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Liquid-phase microextraction combined with high-performance liquid chromatography for the determination of local anaesthetics in human urine

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Abstract

A simple liquid-phase microextraction (LPME) device combined with high-performance liquid chromatography (HPLC) is presented for the simultaneous analysis of local anaesthetics, lidocaine, bupivacaine, and tetracaine, from human urine sample. An organic solvent showed good compatibility with the mobile phase of the HPLC, *o*-dibutyl phthalate, was selected. Local anaesthetics are extracted from 6 ml of the feed aqueous solution and human urine sample into a water-immiscible organic solvent suspended at the needle tip of the microsyringe, then the organic solvent was directly introduced to a reversed-phase HPLC system. The kind of the organic extraction solvent, the stirring rate, the pH value of the aqueous feed solution, and the extraction time have been discussed. Under the optimized extraction conditions, high enrichment factors (more than 86.0-fold) and significant sample clean-up for all of studied local anaesthetics were achieved within 30 min. The detection limits (lower than 0.05 μg/ml) were comparable with previously reported gas chromatography methods. This method was applied to specimen of patient who was treated with extradural anaesthesia of lidocaine, bupivacaine, and tetracaine, and revealed that simultaneous determination of above three local anaesthetics in human urine was possible.

Keywords: Liquid-phase microextraction; High-performance liquid chromatography; Lidocaine; Bupivacaine; Tetracaine; Urine

1. Introduction

Mixtures of local anesthetics may be used to improve onset time, modify block duration, and reduce toxicity. Combination of an amide and ester is suitable because the times of peak levels for the drugs are different, and the toxicity of two drugs in the mixture is not additive [1,2]. Local anesthetics are metabolized in plasma or kidney, with less than 5% of the local anesthetics excreted unmetabolized in urine [1,3]. It is significant to estimate the concentrations of local anesthetics in urine after possible inadvertent intraarterial injection or intrathecal injection or in the case when the administration of an excessive dose of local anesthetic causes systemic toxic reactions and to guide clinical use of local anesthetics. Usually, local anesthetics are present at low concentration in the urine. Therefore, sample preconcentration and clean-up must be carried out on the urine sample before local anesthetics can be determined by high-performance liquid chromatography (HPLC) or gas chromatography (GC). Liquid–liquid extraction (LLE) [3–5], solid phase extraction (SPE) [6–9], and solid phase microextraction (SPME) [10–13] have been used successfully as the preparing method of the local anesthetics. However, LLE and SPE techniques have a number of drawbacks of time and large organic solvent consumption. In SPME techniques, the fiber need to be treated to avoid carry-over effects after sample desorption every time, analytes extracted by SPME must be desorbed into a suitable receiving

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solvent prior to HPLC analysis and the SPME–HPLC interface requires a desorption chamber as part of a switching valve and is thus not as convenient to use [14].

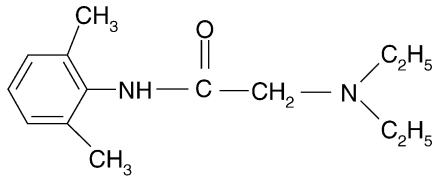
Recently, liquid phase microextraction (LPME) was developed as a novel and disposable method for sample preparation. In 1996, Dasgupta and Liu introduced a drop-based automated liquid–liquid extraction and detection system of optical absorption of the organic phase using a single, microliter-volume, organic drop [15]. At the same year, Jeannot and Cantwell proposed a method of microextraction combined with GC based on an organic solvent microdrop suspended on the tip of either a Teflon rod or microsyringe, which was immersed in the stirred aqueous sample solution [16,17]. The research group of Lee further developed this technique by introducing the concepts of static and dynamic microextraction combined with GC [18–20]. Since the organic solvent microdrop can be directly analyzed with GC after extraction because of the good compatibility of organic solvent with GC, LPME combined with GC has been used successfully to concentrate and enrich the low concentration drugs from biological matrices [21–24]. However, the organic solvent used may not be compatible with HPLC mobile phase or capillary electrophoresis (CE) background electrolyte. In view of this, Lee and Vandecasteele reported that the organic solvent was first dried by nitrogen flow, and then redissolved with methanol or the mobile phase for the subsequent HPLC analysis [25,26]. However, the processes of the organic solvent evaporation and reconstitution were tedious and time-consuming, and some analytes may be lost during this procedure. Lately, Pedersen-Bjergaard and Rasmussen proposed a new liquid–liquid–liquid microextraction (LLLME) method based on a small piece of a porous hollow fiber, impregnated with organic solvent, immersed in a sample vial [27]. Since the aqueous acceptor phase can be directly analyzed with CE or HPLC, some successful LLLME researches combined with HPLC [21] and CE [21,28–31] have been reported on concentrating and enriching the low concentration drugs from biological matrices. However, comparing to the organic solvent drop of 1–3 μl, the volume ratios between the initial feed solution and the acceptor phase basis on the porous hollow fiber LLLME (~25 μl of acceptor phase) are lower, so relatively low enrichment factors are obtained.

