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On-line clean-up and determination of tramadol in human plasma and urine samples using molecularly imprinted monolithic column coupling with HPLC

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ABSTRACT

The applicability of an on-line solid phase extraction method using molecularly imprinted monolithic column was developed for the assay of tramadol (TRD) in urine and plasma samples. The monolithic column was prepared by using TRD as the template, methacrylic acid (MAA) as the functional monomer, ethylene glycol dimethacrylate (EGDMA) as the cross-linker and chloroform as the porogen with in situ molecular imprinting polymerization technique. Various parameters affecting the extraction efficiency of the monolithic column were evaluated. Chromatographic analysis of TRD after on-line clean-up of samples was performed by reversed-phase HPLC on an ACE column with ultraviolet detection at 218 nm. The present work was successfully applied for automated simple analysis of TRD in urine and plasma samples with high recoveries between 90.5–93.1% and 93.3–96.0%, respectively. The results revealed that in concentration up to 500 ng/mL of dextromethorphan (DEX), timolol (TMO) and *O*-desmethyltramadol (M1), the recoveries were not reduced more than 4.3% and 4.0% for plasma and urine samples, respectively. The limit of detection (S/N = 3) and limit of quantification (S/N = 10) for TRD in urine samples were 0.03 ng/mL and 0.10 ng/mL, and in plasma samples were 0.3 and 1.0 ng/mL, respectively. Inter-column precision of the assays (n = 3) for urine and plasma samples at the 100 ng/mL TRD level were 4.0% and 4.2%, respectively.

1. Introduction

Developing new selective and sensitive methods for extracting and isolating components from complex biologic matrices is important. For this purpose, solid phase extraction (SPE) technique is the most widely used for sample pre-treatment due to factors such as convenience, low cost, time saving and simplicity [1,2]. However, the common solid phase extraction materials show lack of selectivity except immunoadsorbents which are very selective but expensive and not suitable for most real samples [3]. Thus rapid and selective clean-up methods are needed. The application of SPE procedures involving molecularly imprinted polymers (MIPs), called MISPE, offering the advanced specificity in comparison with traditional SPE adsorbents. MISPE has received increasing attention over the past decade as an attractive alternative for the analysis of complex samples [4–6].

Traditionally, MIPs have been synthesized in bulk polymerization followed by grinding and sieving process to acquire the desired particle size for chromatographic purposes [7]. This tedious and time-consuming process commonly yields irregular particles in shape and size, which limits the chromatographic efficiency. To overcome these disadvantages, various strategies have been proposed for the preparation of MIPs stationary phases [8]. Monolithic MIPs are expected to be one of promising approach to improve separation, which are prepared by in situ polymerization. The simple preparation and high porosity of MIPs monolith can provide a fast separation with higher column efficiencies [9,10]. Compared with conventional particle columns, the loop monolithic column has attracted significant interest because of their ease of preparation, high reproducibility and rapid mass transport [10]. Moreover, the preparation of this type of MIP is more cost-efficient, because it requires much smaller amount of template molecules [9].

Tramadol hydrochloride (TRD), trans-(±)-2-[(dimethylamino)methyl]-1-(3-methoxyphenyl)-cyclohexanol hydrochloride (Fig. 1), is a synthetic analgesic (pain reliever). TRD is an opioid which has the additional property of inhibiting intersynaptic reuptake of noradrenaline and serotonin, thus giving it a dual mode of analgesic action [11]. TRD, like other narcotics used for the treatment of pain, may be abused. Its therapeutic plasma concentration is in the range of 100–300 $\mu g\,L^{-1}$ [12]. TRD is rapidly and almost completely absorbed after oral administration but its absolute bioavailability is only 65–70% due to first-pass metabolism [13]. The use of MIPs for SPE can involve various modes, including conventional SPE where the MIP is packed into

