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Synthesis of core–shell magnetic molecularly imprinted polymers and detection of sildenafil and vardenafil in herbal dietary supplements

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ABSTRACT

An analytical procedure for selective extraction of sildenafil and vardenafil in herbal dietary supplements (HDSs) has been set up by using the magnetic molecularly imprinted polymers (MMIPs) as the extraction and clean-up materials, followed by high performance liquid chromatography-ultraviolet (HPLC-UV). The MMIPs were prepared by a surface molecular imprinting technique, using Fe_3O_4 magnetite as a magnetically susceptible component, sildenafil as template molecule, 2-(trifluoromethyl) acrylic acid (TFMAA) as functional monomer, ethylene glycol dimethacrylate (EGDMA) as polymeric matrix components. The MMIPs were characterized by transmission electron microscope (TEM), Fourier transform infrared spectrometer (FT-IR), X-ray diffraction (XRD) and vibrating sample magnetometer (VSM), respectively. The heterogeneity of the MMIPs was modeled with the Freundlich isotherm equation. The resulting MMIPs had high recognition ability and fast binding kinetics for sildenafil. The MMIPs were used as dispersive solid-phase extraction (DSPE) materials to selectively extract sildenafil and vardenafil from HDSs, the contents of sildenafil and vardenafil were found to be 8.05 and 3.86 $\mu g\,g^{-1}$, respectively, and the average recoveries in spiked HDSs were 70.91–91.75% with a relative standard deviation (R.S.D.) below 7%. The MMIPs were successfully used to selectively enrich and determine sildenafil and vardenafil from HDSs.

1. Introduction

Since 1998, three inhibitors of phosphodiesterase type-5 (PDE-5) for the treatment of erectile dysfunction (ED) have been approved and marketed: sildenafil (Viagra®), vardenafil (Levitra®) and tadalafil (Cialis®) [1,2].

ED occurs commonly (up to 60–70%) in patients accompanying with hypertension and ischemic heart diseases. Unfortunately, PDE-5 inhibitors show negative interactions with some of the drugs used in the treatment of these diseases (e.g., nitroglycerine, doxazosin and terazosin) [3,4]. Lots of evidences are coming into literature about adulteration of HDSs with synthetic drugs or their congeners, in order to enhance the claims stated on the label. Prolonged or excessive consumption of these supplements containing undeclared amounts of drugs may cause serious adverse health consequences. Many of HDSs on the market have been found to contain synthetic PDE-5 inhibitors as adulterants. There have been reports that not only the approved drugs, but even unapproved analogues of PDE-5 inhibitors have been found in HDSs [5–9]. The illegal products may endanger people's health.

There are a few reports that introduce the strategy for the determination of sildenafil in herbal aphrodisiacs. Various synthetic PDE-5 inhibitors combined with the complex herbal matrix make the detection difficult, even with the most sophisticated instrumentation. Several reported methods for the determination of sildenafil including widely used HPLC technology [10–15], capillary electrophoresis [16], LC-MS [17], together with sample pretreatment methods such as solid-phase extraction (SPE), liquid–liquid extraction (LLE), etc. Among these, SPE can be applied readily to a wide range of compounds because of its broad range of sorbents available on the market. However, one substantial drawback of conventional SPE sorbents is of their low specificity towards a particular target analyte. Therefore, there is an ample scope for further improving SPE sample preparation techniques so as to enhance its selectivity. Luo et al. had compared the prepared molecularly imprinted polymers (MIPs) as SPE sorbents with the commercial SPE column, the chromatogram using prepared MIPs depicts better baselines and selectivity than that obtained after the commercial SPE column [18]. At the same time, SPE as a sample pretreatment method requires a large amount of organic solvents and time-consuming

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operations. DSPE as a new extraction method based on the SPE methodology has became increasingly popular for sample pretreatment. The sorbent is directly added into the extracts and the clean-up is easily carried out by being just shaken and centrifugation [19,20].

The molecular imprinting technique is an attractive method to build selective recognition sites in a stable polymer matrix. But tedious centrifugal process is inevitable. The molecularly imprinted polymers are easily lost in this process. In order to overcome these drawbacks effectively, the magnetic molecular imprinting technique has been developed.

Magnetic separation technology has received considerable attention in recent years thanks to their potential application in cell isolation [21], enzyme immobilization [22], protein separation [23] and pre-concentration of targets from crude samples in a rapid way. The unique and attractive property of magnetic carrier materials is that they can readily be isolated from sample solutions by the application of an external magnetic field. The magnetic materials also can be reused or recycled because they do not agglomerate after the removal of external magnetic field. Selective recognition and removal of targets from the complex matrix using MMIPs have been recently demonstrated [24,25]. But the strategy for the simultaneous determination of sildenafil and vardenafil using MMIPs as the sample pretreatment materials has been seldom reported up-to-date.

