Available online at www.sciencedirect.com
SCIENCE DIRECT
Journal of Chromatography B, 831 (2006) 91–98
JOURNAL OF CHROMATOGRAPHY B
www.elsevier.com/locate/chromb

Liquid chromatography–electrospray mass spectrometry determination of free and total concentrations of ropivacaine in human plasma

Olivier Mathieu [a], Dominique Hillaire-Buys [a,b], Christophe Dadure [c], Franck Barnay [a], Jean Claude Mathieu-Daudé [a], Françoise Bressolle [d,*]

[a] *Department of Medical Pharmacology and Toxicology, Lapeyronie Hospital, Montpellier, France*
[b] *INSERM U 454, Arnaud de Villeneuve Hospital, Montpellier, France*
[c] *Intensive Care Unit, Lapeyronie Hospital, Montpellier, France*
[d] *Clinical pharmacokinetic laboratory, Faculty of Pharmacy, Montpellier I University, B.P. 14491, 15 Avenue Ch. Flahault, 34093 Montpellier Cedex 5, France*

Received 13 September 2005; accepted 21 November 2005
Available online 19 December 2005

Abstract

A specific and sensitive liquid chromatography-electrospray ionization mass spectrometry (LC–ESI–MS) method was developed for the determination of free and total ropivacaine in human plasma. The work-up procedure involved a simple precipitation of plasma proteins with methanol. Etidocaine served as the internal standard. After microscale equilibrium-dialysis, measurement of free ropivacaine levels was performed after direct injection of the dialysate into the chromatograph. The system used a Zorbax eclipse XD8 C8 analytical column packed with 5 μm diameter particles as the stationary phase. The mobile phase consisted of a 15-min gradient (mobile phase A: 0.05% (v/v) trimethylamine in acetonitrile, mobile phase B: 2 mM ammonium formate buffer (pH 3)). Mass spectrometric data were acquired in single ion monitoring mode at *m*/*z* 275 for ropivacaine and *m*/*z* 277 for etidocaine. The drug/internal standard peak area ratios (plasma) or peak areas (dialysate) were linked via a quadratic relationship to concentrations. Precision ranged from 1 to 7.6% and accuracy was between 92.6 and 109%. The lower limits of quantitation were 1 μg/l in plasma and 2 μg/l in the dialysate. This method was found suitable for the analysis of plasma samples collected during a clinical trial performed in 30 infants undergoing epidural anaesthesia or continuous psoas compartment block.

Keywords: Ropivacaine; Local anaesthetics; Equilibrium-dialysis; Plasma; Quantitation LC–ESI–MS

1. Introduction

Ropivacaine, 1-propyl-2′,6′-pipecoloxylidide, is a long-acting amide-type local anaesthetic agent with an onset time and a duration of action comparable with bupivacaine. It is the *N*-propyl homologue of bupivacaine (Fig. 1). Ropivacaine can be used for peripheral blocks and caudal or epidural anaesthesia and analgesia. Unlike bupivacaine, which is a racemate, ropivacaine is the (*S*)-(−)-enantiomer. However, ropivacaine exhibits less toxicity for the central nervous system and the heart [1] compared to bupivacaine. Duration of action for ropivacaine ranges from 2.5 to 5.9 h for epidural block to 8–13 h for peripheral nerve block [2,3]. Ropivacaine is also 10 times less lipid soluble and cleared via the liver more rapidly than bupivacaine [4]. Some studies have shown less motor blocking effects of ropivacaine than that of bupivacaine. Due to its better safety profile and significantly better sensory-motor differentiation, ropivacaine gives a wider safety margin in the daily clinical practice both for single shot and for continuous infusion, intra-operatively during various surgical procedures and for the postoperative pain control in pediatrics.

Ropivacaine is extensively metabolised in the liver by different cytochrome P-450 isozymes. The major metabolite of ropivacaine is 3-hydroxy-ropivacaine, representing approximately 37% of the total administered dose [5]. Other metabolites include 2-hydroxymethyl-ropivacaine and 4-hydroxy-ropivacaine. After intra-venous administration, an elimination half-life of about 2 h was reported [6]. Ropivacaine is extensively bound to plasma proteins (mainly to α_1-acid glycoprotein) with a free fraction around 6% [7]. Because α_1-acid glycoprotein is

* Corresponding author. Tel.: +33 4 67 54 80 75; fax: +33 4 54 80 75.
E-mail address: Fbressolle@aol.com (F. Bressolle).

1570-0232/$ – see front matter © 2005 Elsevier B.V. All rights reserved.
doi:10.1016/j.jchromb.2005.11.035

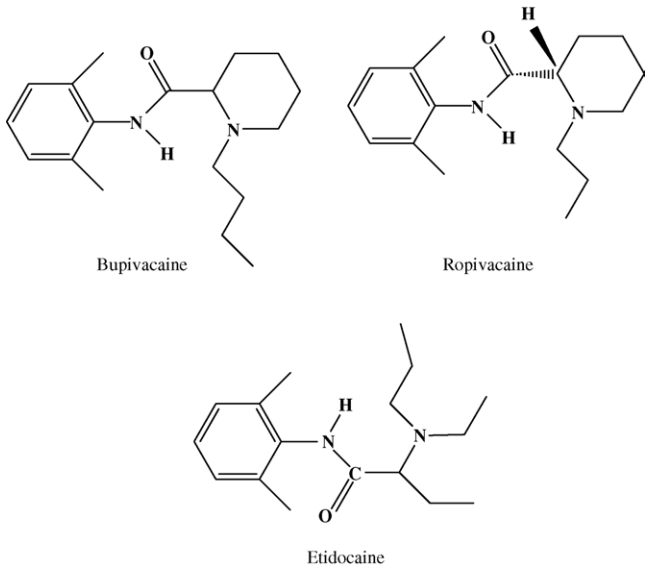

Fig. 1. Chemical structures of bupivacaine, ropivacaine and etidocaine.

a major acute phase protein, its concentration rapidly increases when inflammatory processes develop, particularly during the postoperative period. Since the amount of unbound drug is considered to be related to the pharmacological effect, the knowledge of the unbound fraction may have significant pharmacodynamic implications, especially in a pediatric population [8,9]. Indeed, neonates and infants have a lower α_1-acid glycoprotein concentration in serum as compared with adults, therefore, their free fraction of ropivacaine is increased accordingly [10].

