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Molecularly imprinted solid phase extraction of fluconazole from pharmaceutical formulations

S. Manzoor, R. Buffon, A.V. Rossi *

Institute of Chemistry, University of Campinas, CP 6154, CEP 13083-970 Campinas, Brazil

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ABSTRACT

This work encompasses a direct and coherent strategy to synthesise a molecularly imprinted polymer (MIP) capable of extracting fluconazole from its sample. The MIP was successfully prepared from methacrylic acid (functional monomer), ethyleneglycoldimethacrylate (crosslinker) and acetonitrile (porogenic solvent) in the presence of fluconazole as the template molecule through a non-covalent approach. The non-imprinted polymer (NIP) was prepared following the same synthetic scheme, but in the absence of the template. The data obtained from scanning electronic microscopy, infrared spectroscopy, thermogravimetric and nitrogen Brunauer–Emmett–Teller plot helped to elucidate the structural as well as the morphological characteristics of the MIP and NIP. The application of MIP as a sorbent was demonstrated by packing it in solid phase extraction cartridges to extract fluconazole from commercial capsule samples through an offline analytical procedure. The quantification of fluconazole was accomplished through UPLC–MS, which resulted in LOD $\leq 1.63 \times 10^{-10}$ mM. Furthermore, a high percentage recovery of $91 \pm 10\%$ ($n=9$) was obtained. The ability of the MIP for selective recognition of fluconazole was evaluated by comparison with the structural analogues, miconazole, tioconazole and secnidazole, resulting in percentage recoveries of 51, 35 and 32%, respectively.

1. Introduction

Fluconazole (Fig. 1) is a synthetic triazole antifungal agent commonly used for the treatment of oropharyngeal, oesophageal, and deep candidiasis that works by inhibiting the C-14 demethylation of lanosterol, similar to other antifungal drugs of triazole and the imidazole class. It is predominantly excreted through the kidneys where approximately 11% of the administered dose is eliminated in the form of metabolites [1]. Commonly, the monitoring of the drug level is not necessary; however, it becomes important in patients suffering from renal inefficiency or undergoing dialysis [2]. Fluconazole is commonly commercialised in the form of capsules containing 150 mg of the active pharmaceutical ingredient (API) and excipients, which generally facilitate the release of the API inside the organism [3,4]. Excipients may also sometimes contain impurities having chemical structures similar to that of the API [5].

The methods frequently applied for the analysis of fluconazole in pharmaceutical formulations and biological samples include gas chromatography, high performance liquid chromatography (HPLC) and spectrophotometry [6,7]. Among these techniques, most of the HPLC methods utilise C18 columns for the analysis of fluconazole in samples prepared mainly using liquid–liquid extraction and solid phase extraction [8].

Solidphase extraction (SPE), for the purpose of sample pre-treatment, has gained attention in recent years because of its simplicity and active participation in the field of separation science [9,10]. Different types of SPE sorbents are available, and molecularly imprinted polymerisation is one of the prime and most promising techniques to synthesise sorbents with high selectivity [11,12].

Molecularly imprinted polymers (MIPs) are tailor-made polymeric materials with cavities complementary in shape, size and functional groups to the template molecule and offer highly selective molecular recognition properties [13–15]. Different techniques can be used for the synthesis of MIPs, including bulk, suspension, dispersion, multi-step swelling and precipitation polymerisations [16–19]. The low cost, stability, ease of preparation and increased selectivity of MIPs make them highly useful in SPE as well as several other applications, such as sensors, enantiomeric separations and analytical applications [20,21]. Moreover, the high affinity of these sorbents may result in the efficient extraction of the analyte from different matrices [22]. Keeping in mind the efficacy of this technique, we have successfully synthesised molecularly imprinted sorbents for the analysis of fluconazole in commercial pharmaceutical samples. The proposed scheme of the synthesis is represented in Fig. 1.

* Corresponding author.
E-mail address: adriana@iqm.unicamp.br (A.V. Rossi).

