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Trace analysis of three antihistamines in human urine by on-line single drop liquid–liquid–liquid microextraction coupled to sweeping micellar electrokinetic chromatography and its application to pharmacokinetic study

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ABSTRACT

A rapid and efficient dual preconcentration method of on-line single drop liquid–liquid–liquid microextraction (SD-LLLME) coupled to sweeping micellar electrokinetic chromatography (MEKC) was developed for trace analysis of three antihistamines (mizolastine, chlorpheniramine and pheniramine) in human urine. Three analytes were firstly extracted from donor phase (4 mL urine sample) adjusted to alkaline condition (0.5 M NaOH). The unionized analytes were subsequently extracted into a drop of *n*-octanol layered over the urine sample, and then into a microdrop of acceptor phase (100 mM H_3PO_4) suspended from a capillary inlet. The enriched acceptor phase was on-line injected into capillary with a height difference and then analyzed directly by sweeping MEKC. Good linear relationships were obtained for all analytes in a range of 6.25×10^{-6} to 2.5×10^{-4} g/L with correlation coefficients (r) higher than 0.987. The proposed method achieved limits of detections (LOD) varied from 1.2×10^{-7} to 9.5×10^{-7} g/L based on a signal-to-noise of 3 (S/N = 3) with 751- to 1372-fold increases in detection sensitivity for analytes, and it was successfully applied to the pharmacokinetic study of three antihistamines in human urine after an oral administration. The results demonstrated that this method was a promising combination for the rapid trace analysis of antihistamines in human urine with the advantages of operation simplicity, high enrichment factor and little solvent consumption.

1. Introduction

Capillary electrophoresis (CE) has become an important and widely employed analytical tool because of its powerful separation efficiency, high detection sensitivity, short analysis time, minimal consumption of sample and a strictly limited solvent waste since the 1960s. However, its applications in trace analysis are limited due to the small injection volume [1]. Fortunately, this problem has been circumvented by various on-column preconcentration methods which include large-volume sample stacking (LVSS) [2], field-amplified sample stacking (FASS) [3], dynamic pH junction [4], transient isotachophoresis (t-ITP) [5], micelle to solvent stacking (MSS) [6] as well as sweeping [7].

More recently, sweeping micellar electrokinetic chromatography (sweeping MEKC) has been increasingly recognized as the most efficient and frequently used methodology for trace analysis. The on-column preconcentration efficiency of sweeping MEKC is proven to be increased by the factor which expressed as $k = K\varphi$, where k is the retention factor, K is the partition or distribution coefficient (concentration of the analyte in the pseudostationary/concentration of the analyte in the surrounding liquid phase), and φ is the phase ratio (volume of the pseudostationary/volume of the surrounding liquid phase) [8]. The higher the affinity of the analyte toward the pseudostationary phase (the higher K), the higher on-column preconcentration efficiency (the higher k) [9]. In general, the analytes should be prepared in a matrix with the compatibility and similar conductivity to background solution (BGS). For this reason, sweeping MEKC cannot be appropriate for some practical applications directly. As a result, a necessary matrix-transfer step is required prior to the analysis of the analytes. The microextraction is regard as the prefect pretreatment strategy combined with sweeping MEKC in an off-line or on-line mode [10,11].

Liquid-phase microextraction (LPME) provides a new technique for sample preparation with simple and rapidly operation, little solvent consumption and the convenience to couple with CE [12,13]. However, hydrophobic organic solvents which are used as acceptor phases are not compatible with BGS of CE in LPME. Therefore,

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liquid–liquid–liquid microextraction (LLLME) which uses water-based acceptor phases is adopted in the combination [14]. Single drop liquid–liquid–liquid microextraction (SD-LLLME) is a novel LLLME method which consists of two steps named pre- and back-extraction. It has the advantages of simplicity, cost effectiveness, minimization of organic waste, absence of sample carry-over except for higher extraction selectivity and efficiency, which is suitable for concentration and purification of ionizable compounds in various matrixes [15]. Nowadays, this technique has been widely applied in environmental and biological analysis.

SD-LLLME was adopted as a sample pretreatment method prior to CE analysis. However, most of the SD-LLLME-CE methods were off-line, where analytes were firstly extracted into an organic phase, and then into a droplet of acceptor phase. Finally, the droplet was transferred to a sample vial for injection and CE analysis [16]. The methods of SD-LLLME-CE in on-line mode were also reported, where a thin layer of organic solvent was used to separate a drop of aqueous acceptor phase at the capillary inlet from a bulk of aqueous donor phase [17]. Recently, on-line SD-LLLME coupled with base stacking as a dual sample preconcentration method has been developed [18]. The combinations allowed commercial CE instruments to handle in environmental and biological matrices directly except for further increasing the detection sensitivity.

