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Short Communication

Quantification of ropivacaine and its major metabolites in human urine samples utilizing microextraction in a packed syringe automated with liquid chromatography-tandem mass spectrometry (MEPS-LC-MS/MS)

The determination of ropivacaine and its major metabolites in urine was performed using microextraction in a packed syringe as an on-line sample preparation method with LC and MS/MS. The sampling sorbent utilized was polystyrene polymer. [$^2H_7$]ropivacaine was used as the internal standard. The lower LOQ was 5.0 nmol/L. The calibration curves were obtained within the concentration range 5 – 2000 nmol/L in urine. The regression correlation coefficients for urine samples were ≥0.999 for all runs. The between-batch accuracy and precision values were determined from six replicates of quality control (QC) samples at three different concentrations in human urine. The mean accuracy values for the QC samples, reported as the percentage difference from the nominal value, were in the range of 99 – 115%. The precisions, given as the RSDs, were in the range 1.9 – 11%. The present method is miniaturized and fully automated and can be used for pharmacokinetic and pharmacodynamic studies.

Keywords: LC-MS/MS / Microextraction in packed syringe / Polystyrene sorbent / Ropivacaine and metabolites / Sample preparation

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1 Introduction

Ropivacaine is an amide-type local anaesthetic, mainly used for surgery and for postoperative pain relief. In addition, it has a lower central nervous and cardiotoxic potential than its predecessor, bupivacaine [1]. The major metabolites of ropivacaine are 2,6-pipecoloxylidide (PPX) and 3-hydroxyropivacaine (3-OH-ropivacaine). The determination of ropivacaine and its metabolites has been performed by LC and GC [2 – 5].

The measurement of drug levels in biological fluids is the corner stone for drug discovery and development. Also the analysis of drugs in pharmaceutical products and biological samples is growing in importance, both in the development of more selective and effective drugs and in understanding their therapeutic and toxic effects. Quite often, sampling and sample preparation steps constitute over 80% of the total analysis time, and these steps are important in determining the success of analysing compounds of interest in complex matrices such as biological samples. Therefore, the choice of a sample preparation method greatly influences the reliability and accuracy of the analysis. Sample preparation is frequently done off-line and in fact, this is often a limiting step to perform fast bioanalysis; the introduction of on-line sample pretreatment would greatly speed up the analyses. Furthermore, as the number of samples increases, high-throughput and fully automated analytical techniques are required. Therefore, new on-line and automated sample handling techniques need to be developed. Microextraction in a packed syringe (MEPS) is a new technique for miniaturized SPE that can be connected on-line to GC or LC without any modifications [6 – 11]. In MEPS, approximately 1 mg of solid sorbent material is inserted into a syringe (100–250 μL) as a plug. The urine sample (50 – 1000 μL) is withdrawn through the syringe by an autosampler. When the urine has passed through the solid sorbent, the analytes are adsorbed to the solid phase. The solid phase is then washed once by

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Abbreviations: I.S., internal standard; **MEPS,** microextraction in packed syringe; **PPX,** 2,6-pipecoloxylidide; **QC,** quality control

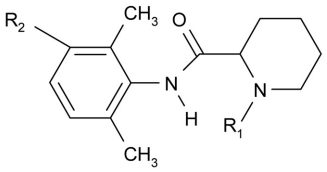
water to remove the salts and other interfering material. The analytes are then eluted with an organic solvent such as methanol or the LC mobile phase (20 – 50 μL) directly into the instrument's injector. The process is fully automated. Different types of adsorption materials such as silica based (C2, C8, C18), restricted access material (RAM), or molecular imprinted polymers (MIPs) can be used.

The aim of this study was to develop and validate a method for the determination of ropivacaine and its major metabolites 3-OH-ropivacaine and deisopropylropivacaine (PPX) in urine samples utilizing MEPS as online sample preparation with LC-MS/MS method.

2 Material and methods

2.1 Reagents

Ropivacaine, 3-OH-ropivacaine, PPX and [$^2H_7$]ropivacaine internal standard (I.S.) (Fig. 1) 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).



Compound	R_1	R_2
Ropivacaine	C_3H_7	H
3-OH-ropivacaine	C_3H_7	OH
PPX	H	H
[$^2H_7$]Ropivacaine	$C_3\,^2H_7$	H

Figure 1. The chemical structures of the studied compounds.

