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# Development of a molecularly imprinted polymer based solid-phase extraction of local anaesthetics from human plasma

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**Abstract**

Molecular imprints selective for a homologous series of local anaesthetics, including bupivacaine, ropivacaine and mepivacaine, were prepared and the resultant polymers were used for solid-phase extraction of human plasma. The template was a structural analogue, pentycaine, which was imprinted in methacrylic acid–ethylene glycol dimethacrylate copolymers. Equilibrium ligand binding experiments using radiolabelled bupivacaine were performed to characterize the imprinted polymers, as well as to identify optimal conditions for selective extraction of plasma samples. Dilution of the plasma prior to extraction with citrate buffer pH 5.0 containing ethanol and Tween 20 was found optimal for selective imprint–analyte binding, and for reduction of non-specific adsorption of lipophilic contaminants to the hydrophobic MIP surface. Wash steps using 20% methanol in water followed by a solvent switch to 10% ethanol in acetonitrile removed contaminants and strengthened the selective imprint–analyte binding. Elution under basic conditions using triethylamine–water–acetonitrile mixtures recovered bupivacaine in 89% yield with superior selectivity over elution under acidic conditions. The final protocol extracted trace levels of ropivacaine and bupivacaine from human plasma and allowed determination of bupivacaine in the range of 3.9–500 nmol $L^{-1}$ and ropivacaine in the range of 7.8–500 nmol $L^{-1}$ with inter-assay accuracies of 94–99 and 95–104%, respectively. This present investigation provides an improved understanding of approaches available for optimization of protocols for molecular-imprint based solid-phase extraction of plasma samples.


*Keywords:* Molecular imprints; Solid-phase extraction; Local anaesthetics

## 1. Introduction

Trace and ultra-trace analysis of complex sample matrices often rely on efficient sample pre-treatment and selective methods where a more efficient sample clean-up, performed either on-line or off-line, simplifies downstream analytical separation and facilitates accurate and sensitive detection. Various formats of solid-phase extraction (SPE) is currently a routine sample preparation technique employed in numerous environmental and bioanalytical applications [1–5]. Separation on most current solid-phase extraction sorbents is based on physicochemical retention on the functionalized surface and the SPE column retains not only the target analyte(s) but also other matrix components. Therefore, a considerable amount of method development work is often spent on optimizing the complete analytical method. More selective SPE materials, such as immunosorbents [6,7] and molecularly imprinted polymers (MIPs) [8–11], rely on affinity interactions and potentially offer a higher degree of sample clean-up efficiency than that achieved using conventional type SPE sorbents. Characteristic for both types of affinity materials are their high ligand selectivity and affinity, where selectivity can

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be pre-determined for a particular analyte by, respectively the choice of antigen used for antibody generation and the choice of template used for MIP preparation.

From an analytical separation point of view, a MIP may be best characterized as a material, which in addition to the imprinted affinity sites contains both polar and lipophilic surface functionality. Thus, chromatographic retention of analyte is due to a mixed-mode mechanism involving both selective affinity binding with imprints and non-specific physicochemical adsorption on polymer surface. Accordingly, the development of a MIP based solid-phase extraction (MISPE) [12–16] method requires a fundamental understanding of the strength and nature of imprint–analyte and polymer surface–analyte interactions, respectively, and how these vary with the type of solvent or buffer employed. Under conditions where physicochemical adsorption mechanisms dominate, non-specific retention of other sample components leads to poor clean-up efficiency. Also, the analyte will be retained primarily through interaction with polymer surface and selectivity of the imprints may remain undetected. Under normal-phase conditions non-specific physicochemical retention is due mainly to polar interactions and under reversed-phase conditions to hydrophobic interaction. Hence, non-specific binding can be eliminated by the choice of appropriate wash and eluent solvents. Also, selectivity of the imprint–analyte binding is tuned by the solvent properties of the surrounding medium, where under normal-phase conditions imprint recognition of polar functionalities of the analyte increases in relative importance and under reversed-phase conditions imprint recognition of lipophilic moieties become more significant [17–19]. Most MIP syntheses are organic solvent based, and studies on imprint rebinding are often conducted using organic solvents as the incubation medium, where establishment of conditions for strong and selective rebinding is fairly well understood. This is not yet true to the same extent for aqueous rebinding, such as in the presence of biofluids, and this present study focuses on optimization of conditions for direct SPE of plasma samples.

Imprinted polymers are made in the presence of high concentrations of template and it is known that despite exhaustive washing trace amounts of imprint species may remain in the MIP and later leak during use [12–16,20], i.e. template bleeding. While being less pronounced for on-line SPE systems where the MIP column is continuously washed by the solvent flow as well as for templates where weaker binding interactions are employed, template bleeding is acknowledged generally as an inherent problem associated with the use of MIPs. For MISPE applications in trace analysis near-complete removal is essential as even very small amounts of remaining template species may interfere with the assay. Hence, template bleeding must be addressed in each MISPE development. An approach to circumvent this problem completely is the alternative template approach [20], which uses a close structural analogue of the analyte(s) of interest for the MIP preparation. In this present study, imprinting of pentycaine produced MIPs selective for a homologous series of local anaesthetics, including mepivacaine, ropivacaine and bupivacaine (Fig. 1) [21]. The resultant MIPs were used for development of a MISPE protocol for pre-concentration of these drugs from plasma samples. To examine imprint–analyte selectivity and to study the influence of incubation medium composition on selective and non-specific binding modes a series of radioligand binding experiments were conducted. These investigations defined a starting point for optimization of the MISPE protocol. The final protocol was evaluated with respect to accuracy and precision of the determination of ropivacaine and bupivacaine in human plasma.