Mizolastine (MLS), chlorpheniramine (CPM) and pheniramine (PHM) are alkylamine derivatives serving as the antihistamines. As the widely available over-the-counter (OTC) drugs, they are the antagonist of histamine H1 receptors to be used in the treatment of seasonal and perennial allergic rhinitis, urticaria, angioedema, localized and systematic allergic reactions. Several methods are reported in the literature for the determination of these compounds in pharmaceuticals and in physiological fluids using high-performance liquid chromatography (HPLC) [19], gas chromatography with mass spectrometric detection (GC–MS) [20], and capillary electrophoresis [21]. The pharmacokinetic studies of CPM have also been researched, which undergo time-consuming derivatizations or tedious several extraction cycles because CPM is in a low concentration in human plasma and urine after an oral administration [22].

The purpose of this work was to develop rapid and efficient dual preconcentration method by combining SD-LLLME with sweeping MEKC in an on-line mode for the analysis of three antihistamines in a trace concentration in human urine. The extract for three antihistamines could be injected directly in MEKC analysis without the need of any time-consuming matrix-transfer step. Several factors affecting SD-LLLME and sweeping MEKC were investigated. To our knowledge, this was the first report of the on-line SD-LLLME coupled to sweeping MEKC for the trace analysis of MLS, CPM and PHM, and this method was successfully applied in the pharmacokinetic study in human urine after an oral administration of three antihistamines. By the proposed method, the analytes could be rapidly and effectively concentrated combined with a sensitive determination.

2. Experimental

2.1. Reagents and materials

Mizolastine (MLS), chlorpheniramine (CPM), pheniramine (PHM), 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 g/L individual stock solutions of the analytes (stable for 3 months at 4 °C) were prepared by dissolving of each standard in deionized water obtained from a Milli-Q water purification system (Milipore, Bedford, USA) and IS was also prepared in methanol at concentration of 1.0 g/L. Working solutions were prepared daily by spiking mixed standard solution and IS standard solution to deionized water during the optimization exercise and filtered with 0.45 μm filters (Xingya, Shanghai, China) before use.

Sodium chloride (NaCl), sodium hydroxide (NaOH), hydrochloric acid (HCl), phosphoric acid (H_3PO_4), sodium dodecyl sulfate (SDS), tetrahydrofuran (THF), methanol (MeOH), acetonitrile (ACN), *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 are of analytical grade.

2.2. Apparatus

Stirring of the solution was carried out by a Hot Plate Stirrer model PC-420D (Corning, USA) and a fisher magnetic stirring bar (rod, 4 mm in diameter and 10 mm in length, Fisher, USA). The conductivity was measured by using a DDS-6700 digital conductometer (Leici, Shanghai, China). A PHS-3CA precision pH meter (Dapu, Shanghai, China) was used in 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 ID × 375 μm OD (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 procedure

A new fused silica capillary was flushed successively with methanol for 20 min, 1 M NaOH for 30 min, 1 M HCl for 30 min, then with deionized water for 30 min and finally the BGS for 30 min. At the beginning of each experiment, the capillary was washed with 1 M NaOH for 15 min, 1 M HCl for 15 min, deionized water for 10 min and the BGS for 15 min. Furthermore, to ensure repeatability, the capillary was washed between each analysis with 1 M NaOH for 3 min, 1 M HCl for 3 min, deionized water for 5 min and the BGS for 6 min. At the end of the day, the capillary was flushed successively with methanol for 20 min, 1 M NaOH for 20 min, then with deionized water for 20 min and it was protected by filling of water over night. The analytes should be conveniently separated in BGS which had the compatibility and similar conductivity to the acceptor phase in back-extraction. Therefore, BGS was composed of 75 mM H_3PO_4, 15 mM SDS and 10% (v/v) THF at pH 2.0. During this process, electrophoresis was performed at a constant voltage of −20 kV with UV detection at 214 nm. All the experiments were run at room temperature (25 ± 1 °C). Samples were injected with a height difference of 15 cm between the inlet and outlet of capillary for 300 s.

