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Full Length Research Paper

A validated HPLC method for the determination of levobupivacaine in plasma and its application to pharmacokinetic studies in Chinese people

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A rapid, simple and sensitive HPLC method for the quantification of levobupivacaine in plasma was developed and validated. The analysis involved a simple liquid-liquid extraction. Plasma was extracted with hexane and the organic extract was then evaporated and the residue was reconstituted in mobile phase. The reconstituted solution was injected into an HPLC system and was subjected to reverse-phase HPLC on a 5 µm C_{18} column at a flow-rate of 1 ml/min. The mobile phase consisted of potassium dihydrogen phosphate (0.01 mol/L) and acetonitrile (85:15 pH = 4.0). Standard curves were linear over the concentration range of 0.0125 to 2 mg/L. The mean predicted concentrations of the quality control (QC) samples deviated by less than 2% from the corresponding nominal values; the intra-assay and inter-assay precision of the assay were within 6% relative standard deviation. The extraction recovery of levobupivacaine was more than 85%. The validated assay was applied to a pharmacokinetic study of levobupivacaine in plasma in Chinese patients with normal renal function or renal disease.

Key words: Levobupivacaine, high-performance liquid chromatography (HPLC), pharmacokinetic study, renal function, anesthetic agent, validation.

INTRODUCTION

Levobupivacaine (1-Butyl-N-(2,6-dimethylphenyl)-iperidine-2-carboxamide CAS: 27262-47-1) is a long-acting amide-type local anesthetic agent with both analgesic and anesthetic properties (Naheed et al., 2011). It is one of a chemical group, the pipecoloxylidides and it is a racemic mixture of two stereo-enantiomers. In clinical, it was indicated for local anesthesia, including infiltration (Zaralidou et al., 2007), perineural techniques (Leeuw et al., 2008), epidural (Koch et al., 2008) and intrathecal administration (Camorcia et al., 2007) and has the clinical advantages of long duration of action and favorable ratio of sensory to motor neural block.

Previous studies in animals, volunteers and patients have demonstrated a significant reduction in symptoms of cardiac or central nervous system (CNS) toxicity with the use of the single (S-)-enantiomer levobupivacaine when compared with equal doses of racemic bupivacaine (Bardsley et al., 1998; Denson et al., 1992; Huang et al., 1998; Mazoit et al., 1993; Valenzuela et al., 1995).

Numerous *in vitro* (Boitquin et al., 2004; Jäppinen et al., 2003) and *in vivo* (Wu et al., 2010; Stehr et al., 2007) methods for the bioanalysis of levobupivacaine have been previously described, including high-performance liquid chromatography (HPLC) with UV. However, these methods were not satisfactory with respect to sensitivity, feasibility and reliability, and were tedious in sample preparation. In addition, pharmacokinetic parameters of Chinese data are rarely reported, especially considering the condition of renal function. Our goal was therefore to optimize these methods to provide more guidance to the reasonable use of this drug and to study the pharmacokinetic profile *in vivo*, a more sensitive HPLC method has been developed (Linmei et al., 2011; Danlami et al., 2011; Mahmood et al., 2011). Based on our method, the pharmacokinetic parameters of levobupivacaine in Chinese volunteers were first reported

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considering two groups of people with normal renal function or renal disease.

MATERIALS AND METHODS

Chemicals and reagents

0.5% levobupivacaine (99% purity, Batch: 052648) was supplied by Henrui Pharmaceutical Co. Ltd. (Guangzhou China). The internal standard (IS), ropivacaine (98% purity), was a gift from Shanghai Institute of Materia Medica, Chinese Academy of Sciences. Potassium dihydrogen phosphate and hexane were of analytical grade, and acetonitrile (Merck, Germany) of HPLC grade. All the other chemicals were purchased from Shanghai chemical reagents company. Purified water from a Milli-Q system (Millipore, Bedford, MA, USA) was used throughout the experiment.

Chromatographic conditions

The amount of levobupivacaine in each sample was determined by HPLC (LC-10A, Shimadzu Co Ltd, Kyoto, Japan). Chromatographic separation was achieved using a Dikma DiamonsilTM C_{18} column (Dikma Co Ltd, Beijing, China, 5 µm, 200 × 4.6 mm) and a precolumn (Nova-Pak, 10 µm, C_{18}, Waters) at 40℃. The mobile phase was a mixture of potassium dihydrogen phosphate (0.01 mol/L) and acetonitrile (85:15 pH = 4.0). The UV absorbance of the effluent was monitored (SPD-10A, Shimadzu Co Ltd, Kyoto, Japan) at a wavelength of 210 nm.

