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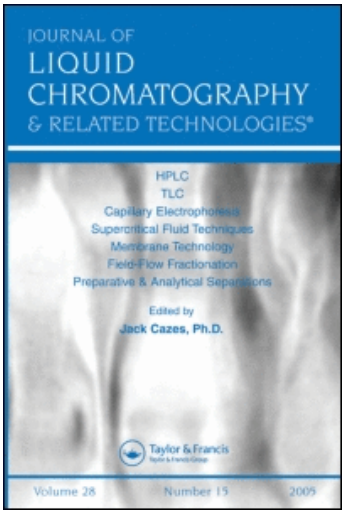

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Evaluation of Monolithic Packed 96-Tips for Solid-Phase Extraction of Local Anesthetics from Human Plasma for Quantitation by Liquid Chromatography Tandem Mass Spectrometry

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Abstract: Polypropylene 96-well tips packed with a chemically bonded monolithic methacrylate plug as sample adsorbent were used for solid-phase extraction (SPE) of ropivacaine and bupivacaine in human plasma samples. Monolithic packed 96-tips are a miniaturized, solid-phase extraction. Packed 96-tips require some microliter volumes of solvent (50–100 μL) for elution. Using packed 96-tips, a 96-well plate could be handled in about 2 minutes. The key aspect of the monolithic phase is that monolithic material processes both high binding capacity and low back-pressure properties. The validation of the methodology showed that the accuracy values of quality control samples were between 101 and 118%, while the precision ranged from 4% to 17%. The standard calibration curves were obtained within the concentration range 2–2000 nM in plasma samples. The coefficients of determination (R^2) for plasma samples were between 0.984 and 0.999.

Keywords: Packed 96-tips, Automated sample preparation, Methacrylate monolith, LCMS/MS, Local anesthetic, Plasma samples

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INTRODUCTION

High throughput and fully automated analytical techniques are becoming more of a requirement in drug discovery and drug development. Current developments in sample handling techniques are directed toward the automation and on+line coupling of sample preparation units and detection systems.[1–7]

In addition, there is a trend toward the development of more selective sorbents for sample clean-up and enrichment. Recently, we introduced a 96-tips set packed with a short plug of a monolithic adsorbent, where the set makes it possible to handle a 96-well plate in only 2 minutes.[7,8] Packed 96-tips sample preparation is a clean, high-throughput, and completely automated sample preparation method. Samples are prepared in a 96-well plate format and the analytes adsorb onto the polymer based monoliths in the extraction step. The next step purifies the sample by washing the sorbent with an appropriate washing solution. In the present work, sample preparation of plasma samples took place, water being used as washing solution to elute proteins, salts, and other polar substances. In a final step, the analyte was directly eluted into a 96-well plate using an appropriate solvent for the analyte and the subsequent instrumental analysis.

The aim of this work has been to develop a method for rapid and fully automated clean-up of samples containing drugs in a plasma matrix. For this purpose a Personal Pipettor robot has been used to handle the 96-tips and the 96-well plate. Each tip has been packed with a small plug of monolithic support for the clean-up procedure.

The methodology was validated using ropivacaine, bupivacaine, and lidocaine in plasma samples as model substances. Ropivacaine, bupivacaine, and lidocaine are amide type local anesthetics and have been used in anesthesiology for many years. They inhibit reversibly the conduction of nerve signals by preventing the passage of sodium ions into the cell membrane.[9–11]

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EXPERIMENTAL

Chemicals and Materials

Ropivacaine, bupivacaine, and $^{2}H_{7}$-ropivacaine (I.S.) (Figure 1) were supplied by the Department of Medicinal Chemistry, AstraZeneca (Södertälje, Sweden). Methanol, acetonitrile, acetone, and formic acid were obtained from Merck (Darmstadt, Germany). Ethylene glycol dimethacrylate (EGDMA), butyl methacrylate (BMA), porogenic solvents (1,4-butanediol and l-propanol) and benzoylperoxide (BPO) were from Sigma-Aldrich (Stockholm, Sweden). All chemicals were of analytical grade.