2.4. Extraction procedure

The microextraction procedures including pre- and back-extraction were performed on a homemade extraction unit consisted of a 5 mL sample vial and a vial cover. The unit was placed in a hot plate stirrer. For the pre-extraction, a 4 mL alkalized urine sample containing analytes and IS (donor phase) was placed in the sample vial. Then, 350 μL of *n*-octanol (organic phase) was delivered and floated on the top of it. Afterwards, the vial was covered and the mixture was stirred at 1150 rpm for 10 min. In this step, the unionized analytes were firstly extracted into *n*-octanol from the urine sample. Fig. S1 in Supplementary was schematic diagrams of the back-extraction and on-line injection steps. After the capillary was filled with BGS, 100 mM H_3PO_4 (acceptor phase) was injected into inlet of the capillary under a constant pressure (30.0 psi) for 10 s (Fig. S1A). Afterwards, the inlet was immersed into *n*-octanol

and then the same backpressure was applied to the outlet of the capillary using the same pressure and time. The acceptor phase was extruded and formed a microdrop suspended from the inlet of capillary inside of *n*-octanol, and then the outlet was immersed in BGS (Fig. S1B). To maintain the shape of the microdrop influenced by siphonage effect, the outlet was lower by 1 cm than the inlet. Moreover, prior to extraction, 1 cm of polyimide coating was removed from the inlet of capillary to prevent the drop from creeping up along the capillary outer wall [16]. Under stirring rate of 550 rpm, the analytes were extracted into the acceptor phase from the *n*-octanol (Fig. S1C). For the injection step (Fig. S1D), the enriched extractant was injected directly by raising the inlet by 15 cm above the outlet. Then, the inlet was removed from the sample vial and inserted into BGS and then the sweeping MEKC was carried out.

2.5. Sample preparation

Fresh urine sample was taken from six healthy volunteers (three males and three females) in the laboratory. To avoid interferences, the volunteers had abstained from any medications during the week preceding the study. In addition, the participant was not allowed to consume any foods in the morning. After an overnight fasting and urine was discharged, the participant drank 100 mL of water and then collected urine samples. 100 mL of water was drunk after collected urine sample each time, until the urine reached 200 mL which was an enough quantity for this work. The collected urine was filtered with 0.45 μm filters. And then, the urine sample was diluted with 1 M NaOH (1:1, v/v) to adjust to alkaline condition, which was used as donor phase throughout this work. All the urine samples stored at 4 °C before use.

The pharmacokinetic of CPM in the urine was studied. After an overnight fasting and urine was discharged, six healthy volunteers took 4 mg CPM (Release Capsules of CONTAC®, TSKF, China) with 200 mL of water and then urine samples were collected at the time of 4, 8, 12, 20, 28, 36, 48, 60, 72, 84, 96 h after oral. In addition, 200 mL of water was drunk after collecting urine sample each time. The volume of urine samples collected each time was recorded. Moreover, the pharmacokinetic studies of the MLS and PHM were also carried out and the details were shown in Supplementary.

2.6. Calculation of enrichment factor

Enrichment factor (EF) was calculated by the equation which expressed as $EF = C_a/C_d$, where C_a and C_d were the final and initial concentrations of the analytes in the acceptor and donor phases, respectively [23]. C_a was obtained from calibration graph of direct injections of standard solutions in 100 mM H_3PO_4 at the range of 1.25×10^{-2} to 2.0×10^{-1} g/L under the optimized electrophoresis conditions mentioned in Section 2.3. And the curves, obtained by plotting the peak areas versus the concentrations of analytes, gave a high level of linearity with correlation coefficients (r) of 0.993–0.996.

3. Results and discussion

3.1. Optimization of sweeping MEKC conditions

To obtain both the best separation efficiency and on-column preconcentration efficiency, 5.0×10^{-2} g/L each of MLS, CPM, PHM and STN (IS) prepared by 100 mM H_3PO_4 as sample matrix was used for all the optimization experiments with −20 kV as the operation voltage and a height difference of 15 cm between the inlet and outlet of capillary for 300 s as injection method. The experimental conditions of BGS concentration, pH, SDS concentration and organic modifier were optimized as follows.

