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Research Article

Dendritic Poly (amidoamine) Functionalized with Magnetic Nanoparticles as Sorbent for Simultaneous Magnetic Solid-Phase Extraction of Miconazole, Clotrimazole and Tioconazole Followed by Determination via HPLC-UV

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ABSTRACT

In this study, an attempt was made to synthesize a new sorbent based on the second generation of silica coated magnetic poly(amidoamine) (Fe_3O_4@SiO_2@PAMAM) to improve the performance of magnetic solid phase extraction (MSPE) of some antifungal drugs including miconazole, clotrimazole and ticonazole in various real samples such as urine and human plasma. The extracted analytes were measured by high performance liquid chromatography equipped with ultraviolet detection (HPLC-UV). Field emission-scanning electron microscopy (FE-SEM), X-ray diffraction analysis (XRD), Thermogravimetric analysis (TGA), Transmission electron microscopy (TEM) and Fourier transform-infrared spectroscopy (FT-IR) were used to study the morphology and structure of the prepared sorbent. The various factors such as: extraction time, sorbent amount, solvent desorption volume, desorption time, ionic strength and pH were studied and optimized. The method was validated according to ICH guidelines with respect to precision, accuracy, linearity, specificity, robustness, and limits of detection and quantification. Under the optimized condition, the linearity of the method was in the range of 1-500 µg L^{-1} (miconazole= 1-200 µg L^{-1}, clotrimazole = 1-500 µg L^{-1} and ticonazole = 1-200 µg L^{-1}). The obtained coefficient of determination (r^2) were between 0.9871-0.9977. The limits of detection (LODs) were also calculated to be 0.14-0.18 µg L^{-1} (miconazole= 0.16 µg L^{-1}, clotrimazole = 0.18 µg L^{-1} and ticonazole=0.14 µg L^{-1}). The limits of quantification (LOQs) were also in the range of 0.46-0.60 µg L^{-1} for the selected analytes. The relative standard deviations (RSDs%), were obtained in the range of 4.6 to 5.9%. Moreover, the calculated enrichment factors were between 85 and 93. The proposed method was employed for the analysis of various real samples such as urine and plasma samples. The obtained recoveries (higher than 92%) indicated that the method was useful and applicable in complicated real samples.

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1. Introduction

Magnetic solid phase extraction (MSPE) technique as a technology to increase the capability of the solid phase extraction (SPE) method has been widely considered since 1999. As a subset of solid-phase microextraction (SPME) methods, MSPE represent an external magnetic field that significantly simplifies the sample preconcentration process without the need for filtration or centrifugation steps. On the other hand, the usual problems of the SPE method, which are related to absorbent packaging, such as blocking the packaging bed or applying high pressure, can be eliminated by the MSPE process. In addition, most of the magnetic sorbents can be easily recycled and used in the next steps, which reduction in costs. On the other hand, it also protects the environment [1-3]. In MSPE, first, a magnetic material is dispersed as a sorbent in a suspension or solution containing the analyte. After that, this magnetic sorbent, extract the desired analytes. Then, the dispersed magnetic materials can be easily separated from the solution or suspension by using an external magnet. In the desorption stage, in order to separate the analyte from the magnetic sorbent, the materials are dispersed in the desorption solvent and allowed to pass for a suitable period of time. After that, with the help of an external magnetic field, the sorbent is removed from the desorption solution and the desorption solvent concentrated with analytes is injected into the analytical device for analysis. The dispersibility feature of the magnetic particles causes mass transfer by increasing the contact surface between the analytes and the magnetic sorbent. Finally, the sample preconcentration process is easier and faster. In addition, magnetic materials are easily activated to increase the selectivity of sorbents for the desired analytes [4-6]. Overall, MSPE is a simple, environmentally friendly and efficient preconcentration method.

In recent years, the surface of magnetic nanoparticles has been modified with organic molecules [7], silica [8], polymers [9], and dendrimers [10], for different applications. Magnetite nanoparticles functionalized with dendrimers are interesting magnetic scaffolds due to their well-defined structure, high dispersibility and functional groups providing more reaction sites in the interior as well as periphery proving their proficiency and beyond these, it can be magnetically recycled for further use.