## 2. Experimental

### 2.1. Materials and methods

(*R*,*S*)-Mepivacaine, (*R*,*S*)-ethycaine, (*S*)-ropivacaine, (*R*,*S*)-bupivacaine and (*R*,*S*)-pentycaine (Fig. 1) were obtained from AstraZeneca R&D Södertälje. Ethylene glycol dimethacrylate (EGDMA), methacrylic acid (MAA), 2-(trifluoromethyl)acrylic acid (TFMAA) and 2,2′-azobisisobutyronitrile (AIBN) were all purchased from Aldrich (Milwaukee, WI, USA) and used as-received. All solvents were HPLC grade and used as-received.

### 2.2. Polymer synthesis

In a typical procedure the free base of pentycaine (template, 0.55 mmol) and AIBN (initiator, 0.73 mmol) were dissolved in toluene (8.7 g) and then EGDMA (33.2 mmol) and either MAA (6.64 mmol) or TFMAA (6.64 mmol) were added. The tubes were placed in an ultrasonic water bath until clear solutions were obtained, then cooled on ice and the solutions sparged with nitrogen. The tubes were placed under a UV-lamp (366 nm) at 4 °C for 7 h and then at room temperature for 20 h. The hard polymers were ground in a laboratory mortar grinder (Retsch, Haan, Germany) under wet conditions. The particles were sieved with water either to collect particles 25–45 μm in size (for solid-phase extraction experiments) or particles that passed through a 25 μm sieve (for radioligand binding experiments). Grinding and sieving were repeated until all material passed the 45 μm sieve. Fines in the <25 μm fraction were removed by repeated sedimentation from ethanol. The particles were washed with methanol–acetic acid (4:1 v/v), methanol, ethanol–water–5 mol $L^{-1}$ NaOH (5:3:2 v/v/v), methanol,

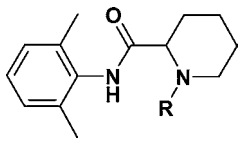


Fig. 1. Structures of the compounds studied. Mepivacaine: R = methyl; ethycaine: R = ethyl; ropivacaine: R = *n*-propyl; bupivacaine: R = *n*-butyl; pentycaine: R = *n*-pentyl.

ethanol–acetic acid–water (18:1:1 v/v/v), methanol and finally dried under vacuum. In parallel, non-imprinted reference polymers were prepared using the same conditions with the exception that template was omitted.

### 2.3. Template leakage test

To glass test tubes were added 5 mg of imprinted polymer, 260 μL of ethanol, 340 μL of NaOH (2.5 mol $L^{-1}$) and 3.4 mL of heptane. The contents of the tubes were mixed by continuous end-over-end rotation for 1 h and then the tubes were centrifuged at 3000 rpm for 10 min. The organic layers were washed with 800 μL of NaOH (2.5 mol $L^{-1}$), and then transferred to borosilicate test tubes and evaporated to dryness. The residues were re-dissolved in 150 μL of heptane–ethanol (9:1 v/v) and 1 μL analyzed by GC.

### 2.4. Organic solvent based radioligand binding experiments

Suspensions of each polymer in the incubation solvent were made and the particles allowed to swell for at least 16 h before use. To Eppendorf test tubes were added [$^3$H]-bupivacaine (specific radioactivity 6 Ci $mmol^{-1}$, 220 GBq $mmol^{-1}$), appropriate volumes of polymer and competing ligand stock solutions, and solvent to a total volume of 1 mL. The tubes were placed on a rocking table for 3 h, centrifuged at 10,000 × *g* and 700 μL of the supernatants analyzed by liquid scintillation counting.

### 2.5. Aqueous buffer based radioligand experiments

Suspensions of each polymer in water were made and the particles allowed to swell for at least 24 h before use. To Eppendorf test tubes were added [$^3$H]-bupivacaine (specific radioactivity 6 Ci $mmol^{-1}$, 220 GBq $mmol^{-1}$), appropriate volumes of polymer and competing ligand stock solutions, buffer, ethanol and detergent to a total volume of 1 mL. The tubes were placed on a rocking table for 3 h, centrifuged at 10,000 × *g* and 700 μL of the supernatants analyzed by liquid scintillation counting.

### 2.6. GC-analysis

The GC system consisted of a Hewlett Packard 5890 gas chromatograph with an NPD detector operated at 260 °C, a Hewlett Packard 7673 auto-sampler and an HP 50 capillary column (25 m × 0.20 mm × 0.31 μm film thickness) (Palo Alto, CA, USA). The oven temperature program was as follows: the initial temperature of 120 °C was kept for 2 min, then increased to 280 °C at a rate of 40 °C/min and the final temperature kept for 6 min. The carrier gas was helium at a flow velocity of 35 cm $s^{-1}$. Injection in the splitless mode was carried out at 260 °C. Data collection was performed using HP Chemstation Software (Palo Alto, CA, USA).

