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Selective extraction of alkaloids in human urine by on-line single drop microextraction coupled with sweeping micellar electrokinetic chromatography

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ABSTRACT

A novel method of on-line single drop microextraction (SDME) coupled with sweeping micellar electrokinetic chromatography (MEKC) for the selective extraction and dual preconcentration of alkaloids was developed. In this technique, analytes of three alkaloids were firstly extracted from 4.0 mL basic aqueous sample solution (donor phase, 500 mM NaOH) into a layer of *n*-octanol at temperature 30 °C with the stirring rate of 1150 rpm, then back-extracted into the acidified aqueous acceptor (acceptor phase, 50 mM H_3PO_4) suspended at the tip of a capillary at 650 rpm. Then, the aqueous acceptor was introduced into capillary by hydrodynamic injection with a height difference of 15 cm between the inlet and outlet of capillary for 300 s, and analyzed directly by on-line sweeping MEKC. With the selective SDME, we were able to extract three alkaloids without any interfering components in human urine samples. Under the optimum conditions, the proposed method achieved limits of detections (LOD) of between 0.2 ng mL^{-1} and 1.5 ng mL^{-1} with 1583–3556-fold increases in detection sensitivity for three analytes, which indicated that it was a promising method for analysis of alkaloids in human urine.

1. Introduction

Over the recent years, capillary electrophoresis (CE) has become a widely employed separation technique and has been successfully used for the analysis of alkaloids due to low samples consumption (fewer nanoliters), high separation efficiency, fast analysis speed and multiple separation modes [1–3]. As an important mode of CE, MEKC had powerful separation efficiency not only of charged analytes as capillary zone electrophoresis (CZE) but neutral ones using commercial CE instruments [4,5]. In MEKC, additives (micelles, polymers and dendrimers) were used as a pseudostationary phase and the differences in interaction between the micelle phase and the aqueous phase promoted the separation of the analytes [4]. At the same time, the poor concentration sensitivity, the main drawback of CE, because of the small injection volume and the short light optical path in the most commonly used ultraviolet–visible (UV–vis) detection limited its application in trace analysis. Although more sensitive detectors such as mass spectrometry (MS) [6,7] and laser-induced fluorescence (LIF) [8,9] can overcome this deficiency, they were more expensive as compared to the cost of CE-UV and not available in many laboratories. Thus, on-column sample preconcentrations methods were often proposed due to the advantage of simplicity, economy and efficiency in CE analysis.

On-column sample preconcentration methods were performed by changing the physico-chemical property of the sample solution relative to the background electrolyte (BGE) in order to preconcentrate the analytes, such as field amplification of enhancement (field-amplified sample stacking (FASS) [10,11] and large-volume sample stacking (LVSS) [12,13]), transient isotachophoresis (t-ITP) [14–16], dynamic pH junction [17,18], transient moving reaction boundary (tMCRBM) [19], micelle to solvent stacking (MSS) [20,21], pH mediated stacking [22,23], acetonitrile stacking [24], analyte focusing by micelle collapse (AFMC) [25] and sweeping [26–31], etc. Since being introduced in 1998 [27], sweeping MEKC has been accepted as an attractive and powerful on-column preconcentration method because of greatly improved concentration sensitivity both for neutral and charged molecules. With the technique, the analyte molecules were picked up and accumulated by a pseudostationary phase (micelles) in the buffer that moved through the sample zone. The concentration sensitivity was found to be increased by a factor of $(1+k)$ [27], where k is the retention factor. Theoretically, this technique can provide almost unlimited increases in detection sensitivity when the interaction between the analytes and the pseudostationary phase was very strong.

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Nevertheless, interfering components would interfere with sample stacking or cause peak integration in real samples (especially in biological samples), and, as a result, a clean-up step was the prerequisite for sample analysis.

