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Microextraction in packed syringe (MEPS) for liquid and gas chromatographic applications. Part II — Determination of ropivacaine and its metabolites in human plasma samples using MEPS with liquid chromatography/tandem mass spectrometry

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A new technique for sample preparation on-line with liquid chromatographic/tandem mass spectrometric (LC/MS/MS) assay was developed. Microextraction in a packed syringe (MEPS) is a new miniaturized, solid-phase extraction technique that can be connected on-line to gas or liquid chromatography without any modifications. In MEPS ~1 mg of the solid packing material is inserted into a syringe (100–250 μl) as a plug. Sample preparation takes place on the packed bed. The bed can be coated to provide selective and suitable sampling conditions. The new method is very promising, very easy to use, fully automated, of low cost and rapid in comparison with previously used methods. This paper presents the development and validation of a method for MEPS on-line with LC/MS/MS. Ropivacaine and its metabolites (PPX and 3-OH-ropivacaine) in human plasma samples were used as model substances. The method was validated and the calibration curves were evaluated by means of quadratic regression and weighted by the inverse of the concentration, $1/x$, for the calibration range 2–2000 nM. The applied polymer could be used more than 100 times before the syringe was discarded. The extraction recovery was between 40 and 60%. The results showed high correlation coefficients ($R^2 > 0.999$) for all analytes in the calibration range studied. The accuracy, expressed as a percentage variation from the nominal concentration values, ranged from 0 to 6%. The precision, expressed as the relative standard deviation, at three different concentrations (quality control samples) was consistently about 2–10%. The limit of quantification was 2 nM. **Copyright © 2004 John Wiley & Sons, Ltd.**

KEYWORDS: on-line sample preparation; microextraction in packed syringe; liquid chromatography/tandem mass spectrometry; ropivacaine; ropivacaine metabolites; plasma; validation

INTRODUCTION

In cases when analytes are present in a complex matrix, e.g. plasma or urine, or in samples of environmental origin, the sample preparation is of crucial importance for the analysis. The purpose of sample preparation is to remove interfering substances and also enrichment of the analytes. The procedure must be highly reproducible, with a high recovery of the target analytes. In addition, an ideal sample preparation method should involve a minimum number of working steps, which should be fully automated. Miniaturization is a growing trend in the area of bioanalysis. Methods that are less time consuming, less labor intensive and more environmentally friendly and economical are ideal.

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Commonly used sample preparation methods are solid-phase extraction (SPE), liquid–liquid extraction (LLE) and solid-phase microextraction (SPME). With LLE it is difficult to obtain a high recovery of polar analytes and it is not easy to automate the methods. SPE gives both high recovery and good chromatography, but takes longer and more steps are required.[1–7] SPME is based on a single equilibrium in which analytes partition between a fiber coating and the matrix. Many factors, such as pH, temperature, salt concentration and stirring, affect the equilibrium constant and the equilibration time.[8–11] The major disadvantages of SPME in quantitative analysis are low recovery (low sensitivity), the frequent inability of the fiber to withstand a complete run (standards + blanks + quality control samples + patient samples) and the impossibility of treating samples in an organic solvent. SPME has shown higher deviations than LLE and SPE techniques.[11] In addition, the quality of the fiber and the fiber length can differ from batch to batch.

Microextraction in a packed syringe (MEPS) is a new technique for miniaturized SPE that can be connected on-line to gas (GC) or liquid chromatography (LC) without any modifications.[12,13] In MEPS, ~1 mg of the solid packing material is inserted into a syringe (100–250 µl) as a plug (Fig. 1). The plasma sample (20–1000 µl) is drawn through the syringe by an autosampler (which pumps the sample up and down). When the plasma has passed through the solid support, the analytes have been adsorbed by the solid phase. The solid phase is then washed once with water (50 µl) to remove the proteins and other interfering material. The analytes are subsequently eluted with an organic solvent such as methanol or the LC mobile phase (20–50 µl) directly into the instrument's injector. The process is fully automated.

