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Optimization of a novel method based on solidification of floating organic droplet by high-performance liquid chromatography for evaluation of antifungal drugs in biological samples

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ABSTRACT

In this study, a simple, rapid, and highly efficient liquid-phase microextraction method based on solidification of floating organic droplet was coupled with high performance liquid chromatography-photo diode array detection (HPLC–PDA) for determination of ketoconazole, clotrimazole, and miconazole as antifungal drugs. Central composite design (CCD) was used for optimization of several factors affecting the extraction efficiency. The optimized conditions were established to be 550 rpm for stirring rate, 35 min for extraction time, 57 °C for extraction temperature, 8.5 for solution pH, 10 μl for organic solvent volume, and 7% (w/v) of NaCl for ionic strength. Limit of detections (LODs) of the extraction method ranged from 0.01 to 0.1 μg L^{-1} and the linear dynamic ranges (LDRs) ranged from 0.1 to 300 μg L^{-1} for the three antifungal drugs. Relative standard deviations (RSDs) of the proposed method were 5–11%. Preconcentration factors in the range of 306–1350 were obtained at extraction time of 35 min. Finally, performance of the proposed method was evaluated for the extraction and determination of the drugs' levels in microgram per liter in samples and satisfactory results were obtained.

1. Introduction

Many different species of fungi are beneficial to food and fermentation industries, but hundreds of them are pathogenic to plants and cause infection in humans [1]. The incidence of mycosis is mainly due to the use of immunosuppressive agents in cancer treatment and in preventing organ rejection in transplant recipients, which modulates host's defense mechanisms. The other underlying cause of mycosis is the inappropriate use of broad-spectrum antibiotics that decrease the bacterial populations competing with fungi. Therefore, development of antifungal drugs has been recently emphasized to find agents which arrive at the infection focus [2,3]. The Food and Drug Administration (FDA) has suggested several antifungal agents belonging to different chemical classes as therapeutic options for fungal infections. Among those, azole antifungal agents are the most rapidly expanding group. Inhibition of fungal growth by azole compounds was investigated in the 1940s. Imidazoles such as clotrimazole (CT), miconazole (MC), and ketoconazole (KC) are the compounds possessing five-membered ring structures containing two nitrogen atoms with a complex side-chain attached to one of the nitrogen atoms. The structures and corresponding log *P* values (octanol–water partition coefficient) of these molecules are illustrated in Fig. 1 [4,5].

The imidazole group is responsible for inhibition of ergosterol biosynthesis, which is the main sterol of the fungi wall. In the present study, three antifungal agents, ketoconazole, clotrimazole, and miconazole, are studied. KC is used in treatment of tinea infections and is a potent inhibitor of cytochrome P-450-dependent steroid hydroxylation in adrenal glands and CT has mechanism of action and activity similar to KC [6]. Also, MC is a typical antifungal drug used topically or intravenously in case of local or systemic mycotic infections [7,8]. Based on previous clinical studies, systemic CT has little efficacy and considerable toxicity compared to other systemic imidazoles (KC and MC). Thus, due to the importance of determining the level of these agents in pharmaceutical preparations and biological fluids and because of the complexity of pharmaceutical matrix, a one-step sample preparation method is required. So far, separation methods such as liquid–liquid extraction [9], ultrasonic extraction [10], acid degradation [11], solid-phase extraction [12], and supercritical fluid extraction [9,13] have been reported to be employed for extraction of these agents. Most of these extraction methods require the use of large amounts of high-purity organic solvents, which are usually hazardous, resulting in the production of toxic laboratory waste.

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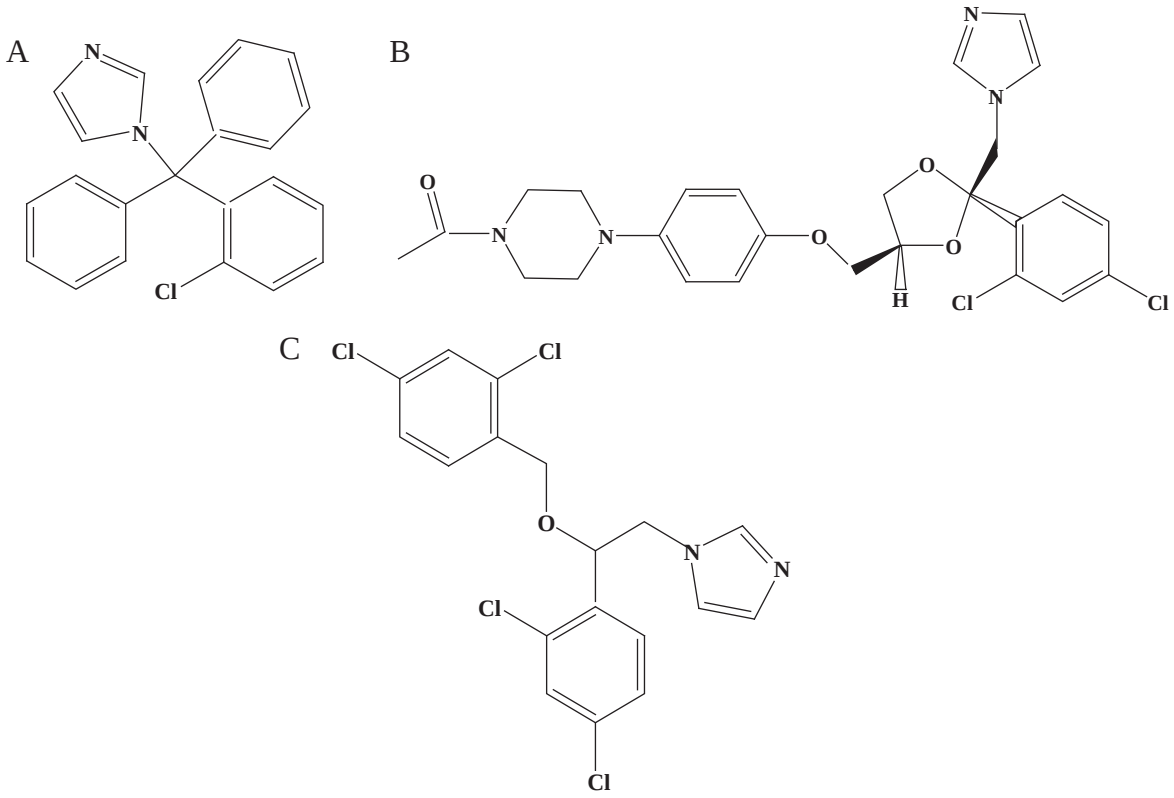

