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ORIGINAL RESEARCH

Liquid chromatographic bioanalytical determination of ropivacaine, bupivacaine and major metabolites

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ABSTRACT: Bioanalytical methods for the determination of ropivacaine, bupivacaine and their major metabolites in urine and blood plasma are presented. Ropivacaine is a new local anaesthetic drug mainly used for surgery and for postoperative pain relief. The samples are hydrolysed and cleaned using solid-phase extraction and analysed using ion-pair reversed-phase liquid chromatography with gradient elution. The analytes are detected using UV at 210 nm. The methods are highly selective and the limits of quantification were 1 μM in urine and 0.1 μM in plasma, respectively. The between-day variance was generally below 3% (RSD). Copyright © 1999 John Wiley & Sons, Ltd.

INTRODUCTION

The new amide local anaesthetic drug ropivacaine belongs to the pipecoloxylidide group, to which the well-established local anaesthetics mepivacaine and bupivacaine also belong. Unlike these two agents, which are used as racemic mixtures, ropivacaine is exclusively the *S*-(−)-enantiomer ((*S*)-(−)-1-propyl-2′,6′-pipecoloxylidide). Ropivacaine, together with bupivacaine and mepivacaine, is extensively metabolized before being excreted, mainly in the urine, in both animals and man (Halldin *et al.*, 1996). The major metabolic pathways include aromatic hydroxylation and *N*-dealkylation mainly by the cytochromes P4503A4 and 1A in the liver (Ekström and Gunnarsson, 1996). *N*-dealkylation will give the same metabolite, pipecoloxylidide (PPX), from the three local anaesthetic agents in this series (Thomas and Meffin, 1972; Dennhardt and Konder, 1980; Halldin *et al.*, 1996). The hydroxylated metabolites of ropivacaine are mainly excreted in the urine as conjugates (sulphates and/or glucuronides). The lack of metabolic racemization for ropivacaine and the metabolites PPX and 3-hydroxy-ropivacaine was confirmed in urine samples from different species, including man, using chiral liquid chromatography with an AGP column (Arvidsson *et al.*, 1995). This finding justifies the use of non-chiral analytical methodology for routine bioanalytical work.

The total and the free concentrations of ropivacaine and bupivacine were determined using gas chromatography (Björk *et al.*, 1990; Engman *et al.*, 1997) and liquid chromatography (Arvidsson and Eklund, 1995), respectively. The metabolites of bupivacaine were determined using gas chromatography (Lesko and Ericson, 1980) or liquid chromatography (Lindberg *et al.*, 1986; Kastrissios *et al.*, 1992; Gupta and Dauphin, 1994) with liquid–liquid extraction or solid-phase extraction as sample pre-treatment. The metabolites of ropivacaine, in man, were determined by ion-pair liquid chromatography using a step gradient of acetonitrile (Halldin *et al.*, 1996) after acid hydrolysis and solid-phase extraction. The present paper will focus on the development and validation of analytical procedures for the determination of ropivacaine, bupivacaine and metabolites including sample preparation (urine and plasma) with cleaving of conjugates, solid-phase extraction and ion-pair liquid chromatography using a continuous gradient of acetonitrile.

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EXPERIMENTAL

Chemicals. Ropivacaine hydrochloride monohydrate and bupivacaine hydrochloride monohydrate were obtained from Astra Production Chemicals (Södertälje, Sweden). Pipecoloxylidide HCl (PPX) was obtained from Nobel Chemicals (Karlskoga, Sweden), 3′-hydroxy-ropivacaine (3-OH-ropivacaine), 4′-hydroxy-ropivacaine (4-OH-ropivacaine), 2′-hydroxy-methyl-ropivacaine (2-OH-methyl-ropivacaine), 3′-hydroxy-PPX HCl (3-OH-PPX), 4′-hydroxy-PPX HCl (4-OH-PPX), 3′-hydroxy-bupivacaine (3-OH-bupivacaine), 4′-hydroxy-bupivacaine (4-OH-bupivacaine), isopropyl-PPX HCl and ethyl-PPX HCl were synthesized at the Department of Medicinal Chemistry, Astra Pain Control AB (Södertälje, Sweden). The chemical structures are given in Fig. 1.

