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Facile synthesis of magnetic molecularly imprinted polymer: Perphenazine template and its application in urine and plasma analysis

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ABSTRACT

Synthesis of magnetic iron oxide nanoparticles and its surface modification with methacrylic acid (MAA) was performed simultaneously by adding Fe^{2+}/Fe^{3+} to an alkaline MAA solution under nitrogen atmosphere. MAA coated magnetite (Fe_3O_4@MAA) has abundant reactive double bonds on the surface that can initiate polymerization. Magnetic molecularly imprinted polymers (MMIPs) were synthesized through distillation–precipitation polymerization of MAA as monomer, perphenazine (PPZ) as template, and ethylene glycol di-methacrylate (EGDMA) as cross linker on Fe_3O_4@MAA, with concise control of experimental conditions in about 90 min. The produced super paramagnetic MMIPs can be separated from the solution in the presence of external magnetic field in less than 1 min. Characterizations of the synthesized particles were performed by electron microscopes, thermo-gravimetric analysis (TGA), vibrating sample magnetometer (VSM), Fourier transform infrared (FT-IR) spectroscopy, and BET. The data showed that Fe_3O_4@MAA was well encapsulated in the polymer shell. The MMIPs showed high porosity. Moreover, MMIPs were used for rapid pre-concentration and separation of PPZ in human plasma and urine without any dilution and pretreatments using high performance liquid chromatography equipped with a photo diode array detector (HPLC-PDA). The calibration curve in urine and plasma has shown the same slope as the external calibration curve. Linear range of 20–5000 ng mL^{-1}, and a detection limit of 5.3 ng mL^{-1} was obtained. The results showed 97.92% recovery along with the relative standard deviation of 6.07% (n = 6) for 1 μg mL^{-1} PPZ. Pre-concentration factor was 13. The MMIPs adsorbed PPZ in 1 min and then desorbed it by MeOH:HOAc in 2 min.

1. Introduction

Selective extraction of analytes from complex sample matrices was revolutionized by the development of molecularly imprinted polymers (MIPs). These polymers act as artificial antibodies with the ability to recognize and bind to the desired molecular target which is called "template". The widespread use of MIPs is for their chemical and thermal stability, possibility of being used in wide pH ranges, high selectivity in adsorption of the analyte and their reusability [1–5]. In common methods, MIPs are synthesized by bulk polymerization, which should be crushed into fine particles for further use. This is a prolix and low yield process and has no control over shape and size. Therefore, the traditional method was rarely ever used [6]. MIPs prepared by surface polymerization show acceptable shape and size. In this procedure, polymerization occurs on the surface of solid particles as core [7,8]. Magnetic core is also used in MIPs preparations and the resulting polymers are called MMIPs. Moreover, MMIPs have been reported as solid phase sorbents in sample pretreatments [9]. The main advantage is the combination of both specific molecular recognition and their separation by an external magnetic field [10–14]. So these particles can be easily used in dispersive magnetic solid phase extraction (DMSPE) [15,16] and eliminate the need for filtration or centrifugation [17]. Magnetic core may be micro or nano in dimension [10]. Fe_3O_4 magnetic nanoparticles (MNPs) constitute the common magnetic core used for MMIPs synthesis due to the super paramagnetic properties, good chemical and physical stability, and simple synthesis methodology [18].

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After the synthesis of MNPs, silanization step is performed to increase stability and protect the particles from aggregation. An expensive functional material such as 3-(Methacryloxy) propyl trimethoxysilane (MPS) is also used to modify the silica layer prior to MIPs preparation. This modification provides surface reactive double bonds which are necessary to start polymerization [19]. Then, the polymer is deposited on the surface of the functionalized silica layer. Silanization step is the main cause of increase in imprinted polymer particle size and reduces the magnetic saturation and adsorption capacities of MMIPs [20–22]. Decrease in the number of synthethic steps and time is also very important.

The objective of this study was to introduce a rapid and economic synthetic protocol for MMIPs preparations along with super paramagnetic and stable particles. Silica modified and functionalized Fe_3O_4 nanoparticles (Fe_3O_4@SiO_2) that are used in traditional MMIP procedures are replaced by Fe_3O_4@MAA. In situ Fe_3O_4 synthesis and MAA modification were performed. FT-IR spectrum confirms that Fe_3O_4@MAA has a surface reactive double bond to promote polymerization. Therefore, Fe_3O_4@MAA was employed for grafting copolymerization of MAA and EGDMA which was imprinted by PPZ as template via distillation precipitation polymerization. After characterization of the resultant MMIPs, their applications in dispersive micro solid phase extraction (DMSPE) cleanup and pre-concentration of PPZ in human plasma and urine were evaluated.

PPZ is an antidepressant and is recently used in the treatment of Parkinson's disease. Thus, the pharmacokinetic data is of primordial help to physicians in order to decide on the dosage of the prescribed drug [23]. The matrices of biological samples are complex and sample treatment is commonly required prior to instrumental analysis [24]. Among different reported sample pretreatments, methods that are simple, economic, fast, environmentally green and easy to automate have greater interest [25,26]. The present MMIP separates PPZ from urine and plasma rapidly and efficiently. The present sample preparation is green and no hazardous solvents are used.

