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Determination of ropivacaine and [$^2H_3$]ropivacaine in biological samples by gas chromatography with nitrogen–phosphorus detection or mass spectrometry

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Abstract

Bioanalytical methods for determining the total concentration of the new local anaesthetic drug ropivacaine in blood plasma, urine and tissues are presented. Ropivacaine is a drug mainly used in connection with surgery and for post-operative pain relief. The biological samples were prepared using liquid–liquid extraction and analysed using capillary gas chromatography with nitrogen–phosphorus detection or mass spectrometry. The methods are highly selective and reliable with a between-day precision, given as the relative standard deviation, generally below 6%. More than 20 000 samples have been analysed using the methods described. © 1998 Elsevier Science B.V.

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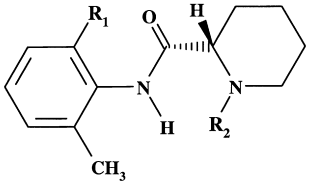
1. Introduction

Ropivacaine hydrochloride monohydrate (ropivacaine, Fig. 1) is a recently developed, long-acting local anaesthetic drug of the amide type structurally related to bupivacaine and mepivacaine. Ropivacaine takes the form of the *S*-enantiomer, whereas bupivacaine and mepivacaine are racemic mixtures. In preclinical studies [1] as well as in studies on human volunteers [2], ropivacaine shows less central nervous system and cardiac toxicity than bupivacaine. Ropivacaine is mainly used for surgical anaesthesia, during labour and for post operative



	R_1	R_2
Ropivacaine	CH_3	C_3H_7
[$^2H_3$]Ropivacaine	C^2H_3	C_3H_7
[$^2H_7$]Ropivacaine	CH_3	$C_3{}^2H_7$
Pentycaine	CH_3	C_5H_{11}

Fig. 1. Chemical structures.

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pain relief. In the development of the new drug, the concentration of ropivacaine in biological fluids and tissues was determined in a number of pharmacokinetic, pharmacodynamic and toxicological studies, e.g., [3–13] using the methods described in this paper.

A number of papers on the bioanalysis of local anaesthetics are available, e.g., including liquid chromatography (LC) with UV detection [14–18] and gas chromatography with nitrogen–phosphorus detection (GC–NPD) or mass spectrometry (GC–MS) [18–21]. The methods, for determination of the free concentration of ropivacaine [22] and the major ropivacaine metabolites [23], based on LC, were previously published. These methods [19,22,23] determined the racemate of ropivacaine. Since ropivacaine is given as the single *S*-enantiomer the lack of metabolic racemisation in vivo has been confirmed [24] by studying urine samples from different species, taken after the administration of ropivacaine.

The present paper will describe the methods used in our laboratory for the determination of the total concentration of the ropivacaine, in blood plasma, urine and tissues, using capillary GC with either NPD or MS, with selective ion monitoring. The methodology is based on a previously published method, including liquid–liquid extraction of ropivacaine, in blood plasma, and GC–NPD [19]. Modifications were made to apply this method to analysis of urine and tissue samples and to increase the sensitivity. Additionally, a more selective mass spectrometric method was developed to be able to distinguish between deuterium-labelled and unlabelled ropivacaine. This method was needed in order to further study the pharmacokinetics of ropivacaine, when labelled and unlabelled substance was administered simultaneously by different routes [25]. This paper will also include a method comparison as well as statistics on their long-term use.

2. Experimental

2.1. Chemicals and reagents

Ropivacaine, (*S*)-(−)-1-propyl-2′,6′-pipecoloxylidide hydrochloride monohydrate (M_r=328.9), [$^2H_3$]ropivacaine, (*S*)-(−)-1-propyl-2′-($^2H_3$)methyl-6′-methyl-pipecoloanilide hydrochloride monohydrate (M_r=331.9), and the internal standards pentycaine, 1-pentyl-2′,6′pipecoloxylidide hydrochloride (M_r=338.9) and [$^2H_7$]ropivacaine and pentycaine (*S*)-(−)-1-($^2H_7$)propyl-2′,6′-pipecoloxylidide hydrochloride monohydrate (M_r=335.9), (Fig. 1), were obtained from Astra Pharmaceutical Chemicals and the department of Medicinal Chemistry at Astra Pain Control (Södertälje, Sweden). Their chemical purities were determined to be 99.9%, 99.4%, 99.8% and 99.9%, respectively, determined by GC or LC. The degrees of labelling determined by mass spectral analysis were 99.7% ^2H and 99.9% ^2H for [$^2H_3$]ropivacaine and [$^2H_7$]ropivacaine, respectively. All the other chemicals were of analytical grade or better.