In this study, a kind of core–shell MMIPs was synthesized by using Fe_3O_4 magnetite as the magnetically susceptible component, sildenafil as template molecule. A simple, specific and sensitive pretreatment method has been developed for simultaneously determining sildenafil and vardenafil in HDSs using MMIPs as magnetic DSPE materials coupled with HPLC. Exhilarating results were obtained.

2. Experimental

2.1. Materials

Sildenafil, vardenafil and tadalafil were purchased from Zhengzhou Lion Biological Technology Co., Ltd., γ-methacryloxypropyl trimethoxysilane (KH570) were obtained from Diamond Advanced Material of Chemical Inc., 2,2′-Azobisisobutyronitrile (AIBN) was purchased from Shanghai No.4 Reagent & H.v Chemical Co., Ltd., TFMAA was acquired from Shanghai Enfujia Technology Co., Ltd., EGDMA was bought from Sigma-Aldrich Inc, tetraethyl orthosilicate (TEOS) was acquired from Sinopharm Chemical Reagent Co., Ltd., all other chemicals used were of analytical grade and were commercially obtained.

2.2. Instrumentation

HPLC was performed with a Shimadzu (Japan) system comprising LC-10ATVP pump, SPD-10AVP UV-detector and HW-2000 chromatographic work station. The identification of target compounds was performed using an Agilent 1200 liquid chromatograph which was coupled to an Agilent 6410B Triple Quad mass spectrometer. The MMIPs were characterized by TEM (JEM1010, JEOL Ltd., Japan), VSM (M27407, Lake Shore Ltd.), XRD (D/max22500, Rigaku Ltd., Japan) and FT-IR (BRUKER Ltd., Germany).

2.3. Samples

HDSs (advertising dietary supplements for kidney-reinforcing, the fundamental composition as described by the manufacturer: epimedium, dodder, cherokee rose, fructus ligustri lucidi, rhizoma cibotii) were purchased from the market.

2.4. Preparation of Fe_3O_4@SiO_2

Iron oxide nanoparticles were first synthesized by the solvothermal reduction method [26–29], and then 300 mg superparamagnetic magnetite nanoparticles were dispersed in 50 mL of 2-propyl alcohol by sonication for 15 min. To this solution, 2 mL of ammonium hydroxide and 2 mL of ultra-pure water were added sequentially, and then 5 mL of TEOS was added at a rate of 20 μL min^{-1}. The mixture was stirred for 12 h at room temperature. The resultant product was collected by an external magnetic field, and rinsed with ultra-pure water to neutral, and dried to powder in vacuum.

2.5. Surface modification of Fe_3O_4@SiO_2

0.1 g Fe_3O_4@SiO_2 was dispersed in 50 mL of toluene solution, 2 mL of KH570 was added under the protection of nitrogen. The solution was stirred for 24 h at 120 °C. The product was collected by an external magnetic field, and dried in vacuum.

2.6. Preparation of MMIPs

0.26 mmol sildenafil, 1.04 mmol TFMAA and 40 mg modified Fe_3O_4@SiO_2 were dispersed into 50 mL of toluene, the mixture was stirred for 1 h at room temperature. 2.08 mmol EGDMA as a polymeric matrix and 20 mg AIBN as an initiator were all dissolved in the above solution. Under the protection of nitrogen, the mixture was stirred for 6 h at 50 °C, 24 h at 60 °C and 8 h at 85 °C. After the polymerization process, the obtained polymers were separated by an external magnetic field and eluted by a mixture of methanol/acetic acid (9:1, v/v) to remove the templates. The MMIPs were finally rinsed with ethanol for one time to remove the remaining acetic acid and then dried in vacuum. As a control, the magnetic non-imprinted polymers (MNIPs) were prepared identically except for the addition of sildenafil. Fig. 1 showed the possible preparation process of molecular imprinting at the surface of 500 nm-sized modified Fe_3O_4@SiO_2.

2.7. Characterization of MMIPs

The size and morphology of MMIPs were measured using TEM instrument. FT-IR spectra were recorded in the range of 4000–400 cm^{-1}. The magnetic properties of MMIPs were measured by VSM. The identification of the crystalline phase of MMIPs was performed using an X-ray diffractometer over the 2θ range of 10–80° [30–32].