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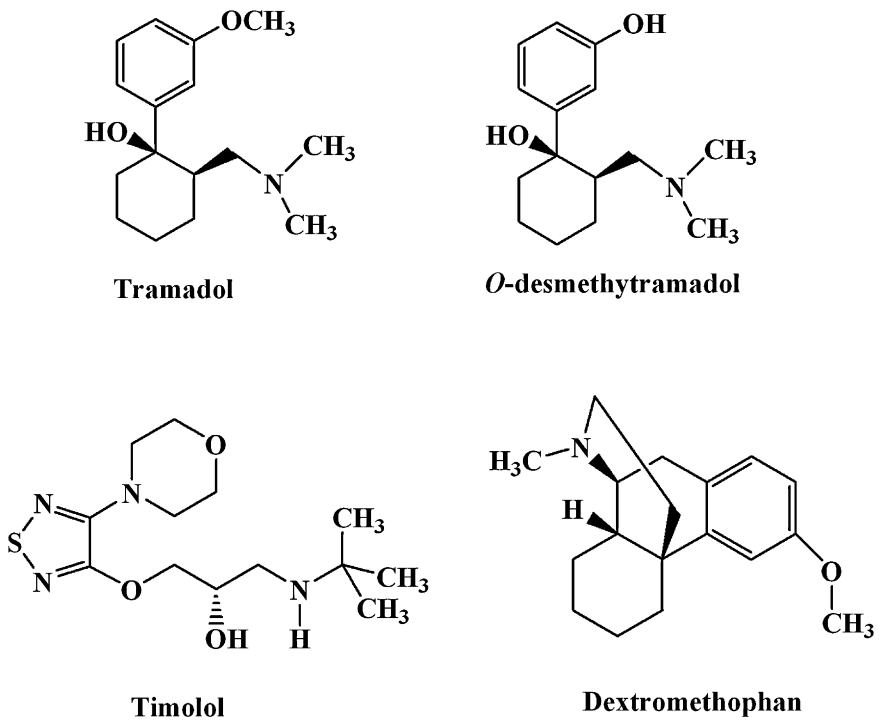

Fig. 1. Chemical structures of investigated drugs in this study.

columns or cartridges [14,15] and batch mode SPE where the MIP is incubated with the sample [16]. 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 [17] and cetirizine [18], SPE of verapamil [19], bromhexine [20] metoclopramide [21], tramadol [22], dipyridamole [23] and dextromethorphan [24] and sustained release of dipyridamol [25,26] and carbamazepine [27,28]. In operation of the on-line SPE-HPLC systems [24], the optimized MIP polymers were packed in polypropylene cartridges, which were incorporated in flow systems prior to the HPLC analytical instrumentation. These cartridges were conditioned and were loaded with samples containing the analyte. Then, the elution phase solution subsequently eluted into the injection loop and each eluted sample was injected into the analytical column and analyzed on HPLC. In this work, new strategy for the first time was developed for on-line sample clean-up and assay of TRD using a loop-monolithic column by in situ molecular imprinting technique. The optimal in situ synthesis conditions and the selectivity of TRD imprinted loop-column were investigated. High performance liquid chromatographic (HPLC) analysis of TRD after on-line clean-up of samples was performed by reversed-phase HPLC on an ACE column with ultraviolet detection at 218 nm.

2. Experimental

2.1. Materials

Methacrylic acid (MAA) from Merck (Darmstadt, Germany) was vacuum distilled 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 (Darmstadt, Germany). Tramadol hydrochloride (TRD) and *O*-desmethyltramadol (M1) (purity > 99%) were gifts from the Food & Drug Organization (Tehran, Iran) and were used for preparing stock and standard solutions. The TRD stock solutions (1000 $\mu g\,L^{-1}$) were prepared weekly and stored at +4 °C. Intermediate standard solution of 100 $\mu g\,L^{-1}$ 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. HPLC apparatus

A DIONEX HPLC instrument was used for chromatographic analysis of TRD. This chromatographic system was composed of a multi solvent gradient pump, a UVD170U 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 C18, 5 μm, 4.6 mm × 250 mm column. For the mobile phase, a degassed mixture of acetonitrile:phosphate buffer (0.01 mol L^{-1}, pH = 5.8) (18:82) was prepared and delivered in isocratic mode at flow rate of 1 mL/min. All of the analyses were carried out at 218 nm and HPLC data were acquired and processed using a PC and Chromeleon Ver. 6.60 chromatography manager software. The retention time of TRD was 15.15 min.

