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Solid-phase extraction of tramadol from plasma and urine samples using a novel water-compatible molecularly imprinted polymer

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ABSTRACT

In this study, a novel method is described for the determination of tramadol in biological fluids using molecularly imprinted solid-phase extraction (MISPE) as the sample clean-up technique combined with high-performance liquid chromatography (HPLC). The water-compatible molecularly imprinted polymers (MIPs) were prepared using methacrylic acid as functional monomer, ethylene glycol dimethacrylate as cross-linker, chloroform as porogen and tramadol as template molecule. The novel imprinted polymer was used as a solid-phase extraction (SPE) sorbent for the extraction of tramadol from human plasma and urine. Various parameters affecting the extraction efficiency of the polymer have been evaluated. The optimal conditions for the MIP cartridges were studied. The MIP selectivity was evaluated by checking several substances with similar molecular structures to that of tramadol. The limit of detection (LOD) and limit of quantification (LOQ) for tramadol in urine samples were 1.2 and 3.5 $\mu g\,L^{-1}$, respectively. These limits for tramadol in plasma samples were 3.0 and 8.5 $\mu g\,L^{-1}$, respectively. The recoveries for plasma and urine samples were higher than 91%.

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1. Introduction

Tramadol hydrochloride, *trans*-(±)-2-[(dimethylamino)methyl]-1-(3-methoxyphenyl)-cyclohexanol hydrochloride, is a synthetic analgesic (pain reliever). Like morphine, tramadol binds to receptors in the brain (opioid receptors) and inhibits reuptake of norepinephrine and serotonin, which appears to contribute to the drug's analgesic effect. Tramadol, like other narcotics used for the treatment of pain, may be abused. Its therapeutic plasma concentration is in the range of 100–300 $\mu g\,L^{-1}$ [1]. Tramadol is rapidly and almost completely absorbed after oral administration but its absolute bioavailability is only 65–70% due to first-pass metabolism [2].

The drug has been quantified using different methods such as UV–vis spectrophotometry [3,4], voltammetry and amperometry [5], elecrophoresis with fluorescence [6] or electrochemiluminescence [7] detection, gas chromatography with flame ionization [8], mass spectrometry [9–11] or nitrogen–phosphorus [12] detection. The methods described for the determination of tramadol in biological samples involve high-performance liquid chromatographic (HPLC) methods with UV [13–15], fluorescence [16–18], electrochemical [19] and mass spectrometry detection [20], but in most cases it combined with a liquid–liquid extraction (LLE) step using organic phases such as ethyl acetate [21–23], n-hexane [24] and *tert*-butyl methyl ether [17], that hindered the degree of automation.

Sample preparation is essential for the analysis of compounds in real samples. Due to unsatisfactory selectivity, the traditional sorbents usually cannot separate analytes efficiently in complex biological or environmental samples. Solid-phase extraction (SPE) is the most popular of clean-up techniques due to factors such as convenience, cost, time saving and simplicity. SPE is the most accepted sample pretreatment method today [25]. A relatively new development in the area of SPE is the use of molecularly imprinted polymers (MIPs) for the sample clean-up [26–29]. MIPs are synthetic polymers possessing specific cavities designed for a target molecule. MIPs are synthesized by the polymerization of different components. In the most common preparation process, monomers form a complex with a template through covalent or non-covalent interactions and are then joined by using a cross-linking agent. After removing of the template by chemical reaction or extraction, binding sites are exposed which are complementary to the template in size, shape, and position of the functional groups, and consequently allow its selective uptake. MIPs are often referred to as 'artificial

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antibodies'. Unlike antibodies, MIPs are stable to extremes of pH, organic solvents and temperature which allows for more flexibility in the analytical methods [26,30]. The use of MIPs for SPE can involve various modes, including conventional SPE where the MIP is packed into columns or cartridges [31,32] and batch mode SPE where the MIP is incubated with the sample [33]. Another major advantage of MIP-based SPE, related to the high selectivity of the sorbent, is the achievement of an efficient sample clean-up.

