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Molecularly imprinted polymer with high-fidelity binding sites for the selective extraction of barbiturates from human urine

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ABSTRACT

In this paper we describe the synthesis of a molecularly imprinted polymer (MIP) by precipitation polymerisation, with barbital as the template molecule, and the application of the barbital MIP as a molecularly selective sorbent in the solid-phase extraction (SPE) of barbiturates from human urine samples. The MIP was synthesised by precipitation polymerisation using 2,6-*bis*-acrylamidopyridine as the functional monomer and DVB-80 as the cross-linking agent. The spherical MIP particles produced were 4.2 ± 0.4 μm in diameter; a non-imprinted control polymer (NIP) in bead form was 4.8 ± 0.4 μm (mean ± standard deviation) in diameter. The particles were packed into a solid-phase extraction cartridge and employed as a novel sorbent in a molecularly imprinted solid-phase extraction (MISPE) protocol. The MIP showed high selectivity for the template molecule, barbital, a feature which can be ascribed to the high-fidelity binding sites present in the MIP which arose from the use of 2,6-*bis*-acrylamidopyridine as the functional monomer. However, the MIP also displayed useful cross-selectivity for other barbiturates besides barbital. For real samples, the MIP was applied for the extraction of four barbiturates from human urine. However, due to the high urea concentration in this sample which interfere the proper interaction of barbiturates onto the MIP, a tandem system using a commercially available sorbent was developed.

1. Introduction

The detection of regulated substances has always been of prime interest in different fields, not only to detect illicit consumption on self-administration [1], but also for forensic purposes to prove that regulated substances have been used to commit crimes such as sexual assaults [2] and suicide [3] or abused as performance-enhancing drugs in sports [4,5], for example.

Regulated drugs of interest are usually present at very low concentration levels in samples, therefore the detection methods used for their quantification must typically be very sensitive. This is the reason why the mostly commonly used separation techniques in this area, such as liquid-chromatography (LC) or gas-chromatography (GC), are often coupled to mass spectrometry (MS) [2,4,6].

In the majority of the cases reported concerning the detection of regulated drugs, pre-treatment of the sample is required. The most widely used sample pre-treatment technique for liquid samples is solid-phase extraction (SPE). SPE is used to preconcentrate the analytes present in the sample and to remove interferences. However, in conventional SPE protocols not only is the target of interest preconcentrated, but also other compounds are often retained by the SPE sorbent too. One way in which the preconcentration of the sample can be made more selective is to use designed sorbents which show molecular selectivity for the analyte of interest; an important class of sorbents which fall into this category are molecularly imprinted polymers (MIPs). When MIPs are applied as SPE sorbents, the technique is termed molecularly imprinted solid-phase extraction (MISPE).

Over the last few years, MISPE protocols have been used widely to facilitate difficult chemical separations. This is because MIPs are very often not particularly difficult to synthesise or obtain and their use can lead to significant enhancements in the selective retention of target analytes, even when low concentrations of analytes are being extracted from complex matrices [7,8]. MISPE typically leads to cleaner samples and lower limits of detection.

MIPs have been used to extract many different substances from a variety of sample types [8,9], including herbicides from vegetables [10], non-steroidal anti-inflammatory drugs [11] from water, naphthalene sulfonates from river water [12], metabolites of illicit drugs from water at clinically relevant concentrations [13], and pharmaceuticals from urine [14].

When designing a MIP, rational selection of the functional monomer is mandatory in order for the MIP to have the desired molecular selectivity when in use. In the majority of published examples where MIPs have been synthesised, commercially