2.3. Preparation and modification of tramadol imprinted monolithic column

TRD imprinted monolithic column was prepared by in situ polymerization method. The template molecule TRD (0.1 mmol), functional monomer MAA (0.8 mmol) were dissolved in chloroform (10 mL) as porogen. The mixture was purged ultrasonically for 30 min and swirled for 6 h, and then cross-linker EGDMA (1 mmol) and initiator AIBN (0.056 mmol) were added into mixture solution. The solution was purged ultrasonically for 15 min before pouring the polymerization mixture into the stainless steel monolithic column (100 mm × 0.46 mm I.D.) sealed with a dead nut at the bottom. The monolithic column was then sealed at the top and polymerization was thermally initiated at 60 °C in a water bath

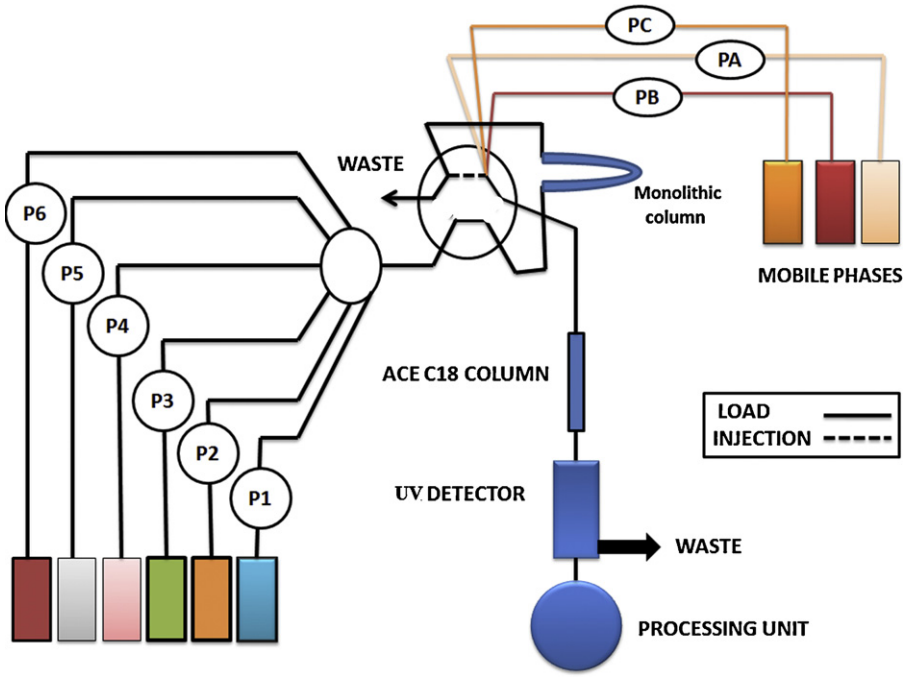

Fig. 2. Schematic representation for operation of the on-line assay of the tramadol by monolithic column coupled with HPLC.

and allowed to continue for 12 h at this temperature. After the polymerization, the seal was removed and the monolithic column was connected to an HPLC pump. The column was washed exhaustively with methanol:phosphate buffer (60:40, v/v) at flow rate of 0.5 mL/min, to remove the template molecule, porogenic solvents and unreacted monomers for at least 2 h, until no template could be detected from the washing solvent by HPLC. It has often been reported that using the same template as the target analyte may lead to false positives if the template molecule is not completely washed off the MIP cavity and may leach during extraction [29].