Pretreatment steps are often adopted to concentrate analytes and clean up sample matrices before CE analysis. Liquid–liquid extraction (LLE) and solid-phase extraction (SPE) were the widely used sample preparation techniques for clean-up of biological samples [32,33]. However, evaporation of solvent to dryness and the reconstitution of the dry residue in a suitable solvent were unavoidable before CE analysis for both techniques, which were time-consuming, tedious and also prone to loss of analytes [34]. Therefore, development of new sample preparation techniques with simplification, miniaturization and automation was the noticeable trend. Solid-phase microextraction (SPME) was introduced as a solvent-free, relatively fast and simple technique which has been used in combination with CE in the off-line or on-line mode [33,35,36]. As extremely low sample volumes were injected, on-line coupling of SPME-CE was more difficult than SPME-LC. Single drop microextraction (SDME), introduced by Jeannot and Cantwell [37], provided an alternative technique for sample preparation. It was a simple, low-cost and virtually solvent-free sample pretreatment procedure and compatible with gas chromatography (GC) as well as high performance liquid chromatography (HPLC). Three-phase SDME was developed with a view to expanding the scope of the applications in CE and reversed-phase HPLC analysis. In the method, analytes of uncharged acidic or basic compounds were initially extracted into the organic phase and then back-extracted into a μL volume of aqueous acceptor immersed in organic phase. With a high volume ratio between the donor phase and acceptor phase, very high enrichment factors (*EF*) can be obtained [38,39]. In 2004, an impressive on-line preconcentration method using three-phase SDME prior to CE analysis was developed by Choi and Chung [40]. In the technique, a drop of aqueous acceptor phase covered with *n*-octanol as a thin organic film was hung at the tip of a capillary and placed into a donor phase for extraction. Enrichment factors of 3 orders of increase were obtained within 30 min. In addition, dual sample preconcentration methods of on-line three-phase SDME coupled with stacking technique were also developed [23,41]. The combinations can not only handle complex matrices directly but also further increase the sensitivity. For example, He et al. [23] utilized pH mediated stacking (base stacking) to concentrate anionic analytes in a low conductive and field-amplified zone which resulted from the injection of hydroxyl ions into an enriched acceptor phase. Chen et al. [41] relied on large-volume sample stacking combined sweeping (LVSS-sweeping) without polarity switching to further concentrate the target analyte after SDME.

In this work, a novel method of on-line SDME coupled with sweeping MEKC for the direct analysis of alkaloids at trace level in human urine samples was presented. Several factors that affect the sweeping MEKC separation and the extraction efficiency were investigated. The presented method provided a new strategy for sample treatment of urine.

2. Experimental

2.1. Reagents and materials

Berberine hydrochloride (BBR), palmatine hydrochloride (PMT), tetrahydropalmatine (THP) and strychnine (STN) (internal standard, IS) were purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). The 1.0 mg mL^{-1} individual stock solutions of the analytes and IS were prepared by dissolving of each standard in methanol. The stock solutions were stored at 4 °C. Working solutions were prepared daily with deionized water obtained from a Milli-Q water purification system (Millipore, Bedford, USA) and filtered with 0.45 μm filters (Xingya, Shanghai, China) before use.

Sodium chloride (NaCl), sodium hydroxide (NaOH), hydrochloric acid (HCl), orthophosphoric acid (H_3PO_4), sodium dodecyl sulfate (SDS), tetrahydrofuran (THF), isopropyl alcohol (IPA), methanol, acetonitrile, *n*-hexane, *n*-pentanol and toluene were purchased from Sinopharm Chemical Reagent Co. Ltd. (Shanghai, China). Ethyl acetate and *n*-octanol were purchased from Guangzhou Chemical Reagent Plant (Guangzhou, China). All reagents were of analytical grade.