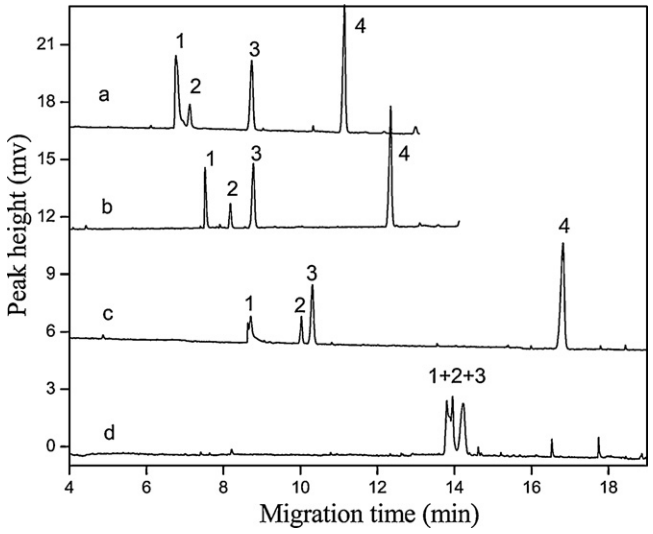

Fig. 1. Influence of the concentration of THF: (a) 8%, (b) 10%, (c) 12%, (d) 15%. Peak identification: (1) MLS, (2) CPM, (3) PHM, (4) STN (IS). MEKC conditions: sample containing 5.0×10^{-2} g/L each of analytes and IS prepared by 100 mM H_3PO_4; BGS: 75 mM H_3PO_4, 15 mM SDS, pH 2.0; −20 kV; injection: a height difference of 15 cm between the inlet and outlet of capillary for 300 s.

3.1.1. Influence of the BGS concentration

The H_3PO_4 concentration in the BGS was tested by changing its concentration to 50, 75, 100, and 120 mM, respectively. The results demonstrated that the migration time of the analytes increased and the resolution almost remained unchanged with increasing H_3PO_4 concentration. However, when the concentration exceeded 75 mM, baseline noise and repetitiveness were worse. Therefore, 75 mM H_3PO_4 was employed as BGS concentration for subsequent investigations.

3.1.2. Influence of the BGS pH

In sweeping MEKC, the BGS pH can directly affect separation efficiency. The BGS at different pH values (1.5, 2.0, 2.5 and 3.0) were examined. The migration time of the analytes increased sharply when pH increased (from 10 min for pH 1.5 to 24 min for pH 3.0). In addition, the analytes could be well separated at pH 2.0 (the resolution values: 3.98 between MLS and CPM, 2.85 between CPM and PHM, respectively). Therefore, pH 2.0 was chosen as the optimized condition.

3.1.3. Influence of the organic modifier

Addition of organic modifier in the BGS can influence the preconcentration efficiency and resolution. In this work, the analytes cannot be separated completely when the BGS without any organic solvent. Therefore, the effects of ACN, MeOH and THF were investigated by changing their concentrations to 8, 10, 12, and 15% (v/v), respectively. The analytes could not be well separated with the addition of ACN or MeOH. As shown in Fig. 1, the analytes had sufficient resolution meanwhile their peaks showed sufficient height with the addition of 10% THF. As the comparisons both of resolutions and peak heights, the 10% (v/v) THF was chosen for subsequent optimizations.

3.1.4. Influence of SDS concentration

In general, a higher SDS concentration can provide a higher on-column preconcentration efficiency. A series of BGS containing 5, 10, 15 and 20 mM SDS were examined, respectively. The results indicated that there was the best on-column preconcentration efficiency of analytes when SDS concentration was 15 mM. Meanwhile, the migration time was decreased with increasing the SDS (from

25 min for 5 mM to 11 min for 20 mM). Therefore, 15 mM SDS was selected.

After testing various factors which influenced on-column preconcentration efficiency in sweeping MEKC and separation of analytes, the optimized conditions of electrophoresis procedure had been decided as follows: 75 mM H_3PO_4, 15 mM SDS and 10% (v/v) THF at pH 2.0 as BGS.

3.2. Optimization of extraction conditions

For the optimization of the SD-LLLME, the factors influencing the sample extraction efficiency including the concentrations of NaOH and NaCl in urine phase, organic solvent, extraction time, temperature stirring rate, acceptor phase and injection time were studied. Initial conditions of optimization experiments were set as follows: 4 mL of urine sample (no salt) containing 2.5×10^{-5} g/L each of analytes and IS as donor phase; 350 μL of *n*-octanol as organic phase, 100 mM H_3PO_4 formed under 30 psi in 10 s and then the same backpressure in same time was selected as the acceptor phase; 10 min at 1150 rpm for pre-extraction; 10 min at 550 rpm for back-extraction; 30 °C; and injection: a height difference of 15 cm between the inlet and outlet of capillary for 300 s.