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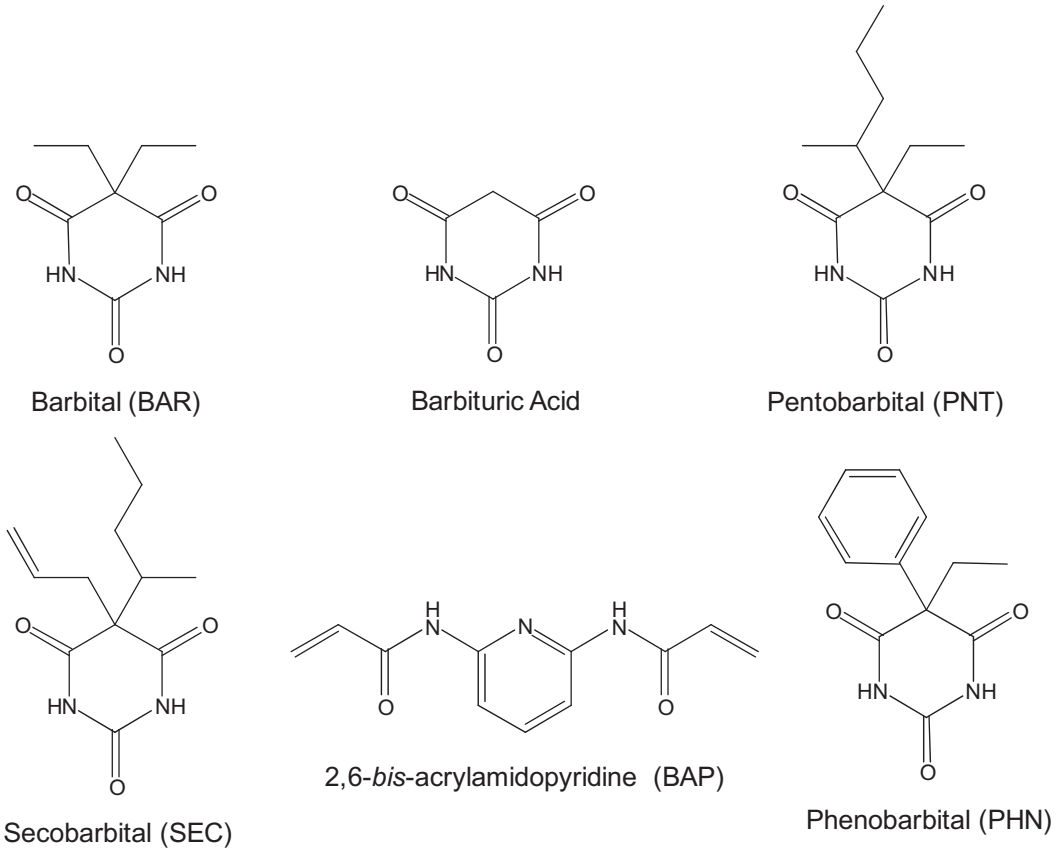

Fig. 1. Molecular structures of the barbiturates and the functional monomer used in this study.

available functional monomers are normally used since there is a wide range of monomers to select from. However, for some templates even better molecular recognition outcomes may be realisable through the synthesis and implementation of designer functional monomers which are tailor-made for the analyte of interest [15]. In this context, in the present study we synthesised a functional monomer which was tailor-made to complement the hydrogen bonding motif found in barbital, which was our analyte of interest. This functional monomer, which can interact with barbital though a triple hydrogen bonding array, offered the prospect of novel MIPs being synthesised and used in MISPE, where the MIPs have high-fidelity binding sites in place and correspondingly reduced levels of non-specific binding when used in MISPE [16].

Recently, Haginaka et al. described the synthesis and application of a MIP with size-exclusion properties (a RAM-MIP; RAM = restricted-access material) for the extraction of barbiturates from river water samples [17]. Here, the authors synthesised their MIP by multi-step swelling polymerisation, with cyclobarbital as template molecule and a commercially available monomer (4-vinylpyridine) as the functional monomer. The RAM-MIP material was packed into a column and connected on-line to a chromatographic system coupled to a mass spectrometer. This set-up enabled the quantification of amobarbital, cyclobarbital, phenobarbital and phenytoin from river water samples (50 mL sample volumes).

In the present work, not only did we target a designer functional monomer for barbital to enhance the molecular recognition profile of the MIP, but by performing the template polymerisations under precipitation polymerisation conditions we were keen to synthesise spherical polymer particulates (beads) in a single preparative step [18]. The latter objective potentially delivers polymer particles which ought to perform rather well when applied as sorbents in packed columns in flow-through applications, due to their low particle size and narrow particle size distributions. A further objective was to use the novel materials for the selective extraction of barbital and related compounds from human urine samples to demonstrate their value in a challenging and practically useful chemical separation and analysis process.

2. Experimental

2.1. Reagents and standards

The compounds used to synthesise the functional monomer, 2,6-*bis*-acrylamidopyridine (BAP), were 2,6-diaminopyridine, acryloyl chloride and triethylamine (all from Aldrich, Steinheim, Germany); all were used as received.

The compounds used in the synthesis of the MIP and NIP were barbital (template molecule) divinylbenzene-80 (DVB-80; cross-linking agent) and 2,2′-azobisisobutyronitrile (AIBN) (free radical initiator), both from Acros Organics (Geel, Belgium). Prior to use in polymerisations, DVB-80 was passed through a short column packed with neutral alumina, to remove polymerisation inhibitor, and AIBN was recrystallised from methanol.