A number of papers on the analysis of ropivacaine in biological matrices (blood, plasma, urine and brain) are available. They include gas chromatography with nitrogen–phosphorus or mass spectrometry (MS) detection [11–13] and liquid chromatography (LC) with ultra-violet [14–19] or MS [19,20] detection. Recently, three methods [19,21,22] by LC–MS–MS have been published; two of them allowed the quantitation of both ropivacaine and some of its metabolites in plasma [22] or urine [19]. These methods involved tedious and time-consuming sample pretreatment by liquid–liquid or solid-phase extraction of the biological samples. An on-line LC–MS–MS method has been also published [22]. These published methods allowed for the quantification of either total ropivacaine [11,13,18,21,23], the total and free ropivacaine [15,20] or only the free drug [12,14,17,22]. High sample volumes are usually required, from 400 μl to 1 ml; however, for both ethical and practical reasons, it is essential to limit the volume of the blood sampling in the pediatric population.

This study was initiated by the need to determine the free and total concentrations of ropivacaine in small plasma sample volumes from pediatric patients. The aim of this paper was to develop reliable, specific and sensitive LC–MS methods for the quantitation of the free and total fractions of ropivacaine in human plasma. The determination of total drug concentration in plasma samples involved a simple precipitation with methanol allowing rapid therapeutic drug monitoring of patients treated with ropivacaine. The determination of the free concentration was performed after equilibrium-dialysis. These methods were validated according to validation procedures, parameters and acceptance criteria based on USP XXIII guidelines and FDA guidance [24–27].

2. Experimental

2.1. Materials and reagents

Reference substances of the local anaesthetics, (*S*)-ropivacaine hydrochloride (Naropein®, ampoules 10 mg/ml, ropivacaine base, M_w 274.4 g/mol) and the internal standard, (*S*)/(*R*)-etidocaine hydrochloride or (+/−)-(*N*-ethyl propylamino)-2 dimethyl-2′-6′butyramilide-(*RS*) chlorhydrate (Duranest®, ampoules 10 mg/ml, etidocaine base, M_w 276.4 g/mol), were purchased from AstraZeneca (Nanterre, France). Ampoules contained drug in normal saline (isotonic concentration) with no other substances. Acetonitrile, methanol, formic acid, ammonium formate, potassium dihydrogen phosphate, dipotassium hydrogen phosphate and trimethylamine were obtained from Merck (Nogent/Marne, France). All chemicals were of analytical grade. The formate buffer solution (pH 3) consisted of ammonium formate (126 mg/l) in purified water. The buffer (pH 7.4, ionic strength 0.2) consisted of dipotassium hydrogen phosphate (0.066 M) and potassium dihydrogen phosphate (0.066 M) in purified water. Purified water was generated by a Milli-Q reagent water system (Millipore corporation, Bedford, MA).

For the validation of the method, blood samples from healthy volunteers (Etablissement Français du sang, Montpellier, France) were collected in heparinized tubes and plasma was obtained by centrifugation at 1000 × *g* for 10 min. Pooled drug-free plasma samples were aliquoted, frozen at −20 °C, and then used during the study for the preparation of calibration standards and quality control (QC) samples. Eleven different batches of drug-free human plasma were used. One batch was used for the preparation of calibration curve standards and quality control samples and ten other batches were used to check the specificity of the method.

Stock solutions of ropivacaine (100 mg/l) and of the internal standard (2.5 mg/l) were prepared in purified water then stored at +4 °C. These solutions were analysed immediately after preparation (reference values) and at selected time intervals after storage over the study period. Thus, stock solutions could be stored at +4 °C for 4 months. For each compound, two separate stock standard solutions were prepared; one which was used for the preparation of calibration curve standards and the second which was used for the preparation of quality control samples. The working solutions of ropivacaine (calibration curve standards: 50, 100, 250 and 500 μg/l; 2.5, 5, 25, 50 and 75 mg/l; QC standards: 50, 100, 400 μg/l, 10 and 62.5 mg/l) were prepared fresh daily by diluting stock solutions with water.

2.2. Equipment and chromatographic conditions

The LC–MS analysis was performed using a Hewlett Packard (Les Ulis, France) Agilent G 1946A quadrupole mass spectrometer (mass range: *m*/*z* 50–2000) equipped with an electrospray

interface and a data acquisition station (HPChem software, version 08.04). The mass spectrometer was coupled to a Hewlett Packard LC system (1100 model) equipped with a quaternary pumping unit and an autosampler fitted with a rheodyne loading valve and set at 20 °C.

Optimization of various experimental parameters including nature of the stationary phase, composition of the eluent, nature of the organic modifier, capillary voltage, nebulizer pressure and sampling cone voltage was carried out (data not shown).