2.2. Instruments

Stirring of the solution was carried out by a Hot Plate Stirrer model PC-420D (Corning, USA) and a magnetic stirring bar (10 mm × 4 mm). A PHS-3CA precision pH meter (Dapu, Shanghai, China) was used throughout the experiment. A CL1030 capillary electrophoresis system (Cailu, Beijing, China) equipped with a UV detector was employed throughout the experiment. A fused silica separation capillary of 70 cm (41 cm effective length) ×50 μm I.D. ×375 μm O.D. (Yongnian, Hebei, China) was used throughout the study. The data acquisition was carried out with a HW-2000 Chromatography Workstation (Qianpu, Shanghai, China).

2.3. Electrophoresis conditions

The analytes were conveniently separated in a sweeping MEKC separation solution that is compatible with the acceptor in back extraction. This solution was 100 mM H_3PO_4, 15 mM SDS and 12% (v/v) THF at pH 1.8. A new fused silica capillary was consecutively rinsed with 1 M NaOH for 15 min, 1 M HCl for 15 min, then with deionized water for 10 min and finally the running buffer for 15 min. To ensure repeatability, the capillary was treated by rinsing it with 1 M NaOH for 5 min, 1 M HCl for 5 min, deionized water for 5 min and the running buffer for 5 min between runs. During the analysis process, the voltage was operated at −28 kV and the detection wavelength was set to 265 nm. Samples were introduced into the capillary with 15 cm height difference for 300 s.

2.4. Extraction procedure

The procedures were performed on a homemade extraction unit consisted of a sample vial and a vial cover (Supplementary Fig. Sa). For the first step, a 4 mL basified sample solution (see Section 2.5) containing analytes and IS was placed in a 5 mL sample vial. Then, 350 μL of *n*-octanol was delivered on the top of sample solution. Afterwards, the vial was covered with a piece of aluminum foil and the mixture was agitated at 1150 rpm for 5 min as pre-extraction. For the later step, 1 cm of polyimide coating was removed from the tip to prevent the drop from creeping up along the capillary outer wall [40]. After the capillary was filled with the running buffer (about 1.37 μL), acceptor phase (50 mM H_3PO_4) was injected into inlet of the capillary at a forward pressure of 60 psi for 5 s. Then, the inlet end of the capillary was immersed in *n*-octanol and a backpressure was applied to the outlet of capillary using the same pressure and time as forward pressure to create a droplet (0.3 μL) hanging at the capillary tip (Supplementary Fig. Sb). The stirring rate was adjusted at 650 rpm for 10 min as back-extraction. To maintain the drop's shape, the inlet end of the capillary was lowered by 3 cm from the outlet during back-extraction. For the final step, about 0.24 μL of the enriched extractant was injected hydrodynamically by raising the injection end of the capillary 15 cm above the detection end

for 300 s. Then, the capillary inlet was removed from the sample vial and inserted into buffer solution to carry out CE separation.

2.5. Sample preparation

Blank urine samples were provided by healthy volunteers. The collected urine samples were centrifuged for 10 min at 4000 rpm and filtered with 0.45 μm filters. Then, the samples were alkalized with solid NaOH at concentration of 500 mM (pH 13.7). All the samples were stored at 4 °C before use.

3. Results and discussion

3.1. Basic principles of SDME and sweeping MEKC

There were two preconcentration procedures consisted in this study: SDME; and sweeping MEKC. In SDME, the unprotonated alkaloids (B) in the donor phase (d) were first extracted into the organic phase (o) and then back-extracted into the acceptor phase (a) to produce ammonium ions, which are given as follows [42]:

$$B_d \rightleftharpoons B_o \rightleftharpoons BH_a^+ \quad (1)$$

The enrichment factor (EF_e) in this procedure was calculated by the following Eq. [43]:

$$EF_e = \frac{C_{a_2}}{C_{a_1}} = \frac{1}{K_2/K_1 + K_2V_o/V_{a_1} + V_{a_2}/V_{a_1}} \quad (2)$$

