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Sensitive bioassay of bupivacaine in human plasma by liquid-chromatography-ion trap mass spectrometry

Guillaume Hoizey [a,*], Denis Lamiable [a], Arnaud Robinet [a], Hugues Ludot [b], Jean-Marc Malinovsky [b], Matthieu L. Kaltenbach [c], Laurent Binet [a], Christian Boulanger [a], Hervé Millart [a]

[a] *Laboratoire de Pharmacologie et Toxicologie, Hôpital Maison Blanche, CHU de Reims, 45, rue Cognacq-Jay, 51092 Reims Cedex, France*
[b] *Département d'Anesthésie-Réanimation, CHU de Reims, Reims, France*
[c] *Laboratoire de Pharmacologie et de Pharmacocinétique, UFR de Pharmacie, Reims, France*

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Abstract

A sensitive high-performance liquid chromatography–tandem mass spectrometric (HPLC–MS–MS) method, using an ion trap spectrometer, was developed for quantitation of bupivacaine in human plasma. Bupivacaine and an internal standard (ropivacaine) were extracted in a single step from 100 μL of alkalinized plasma with diethyl-ether. The mobile phase consisted of acetonitrile with 0.1% formic acid (50:50, v/v), and was delivered at a flow rate of 0.3 mL/min. The effluent was detected by MS–MS in positive ion mode. Ionisation was performed, using an electrospray ion source, operating at 200 °C. The selected reaction monitoring transitions m/z 289 → m/z 140 and m/z 275 → m/z 126 were chosen for bupivacaine and ropivacaine, respectively. Calibration curves were linear over the concentration range of 3.90–500 μg/L with determination coefficients >0.996. The method is accurate (bias <10%) and reproducible (intra-assay and inter-assay precision <15%), with a quantitation limit of 3.90 μg/L, using only 100 μL of plasma. The high specificity and sensitivity, achieved by this fast method (total run-time <3 min), allowed the determination of bupivacaine plasma levels in pediatric patients, following epidural administration of bupivacaine.
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1. Introduction

Bupivacaine is a long-acting aniline local anesthetic agent frequently used via epidural routes for major pain control and regional anesthesia [1]. Similar to other amino-amide local anesthetics, it acts by blocking the conduction of impulses in target nerve structures, primarily located within the subarachnoid space. Upon epidural injection, bupivacaine enters the general circulation where it may induce systemic toxicity, mainly on the central nervous and cardiovascular systems [2]. It may be thus necessary to monitor blood levels over time following epidural administration, especially during long-term continuous epidural infusion in children [1,3].

A number of methods using HPLC with UV [4–6] or mass spectrometry (MS) detection [7,8], capillary electrophoresis [9], and gas chromatography (GC) with [10,11] or without MS [12], have been developed to measure bupivacaine levels in biological samples. These methods differ mainly by their extraction procedure, limits of quantitation, volume of sample or total run-time.

In this work, we propose an alternative procedure for the determination of bupivacaine levels in biological fluids. A particular attention has been paid to optimize the method for both speed of the analytical run and sample volume, as required for pharmacokinetic studies in young children from whom only a limited volume of plasma can be obtained. To this end, a fast, sensitive and highly selective bioassay of bupivacaine by liquid chromatography-ion trap mass spectrometry (LC–MS–MS) was set up and validated in order

* Corresponding author. Tel.: +33 3 2678 7530; fax: +33 3 2678 8456.
E-mail address: ghoizey@chu-reims.fr (G. Hoizey).

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to monitor plasma concentrations after continuous epidural infusion in pediatric patients.

Although bupivacaine is used clinically as a racemic mixture of *R*- and *S*-enantiomers, our assay does not resolve the enantiomers of that drug. Enantioselective activity and pharmacokinetics have been described for bupivacaine [13–15]. Yet, determination of the sum of the enantiomers concentrations after epidural administration of the racemate can still provide information of interest [4].