Standard solutions

A standard stock solution of levobupivacaine (400 mg/L) was prepared by dissolving the drug in purified water. The solution was then successively diluted with purified water to prepare working standard solutions in the concentration range of 0.0125 to 2 mg/L for analyte. The IS stock solution (200 mg/L) was prepared with purified water as solvent. All the solutions were stored at 4℃ and were brought to room temperature before use. For preparation of plasma calibration curves samples, each of the working solution within the proper concentration range was evaporated in tubes under gentle stream of nitrogen at 50℃. After addition of 0.5 ml blank plasma and it was mixed for 10 s on a vortex mixer, the samples were subjected to extraction and analysis. Plasma concentrations were 0.0125, 0.025, 0.1, 0.25, 0.5, 1 and 2 mg/L for analyte. Plasma samples were quantified using the ratio of peak area of levobupivacaine to that of the IS. Peak area ratios were plotted against concentrations equation of the least squares regression line calculated. All the quality control samples (QCs) used in the validation and during the pharmacokinetic study were prepared in the same way as the calibration standards before analysis. Plasma concentrations of QCs were 0.1, 0.5 and 2 mg/L for levobupivacaine.

Sample preparation

Plasma samples were thawed in a water-bath at 37℃. 0.5 ml volume of the plasma sample was transferred to a 5 ml plastic test tube together with 50 µl of IS solution (40 mg/L). After vortex shaking for 1 min (Eppendorf, 5432 vortex mixer, Germany), 3 ml of hexane (with 5% isopropyl alcohol) was added and the mixture was vortexed for 2 min. After centrifugation at 3500 rpm for 10 min (TGL-16G, Shanghai, China), the upper organic layer was quantitatively transferred to a 10 ml glass tube and evaporated to dryness using evaporator at 50℃. The residue was reconstituted in 100 µl of the mobile phase, and then vortex-mixed. After centrifugation at 12000 rpm (Thermo IEC, Micromax, USA) for 10 min, 20 µl aliquot of the solution was injected into the HPLC system for analysis.

Validation test

Specificity, linearity and sensitivity

The specificity of the method was assessed by comparing the chromatograms of six different batches of blank plasma. Peak areas of endogenous compounds co-eluting with the analyte should be less than 20% of the peak area of the limit of quantitation (LOQ) standard. The deviation from the nominal concentration for the LOQ in these six plasma batches should be within ±20% (FDA, 2001).

The calibration standards were prepared and assayed in duplicate on three different days to demonstrate the linearity of this method. Peak area ratios were plotted against analyte concentrations, and calibration curves were calculated with a weighted ($1/C^2$) least square linear regression method. The acceptance criterion for each back-calculated standard concentration was ±15% deviation from the nominal value except at LOQ.

The LOQ was defined as the lowest concentration at the calibration curve, at which the signal-to-noise (S/N) ratio was not less than 10, with precision less than or equal to 20% and accuracy within ±20%. The LOQ was determined in six replicates on three consecutive days.

Precision and accuracy

The precision and accuracy of the assay were obtained by comparing the predicted concentration (obtained from the calibration curve) to the actual concentration of levobupivacaine spiked in blank plasma. Intra-day precision was determined by repeated analysis of each QC sample on one day (n = 5), and inter-day precision was determined by repeated analysis on five consecutive days (n = 1 series per day). The precision was expressed as the inter-day and intra-day coefficient of variation [RSD = (S.D./mean of the recoveries) × 100%]. Accuracy was defined as the relative deviation in the computed value (E) of a standard from that of its true value (T) expressed as a percentage (RE%). It was calculated using the formula RE (%) = (E−T)/T × 100.

Freeze and thaw stability

The freeze and thaw stability study samples were obtained by adding the standard solution in the blank rat plasma at three QC levels. These samples were frozen at -20℃ for 7 days, and then thawed at room temperature. After being allowed to completely thaw, the samples were refrozen for 24 h under the same conditions. This freeze-thaw cycle was repeated three times before these samples were analyzed.

Recovery determination

The absolute recovery of levobupivacaine was determined by direct comparison of peak areas from extracts versus spiked post-extraction samples at 0.1, 0.5 and 2 mg/L.

Pharmacokinetic study

We performed an open, parallel-group, single-dose pharmacoki-

inetic study in 8 patients suffering from impaired renal function and 8 healthy volunteers, that is, two groups with 8 study subjects each. The study protocol was approved by the Coordinating Ethics Committee. Written informed consent was obtained from all study subjects. The subjects were divided into two groups according to their creatinine clearance (CLCR) values, calculated at the enrolment visit 6 weeks before the study, as follows: Group 1, CLCR>80 ml/min (healthy volunteers); Group 2, CLCR<40 ml/min. CLCR values were calculated using the Cockroft-Gault formula (Cockcroft and Gault 1976).