It was the aim of our study to perform sample preparation with a minimum of equipment and HPLC simultaneous analysis of local anesthetics, lidocaine, bupivacaine, and tetracaine, using ultraviolet (UV) spectrophotometric detection. To determine urine levels of each component of such a mixture simultaneously by HPLC is not found in the previous studies. In the present investigation, simple two phase microextraction device was still used, i.e., the organic solvent drop suspended at the tip of the microsyringe needle was drawn back into the microsyringe and directly analyzed with HPLC after exposed in the aqueous feed solution for a period of time. An organic solvent showed good compatibility with the mobile phase of the HPLC, *o*-dibutyl phthalate, had been selected. Since the organic drop used in this LPME can be directly introduced to a reversed-phase HPLC system, this method is more convenient than the combination of SPME with HPLC. The method is verified to be sensitive, repeatable, and linear over a wide range and requires only small volumes of organic solvent as well as samples. High enrichment factor and significant sample clean-up have been achieved. In addition, specimen of patient who was treated with extradural anaesthesia of lidocaine, bupivacaine, and tetracaine, was analyzed with the present method.

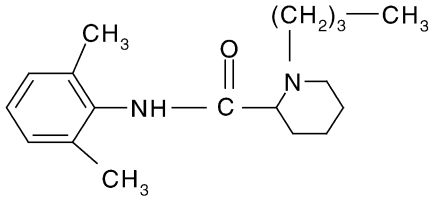
2. Experimental

2.1. Reagents

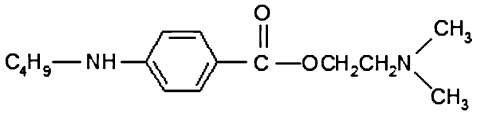
Lidocaine hydrochloride, bupivacaine hydrochloride, and tetracaine hydrochloride (shown in Fig. 1) were purchased from the Chinese National Institute for the Control of Pharmaceutical and Biological Products (Beijing, PR China). HPLC-grade acetonitrile was obtained from Tedia Company, Inc. (Fairfield, OH, USA). Analytical grade formic acid, sodium hydroxide, phosphoric acid, *n*-butyl alcohol, *n*-octyl alcohol, cyclohexane, benzene, toluene, *p*-xylene, carbon tetrachloride, chloroform, and *o*-dibutyl phthalate were bought from Chemical Reagent Co., Ltd. of Hunan, Changsha, PR China. The water used was purified on a Milli-Q Ultrapure water purification system (Millipore, Bedford, MA, USA).



Lidocaine, pK_a=7.9



Bupivacaine, pK_a=8.1



Tetracaine, pK_a=8.6

Fig. 1. Structure and pK_a of lidocaine, bupivacaine, and tetracaine.

2.2. Preparation of standard solutions

Stock solutions of lidocaine hydrochloride (2 mg/ml), bupivacaine hydrochloride (0.75 mg/ml), and tetracaine hydrochloride (1 mg/ml) were prepared by dissolving calculated amounts of lidocaine hydrochloride, bupivacaine hydrochloride, and tetracaine hydrochloride in water, respectively. They were stored at 4 °C. Standard working solution containing of 5 μg/ml of lidocaine hydrochloride, bupivacaine hydrochloride, and tetracaine hydrochloride, was prepared by dilution of the stock solutions in water once every week and also stored at 4 °C. The pH was adjusted to an expectant value with 1.0 mol/l sodium hydroxide solution just before the extraction experiment beginning.

2.3. Urine samples

The human urine was obtained from a health female. Spiked urine samples containing lidocaine, bupivacaine, and tetracaine were prepared by diluting the stock solution of local anaesthetics in urine.

A real patient urine sample from a female patient treated with cholecystectomy was collected for administration start time 0–4 h. Drug-free urine just before local anaesthetics administration was used as the patient blank urine sample. Over a 4-h period of administration, local anaesthetics were administered with extradural anaesthesia as follows: 630 mg of lidocaine hydrochloride, 157.5 mg of bupivacaine hydrochloride, and 17.2 mg of tetracaine hydrochloride. All urine samples were stored at 4 °C.