2. Materials and methods

2.1. Materials

Iron (III) chloride hexahydrate ($FeCl_3{\cdot}6H_2O$), iron (II) chloride tetrahydrate ($FeCl_2{\cdot}4H_2O$), potassium dihydrogenphosphate (KH_2PO_4), ethanol (EtOH), hydrochloric acid, glacial acetic acid (HOAc), and cetyltrimethyl ammonium bromide (CTAB) were purchased from Merck (Darmstadt, Germany) and used without additional purification. Ethylene glycol dimethacrylate (EGDMA), methacrylic acid (MAA) and HPLC-grade acetonitrile (ACN) were also obtained from Merck (Darmstadt, Germany). Perphenazine (PPZ) and 2,2-azobisisobutyronitrile (AIBN) were bought from Sigma–Aldrich (St. Louis, MO, Germany). Thioridazine hydrochloride (TRZ), amitriptyline hydrochloride (AML), chlorpromazine hydrochloride (CPZ) and promethazine hydrochloride (PHZ) were supplied by Minoo-pharmaceutical, Darouopakhsh, and Tehran Chemie companies (Tehran, Iran), respectively. HPLC-grade methanol (MeOH) was supplied by Fanavaran Petrochemical Company (Tehran, Iran). Stock solutions (1000 mg L^{-1}) of all standards were prepared in MeOH:HOAc (9:1) and stored at 4 °C, protected from light. Working standards were prepared by serial dilution of these stock solutions.

2.2. Apparatus

TEM images were captured by an EM 10C-80KV transmission electron microscope (Zeiss, Germany). FESEM was obtained by Mira3-XMU FESEM (Tescan, USA). CHN analysis was performed by an ECS4010 Costech elemental analyzer (Italy). A vertex 70 FTIR spectrometer (Bruker, Germany) was used to record the spectrums. TGA analysis was done using simultaneous thermal analyzer STA503 (BÄHR-Thermoanalyse GmbH, Germany). Specific surface area was determined by NanoSORD BET (Toseye Hesgarsazan Asia Co., Iran). Magnetic properties of the synthesized particles were recorded by MDK-VSM system (Meghnatis Daghigh Daneshpajouh Co., Iran). Suspensions of the particles were lyophilized using Operon freeze dryer (Korea). Jasco 7850 spectrophotometer (Japan) was used whenever required.

HPLC was performed on Waters 600 instrument comprising of two reciprocating pumps, an AF in-line degasser, a high-pressure manual Rheodyne injection valve (20-μL injection loop), and a Waters 2998 photodiode array detector (PDA). Data acquisition and processing were performed using Empower2 software. The sample components were separated on a RP-C18 Waters ODS2 column (25 cm × 4.6 mm, 5-μm) equipped with a 1-cm guard column having the same stationary phase. The mobile phase composed of (A) 0.01 M phosphate buffer containing 1 mM CTAB, and (B) acetonitrile. Binary gradient elution profile of the mobile phase was: t = 0, 100% A; t = 6, 80% A; t = 10, 50% A; t = 20, 20% A; t = 25, 100% A. The mobile phase flow rate was 1 mL min^{-1}. The mean retention time of PPZ was 14.22 min.

2.3. Preparation of methacrylic acid coated Fe_3O_4 nanoparticles

The preparation protocol is shown in Fig. 1A. At first, Fe_3O_4@MAA nanoparticles were prepared using common chemical co-precipitation methods with little modification [27,28]. It was a one-pot process in which iron oxide nanoparticles production and their surface modification were simultaneously performed using alkaline MAA solution instead of NaOH. To briefly describe, 10 mmol $FeCl_3{\cdot}6H_2O$ and 5 mmol $FeCl_2{\cdot}4H_2O$ were dissolved in 50 mL of 1 mol L^{-1} HCl. Then 2 mL MAA was added to 100 mL of 2 mol L^{-1} NaOH and degassed for 15 min. Finally, the Fe^{2+}/Fe^{3+} solution was added drop-wise to stirring alkaline MAA solution (800 rpm) under nitrogen atmosphere at reflux conditions at 60 °C. The obtained black precipitates (Fe_3O_4@MAA nanoparticles) were magnetically collected and washed repeatedly with deionized water until the pH of the eluents became neutral. Finally, it was dispersed in acetonitrile (20% V/V) for next use.

2.4. Preparation of MMIPs

A mixture of 0.5 mmol PPZ and 4 mmol MAA in 50 mL acetonitrile was first stirred in a three-necked round-bottomed flask for 30 min to form a template–monomer complex [29]. Then 100 mL of Fe_3O_4@MAA suspension (20% V/V) was added into the complex solution followed by adding 25 mmol EGDMA and 1 mmol AIBN. The mixture was stirred at 500 rpm. It was purged with nitrogen gas to displace oxygen while the solution temperature reached 80 °C under distillation condition. This temperature was maintained by keeping oil bath at 90 °C.

The mixture was then kept around boiling point till about 70 mL of the acetonitrile was distilled out within 90 min [20,30]. The final mixture was cooled to ambient temperature and the resultant Fe_3O_4@MAA@MIPs composite was separated by an external permanent magnet (1.4 T). The produced MMIPs were washed several times by re-dispersion in acetonitrile and collection with magnet. After that, the particles were further washed with MeOH:HOAc (9:1, v/v) five times in order to remove template molecule (PPZ) completely. The template removal was monitored by both UV–vis spectrophotometer and HPLC at 253 nm. The MMIPs were washed with deionized water until the eluents became neutral. Finally, it was re-dispersed in deionized water to make a 50 mg mL^{-1} MMIPs