Dendrimers, a member of hyperbranched polymers, are three-dimensional macromolecules that are formed by nano-sized molecular frameworks [11]. Also, they have received great attention in a variety of fields including drug delivery, sensors, materials science, and catalysis [12,13] owing to their three-dimensional cauliflower shape with numerous chelating sites. Among the various dendrimers, polyamidoamine (PAMAM) dendrimer owes their symmetrically dispersed structure to numerous functional groups like terminal amine groups and interior amide groups that have been employed for diverse applications [14]. PAMAM is the most common type of these materials, which have unique properties such as low toxicity, regular 3D structure, and good extraction efficiency. It is worth noting that although prepared PAMAM sorbents can be successfully used for the separation and enrichment of various compounds, most of these sorbents have problems such as low adsorption capacity, prolonged adsorption time, and difficult separation and recovery from the system [15,16]. Therefore, magnetic nanoparticles can be a good idea to overcome these problems.

This study aims to prepare a new sorbent and environmentally friendly coating for MSPE application. Here, PAMAM dendrimers were

synthesized by Michael addition and amidation reaction. It is expected that the prepared sorbent show excellent performance for the preconcentration and extraction of very small amount of antifungal drugs such as miconazole, clotrimazole and ticonazole in a complicated matrix such as urine and human plasma samples. Miconazole belongs to the group of antifungal drugs and the category of imidazoles. Miconazole is prescribed to treat fungal skin diseases such as Taenia Versicolor. The side effects of miconazole can be skin rashes, blisters, burning, redness, or other symptoms of skin irritation that did not exist before the topical use of miconazole [17]. Ticonazole is an antifungal drug from the imidazole class used to treat infections caused by fungi or yeast. This drug belongs to the group of drugs that inhibit the growth of fungi and kill them. Doctors prescribe this medicine to treat fungal diseases such as ringworm in the hair-growing parts of the body or to remove infections of the female genital tract. One of the common side effects of using this medicine is burning and itching the skin [18]. Clotrimazole is an antifungal that is used in the treatment of fungal infections caused by dermatophytes, yeasts, and also in the treatment of fungal infections around the nails and ringworm of the beard and head. Clotrimazole is a drug that prevent the growth of fungi and is very effective in the treatment of fungal infections, but depending on the amount used, it can also kill the fungus. Clotrimazole is also used in the form of ointment, tablets and drops. But in general, the best way to use this medicine is topically [19].

2. Experimental

2.1. Materials, Methods and Preparation of real Samples

Details of the prepared materials, reagents, preparation of standard solutions, chemicals, and instrumentation that is used in the current study can be found in the part entitled Supporting Data. Moreover, in order to approve the performance of the presented method, real samples such as urine and plasma samples were prepared. The real sample preparation information, which is used in the current study are written in the part entitled as Supporting Data.

2.2. Preparation of PAMAM dendrimer on the silica coated iron oxide nanoparticles (Fe_3O_4@SiO_2@PAMAM)

The synthesis of the Fe_3O_4@SiO_2@PAMAM was accomplished according to the procedure described in detail in the literature [20]. Briefly, the precursors of $FeCl_2 \cdot 4H_2O$ and $FeCl_3 \cdot 6H_2O$ with a molar ratio of 1:2 were dissolved in 20 mL of deionized water. 5 mL of NH_3 was added dropwise with continuous stirring at room temperature (1 h) in order to increase the pH of the solution to 10. Then, magnetite nanoparticles were separated using an external magnetic field. Magnetically separated nanoparticles were washed twice with deionized water, acetone and dried in a vacuum oven at 70 ° C for 6 h.

Magnetic nanoparticles (0.5 g) were sonicated in a mixture of 40 mL ethanol and 10 mL of deionized water for 20 min, followed by the addition of 2 mL aqueous ammonia (25 (%w/w)). Then, tetraethoxysilane (TEOS) (2 mL) was added dropwise to the reaction mixture and stirred for 12 h. Finally, the products (Fe_3O_4@SiO_2) were collected by an external magnet and washed three times with deionized water and ethanol and dried at 50 °C. Fe_3O_4@SiO_2 nanoparticles (0.5 g) were dispersed in 100 mL of dry toluene by ultrasonic for 20 min. Next, a solution of 3-aminopropyl-trimethoxysilane (APTES) (3 mL) was added under vigorous stirring and heated at reflux temperature for 48 h. After cooling, the product (Fe_3O_4@SiO_2@Pr-NH_2) was separated by an external magnet and washed with dry methanol and

acetone (three times) and dried at 50 °C in an oven for 8 h.