3.2.1. Influence of NaOH concentration

To increase the extraction efficiency, the urine sample was strongly alkalized to keep the analytes in their neutral forms. The urine was diluted with NaOH (1:1, v/v) and the NaOH concentrations in urine samples at range of 0, 0.25, 0.5 and 0.75 M were tested. As shown in Fig. S2A, the maximum extraction efficiency was achieved at 0.5 M NaOH (pH 13.7). Thus, NaOH concentration in the donor phase was selected as 0.5 M for the subsequent extractions.

3.2.2. Influence of NaCl concentration

With the addition of salt, the solubility of analytes in the aqueous sample phase will decrease so that extraction efficiency will enhance due to the salting-out effect. However, the extraction efficiency will decrease with increasing the ionic strength of the sample by adding salt. Thus, NaCl was added into the urine sample at a concentration at 0, 1, 5, 10, 20% (w/w) and the results were shown in Fig. S2B. The extraction efficiencies of analytes decreased with increasing salt concentration. This phenomenon could be explained as follows: The addition of salt increased the viscosity of the urine sample, which reduced the diffusion rate of analytes from the urine sample to the organic phase. Taking into account above ingredients, we decided not to add NaCl to obtain maximum extraction efficiency.

3.2.3. Influence of extraction temperature in SD-LLLME

A high temperature can enhance the rates of diffusion and partition, which can accelerate the extraction. However, the high temperature will enhance the loss of the organic phase, which should be avoided. In this work, the effect of temperature was tested at 25, 30, 35, 40, 45 °C, respectively. The result showed that peak areas of analytes were the largest at 30 °C. Furthermore, when temperature exceeded 30 °C, the microdrop of the acceptor phase easily fell off from capillary inlet because of the changes of its surface tension and viscosities. As a compromise between the stability and extraction efficiency, the optimum extraction temperature was chosen at 30 °C.

3.2.4. Influence of stirring rate in SD-LLLME

To shorten the extraction time to reach equilibrium, the stirring aqueous sample phase is necessary. Maximum rate (1150 rpm) of the magnetic stirrer used was selected to accelerate extraction rate in the pre-extraction procedure.

In the back-extraction procedure, the microdrop easily fell off the capillary inlet when the string was too fast. Meanwhile, the *n*-octanol (350 μL) was difficult to form a drop when the string was too slow. Therefore, the stirring rate was tested in the range of 450–750 rpm in intervals of 100 rpm. As shown in Fig. S2C, there was the best extraction efficiency when the stirring rate was 550 rpm. The reason for the decrease of extraction efficiency was that the stirring rate exceeding 550 rpm caused the loss of acceptor phase. As a result, the stirring rate at 550 rpm was chosen in this step.

3.2.5. Influence of the organic phase

The organic solvent which was in the surface of the urine sample must have a low density, a low solubility in aqueous solution and a high extraction capability for analytes. In an addition, high viscosity and low volatility were also required to prevent loss during the extraction. Based on these standards, 350 μL of *n*-hexane, *n*-pentanol, *n*-octanol and toluene was tested, respectively. The organic phase of toluene, *n*-hexane and *n*-pentane had much loss in 10 min due to their high volatility. Therefore, *n*-octanol with high viscosity, low solubility and better extraction capability was selected as organic phase for the further experiments.

A lower volume of organic phase is a precondition to reach satisfactory extraction efficiency. Therefore, the volume of the *n*-octanol in the range of 325–450 μL in intervals of 25 μL was investigated. The lower volume of organic phase led to the higher extraction efficiency. However, extraction could not be carried out at 325 μL because the microdrop of acceptor phase easily fell off due to the larger rotational moving of *n*-octanol. Therefore, 350 μL of *n*-octanol was chosen as the organic phase in the following experiments.

3.2.6. Influence of acceptor phase

Two factors should be considered in the selection of the acceptor phase: firstly, it must have the compatibility and similar conductivity to the BGS of sweeping MEKC; secondly, it must ensure to provide appropriate extraction efficiency. According to the study of Lin et al., an acceptor phase conductivity of 1.2–1.4 times conductivity of BGS gave the optimum focusing effect of sweeping [24]. Taking the above factors into account, 100 mM H_3PO_4 (0.40 ms/cm) with 1.2 times conductivity of BGS (0.34 ms/cm), was chosen as acceptor phase component.

