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Evaluation of water-compatible molecularly imprinted polymers as solid-phase extraction sorbents for the selective extraction of sildenafil and its desmethyl metabolite from plasma samples

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Abstract

The evaluation of molecularly imprinted polymers (MIPs) as selective sorbents for the solid-phase extraction of sildenafil and its principal metabolite, desmethylsildenafil, was investigated. Two MIPs were synthesised using structural analogues of sildenafil as templates, and a comparison of the performance of the two MIP sorbents in organic and aqueous media was performed. Additionally, the feasibility of applying molecularly imprinted solid-phase extraction (MISPE) to the clean-up of plasma samples containing sildenafil and desmethylsildenafil was investigated. A preliminary, quantitative MISPE for the determination of both compounds in plasma was also performed. The results showed that the MIPs used for the selective extraction of sildenafil gave better compound recovery when aqueous samples were used in comparison to organic-based samples. A preliminary, quantitative MISPE of sildenafil and desmethylsildenafil indicated that the imprinted materials could be used successfully as SPE sorbents for sample pre-treatment for the determination of sildenafil, and related compounds, in plasma.

Keywords: Molecularly imprinted polymers; Sildenafil; Solid-phase extraction

1. Introduction

Molecularly imprinted polymers (MIPs) are synthetic, polymeric materials designed to express high affinity and selectivity towards a single compound (template) or a group of compounds related structurally to the template. This highly desirable feature of imprinted materials arises as a result of the existence, within the crosslinked polymer network, of binding sites capable of molecular recognition of the template. The binding sites are created during the polymer synthesis, which is performed in the presence of the template molecule itself. Post-synthesis, the template is removed from the polymer network, yielding a material with attractive molecular recognition properties [1]. The high molecular specificity of MIPs has been evaluated extensively and MIPs have been applied in a wide range of applications, including antibody binding mimics, catalysts, sensors, selective beds in chromatography and electrochromatography, as well as SPE sorbents [2–14].

The main goal in sample preparation is to obtain clean extracts that are compatible with the analytical method used for the analyte determination. Drugs are becoming more potent and there is a need to increase the sensitivity of analytical methods in order to monitor low drug concentrations in biological matrices. Biological samples contain impurities and endogenous components that can interfere with the quantification of analytes. Nowadays, the most popular sample clean-up methods for biological samples are protein precipitation, liquid–liquid extraction and solid-phase extraction (SPE), with SPE being used routinely in pharmaceutical laboratories. SPE is usually the method of choice

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due to its simplicity and potential to deliver cleaner extracts. Additionally, SPE can be applied readily to a wide range of compounds, due to the broad range of sorbents available on the market, and it can be automated in a facile manner. However, one substantial drawback of conventional SPE sorbents is their low specificity towards a particular target analyte. Therefore, there is considerable scope for further improving SPE sample preparation techniques, leading to an enhancement of its selectivity.

In this paper, we report upon the application of molecularly imprinted polymers as solid-phase extraction sorbents. In recent years, the development of molecularly imprinted solid-phase extraction (MISPE) has been reported extensively in the areas of environmental and pharmaceutical analysis [15,20–23], including the use of MIPs as sample preparation sorbents for the extraction of different classes of compound from various biological matrices, including plasma and urine [1,24–28]. However, there are two general concerns that arise over the practical application of MIPs for particular sample preparations. The first concern relates to the poor level of recognition of the target compound (template) in aqueous environments [29]. As a result, applications of MIPs for aqueous samples (most common in biological analysis) are limited. The second concern that can arise when MIPs are applied as sorbents in SPE is the possibility of leaking (bleeding) of residual template molecule that remains trapped in the polymer after it has been synthesised and washed extensively. If the MIP is not completely template free, then its use might compromise quantitation if trace level analysis is required [30].

The specific aim of the present work was to evaluate the molecular recognition properties of two MIPs in their application as SPE sorbents for the selective extraction of sildenafil, the citrate salt of which is an active component of Viagra®, a drug used widely in the treatment of male erectile dysfunction [31,32]. The polymers were designed from the outset to circumvent the problems of analyte bleeding and non-selective binding that can arise when low analyte concentrations and aqueous samples are involved. In addition, the extraction of plasma samples containing sildenafil and its main metabolite was investigated using the two polymers. Finally, the potential application of the materials for quantitative analysis is discussed.

2. Experimental

2.1. Materials

Sildenafil, its principal metabolite (desmethylsildenafil), Analogues **1** and **2** (Fig. 1) were synthesised at Pfizer Global R&D, Sandwich, UK. Analogues **1** and **2** were used as templates in the production of MIP1 and MIP2, respectively.