Fe_3O_4@SiO_2@Pr-NH_2 (0.5 g) was dispersed in 20 mL of anhydrous methanol and sonicated for 30 min. Then, 1 mL of methyl acrylate was slowly added at room temperature. Afterward, the temperature of the reaction mixture was gradually raised to 60 °C and stirred for 3 days. Next, the mixture was cooled to room temperature. The nanoparticles (Fe_3O_4@SiO_2@PAMAM-G0.5) were separated by an external magnetic field and washed two times with the dry methanol and acetone and dried at 50 °C in the oven for 8 h.

0.5 g of Fe_3O_4@SiO_2@PAMAM-G 0.5 was dispersed in 20 mL of anhydrous methanol and was sonicated for 30 min. Then, 2 mL of ethylenediamine was slowly added at room temperature. Afterward, the temperature of the reaction mixture was gradually raised to 60°C and stirred for 3 days. The mixture was cooled to room temperature. The nanoparticles (Fe_3O_4@SiO_2@PAMAM-G1) were separated by an external magnetic field and washed two times with the dry methanol and acetone and dried at 50 °C in the oven for 8 h. By repeating the above two steps alternately, Fe_3O_4@SiO_2@PAMAM-G1.5 and Fe_3O_4@SiO_2@PAMAM-G2 were prepared.

2.3. MSPE procedure

In order to perform the extraction, first, 15 mg of the prepared sorbent was added in 10 mL of the test sample solution with a concentration of 100 µg L^{-1} and 15 % w/v of NaCl, which pH had reached at 6 with the addition of 1 mM HCl, and this solution was sonicated for 15 min at room temperature. After the extraction time, the magnetic sorbent containing the target analytes was separated by the aid of an external magnet (1.6 Tesla), and the supernatant was decanted. To desorb the extracted analytes, 50 µL of methanol (as desorption solvent) was added to the sorbent and subjected to ultrasonic waves for 5 min. Finally, 25 µL of the desorbed solvent was injected into the HPLC-UV.

3. Results and discussion

3.1. Characterization of the synthesized Fe_3O_4@SiO_2@PAMAM

After successful synthesis, these nanoparticles were characterized by FT-IR, SEM, TEM, and TGA. The growth of PAMAM dendrimer on the silica-coated iron oxide nanoparticle was confirmed from FT-IR spectra (Fig. 1A-a, b).

FT-IR spectra show a very strong stretching absorption band at 580 cm^{-1} for Fe–O–Fe and an absorption band at 3450 cm^{-1} observed for peripheral –OH stretching of iron oxide nanoparticles. Similarly, the appearance of a new absorption band at 1083 cm^{-1} confirms the formation of a silica shell around the magnetite core (Fig. 1A-a). Broad intense peak around 3444 cm^{-1} corresponds to the –OH and –NH stretching present on the silica-coated iron oxide nanoparticles and magnetite cored silica coated PAMAM dendrimer (Fig. 1A-b). Well defined peak around 1722 cm^{-1} was observed for the carbonyl stretching in the ester groups of Fe_3O_4@SiO_2@PAMAM G1.5 (Fig. 1A-b), whereas these peaks disappeared after the formation of the amide group in the full generation of Fe_3O_4@SiO_2@PAMAM G2 (Fig. 1A-b).

The surface topography of Fe_3O_4@SiO_2@PAMAM G2 was studied by SEM and TEM (Figure 1-B, C). The attained SEM micrographs verified the spherical shape of the prepared structure which consists of particles in the domain of the nanometer scale.

The thermogram of Fe_3O_4@SiO_2@PAMAM (G2) is depicted in Figure 1-D. Regarding the TGA of Fe_3O_4@SiO_2@PAMAM (G2), MNPs had a partial mass loss (0.99%), which should be the loss of water molecules on the surface of nanoparticles. Also, a weight loss (53.72%), can be due to the degradation

of the organic layer (PAMAM) coating on the nanoparticle surface.

