Short communication

High-performance liquid chromatographic determination of ropivacaine, 3-hydroxy-ropivacaine, 4-hydroxy-ropivacaine and 2′,6′-pipecoloxylidide in plasma

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Abstract

A sensitive HPLC method has been developed for the determination of ropivacaine, 3-hydroxy-ropivacaine, 4-hydroxy-ropivacaine and 2′,6′-pipecoloxylidide in plasma. The procedure involved extraction from plasma with a mixture of *n*-heptane-ethyl acetate and a back-extraction into an acidified aqueous solution. The chromatography was achieved using a LiChrospher RPB C_8 column with a mobile phase consisting of a mixture of acetonitrile and pH 2.1, 0.01 M potassium dihydrogenphosphate, the latter phase containing 0.005 M 1-heptanesulfonic acid for ropivacaine metabolites analysis. The extraction yields of ropivacaine, 3-hydroxy-ropivacaine, 4-hydroxy-ropivacaine and 2′,6′-pipecoloxylidide were 94.7%, 79.4%, 79.4% and 77.7%, respectively. The limits of detection of ropivacaine, 3-hydroxy-ropivacaine, 4-hydroxy-ropivacaine and 2′,6′-pipecoloxylidide in plasma samples were 0.9 ng/ml, 3 ng/ml, 5 ng/ml and 1 ng/ml, respectively. © 1998 Elsevier Science B.V. All rights reserved.

Keywords: Plasma; Ropivacaine; Hydroxyro-pivacaine; 2′6′-Pipecoloxylidide

1. Introduction

Ropivacaine, a new amide-type local anaesthetic, is the *N*-propyl homologue of rac-bupivacaine used as the (*S*)-(−)-enantiomer. Because of similar pharmacodynamic properties, ropivacaine and bupivacaine are both used in epidural anaesthesia and post-operative pain relief [1]. They have a similar efficacy in these indications but ropivacaine has a lower cardiac toxicity [2]. Ropivacaine is extensively bound to plasma proteins with a free fraction around 6 % [3].

To investigate the pharmacokinetic properties and plasma protein binding of ropivacaine, a sensitive analytical method is needed. High-performance liquid chromatographic methods for the determination of ropivacaine in plasma with detection limits between 10 to 200 ng/ml have been reported [4–6] as well as two gas chromatographic methods with detection limits of 10 ng/ml [7] and 11 ng/ml [8].

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Arvidsson and Eklund described a method using a coupled-column system of a reversed-phase and an ion-exchange column for the determination of ropivacaine in plasma ultrafiltrate with a limit of determination around 3 ng/ml [9]. A method offering the advantage of a direct injection of the plasma sample into the chromatographic system has been described by Yu and Westerlund [5], but the limit of detection is about 100 ng/ml.

Ropivacaine is extensively metabolised in the liver by different cytochrome P450 isozymes. Based on urinary analysis in humans, 3-hydroxy-ropivacaine, 4-hydroxy-ropivacaine, 2′,6′-pipecoloxylidide, 3-hydroxy-2′,6′-pipecoloxylidide and 2-hydroxymethyl-ropivacaine have been identified as metabolites, the former being the main metabolite in humans [9–11] as in several animal species [12]. Biotransformation to 3-hydroxy-ropivacaine was catalysed by CYP4501A [11, 12] while the formation of 4-hydroxy-ropivacaine, 2′,6′-pipecoloxylidide and 2-hydroxymethylropivacaine was catalysed by CYP4503A [11]. Arvidsson et al. described a chiral HPLC method for enantioselective analysis of ropivacaine, 3-hydroxy-ropivacaine and 2′,6′-pipecoloxylidide in urine samples in order to examine the racemisation of S-(−)-ropivacaine [12]. Oda et al. described a HPLC method for metabolite analysis in hepatic microsomes [10]. Halldin et al. described a HPLC method for metabolite analysis in plasma and urine but the plasma concentrations of the metabolites were below or just above the limit of quantitation [11].

The current paper describes a HPLC method allowing a sensitive assay of ropivacaine and three of its metabolites i.e., 3-hydroxy-ropivacaine, 4-hydroxy-ropivacaine and 2′,6′-pipecoloxylidide in plasma. Different extraction procedures and chromatographic conditions were needed for the analysis of ropivacaine and its metabolites as a result of differences in physicochemical properties.