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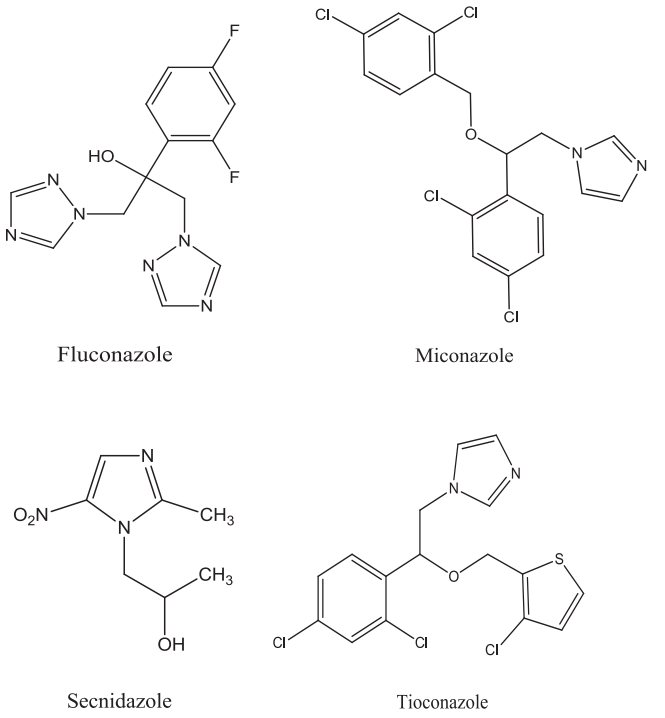

Fig. 1. Molecular structures of (a) fluconazole, (b) miconazole, (c) secnidazole and (d) tioconazole.

2. Experimental

2.1. Reagents

Fluconazole (98%), methacrylic acid (MAA, 99%), ethyleneglycoldimethacrylate (EGDMA, 98%) and 2,2′-azo-bis-isobutyronitrile (AIBN, 98%) were obtained from Sigma-Aldrich (St. Louis, USA). Miconazole (99.6%) and tioconazole (99.8%) were supplied by Evonik Degussa Brasil Ltda (São Paulo, Brazil), whereas secnidazole (98.8%) was supplied by Galena (Campinas, Brazil). Methanol (99.8%) was purchased from Synth (São Paulo, Brazil) and acetonitrile (99.5%) was purchased from Vetec (Duque de Caxias, Brazil). Methanol and acetonitrile were of ultra-high performance liquid chromatography (UPLC) grade and dimethylsulfoxide (DMSO) was purchased from Merck (Schwalbach, Germany) with 99% purity. Ultrapure water (18.3 MΩ × cm) was generated by a Milli-Q Plus system (Millipore, Billerica, USA).

2.2. Preparation of polymers

Fluconazole imprinted polymer (FLUMIP) was synthesised through the precipitation polymerisation method. The template (1 mmol), the monomer MAA (4 mmol), the cross-linker EGDMA (20 mmol) and the initiator AIBN (0.07 mmol) were dissolved in 25 mL of acetonitrile in a Schlenk flask. The solution was purged with argon for 5 min, and the flask was sealed with a septum. The polymerisation was performed at 60 °C for 24 h in a thermostatic bath. The polymer, obtained in the form of precipitates, was washed with methanol in a Soxhlet apparatus for 24 h to remove fluconazole and unreacted monomers.

The non-imprinted polymer (NIP) was synthesised using the same procedure mentioned above, but without adding the template molecule.

2.3. Characterisation

Infrared (IR) spectra from 4000 to 400 cm^{-1} were obtained on an FTIR BOMEM NB instrument in transmission mode. Thermogravimetric analysis (TGA) was performed using a TA instruments TGA 2050 using 4.3 mg of MIP and NIP at a heating rate of 5 °C/min. Scanning electron microscopy (SEM) analysis was performed using a JEOL SEM 6360-LV microscope. Nitrogen adsorption/desorption isotherms were measured using a Nova 4200 apparatus using 500 mg of each polymer dried under vacuum at 120 °C for 5 h before analysis. The Brunauer–Emmett–Teller (BET) plot and the Barrett–Joyner–Halenda (BJH) method were used to determine the specific area, the pore volume and the pore size distribution.

2.4. MIP–SPE conditions

A FLUMIP–SPE cartridge was prepared by packing 50 mg of FLUMIP in an empty SPE cartridge. A NIP SPE cartridge was also prepared following the same procedure. Thereafter, the cartridges were conditioned with 2 mL of acetonitrile, followed by 2 mL of an acetonitrile/water (1:15 v/v) mixture.

The washing conditions for the FLUMIP–SPE were studied. For this purpose, washing solutions were prepared by adjusting the pH of deionised water from 4 to 8 by the dropwise addition of 0.001 M HCl or NaOH solutions as required. The addition of a 2% (v/v) aqueous DMSO solution was also tested. Elution was performed using 1 mL of methanol, and the eluate was analysed using UV spectroscopy. Five replicates for each washing condition were performed.

2.5. MIP–SPE analysis

A fluconazole capsule (local pharmaceutical commercial sample) was uncoated, and 6.00 mg of the powder (containing 2.5 mg of API) was dissolved in 3 mL of acetonitrile and then diluted to 50 mL with deionised water in a volumetric flask. The solution was filtered through a 0.5 μm Millipore filter, and 1000 mL was then loaded onto the FLUMIP and NIP cartridges. The cartridges were washed with 0.50 mL of 2% aqueous DMSO solution and then eluted with 1 mL of methanol. Three replicates were performed.