2.4. Operation of the on-line assay by monolithic column coupled HPLC system

The MIP monolithic column was prepared by using in situ molecular imprinting polymerization technique and was incorporated in a flow system prior to the HPLC analytical instrumentation (Fig. 2). The principle of sequential injection was utilized for a rapid automated and efficient SPE procedure on the MIP. Samples, buffers and washing solvents were introduced to the monolithic column via six Waters 515 peristaltic pumps (P1 to P6 in Fig. 2). The sequential injection manifold was comprised of a micro-electrically actuated 10-port Valco valve. The flow system used 0.7 mm i.d. PTFE tubing through out. Firstly this monolithic column was conditioned via P1 to P3 pumps with 1.0 mL methanol, 1 mL ultra-pure water and 1 mL 25 mM ammonium phosphate, adjusted to pH 3.0. Extraction experiments involved loading the monolithic column with 1.0 mL of sample containing 100 ng/mL TRD at a flow rate of 1.0 mL/min with P4 pump. After loading, the monolithic column for biological based matrixes were washed with 200 μL HCl 0.1 M and 200 μL ultra-pure water with P5 and P6 pumps, respectively. For analysis of matrix based samples, matrix based calibrators were used to calibrate the instrument. Thus, a 100 ng/mL TRD sample prepared in urine or plasma matrix was used for extraction experiments. This stage was performed at a constant flow rate of 1.0 mL/min. After retaining TRD in monolithic column, the elution phase was performed by steady changes of the mobile phase composition during the chromatographic run. This was done through passing 500 μL methanol:phosphate buffer (60:40) via P_A peristaltic pump. After that, each eluted compound was passed through analytical HPLC column by an isocratic elution of mobile phases of acetonitrile:phosphate buffer (18:82) via P_B and P_C peristaltic pumps (Fig. 2) as mentioned in Section 2.2.

2.5. Evaluation of retention and selectivity factors

The retention factors (k') for analytical column were measured as $((t_R - t_0)/t_0)$ where t_R was the retention time of the eluted substance and t_0 the retention time of the void marker (acetone). Selectivity factor (α) was calculated as $\alpha = k'_1/k'_2$, where k'_1 is the retention factor of the template molecule and k'_2 is the retention factor of dextromethorphan (DEX), timolol (TMO) and *O*-desmethyltramadol (M1) (Fig. 1). The retention factors and the selectivity factors were measured by injecting 1.0 mL of a 100 ng/mL samples separately onto each monolithic column which were prepared in plasma matrix. The mobile phase was methanol:phosphate buffer (60:40).

Furthermore, to accurately assess MIP monolithic selectivity, recovery studies of TRD (50 ng/mL) in binary mixtures in the presence of DEX and TMO (50–500 ng/mL) were performed and compared with the results for NIP monolithic column.

2.6. 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. Due to possibility of protein-bonding for TRD and reducing the recoveries in solid-phase extraction processes, it is necessary to pre-treat the plasma before extraction with MIP monolithic column. So, the plasma samples was diluted with 25 mM ammonium acetate (pH 5.0), then centrifuge 20 min at 8000 rpm to remove excess of proteins. Then the supernatant was filtered through a cellulose acetate filter (0.20 μm pore size, Advantec MFS Inc., CA, USA). The filtrate was collected in glass containers and stored at −20 °C until the analysis was performed. 1.0 mL of the

Table 1
Compositions and comparisons of the monolithic MIP and the recovery percent of the polymers.

MIP	MAA (mmol)	Tramadol (mmol)	EGDMA (mmol)	AIBN (mmol)	Recovery(%) (mean ± SD)[a]
MIP1	0.2	0.1	1.0	0.056	52 (±2.3)
MIP2	0.4	0.1	1.0	0.056	59 (±2.2)
MIP3	0.6	0.1	1.0	0.056	65 (±3.1)
MIP4	0.8	0.1	1.0	0.056	96 (±2.8)
MIP5	1.0	0.1	1.0	0.056	82 (±2.0)
NIP1	0.2	0	1.0	0.056	30 (±1.9)
NIP2	0.4	0	1.0	0.056	31 (±2.1)
NIP3	0.6	0	1.0	0.056	33 (±1.8)
NIP4	0.8	0	1.0	0.056	32 (±2.1)
NIP5	1.0	0	1.0	0.056	33 (±1.9)

[a] Average of three determinations.

filtered supernatant was collected to be directly injected through the molecularly imprinted monolithic column.