Methanol LiChrosolv, HPLC quality, and acetonitrile LiChro-

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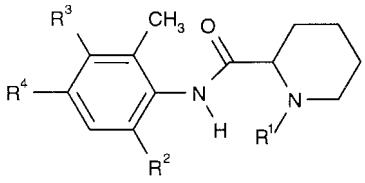

Compound	R^1	R^2	R^3	R^4
Ropivacaine	C_3H_7	CH_3	H	H
3-OH-PPX	H	CH_3	OH	H
4-OH-PPX	H	CH_3	H	OH
4-OH-ropivacaine	C_3H_7	CH_3	H	OH
3-OH-ropivacaine	C_3H_7	CH_3	OH	H
2-OH-methyl-ropivacaine	C_3H_7	CH_2OH	H	H
Ethyl-PPX (IS)	C_2H_5	CH_3	H	H
Isopropyl-PPX (IS)	$CH(CH_3)_2$	CH_3	H	H
PPX	H	CH_3	H	H
Bupivacaine	C_4H_9	CH_3	H	H
4-OH-bupivacaine	C_4H_9	CH_3	H	OH
3-OH-bupivacaine	C_4H_9	CH_3	OH	H

Figure 1. Chemical structure of ropivacaine, bupivacaine and metabolites.

solv, HPLC quality, were obtained from E. Merck (Darmstadt, Germany). All the other chemicals were of analytical grade or better and were obtained from commercial sources.

Equipment. An HETO DT1 thermostat water-bath from Heto Lab Equipment (Alleroed, Denmark) was used for hydrolysis of conjugated metabolites in the urine. An ASPEC system from Gilson (Villiers-LeBel, France) was used for solid-phase extraction.

A Turbovap LV Nitrogen Degasser from Zymark (Hopkinton, Mass) was used for evaporation. The chromatographic system consisted of two LC-10AD pumps with a 0.5 mL or a 1.7 mL gradient mixer (Shimadzu, Kyoto, Japan) connected to a CMA/200 autosampler (CMA/Micro-dialysis, Stockholm, Sweden). For ropivacaine in urine samples the column was a Symmetry™ C8 (150 × 3.9 mm i.d., 5 μm), for ropivacaine in plasma samples a Symmetry™ C8 (150 × 2.1 mm i.d., 5 μm) and for bupivacaine a Symmetry™ C18 (150 × 3.9 mm i.d., 5 μm), and in all systems a Symmetry™ C8 guard column (20 × 3.9 mm i.d., 5 μm) was used (Waters, Milford, MA, USA). The column was thermostatted using a Jones chromatography model 7961 column oven (Mid Glamorgan, UK). The detector was a Spectra 100 UV–vis detector (Spectra Physics, San José, CA, USA). The chromatographic data were collected and processed with the PE Nelson Access* Chrom (Perkin-Elmer Nelson System Inc., Cupertino, CA, USA).

Procedures. Phosphate buffer pH 2 ($I = 0.1$) was prepared by diluting 158 mL of 1 M phosphoric acid and 100 mL of 1 M sodium dihydrogen phosphate to 1000 mL with deionized water. The eluents were prepared by mixing specified volumes of acetonitrile and buffer pH 2 ($I = 0.05$), containing octanesulphonic acid. Stock solutions of ropivacaine, bupivacaine and the metabolites in the concentration range 500 –3500 μM were prepared in phosphate buffer pH 2 ($I = 0.1$) and stored at +4°C. Three stock solutions of each substance were prepared. Standards were prepared from two separate stock solutions by dilution, using drug-free urine or plasma. Control samples of urine or plasma were prepared by adding ropivacaine and its metabolites or bupivacaine and its metabolites from a separate stock solution to pooled blank human urine or plasma. The control samples were stored frozen at −20°C. The internal standard for the urine samples was isopropyl-PPX at a concentration of 17 μM. The internal standard for the plasma samples was ethyl-PPX at a concentration of 1.5 μM.