Fig. 1. Chemical structures and their corresponding log P values (A: clotrimazole (CT), pK_a = 6.02, log P = 4.1, B: ketoconazole (KC), pK_a = 6.51, 2.94, log P = 4.45, and C: miconazole (MC), pK_a = 6.5, log P = 5.69).

Also, various analytical methods have been reported to be used for determination of CT, KC, and MC level including high-performance liquid chromatography (HPLC) [14–17], capillary zone electrophoresis chromatography [18], UV–visible spectrophotometry [19], spectrofluorimetry [20], and high-performance thin-layer chromatography (HPTLC) [21].

Recently, a novel liquid-phase microextraction method called solidification of floating organic droplet solvent on solution has been developed initially for extraction of polycyclic aromatic compounds from aqueous samples [22]. This method is simple, fast, and efficient, consumes low-toxic organic solvents, and can be directly used for biological samples.

In this study, the use of a liquid-phase microextraction method based on solidification of floating organic drop microextraction (SFODME) was investigated for the extraction and determination of the three above-mentioned antifungal drugs in biological fluids (plasma and urine). Several factors such as extraction time, temperature, organic solvent volume, ionic strength, stirring rate, and solution's pH were optimized by central composite design (CCD). Then, the proposed method was successfully applied to the extraction and determination of the level of these three drugs in biological samples.

2. Experimental

2.1. Materials

The three antifungal drugs were obtained from Darou Pakhsh Pharmaceutical Company (Tehran, Iran) and were used without further purification. HPLC-grade acetonitrile, sodium acetate, sodium chloride, 1-undecanol, undecane, 1-dodecanol, cyclohexanol, hexadecane, and heptadecane were purchased from Merck (Darmstadt, Germany). The biological samples were obtained from the Pharmaceutical Center of Tehran University (Tehran, Iran) and kept in glass tubes at 4 °C in fridge. Working solutions of antifungal drugs (200 mg L^{-1}) were prepared in acetonitrile and all other standard solutions were prepared in double-distilled water and kept at +4 °C. Aqueous standard solution stability has been tested at +4 °C for 3 days. The results showed that the solution was stable in this period of time.

2.2. Instrumentation

Analysis of the standard and test samples was performed by Shimadzu SCL-10AVP HPLC instrument from Shimadzu Company (Tokyo, Japan) combined with an LC-10AVP pump, SPD-M10AVP diode array detector (DAD), a Rheodyne 7725i (PerkinElmer, USA) injector, along with a 10-μl sample loop. The Class VP program for LC was used to perform data processing. A capital HPLC column (Scotland, UK) ODS-H C_{18} (250 mm × 4.6 mm, i.d. 5 μm) was employed for all separations. The mobile phase was a mixture of sodium acetate buffer (0.01 M, pH = 4.0) and acetonitrile (10:90, v/v) running at 1 ml min^{-1} in isocratic mode with the detector's wavelength set at 212 nm. Three blank samples of standard solution were analyzed after injection of high concentration of the drugs. The results showed no memory effect in the HPLC column.

Hettich centrifuge model EBA 20 (Oxford, England) was employed for phase separation.

2.3. Extraction procedure

The pH of sample solution was adjusted in the range of 8–8.5 by stepwise addition of sodium hydroxide solution (1 N), and the ionic strength was adjusted at 7% (w/v) of NaCl. Subsequently, 8 ml of the solution was transferred to an 11-ml vial. About 10 μl of organic solvent was floated on the surface of sample solution and

Table 1
The experimental variables and levels of the central composite design.

Factors	Level			Star points ($\alpha = 2.0$)	
	Lower	Central	Upper	$-\alpha$	$+\alpha$
A: stirring rate (rpm)	200	400	600	0.0	800.0
B: solution pH	6.5	8.5	10.5	4.5	12.5
C: extraction time (min)	10	20	30	0.0	40.0
D: extraction temperature (°C)	25	40	55	10.0	70.0
E: organic solvent volume (μL)	6	10.5	15	1.5	19.5
F: ionic strength (%)	3	6	9	0.0	12.0

stirred with an 8 mm × 4 mm magnetic stirring bar for 35 min at 55 °C. After extraction, the vial was transferred to an ice bath and the organic solvent was solidified in 5 min. The solvent was then transferred to a conical vial and was allowed to melt down. The total amount of organic solvent was directly injected to HPLC using a 10-μl Hamilton syringe (USA) for analysis.