3. Results and discussion

3.1. Optimal monolithic MIP formulation and porogenic 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 [30]. 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 methanol in aqueous environment (80:20, v/v). The optimum ratio of functional monomer to template for the specific rebinding of TRD was 13:1 (MIP4, Table 1), which had the best recovery of 96%, while that of the corresponding NIP was low at 32%. Percent recovery of TRD was calculated from the following equation:

$$\text{Recovery } \% = \frac{C_i - C_f}{C_i} \times 100 \quad (1)$$

In which C_i and C_f are the concentrations of TRD before and after injection to the monolithic column. This recovery data were obtained from non-matrix calibration curve as described in Section 2.4.

3.2. Effect of eluent solvent

The eluent containing methanol and phosphate buffer (0.01 mol L^{-1}, pH = 5.8) at different ratio (40:60, 50:50, 60:40 and 70:30 for methanol:phosphate buffer, respectively) was investigated and the results showed that with a degassed mixture of two solvent methanol:phosphate buffer (60:40) the recovery of TRD in the monolithic column reached 96%. The retention time for TRD was 15.15 min.

3.3. Effect of pH

The effect of pH on the rebinding efficiency of TRD was investigated by varying the solution pH from 4.0 to 9.0. For this section, 1.0 mL of non-matrix sample containing 100 ng/mL TRD at a flow rate of 1.0 mL/min under the desired range of pH was loaded on the MIP monolithic column. After loading, the TRD-MIP monolithic was eluted with 500 μL methanol:phosphate buffer (60:40, v/v). It was observed that TRD underwent complete elution at pH 7.6. The lower responses observed at lower and higher pHs may be attributed to the protonation of the amine group of TRD and deporotonation of carboxyl groups of the polymer, respectively. Tramadol hydrochloride has a pK_a value of 9.41.

3.4. Study of analytical and monolithic columns selectivity

To survey the analytical column selectivity, some typical drugs such as DEX (related structure), TMO (non-related structure) and *O*-desmethyltramadol (main metabolite) (Fig. 1) were selected. It must be noted that selective and simultaneous analysis techniques for determination of drugs in biological fluids are very important. It can be helpful in control of drug interactions and also in clinical laboratories for diagnostic purposes and drug abuse [31].

Solutions of these compounds were prepared individually with the concentration of 100 ng/mL in aqueous solution. The retention times and selectivity factors for the column is shown in Table 2. The selectivity factors for DEX, TMO and M1 showed the ability of this procedure for selective extraction of TRD.

Possible interference by DEX, TMO and M1 with the clean-up monolithic column was investigated by the addition of the interfering compounds to the urine and plasma samples in binary mixtures containing 50 ng/mL TRD under the optimized conditions. Recovery of the results was checked and compared with that obtained by NIP monolithic column. The results reveal that in concentration up to 500 ng/mL of DEX, TMO and M1, the recoveries were not reduced more than 4.3 and 4.0 percent for plasma and urine samples, respectively (Table 3).

Additionally, in NIP monolithic column based procedure, the recovery of TRD in binary mixtures in the presence of 500 ng/mL of interference in plasma media showed the recoveries of 19, 17 and 17.6% in the presence of DEX, TMO and M1, respectively and 32% for TRD solution alone. These result also confirmed the specific adsorption of TRD by means of the MIP monolithic column in complex biological media.

3.5. Tramadol assay in human plasma and urine samples

To demonstrate the potential of MIP monolithic for the selective clean-up of analyte, the MIP monolithic column was applied to the purification of spiked TRD in human plasma and urine. Diluted biological fluids were employed for the loading solution and the wash procedure was assessed for obtaining maximum recovery of the analyte. The chromatograms obtained for urine samples for MIP monolithic column were compared in Fig. 3. Those for plasma samples were illustrated in Fig. 4. HPLC chromatogram obtained for blank plasma sample for comparison was shown in Fig. 5. The results showed that the procedure can wash interferences and avoid contaminating HPLC column. The monolithic columns in this 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 using the MIP monolithic, calibration curve of TRD for plasma and urine

Table 2
Separation performance for the investigated drugs.