2.2. Analytical method

2.2.1. Sample preparation

2.2.1.1. *Determination of ropivacaine in plasma and urine.* To the plasma or urine sample (0.250–1.00 ml) an internal standard solution (pentycaine or [$^2H_7$]ropivacaine) and 0.5 ml of 10% (w/v) sodium carbonate solution were added to give a final aqueous volume of less than 2 ml. A liquid–liquid extraction was performed using 4 ml of *n*-heptane–methylene chloride (4:1, v/v), under slow rotation for 20 min. After centrifugation for 10 min at 1000 *g*, the organic phase was evaporated to dryness under a stream of nitrogen at 40°C (Turbo Vap, Zymark, Hopkington, MA, USA). The residue was redissolved in 200 μl of *n*-heptane–ethanol (9:1, v/v), and an aliquot of 1 μl was injected onto the GC system using either NP or MS detection.

2.2.1.2. *Determination of ropivacaine in rat tissues.* The tissue (300–800 mg) was completely homogenised mechanically (Ultra Turrax T25, IKA-Larbortechnik, Staufen, Germany) together with 0.5 ml of an internal standard solution (pentycaine) in 5 ml of cold acetic acid diluted in water (brain and heart) or acetone (spleen, kidney, liver and lung) to give a concentration of 1 *M*. To ensure complete transfer of the homogenate, the piston was washed with 1–2 ml of water. The homogenate was centrifuged for 10

min at 1000 *g* and the supernatant was washed with 5 ml of *n*-heptane. The aqueous phase was alkalised with 1 ml of 10 *M* NaOH, extracted with 6 ml of *n*-heptane–methylene chloride solution (4:1, v/v) and further processed as described above. The samples were analysed by GC–NPD according to the previously published method [19].

2.2.2. GC–NPD

The gas chromatograph, HP5890 series II (Hewlett-Packard, Palo Alto, CA, USA), was equipped with a splitless injector and a nitrogen–phosphorus sensitive detector. The fused-silica capillary column, 25 m×0.32 mm I.D., was crosslinked with a methyl siloxane phase, 0.17 μm (Ultra-1, Hewlett-Packard). The splitless injections were performed using an automatic sampler (7673, Hewlett-Packard). The injector temperature was set at 260°C and the purge activation time was set at 1 min. The column temperature was set at 80°C for 1 min, raised to 280°C at a rate of 40°C/min and then kept at a constant temperature for 3 min. The temperature of the detector was set at 300°C. Helium was used as carrier gas and the linear velocity was 56 cm/s at 80°C, with an inlet pressure of 100 kPa. The chromatograms were recorded and integrated and the results were calculated on-line using P.E. Nelson Access Chrom (Perkin-Elmer Nelson Systems, Cupertino, CA, USA).

2.2.3. GC–MS

The mass spectrometer was a single-stage quadrupole, SSQ 710 (Finnigan MAT, San Jose, CA, USA), coupled with a gas chromatograph (HP 5890 series II). The fused-silica capillary column, 25 m× 0.2 mm I.D., was crosslinked with a 5% phenyl methyl siloxane phase, 0.25 μm (BPX5, SGE Analytical products, USA). Splitless injections were performed with an automatic sampler (CTC A 200 S, Finnigan MAT). The injector temperature was set at 250°C and the purge activation time was set at 1 min. The column temperature was set at 90°C for 1 min, raised to 260°C at a rate of 40°C/min and maintained for 4 min. Helium was used as carrier gas and the linear velocity was 17 cm/s at 90°C, with an inlet pressure of 76 kPa.

The mass spectrometer was operated in the chemical ionisation (CI) mode with NH_3 (g) at an ion source pressure of 1.1–1.2 kPa. The temperatures of the transfer line and the ion source were set at 290°C and 120°C, respectively. The data was recorded in selected-ion monitoring (SIM) mode at an electron energy of 150 eV. The $[M+H]^+$ ions of interest were *m/z* 275, *m/z* 278 and *m/z* 282 for ropivacaine, $[^2H_3]$ropivacaine and $[^2H_7]$ropivacaine, respectively. The chromatograms were interpreted with a DEC workstation, with the Ultrix operating system and ICIS II application software.

2.2.4. Standards and quality-control samples

Two standard stock solutions (about 7 mg/50 ml) of ropivacaine were prepared in deionised water and stored in a refrigerator. The standards and control samples were prepared by adding diluted standard stock solution in drug-free plasma or urine. For GC–NPD the standard concentration level (single-point calibration, $n=6$) was about 1000 n*M*. For GC–MS the range of the standard concentrations was about 30–4000 n*M*. The quality-control samples were prepared in blank plasma or urine at three concentration levels in the range of 30–5000 n*M*. In determination of tissue samples the standards were prepared in water and quality control samples were prepared in blank plasma.