2.4. Real sample preparation

2 ml of the biological fluids (plasma or urine) was mixed with acetonitrile at 1:2 ratio. The solution was afterwards stirred for 10 min at 1200 rpm and centrifuged for 15 min at 5000 rpm. The transparent solution was transferred to a sample vial and diluted to 8 ml with deionized water. The ionic strength and pH of the solution were adjusted at 7% (w/v) and 8.5, respectively. The resulting solution was used for subsequent analysis.

2.5. Experimental design

Central composite design was used to optimize the conditions in this extraction technique. According to preliminary studies, the following six variables were selected: stirring rate, solution pH, ionic strength, organic solvent volume, extraction time, and temperature. The levels of the factors selected on the basis of the results of previous studies [23] and are shown in Table 1. As indicated in Table 2, the experiments were designed by Design-Expert software version 6 and conducted in 33 experiments (16 experiments as the cube points: $2^{(6-2)}$, 12 experiments as the star points, and 5 experiments as the center points). The star points are located at $+\alpha$ and $-\alpha$ from the center of the experimental domain. An axial distance α was selected with a value of 2 in order to establish the rotatability condition of the central composite design.

Analysis of variance (ANOVA) was used to evaluate the model and to obtain response surface methodology (RSM).

3. Results and discussion

3.1. Organic solvent selection

The type of extracting solvent is the most significant parameter in this method. The extracting solvent should possess the following properties: (1) immiscibility with aqueous solution, (2) having high boiling point, so that loss of solvent during extraction is avoided, (3) having melting point near room temperature (10–30 °C), (4)

Table 2
Order of experiments in CCD.

Std	Run	A	B	C	D	E	F
5	1	200.00	10.50	10.00	55.00	15.00	9.00
16	2	200.00	6.50	10.00	25.00	6.00	3.00
3	3	600.00	10.50	10.00	55.00	6.00	9.00
21	4	400.00	8.50	0.00	40.0	10.50	6.00
33	5	400.00	8.50	20.00	40.0	10.50	6.00
8	6	600.00	10.50	10.00	25.00	15.00	9.00
2	7	600.00	10.50	30.00	25.00	15.00	3.00
24	8	400.00	8.50	20.00	70.00	10.50	6.00
7	9	200.00	10.50	30.00	25.00	6.00	3.00
17	10	0.00	8.50	20.00	40.0	10.50	6.00
11	11	200.00	10.50	10.00	25.00	6.00	9.00
25	12	400.00	8.50	20.00	40.00	1.50	6.00
4	13	600.00	6.50	30.00	25.00	15.00	9.00
28	14	400.00	8.50	20.00	40.00	10.50	12.00
6	15	600.00	6.50	30.00	55.00	6.00	9.00
15	16	200.00	10.50	30.00	55.00	15.00	3.00
31	17	400.00	8.50	20.00	40.00	10.50	6.00
27	18	400.00	8.50	20.00	40.00	10.50	0.00
26	19	400.00	8.50	20.00	40.00	19.5	6.00
19	20	400.00	4.50	20.00	40.00	10.50	6.00
1	21	600.00	10.50	30.00	55.00	6.00	3.00
20	22	400.00	12.50	20.00	40.00	10.50	6.00
32	23	400.00	8.50	20.00	40.00	10.50	6.00
29	24	400.00	8.50	20.00	40.00	10.50	6.00
30	25	400.00	8.50	20.00	40.00	10.50	6.00
13	26	200.00	6.50	10.00	55.00	10.50	3.00
9	27	600.00	6.50	10.00	55.00	6.00	3.00
14	28	200.00	6.50	30.00	55.00	15.00	9.00
23	29	400.00	8.50	20.00	10.00	10.50	6.00
22	30	400.00	8.50	40.00	40.00	10.50	6.00
18	31	800.00	8.50	20.00	40.00	10.50	6.00
12	32	600.00	6.50	10.00	25.00	15.00	3.00
10	33	200.00	6.50	30.00	25.00	6.00	9.00

Table 3
Results of analysis of variance.

Factors	Sum of squares	DF	Mean square	*F* value	Prob > *F*	
		23				
A	110.69	1	110.69	780.25	<0.0001	Significant
B	0.34	1	0.34	2.4	0.1555	
C	2.22	1	2.22	15.67	0.0033	
D	8.61	1	8.61	60.7	<0.0001	
E	585.05	1	585.05	4124.02	<0.0001	
F	1.24	1	1.24	8.76	0.016	
AB	22.75	1	22.75	160.40	<0.0001	
AC	13.2	1	13.2	93.02	<0.0001	
AD	55.35	1	55.35	390.18	<0.0001	
AE	17.9	1	17.9	126.19	<0.0001	
BC	7.6	1	7.6	53.57	<0.0001	
BD	23.06	1	23.06	162.56	<0.0001	
BE	1.82	1	1.82	12.85	0.0059	
BF	19.26	1	19.26	135.75	<0.0001	
CD	13.64	1	13.64	96.18	<0.0001	
CE	72.29	1	72.29	509.61	<0.0001	
CF	0.5	1	0.5	3.49	0.0945	
EF	1.49	1	1.49	10.52	0.0101	
Residual	1.28	9	0.14			
Lack of fit	1.02	5	0.20	3.20	0.1414	Not significant
Pure error	0.26	4	0.064			

having lower density compared to water, and (5) showing suitable chromatographic behavior. In this work, six solvents including 1-undecanol, cyclohexanole, hexadecane, heptadecane, undecane, and 1-dodecanol were used as extracting solvents. The results demonstrated that 1-dodecanol was the best solvent.