Sample collection and storage. Human and sheep urine was collected via catheters into plastic bottles and thereafter transferred to polypropylene tubes (Halldin *et al.*, 1996; Arvidsson *et al.*, 1995). The urine samples were frozen and stored at −20°C until analysis. Human blood was collected in heparinized Venoject tubes and centrifuged within 1 h (Halldin *et al.*, 1996). The plasma was transferred to polypropylene tubes and frozen within 30 min. The plasma samples were stored at −20°C until the analysis.

Sample preparation (urine). The urine samples with internal standard added (1.0 mL) were mixed with 1.0 mL of 6 M hydrochloric acid and placed in a water-bath at 95°C for hydrolysis, 1 h for ropivacaine and 2 h for bupivacaine, respectively. The hydrolysate (250 μL) was diluted with 1.0 mL deionized water.

Table 1. ASPEC procedure for the extraction of urine and plasma samples

Liquid dispensed	Dispensing flow rate (μL/se)	Pressuring air volume (μL)	
		Urine	Plasma
Conditioning			
1. Methanol	100	250	200
2. Buffer pH 2, $I = 0.01$, 1000 μL	100	250	200
Loading			
1.3–1.5 mL samples	12	400	1000
Washing			
1. Buffer pH 2, $I = 0.01$, 1000 μL	100	300	600
2. Buffer pH 2, $I = 0.01$ + methanol (1:1, v/v), 2000 μl	100	300	600
Elution			
2 M ammonia + methanol (1:4, v/v), 2000 μL	12	600	600

Table 2. Gradients of acetonitrile used for separation of ropivacaine, bupivacaine and metabolites in urine and blood plasma

	Ropivacaine urine sample		Ropivacaine plasma sample		Bupivacaine urine sample	
	Time (min)	Eluent II (%[a])	Time (min)	Eluent II (%[a])	Time (min)	Eluent II (%[a])
Sample injected	0	30	0	30	0	30
Isocratic run	10	30	10	30	10	30
Gradient	40	54.3	44	56	45	58.2
Step gradient	40	90	44	90	45	90
Condition	43	30	48	90	48	30
Next sample	65		71		71	

[a] Eluent I: 10 mM octanesulphonic acid in acetonitrile–phosphate buffer (3:97, v/v). Eluent II: 10 mM octanesulphonic acid in acetonitrile–phosphate buffer pH 2 (50:50, v/v). Flow rate = 1 mL/min.

Sample preparation (plasma). Unconjugated metabolites: to the plasma sample (500 μL), 100 μL internal standard, 100 μL of phosphate buffer pH 2 ($I = 0.1$) and 800 μL of phosphate buffer pH 6 ($I = 0.1$) were added, mixed and then centrifuged.

Total amount of conjugated and unconjugated metabolites: to the plasma sample (500 μL), 50 μL internal standard solution, 150 μL of 1 M phosphoric acid and 50 μL of phosphate buffer pH 2 ($I = 0.1$) were added. The mixture was filtered using Centrisart 1, 20,000 cut-off (Sartorius AG, Göttingen, Germany) at 1250 ***g*** for 15 min. To the ultrafiltrate (250 μL) an equal volume of 6 M hydrochloric acid was added. The mixture was hydrolysed in a water-bath at 95°C for 1 h. The hydrolysed sample was diluted with 1 mL of deionized water.

Solid-phase extraction. The total volume obtained from the sample preparation was used for solid-phase extraction on a Bond Elut SCX, 100 mg. The procedure was automated using an ASPEC system and the sequences used are described in Table 1 for the extraction of urine and plasma samples. After extraction the eluate is evaporated to dryness with nitrogen at 40°C. The dry sample is reconstituted in 150–200 μL of a mixture of eluents I and II (7 + 3).

Liquid chromatography. Chromatography was performed at 45°C using an acetonitrile gradient in an ion-pair liquid chromatographic system. The columns used were a Symmetry C8 for ropivacaine and a Symmetry C18 for bupivacaine. Two eluents were mixed in the gradient system. The first eluent consisted of 10 mM octanesulphonic acid in acetonitrile-phosphate buffer pH 2 ($I = 0.05$) (3:97, v/v) and the second eluent of 10 mM octanesulphonic acid in acetonitrile–phosphate buffer pH 2 ($I = 0.05$) (50:50, v/v), respectively. Three different gradients were used (Table 2) depending on sample type and analytes. The flow rate was 1 mL/min.