2. Experimental

2.1. Reagents

Bupivacaine and ropivacaine (internal standard) hydrochlorides were purchased from SIGMA (Saint-Quentin Fallavier, France). Organic solvents and reagents were all of analytical grade. Acetonitrile and diethyl ether were supplied by SDS (Peypin, France). Methanol and formic acid were obtained from Merck (Darmstadt, Germany). Purified water was prepared on a Milli-Q Waters purification system (Millipore, Saint-Quentin en Yvelines, France).

2.2. Biosamples

Blank human plasma samples were supplied from our local blood bank. Clinical blood samples were collected from pediatric patients undergoing continuous epidural infusion of bupivacaine for postoperative pain relief, following surgery.

2.3. Standard solutions and calibration curves

Stock standard solutions of bupivacaine and its internal standard (IS) were prepared in methanol at a concentration of 1 mg/mL, and stored at +4 °C. These were further diluted in methanol to give appropriate working solutions used to prepare the calibration solutions. Standard curves were prepared in human plasma (100 μL) to yield final concentrations of 3.90, 7.81, 15.63, 31.25, 62.5, 125, 250 and 500 μg/L.

2.4. Sample preparation

Plasma sample (100 μL) was extracted with 3.0 mL of diethyl-ether after addition of 10 μL IS solution (0.5 mg/L ropivacaine in methanol) and 20 μL of carbonate buffer (pH 9.0). The mixture was vortex-mixed for 1 min, and then centrifuged at 3000 × *g* for 5 min. The organic layer was transferred into conical glass tubes and evaporated to dryness under a nitrogen stream at 40 °C. The residue was finally dissolved in 200 μL of 0.1% formic acid:acetonitrile (50:50, v/v), and 10 μL were injected into the LC column.

2.5. Liquid chromatography-mass spectrometry

2.5.1. Equipment and chromatographic conditions

The LC–MS–MS system consisted of a Thermofinnigan Surveyor® LC system (Les Ulis, France) equipped with an autosampler. Compounds were screened for, identified, and quantified in plasma, using a Thermofinnigan LCQ Advantage® trap ion mass spectrometer, and the Thermofinnigan Xcalibur® data system. Chromatographic separations were carried out by using a 5 μm particle size Hypurity C18 column (150 × 2.1 mm i.d., ThermoHypersil-Keystone, Les Ulis, France) whose temperature was maintained at 30 °C. Samples were eluted with a mobile phase consisting of acetonitrile:0.1% formic acid in purified water (50:50, v/v) delivered at a flow-rate of 0.3 mL/min. The entire flow was directed into the source without splitting. During use, the mobile phase was degassed by an integrated Surveyor® series degasser. In order to optimize the MS–MS parameters and to create a spectra library, infusion experiments were carried out with a 500 μL syringe connected to a pump with a flow-rate of 5 μL/min.

2.5.2. Mass spectrometry conditions

The ionization technique used was electrospray ionization (ESI) in the positive-ion mode for both compounds. The spray needle was set at a potential of 4 kV. The heated capillary was set at 200 °C, and the stainless-steel capillary held at a potential of 10 V. Nitrogen was used as drying and nebulising gas. The sheath gas flow-rate of nitrogen was set at 40 (arbitrary units). The tube lens offset was set at 40 V and the electron multiplier voltage set at 400 V peak-to-peak. Helium was used in the trap as damping and collision gas. The instrument was set to acquire 3 microscans, and ion injection time into the trap was optimized by using the integrated automatic gain-control software.

2.5.3. MS conditions for identification

The detection of bupivacaine and ropivacaine was performed by LC–MS–MS in full MS–MS scan mode (*m*/*z* 100–400). Full scan MS–MS spectra were produced by collision-induced dissociation (CID) of each molecular ion, using a normalized collision energy of 50%. Two alternating scan events, generating fragment ions of the molecular ion through CID, were carried out at *m*/*z* 289 and *m*/*z* 275, corresponding to the protonated molecular ions ($[M+H]^+$) of bupivacaine and ropivacaine (IS), respectively.

The reference MS–MS spectra of compounds of interest were previously collected individually using direct injection via the integrated syringe pump. Those spectra were obtained by using a normalized collision energy of 50%, and were included in a custom full MS–MS library. Positive peaks were identified by searching and comparing the underlying ESI mass spectra with the reference spectra of our MS–MS library.