2. Materials and methods

2.1. Materials

(S)-Ropivacaine-HCl,   (S)/(R)-mepivacaine-HCl and (S)/(R)-etidocaine-HCl were supplied by Astra (Nanterre, France) and 3-hydroxy-ropivacaine, 4-hy-
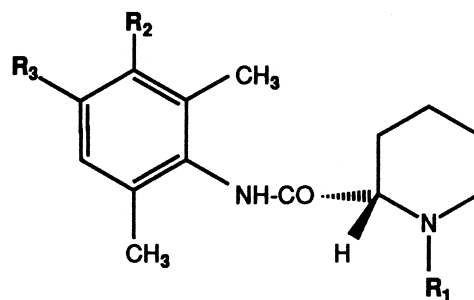
R_1	R_2	R_3	Product
C_3H_7	H	H	Ropivacaine
C_3H_7	OH	H	3-hydroxy-ropivacaine
C_3H_7	H	OH	4-hydroxy-ropivacaine
H	H	H	2′,6′-pipecoloxylidide

Fig. 1. Chemical structures of ropivacaine and metabolites i.e., 3-hydroxy-ropivacaine, 4-hydroxy-ropivacaine and 2′,6′-pipecoloxylidide.

droxy-ropivacaine and 2′,6′-pipecoloxylidide-HCl were provided by Astra Pain Control (Södertälje, Sweden). All chemicals were of analytical grade and were purchased from Merck (Darmstadt, Germany). The chemical structures of ropivacaine, 3-hydroxy-ropivacaine, 4-hydroxy-ropivacaine and 2′,6′-pipecoloxylidide are presented in Fig. 1.

2.2. Spectrophotometric method

The absorption maximum of the drugs were evaluated between 200 and 350 nm on a Lambda 3B (Perkin Elmer, Norwalk, CO, USA) in order to determine the optimal detection wavelength. Compound solutions were prepared in the mobile phase at a concentration of 10 μg/ml.

2.3. Chromatographic conditions

The HPLC system consisted of a Waters Model 6000 A pump (Waters, Millford, MA, USA) equipped with a Waters 717 autosampler, a Milton Roy variable wavelength detector, Model Spectro-Monitor 3100 (LDC Milton Roy, Riviera Beach, FL,

USA) set at 205 nm and a Delsi integrator, Model Enica 21, (Delsi, Suresnes, France).

The determination of ropivacaine was performed with a Lichrospher RP-SelectB-C8 column (125×4 mm I.D., 5-μm particle size) (Interchim, Montluçon, France) maintained at 30°C. The mobile phase was a mixture of pH 2.1, 0.01 *M* potassium dihydrogenphosphate and acetonitrile 80:20 (v/v) at a flow-rate of 1 ml/min. For the separation of the metabolites of ropivacaine, the mobile phase used was a 85:15 (v/v) mixture of pH 2.1, 0.01 *M* potassium dihydrogenphosphate containing 0.005 *M* 1-heptane sulfonic acid and acetonitrile.

2.4. General procedure for sample preparation

For the analysis of ropivacaine, etidocaine was used as internal standard. The spiked plasma sample (0.5 ml) was alkalinized by adding 50 μl of 1 *M* NaOH. Three millilitres of a mixture of *n*-heptane and ethyl acetate were added. After agitation (3 min) and centrifugation (5 min at 3500 rpm), the organic phase was transferred to a conical vial and 50 microlitres of 0.1 *M* H_2SO_4 were added. After agitation (3 min) and centrifugation (5 min at 3500 rpm), the organic phase was discarded. The aqueous acid phase was buffered with 0.2 *M* sodium acetate and 40 μl were injected into the chromatographic system.

For the analysis of ropivacaine metabolites, mepivacaine was used as internal standard. The extraction procedure of the metabolites was slightly different to that described above. The second extraction step was achieved by using 500 μl of 0.5 *M* formic acid. After agitation (3 min) and centrifugation (5 min at 3500 rpm), the organic phase was discarded and the acid aqueous phase was evaporated to dryness under a gentle stream of nitrogen. The residue was dissolved in 50 μl of the mobile phase and 40 μl were injected into the chromatographic system.