2.8. Adsorption experiment

20 mg MMIPs or MNIPs were added into a glass tube containing sildenafil standard solution which was prepared in toluene/methanol (5:5, v/v) varied in the concentration of 0.06–3 mmol L^{-1}. After the samples were shaken at 25 °C for 24 h, the solution was separated by an external magnetic field and measured by HPLC at 290 nm. The amount of sildenafil binding to the MMIPs was calculated by subtracting the amount of free sildenafil from the amount of sildenafil initially added.

The selectivity of the MMIPs or MNIPs was investigated using vardenafil and tadalafil as the structurally related compounds in three different concentrations.

2.9. Chromatographic conditions

The HPLC column was a Diamonsil® C_{18} column (5 μm, 150 mm × 4.6 mm), the mobile phase consisted of methanol/water

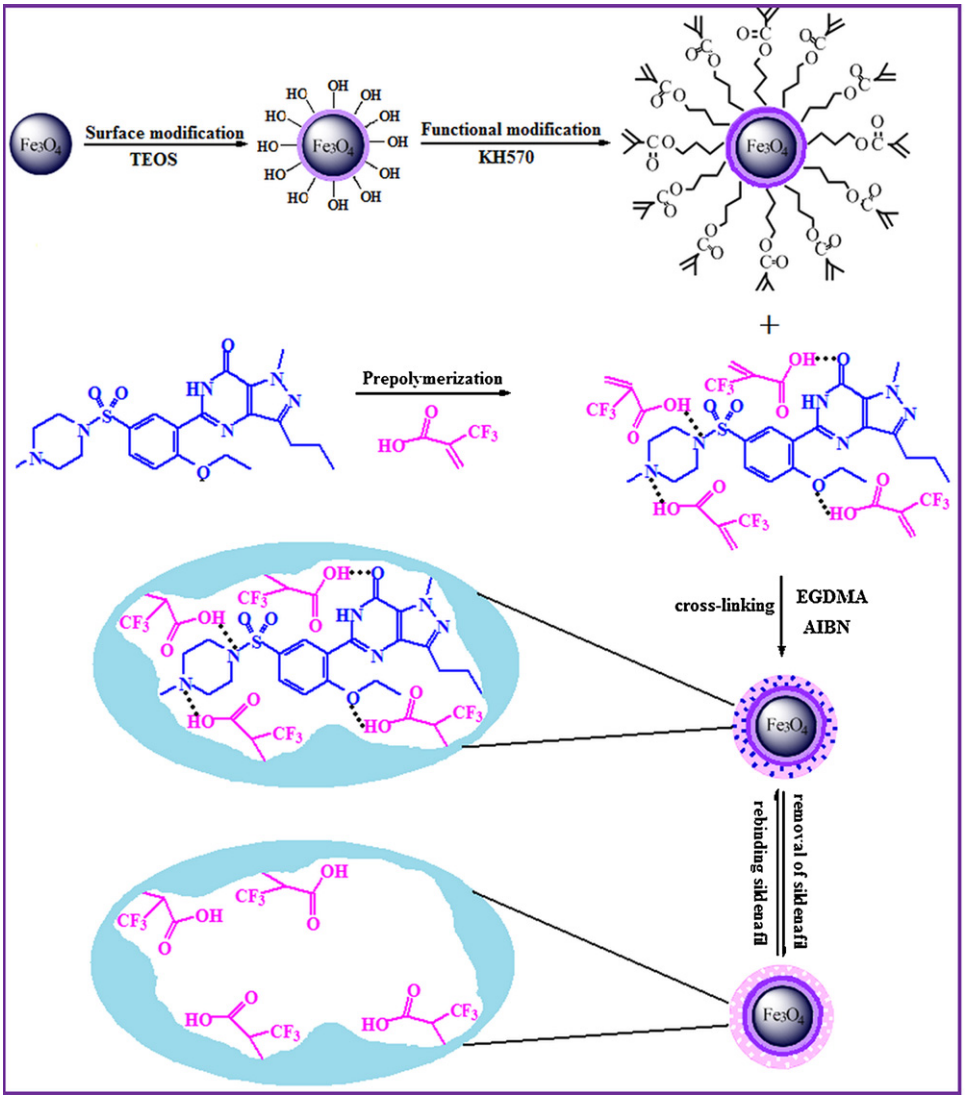

Fig. 1. Schematic representation of the possible process of molecular imprinting at the surface of modified Fe_3O_4@SiO_2.

(68:32, v/v) at a flow rate of 1.0 mL min^{-1}. The injection volume was 20 μL. The detection wavelength was set at 290 nm.