The solvents/porogens used for the polymerisations were acetonitrile (ACN) and toluene (both were of analytical grade and supplied by Riedel-de-Haën, Seelze, Germany). The solvents used in the chromatographic evaluations and MISPE experiments were ACN, methanol (MeOH), dichloromethane (DCM), chloroform, ethyl acetate (EtOAc), acetic acid (AcOH) and isopropanol; all were of HPLC grade and were supplied by SDS (Peypin, France). Phosphoric acid (H_3PO_4) was from Merck (Darmstadt, Germany), and water was sourced from a Milli-Q purification system (Millipore, Molsheim, France).

The analytes used during the study were barbituric acid (BA), barbital (BAR), phenobarbital (PHN), pentobarbital (PNT) and secobarbital (SEC), and were sourced from Sigma Aldrich (St. Louis, MO, USA). The chemical structures of the analytes are presented in Fig. 1. A stock solution (1000 mg L^{-1}) for each analyte was prepared in water:MeOH (1/1 (v/v)) and stored in a fridge when not in use; diluted solutions were daily prepared from the stock solution.

To remove the urea present in the urine samples, a tandem cartridge system using a selection of commercially available sorbents was developed. In this regard, the sorbents tested were: Oasis® HLB (hydrophilic–lipophilic styrene-divinylbenzene:4-vinylpyrrolidone copolymer, 200 mg) from Waters (Milford, MA, USA); Strata™ SDB-L (styrene–divinylbenzene copolymer, 200 mg), from Phenomenex (Torrance, CA, USA); Bond Elute LRC® (C_{18}, 200 mg) from Varian (Harbor City, CA, USA).

2.2. Synthesis of the functional monomer

2,6-*bis*-Acrylamidopyridine was synthesised according to a method reported by Yano et al. [19]. Briefly, 2,6-diaminopyridine (5.46 g, 50 mmol) was suspended in chloroform (150 mL) and acryloyl chloride (10.90 g, 120 mmol) added dropwise to the solution in the presence of triethylamine (12.1 g, 120 mmol) at ice-bath temperature and left to stir overnight at 0 °C. After this time, the volatiles were removed under reduced pressure and the residue was dissolved in MeOH (100 mL). 50 mL of the methanol solution was then added slowly to deionised water (800 mL) with stirring. The product which precipitated was collected by vacuum filtration, washed with deionised water and then dried *in vacuo* at 40 °C to constant mass. The NMR assignments were consistent with the NMR assignments in the literature, and the FTIR spectroscopic and MS data were in agreement with the proposed structure, thus the functional monomer was used directly without any further purification being undertaken.

2.3. Synthesis and characterisation of the molecularly imprinted polymer

The barbital MIP was synthesised by precipitation polymerisation using a non-covalent molecular imprinting approach. BAR (0.095 g, 1.3 mmol), BAP (0.209 g, 2.6 mmol), DVB-80 (1.3405 g, 26.5 mmol) and AIBN (0.112 g; 3 mol% relative to the number of moles of polymerisable double bonds) were dissolved in a 3:1 (v/v) mixture of ACN:toluene (40 mL; 4% (w/v) of total monomer w.r.t. solvent) in a 100 mL polypropylene bottle. The pre-polymerisation mixture was deoxygenated by sparging with oxygen-free N_2 for 5 min in an ice bath, the bottle sealed under a N_2 atmosphere and the mixture left to polymerise while rotating the bottle slowly about its long axis on a low-profile roller (Stovall, Greensboro, NC) in a temperature-controllable incubator (Stuart Scientific, Surrey, UK). The temperature was ramped from room temperature to 60 °C over a time period of approximately 2 h and was held at this temperature for a further 46 h. Once the reaction was complete, the particles were recovered by vacuum filtration on a nylon membrane filter prior to overnight Soxhlet extraction with methanol to remove template and unreacted starting materials. The yield of product was 53%.

A non-imprinted control polymer (NIP) was synthesised in a fashion analogous to that used for the MIP, but without the addition of the template molecule. In this case, the yield of product was 78%.

The particles obtained were imaged by SEM using a JEOL JM-6400 Scanning Electron Microscope (Peabody, MA). The particle size distributions were measured using Image J Launcher software [20].

The specific surface areas of the MIP and NIP were measured by nitrogen sorption porosimetry using an ASAP 2020 Accelerated Surface Area and Porosimetry Analyzer (Micromeritics Instrument Corporation, Norcross, GA) followed by application of BET theory.