2.2.5. Determination of unknown samples

In the GC–NPD method the calibration is based on a single-point calibration. The mean value of the peak-height ratios of ropivacaine and pentycaine from six standards at one concentration level was used for calibration. In the GC–MS method a standard curve was constructed using peak-area ratios of either ropivacaine or $[^2H_3]$ropivacaine and $[^2H_7]$ropivacaine versus the concentration. The concentrations of unknown samples were calculated by means of unweighted linear regression analysis. In addition, in each batch for analysis-quality control samples at three different concentration levels were determined in duplicate. In the GC automatic sampler the standards and quality-control samples were spread among the unknown samples.

2.3. Sampling and storage

Blood samples were collected in heparinised Venoject tubes with green stoppers. The blood was

centrifuged within 1 h of collection. The plasma was transferred to polypropylene tubes, frozen within 30 min and stored at −20°C until analysis. Urine samples were collected via catheters, and stored in polypropylene vessels at −20°C until analysis. Tissue samples were also stored at −20°C until analysis.

2.4. Method validation

The stability was tested in samples where ropivacaine was added to drug-free biological fluid. The samples were kept at room temperature and in a refrigerator (+4°C) for one week, as well as being frozen at −20°C for at least seven months.

The recovery of ropivacaine from plasma and urine was determined by comparing the ratio of the slopes from calibration curves, using two different experimental conditions. In the first experiment, the standards in plasma or urine were processed according to the method, while in the second experiment drug-free plasma and urine including internal standard was extracted and ropivacaine was added just after the evaporation step, in exactly the same amount as in the first experiment. The recovery from rat tissues was determined by the ratio of concentrations obtained after extraction of tissues with ropivacaine added and unextracted ropivacaine standard solution. In the first experiment a known amount of ropivacaine was added to the tissues before homogenisation and then the samples were processed according to the analytical method. After the evaporation a fixed volume of pentycaine in ethanol was added. In the second experiment the ropivacaine standard solution was evaporated and redissolved in the same fixed volume of pentycaine as the extracted samples.

Linearity was checked in the concentration ranges of 30–6000 n*M* (0.5 ml sample) and 30–4000 n*M* (0.5 ml plasma), for GC–NPD and GC–MS, respectively. The limit of detection (LOD) was set at three-times the noise of the detection signal. Selectivity was tested in drug-free plasma samples from human, dog, rat, rabbit, sheep, cat, mouse, in drug-free human urine and in drug-free tissues from rat.

The within-day precision and accuracy were determined from quality-control samples ($n=5$–9), usually at three different concentration levels. The between-day precision and accuracy over three days were determined from quality-control samples prepared at three different concentration levels.

2.5. Method comparison

To compare the GC–NPD and the GC–MS methods 61 plasma samples from dosed subjects were determined. The difference between the sample concentrations obtained by GC–MS and GC–NPD was plotted against the mean of each pair of samples. Linear regression (Deming regression [26]), paired *t*-test and corresponding confidence interval, were used to assess potential systematic differences between the methods.

3. Results and discussion

The method described in this paper is based on a previously published method from our laboratory [19]. Some modifications of the extraction procedure and the GC were made, which resulted in improved precision and improved sensitivity compared to the old method in determination of plasma samples. Additionally, the developed sample work-up procedures for tissue samples is described and applied to the method [19]. MS detection was implemented for simultaneous determination of ropivacaine and [$^2H_3$]ropivacaine in plasma samples.

3.1. Method development

As ropivacaine is a hydrophobic amine (pK_a 8.0), it is easily extracted from an alkaline aqueous solution by organic solvents. In the modified method, heptane was replaced by hexane in the extraction procedure. The shift was made due to working environmental reasons and no change in the performance of the method was observed. The extraction recovery from blood plasma and urine were 93% and 96%, respectively, which is similar to that reported for plasma in the previously published method [19]. To minimise emulsion and to obtain a high recovery in the extraction procedure the tubes were slowly rotated for 20 min. In the development of extraction conditions for rat tissues the homogenates were acidified to release ropivacaine from the

tissues, and for some tissues acetone was added to increase the recovery. The extraction recovery was in the range 60–100%, depending on the tissue. Typical chromatograms obtained from human urine (Fig. 2) and rat liver tissue (Fig. 3) after administration of ropivacaine, show that ropivacaine is easily resolved using the previously published GC conditions [19]. However, the sensitivity of the method was enhanced by increasing the column diameter and the purge activating time. To concentrate the analyte at the top of the column, the initial column temperature was lowered to 80–90°C and to shorten the analysis time, the final temperature was increased to 260–280°C. The choice of column is not critical, i.e., most kinds of non-polar columns can be used with this method. Using the GC conditions described, ropivacaine is easily selectively resolved from human plasma (Fig. 4).