2.10. Extraction procedure and calibration

100 mg of pulverized samples were vortex-mixed with 10 mL of acetonitrile and ultrasounded for 20 min. After that, the solids were separated by filtration, and the filtrating was kept 1 mL of filtration was evaporated to dryness under nitrogen gas at room temperature, 20 mg MMIPs were added to the residues, reconstituted with 1 mL of toluene/methanol (5:5, v/v), shook at room temperature for 15 min, the MMIPs were separated by an external magnetic field, then washed with 1 mL of methanol/acetic acid (9:1, v/v) by sonication for 12 min. 0.8 mL supernatants were obtained and evaporated to dryness under nitrogen gas at 40 °C. Finally, the residues were redissolved in 0.8 mL of the mobile phase for a further HPLC-UV analysis.

The linearity of the analytical method was evaluated by a calibration curve in the range of 0.0970–1.946 and 0.0480–1.916 μmol L^{-1} for sildenafil and vardenafil, respectively. The limits of detection (LOD) were defined as three times ratio of signal to noise. For recoveries, 1 mL extracts were spiked with sildenafil and vardenafil standard solutions at three different levels and tested by the method built (n = 3).

3. Results and discussions

3.1. Characterization of the MMIPs

TEM image (Fig. 2) indicated the MMIPs were of uniform spherical morphology with about 500 nm in size, and the imprinting layer thickness of MMIPs was about 25 nm.

Fig. 3a compared the XRD patterns of Fe_3O_4, Fe_3O_4@SiO_2 and MMIPs which displayed several relatively strong reflection peaks in the 2θ region of 10–80°, the crystalline structure of the Fe_3O_4, Fe_3O_4@SiO_2 and MMIPs was essentially maintained.

FT-IR spectra were obtained for Fe_3O_4@SiO_2, modified Fe_3O_4@SiO_2, MMIPs and MNIPs, respectively. The peaks at 799, 954 and 1096 cm^{-1} attributed to the stretching of Si–O, Si–O–H and Si–O–Si, respectively. The modified Fe_3O_4@SiO_2 displayed the relatively strong band of carbonylic groups at 1723 cm^{-1} and bands of methyl and methylene at 2987, 2922 and 2848 cm^{-1}. The peaks of C–F band in MMIPs and MNIPs were not obvious in Fig. 3b.

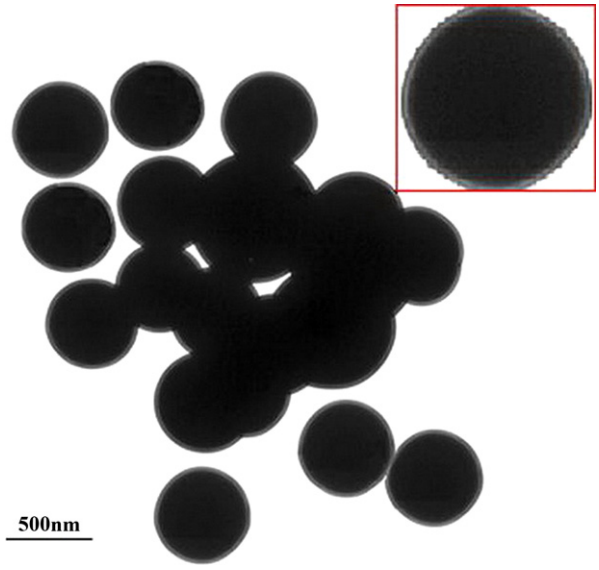

Fig. 2. TEM images of MMIPs.

