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Short communication

# Simultaneous determination of procaine, lidocaine, ropivacaine, tetracaine and bupivacaine in human plasma by high-performance liquid chromatography

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ABSTRACT

A simple and sensitive high-performance liquid chromatography with ultraviolet detection (HPLC-UV) method has been developed and validated for simultaneous quantification of five local anesthetics in human plasma: procaine, lidocaine, ropivacaine, tetracaine and bupivacaine. In an ice-water bath, 500 μL plasma sample, containing 100 μg/mL neostigmine methylsulfate as anticholinesterase, was spiked with carbamazepine as internal standard and alkalized by sodium hydroxide. Liquid–liquid extraction with ethyl ether was used for plasma sample preparation. The chromatographic separation was achieved on a Kromosil ODS C18 column with a mobile phase consisting of 30 mM potassium dihydrogen phosphate buffer (0.16% triethylamine, pH adjusted to 4.9 with phosphoric acid) and acetonitrile (63/37, v/v). The detection was performed simultaneously at wavelengths of 210 and 290 nm. The chromatographic analysis time was 13 min per sample. The calibration curves of all five analytes were linear between 0.05 and 5.0 μg/mL ($r^2 \geq 0.998$). Precision ranged from 1.4% to 7.9% and accuracy was between 91.7% and 106.5%. The validated method is applicable for simultaneous determination of procaine, lidocaine, ropivacaine, tetracaine and bupivacaine for therapeutic drug monitoring and pharmacokinetic study.



## 1. Introduction

Procaine, lidocaine, ropivacaine, tetracaine and bupivacaine are often used in combinations to achieve appropriate onset time and duration of action for local anesthesia [1,2]. Several analytical methods have been published for simultaneous determination of some of these compounds in human plasma [3–7]. These five local anesthetics can be chemically divided into two groups, the amino-amides (lidocaine, ropivacaine and bupivacaine) and the amino-esters (procaine and tetracaine) [8]. Some challenges existed in simultaneous determination of these five anesthetics, such as that the amino-esters in human plasma is easy to degrade, and that the ultraviolet absorbance of the two groups peaks at 210 and 290 nm, respectively [4,7]. As far as we know, only two published methods simultaneously determined both the amino-amides and the amino-esters [4,7]. However, neither of the two methods covered all these five common local anesthetics, and both methods had some analytical limitations which need to be improved. The GC–MS method required a 3-h solid phase extraction procedure for sample preparation and the HPLC method demanded a 30 min gradient elution for chromatographic analysis [4,7].

The purpose of our study is to develop a reliable, rapid, sensitive and selective analytical method for simultaneous determination of these five local anesthetics in human plasma. Compared to other methods that have been published, the advantages of our validated technique are its simplicity, rapidity, and capability to simultaneously measure these five anesthetics under the same sample preparation procedure and chromatographic conditions.

## 2. Experimental

### 2.1. Regents and materials

Bupivacaine hydrochloride and ropivacaine hydrochloride were provided by Sunve Pharm (Shanghai, China) and AstraZeneca (Wuxi, China), respectively. Tetracaine hydrochloride, lidocaine and procaine hydrochloride were obtained from the China National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Carbamazepine (internal standard, IS) was obtained from Sigma–Aldrich (St Louis, USA). Neostigmine methylsulfate was provided by Sine-Jinzhu Pharmaceutical. Co., Ltd. (Shanghai, China). HPLC-grade methanol and acetonitrile were obtained from Promptar (Elk Grove, USA). The following reagents

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were analytical grade: potassium dihydrogen phosphate (Jinsha Chemical, Shantou, China), sodium hydroxide (Jinlu Chemical, Shanghai, China), triethylamine and ethyl ether (Lingfeng Chemical, Shanghai, China). Ultra pure water was prepared by the Milli-Q Academic water-purification system (Millipore, Milford, USA). Drug-free human plasma was supplied by the Blood Bank of Huashan Hospital, Fudan University (Shanghai, China).

### 2.2. Preparation of stock solutions, calibration standards and quality-control samples

Standard stock solutions of each analyte (1 mg/mL) and the IS (1 mg/mL) were prepared separately by dissolving appropriate amounts in water–methanol (50/50, v/v). And each stock solution was further diluted with water–methanol (50/50, v/v) for the preparation of working solutions at a series of concentrations. A stock solution of neostigmine (20 mg/mL) was prepared in water–methanol (50/50, v/v). Working solutions were prepared daily and stock solutions were stored at 4 °C.

