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Lack of Metabolic Racemisation of Ropivacaine, Determined by Liquid Chromatography Using a Chiral AGP Column

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ABSTRACT Ropivacaine hydrochloride monohydrate (ropivacaine) is a new local anaesthetic agent which is administered exclusively as the (−)-(S)-form. The aim of the study was to determine whether metabolic racemisation of (−)-(S)-ropivacaine occurs. This was tested in man, rat, dog, and sheep after different routes of administration. The enantiomers of ropivacaine and two of the major metabolites, 3-hydroxy-ropivacaine and 2′,6′-pipecoloxylidide (PPX), were determined in urine samples by liquid chromatography on a Chiral AGP column after liquid–liquid extraction. It was possible to detect <1% of the (+)-(R)-enantiomer of both ropivacaine and the two major metabolites. In the samples examined, no trace of metabolic racemisation was observed. In pharmacokinetic, pharmacodynamic, toxicological, and metabolic studies, therefore, nonchiral assays are considered to be adequate.

KEY WORDS: local anaesthetics, enantiomers, stereoselective, liquid chromatography, Chiral AGP column, bioanalysis, urine samples

(−)-(S)-Ropivacaine hydrochloride monohydrate (ropivacaine) is a new local anaesthetic compound of the amide type, with structural similarities to two local anaesthetic agents on the market, mepivacaine and bupivacaine. Unlike these agents, which are racemic mixtures, ropivacaine is marketed exclusively as the (−)-(S)-enantiomer. Studies using the different isomers of these local anaesthetics have shown that the (S)-form is less toxic and has a longer duration of blockade, especially in infiltration anaesthesia.[1–3] Due to the differences in toxicity between the enantiomers, it is of importance to establish whether ropivacaine undergoes metabolic inversion to the (+)-(R)-form in vivo. It has previously been shown that ropivacaine can racemise under extreme conditions, e.g., at pH 5.5 and 100°C. However, no racemisation occurs below pH 3.[4] Ropivacaine is extensively metabolised in the liver by the cytochrome P-450 enzymes and the principal metabolite produced is 3-hydroxy-ropivacaine in various animal species, including man.[5] This metabolite is conjugated and excreted in both urine and bile. Oxidative dealkylation yields 2′,6′-pipecoloxylidide (PPX), which is also an important metabolite (Fig. 1).

The aim of this study is to describe analytical methodology for the separation and determination of the different enantiomers of ropivacaine and its two metabolites, PPX and 3-hydroxy-ropivacaine, and to establish whether chiral inversion occurs in vivo after the administration of ropivacaine by analysing urine samples from rat, dog, sheep, and man.

The enantiomers of other local anaesthetic agents have been determined in biological samples. The enantiomers of prilocaine in plasma were determined by liquid chromatography on a Chiracel OD column after liquid–liquid extraction.[6] The determination of (+)-(R)- and (−)-(S)-bupivacaine is described in several papers incorporating liquid chromatography, using columns with immobilised α_1-acid glycoprotein, e.g., the Chiral AGP column.[7–11] The enantiomers of bupivacaine were also determined using capillary zone electrophoresis with modified cyclodextrins.[12] The sample preparation included liquid–liquid extraction,[7,9,11,12] solid-phase extraction,[8] and coupled-column liquid chromatography.[7,10]

In the present paper the enantiomers of ropivacaine, PPX and 3-hydroxy-ropivacaine, were determined on a Chiral AGP column after liquid–liquid extraction of the urine samples. The method described is suitable to test for metabolic racemisation but not for quantitative determination. In our laboratory the methods for the determination of ropivacaine in biological samples includes nonchiral separation systems using gas and liquid chromatography.[5,13,14] This paper is presented to justify the nonchiral methods.

MATERIALS AND METHODS

Animal and Human Studies

Rat. Four male animals were given either a single intravenous or subcutaneous dose of 10 μmol/kg ropivacaine. The 24 h urine samples were collected and analysed.