Table 3. Concentration ranges in typical standard curves

	Concentration (μM)	
Analyte	Urine	Plasma
3-OH-PPX	1–30	
PPX	1–70	0.1–7.5
3-OH-ropivacaine	1–150	0.1–7.5
4-OH-ropivacaine	1–20	
2-OH-methyl-ropivacaine	1–15	
Ropivacaine	1–25	0.1–10.0
3-OH-bupivacaine	1–150	
Bupivacaine	1–25	

Calibration. The samples were quantified using internal standardization. Standard curves were prepared using five to eight different concentrations for each analyte. The concentration range of each individual standard curve was chosen according to the actual concentration in the samples. Typical concentration ranges of the standard curves are given in Table 3. Non-weight linear regression was used for quantification.

Accuracy and precision. The between-day precision and the accuracy for ropivacaine, bupivacaine and their metabolites were determined from quality-control samples prepared at two or three different concentration levels. The accuracy was determined as the recovery, stated in per cent, and the precision as the relative standard deviation (RSD).

Stability. The stability of ropivacaine and its metabolites in human and sheep urine and in human plasma was tested in samples from dosed subjects and in control samples where the analytes were added to blanks. The samples were stored at −20°C and analysed at regular intervals up to a storage time of 3 years. The stability of bupivacaine and its metabolites in sheep urine was tested from dosed subjects. The samples were stored at −20°C and analysed at regular intervals up to a storage time of 22 months.

Extraction recovery. The extraction recovery was determined by comparing extracted standard samples with a standard diluted in the same way as the actual dilution during the extraction. The non-extracted standard was injected directly into the chromatographic system. Three concentration levels were studied.

RESULTS AND DISCUSSION

Ropivacaine and bupivacaine are both local anaesthetics of the amide type. They are basic amines with a pK_a of