Plasma-based calibration standards for the analytes at 0.05, 0.1, 0.25, 1, 2.5, 5.0 μg/mL, together with plasma quality control (QC) samples at 0.15, 2, 4 μg/mL, were prepared by appropriate dilutions of the respective working solution with drug-free plasma. To block esterase activity in the plasma, neostigmine methylsulfate (final 100 μg/mL) was mixed before the addition of procaine and tetracaine.

### 2.3. Sample preparation

Patients' blood samples (3 mL) were collected in tubes containing ethylenediaminetetraacetic acid (EDTA) and neostigmine methylsulfate (final 100 μg/mL). The samples were stored in ice-water bath for 10–15 min and then centrifuged at 1300 × *g* at 4 °C for 5 min to obtain plasma. Aliquots of 500 μL plasma samples (blank, standard, control, or patient sample) were mixed with 100 μL IS solution (5 μg/mL), 100 μL sodium hydroxide aqueous solution (1 M) and 3 mL ethyl ether in an ice-water bath. Then the mixtures were vigorously vortexed for 2 min and centrifuged at 1300 × *g* for 10 min under the room temperature. The organic phase was transferred to a 10 mL polypropylene conical tube and evaporated to dryness in a 40 °C water bath under a gentle stream of nitrogen. The residue was dissolved in 100 μL water–methanol (50/50, v/v) and was vortex-mixed for 30 s. The mixture was transferred to a clean polypropylene tube and then centrifuged at 13,800 × *g* for 10 min. The supernatant was transferred to an auto-sampler vial and 30 μL was injected into the HPLC system.

**Table 1**
Drugs potentially co-administered with procaine, lidocaine, ropivacaine, tetracaine and bupivacaine were examined for possible interference with this method.

| Compound | RT (min) | Compound | RT (min) |
|---|---|---|---|
| Acetaminophen | 2.8 | Midazolam | 4.6 |
| Adefovir | n.d. | Naproxen | n.d. |
| Anectine | n.d. | Neostigmine | n.d. |
| Antipyrine | n.d. | Nevirapine | n.d. |
| Atenolol | 3.3 | Nifedipine | 12.9 |
| Cephazoline | n.d. | Nimodipine | 17.7 |
| Cefotiam | n.d. | Nitrazepam | 4.6 |
| Cefuroxime | n.d. | Ofloxacin | n.d. |
| Chlorzoxazone | 14.3 | Penciclovir | n.d. |
| Ciprofloxacin | n.d. | Phenacetin | n.d. |
| Clindamycin | n.d. | Phenytoin | n.d. |
| Clonazepam | 4.3 | Propafenone | n.d. |
| Dexamethasone | n.d. | propofol | n.d. |
| Diazepam | 7.2 | Propranolol | 10.4 |
| Digoxin | n.d. | Pseudoephedrine | 2.9 |
| Diltiazem | n.d. | Raubasine | n.d. |
| Fentanyl | 13.1 | Ribavirin | n.d. |
| Ganciclovir | n.d. | Topiramate | n.d. |
| Gentamicin | n.d. | Tramadol | n.d. |
| Ibuprofen | n.d. | Valaciclovir | n.d. |
| Indometacin | n.d. | Valganciclovir | n.d. |
| Metoprolol | 6.9 | Vecuronium | n.d. |

RT: retention time; n.d.: not detectable within 20 min elution.

### 2.4. Chromatographic system

The chromatographic system was Waters 2690 series (Waters, USA) equipped with a quaternary pump, a temperature controlled auto injector, and a temperature controlled column compartment. The Waters 2487 dual-wavelength ultraviolet detector was set

