstation (CHI Co., Shanghai, China). A traditional three-electrode system was used with a carbon fiber micro-column electrode (diameter: 8 μm, length: 60 μm) as a working electrode, a platinum wire as an auxiliary electrode, and an AgCl/Ag electrode (made of chlorinated Ag wire and its formal potential was ca. 0.5 V) as a reference one against which all potentials were recorded.

HPLC analysis was carried out on a Waters 2695 Separations Module equipped with a vacuum degasser, a quaternary pump and an auto-sampler, and a 996 UV–vis photodiode-array detector (PDA) (Waters, Milford, USA). The separation was controlled and the chromatograms were recorded by a Waters Millinium^{32} chromatography manager system.

2.3. Preparation of PDMS microchip and working electrode

The cross-type PDMS microchip with a 4.2-cm long separation channel (effective separation length, 4.0 cm) and 1.0-cm long injection channel was fabricated using a positive GaAs channel master (no. 55, Electronic Institute, Nanjing, China). PDMS monomer and curing agent were thoroughly mixed with 10:1 ratio, degassed and poured over the master. After curing at 70 °C for 2 h and cooling at room temperature, the PDMS was peeled off from the master, cut to the proper size by a blade and holes (3 mm in diameter) were punched into the cure polymer to create access reservoirs. Scanning electron microscopy (Hitachi X-650, Japan) showed that the channel was 50 μm wide at the top, 65 μm wide at the bottom, and 17 μm in depth. A flat piece of PDMS was molded in the same procedure using glass as a master. The PDMS with micro-channels was sealed against the flat one after both halves were ultrasonically cleaned with methanol and water. Once dried by infrared lamp, the two pieces were put together and formed a reversible seal.

The fabrication of working electrode was according to the previous report [23]. Briefly, a glass capillary with inner diameter of 0.5 mm was pulled to form a 0.3 mm tip by a multifunctional glass microelectrode puller (Shanghai Biological Institute, Shanghai, China). A single carbon fiber with a diameter of 8 μm was carefully inserted into the tip and fixed with epoxy. The length of carbon fiber out of tip was taken for ca. 60 μm. A copper wire was connected with the carbon fiber through carbon powder on the other end of capillary and then fastened with epoxy. Before use, the electrode was electrochemically activated at 2.0 and −1.0 V for 60 s, respectively.

2.4. Electrophoresis procedures

The running buffer solution of PBS (pH 6.6, 40 mM)–β-CD (20 mM)–acetonitrile (30%, v/v) was freshly prepared daily. An easy channel outgas technique [25] was used here to fill PDMS channel with water. The water in microchannel was then replaced by the running buffer solution under high voltage. Then the device was flushed for 15 min with the running buffer at high voltage of 1000 V. Sample was injected into the separation channel using a cross arrangement.

2.5. Sample preparation

Urine sample was obtained from a healthy volunteer after oral administration of 25 mL cough syrup containing codeine phosphate (1 mg/mL). Blank sample was obtained from the healthy volunteer with a normal diet as control. SPE was applied to extract the analytes from urine. The procedure was as follows: SPE cartridge was activated with 5 mL methanol and 5 mL water. Then 2 mL urine was introduced to SPE cartridge and washed consecutively with 1 mL 0.01 M HCl and 5 mL water. Followed vacuum about 2 min, the analytes were eluted with 1 mL methanol and the extract was then dried under a stream of nitrogen at 40 °C. The residue was dissolved in 1 mL running buffer solution for assay.

3. Results and discussion

3.1. Selection of the detection potential

In EC detection, the detection potential was necessary to be optimized because it greatly affects the electrochemical behavior of analytes. The hydrodynamic voltamograms for morphine and codeine was illustrated in Fig. 1. It was observed that when the potential was lower than 0.8 V, the analyte signals for morphine and codeine were relatively small. When the detection potential was above 1.4 V, the background current increased apparently though the analyte signals of the two analytes increased as well. In order to obtain relative high signal-to-noise ratio, 1.4 V was selected as the optimum detection potential.

3.2. Selection of the running buffer

3.2.1. Effect of buffer modifier

In the experiment, the separation of morphine and codeine was found to be impossible in PBS in PDMS micro-channel (Fig. 2, curve 1). It was reported that surfactants, organic modifiers and cyclodextrins could be used as additives of the electrophoresis buffer to enhance the selectivity of similar compounds [26–30]. Thus many additives including surfactants, organic modifiers and cyclodextrin were tested in order to obtain good separation efficiency of morphine and codeine. It was found that the combination use of β-CD and acetonitrile was the most effective modifier.