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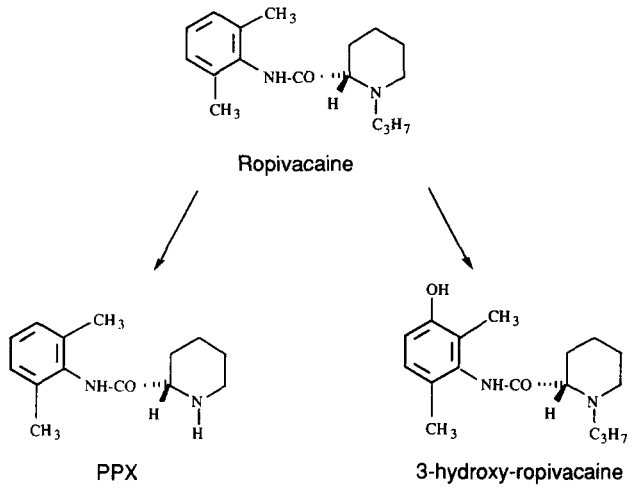

Fig. 1. Chemical structures of ropivacaine and metabolites.

Dog. Four animals (3 males and 1 female) were given either a single intravenous or subcutaneous dose of 10 μmol/kg ropivacaine or a rectal dose of 20 μmol/kg ropivacaine in a gel formulation. The 24 h urine samples were collected and analysed.

Sheep. One nonpregnant and one pregnant ewe were given an intravenous infusion of about 6 μmol/kg ropivacaine over 15 min. The 30 min urine samples were collected and analysed.

Humans. Out of 22 male volunteers one group of six was given an intravenous infusion of 150 μmol ropivacaine over 15 min and another group of sixteen 300 μmol ropivacaine rectally in a gel formulation. The 0–2 h urine was collected in the iv study and the 2–4, 4–6, and 6–8 h urine samples were collected after the rectal dose. Metabolic racemisation of ropivacaine was tested in urine samples from all the species described above, whereas metabolic racemisation of the metabolites, 3-hydroxy-ropivacaine and PPX, was tested only in urine samples from humans.

Chemicals

All chemicals were of analytical grade or better and were used without further purification. Acetonitrile of LiChrosolve grade was obtained from E. Merck (Darmstadt, Germany). The standards of ropivacaine, 2′,6′-pipecoloxylidide hydrochloride (PPX · HCl) and 3′-hydroxy-1-propyl-2′,6′-pipecoloxylidide (3-hydroxy-ropivacaine), each as the separate (+)-(R)- and (−)-(S)-enantiomers as well as the racemic mixture, were synthesised at the Department of Medicinal Chemistry at Astra Pain Control AB (Södertälje, Sweden) (Fig. 1).

Instrumentation

The liquid chromatographic equipment was an LC-9A solvent pump (Shimadzu Co, Kyoto, Japan) connected to a CMA/200 autoinjector (CMA/Microdialysis AB, Stockholm, Sweden). The column was thermostated using a column oven BAS LC-22C temperature controller (Bioanalytical Systems Inc, West Lafayette, IN). The detector was a Spectra 100 UV detector (Spectra Physics Inc, San José, CA). The chromatographic data were collected and processed with the PE Nelson Access *Chrom (Perkin-Elmer Nelson System Inc. Cupertino, CA).

Chromatographic System

Two chromatographic systems were used, system I for the determination of the enantiomers of ropivacaine and system II for the determination of the enantiomers of the metabolites.

System I. The guard column was Chiral AGP (10 × 4 mm i.d., 5 μm) (Chrom Tech AB, Stockholm, Sweden) and the analytical column was a Chiral AGP (100 × 4 mm i.d., 5 μm). The eluent was 6% acetonitrile in phosphate buffer pH 6.0 (I = 0.05). The flow rate was 0.9 ml/min and the column temperature was 55°C.

System II. The analytical column was Chiral AGP (100 × 2 mm i.d., 5 μm) used with an eluent of 6% acetonitrile in phosphate buffer pH 7.3 (I = 0.05) at a flow rate of 0.25 ml/min and the column temperature was 50°C.

The eluate from both systems was detected by UV at 210 nm. The buffer solutions at different pH were prepared by mixing different volumes of 1 M phosphoric acid and 1 M sodium hydroxide to the pH specified.

