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Separation of caffeine and theophylline in poly(dimethylsiloxane) microchannel electrophoresis with electrochemical detection

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Abstract

A method for the rapid separation and sensitive determination of caffeine and theophylline was presented in poly(dimethylsiloxane) (PDMS) microchannel electrophoresis integrated with electrochemical detection. By using methanol as an additive, the peak shape and resolution were essentially improved. The analytes were well separated within only 40 s in the running buffer of 5.0 mM borate solution (pH 9.2) containing 10% (v/v) methanol. The linear ranges were from 6 μM to 0.6 mM and the detection limits were 4 μM for caffeine and theophylline, respectively. The proposed method has been successfully applied to determine caffeine and theophylline in rat serum and urine.
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Keywords: PDMS microchannel electrophoresis; Electrochemical detection; Caffeine; Theophylline

1. Introduction

Caffeine and its major metabolite, theophylline, are widely distributed in plant products. Similar to other methylxanthine derivatives, they can cause various physiological effects such as relaxation of bronchial muscle, stimulation of the central nervous system, gastric acid secretion and diuresis. And their concentration in vivo is a key mark for various disorders including heart disease, carcinogenesis, kidney malfunction, and asthma [1]. Therefore, establishing a rapid and cheap analytical method for their simultaneous determination is of interest in clinical diagnosis, pharmaceutical analysis and doping control, etc.

The separation of caffeine and its metabolites in biological fluids and drugs has been performed with HPLC [2–4], and capillary electrophoresis (CE) [1,5–9], etc. The device and maintenance of HPLC are usually expensive. As an alternative, conventional CE can provide simple operation for the separation. But it often takes thousands of seconds for a separation process. In the past decades, microchip CE has quickly become a key area in analytical and biomedical separation for the following reasons. Firstly, the separation period on a microchip can be greatly decreased to be decades of seconds. Secondly, the injection and separation in the microchannel can be carried out simply and automatically. Thirdly, the consumption of reagents and energy is greatly reduced so that the impact of analytical behavior on environment can be ignored. Therefore, CE on microchip has become a trend in the development of the next generation of CE [10–12].

Chiem and Harrison [13,14] reported the separation of theophylline in serum samples on glass CE microchip integrated with fluorescence detection based on immunoassay. However, the glass microchip is somewhat expensive ($200 for a research chip). The polymer of PDMS has recently become an important alternative due to its low price and simple operation for the fabrication of microchip, and PDMS microchip CE integrated with electrochemical detection have been widely reported in the recent literatures because of the easy miniaturation of detection instrument without loss of sensitivity [15–22].

In the present work, separation of caffeine and theophylline were carried out in PDMS microchip CE system with electrochemical detection. The factors that influenced the separation and detection were optimized. Caffeine and theophylline in urine and serum of rats were successfully separated and quantified, and the results were in accordance with those obtained using HPLC.

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2. Experimental

2.1. Reagents and materials

All reagents were of analytical grade. Caffeine and theophylline were the gifts from China Doping Control Center. Sodium hydroxide, hydrochloric acid, borate and methanol were purchased from Shanghai Chemical Reagents Factory (Shanghai, China). Sylgard 184 (PDMS) was from Dow Corning (Midland, MI, USA). SD rats (5 weeks old, 180–200 g) used for animal experiments were supplied by Animal Center of Nanjing Medical University. SPE cartridge columns (C_{18}, 250 mg) used for pretreating the biological fluids of rats were from Hanbang Science and Technology Co. Ltd. (Huaiyin, China). All solutions were prepared with doubly distilled water and passed through a 0.22-μm cellulose acetate filter (Xinya Purification Factory, Shanghai, China).

2.2. Apparatus

The PDMS chip was horizontally fixed on a laboratory-made plexiglass microchip holder as we reported previously [17]. 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 [24] with the help of a XTB-1 microscope (Jiangnan Optical Instrument Factory, Nanjing, China). Electrochemical detection was performed with a CHI 832 electrochemical workstation (CHI Co., Shanghai, China). A traditional three-electrode system was used with a carbon fiber microdisk electrode with a diameter of 8 μm as working electrode, a platinum wire as auxiliary electrode, and an AgCl/Ag electrode (it was made of chlorinated Ag wire and its formal potential is 0.5 V (versus SHE)) as reference one against which all potentials were measured.