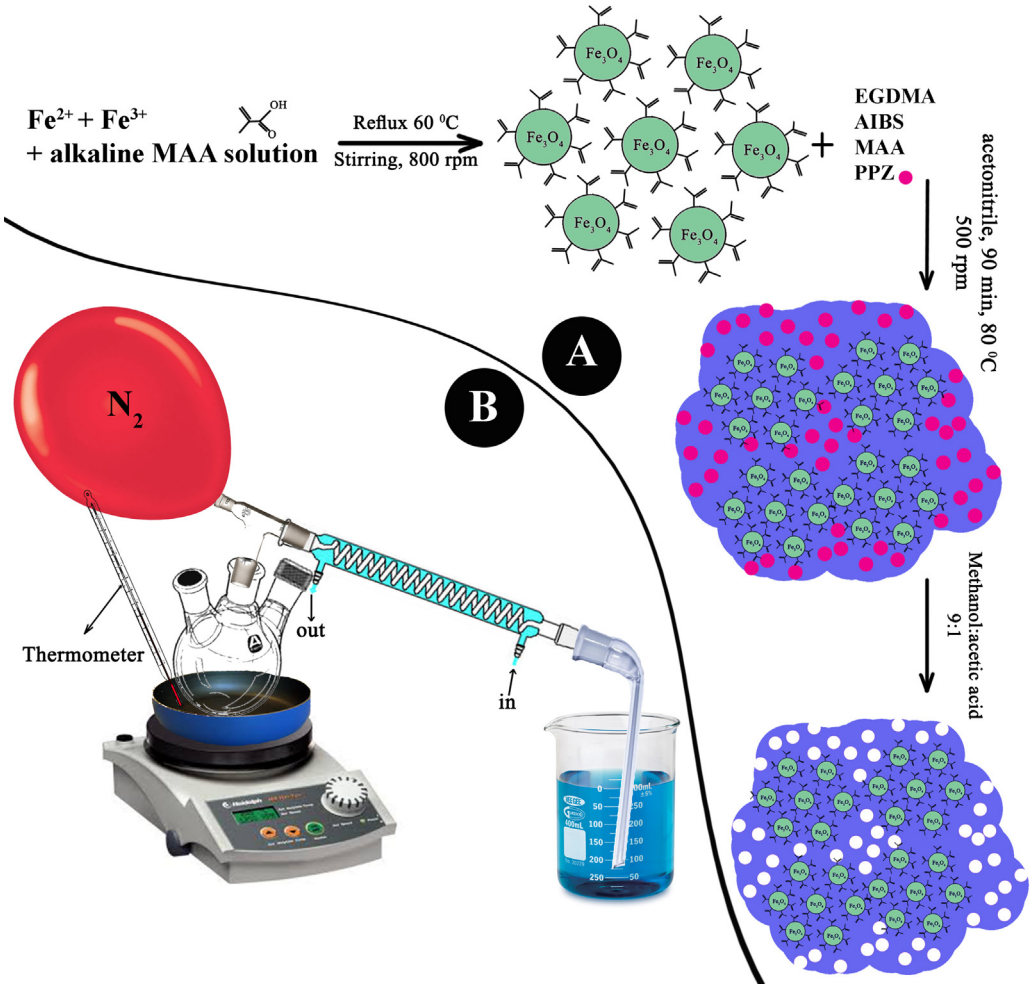

Fig. 1. Schematic representation of A) the Fe_3O_4@MAA synthesis B) the reactions used for preparation of MMIPs and C) the system set up.

suspension and stored at 4 °C for next use. Schematic representation of the MMIPs synthesis set up is shown in Fig. 1B. A photograph of the system setup is also presented in the Supplementary file.

In order to check the MMIPs selectivity in PPZ separation, magnetic non-imprinted polymers (MNIPs) were also prepared with the same procedure in the absence of the target molecule. MMIPs and the other synthesized particles were lyophilized for particles characterizations.

2.5. Analysis of human urine and plasma samples

Drug-free human plasma was obtained from Golestan hospital (Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran) and stored at −80 °C before use. Urine was collected from healthy volunteers (males, 35–40-year-old). Both types of samples were used for PPZ extraction without any sample pretreatments and/or dilution. Urine and plasma calibration curves were also prepared in drug free samples.

2.6. Extraction of PPZ

At first 100 μL of suspension of the prepared MMIPs (containing 5 mg of dried particles) was placed in the sample cell, completely washed by 2 mL deionized water and decanted under magnetic field. Then 5 mL of the sample or standard PPZ solution at pH 4.5 was added and the resulting mixture was stirred for 1 min. Next, the solution was decanted by applying the magnetic field. The separated particles were washed by 2 mL deionized water to remove any possible interferences. PPZ was extracted from MMIPs shell by eluting the adsorbent twice with 200 μL of MeOH:HOAc (9:1, v/v) followed by sonication for about 1 min. Both solvents were mixed and 20 μL of the mixture was injected to HPLC for PPZ analysis. The pre-concentration factor was about 13.

3. Results and discussion

3.1. Synthesis and characterization of MMIPs

Both the synthesis and the following stabilization/functionalization were achieved in one step upon addition of mixture of Fe^{2+} and Fe^{3+} to an alkaline MAA solution. For comparison, nonfunctionalized Fe_3O_4 was also synthesized. The typical TEM images of the synthesized Fe_3O_4 and Fe_3O_4@MAA nanoparticles are shown in Fig. 2. Fe_3O_4@MAA (Fig. 2B) with a mean size of about 5 nm, is approximately smaller in size compared to Fe_3O_4 (MNPs, Fig. 2A). It seems that modification slightly stabilizes the nanoparticles and lee aggregation happens. This modification was confirmed by FTIR and TGA. The IR spectrum of Fe_3O_4@MAA nanoparticles in Fig. 3B indicates vinyl group peaks around 1631 cm^{-1}. The bands around 2923 and 2855 cm^{-1} were attributed to the stretching vibrations of C—H (—CH_2 and —CH_3 of MAA). Compared with Fig. 3A, the peak location of Fe—O has shifted from about 589.26 cm^{-1} to 582 cm^{-1} and that of OH stretching band from 3388 cm^{-1} (in Fe_3O_4) to 3433 cm^{-1} (in Fe_3O_4@MAA).