2.4. HPLC system

The HPLC system consisted of two Jiangshen (Jiangshen, Dalian, PR China) LC-6A pumps, a LC-10 UV detector and a U6K injection valve. Separation was accomplished using a 250 mm × 4.6 mm Johnsson spherigel C_{18} analytical column (Jiangshen, Dalian, PR China). The mobile phase consisted of (A) a mixture of acetonitrile and triethylamine aqueous solutions (11 mM)–0.1% phosphoric acid aqueous solutions (10/90, v/v) and (B) a mixture of acetonitrile and triethylamine aqueous solutions (20 mM)–0.1% phosphoric acid aqueous solutions (50/50, v/v). The gradient elution profile was as follows: 0–15 min, 100–30% A, 15–30 min, 30–0% A. The flow-rate was 1.0 ml/min. The detection wavelength was 210 nm. Prior to use the mobile phase was filtered through a 0.45 μm membrane and degassed for 10 min.

2.5. Extraction procedure

The extraction procedure was carried out using a Hamilton 801RN 10-μl microsyringe with a flat-cut needle tip (Hamilton, Reno, NV, USA), a 10 mm × 2 mm magnetic stir bar and a silanized 10-ml extraction vial with a PTFE-silicon septum (constructed in the laboratory) shown in Fig. 2. For an extraction, the microsyringe was rinsed with organic solvent for at least five times to ensure that no air bubble was left in the barrel and the needle. Three microliters of organic solvent was drawn into the syringe, then the needle tip was pulled out of the organic solvent, and the plunger was depressed to 1 μl. The needle was then inserted through the septum of the sample vial and immersed in 6 ml of the aqueous feed solution (pH 11). The distance between the tip and the stir bar should be kept consistently (ca. 1 cm) for all experiments to ensure good precision. To form the extraction drop, the plunger was depressed, causing the 1 μl of organic solvent to be suspended from the needle tip, and the aqueous feed solution began to be stirred at 160 rpm. The drop was exposed to the aqueous feed solution for 30 min after which the drop was drawn back into the microsyringe with the needle still immersed in the aqueous feed solution. The microsyringe was then retracted from the sample vial and the extraction solvent with local anaesthetics was injected into the HPLC directly. The microsyringe was cleaned using methanol, water, methanol, and organic solvents after each experiment, respectively, to insure the microsyringe can be used repeatedly. The experimental temperature was 30 ± 0.5 °C.

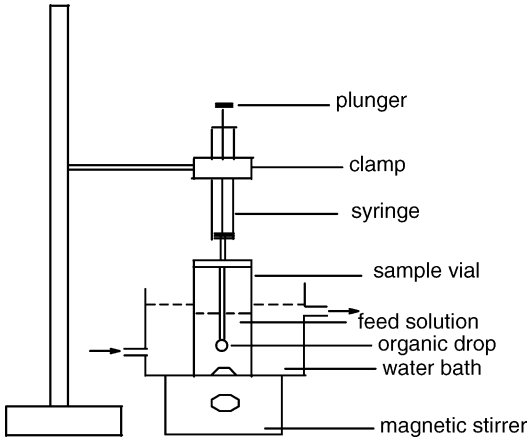


Fig. 2. Schematic diagram of the LPME system.

2.6. Validation

To estimate the validity of the present method, urine samples spiked with local anaesthetics at concentrations ranging from 0.1 to 10 μg/ml were prepared and analyzed using the above procedure. Repeatability was evaluated on six replicate experiments with spiked urine samples containing 1.0 μg/ml of three mixed local anaesthetics under the optimized conditions. The calibration curves were obtained by plotting the peak area of the analytes versus their corresponding concentrations in spiked urine samples. The linearity of the calibration curve was evaluated using least-squares linear regression analysis. Limit of detection (LOD) was defined as the concentration that yielded a signal-to-noise ratio of approximately 3. The recoveries of the studied local anaesthetics were determined by the standard addition method. To determine recoveries, spiked

urine samples with three mixed local anaesthetics at the concentration of 5.0 and 0.5 μg/ml were prepared and analyzed on the same day (intra-day recovery) and seven consecutive days (inter-day recovery). Recovery is defined as follows:

$$R = \frac{c_{\text{determine}}}{c_{\text{initial}}} \times 100\%$$

where $c_{\text{determine}}$ and c_{initial} are the determined concentration of three local anaesthetics with the present method and their initial concentration in the spiked urine sample, respectively.

Extractions were repeated three times for each concentration to obtain statistical mean of peak area except in repeatability experiment.

3. Results and discussion

3.1. Optimization experiments of LPME

Since lidocaine, bupivacaine, and tetracaine are basic substances (Fig. 1), the feed solution is made alkaline to deionized local anaesthetics and consequently reduces the solubility of local anaesthetics in the feed solution. Then deionized local anaesthetics were extracted from the feed solution into the organic solvent because of their affinity to the organic phase. The stirring can enhance the mass transfer. Because of the lipophilic properties of lidocaine, bupivacaine, and tetracaine and the low volume ratio between the organic solvent and the initial aqueous feed solution (1:6000), local anaesthetics were preconcentrated in the organic phase.