2.5.4. MS conditions for quantitation

For quantitation, the selected reaction monitoring (SRM) precursor-product ion transitions *m*/*z* 289 → *m*/*z* 140 and *m*/*z* 275 → *m*/*z* 126 were monitored for bupivacaine and ropivacaine, respectively.

Table 1
Intra-day ($n = 10$) and inter-day ($n = 12$) precision, and accuracy of the LC–MS–MS (SRM mode) assay for bupivacaine

Analyte	Spiked LOW MEDIUM HIGH (μg/L)	Mean measured (μg/L)		R.S.D.[a] (%)		Bias[b] (%)	
		Intra-day	Inter-day	Intra-day	Inter-day	Intra-day	Inter-day
	3.90	4.00	4.27	11.5	14.7	2.6	9.4
Bupivacaine	62.5	68.4	63.9	13.8	11.8	9.4	2.4
	250	228.9	259.2	5.2	10.3	−8.4	3.7

[a] Relative standard deviation.
[b] Expressed as [100 − (mean calculated concentration/spiked concentration) × 100].

2.5.5. Calculations

The calibration curves were calculated by unweighted least-squares linear regression analysis of the concentrations of the analyte versus the peak areas ratio of the target ion of bupivacaine (m/z 140) to that of the IS (m/z 126). Concentrations of unknown samples were determined by applying the linear regression equation of the standard curve to the unknown sample's peak area ratio.

2.6. Method validation

2.6.1. Quality control

Quality controls were prepared from a pool of blank human plasma spiked with three different amounts of bupivacaine corresponding to the LOW, MEDIUM and HIGH concentrations given in Table 1. Plasma aliquots were stored at −20 °C until assayed.

2.6.2. Precision and accuracy

Precision and accuracy of the assay were assessed by replicate analysis of quality control samples of bupivacaine. Ten and twelve separate samples were assayed for intra- and inter-day evaluations, respectively. Precision is reported as relative standard deviation (% R.S.D.) of the estimated concentrations and accuracy (bias) expressed as [100 − (mean calculated concentration/spiked concentration) × 100].

2.6.3. Limits of detection and quantitation

The limit of quantitation of the bupivacaine assay was defined as the lowest concentration of the calibration curve. The limit of detection was determined from the detector response after direct injection of decreasing amounts of bupivacaine. A signal-to-noise ratio of 10 was taken as the limit of detection.

2.6.4. Recoveries

Absolute recoveries from human plasma were evaluated at LOW and HIGH concentrations levels ($n = 5$). The samples were extracted without IS, according to the procedure described above. 10 μL of IS solution (0.5 mg/L ropivacaine in mobile phase) were added to the organic phase, and evaporated to dryness. The residue was dissolved in 200 μL of mobile phase prior to analysis. As controls ($n = 5$), bupivacaine solutions in mobile phase at the LOW and HIGH levels to which were added 10 μL of IS solution were gently evaporated. The residues were then dissolved in 200 μL of mobile phase and analyzed. Recoveries were calculated by comparing peak areas of controls to those of spiked plasma samples.

2.6.5. Patients' samples

This technique has been applied to measure bupivacaine concentrations in plasma samples collected from pediatric patients who received continuous epidural bupivacaine for pain control, following surgery. Briefly, a loading dose of 1.25 mg/kg of bupivacaine was injected epidurally, followed 60 min later by an infusion of 0.25 mg/kg/h, delivered via a volumetric infusion pump. The infusion was maintained at the same rate for 71 h during the post-operative period. Blood samples were collected before dosing (T0), and at 5, 10, 15, 20, 30, 45, 60, 120, 180, and 240 min, and then at 24, 48 and 72 h after the start of infusion. After centrifugation, plasma samples were frozen and stored at −20 °C, until analysis.

