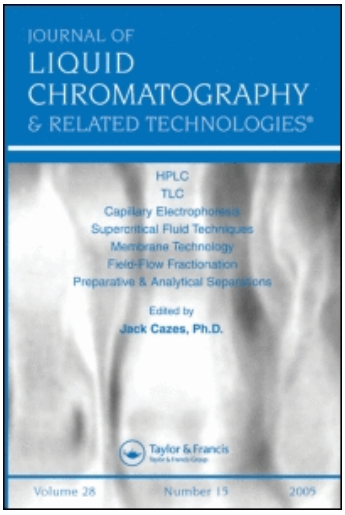

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Microextraction in Packed Syringe Online with Liquid Chromatography-Tandem Mass Spectrometry: Molecularly Imprinted Polymer as Packing Material for MEPS in Selective Extraction of Ropivacaine from Plasma

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Microextraction in Packed Syringe Online with Liquid Chromatography-Tandem Mass Spectrometry: Molecularly Imprinted Polymer as Packing Material for MEPS in Selective Extraction of Ropivacaine from Plasma

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Abstract: The excellent performance of a new sample preparation method, microextraction in packed syringe (MEPS), was recently illustrated by online LC-MS and GS-MS assays of local anaesthetics in plasma samples. In the method, approximately 1 mg of solid packing material was inserted into a syringe (100–250 μL) as a plug. Sample preparation took place on the packed bed. The new method was easy to use, fully automated, of low cost, and rapid in comparison with previously used methods. This paper presents the use of molecularly imprinted polymers (MIPs) as packing material for higher extraction selectivity. Development and validation of a method for MIP-MEPS online with LC-MS-MS using ropivacaine in plasma as model compound were investigated. A bupivacaine imprinted polymer was used. The method was validated and the standard curves were evaluated by means of quadratic regression and weighted by 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 60%. The results showed high correlation coefficients ($R^2 > 0.999$) for all runs. The accuracy, given as a percentage deviation from the nominal concentration values, ranged from −6% to 3%. The precision, given as the relative standard deviation, at three

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different concentrations (QC samples) was consistently about 3% to 10%. The limit of quantification was 2 nM.

Keywords: Column liquid chromatography-tandem mass spectrometry, Molecularly imprinted polymers, Microextraction in packed syringe, Sample preparation, Ropivacaine

INTRODUCTION

In bioanalysis, sample preparation is of crucial importance. An ideal sample preparation method should involve a minimum number of working steps, which should be fully automated. The method should also be rapid, environmentally friendly, and of low cost. In addition, the procedure must be highly reproducible, with a high recovery of the target analytes. As miniaturization is also a growing trend in bioanalysis, the new method may be considered a step in this development.

Microextraction in packed syringe (MEPS) is a new technique for miniaturized, solid-phase extraction that can be connected online to GC or LC without any modifications.[1–4] In MEPS, approximately 1 mg of a solid packing material is inserted into a syringe (100–250 μL) as a plug inside the syringe, or between the barrel and the needle (Figure 1A). The plasma sample (20–250 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 to the solid phase. The solid phase is then washed once by water (50 μL) to remove the proteins and other interfering materials. The analytes are then eluted directly with an

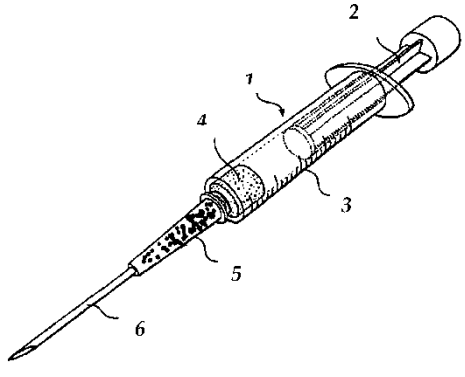

Figure 1. (A) A syringe (1) with a plunger (2) having a syringe barrel (3) slidable within the barrel and a hollow needle (6) extending from the barrel, through which needle the liquid sample is drawn into the syringe barrel such that a solid phase or coating material (4, 5) is provided in the syringe barrel (4) or between the barrel and the needle (5). (B) Schematic diagram of MEPS (the process is fully automated).