where C_{a_1} and C_{a_2} were the equilibrium concentration of analytes in the donor phase and acceptor phase, respectively; V was the volume of the phase denoted by the subscript; K_1 was the equilibrium distribution coefficient between the donor phase and the organic phase; K_2 was the equilibrium distribution coefficient between the organic phase and the acceptor phase. In sweeping MEKC, the micelles in buffer solution would enter the sample zone during the application of voltage and sweep the charged alkaloids into thin concentrated zones. The length of sample zone after sweeping was narrowed down by a factor of $(1 + k)$ of initial length of sample zone. According to the literature [27], the enrichment factor (EF_s) in this procedure was given by:

$$EF_s = \frac{C_{a_3}}{C_{a_2}} = 1 + k \quad (3)$$

where C_{a_3} was the resulting concentration after sweeping. The total enrichment factor (EF_t) can be calculated by:

$$EF_t = EF_e \cdot EF_s = \frac{1 + k}{K_2/K_1 + K_2V_o/V_{a_1} + V_{a_2}/V_{a_1}} \quad (4)$$

Therefore, in order to have higher EF_t for the alkaloids (positively charged) with SDS micelles, it was necessary to adjust the composition and volume of the donor, middle and acceptor phases in SDME and to have higher retention factor in sweeping MEKC.

3.2. Optimization of CE conditions

In this study, the sweeping MEKC was used to separate and concentrate three alkaloids. The low pH buffer was generated by 100 mM H_3PO_4 to suppress electroosmotic flow (EOF). The pH of buffer was adjusted to 1.8 before use. When sample zone was injected into capillary, the negative micelles (15 mM SDS) in buffer penetrated it upon application of voltage (−28 kV) and swept analytes into thin concentrated zones. The effect of organic additives and their concentrations on separation efficiency was tested. It was found that 12% THF (v/v) had better baseline separation. The effect of injection time in the range of 60–360 s was tested, and 300 s was chosen as the optimum time due to higher sensitivity and better resolution, as depicted in Fig. 1.

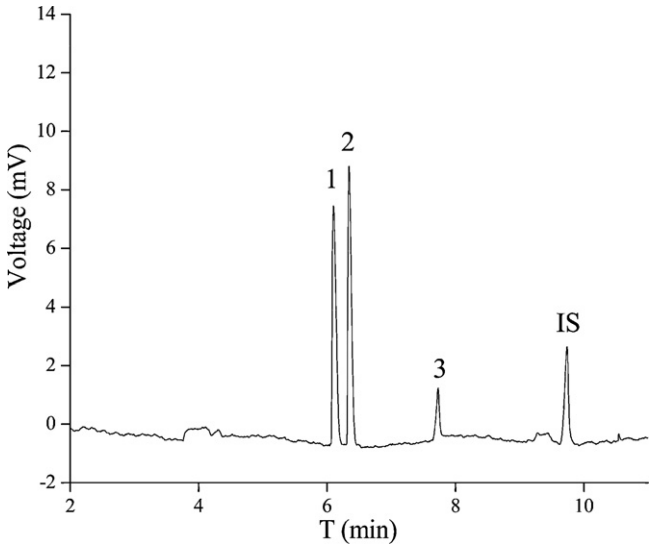

Fig. 1. Electropherogram of 5 μg mL^{-1} of the standard solution. CE conditions: running buffer, 100 mM H_3PO_4, 15 mM SDS and 12% (v/v) THF at pH 1.8; applied voltage, −28 kV; injection time, 300 s; UV detection 265 nm. Peak identification: (1) PMT, (2) BBR, (3) THP.

3.3. Optimization of extraction conditions

3.3.1. Extraction solvent

Several organic solvents (*n*-hexane, *n*-pentanol, *n*-octanol, ethyl acetate and toluene) with the characteristics of low solubility in water and low density than water were investigated. For toluene and *n*-hexane, the acceptor phase was easily detached from the capillary during back-extraction. When ethyl acetate was tested, no organic phase was observed after 5 min pre-extraction. The maximum extraction efficiency was given by *n*-octanol due to large equilibrium distribution coefficient of the analytes between *n*-octanol and sample. In addition, *n*-octanol has high viscosity. Thus, in the case of *n*-octanol as extraction solvent, the acceptor phase was stable during on-line SDME. So it was selected as the extraction solvent.