2.5. Validation of the method

Stock solutions of ropivacaine-HCl, etidocaine-HCl, mepivacaine-HCl and 2′,6′-pipecoloxylidide-HCl were prepared in distilled water. Stock solutions of 3-hydroxy-ropivacaine and 4-hydroxy-ropivacaine were prepared in 1 *M* HCl aqueous solution. The concentration of stock solutions was 1 mg/ml and solutions were kept at +4°C. The linearity of the method of determination of ropivacaine was investigated in the range from 1 to 2000 ng/ml and checked by a least-squares linear regression fitting. The limit of detection in plasma samples were studied in the range from 0.2 to 200 ng/ml. The extraction yield was calculated by comparing the peak surface area of the drugs at a concentration of 100 ng/ml plasma obtained from plasma extracts with those obtained with drug solutions (1 μg/ml) in 0.1 *M* H_2SO_4 buffered with 0.2 *M* sodium acetate. The within-day and between-day reproducibilities were checked at a concentration of 100 ng/ml plasma (*n*=10). For the determination of the between-day reproducibility, spiked plasma samples were stocked at −20°C until analysis.

The linearity of the method for the determination of the metabolites 3-hydroxy-ropivacaine, 4-hydroxy-ropivacaine and 2′,6′-pipecoloxylidide was studied in the range from 0.5 to 200 ng/ml.

2.6. Protein binding

Determination of the free fraction in human plasma was investigated by ultrafiltration using the Ultrafree CL model UFC 4 LTK device (Millipore, Bedford, MA, USA). Protein binding was studied in a human plasma sample spiked with ropivacaine in the concentration range from 50 to 3000 ng/ml. Spiked plasma aliquots were incubated at 37°C for 30 min, 2 ml were poured into the ultrafiltration device and then centrifuged at 2500 *g* for 50 min leading to a 500-μl ultrafiltrate.

2.7. Pharmacokinetic application

Two milligrams of ropivacaine-HCl were injected intravenously in a rabbit. Blood samples were collected until 60 min post dosing. Blood samples were centrifuged immediately after collection and plasma was stored frozen at −20°C until analysis.

3. Results and discussion

Since the spectrum of absorption of the compounds did not show any characteristic absorption

maxima, the detection wavelength was set at 205 nm since absorption increased continuously with the decrease in wavelength.

The extraction yield of ropivacaine was about 90% and not significantly influenced by the composition of the extraction solvent mixture. The extraction yield of the internal standard etidocaine was more influenced by variation of the extraction solvent compared to ropivacaine and was generally lower than that of ropivacaine. The maximum extraction of ropivacaine was reached with a 90:10 (v/v) mixture of n-heptane–ethyl acetate. The mean ($\pm$ S.D., $n=$ 10) extraction yield of ropivacaine and etidocaine were 94.7$\pm$5.7% and 84.2$\pm$17.2%, respectively.

Extraction with pure ethyl acetate produced the highest extraction yield for 3-hydroxy-ropivacaine and 4-hydroxy-ropivacaine while the extraction yield of 2′,6′-pipecoloxylidide and mepivacaine increased as the percentage of n-heptane increased. The extraction procedure described for ropivacaine led to very low extraction yields for the three metabolites. As a result of a rather low back-extraction of the metabolites in the aqueous acid phase, an increase in the extraction yield was obtained by using a high volume of acid phase (0.5 ml). A formic acid aqueous solution was chosen because of the possibility to evaporate formic acid to dryness. The extraction solvent leading the highest extraction yields (about 80%) for all the metabolites and the internal standard was a 25:75 (v/v) mixture of n-heptane–ethyl acetate (Fig. 2). The extraction yield of 3-hydroxy-ropivacaine, 4-hydroxy-ropivacaine, 2′,6′-pipecoloxylidide and mepivacaine was 79.4%, 79.4%, 77.7% and 81.2%, respectively.

We used different reversed-phase (C_8 and C_{18}) chromatographic columns in order obtain a simultaneous separation of all compounds in a single run. However, it was not possible to separate the different compounds in a suitable duration of analysis. By performing ion-pair reversed-phase liquid chromatography, the chromatographic patterns of the ropivacaine metabolites became closer, but the ropivacaine elution was too late ($k'=25$) to be detected under the same conditions. The use of either 1-heptane sulfonic acid or 1-octane sulfonic acid did not solve this problem. The capacity factors of the compounds separated by a mobile phase containing 15 % of acetonitrile and 0.005 M 1-heptane sulfonic
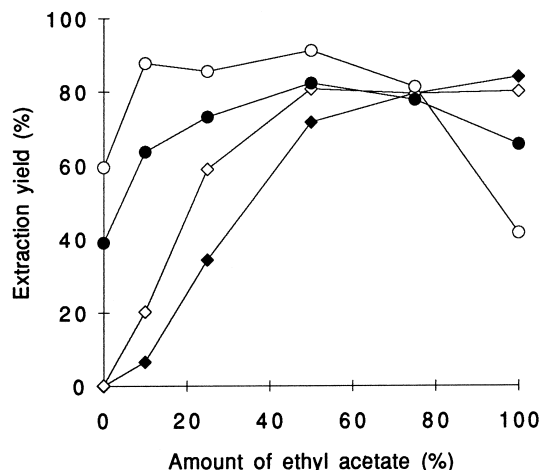