Standard Preparation

Stock solutions of ropivacaine containing 2500 μM of (−)-(S)-ropivacaine and 700 μM (+)-(R)-ropivacaine were prepared in phosphate buffer pH 2 (I = 0.05) and stored at +4°C. Urine standards at low concentrations were prepared from the stock solutions. The concentration of (−)-(S)-ropivacaine was 5–15 μM and that of (+)-(R)-ropivacaine 0.03–0.07 μM. The (+)-(R)-ropivacaine concentration was 0–6% of (−)-(S)-ropivacaine.

Stock solutions of the metabolites in separate enantiomers as well as the racemic mixture were of about 1000 μM and were prepared in phosphate buffer pH 2 (I = 0.05) and stored at +4°C. Reference solutions of *rac*-3-hydroxy-ropivacaine (8 μM) and *rac*-PPX (12 μM) were prepared in phosphate buffer of the same pH as the eluent. Standards of PPX and 3-hydroxy-ropivacaine were extracted and treated in the same way as the urine samples. To confirm the enantiomer separation in the urine samples tested, about 5% of the (R)-form was added and determined separately.

Sample Preparation

Ropivacaine. For the determination of ropivacaine an aliquot of urine sample, equivalent to 4–7 nmol ropivacaine (0.3–3 ml), was extracted with 4–6 ml n-heptane for 20 min after the addition of 0.5 ml of 2 M sodium hydroxide. The samples were centrifuged for 10 min. The organic phase was evaporated at 40°C under N_2, the residue was dissolved in 200 μl of phosphate buffer, pH 6 (I = 0.05), and 100 μl was injected into chromatographic system I.

The procedure described was suitable for the determination of metabolic racemisation of ropivacaine in urine samples obtained from man, sheep, and rat. However, the dog urine needed an additional purifying stage since interfering peaks occurred in the chromatogram. After extraction (as described above), the residue was dissolved in 200 μl of phosphate buffer, pH 6. Of the residue, 170 μl was injected into a

TABLE 1. Retention and selectivity of (−)-S- and (+)-R-ropivacaine at various pH and acetonitrile content in the eluent[a]

pH	Acetonitrile %	k' (−)-(S)-Ropivacaine	α	R_S^b
3.0	1	0.7	1.3	1.0[c]
6.0	5	8.6	1.24	2.3
6.0	7	5.7	1.21	1.6
7.0	7	19.1	1.28	2.5
7.0	9	10.6	1.26	2.2
7.5	10	17.4	1.29	2.6
7.5	14	9.5	1.25	2.3

[a]Column: Chiral-AGP (100 × 4 mm i.d., 5 μm); eluent: acetonitrile in phosphate buffer; column: temperature: 55°C; flow rate: 0.9 ml/min.
[b]R_s is calculated from chromatograms of the racemate.
[c]Flow rate: 0.5 ml/min.

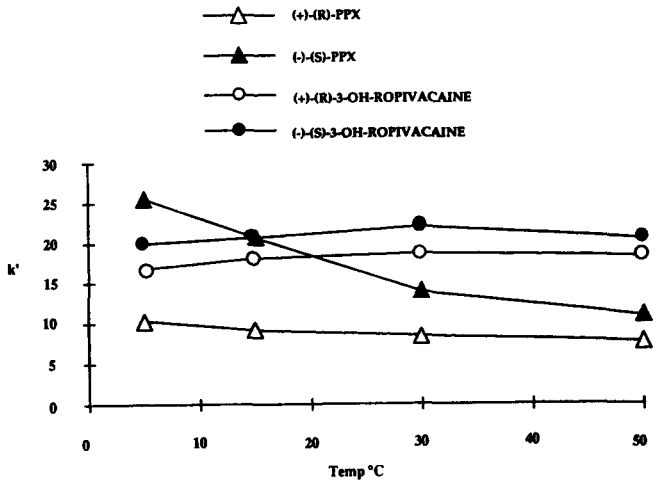

Fig. 2. Influence of temperature on the retention of ropivacaine metabolites. Chromatographic conditions: column: Chiral-AGP (100 × 2 mm i.d., 5 μm); eluent: 6% acetonitrile in phosphate buffer pH 7.3; flow rate: 0.25 ml/min. Samples: *rac*-PPX 12 μ*M* and *rac*-3-hydroxy-ropivacaine (3-OH-ropivacaine) 8 μ*M* in phosphate buffer pH 7.3.

chromatographic system with a nonchiral column (Superspher Select B 125-4, 119 × 4 mm i.d., 4 μm), mobile phase 33% (v/v) acetonitrile in phosphate buffer, pH 6.6 (I = 0.1), containing 5 m*M* sodium octyl sulphate. The column temperature was 45°C.

