Determination of Ropivacaine and Its Metabolites in Human Plasma Using Solid Phase Microextraction and GC-NPD / GC-MS

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Abstract: The performance of solid-phase microextraction (SPME) in combination with capillary gas chromatography (CGC) to quantify ropivacaine and its metabolites in human plasma was investigated. The analysis was performed using either a nitrogen phosphorus detector (NPD) or a mass-spectrometric detector. For extraction, Carbowax/divinylbenzene, polyacrylate and polydimethylsiloxane fibers were tested. Absorption and desorption times were studied for all analytes separately. The Carbowax/divinylbenzene fiber gave the highest recovery in plasma samples as compared to the other fibers. The effects of temperature, addition of salt, and agitation of the sample were studied. The validation of the method showed that the chromatographic selectivity was satisfactory and all metabolites were well separated. SPME gave higher deviation as compared to published data for solid-phase and liquid-liquid extraction as sample preparation methods but the acceptance criteria for the study validation were well in line with the international criteria. The major disadvantage of SPME in quantitative bioanalysis is that the fiber does not withstand a complete run (standards + blanks + QC samples + patient samples). Also, the quality of fiber and the fiber length can differ from batch to batch. J Micro Sep 13: 313–321, 2001

Key words: *SPME; GC-NDP; GC-MS; ropivacaine and metabolites*

1. INTRODUCTION

Ropivacaine is a new amide-type local anaesthetic drug, mainly used for surgery and for postoperative pain relief. Also, it has lower central nervous and cardiotoxic potential than its predecessor, bupivacaine [1].

Solid-phase microextraction, SPME, is a simple, solvent-free sample preparation method for gas chromatography, GC, and liquid chromatography, LC. This extraction technique is rapid and easy to handle. It is also easily automated and it shows good linearity for many analytes. SPME has been introduced as an alternative to current sample-preparation technology. Today the technique is applied to extract a wide range of analytes in many areas. The extraction is based on the partitioning of the analyte between the organic phase on the fused silica fiber and the matrix. Many factors, such as pH, temperature, salt concentration, and stirring, affect the equilibrium constant and the equilibration time [2]. Number of publications on SPME involving drugs and their metabolites in human urine or plasma have been reported [2–18]. However, only a few publications deal with optimization of SPME for plasma analysis [11–13,16–18].

Sample preparation is an important part in the analysis of pharmaceutical compounds in body fluids. The aim of a sample-preparation method is to eliminate interfering substances from biological samples. Further, it should be reproducible, easy to handle, easy to automate and should require a minimum number of steps. Liquid-liquid extraction (LLE)

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and solid-phase extraction (SPE) take longer times and they involve more steps as compared to SPME [19,20].

The extraction of drug metabolites from biological material is often difficult due to the high polarity of metabolites. The aim of the present investigation was to evaluate SPME as a sample-preparation tool to determine ropivacaine and its metabolites in human plasma by GC-NPD and GC-MS. The effects of different fibers with different polarities on the recovery were studied. The effects of agitation, pH, temperature, and salt addition on the amount extracted were also investigated.

2. EXPERIMENTAL

2.1. Instrumentation. The gas chromatographic analysis was performed using a Hewlett-Packard model HP 5890 Series II, equipped with a split-splitless injector and a nitrogen-phosphorus detector (NPD). A Varian 8200 CX autosampler with SPME III was used. Sample agitation was applied. The autosampler was equipped with an autotherm controller (Strumenti Scientific, Padova, Italy) with a temperature range of 5–70°C. The autotherm controller was used to control the sample temperature. A ChemStation data system (HP 3365 Series II version A.06.03) was used for data processing. The GC oven temperature was programmed for an initial hold of 3 min at 80°C to focus the ropivacaine and its metabolites in the beginning of the column, after which the temperature was increased at 40°C min^{-1}, to 280°C. The injector and detector temperatures were 250°C and 300°C, respectively.