### 2.7. Optimization of MIP extraction elution conditions

#### 2.7.1. Experiment 1

Plasma samples (400 μL) containing 800 nmol $L^{-1}$ bupivacaine, 80 nmol $L^{-1}$ [$^3$H]-bupivacaine and 800 nmol $L^{-1}$ ropivacaine were diluted to 1 mL with citrate buffer pH 5.0 in a final concentration of 0.2 mol $L^{-1}$ citrate, 5% ethanol and 0.05% Tween 20. Samples were loaded on pentycaine-MIP columns and columns washed with 1 mL of water–ethanol (4:1 v/v). Analytes were eluted with 5 × 1 mL of eluent as listed in Table 1. Radioactivity was counted in 10 vol.% of fractions 1 and 2, and in whole fractions 3–5. The remaining 90 vol.% of fractions 1 and 2 were evaporated to dryness, re-dissolved in heptane–ethanol (9:1 v/v) and analyzed by GC. Recovery in each fraction is reported as percent of total radioactivity added to sample.

#### 2.7.2. Experiment 2

Plasma samples (400 μL) containing 20 nmol $L^{-1}$ [$^3$H]-bupivacaine, 1000 nmol $L^{-1}$ ropivacaine and 2000 nmol $L^{-1}$ ethycaine were diluted to 1 mL with citrate buffer as above and loaded on bupivacaine-MIP columns. The columns were washed with 2× 1 mL of water–methanol (4:1 v/v) followed by 2× 1 mL acetonitrile. Analytes were eluted with 2× 1 mL of eluent as listed in Table 2. Radioactivity was counted in 10 vol.% of each fraction and the remaining 90 vol.% of each fraction were evaporated to dryness, re-dissolved in heptane–ethanol (9:1 v/v) and analyzed by GC. Recovery in each fraction is reported as percent of total radioactivity added to sample.

### 2.8. Solid-phase extraction on MIP and non-imprinted reference polymer

MIP and reference polymer stock suspensions in water were prepared at a concentration of 40 mg $mL^{-1}$. Appropriate volumes of stock suspension were transferred into empty solid-phase extraction columns and particles packed

Table 1
Typical recovery of bupivacaine with elution using methanol, ethanol or acetonitrile based elution solvents

| Eluent | First (%) | Second (%) | Third (%) | Fourth (%) | Fifth (%) | Total recovery (%) |
|---|---|---|---|---|---|---|
| TEA in methanol | 67.7 | 13.1 | 4.5 | 1.7 | 0.94 | 87.8 |
| TEA in ethanol | 72.7 | 13.1 | 4.9 | 2.6 | 1.2 | 94.5 |
| TEA in acetonitrile | 61.3 | 8.9 | 3.4 | 1.5 | 1.0 | 76.1 |

Samples were applied to columns as described in Section 2.7.1. The columns were washed with 1 mL of 20% ethanol in water and eluted with 5× 1 mL of varying amounts of TEA in solvent.

Table 2
Recovery of bupivacaine as a function of water in TEA–acetonitrile or acid in acetonitrile

| Elution solvent | First (%) | Second (%) | Total recovery (%) |
|---|---|---|---|
| 2% TEA in acetonitrile | 41.5 | 20.8 | 62.3 |
| 2% water + 2% TEA in acetonitrile | 63.8 | 13.8 | 77.6 |
| 4% water + 2% TEA in acetonitrile | 71.2 | 11.0 | 82.2 |
| 6% water + 2% TEA in acetonitrile | 76.8 | 8.9 | 85.7 |
| 10% water + 2% TEA in acetonitrile | 75.3 | 9.1 | 84.4 |
| 15% water + 2% TEA in acetonitrile | 78.1 | 8.1 | 86.2 |
| 20% water + 2% TEA in acetonitrile | 74.2 | 7.2 | 81.4 |
| 30% water + 2% TEA in acetonitrile | 76.1 | 7.2 | 83.3 |
| 2% TFA in acetonitrile | 72.3 | 10.6 | 82.9 |
| 10% TFA in acetonitrile | 81.4 | 6.5 | 87.9 |
| 2% formic acid in acetonitrile | 49.1 | 15.5 | 64.6 |
| 10% formic acid in acetonitrile | 65.5 | 13.5 | 79.0 |
| 2% acetic acid in acetonitrile | 13.6 | 11.1 | 24.7 |
| 10% acetic acid in acetonitrile | 36.9 | 12.7 | 49.6 |

Samples were applied to columns as described in Section 2.7.2. The columns were washed with 2× 1 mL of 20% methanol in water, then 2× 1 mL of acetonitrile and finally eluted with 2× 1 mL of elution solvent.

by help of vacuum and pressure. The columns were stored in the dry state until use. Prior to use columns were activated by treatment with 1 mL methanol and 1 mL water. Plasma samples (400 μL) were diluted with 100 μL of ethycaine internal standard solution and 500 μL of 0.4 mol $L^{-1}$ citrate buffer pH 5, containing 0.1% Tween 20 and 10% ethanol, and applied onto the columns. After washing the columns with 2 mL of water–methanol (4:1 v/v) and 0.5 mL of acetonitrile–ethanol (9:1 v/v), the analyte was eluted with 2 mL of acetonitrile–water–triethylamine (46:3:1 v/v/v). The eluates were evaporated to dryness, the residues re-dissolved in 150 μL of heptane–ethanol (9:1 v/v) and 5 μL analyzed by GC.