The eluate fraction containing ropivacaine, eluted at 14.9–16.4 ml, was collected and evaporated, after addition of 0.5 ml ethanol, at 40°C under N_2. The residue was dissolved in 200 μl of phosphate buffer, pH 6.0, and 120 μl was injected on the Chiral AGP column. Standard urine samples with the (−)-(S)- and (+)-(R)-enantiomers were treated in the same way as the dog samples.

3-Hydroxy-ropivacaine. For the determination of 3-hydroxy-ropivacaine, 1 ml of the urine sample was heated with 5 *M* hydrochloric acid at 90°C for 60 min to hydrolyse conjugates. The hydrolysate (0.25 ml) was adjusted to pH 10 by addition of 0.5 ml 2 *M* sodium hydroxide and was extracted with 5 ml *n*-heptane/ethyl acetate (9:1) at ambient temperature for 20 min. The samples were centrifuged (10 min) and the organic phase was evaporated at 40°C with N_2. The residue was dissolved in 150 μl of phosphate buffer, pH 6 (I = 0.05), and 10 μl was injected into chromatographic system II.

PPX. PPX was extracted in the same way as ropivacaine, but in general about 0.5–1 ml of urine was used. After evaporation of the organic phase the residue was dissolved in 150 μl of phosphate buffer, pH 6 (I = 0.05), and 10 μl was injected into chromatographic system II.

RESULTS AND DISCUSSION

Knowledge of the biotransformation of amide type local anaesthetics in various species, including man, is very limited, with the exception of lidocaine. It is apparent from the literature that aromatic hydroxylation and dealkylation are minor metabolic pathways in man.[15] No metabolic inversion of these compounds or their metabolites has been reported. We have shown that ropivacine is extensively metabolised by the different cytochrome P-450 isozymes before being excreted in the urine.[5] Aromatic hydroxylation is far more dominant in vivo than in vitro using liver microsomes, where depropylation predominates. This difference could be explained by a substrate-dependent metabolism similar to that seen for lidocaine.[16] Between 33 and 41% of an iv dose of ropivacaine in man has been identified as 3-hydroxy-ropivacaine, mainly conjugated, and 1 to 4% as ropivacaine and PPX, respectively.[5] Only a very small amount of the dose (about 0.5%) was excreated as 4-hydroxy-ropivacaine. Similar patterns have been observed in various species, including rat, dog, and sheep.

Regulation of Retention and Selectivity on the Chiral AGP Column

Enantiomers of local anaesthetics of the amide type, e.g., mepivacaine and bupivacaine, are generally easily separated on the Chiral AGP column.[17] Similarly, the enantiomers of ropivacaine and the enantiomers of its metabolites 3-hydroxy-ropivacaine and PPX are easily separated. However, in practice if less than 1% of the (+)-(R)-enantiomer is detected, optimisation of the separation conditions is needed.

The retention of ropivacaine and its metabolites is regulated by the content of organic modifier (e.g., acetonitrile), pH, and the column temperature. The retention decreases with increased content of acetonitrile in the eluent and increases with increased pH of the eluent. In general the retention of ropivacaine is 3–5 times higher than the retention of the metabolites. To obtain suitable retention times, and because the concentration of the metabolites in urine samples is generally much higher compared to ropivacaine, two distinct separation systems were developed, one for ropivacaine and the other for the metabolites. For ropivacaine, complete resolution was obtained in the pH range 6–7.5. The separation factor was 1.2–1.3 within the pH interval 3–7.5. At pH 3 the retention was too low to obtain complete resolution (Table 1). At increased column temperature the column efficiency increases and a temperature of about 50°C was chosen in the analytical procedure. To fully optimise the separation, a chemometric approach may be adequate; however, in the present study this was not necessary in order to obtain suitable conditions.