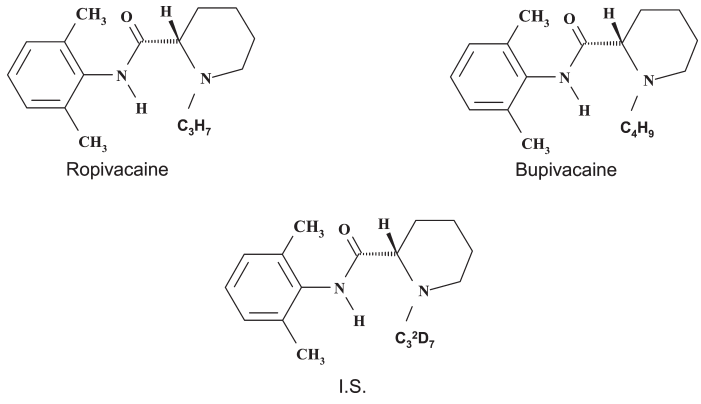

Figure 1. Chemical structure of ropivacaine, bupivacaine, and internal standard (I.S.).

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Apparatus

The high performance liquid chromatography (HPLC) instrument included two pumps, Shimadzu (Kyoto, Japan), and a CTC-Pal Crelab (Knivsta, Sweden) autosampler with a 20 μL sample loop. A Symmetry (50 × 2.1 mm, C_8, 3.5 μm) column obtained from Waters (Mass., USA) was used as an analytical column connected to an Optiguard (C_8, 10 × 1 mm) as a guard column (Optimize Technologies, Oregon, USA). The Milli-Q water was obtained using a Reagent Grade Milli-Q Plus water purification system from Millipore Corporation (Bedford, Mass., USA). A centrifuge, Hettich Rotanta/AP (Tuttlingen, Germany), was used for plasma centrifugation.

A gradient HPLC instrument was used with a mixer volume of 0.1 mL. Mobile Phase A was 0.1% formic acid in acetonitrile and water (10:90, v/v) and mobile phase B contained 0.1% formic acid in acetonitrile and water (80:20, v/v). The gradient started from 0% of phase B up to 80% from 1 to 5 min and then from 5 to 6 min isocratic at 80% of phase B, and at 6.1 min phase B was reset to 0%.

All experiments were conducted using a triple quadrupole mass spectrometric instrument, Micromass QII Z-spray (Manchester, UK), equipped with a Z-electrospray interface and operated in positive ion mode. The parameter settings used were: capillary voltage of 3.1 kV, cone voltage of 38 V, extractor of 5 V, RF lens of 0.2 V, and source block and desolvation temperatures of 150°C and 300°C, respectively. Nitrogen was used both as a drying (400 L/h) and a nebulizing gas (20 L/h) and the vacuum was 2×10^{-5} mbar in the mass analyzer and 2×10^{-3} mbar in the collision cell. Argon was used as collision gas and the collision energy was 25 eV. The gases were from ScanGas (Stockholm, Sweden).

The scan mode was multiple reaction monitoring using the precursor ion at m/z (M + 1) (m/z: 275, 289, and 282) and after collisional dissociation the product ions 126, 140, and 133 were used for quantitation of ropivacaine, bupivacaine, and $^{2}H_{7}$-ropivacaine (I.S.), respectively. The data were collected and processed using MassLynx version 3.5 and all calculations were based on peak area ratios.