2.4. Chromatographic analysis

For the chromatographic evaluation of the polymers, two stainless steel LC columns (50 mm × 4.6 mm ID) were slurry packed with ~0.5 g of both MIP and NIP particles using an air-driven, fluid pump (Alltech, Model 1666) with acetone as the slurrying and packing solvent. These columns were applied as LC stationary phases to confirm the molecular recognition character of the MIP. 20 μL aliquots of 0.5 M standard solutions of BA, BAR, PHN, PNT and SEC, as well as acetone (void marker), were injected sequentially onto either the MIP or the NIP. The mobile phase was ACN:H_2O (25:75) (adjusted to pH ~ 3 with H_3PO_4) at a flow rate of 1 mL min^{-1}, using a UV detection set at 210 nm. From the data obtained through the various LC experiments, the retention factors and imprinting factor (IF) were calculated.

When the MIP was used for MISPE studies, a commercially available LC column (Kromasil 5 μm, 100 Å C_{18}, 250 mm × 4.6 mm from Teknokroma) was used to separate the analytes. A gradient elution profile with ACN and acidified water (adjusted to pH ~ 3 with H_3PO_4) was applied. The gradient started with 20% ACN and increased to 60% in 4 min. From minute 4 to minute 8 the gradient was increased again from 60% to 70% ACN and reached 100% ACN at minute 10. From minute 10–13 the ACN content was decreased again to the initial conditions (20% ACN). The column temperature was maintained at 30 °C and the UV detector set to monitor at 210 nm.

For all the chromatographic studies, the chromatographic instrument used was a Shimadzu LC-10AD binary liquid chromatograph equipped with a DGU-14A degasser, an injection loop of 20 μL, a CTO-10A oven and an SPD-10A UV detector. All of the LC modules were from Shimadzu (Tokyo, Japan).

2.5. MISPE conditions

For the MISPE analyses, ~60 mg of both MIP and NIP polymers were packed into two empty 6 mL polyethylene cartridges (Symta, Madrid, Spain). Given the particle sizes of the MIP and NIP, the upper frit was polyethylene with a pore size of 10 μm (Applied Separations, Allentown, PA, USA) whereas the bottom frit was a metallic frit with a pore size of 2 μm (Supelco, Bellefonte, PA, USA). The cartridges were connected to a 12-port SPE manifold (Teknokroma, Barcelona, Spain) which was connected, in turn, to a vacuum pump.

When analysing urine samples, the urea was removed from the samples prior to the MISPE protocol. This involved the percolation of 2 mL of human urine (previously adjusted to pH ~ 3 with H_3PO_4 and filtered using a 0.22 μm pore size nylon) through an Oasis HLB cartridge; the cartridge was then flushed with 15 mL of acidified water (adjusted to pH ~ 3 with H_3PO_4) and eluted with EtOAc (5 mL). The eluted fraction was evaporated under a stream of N_2 and reconstituted in 1 mL of acidified water (adjusted to pH ~ 3 with H_3PO_4) prior to percolation through the MIP and NIP cartridges.

The MISPE protocol involved activation of the MIP cartridge with 15 mL of acidified water (adjusted to pH ~ 3 with H_3PO_4), loading of the cartridge with the sample at pH ~ 3 (adjusted with H_3PO_4), cleaning of the sample using 1 mL of EtOAc and elution from the cartridge with 5 mL of MeOH (containing 1% AcOH).

The eluted fraction was evaporated to dryness under a stream of N_2 before reconstitution in 1 mL of acidified water (adjusted to pH ~ 3 with H_3PO_4). The reconstituted sample was then injected onto the chromatographic system.

Both before and after the percolation of the cleaning solvent, the cartridges were left to dry as completely as possible under high vacuum.

3. Results and discussion

3.1. Synthesis and evaluation of the MIP

In this study, the global objective was to use a MIP to extract barbiturates from highly complex matrices in an efficient manner. To this end, a MIP was synthesised by a precipitation polymerisation protocol because of the advantages that precipitation polymerisation has over other synthetic strategies [21].

The template molecule selected was BAR since we were interested in developing cavities for allocating different barbiturates so we considered barbital as a compromise structure for all of them in the present study.

In an attempt to engineer group selectivity into the MIP, a second synthetic strategy adopted was to use a functional monomer which was complementary to the core structure common to all the barbiturates of interest. As can be seen in Fig. 1, barbital, barbituric acid, pentobarbital, secobarbital and phenobarbital all share a common ADA (acceptor–donor–acceptor) hydrogen bonding array motif, so selection and use of a functional monomer which complements this hydrogen bonding pattern ought to lead to a MIP with good selectivity for the target analytes [22]. A good candidate in this regard, i.e., a functional monomer which has a DAD hydrogen bonding motif, is 2,6-*bis*-acrylamidopyridine (BAP) (Fig. 1), a functional monomer which has already been applied within the field of molecular imprinting [23].