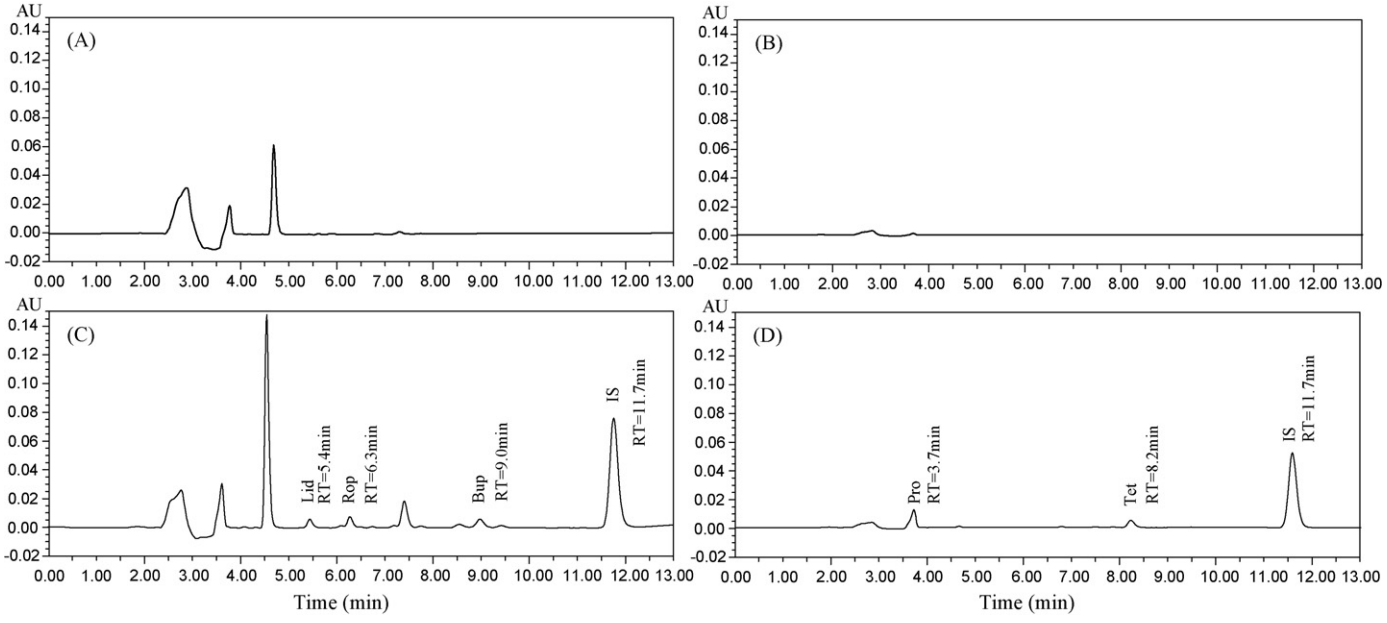


**Fig. 1.** Representative chromatograms of a pooled blank plasma at 210 nm (A) and 290 nm (B) and representative chromatograms of the lower limit of quantification (0.05 μg/mL) of procaine (Pro), lidocaine (Lid), ropivacaine (Rop), tetracaine (Tet) and bupivacaine (Bup) in human plasma spiked with 1 μg/mL internal standard (IS) at 210 nm (C) and 290 nm (D), respectively.

**Table 2**
The precision and accuracy of the assay for procaine, lidocaine, ropivacaine, tetracaine and bupivacaine in human plasma.

| | Concentration added (μg/mL) | Intra-batch ($n$ = 6) | | | Inter-batch ($n$ = 6) | | |
|---|---|---|---|---|---|---|---|
| | | Concentration found (mean ± SD, μg/mL) | Accuracy (%) | Precision (%) | Concentration found (mean ± SD, μg/mL) | Accuracy (%) | Precision (%) |
| Procaine | 0.05 | 0.052 ± 0.002 | 104.1 | 4.5 | 0.051 ± 0.006 | 102.5 | 11.3 |
| | 0.15 | 0.160 ± 0.006 | 106.5 | 3.6 | 0.156 ± 0.008 | 104.1 | 5.1 |
| | 2 | 1.98 ± 0.03 | 98.8 | 1.4 | 1.97 ± 0.04 | 98.7 | 1.8 |
| | 4 | 3.84 ± 0.09 | 96.0 | 2.4 | 3.86 ± 0.11 | 96.5 | 2.8 |
| Lidocaine | 0.05 | 0.048 ± 0.005 | 96.3 | 10.1 | 0.051 ± 0.001 | 101.8 | 2.6 |
| | 0.15 | 0.157 ± 0.006 | 104.6 | 4.0 | 0.155 ± 0.008 | 103.7 | 5.3 |
| | 2 | 1.92 ± 0.10 | 96.2 | 5.1 | 1.99 ± 0.10 | 99.4 | 4.8 |
| | 4 | 3.86 ± 0.15 | 96.5 | 4.0 | 3.91 ± 0.15 | 97.7 | 3.8 |
| Ropivacaine | 0.05 | 0.048 ± 0.004 | 95.4 | 8.3 | 0.052 ± 0.005 | 104.8 | 9.8 |
| | 0.15 | 0.152 ± 0.006 | 101.2 | 4.0 | 0.153 ± 0.006 | 102.2 | 3.9 |
| | 2 | 1.94 ± 0.07 | 96.9 | 3.6 | 1.97 ± 0.07 | 98.4 | 3.3 |
| | 4 | 3.84 ± 0.13 | 96.1 | 3.4 | 3.84 ± 0.10 | 96.0 | 2.7 |
| Tetracaine | 0.05 | 0.054 ± 0.005 | 107.1 | 8.8 | 0.053 ± 0.004 | 105.3 | 7.2 |
| | 0.15 | 0.149 ± 0.004 | 99.6 | 2.5 | 0.153 ± 0.006 | 102.0 | 3.7 |
| | 2 | 1.83 ± 0.08 | 91.7 | 4.6 | 1.84 ± 0.09 | 92.2 | 4.7 |
| | 4 | 3.77 ± 0.13 | 94.2 | 3.5 | 3.79 ± 0.10 | 94.9 | 2.7 |
| Bupivacaine | 0.05 | 0.052 ± 0.004 | 103.4 | 7.5 | 0.051 ± 0.005 | 102.6 | 9.5 |
| | 0.15 | 0.149 ± 0.010 | 99.3 | 6.8 | 0.147 ± 0.012 | 98.2 | 7.9 |
| | 2 | 1.89 ± 0.06 | 94.7 | 3.1 | 1.93 ± 0.06 | 96.5 | 3.2 |
| | 4 | 3.84 ± 0.13 | 96.1 | 3.4 | 3.85 ± 0.10 | 96.3 | 2.7 |