Separation of the analytes was performed on a Zorbax eclipse XD8 C8 column 150 × 4.6 mm, i.d., packed with particles of 5 μm-size (Agilent technologies, Palo Alto, CA). The column temperature was 25 °C. A C_{18} symmetry column (20 × 3.9 mm, i.d., 5 μm-size) obtained from Waters (Paris, France) was used as guard column. A 15-min mobile phase gradient was used. Mobile phase A was 0.05% (v/v) trimethylamine in acetonitrile and mobile phase B consisted of 2 mM ammonium formate buffer (pH 3). The gradient started from 15% of phase A for 5 min and then went up to 35% from 5 to 10 min. It increased directly to 50% in the next 0.1 min and from 10.1 to 15 min was isocratic at 50% of phase A. The column was then washed for 1 min with 80% of phase A, brought back to the initial conditions over 1 min and re-equilibrated for 4 min. The flow rate started at 1 ml/min then decreased to 0.5 ml/min from 1 to 5 min and remained unchanged for 5 min. It increased directly to 1 ml/min in the next 0.1 min and then remained stable. The injection volume was 20 μl.

The mass spectrometer was calibrated in the positive ion mode (ESI^+) using a mixture of NaI and CsI (peak width of the mass: 0.6–0.7 amu). The MS system was operated with a capillary voltage of 4.0 kV and a cone voltage of 80 V. The drying gas temperature and flow were maintained at 350 °C and 10 l/min, respectively and the nebulizer pressure was set at 13 psi. From the full-scan spectra (Fig. 2), ropivacaine was characterized by the protonated molecule $(M + H)^+$ at *m*/*z* 275 and by the fragment ion at *m*/*z* 126 corresponding to the propane-1-piperidinyl group; the internal standard was characterized by the protonated molecule $(M + H)^+$ at *m*/*z* 277 and by the fragment ion at *m*/*z* 128 corresponding to the dipropyl-ethyl amine group. The selected ion monitoring (SIM) mode was used (SIM dwell time: 98 ms) and following ions were monitored *m*/*z* 275 for ropivacaine and *m*/*z* 277 for etidocaine.

2.3. Equilibrium dialysis

Separation of free drug was done by microscale equilibrium-dialysis using an equilibrium dialyser (Dianorm 50 apparatus, Munchen, Germany). The sample and buffer compartments were separated by a regenerated hemicellulose membrane (molecular mass cut-off: 2000 Da) and filled with 200 μl patient or control plasma and 200 μl potassium phosphate buffer pH 7.4, respectively. Dialysis semipermeable membranes were washed successively four-fold in distilled water for 15 min and two-fold in the phosphate buffer for 15 min before each experiment.

The adsorption of ropivacaine to the cellulose membrane of the microdialysis device was firstly studied at concentrations of 40–2000 μg/l in phosphate buffer (pH 7.4). Ropivacaine was

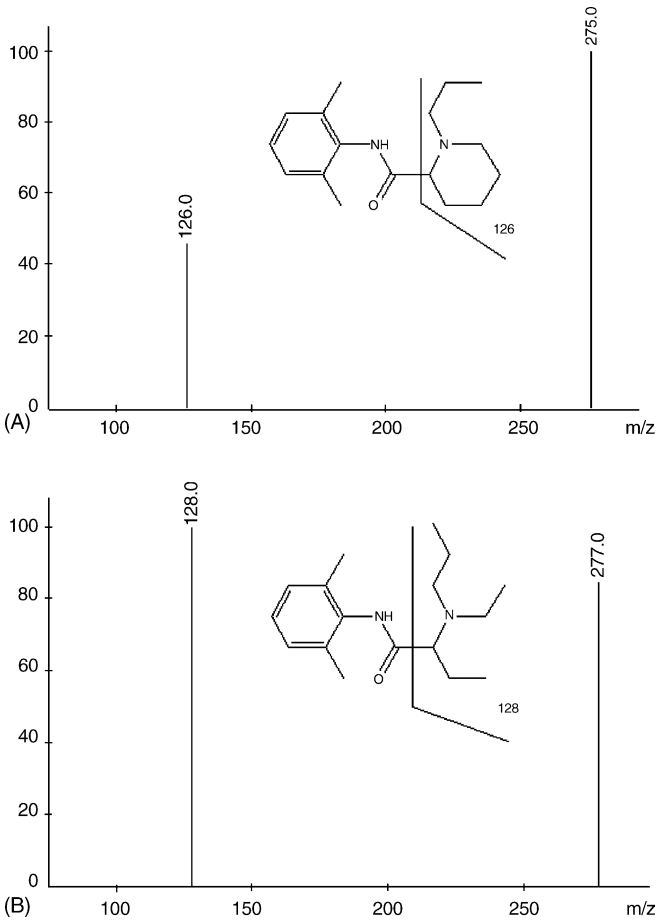

Fig. 2. Mass spectra (scan mode) of: ropivacaine (A) and etidocaine (B).

quantified before and after dialysis. The binding of ropivacaine to the membrane, computed by comparison of the total drug concentration added to the concentrations of ropivacaine on the two sides of the membrane was considered negligible and averaged 1%. The time necessary for achievement of equilibrium of ropivacaine binding to plasma proteins was determined in a preliminary study at 1000 and 2000 μg/l for dialysis times of 5–20 min. Stable equilibrium of ropivacaine binding to plasma proteins was reached after 15 min at 37 °C.

The concentration of ropivacaine on both sides of the dialysis membrane was determined by LC–MS. For the dialysate, a 20-μl volume was directly injected into the LC–MS system.