2.6. UPLC–MS analysis

The liquid chromatographic system consisted of an Ultra Performance Liquid Chromatograph Acquity-Waters with a C18 column (Acquity BEH; length 5.0 × 2.1 mm and particle diameter 1.7 μm) coupled with a mass detector Quattro Micro API Waters.

The column oven temperature was maintained at 40 °C. The mobile phase consisted of 0.1% (v/v) formic acid in water (A) and methanol (B). The elution gradient is described in Table 1.

The flow rate was set to 0.35 mL/min and an injection volume of 3 μL was used. The mass spectrometer was operated in positive mode with 1000 V spray voltage. The electrospray ion source was heated to 150 °C, and an ion ratio (m/z) of 307 was observed.

2.7. Cross-reactivity study

The selectivity of the FLUMIP was evaluated by comparing the percentage recovery of fluconazole with that of structurally related analogues, i.e., miconazole, tioconazole and secnidazole (Fig. 1),

Table 1
Description of gradient elution chromatography UHPLC–MS.

Time (min)	Solvent A (% v/v)	Solvent B (% v/v)
0	97	3
4.0	5	95
4.2	5	95
5.0	97	3

using the FLUMIP and NIP cartridges. The MIP–SPE conditions optimised for fluconazole (Section 3.2) were adopted for these tests with six replicates for each compound. The eluates obtained from the MIP–SPE cartridge were then quantified through UV spectroscopy.

2.8. Comparison with C18

The FLUMIP–SPE cartridge was also compared to the commercially available C18 SPE cartridge (particle diameter of 47–60 μm, Varian). For this purpose, the percentage recoveries of the four antifungals were determined using both cartridges. The analytical procedure followed by Inagaki et al. [23] for the analysis of fluconazole was adopted with some modifications. An empty cartridge was packed with 50 mg of synthesised MIP sorbent and preconditioned using 1 mL of methanol, followed by 1 mL of 0.1 M sodium phosphate buffer (pH 6). In the next step, standard solutions of fluconazole were prepared in 3 mL of acetonitrile with further dilution with sodium phosphate buffer (pH 6). An aliquot of 1 mL of the standard solution percolated the conditioned cartridge, followed by washing it with 1 mL of sodium phosphate buffer solution and then 1 mL of deionised water. Subsequently, the elution was performed using 1 mL of methanol, and the quantification of the analyte in the eluate was achieved through UV spectroscopy.

In the cases of miconazole, tioconazole and secnidazole, a higher volume of acetonitrile, i.e., 9 mL, was required for the preparation of standard solutions because of their low solubility in phosphate buffer. The remainder of the procedure followed the same steps as those employed for the analysis of fluconazole with six replicates for each compound.

3. Results and discussion

The chemical structure of the fluconazole molecule (Fig. 1) suggests the probable formation of hydrogen bonds with a functional monomer, such as methacrylic acid. Hence, it is possible to adopt a non-covalent approach for the formation of the pre-polymerisation complex of the template molecule with methacrylic acid. Therefore, the non-covalent approach was taken into account, and the schematic representation of the imprinting process is shown in Fig. 2.

The synthesis of FLUMIP and NIP involved precipitation polymerisation; therefore, a polymer in the form of precipitate was obtained, as observed in the SEM images. The images obtained at 10,000 × magnification are shown in Fig. 3.

It can be observed from Fig. 3 that there was a significant difference in the appearance of both precipitates, which suggests that the presence and absence of the template molecule prominently affected the morphology of FLUMIP and NIP.

The values of the surface area, pore volume and average pore diameter are summarised in Table 2. The data infer that the surface area and the porosity of the MIP were also significantly affected by the presence of the template molecule. This can be observed from the nitrogen adsorption/desorption isotherms applied for the comparative study of the textural properties of both polymers (FLUMIP and NIP). The results showed that FLUMIP was characterised by higher surface area and pore volume (Table 2), which is indicative of greater binding capacity. Moreover, the isotherms (Fig. 4) obtained through BET plot were of type II profile. According to the classification of IUPAC, type II isotherms are generally related to the presence of non-porous or macroporous surfaces [24].