Recently, we applied MIPs as new sensing material in potentiometric detection of hydroxyzine [34], cetirizine [35], and SPE of verapamil [36], bromhexine [37] and metoclopramide [38]. In this study we present a novel method for the performance evaluation of tramadol based MIPs as selective SPE sorbents for efficient sample clean-up and followed determination of tramadol from complex matrices by high-performance liquid chromatography. This scheme as MISPE allows the sensitive, simple and inexpensive extraction and detection of the analyte in human plasma and urine samples.

2. Experimental

2.1. Reagents

Methacrylic acid (MAA) from Merck (Darmstadt, Germany) was distilled in vacuum prior to use in order to remove the stabilizers. Ethylene glycol dimethacrylate (EGDMA) and 2,2′-azobis isobutyronitrile (AIBN) from Sigma–Aldrich (Steinheim, Germany) were of reagent grade and were used without any further purification. All solvents used in chromatography analyses were HPLC grade and supplied by Merck. Tramadol hydrochloride was used for preparing stock and standard solutions. Bromhexine HCl, dextromethorphan HBr, diphenhydramine HCl, morphine, acetaminophen, aspirin and ephedrine were obtained from the Ministry of Health and Medical Education (Tehran, Iran). The tramadol stock solutions (1000 μg L^{-1}) were prepared weekly and stored at +4 °C. Intermediate standard solution of 100 μg L^{-1} was prepared by the dilution of stock solutions with water. Working standard solutions of different concentrations were prepared daily by diluting the intermediate standard solution with mobile phase solution.

2.2. Apparatus

An Alliance HPLC instrument from Waters Company was used to separate and analyze tramadol in plasma and urine samples. The chromatographic system was composed of an isocratic Waters pump, a Waters 2996 photodiode array detector and an online degasser. A Rheodyne model 7725i injector with a 20 μL loop was used to inject the samples. Chromatographic separation was achieved on an ACE 5 μm, C18 4.6 mm × 250 mm column. HPLC data were acquired and processed using a PC and Millennium 2010 Chromatogram Manager software (Version 2.1 Waters). pH of solutions were adjusted using a model 630 digital Metrohm pH meter equipped with a combined glass-calomel electrode. IR spectra of grounded polymer were recorded on a Shimadzu IR-460 spectrometer (Kyoto, Japan) using KBr pellets in the range of 400–4000 cm^{-1}.

2.3. Procedures

2.3.1. MIP and NIP preparation with bulk polymerization

For the preparation of the tramadol imprinted polymer (Fig. 1), the template (65.8 mg, 0.25 mmol) was dissolved in chloroform in a 25-mL thickwalled glass tube. The functional monomer (MAA) (0.129 mL, 1.5 mmol), the cross-linking monomer (EGDMA) (2.96 mL, 16 mmol), and the initiator (AIBN) (12 mg, 0.082 mmol) were then added to the above solution. The mixture was uniformly dispersed by sonication for 40 min to remove oxygen. Then the solution was placed in a water bath at 60 °C. The reaction was allowed to proceed for 18 h. The hard polymers that were obtained were crushed. After the polymerization procedure and drying, the polymer particles were washed with methanol and acetic acid (10:1, v/v, of 98% methanol and pure acetic acid) for three times and with distilled water for two times. The complete removal of template was followed by HPLC-UV. In order to verify that retention of template was due to molecular recognition and not to non-specific binding, a control, non-imprinted polymer (NIP) was prepared as the same procedure, including washing, but with the omission of the target molecule, tramadol. The size of the particles used after crushing and sieved was between 15 and 40 μm.

2.3.2. MISPE conditions

MIP and NIP columns were prepared by packing 60 mg of the polymer into 1 mL empty SPE cartridges. The cartridge was conditioned sequentially with 1 mL methanol, 1 mL of ultra-pure water and 1 mL 20 mmol L^{-1} ammonium phosphate at pH 3.0, loading with 5 mL of the water sample (50 μg L^{-1}) at pH 8.0. After loading, column was washed with 1 mL acetonitrile:acetone (1:3, v/v) and 1 mL dichloromethane. We need to dry the SPE cartridge thoroughly after the washes by vacuum application. Finally, the elution was performed by passing 3 × 1 mL methanol:acetic acid (10:1, v/v). All fractions were evaporated to dryness at 20 °C under a stream of nitrogen and finally recovered in 1 mL of mobile phase. Then, 20 μL of each sample was injected onto the analytical column of HPLC.