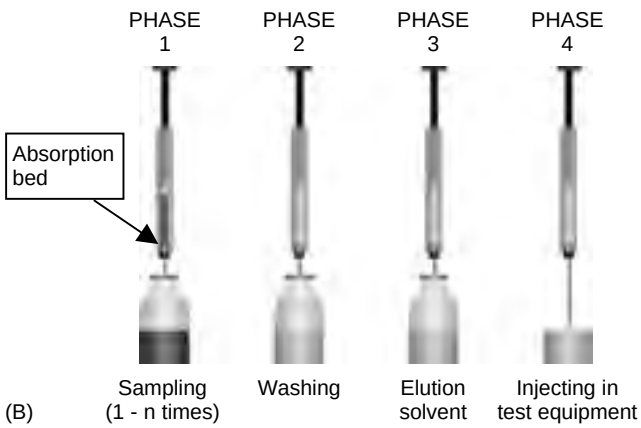
MEPS is based on multiple extractions in which the sample flows through a bed of solid extractant. To allow this, the bed of solid extractant and the particle size of the extractant must be as small as possible. This speeds up the mass transfer of analytes from the liquid sample to the solid phase. Very close contact between the aqueous sample and the surface of the solid phase is also very important. So as not to exceed the capacity of the method, a balance between the amount of the sorbent, the loading volume and the volume of the elution is necessary.

Recently, an application of MEPS to the analysis of local anesthetics (mepivacaine, lidocaine, prilocaine and ropivacaine) in human plasma samples using GC/mass spectrometry (MS) was published.[13] The extraction recovery was 60–80%. The results showed close correlation coefficients ($R > 0.999$) for all analytes in the calibration range studied. The accuracy of MEPS/GC/MS was between 99 and 115% and the inter-day precision ($n = 3$ days), expressed as the relative standard deviation (RSD), was 3–10%. The applied polymer could be used more than 100 times before the syringe was discarded.[13]

The MEPS technique differs from commercial SPE in that the packing is inserted directly into the syringe, not into a separate column. Hence there is no need for a separate robot to apply the sample into the solid phase as with conventional SPE. The packed syringe can also be used several times, more than 100 times for plasma or urine samples and more than 400 times for water samples, whereas a conventional SPE column can be used only once. MEPS can handle both small sample volumes (10 µl of plasma, urine or water) and large volumes (1000 µl) and can be used for GC and LC applications.

Compared with SPME, the new technique is more robust. In SPME the sampling fiber is fairly sensitive to the nature of the sample matrix. The new technique can be used for complex matrices without problems (such as plasma, urine and organic solvents), which is not always the case with SPME. Also, a much higher extraction recovery can be obtained (>60%)[13] than with SPME (1–10%). Small sample volumes can be treated (10 µl) compared with SPME (≥1000 µl). In this work, MEPS was tested and validated with LC/MS, using ropivacaine and its metabolites as model substances. Ropivacaine is an amide-type, local anesthetic drug, mainly used for surgery and for postoperative pain relief. It also has a lower central nervous and cardiotoxic potential than its predecessor bupivacaine.[14] The major metabolites of ropivacaine are PPX and 3-OH-ropivacaine.

(A)



(B)

Figure 1. (A) Packed syringe; (B) schematic drawing of MEPS (the process is fully automated).

EXPERIMENTAL

Reagents and materials

Ropivacaine, PPX, 3-OH-ropivacaine (Fig. 2) and [$^2H_7$]ropivacaine (internal standard (IS)), in hydrochloride form, were supplied by the Department of Medicinal Chemistry, AstraZeneca (Södertälje, Sweden). Methanol (LiChrosolv grade), formic acid and ammonium hydroxide were obtained from Merck (Darmstadt, Germany).