Enrichment factor (E_e) was used to evaluate the extraction efficiency under different conditions. E_e signifies the number of times that the extraction process increase the analyte concentration. It is defined as:

$$E_e = \frac{c_o}{c_{f,i}} \quad (1)$$

where c_o is the concentration of the analyte in the organic phase at time t and $c_{f,i}$ is the concentration of the analyte in the initial aqueous feed solution, respectively.

3.1.1. The kind of the organic extraction solvent

One of the critical steps in LPME is to select an organic solvent for enrichment target compounds. Nine kinds of inexpensive, immiscible with water organic solvents, which have different chemical characteristics (specific density and solubility in water) were tested. The specific density (ρ), the solubility in water of the interest organic solvents [32], the enrichment factor of local anaesthetics and the relative standard deviation (R.S.D.) were collected in Table 1. As can be seen in Table 1, all of the interest organic solvents have very low solubility in water except *n*-butyl alcohol. *n*-Butyl alcohol, cyclohexane, carbon tetrachloride, and chloroform are not suitable for the LPME because of the difficulty of holding their respective microdrops at the tip of the microsyringe for a considerable time (≥10 min). The stability of the organic solvent drop is correlative with three factors, i.e. upward floating force ($F_f = V_o\rho_w g$), downward gravity ($F_g = V_o\rho_o g$), and adhesion forces (F_a) resulted from the interfacial tension [33], where V_o, ρ_o, ρ_w and g were the volume of the organic solvent drop, the density of the organic solvent, the density of the aqueous feed solution, and the acceleration of gravity, respectively. Therefore, the dropsize and the stability of the organic solvent drop suspended at the microsyringe needle tip are correlated to the interfacial tension and the specific density of the extraction solvent and the specific density of the aqueous feed solution. *n*-Octyl alcohol, benzene, toluene, *p*-xylene, and *o*-dibutyl phthalate can enrich local anaesthetics effectively, and no interfere peak was found at the elution time of local anaesthetics. However, the R.S.D. values were higher than 13% with *n*-octyl alcohol, benzene, toluene, and *p*-xylene as the organic solvent. The reasons resulted in high R.S.D. may be as follows: one is the poor compatibility of benzene, toluene, and *p*-xylene with the mobile phase of HPLC because of their typical non-polar character. Obvious peak bandbroadening and tail have been found in the chromatograms with benzene, toluene, and *p*-xylene as the extraction solvent. This will lead to the high error in integrating the peak area. The other reason may be the slight dissolution of organic drop with *n*-octyl alcohol, benzene, toluene, and *p*-xylene as the extraction solvent. The volumes of organic drops decrease to

Table 1
The values of the specific density, the solubility in water of the interested organic solvents, the enrichment factor of local anaesthetics, and the relative standard deviation

Organic solvent	ρ (g/cm^3)	Solubility[a] (%)	E_e-fold (R.S.D.)		
			Lidocaine	Bupivacaine	Tetracaine
n-Butyl alcohol	0.8098	9^{15}	Unstable drops suspension	Unstable drops suspension	Unstable drops suspension
n-Octyl alcohol	0.8270	0.054^{20}	82.4 (13%)	172.3 (14%)	184.3 (16%)
Cyclohexane	0.7785	i	Unstable drops suspension	Unstable drops suspension	Unstable drops suspension
Benzene	0.8786	0.07^{22}	71.3 (14%)	154.5 (16%)	161.4 (18%)
Toluene	0.8669	i	76.6 (16%)	163.2(19%)	170.7(19%)
p-Xylene	0.8611	i	76.5 (20%)	161.1 (21%)	169.2 (23%)
Carbon tetrachloride	1.5940	0.08^{20}	Unstable drops suspension	Unstable drops suspension	Unstable drops suspension
Chloroform	1.4832	0.82^{20}	Unstable drops suspension	Unstable drops suspension	Unstable drops suspension
o-Dibutyl phthalate	1.047	0.04^{25}	88.1 (4.3%)	178.0 (7.9%)	189.2 (5.6%)

[a] The superscript is the measurement temperature (°C) and i expresses the indissolubility in water. The stirring rate is 160 rpm and the extraction time is 30 min.

ca. 0.9 μl when the extractions were over (30 min). It is difficult to explain this phenomenon using the solubility of *n*-octyl alcohol, benzene, toluene, and *p*-xylene in the aqueous feed solution, because solubilities of above organic solvents in water are very low. However, good repeatabilities (lower than 7.9% of R.S.D.) with *o*-dibutyl phthalate as the extraction solvent may indicate a good compatibility of *o*-dibutyl phthalate with the mobile phase because *o*-dibutyl phthalate is a polar solvent, and hardly any *o*-dibutyl phthalate leaves the drop due to its adjacent specific density with the aqueous feed solution and suitable interfacial tension. *o*-Dibutyl phthalate, with lightly higher enrichment factors than the other researched solvents and low R.S.Ds., was selected as the organic solvent of LPME combined with HPLC for extracting local anaesthetics.