2.3.3. Chromatographic conditions

A simple, sensitive, and reproducible HPLC method has been developed for the determination of tramadol employing reversed phase high-performance liquid chromatography with UV detection [39]. The HPLC was carried out at +40 °C. A degassed mixture of acetonitrile:phosphate buffer (0.01 mol L^{-1}, pH 5.8) (18:82, v/v) was prepared and delivered in isocratic mode at flow rate of 1 mL min^{-1}. All of the analyses were carried out at an operation wavelength of 218 nm and HPLC data were acquired and processed using a PC and Millennium 2010 chromatogram manager software (Version 2.1 Waters). The mean retention time of tramadol was 3.92 min.

2.3.4. Rebinding experiments

Batch adsorption experiments were used to evaluate the binding affinity of the imprinted polymer as reported before [40]. The general procedure for the extraction of tramadol by the MIP was as follows: the polymer beads were suspended in aqueous solutions and the pH was adjusted at 8.0. Then 50 mg of polymer particles were added in 5-mL flask containing tramadol solutions of various concentrations. The mixtures were thermostated at 25 °C for 2 h under continuous stirring and then was filtrated on a paper filter (flow rate = 50 mL min^{-1} by applied vacuum). The free concentration of tramadol after the adsorption was recorded by HPLC-UV at 218 nm. Three replicate extractions and measurements were performed for each aqueous solution. The adsorbed tramadol was desorbed from the MIP by treatment with 2 mL of methanol and acetic acid (10:1, v/v, of 98% methanol and pure acetic acid). The imprinted polymer containing tramadol was placed in the desorption medium and stirred continuously at 600 rpm and room temperature for designated time. The final tramadol concentration in the aqueous phase was determined. The same procedure was followed for NIP particles.

2.3.5. Extraction procedure for human plasma and urine samples

Drug-free human plasma was obtained from the Iranian blood transfusion service (Tehran, Iran) and stored at −20 °C until use after gentle thawing. Urine was also collected from healthy volunteers (males, around 30-year-old). Stock standard solutions of tramadol were prepared in water. For urine, solutions were prepared by adding 5 mL volumes of tramadol solution to a 10 mL