3.2. Mass spectrometry

Ropivacaine and the deuterium-labelled analogue, [$^2H_7$]ropivacaine, used as internal standard may be determined by SIM either by chemical ionisation (CI) or by electron impact ionisation (EI). In the CI mode the pseudomolecular ion, $[M+H]^+$, appeared as the most abundant ion in the mass spectra of both unlabelled and labelled ropivacaine, m/z 275 and 282, respectively. In addition to the pseudomolecular ions, the mass spectra showed one fragment ion of relative intensity >2% (except for natural isotopes), at m/z 126 and at m/z 133, for ropivacaine and [$^2H_7$]ropivacaine, respectively. This means that the deuterium-label is in the fragment ion of [$^2H_7$]ropivacaine. When carrying out EI of these compounds, the molecular ion is not detectable and the base peaks are the similar fragment ions at m/z 126 for ropivacaine and m/z 133 for [$^2H_7$]ropivacaine, respectively. On the other hand, when there is a need to measure ropivacaine and [$^2H_3$]ropivacaine simultaneously, the best way is to monitor the $[M+H]^+$ ions (m/z 275 and 278) in the CI mode, since in the EI mode the fragment ion m/z 126 (base peak) of [$^2H_3$]ropivacaine does not contain the deuterium label. Consequently, it is not possible to distinguish it from unlabelled ropivacaine in the EI mode. Typical mass chromatograms in the CI

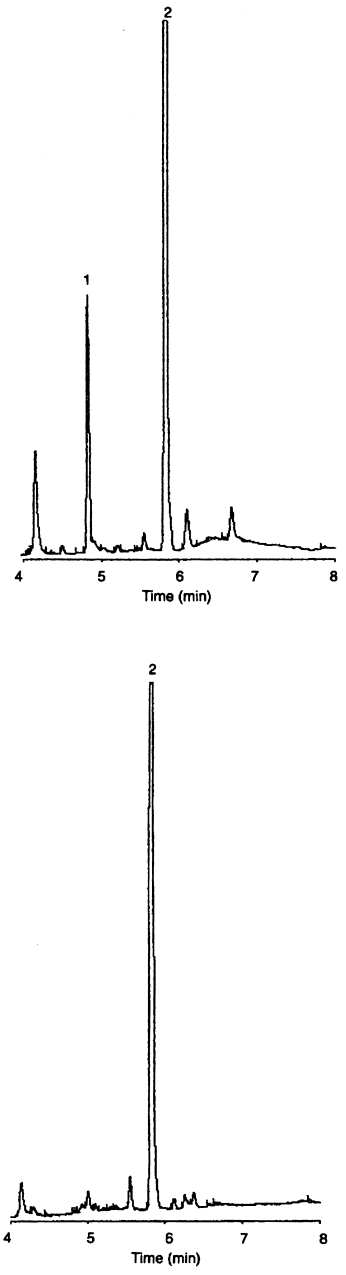

Fig. 2. Gas chromatograms of urine samples. The sample preparation conditions were according to the analytical method and the GC conditions were similar to Ref. [19]. Column: fused-silica capillary column, 12 m×0.22 mm I.D., crosslinked with a methyl siloxane phase, 0.33 μm (HP-1, Hewlett-Packard). Temperature program: 150°C for 1 min, raised to 250°C at a rate of 40°C/min and then kept at a constant temperature for 5 min. Upper: human urine taken after i.v. administration of ropivacaine, ropivacaine 0.76 μ*M*. Lower: drug-free human urine with internal standard. Peaks: 1=ropivacaine, 2=pentycaine (internal standard).

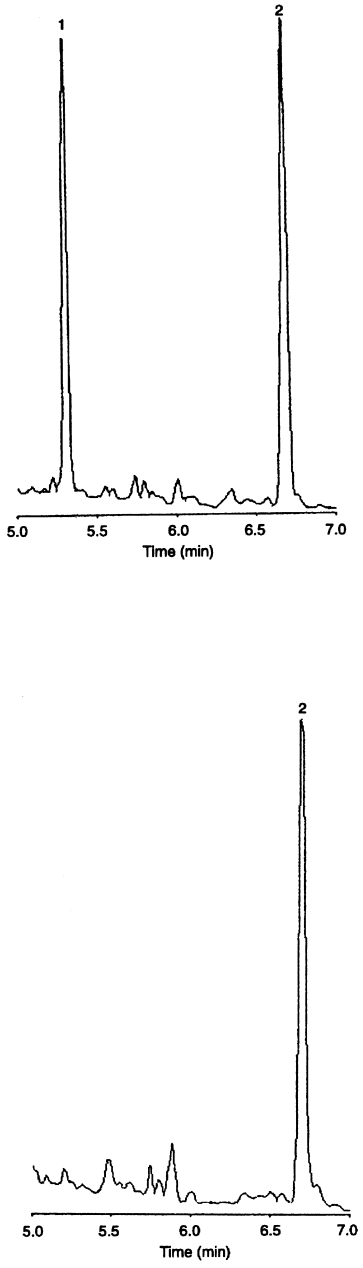

Fig. 3. Gas chromatograms of rat liver samples. The sample preparation conditions were according to the analytical method and the GC conditions were similar to Ref. [19]. Column: fused-silica capillary column, 12 m×0.22 mm I.D., crosslinked with a methyl siloxane phase, 0.33 μm (HP-1, Hewlett-Packard). Temperature program: 150°C for 1 min, raised to 250°C at a rate of 40°C/min and then kept at a constant temperature for 5 min. Upper: rat liver tissue taken after i.v. administration of ropivacaine to rat, ropivacaine 1.70 μ*M*. Lower: drug-free rat liver tissue with internal standard. Peaks: 1=ropivacaine, 2=pentycaine (internal standard).

mode of the selected ions, *m*/*z* 275, 278 and 282, are shown in Fig. 5.