Methacrylic acid (MAA), 2-hydroxyethyl methacrylate (HEMA), ethylene glycol dimethacrylate (EGDMA), 2,2′-azobisisobutyronitrile (AIBN) and 1,1,1-trichloroethane (TCE) were purchased from Sigma–Aldrich, Germany. Methanol, NaCl, $MgSO_4$, acetone and alumina were also obtained from Sigma–Aldrich, Germany. Trifluoroacetic acid and acetic acid (Fisher Scientific, UK), and acetonitrile (Sigma–Aldrich, Germany) were used for washing, conditioning and elution of the MIP cartridges. Ammonium acetate, used for preparation of the HPLC buffer, was purchased from Fisher Scientific, UK. All the solvents used were of analytical grade. High purity Milli-Q-plus purified water was used for solutions and HPLC buffer preparations.

2.2. Polymer synthesis

MAA and HEMA were purified by passing them through a short column of neutral alumina, followed by distillation under reduced pressure. EGDMA was washed three times with a saturated aqueous solution of NaCl, dried over $MgSO_4$ and then distilled under reduced pressure. AIBN was recrystallised from methanol.

The template molecule (Analogues **1** or **2**) (Analogue **1**: 0.230 g, 0.46 mmol; Analogue **2**: 0.211 g, 0.46 mmol), MAA

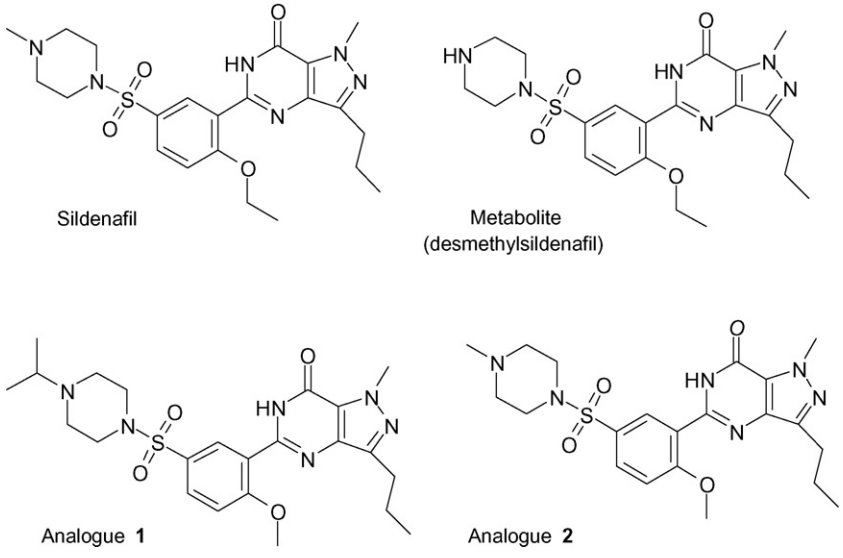

Fig. 1. Chemical structures of sildenafil, desmethylsildenafil, Analogues **1** and **2**.

(0.473 g, 5.50 mmol), HEMA (0.239 g, 1.83 mmol), EGDMA (1.038 g, 5.23 mmol) and AIBN (0.025 g, 0.18 mmol) were dissolved in dry porogen (TCE) (2.33 mL) in a thick-walled, glass polymerisation tube (Kimax). Each individual solution was then purged with nitrogen gas for 10 min at 0 °C to remove dissolved molecular oxygen, and the reaction vessels sealed under nitrogen. The polymerisations were carried out under UV initiation at 0 °C for 24 h using a Blak-Ray Ionwave UV lamp (model B-100A) followed by a thermal cure at 60 °C for a further 24 h. A non-imprinted polymer (NIP) was prepared under identical conditions to MIP1 and MIP2, albeit in the absence of template. The hard, glassy polymer monoliths obtained were ground mechanically using a ball mill and the particles separated by size through wet sieving in acetone using 25 and 38 μm sieves. The yields of polymer particulates, with the size range desired, obtained after grinding, sieving, repeated sedimentation in acetone to remove the fines and drying *in vacuo* were 11, 16 and 13% for MIP1, MIP2 and NIP, respectively.

2.3. Preparation of solutions and plasma samples

Stock solutions of sildenafil, desmethylsildenafil, Analogues **1** and **2** were prepared by weighing appropriate amounts of each compound and dissolving them in methanol in 10 mL volumetric flasks. Working solutions were prepared by transferring appropriate volumes of stock solution to 10 mL volumetric flasks and diluting with acetonitrile to give final concentrations of 10 μg/mL sildenafil and metabolite (WS1), 10 μg/mL Analogues **1** and **2** (WS-IS), and 1 μg/mL sildenafil and metabolite (WS2). WS1 was diluted further to 500 ng/mL solutions of sildenafil and metabolite in acetonitrile and water.

Plasma samples spiked with sildenafil, desmethylsildenafil, and Analogues **1** and **2** at 500 ng/mL were prepared by adding an appropriate volume of WS1 to 0.5 mL of plasma. Plasma samples used for preliminary, quantitative validation tests (calibration lines and quality control samples) were prepared by adding appropriate volumes of solution WS1 or WS2 to 0.5 mL plasma to reach the required concentration level. WS-IS was added to the spiked samples to obtain an I.S. concentration of 250 ng/mL. The samples were mixed and protein precipitation was carried out by adding 1.5 mL CH_3CN to the sample, mixing and centrifuging (10 min, 3000 rpm, 22 °C). 1.5 mL of supernatant was collected and evaporated under nitrogen at 40 °C. The sample was then reconstituted with 0.5 mL acetonitrile and loaded onto the MIP sorbent. The reconstitution solvent consisted of 0.5 mL water/acetonitrile 3/1 (v/v).