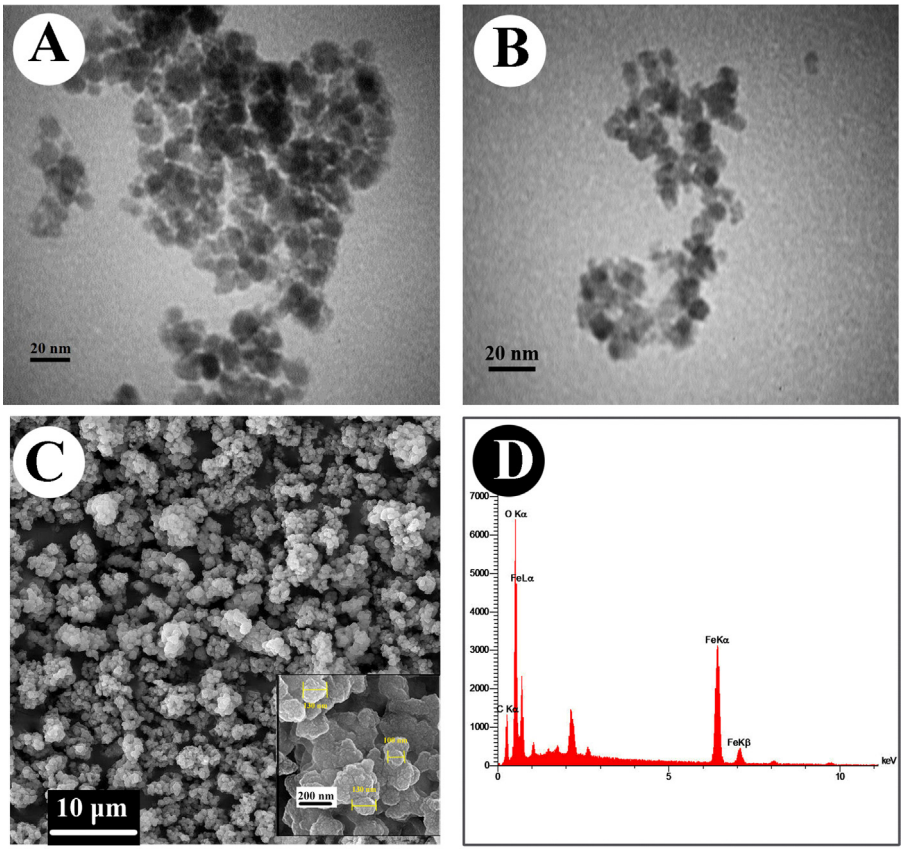

Fig. 2. TEM images of A: Fe_3O_4 and B: Fe_3O_4@MAA nanoparticles, C: FESEM image and D: EDX spectra of synthesized MMIPs.

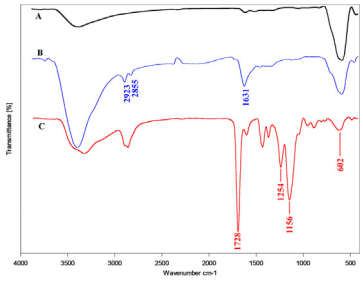

Fig. 3. FT-IR spectra of (A) bare Fe_3O_4 nanoparticles, (B) MAA functionalized Fe_3O_4 nanoparticles, (C) magnetic molecularly imprinted polymers (MMIPs) in KBr.

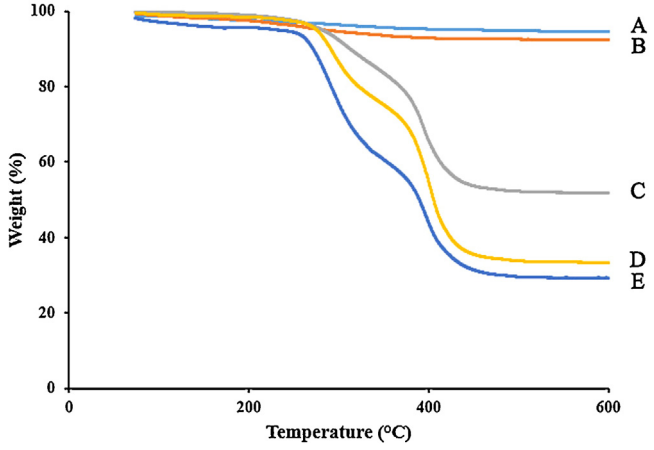

Fig. 4. TGA analysis of (A) Fe_3O_4, (B) Fe_3O_4@MAA, (C) MNIPs, (D) MMIPs and (E) MMIPs + PPZ.

This suggests that MAA molecules were coated on the surface of Fe_3O_4 by interaction between —COO^- of MAA and hydroxyl functional groups on the surface of Fe_3O_4 [31]. Additionally, Fe—O stretching peak intensity was decreased in Fe_3O_4@MAA, indicating that the modification successfully happened [32]. Thermal behavior of materials such as thermal mass differs from one compound to the others. TGA analyses identify temperature ranges that a compound is stable through recording the weight loss. This weight loss is due to deformations of the compounds. However, every compound has its own TGA pattern. TGA pattern of Fe_3O_4 and Fe_3O_4@MAA (Fig. 4) demonstrates thermal stability of Fe_3O_4 particles in the studied temperature ranges. Curves A and B indicate weight loss of about 2.36% above 220 °C for Fe_3O_4@MAA compared to Fe_3O_4. Fe_3O_4@MAA particles convert to Fe_3O_4 above 220 °C. This confirms that a thin layer of MAA was grafted on the surface of Fe_3O_4.

