

Ultrasound-enhanced surfactant-assisted dispersive liquid–liquid microextraction and high-performance liquid chromatography for determination of ketoconazole and econazole nitrate in human blood

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Abstract A simple and efficient method, based on ultrasound-enhanced surfactant-assisted dispersive liquid–liquid microextraction (UESA-DLLME) followed by high-performance liquid chromatography (HPLC) has been developed for extraction and determination of ketoconazole and econazole nitrate in human blood samples. In this method, a common cationic surfactant, cetyltrimethylammonium bromide (CTAB), was used as dispersant. Chloroform (40 μL) as extraction solvent was added rapidly to 5 mL blood containing 0.068 mg mL^{-1} CTAB. The mixture was then sonicated for 2 min to disperse the organic chloroform phase. After the extraction procedure, the mixture was centrifuged to sediment the organic chloroform phase, which was collected for HPLC analysis. Several conditions, including type and volume of extraction solvent, type and concentration of the surfactant, ultrasound time, extraction temperature, pH, and ionic strength were studied and optimized. Under the optimum conditions, linear calibration curves were obtained in the ranges 4–5000 $\mu\text{g L}^{-1}$ for ketoconazole and 8–5000 $\mu\text{g L}^{-1}$ for econazole nitrate, with linear correlation coefficients for both >0.99 . The limits of detection (LODs, $S/N=3$) and enrichment factors (EFs) were 1.1 and 2.3 $\mu\text{g L}^{-1}$, and 129 and 140 for ketoconazole and econazole nitrate, respectively. Reproducibility and recovery were good. The method was successfully applied to the determination of ketoconazole and econazole nitrate in human blood samples.

Keywords Ultrasound-enhanced surfactant-assisted dispersive liquid–liquid microextraction · Ketoconazole · Econazole nitrate · High-performance liquid chromatography · Human blood sample

Introduction

Ketoconazole and econazole nitrate are azole antifungal pharmaceuticals used for systemic and local infections. They inhibit ergosterol biosynthesis and change the composition of cell membrane lipid substances [1–4]. Clinical studies have revealed that, in the event of substantial amounts of the residues in the human body, azoles participate in interactions with many drugs [5–7] and thus pose a possible threat to human health. It is, therefore, necessary to perform therapeutic drug monitoring of azoles in human blood, for which rapid, accurate, and simple methods are indispensable.

Many analytical methods have been used to determine azole antifungal pharmaceuticals, including high-performance chromatography [8–10], spectrofluorimetry [11], capillary zone electrophoresis [12], and high-performance thin-layer chromatography (HPTLC) [13]. Because of the complexity of biological matrices and the low levels of the target analytes in the samples, sample pretreatment is required. Numerous sample-preparation methods have been proposed for extraction and preconcentration of azole residues, including liquid–liquid extraction (LLE) [14], ultrasonic extraction (UE) [15], and solid-phase extraction (SPE) [16, 17]. However, most of these require large amounts of hazardous solvents, which are potentially harmful to human health. Hence, to solve the problem, much effort is being devoted to development of miniaturized extraction techniques, for example liquid-phase microextraction (LPME) [18] and

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solid-phase microextraction (SPME) [19, 20], which not only reduce organic solvent consumption but also improve extraction efficiency. However, the fiber in SPME is quite expensive and the polymer coating is fragile, so LPME techniques, for example single-drop microextraction [21], solvent-bar microextraction [22], continuous-flow LPME [23], and dynamic LPME [24] have recently attracted increasing interest.

Very recently, a novel LPME technique, termed dispersive liquid–liquid microextraction (DLLME), was developed and introduced by Assadi et al. [25]. The method is based on a ternary component solvent system in which an appropriate mixture of organic extractant and dispersive solvents is rapidly injected into the aqueous sample by means of a syringe, resulting in a cloudy solution. In fact, the aforementioned ternary component solvent system consists of fine particles of extraction solvent which are dispersed throughout aqueous phase. This method has many advantages (simplicity of operation, speed, low cost, and high extraction efficiency), and many researchers have attempted to explore alternatives to further improve it. This led Huang et al. to develop the ultrasound-assisted dispersive liquid–liquid microextraction method (UDLLME) [26] on the basis of DLLME, shortening the extraction time by use of ultrasonic radiation. Yamini et al. used surfactant-assisted dispersive liquid–liquid microextraction (SA-DLLME) for sample preparation in the analysis of chlorophenols in water samples [27]. Surfactants are organic compounds that contain both hydrophobic and hydrophilic groups. Therefore they are soluble in both organic solvent and water. From the standpoint of solubility in both organic and aqueous phases, there is a similarity between the disperser solvent in DLLME and surfactant. Furthermore, surfactants can reduce interfacial tension and, hence, increase the contact area between the organic and water phases. So extraction efficiency can be greatly improved.

The objective of this work was to develop a simple and efficient method called ultrasound-enhanced surfactant-assisted dispersive liquid–liquid microextraction (UESA-DLLME) by combining the advantages of UDLLME and SA-DLLME. We applied the method to the extraction and determination of ketoconazole and econazole in blood samples by using high-performance liquid chromatography–diode array detection (HPLC–DAD). To the best of our knowledge, this is the first report of the development and application of UESA-DLLME for simultaneous determination of twoazole antifungal pharmaceuticals in blood samples.