2.4. MISPE

SPE cartridges were packed manually by weighing 30 mg of MIP1, MIP2 or NIP and placing the sorbent into 1 mL polypropylene cartridges (IST, UK). Polyethylene frits (10 μm) were used (top and bottom) to prevent loss of the bed particles. Cartridges were pre-conditioned with 10 mL of 5% trifluoroacetic acid in CH_3CN and 10 mL of 5% acetic acid in CH_3CN in order to remove potential contaminants, including any template still present in the imprinted material.

The imprinted polymers, MIP1 and MIP2, and the NIP, were evaluated by off-line solid-phase extraction. The extraction was performed using 500 ng/mL sildenafil (or metabolite) solubilised in CH_3CN or H_2O. The SPE extraction used to investigate the imprinting effect of the polymer involved: conditioning with 1 mL 5% acetic acid in CH_3CN and 1 mL CH_3CN; loading with 0.5 mL 500 ng/mL of sildenafil in water or acetonitrile; washing/eluting with 1 mL CH_3CN, followed by 1 mL of 0.1, 0.25, 0.5, 0.75, 1, 1.25, 1.5, 1.75, 2, 3, 4% acetic acid in CH_3CN, and 3 mL × 1 mL 5% acetic acid in CH_3CN. The optimal extraction conditions were implemented in the final SPE procedure. In this regard, while the conditioning, loading and washing steps remained the same, the elution was carried out using 3 mL × 1 mL of 5% acetic acid in CH_3CN. Using this procedure, sildenafil and its metabolite were extracted from CH_3CN and water. The same MISPE protocol was applied to the extraction of sildenafil from plasma samples after protein precipitation, followed by evaporation and reconstitution of the residue with 0.5 mL of CH_3CN. Blank plasma samples were extracted in parallel with sildenafil spiked plasma samples. The evaluation of MIP1 and MIP2 was performed using sildenafil in solution. All loading, washing and elution fractions were collected, evaporated and reconstituted with 0.2 mL of 3/1 H_2O/CH_3CN (v/v). In the case of the quality control samples, the elution fractions were combined, evaporated and reconstituted with the same mixture. The quantitation of sildenafil and its metabolite was carried out by HPLC-UV. Every experiment was performed at least three times (R.S.D. less than 5%).

2.5. HPLC-UV analysis

An HP1100 series binary HPLC pump (Agilent, UK) delivered the mobile phase at a flow-rate of 1 mL/min onto a Thermohypersil-Keystone 100 C18, 5 μm packing, 50 mm × 4.6 mm I.D. column. The elution of the analytes occurred in gradient mode using a mobile phase consisting of 30 mM ammonium acetate/CH_3CN (0–1 min, 90/10; 1–12 min, 90/10–55/65; 12–13 min, 55/65–90/10; 13–14.9 min, 90/10 30 mM ammonium acetate/CH_3CN). A Jasco AS-950-10 autosampler (JASCO, Japan) injected 100 μL of reconstituted material. An HP1100 series UV detector (Agilent, UK), set at a wavelength of 230 nm, was used for the detection of the analytes. The retention time of the sildenafil metabolite, Analogue **2**, sildenafil and Analogue **1** were approximately 8.9, 9.6, 10.6 and 12.6 min, respectively.

3. Results and discussion

3.1. MIP synthesis

The design criteria governing the production of the MIPs dictated that it was necessary to obtain imprinted materials able to bind sildenafil selectively from aqueous samples, but also materials able to circumvent the problem of template bleeding. Two scenarios to deal with the uncontrolled release of template from the imprinted materials were envisaged. The first scenario involved the removal of the template by using different extrac-

tion techniques, including Soxhlet extraction, supercritical fluid extraction, microwave-assisted extraction, application of potent washing solvents and the *in situ* chemical transformation of template [30]. The second option was to use structural analogues of the analytes as templates in the synthesis of the molecularly imprinted polymers [27]. Assuming that the analogue can be separated from the analyte during the post-MISPE chromatographic step and that its affinity to the polymer is similar to the compound of interest, the second option can be a viable alternative for the production of imprinted polymers where template bleeding is no longer an issue. In our study, two MIPs imprinted with analogues of sildenafil (Fig. 1) were prepared and examined. The analogues selected for this purpose were structurally similar to sildenafil, giving imprinted materials that retained high affinity towards the compounds of interest. Furthermore, the templates could be baseline-separated by reversed-phase HPLC from sildenafil and its metabolite.