The FESEM image of the MMIPs particles is shown in Fig. 2C. This figure illustrates that Fe_3O_4@MAA cores are surrounded by a polymer layer. It also clearly indicates that the MMIPs nanoparticles have high porosity with an estimated diameter between 120 and 160 nm. To further confirm polymerization, both EDX and CHN analyses were performed. EDX spectra (Fig. 2D) indicate the presence of Fe, O and C, confirming that polymerization occurs on the surface of Fe_3O_4@MAA. The CHN elemental analysis data for MMIPs showed 41.76 and 5.23 Wt% of C and H, respectively. The high carbon content of the MMIPs also identifies the extent of surface polymerization [20].

More evaluation of the MMIPs was done by recording its FTIR spectrum (Fig. 3C). The present MMIPs showed main absorption bands at around 602, 1728, 1254 and 1156 cm^{-1} which were assigned to Fe—O bond for Fe_3O_4 MNPs, C=O stretching vibration

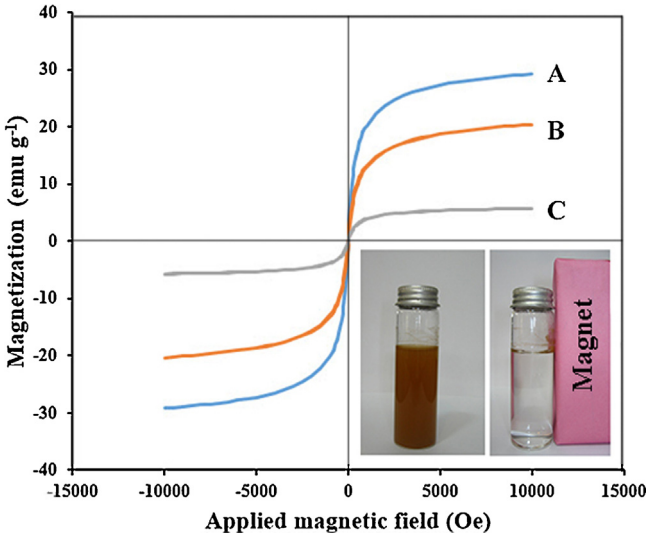

Fig. 5. The hysteresis loop of (a) Fe_3O_4, (b) Fe_3O_4@MAA and (c) MMIPs. The inserted photograph shows the separation and re-dispersion process of a solution containing MMIPs in the presence (left) and absence (right) of an external magnetic field.

of carboxyl, C—O symmetric, and asymmetric stretching vibration of ester, respectively [1].

The weight loss difference between MNIPs and MMIPs, curves C and D (Fig. 4), may be attributed to thickness of polymer layer and the template molecules residue that remains in polymer backbone even after several washing steps. This difference showed that template molecules were successfully grafted into the MIP shell.

The MMIPs were brought into contact with 100 ppm of PPZ standard solution (10 mL) for 1 min, then washed with deionized water, freeze dried, and abbreviated as MMIPs + PPZ. Fig. 4 illustrates that the weight retention at 420 °C for MMIPs (Curve D) and MMIPs + PPZ (Curve E) is ~41.25% and ~35.92%, respectively. About 5% differences between weight losses of the two mentioned particles at 420 °C is attributed to the adsorption of PPZ by MMIP [33,34]. This amount (5%, 50 mg g^{-1}) can be regarded as MMIPs adsorption capacity of PPZ.

The extent of magnetization can also be determined by the per cent of Fe_3O_4 in the MMIPs. The TGA curve of MMIP can identify its magnetite contents. However, weight retention of 41.25% in curve E (Fig. 4) shows relatively high amounts of Fe_3O_4 in the MMIPs. This also predicts super paramagnetic properties of the MMIPs.

The specific surface area of the Fe_3O_4@MAA and MMIPs were 50.03 and 31.32 m^2/g, accordingly. Although MMIPs size is about 40 times higher than that of Fe_3O_4@MAA, the specific surface area is still in an acceptable range. This may be due to high porosity of the MMIPs.

Potential use of the adsorbent in magnetic separation technology can be judged by their room temperature magnetic hysteresis loops. This feature illustrated the extent of the materials response

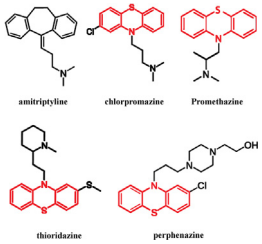

Fig. 6. Chemical structures of perphenazine and its structural analogues.

to an external magnetic field. Fig. 5 shows that there were no hystereses for the three curves. The saturation magnetization of Fe_3O_4, Fe_3O_4@MAA and MMIPs is 30, 20.4 and 5.7 emu g^{-1}, respectively. These decreases in saturations are expected since the amount of Fe_3O_4 in the particles has reduced consequently. However, the MMIPs have still enough magnetization and can be used as good magnetic adsorbents. Therefore, elimination of silanization steps in the preparation of MMIPs led to achieving good magnetic properties in the final adsorbent and, as a result, effective separation from the solution became possible. Chen et al. [12] reported preparation of MMIPs using hydrophobic Fe_3O_4 magnetite as the magnetically susceptible component and eliminated silica based materials. In this work, oleic acid coated magnetite nano particles were used as core in the synthesis of MMIPs. The obtained saturation magnetization of their final MMIPs was 5.3 (MZ_{MMIPs} = 5.3). Because of the differences in definitive values measured by various VSM devices, the extent of magnetization was determined by MZ $_{MMIPs}$/MZ_{Fe3O4} ratio. The larger the value, the higher the magnetization. This ratio was about 0.11 for the Chen's study, 0.19 for the present MMIPs, and 0.12 for Kong product (Fe_3O_4@SiO_2@IPs) [19]. The thickness of the polymer layer or encapsulation ratio of Fe_3O_4 in polymer backbone has also considerable effect on the extent of saturation magnetization [35]. TGA data (Fig. 4) identifies about 40% of Fe_3O_4 in the prepared MMIPs. However, our synthesized MMIPs have higher magnetic properties. In the inserted photograph of Fig. 6a, brown homogeneous dispersion is presented in the absence of the external magnetic field. On applying the external magnetic field (1.4 T), the brown particles were attracted to the vial wall in less than 10 s.