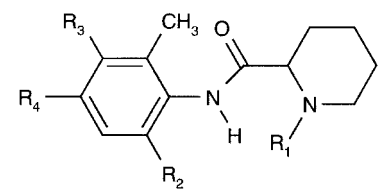
The GC-MS system consisted of a HP 6890-Plus gas chromatograph and a mass selective detector model 5973 (Palo Alto, CA, USA) equipped with a programmed temperature vaporizer (PTV) and Combi Pal SPME autosampler (CTC Analytics AG, Zwingen, Switzerland). The PTV system was an OPTIC 2 (ATAS International, Veldhoven, the Netherlands). The injection mode was splitless at three minutes. Injector temperature was set at 90°C and after ten seconds increased by 5°C s^{-1} to 280°C. The GC oven temperature was programmed for an initial hold of 3 min at 90°C, the temperature was increased at 50°C min^{-1}, to 280°C. The initial and transfer pressure was 8 psi with a transfer time of one minute and the final pressure was set to 18 psi. Injector temperature programming was used, as a manufacturer recommendation, to extend the fiber lifetime and also for thermally labile substances.

Conditions for MS measurements were MS transfer line at 280°C, ion source at 230°C, electron impact ionization at 70 eV, SIM mode with dwell time 50 ms, solvent delay, 8 min. The ions corresponding to ropivacaine, 3-OH-ropivacaine, 4-OH-ropivacaine, PPX and internal standard are m/z: 126, 126, 126, 84, and 133, respectively. A MSD ChemStation data system (version B.01.00) was used for data processing. The column used (for GC-NPD and GC-MS) was an HP50 (50% phenyldimethylpolysiloxane) fused-silica capillary column (25 m × 0.25 mm i.d., 0.31 μM thickness) obtained from Hewlett-Packard (Palo Alto, CA, USA).

Helium was used as carrier and as makeup gas. Helium, air and hydrogen were of high purity grade and obtained from AGA (Lidingö, Sweden). The gas flow rates were 2 ml/min (helium), 4 ml/min (hydrogen), 100 ml/min (air) and the makeup gas was 27 ml/min (nitrogen). The flow rates were measured using a digital flow meter (J & W Scientific Fisons, USA).

Columns. For method development for GC, seven fused silica capillary columns from different manufacturers covering a wide range of polarity were tested. DB17 (50% phenyldimethylpolysiloxane) was purchased from J & W Scientific (CA, USA). CP-Sil 5CB (dimethylpolysiloxane) was obtained from Chrompack Nederland BV (the Netherlands). BPX70 (70% cyanopropyl-equivalent polysilphenylene-siloxane) was purchased from SGE (Texas, USA), while Ultra1 (dimethylpolysiloxane), Ultra2 (5% phenyldimethylpolysiloxane), HP35 (35% phenyldimethylpolysiloxane) and HP50 (50% phenyldimethylpolysiloxane) columns were obtained from Hewlett-Packard (Palo Alto, CA, USA). All the columns used were of fused silica and were crosslinked.

2.2. Reagents and materials. Ropivacaine and metabolites (Figure 1), in hydrochloride form, were supplied by the Department of Medicinal Chemistry, AstraZeneca (Södertälje, Sweden). Methanol LiChrosolv grade, sodium hydroxide (NaOH) and



Compound	R_1	R_2	R_3	R_4
Ropivacaine	C_3H_7	CH_3	H	H
PPX	H	CH_3	H	H
3-OH-ropivacaine (LEA 145)	C_3H_7	CH_3	OH	H
4-OH-ropivacaine (LEA 144)	C_3H_7	CH_3	H	OH
$^2H_7$-Ropivacaine	$C_3{}^2H_7$	CH_3	H	H
Pentycaine	C_5H_{11}	CH_3	H	H

Figure 1. *Structure of ropivacaine, its metabolites and the interal standard ($^2H_7$-ropivacaine).*

sodium chloride (NaCl) were obtained from Merck (Darmstadt, Germany). Pentycaine and [$^2H_7$]ropivacaine (AstraZeneca, Södertälje) were used as internal standards for GC-NPD and GC-MS, respectively (Figure 1).

