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Efficient sample pre-concentration of bupivacaine from human plasma by solid-phase extraction on molecularly imprinted polymers

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The ability to use imprinted polymers for solid-phase extraction is demonstrated in a model pre-concentration of bupivacaine from human plasma samples prior to gas chromatography. Imprinting of the structural analogue pentycaine yielded a sorbent which efficiently extracted analyte and internal standard, while possible interference on analyte quantification from leakage of remaining template molecules was eliminated. Human plasma samples were diluted with citrate buffer pH 5, and applied onto solid phase extraction columns containing 15 mg of imprinted sorbent. Wash steps with 20% methanol in water followed by acetonitrile preceded elution with 2% triethylamine in acetonitrile. A direct comparison with conventional sample pre-treatment methods showed the high selectivity of the imprinted sorbent resulted in distinctly cleaner chromatographic traces than were obtained both after liquid–liquid extraction and C18-based solid-phase extraction.

Introduction

Trace analysis of biofluids and other complex matrices often rely on efficient sample pre-treatment and selective methods. A more efficient sample clean-up, either on-line or off-line, simplifies downstream analytical separation and facilitates accurate and sensitive detection. A need for short turnaround time and high sample through-put requires fast methods amenable to automation. An ever increasing number of analysis problems requires strategies which support fast method development. Recently, a novel approach to solid-phase extraction based on the use of highly selective molecularly imprinted polymers (MIPs)[1–3] has been introduced.[4–15] The high analyte selectivities and affinities which can be achieved and the fact that sorbent selectivity can be pre-determined for a particular separation problem make imprinted polymers attractive alternatives to more traditional solid-phase extraction sorbents. The approach has variously been referred to as MIP-SPE or MISPE and has been reviewed recently.[16,17] Being a novel type of sorbent, however, there is a need for development of robust method development strategies.

Most MIP syntheses are organic solvent based, and studies on imprint rebinding are very often conducted using organic solvents as the incubation medium, where establishment of conditions for strong and selective rebinding is fairly well understood. The same is not yet true for aqueous rebinding and especially not for biofluids. The total binding to a MIP observed is the sum of the specific binding to the imprints and the non-specific binding to the polymer. If the non-specific element dominates, any selectivity shown by the imprints will remain undetected. Due to the hydrophobic nature of the polymer, problems with non-specific adsorption are often encountered under aqueous conditions. Hence, the effective use of MIPs in water relies on elimination of the non-specific binding while strengthening, or at least leaving unaffected, the specific imprint–analyte interaction. This present study focused on optimisation of conditions for aqueous rebinding, including direct solid-phase extraction of plasma samples. As a model system a MIP selective for a homologous series of compounds, including the local anaesthetics mepivacaine, ropivacaine and bupivacaine (Fig. 1), was used. Preliminary data show that direct sample clean-up of plasma samples on the MIP column yields more pure extracts than are obtained using well-established, robust methods based on conventional SPE on C18-columns and liquid–liquid extraction.

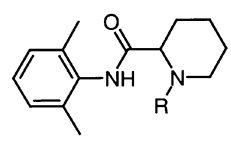
Materials and methods

MIP preparation

In a typical preparation pentycaine free base (1 mmol) and AIBN (1.3 mmol) were weighed into a Kimax test tube and dissolved in 15.6 g of dry toluene by help of ultra-sonication. Methacrylic acid (12 mmol) and ethylene glycol dimethacrylate (60 mmol) were added. A non-imprinted reference polymer, with the same composition except for the absence of pentycaine, was prepared in parallel. The clear solutions were cooled on ice and 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 16 h. The hard polymers were ground in a laboratory mortar grinder (Retsch, Haan, Germany) and sieved to collect particles 25–45 μm in size. Grinding and sieving were repeated until all material passed the 45 μm sieve. The particles were washed with methanol–acetic acid (4 : 1; v/v), methanol, methanol–2 M NaOH (1 : 1; v/v), methanol–water–acetic acid (18 : 1 : 1; v/v/v), methanol and finally dried under vacuum. Particles were suspended in water and packed into empty solid-phase extraction cartridges. The columns were stored in the dry state until use.