2.4. Preparation of calibration curves and quality-control samples

Calibrators were prepared by spiking appropriate volumes of working solutions into 0.1 ml of drug-free plasma. The effective concentrations of ropivacaine in plasma (expressed in free base equivalent) were 1, 5, 10, 50, 100, 500, 1000, 2000 and 3000 μg/l.

The calibration standards were prepared in the phosphate buffer (pH 7.4) within the concentration range of 2–2000 μg/l (8 calibration points). The standards were injected directly into the LC–MS system.

QC samples used in the validation were prepared in the same way as the calibration standards, by mixing drug-free plasma samples or phosphate buffer with appropriate volumes of working solutions to achieve four different concentrations (lower limit of quantitation (LLOQ), 8, 200 and 1250 μg/l). They were used to measure intra- and inter-assay precision and accuracy.

2.5. Sample preparation procedure

A 100 μl aliquot of plasma was mixed with 40 μl of internal standard (2.5 mg/l), then a volume of 460 μl of methanol was added. The mixture was vortex-mixed (10 s) then centrifuged (4 °C) at 2000 × *g* for 5 min. The supernatant (450 μl) was collected and evaporated to dryness under a stream of nitrogen at 40 °C. The dried residue was reconstituted in 100 μl mobile phase (15% of phase A, 85% of phase B), then vortex-mixed to homogenise. Prior to analysis by LC–MS, samples were transferred to a 200-μl insert of the LC autosampler vial and 20 μl was injected into the LC column.

2.6. Data analysis

In plasma, the ratio of the peak area of ropivacaine to that of internal standard was used as the assay parameter. The dialysate being directly injected into the LC–MS system without addition of the internal standard, the peak area of ropivacaine was used as the assay parameter. In both cases, the response was linked to the concentrations of ropivacaine according to a quadratic process as $Y = aX^2 + bX + c$. The regression curve was not forced through zero. Calibration curve equations were used to calculate "back-calculated" concentrations for the calibrators. The "back-calculated" values were statistically evaluated. The normal distribution of the residuals (difference between nominal and back-calculated concentrations) was verified. Moreover, the mean residual values (mean predictor error) was computed and compared to zero (Student's *t*-test); the 95% confidence interval was also computed.

2.7. Ion suppression study

The absence of ion suppression was demonstrated by the method of Matuszewski et al. [28]. To investigate potential ion suppression effects attributable to the matrix, seven different batches of drug-free human plasma were treated as described above. the reconstituted extracts (100 μl mobile phase, 15% A:85% B) were then enriched with ropivacaine and the internal standard to final nominal concentrations of 5, 100 and 1000 μg/l (ropivacaine) and 1 mg/l (internal standard). A reference solution comprising 100 μl of mobile phase was also enriched with the two drugs to the same nominal concentrations. The reconstituted extracts and reference solutions were injected into the LC–MS system. Peak areas obtained from the reconstituted extracts were compared with the corresponding peak areas produced by the reference solutions. The mean area ratios (reconstituted extracts/reference solutions) were as follows: ropivacaine 1.00 (R.S.D., 9%) and etidocaine 0.98 (CV: 3.5%). Thus, no ion-suppression was observed.

2.8. Validation procedure

The specificity of the method was investigated by analyzing ten different batches of drug-free human plasma from healthy volunteers to determine whether endogenous constituents coeluted with the different analytes. The possible interference by other drugs, which might be taken concomitantly with the test drug, was also verified. The following drugs were checked: mepivacaine, lidocaine and its main metabolites, bupivacaine, niflumic acid, nalbuphine, tramadol, morphine and paracetamol.

Within- and between-day precision and accuracy of the assay were assessed by performing replicate analyses of QC samples at the above mentioned four concentrations against calibration curves. The procedure was repeated on different days ($n = 6$) on the same spiked standards to determine between-day repeatability. Intra-day repeatability was determined by treating spiked samples in replicate ($n = 6$) the same day. The accuracy was evaluated as [mean found concentration/theoretical concentration] × 100. Precision was given by the percent relative standard deviation (R.S.D).

The extraction recovery of ropivacaine from plasma was measured three times at all concentrations of calibration standards, based on the comparison of the areas under the peaks of the extracted samples with those of the authentic (unextracted) standards in the relevant concentration range prepared in the mobile phase. The extraction recovery was also calculated for the internal standard. In all cases, the means and standard deviations (S.D.) were calculated.

The lower limit of quantitation (LLOQ) was defined as the lowest drug concentration which can be determined with a R.S.D. ≤20% and an accuracy of 100 ± 20% on a day-to-day basis [24–27]. Accuracy and precision at the LLOQ were estimated using QC samples. Each QC sample was analyzed versus a calibration curve to measure intra- and inter-assay precision and accuracy.

2.9. Stability assays

The stability of ropivacaine in plasma and at various steps of the analysis was explored to assess the substance integrity throughout the procedure, starting from sampling to processing in the laboratory. For stability studies carried out in plasma, QC samples representing the low, middle and high concentrations (8, 200 and 1250 μg/l) were used. QC samples were analyzed immediately after preparation (reference values) and after storage. Each determination was performed in triplicate. Concentrations of each analyte were determined against a calibration curve. The stability assays were carried out as follows:

(i) The stability of ropivacaine was assessed at 12 and 48 h after bench-top storage at both ordinary laboratory temperature (20 °C) and in a refrigerator at 4 °C.

(ii) The protocol was repeated after 2, 5, 15 and 30 days storage in a freezer at −20 °C. Prior to their analyses, samples were brought to room temperature and vortex-mixed well.
(iii) Run-time stability at room temperature (20 °C) for 48 h of processed samples after extraction was determined for each calibration point.

Compounds were considered as stable when losses were <15%.