2.2 Apparatus and chromatographic conditions

The HPLC instrument included two pumps, Shimadzu LC-10ADvp, Shimadzu corporation (Kyoto, Japan). The mass spectrometer was a Micromass Z-spray QII mass spectrometer from Waters (Manchester, UK) and the chromatographic data system was MassLynx version 3.4. An Optiguard (C8, 1 mm × 20 mm) obtained from Optimize Technologies (Oregon, USA) was used and was connected to the mass spectrometer. An isocratic HPLC 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 and the sample volume (elution volume from MEPS) was 20 μL.

All experiments were conducted using a triple quadrupole mass spectrometric instrument equipped with a Z-electrospray interface (ESI) operated in 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 the collision gas. The data were collected using MassLynx version 3.4 and all calculations were based on peak area ratios.

The scan mode was multiple reaction monitoring using the precursor ion at *m/z* (M + 1) (*m/z*: 275, 291, 233 and 282), and after collisional dissociation the product ions 126, 126, 84 and 133 were used for quantification of ropivacaine, 3-OH-ropivacaine, PPX and [$^2H_7$]ropivacaine (I.S.), respectively.

2.3 Preparation of samples

Stock solutions (300 μM) ropivacaine, 3-OH-ropivacaine and PPX in 0.1% HCOOH in methanol/water (1 : 1) were prepared. Spiked urine samples were prepared by adding a few microlitres of analytes to 1.0 mL of urine and 25 μL of the I.S. (10 μM) was added. The concentration range of the standard curve was between 5 and 2000 nM.

2.4 MEPS conditions

The sorbent used was a polystyrene polymer, ISOLUTE ENV+, from Argonaut (Mid Glamorgan, UK). This sorbent has irregular particles with an average size of 50 μm and nominal 60 Å porosity. One milligram of the solid material was manually inserted inside the syringe as a plug. The sorbent material was tightened by filters to avoid movement inside the syringe. The packed syringe is conditioned first with methanol and then with water (50 μL) before being used for the first time. The urine sample is drawn through the syringe three times (50 μL each time from the same portion) 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 salts and other sources of interference. The analytes are then eluted with 20 μL (0.2% NH_4OH in methanol) directly into the LC injector. The multiple pulling/pushing of the sample by the syringe increases the extraction yield. Also, using a small amount of the adsorbent (1 mg) makes it easy to wash and use the same syringe many times. In MEPS, a standard syringe with removable needle is used and no modifications are needed either for the autosampler or for LC. The packed syringe can be normally used for 100 injections without repacking.

3 Results and discussion

3.1 Validation

Calibration standard solutions with a concentration range of 5 – 2000 nM in human urine were prepared.