3.1.2. Stirring rate

Based on the film theory of convective-diffusive mass transfer [16,17], at steady state, the mass transfer coefficient in the aqueous phase increases with increasing stirring rate because faster agitation can decrease the thickness of the diffusion film in the aqueous phase. The influence of three stirring rates, 80, 160, and 250 rpm, on the extraction of local anaesthetics was researched as shown in Fig. 3. The results indicated the enrichment factor increased with the increase of stirring rate. However, high stirring speed gives rise to instability of the *o*-dibutyl phthalate drop. It was found that a 1-μl *o*-dibutyl phthalate drop was unstable when stirring speed was over 250 rpm. On the basis of this, the stirring rate of 160 rpm was selected.

3.1.3. pH value of the aqueous feed solution

The pH of the aqueous feed solution is known to play an essential role in the extraction of basic drugs. The pH of the aqueous feed solution should be higher than the pK_a of analytes, so that analytes are largely neutral and therefore extractable. Four pH values of the aqueous feed solution, 9.0, 10.0, 11.0, and 12.0, have been explored shown in Fig. 4. The result indicates that high enrichment factors are obtained at pH 11.0 and 12.0. Then pH 11.0 was selected as the pH value of the aqueous feed solution in the later experiments.

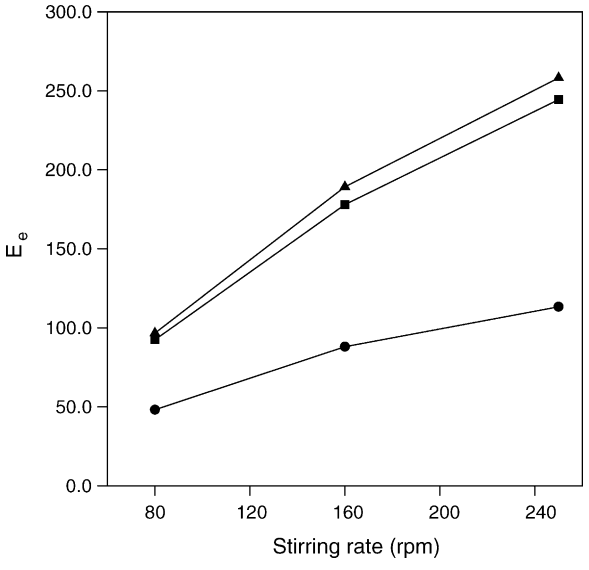


Fig. 3. Effect of the stirring rate on the enrichment factor of local anaesthetics: (●) lidocaine; (■) bupivacaine; (▲) tetracaine.

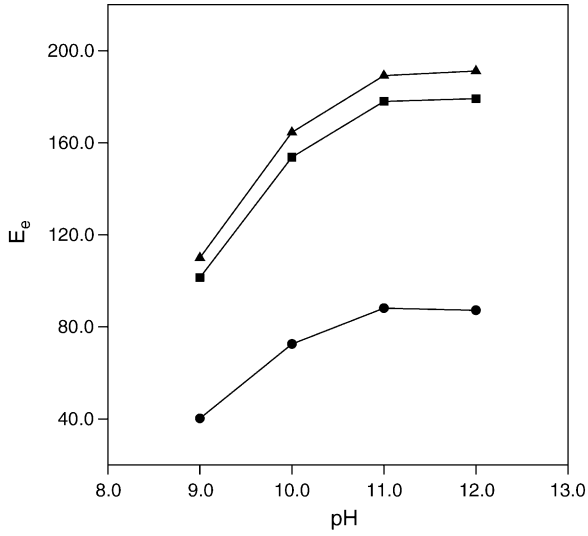


Fig. 4. Effect of the pH value of the aqueous feed solution on the enrichment factor of local anaesthetics: (●) lidocaine; (■) bupivacaine; (▲) tetracaine.

3.1.4. Extraction time

In LPME, the concentration of analyte in the organic solvent drop at time t, c_o, is described by Eq. (2) [16,17].

$$c_o = c_{o,eq}(1 - e^{-kt}) \quad (2)$$

After substitution from Eq. (1), Eq. (2) translates into:

$$E_e = E_{e,eq}(1 - e^{-kt}) \quad (3)$$

where k is the first-order extraction rate constant, $c_{o,eq}$ is the equilibrium concentration of analyte in the organic solvent drop, which is reached after a long time, and $E_{e,eq}$ is the equilibrium enrichment factor of analyte, respectively. Eq. (3) demonstrates that E_e increases with the increase of the extraction time.