## 3. Results and discussion

### 3.1. Polymer synthesis and initial characterization

MIPs were synthesized by copolymerization of ethylene glycol dimethacrylate (EGDMA) with either methacrylic acid (MAA) or 2-(trifluoromethyl)acrylic acid (TFMAA) in the presence of pentycaine, called $MIP_{MAA}$ and $MIP_{TFMAA}$, respectively. Following grinding and sieving particles of 25–40 μm were collected for use in solid-phase extraction and particles <25 μm for radioligand binding experiments. Template removal was effected by an extensive wash protocol, which included both acidic washes using acetic acid in methanol and basic wash steps using a mixture of ethanol and 2 mol $L^{-1}$ sodium hydroxide.

The use of a structural analogue as an alternative template eliminated problems with template leakage during solid-phase extraction, however, it was found that sensitivity and reproducibility of the radioligand binding experiments below were improved when template leakage was minimized. Therefore, each new batch of MIP was evaluated with respect to template bleeding by a standardized leakage assay developed for this purpose. A known, fixed amount of MIP was treated with a mixture of sodium hydroxide, ethanol and heptane, where ethanol and heptane causes swelling of the polymer and the strong base forces release of the template, which then immediately partitions into the organic layer. The organic layer was collected and the amount of pentycaine quantified by GC-analysis. A general observation was that a more thorough washing scheme gave less template leakage. Whereas template leakage was found to be 230 nmol $g^{-1}$ for $MIP_{MAA}$ treated with acidic wash steps only, an additional basic wash step reduced the template leakage to $18 \pm 3$ nmol $g^{-1}$ ($n$ = 4 batches of $MIP_{MAA}$). Similarly, for $MIP_{TFMAA}$ acidic washes only gave a leakage of 75 nmol $g^{-1}$, while following treatment with the optimized wash protocol pentycaine leakage could not be detected. A comprehensive study into the influence of various post-polymerization treatments on bleeding of residual template compared extraction techniques such as thermal annealing, microwave assisted extraction, Soxhlet extraction and supercritical fluid desorption [22]. While microwave assisted extraction using trifluoroacetic acid or formic acid was found to be the most efficient extraction technique also polymer degradation and loss of selectivity were observed. None of the treatments eliminated template bleeding completely and it was concluded [22] that the bleeding problem appears to be best solved by the use of an analogue of the analyte as the template [20]. The levels of template bleed found here are on a par with the best extraction methods reported in that study.

The MIP preparations were characterized initially by equilibrium binding experiments where incubations with polymer and radiolabelled bupivacaine were carried out using a mixture of heptane, dichloromethane and ethanol. To prevent non-specific interactions with randomly incorporated carboxylic acid residues, a low percentage of acetic acid was added to the incubation solvent. Binding isotherms were, however, essentially identical to those in the same solvent mixture without acetic acid, and in both instances confirmed the presence of imprints in both $MIP_{MAA}$ and $MIP_{TFMAA}$ (Fig. 2). The polymer concentrations required for binding 50% of the added radioligand, $PC_{50}$, were calculated to be 3.5, 1.8 and 7.3 mg $mL^{-1}$ for $MIP_{MAA}$, $MIP_{TFMAA}$ and $REF_{TFMAA}$, respectively. Very weak binding to $REF_{MAA}$, which was too low to allow calculation of a $PC_{50}$-value, was observed. A $PC_{50}$-value is a function of both imprint affinity and imprint density, for the population of imprints probed under the particular experimental conditions used, and can be used as an estimate of imprinting efficiency. The $PC_{50}$-concentration is a direct readout from the binding isotherm and requires no assumption with respect to binding site affinity distribution model. For a discussion on heterogeneous models for characterization of the binding properties of MIPs, see [23]. Hence, $PC_{50}$-values can be used to compare imprinting recipes as well as to compare re-binding efficiency between varying incubation conditions. Generally, differences