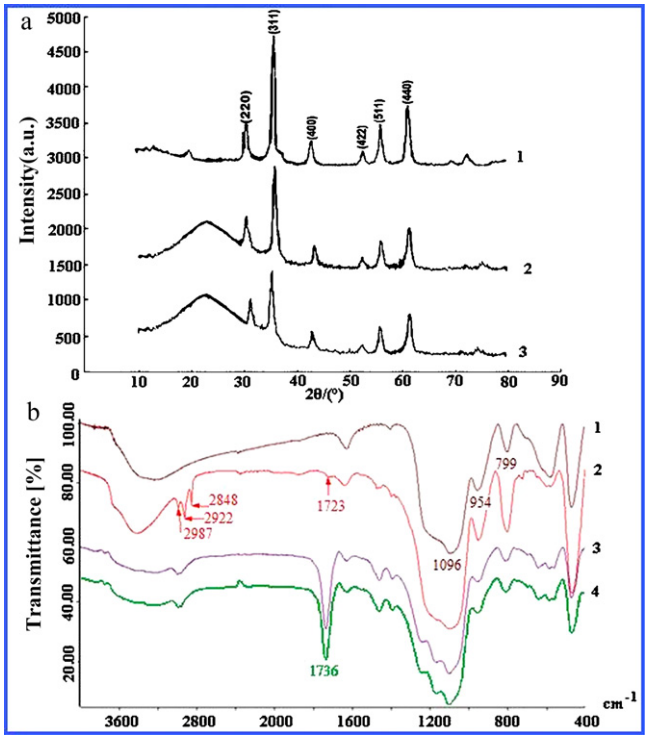

Fig. 3. (a) XRD patterns of Fe_3O_4 (curve 1), Fe_3O_4@SiO_2 (curve 2) and MMIPs (curve 3); (b) FT-IR spectra of Fe_3O_4@SiO_2 (curve 1), modified Fe_3O_4@SiO_2 (curve 2), MMIPs (curve 3) and MNIPs (curve 4).

But a strong adsorption at 1736 cm^{-1} indicated the existence of the C=O group in MMIPs and MNIPs.

VSM was employed to study the magnetic properties of MMIPs. Fig. 4 showed the magnetic hysteresis loops of MMIPs. This feature illustrated that the materials responded magnetically to an external magnetic field and this response vanished upon the removal of the magnetic field. The saturation magnetization of Fe_3O_4, Fe_3O_4@SiO_2 and MMIPs was 85.43, 12.34 and 0.69 emu g^{-1}, respectively. The MMIPs kept enough magnetic response to meet the need of magnetic separation. In the inserted photograph of Fig. 4, without the external magnetic field, a brown homogeneous dispersion existed;

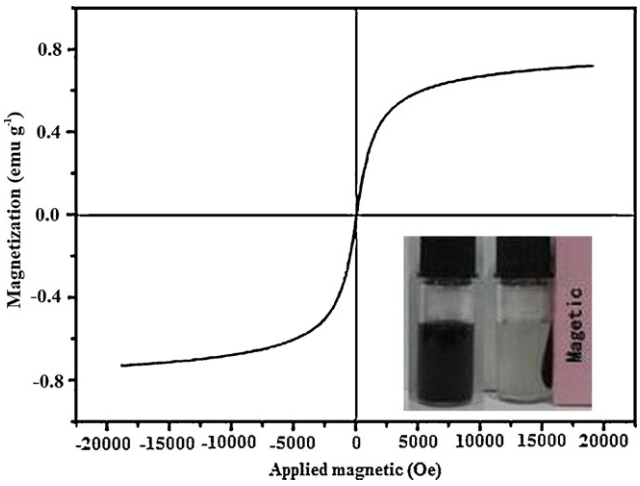

Fig. 4. The hysteresis loop of MMIPs, the inserted photograph shows the separation and redispersion process of a solution of MMIPs in the presence (right) and absence (left) of an external magnetic field.

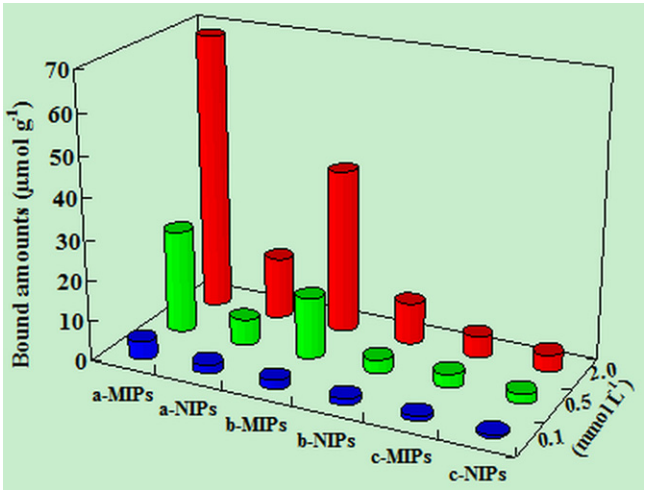

Fig. 5. The selective recognition property of each compound with MMIPs and MNIPs at the concentration of 0.1, 0.5 and 2 mmol L^{-1} (a: sildenafil, b: vardenafil and c: tadalafil).

with the external magnetic field, the brown particles were attracted to the wall of vial.

