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 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 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'-pipercoloxylidide (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 α1-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.
The standards of ropivacaine, 2', 6'-pipecoloxylidide hydrochloride samples from humans. 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 human urine samples. 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.

**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 x 4 mm i.d., 5 µm) (Chrom Tech AB, Stockholm, Sweden) and the analytical column was a Chiral AGP (100 x 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 x 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 elute 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 the racemic mixture.

Stock solutions of the metabolites in separate enantiomers as well as the racemic mixture were prepared at 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 N₂, the residue was dissolved in 200 µl of phosphate buffer, pH 6 (I = 0.05), and 100 µl was injected into a chromatographic system I.

The procedure described was suitable for the determination of racemic metabolisation 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

<table>
<thead>
<tr>
<th>pH</th>
<th>Acetonitrile</th>
<th>k'</th>
<th>α</th>
<th>R'</th>
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</thead>
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<td>1</td>
<td>0.7</td>
<td>1.3</td>
<td>1.0'</td>
</tr>
<tr>
<td>6.0</td>
<td>5</td>
<td>8.6</td>
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<td>5.7</td>
<td>1.21</td>
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</tr>
<tr>
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<td>14</td>
<td>9.5</td>
<td>1.25</td>
<td>2.3</td>
</tr>
</tbody>
</table>

Column: Chiral-AGP (100 x 4 mm i.d., 5 μm); eluent: acetonitrile in phosphate buffer; column: temperature: 55°C; flow rate: 0.9 ml/min.

 regulates 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.

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**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. No metabolic inversion of these compounds or their metabolites has been reported. We have shown that ropivacaine is extensively metabolised by the different cytochrome P-450 isozymes before being excreted in the urine. 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. 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. Only a very small amount of the dose (about 0.5%) was excreted 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. Similarly, the enantiomers of ropivacaine and the enantiomers of its metabolites 3-hydroxyropivacaine 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.
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-rospivacaine 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-rospivacaine 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 rospivacaine is approx. 1 pmol. If less than 1% of the (+)-(R)-enantiomer of rospivacaine 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 rospivacaine and the metabolites in urine samples. 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 rospivacaine 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 rospivacaine 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-rospivacaine conjugate was available, for which reason the metabolite was hydrolyzed under acidic conditions prior to analysis. The hydrolysate was alkalised to pH 10 and 3-hydroxy-rospivacaine 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-rospivacaine was higher with increased amounts of ethyl acetate in the organic phase. However, the
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 and the free concentration (i.e., non-protein-bound concentration) is determined by ultrafiltration and coupled-column liquid chromatography using ion-exchange and reversed-phase columns. The metabolites of ropivacaine are determined using nonchiral reversed-phase liquid chromatography after solid-phase extraction.

REFERENCES

5. Unpublished data. Astra Pain Control AB.