In general, the extraction efficiency increases when the volume ratio between organic phase and acceptor phase increases. The volume ratio could be changed through a constant pressure and time in this work. The pressure time from 5 to 20 s were examined under the constant pressure (30 psi). The results indicated that the highest extraction efficiency achieved when the pressure time at 10 s was applied. Considering above factors, 100 mM H_3PO_4 was injected into inlet of the capillary under 30 psi in 10 s and then the same backpressure in same time was selected in this work.

3.2.7. Influence of extraction time in SD-LLLME

In the pre-extraction procedure, the extraction efficiency reached maximum after extracting for 10 min at stirring rate of 1150 rpm. Moreover, it would cause the unacceptable loss of *n*-octanol for volatilizing when the extraction time was more than 10 min. Therefore, 10 min was selected as the optimized extraction time in pre-extraction procedure.

In the back-extraction procedure, the extraction time was investigated in the range of 7–12 min at intervals of 1 min. As shown in Fig. S2D, the extraction efficiency reached maximum with extracting for 10 min. Moreover, the extraction efficiency decreased with the time longer than 10 min, which may result from the small quantity loss of acceptor phase dissolving into organic phase.

Table 1
Performance of the proposed analysis procedure.

Analyte	Calibration equation[a]			LR[b] ($\times 10^{-6}$ g/L)	Lack-of-fit		LOD[c] ($\times 10^{-6}$ g/L)	EF[d]
	Slope ± S_a	Intercept ± S_b	r		F-Value	p-Value		
MLS	0.0033 ± 0.00084	0.097 ± 0.0013	0.987	6.25–250	9.39	0.066	0.68	900
CPM	0.0019 ± 0.00024	0.098 ± 0.0068	0.993	6.25–250	4.02	0.14	0.95	751
PHM	0.016 ± 0.0036	0.098 ± 0.0025	0.990	6.25–250	5.27	0.18	0.12	1372

[a] S_a, standard deviation of the slope; S_b, standard deviation of the intercept; r, correlation coefficient.
[b] LR, linear range.
[c] LOD, limits of detections.
[d] EF, enrichment factor.

Therefore, we selected 10 min as the optimized extraction time in this step.

3.2.8. Influence of injection time

The acceptor phase should be injected into capillary as much as possible to increase the detection sensitivity. Meanwhile, *n*-octanol should be avoided being injected into capillary for it easily caused the baseline drift even broke off MEKC. The injection time was investigated by changing it to 180, 300, 420 and 540 s at the height difference of 15 cm between the inlet and outlet of capillary, respectively. There were the optimal sensitivities of analytes when the injection time was 300 s. Therefore, 300 s were selected.

Over all, the optimized conditions of extraction procedure were: 4 mL urine sample (no salt addition, 0.5 M NaOH) as donor phase; 350 μL of *n*-octanol as organic phase with the stirring rate of 1150 rpm and 10 min in pre-extraction; a microdrop of 100 mM H_3PO_4 formed under 30 psi in 10 s and then the same backpressure in same time was selected as the acceptor phase with the stirring rate of 550 rpm and 10 min in back-extraction, 30 °C, and the acceptor phase was injected into capillary for 300 s at a height difference of 15 cm between the inlet and outlet of capillary.

3.3. Method validation

Under the optimized experimental conditions described above, the resolution values were 3.98 between MLS and CPM, 2.85 between CPM and PHM, respectively. Meanwhile, the parameters including specificity, linearity, limit of detection (LOD), EF, repeatability, precision and accuracy were evaluated in urine samples. The representative electropherograms of the extract of urine samples from blank and spiking of three antihistamines were shown in Fig. 2.

3.3.1. Specificity

To evaluate specificity, urine samples from six different sources containing each analyte at 2.5×10^{-5} and 1.0×10^{-4} g/L. The relative recoveries (RR) of analytes were in the range of 85–113% and 87–118% and the relative standard deviations (RSD, $n = 6$) were lower than 7%, which indicated that the proposed method had sufficient specificity. No significant batch-to-batch variation was observed because some endogenous components in urine could be cleaned up via extraction. Moreover, the effect of concomitant medicines (caffeine and ascorbic acid) at a similar concentration to the analytes in the urine sample has been evaluated. No interference from these compounds was found.