3.2. Selectivity of MMIPs

Fig. 5 showed the adsorption capabilities of MMIPs and MNIPs for three PDE-5 inhibitors, the tests were investigated using the standard solution at 0.1, 0.5 and 2 mmol L^{-1} levels. It was obvious that the binding capacity of the MMIPs to sildenafil was 1.7 and 12 times that of vardenafil and tadalafil. The interaction between MMIPs and template could be used for selective adsorption of sildenafil and its analogues. The degree of molecular analogy to the template was relative to the extraction yields of MMIPs, and it referred not only the hydrogen bonding between functional monomer and target but also the similarity between the target and templates in size and shape.

3.3. Adsorption isotherms

The experimental binding data for this study were modeled with the Freundlich isotherm (FI) equation (see Eq. (1)) in the concentration range of 0.03–1.4 g L^{-1} (Fig. 6a). Where Q (mg g^{-1}) was the

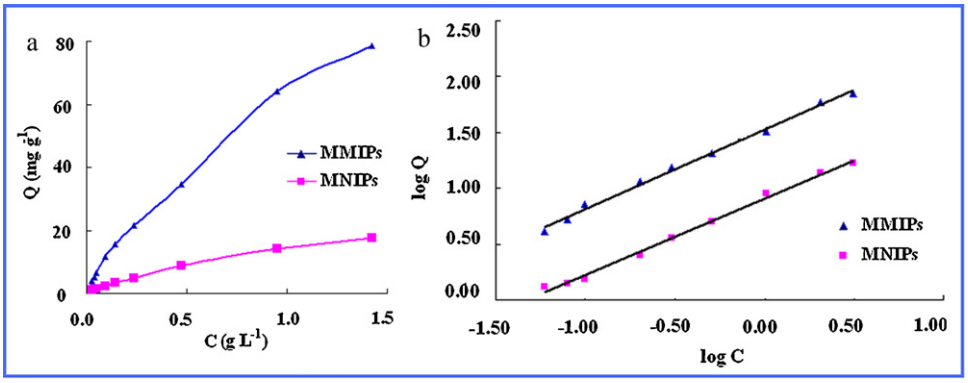

Fig. 6. (a) Adsorption isotherm of sildenafil on MMIPs and MNIPs and (b) fitting to the Freundlich isotherm.

amount of adsorbed analyte per unit of polymer mass, and C (g L^{-1}) was the concentration of the analyte in solution. The constant α was a measure of the capacity and the average affinity. The parameter m was the heterogeneity index, with values from 0 to 1, indicating homogeneity of the sites, m became closer to 1 as heterogeneity decreases, being $m = 1$ for a homogeneous system [33]. The Freundlich constants α and m could be calculated by plotting log Q versus log C by a linear regression (Fig. 6b). The broad applicability of the FI to noncovalent MIPs has been demonstrated recently [34–36].

Two additional binding parameters could be calculated: the number of binding sites per gram of material ($N_{K_{min}-K_{max}}$; see Eq. (2)) and the apparent average association constant ($\bar{K}_{K_{min}-K_{max}}$; see Eq. (3)).

$$\log Q = \log \alpha + m \log C \quad (1)$$

$$N_{K_{min}-K_{max}} = \alpha(1 - m^2)(K_{min}^{-m} - K_{max}^{-m}) \quad (2)$$

$$\bar{K}_{K_{min}-K_{max}} = \left(\frac{m}{m-1}\right)\left(\frac{K_{min}^{1-m} - K_{max}^{1-m}}{K_{min}^{-m} - K_{max}^{-m}}\right) \quad (3)$$

The values for these parameters could be calculated for any range of binding affinities within the limits of the K_{min} and K_{max} equal to the reciprocal corresponding concentrations $K_{min} = 1/C_{max}$ and $K_{max} = 1/C_{min}$. The calculated results are listed in Table 1.

The data also showed that $N_{K_{min}-K_{max}}$ in MMIPs was about 4 times higher than in MNIPs. This means that the number of sites with adequate geometry and good accessibility to sildenafil were higher in MMIPs than in MNIPs, demonstrating the imprinting phenomenon.

3.4. Adsorption and desorption kinetics

The adsorption kinetics was carried out in three different volumes (1 mL, 2 mL and 5 mL; 100 ng mL^{-1} sildenafil equivalent to the concentration in real samples). The MMIPs reached adsorption equilibrium at about 15 min in 1 mL or 2 mL, while at 35 min in 5 mL (Fig. 7a). The sample volume had an obvious effect on the adsorption time and the bounding amount of targets. 1 mL was chosen as the adsorption volume in following experiments for its better adsorptive capacity for sildenafil.