Table 1 summarize some recent MMIP synthetic procedures concerning the degree of magnetization, the total reaction and extraction time, the total number of synthesis steps, the extent of magnetization, and the final MMIPs average particle size. Table 1 indicates that polymerization occurs slowly in all methods except for the microwave assisted synthesis. No magnetization was reported for this method. Having considered the other listed parameters, the present method is preferable. Additionally, MAA is chemically bonded to the Fe_3O_4 surface. Therefore, the particles' stability is high.

Table 1
Some recently MMIPs synthesis methods.

Analyte	Num. of steps in synthesis	Time of polymerization (h)	Time of extraction (min)	Average Size (nm)	Extent of magnetization	Ref.
Sulfadiazine	4	24	2.66	300	–	[26]
estrogens	4	38	20	515	0.5	[25]
β-sitosterol	3	1.1[a]	40	11×10^4	–	[11]
4-nitrophenol	4	24	150	130	–	[13]
tizanidine	4	24	15	80	–	[17]
sulfonamide	4	2.5	0.5	1.3×10^3	–	[20]
tetracycline	3	24	15–20	1.1×10^3	0.11	[12]
ppz	2	1.5	1	140	0.19	This study

[a] Microwave assisted polymerization.

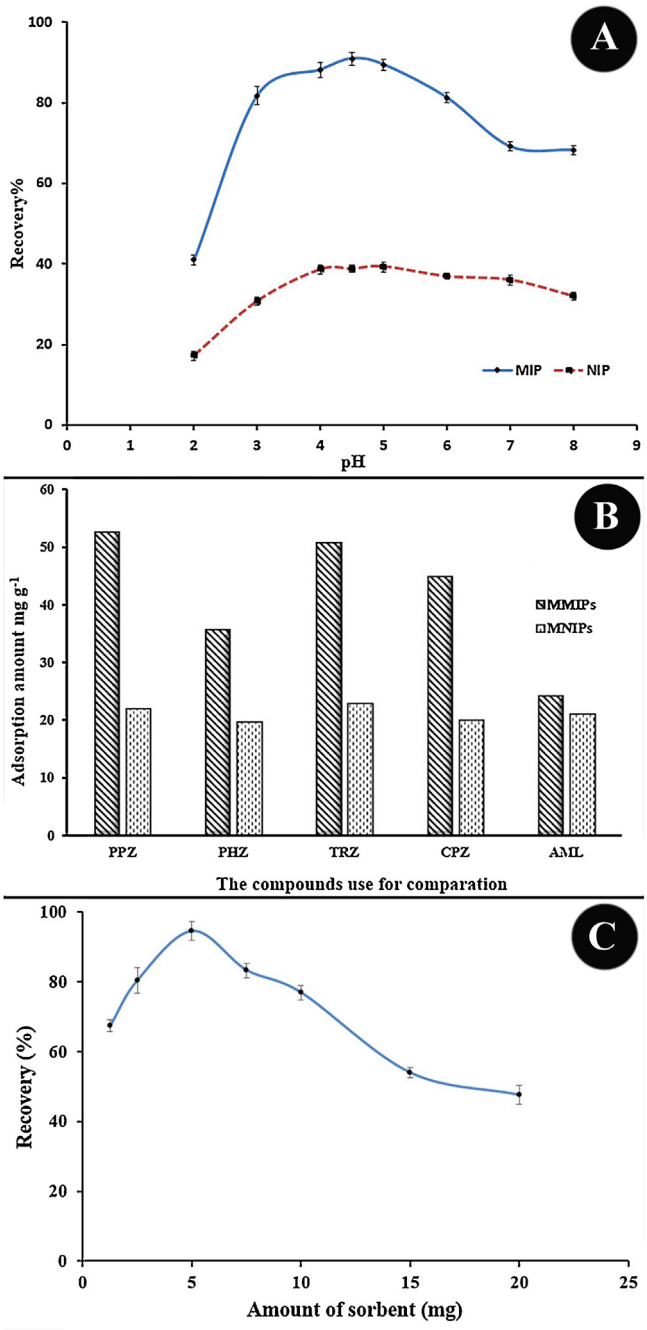

Fig. 7. A) The adsorption amounts of PPZ, TRZ, CPZ, PHZ and AML on magnetic MIPs and NIPs at the concentration of 100 mg L^{-1}. The effect of B) pH on the extraction recovery of PPZ by the MSPE method for both MMIPs and MNIPs. (PPZ concentration was 1 μg mL^{-1} and 10 mg adsorbent) and C) The amount of sorbent on extraction yield of PPZ by the present MMIPs.

3.2. Selectivity experiments

Selectivity of the MMIPs was investigated for other phenothiazines such as TRZ, CPZ, PHZ and AML. They are the structural analogues of the PPZ template (Fig. 6). Experiments were carried out as described under PPZ assay (Section 2.6). The phenothiazine concentrations were 100 mg L^{-1}. The solution was incubated for 1 h at room temperature, and then the supernatant was separated and analyzed by HPLC.