The effect of the extraction time on the enrichment factor was investigated by monitoring the variation of enrichment factor with exposure time. As the *o*-dibutyl phthalate drop was exposed to the aqueous feed solution, more and more local anaesthetics were transferred into the *o*-dibutyl phthalate drop from the aqueous feed solution. Fig. 5 illustrates that the enrichment factor increases with exposure time in the range of 1–60 min. By fitting the data points to Eq. (3) using the software package Sigmaplot, the values of $E_{e,eq}$ and k are obtained. The $E_{e,eq}$ values for lidocaine, bupivacaine, and tetracaine are 306 ± 36, 529 ± 71, and 558 ± 48, respectively, and the k values for lidocaine, bupivacaine, and tetracaine are $(1.11 \pm 0.17) \times 10^{-2}$, $(1.32 \pm 0.24) \times 10^{-2}$, and

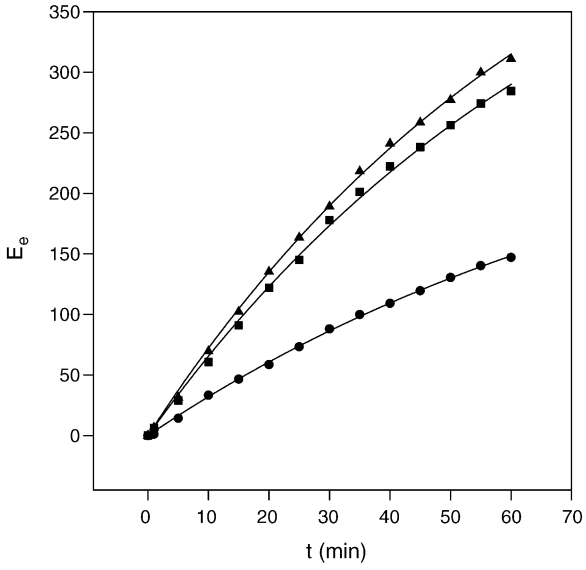


Fig. 5. Effect of the extraction time on the enrichment factor of local anaesthetics: (●) lidocaine; (■) bupivacaine; (▲) tetracaine.

$(1.39 \pm 0.16) \times 10^{-2}\,\text{min}^{-1}$, respectively. The results indicate that equilibrium is not reached even at 60 min of exposure time. Compared to other LPME work [17,20,21], the equilibrium time is long. The reason is probably due to the high volume ratio of sample-to-extract and the low stirring rate. However, longer extraction time will result in the organic solvent drop dissolving in the aqueous phase, especially under stirring. Moreover, it is not considered practicable for exposure time to be excessively long to allow equilibrium to be reached. On the basis of these, an exposure time of 30 min is selected for later experiments. From Fig. 5 the different E_e values of local anaesthetics have been obtained under the same experimental condition. This result demonstrates E_e is related to the structure of the analyte. The analyte with relatively bulky hydrophobic group, such as tetracaine and bupivacaine exhibited high E_e, and lidocaine with relatively small hydrophobic group showed low E_e. This result has been supported by octanol–water partition coefficients ($\log P_{o/w}$ values). $\log P_{o/w}$ values are 3.40, 4.05, and 4.32 for lidocaine, bupivacaine, and tetracaine, respectively [34]. From E_e, the transport ability of analytes through the organic solvent layer can be doped out.

According to the experiments discussed above, the optimal LPME conditions were 6 ml of the feed solution (pH 11), 1 μl of *o*-dibutyl phthalate as the organic phase, 160 rpm of the stirring rate, and 30 min of the extraction time.

3.2. Urine sample analysis

In order to explore the influence of biological fluid on our method, the blank urine sample, the blank urine sample extract, and the spiked urine sample extract (5 μg/ml of local anaesthetics) were analyzed under the optimal experimental conditions shown in Fig. 6. The pH of the feed solution was adjusted to 11.0 with 1.0 mol/l sodium hydroxide solution just before the extraction beginning. Fig. 6(A) illustrated that there were some potential interfering peaks in the blank urine sample. However, the interesting result, the clean chromatogram in a range of elution time 9.5–23.0 min with the blank urine sample extract, has been obtained shown in Fig. 6(B). Fig. 6(B) demonstrates this LPME set-up can serve as a method for sample clean-up and provide very clean extract since the endogenic substances in the urinary matrix have no interference to the determination of the local anaesthetics. As illustrated in Fig. 6(C), three local anaesthetics were effectively preconcentrated from the urine sample. The high enrichment factors, 86.0, 175.3, and 184.5, for lidocaine, bupivacaine, and tetracaine, respectively, have been obtained. An interesting phenomenon has been found that the enrichment factors from water and urine are almost comparable. This result indicates again that no matrix effects occur during the extraction.