Theoretically, when β-CD was added to the running buffer, stable inclusion β-CD complexes can be formed according to the molecular structures of morphine and codeine including size, hydrophobic property, and relative position of substituent groups. Fig. 2 presents the effects of β-CD concentration on the migration behaviors of morphine and codeine. As can be seen, an increase of β-CD concentration increased the migration time of both analytes and enhanced the resolution, which was due to relatively small decrease in analyte mobility from specific β-CD

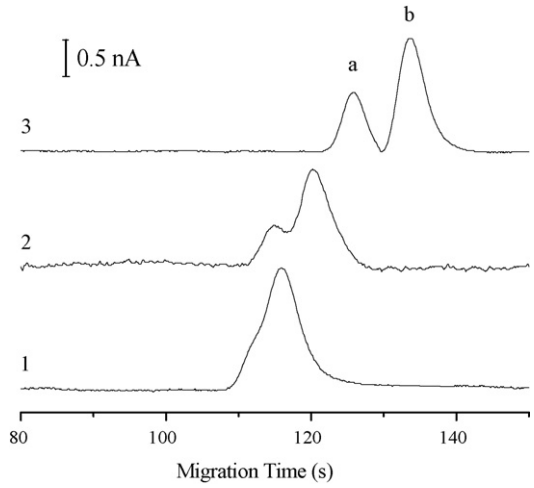


Fig. 2. Electropherograms of morphine (a) and codeine (b) of different β-CD concentration. (1) 0 mM, (2) 10 mM and (3) 20 mM. Detection potential, 1.4 V (vs. AgCl/Ag). Other conditions were the same as Fig. 1.

binding superimposed and increase in buffer viscosity with β-CD addition. The addition of 20 mM β-CD yielded the optimal approach to separate morphine and codeine. Further increasing the concentration of β-CD was not appropriate for the insolubility of β-CD and a relative long separation time.

Organic modifiers can affect the interaction between analytes and microchannel, the dielectric constant of the solution and zeta potential of electrical double layer of the microchannal. This provided a confirmation that they can modify the electroosmotic flow (EOF) and improve the separation. Several papers had indicated the effective use of organic modifiers in CE [28,31,32]. Acetonitrile, methanol and ethanol were tested and acetonitrile exhibited the best performance. A series of experiments with different concentration of acetonitrile from 0% to 40% were investigated (Fig. 3). It was demonstrated that higher concentration of acetonitrile increased the analysis time, sharpened peaks and enhanced the resolution. Additionally, when 40% (v/v) acetontrile was added, bubbles were often observed

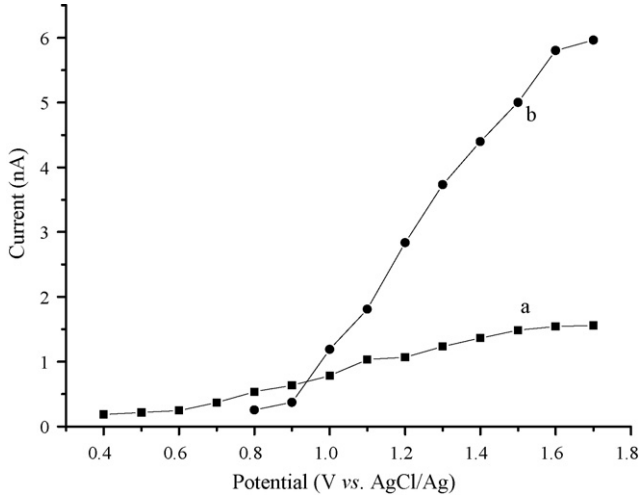


Fig. 1. Hydrodynamic voltammograms for 118 μM morphine (a) and 322 μM codeine (b). Separation voltage, 1000 V; sample injection, at 600 V for 6 s; running buffer, PBS (pH 6.6, 40 mM)–β-CD (20 mM)–acetonitrile (30%, v/v).