(*Continued*)

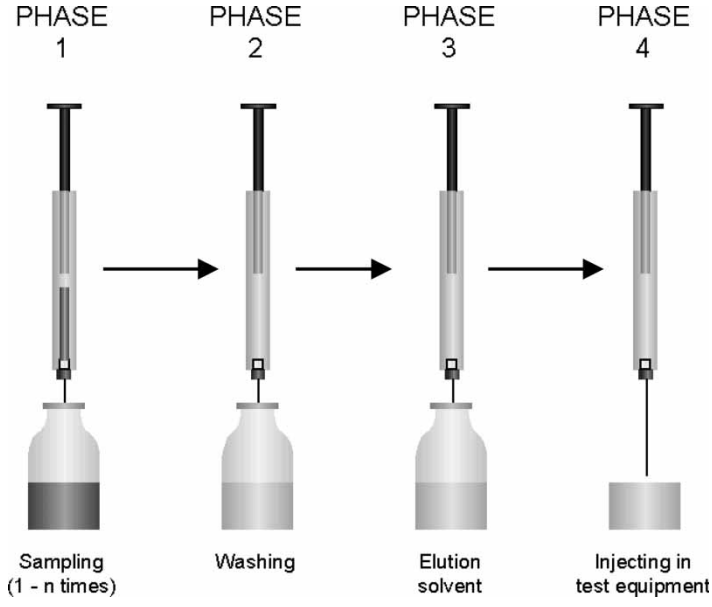

Figure 1. Continued.

organic solvent, such as methanol or the LC mobile phase (20–50 μL), directly into the instrument's injector (Figure 1B). The process is fully automated.

The MEPS technique differs from commercial solid-phase extraction (SPE) in that the packing is inserted directly into the syringe, not into a separate column. Thus, 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 is only used once. MEPS can handle small sample volumes (10 μL of plasma, urine, or water) as well as large volumes (250 μL), and can be used for GC and LC applications. Compared with liquid-liquid extraction (LLE) and solid-phase extraction (SPE), MEPS will reduce sample preparation time and organic solvent consumption. MEPS is fully automated and each sample takes only about one minute to prepare. Compared with solid-phase microextraction (SPME), the new technique is more robust. In SPME, the sampling fibre of SPME is rather sensitive to the nature of the sample matrix. The new technique can be used for complex matrices (such as plasma, urine, and organic solvents) without problems, which is not always the case with SPME.

Molecularly imprinted polymers (MIPs) have been used as selective separation materials in several analytical techniques, including liquid chromatography, capillary electrophoresis, capillary electrochromatography, solid-phase extraction, and immunoassay. An advantage of this type of sorbent is

the possibility to synthesise polymers with selectivity predetermined for a particular analyte. The key step of the technique is the polymerization of functional and cross-linking monomers in the presence of a template or imprint species. The selective interactions between the template and monomers, as well as between template and imprint, are based upon hydrogen bonds, ionic bonds, and hydrophobic interactions. Already, a number of highly selective extractions of biological and environmental samples have been reported, where several studies have made direct comparisons with conventional extraction materials and demonstrated superior cleanup using the MIP materials.[5–9] Imprinted polymers have excellent stability over a wide range of buffer pH, solvent, temperature, and pressure conditions,[10] which provide flexibility in finding the best experimental conditions for sample cleanup.

The present study used an MIP prepared against bupivacaine, which is selective for a homologous series of local anaesthetics, including mepivacaine, ropivacaine and bupivacaine.

The compound studied, ropivacaine, is an amide type local anaesthetic drug, mainly used for surgery and for postoperative pain relief. It also has a lower central nervous and cardiotoxic potential than its predecessor bupivacaine.[11]

EXPERIMENTAL

Reagents

Ropivacaine, [$^2H_7$] ropivacaine (IS), and bupivacaine (Figure 2), were supplied by the Department of Medicinal Chemistry, AstraZeneca (Södertälje,

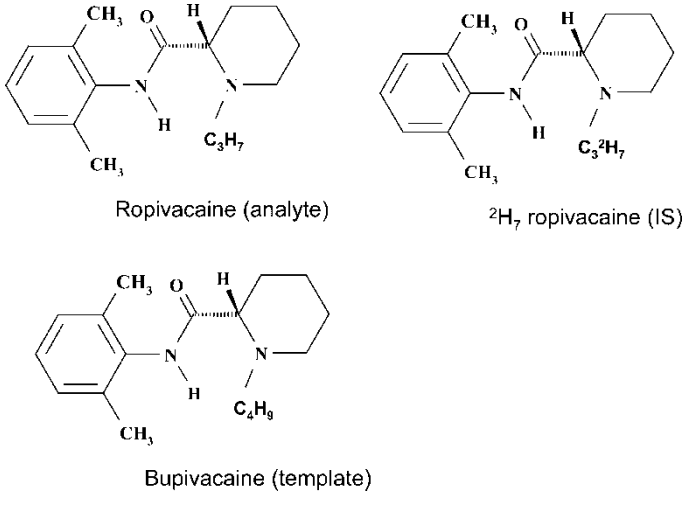

Figure 2. Structure of ropivacaine and internal standard.