3.3.2. Linearity, LOD and EF

The vertical coordinate of the calibration equations showed the ratio between peak areas of analytes and that of IS, and the abscissa reflected the change of the concentration of analytes. The important parameters of calibration equations were presented in Table 1. Moreover, the calibration equations were evaluated for linearity by the lack-of-fit test [25]. The linear model for the relationship between concentration and response was considered to be appropriate as no significant lack-of-fit was observed. LOD was calculated based on the analytical responses of the background noise for 3 times (S/N = 3). EF was calculated by analysis of urine sample containing 2.5×10^{-5} and 1.0×10^{-4} g/L each of analytes for five times. The details were shown in Table 1. Compared to the previous works (LOD: 4.0×10^{-4} g/L by CZE for MLS [26]; 2.5×10^{-4} g/L by CZE for CPM [27]; 5.2×10^{-6} g/L by CZE for PHM [28]), the results of this work were satisfactory.

3.3.3. Repeatability, precision and accuracy

The repeatability studies of sweeping MEKC and the proposed method were both carried out by repeating intra- and inter-day analysis for five times. For sweeping MEKC, the repeatability was checked by injecting spiked samples (analytes at 5.0×10^{-2} and 1.5×10^{-1} g/L) in the same day (intra-day) and on three consecutive days (inter-day). For the proposed method, the repeatability was established by performing determinations of urine samples (the analytes at 2.5×10^{-5} and 1.0×10^{-4} g/L) in the same day (intra-day) and on three consecutive days (inter-day). As shown in Table 2, there were acceptable RSD values (<5%) for the migration times and response ratios, which indicated that sweeping MEKC and the proposed method had the good intra- and inter-day repeatability. Meanwhile, the repeatability between different capillaries was also determined by analysis of the same urine sample (the analytes at 1.0×10^{-4} g/L) in three capillaries. The acceptable RSD values ($n = 3$) for the migration times and response ratios were 2.82% and 2.12%, indicating that the proposed method had the acceptable inter-capillary repeatability.

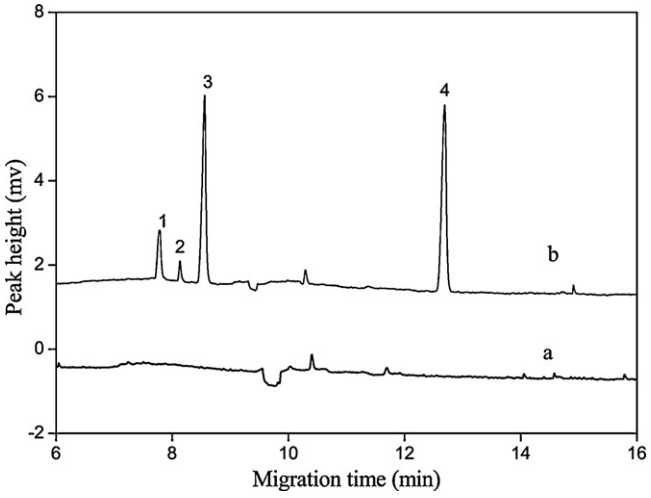

Fig. 2. Electropherograms of urine from blank (a) and after spiking at concentration level of 5.0×10^{-5} g/L each of analytes and IS (b). Peak identification: (1) MLS, (2) CPM, (3) PHM, (4) IS. 10% THF as organic modifier and other sweeping MEKC conditions were the same as in Fig. 1. Extraction conditions: 4 mL of urine sample (no salt addition, 0.5 M NaOH) containing 2.5×10^{-5} g/L each of analytes and IS as donor phase, 10 min at 1150 rpm for pre-extraction and other extraction conditions were the same as in Fig. 1.

Table 2
Repeatability for intra- and inter-day analysis.

Analyte	RSD[a] (%) of intra-day ($n=5$)				RSD[a] (%) of inter-day ($n=3$)			
	T_{m1} [b]	R_{m1} [c]	T_{m2} [d]	R_{m2} [e]	T_{m1} [b]	R_{m1} [c]	T_{m2} [d]	R_{m2} [e]
MLS	1.92	2.31	2.14	3.31	2.07	2.78	2.56	3.13
CPM	2.64	1.47	3.24	3.27	3.28	3.16	3.83	3.72
PHM	2.47	3.25	2.64	4.93	3.91	4.45	3.95	4.83

[a] RSD, the average of the five determinations of the analytes at two concentration level in the same day (intra-day) or on three consecutive days (inter-day).
[b] T_{m1}, migration time. Spiked sample containing the analytes at 5.0×10^{-2} and 1.5×10^{-1} g/L was injected and separated by sweeping MEKC.
[c] R_{m1}, response ratio between peak area of the analyte and the mean peak area of the STN (IS). Spiked sample containing the analytes at 5.0×10^{-2} and 1.5×10^{-1} g/L was injected and separated by sweeping MEKC.
[d] T_{m2}, migration time. Urine sample containing the analytes at 2.5×10^{-5} and 1.0×10^{-4} g/L was determined by the purposed method.
[e] R_{m2}: response ratio between peak area of the analyte and the mean peak area of the STN (IS). Urine sample containing the analytes at 2.5×10^{-5} and 1.0×10^{-4} g/L was determined by the purposed method.