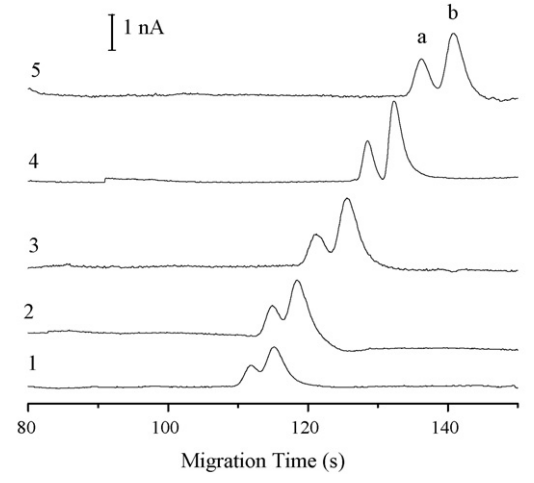


Fig. 3. Electropherograms of morphine (a) and codeine (b) of different acetonitrile concentration (v/v). (1) 0%, (2) 10%, (3) 20%, (4) 30% and (5) 40%. Detection potential, 1.4 V (vs. AgCl/Ag). Other conditions were the same as Fig. 1.

in PDMS microchannel, which resulted in interrupting the separation. The 30% (v/v) acetonitrile was selected in the further experiment.

3.2.2. Effect of buffer pH and concentration

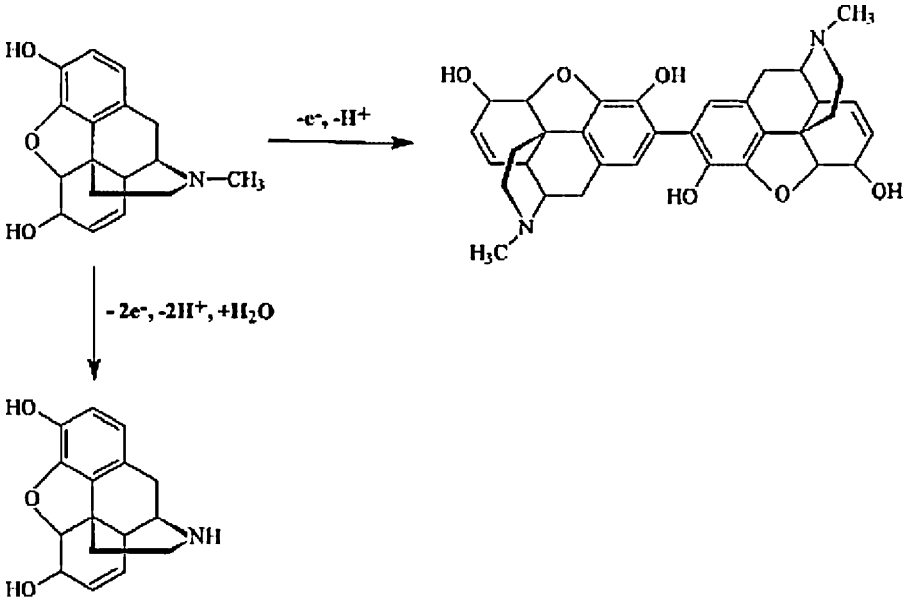
The kind, pH and concentration of buffer solution, just as those in conventional CE, also play important roles in separation and detection of morphine and codeine. At the same conditions, PBS gave the better resolution and more sensitive detction for morphine and codeine than borate buffer solution. Therefore, PBS was chosen in further experiment.

In PDMS microchip CE, the pH value of PBS affected not only EOF but also the ionization of morphine and codeine. Garrido et. al. [33,34] had reported that the complex oxidation mechanisms of morphine and codine were as follows:

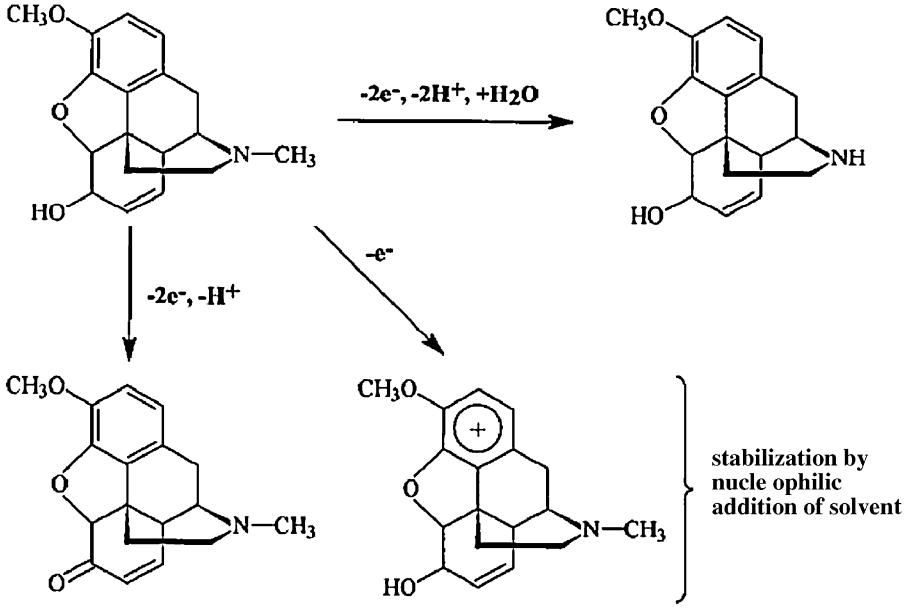
It was clear that proton played an important role in the oxidation of morphine and codeine. In this work, the pH effect on the electrophoresis analyte signals was shown in Fig. 4. The buffer of pH 6.6 was found to be optimal for electrochemical detection and separation of morphine and codeine in PDMS micochannel and thus chosen for further experiments.