The selection of Analogues **1** and **2** as template molecules was made on the basis of the results obtained in a previous study, in which 17 compounds, all related structurally to sildenafil, were evaluated for their selective affinity and binding to a sildenafil imprinted polymer. In this study, a sildenafil imprinted polymer was applied as a stationary phase in LC to screen the cross-selectivity of compounds present in a drug library. The binding affinity of the compounds was assessed using the normalised retention index (RI). Analogues **1** and **2** were found to bind even more strongly (RI > 1) than sildenafil to the imprinted column [33].

The evaluation of the hydrophilic, HEMA-containing molecularly imprinted polymers MIP1 and MIP2 in the MISPE of sildenafil was carried out in a non-aqueous environment initially and then in an aqueous environment, including the use of the MIPs for the extraction of the drug from plasma samples. Historically, the first generation of MIPs applied in SPE relied on the selectivity being driven by specific, electrostatic interactions between polymer and template, with the selectivity being particularly pronounced in organic solvents [15]. However, when changing the sample from organic to aqueous, in many cases this leads to a decrease in the selective binding of the analyte to the polymer because of a corresponding increase in non-specific, hydrophobic interactions. To obtain a polymer able to bind the analyte selectively in aqueous samples, polar porogens, hydrophilic comonomers and/or crosslinkers are used for the polymer synthesis [29,34]. The optimal chemical composition of MIP1 and MIP2 were obtained in a previous study focussing on the optimisation of water-compatible MIPs, imprinted non-covalently with sildenafil in the miniMIP format using a high-throughput synthesis approach [35]. In this study, the porogens (acetonitrile, 1,1,1-trichloroethane, toluene), the monovinyl monomers (MAA and HEMA) and the crosslinker (EGDMA) were varied systematically to give 45 chemically distinct sildenafil imprinted materials and 45 non-imprinted reference (control) polymers. Sildenafil binding to the MIPs was then evaluated using equilibrium rebinding methods. The seven polymers that exhibited the most promising sildenafil binding properties in the equilibrium rebinding study were synthesised on a larger (multi-gram) scale and evaluated, where feasible practically, in chromatographic (LC) experiments. The imprinted polymers constituted the stationary phases in in-house packed, stainless-steel LC columns. The polymer prepared with a sildenafil/MAA/HEMA/EGDMA mole ratio of 1/12/3/10.7, with 1,1,1-trichloroethane as the porogenic solvent was the most promising imprinted material, both in terms of its affinity towards sildenafil and its suitability for flow-through applications. The inclusion of HEMA as a hydrophilic comonomer makes the material less hydrophobic and therefore less susceptible to non-selective, hydrophobic binding events [29]. The optimised synthesis conditions were then applied to the synthesis of polymers MIP1, MIP2 and NIP.

3.2. Evaluation of MIPs

The testing of the selective binding of sildenafil to the MIPs was carried out off-line using MISPE cartridges that were manually packed in house with the imprinted material. The chromatograms obtained from the fraction collected after the ultimate washing step revealed the presence of traces of the template, however no interfering peaks were present at the retention times corresponding to sildenafil and its metabolite. CH_3CN was used for conditioning and washing, and it was chosen due to its miscibility with water and its versatility in laboratory work. Solutions of acetic acid in CH_3CN were used to desorb the selectively bound analytes from the imprinted polymers [27,28].

The first step in the present work was to evaluate the selective interaction of sildenafil with MIP1 and MIP2. The concentration profile obtained from sildenafil released from the polymers after loading in CH_3CN was compared with the concentration profile obtained from the reference, non-imprinted polymer (NIP).

At physiological pH, sildenafil is a relatively lipophilic ($\log D_{7.4} = 2.7$) compound with two aromatic rings. It bears two ionisable groups, a tertiary amine in the piperazine ring (pK_a 6.78) and an acidic group in the pyrimidinone fragment (pK_a 9.12) [36]. However, at neutral pH, the tertiary amine is only partially ionised and the pyrimidinone fragment remains neutral. As a consequence, the extraction of sildenafil from water and plasma samples was carried out without pH adjustment.

The sildenafil sample was loaded onto cartridges containing MIP1, MIP2 and the NIP, and the cartridges washed with increasing amounts of acetic acid in CH_3CN. Fig. 2 shows that there are significant differences in binding between the MIPs and the NIP. Sildenafil was retained only to a limited extent by the non-imprinted material during the loading step.