Fig. 7A shows the adsorption amounts of PPZ, TRZ, CPZ, PHZ and AML by the magnetic MIPs and NIPs. Obviously, MMIPs adsorption capacity of PPZ and its analogues were higher than those for the magnetic NIPs. It was also indicated that the MMIPs provided high selectivity toward PPZ and its structural analogues. The high selectivity is mainly due to the molecular size recognition of MIPs to template molecule and the hydrogen bonding interactions between the carboxylic group in the MIPs, and functional groups in four phenothiazines at identical positions. AML that is far different in chemical structure among the phenothiazines did not show good adsorption relative to the other tested compounds. The adsorption capacity for PPZ was 52.7 mg g^{-1}. This is in good agreement with the results obtained by TGA (50 mg g^{-1}).

3.3. Optimization of conditions for MSPE

To achieve the best separation conditions for the target drug, various parameters such as pH, an amount of adsorbent, extraction time, desorption time and desorption solvent were studied and optimized for PPZ pre-concentration and clean-up.

3.3.1. Effects of eluent type and volume

ACN, MeOH, water, ACN:HOAc (9:1) and MeOH:HOAc (9:1) were tested as eluent for desorption of PPZ from the MMIPs sorbent. Using 400 μL of these eluents resulted in recoveries of 47.5, 20, 0, 66.7 and 89, respectively (Fig. S1 in the Supplementary file). Therefore, MeOH:HOAc (9:1) solution was selected as the eluent and its volume were optimized. The results showed the highest recovery for the two steps elution with 200 μL of eluent. Moreover, water was selected as washing solvent because it was unable to desorb PPZ and its analogues.

3.3.2. Effects of pH

The extent of PPZ extraction depends on its interaction with carboxylic groups of the MIPs. Therefore, the acidity of the media is an important factor. The pH of the solutions was maintained over the range of 2.0–8.0 by 10 mM phosphate buffer. As shown in Fig. 7B, the maximum extraction recovery for PPZ was obtained at pH 4.5. There is a competition between proton and the drug for carboxyl site in the acidic solutions. Moreover, carboxyl acid groups of polymer might be protonated at lower pH. As a result, extraction recovery was decreased in pH less than 4.5.

3.3.3. Effect of extraction and desorption time

The adsorption and desorption kinetic of MMIPs were also studied at different time intervals. The results showed that the adsorption of PPZ by MMIPs was fast (1 min). Desorption time had no significant influence on the extraction efficiency. High porosity of the sorbent and available adsorption site in the polymer backbone is the main reason for the efficient and short extraction time. This shortens the analysis time. Similarly, the results indicated that 1 min contact time was enough for the extraction of PPZ from MMIP shell.

3.3.4. Effect of adsorbent amount

Adsorbent amount is a significant parameter in MSPE method. Irrational increase and/or decrease in the adsorbent amount may decrease the extraction efficiency. In this study, a 50 mg mL^{-1} aqueous suspension of adsorbent was used for dose response studies. The results shown in Fig. 7C indicate that the recovery of the PPZ has increased following an increase in the MMIPs amount up to 5 mg due to increases in available adsorption sites. Therefore, 100 μL of the adsorbent suspension (equal to 5 mg dried MMIP particles) was suitable for the extraction of PPZ from the aqueous matrixes. Further increase in the amount of the MMIPs decreased the extraction yields of PPZ because of incomplete desorption. Multiple PPZ sample dilutions or increase in the eluent volume can adjust the recovery.

Table 2
Method validity for PPZ determination in urine and plasma by the present method.

Data	External calibration	In urine calibration	In plasma calibration
Liner range ($\mu g\,L^{-1}$)	20–5000	20–5000	20–5000
Regression equation	y = 1E + 06x + 172534	y = 1E + 06x + 29320	y = 1E + 06x + 166447
Correlation coefficient	0.9998	0.9994	0.9993
Inter-day precision (%)	6.07	5.68	4.25
Intra-day precision (%)	5.93	–	–
LOD ($\mu g\,L^{-1}$)	5.3	5.3	5.4
LOQ ($\mu g\,L^{-1}$)	17.9	17.8	18.0

3.4. Reusability of the MMIPs

The main advantage of a certain sorbent in MSPE is its reusability especially when it is intended to be used in clean up and preconcentration of a target analyte in complex matrixes such as urine and plasma. Reusability of MMIPs was investigated by performing ten successive adsorption and desorption cycles of the aqueous solution of 1 mg L^{-1} PPZ. To do this, MMIP particles were subjected to several loading and elution cycles, under the same experimental conditions (i.e., amount of MMIPs, 5 mg; sample volume, 5 mL; pH, 4.5). After ten cycles, the minimum PPZ recovery was 95.70%. The details are shown in Fig. S2 (supplementary file). Super paramagnetic properties of MMIPs prevent smaller particles from getting lost during filtration and separation. So super paramagnetic properties and high chemical stability of the particles predicts high reusability. The prepared MMIPs were stored for 15 months in our laboratory. No significant decrease in the sorbent affinity was observed over this period of time. For more details, see Fig. S3 in the supplementary file. Therefore, the particles have high shelf life and can be used in clinical laboratory without considerable changes in their performance.