Figure 1-E shows the results of the VSM analysis of the synthesized sorbent. According to the figure, this magnetic material has a saturation magnetism equal to 24 emu g^{-1}, and its steep slope indicates the appropriate and high response of this material to the magnetic field.

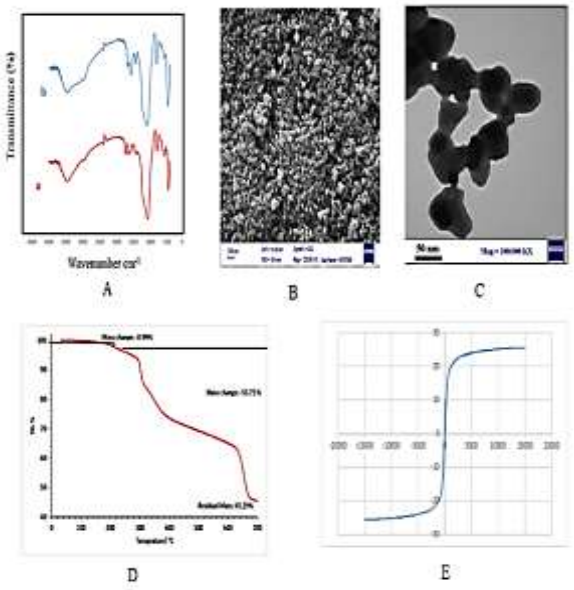

Figure 1. FT-IR spectra of Fe_3O_4 and Fe_3O_4@SiO_2@PAMAM-G2 (A), FE-SEM images of Fe_3O_4@SiO_2@PAMAM-G2 (B), TEM images of Fe_3O_4@SiO_2@PAMAM-G2 (C), TGA of Fe_3O_4@SiO_2@PAMAM-G2 (D), VSM of Fe_3O_4@SiO_2@PAMAM-G2 (E).

3.2. Optimization of eluent solvent type

In order to reach the most suitable desorption solvent, various desorption solvents such as methanol, ethanol, isopropanol and acetonitrile were investigated (n=3). According to the obtained results (Figure 2), the best desorption was done by methanol. Therefore, methanol was chosen as the desorption solvent for the next steps. At this stage, the memory effect was also examined. According to the obtained results, there is no memory effect. Each experiments were done triplicate.

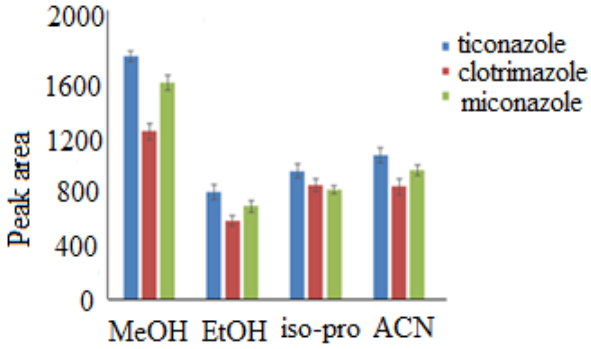

Figure 2. Effect of desorption solvent type on the extraction efficiency.

3.3. Optimization through experimental design

In order to achieve the best extraction efficiency, all effective parameters including desorption time, the amount of added salt, the pH of the sample solution, the extraction time, the volume of the desorption solvent, and the amount of sorbent were optimized.

At the beginning of the work, the identified factors were determined with the help of the Placket-Berman design. The screening and the parameters that had the highest effect on the extraction process were determined by the Minitab software. Each of the selected factors were coded at two levels: maximum (+1) and minimum (-1). Each of the factors along with the selected maximum and minimum levels can be seen in Table S1 and Table S2. After the experiments, the obtained results were investigated with the help of the analysis of variance method and finally, the effective parameters in the extraction process were determined by the Pareto chart (Figure S1). According to the results of this graph with a confidence level of 95% (Figure S1), the amount of added salt has the greatest effect on the extraction process. The next most important variables are the volume of the solvent and the amount of sorbent. Extraction time, desorption time and pH of the solution do not show a significant effect in the analyte extraction process. After the screening stage, these three parameters were considered constant in suitable values for further