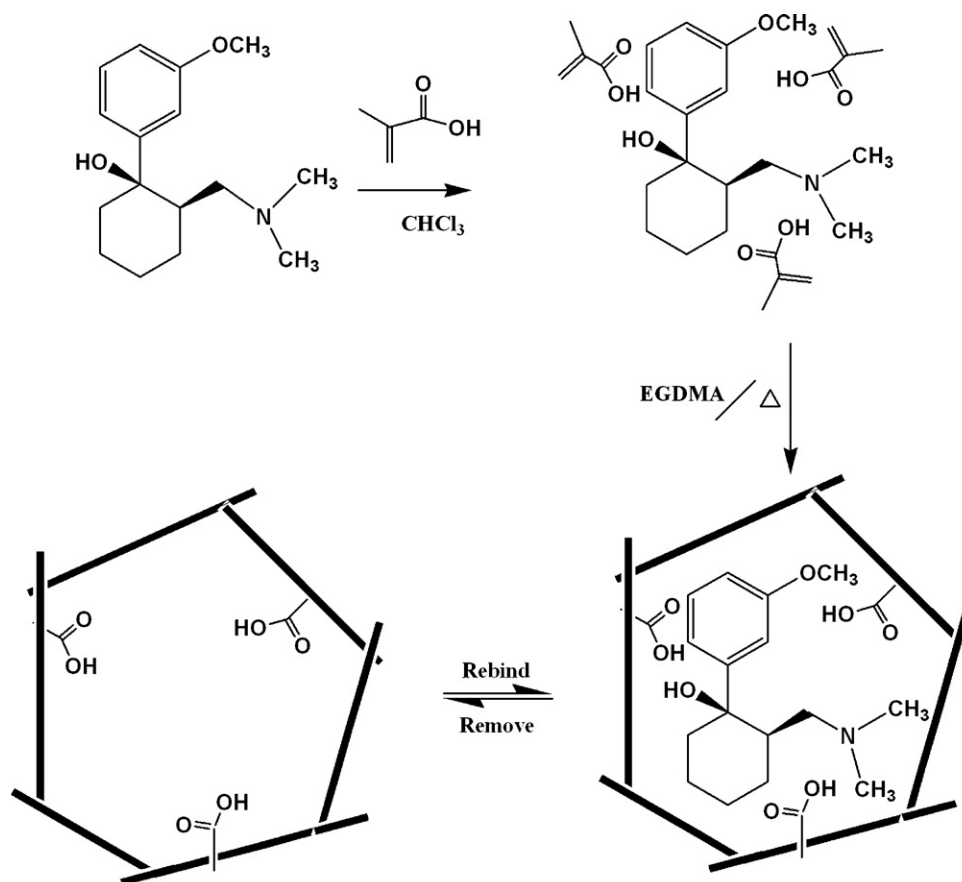

Fig. 1. Schematic representation of the MIP synthesis.

volumetric flask and the solution was diluted to the mark with buffer (pH 3) and vortexed for 5 min and were centrifuged for 20 min at 8000 rpm. For plasma, we suggest it dilute with 25 mM ammonium acetate (pH 5), then centrifuge to remove excess of proteins. 2 mL of the plasma and urine samples spiked by tramadol were diluted with 2 mL buffer pH 8.0 and were centrifuged for 20 min at 8000 rpm and then filtered through a cellulose acetate filter (0.20 μm pore size, Advantec MFS Inc., CA, USA). The filtrate were collected in glass containers and stored at −20 °C until analysis was performed. 2 mL of the filtered supernatant was collected to be directly percolated through the MIP or the NIP cartridges.

3. Results and discussion

3.1. Characterization

The IR spectra of NIP, the unleached and leached MIPs displayed similar characteristic peaks, indicating the similarity in the backbone structure of the different polymers. As a result of the hydrogen binding with the –COOH group of MAA, the O–H stretching and the bending vibrations at 3494 cm^{-1} and 1398 cm^{-1} in the leached MIP materials were shifted to 3476 cm^{-1} and 1386 cm^{-1} in the corresponding unleached MIP, respectively. Furthermore, there were two other distinct differences between the IR spectra of the leached and unleached MIPs. In the leached polymer, there were one broad band around 2704 cm^{-1} and one sharp band around 2956 cm^{-1}. The first was shifted to 2636 cm^{-1} and another appeared around 2987 cm^{-1}. Other absorption peaks match both those of MIP, as well as NIP: 1731 cm^{-1} (stretching vibration of C=O bonds on carbonyl groups); 1636 cm^{-1} (stretching vibration of residual vinylic C=C bonds) and vibrations at 1258, 1159, 954, 875, 817 and 756 cm^{-1}.

3.2. Optimal MIP formulation and progenic solvent

There are several variables, such as amount of monomer or nature of cross-linker and solvent that affects the final characteristics of the obtained materials in terms of capacity, affinity and selectivity for the target analyte. Thus, by achieving an optimum combination of cross-linker and functional monomer non-specific binding should be able to be minimized. Primary experiments revealed that the imprinted polymers prepared in chloroform show better molecular recognition ability than acetonitrile (AN) and dimethyl formamide (DMF) in aqueous environment (Fig. 2). Thus, in chloroform, different formulations for the obtainment of MIPs with improved molecular recognition capabilities have been used. Generally, proper molar ratios of functional monomer to template are very important to enhance specific affinity of polymers and number of MIPs recognition sites. High ratios of functional monomer to template result in high non-specific affinity, while low ratios produce fewer complexation due to insufficient functional groups [41]. Four molar ratios of the monomer MAA to the template of 2:1, 4:1, 6:1 and 8:1 were used in the experiments. The optimum ratio of functional monomer to template for the specific rebinding of tramadol was 6:1 (Table 1), which had the best specific affinity and the highest recovery of 94%, while that of the corresponding NIPs was low at 25%. The specific adsorption recovery of tramadol at 6:1 was 69%, while those at 2:1, 4:1 and 8:1 were 26%, 37% and 36%, respectively. Therefore, a typical 1:6:64 template:monomer:cross-linker molar ratio was used for further studies.