3.3. Method validation

3.3.1. Repeatability, linearity, and limit of detection

To evaluate the practical applicability of the proposed LPME technique, repeatability, linearity, and the limit of detection in the spiked urine sample were investigated under the optimal extraction condition. The relative standard deviations were lower than 5.5%. All local anaesthetics exhibited good linearity over the concentration range studied. Coefficients of correlation (r^2) were better than 0.998. The limits of detection were 0.05, 0.03, and 0.05 μg/ml for lidocaine, bupivacaine, and tetracaine, respectively. These values are better than those that can be obtained by SPE and LLE coupled of HPLC–UV system [5,8,9] and comparable with the data obtained by GC–MS [11] although the sensitivity of MS is generally higher than that of UV detection. Table 2 summarizes the analytical data obtained.

3.3.2. Recovery

The recoveries of local anaesthetics (intra-day and inter-day) in two concentrations of spiked urine sample (5.0 and 0.5 μg/ml) stored at 4 °C for seven days were explored. The results collected in Table 3 show the intra-day recoveries of local anaesthetics were more than 93.3%. The inter-day recoveries for lidocaine and bupivacaine were more than 88.9%. These indicate that lidocaine and bupivacaine in the spiked urine sample are stable stored at 4 °C for seven days. However, the inter-day recoveries for tetracaine were only 70.1 and 68.6% for the concentrations of 5.0 and 0.5 μg/ml. The reason may be the hydrolyzation of the ester bond in tetracaine molecule in the spiked urine sample [35]. At the same time, the stability of lidocaine, bupivacaine, and tetracaine in the standard solution (5.0 μg/ml) were studied with the standard solution stored at 4 °C for four weeks. The recoveries of lidocaine (100.3%), bupivacaine (95.0%), and tetracaine (100.1%) demonstrate that the three studied local