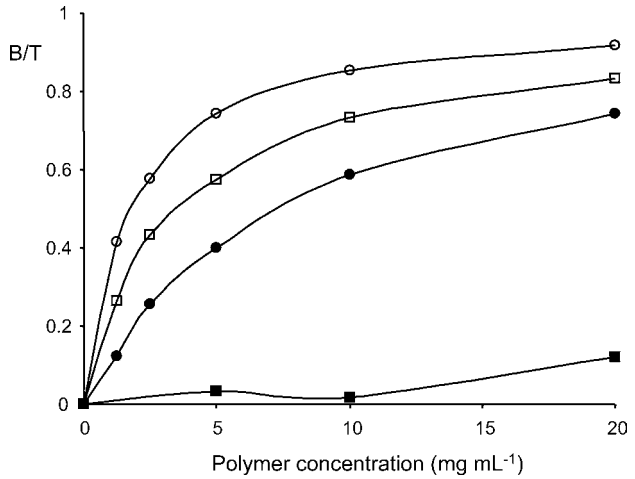


Fig. 2. Binding of [$^3$H]-bupivacaine (7 nmol L$^{-1}$) to $MIP_{MAA}$ (open squares), $MIP_{TFMAA}$ (open circles), $REF_{MAA}$ (filled squares) and $REF_{TFMAA}$ (filled circles) as a function of polymer concentration. The solvent of incubation was heptane–methylenechloride–ethanol (36:9:5 v/v/v) containing 0.5% acetic acid.

in $PC_{50}$-value relative to that recorded for a non-imprinted reference polymer is analyzed. The combination of a low $PC_{50}$-concentration for the MIP and a high $PC_{50}$-value for the reference polymer, such as that seen for the MAA system, indicates the presence of a high density of high-affinity binding sites in the MIP. In contrast, a low $PC_{50}$-value for a non-imprinted reference polymer and a small difference between reference polymer and MIP, as is the case for the TFMAA system, indicates strong non-specific binding under the conditions used and a low number of imprints in the MIP. Hence, the methacrylic acid system is the more efficient one for imprinting of pentycaine.

### 3.2. Investigation into aqueous re-binding conditions

A series of radioligand binding experiments to optimize the conditions for selective analyte binding from plasma samples was performed. The first experiment, which employed phosphate buffer of pH 7.4 containing 10% ethanol as the incubation medium, resulted in binding isotherms for $MIP_{MAA}$ and $REF_{MAA}$ with $PC_{50}$-values of 0.27 and 0.47 mg mL$^{-1}$ (Fig. 3A), demonstrating a strong non-specific hydrophobic adsorption under these conditions. The binding isotherms obtained for $MIP_{TFMAA}$ and $REF_{TFMAA}$ were essentially identical with $PC_{50}$-values of 0.27 and 0.26 mg mL$^{-1}$. The objective of subsequent experiments was to reduce this substantial non-specific binding initially observed, while the selective imprint–analyte binding component should remain unaffected.

The effect of buffer pH on bupivacaine binding was investigated over the range of pH 3–9 for the MAA system and pH 1.5–9 for the TFMAA system (Fig. 4). For both types of MIP binding increased with increased pH, as did binding to the respective reference polymers, and the maximal difference between binding to MIP and reference polymer occurred at

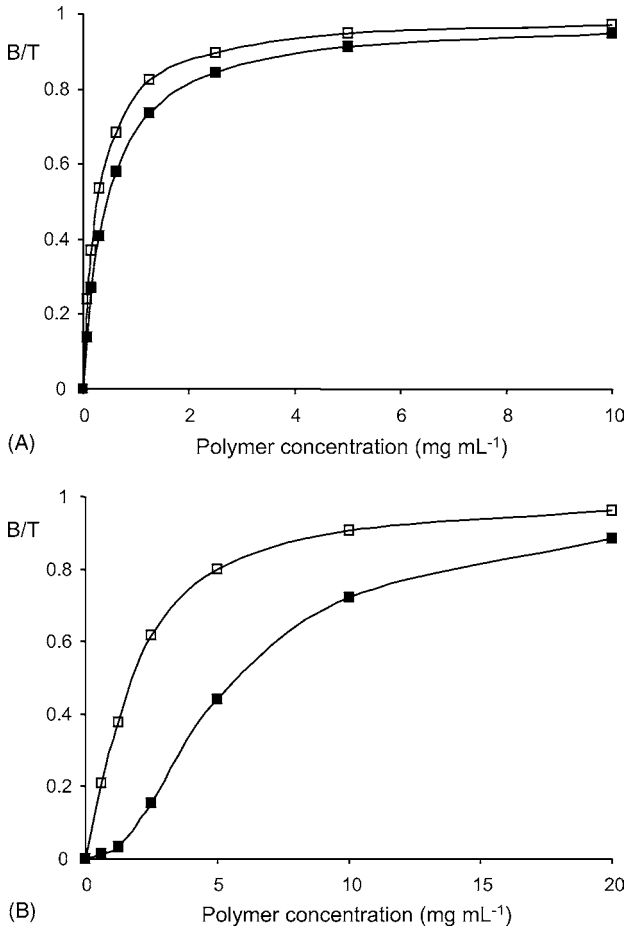


Fig. 3. Binding of [$^3$H]-bupivacaine (7 nmol L$^{-1}$) to $MIP_{MAA}$ (open squares) and $REF_{MAA}$ (filled squares) as a function of polymer concentration. The buffer of incubation was (A) 0.1 mol L$^{-1}$ sodium phosphate, pH 7.4, containing 10% ethanol, and (B) 0.1 mol L$^{-1}$ sodium citrate, pH 5.0, containing 5% ethanol and 0.5% Tween 20.

pH 5 for the MAA system and at pH 3 for TFMAA system. Since the separation between MIP and reference polymer was significantly greater for the MAA system, it was decided to proceed with the MAA polymers only and no subsequent studies were made on the TFMAA system. Acetate and citrate/phosphate buffers gave marginally higher non-specific binding than citrate buffer (all at pH 5.0), and citrate buffer was used in subsequent experiments.