The use of a deuterium-labelled internal standard, which is chemically very similar to the analytes, gives a high accuracy of measurement. A lack of this type of internal standard may cause problems relating to sensitivity or accuracy, reported for determinations of lidocaine and bupivacaine [27]. The contribution of unlabelled ropivacaine from the deuterium-labelled analogues was determined to be <0.5% while the contribution of naturally occurring isotopes from the unlabelled ropivacaine to [$^2H_3$]ropivacaine was <0.4%. To avoid contribution from the internal standard in the assay, the concentration of internal standard should be less than 0.5 μ*M*. In order to get a good response and still avoid deuterium exchange it was important to optimise the temperature of the ion source. At a high temperature (250°C) there was an increased response but also an exchange of deuterium.

3.3. Method validation

The results of the stability studies indicated no substantial degradation of ropivacaine. Ropivacaine and [$^2H_3$]ropivacaine in blood plasma was stable at room temperature and at 4°C for at least four days and at −20°C unlabelled ropivacaine was stable for at least 30 months and [$^2H_3$]ropivacaine for at least seven months, respectively. Ropivacaine in urine was stable at room temperature and at 4°C for at least seven days and at −20°C for at least 30 months.

A GC–NPD calibration curve in the concentration range 30–6200 n*M* was well described by a linear function, with a coefficient of determination $r^2 \geq$ 0.99, and the back-calculated concentrations compared to nominal concentrations were within ±6% for ropivacaine. The GC–MS calibration in the concentration range 30–4000 n*M* was best described by two straight lines (30–300 n*M* and 300–4000 n*M*), $r^2 \geq 0.99$. The back-calculated concentrations compared to nominal concentrations were within ±7% for [$^2H_3$]ropivacaine and within ±3% for ropivacaine, respectively.

Selectivity was obtained and is shown in chromatograms of samples from drug-free plasma, urine and rat liver tissue and of samples from dosed subjects (Figs. 2–4). Similar results, i.e., no peak liable to interference with ropivacaine were found in