optimization. pH was equal to 6, desorption time was equal to 5 min and extraction time was equal to 15 min. After finalizing the screening process and finding the effective parameters, these factors were optimized using the Box-Behnken design. The effective factors along with their levels and coded expressions are specified in Table S3. These tests, which are designed to implement the Box-Behnken design, were compiled in Table S4. According to this design, 15 experiments were conducted. The surface area of each peak related to each analyte was considered the final answer. The number of experiments is obtained by the relation $N = 2k (k-1) + C_0$, where N is defined as the number of experiments, k is the number of parameters, and C_0 is the number of central points. The results were investigated with the help of the ANOVA (Table S5). In this table, using F and p values, the importance of each factor was determined. The value of p is the probability by which the coefficient of each factor can be zero. As the coefficient of each factor increases above the value of 0.05, the effect of the corresponding factor on the model (with a confidence level of 95%) is not significant and that factor is removed. ANOVA results show that the effect of all parameters will be significant at $P<0.05$. Using the results of the BBD design, the desired line equation was obtained under the influence of each of the effective factors (1).

$$Y= 3356.67 + 511.25 A + 161.25 B - 205.00 C + 121.667 A^2 - 218.333 B^2 - 340.833 C^2 + 245.00 AC + 212.500 CB + 162.500 DC \quad \textbf{(1)}$$

In this obtained equation, the total peak area (Y) is dependent on screened parameters such as the amount of salt added (A), the volume of the eluent (B) and the amount of the sorbent (C). In addition, by using the software data, the coefficients for the BBD are given in Table S6. The obtained values confirm the good and appropriate correlation between experimental and theoretical results. Figure S2 shows the response surface plots for each pair of independent parameters. If the amount of sorbent which is used in the extraction procedure is selected appropriately, it will lead to the achievement of suitable extraction efficiency and a high preconcentration factor of the target analytes. On the other hand, the amount of extracted analytes, which can affect the efficiency of MSPE method, depends on the amount of sorbent. Now, to investigate the effect of the sorbent amount on the efficiency of extraction, the sorbent amount was changed from the lowest amount (5 mg) to the highest amount (15 mg). According to the obtained results, with an increasement in sorbent amount, the peak area increases to some extent and then decreases. The results of the experiment design showed that the sorbent amount of 5.1 mg is the optimal value of this parameter. The desorption solvent volume is one of the important factors affecting the preconcentration factor. In order to obtain a high pre-concentration factor, the effect of the desorption solvent volume on the extraction recovery of target analytes in a certain range in terms of microliters was investigated.

To investigate the effect of the desorption solvent volume on the efficiency, the desorption solvent volume was changed from the lowest value of 10 µL to the highest value of 50 µL. According to the obtained results, the efficiency of the extraction procedure increased with the increase in the volume of the desorption solvent. The results of the design of the experiment showed that the optimal volume of the desorption solvent is 50 µL. By adding the salt to the investigated solution, the decrease in the solubility of organic analytes in the sample solution is observed due to the increase in the ionic strength and as a result, the extraction efficiency is also increased. On the other hand, adding salt increases the viscosity of the solution and thus decreases the

diffusion coefficient and reduces the efficiency of extraction.

Table1. Analytical performance characteristics of the proposed method in water

Analyte	Linear range [a]	Coefficient of determination (r^2)	Precision (%)[b]						LOD	LOQ	EF	RSD %
			Inter-day			Intra-day						
			5	50	100	5	50	100				
Miconazole	1-200	0.9977	5.6	5.3	5.1	5.3	4.8	4.6	0.16	0.53	85	5.3
Clotrimazole	1-500	0.9922	5.8	5.6	5.1	5.7	5.4	5.0	0.18	0.60	89	5.8
Ticonazole	1-200	0.9871	5.7	5.3	4.9	5.9	5.6	5.3	0.14	0.46	93	4.9

[a] All concentration units are in μg L^{-1}

[b] Relative standard deviation (RSD) (n = 3).

To investigate and study the effect of adding salt to the sample solution, NaCl was added in the range of the lowest amount from 10% w/v to the highest amount of 15% w/v. The results of the experiment design showed that the added salt amount of 15% w/v is the most optimal value for this parameter.