Attenuation of non-specific hydrophobic interactions can be achieved through the use of organic modifiers, such as methanol, ethanol and acetonitrile [17,18]. Analogously to that found for propranolol [17] and bupivacaine [18] MIPs, addition of increasing concentrations of ethanol to the buffer induced a non-uniform reduction in binding to the two polymers. While the non-specific binding was reduced dramatically, the level of selective binding was found affected much less. Ethanol concentrations of 4–16% resulted in a slight increase in relative selectivity for the MIP. The upper limit of ethanol content is, however, limited as precipitation of plasma proteins may occur at high ethanol concentrations, and an ethanol concentration of 5% was used in subsequent

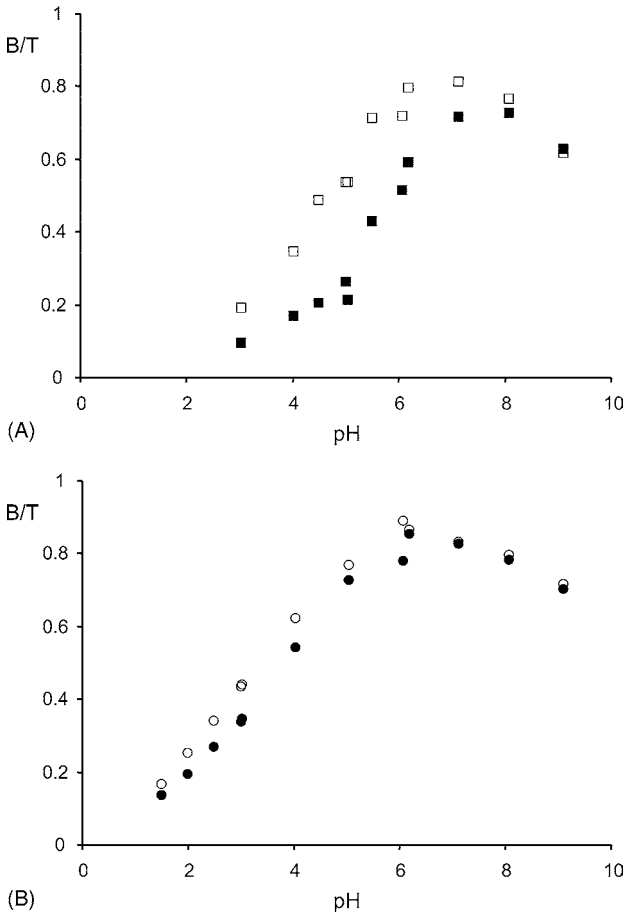


Fig. 4. . Binding of [$^3$H]-bupivacaine to: (A) $MIP_{MAA}$ (open squares), $REF_{MAA}$ (filled squares), and (B) $MIP_{TFMAA}$ (open circles), $REF_{TFMAA}$ (filled circles) as a function of pH. The incubation mixtures consisted of 1.2 mg mL$^{-1}$ polymer, 7 nmol L$^{-1}$ [$^3$H]-bupivacaine, 0.1 mol L$^{-1}$ buffer and 5% (v/v) ethanol. The buffers were citrate/HCl (pH 1.5–3), citrate (pH 3–6), phosphate (pH 6–8) and carbonate (pH 9). *B*/*T* is the ratio of the amount of radioligand bound (*B*) to the total activity (*T*) added to the test tubes. For each pH the precise total activity, *T*, was determined, in tubes without polymer but otherwise treated identically to samples, in order to confirm that the ligand did not adsorb to the tube walls and was found constant for pH-values of 7 and below, above pH 7 adsorption increased with increasing pH.

experiments. A second means of reducing non-specific hydrophobic interactions is the use of detergents. Non-charged detergents, such as Tween 20, Triton X-100 and Brij 35, efficiently reduce non-specific binding while leaving selective imprint–analyte binding unaffected [24]. The influence of combinations of Tween 20 and ethanol on imprint–analyte binding for a bupivacaine-MIP has been studied in detail and maximal level of selective binding was found obtained with 0.05% Tween 20 and 5% ethanol [18]. Using citrate buffer of pH 5 with this combination the binding isotherms for MIP and reference polymer could be well separated with $PC_{50}$-values of 1.8 and 6.1 mg mL$^{-1}$ for $MIP_{MAA}$ and $REF_{MAA}$, respectively (Fig. 3B). Finally, it was confirmed that the presence of 40% (v/v) plasma in the buffer did not affect binding. Both in the absence and presence of plasma a 10 mg mL$^{-1}$ MIP suspension bound 91% of the radioactivity from a 7.5 nmol L$^{-1}$ solution and 82% from a 2.9 μmol L$^{-1}$ solution.