Solid-phase extraction on MIP and non-imprinted reference polymer

Prior to use the MIP or reference columns, containing 15 mg of sorbent, were activated by treatment with 5 ml of methanol followed by 10 ml of water. Plasma samples (400 μl) were diluted with 100 μl of ethycaine internal standard solution (8 μM in water) and 500 μl of citrate buffer (0.4 M, pH 5.0),



R = methyl; Mepivacaine
R = ethyl; Ethycaine
R = n-propyl; Ropivacaine
R = n-butyl; Bupivacaine
R = n-pentyl; Pentycaine

Fig. 1 Structures of the compounds studied. Pentycaine was used as the imprint species, and solid-phase extraction of bupivacaine was studied using ethycaine as the internal standard.

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) followed by 2 ml of acetonitrile, the analyte was eluted with 2 ml of acetonitrile–triethylamine (49:1; 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 analysed by GC.

Solid-phase extraction on C18-silica

Prior to use 50 mg C18-columns (Isolute, IST, UK) were activated by treatment with 1 M HCl (1 ml), methanol (2 ml) and water (1 ml). Plasma samples (400 μl), were diluted with 100 μl of ethycaine internal standard solution (8 μM in water) and 500 μl of citrate buffer (0.4 M, pH 5.0) , and applied onto the columns. After washing the columns with 2 ml of water followed by 1 ml of acetonitrile, the analyte was eluted with 1 ml of methanol–acetic acid (49:1; 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 analysed by GC.

Liquid–liquid extraction

To glass test tubes were added 400 μl of plasma sample, 100 μl of ethycaine internal standard solution (8 μM in water), 500 μl of NaOH (2.5 M), 1 ml of water and 4 ml of heptane–methylene chloride (4:1; v/v). The contents of the tubes were mixed by continous end-over-end rotation for 35 min and then the tubes were centrifuged at 3000 rpm for 10 min. The organic layers were 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 5 μl analysed by GC.

Results and discussion

A MIP selective for a homologous series of compounds, including the local anaesthetics mepivacaine, ropivacaine and bupivacaine, was prepared by molecular imprinting of a higher analogue pentycaine (Fig. 1). The objective was to study its use as an imprinted SPE sorbent for pre-concentration of any of these local anaesthetics from biofluids prior to the analytical separation. Since it is known from previous studies that despite exhaustive washing during preparation traces of the imprint species may remain in the MIP,[5,7,13] the structural analogue pentycaine was used as an alternative template molecule. This approach eliminates potential problems associated with release of remaining imprint molecules during solid-phase extraction. Any leaking pentycaine is easily separated from the analyte by the subsequent GC-analysis and does not interfere with the quantification.

In inital experiments 12.5, 15, 25 and 35 mg of MIP were packed into empty 1 ml solid-phase extraction columns, and their ability to quantitatively extract bupivacaine from diluted plasma samples tested. Human plasma was spiked with known amounts of tritium-labelled bupivacaine, aliquots of 400 μl diluted with buffer to a total volume of 1 ml and applied onto the columns. Slight break-through 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 1 ml of diluted plasma sample, at least in concentrations up to a few μmol l^{-1}. In subsequent experiments the 15 mg columns were used.

Human plasma samples from several individuals were spiked with known amounts of bupivacaine and subjected to SPE on the MIP column. It was found that with the appropriate washing protocol essentially clean extracts could be obtained (Fig. 2). The corresponding GC-traces following solid-phase extraction on a non-imprinted reference polymer showed minimal peaks for analyte and internal standard (Fig. 2), which demonstrates that the retention is based on selective binding to the imprints. Optimisation of binding, washing and elution conditions is built on the fact that the retention of analyte (and contaminants) on a MIP column is due to both specific binding to the imprints and non-specific interaction with the polymer, where the non-specific element leads to compromised selectivity of the sample clean-up. The polymer matrix is essentially hydrophobic in nature. A mixed mode non-specific retention mechanism operates. Whereas in organic solvents non-specific retention due to polar type interactions dominates, possibly with randomly distributed non-templated carboxylic acid residues, in aqueous media hydrophobic interactions increase in significance and often very strong binding is observed. In an analogous manner the imprint selectivity observed is tuned by the surrounding medium.[18] In organic solvents the imprints recognise subtle differences in polar functionalities of the analyte, and in aqueous media recognition of non-polar parts of the molecule is efficient.