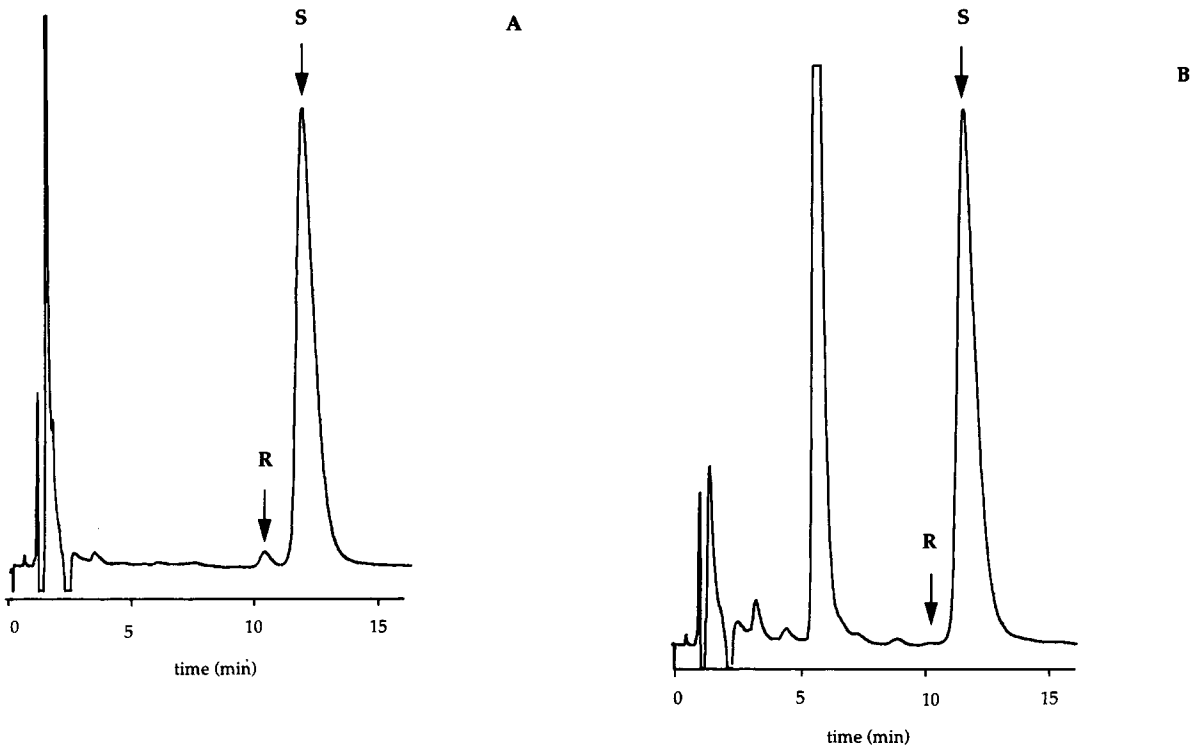

Fig. 3. Chiral separation of ropivacaine. **(A)** (R/S:2/98)-Ropivacaine 5 μ*M* in blank human urine. **(B)** Urine sample after rectal administration of (−)-(S)-ropivacaine to man, 3.4 μ*M* ropivacaine. Column: Chiral AGP (100 × 4 mm i.d., 5 μm); eluent: 6% acetonitrile in phosphate buffer pH 6.0; detection: UV 210 nm.

In the separation of the enantiomers of the metabolites some unexpected results were obtained. It was found that the retention of (−)-(S)-PPX was affected by the temperature to a higher extent than to the retention of the (+)-(R)-PPX and the 3-hydroxy-ropivacaine enantiomers (Fig. 2). At low temperature (5°C), the separation factor of the enantiomers of PPX is high ($\alpha = 2.5$), whereas at 50°C the separation factor is lower ($\alpha = 1.4$); however, complete resolution of the enantiomers is obtained. At room temperature (−)-(S)-PPX and (+)-(R)-3-hydroxy-ropivacaine coelute. However, above 30°C the two enantiomer pairs as well as the individual enantiomers are completely separated (Fig. 2).