The stability of the free concentration of ropivacaine in a plasma sample from a 3-year-old patient entering the clinical study was assessed after 8 days of storage at both 20 and −20 °C.

2.10. Clinical study

With ethics approval and parental consent, 30 infants (aged 1–12 years) undergoing epidural (EP) anaesthesia or continuous psoas compartment block (CPCB) were recruited to the study (15 patients in each arm). Ropivacaine was administered by bolus (1.875 mg/kg) followed by a 48-h continuous infusion (0.4 and 0.2 mg/kg/h for EP and CPCB, respectively). Blood samples (350 μl) were collected in heparinized tubes and were obtained 0.5, 6, 24 and 48 h (end of infusion) after the start of the loading dose. Samples were immediately centrifuged at 4 °C, then the plasma was aliquoted into two fractions. The first fraction was frozen at −20 °C and was used to quantify the total

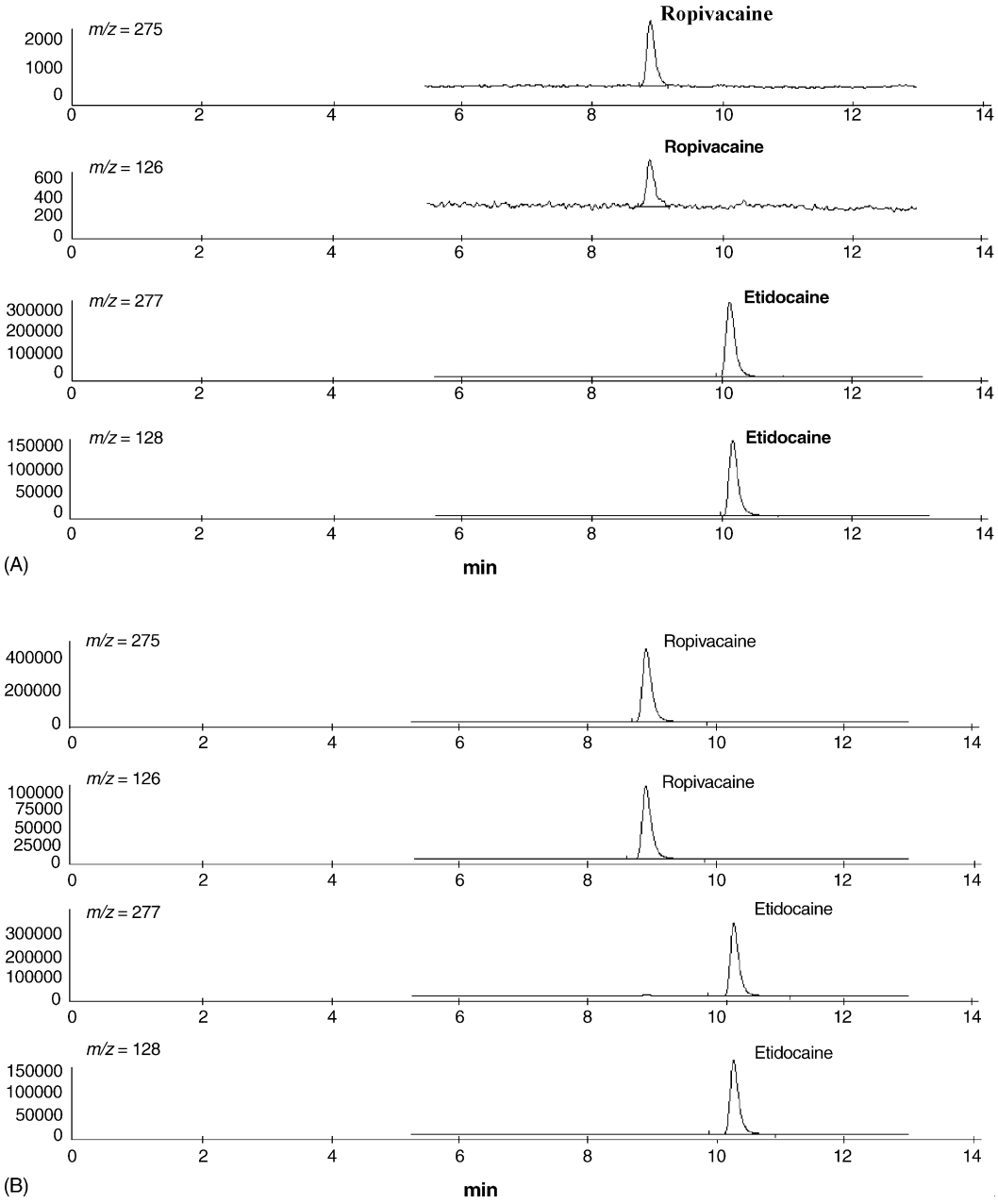

Fig. 3. Typical chromatograms (single-ion monitoring mode) of: drug-free human plasma enriched with ropivacaine at 8 μg/l (A) and of plasma from an authentic infant treated with ropivacaine (concentration, 2.7 mg/l) (B). For LC–MS conditions see Section 2.2. Concentrations are expressed in free base equivalent.

ropivacaine. The second fraction was used for the separation of the free drug that was immediately done after sample collection by equilibrium-dialysis.

2.11. Statistical analysis

A comparison of ropivacaine concentrations in the two arms of the clinical study was performed by the box-and-whisker plots (BWP) procedure. The BWP procedure combines statistical techniques and graphical displays to study symmetry, check distributional assumptions and detect outliers. Box-plots divide the data into three classes according to their quartiles. A central line inside the box indicates the median value. The box encloses the lower and upper quartiles (middle 50% of data values). The whisker extends from the ends of the box to the lower and upper limits of the data, respectively. Extreme data lying outside the whiskers are identified as outliers and allow the identification of patients with unusual values. Analyses were performing using the computer program Sigma-Plot.