The low amount of sildenafil that bound with the loading step to NIP was washed completely off the polymer by the first washing step (CH_3CN alone). Subsequent washings of the non-imprinted cartridge with increasing percentages of acetic acid in the washing solvent did not lead to any further release of sildenafil. In contrast, MIP1 and MIP2 retained a high percentage of sildenafil in the loading step, and it was necessary to have acetic acid present in the washing step to desorb sildenafil from these materials effectively. Hence, MIP1 and MIP2 show good affinity for sildenafil. The imprinting effect was also observed when sildenafil was extracted from water. Fig. 3 shows that sildenafil is bound quantitatively to the MIPs during the loading step

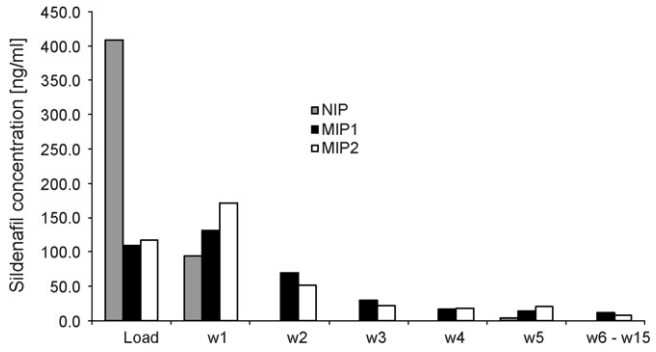

Fig. 2. The concentration elution profile of sildenafil from MISPE fractions collected after loading of a 0.5 mL 500 ng/mL sildenafil sample in CH_3CN (load) on NIP, MIP1 and MIP2 cartridges and washing with increasing amounts of acetic acid in acetonitrile (w1 – 0%, w2 – 0.1%, w3 – 0.25%, w4 – 0.5%, w5 – 0.75%, w6 – 1%, w7 – 1.25%, w8 – 1.5%, w9 – 1.75%, w10 – 2%, w11 – 3%, w12 – 4%, w13–15 – 5% acetic acid in CH_3CN). Wash volumes: w1–15, all 1 mL.

(no breakthrough of sildenafil was observed) and that it can be eluted, subsequently, with solutions of acetic acid in CH_3CN.

The major challenge of the present work was the application of the MISPE technology to the validation of an analytical method for determination of sildenafil in plasma. Hence, further experiments were performed to adapt the extraction procedure for more practical uses. After sample loading, the cartridges were washed with 1 mL of CH_3CN and the elution step was performed using 3 mL × 1 mL of 5% acetic acid in CH_3CN (c.f. w13–15 in Fig. 3). The corresponding data obtained from MISPE using NIP, MIP1 and MIP2 as the sorbents, where sildenafil is loaded either in CH_3CN or water, are presented in Table 1.

A significant difference is apparent when comparing the relative extraction recovery (calculated relative to the initial concentration of sildenafil) for MIP1, MIP2 and NIP. The relative recovery of sildenafil on the NIP is similar for each step of the extraction, irrespective of the solvent applied. The majority of the analyte is released from the reference polymer (NIP) during the loading and washing steps. In contrast, MIP1 and MIP2 both show high affinity towards the target analyte. However, the relative recovery from water is higher than that from CH_3CN for both MIP1 and MIP2. When the sample is loaded from CH_3CN, sildenafil is eluted from the polymers during the washing step to some extent. When the sample is loaded from water, sildenafil remains on the polymers throughout the CH_3CN washing step and most of the drug is released from the polymers only during the elution step. Thus, imprinted materials have been produced that are not only aqueous-compatible (water wettable, *etc.*) but which can bind sildenafil efficiently when sildenafil is present in fully-aqueous samples.

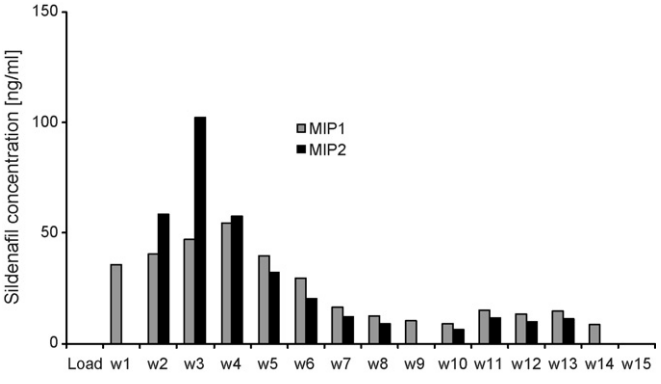

Fig. 3. The concentration elution profile of sildenafil from MISPE fractions collected after loading of a 0.5 mL 500 ng/mL sildenafil sample in water (load) on MIP1 and MIP2 cartridges and washing with increasing amounts of acetic acid in acetonitrile (w1 – 0%, w2 – 0.1%, w3 – 0.25%, w4 – 0.5%, w5 – 0.75%, w6 – 1%, w7 – 1.25%, w8 – 1.5%, w9 – 1.75%, w10 – 2%, w11 – 3%, w12 – 4%, w13–15 – 5% acetic acid in CH_3CN). Wash volumes: w1–15, all 1 mL.