Prior to SPE the plasma samples were diluted by addition of 1.5 volumes of citrate buffer. In a separate radioligand binding

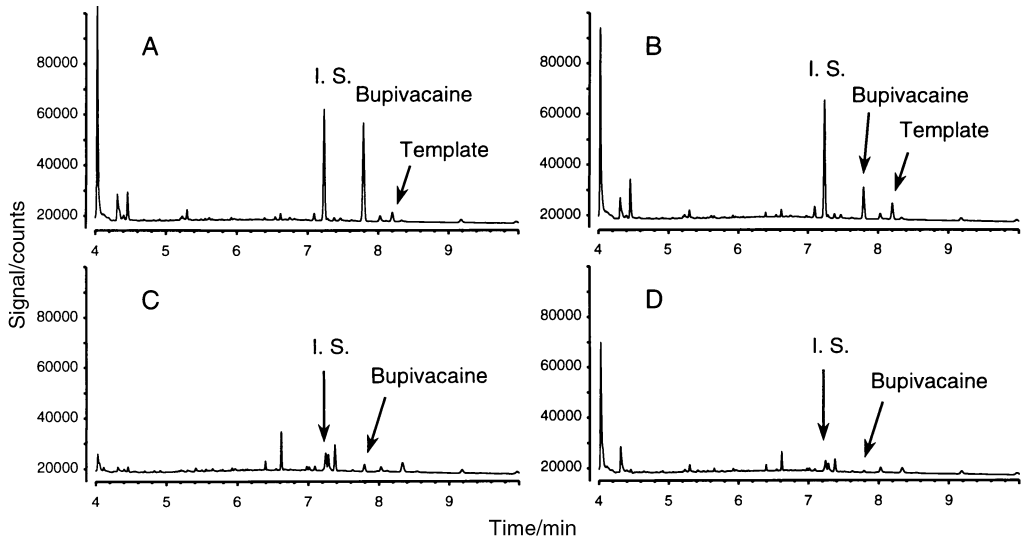


Fig. 2 Representative GC traces of spiked human plasma subjected to solid-phase extraction on MIP (A and B) and non-imprinted reference polymer (C and D). The retention times for internal standard and bupivacaine are, respectively, 7.24 and 7.79 min. Plasma was spiked with 735 (A and C) and 160 nM (B and D) bupivacaine. Relative peak areas for major peaks in chromatogram A are: 204% 4.03 min; 33% 4.32 min; 27% 4.46 min; 100% 7.24 min; 100% 7.79 min.

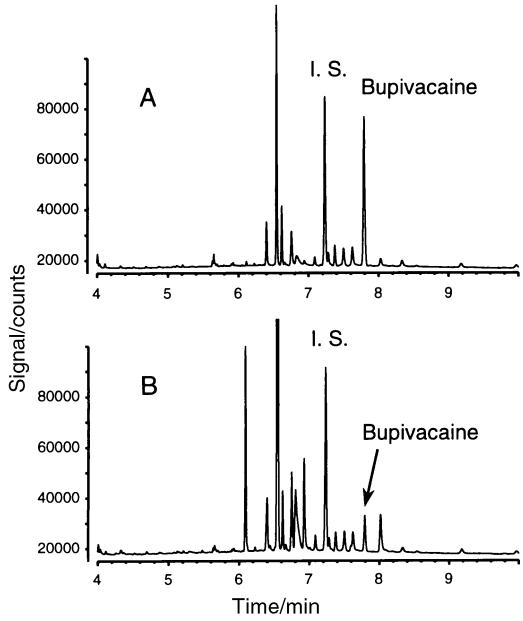


Fig. 3 Representative GC traces of spiked human plasma subjected to solid-phase extraction on C18 column. The plasma samples were identical to those in Fig. 2 and spiked with 735 (A) and 160 nM (B) bupivacaine. The extraction was performed according to a previously published method.[20] Relative peak areas for major peaks in chromatogram A are: 20% 6.40 min; 107% 6.55 min; 27% 6.62 min; 21% 6.76 min; 104% 7.24 min; 100% 7.79 min.