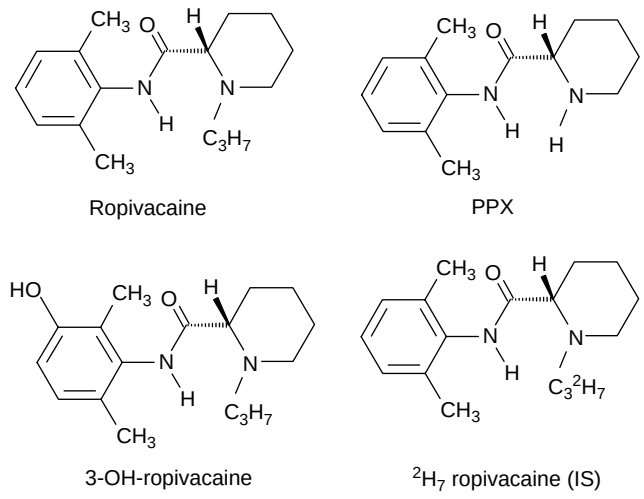


Figure 2. Structures of ropivacaine and its metabolites.

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Instrumentation

The LC apparatus included two LC10Advp pumps (Shimadzu, Kyoto, Japan) and a CTC-Pal autosampler obtained from Crelab (Knivsta, Sweden).

Chromatographic system

Only an Optiguard column (C_8, 1 × 10 mm), obtained from Optimize Technologies (OR, USA), was used and mounted on the mass spectrometer. An isocratic high-performance liquid chromatographic system was used. The mobile phase was 0.1% formic acid in methanol–water (1 : 1, v/v). The flow-rate was 0.2 ml min^{-1} and the sample volume (elution volume from MEPS) was 20 µl.

All the experiments were conducted using a QII triple-quadrupole mass spectrometer instrument (Micromass, Manchester, UK) equipped with a Z-electrospray interface (ESI) operated in the positive ion mode. The source block and desolvation temperatures were 150 and 250 °C, respectively. Nitrogen was used as both drying and nebulizing gas and argon was used as collision gas. The data were collected using MassLynx version 3.4. All calculations were based on peak area ratios. Prior to each batch of analyses, a test sample containing all the metabolites was analyzed in order to check the sensitivity and to set integration parameters. The scan mode was multiple-reaction monitoring using the precursor ion at m/z (M + 1) (m/z 275, 233, 291 and 282), and after collisional dissociation the product ions at m/z 126, 84, 126 and 133 were used for the quantification of ropivacaine, PPX, 3-OH-ropivacaine and [$^2H_7$]ropivacaine (IS), respectively.

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 of plasma, after which 50 µl of the IS solution (0.5 M) was added. The concentration range of the calibration curves was between 2 and 2000 nM.

Validation

Calibration standard solutions with a concentration range of 2–2000 nM in plasma were prepared. Finally, the IS was added. A calibration curve with at least seven standard concentrations and one zero concentration was prepared. The peak area ratios of solutes and the IS were measured and a calibration curve without the zero concentration was constructed. Calibration curves were typically described by the equation

$$y = Ax^2 + Bx + C$$

where y is the peak area ratio, x is the concentration, B and C are the slope and intercept, respectively, and A is the curvature. The calibration curves were weighted ($1/x$). Quality control (QC) samples were treated in the same way as the standards. The intra- and inter-assay variations were determined by using three levels of concentrations (QC: low, medium, and high), which were 40, 400 and 1500 nM ($n = 6$). Selectivity, linearity, accuracy, precision, recovery and limit of quantification were studied according to Shah *et al*.[15]

RESULT AND DISCUSSION

Method development

The aim of the present study was to test and validate MEPS as a new sample preparation technique using ropivacaine and its metabolites as model compounds. One milligram of solid-phase material was inserted into a 250 µl syringe. The packed syringe was conditioned first with methanol and then with water (2 × 50 µl) before being used for the first time. The plasma sample is drawn through the syringe (50 µl) by the autosampler (which pumps the sample up and down three times). The solid phase is then washed once with water (50 µl) to remove the proteins and other interferents. The analytes are then eluted directly (with 20 µl of methanol–water (95 : 5)) into the LC injector. The washing and elution steps were studied and optimized. Also, different sorbent types (C_2 and C_8) were investigated.

Washing solution

Different washing and elution solutions were tested. Water, methanol–water and acetonitrile–water in different proportions were tested. The washing volume used was 50 µl. Figure 3 shows the effect of washing on the recovery, using different proportions of organic solvent. Using 10% methanol in water reduced the recovery by about 10% compared with water alone, although cleaner extracts could be obtained.