Table 1
Relative recovery (%) of sildenafil from fractions collected after MISPE extraction of 0.5 mL 500 ng/mL sildenafil in acetonitrile and water using NIP, MIP1 and MIP2 (wash – 1 mL CH_3CN; e1–e3 – elution with 3 mL × 1 mL 5% acetic acid in CH_3CN)

	MIP1	MIP2	NIP
CH_3CN			
Load	6.2	9.6	1.0
Wash	24.6	46.2	82.2
e1	49.0	38.2	13.8
e2	8.2	4.4	0.0
e3	0.0	0.0	0.0
Total	57.2	42.6	13.5
Water			
Load	0.0	1.0	0,0
Wash	0.0	7.6	72.9
e1	52.8	60.5	11.0
e2	6.1	8.5	0.7
e3	1.7	2.6	0.0
Total	60.6	71.6	11.7

3.3. Extraction of sildenafil from plasma

The ultimate application for MIP1 and MIP2 is their use as SPE sorbents for the direct extraction and quantification of sildenafil, and its metabolite, in plasma samples. Preliminary experiments involving the direct loading of plasma onto the MISPE cartridges revealed that the direct application of plasma to the polymers was difficult to carry out, as blockage of the cartridges gave rise to high inconsistencies in the extraction results. As a consequence, it was decided to introduce a protein precipitation (PP) step prior to the sample loading on the polymers.

The elution of analyte from the cartridges was performed with 5% acetic acid in CH_3CN. The MISPE extraction was performed using 0.5 mL plasma samples, similarly to the extraction study using pure solvents. Additionally, in order to examine the influence on the extraction efficiency of the presence of residual proteins or endogenous components still present in the sample after the protein precipitation, the sample volume was reduced to 0.1 mL. For comparison purposes, the same extraction was carried out on a 0.1 mL solution of sildenafil in water at the same nominal concentration (Table 2).

The total relative extraction recovery of sildenafil from 0.5 mL plasma samples using both MIPs was much lower (27–33%, Table 2) than in the case of sample loaded from neat

Table 2
Relative recovery (%) of sildenafil from fractions collected after MISPE extraction of 0.5 mL (500 ng/mL) and 0.1 mL (2500 ng/mL) plasma samples and 0.1 mL (2500 ng/mL) water samples using MIP1 and MIP2 (wash – 1 mL CH_3CN; e1–e3 – elution with 3 mL × 1 mL 5% acetic acid in CH_3CN)

	0.5 mL plasma	0.1 mL plasma	0.1 mL water
MIP1			
Load	2.0	2.7	0.0
Wash	8.6	11.3	6.3
e1	25.4	38.3	42.7
e2	1.6	1.8	5.2
e3	0.0	1.5	0.0
Total	27.0	41.6	47.9
MIP2			
Load	0.0	0.0	0.0
Wash	5.6	19.6	12.4
e1	29.6	42.3	51.6
e2	3.2	3.7	7.3
e3	0.0	1.8	2.6
Total	32.8	47.8	61.5

solvent (Table 1). A reduction in the plasma sample volume from 0.5 to 0.1 mL resulted in about a 15% improvement in the total relative recovery for both polymers (MIP1 42%, MIP2 48%). Slightly improved total relative recovery (6% MIP1 and 14% MIP2) was noted for 0.1 mL water samples in comparison to the 0.1 mL sildenafil spiked plasma sample. This suggests that the presence of endogenous plasma components affects the total relative recovery of sildenafil, probably as a result of the drug binding to proteins.

The determination of a drug profile in body fluids is often accompanied by monitoring of its metabolites. In this regard, MIP1 and MIP2 were evaluated for the simultaneous extraction of sildenafil and its main metabolite, desmethylsildenafil [37]. Table 3 shows the relative extraction recovery for desmethylsildenafil from CH_3CN and water. Overall, the relative recovery

Table 3
Relative recovery (%) of desmethylsildenafil from fractions collected after MISPE extraction of 0.5 mL 500 ng/mL metabolite in acetonitrile and water using MIP1 and MIP2 (wash – 1 mL CH_3CN; e1–e3 – elution with 3 mL × 1 mL 5% acetic acid in CH_3CN)

	MIP1	MIP2
CH_3CN		
Load	12.9	16.2
Wash	31.9	49.9
e1	26.0	32.2
e2	5.8	4.7
e3	1.4	4.5
Total	33.2	41.3
Water		
Load	1.0	0.0
Wash	8.2	0.9
e1	46.0	68.3
e2	3.8	4.4
e3	1.7	0.6
Total	51.5	73.4

Table 4
Relative recovery (%) of desmethylsildenafil (M, on MIP1 and MIP2), Analogue **1** (A1, on MIP2) and Analogue **2** (A2, on MIP1) from fractions collected after MISPE extraction of drug samples (0.5 mL 500 ng/mL) in plasma (wash – 1 mL CH_3CN; e1–e3 – elution with 3 mL × 1 mL 5% acetic acid in CH_3CN)

	Desmethylsildenafil	Analogue **2**
MIP1		
Load	2.1	6.6
Wash	10.6	9.7
e1	22.8	20.2
e2	3.4	2.9
e3	1.5	1.3
Total	27.7	24.4
	Desmethylsildenafil	Analogue **1**
MIP2		
Load	0.0	5.8
Wash	8.9	13.5
e1	26.4	8.7
e2	2.1	2.0
e3	0.9	1.6
Total	29.4	12.3

from CH_3CN is lower (33% MIP1, 41% MIP2) than from the water sample (52% MIP1 and 73% MIP2).