Sweden). Methanol LiChrosolv grade, formic acid, and ammonium hydroxide were obtained from Merck (Darmstadt, Germany). The MIP was prepared as described using bupivacaine as the template.[12]

Apparatus and Chromatographic Conditions

The HPLC instrument included two pumps, Shimadzu LC10Advp, Shimadzu corporation (Kyoto, Japan). The mass spectrometer was a Micromass Z-spray QII mass spectrometer from Micromass UK Limited (Manchester, UK) and the chromatographic data system was MassLynx version 3.4.

An Optiguard (C8, 1 × 10 mm) obtained from Optimize Technologies Inc. (Oregon, USA) was used, and was connected to the mass spectrometer. An isocratic high performance liquid chromatography 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 sample volume (elution volume from MEPS) was 20 μL.

All experiments were conducted using a triple quadrupole mass spectrometric instrument Micromass (Manchester, UK), equipped with a Z-electrospray interface (ESI) operated in positive ion mode. The source block and desolvation temperatures were 150°C 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 ropivacaine was analysed in order to check 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 and 282), and after collisional dissociation the product ions 126 and 133 were used for quantification of ropivacaine and [$^2H_7$] ropivacaine (I.S), respectively.

Preparation of Samples

Stock solutions (300 μM) of ropivacaine, and $^2H_7$-ropivacaine (I.S) in 0.1% HCOOH in methanol/water (1:1) were prepared. Spiked plasma samples were prepared by adding a few microlitres of analytes to 1.0 mL of plasma, and 25 μL of the I.S was added. The plasma was diluted with water (1:1). The concentration range of the standard curve was between 2 nM and 2000 nM.

MEPS Conditions

About 1 mg of MIP material was inserted into a 250 μL syringe as a plug with a filter from both sides and fitted manually into the syringe. The plug is fixed tightly in the syringe to keep it inside the syringe. Any adsorption material

such as silica based (C_2, C_8, C_{18}) or restricted access material (RAM) can be used. The packed syringe is conditioned first with methanol and then with water (50 μL) before being used for first time. The plasma sample is drawn through the syringe three times (50 μL each time) 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 sources of interference. The analytes are then eluted with 20 μL (0.4% NH_4OH in methanol) directly into the LC injector. The multiple pulling/pushing of the sample by the syringe increases the extraction recovery. Also, using a small amount of the adsorbent (1 mg) makes it easy to wash, and the same syringe can be used many times. In MEPS, a standard syringe with removable needle is used and no modifications are needed, either for the autosampler or for LC. In the case of MEPS, the plasma quality is very important. If the plasma is thick, it has to be diluted with water at least 1:1 (v/v), otherwise clogging may occur. If the needle is plugged, water has to be pushed through the syringe from the upper side. The syringe can be normally used for one hundred injections.

Validation

Calibration standard solutions with a concentration range of 2–2000 nM in human plasma were prepared. Finally, the internal standard was added. A standard curve with at least eight 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 equation:

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

where y is the peak area ratio, x is the concentration, B is the slope, C is the intercept, and A is the curvature. The calibration curves were weighted ($1/x$). The quality control (QC) samples were treated in the same way as the standards. The intra- and inter-assays 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 investigated according to Shah et al.[13]

RESULTS AND DISCUSSION

Method Development

MIP material (0.75 mg) was inserted into a 250 μL syringe. The packed syringe was conditioned first with methanol (50 μL) and then with water (50 μL) before being used for the first time. The plasma sample was drawn through the syringe

(50 μL) by the autosampler, which pumps the sample up and down three times. The solid phase was then washed once with water (50 μL) to remove the proteins and other sources of interference, after which the analytes were eluted directly (by 20 μL 0.4% NH_4OH in methanol) into the LC injector. Washing and elution steps were studied and optimized.