The subjects then received an intravenous (i.v.) infusion of levobupivacaine 2 mg/kg over 30 min using a volume-controlled infusion pump. 3 ml blood samples were collected immediately before and at 10, 20, 30, 45, 60, 90, 120, 210, 300, 420, 540, 660 and 840 min after drug administration. The blood samples were withdrawn into heparinized Eppendoff tubes, and were centrifuged at 12000 rpm for 10 min at 4℃. A 0.5 ml volume of plasma was obtained and stored at -20℃ until analysis. Pharmacokinetic parameters were calculated from the plasma concentration-time data. The elimination half-life ($T_{1/2}$) was determined by linear regression of the terminal portion of the plasma concentration-time data. The area under the plasma concentration-time curve from zero to the last measurable plasma concentration point (AUC_{0-t}) was calculated by the linear trapezoidal method. Extrapolation to time infinity ($AUC_{0-\infty}$) was calculated as follows: $AUC_{0-\infty} = AUC_{0-t} + Ct/ke$, where Ct is the last measurable plasma concentration and ke is the terminal elimination rate constant. The results were expressed as mean ± SD. Student t-test was used to test the differences between the normal renal function and renal disease group. Differences were considered statistically significant when $p < 0.05$.

RESULTS

The representative chromatograms of blank plasma (A) and spiked plasma samples (0.5 mg/L) (B) are as shown in Figure 1. The analytical peaks of levobupivacaine and internal standard were resolved with good symmetry, the retention time of levobupivacaine and internal standard were 13.2 and 8.1 min, respectively, no endogenous sources of interference were observed at the retention time of the analyte. A sample from a subject after intake of levobupivacaine (t = 2 h) is also shown in Figure 1.

Good linearity was observed over the concentration range of 0.0125 to 2 mg/L plasma. A representative regression equation was: y = 5.46x - 0.3216, where y indicates the ratios of the peak areas of levobupivacaine to IS and x indicates the plasma concentrations. The limit of quantitation (LOQ) was 0.01 mg/L (S/N = 10). The RSD (n = 5) of the slope calculated with calibration curve data was 0.14%, showing a good repeatability (Table 1). The intra-/inter-day precision and accuracy are as shown in Table 2. The RSD of levobupivacaine ranged from 3.06 to 5.55% for intra-day and 3.41 to 5.88% for inter-day, respectively. The RE of levobupivacaine ranged from -1 to 1.2% for intra-day and -0.4 to 2% for inter-day, respectively.

Freshly prepared solutions showed no evidence of degradation for either levobupivacaine or the internal standard. No significant degradation was observed for any analytes during the sample processing and extraction including the dry down procedure. The stability of the sample solution in the auto sampler at 4℃ was also assessed. Levobupivacaine in sample solution was found