The efficiency of extraction of desmethylsildenafil from 0.5 mL plasma samples is comparable for the two materials (28% MIP1 and 30% MIP2, Table 4), as for sildenafil (Table 2). Additionally, Table 4 shows the efficiency of extraction of Analogue **2** using MIP1, and Analogue **1** using MIP2, from plasma samples.

The total relative extraction recovery of Analogue **2** on MIP1 was similar to that reported for sildenafil (0.5 mL plasma, Table 2) and its metabolite, whereas in the case of the extraction of Analogue **1** on MIP2 the recovery was a little lower. However, it suggested that it is possible to use either MIP1 or MIP2 for the SPE extraction of sildenafil, its metabolite and structurally related compounds (*e.g.*, other metabolites or analogues) from plasma samples.

A preliminary validation test for quantifying sildenafil and its metabolite using the MIP1- and MIP2-based MISPE was per-

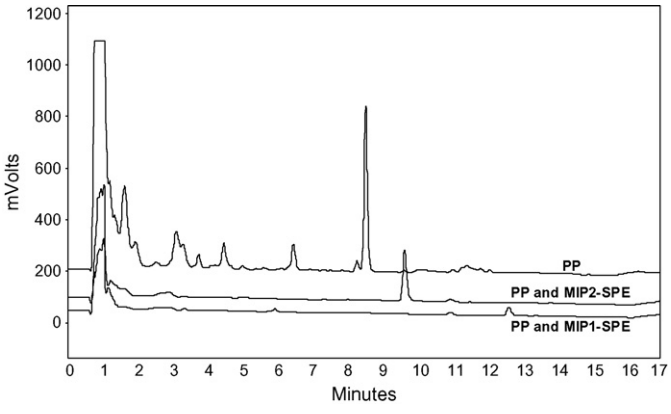

Fig. 4. Chromatograms of elution fraction of 0.5 mL blank plasma obtained after protein precipitation (PP), and protein precipitation followed by MISPE with MIP1 and MIP2, respectively.

Table 5
Calibration line parameters obtained after protein precipitation followed by MISPE on MIP1 and MIP2 and HPLC-UV analysis of sildenafil and metabolite in plasma ($n = 7$)

	Regression equation Weighting: 1/(Response)^2	r^2	Linear range (ng/mL)
MIP1			
Sildenafil	$y = 0.0025x + 0.0839$	0.9968	50–2500
Metabolite	$y = 0.0025x + 0.1858$	0.9946	50–2500
MIP2			
Sildenafil	$y = 0.0022x + 0.0557$	0.9968	50–2500
Metabolite	$y = 0.0020x + 0.0284$	0.9796	50–2500

formed. The method consisted of protein precipitation of the plasma sample, followed by MISPE and HPLC monitoring of sildenafil and its metabolite using UV detection. Analogue **2** was used as internal standard (I.S.) in the case of extractions carried out using MIP1 whereas Analogue **1** was the I.S. for extractions performed using MIP2. Chromatograms obtained for 0.5 mL blank plasma samples after protein precipitation, and protein precipitation followed by MISPE with MIP1 or MIP2, revealed that the samples were significantly cleaner after the MISPE had been applied (Fig. 4). In Fig. 5, examples of representative chromatograms of plasma samples containing 100 and 2500 ng/mL sildenafil and metabolite after protein precipitation and MISPE with either MIP1 or MIP2 are shown.

In the validation test, the sample obtained after protein precipitation was evaporated and reconstituted with a mixture of water and acetonitrile, taking into account the sildenafil solubility, the rate of sample passage through the MISPE cartridge and the polymer extraction efficiency. The dynamic range of the assay was 50–2500 ng/mL. As can be seen in Table 5, the linearity of the calibration lines for both sildenafil and metabolite determination in plasma using MIP1 and MIP2 was satisfactory.

Quality control (QC) samples were spiked at concentrations of 100, 700 and 1500 ng/mL for sildenafil and 100, 500 and 1200 for desmethylsildenafil (Table 6).