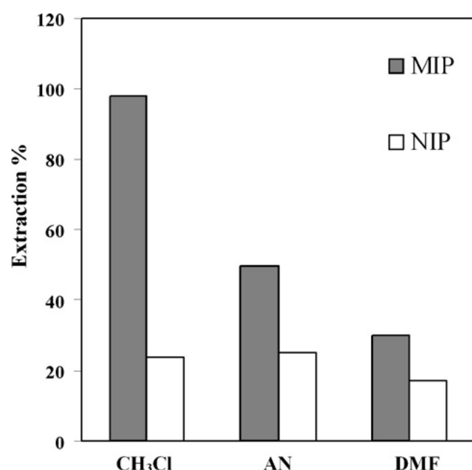

Fig. 2. Recoveries obtained using the MIP and NIP polymers synthesized in different organic solvents. Batch experiments with 50 mg of polymer particles; sample volume, 5 mL; pH, 8.0; tramadol concentrations, 50 μg L^{-1}.

Table 1
Compositions and comparisons of the extraction of tramadol from tramadol standard solution (5 mL, 100 μg L^{-1}) using 50 mg of various polymers as sorbents at pH 8.0, elute: 2 mL methanol and acetic acid (10:1, v/v).

MIP	MAA (mmol)	Tramadol (mmol)	EGDMA (mmol)	AIBN (mmol)	Extraction (%) (mean ± SD)[a]
MIP1	0.5	0.25	16	0.082	52 (±2.4)
MIP2	1.0	0.25	16	0.082	65 (±2.6)
MIP3	1.5	0.25	16	0.082	94 (±3.1)
MIP4	2.0	0.25	16	0.082	70 (±3.0)
NIP1	0.5	0	16	0.082	26 (±1.9)
NIP2	1.0	0	16	0.082	28 (±2.1)
NIP3	1.5	0	16	0.082	25 (±2.1)
NIP4	2.0	0	16	0.082	34 (±2.8)

[a] Average of three determinations.

3.3. Effect of pH

It has been demonstrated that efficient imprint rebinding is possible in aqueous buffer solutions, showing high binding affinity and selectivity as a result of hydrophobic interactions. The effect of pH on the rebinding efficiency of tramadol was investigated by varying the solution pH from 4.0 to 9.0 (Fig. 3). Several batch experiments were performed by equilibrating 50 mg of the imprinted particles with 5 mL of solutions containing 50 μg L^{-1} of tramadol under the desired range of pH. It was observed that tramadol underwent complete rebinding/elution at pH 8.0. The lower responses observed at lower pHs may be attributed to the protonation of the amine group of tramadol.

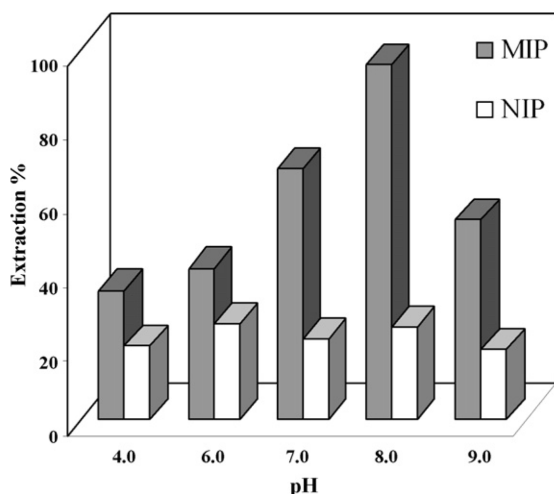

Fig. 3. Effect of pH. Batch experiments with 50 mg of polymer particles; sample volume, 5 mL; tramadol concentrations, 50 μg L^{-1}.