2.3. Preparation of samples. Stock solutions of the analytes in methanol were prepared. Spiked plasma samples were prepared by adding a few microliters of analyte standard to 1.0 ml plasma. The pH of the plasma sample was adjusted to about 9.5 by adding 15 μL NaOH (2 M), after which 50 μL of the internal standard (0.5 M), was added. The ionic strength of the sample was increased by adding 20% NaCl (w/v). The concentration range of standard curves was between 0.005 and 15 μM.

2.4. SPME extraction procedure. Polyacrylate, polydimethylsiloxane (PDMS) and Carbowax/divinylbenzene (CW/DVB) coated fused-silica fibers (Supelco Inc., Bellefonte, USA) with film thicknesses of 85, 100, and 65 μM, respectively, were tested. The sample volume selected was 1.0 mL (in 2-ml vial). The type of sampling was aqueous, not headspace. Prior to the first extraction, the fibers were conditioned at 250°C for one hour according to the manufacturer's recommendation.

2.5. Validation. The optimum conditions for SPME were applied to the plasma samples for a validation study. Calibration standard solutions (6–7) with a concentration range of 0.03–15 μM (NPD) and 0.005–13 μM (MSD) in plasma were prepared. The internal standard was added and the pH was adjusted to about 9.5 by adding 15 μL NaOH (2 M) to 1.0 mL of sample. Finally, 20% NaCl (w/v) was added and the sample was transferred to the SPME autosampler. The temperature was fixed at 35°C and agitation was used. A standard curve with at least six standard concentrations and one zero concentration was prepared. The peak area ratios of solutes and the internal standard were measured and a standard curve without the zero concentration was constructed. Calibration curves were typically described by the formula

$$y = Bx + A;$$

where y is the peak-area ratio, x is the concentration and A and B are the intercepts and slopes, respectively. The linear regression equation was weighted ($1/x$). The quality control samples (1.0 ml) were treated in the same way as the standards. Selectivity, linearity, accuracy, precision, recovery, and limit of quantitation were studied according to Shah et al. [21].

3. RESULTS AND DISCUSSION

3.1. Method development. The chromatographic behavior of ropivacaine and its metabolites, in underivatized form, was investigated using capillary gas chromatography (CGC). On most of the used columns separation of the analytes was obtained, however the performance with respect to peak symmetry varied extensively. An improvement in peak symmetry and resolution was observed using an Ultra2 column compared to CP-SIL-5CB, despite the fact that both column types are nonpolar. The highly polar column (BPX70) could only separate two out of four compounds. Generally, the intermediate polar columns (DB17 and HP50) showed the best performance with respect to peak symmetry (Table I). HP50 was chosen as the column for detailed studies in our SPME investigation.

3.2. SPME. To use SPME-GC, it was necessary to optimize the extraction conditions for the metabolites. In this study, some factors affecting the extraction using three different SPME fibers were examined (Table II). The factors studied were fiber coating, absorption–desorption time, agitation, pH, temperature and ionic strength. The methodology under optimized conditions using a Carbowax/divinylbenzene fiber was used for method validation.

3.2.1. Effect of different fiber coating. Polyacrylate, PDMS, and CW/DVB fibers were tested and the peak areas of the analytes were compared (Figure 2). The ropivacaine response increased by about ten and four times using CW/DVB as compared to PDMS and polyacrylate, respectively. However, a number of coextracted compounds were observed when polyacrylate fiber was used. That is, this phase

Table I. *Asymmetry factors of ropivacaine and its metabolites.*

Compound	CP-SIL-5CB	Ultra1	Ultra2	HP35	HP50	DB17	BPX70
PPx	1.3	1.03	0.96	0.9	1.0	0.94	0.99
Ropivacaine	0.8	0.9	0.8	0.8	0.9	0.8	1.6
3-OH-Ropivacaine	1.02	1.1	0.9	1.1	0.92	0.95	[a]
4-OH-Ropivacaine	2.03	1.7	1.1	1.2	1.04	0.94	[a]

[a]No peak detected.