Given the facts that BAP interacts with the template (BAR) by three hydrogen bonds (Fig. 2), leading to a high association constant, and that two equivalents of BAP are required to satisfy completely the hydrogen bonding requirements of BAR, an efficient molecular imprinting outcome can be expected in terms of the affinity of the MIP for the analytes. One further advantage which accrues from the use of highly complementary functional monomers such as BAP is that a stoichiometric, non-covalent molecular imprinting approach can be adopted. In practical terms, this means that the use of an excess of functional monomer is avoided which ought to lead to an imprinted product with a reduced propensity for non-selective binding, i.e., the MIP should have higher selectivity. In the present work, the mole ratio of the template (BAP) to functional monomer (BAR) was fixed at 1:2. We were interested in keeping this ratio as low as possible in order to diminish the non-specific interactions while keeping a high degree of complexation between the template molecule and the functional monomer.

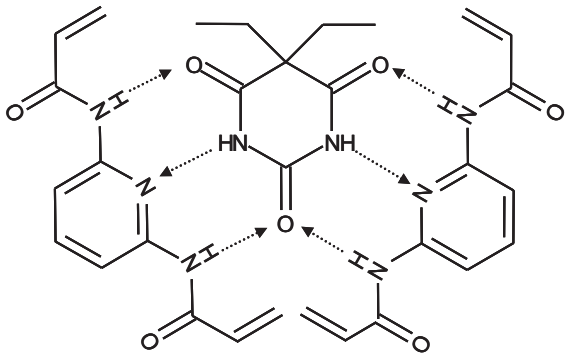

Fig. 2. Hydrogen bonding interactions between the template (BAR) and the functional monomer (BAP). Mole ratio of BAR to BAP = 1:2.

Once both the MIP and the NIP were obtained (see Section 2 for details), the polymers were imaged by SEM. As can be seen in Fig. 3, high quality spherical particles with narrow particle size distributions were obtained. The sizes of the particle were 4.2 ± 0.4 μm and 4.8 ± 0.4 μm (mean ± standard deviation) for the MIP and the NIP, respectively. Nitrogen sorption porosimetry analysis verified that both polymers were microporous with some mesoporosity and an average pore diameter of 26.8 Å and 26.4 Å for MIP and NIP, respectively. The pore volumes were of 0.38 cc g^{-1} and 0.36 cc g^{-1} for the MIP and the NIP, respectively, and the specific surface areas were 685 ± 15 m^2 g^{-1} and 654 ± 15 m^2 g^{-1} in the same order as above.

After the polymers had been synthesised, they were packed into empty LC columns and an LC analysis performed to probe their molecular recognition character and verify the molecular imprinting outcome. The imprinting factors (IFs) for the different compounds under study were thereby obtained: 8.64 for barbituric acid; 5.13 for BAR; 4.28 for PNT; 3.12 for PHN; 2.93 for SEC. The relatively high IF values obtained for all the compounds demonstrate that retention of the compounds by the MIP was through selective interactions. It is also noteworthy that steric factors appear to have some influence on the molecular recognition; PNT, PHN and SEC are all more sterically bulky than BAR and give lower IF values in the chromatographic analysis than BAR. These results are in good agreement with results reported recently by Hoshina et al. [17], where a MIP displayed highest affinity towards the template molecule and a somewhat lower affinity for analytes with bulkier pendent groups.

As a final note at this point, although barbituric acid was included as an analyte when measuring the IF values, since this compound does not have any therapeutic effect it was omitted when real samples were studied.

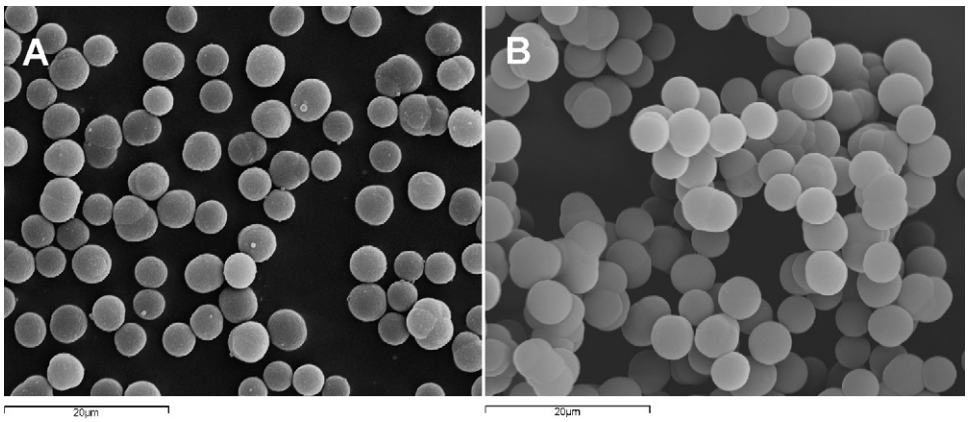

Fig. 3. SEM images of the MIP (A) and NIP (B).