3. Results and discussion

3.1. LC–MS–MS analysis

Under our analytical conditions, bupivacaine is not fully chromatographically separated from the IS, with retention times of 1.75 and 1.80 min for ropivacaine and bupivacaine, respectively. However, due to the high selectivity of tandem MS, complete chromatographic separation is not necessary any more. By using an isocratic elution of compounds, total run-time was shorter than 3 min per sample. For application to pharmacokinetic studies, the single-step extraction procedure combined to a short chromatographic run-time could be considered as a major advantage.

Fig. 1 (top) shows the full MS spectra of bupivacaine and ropivacaine. These show the abundant molecular cations ($[M + H]^+$) which were used as precursor ions for MS–MS analysis. We preferred the ESI source over the atmospheric pressure chemical ionization (APCI) source, since the latter resulted in bad ionisation of the compounds of interest under our experimental conditions. Similarly, positive-ion mode was chosen in order to obtain the most intense signal of the molecular ion. Corresponding full MS–MS spectra (bottom part of Fig. 1) allowed fast identification of the compounds via our home-made full MS–MS reference library.

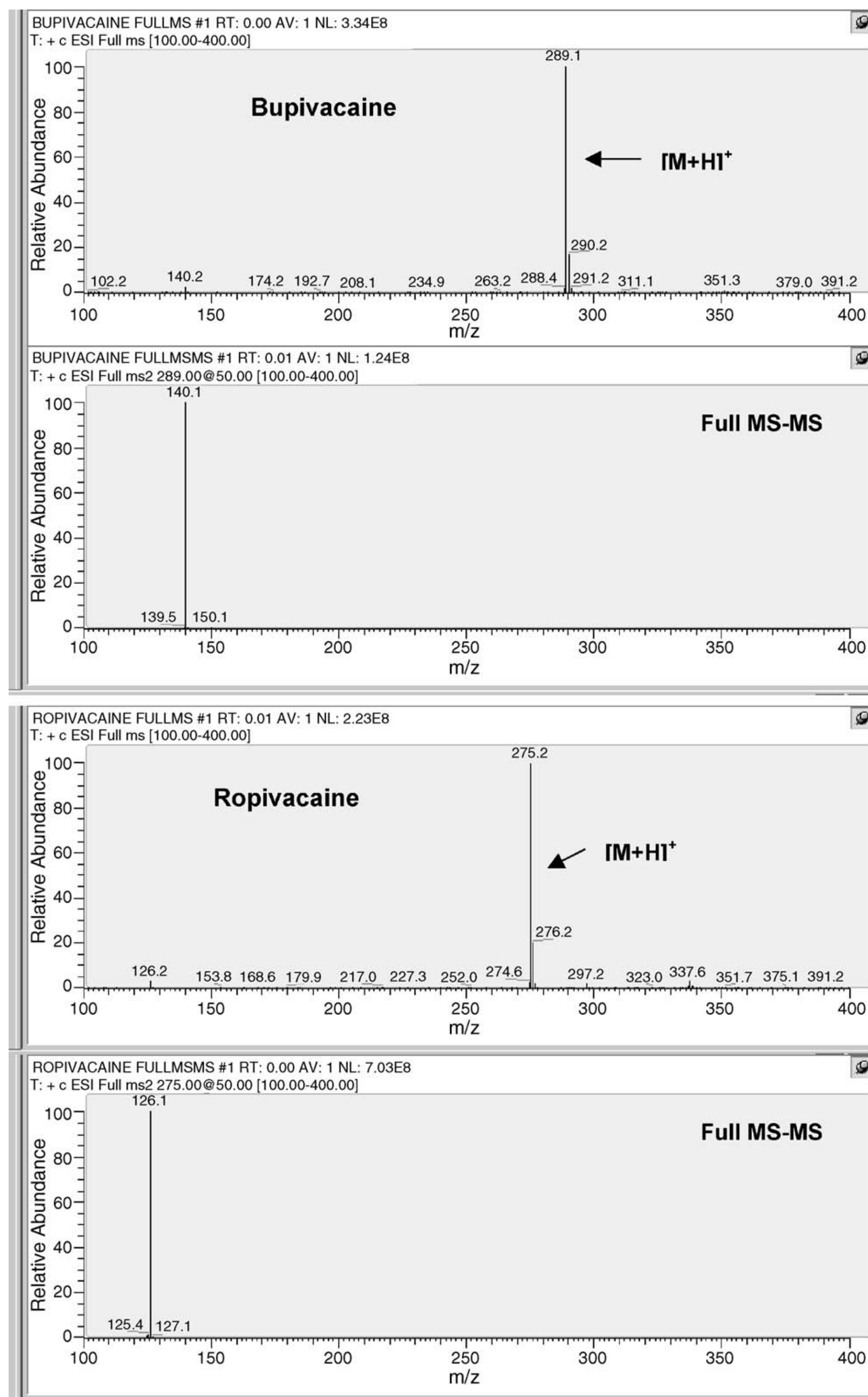

Fig. 1. ESI–MS (top) and ESI–MS–MS (bottom) spectra of bupivacaine and ropivacaine (IS). *x*-axis: *m*/*z* values, *y*-axis: relative abundance.

Quantitation of bupivacaine was performed in the SRM mode using the molecular ions of both bupivacaine and its IS as target ions. The most favourable transitions were selected for each drug, and product ions (i.e. *m*/*z* 140 and *m*/*z* 126 for bupivacaine and ropivacaine, respectively) were chosen by taking into account the fragment ions with the most intense signal. This approach resulted in a high sensitivity and specificity. Additionally, the high sensitivity of the

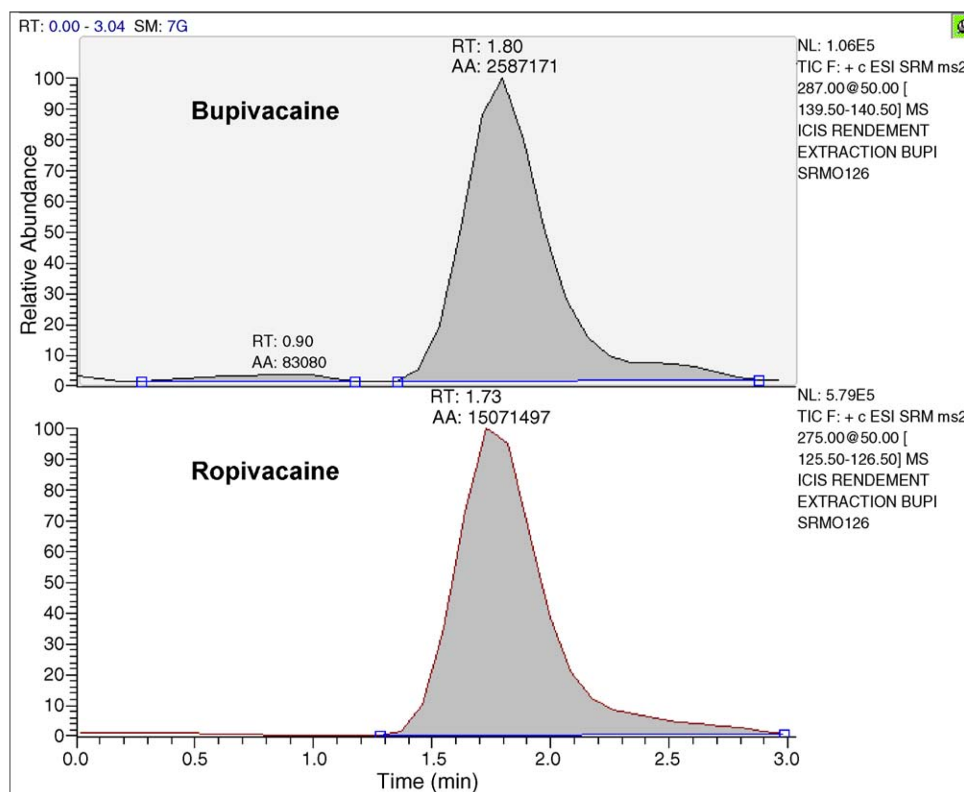

Fig. 2. LC–ESI–MS–MS–SRM smoothed chromatograms of a blank plasma sample, spiked with bupivacaine (31.25 μg/L) and ropivacaine (IS).

method allowed us to use a small sample volume (ca. 100 μL plasma), which undoubtedly constitutes another important benefit for repeated measurements of drug concentrations in paediatric patients. Fig. 2 shows a representative SRM-smoothed chromatogram from blank plasma spiked with bupivacaine (15 μg/L) and IS.