simultaneously at wavelengths of 210 nm for lidocaine, ropivacaine and bupivacaine, and 290 nm for procaine and tetracaine. The separation was performed at 40 °C on a 4.6 mm × 250 mm, 5 μm Kromosil ODS C18 analytical column (Eka Chemicals, Sweden). The data collection and analysis were run with the Millennium$^{32}$ Chemstation software (Waters, USA). The isocratic mobile phase composition was a mixture of 30 mM potassium dihydrogen phosphate buffer (0.16% triethylamine, pH adjusted to 4.9 with phosphoric acid)/acetonitrile (63/37, v/v), which was pumped at a flow rate of 1.0 mL/min.

### 2.5. Method validation

The method validation assays were carried out according to the bioanalytical method validation guidance of United States Food and Drug Administration (FDA, 2001) [9].

To evaluate potential chromatographic interference by endogenous substances and potential co-administered drugs, eight blank human plasma samples from different sources and drug-containing methanol standards (10 μg/mL) listed in Table 1, were tested.

Calibration curves were analyzed at the concentration range of 0.05–5.0 μg/mL for each analyte ($n$ = 6) and were generated by using the ratios of the analyte peak area to the IS peak area ($y$) versus concentrations ($x$) and were fitted into the equation of $y = bx + a$ by weighted least squares regression.

The precision and accuracy were determined by analyzing six spiked plasma samples at each QC level within one run and by analyzing three spiked plasma samples at each QC level on six consecutive runs. The concentration of each sample was calculated by using the standard curve prepared and analyzed on the same run. The precision was calculated as the coefficient of variation (C.V. %) within one run (intra-batch) and between different runs (inter-batch), and the accuracy as deviation between nominal and measured concentrations. Each back-calculated concentration of QC samples should be with the precision within 15% and the accuracy within 85–115%. The lower limit of quantification (LLOQ) for each analyte in plasma was experimentally chosen and the response to the analyte was at least five times greater than the blank with precision within 20% and accuracy within 80–120%.

The pre-preparative stability was assessed by determining QC plasma samples kept in an ice-water bath for 4 h. The post-