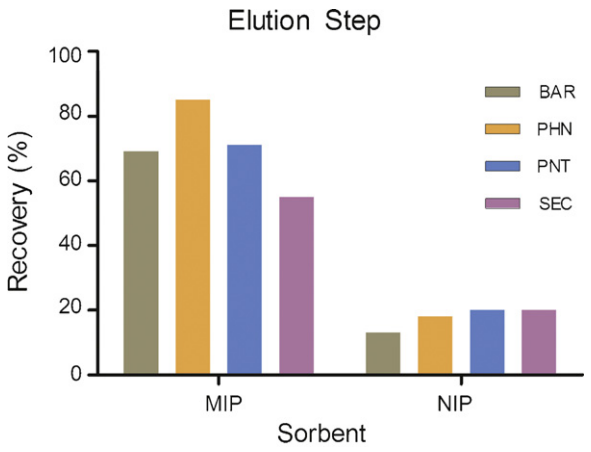

Fig. 4. Recoveries obtained for the MIP and the NIP after the MISPE protocol. Both sorbents were loaded with 5 mL of acidified water spiked at 5 mg L^{-1} with each barbiturate, cleaned with 1 mL of EtOAc and eluted with 5 mL of MeOH (1% AcOH).

3.2. MISPE experiments

For the MISPE experiments, 5 mL volumes of several different loading solvents (acidified water [adjusted to pH ~ 3 with H_3PO_4], ACN, MeOH, isopropanol and DCM) spiked at 0.5 mg L^{-1} with each barbiturate were percolated through the MIP. Water at basic pH was not included in the loading study since the analytes may be hydrolysed under basic conditions. For ACN and MeOH, retention of the compounds under study was negligible (<5%), whereas when using either DCM or isopropanol the retention of the analytes was lower than 40% and 75%, respectively. However, for water [adjusted to pH ~ 3 with H_3PO_4], complete retention of all the compounds was achieved (>99%) so water was selected as the optimal loading solvent.

Even though the MIP was synthesised using BAR as template molecule and this compound was also amongst the target molecules to be extracted, there was no misquantification for this compound due to the possible template bleeding effect sometimes observed in MIP, since the blank runs between analysis did not show any chromatographic signal of BAR. In any case, if there was any template remaining in the MIP, it was at concentration levels below the sensitivity of the UV detection system used.

Given the data obtained in the loading step study, MeOH was selected as eluting solvent because MeOH gave rise to the lowest retention of the analytes on the MIP when this solvent was used as a loading solvent. However, in order to optimize the total volume of MeOH used in this step, the addition of an organic modifier, in this case acetic acid (AcOH), was studied. Inclusion of an organic modifier is sometimes needed because it facilitates the elution of retained compounds from a polymer. Eventually, the optimum volume and composition of MeOH for eluting the compounds from the MIP was set at 5 mL MeOH containing 1% (v/v) of AcOH, since all the barbiturates were removed completely from the MIP under these conditions (recoveries >99%), whereas 5 mL of MeOH without acetic acid was insufficient to elute all the compounds retained on the MIP.

In any MISPE protocol, but especially those using water matrices, a cleaning step is highly desirable in order to remove as many of the non-specifically retained compounds from the MIP as possible, thus obtaining a cleaner extract. Moreover, to assess the selectivity of the imprinted binding sites in the MIP, a comparison between the MIP and the NIP was performed. To this end, 1 mL of toluene or 1 mL of EtOAc were used to perform the clean-up step. In the case of toluene, after eluting both sorbents there was no difference between the MIP and the NIP, so toluene was unable to discern the selective interactions on the MIP from the non-selective interactions on the NIP. However, and as it is shown in Fig. 4, percolation of 1 mL of EtOAc had the desired effect. For the NIP, 1 mL of EtOAc was enough to remove over 80% of each barbiturate from the MISPE cartridge whereas for the MIP the eluted fraction was less than 30% for BAR, PHN and PNT, and below 42% for SEC. Given these observations, which further support the molecular recognition/imprinting conclusions drawn from the LC study, we decided to use 1 mL of EtOAc as cleaning solvent.