Performance	Tramadol	Timolol	Dextromethorphan	*O*-Desmethyl tramadol
Retention factor (k')	5.21	2.56	2.26	2.60
Selectivity factor (α)	–	2.04	2.30	2.00

Table 3
The recovery of TRD in binary mixtures containing 50 ng/mL TRD in human plasma and urine by means of the monolithic columns coupled with HPLC.

Sample	Monolithic column	Recovery%					
		TMO (ng/mL)		DEX (ng/mL)		M1 (ng/mL)	
		50	500	50	500	50	500
Human plasma	MIP	95.1[a]	94.0	95.2	93.0	93.4	91.7
	NIP	23.2	17.0	22.5	19.0	18.4	17.6
Human urine	MIP	94.5	93.0	93.9	93.7	93.0	92.0
	NIP	25.1	18.0	24.5	20.8	18.1	16.6

[a] Average of three determinations.

Table 4
Determination of tramadol in human plasma and urine by means of the monolithic column coupled with HPLC.

Sample	Spiked concentration (ng/mL)	Calculated concentration (ng/mL)	Recovery (%)	RSD
Human plasma	2.0	1.81[a]	90.5	3.8
	10	9.1	91.4	2.6
	30	27.9	92.5	2.8
	50	46.5	93.1	3.0
	200	184	92.0	3.2
Human urine	0.5	0.47	94	3.5
	10	9.3	93.3	3.0
	30	27.8	95.2	2.9
	50	48.0	96.0	3.1
	200	191	95.5	3.9

[a] Average of three determinations.

samples are linear in the ranges 1.0–350 ng/mL and 0.1–300 ng/mL with good precision (3.8% for 10.0 ng/mL and 3.1% for 25.0 ng/mL) and recoveries (between 90.5–93.1% and 93.3–96.0%), respectively (Table 4). The limit of detection (S/N = 3) and limit of quantification (S/N = 10) for TRD in urine samples were 0.03 ng/mL and 0.10 ng/mL, and in plasma samples were 0.3 and 1.0 ng/mL, respectively.

For evaluation of reproducibility of the method, batch-to-batch consistency for many preparations of MIP monolithic columns for TRD was done. Batch-to batch reproducibility was investigated by running one identical sample (1.0 mL of 100 ng/mL TRD) on three monolithic columns from three different manufacturing batches. Reproducibility RSDs of batches for urine and plasma samples were found to 4.0% and 4.2%, respectively.

The monolithic column in this work was used for on-line sample extraction 'multiple use'. The results revealed that in concentration of 100 ng/mL of TRD, the recoveries were not reduced more than 5.0% after four on-line monolithic column coupled HPLC runs (three samples in each analytical run).