**Table 3**
The stability of procaine, lidocaine, ropivacaine, tetracaine and bupivacaine in human plasma.

| Concentration added (μg/mL) | | Concentration found (mean ± SD, μg/mL) | | |
|---|---|---|---|---|
| | | Pre-preparative (ice-water bath × 4 h), $n$ = 6 | Post-preparative ($T$ = 4 °C × 24 h), $n$ = 6 | Freeze–thaw stability (3 circles, −20 °C × 24 h-ice-water bath × 1 h), $n$ = 3 |
| Procaine | 0.15 | 0.160 ± 0.006 | 0.158 ± 0.005 | 0.159 ± 0.004 |
| | 4 | 3.88 ± 0.04 | 3.83 ± 0.09 | 3.83 ± 0.07 |
| Lidocaine | 0.15 | 0.159 ± 0.007 | 0.155 ± 0.007 | 0.155 ± 0.009 |
| | 4 | 3.87 ± 0.09 | 4.08 ± 0.21 | 3.90 ± 0.14 |
| Ropivacaine | 0.15 | 0.154 ± 0.008 | 0.158 ± 0.005 | 0.151 ± 0.005 |
| | 4 | 3.81 ± 0.07 | 3.91 ± 0.08 | 3.83 ± 0.12 |
| Tetracaine | 0.15 | 0.155 ± 0.006 | 0.160 ± 0.005 | 0.153 ± 0.007 |
| | 4 | 3.83 ± 0.09 | 3.79 ± 0.13 | 3.79 ± 0.11 |
| Bupivacaine | 0.15 | 0.144 ± 0.013 | 0.157 ± 0.010 | 0.147 ± 0.008 |
| | 4 | 3.82 ± 0.07 | 3.92 ± 0.09 | 3.88 ± 0.11 |

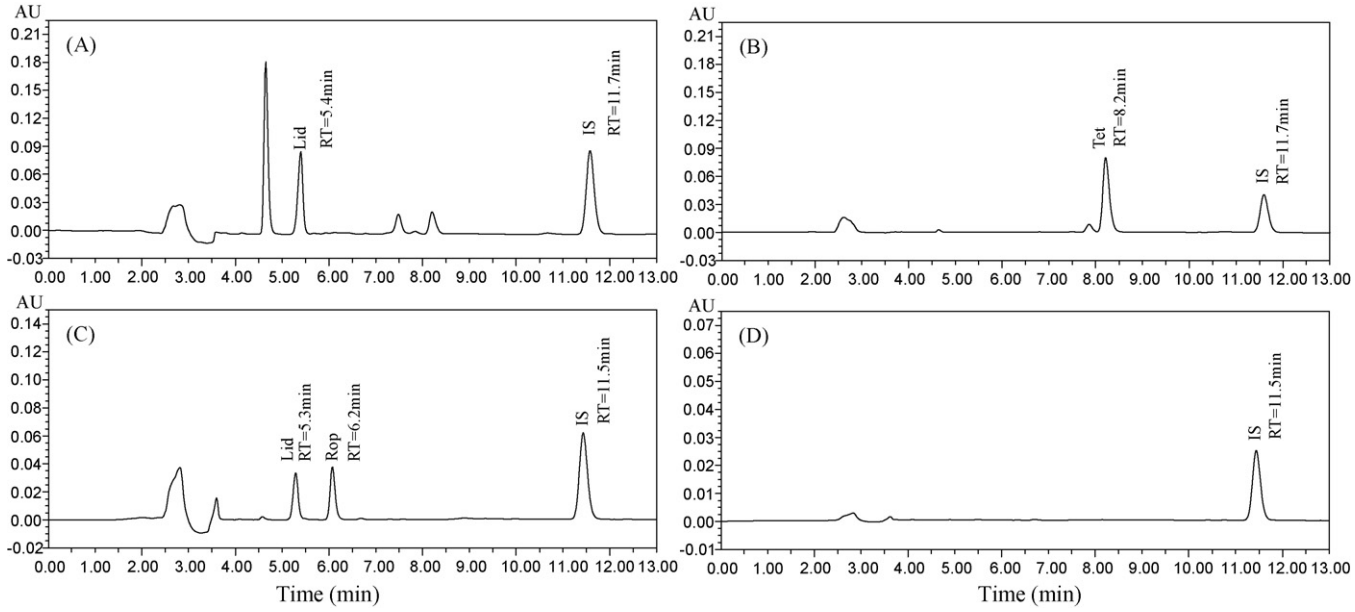


**Fig. 2.** Representative chromatograms of a plasma sample obtained at 20 min from a patient after a single injection of 2 mg/kg lidocaine and 2 mg/kg tetracaine at 210 nm (A) and 290 nm (B), respectively; the concentration was determined to be 1.62 μg/mL for lidocaine and 1.55 μg/mL for tetracaine. Representative chromatograms of a plasma sample obtained at 10 min from a patient after a single injection of 2 mg/kg lidocaine and 2 mg/kg ropivacaine at 210 nm (C) and 290 nm (D), respectively; the concentration was determined to be 0.94 μg/mL for lidocaine and 0.79 μg/mL for ropivacaine.

preparative stability was evaluated by determining the extraction of processed QC plasma samples kept at 4 °C for 24 h. The freeze–thaw stability was tested by analyzing QC plasma samples undergoing three freeze (−20 °C)–thaw (ice-water bath) cycles on consecutive days. The stock solution stability of each analyte and the IS were evaluated by analyzing their working solutions (concentrations in three QC levels for each analyte and 5 μg/mL for the IS) kept at room temperature for 6 h, respectively. After each storage period, samples were regarded as stable if the deviation from the initial condition was within ±15%.