The data of desorption kinetics experiments showed that desorption could reach equilibrium in 12 min (Fig. 7b), and the MMIPs could be isolated in a short time (about 30 s) by an external magnetic field.

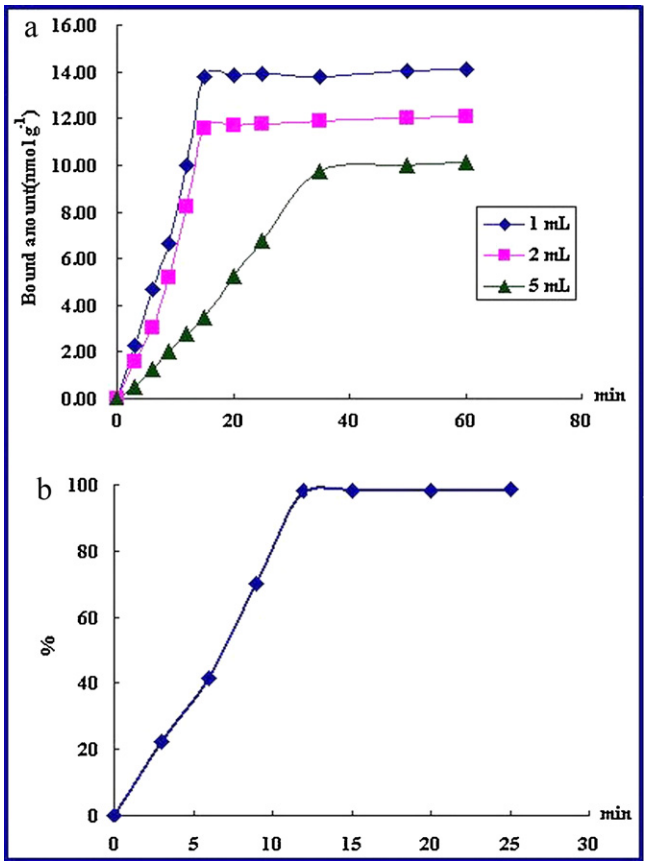

Fig. 7. (a) Dynamic adsorption isotherms of MMIPs for sildenafil in three sample volumes; (b) desorption dynamics of MMIPs.

Luo et al. had compared the prepared MMIPs as DSPE sorbents with commercial SPE column [34]. The results showed that the recoveries of the targets on MMIPs were higher than that of SPE column. Furthermore, MMIPs could be easily separated by the external magnetic field to avoid the complicated process of filling column and pretreatment in traditional SPE.

Table 1
Freundlich fitting parameters, weighted average affinity, and number of sites for MMIPs and MNIPs.

	m	α [(mg g^{-1})(L g^{-1})^m]	R^2	$N_{K_{min}-K_{max}}$ (mg g^{-1})	$\bar{K}_{K_{min}-K_{max}}$ (L g^{-1})
MMIPs	0.76 ± 0.02	36.94 ± 1.4	0.9960	21.19 ± 1.52	2.93 ± 0.97
NMIPs	0.72 ± 0.05	8.25 ± 0.5	0.9893	4.82 ± 0.33	3.92 ± 0.71

Table 2
Determination of sildenafil and vardenafil in HDSs ($n = 3$).

Compound	Content in real sample ($\mu g\,g^{-1}$)	Spiked amount ($\mu g\,g^{-1}$)	Recovery (%)	R.S.D. (%)	Linearity (r)
Sildenafil	8.05	4.27	90.12	5.06	0.9992
		8.54	89.72	6.32	
		12.81	91.75	4.08	
Vardenafil	3.86	1.95	70.91	6.44	0.9996
		3.90	77.61	3.00	
		5.85	78.17	3.78	