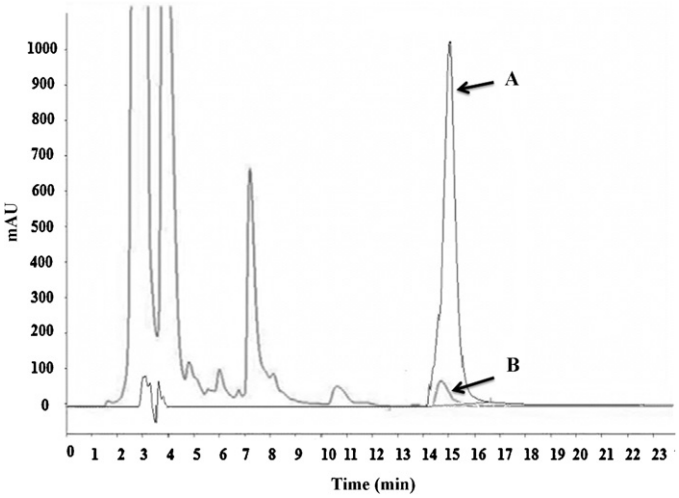

Fig. 3. HPLC chromatograms obtained after percolation of 1.0 mL urine sample spiked with 50.0 ng/mL of tramadol with a clean-up step comprising (A) the imprinted monolithic column (B) NIP, monitored at 218 nm. Conditions: column ACE 5 μm, C18 4.6 mm × 250 mm at +40 °C, eluent methanol:phosphate buffer (0.01 mol L^{-1}, pH 5.8) (60:40, v/v) at flow rate of 1.0 mL/min.

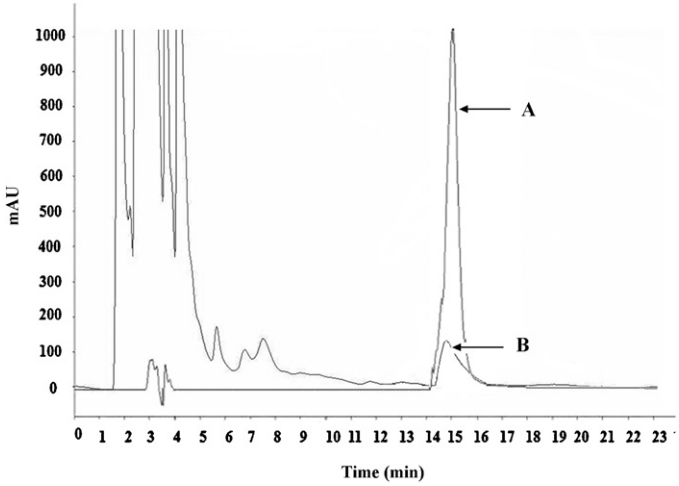

Fig. 4. HPLC chromatograms obtained after percolation of 1.0 mL plasma sample spiked with 50.0 ng/mL of tramadol with a clean-up step comprising (A) the imprinted monolithic column (B) NIP, monitored at 218 nm. Conditions: column ACE 5 μm, C18 4.6 mm × 250 mm at +40 °C, eluent methanol:phosphate buffer (0.01 mol L^{-1}, pH 5.8) (60:40, v/v) at flow rate of 1.0 mL/min.

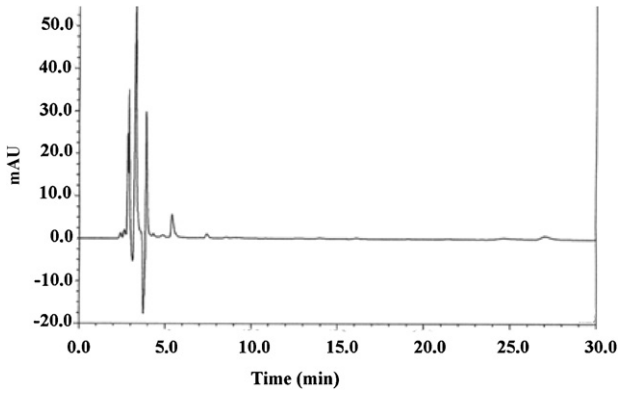

Fig. 5. HPLC chromatogram obtained for blank plasma with a clean-up step comprising the imprinted monolithic column monitored at 218 nm. Other conditions are similar to Fig. 4.

4. Conclusions

In this work, the TRD imprinted monolithic column was directly prepared in a stainless steel loop-column by in situ polymerization for the first time using MAA and EGDMA as the monomer and cross-linker, respectively. The resultant TRD-MIP monolithic column was used as a clean-up pre-column coupling with HPLC directly to determine the TRD contents in human urine and plasma samples. There was a good separation between TRD and other constituents in the biological complex matrices. Moreover, the results showed the recovery of TRD not affected in the presence of other investigated interferences such as DEX, TMO and M1. The obtained monolithic column showed good flow-through and pretreatment property for complex fluids such as human plasma and urine samples. This automated clean-up method is easy with wide linear dynamic range.

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