3. Results

3.1. Retention times and specificity

Representative chromatograms are shown in Fig. 3. Under the chromatographic conditions described above, peaks were adequately separated ($R_s = 3.4$). During the 3 months of validation, observed retention times (t_r) were 8.9 min (R.S.D. = 1.1%) for ropivacaine and 10.1 min (R.S.D. = 1.0%) for etidocaine. The mean retention factors (k') were 5.4 and 6.1, respectively. The separation factor (α) was 1.13. Under these chromatographic conditions, ropivacaine and etidocaine were adequately separated from other local anaesthetic agents, bupivacaine ($t_r = 10.7$ min), mepivacaine ($t_r = 6.4$ min), and lidocaine ($t_r = 6.4$ min) and its two metabolites (monoethylglycine xylidide (MEGX, $t_r = 6.1$ min) and glycine xylidide (GX, $t_r = 5.1$ min)).

As shown in Fig. 4, no peaks due to the matrix interfered at the retention time of the analytes. No interference was found with the following metabolites of ropivacaine: 3-hydroxy ropivacaine, 2-hydroxy-methyl-ropivacaine and 4-hydroxy ropivacaine, and with all drugs tested that could be co-administered.

3.2. Drug/detector response relationship

The quadratic regression indicated a mean coefficient of determination of 0.998. Mean parameters of the quadratic equation are given in Table 1. For each point on the calibration curves, the concentrations were back-calculated from the corresponding quadratic equation parameters and mean ± S.D. values were computed. Results are presented in Table 2. A linear regression of the back-calculated concentrations versus the nominal ones provided a unit slope and an intercept equal to 0. The distribution of the residuals showed random variations, the number of positive and negative values being approximately equal. Moreover, they were normally distributed and centred around zero. The mean values of residuals were not statistically different from zero and the 95% confidence intervals included the zero value.

3.3. Precision, accuracy, extraction efficiency and LLOQ

The accuracy and precision results are shown in Table 3. For ropivacaine, the mean extraction recovery from plasma was 90% (R.S.D. = 7%, $n = 24$); it was not statistically different over the range of concentrations studied. We also determined the extraction recovery of the internal standard, which was 80% (R.S.D. = 6%, $n = 18$).

Using 100 μl of plasma, the LLOQ was established as 1 μg/l (3.6 nM). At this concentration, inter-day accuracy and precision

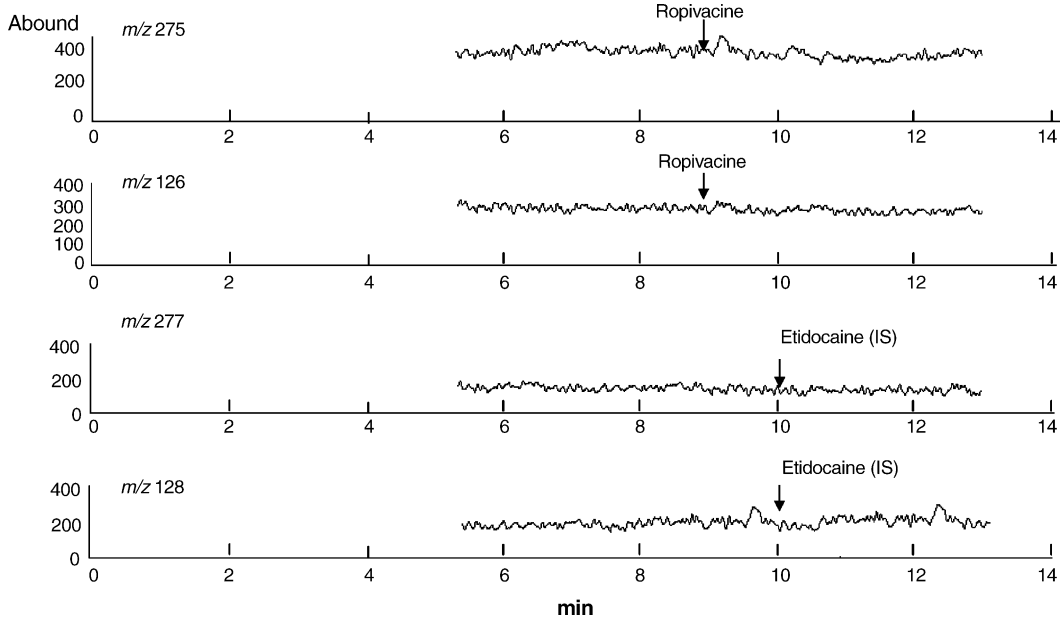

Fig. 4. Mass chromatogram obtained from 0.1 ml of blank human plasma.

Table 1
Results of calibration curves ($n = 6$)[a]

	Range (μg/l)	*a* (mean)	*b* (mean)	*c* (mean)	r^2
Plasma, inter-assay reproducibility ($n = 6$)	1–3000	−0.074 (R.S.D. = 14.0%)	0.692 (R.S.D. = 6.0%)	0.0006	0.998 (R.S.D. = 0.10%)
Plasma, intra-assay reproducibility ($n = 6$)	1–3000	−0.08 (R.S.D. = 12.0%)	0.723 (R.S.D. = 2.0%)	0.0006	0.998 (R.S.D. = 0.07%)
Buffer, inter-assay reproducibility ($n = 6$)	2–2000	-45×10^4 (R.S.D. = 11.0%)	331×10^4 (R.S.D. = 3.0%)	0.0006	0.999 (R.S.D. = 0.06%)

n: number of replicates.
[a] $Y = aX^2 + bX + c$.