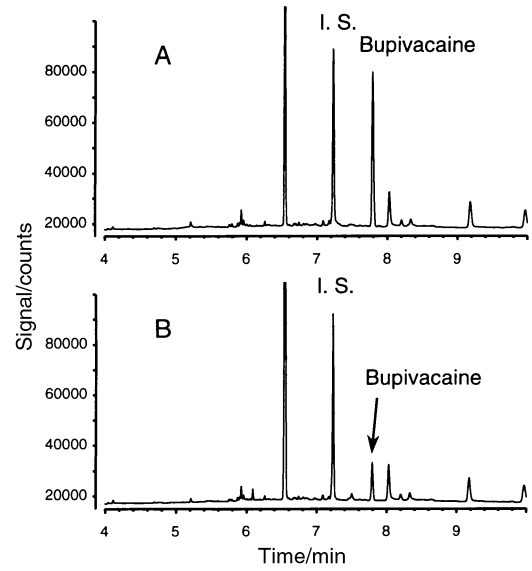


Fig. 4 Representative GC traces of spiked human plasma subjected to liquid–liquid extraction. The plasma samples were identical to those in Fig. 2 and spiked with 735 (A) and 160 nM (B) bupivacaine. The extraction was performed according to a previously published method.[21] Relative peak areas for major peaks in chromatogram A are: 225% 6.55 min; 106% 7.24 min; 100% 7.80 min; 31% 8.03 min; 25% 9.18 min.

experiment the effect of buffer pH on bupivacaine binding was investigated. The maximal difference between binding to the MIP and non-specific binding to the non-imprinted reference polymer was found to occur at pH 5 (data not shown). The buffer contained 5% ethanol and 0.05% Tween 20 (final composition in sample), both of which were added to reduce hydrophobic type non-specific retention. The first wash step used 20% methanol in water, as it has previously been shown for other systems that addition of alcohol to the buffer reduces the non-specific portion of binding while the specific imprint-analyte binding remains essentially unaltered.[18] This was followed by a second wash step using acetonitrile to further remove contaminants retained by hydrophobic interactions. It is known from previous studies that imprint binding of amino acid amides, the same structural elements as are present in both analyte and internal standard (Fig. 1), is strong in pure acetonitrile.[19] Elution was affected with 2% triethylamine in acetonitrile. The over all recovery from the pre-concentration was 65–75%, and 10–15% of the bupivacaine remained on column. Increasing the triethylamine content up to 10% did only marginally improve recovery. Elution with trifluoroacetic acid in acetonitrile did not increase recovery and the extracts were less pure (data not shown).

A comparison with existing methods was made where samples were aliquoted and subjected to extraction using the MIP method, a C18-based solid-phase extraction method[20] and a liquid–liquid extraction method.[21] The latter methods are robust, well-established routine methods, which have been optimised through their long-term use for extraction of local anaesthetics from plasma samples, and each method was performed using the established conditions. For all samples tested the MIP method produced the purest extracts (Figs. 2–4). Relative peak areas for major peaks recorded for the 735 nM sample, where the peak area for bupivacaine is set to 100%, are presented in the legends to Figs. 2-4. The MIP method uses addition of ethanol and detergent to the plasma diluent buffer. The same buffer composition was tested for the C18 method and preliminary data indicate a reduction in most contaminant peak areas and elimination of some minor contaminant peaks (data not shown); however, the general picture remained unaltered.

The present study has addressed problems encounted with direct extraction of biosamples and established conditions under which efficient pre-concentration of bupivacaine from buffer-diluted plasma is possible. The selectivity of the MIP–bupivacaine binding led to improved sample clean-up, which may facilitate down-stream analytical separation and quantitation. The results presented indicate that imprinted polymers may be a viable alternative to the more traditional sorbents for biosample pre-concentration.

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