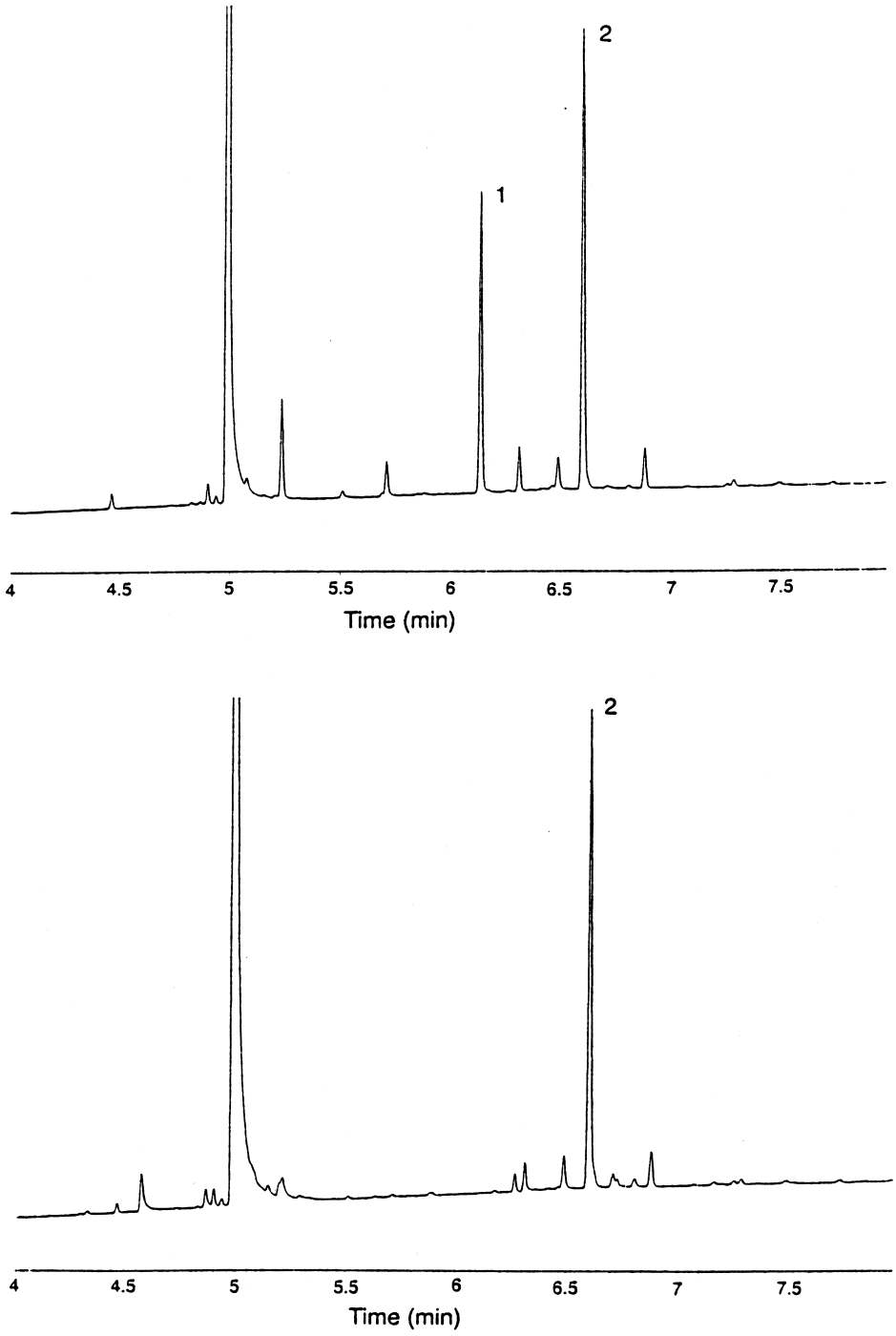

Fig. 4. Gas chromatograms of blood plasma samples. The experimental conditions were according to the analytical method. Upper: human blood plasma taken after epidural administration of ropivacaine to man, ropivacaine 2.0 μ*M*. Lower: drug-free human blood plasma with internal standard. Peaks: 1=ropivacaine, 2=pentycaine (internal standard).

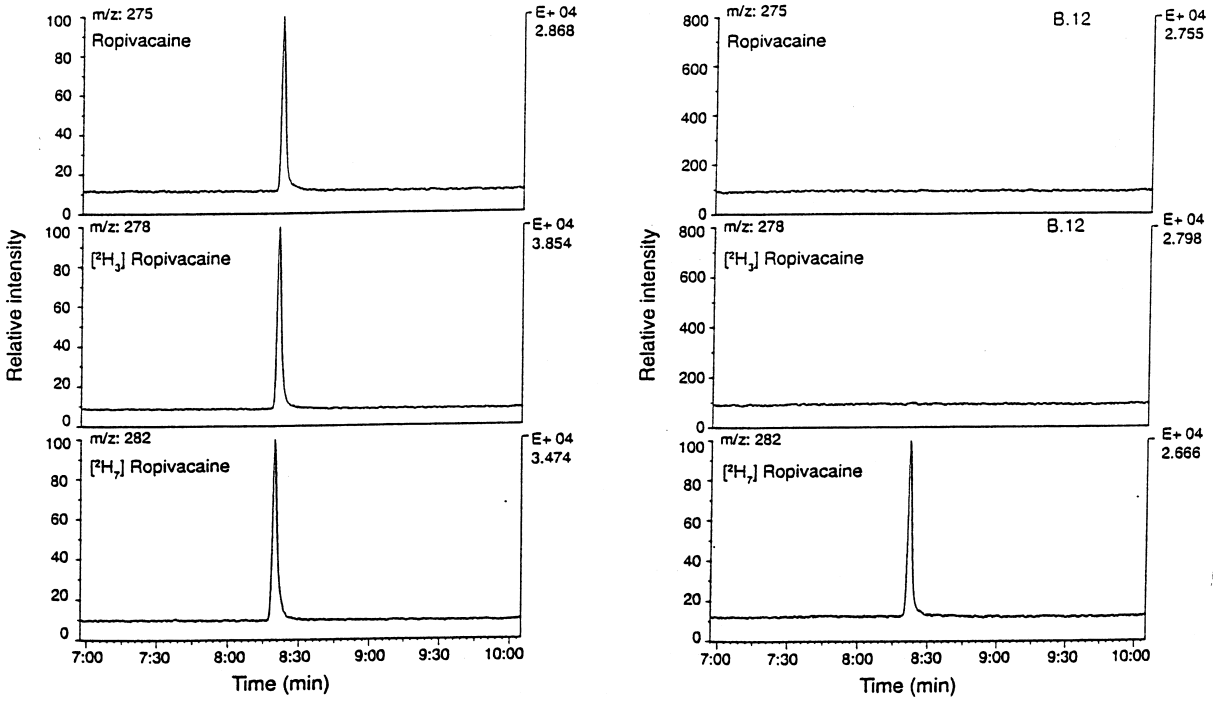

Fig. 5. GC–MS selected-ion monitoring chromatograms of human plasma samples. The experimental conditions were according to the analytical method. Left: human drug-free plasma after addition of 0.43 μ*M* of ropivacaine (*m*/*z* 275), 0.54 μ*M* of [$^2H_3$]ropivacaine (*m*/*z* 278) and 0.50 μ*M* of [$^2H_7$]ropivacaine (*m*/*z* 282, internal standard). Right: human drug-free plasma sample with 0.38 μ*M* of [$^2H_7$]ropivacaine (internal standard).

drug-free plasma from man, rat, dog, rabbit, sheep and mouse as well as in drug-free urine from man and drug free tissues from rat.