Solid phase type and amount and elution solution

Different solid phases and elution solutions were investigated. The elution volume used was 20 µl. Figure 4 shows that the C_2 phase gave a higher recovery than the other phases. In addition, methanol–water

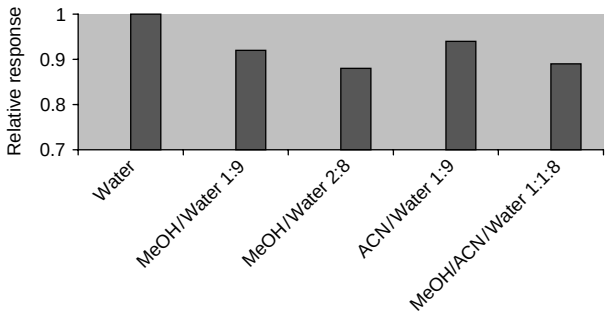

Figure 3. Effect of washing solution on the ropivacaine response.

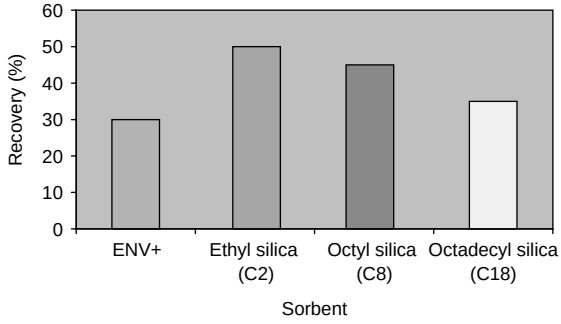

Figure 4. Effect of type of solid phase on the recovery of ropivacaine.

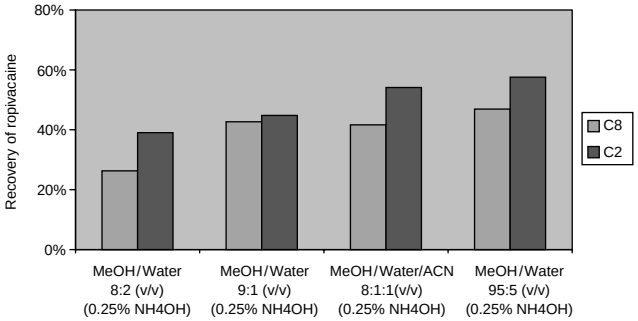

Figure 5. Effect of elution solvent on the ropivacaine response.

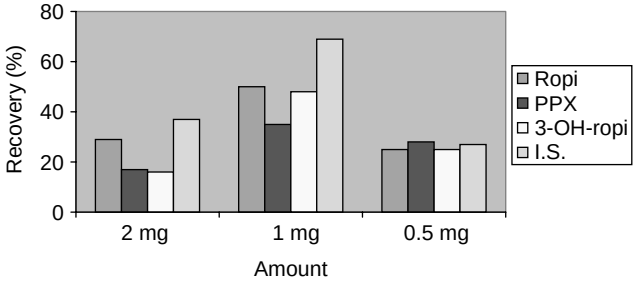

Figure 6. Effect of amount of solid phase (C_2) on the recovery.

and methanol–acetonitrile–water in different proportions were used as elution solutions. The maximum recovery was obtained using 0.25% ammonium hydroxide in water–methanol (95 : 5) (Fig. 5). The effect of different amounts of solid-phase packing on the recovery was studied. Figure 6 illustrates that 1 mg is suitable for a concentration range of 2–5000 nM. At concentrations higher than 5000 nM, the amount of packing material should be increased.

Selectivity

When plasma spiked with a mixture of analytes and the IS was analyzed and compared with blank plasma, no interfering compounds were detected at the same retention times as the studied compounds. Figure 7 show good selectivity for MEPS as a sample preparation method.