3.2. Response surface methodology

The influence of selected parameters on calculation of the desirability function was evaluated by a central composite design. Mathematical modeling for extraction of the antifungal drugs was carried out by following equation, where the *Y* minus power indicates that the lower the curve point is, the better the extraction process occurs.

$$Y^{-0.75} = (\text{relative desirability function} + 0.01)^{-0.75}$$
$$= +2.83 - 2.15 \times A - 0.53 \times C - 1.04 \times D - 8.55 \times E - 0.39 \times F + 0.8 \times A^2 + 0.25 \times C^2 + 3.86 \times E^2 - 0.22 \times F^2 + 1.19 \times AB + 0.91 \times AC - 3.22 \times AD + 1.83 \times AE - 1.19 \times BC + 1.2 \times BD + 0.34 \times BE + 1.9 \times BF + 0.92C \times D + 2.13 \times CE - 0.31 \times EF$$

The ANOVA results indicate that all the effect of all the six factors are significant at $P < 0.1$ (Table 3). *R*-square and adjusted *R*-square (with values 0.9994% and 0.9977%, respectively) demonstrated a good correlation between experimental and theoretical results.

The six selected variables were stirring rate (A), solution pH (B), extraction time (C), extraction temperature (D), organic solvent volume (E), and ionic strength (F).

In addition to the influence of stirring rate (A), which caused an increase in mass transfer to organic solvent, the stirring rate of the solution enhanced the effectiveness and improved the extraction efficiency. Nevertheless, at higher stirring rates, the organic solvent dispersed in solution and made some problems for collecting the solvent.

The effect of solution pH (B) on extraction recovery was investigated in the pH range of 4.5–12.5. The solution pH was adjusted by either diluted hydrochloric acid or sodium hydroxide solution. It can be explained by the fact that at higher pH values ($\geq pK_a + 2$), the analytes would be ionized; hence a decrease in the molecular concentration occurred. Also, when pH = pK_a, just 50% of molecules were extracted by ionization. It should also be noted that the value of this parameter is affected by the ionic strength.

Extraction time (C) is one of the most influential parameters in this extraction method. It improved the precision and sensitivity of the technique and helped to maintain equilibrium between the aqueous sample and the organic solvent. Based on the results of ANOVA test (Table 3), by increasing the extraction time, the extraction efficiency was increased; but the extraction time was correlated with other parameters, as well. The results demonstrated that the optimal extraction time was 35 min. At longer times, the response was almost constant and no significant increase was obtained because of the achievement of extraction equilibrium between the two phases.

The effect of temperature on extraction efficiency (D) was studied by varying the temperature in the range of 10–70 °C. The results showed that by increasing temperature, extraction efficiency increased as a result of the increase in mass transfer. On the other hand, due to higher solubility of the organic solvents in the aqueous media, at very high temperatures the extraction efficiency decreased; thus 55 °C was chosen for subsequent experiments.

To examine the effect of organic solvent volume (E) on the extraction efficiency, we changed it in the range of 6–15 μl. The results showed that it has an important effect on preconcentration factor. But at large volumes, enrichment factor decreased because of the dilution effect. So, 10 μl organic solvent was selected for extraction.

Due to salting-out effect, increasing the salt content of solution improved the extraction efficiency in majority of extraction methods [22]. In order to study the influence of ionic strength on the extraction efficiency, NaCl was added to the aqueous solution in the range of 3–9 (w/v). Extraction efficiency increased with increasing salt content. Yet, the pattern reversed at higher values of ionic strength, which could be attributed to increased sample viscosity and the resulted difficulty in diffusion of analytes into the organic solvent. According to the results of the experimental design, the best amount of salt to be added was determined to be about 6.5–7.5% (w/v).

According to CCD optimization, the most influential parameters were the two-factor effects including AB, AC, AD, BC, BD, BF, and CD (A: stirring rate, B: pH of solution, C: time of extraction, D: temperature, E: volume of organic solvent, and F: ionic strength).