Table 2
Back-calculated concentrations from calibration curves

Theoretical concentration[a] (μg/l)	Inter-assay reproducibility in plasma		Intra-assay reproducibility in plasma		Inter-assay reproducibility in phosphate buffer	
	R.S.D. (%)	Recovery (%)	R.S.D. (%)	Recovery (%)	R.S.D. (%)	Recovery (%)
1	1.18	100	5.6	96.8	–	–
2	–	–	–	–	2.6	98.3
5	10.3	99.0	8.9	102	4.9	102
10	5.0	103	4.0	102	1.3	99.9
50	4.4	102	3.1	104	1.2	104
100	1.2	99.5	4.0	102	0.0	100
500	5.4	95.4	3.6	96.4	1.7	96.7
1000	6.8	93.8	3.0	92.5	2.9	91.5
2000	3.7	94.5	4.8	92.5	3.2	95.5
3000	5.2	97.0	6.2	94.7	–	–

[a] Expressed in free base equivalent.

values were 101 and 1.5%, respectively. In the dialysate, the LLOQ was 2 μg/l (7.3 nM).

3.4. Stability

Frozen QC plasma samples tested over a 1 month period showed no sign of either degradation or losses. For all concentrations no significant difference appeared between times 0, 2, 5, 15 and 30 days ($p > 0.05$).

Plasma samples spiked with ropivacaine allowed to stand at room temperature or at 4 °C for 48 h showed no sign of decrease in the nominal starting concentration.

Ropivacaine and etidocaine were stable during the evaporation process. Run-time stability at 20 °C of reconstituted extracts originating from plasma (i.e., in the mobile phase) was determined for each concentration of calibration standard in replicate ($n = 5$). After 48 h, no significant losses occurred.

In plasma, after 8 days of storage at both 20 and −20 °C, an increase of the free concentration of ropivacaine was observed, from 3.4 to 8.5%. Thus, there was a displacement of the equilibrium during storage. Consequently, the determination of the unbound fraction must be performed extemporaneously.

3.5. Clinical trial

For both arms of the study, results are presented in Fig. 5. After EP administration a constant increase in drug concentration occurred during the 48-h infusion period. The absorption rate constant from the site of administration could be lower than the elimination rate constant (0.35 h^{-1}) [6]; indicating that absorption is the rate-limiting step in elimination of ropivacaine after EP administration [6]. After a CPCB, plasma concentrations of ropivacaine decreased from 1.5 to 6 h then increased until 48 h (end of infusion) (Fig. 5). A biphasic absorption pro-

Table 3
Accuracy and precision of the method

Theoretical concentration[a] (μg/l)	Inter-assay reproducibility in plasma		Intra-assay reproducibility in plasma		Inter-assay reproducibility in phosphate buffer		Intra-assay reproducibility in phosphate buffer	
	Precision (%)	Accuracy (%)	Precision (%)	Accuracy (%)	Precision (%)	Accuracy (%)	Precision (%)	Accuracy (%)
1	1.5	101	1.6	99.0	–	–	–	–
2	–	–	–	–	2.4	100	2.7	98.0
8	7.4	102	6.0	108	4.3	98.6	4.3	106
200	7.6	105	4.7	109	2.8	103	1.0	108
1250	7.6	95.6	4.3	101	2.6	92.6	5.0	106

[a] Expressed in free base equivalent.

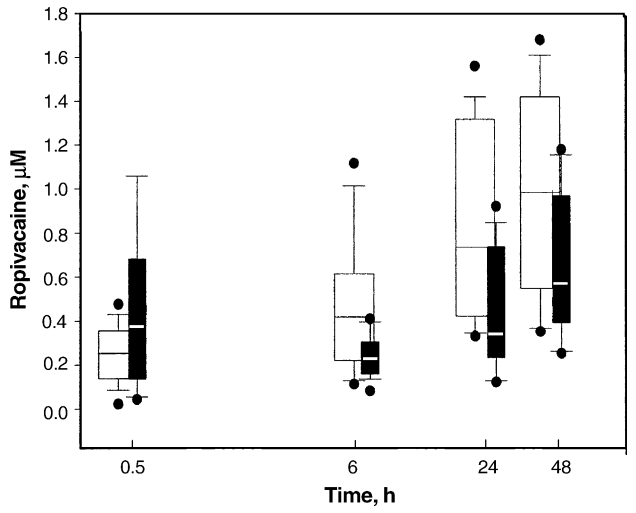

Fig. 5. Box plots presenting the variations of total ropivacaine concentrations (bound and unbound) after epidural (EP) anaesthesia (□) and continuous psoas compartment block (CPCB) (■) (15 patients in each arm). Ropivacaine was administered by bolus (1.875 mg/kg) followed by a 48-h continuous infusion (0.4 and 0.2 mg/kg/h for EP and CPCB, respectively). The inner boxes cover 50% of all values with marks indicating medians (—). Circles outside the whiskers defined by the adjacent values are plotted as outliers.

cess from the site of administration could be considered with a rapid initial phase followed by a slower phase with an half-life higher than the true elimination half-life of ropivacaine. The extent of unbound fraction averaged 3.4%.