The influence of *n*-octanol volume on extraction efficiency was investigated in the range of 350–550 μL in 50 μL intervals. The experimental results showed that the extraction efficiency decreased with increasing the volume of *n*-octanol. The volume less than 350 μL was not investigated, because no three-phase system was observed. Therefore, 350 μL of *n*-octanol was chosen for the next study.

3.3.2. Drop formation

Drop formation was a decisive step for on-line SDME. The acceptor phase (50 mM H_3PO_4) was injected into inlet of the capillary at the pressure of 60 psi. Then, a droplet was formed at the tip of the capillary inlet by applying a same backpressure for same time as the former step from outlet to inlet. The pressure time was used to control volumes of acceptor phase injected into or pushed out of capillary [41]. In this study, pressure time from 5 to 25 s was examined. The results indicated that the highest extraction efficiency was achieved when a pressure time of 5 s was applied (Fig. 2). The pressure time less than 5 s could not meet the requirements of injection. As a compromise, 5 s was selected as pressure time in drop formation step.

3.3.3. Stirring rate in pre- and back-extraction

The stirring donor phase could accelerate the kinetics of extraction and attain maximum extraction efficiency within a relatively short period of time. Therefore, working solutions were extracted

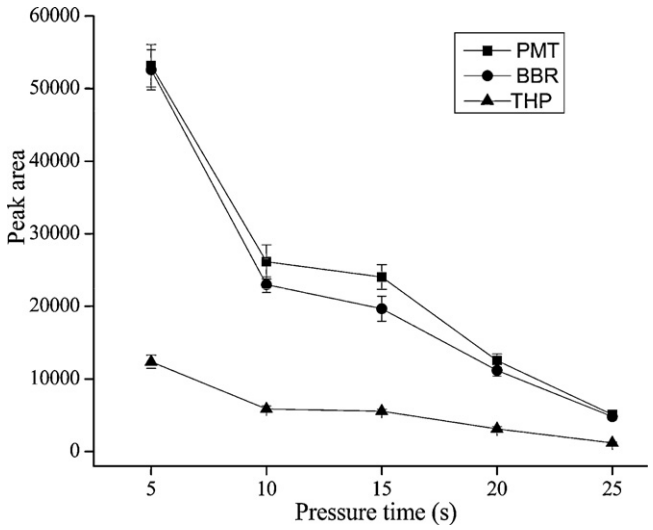

Fig. 2. Effect of pressure time on the extraction efficiency. Extraction conditions: 5 ng mL^{-1} of PMT and BBR, 50 ng mL^{-1} of THP; 350 μL of *n*-octanol as organic phase; 4.0 mL of urine alkalized with NaOH at concentration of 500 mM; pre-extraction, 5 min at 1150 rpm; back-extraction, 5 min at 650 rpm; acceptor phase, 100 mM H_3PO_4; 25 °C and no salt addition.