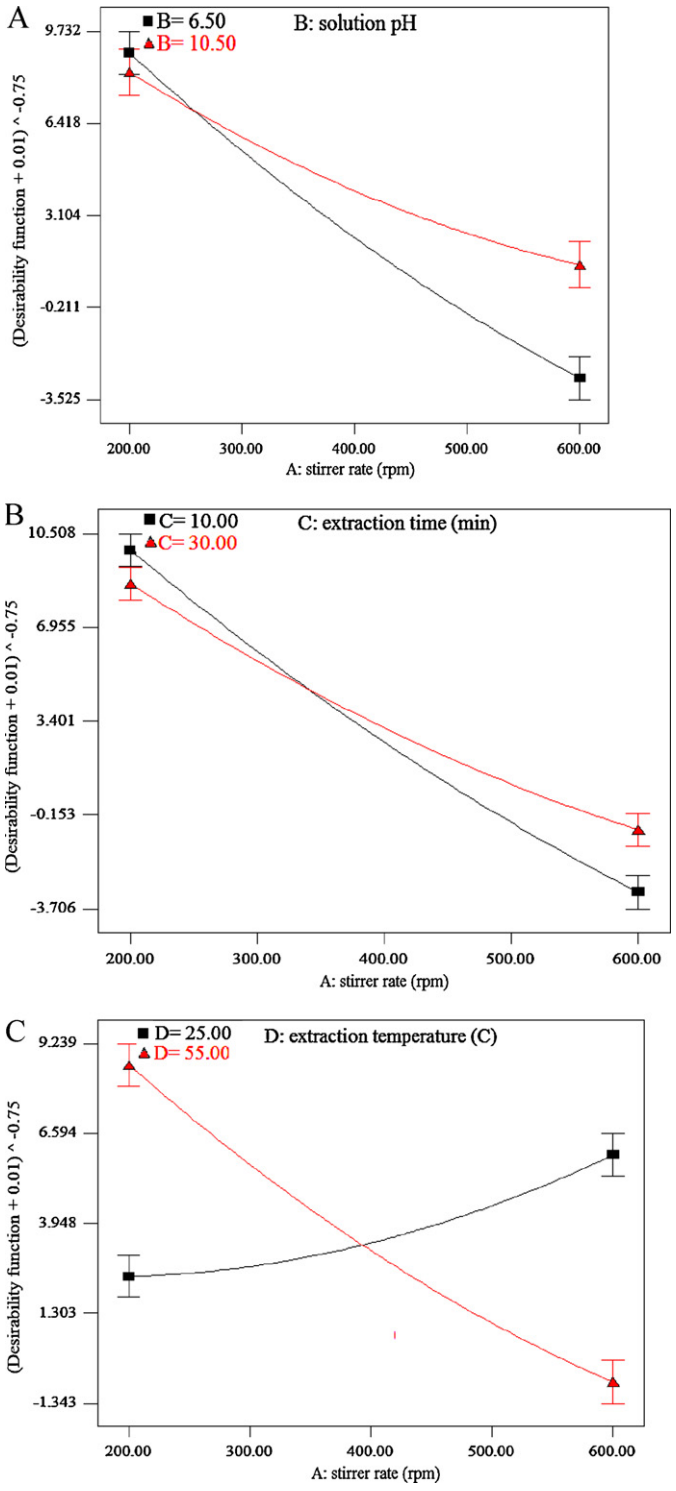

Fig. 2. The interactive effects of stirring rate and pH solution (A), extraction time (B), and extraction temperature (C) were obtained such that in each figure other parameters were constant at the best point.

Stirring rate (A) was correlated with pH of the solution (B), time (C), and temperature (D). By increasing the stirring rate in the two pH values of 6.5 and 10.5, the extraction efficiency increased (Fig. 2A). The results showed that this effect is more noticeable at lower pH. In the other words, at pH = 6.5, increasing the stirring rate caused a higher increase in the extraction efficiency than that at pH = 10.5. Similar phenomenon was observed for time of extraction (C) and stirring rate (A). Increasing the stirring rate to

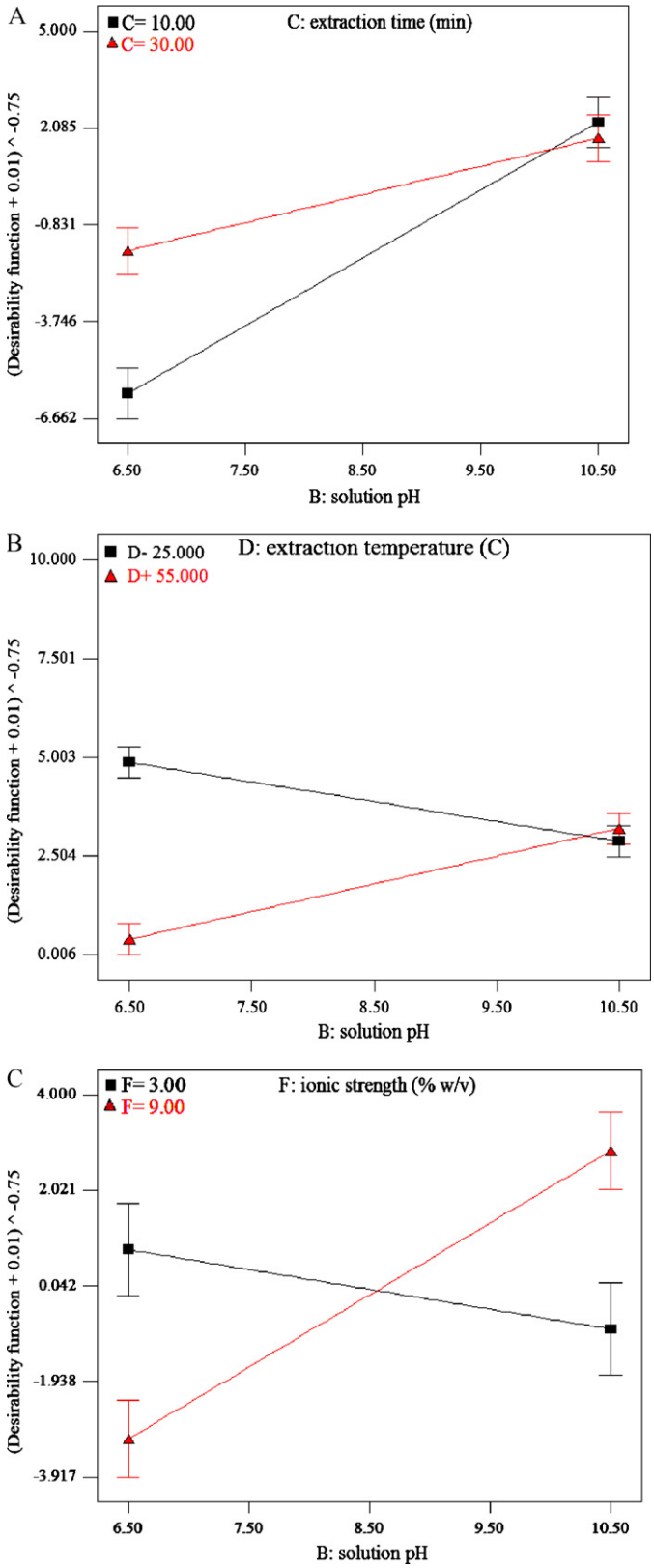

Fig. 3. The interactive effects of solution pH with extraction time (A), temperature (B), and ionic strength (C) were obtained such that in each figure other parameters were constant at the best point.