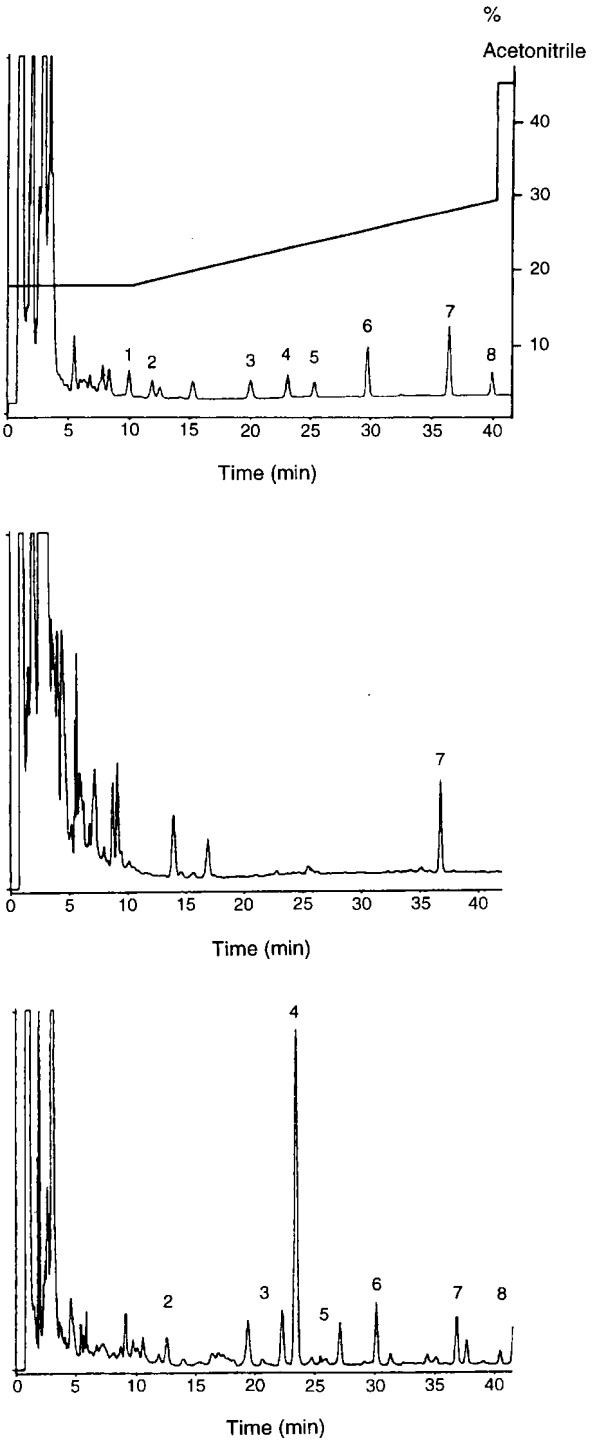

Figure 2. Chromatogram of ropivacaine and metabolites from human urine samples. Peaks: (1) 4-OH-PPX, (2) 3-OH-PPX, (3) 4-OH-ropivacaine, (4) 3-OH-ropivacaine, (5) 2-OH-methyl-ropivacaine, (6) PPX, (7) isopropyl-PPX (IS), 8. ropivacaine, Upper: ropivacaine and metabolites added to blank human urine in the concentration range 5–15 μM. Middle: hydrolysed blank human urine sample. Lower: hydrolysed human urine sample taken after 12–24 h during continuous epidural infusion of ropivacaine (30 mg/h for 72 h). Analytes: (2) 3-OH-PPX 10.0 μM, (4) 3-OH-ropivacaine 118 μM, (6) PPX 23.2 μM, (8) ropivacaine 4.7 μM.

about 8. The basic routes of metabolism are aromatic hydroxylation and *N*-dealkylation (Fig. 1). Both the parent compound and the metabolites are positively charged at physiological pH and at low pH, which usually occurs in urine samples. Due to the polar character of the metabolites, liquid chromatography was the methodology of choice. The analytes have no distinct chromophores, hence in order to obtain high sensitivity in the assay, UV detection at 210 nm was chosen. However, detection is non-specific and considerable demands are put on the selectivity of the sample work-up procedure as well as the selectivity of the chromatographic system.

Sample preparation

The hydroxylated metabolites are mainly conjugated (sulphate and/or glucuronide) in the urine. However, no synthesized reference standards for the conjugated metabolites were available and the urine samples were therefore hydrolysed to cleave the conjugate. To obtain full recovery at hydrolysis, it was necessary to hydrolyse samples containing ropivacaine for 1 h and samples of bupivacaine for 2 h. The recovery of hydrolysis of the conjugate of 3-OH-bupivacaine was only about 85% after 1 h.

To isolate the analytes from the complex matrix of hydrolysed urine, an extensive work-up procedure was necessary. Initially different liquid–liquid extraction procedures were tested, but it proved difficult to obtain both high recoveries for all analytes and clean chromatograms. Similar problems occurred using liquid–solid extraction with reversed-phase columns (C8 or C18). However, by means of cation-exchange extraction columns both a high selectivity and acceptable recoveries were obtained. Despite the high ionic strength of the hydrolysed urine samples, it was possible, after a fourfold dilution, to adsorb both ropivacaine, bupivacaine and their metabolites to the cation-exchange column. The sample was cleaned by washing it with a mixture of methanol and buffer with a low ionic strength. In the last step the analytes were eluted from the column, as the amines in uncharged form, by a solution of ammonia–methanol at high pH. The extraction recoveries of all the analytes and the internal standard were similar, about 70–80%.

It was also possible to use the extraction system for blood plasma samples, obtaining similar recoveries. However, particulate matter was occasionally found in the extract after redissolution of the sample, but after centrifugation of the vial it was possible to inject the sample in the chromatograph. The particulate matter possibly consisted of plasma proteins passing through the extraction system, although no further optimization was made.