Washing Solution

Different washing and elution solutions were tested. Water and methanol/water in different compositions were tested. The washing volume used was 50 μL. The use of methanol in the washing mixture increased the leakage and decreased the recovery; however, clean extract was obtained. The lowest amount of leakage, with no interference and the highest recovery, was obtained with 20% methanol in water.

Test of MIP Amount and Elution Solution

Varying MIP amounts in the range of 0.5–1.5 mg were tested and 0.75 mg was found to be suitable for a concentration range of 2–5000 nM. At analyte concentrations higher than 5000 nM, the amount of packing material should be increased. In addition, different elution solutions were investigated (50 μL methanol/water at different percentages) and methanol/water/acetonitrile were examined as elution solutions. Also, different percentages of ammonium hydroxide in methanol were tested. The maximum recovery was obtained using 0.4% ammonium hydroxide in methanol.

Selectivity

When plasma spiked with ropivacaine and the internal standard were analysed and compared to blank plasma, no interfering compounds were detected at the same retention times as the studied compounds. Figures 3 and 4 show good selectivity when using MIP for MEPS as a sample preparation method.

Calibration

For the construction of the calibration curve, 9 levels of the analyte in human plasma were used. The method was validated using [$^2H_7$] ropivacaine as internal standard. 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 coefficient (R^2) values obtained were over

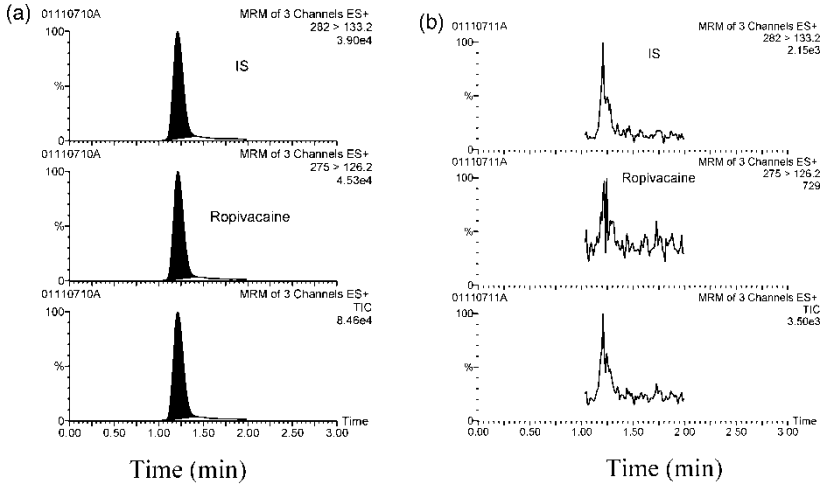

Figure 3. Mass chromatograms obtained from human plasma spiked with ropivacaine and internal standard on molecularly imprinted polymer, MIP (A), and on non-imprinted polymer, NIP (B).

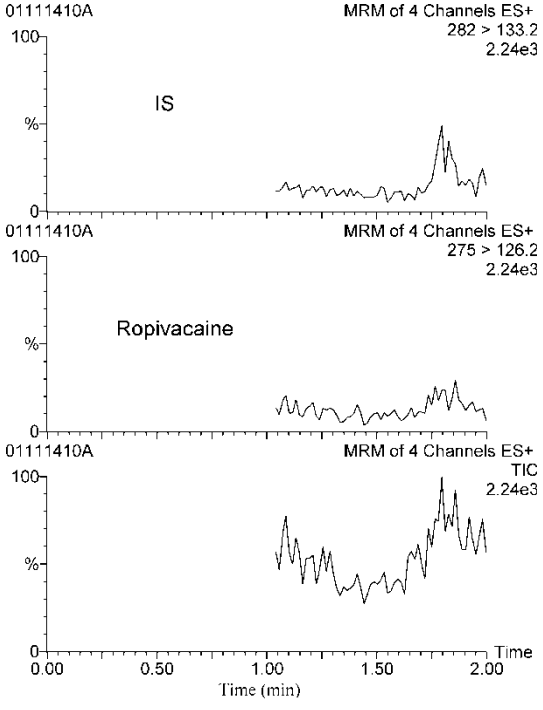

Figure 4. Mass chromatograms obtained from human blank plasma.