3.4. Method Evaluation

The figures of merit of the method including the linear dynamic range (LDR), the limits of detection (LODs), the limits of quantification (LOQs), and relative standard deviations (RSDs) were obtained. To calculate the LDR, different concentrations of the selected analytes were prepared in the concentrations of 1, 2, 5, 10, 20, 50, 100, 200, and 500 μg L^{-1} and were extracted using the desired sorbent in optimal condition. Using the obtained results, clotrimazole in the concentration range of 1 to 500 μg L^{-1}, miconazole in the concentration range of 1 to 200 μg L^{-1} and ticonazole in the concentration range of 1 to 200 μg L^{-1} are linear. In addition, the coefficient of determination, which is related to the linear range of the experimental analytes, have been obtained between 0.9871 and 0.9977. LODs and LOQs were calculated according to the signal-to-noise ratio of 3 and 10 respectively.

The LODs and LOQs of the method for the target analytes in the water matrix were found between 0.14-0.18 μg L^{-1} and 0.46-0.60 μg L^{-1}, respectively. The RSDs were determined to indicate the reproducibility of the MSPE-HPLC-UV procedure in different concentration levels of 5, 50 and 100 μg L^{-1} and the results are shown in Table 1. The calculated RSDs (n=3) were in the range of 4.5-5.9%. In order to indicate the RSDs, each experiments were performed three times.

Moreover, a comparison was made between the present method, and other published extraction methods based on the obtained figures of merit such as extraction time, LOD and RSD which are summarized in Table 2. As can be observed, some variables of the developed method are better or comparable with the other reported methods. Moreover, the presented method is cheap, simple, and rapid with low maintenance and no need for filtration or centrifugation. The results confirmed that the present method has desirable RSDs% and LODs that are comparable to that of other published methods. The developed method has low LODs and wide linear ranges. The overall results confirmed that our proposed method is simple, easy, relatively fast, repeatable and sensitive. Consequently, the obtained results confirmed that the proposed method could be used successfully as an alternative for the extraction and quantification of various analytes [21-24].

Table 2. Comparison of the presented method with other MSPE methods used for the extraction and determination of target analytes.

Method	Instrument	Analytes	RSD (%)	LOD ($\mu g L^{1}$)	Ref
MSPE	LC-MS/MS	Miconazole, clotrimazole	3.1-12	0.11-0.32	21
SPE	HPLC-UV	12 azole	8.45	0.05-5.00	22
FPSE	HPLC-UV	Miconazole, clotrimazole, tioconazole	13.9	0.4-0.5	23
LLC	HPLC-UV	Miconazole, clotrimazole, tioconazole	1.2-2.5	0.05	24
MSPE	HPLC-UV	Miconazole, clotrimazole, tioconazole	4.9-5.8	0.14-0.18	This work

Table 4. The results of the obtained SR(%)

Real sample	Analyte	Added	SR (%)
Urine 1	Miconazole	2	105
	Clotrimazole	20	102
	Ticonazole	200	97
Urine 2	Miconazole	2	98
	Clotrimazole	20	96
	Ticonazole	200	95
Plasma 1	Miconazole	2	102
	Clotrimazole	20	98
	Ticonazole	200	94
Plasma 2	Miconazole	2	98
	Clotrimazole	20	96
	Ticonazole	200	102

3.5. Real Sample Analysis

To demonstrate the application of MSPE-HPLC-UV to extract and determine the amount of drugs in real samples, this method was used to determine miconazole, clotrimazole and ticonazole in urine and plasma samples. Quantitative analysis was performed using the standard addition method. In addition, to study the accuracy of the method, each of the samples were spiked at three concentration levels of 5 μg L^{-1}, 10 μg L^{-1}, and 20 μg L^{-1} of the standard samples of the analytes and spiking recovery was

calculated through the following equation:

$$\text{Spiking recovery (\%)} = \frac{C_{found} - C_{real}}{C_{added}} \times 100 \quad (2)$$

The results of measuring real samples were presented in Table 3. Moreover, as it was mentioned, the standard addition method was applied for quantification in real samples. The standard addition is also an accurate quantification method and especially useful when the matrix of the sample is complex. The advantage of standard addition method is that the sample matrix effects can be compensated. In addition, the relative recovery has been calculated at a concentrations low, medium, and higher range of linear dynamic rang. The results are as summarized in Table 4.