3.5. Method validation and real sample analysis

In order to check applicability of the prepared MMIP in perphenazine determination, the linearity, detection limit, and limits of quantification were investigated. The results in Fig. S4 (in the supplementary file) indicated good linearity in the range of 20–5000 ng mL^{-1} (six selected concentrations and three replicates) with correlation coefficient (R^2) of 0.999. LOD value was defined as the sample concentration in which S/N = 3 while LOQ was that of S/N = 10. The obtained LOD and LOQ (n = 21) were 5.3 and 17.9 ng mL^{-1} for the target molecule, respectively. Six replicate analyses of 1 $\mu g\,mL^{-1}$ PPZ at the optimized conditions resulted in a mean recovery of 97.92% with a mean CV of 6.07%. Calibration curves were drawn in both urine and plasma. Linear range and slope of the calibration curves are the same as it can be seen in Table 2. This confirms the high clean up efficiency of the present MMIP. Interday and intraday precision (n = 6) are also in the acceptable ranges. Tu et al. introduced a dispersive liquid-liquid micro extraction technique (DLLME) [36] prior to capillary electrophoretic determination of PPZ. In their report, dispersion and extraction solvents were chloroform and THF, respectively. The solvent was changed to 50% MeOH before injection. In case of plasma, pretreatments were required before PPZ extraction. As they also implied in their manuscript, chloroforms are among the hazardous solvents and more caution is required while using it. In immunoassay methods of PPZ analysis, the hapten and immunogen preparation takes days and the product should be at −20 °C before use [37]. The practical application of these methods is not simple and safe although their LOD is low. The present method is fast, economic, green and the determination range is acceptable for pharmacokinetic study. In addition, lower LOD can be obtained if the elution solvent evaporates and then the residue re-disperse in lower volume of the solvent. Increasing the original sample volume is another solution to decrease the detection limit.

Table 3
Analytical results for the determination of PPZ in clinical samples by the proposed method, under the optimized conditions (mean ± S.D., n = 3).

Samples	Added ($\mu g\,mL^{-1}$)	Found ($\mu g\,mL^{-1}$)	Recovery (%)
Human urine	–	ND	–
	0.5	0.474 (±0.042)	94.95
	1	1.026 (±0.070)	102.60
	3	3.086 (±0.058)	102.86
Human plasma	–	ND	–
	0.5	0.461 (±0.024)	92.25
	1	1.018 (±0.065)	101.88
	3	3.015 (±0.037)	100.50

The applicability and reliability of the method were also evaluated by spiking urine and plasma samples with PPZ at three levels, namely 0.5, 1 and 3 $\mu g\,mL^{-1}$. The spiked samples were subjected to PPZ separation by the MMIPs adsorbent after equilibration for about 1 min. The results are summarized in Table 3. It is to be mentioned that no sample pretreatments and/or dilution for both plasma and urine are required. As the results indicate, the recoveries for the spiked biological samples are in the acceptable range. Therefore, the method can be used successfully for PPZ determinations in clinical samples. A typical HPLC chromatogram for human plasma extracted by the present method before and after spiking 1 $\mu g\,mL^{-1}$ PPZ is presented in Fig. 8.

4. Conclusion

In conclusion, the main advantage of the present MMIPs synthetic methodology is its simplicity, considerable reduction in the number of synthesis steps and time, and economical cost compared to those reported before as well as the preparation of more stable MMIPs. MAA is used for both nanoparticle surface modification and as monomer in the polymerization step. Additionally, magnetization is improved by the presence of a small molecule, MAA, as the source of double bond on the surface of magnetite nanoparticles. This method can also be extended to the synthesis of MMIPs for templates other than PPZ. It was successfully applied to the simultaneous clean-up and pre-concentrations of PPZ in urine and plasma without any dilution and pre-treatments. However, ample sample pretreatment steps in the analysis of a target compound in complex biological matrixes are omitted.

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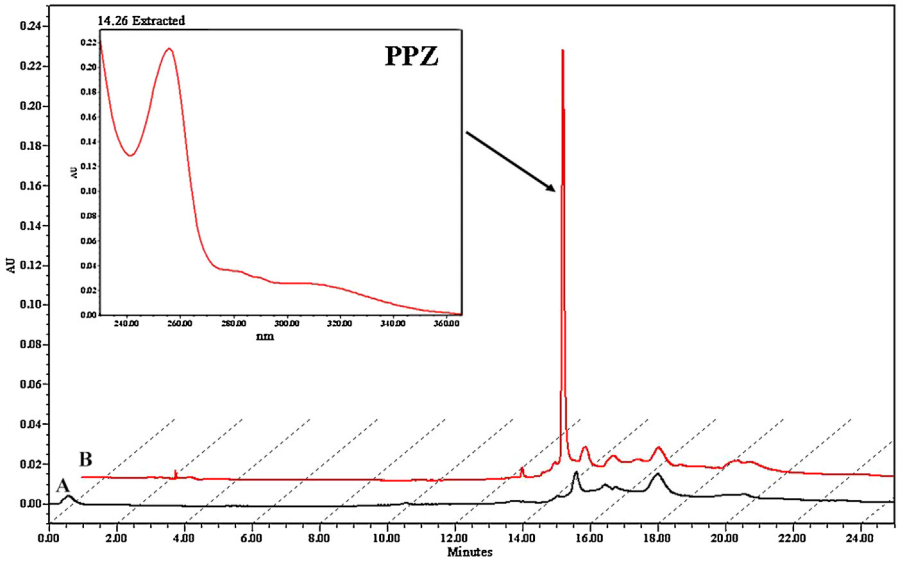

Fig. 8. HPLC chromatograms obtained for PPZ extracted from plasma by the proposed method A) before and B) after spiking with 1 μg mL^{-1} PPZ; experimental condition: amount of MMIPs, 5 mg; sample volume, 5 mL; pH, 4.5, contact time, 1 min.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.chroma.2016.05.083.

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