Influence of Sample Size

The Chiral AGP column has a limited capacity, i.e., if the injected sample amount is too high, the column will be overloaded, the peak efficiency will decrease, and the resolution will deteriorate. In the present study the injected amount has been limited to approx. 2–7 nmol. In the analytical procedure the limit of determination of ropivacaine is approx. 1 pmol. If less than 1% of the (+)-(R)-enantiomer of ropivacaine and metabolites is to be determined, each sample needs to be treated individually. It is convenient if the total concentrations of the analytes are known (e.g., determined by a nonchiral method) in order to set the conditions for the sample preparation. In general, there is a large variation of the concentration of ropivacaine and the metabolites in urine samples. If the concentration in the urine sample is too low, less than 1% of the (+)-(R)-form cannot be determined, and if the concentration in the urine sample is too high, the separation will be incomplete. In the latter case, sample dilution is required.

Sample Preparation and Selectivity

A selective extraction method is required since UV detection at 210 nm is needed to obtain high sensitivity for ropivacaine and its metabolites. High selectivity is more important than high recovery because the (−)-(S)-form can be regarded as an internal standard. Ropivacaine and PPX were extracted with *n*-heptane under alkaline conditions. Recovery was greater than 90% and about 70% for ropivacaine and PPX, respectively.

The chromatograms of urine samples were free from interference by endogenous compounds at the retention times of the enantiomers of ropivacaine and PPX. Extraction with toluene or a mixture of *n*-heptane and dichloromethane (4:1) resulted in chromatograms with several interfering peaks. Pretreatment with solid-phase extraction with C_{18} or SCX columns resulted in wide fronts and several interfering peaks in the chromatograms.

No standard of the 3-hydroxy-ropivacaine conjugate was available, for which reason the metabolite was hydrolyzed under acidic conditions prior to analysis. The hydrolysate was alkalinised to pH 10 and 3-hydroxy-ropivacaine was extracted with *n*-heptane/ethyl acetate (9:1). The selectivity was high but the extraction recovery was only 45%. The extraction recovery of 3-hydroxy-ropivacaine was higher with increased amounts of ethyl acetate in the organic phase. However, the