Table II. *Factors affecting the extraction recovery of ropivacaine and its metabolites.*

Factor	Range	Optimum recovery
Adsorption time	10–40 min	40 min
Desorption time	1–10 min	3 min
Temperature	25–50°C	35°C
pH	3–9.5	pH 9.5
Sample ionic strength	1–30% NaCl (w/v)	20% NaCl (w/v)
Fiber coating	CW/DVB Polyacrylate PDMS	CW/DVB > polyacrylate > PDMS

is less selective and other solutes from plasma were adsorbed to it. The CW/DVB and PDMS phases are liquid polymer phases and the analytes will be distributed between the plasma and these phases. The CW/DVB fiber is much more polar than the PDMS fiber. The distribution of the metabolites is higher in the CW/DVB phase than the nonpolar PDMS phase. The CW/DVB phase gave the highest peak areas as well as high selectivity and was therefore chosen as the fiber for detailed studies.

3.2.2. Absorption and desorption times profile. The extraction time was reduced by a factor of two by the application of agitation. Different extraction times (10–40 minutes) were tested with agitation for ropivacaine and its metabolites using three CW/DVB. Figure 3 shows extraction profile using a Carbowax/divinylbenzene (CW/DVB) coated fiber. Desorption times (1–10) minutes were tested for the analytes in the injection port of the GC (250°C). The minimum time for complete desorption was three

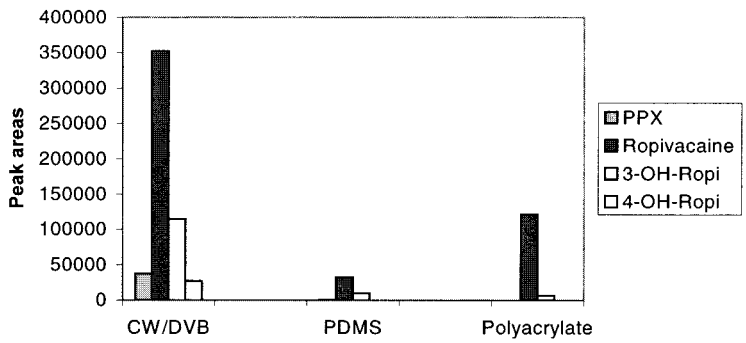


Figure 2. *The effect of fiber type on the amount extracted. Sample concentration: 300–400 nM. SPME conditions: pH, 9.5; salt concentration, 20% of NaCl (w / v); extraction time, 30 min and extraction temperature, 35°C.*

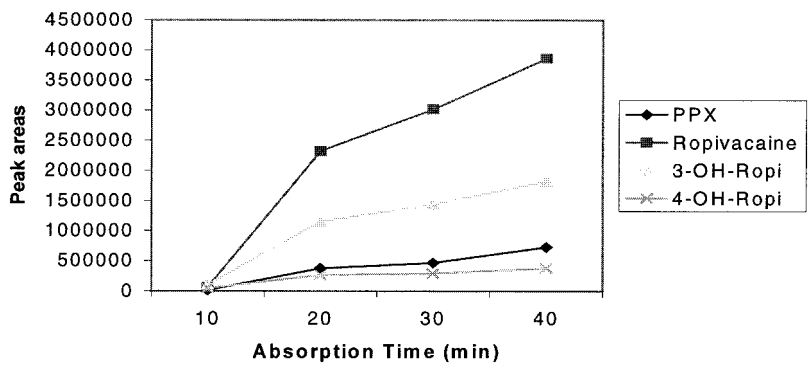


Figure 3. *The effect of extraction time on extraction efficiency on an HP50 (25 m). Sample concentration: 3–4 μM; extraction times: 10–40 min.*

minutes, although the fiber was allowed to desorb for five minutes in the injector port to avoid memory effects from possible impurities from plasma. Carry-over was tested by injecting blank plasma after the highest standard concentration. Carry-over of the ropivacaine was about 1% with the five-minute post-desorption time for GC-MS (for GC-NPD carry-over was not observed).

3.2.3. Effect of pH. Using CW/DVB, the peak areas of ropivacaine and its metabolites in plasma increased by a factor of 2–8 when the pH increased from pH 4 to pH 9 (Figure 4). At high pH the analytes are in neutral form, which shifts the equilibrium towards the coated fiber phase. The pK_a values for ropivacaines and its metabolites are between 7 and 8. The amount of neutral analyte extracted increases by increasing the pH above pK_a. However, when the pH is about 10, the fiber coating becomes unstable.