Once the entire MISPE protocol had been established, we also determined the maximum volume of sample which could be percolated through the MIP without significant breakthrough. Under the optimal conditions, the recoveries of analytes after percolation of 25 mL of ultra-pure acidified water were similar to those reported previously (Fig. 4), whereas when higher volumes of sample were used, for instance 50 mL, recoveries were lower than 50% for every barbiturate.

3.3. MISPE of human urine

Once the performance of the MIP in respect of the extraction of barbiturates had been demonstrated, we moved to extract barbiturates from urine samples. The motivation for targeting this matrix arose because urine analysis constitutes a convenient way of monitoring the intake of drugs since urine is one of the main routes of excretion in the body.

Due to the high complexity of real samples, the recoveries of analytes from real samples are normally always lower than those obtained from the standard solutions used during the optimization of the method. Hence, we started using 5 mL of human urine instead of the maximum volume achieved when using ultra-pure water.

In spite of the high affinity displayed by the MIP towards the barbiturates, no retention was observed when extracting 5 mL of human urine. Even when the sample volume was decreased to 2 mL the recoveries of all the barbiturates under study were still below 15%. This is surprising, but only until one appreciates that the low retention of barbiturates on the MIP could be due to the high concentration of urea in urine samples (bearing Fig. 2 in mind, urea may be capable of establishing three simultaneous hydrogen bonds with an imprinted binding site, i.e., it is potentially a potent competitor to the barbiturates for binding to the MIP).

Since our goal was to extract barbiturates from human urine samples and our hypothesis was that it was the urea present in urine which prevented barbiturates from being retained efficiently by the MIP, we decided to establish a tandem system using a commercial sorbent to remove the urea present in the sample. If successful, this strategy would confirm our hypothesis and, more importantly, enable the subsequent quantification of barbiturates in urine. In this regard, we tested three conventional SPE sorbents to allow the separation of urea from barbiturates in urine prior to the MISPE stage.

5 mL samples of acidified water spiked at 2 mg L^{-1} of each barbiturate were percolated through a hydrophobic C_{18} sorbent (Bond Elut LCR), a styrene–divinylbenzene copolymer (Strata SDB-L) and a divinylbenzene:4-vinylpyrrolidone copolymer (Oasis HLB). From this comparison, both the C_{18} and styrene–divinylbenzene SPE cartridges were discarded since losses of up to 80% and 35%, respectively, were observed during the loading step. Oasis HLB was eventually selected because this sorbent allowed recoveries higher than 97%, and the barbiturates retained on this sorbent could also be eluted using 5 mL of EtOAc.

For urine samples, the Oasis HLB cartridge was loaded with 2 mL of human urine spiked at 5 mg L^{-1} with each barbiturate, and flushed with 15 mL of acidified water to remove the urea present while keeping all the compounds of interest retained on the sor-

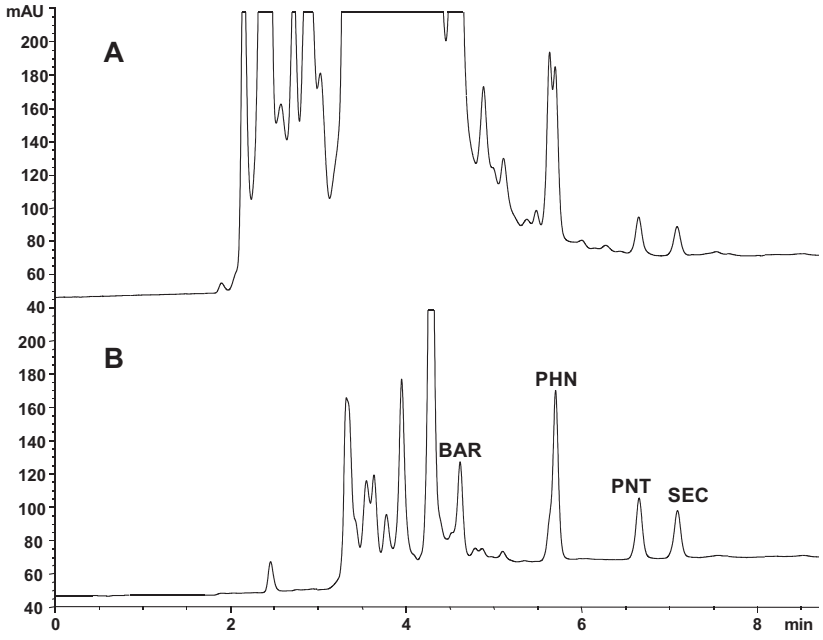

Fig. 5. Comparison of chromatograms obtained after the SPE of 2 mL of human urine spiked with 5 mg L^{-1} of each barbiturate using Oasis-HLB (A) or the tandem system (B).

bent. The sorbent was then eluted with 5 mL of EtOAc since this solvent was capable of stripping the barbiturates from the Oasis HLB cartridge. This eluted fraction was then evaporated to dryness under a stream of N_2 and reconstituted in 1 mL of acidified water prior to its percolation through the MIP. Once the MIP was loaded with this urea-free fraction, a clean-up step using 1 mL of EtOAc was performed, and the MIP eluted subsequently with 5 mL of MeOH (1% AcOH). This methanolic fraction was also evaporated to dryness under a stream of N_2 and quantified by LC thereafter and recoveries of barbiturates from this tandem system are shown in Table 1.