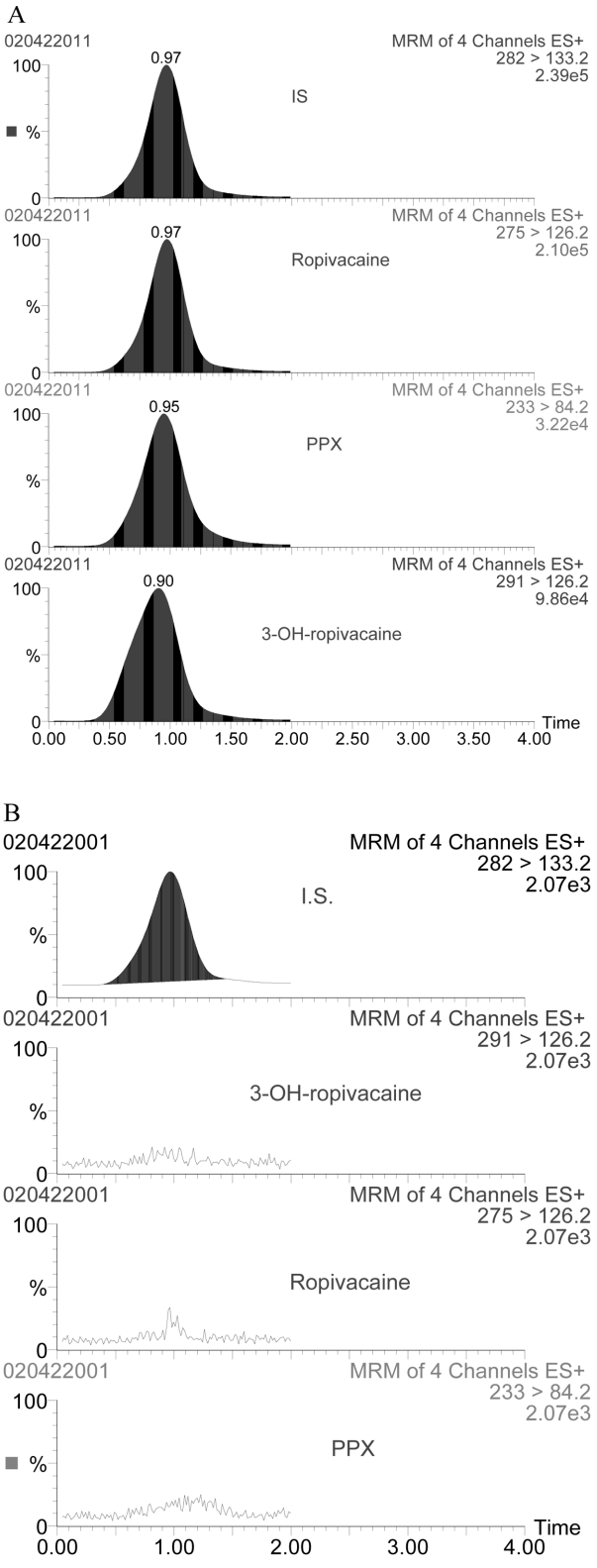


Figure 2. Mass chromatograms obtained from (A) human urine spiked with ropivacaine, 3-OH-ropivacaine and PPX (2000 nmol/L) and [$^2H_7$]ropivacaine as I.S.; (B) blank human urine with I.S.

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

$$y = ax^2 + bx + c \quad (1)$$

where y is the peak area ratio, x the concentration, b the slope, c the intercept and a 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 interassays were determined by using three levels of concentrations (QC: low, medium and high), which were 25, 700 and 1400 nM (n = 6). Selectivity, linearity, accuracy, precision, recovery and LOQ were investigated according to Shah *et al.* [12].

3.2 Selectivity

When urine spiked with ropivacaine and its metabolite was analysed and compared to blank plasma, no interfering compounds were detected at the same retention times as the studied compounds. Figure 2A and B shows good selectivity when using MEPS as a sample preparation method.

3.3 Calibration

For the construction of the calibration curve, eight levels of the analytes in human urine were used. The results showed a close relationship between the concentrations and relative peak areas for the analytes studied in the concentration range 5–2000 nM. The correlation coefficient (R^2) values obtained were over 0.999 for all runs.

3.4 Accuracy and precision

The accuracy was determined by the ratio of the found and theoretical concentrations for human urine control samples, at three different levels. The precision is a measure of the random error and was determined by the percentage coefficient of variation (CV%) of the within- and between-day variations (intra- and interday) at three levels. The intra- and interday variations were determined by analysis of QC samples at three different concentrations 25, 700 and 1400 nM. The results are shown in Table 1. The CV% values were between 1.9 and 15% for intraday and 2.1–7.9% for interday. The accuracy varied from 99 to 115% (n = 18). Validation of the methodology showed that the method is highly selective for ropivacaine and its metabolites in urine samples. The accuracy and precision were well in line with international criteria [12].

Table 1. Intra- and interday precision and accuracy for ropivacaine and its metabolites in human urine (QC samples)

Analyte	Concentration (nM)	Accuracy (RSD%) $n = 18$	Intraday (RSD%) $n = 6$	Interday (RSD%) 3 days
Ropivacaine	25	115	4.9	7.9
	700	99	3.2	2.1
	1400	106	1.9	2.9
PPX	25	103	15	4.6
	700	107	3.8	3.1
	1400	107	3.5	4.5
3-OH-ropivacaine	25	113	11	5.6
	700	107	3.6	2.5
	1400	110	3.2	6.1

3.5 Extraction degree, 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 and 40% for the metabolites. The LOQ for the analytes studied was 5 nmol/L. 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 0.1%. The syringe-to-syringe variations were also tested. The variations were less than 2%.

4 Concluding remarks

A new selective and accurate on-line sample preparation technique, based on the use of a small amount of polystyrene polymer packed in a syringe, was developed and validated for the determination of ropivacaine and two of its major metabolites in human urine 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 [2, 3]. Sample preparation takes only 1 min for each sample compared with 10 – 20 min with earlier methods (SPE and LLE).

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