Calibration

For the construction of the calibration curve, 7–9 levels of the analytes in human plasma were used as the analytes. The method was validated using [$^2H_7$]ropivacaine as IS. The results showed a close relationship between the concentrations and relative peak areas for the analytes studied in the concentration range 2–2000 nM. Regression parameters for all the calibration curves are given in Table 1. The correlation coefficients (R^2) obtained were >0.999. The calibration curves indicated that the method is suitable for quantitative analysis. The back-calculated values of the calibration points showed good agreement with the theoretical concentrations (Table 2). No deviation outside ±10% of the nominal concentrations was observed.

Table 1. Regression parameters for calibration curves ($n = 3$)

Analyte	Curvature A ($\times 10^{-8}$)	Slope B	Intercept C	R^2
Ropivacain	199.2	0.00120	0.0087	0.9998
PPX	64.71	0.00041	0.0085	0.9997
3-OH-ropivacaine	147.8	0.00091	0.0064	0.9996

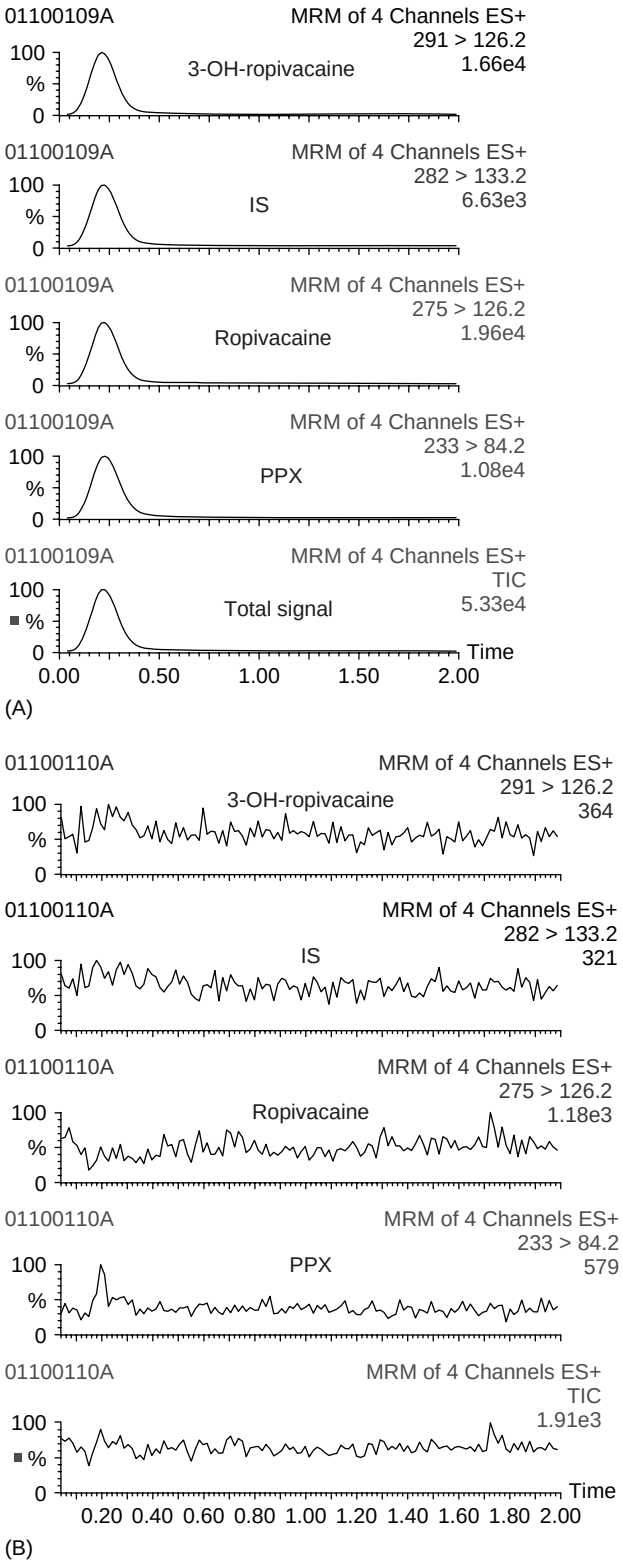

Figure 7. (A) Mass chromatograms obtained from human plasma spiked with analytes (*m/z* 291, 182, 275 and 233 for 3-OH-prilocaine, IS, ropivacaine and PPX, respectively). (B) Mass chromatograms obtained from human blank plasma.