Table 4
Limits of detection, regression equations, correlation of determinations, dynamic linear ranges, and preconcentration factors for extraction method.

Analyte	Regression equation	R^2	LOD ($\mu g\,L^{-1}$)	DLR ($\mu g\,L^{-1}$)	Preconcentration factor
Ketoconazole	$Y = 98.73\,X + 0.41$	0.9955	0.014	0.1–200	306
Clotrimazole	$Y = 60.30\,X + 0.44$	0.9987	0.01	0.1–100	860
Miconazole	$Y = 11.34\,X + 0.53$	0.9875	0.1	1–300	1350

30 min and especially to 10 min caused an increase in extraction efficiency (Fig. 2B). Also, some bilateral interactions between the stirring rate and temperature (AD) was observed, indicating that with an increase in stirring rate at 25 °C, the extraction efficiency decreased, while at 55 °C the efficiency increased by enhancing the stirring rate (Fig. 2C). This observation may be related to earlier saturation of extraction solvent at lower temperature.

The solution pH (B) showed some complex correlation with extraction time (C), temperature (D), and ionic strength of the solution (F). However, at higher pH, longer extraction time led to higher extraction efficiency, and shorter extraction time resulted in lower efficiency. It can be concluded that it would be better to choose either lower values for pH and extraction time or higher values for both (Fig. 3A). The results demonstrated that at higher pH, the efficiency increased by decreasing the temperature and vice versa (Fig. 3B). As it was observed, at the highest pH value, the extraction efficiencies at both temperatures were identical. The best results may be obtained at higher temperature and lower pH. The results demonstrated that at higher solution pH, the extraction efficiency increased by decreasing the temperature and vice versa. The final influential two-factor effect related to the correlation between ionic strength and the solution pH (BF). Regarding this correlation, higher extraction yield may be obtained at higher pH and lower ionic strength or lower pH and higher ionic strength (Fig. 3C). This means that since at higher ionic strengths enough amounts of ions exist to limit the ionization, there is therefore no material with similar pH and pK_a. But in the case of lower ionic strength, the solution pH should be increased to demand the ionization process.

The results showed some correlation between the extraction time and temperature (CD) such that with increasing the extraction time, the extraction efficiency was increased. On the other hand, at higher temperatures, longer extraction time resulted in lower efficiency (Fig. 4). However, 30-min extraction time at both higher and lower temperatures was observed. These can be illustrated by slower saturation phenomenon at lower temperature and better solution followed by faster saturation at higher temperatures. So, at higher temperatures, by increasing the extraction duration, the extraction efficiency increased faster. RSM is demonstrated in Fig. 5 for some interactive effects between the factors, and in each figure other parameters were considered to be constant at their optimum points.

To summarize, the highest extraction yield may be obtained at higher time, temperature, ionic strength, and stirring rate and lower solution pH. However, the noticeable point is that with very high stirring rate, drop of organic solvent is dispersed and it is very hard to collect the solvent.

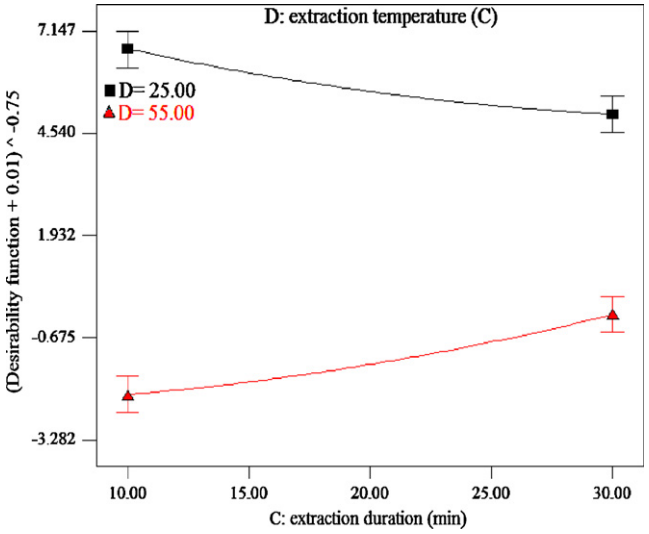

Fig. 4. The interaction effect of extraction time and temperature was obtained such that other parameters were constant at the best point.

3.3. Analytical performance

The optimized method combined with HPLC–PDA was used for extraction and determination of the level of antifungal drugs in plasma and urine samples. In another study, the schematic diagram of extraction was reported [22]. Extraction efficiency (EF) and preconcentration factor (PF) were calculated using the following equations:

$$EF = \left(\frac{C_{org}V_{org}}{C_{aq,\,initial}V_{aq}}\right) \times 100$$

$$PF = \frac{C_{org}}{C_{aq}}$$

Table 5
Determination of anti-fungal compounds in plasma and urine samples.