3.1. IR and thermogravimetric studies

IR spectra (Fig. 5) were obtained to compare the structural characteristics of FLUMIP and NIP. The spectra of both polymers showed similar characteristic bands, indicating the presence of similar chemical structures. A band at 3444 cm^{-1} is characteristic of an –OH group, whereas those observed at 2948 and 1460 cm^{-1} can be attributed to methyl asymmetric/symmetric stretching and asymmetric/symmetric bending, respectively. The absorption

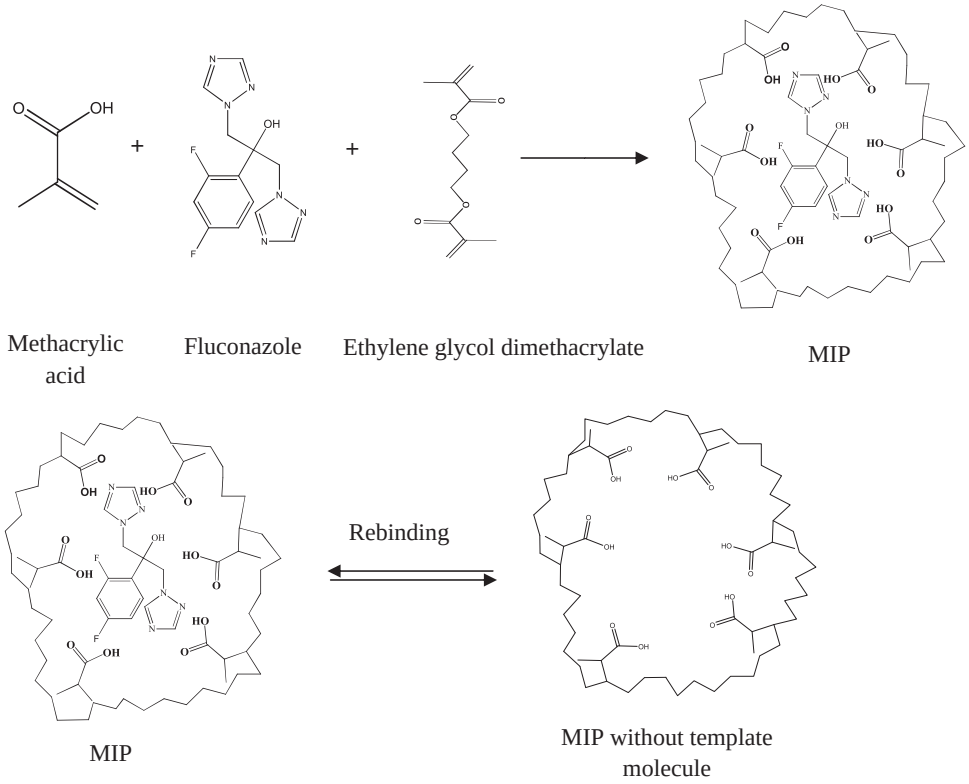

Fig. 2. Synthetic scheme of MIP, suggesting its molecular structure.

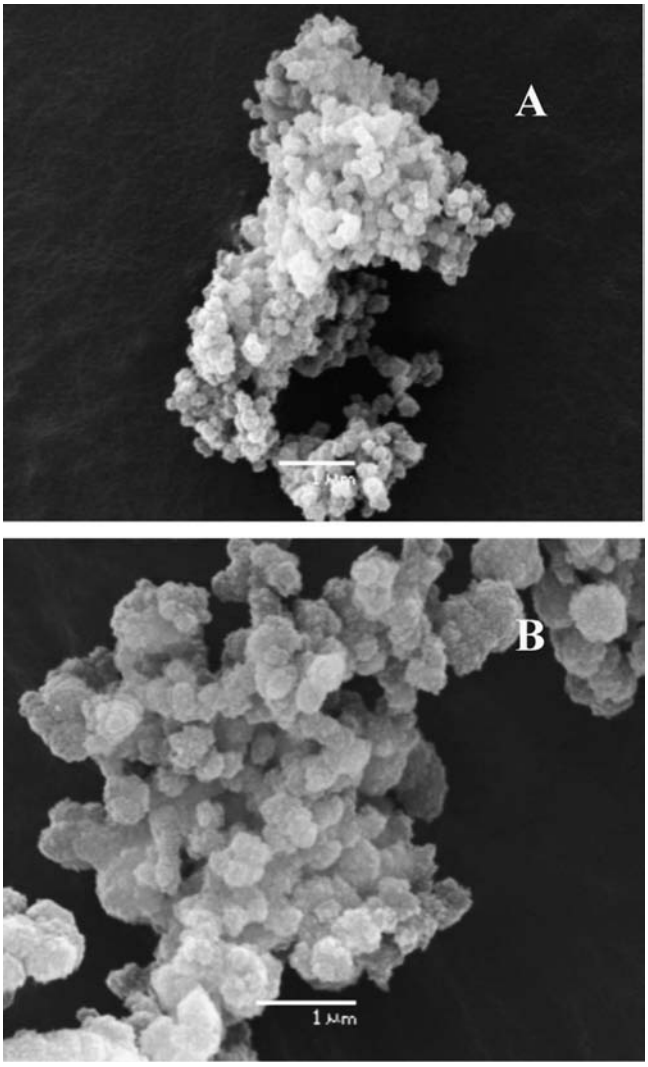

Fig. 3. SEM micrographs of (a) FLUMIP and (b) NIP at 10,000× magnification.