The lowest concentration determined by the method was generally set at 30 n*M* (0.5 ml plasma) but for tissues at 40–100 nmol/kg (0.5 g tissue). The relative standard deviations (R.S.D.s) of between-day precision at this concentration level (for standards) were usually below 10%. Generally, no attempt was made to reach a lower level because in most cases there was no need for determination of lower concentrations in clinical and preclinical studies. The LOD for the methods is below 2 n*M* in plasma, and if it is necessary to determine lower concentrations, the sample amount may be increased, the redissolving volume decreased or the injected volume increased.

The between-day accuracy given in per cent of nominal concentration of quality-control samples in plasma and urine was close to 100% and the R.S.D. was less than 10% (Table 1). Validation of the GC–MS method for determination of [$^2H_3$]ropivacaine in plasma shows similar results as for ropivacaine (Table 1). In the concentration range 30–2700 n*M* the accuracy for [$^2H_3$]ropivacaine was close to 100% and the between-day precision was 1–6% (R.S.D.). In analysis of tissue samples the standards were made in water and the quality control samples were made in blood plasma. The accuracy of the procedure was confirmed in the validation i.e., the same results were obtained after addition of ropivacaine to water, blood plasma and tissue samples. The within-day precision (R.S.D.) in all of the tissue sample types investigated were below 13% (Table 2).

3.4. Method comparison

The results obtained, from the GC–MS and GC–NPD methods, in the concentration range 180–5300

Table 1
Between-day precision and accuracy of ropivacaine and [$^2H_3$]ropivacaine in blood plasma and urine

Method	Analyte	Sample type	Nominal concentration (μM)	Accuracy (%)	R.S.D. (%)	*n*
GC–NPD	Ropivacaine	Plasma	0.10	96	5.7	19
			2.10	96	2.5	20
			5.14	98	3.2	20
GC–NPD	Ropivacaine	Urine	0.12	105	9.4	21
			1.26	100	6.7	23
			5.04	100	5.9	14
GC–MS	Ropivacaine	Plasma	0.04	101	3.8	29
			0.40	100	2.2	29
			3.97	97	2.5	22
	[$^2H_3$]Ropivacaine	Plasma	0.03	98	6.5	30
			0.27	100	1.4	29
			2.74	101	2.2	29

n*M* were examined to assess various types of differences. Plots of the differences between the two methods against their average, both for original data and log-transformed data (Fig. 6) [28], do not reveal any systematic differences. A linear regression minimising the perpendicular distances (Deming regression [26]) was performed on the log-transformed data. The estimated regression equation was log $C_{MS} = -0.011 + 1.00 \cdot \log C_{NPD}$, where C_{MS} and C_{NPD} are the concentrations obtained from the GC–MS and GC–NPD methods, respectively. The intercept and slope were not significantly different from zero or one (95% confidence intervals −0.022 to 0.001 and 0.97 to 1.03), indicating that there are no

Table 2
Within-day precision and accuracy of ropivacaine determined in different tissues by GC–NPD

Sample type	Added amount of ropivacaine (pmol)	Accuracy (%)	R.S.D. (%)	*n*
Rat brain (whole)	176	96	7.2	6
	440	98	2.6	5
Rat heart (whole)	41	94	8.5	6
	407	95	5.7	5
Rat lung (one out of two)	41	81	7.9	4
	1220	94	2.8	5
Rat liver (1/4)	41	109	5.5	6
	1220	90	2.8	6
Rat spleen (whole)	102	116	12.7	5
	1220	100	4.1	5
Rat kidney (one out of two)	82	88	9.5	5
	328	96	6.9	6