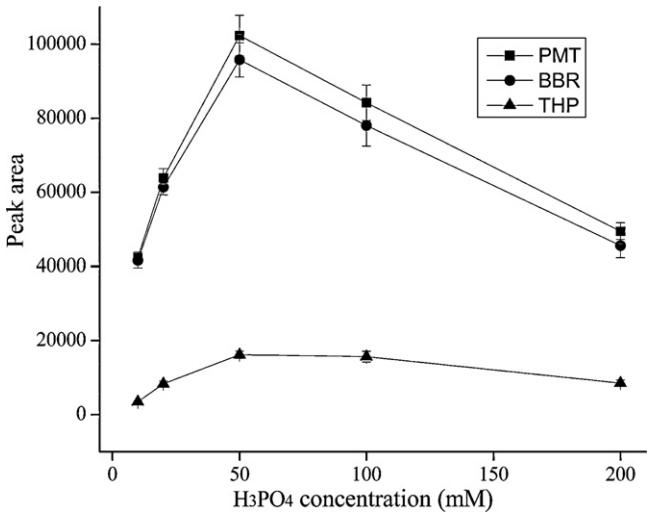

Fig. 3. Effect of H_3PO_4 concentration on the extraction efficiency. Extraction conditions: 5 ng mL^{-1} of PMT and BBR, 50 ng mL^{-1} of THP; 350 μL of *n*-octanol as organic phase; 4.0 mL of urine alkalized with NaOH at concentration of 500 mM; pre-extraction, 5 min at 1150 rpm; back-extraction, 10 min at 650 rpm; 30 °C and no salt addition.

at maximum rate (1150 rpm) for 5 min during pre-extraction procedure. For back-extraction, the stirring rate was tested in the range of 500–700 rpm in 50 rpm intervals for 5 min. The results indicated that the maximum extraction efficiency was obtained at 700 rpm. However, the stirring rate of 650 rpm was chosen due to better repeatability and extraction efficiency.

3.3.4. Extraction time in pre- and back-extraction

It would take a period of time for analytes to achieve the equilibrium concentration both in pre- and back-extraction. The pre-extraction times in the range of 1, 3, 5, 8 and 10 min at 1150 rpm were tested, and 5 min was chosen as the optimum pre-extraction time. The effect of back-extraction time on the extraction efficiency in the range of 5–25 min was also investigated. The extraction efficiency increased with increasing extraction time from 5 to 10 min, and then decreased when the time was longer than 10 min. The decrease for extraction efficiency might result from the drop (acceptor phase) dissolution and loss [44]. Therefore, 10 min was selected as the optimum back-extraction time.

3.3.5. Sample temperature and NaCl concentration

Mass transfer coefficient and distribution constant can be enhanced by increasing the extraction temperature. The extraction temperature from 30 to 60 °C was investigated. It was found that the extraction efficiency increased slightly in the studied range. However, with the temperature higher than 30 °C, the acceptor phase was easily detached. So we had to choose 30 °C as extraction temperature.

Addition of salt has been used to enhance the extraction of analytes due to the salting-out effect [45]. Thus, a series of experiments were performed on different concentrations of NaCl in donor solutions. By increasing the salt concentration from 0 to 20% (w/v), the extraction efficiency decreased instead. The decrease in extraction efficiency may attributed to the addition of salt increased the viscosity of sample solution [46] leading to decrease in the diffusion rate of analytes. Hence, no salt was added in the following study.

3.3.6. Composition of donor and acceptor phase

The analytes' charge status depended on their pK_a values and the pH of the donor phase. Most alkaloids have basic properties with pK_a values ranging from 6 to 12 [47]. Thus, the donor phase should