In the determination of conjugated metabolites in blood plasma it became necessary to remove the plasma

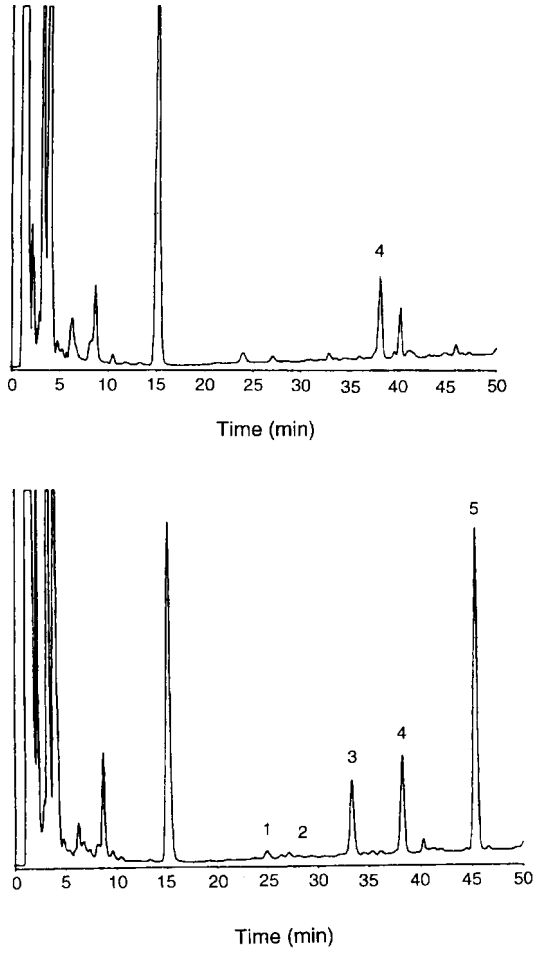

Figure 3. Chromatograms of ropivacaine and metabolites from human plasma samples. Peaks: (1) 3-OH-ropivacaine, (2) 2-OH-methyl-ropivacaine, (3) PPX, (4) Ethyl-PPX (IS), (5) ropivacaine. Upper: blank human plasma sample. Lower: hydrolysed human plasma sample taken 72 h after continuous epidural infusion of ropivacaine (20 mg/h for 72 h). Analytes: (3) PPX 1.5 μM, (5) ropivacaine 4.9 μM.

proteins prior to hydrolysis in order to obtain acceptable chromatographic conditions. Hydrolysis of plasma proteins resulted in a number of disturbing peaks in the chromatogram. The plasma proteins were removed by ultrafiltration and, prior to the ultrafiltration, the sample was acidified with phosphoric acid to eliminate the protein binding of the analytes (Arvidsson, 1988).

Liquid chromatographic system

To obtain a selective determination of the analytes, considerable effort is needed where both the sample work-up and the chromatography are concerned. Generally, ropivacaine, bupivacaine and their metabolites are easily resolved using reversed-phase liquid chromatography. Using a simple system with a C8 column and an eluent consisting of acetonitrile and buffer, all the analytes were resolved within 15 min. However, when