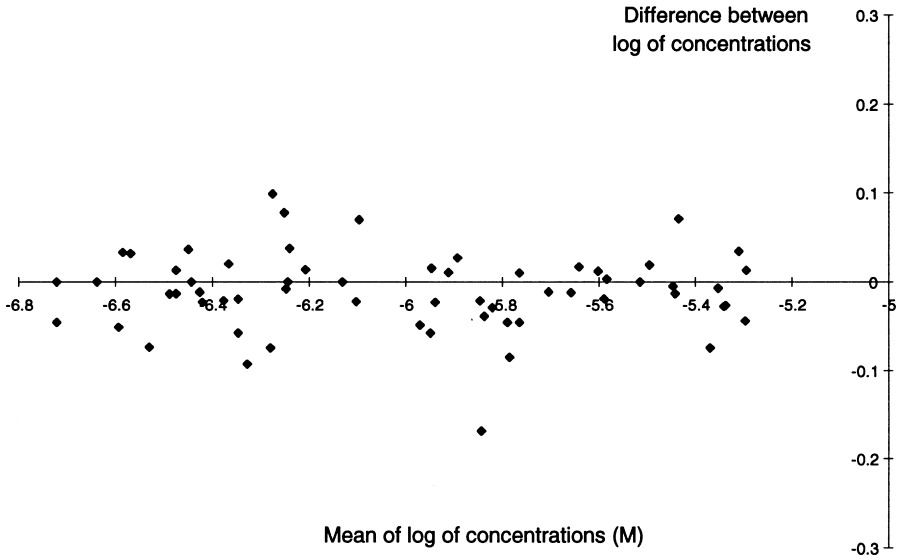

Fig. 6. Comparison between the GC–MS and GC–NPD methods.

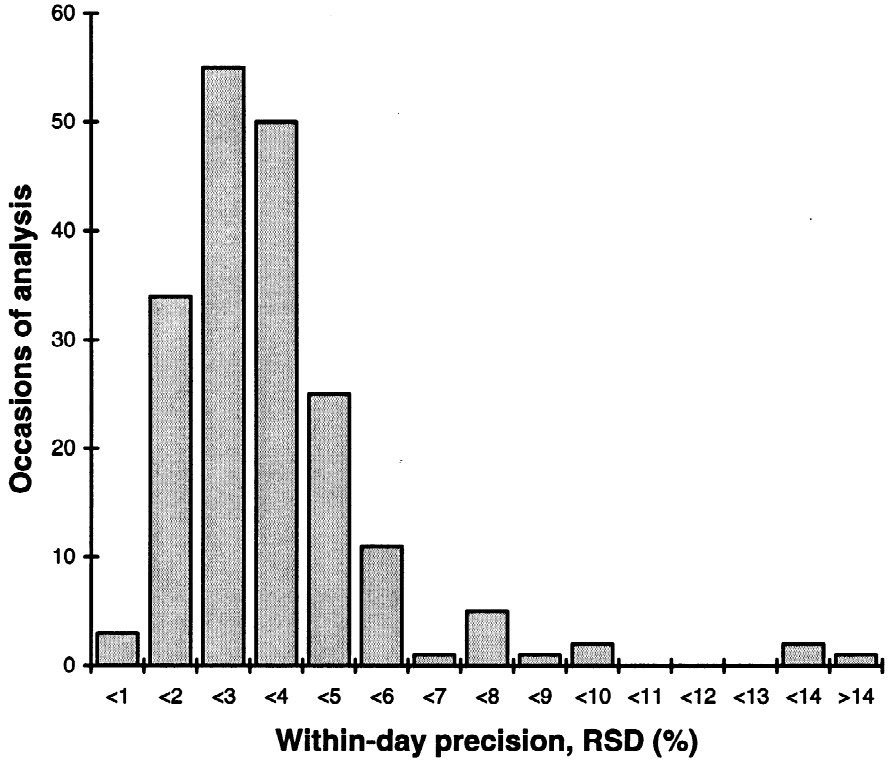

Fig. 7. Within-day precision (R.S.D.) during long-term use of the GC–NPD method.

noticeable proportional or non-linear differences between the methods. Furthermore, paired *t*-tests were performed both on the log-transformed data (mean difference = −0.011, confidence interval −0.022 to 0.0004, $p = 0.06$) and on the original data (mean difference = −0.037, confidence interval −0.086 to 0.011, $p = 0.13$). These results confirm the absence of substantial proportional and additive differences between the methods.

3.5. Application of method

The GC–NPD method for the determination of ropivacaine was applied in several preclinical and clinical studies, e.g., [3–13,23]. Until now the method has been used for more than 20 000 plasma samples, 500 urine samples and about 200 tissue samples, respectively. Each individual batch of analysis consists of 35–50 unknown samples, 6–8 quality-control samples as well as standards and blanks. In the acceptance criteria for the method the results of the quality-control samples should be within ±10% of their nominal concentration, which was somewhat stringent compared to the routines described by Shah et al. [29]. Generally, more than 95% of the batches of analysis were accepted directly using this acceptance criteria. The method is robust and reliable in long-term usage. Results from about 200 batches for analysis (Fig. 7) show that the within-day precision (R.S.D.) was generally less than 5%.

The GC–MS method was applied in determination of ropivacaine and [$^2H_3$]ropivacaine simultaneous [25] and in a few other studies for determination of ropivacaine e.g., [7] and totally about 700 samples have been determined so far using this method.

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