Table 2
Surface area, pore volume and pore size of MIP and NIP.

Polymer	Surface area ($m^2\ g^{-1}$)	Pore volume ($cm^3\ g^{-1}$)	Average pore diameter (nm)
MIP	72	0.11	66
NIP	28	0.06	87

at 1727 cm^{-1} can be assigned to ν(C=O), which is characteristic of an ester.

The TGA plots (Fig. 6) were obtained to investigate the thermal stability of the polymers. A significant decomposition of the polymers began at approximately 250 °C, and the maximum weight losses occurred at approximately 390 °C and 395 °C in the case of FLUMIP and NIP, respectively. These results indicate that these polymers possess adequate thermal stability for analytical applications below 250 °C.

3.2. Adjustments of the FLUMIP–SPE procedure

The pH is an important factor that can affect the selectivity of MIPs. The pH of the matrix may alter the ability of MIPs to interact with the analyte because of the variation in the surface properties [25]. Therefore, the effect of the pH of the washing solution has

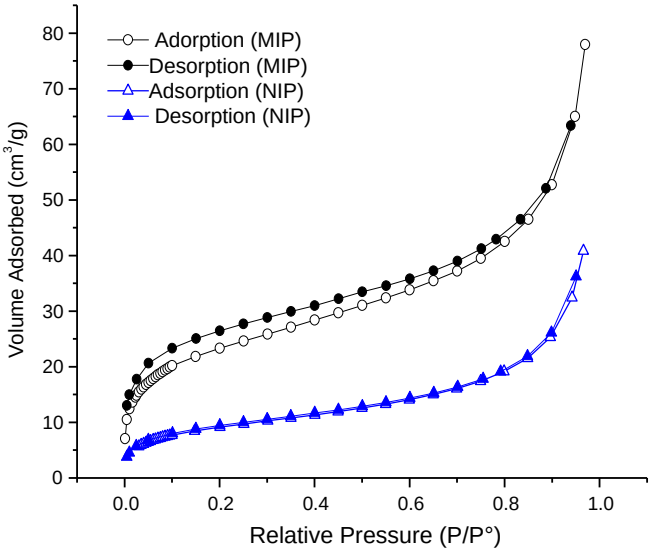

Fig. 4. Nitrogen adsorption/desorption isotherms of FLUMIP and NIP.

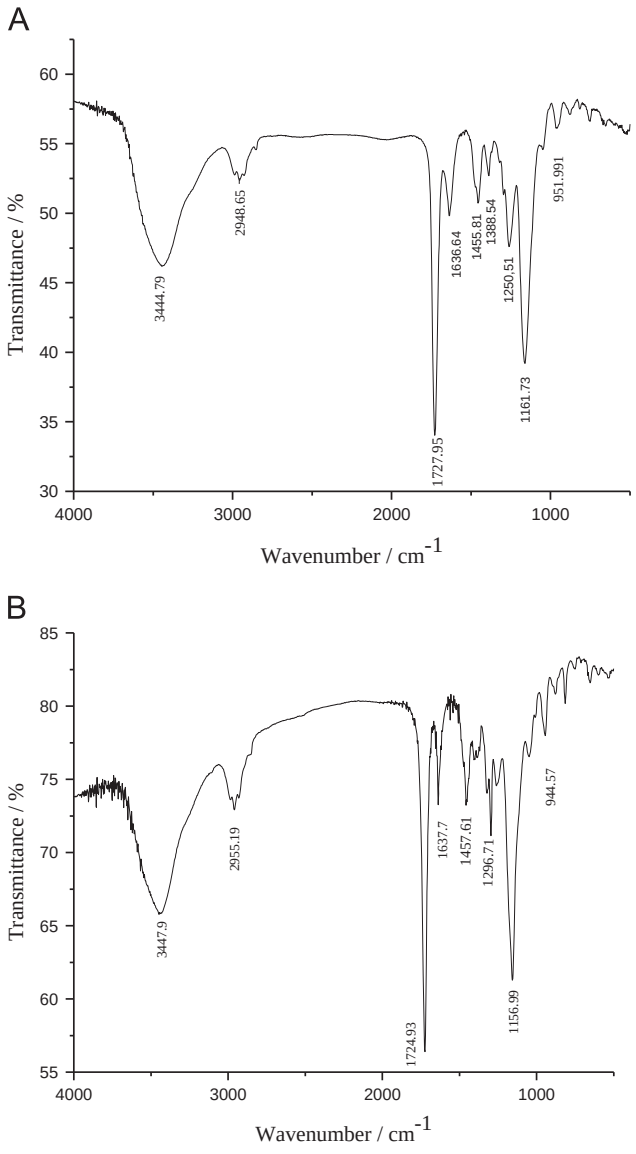

Fig. 5. IR spectra of (a) FLUMIP and (b) NIP.