The absolute extraction recovery of each analyte was assessed by comparing the peak areas from extracted plasma samples at each QC level with those from standard solutions at the same concentrations ($n = 6$). The absolute recovery of the IS was assessed by comparing the peak areas from extracted plasma samples at 1 μg/mL with those from standard solutions at the same concentrations ($n = 6$).

## 3. Results and discussion

No visible interferences were observed at the retention times of these five analytes and IS at both 210 and 290 nm. All the commonly administrated drugs shown in Table 1 were not found to be interfering with the assay. The chromatogram of pooled blank plasma was shown in Fig. 1A and B. The analytical method presented linear response over the concentration range of 0.05–5.0 μg/mL by weighted ($1/x$) least-squares regression analysis. The regression coefficients ($r^2$) were stable and all ≥0.998. The precision and accuracy results obtained from QCs were presented in Table 2. The data revealed good precision and accuracy. As all values of the precision were less than 12% and that of the accuracy were within ±9%, 0.05 μg/mL was chosen as the LLOQ for each analyte. Although the LLOQ is less sensitive compared to the 1 ng/mL by the LC–MS/MS method [6], it is comparable to the 0.1 and 0.05 μg/mL by the HPCL-UV method and the GC–MS method, respectively, which simultaneously determined both the amino-amides and the amino-esters [4,7]. The representative chromatogram of LLOQ samples was shown in Fig. 1C and D.

The amino-esters can be easily hydrolyzed by the esterase in human plasma. One of critical conditions for determination of the amino-esters is to inhibit their degradation, which has been reported by previous studies [4,7]. Table 3 summarized the results of the pre-preparative, post-preparative and freeze–thaw stability of the five anesthetics. The data showed that the degradation of the amino-esters in human plasma was well controlled by combined use of the neostigmine methylsulfate and the ice-water bath. Based on the data obtained, the working solutions of the five anesthetics and the IS were intact within 6 h at room temperature.

The absolute extraction recovery was (82.8 ± 7.8) – (84.9 ± 6.9)% for procaine, (80.7 ± 7.9) – (84.5 ± 8.8)% for lidocaine, (82.6 ± 7.3) – (83.8 ± 8.4)% for ropivacaine, (77.0 ± 9.2) – (82.8 ± 7.0)% for tetracaine, and (79.8 ± 8.3) – (83.2 ± 6.3)% for bupivacaine at the plasma concentration range of 0.15–4 μg/mL. The recovery of the IS was (92.1 ± 5.5)% at the plasma concentration of 1 μg/mL. Ethyl ether was proven to be successful in terms of the high extraction recovery and the absence of endogenous interference in the chromatogram in this study. However, due to the toxicity concern of ethyl ether [10], devices for ethyl ether collection and disposal are recommended when this analytical method is applied.

## 4. Clinical application

This method was applied to a preliminary pharmacokinetic study in which 10 Chinese adult patients administrated with either a mixture (A) of lidocaine (2 mg/kg) and ropivacaine (2 mg/kg) or a mixture (B) of lidocaine (2 mg/kg) and tetracaine (2 mg/kg) as local anesthetics during brachial plexus block. The study protocol was approved by the Institutional Ethics Committee of Huashan Hospital and was conducted according to the recommendations described in the Declaration of Helsinki. All the patients gave written informed consent before enrollment. After fasted for at least 8 h, patients were administrated with a single injection of either mixture A or mixture B. Serial arterial blood samples were collected at 1, 2.5, 5, 10, 15, 20, 30, 45, 60, 90, 120, and 180 min post-dose. Representative chromatograms of plasmas from patients were presented in Fig. 2A–D, respectively.

## 5. Conclusions

The present paper first described a simple and reliable HPLC-UV method for rapid simultaneous determination of procaine, lidocaine, ropivacaine, tetracaine and bupivacaine in human plasma. The method has been proven to be applicable in a pharmacokinetic study of procaine, lidocaine, ropivacaine, tetracaine and bupivacaine.

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