### 3.3. Optimization of solid-phase extraction protocol

Studies into adsorption, washing and elution steps of the solid-phase extraction protocol were done using human plasma spiked with varying amounts of tritium-labelled bupivacaine. Aliquots of 400 μL were diluted with the buffer described above to a total volume of 1 mL and applied onto MIP columns. Then, several 1 mL aliquots of wash solvent and elution solvent followed. For application and each wash step analyte breakthrough was measured by radioactivity counting, and following elution each eluate volume was split into 100 μL for measurement of recovery by radioactivity counting and 900 μL for analysis of chromatographic purity by gas chromatography.

Having established conditions for efficient and selective MIP–analyte binding the next series of experiments were performed to find optimal SPE column size. MIP particles, 12.5, 15, 25 or 35 mg, were packed into empty 1 ml solid-phase extraction columns, and their ability to quantitatively extract bupivacaine from plasma samples investigated. Slight breakthrough was observed for the 12.5 mg columns only, on which occasionally a few percent (<6%) of the applied radioactivity were found non-retained. It was concluded that 15 mg of MIP was sufficient for complete extraction of bupivacaine from the samples, at least in concentrations up to a few μmol L$^{-1}$. Investigation of breakthrough was initially done using small particles (<25 μm) and it was later confirmed that analyte breakthrough was absent (<1%) also for columns packed with 15 mg of 25–40 μm particles. In all subsequent experiments 15 mg columns were used. Following packing columns were stored in the dry state, and activation of the columns immediately prior to use was required. Since MIPs need to swell for the imprints to efficiently bind analyte, initially large volumes of methanol and water were used. Later, a thorough investigation into influence of activation on calibration graph linearity concluded that an activation protocol using 1 mL of methanol and 1 mL of water is sufficient.

Following loading of sample, subsequent wash steps serve to remove as much as possible of sample contaminants while analyte and internal standard remain on column. Mixtures of water and methanol or ethanol over the whole concentration range of 0–100% of solvent additive were tested. Higher concentrations of organic modifier yielded cleaner chromatograms with less and smaller contaminant peaks, where methanol was found more effective than ethanol. Breakthrough of analyte increased, however, with increasing percentage of alcohol. Addition of up to 20% of either methanol or ethanol could be permitted without loss of recovery and the final MIP extraction method uses an aqueous wash with 2× 1 mL of water–methanol (4:1 v/v) with loss of analyte being less than 1%. Alternative washes using water containing acetic acid in concentrations of 0.1–3% did not improve chromatograms and gave poor recovery.

A second wash step using 2 mL of acetonitrile significantly reduced remaining contaminant peaks. This solvent switch from water to acetonitrile causes a switch in binding mechanism and removal of hydrophobically bound contaminants. In addition, the solvent switch re-distributes the fraction of analyte, which is non-specifically adsorbed to polymer surface to all analyte molecules being selectively bound in imprints. Addition of ethanol to the acetonitrile wash solvent was found to remove the few remaining small contaminating peaks, however, at the expense of some leakage of analyte. Therefore, both composition and volume of this mixture had to be optimized, where it was found that 10% ethanol was required to eliminate contaminants, while the volume must be 0.5 mL or less to reduce loss of analyte to below 5%. A technical hint is that, in order to avoid loss of analyte, it was found advantageous to dry columns of inter-particle liquid between washes, in particular when switching from water–methanol to acetonitrile–ethanol mixtures.

Initially, strong imprint–analyte binding prevented a high over all extraction recovery [21], and a series of experiments was performed to study elution solvent composition. Comparison of methanol, ethanol and acetonitrile based eluents containing 1, 2, 3, 5 and 10% TEA showed that ethanol gave higher recovery than methanol and acetonitrile. Varying TEA in this concentration range did not seem to affect elution strength and recoveries reported in Table 1 are means for respective solvent system. Five 1 mL aliquots were used and significant recoveries also were found in second and third fractions. Methanol–TEA mixtures were found to yield turbid eluates, also after evaporation and re-dissolution in heptane–ethanol for GC-analysis. Although ethanol–TEA gave higher recovery than acetonitrile–TEA this was accompanied with a higher number of contaminant peaks, and, therefore, it was decided to use acetonitrile in subsequent experiments. In order to increase recovery, water was added to the acetonitrile–TEA mixture and recovery was found to increase with increasing water content up to 6% water then remained constant at 85% (Table 2). Also, elution occurred increasingly in first aliquot. The reason for lower recovery and later elution for 2% TEA in acetonitrile in Table 2 compared with Table 1 is most likely due to an intermediate acetonitrile wash step, used in experiments reported in Table 2. This strengthens imprint–analyte binding as discussed above. For elution solvents containing ≥15% of water it was difficult to remove the water completely during evaporation, and for this reason these were incompatible with GC-analysis. Elution using acidic solvents, such as acetic acid, formic acid and trifluoroacetic acid in acetonitrile, showed that a higher content of acid increased recovery and that TFA was a stronger eluent than formic acid and acetic acid (Table 2). In all instances, the GC traces contained a higher number of contaminating peaks compared with that recorded for TEA–water–acetonitrile based solvents (data not shown). It was decided to use a mixture of 2% TEA and 6% water in acetonitrile, which gave both high extraction yield and eluates containing essentially pure analyte plus internal standard (Fig. 5).