Fig. 2. Extraction yield (%) of 3-hydroxy-ropivacaine ($\diamond$), 4-hydroxy-ropivacaine ($\blacklozenge$), 2′,6′-pipecoloxylidide ($\bullet$) and mepivacaine ($\bigcirc$) from plasma with a mixture of n-heptane and ethyl acetate.

acid were quite similar to those resulting from a separation by a mobile phase composed of 20 % acetonitrile and 0.005 M 1-octane sulfonic acid. Because of the better separation characteristics, the first mobile phase was chosen for further study. Hence, due to the difference in physicochemical properties between ropivacaine and its metabolites, it was not possible to determine ropivacaine and its metabolites by using the same chromatographic conditions. The chromatographic separation of the different compounds is illustrated by the chromatograms in Fig. 3 and the chromatographic separation parameters are presented in Table 1.

Calibration curves of ropivacaine and etidocaine were linear between 1 and 2000 ng/ml ($r^2>0.999$). The linearity of the calibration curves of the metabolites was checked over a narrower concentration range because no data on in vivo plasma concentrations were available. Calibration curves were linear ($r^2>0.999$) between 5 and 200 ng/ml for 4-hydroxy-ropivacaine, 3-hydroxy-ropivacaine and 2′,6′-pipecoloxylidide.

The limit of detection (signal-to-noise ratio of 3:1) of ropivacaine, 4-hydroxy-ropivacaine, 3-hydroxy-
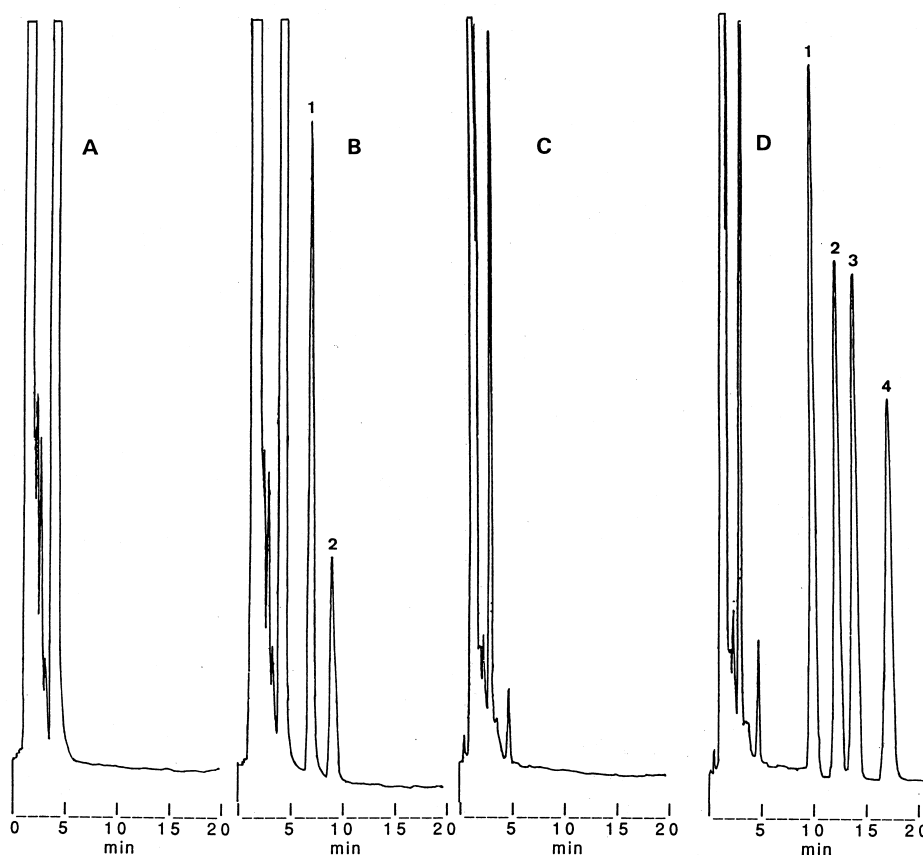
Fig. 3. Chromatograms of plasma samples spiked with ropivacaine, 3-hydroxy-ropivacaine, 4-hydroxy-ropivacaine and 2′,6′-pipecoloxylidide. See Section 2.3 for analytical conditions. (A) Blank plasma sample for ropivacaine analysis. (B) Spiked plasma sample for ropivacaine analysis, 1=ropivacaine (100 ng/ml), 2=etidocaine (internal standard). (C) Blank plasma sample for metabolite analysis. (D) Spiked plasma sample for metabolite analysis, 1=4-hydroxy-ropivacaine (200 ng/ml), 2=3-hydroxy-ropivacaine (200 ng/ml), 3=2′,6′-pipecoloxylidide (200 ng/ml) and 4=mepivacaine (internal standard). Detector setting 0.005 AU full scale.