Preparation of Monolithic Packed 96-Tips

The preparation of a poly(EGDMA-BMA) monolithic sorbent plug has been described in previous publications.[7,8] A solution containing EGDMA (70%), BMA (30%), BPO (2wt% with respect to monomers), 1,4-butanediol (25%), 1-propanol (65%), and water (10%) was vortexed for 10 min and purged with nitrogen for 10 min in order to remove oxygen. Using a microsyringe, the tips were filled with about 10 μL of the polymerization mixture and placed vertically inside the polymerization apparatus, calibrated at 254 nm UV light. The polymerization was allowed to proceed initially for 80 min with the sharp end of the tip down at a distance of 15 cm from the lamps, and then for 50 min with the sharp end up at a distance of 5 cm from the lamps. On completion of polymerization, the tips were removed, inspected under the microscope for bubbles, and washed with an appropriate washing solution to remove the porogenic solvents and other compounds remaining in the monolith. Before use, the tips were thoroughly washed with methanol and then conditioned with the wetting solution (water), to ensure optimum adsorption of the analytes. The setup is shown in Figure 2.

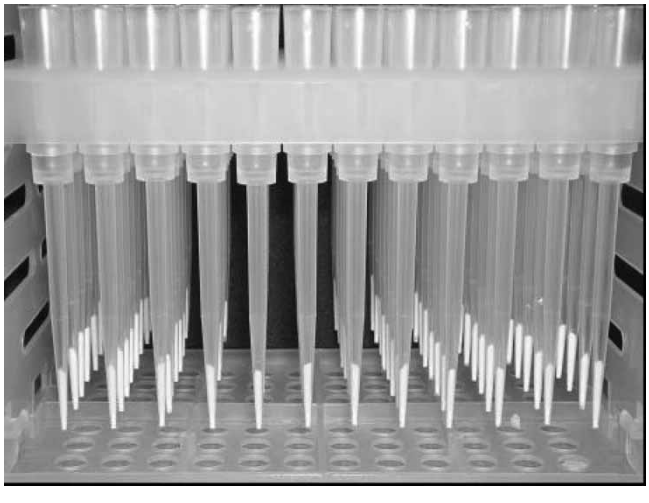

Figure 2. UV-polymerized 96-tips packed with monolithic methacrylate polymer.

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Sample Preparation

Plasma samples were stored at −20°C. Before use, the plasma was thawed at room temperature and centrifuged (3500 rpm for 10 min). Stock solutions of ropivacaine and bupivacaine were dissolved in methanol/water, 1:1 (20 μM). The spiked human plasma was diluted with water (1:5) and 20 μL of I.S. (10 μM) was added in a 96-well plate format. The concentration range of the standard curve was between 2 and 2000 nM. A Personal Pipettor robot (PP-550 N-MS) obtained from Apricot Designs, Inc. (Monrovia, Calif., USA) was used to handle the 96-tips. A plasma sample of 100 μL from a 96-well plate was sampled by the robot. After the plasma had passed through the monolithic plug, the analytes had been adsorbed to it. The solid phase was then washed once in water (100 μL) to remove the proteins and other interferences. Finally, the analytes were eluted with 100 μL of 95% methanol in water directly into a clean 96-well plate.

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Method Validation

Each calibration curve consisted of eleven calibration points covering the concentration range 2–2000 nM. Blank samples were run simultaneously. The plasma used was collected and pooled from six different sources. The peak area ratios for analyte and internal standard were measured and a standard curve without zero concentration was constructed. The calibration curves were described by the equation:

$$y = ax^2 + bx + c,$$

where y is peak area ratio, x is the concentration, a is the curvature, b is the slope, and c is the intercept. Due to the relatively low capacity of the monolithic polymer used here and the nature of the matrix, the calibration curves were quadratic and the weight was 1/x. The quality control (QC) samples were prepared with the concentrations 8.0, 800 nM, and 1500 nM. The accuracy and precision were calculated for the QC samples at three different concentration levels. The method was validated under optimized conditions.