3.2. Validation data

3.2.1. Precision and accuracy

Table 1 summarizes mean values, precision, and accuracy of intra- and inter-assay analyses. Precision and accuracy were within the ranges acceptable for bio-analytical purposes. Intra-day precision ranged from 5.2 to 11.5 %, and accuracy (bias) was less than 10%. Inter-day precision did not exceed 15% over the three quality control samples investigated. The accuracy of the technique was considered satisfactory, since between-day bias over the concentration range studied was found to be less than 10%.

3.2.2. Linearity and limit of quantitation

Calibration curves for bupivacaine in human plasma exhibited good linearity over the concentration range studied (i.e. 3.90–500 μg/L). Using unweighted linear regression analysis, they were best described by the equation $y = 0.984x - 7.156$ ($r^2 = 0.998$), where y is the peak-area ratio of bupivacaine to IS, and x is the bupivacaine concentration. Values of the coefficients of determination were all >0.996.

The limit of quantitation (LOQ) was chosen as the lowest calibration standard concentration (3.90 μg/L). As shown below, this concentration is largely sufficient for the purpose of pharmacokinetic studies of bupivacaine in pediatric patients, and is well above the limit of detection of bupivacaine (0.05 μg/L). Considering the small volume of plasma used for analysis (100 μL), we should stress that the LOQ

Table 2
LOQ, linearity and absolute recovery of bupicavaine in human plasma

Analyte	LOQ (μg/L)	Linearity (μg/L)	Coefficients of determination (R^2)	Recovery	
				Concentration (μg/L)	(%)
Bupivacaine	3.90	3.90–500	0.998	31.25	86.8
				125	93.6

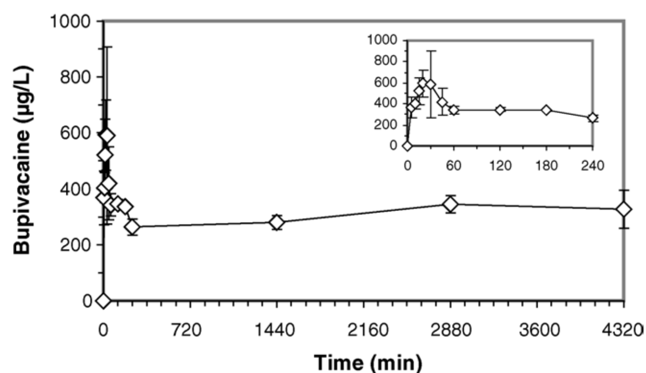

Fig. 3. Plasma concentration–time profile of bupivacaine (mean ± S.D.) after epidural administration of a loading dose of 1.25 mg/kg, followed 60 min later by an infusion of 0.25 mg/kg/h over 71 h in pediatric patients ($n = 3$).

could be considerably improved by using larger sample volume, if necessary.

3.2.3. Recoveries

Bupivacaine recoveries from human plasma at two levels of concentration are summarized in Table 2. These results indicate that the single-step extraction procedure used in this assay is sufficient to ensure satisfactory extraction recovery (i.e. >85%).

3.2.4. Patients' samples

The mean concentration–time profile for bupivacaine in plasma obtained from three pediatric patients participating in an ongoing pharmacokinetic study is shown in Fig. 3. Detailed pharmacokinetic data for all subjects ($n = 15$) enrolled in the clinical study will be reported in a separate article.

In conclusion, we developed an LC–MS–MS method to quantify bupivacaine in human plasma for application to pharmacokinetic studies in children. In addition to its high specificity, this assay demonstrates good precision and accuracy, has a short analysis run-time, and only requires a 100 µL sample. It has been successfully applied to the analysis of plasma samples from paediatric patients following epidural administration of bupivacaine. Finally, with additional validation, our method could be employed in the determination of ropivacaine levels using bupivacaine as an IS.

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