2.3. Preparation of PDMS microchip and working electrode

The method used to create PDMS microchip was based on our previously published procedure [23]. The cross-type microchip has a 42-mm long separation channel (effective separation length, 39 mm) and a 10-mm long injection channel. Scanning electron microscopy (Hitachi X-650, Japan) showed that the cross-section of the channel was 50 μm wide at the top, 65 μm wide at the bottom, and 17 μm in depth.

The fabrication of working electrode was the same as our previous work [24]. 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 of 5.0 mM borate solution (pH 9.2) containing 10% (v/v) methanol was freshly prepared every day. 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 the high voltage of 1500 V. Sample was injected into the separation channel using a cross arrangement.

2.5. Sample preparation

Blood and urine of healthy SD rats were collected 60 and 120 min after they were oral administration of theophylline and caffeine, respectively [26]. Blank sample was obtained from the rats with a normal diet as control. Blood sample was stayed for 30 min and then centrifuged at 3500 rpm for 15 min. The supernatant (serum) was collected. SPE was applied to pretreat the serum and urine. The procedure was as follows: SPE columns were washed with 5 mL methanol and 5 mL water. One millilitre of serum or urine was diluted to 2 mL with water, then was introduced to SPE columns and washed consecutively with 0.01 M HCl and 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 running buffer solutions

Similar to conventional CE, the running buffer solution has a considerable effect on the separation in microchip CE because it influences the total charge of analytes and the generation of electroosmotic flow (EOF). It was firstly optimized in our experiment. Fig. 1 showed the electropherograms of caffeine and theophylline in 5.0 mM borate buffer solution with different pH values of 8.6, 9.2, and 10.0. It can be observed that the resolution of the analytes was poor at pH 8.6. When the running buffer pH was over 9.2, the resolution was satisfactory. Meanwhile, the separation time increased with the increase pH of running buffer. The concentration of borate buffer solution was investigated in

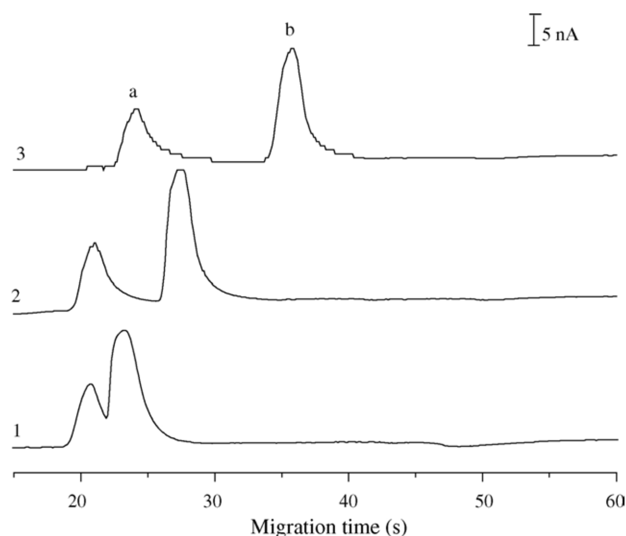

Fig. 1. Effect of pH on the electropherograms of caffeine (a) and theophylline (b). Separation voltage, 1000 V; sample injection, at 1000 V for 4 s; working electrode, carbon fiber microdisk electrode; detection potential, 1.5 V; running buffer solution, borate buffer solution. (1) pH 8.5, (2) pH 9.2, (3) pH 10.0.