3.2.4. Effect of temperature. Using CW/DVB, the amounts extracted increased with temperature up to 35°C. When the temperature increased from 25 to 35°C, the response increased twofold for ropivacaine and by about 30% for the metabolites. When the temperature was 50°C, the response decreased rapidly for all the solutes. As is known, increasing the temperature can increase mass transport between the fiber phase and the solution, resulting in higher recovery. In addition, the higher temperature can increase the free fraction of the drug, which results in higher recovery. However, the relative peak area decreased when the temperature was increased to 50°C. This may be due to the transfer of the analyte from the liquid to the gas phase.

3.2.5. Effect of salt addition on analyte. Addition of a soluble salt into the sample can increase or decrease the amount extracted, depending on the compound polarity and salt concentration [22]. Different amounts of NaCl from 1% to 30% (w/v) were added to plasma samples. The addition of NaCl up to a concentration of 20% increased the recovery of the analytes studied using CW/DVB fiber. When the salt concentration was increased to 30%, the solution became saturated and the recovery decreased. The addition of NaCl will increase the ionic strength and change the viscosity of the plasma sample. The diffusion of the analytes in plasma will be decreased, but the partitioning of the analytes will be shifted towards the coated fiber [23].

3.3. Method validation

3.3.21. Selectivity and linearity. Plasma spiked with a mixture of ropivacaine, metabolites and the internal standard were analyzed and compared to blank plasma (Figures 5, 6). No interfering compounds were detected at the same retention times of metabolites. All the metabolites and internal standard were separated. Figures 5 and 6 shows that the SPME technique is satisfactorily selective as a sample-preparation tool for ropivacaine and its metabolites. The results showed a linear response for all analytes in the calibration range studied. The coefficients of determination (R^2) were between 0.997 and 0.999 for GC-NPD and 0.999 to 1.000 for GC-MS using plasma samples (Tables III, IV).

3.3.2. Accuracy and precision. The accuracy is determined by the ratio of the found and the theoretical concentration for two concentrations for ropivacaine and its metabolites (between 0.16 and 2.2 μM, $n = 12$) using GC-NPD, while for GC-MS the concentrations were 0.08 and 1.1 μM ($n = 12$). The accuracy ranged from 98 to 105% for GC-NPD and from 100 to 110% for GC-MS (Table V). The precision is determined by the percentage coefficient of variation of the within- and between-day variations at two different concentrations. The within-day precision was consistently about 3.3% to 13.4% using GC-NPD and from 5.2 to 26.3% for GC-MS. The data of between-day variation of the

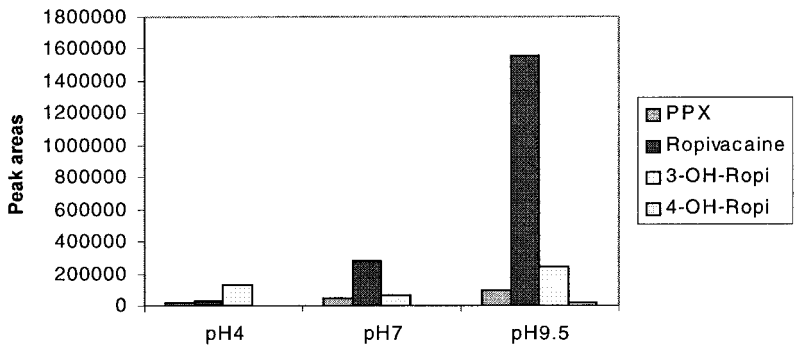


Figure 4. *The effect of pH on the amount extracted using CW / DVB. Sample concentration: 300–400 nM. SPME conditions: salt concentration, 20% of NaCl (w / v); extraction time, 30 min and extraction temperature, 35°C.*