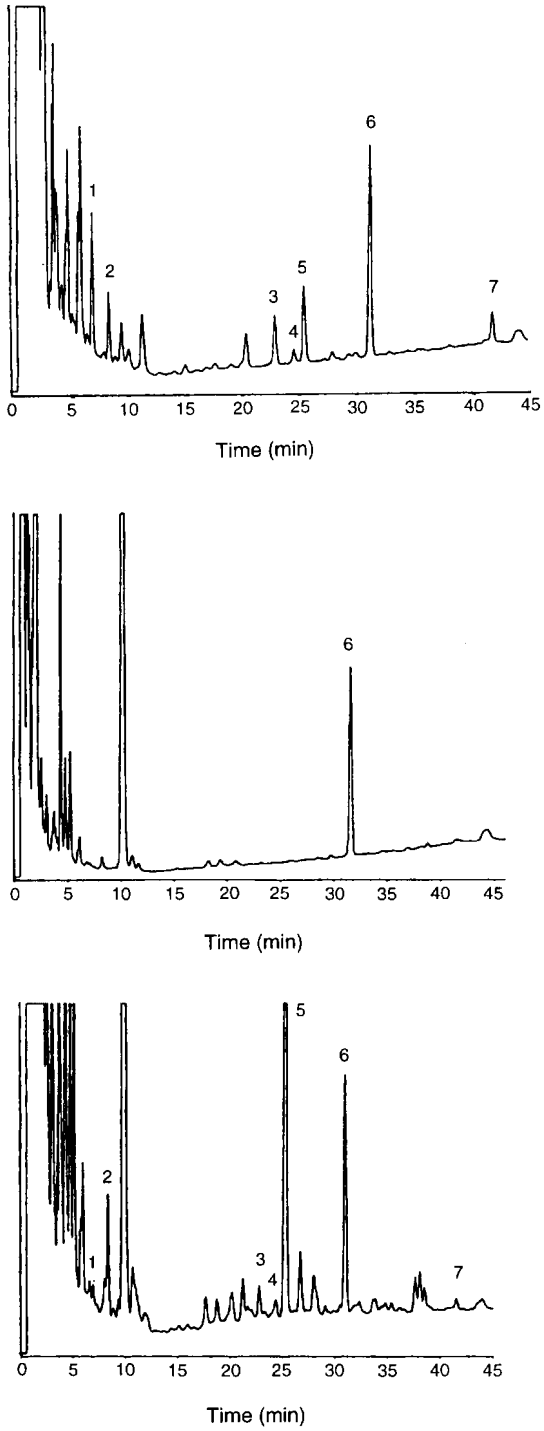

Figure 4. Chromatograms of bupivacaine and metabolities from urine samples. Peaks: (1) 4-OH-PPX, (2) 3-OH-PPX, (3) 4-OH-bupivacaine, (4) PPX, (5) 3-OH-bupivacaine, (6) Isopropyl-PPX (IS), (7) bupivacaine. Upper: bupivacaine and metabolites added to blank human urine in the concentration range 1–17 μM. Middle: hydrolysed blank sheep urine sample. Lower: hydrolysed sheep urine sample taken 30 min after i.v. infusion of bupivacaine (6.28 μmol/kg). Analytes: (1) 4-OH-PPX <3 μM, (2) 3-OH-PPX 6 μM, (3) 4-OH-bupivacaine 3 μM, (4) PPX 1.4 μM and (5) 3-OH-bupivacaine 39 μM, (7) bupivacaine <1.0 μM.

Table 4. Between-days precision and accuracy of ropivacaine and metabolites in human urine

Analyte	Concentration (μM)	Accuracy (%)	Precision (RSD%)	*n*
3-OH-ropivacaine	103	101	1.0	28
	8.2	99	1.1	24
	4.1	96	2.9	28
2-OH-methyl-ropivacaine	9.1	100	1.1	28
	1.8	102	4.8	24
	0.9	103	8.5	28
PPX	43.7	100	0.9	28
	7.3	102	1.3	24
	3.6	102	2.9	28
3-OH-PPX	17.2	101	1.3	28
	4.3	101	1.4	24
	2.1	103	3.8	28
Ropivacaine	19.0	101	0.5	28
	3.9	100	0.8	24
	1.9	100	1.4	28

applying a urine sample to this system, no resolution from the matrix was obtained. The addition of an ion-pair reagent, octanesulphonic acid, to the eluent selectively increases the retention of the positively charged analytes and resolution from the urine matrix was obtained. The retention of the most polar analyte had to be in the range of about $k' = 5–10$ for it to be resolved, which resulted in a very high retention of the parent drug (>2 h). Initially a step gradient of acetonitrile was utilized to speed up the retention of ropivacaine (Halldin *et al.*, 1996). However, with the step-gradient approach it was occasionally difficult to resolve all the analytes, but by changing to a continuous gradient of acetonitrile, all the metabolites were generally resolved (Fig. 2).

The retention of analytes in ion-pair liquid chromatographic systems is generally sensitive to changes in column temperature. At night the temperature in the lab often falls because of the ventilation system, often causing a shift in retention times. Accordingly, in order to obtain reliable quantification, it is of the highest importance to control the column temperature. A higher column temperature is also beneficial since the retention time decreases and the peak efficiency increases. At 45°C the retention of ropivacaine decreases by about 20% compared with ambient temperature and during a normal batch of analysis, over 36 h, a low variation of retention times (RSD < 0.5%) was generally observed.