Sample	Analyte	C_{added} ($\mu g\,L^{-1}$)	C_{found} ($\mu g\,L^{-1}$)	RSD%	Recovery%
Plasma	Ketoconazole	50	46.8	8.6	93.6
		200	195.4	6.1	97.7
	Coltrimazole	50	44.7	9.1	89.4
		100	94.2	5.1	94.2
	Miconazole	50	40.3	10.2	80.6
		200	187.4	6.7	93.7
Urine	Ketoconazole	50	48.1	7.1	96.2
		200	196.3	4.7	98.15
	Coltrimazole	50	46.3	5.8	92.6
		100	95.3	3.4	95.3
	Miconazole	50	45.1	8.3	90.2
		200	190.7	7.3	95.35

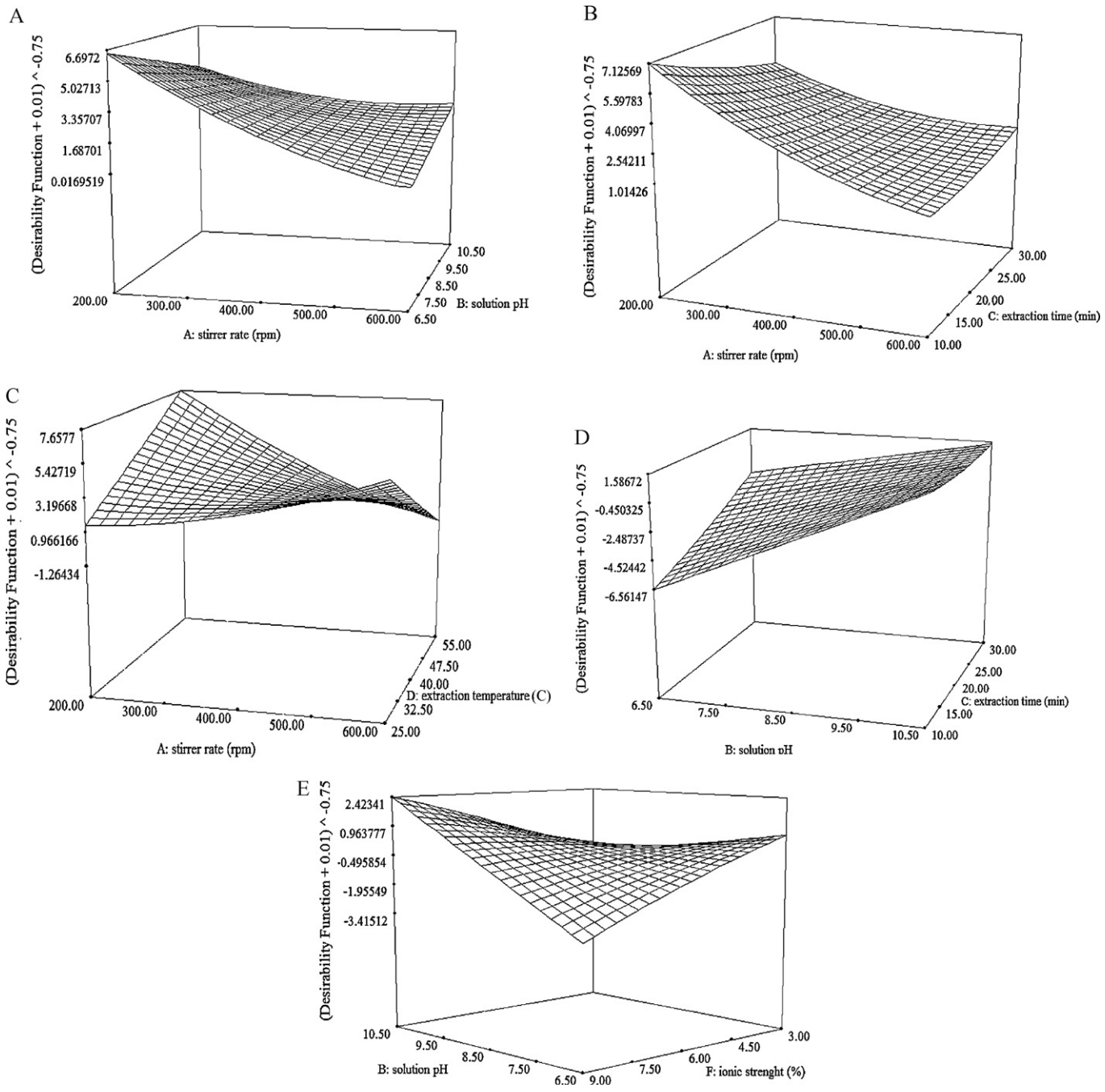

Fig. 5. RSM for some interactive effects between factors such that in each figure other parameters were constant in the best point. (A) The interactive effect of stirring rate (rpm) with solution pH. (B) The interaction effect of stirring rate (rpm) with extraction time (min). (C) The interaction effect of stirring rate (rpm) with extraction temperature (°C). (D) The interaction effect of solution pH with extraction time (min). (E) The interaction effect of solution pH with ionic strength (%).

where V_{org} and C_{org} are the volume and concentration in organic droplet extractant and V_{aq} and C_{aq} are the volume and concentration in aqueous sample solution, respectively.

The analytical characteristics of the extraction method were evaluated by calculating parameters such as calibration curve, limit of detection (LOD), limit of quantification (LOQ), precision, accuracy, correlation coefficient, preconcentration factor, and recovery under optimum conditions as listed in Table 4.

LOD was determined using equation $3S_b/m$; where S_b is the standard deviation of the blank signal and m is the slope of calibration curve after extraction, which was in the range of 0.01–0.1 $\mu g\,L^{-1}$. Also, LOQ was obtained using equation $10S_b/m$, in the range of 0.1–1 $\mu g\,L^{-1}$. Correlation coefficient (r^2) of the method was >0.98 for each of the three antifungal drugs.