The chromatogram obtained by adding 10 µg L^{-1} of the standard sample including miconazole, clotrimazole and ticonazole to the urine sample is given in Figure 3. The obtained results showed that MSPE-HPLC-UV can be used as an effective alternative method to other methods for the extraction and determination of drugs in complex matrices

4. Conclusion

A new type of sorbent was synthesized based on Fe_3O_4@SiO_2@PAMAM-G_2 for simultaneous extraction and measurement of three antifungal drugs including miconazole, clotrimazole and ticonazole from urine and human plasma samples were used by the MSPE-HPLC-UV method. The driving forces of target analytes on Fe_3O_4@SiO_2@PAMAM-G_2 were primarily due to coordination bonds, intermolecular hydrogen bonding, and hydrophobic effects. On the other hand, higher extraction efficiency was obtained by multiple interactions between the adsorbent and the target analytes.

After optimizing the extraction condition for selected antifungal drugs, a relatively low detection limit, wide linear range, effective sample cleaning, acceptable accuracy and reproducibility were obtained.

The proposed method has advantages such as good precision and accuracy, simplicity and low cost. In addition, the prepared sorbent can potentially be used for more applications in the field of environment, food safety, and drug analysis.

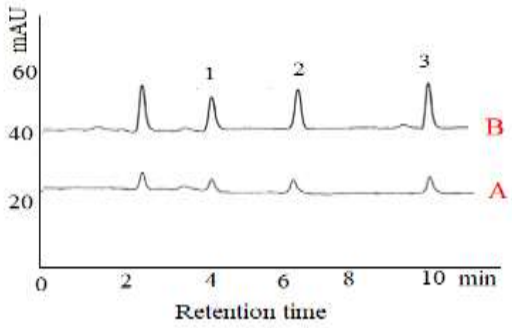

Figure 3. Chromatograms obtained after extraction of the urine sample using the presented method before (A) and after spiking of 10 µg L^{-1} of each analytes (B)

Table 3. Summary of the results from analysis of target analytes in different real samples by presented method.

	Urine 1						Urine 2					
	Measured	**Added**	**Found**	**SR (%)**	**RR[a] (%)**	**RSD% n =3**	**Measured**	**Added**	**Found**	**SR (%)**	**RR[a] (%)**	**RSD % n =3**
Miconazole	**4.9**	**5**	10.2	106	98	**5.3**	2.7	**5**	7.5	96	95	**4.9**
		10	15.0	101	95			**10**	12.5	98	94	
		20	24.6	98	94			**20**	23.0	102	93	
Clotrimazole	3.4	**5**	9.2	94	95	**6.2**	**2.5**	**5**	7.6	102	94	**5.3**
		10	14.0	97	94			**10**	12.3	98	92	
		20	24.5	101	97			**20**	22.8	101	95	
Tioconazole	2.6	**5**	7.5	98	95	**5.9**	1.6	**5**	6.2	92	90	**4.8**
		10	12.3	97	93			**10**	11.3	97	95	
		20	22.1	97	92			**20**	21.5	99	96	
	Plasma 1						**Plasma 2**					
	Measured	**Added**	**Found**	**SR (%)**	**RR[a] (%)**	**RSD% n =3**	**Measured**	**Added**	**Found**	**SR (%)**	**RR[a] (%)**	**RSD % n =3**
Miconazole	ND	**5**	4.8	96	92	**4.9**	ND	**5**	5.1	102	95	**5.6**
		10	9.7	97	90			**10**	9.8	98	96	
		20	20.6	103	96			**20**	19.8	99	95	
Clotrimazole	ND	**5**	4.8	96	94	**5.9**	ND	**5**	4.7	94	92	**6.2**
		10	9.7	97	92			**10**	10.2	102	95	
		20	20.2	101	95			**20**	19.8	99	95	
Tioconazole	ND	**5**	5.2	104	98	**4.8**	ND	**5**	4.7	94	92	**5.3**
		10	9.8	98	96			**10**	9.7	97	95	
		20	19.7	98	95			**20**	20.8	104	97	

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Conflicts of Interest

The author declares that there is no conflict of interest regarding the publication of this manuscript. In addition, the authors have entirely observed the ethical issues, including plagiarism, informed consent, misconduct, data fabrication and/or falsification, double publication and/or submission, and redundancy.

Appendixes

Appendixes appear after Conflicts of Interest.

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