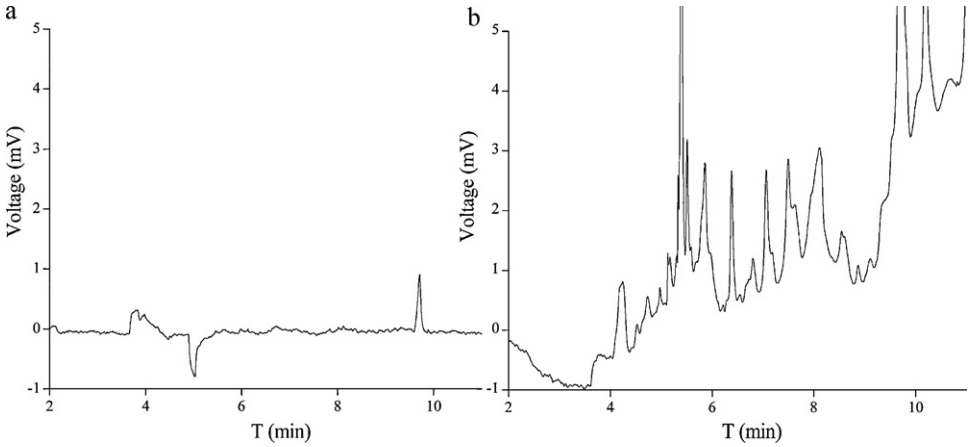

Fig. 4. Electropherograms of the blank urine sample extract (a) and the blank urine sample (b). Extraction conditions: donor phase, 4.0 mL of urine alkalized with NaOH at concentration of 500 mM; pre-extraction, 5 min at 1150 rpm; back-extraction, 10 min at 650 rpm; acceptor phase, 50 mM H_3PO_4; 30 °C and no salt addition. CE conditions are same as shown in Fig. 1.

Table 1
Performance of the proposed method for the determination of analytes in urine samples.

Alkaloids	Linear range (ng mL^{-1})	Calibration curves			LOD[b] (ng mL^{-1})	LOQ[c] (ng mL^{-1})	EF_s[d]	EF_e[e]	EF_t[f]
		Slope	Intercept	r[a]					
PMT	0.5–200	0.0499	0.1978	0.9981	0.5	1.5	31	115	3556
BBR	0.5–200	0.0685	0.2454	0.9944	0.2	0.7	21	101	2114
THP	5–2000	0.0121	−0.0536	0.9997	1.5	4.8	44	36	1583

[a] r: regression coefficients.
[b] LOD: limits of detections for a S/N = 3.
[c] LOQ: limits of quantifications for a S/N = 10.
[d] EF_s: enrichment factors in sweeping MEKC.
[e] EF_e: enrichment factors in SDME.
[f] EF_t: total enrichment factors.

be sufficiently basic to facilitate the deprotonation of the alkaloids and consequently reduced their aqueous solubility. Various concentrations of NaOH solutions in the range of 10–1000 mM (pH from 12 to 14) were evaluated to determine their effect on extraction efficiency. The extraction efficiency enhanced by increasing the concentrations of NaOH from 10 to 500 (pH from 12 to 13.7). When 1000 mM NaOH was used, the transparent *n*-octanol was changed into milky organic solvent under strongly alkalized condition after pre-extraction and the accepter was readily detached from the capillary tip during back-extraction. Accordingly, 500 mM NaOH was used as donor phase.

In this study, the acceptor phase should not only protonate the analytes as traditional SDME, but also need to meet the requirements of sweeping MEKC proposed by Quirino and Terabe [27]. Herein, H_3PO_4 as acceptor phase at five different concentrations (10, 20, 50, 100 and 200 mM) was examined. As can be seen in Fig. 3, 50 mM H_3PO_4 provided the optimum extraction efficiency. The reason may be as follows: in SDME, the unprotonated alkaloids in the organic phase could be easily protonated by 50 mM H_3PO_4 and then analytes enter rapidly into the acceptor. The low concentration of H_3PO_4 (10 and 20 mM) had little ability to protonate analytes while the high concentration of H_3PO_4 (100 and 200 mM) reduce diffusion rate of analytes due to the increased viscosity of H_3PO_4; in sweeping MEKC, the acceptor (50 mM H_3PO_4) having similar conductivity to that of the running buffer gave higher EF_s under suppressed EOF conditions [27,48].