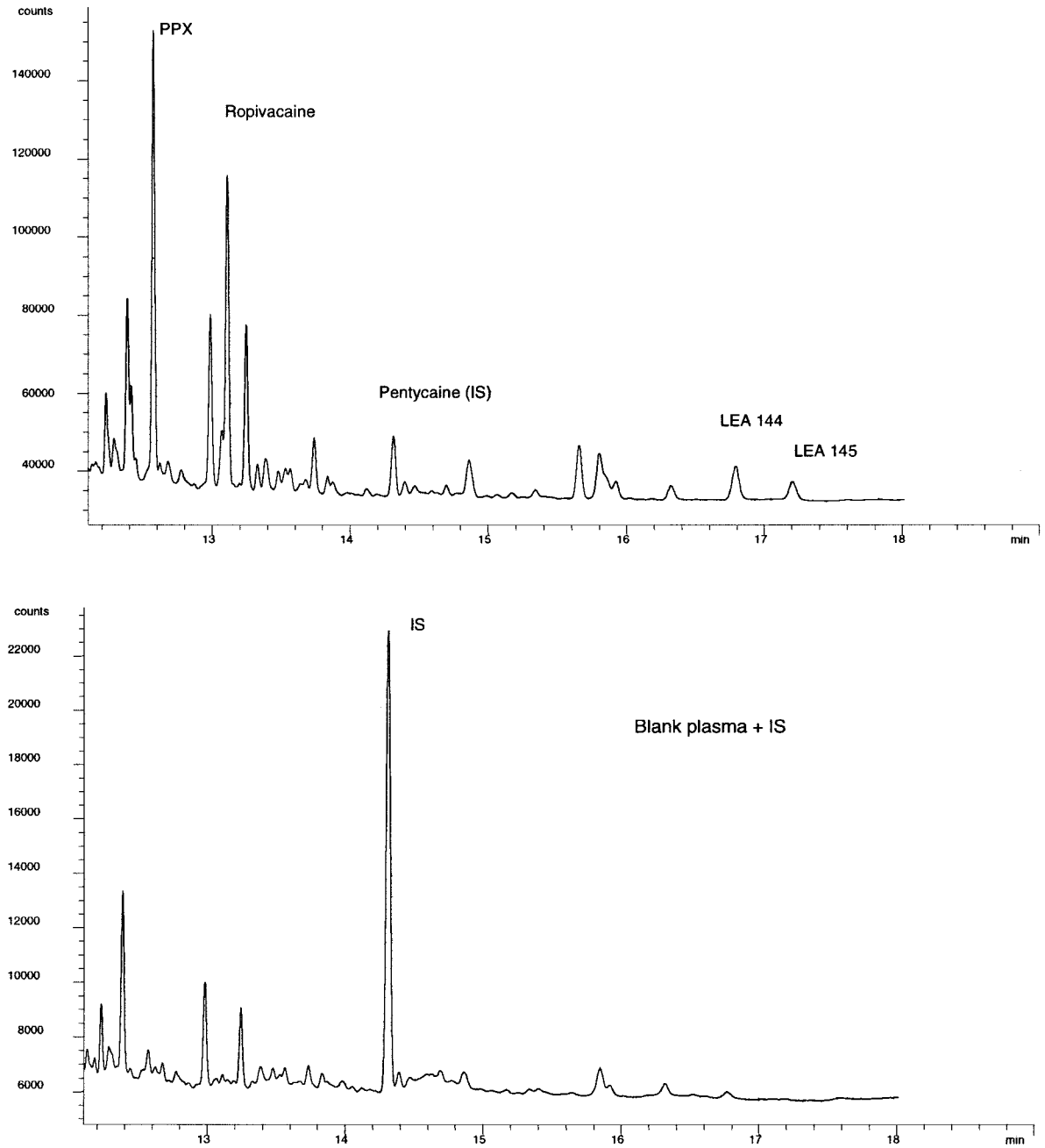


Figure 5. *Chromatograms (NPD) for spiked and blank plasma of ropivacaine and its metabolites on an HP50 (25 m) column. Peak identification: (1) PPx. (2) ropivacaine. (3) 4-OH-ropivacaine (LEA144). (4) 3-OH-ropivacaine (LEA145).*

precision was in the range 5.6–17.1% for GC-NPD and 4.8–25% for GC-MS. The precision results are summarized in Table V.