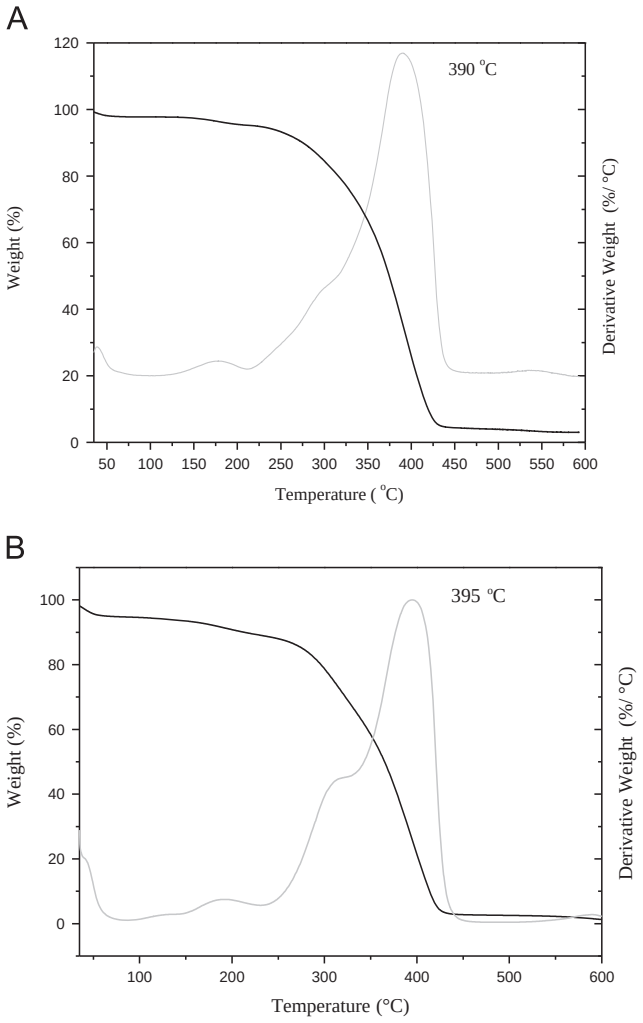

Fig. 6. TG/DTG curves of (a) MIP and (b) NIP.

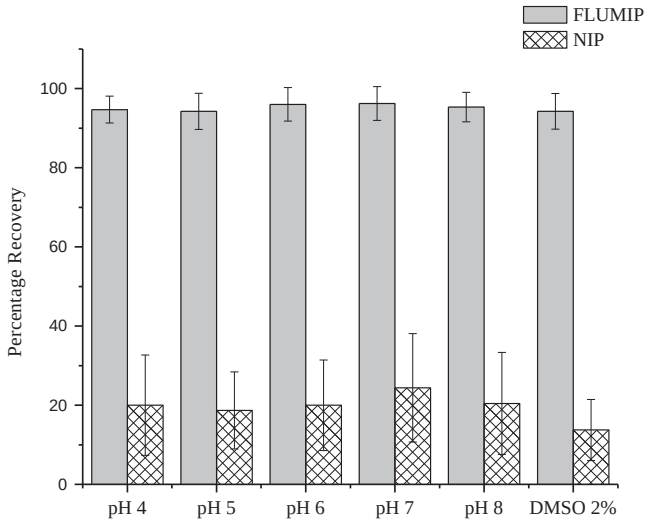

Fig. 7. Effect of washing solution pH on the percentage recovery of fluconazole by MIP–SPE (five replicates).

also been studied and is shown in Fig. 7. The results show that no appreciable change in the values of percentage recoveries was observed between pH values of 4 to 8. Hence, it can be suggested that the change in pH did not alter the ability of the analyte to interact with the synthesised sorbents, and thus, no further pH adjustment was performed.

The use of organic solvents, such as DMSO, has also been recommended in the literature for washing purposes [26]. Therefore, an aqueous DMSO solution (2% v/v) was also tested and was found to decrease the percentage recovery of the analyte from NIP SPE without affecting the FLUMIP–SPE. This result may be because of the disruption of non-specific interactions between the analyte and the sorbent. Hence, this aqueous DMSO solution was selected as the washing solution for the subsequent FLUMIP–SPE tests.