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Table 2. Back-calculated values for calibration of the plasma samples

Analyte	Nominal concentration (nM)	Mean accuracy[a] (%) ($n = 3$)
Ropivacaine	2	9.0
	5	1.0
	10	−2.0
	50	2.0
	100	−6.0
	300	0.0
	500	1.0
	1000	0.0
	2000	0.0
PPX	2	−10
	5	−8.0
	10	2.0
	50	6.0
	100	9.0
	300	−3.0
	500	−1.0
	1000	0.0
	2000	0.0
3-OH-ropivacaine	2	0.0
	5	3.0
	10	−6.0
	50	−1.0
	100	5.0
	300	1.0
	500	−3.0
	1000	1.0
	2000	0.0

[a] Mean accuracies reported as the percentage difference from the nominal value.

Accuracy and precision

The accuracy was determined by the ratio of the found and theoretical concentrations for human plasma control samples at three different levels. The precision is a measure of the random error and is determined by the relative standard deviation (RSD) of the within- and between-day variations (intra- and inter-assays) at three levels. The intra- and inter-assay variations were determined by analysis of QC samples at three different concentrations, 40, 400 and 1500 nM. The results are given in Table 3. The RSDs are between 2 and 10% for both inter-assay and intra-assay data. Validation of the methodology showed that the method is highly selective for ropivacaine and its metabolites in plasma samples. The results showed high correlation coefficients (>0.999) for all the analytes in the calibration range studied. The accuracy and precision were well in line with international criteria (Table 3).[15]

Extraction degree, limit of quantification (LOQ) and carry-over

The extraction degree was determined by comparing the peak area after extraction at two different concentrations (low- and high-quality control samples) with the peak area obtained from the direct injection of the same concentration in water. The extraction recoveries for the ropivcaine and the IS were 60% and for the metabolites were 40%. The effect of the co-extracted biological material was studied by comparing the responses of extracted QC samples in plasma and in blank matrix (water). The recoveries (plasma/water) were 80% and 70% for ropivacaine and the metabolites, respectively. The LOQ for the analytes studied was 2 nmol l^{-1} and in our case was satisfactory. The precision of the LOQ (given as RSD) was 4–6% ($n = 4$) for the analytes studied. The accuracy of the LOQ was 104, 106 and 101% ($n = 4$) for ropivacaine, PPX and 3-OH-ropivacaine, respectively. The carry-over was tested by injecting the LC mobile phase after the highest standard concentration. To reduce the memory effect, the MEPS was washed four times with methanol and four times with water after every injection. The carry-over was <0.01%.

CONCLUSIONS

A new sensitive, selective and accurate on-line sample-preparation technique was developed and validated for the determination of ropivacaine and its metabolites. MEPS is more easily automated than SPE and is more rugged than SPME. It takes only 1 min for each sample compared with 10–20 min with earlier methods (SPE and LLE). Compared with SPME, the new technique is more stable and has a high recovery. In SPME the sampling fiber is fairly sensitive to the nature of the sample matrix. The new technique can be used for complex matrices such as plasma, without problems.

Table 3. Intra- and inter-assay precision using C_2 as sorbent

Analyte	Concentration in plasma (nM)	Accuracy (%)	Intra-assay RSD (%) ($n = 6$)	Inter-assay RSD (%) (3 days, $n = 18$)
Ropivacaine	40	100	10	10
	400	101	4	5
	1500	102	3	6
PPX	40	106	6	6
	400	103	4	5
	1500	100	3	5
3-OH-ropivacaine	40	102	9	10
	400	104	2	5
	1500	104	3	5

Also, a much higher extraction recovery for ropivacaine and its metabolites can be obtained (40–60%) compared with SPME (1–10%). Small sample volumes can be treated (10 µl) compared with SPME (≥1000 µl).

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