3.3.3. Limit of quantification. The limits of quantification (LOQ) for ropivacaine, PPX, 3-OH-Ropivacaine and 4-OH-Ropivacaine were 0.03, 0.1, 0.1, and 0.1 μM, respectively (GC-NPD), while LOQ were 0.005 μM for all analytes using GC-MS.

3.3.4. Method comparison. In Table VI the results of accuracy and precision for ropivacaine from the present study are compared with the results from the literature. The results from this study showed higher deviation compared to earlier published data [19,20].

3.4. Fiber lifetime. Fiber lifetime is an important issue. According to the manufacturer, a fiber can be

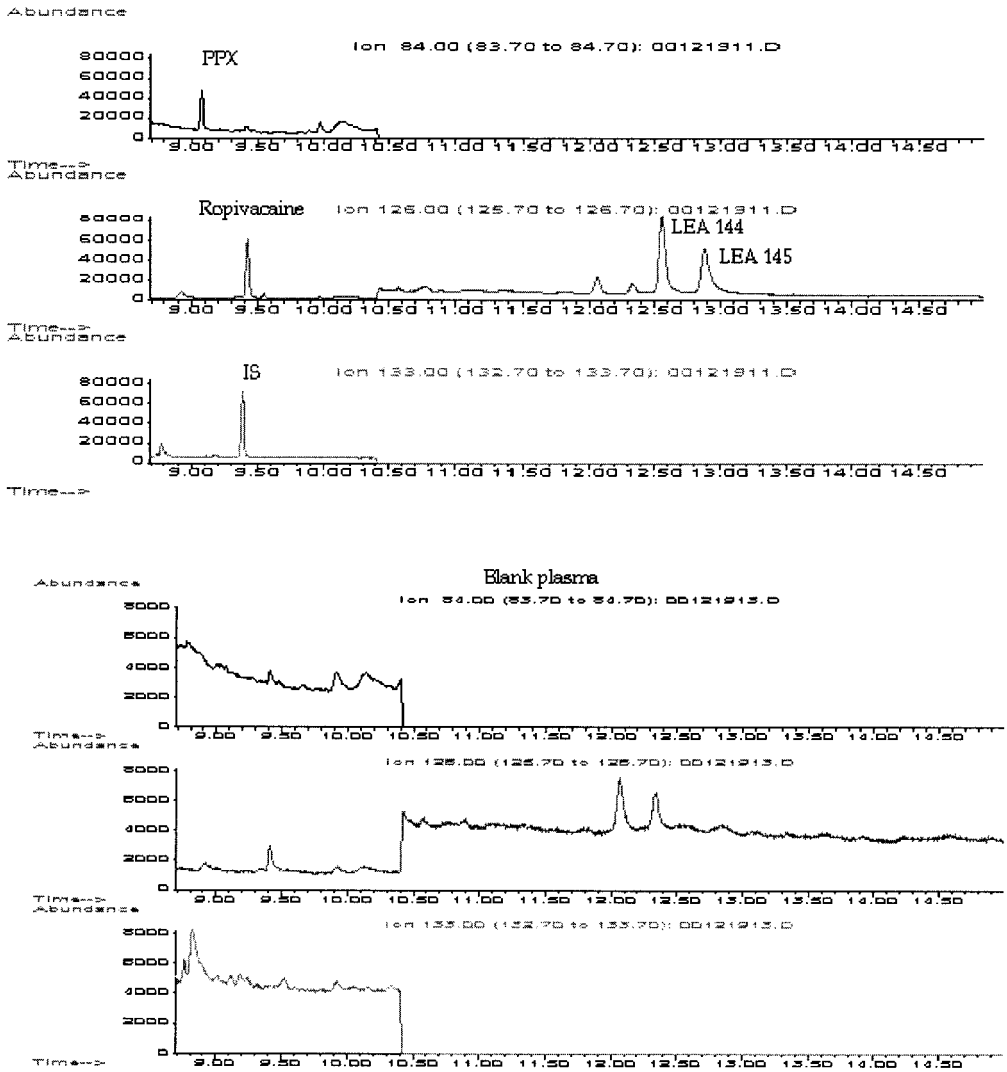


Figure 6. *Mass chromatograms for spiked and blank plasma of ropivacaine and its metabolites on HP50 (25 m) column. Sample concentration: 200–300 nM. Peak identification: (1) PPx. (2) ropivacaine. (3) 4-OH-ropivacaine (LEA144). (4) 3-OH-ropivacaine (LEA145).*