RESULTS AND DISCUSSION

Selectivity

LC–MS/MS analysis of the blank plasma samples showed no significant interference peaks with the quantitation of ropivacaine and bupivacaine. Using six individual sources of blank matrix, the interferences from endogenous plasma compounds were less than 20% of the response of the lower limit of quantitation (LLOQ). The selectivity of the assay was evaluated, with and

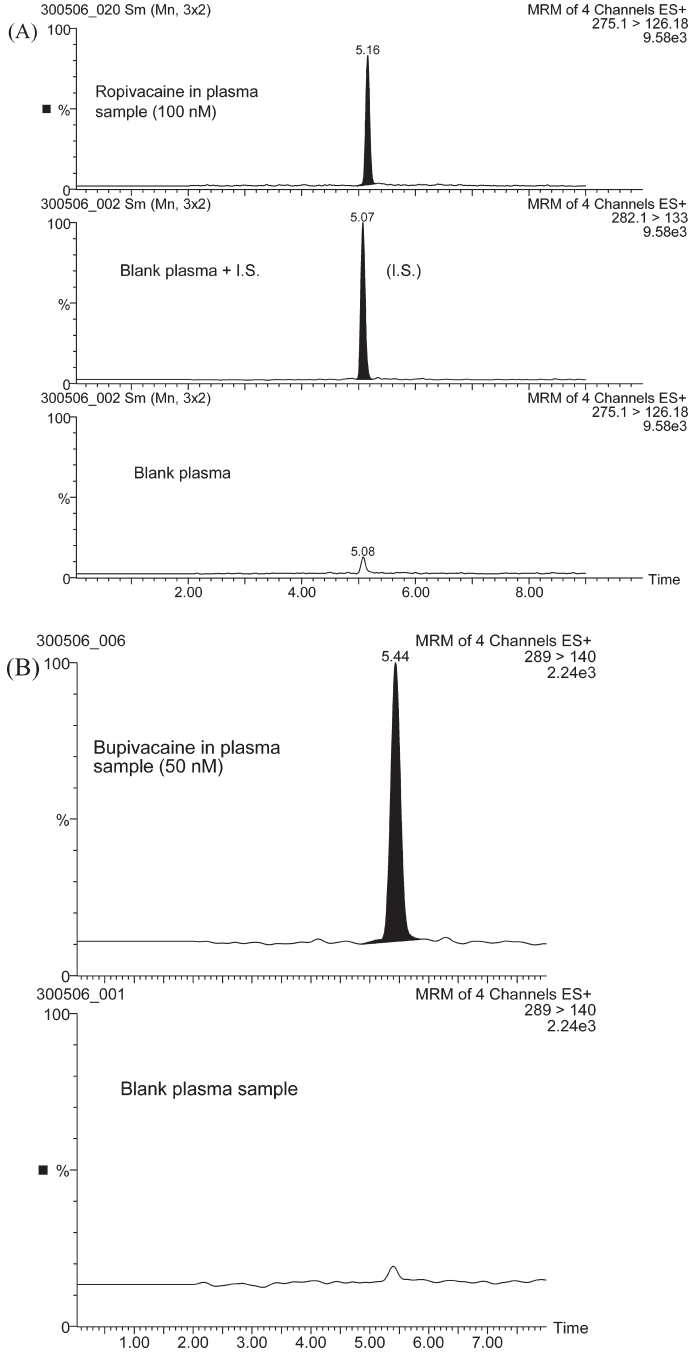

Figure 3. Mass chromatogram obtained from spiked and blank plasma of (A) ropivacaine (100 nM) and (B) bupivacaine (50 nM).

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Table 1. Regression parameters for calibration curves

Analyte	Curvature a (10^{-7})	Slope	Intercept	R^2 (n = 3)
Ropivacaine	1.51E-08	2.4E-04	1.2E-03	0.999
Bupivacaine	2.11E-07	8.7E-04	3.1E-03	0.984

without addition of internal standard (I.S.) ropivacaine-d_7 containing about 1% ropivacaine. Representative chromatograms of blank human plasma and plasma spiked with ropivacaine and bupivacaine are presented in Figure 3.