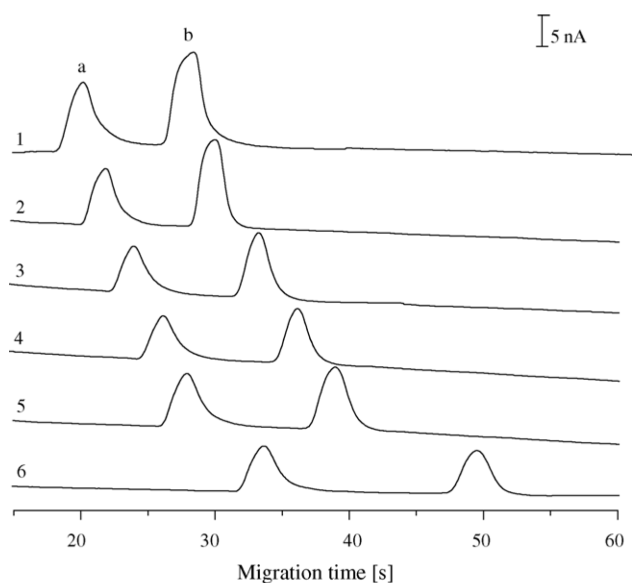

Fig. 2. Electropherograms obtained at different methanol concentration in 5.0 mM borate solution (pH 9.2). (1) 0% methanol; (2) 5% methanol; (3) 10% methanol; (4) 15% methanol; (5) 20% methanol; (6) 30% methanol. Other conditions were the same as in Fig. 1.

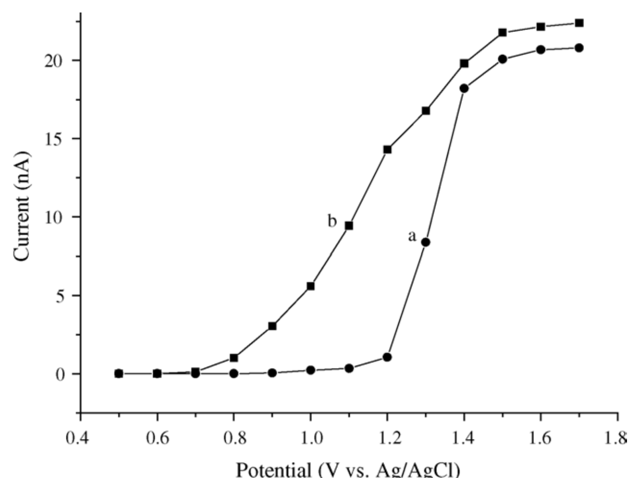

Fig. 3. Hydrodynamic voltammograms for 0.2 mM caffeine (a) and 0.2 mM theophylline (b). Other conditions were the same as in Fig. 1.

the range of 3.0–20.0 mM. 5.0 mM gave the best performance. So 5.0 mM borate buffer solution (pH 9.2) was chosen for the further experiment.

Fig. 2 (curve 1) showed the electropherograms of caffeine and theophylline in 5.0 mM borate buffer solution (pH 9.2). It can be seen that the peak shape is not very good, especially for caffeine. Organic modifiers have been reported to influence the EOF, the viscosity and the conductivity of the running buffer, and can improve the peak shape [27,28]. Thus, conventional organic additives such as methanol, ethanol and acetonitrile have been tested. Methanol was presented to improve the peak shape essentially and the resolution could be gradually improved with the increase of its concentration (Fig. 2 curve 2–6). However, too high methanol concentration leads the decrease of peak height. Considering the sensitivity and resolution, a concentration of 10% (v/v) methanol was selected.

3.2. Electrochemical characteristic of the analytes

Fig. 3 illustrated the hydrodynamic voltammograms of caffeine (a) and theophylline (b) detected by carbon fibre microdisk electrode with the detection potential from 0.5 to 1.7 V under the separation voltage of 1000 V. As shown in the figure, when the detection potential was over 1.5 V, the peak height of both analytes became steady. Additionally, the noise of the electrochemical baseline increased with the increase of the potential. Thus 1.5 V was chosen as the optimal detection potential.

3.3. Effects of separation voltage

Increase separation voltage from 600 to 1000 V resulted in shorter migration time and higher separation efficiency. However, the separation efficiency decreased when further increased the separation voltage, which was attribute to Joule-heat effect. Therefore a separation voltage of 1000 V was selected.

3.4. Validation of the method

Under the optimized experimental conditions, caffeine and theophylline can be well separated within 40 s. Linear data was attained from peak height of caffeine and theophylline. Both of the analytes have the linear range from 6 μM to 0.6 mM. The regression equations are $Y = 0.00215 + 10.05X$ ($R = 0.999$) and $Y = 0.0347 + 8.94X$ ($R = 0.998$) for caffeine and theophylline, respectively, where Y represents the peak current (nA) and X is the concentration of the analyte (mM). The detection limit was found to be 4 μM for both analytes (at S/N = 3). Seven repeated injections of 0.2 mM caffeine and theophylline demonstrated that the run-to-run RSD of peak current was 3.8 and 4.9%, and the RSD of migration time was 1.0 and 0.9% for caffeine and theophylline, respectively, showing a good reproducibility.