3.4. Choice of loading, washing and eluent solution

Generally, the polymers have binding ability with both specific and non-specific interactions. The specific interactions may originate mainly from the imprinting procedure, which creates selective recognition sites for the template. The non-specific interactions were assessed by measuring the binding of the non-imprinted polymer. At first, cartridges were conditioned with 1 mL methanol, 1 mL of ultra-pure water and 1 mL 20 mmol L^{-1} ammonium phosphate at pH 3.0. Water samples were then loaded onto the cartridges at a flow rate of 1 mL min^{-1} and the wash procedure was assessed for obtaining maximum recovery of the analytes using a variety mixtures including; acetonitrile, acetone, tetrahydrofuran, dimethyl formamide (DMF), acetonitrile–acetone, dichloromethane (DCM), acetonitrile–methanol and hydrochloric acid. In order to investigate the usefulness of the washing step, 5 mL of 50 μg L^{-1} tramadol aqueous solution (pH 8.0) was loaded on the MIP and NIP cartridges separately, followed by desorption with the washing solvent. The results were showed that washing with 2 mL of acetone, tetrahydrofuran and dichloromethane had no obviously effect on the retention of tramadol on both MIP and NIP cartridges. In contrast, polar organic solvents, such as methanol, dimethyl formamide and acetonitrile had clearly effect on the retention of tramadol on both MIP and NIP cartridges. With 2 mL acetonitrile:acetone (1:3, v/v), the recovery of tramadol in NIP cartridge was decreased to 14%, while the recovery of tramadol by the MIP cartridges was not reduced (94.0%). For plasma samples, extra wash in the SPE protocol was needed. In this case, by using 1 mL hydrochloric acid (0.01 mol L^{-1}), 1 mL acetonitrile:acetone (1:3, v/v) and following 1 mL DCM, the recovery of tramadol in NIP cartridge was decreased to 6%, while the recovery of tramadol by the MIP cartridges was not reduced noticeably (91.0%). It is important to note that hydrochloric acid wash is necessary for the removal of metal ions. DCM wash should be done prior to elution to have selective retention of tramadol. Note that we need to dry the SPE cartridge thoroughly after the washes by vacuum application. For the recovery of strongly bound tramadol, the polymer were eluted with 3 × 1 mL of methanol/acetic acid (10:1, v/v). As a result, recovery after the whole SPE process avoids contaminating HPLC column. Meanwhile, increasing of the sample volume up to 100 mL, just had a light effect on the extraction of tramadol.

The reproducibility and repeatability of the method were evaluated from run-to-run MISPE experiments (5 μg L^{-1} standard solution, $n = 6$) and different batch experiments (five batches) and RSDs of 3.2% and 5.4% for the extraction amounts of tramadol were obtained, respectively.

3.5. Adsorption capacity

The capacity of the sorbent is an important factor that determines how much sorbent is required to remove a specific amount of drug from the solution quantitatively. In the measurement of the adsorption capacity of MIP and NIP absorbents, the absorbents (100 mg) were added into 5 mL of tramadol solutions at concentrations of 1.0–300 mg L^{-1}, and the suspensions were mechanically shaken at room temperature, followed by centrifugally removing of the absorbents. The remained tramadol in the supernatant was measured by HPLC, and the isothermal adsorptions are plotted in

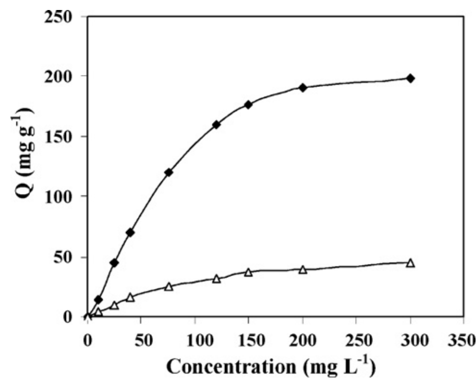

Fig. 4. Effect of tramadol concentrations on the retention capacities of MIP and NIP particles at pH 8.0.

Fig. 4. According to these results, the maximum amount of tramadol that can absorbed by MIP was found to be 190 mg g^{-1} at pH 8.0. For higher tramadol amounts, a slight increase of retained tramadol was observed on MIP capacity curve. As all the accessible specific cavities of the MIP are saturated, the retention of the analyte is only due to non-specific interactions which can be approximatly identical for MIP and NIP polymers.