Fig. 5 shows a comparison of the chromatogram obtained after the extraction of 2 mL of human urine spiked at 5 mg L^{-1} with barbiturates when using only Oasis HLB (Fig. 5A) and after the tandem Oasis HLB-MIP (Fig. 5B). As can be seen in the figure, when using only Oasis HLB, quantification of BAR and PHN was not possible due to the presence of many interfering compounds whereas, after the tandem system, quantification of all the barbiturates was easily performed. Even though the tandem system obviously brings an extra cleaning effect in the sample, it also enables a proper removal of the urea present in the sample what was the key factor for enabling a proper retention of barbiturates onto the MIP.

Despite recoveries for barbiturates were not so high, one must bear in mind that MIPs are not only synthesised to achieve high recoveries for the target analyte but MIPs can also be used to enable selective extractions that would be much more difficult to achieve otherwise. The MIP presented herein not only shown a strong affinity for the target analytes but also overperformed many commercially available sorbents for extracting barbiturates from aqueous matrices. Moreover, considering the high urea content (which is a strong competitor of barbiturates for being retained onto the sorbent) in urine, the cleaner chromatogram obtained in the present study is a clear advantage of the MIP synthesised over already existing SPE sorbents when detecting barbiturates in urine.

Once the feasibility of the tandem system had been established, we moved to validate the method in the range from 0.5 to 25 mg L^{-1} for each barbiturate, which is relevant for the level of barbiturates normally found in urine samples, as reported by Jiang et al. [24] in a determination of barbiturates by means of capillary electrophoresis. The linearity was established by spiking 2 mL of human urine between 0.5 and 25 mg L^{-1} with each barbiturate; after the tandem protocol, the determination coefficient (R^2) was above 0.996 for all of the barbiturates under study. The intraday repeatability was set using four consecutive replicates of 2 mL of human urine spiked at 1 mg L^{-1}; the values for this parameter were 8.8%, 8.1%, 4.4% and 3.6% for BAR, PHN, PNT and SEC, respectively. Reproducibility between days was also determined under the same conditions over four different days; the values obtained were 10.5%, 11.2%, 6.7% and 5.8% for all barbiturates under study, and in the same order as above. The LOD was set using a signal to noise ratio of 3; the values were below 0.2 mg L^{-1} in all cases.

Another important advantage of the present MIP is the improvement in the limit of detection achievable. As reported in literature, barbiturates are generally detected by either LC or GC using highly sensitive detection systems, such as MS, and the limits of detection normally reported with these techniques are at the same range as the limits of detection reported in the present study but using a UV detector instead. Therefore, using the present MIP with a more sensitive detector, lower limits of detection would be expected.

Overall, the novel tandem system developed, which had a MIP as a key component, has enabled the accurate quantification of several barbiturates in human urine samples at clinically relevant concentrations.

Table 1
Recovery values for barbital (BAR), phenobarbital (PHN), pentobarbital (PNT) and secobarbital (SEC) after the tandem system ($n = 3$, RSD < 7.8%).

	Recoveries (%)
Barbital	40
Phenobarbital	65
Secobarbital	47
Pentobarbital	35

4. Conclusions

A MIP which can recognise barbiturates has been prepared in good yield. It was synthesised using a precipitation polymerisation protocol to yield a porous, beaded product, and included a designer

monomer in its production to maximise affinity and minimise non-selective binding events when the MIP is in use.

The high-fidelity of the binding sites in the MIP for the core structure common to all the barbiturates under study enabled a high recovery of not only the template molecule but also closely related barbiturate compounds.

When applying the MIP to real urine samples a MISPE protocol was developed which included a tandem system with a commercial sorbent. This tandem system was implemented to remove the urea present in urine samples because it was found that urea suppressed the binding of barbiturates to the MIP.

The use of such a tandem system proved to be a very attractive alternative for discarding undesirable endogenous compounds which may be present in a sample and complicate the extraction of the compound(s) of interest.

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