Calibration

The constructed calibration curve consisted of eleven levels of human plasma samples in the concentration range 2–2000 nM. The coefficients of determination (R^2) for plasma samples were between 0.98 and 0.99 for all runs of ropivacaine and bupivacaine (Table 1).

Within- and Between-Batch Accuracy and Precision

Accuracy is reported as the percentage difference from the nominal value and precision is stated as the relative standard deviation. Within-batch accuracy and precision were determined by analysis of six repeat QC samples at three concentration levels over the calibration range tested, in one analytical run. Between-batch accuracy and precision were determined by analysis of six repeat QC samples at three concentration levels over the calibration range tested, in three separate analytical runs.

Table 2. Accuracy and precision for ropivacaine and bupivacaine

			Precision	
Substance	Conc. (nM)	Accuracy (%) n = 18	Within-batch (R.S.D.%) n = 6	Between-batch (R.S.D.%) n = 18
Ropivacaine	8	112	12	14
	800	112	11	17
	1500	111	4	12
Bupivacaine	8	116	10	15
	800	118	9	16
	1500	101	14	11

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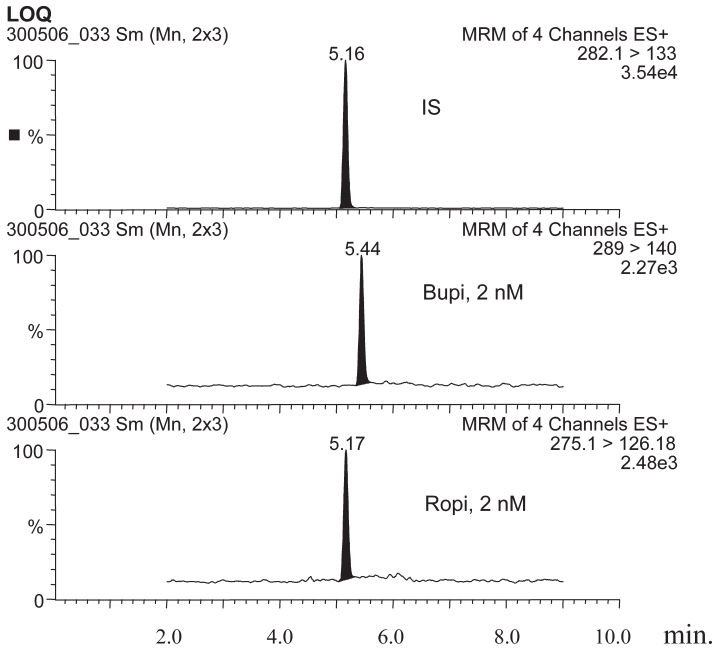

Figure 4. Representative calibration sample in human plasma at LLOQ, 2.0 nM with internal standard.

The within-batch precisions (R.S.D.) were about 4–14% (n = 6) for studied analytes in plasma samples, while the between-batch precisions (R.S.D.) were in the range 10–17% (n = 18). The accuracy varied from 101% to 118% (n = 18). The accuracy and precision data are summarized in Table 2.

LLOQ

The LLOQ for the analytes studied was set to 2 nM for all studied analytes. At this concentration, the accuracy of the LLOQ was between 90% and 110% and the precision had a maximum deviation of 10% (n = 6) for all studied solutes (Figure 4).

CONCLUSIONS

Factors such as miniaturization and high sample throughput were emphasized. Because of the bimodal pore size distribution for monolithic material as prepared in this study, this facilitates a low pressure drop even at high flow rates and provides high binding capacity. These properties are crucial for successful extraction of analytes in complex matrices. Using 96 polypropylene tips containing a monolithic methacrylate bed as sorbent, 96 samples could

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be extracted in about 2 minutes and microliter volumes of solvent were required for elution. The present method provides good accuracy and precision within the range of the calibration curve. Furthermore, our method reduces the sample preparation time and can handle small sample volumes, which is of great importance in bioanalysis.

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