Quantitative determination

The concentration of metabolites in urine samples normally varies a lot, depending on the volume of urine excreted during a given sampling interval. For example, during a 2-h sampling period the volume can vary between 70 and 800 mL (Halldin *et al.*, 1996). This must be taken into consideration when planning the analytical protocol. Often the samples need to be diluted prior to analysis in order to fall within the linear range of the method, since a very high concentration may occur, e.g. concentrations above 1 mM of 3-OH-ropivacaine were found.

The major question of interest in metabolic studies is to determine the amount of metabolites and parent drug excreted in different time intervals after sampling. The result is often given as a molar percentage of the given dose. The metabolism is followed in fractions >0.1% of the given dose and the requirements of the bioanalytical method were set accordingly. The limit of quantification (LOQ) was set to allow the possibility of determining 0.1% of a given dose. In urine samples the LOQ was about 1 μM for all the analytes. The limit of detection is usually at least 10 times lower, i.e. the urine matrix will give a high background which results in an increased LOQ and the LOQ will vary with the water content of the urine sample. In plasma samples the background from the matrix was smaller and the LOQ was about 0.1 μM for all of the analytes.

Validation

The method is generally highly selective for both the parent drug (ropivacaine and bupivacaine) and their metabolites studied. Chromatograms where known metabolites are added are shown for ropivacaine metabolites (Fig. 2 upper) and bupivacaine metabolites (Fig. 4 upper). The blank samples generally show no peak liable to interfere with the analytes (Fig. 2 middle, Fig. 3 upper and Fig. 4 middle). The LOQ was set at 1 μM for all of the analytes in urine samples and at 0.1 μM in plasma samples. Typical chromatograms from dosed subjects are given in Fig. 2 lower (ropivacaine, human urine), Fig. 3 lower (ropivacaine, human plasma) and Fig. 4 lower (bupivacaine, sheep urine).

The method is linear, at least within the concentration

Table 5. Between-day precision and accuracy of ropivacaine and metabolites in human plasma

Analyte	Concentration (μM)	Accuracy (%)	Precision (RSD%)	*n*
3-OH-ropivacaine	2.9	103	1.1	6
	2.1	102	1.0	6
	0.8	105	2.7	6
PPX	14.6	102	0.9	6
	7.3	101	1.2	6
	1.5	102	2.8	6
Ropivacaine	19.3	101	0.9	6
	7.7	102	1.9	6
	1.9	101	3.0	6

Table 6. Between-day precision and accuracy of bupivacaine and metabolites in sheep urine

Analyte	Concentration (μM)	Accuracy (%)	Precision (RSD%)	*n*
3-OH-bupivacaine	180.9	102	2.2	35
	10.1	102	5.8	36
PPX	10.3	98	2.0	35
	3.1	99	3.3	36
3-OH-PPX	27.2	98	2.2	35
	4.1	98	4.5	36
Bupivacaine	19.8	100	1.9	35
	2.9	102	4.1	36

ranges described in Table 2. The standard curves used for urine samples show a very good performance and the back-calculated concentrations were generally within ±3% of the nominal concentration values. A slightly higher variation was obtained in plasma standard curves where the back-calculated concentrations often were within ±5% for non hydrolysed standards and ±10% for hydrolysed standards of their nominal values, respectively. The accuracy and precision of the methods are high (Tables 4–6), and for all the analytes the recovery was close to 100% and the precision (RSD) often less than 2%.

During storage of the samples at −20°C no significant losses of analytes were observed, i.e. ropivacaine and its metabolites studied were stable for at least 3 years in both human urine and human plasma, whereas bupivacaine and its metabolites studied were stable for at least 22 months in sheep urine.

The method has successfully been applied in metabolic studies of ropivacaine in man and bupivacaine in sheep and so far more than 1000 urine samples and hundreds of plasma samples have been analysed. The method is highly accurate and selective; however, as each chromatogram takes 1 h, the sample throughput is low.

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