3.6. Study of MIP selectivity

Chromatographic evaluation and equilibrium batch rebinding experiments are the methods most commonly used to investigate the selectivity of the imprinted materials [42]. For equilibrium batch rebinding experiments, a known mass of template in solution is added to a vial containing a fixed mass of polymer. Once the system has come to equilibrium, the concentration of free template in solution is measured and the mass of template adsorbed to the MIP calculated [43]. The initial concentrations of drugs (100 μg L^{-1}, 5 mL) were extracted by 50 mg of imprinted material at pH of 8.0 on MIP and NIP. The distribution ratio (mL g^{-1}) of tramadol between the MIP particles and aqueous solution was determined by following equation:

$$K_D = \frac{(C_i - C_f)V}{C_f m} \quad (1)$$

where V is the volume of initial solution and m is the mass of MIP materials. Selectivity coefficients for tramadol ion relative to for-

Tramadol Dextromethophan Bromhexine

Diphenhydramine Morphine Acetaminophen

Ephedrine Aspirin

Fig. 5. Chemical structure of investigated drugs in this study.

Table 2
Recoveries (%) obtained from after the loading of the MIP and NIP cartridges with 5 mL of water solution containing 50 $\mu g L^{-1}$ tramadol (pH 8.0).

Drug	K_D (MIP) ($mL g^{-1}$)	K_D (NIP) ($mL g^{-1}$)	k^{sel} (MIP)	k^{sel} (NIP)	k'
Tramadol	1567	33	–	–	–
Acetaminophen	72	30	21.8	1.10	19.8
Diphenhydramine	43	34	36.4	0.97	37.5
Dextromethorphan	38	35	41.5	0.94	44.1
Bromhexine	45	39	34.8	0.85	40.9
Aspirin	59	40	26.7	0.93	28.7
Morphine	36	30	43.5	1.10	39.5
Ephedrine	92	32	17.0	1.03	16.5

eign compounds are defined as:

$$k^{sel}_{Tramadol/j} = \frac{K_D^{Tramadol}}{K_D^j} \tag{2}$$

where $K_D^{Tramadol}$ and K_D^j are the distribution ratios of tramadol and foreign compound, respectively. The previous MISPE protocol was applied also to these drug molecules on NIP particles. The relative selectivity coefficient (k') was also determined by following equation:

$$k' = \frac{k(\text{MIP})}{k(\text{NIP})} \tag{3}$$

Acetaminophen, diphenhydramine, bromhexine, aspirin, morphine and ephedrine were selected to investigate the selectivity of the MIP. Their molecular structures are shown in Fig. 5. Solutions of all compounds were prepared individually with the concentration of 100 $\mu g L^{-1}$. Distribution ratio (K_D), selectivity coefficient (k^{sel}) and relative selectively coefficient (k') values of MIP and NIP material for these different drugs were listed in Table 2. The data in Table 2 shows that MIP exhibit moderately affinity for ephedrine and acetaminophen with the relative selectively coefficient of 16.5 and 19.8, respectively. This could be simply clarified by their close similarity to tramadol in the way of the arrangement of the functional groups or the size of the three-dimensional structure. For aspirin, based on the structural difference, the MIP shows higher relative selectively coefficient (28.8) than those of ephedrine and acetaminophen. Bromhexine, dextromethorphane, morphine and diphenhydramine exhibited much lower affinity for MIP and k' values of 40.9, 44.1, 39.5 and 37.5 were obtained, respectively.

3.7. Tramadol assay in human plasma and urine samples

To demonstrate the potential of MIP for the selective clean-up of analyte, the MIP was applied to the purification of spiked tramadol in human plasma and urine. Aqueous media was employed for the loading solution and the wash procedure was assessed for obtaining maximum recovery of the analyte as said by Sections 2.3.4 and 3.4. It was seen that the procedure can elute interferences and avoid contaminating HPLC column. The chromatograms obtained for plasma and urine samples were compared in Figs. 6 and 7. This efficient method allowed cleaner extracts to be obtained and interfering peaks arising from the complex biological matrices to be suppressed. Results from the HPLC analyses showed that the MIP extraction of tramadol for plasma and urine samples are linear in the ranges 5–350 $\mu g L^{-1}$ and 2.0–300 $\mu g L^{-1}$ with good precision (3.8% for and 3.1% for 25.0 $\mu g L^{-1}$) and recoveries (between 91–94% and 93–96%), respectively (Table 3). Typical chromatograms presented in Figs. 6 and 7 reveal that the MIP can be used for clean-up and when new MIP sorbent was used, a board peak in chromatogram was omitted. The limit of detection (LOD) and limit of quantification (LOQ) for tramadol in urine samples were 1.2 and 3.5 $\mu g L^{-1}$ respectively. These limits for tramadol in plasma samples were 3.0 and 8.5 $\mu g L^{-1}$, respectively.