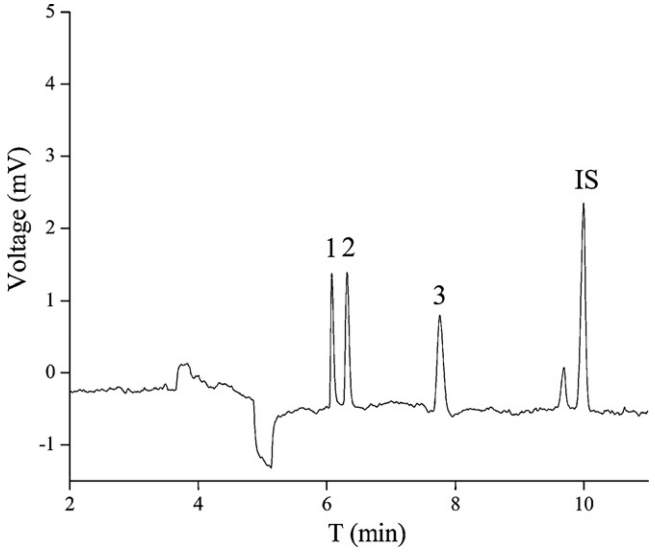

Fig. 5. Electropherogram of analytes (PMT and BBR at 5 ng mL^{-1}, THP at 50 ng mL^{-1}) and IS of the spiked urine sample extract. Other conditions are same as shown in Fig. 4.

Table 2
Recoveries of analytes spiked at different levels in urine samples.

Alkaloids	Concentration (ng mL^{-1})	Recoveries (%)	RSD[a] (%)
PMT	0.5	92.0	8.2
	2	95.0	7.3
	5	88.5	12.4
BBR	0.5	105.5	4.6
	2	105.8	7.7
	5	107.7	8.8
THP	5	93.1	9.0
	20	109.9	7.9
	50	115.5	6.4

[a] RSD: relative standard deviations; n = 3.

3.4. Application to real samples

To evaluate the practical applicability of the technique in real sample, blank urine was chosen as the model sample. It was found that there was nearly no interfering peak on the electropherotogram of blank urine extract (Fig. 4a) as compared with blank urine (Fig. 4b). Fig. 5 illustrates the electropherogram of blank urine spiked with three analytes (PMT and BBR at 5 ng mL^{-1}, THP at 50 ng mL^{-1}) and IS at 20 ng mL^{-1}. Although one unknown peak of the urine extract was observed, it was separated on baseline and did not affect the alkaloids analysis, which indicated the proposed method can afford effective clean-up in human urine.

The linearity of this method was further validated using a series of concentration with analytes (0.5–200 ng mL^{-1} for PMT and BBR, 5–2000 ng mL^{-1} for THP) in human urine under the optimized experimental conditions. As shown in Table 1, good correlation coefficients (0.9944–0.9997) were exhibited in the concentration range. The enrichment factors in sweeping MEKC (EF_s) and SDME-sweeping MEKC (EF_t), calculated as described in reference [49], were 21–44 and 1583–3556, respectively. Theoretically, according to Eq. (4), the enrichment factors in SDME (EF_e) were 115, 101 and 36 for PMT, BBR and THP, respectively. The LOD varied from 0.2 to 1.5 ng mL^{-1} at a signal-to-noise (S/N) of 3 and the LOQ varied from 0.7 to 4.8 ng mL^{-1} at a signal-to-noise (S/N) of 10. These values showed better in sensitivity as compared with those of former literatures [50–53], which indicated that the proposed method was sufficiently sensitive to detect the analytes in human urine. Good recoveries were obtained at three concentration levels (Table 2). The relative standard deviations (RSD, n = 3) of peak areas were 4.6–12.4%. The validation study showed that the developed method was accurate and robust in determination of three alkaloids in human urine.

4. Conclusions

An automatic two-step preconcentration method of on-line combination of SDME with sweeping MEKC has been applied for direct analysis of alkaloids in human urine samples. The proposed

method afforded 1583–3556 fold improvements in sensitivity for analytes within 20 min. Besides, all of the analytes were selectively extracted and no interference was observed. We believe that this method would be a simple and effective analytical technique to monitor the alkaloids in other biological samples as well.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.chroma.2011.06.074.

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