The focus of this work was the analysis of fluconazole in commercial pharmaceutical capsules using FLUMIP as a sorbent with further quantification through UPLC procedures. Based on the optimised FLUMIP–SPE conditions, the sample solution (Section 2.5) was percolated through the FLUMIP–SPE cartridge, followed by washing with aqueous DMSO solution (2% v/v) and elution with methanol. The eluate was then analysed by UPLC–MS, and the obtained chromatograms are shown in Fig. 8A and B.

Several analytical figures of merit, such as percentage recovery, limit of detection (LOD) and limit of quantification (LOQ) were determined. The percentage recovery was calculated by spiking the sample with standard fluconazole and a value of 91 ± 10% with nine replicates (Table 3) was achieved.

A linear response (r^2=0.9998) was observed over the concentration range of 1.17×10^{-5} to 1.27×10^{-4} mM. The use of UPLC–MS for the quantitative determination of fluconazole resulted in a

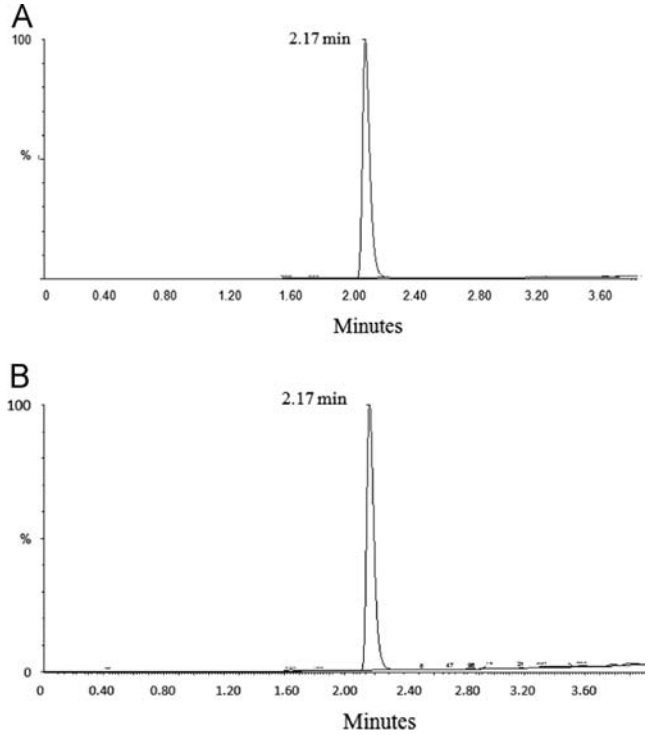

Fig. 8. UPLC–MS chromatograms of commercial samples of fluconazole (a) before and (b) after MIP–SPE based method.

Table 3
Percentage recoveries of sample by FLUMIP SPE at different spiking levels.

Spiked concentration (μg/mL)	Recovery 1 (%)	Recovery 2 (%)	Recovery 3 (%)	Mean (%)	Relative standard deviation (%)
1.00	90.8	72.2	81.9	90.8	10
2.00	94.6	96.5	99.8		
3.00	95.6	99.0	86.5		

very low LOD because of the high sensitivity of mass spectrometry without compromising the peak profile in the chromatogram. To calculate the LOD, the criteria of signal to noise ratio of 3:1 was applied [27]. Because the intensity of instrumental noise was very low, the ratio was not achieved even at a concentration of 1.63×10^{-10} mM (in three replicates). Thus, the possibility that the LOQ has this order of magnitude cannot be ruled out because the detectability of mass spectrometry is very high. A literature survey revealed that the LOQs achieved for fluconazole in some previous studies were 4.08×10^{-4} [28] and 4.5×10^{-5} mM [29]. Comparing these literature values with the results obtained in this work showed that the application of FLUMIP–SPE, followed by analysis with UPLC–MS, is potentially interesting for the quantification of fluconazole at lower concentrations.

3.3. Selectivity studies

To determine the selectivity of FLUMIP, some fungicides with similar structures and functional groups were selected. These studies involved the comparison of the percentage recoveries of secnidazole, miconazole and tioconazole with fluconazole through MIP–SPE. The molecular structures of these compounds are shown in Fig. 1.

The percentage recovery of each compound was determined using the FLUMIP–SPE cartridges with six replicates. Percentage recoveries of 93.3 ± 1.1, 32.2 ± 4.4, 51.3 ± 6.0 and 35.4 ± 3.7% were obtained for fluconazole, secnidazole, miconazole and tioconazole, respectively (Fig. 9).

The higher percentage recovery for miconazole demonstrated a high affinity for FLUMIP in comparison to the other analogues of fluconazole under investigation. This result suggests that the FLUMIP can be considered a group-selective sorbent. Moreover, the low recovery of the fungicides on NIP SPE cartridge revealed that even in small extensions, the non-specific binding sites also play a role in inducing such interactions. Hence, the ability of FLUMIP to interact with fluconazole analogues is not solely a result of their spatial arrangement in the imprinted cavities.