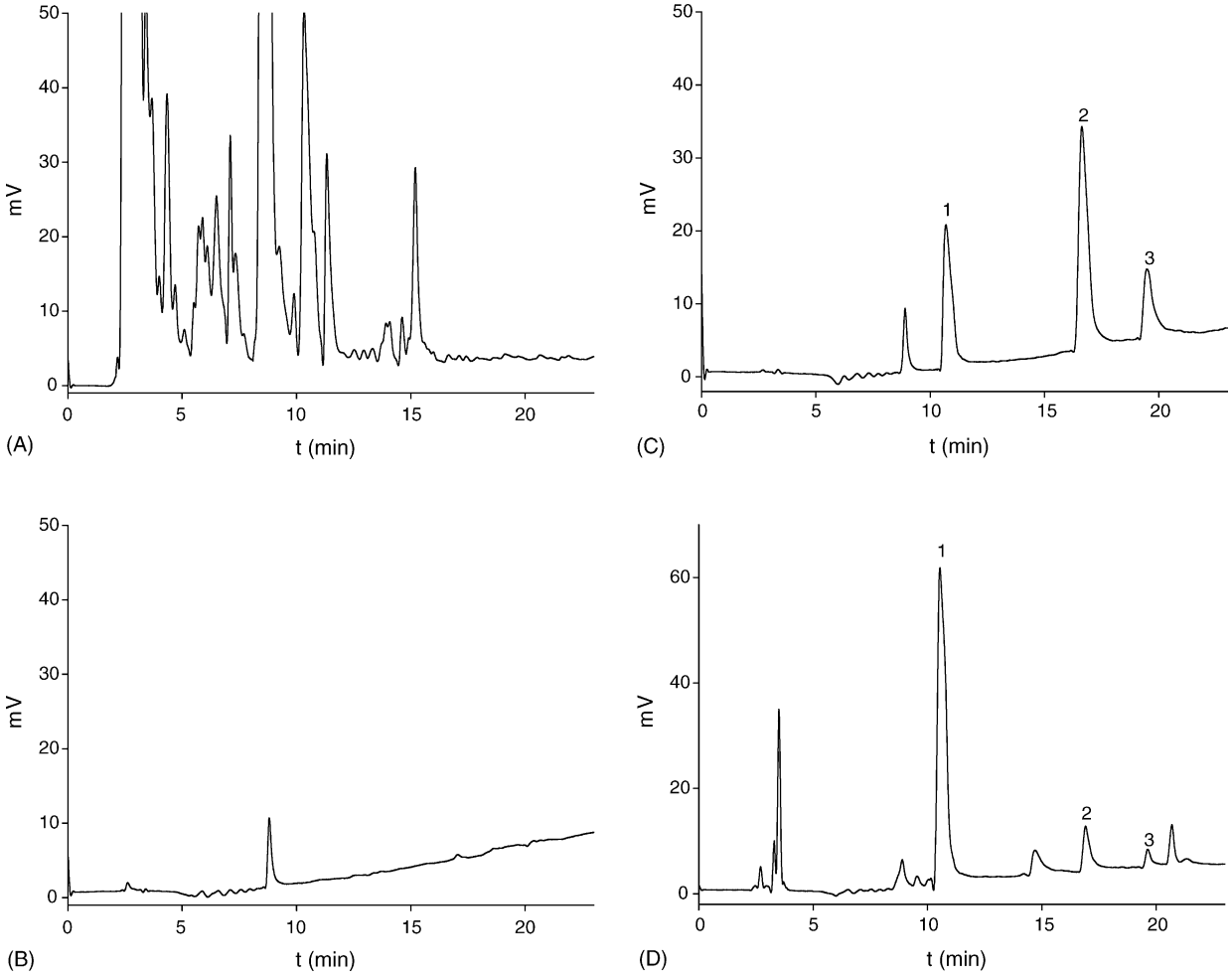


Fig. 6. The chromatograms of the blank urine sample (A), the blank urine sample extract (B), the spiked urine sample extract (5 μg/ml) (C), and the real patient urine sample extract (D).

anaesthetics are stable in the standard solution stored at 4 °C for four weeks.

3.4. Patient urine sample analysis

A real urine sample from a female patient treated with cholecystectomy was investigated. Since the chromatogram of the patient blank urine sample extract is almost same with that shown in Fig. 6(B), only the chromatogram of the real patient urine extract was shown in Fig. 6(D). Fig. 6(D) shows an unknown component (retention time 21 min) in the real patient urine sample can also be extracted in organic phase with the analytes. However, the unknown component does not interfere with the determination of lidocaine, bupivacaine, and tetracaine. The metabolites of the studied local anaesthetics contain three kinds of substances, i.e. acid, phenol, and dimethyl ethanolamine [36]. Under the experimental conditions (pH 11), acid and phenol can't be extracted in *o*-dibutyl phthalate since they are ionized. The metabolite, dimethyl ethanolamine has a large solubility in water, and its retention time in the present HPLC condition is 3.2 min. Therefore, the metabolites of the studied local anaesthetics have no effect on the determination of the local anaesthetics. The concentrations of lidocaine, bupivacaine, and tetracaine

Table 2
Summary of results of the calibration curves for determining local anaesthetics in the spiked urine sample under the optimal LPME condition

Analytes	Linearity range (μg/ml)	Correlation coefficient (r^2)	Limit of detection (μg/ml)	R.S.D. (%) ($n = 6$)
Lidocaine	0.1–10.0	0.999	0.05	5.5
Bupivacaine	0.1–10.0	0.998	0.03	4.4
Tetracaine	0.1–10.0	0.996	0.05	4.9

Table 3
Recovery of local anaesthetics in two concentrations of spiked urine sample (5.0 and 0.5 μg/ml) in intra-day and inter-day measurements ($n = 6$)

Analytes	Recovery ± S.D.[a] (%)			
	5.0 μg/ml		0.5 μg/ml	
	Intra-day	Inter-day	Intra-day	Inter-day
Lidocaine	97.3 ± 4.3	96.4 ± 4.9	99.0 ± 5.1	97.5 ± 4.6
Bupivacaine	95.2 ± 3.9	89.8 ± 3.5	93.3 ± 3.7	88.9 ± 3.7
Tetracaine	95.6 ± 4.1	70.1 ± 3.5	94.1 ± 3.5	68.6 ± 3.8

[a] S.D. is standard deviation.

in the real patient urine, were determined to 13.9, 0.61, and 0.42 μg/ml, respectively. The result indicates the method is high selective and sensitive enough to allow urine concentration determinations of lidocaine, bupivacaine, and tetracaine, following local anaesthetic induced by these three drugs.

4. Conclusion

The present work has outlined the successful development and application of the LPME method for the simultaneous analysis of lidocaine, bupivacaine, and tetracaine from human urine sample prior to HPLC by utilizing a microsyringe as the extraction device. A compatible organic solvent with the mobile phase of the HPLC, *o*-dibutyl phthalate, was selected. Higher enrichment factor and significant sample clean-up were achieved. The method is sensitive, repeatable, and linear over a wide concentration range and requires only small volume of organic extranctant as well as sample. This method was applied to specimen of patient who was treated with extradural anaesthesia of lidocaine, bupivacaine, and tetracaine, and revealed that simultaneous determination of above three local anaesthetics in human urine was possible. Although legal practices specimens were not analyzed, this present method may be applicable for specimens in medico–legal practices and as a useful tool for forensic toxicology and drug monitoring.

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