In order to investigate the recovery of the proposed method for biological samples, the blank serum was spiked with different concentration standard compounds. The recovery was estimated by comparing the peak current of caffeine and theophylline obtained from the spiked serum with that obtained from standard solution of the same concentration. The results listed in Table 1 indicated the satisfactory reliability.

Table 1
The results of recovery in this method ($n = 3$)

Compound	Concentration (mM)	Recovery (%)	RSD (%)
Caffeine	0.10	95.1	3.7
	0.30	107.6	4.5
	0.50	86.4	2.2
Theophylline	0.10	80.1	3.2
	0.30	88.3	0.8
	0.50	108.3	5.2

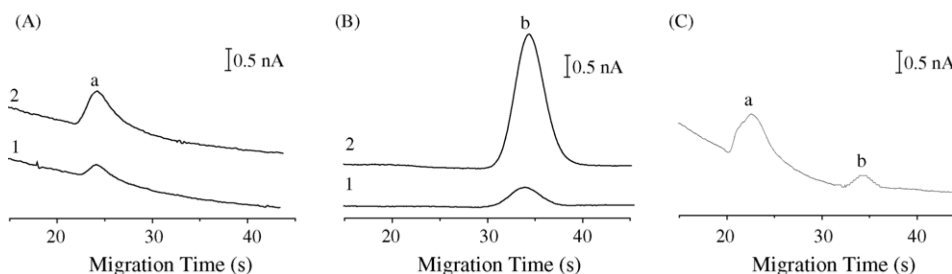

Fig. 4. Electropherograms of real samples. (A) (1) serum (rat was fed with 30 mg caffeine); (2) (1) + 30 μmol caffeine; (B) (1) serum (rat was fed with 20 mg theophylline); (2) (1) + 50 μmol theophylline; (C) urine (rat was fed with 30 mg caffeine); Peaks, caffeine (a); theophylline (b). Experimental conditions were the same as in Fig. 1.

Table 2
Assay results of the method used to determine the analytes in rat fluid ($n = 3$)

Samples	Compounds	Found (μg/mL)	RSD (%)	HPLC found (μg/mL)
Urine 1	Caffeine	48.9	1.4	48.7
	Theophylline	2.6	2.0	1.9
Serum 2	Theophylline	121.9	2.1	121.7
Serum 3	Caffeine	56.8	7.4	
Serum 4	Caffeine	56.7	3.5	
Serum 5	Theophylline	33.9	1.3	
Serum 6	Theophylline	96.3	1.9	

3.5. Analytical application

The presented method has been applied for the separation of caffeine and theophylline in rat serum and urine. Electropherograms of the samples were shown in Fig. 4. Samples A (serum) and C (urine) were obtained from a healthy rat 2 h after an oral dose of 30 mg caffeine. Sample B (serum) was obtained from another healthy rat 1 h after an oral administration of 20 mg theophylline. The samples were pretreated as Section 2.5. The identification of analytes was by the comparison of migration time and spiking with the standard compounds. As one of the important metabolite of caffeine, theophylline was found in sample C.

In order to avoid the contamination each other, a rinsing channel procedure with running buffer should be carried out after every sample has been determined for three times. The determination results are listed in Table 2. Urine 1 and serum 2 were validated by HPLC, which was carried out on a Kromasil C18 column using methanol–acetonitrile–acetic acid–water (15:15:20:50, v/v/v/v) at a flow-rate of 1.0 mL/min and detection by UV absorbance at a wavelength of 270 nm. As can be observed from Table 2, the obtained results were in accordance with those obtained by HPLC, indicating that our method was accurate and convincing.

4. Conclusion

The PDMS-microchip CE integrated with electrochemical detection has been demonstrated to be a new alternative for rapid separation and sensitive determination of caffeine and theophylline. The miniaturized CE-EC system has been successfully applied to analysis the biological fluids of rats. The good agreement of the results obtained with the miniaturized system and those obtained with HPLC indicated the applicable prospect of such a microsystem for some bioassays. Moreover, the microsystem has many attractive advantages such as cheapness, simplification, good resolution, and low reagent consumption. Future works will be focused on the development of online pretreatment of real sample in microchip.

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