The effect of the concentration of pH 6.6 PBS on the separation of morphine and codeine was investigated in the range of 20–50 mM. It was found that EOF in PDMS microchannel decreased with the increase of PBS concentration, which resulted in the increase of migration time of the two analytes and their resolution. The 40 mM PBS was chosen due to the relatively short analysis time and high resolution. When PBS concentration was over 40 mM, too broad peak of the analytes was observed for the high Joule heat.

(1) Oxidation mechanism of morphine:



(2) Oxidation mechanism of codeine:



3.3. Effect of separation voltage

The effect of the separation voltage was examined (Fig. 5). As had been reported that when the separation voltage increased, migration time of morphine and codeine decreased and the peak shapes became sharper. However, the electric current in the microchannel increased obviously. A too high electric current in the microchannel will result in peak broadening and non-ideal stability of separation. On the basis of separation speed, efficiency and the stability, 1000 V was chosen as the separation voltage.

3.4. Linearity, detection limits and reproducibility

Under the optimized conditions, morphine and codeine were well separated within 140 s. The analyte signals were linearly on the concentrations in the range of 11–580 μM for codeine and 7.0–570 μM for morphine, respectively. The regression equations were $Y = 0.0608 + 0.00375X$ ($R = 0.998$) for codeine and $Y = 0.243 + 0.00551X$ ($R = 0.998$) for morphine, where Y represented the analyte signal (nA) and X represented the concentration of the analyte (μM). Based on $S/N = 3$, the detection limits for morphine and codeine were 0.2 and 1 μM, respectively.

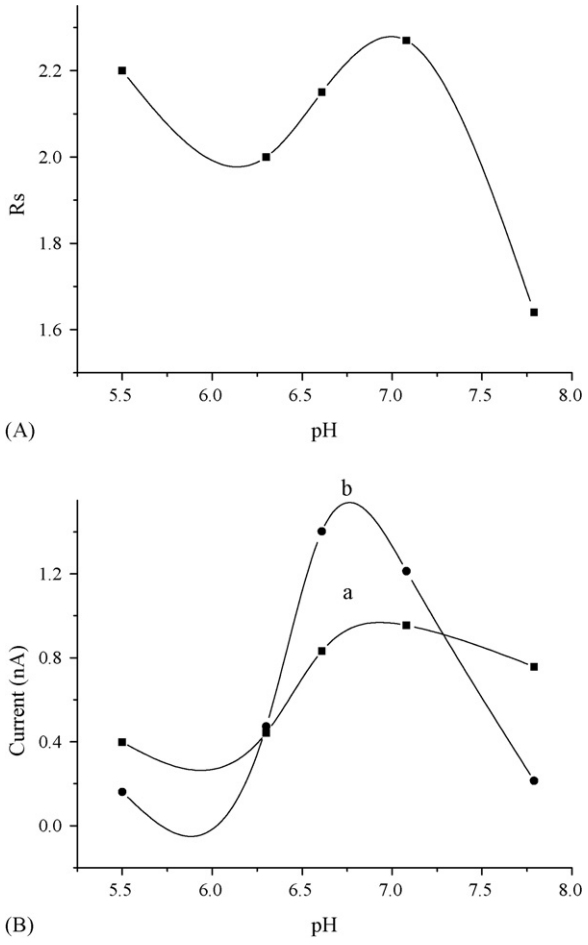


Fig. 4. Effect of PBS buffer pH on the resolution (A) and analyte signal (B) of morphine and codeine. Detection potential, 1.4 V (vs. AgCl/Ag). Other conditions were the same as Fig. 1.