ropivacaine and 2′,6′-pipecoloxylidide in plasma were 0.9 ng/ml, 5 ng/ml, 3 ng/ml, and 1 ng/ml, respectively.

The within-day and between-day reproducibilities for ropivacaine at a plasma concentration of 100 ng/ml were 4.1% (n=10) and 1.9 % (n=5), respectively.

The protein binding of ropivacaine in human plasma was found to be non-linear, with a free fraction ranging from 2 % to 9 % with the increase in drug concentration from 50 to 2000 ng/ml.

In our preliminary pharmacokinetic investigation

in rabbits, 2′,6′-pipecoloxylidide and 3-hydroxy-ropivacaine were measured in plasma as well as an unknown potential metabolite, but 4-hydroxy-ropivacaine was not detected. Given that aromatic hydroxylation in position 4 is a minor pathway, the lack of measurement of the 4-hydroxy-ropivacaine was not unexpected. The concentration–time curve of ropivacaine, 2′,6′-pipecoloxylidide and 3-hydroxy-ropivacaine in plasma is illustrated in Fig. 4.

The present method allows a sensitive determination of ropivacaine and it would be of interest to investigate its pharmacokinetics and non-linear pro-

Table 1
Chromatographic separation parameters of (i) ropivacaine and etidocaine using a mobile phase composed of a 20:80 (v/v) mixture of acetonitrile and pH 2.1, 0.01 M potassium dihydrogenphosphate and of (ii) 3-hydroxy-ropivacaine, 4-hydroxy-ropivacaine, 2′,6′-pipecoloxylidide and mepivacaine using a mobile phase composed of a 15:85 (v/v) mixture of acetonitrile and pH 2.1, 0.01 M potassium dihydrogenphosphate containing 0.005 M 1-heptane sulfonic acid

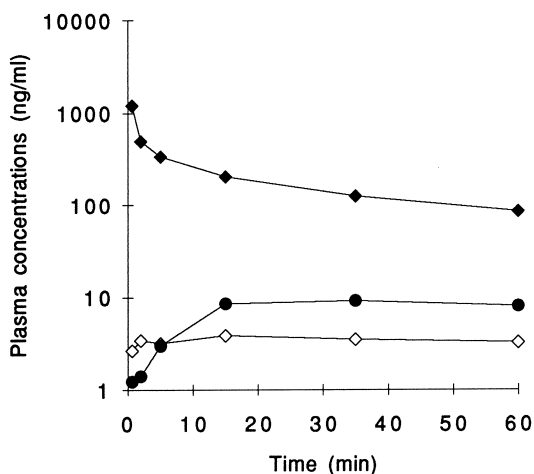|	k'	α	R_s
Ropivacaine	11.3		
		1.4	3.3
Etidocaine	15.8		
4-Hydroxy-ropivacaine	9.4		
		1.2	2.9
3-Hydroxy-ropivacaine	11.5		
		1.1	1.8
2′,6′-Pipecoloxylidide	12.9		
		1.2	3.1
Mepivacaine	15.9		

Fig. 4. Plasma concentration–time profiles of ropivacaine (♦), 3-hydroxy-ropivacaine (◇) and 2′,6′-pipecoloxylidide (●) in a rabbit following i.v. administration of ropivacaine hydrochloride (2 mg).

tein binding. Furthermore, it could be applied to the plasma metabolic investigation of ropivacaine.

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