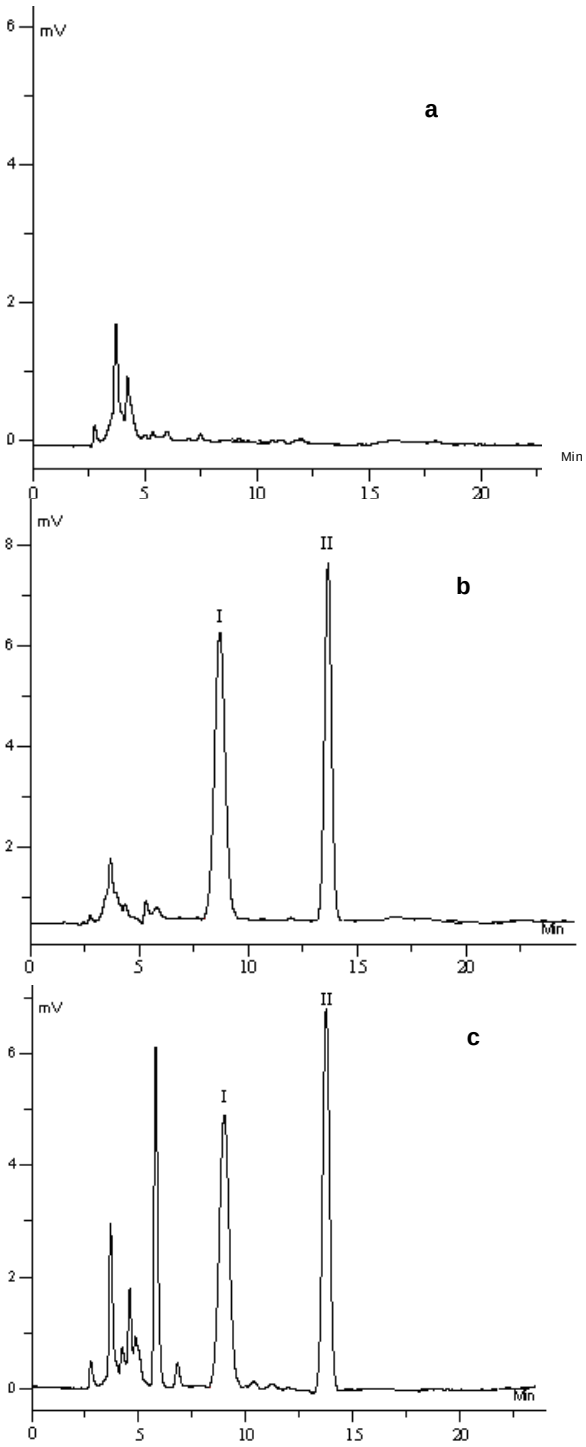

Figure 1. HPLC chromatograph of levobupivacaine. A: Blank serum; B: Blank serum added with internal standard; C: sample; I: ropivacaine; II: levobupivacaine.

Table 1. Inter-day precision in the slope and intercept of standard curves (r = 0.9993 - 0.9997).

Days	Slope	Intercept	Correlation
1	0.3115	0.0063	0.9994
2	0.3119	0.0062	0.9996
3	0.3116	0.0061	0.9993
4	0.3123	0.0059	0.9997
5	0.3111	0.0067	0.9994
Mean ± SD	0.3117 ± 0.0004	0.0062 ± 0.0003	0.9994 ± 0.0002
RSD (%)	0.1442	4.75	0.02

Table 2. Intra- and inter-day precision, accuracy and extraction recovery of levobupivacaine spiked in plasma by HPLC (n = 5).

Concentration (mg/L)	Intra-day (mg/L)	RSD (%)	RE (%)	Inter-day(ng/ml)	RSD (%)	RE (%)	Extraction recovery (%)
0.1	0.098 ± 0.003	3.06	-2.00	0.102 ± 0.006	5.88	2.00	85.63
0.5	0.506 ± 0.024	4.74	1.20	0.498 ± 0.017	3.41	-0.40	88.51
2	1.98 ± 0.11	5.55	-1.00	2.01 ± 0.11	5.47	0.50	89.47

to be stable for approximately 24 h since the found concentrations were within 93 to 102% of the initial concentrations. The results obtained after three freeze-thaw cycles demonstrated that 97.7 ± 3.6% of the initial content of levobupivacaine were recovered and that the analytes were stable under these conditions. Plasma samples collected from studies of levobupivacaine were evaluated before and after storage at -18℃ for sta bility and were found to be stable for at least 3 months. The mean absolute recoveries for levobupivacaine were 85.63, 88.51 and 89.47% at the 0.1, 0.5 and 2 mg/L concentration, respectively (n = 5). The mean absolute recovery for internal standard was 80.19% at the 40 mg/L concentration (n = 5).

The present HPLC method of levobupivacaine was for the first time employed to determine the pharmacokinetic parameters of levobupivacaine in Chinese plasma samples. After a single dose of 2 mg/kg levobupivacaine in patients, concentration versus time profiles were constructed for up to 14 h. Figure 2 showed the mean ± SD plasma concentration-time profile of levobupivacaine with normal renal function or renal disease. Pharmacokinetic parameters were estimated using standard non-compartmental methods. The main pharmacokinetic parameters of two groups were showed in Table 3. For the pharmacokinetic analysis of plasma, the mean (SD) values obtained for the levobupivacaine of two groups were as follows: Cmax, 1.18 (0.37) and 1.27 (0.91) mg/L; Tmax, 0.65 (0.17) and 0.76 (0.21) h; t1/2, 4.02 (1.63) and 4.78 (1.56) h; AUC0-14 h, 4.96 (1.5) and 5.77 (1.17) mg·h/L and AUC0-∞, 5.19 (1.46) and 6.09 (1.22) mg·h/L, respectively.

DISCUSSION

In this study, a rapid, simple and sensitive HPLC method for the quantification of levobupivacaine in plasma was developed and validated. The analysis involved a simple liquid-liquid extraction. Plasma was extracted with hexane and the organic extract was then evaporated and the residue was reconstituted in mobile phase.