Table 3
Precision and accuracy for intra- and inter-day analysis.

Analyte	Added[a] ($\times 10^{-6}$ g/L)	Intra-day		Inter-day	
		RR[b] (%)	RSD[c] (%, $n=5$)	RR[b] (%)	RSD[c] (%, $n=3$)
MLS	25	93	2.86	96	2.19
	100	103	4.16	109	4.43
CPM	25	97	3.05	100	2.93
	100	104	3.64	107	4.01
PHM	25	98	3.12	103	3.78
	100	106	5.73	104	5.89

[a] The concentrations were the added analytes in the urine sample.
[b] RR, relative recoveries, calculated as the average of the five determinations.
[c] RSD, relative standard deviation for five times in the same day (intra-day) or on three consecutive days (inter-day).

Precision was expressed as RSD while accuracy as RR, and they were studied by intra- and inter-day analysis for five times. The RR was calculated by dividing the measured quantities with the nominal (spiked) quantities in urine sample. As shown in Table 3, the good precision (<6%) and accuracy (93–109%) indicated the proposed method could be applicable for urine sample analysis.

3.4. Pharmacokinetics of three antihistamines in human urine

The concentrations of CPM in the urine samples were calculated by its peak areas according to linear regression equation. The excretion amount of CPM was calculated by the concentration of CPM in the human urine multiplying by the urine volume. Fig. 3 presented the relationships between concentration and time (line a), excretion amount and time (line b), respectively, where the time in *X*-axis was the midpoint time of urine collection. The concentration of CPM achieved a maximum (1.02×10^{-4} g/L) at 28 h after oral administration. The increase of excretion amount slowed down at 72 h after oral. Moreover, the excretion amount was 0.558 mg and the excretion level of CPM in unchanged form was 14.0% within 96 h. Compared to the previous study, the result was consistent with the pharmacokinetic characteristics of CPM [29,30]. Moreover, the pharmacokinetic studies of the MLS and PHM were shown in Supplementary. According to results, the excretion amount of MLS in unchanged form was 0.044 mg (0.44%) within 48 h and that of PHM in unchanged form was 0.577 mg (5.77%) within 96 h, which were also consistent with the pharmacokinetic characteristics of MLS and PHM [31,32].

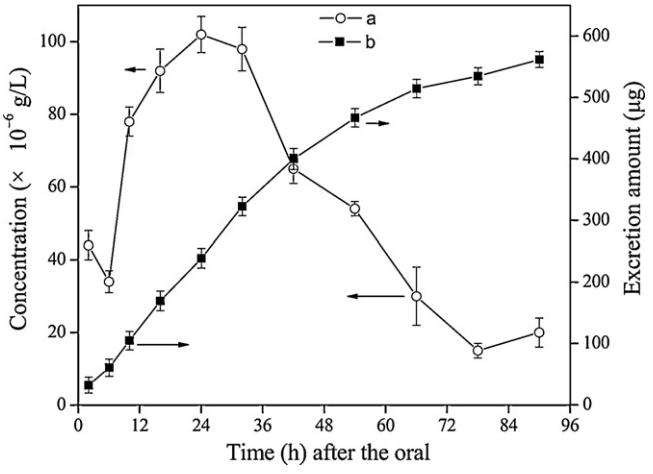

Fig. 3. Concentrations (a) and excretion amount (b) of CPM change in different time in urine samples after an oral of 4 mg CPM. The conditions are the same as Fig. 2.

4. Conclusion

In the present work, on-line SD-LLLME coupled to sweeping MEKC as a rapid and efficient dual preconcentration method has been successfully developed. Because of the compatibility between acceptor phase and BGS, SD-LLLME and sweeping MEKC were ideally coupled in an on-line mode for trace analysis of three antihistamines in urine matrix without any time-consuming matrix-transfer step. The proposed method was successfully used in the pharmacokinetic study of three antihistamines in human urine. The results indicate that the proposed method is a promising combination for the trace analysis of various antihistamines in urine matrix with the advantages of with operation simplicity, rapid detection, high enrichment factor and little solvent consumption.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jchromb.2012.07.032.

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