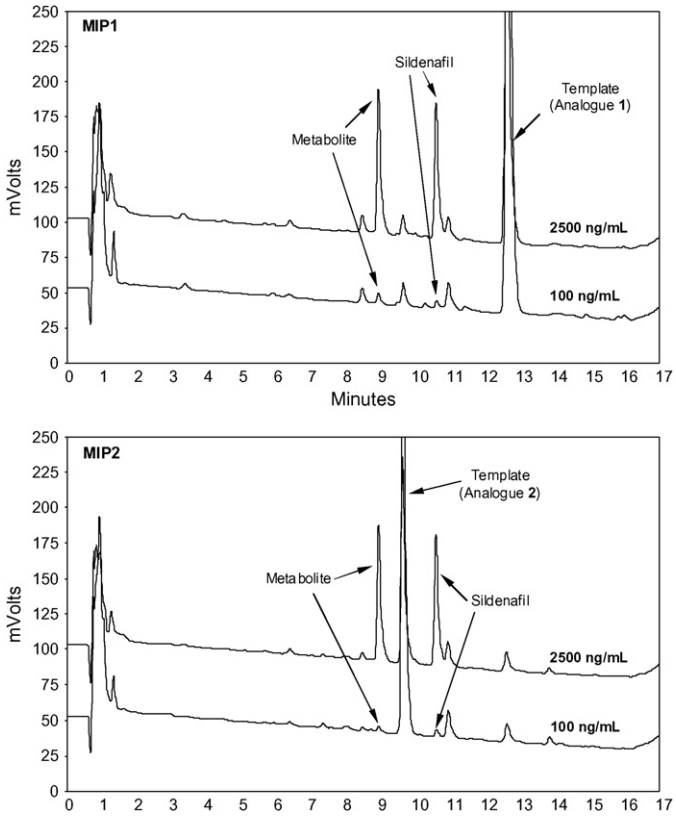

Fig. 5. Chromatograms of elution fraction of 0.5 mL 100 ng/mL and 2500 ng/mL sildenafil and metabolite plasma samples obtained after protein precipitation (PP), and protein precipitation followed by MISPE with MIP1 and MIP2, respectively.

The validation results obtained for the QC samples were very variable in terms of imprecision, inaccuracy and recovery and would not have passed the criteria applied normally in our laboratory for method validation. The inconsistency in the results was probably due to the fact that all of the QC extractions were performed using only one, manually packed cartridge for both MIP1 and MIP2. We anticipate that the use of a machine-packed

Table 6
Quality control results obtained after protein precipitation followed by MISPE on MIP1 and MIP2 and HPLC-UV analysis of sildenafil and metabolite in plasma ($n = 3$)

	Nominal concentration (ng/mL)	Found concentration (ng/mL)	Inaccuracy (%)	Imprecision (%)	Recovery (%)
MIP1					
Sildenafil	100	113.7	13.7	22.1	70.6
	700	739.7	5.7	16.6	64.7
	1500	1770.6	18.0	7.9	49.5
Metabolite	100	105.7	5.7	35.0	58.9
	500	509.9	2.0	21.8	41.1
	1200	1368.8	14.0	7.7	39.0
MIP2					
Sildenafil	100	108.5	8.4	6.1	56.6
	700	706.6	0.9	5.6	56.4
	1500	1573.8	4.9	17.6	38.0
Metabolite	100	98.1	1.8	13.5	38.1
	500	667.8	33.5	11.9	42.7
	1200	1275.7	6.3	28.1	26.5

96-well plate extraction block containing MIP1 or MIP2 for method validation would improve consistency in this regard. Nevertheless, the validation testing performed for the calibration lines gave sufficient indication that the polymers investigated could be used as sorbents for the solid-phase extraction of sildenafil and its metabolite directly from plasma samples. The use of imprinted polymers for the selective extraction of drugs from plasma or urine samples has increased in the last years [4,16–19,25,27]. The novelty of the present work is the use of water-compatible polymeric materials which allowed the recognition of sildenafil and its metabolite desmethylsildenafil in an aqueous environment and the use of an analogue of the analytes of interest as the template molecule. The choice of the analogue as template avoided the tedious problem of interferences from the template, as well as potential template-impurities or degradation products which could compromise the quantitation of the analytes at low concentrations.

4. Conclusions

The results obtained from the application of molecularly imprinted sorbents in the SPE extraction of sildenafil and its principal metabolite, desmethylsildenafil, from aqueous and plasma samples were presented. The molecularly imprinted polymers prepared proved to be very efficient materials for the selective extraction of sildenafil from plasma samples. It has been demonstrated that by exerting judicious choice and control over polymer composition, the polymerisation conditions and the analyte analogues used as templates, it is possible to overcome the practical obstacles that arise frequently during the application of MIPS to the trace analysis of small molecules. The polymer compatibility when aqueous samples are analysed was improved significantly by using a hydrophilic comonomer (HEMA) in the polymer syntheses. This yielded polymers capable of binding selectively to analytes in water, simultaneously diminishing the non-specific, hydrophobic binding of the drug and the endogenous compounds present in the samples. The use of analogues of sildenafil as templates, in combination with a chromatographic separation of the analytes in the sample, overcame the problem of the template bleeding. The extraction recovery of the analytes from aqueous samples was satisfactory. However, during the extraction of plasma samples a cartridge blockage problem arose. To overcome this problem, a protein precipitation step was included before the MISPE step. Data generated as part of a preliminary, quantitative analysis of the MISPE protocol indicates that the imprinted polymers can be used for the quantitative determination of sildenafil, its principal metabolite and related compounds in plasma. Finally, we believe that the analytical protocols can be further optimised and streamlined through the introduction and implementation of automated 96-well plate methodologies.

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