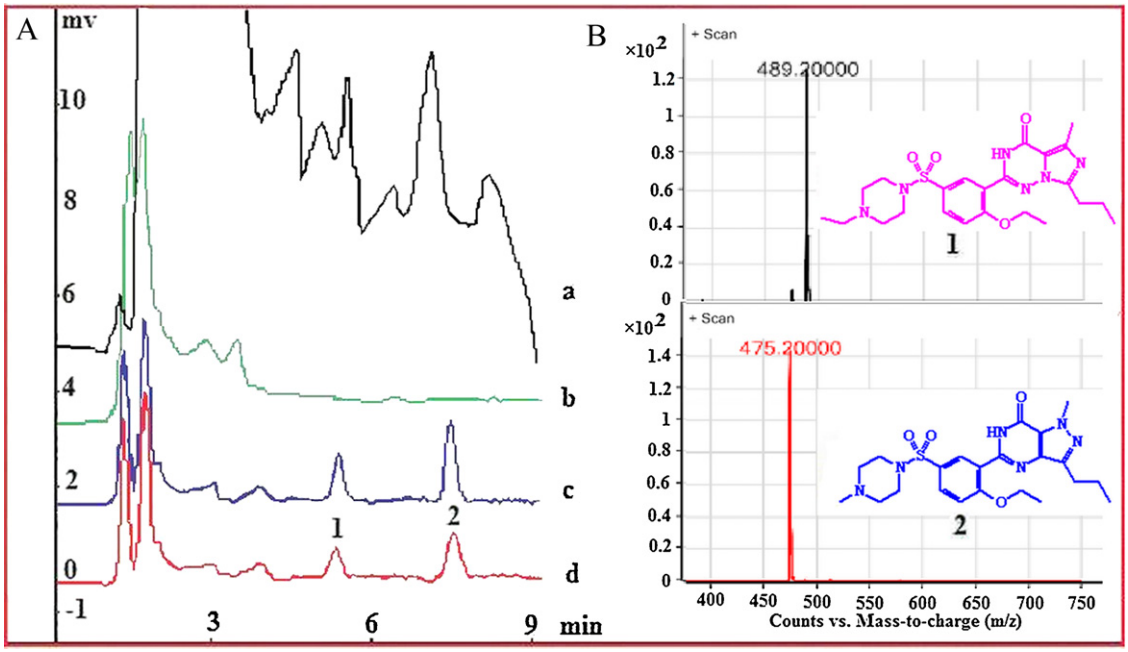

Fig. 8. (A) Chromatograms of sildenafil and vardenafil in HDSs (a) with LLE, (b) with MNIPs, (d) with MMIPs, and (c) spiked samples with MMIPs; (B) Mass spectrum of sildenafil and vardenafil in HDSs by LC–MS (1: vardenafil, 2: sildenafil).

3.5. Method validation

To validate the analytical methodology, the linearity, precision and LOD were investigated for evaluating the proposed method. The regression equation was $A = 165753C + 3921.7$ for sildenafil and $A = 174836C + 1107.1$ for vardenafil, where C was the concentration of the studied compounds, and A was the peak area. The LOD ($S/N = 3$) obtained for the studied compounds were 3.66 and 4.15 $\mu g\,L^{-1}$ for sildenafil and vardenafil, respectively.

The repeatability and accuracy were assessed by analyzing HDSs spiked with sildenafil and vardenafil at three concentration levels. The results were summarized in Table 2. The recoveries for sildenafil and vardenafil were 89.72–91.75% and 70.91–78.17%, respectively, and the RSD% of sildenafil and vardenafil was 4.08–6.32% and 3.00–6.44%, respectively. It indicated that the method was accurate, selective and practical for the determination of sildenafil and vardenafil in complex HDSs.

3.6. Sample analysis

To demonstrate the application of the method, real HDSs were analyzed. Fig. 8A showed the chromatogram of direct injection of LLE, the HDSs sample extracted by MMIP and MNIPs, the spiked HDSs (4.27 $\mu g\,g^{-1}$ sildenafil and 1.95 $\mu g\,g^{-1}$ vardenafil) extracted by MMIP. As shown in Fig. 8A (a), the chromatogram was complex and the target could not be detected by HPLC without enrichment due to the complicated matrix effects. The comparison over MMIPs, MNIPs and LLE clearly demonstrated the advantages of the MMIPs as a selective sorbent for the determination of sildenafil and vardenafil in HDSs. The contents of sildenafil and vardenafil were found to be 8.05 and 3.86 $\mu g\,g^{-1}$, respectively (Table 2). The mass spectrometry as a confirmatory method which worked in a full-scan mode was a useful approach to provide unequivocal identification of target compounds in complex samples. The structural information of sildenafil and vardenafil in HDSs after being extracted with MMIPs was achieved from their full-scan mass spectrograms (Fig. 8B).

4. Conclusions

In this study, MMIPs were synthesized and successfully applied to the separation of sildenafil and vardenafil from HDSs, followed by a HPLC-UV analysis. It took a short time to reach the adsorption and desorption equilibrium, at the same time, the adsorbing analytes of magnetic polymers were easily collected by an external magnetic field without any additional centrifugation or filtration. The technique is simple compared with the classical sample preparation procedures (solvent extraction, centrifugation, and subsequent clean-up and concentration by SPE), with the minimum sample handling and less solvent consumption, and is promising for the monitoring of illegal products in HDSs as well.

Acknowledgments

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