In the early stage of the method development, a protein precipitation method was used, and two widely used precipitating agents (acetonitrile and methanol) were tested. However, the extraction efficiency was low and many endogenous compounds were extracted simultaneously. In the following, liquid-liquid extraction was adopted. Six organic extraction solvents were evaluated: ethyl ether, acetone, chloroform, ethyl acetate, dichloromethane and hexane. It was found that hexane could give a high recovery for levobupivacaine without any significant interference. Therefore, hexane was chosen as the most suitable extraction solvent.

The validated assay was applied to a pharmacokinetic study of levobupivacaine in plasma in Chinese patients with normal renal function or renal disease. There were no adverse events during the conduct of the study. A lower limit of quantification of 0.01 mg/L was achieved with this method, which is sensitive enough for the determination of levobupivacaine concentration in plasma. Moreover, the sample extraction procedure is quite simple. Only 0.5 ml of plasma was extracted by organic solvent and chromatograms showed no evidence of degradation for either levobupivacaine or the internal standard.

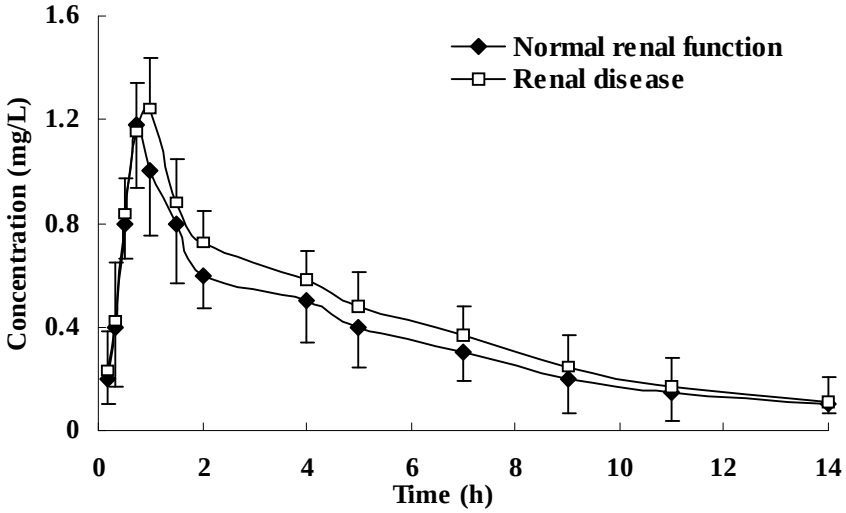

Figure 2. Mean plasma concentration-time profile of levobupivacaine after i.v. infusion (Mean ± SD).

Table 3. Pharmacokinetic parameters of levobupivacaine after i.v. infusion (2 mg/kg, n = 8).

Parameter	Normal renal function	Renal disease
T_{max} (h)	0.65 ± 0.17	0.76 ± 0.21
C_{max} (mg/L)	1.18 ± 0.37	1.27 ± 0.91
$t_{1/2}$ (h)	4.02 ± 1.63	4.78 ± 1.56
$AUC_{0\text{-}14h}$ (mg·h/L)	4.96 ± 1.50	5.77 ± 1.17
$AUC_{0-\infty}$ (mg·h/L)	5.19 ± 1.46	6.09 ± 1.22

There was no difference between two groups. $P > 0.05$; T_{max} (h) = the maximum of concentration; C_{max}(mg/l) = the time of maximum plasma concentration.

The developed method adopts a simple preparation, offers sufficient sensitivity, satisfactory selectivity and good reproducibility. So, it is expected that it can be successfully applied to pharmacokinetic studies. 16 Chinese volunteers received a single dose of levobupivacaine 2 mg/kg, the calculated pharmacokinetic parameter values agreed well with previously reported values (Crews et al., 2002). Crews first described the pharmacokinetic profiles of levobupivacaine in patients after i.v. infusion under normal renal function or renal disease two conditions. Their results regarding C_{max} (the maximum of concentration), T_{max} (the time of maximum plasma concentration) and $T_{1/2}$ of levobupivacaine agree with the present report. But, the difference in AUC is significant. These differences may due to the use of different patient condition and dose of administration. This study demonstrates the clinical efficacy and equivalence of the pharmacokinetic characteristics of 0.5% levobupivacaine in patients with normal renal function or renal disease.

Conclusively, the established HPLC method is very sensitive, precise, selective and useful to monitor low plasma levels of levobupivacaine. So, the method is suitable for quantitative analysis and is required in human pharmacokinetic studies.

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