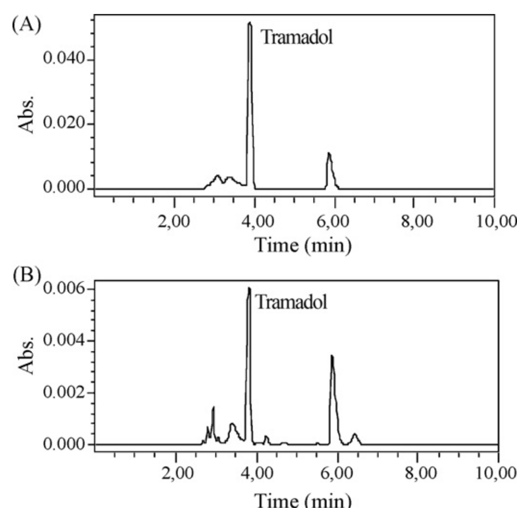

Fig. 6. HPLC chromatograms obtained after percolation of 2 mL urine spiked with 50.0 $\mu g L^{-1}$ of tramadol with a clean-up step comprising the (A) MIP and (B) NIP. Monitored at 218 nm; conditions: column ACE 5 μm, C18 4.6 mm × 250 mm at +40 °C, eluent acetonitrile:phosphate buffer (0.01 mol L^{-1}, pH 5.8) (18:82, v/v) at flow rate of 1.0 mL min^{-1}.

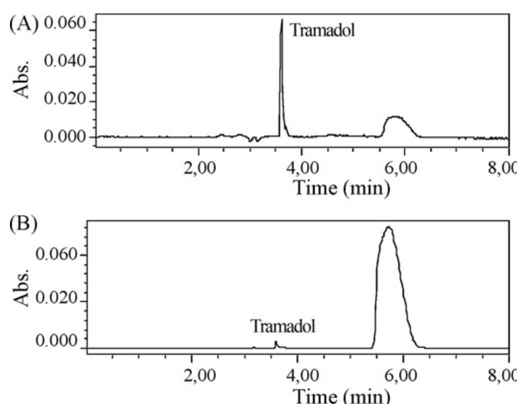

Fig. 7. HPLC chromatograms obtained after clean-up a 50.0 $\mu g L^{-1}$ solution of tramadol in plasma samples with the (A) MIP and (B) NIP monitored at 218 nm; conditions: column ACE 5 μm, C18 4.6 mm × 250 mm at +40 °C, eluent acetonitrile:phosphate buffer (0.01 mol L^{-1}, pH 5.8) (18:82, v/v) at flow rate of 1.0 mL min^{-1}.

Table 3
Assay of tramadol in human plasma and urine by means of the described SPE-HPLC protocol.

Sample	Spiked value ($ng mL^{-1}$)	Proposed SPE-HPLC protocol (recovery% ± SD)[a]	
		MIP	NIP
Human plasma	10	91 ± 3.0	8.1 ± 1.7
	20	94 ± 3.4	9.0 ± 1.4
	50	93 ± 2.9	8.4 ± 1.0
Human urine	10	94 ± 3.3	24 ± 2.6
	25	93 ± 3.1	26 ± 2.4
	50	96 ± 3.4	25 ± 2.8

[a] Average of three determinations.

4. Conclusions

In this paper for the first time, a novel tramadol MIP was prepared by bulk polymerization. The tramadol MIP showed higher

molecular recognition than NIP on chromatographic evaluation. A SPE-HPLC method based on MIP has been developed for the extraction of tramadol from aqueous solutions. Furthermore, the MIP particles as new sorbents in SPE were successfully investigated for the clean-up of human plasma and urine samples with an optimized procedure. This efficient method allowed cleaner extracts to be obtained and interfering peaks arising from the complicated biologic samples to be suppressed. The method was applied to the trace tramadol determination at three levels, and the recoveries for the spiked human plasma and urine samples were higher than 91% in the 10–50 $\mu g\,L^{-1}$. It could be concluded that the technique has great potential in developing selective extraction method for other compounds.

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