4. Discussion and conclusion

A specific and sensitive LC–MS method for the determination of total and free ropivacaine concentration in human plasma was described. Chromatographic conditions have been optimized to further quantify either bupivacaine, ropivacaine, mepivacaine, lidocaine and its two metabolites (monoethylglycine xylidide and glycine xylidide), and etidocaine under the same conditions. Indeed, in clinical practice, drugs with rapid onset are widely used in combination with long-acting local anesthetics. Distinct advantages of the present method include the simplicity and rapidity for sample preparation and chromatography. Indeed, the sample pretreatment procedure to quantify the total drug in plasma required a simple precipitation of proteins with methanol. This method has an enhanced precision due to the use of an internal standard. After equilibrium-dialysis (15 min), measurement of free ropivacaine levels was performed after direct injection of the dialysate into the LC–MS system. An increase of the free fraction was observed during storage of plasma samples at both 20 and −20 °C. So, the determination of the unbound fraction must be immediately performed after sample collection. However, these results were not in accordance with those found by Arvidsson and Eklund [14]. Assay performance of the present method was assessed both on the basis of the statistical characteristics of individual calibration curves and from the results of QC samples. From a 100-μl sample volume of plasma and a 20-μl sample volume of dialysate, the lower limits of quantitation reported in the present study (3.6 nM in plasma and 7.3 nM in the dialysate) were lower than that reported in most of the published methods but higher than that published by Altun et al. [11]. These authors used a on-line microextraction packed syringe-LC–MS–MS method (LLOQ, 2 nM) from a 1-ml sample volume of plasma. This described LC–MS method was found suitable for the analysis of plasma samples collected during a clinical trial performed in 30 infants undergoing epidural anaesthesia or continuous psoas compartment block.

References

[1] D.B. Scott, A. Lee, D. Fagan, G.M. Bowler, P. Bloomfield, R. Lundh, Anesth. Analg. 69 (1989) 563.
[2] T.G. Costello, J.R. Cormack, C. Hoy, A. Wyss, V. Braniff, K. Martin, M. Murphy, J. Neurosurg. Anesthesiol. 16 (2004) 147.
[3] I. Kaloul, J. Guay, C. Cote, A. Halwagi, F. Varin, Can. J. Anaesth. 51 (2004) 52.
[4] G. Lagan, H.A. McLure, Curr. Anaeasth. Crit. Care 15 (2004) 247.
[5] B.M. Emanuelsson, C. Norsten-Höögen, R. Sandberg, J. Sjövall, Eur. J. Pharm. Sci. 5 (1997) 171.
[6] M.J. Jokinen, Best Pract. Res. Clin. Anaeasthesiol. 19 (2005) 269.
[7] A. Lee, D. Fagan, M. Lamont, G.T. Tucker, M. Halltin, D.B. Scott, Anesth. Analg. 69 (1989) 736.
[8] J.X. Mazoit, B.J. Dalens, Clin. Pharmacokinet. 43 (2004) 17.
[9] C. Ecoffey, Minerva Anestesiol. 71 (2005) 357.
[10] J.X. Mazoit, D.D. Denson, K. Samii, Anesthesiology 68 (1988) 387.
[11] Z. Altun, M. Abdel-Rehim, L.G. Blomberg, J. Chromatogr. B 813 (2004) 129.
[12] M. Abdel-Rehim, K.A. Svensson, Y. Askemark, K.J. Petersson, J. Chromatogr. B 755 (2001) 253.
[13] M. Engman, P. Neidenström, C. Norsten-Höög, S.J. Wiklund, U. Bondesson, T. Arvidsson, J. Chromatogr. B 709 (1998) 57.
[14] T. Arvidsson, E. Eklund, J. Chromatogr. B 668 (1995) 81.
[15] S. Reif, P. Le Corre, G. Dollo, F. Chevanne, R. Le Verge, J. Chromatogr. B 719 (1998) 239.
[16] J.A. Jönsson, M. Andersson, C. Melander, J. Norberg, E. Thodarson, L. Mathiasson, J. Chromatogr. A 870 (2000) 151.
[17] Y.C. Kau, K.M. Wang, M.H. Shyr, Y.H. Lee, T.H. Tsai, J. Chromatogr. B 760 (2001) 107.
[18] M. Zuo, G.L. Duan, Z.G. Ge, Biomed. Chromatogr. 18 (2004) 752.
[19] M. Abdel-Rehim, M. Bielenstein, Y. Askemark, Anal. Chim. Acta 482 (2003) 253.
[20] M. Stumpe, N.S. Morton, D.G. Watson, J. Chromatogr. B 748 (2000) 321.
[21] A. Koehler, R. Oertel, W. Kirch, J. Chromatogr. A 1088 (2005) 126.
[22] S.K. Bergström, K.E. Markides, J. Chromatogr. B 775 (2002) 79.
[23] M. Baniceru, O. Croitoru, S.M. Popescu, J. Pharm. Biomed. Anal. 35 (2004) 593.
[24] US Food and Drug Administration. Guidance for industry. Bioanalytical method validation. May 2001. http://www.fda.gov/cder/guidance/4252fnl.htm (accessed May 2004).
[25] United States Pharmacopoeia XXXIII, The United States Pharmacopeia Convention, Rockville, MD, 2003. p. 2439.
[26] V.P. Shah, K.K. Midha, S. Dighe, I.J. McGilveray, J.P. Skelly, A. Yacobi, T. Layloff, C.T. Viswanathan, C.E. Cook, R.D. McDowall, K.A. Pittman, S. Spector, J. Pharm. Sci. 81 (1992) 309.
[27] F. Bressolle, M. Bromet-Petit, M. Audran, J. Chromatogr. B 686 (1996) 3.
[28] B.K. Matuszewski, M.L. Constanzer, C.M. Chavez-Eng, Anal. Chem. 70 (1998) 882.