used successfully for 50 to 100 times under mild conditions. The main strain on the fiber is during the desorption step. Here type of polymer, temperature, duration and additives coming from the sample solution influence the stability of the coating. It should be noted that additives such as sodium hydroxide and salt could catalyze polymer thermal degradation. In an earlier work, anaestetics were sampled from plasma at pH 7.4 (no salt added), desorption was 250°C for 5 min [24]. Under these conditions, the sampling rods, CW/DBV, could be successfully used for more than 80 runs. In the present work, we added sodium chloride (20%) and the pH was increased to 9.5 by addition of sodium

Table III. *Linear calibration range and correlation coefficient (GC-NPD).*

Analyte	Calibration range (μM)	Slope ($n = 4$)	Intercept ($n = 4$)	R^2 ($n = 4$)
Ropivacaine	0.03–15	0.0004	0.029	0.998
PPx	0.10–15	0.0006	0.009	0.997
3-OH-Ropivacaine	0.10–15	0.0001	0.004	0.999
4-OH-Ropivacaine	0.10–15	0.00001	0.0007	0.998

Table IV. *Linear calibration range and correlation coefficient (GC/MS).*

Analyte	Calibration range (μM)	Slope ($n = 4$)	Intercept ($n = 4$)	R^2 ($n = 4$)
Ropivacaine	0.005–13	0.0005	0.004	0.999
PPx	0.005–13	0.0003	0.005	1.000
3-OH-Ropivacaine	0.005–13	0.00005	0.00005	1.000
4-OH-Ropivacaine	0.005–13	0.00003	0.0002	1.00

Table V. *The accuracy and precision (within- and between-day) at various concentrations in plasma.*

	Concentration (μM)		Accuracy ($n = 12$) (%)		Intra-within-day ($n = 5$) (RSD%)		Inter-between-day ($n = 4$ days) (RSD%)	
Analyte	NPD	MS	NPD	MS	NPD	MS	NPD	MS
Ropivacaine	0.16	0.08	98	110	13.4	7.2	17.1	6.3
	2.2	1.1	101	110	8.2	5.5	9.1	4.8
PPx	0.47	0.08	98	111	9.5	20.6	14.2	22.9
	1.9	1.1	98	100	6.8	5.2	6.9	20.6
3-OH-ropivacaine	0.47	0.08	101	114	7.1	9.0	8.7	23.0
	1.9	1.1	101	108	4.4	8.2	5.6	13.3
4-OH-ropivacaine	0.47	0.08	103	103	12.9	12.7	11.1	22.3
	1.9	1.1	105	106	3.3	26.3	6.2	25.0

Table VI. *Comparison of accuracy and precision between this study and earlier study.*

Method	Ropivacaine (μM)	Accuracy %	Precision (RSD%) (Inter-between-day)	Reference
SPME/GC-NPD	0.16	98	17.1	Present study
GC-MS	0.08	110	6.3	
LLE/GC-NPD	0.10	96	5.7	[19]
GC-MS	0.04	101	3.8	
SPE/LC-UV	1.90	101	3.0	[20]

hydroxide; desorption was as before. Now fiber lifetime was decreased to about 20 samplings. In an attempt to increase fiber lifetime, a rinsing step was introduced after every five samplings. The rinsing was in a vial containing water and after that the fiber was immersed in plasma. The plasma treatment was to return to a similar sampling condition as before the rinsing with water. With the rinsing step, a fiber could be used for sampling for about 40 times. Some aspects of fiber cleaning were recently briefly discussed by Lord and Pawliszyn [2].

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