In order to calculate the preconcentration factors for the abovementioned drugs, five replicate extractions were conducted for the solutions containing 0.1 $\mu g\,L^{-1}$ and 10 $\mu g\,L^{-1}$ of the drugs. It was found that under optimal conditions, preconcentration factors in the range of 306–1350 were obtained.

3.4. Analysis of antifungal drugs in plasma and urine

Plasma and urine samples were obtained from the Pharmaceutical Center of Tehran University (Tehran, Iran) and analyzed using

Table 6
Comparisons of LODs and RSDs for three anti-fungal drugs from different samples using SFODME, ultrasonic extraction, SPE, and SFE methods.

Extraction method	Ketoconazole			Clotrimazole			Miconazole			Reference
	LOD ($\mu g L^{-1}$)	RSD %	Recovery %	LOD ($\mu g L^{-1}$)	RSD %	Recovery %	LOD ($\mu g L^{-1}$)	RSD %	Recovery %	
SFODME	0.014	8.6	93.6	0.01	9.1	89.4	0.1	10.2	80.6	Proposed method
Ultrasonic extraction	About 40	<1.24	98.7<	–	–	–	–	–	–	[10]
SPE	20	3.1–6.25	81.2–98.8	–	–	–	120×10^3 (LOQ)	1.8	98.5	[12]
SFE	2310	<2.8	92.2<	420	<3.98	90.8<	120×10^3 (LOQ)	2.15	97.3	[9,13]

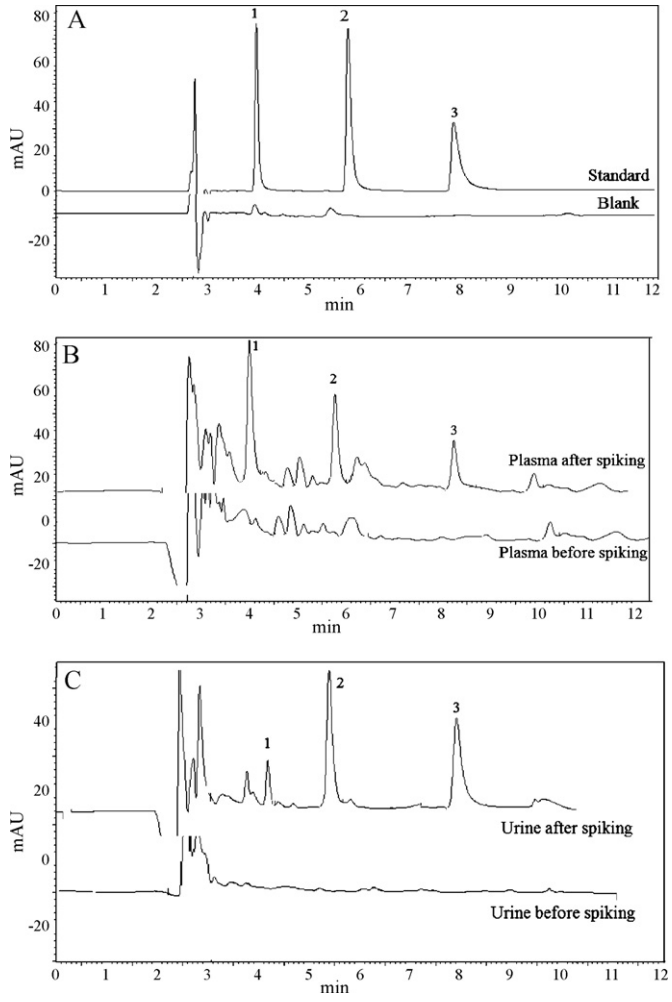

Fig. 6. (A) Chromatogram of the standard solution ($10 \mu g L^{-1}$) of anti-fungal compounds after extraction under the optimum conditions with blank solution; (1) ketoconazole; retention time = 4.04 min, (2) clotrimazole; retention time = 5.68 min, (3) miconazole; retention time = 8.12 min, (B) and (C) chromatograms of the plasma and urine samples of the Pharmaceutical Center of the Tehran University after extraction at optimum conditions.

the proposed method. Analysis of all samples showed that they were free of antifungal drugs. All of them were spiked with 50 and $200 \mu g L^{-1}$ of antifungal compounds. The amount of each antifungal drug was subsequently detected by HPLC (Table 5). Fig. 6 exhibits a chromatogram of the standard solution ($10 \mu g L^{-1}$) belonging to the antifungal drugs after extraction under optimal conditions. Chromatograms of the plasma and urine samples obtained from the Pharmaceutical Center of Tehran University after extraction under optimal conditions is shown in Fig. 6B and C.

3.5. Comparing SFODME with other methods

The proposed SFODME method was compared with other previously reported methods, i.e., LLE–SFE, ultrasonic, and SPE [9,10,12,13]. The LOD and RSD of each method are provided in Table 6. It is obvious that LODs of the proposed method had an important difference ratio compared with other methods. Moreover, SFODME method is the ultra-preconcentration technique with lower LOD.

4. Conclusion

This study was conducted to introduce and validate a novel liquid-phase microextraction method for extraction of antifungal drugs. The optimized conditions were derived using central composite design. Furthermore, influences of parameters and interactions were investigated by response surface methodology. By using this extraction method, not only the analysis of trace levels of antifungal drugs in real samples was achieved, but also a fast and easy extraction method with little consumption of organic solvent was developed which increases preconcentration factor. Also, this method can be directly carried out using all chromatographic instruments with no need to perform dilution. Finally, this extraction technique was examined for extraction of the drugs from plasma and urine samples, with best results for preconcentration factor and efficiency, as well as lower RSD.

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