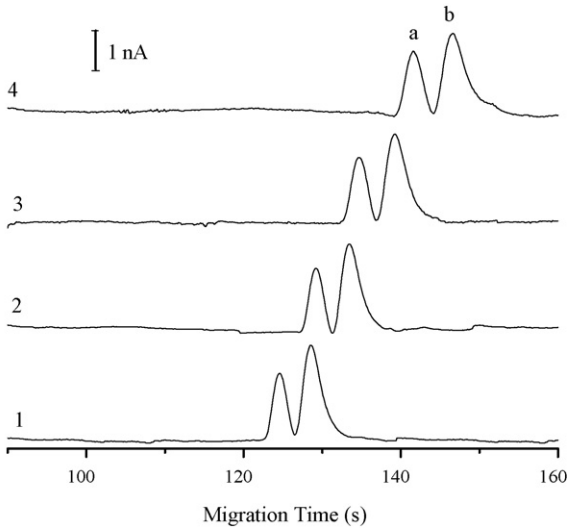


Fig. 5. Effect of separation voltage on the electropherograms of morphine (a) and codeine (b). (1) 1100 V, (2) 1000 V, (3) 900 V and (4) 800 V. Detection potential, 1.4 V (vs. AgCl/Ag). Other conditions were the same as Fig. 1.

Nine replicate injections of a mixture of 0.2 mM morphine and codeine resulted in R.S.D. of 4.2% and 5.7% for analyte signal and 1.0% and 0.9% for migration time respectively, demonstrating relatively good reproducibility.

4. Application

Urine sample was obtained from a healthy volunteer after oral administration of 25 mL cough syrup, which contained codeine phosphate (labeled concentration 1 mg/mL). Urine sample was treated as described in Section 2.5. Codeine can be metabolized by demethylation to its active metabolite morphine. Thus after oral administration of cough syrup, morphine was present in low concentrations in urine. Fig. 6 was the electropherogram of the urine sample. Peak a was identified as morphine and peak b as codeine by the migration time and spiking each standard of the two analytes into the urine sample. The quantitative analytical results were summarized in Table 1. As can be seen, the concentration of morphine and codeine were detected to be 4.8 and 18.1 μM by the modified PDMS microchip CE. Meanwhile, urine sample was analysed by HPLC, which was carried

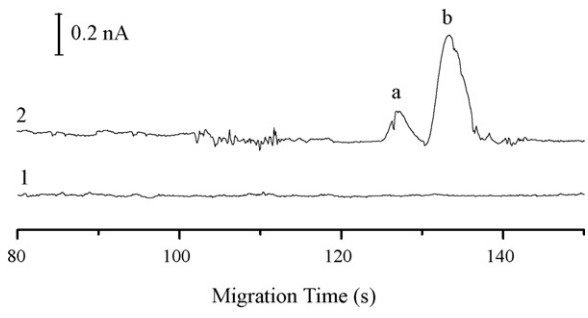


Fig. 6. Electropherograms of morphine (a) and codeine (b) in humam urine. (1) Blank urine, (2) urine after 1 h oral administration of drug containing phosphate codeine. Detection potential, 1.4 V (vs. AgCl/Ag). Other conditions were the same as Fig. 1.

Table 1
Analytical results of urine sample ($n = 3$)

Method	Analyte	Migration time (s)	Found concentration (μM)	R.S.D. (%)
Microchip CE	Morphine	125.7	4.8	5.7
	Codeine	133.7	18.1	4.6
HPLC	Morphine	546.3	5.0	1.7
	Codeine	1700.1	17.6	1.3

out on a Kromasil C18 column using CH_3OH–KH_2PO_4 (pH 6.9, 10 mM, containing 2 mM triethylamine)–H_2O (20:50:30, v/v/v) at a flow-rate of 1.0 mL/min and detection by UV absorbance at a wavelength of 283 nm. The results of HPLC were also listed in Table 1. There was no significant difference of the detection results between the presented method and HPLC. However, the analysis time of microchip CE was 10 times shorter than that of HPLC.

Recovery experiments were also carried out. The recovery was evaluated by comparing the analyte signals of morphine and codeine obtained from the spiked urine with those of the same concentration standard solution ($n = 5$). The average recovery was 96.2% for morphine with R.S.D. of 4.4% and 105.7% for codeine with R.S.D. of 5.1% ($n = 3$), which indicated the accuracy of the method.

In conclusion, the presented work had demonstrated the success of β-CD and acetonitrile modified PDMS microchip CE with EC detection mode for separation and detection of trace morphine and codeine in human urine and the results were in good agreement with those of HPLC. The advantages of this method such as low price, negligible sample consumption and waste production, and small size of device, can offer great promise for rapid determination of morphine and codeine in biofluidic sample.

Acknowledgements

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