Table 1. Regression parameters for calibration curves

Analyte	Curvature A (10^{-8})	Slope B	Intercept C	R^2
Ropivacaine	5.36	0.00043	0.014	0.9999
	2.62	0.00048	0.0059	0.9998
	4.93	0.00047	0.0064	0.9999

0.999. The calibration curves indicated that the method is suitable for quantitative analysis of ropivacaine. The back calculated values of the calibration points showed good agreement with the theoretical concentrations (Table 2). No deviations outside $\pm 6\%$ of the nominal concentrations were observed.

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 was determined by the percentage coefficient variation of the within- and between-day variations (intra- and inter-assays) at three levels. The intra- and inter-assays were determined by analysis of QC samples at three different concentrations, 40, 400, and 1500 nM. The results are shown in Table 3. The CV % values were between 3 and 10% for both inter-assay and intra-assay. Validation of the methodology showed that the method is highly selective for ropivacaine in plasma samples. The accuracy and precision were well in line with international criteria.

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

Solute	Nominal concentration (nM)	Mean accuracy[a] (n = 3) (%)
Ropivacaine	2	2.0
	5	0.0
	10	−3.0
	50	2.0
	100	4.0
	300	−6.0
	500	−1.0
	1000	3.0
	2000	1.0

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

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

Analyte	Concentration (nM) in plasma	Accuracy (%) (n = 18)	Intra-assay (RSD%) (n = 6)	Inter-assay (RSD%) (3 days, n = 18)
Ropivacaine	40	99	10	10
	400	99	3	4
	1500	102	5	6

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 QC samples), with the peak area obtained after adding the concentrations to the LC mobile phase. The extraction was 60% for ropivacaine, whereas extraction on a non-imprinted polymer prepared in the absence of template gave a recovery of 5%. The LOQ for the analytes studied was 2 nmol L^{-1}, and in our case,

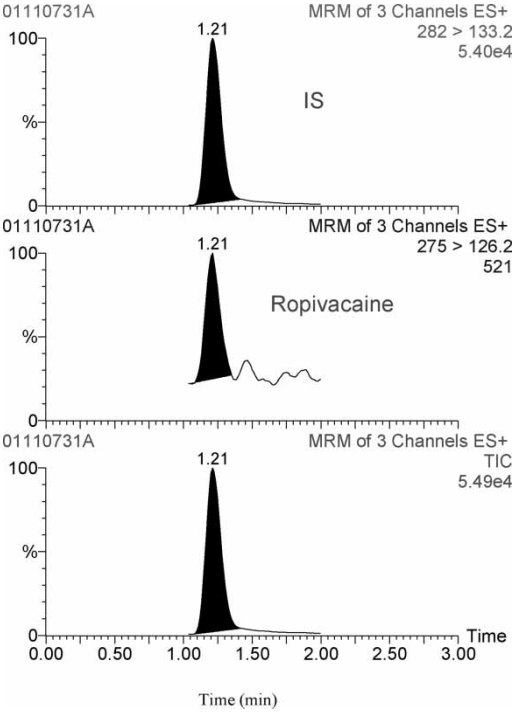

Figure 5. Typical mass chromatogram obtained from human plasma spiked with the LOQ for ropivacaine (2.0 nM with internal standard).

the LOQ was satisfactory. Figure 5 shows a calibration sample in human plasma at the LOQ, 2 nM of ropivacaine with internal standard. The precision of the LOQ (given as RSD%) was $<10\%$ ($n = 6$). To minimize memory effects, the MEPS was washed four times with methanol and four times with water after every injection. The carry over was less than 0.01%. The syringe-to-syringe variations were also tested. The variations were less than 2%.

CONCLUSIONS

A new, selective, and accurate online sample preparation technique, based on the use of a small amount of imprinted polymer packed in a syringe, was developed and validated for the determination of ropivacaine in human plasma samples. Compared with other extraction techniques, such as LLE and SPE, the new MEPS based method reduced sample preparation time and organic solvent consumption. Previously, we have demonstrated that both small and large sample volumes can be handled and that MEPS is more easily automated than SPE and more rugged than SPME.[1,2] It takes only one minute for each sample compared with 10–20 min with earlier methods (SPE and LLE). Compared with solid-phase microextraction (SPME), the system is more stable and often has a higher recovery. In SPME, the sampling fibre is rather sensitive to the sample matrix. The use of an MIP provides high selectivity of the extraction and the new technique can be used for complex matrices, such as plasma, urine, and organic solvents, without problems of contaminant peaks, which is not the case with SPME. In addition, a much higher extraction recovery for ropivacaine can be obtained (60%) compared to SPME ($>10\%$).

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