3.4. Comparison with C18 SPE cartridge

Commercial SPE cartridges generally employ conventional sorbents, such as C18, for the extraction of various analytes from their respective matrices, and their application in evaluating the efficiency of MIP–SPE cartridges has been shown in several studies [30,31]. To test the FLUMIP–SPE cartridge, its performance was

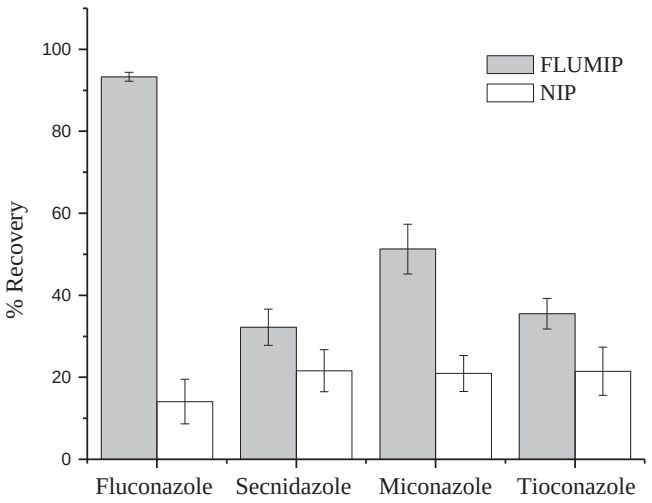

Fig. 9. Selectivity studies of FLUMIP (a) and NIP (b) comparing the data obtained by UV spectroscopy for fluconazole, secnidazole, miconazole and tioconazole samples (six replicates).

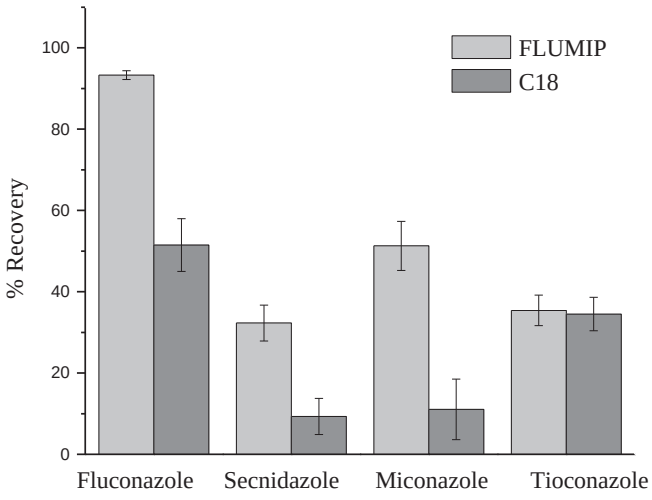

Fig. 10. Percentage recovery of fluconazole, secnidazole, miconazole and tioconazole by MIP–SPE and C18-SPE using UV spectroscopy for quantification at 260, 310, 235 and 204 nm, respectively (six replicates).

compared with the C18 SPE cartridge by comparing the percentage recoveries of fluconazole and its structural analogues. The percentage recoveries obtained through C18 SPE cartridges were 51.5 ± 6.5, 9.3 ± 4.4, 11.0 ± 7.4, and 34.5 ± 4.1% for fluconazole, secnidazole, miconazole and tioconazole, respectively, with six replicates each. Analogous to FLUMIP, C18 showed a higher recovery for fluconazole among the fungicides studied. However, Fig. 10 shows a recovery of 93.3% for fluconazole using FLUMIP, which was nearly two times higher than that obtained by the C18 cartridge. This demonstrates the greater binding capacity of this newly synthesised sorbent to the analyte and better molecular recognition ability compared to the commercial C18 sorbent.

4. Conclusions

This study involved the successful synthesis of an MIP through precipitation polymerisation using a non-covalent approach, which is a simple technique. FLUMIP was applied for the extraction of fluconazole from commercial pharmaceutical capsules using FLUMIP–SPE and a percentage recovery of 91% (RSD = 10%, nine replicates) was obtained. The use of UPLC–MS resulted in a very low detection limit, less than 1.63×10^{-10} mM. Comparing the percentage recoveries for the structurally similar compounds resulted in highly selective imprinting of the FLUMIP for the analyte fluconazole. The high affinity of FLUMIP for fluconazole most likely results from the efficient molecular imprinting in the polymer matrix, as suggested by SEM and nitrogen adsorption/desorption techniques. Hence, the obtained results demonstrate the application of MIP for the analysis of fluconazole in pharmaceutical samples and others at lower concentrations.

Acknowledgements

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