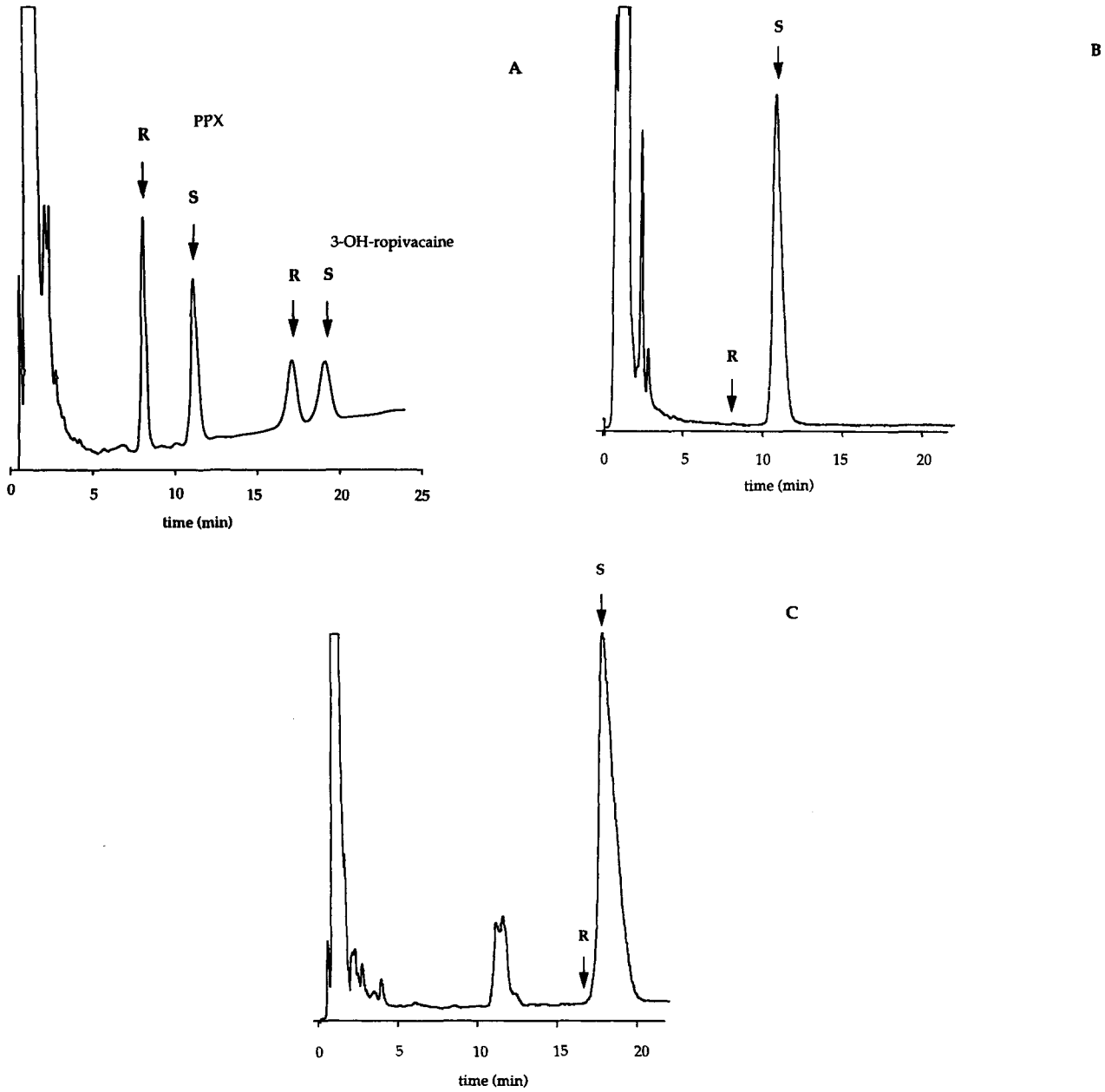

Fig. 4. Chiral separation of PPX and 3-hydroxy-ropivacaine. **(A)** *rac*-PPX (12 μ*M*) and *rac*-3-hydroxy-ropivacaine (8 μ*M*) in phosphate buffer pH 7.3. **(B)** Urine sample after rectal administration of (−)-(S)-ropivacaine to man, 15 μ*M* PPX. **(C)** Same as **B**, 300 μ*M* 3-hydroxy-ropivacaine. Column: Chiral AGP (100 × 2 mm i.d., 5 μm); eluent: 6% acetonitrile in phosphate buffer pH 7.3; detection: UV 210 nm.

increased recovery brought no benefit since the number of interference peaks increased.

Test of Metabolic Racemisation

The metabolic racemisation of ropivacaine was examined after different routes of administration (iv, subcutaneously, and rectally) in urine samples from man, rat, dog, and sheep. In all the samples tested no metabolic racemisation of ropivacaine was observed. A typical chromatogram is shown in Figure 3B. The limit of determination of the (+)-(R)-form was 0.2%. A typical chromatogram of a urine standard containing (R/S:2/98)-ropivacaine added to blank urine is shown in Figure 3A.

The analysis of metabolic racemisation of the metabolites in man shows similar results as for ropivacaine, i.e., no racemisation. Typical chromatograms of PPX and 3-hydroxy-ropivacaine in urine sample are shown in Figure 4B and C. A chromatogram of a standard solution of *rac*-PPX and *rac*-3-hydroxy-ropivacaine is given in Figure 4A. The limit of determination of the (+)-(R)-forms of the metabolites was set at 1%.

CONCLUSIONS

The method described is suitable for the determination of metabolic racemisation of (−)-(S)-ropivacaine and the major metabolites PPX and 3-hydroxy-ropivacaine in urine samples. Each substance is determined separately, i.e., the method is not optimised for routine analysis. No evidence of metabolic racemisation was observed in any of the samples determined in the study. In the determination of ropivacaine in pharmacokinetic, pharmacodynamic, toxicological, and metabolic studies, nonchiral bioanalytical assays are considered to be appropriate. The total concentration of ropivacaine is generally determined by capillary gas chromatography with nitrogen-phosphorus detection after liquid–liquid extraction[13] and the free concentration (i.e., non-protein-bound concentration) is determined by ultrafiltration and coupled-column liquid chromatography[14] using ion-exchange and reversed-phase columns. The metabolites of ropivacaine are determined using nonchiral reversed-phase liquid chromatography after solid-phase extraction.[5]

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