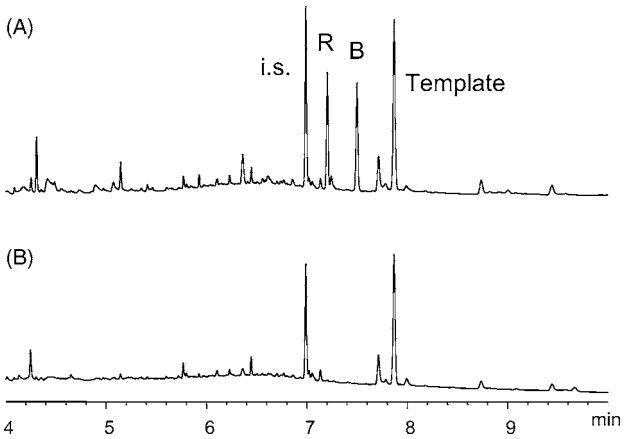


Fig. 5. Chromatograms of: (A) plasma spiked with 300 nmol $L^{-1}$ ropivacaine and 300 nmol $L^{-1}$ bupivacaine and (B) blank plasma. B: bupivacaine; R: ropivacaine; i.s.: internal standard.

The investigations above resulted in a final extraction protocol, which is presented in Table 3, for extraction of ropivacaine and bupivacaine using ethycaine as the internal standard. The recovery of bupivacaine was 89%. In contrast, extraction on a non-imprinted reference polymer gave both significant breakthrough during sample application, as well as loss of most of the retained bupivacaine during the solvent switch wash step (Table 3). This indicates that in parallel with selective imprint binding a significant fraction of the bupivacaine is retained from the aqueous sample through non-specific physicochemical retention on the polymer surface. This fraction is re-distributed to selective binding with imprinted sites during the solvent switch when hydrophobically bound structures are washed off and electrostatic and hydrogen bonding interactions are strengthened.

### 3.4. Evaluation of MISPE method

The extraction is highly selective, yielding GC traces with essentially only peaks derived from analyte and internal standard (Fig. 5). An additional fourth peak is, however, due

Table 3
Final MISPE extraction method

| Extraction step | Recovery MIP (%) | Recovery REF (%) |
|---|---|---|
| Column activation: 1 mL methanol then 1 mL water | | |
| Application of sample: 1 mL of 400 μL plasma in 0.2 mol $L^{-1}$ citrate buffer, pH 5, containing 0.05% Tween 20 and 5% ethanol | 0.8 | 19.6 |
| Wash 1: 2 mL of 20% methanol in water | 0.8 | 11.1 |
| Wash 2: 0.5 mL of 10% ethanol in acetonitrile | 4.5 | 58.8 |
| Elution 1: 2% TEA and 6% water in acetonitrile | 79.0 | 8.0 |
| Elution 2: 2% TEA and 6% water in acetonitrile | 10.0 | 0.6 |
| Total recovery | 95.1 | 98.1 |

Table 4
Method characteristics

| Analyte | Concentration (nmol $L^{-1}$) | Accuracy (%) | Intra-assay imprecision[a] (%) | Inter-assay imprecision[b] (%) |
|---|---|---|---|---|
| Bupivacaine | 4.5 | 95 | 9 | 17 |
| | 10.3 | 96 | 5 | 9 |
| | 39.9 | 99 | 6 | 7 |
| | 300 | 94 | 6 | 6 |
| Ropivacaine | 10.0 | 99 | 15 | 21 |
| | 39.9 | 104 | 9 | 13 |
| | 300 | 95 | 2 | 7 |

[a] Calculation based on six replicates.
[b] Calculation based on three assays with two replicates in each assay.

to leaking template molecules. Radiolabelled bupivacaine is extracted with high recovery (Table 3). Spiked human plasma samples with known concentrations of ropivacaine and bupivacaine were analyzed and inter- and intra-assay precisions and accuracies are presented in Table 4. The figures of merit show the extraction protocol can be used to support GC determination of bupivacaine in the concentration range of 3.9–500 nmol $L^{-1}$ and ropivacaine in the range of 7.8–500 nmol $L^{-1}$. This is sufficient for determination of both drugs in plasma samples from pharmacokinetic studies [25–27].

## 4. Conclusions

This present investigation provides an improved understanding of the approaches available for optimization of molecular-imprint based solid-phase extraction of plasma samples. The use of appropriate pH and buffer additives, such as ethanol and detergents, reduce non-specific adsorption of hydrophobic compounds to the hydrophobic MIP surface. While significant non-specific adsorption of analyte is still present, this is, however, probably an advantage as it facilitates quantitative retention on a small column. A subsequent solvent switch from water–methanol to acetonitrile–ethanol washes off hydrophobically adsorbed contaminants, as well as redistributes non-specifically retained analyte molecules to being selectively bound in imprinted sites. Finally, elution under basic conditions using TEA–water–acetonitrile was found more selective than elution under acidic conditions. The previously rather poor recovery, which is due to strong imprint–analyte binding, could be increased dramatically by addition of some water in the elution solvent. The extraction protocol developed gave highly efficient and selective clean-up of plasma samples, resulting in chromatographic traces with few and small contaminating peaks.

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