Experimental

Chemical and materials

Ketoconazole and econazole nitrate were obtained from Baijinyu Pharmaceutical (Nanjing, China). Chloroform,

dichloromethane, chlorobenzene, and tetrachloromethane of analytical-reagent grade were purchased from Tokyo Chemical Industry (Tokyo, Japan). Tween 80, Triton X-100, sodium dodecyl sulfate (SDS), and cetyltrimethylammonium bromide (CTAB) were chemically pure and purchased from Sinopharm Chemical Reagent (Shanghai, China). HPLC-grade methanol and acetonitrile were obtained from Tedia, USA. Sodium chloride was procured from Zhanyun Chemical (Shanghai, China). The water used was ultrapure (Millipore Simplicity 185, Billerica, MA, USA).

Preparation of standard solutions

A mixed stock solution containing ketoconazole and econazole nitrate at $100 \mu\text{g mL}^{-1}$ was prepared in methanol. A series of standard solutions was obtained by further diluting the stock solution with diluted blank serum (300 μL blank serum was diluted to 5 mL with ultrapure water). All solutions were stored at 4°C.

Instruments

HPLC analysis was performed with an Agilent 1200 liquid chromatograph (Agilent Technologies, USA) equipped with an online degasser, a quaternary pump, a diode array detector (DAD), a 20- μL sample loop, and an analytical ChemStation. An Agilent HC⁻C18 column (250 mm \times 4.6 mm, 5 μm) was used for separation of the analytes. The mobile phase was a mixture of acetonitrile–water (75:25, v/v) and the flow rate was 1.0 mL min^{-1} . The column temperature was 25°C and the detection wavelength was 220 nm. The injection volume was 10 μL . Ultrasonic instrument KQ-100DE was purchased from Kunshan Ultrasonic Instrument (Jiangsu, China) and an 80–2 centrifuge used for phase separation was produced by Changzhou Guohua Electric Appliance (Jiangsu, China).

UESA-DLLME procedure

In the procedure, 5 mL sample solution was placed in a 10-mL glass conical tube. The pH of the sample solution was adjusted to an appropriate value (pH 6) by use of NaH_2PO_4 –NaOH buffer solution. Chloroform as extraction solvent (40 μL) and CTAB as disperser (the concentration of CTAB in the 5 mL sample solution was 0.068 mg mL^{-1}) were then added to the sample solution. The mixture was sonicated for 2 min at $25 \pm 2^\circ\text{C}$. An emulsion was formed in the conical tube. The tube was then centrifuged for 3 min at 1,500 g and the organic phase sedimented to the bottom of the centrifuge tube. The sedimented organic phase was entirely transferred into another conical tube by use of a 20- μL HPLC syringe, and then evaporated to dryness under a stream of nitrogen

(99.999%). The residue was dissolved in 40 μL acetonitrile and 10 μL was injected for HPLC analysis.

Blood sample preparation

Blood samples from five healthy people and ten patients treated with the two azole antifungal pharmaceuticals were obtained from Wuhan Eleventh Hospital. (Hubei, China). Ethical approval for the study was obtained from the Ethics Committee of Wuhan Eleventh Hospital before collection and analysis of human blood samples. The blood samples were stored at 4°C and were not further pretreated before use. In the serum analysis, 500 μL acetonitrile was added to 100 μL serum to remove the protein and some impurities. The serum supernatant (300 μL) was diluted to 5 mL with ultrapure water as the sample solution for simultaneous determination of ketoconazole and econazole. The extraction procedure was the same as that described above for UESA-DLLME.

Results and discussion

In optimization of UESA-DLLME method, different experimental conditions were investigated, for example type and volume of extraction solvent, type and concentration of surfactant, pH, ultrasound extraction time, and the salt effect. All the experiments were performed in triplicate.

Effect of type and volume of extraction solvent

Selecting an appropriate extractant is essential for UESA-DLLME. It should have low solubility in water, higher density than water, high affinity for the analytes, and should form a stable emulsion with the emulsifier under the action of ultrasound. Four solvents with these properties were tested: chlorobenzene, chloroform, dichloromethane, and tetrachloromethane. The extraction solvent creates a cloudy solution when the container is shaken in the presence of cationic surfactant. The compatibility of these solvents with the UESA-DLLME technique was studied by adding 50 μL of each solvent to 5 mL sample solution containing 200 $\mu\text{g L}^{-1}$ azoles and 0.102 mg mL^{-1} CTAB as dispersing agent. The mixture was sonicated for 2 min to complete the extraction process. After centrifugation of the cloudy solutions, 20 μL of the separated organic phase was collected for subsequent analysis. The enrichment factors (EFs) obtained by use of different solvents are shown in Fig. 1. The results showed that chloroform has the highest EFs. Therefore, chloroform was selected as extraction solvent in subsequent experiments.

The effect of chloroform volume on extraction efficiency for ketoconazole and econazole nitrate was also investigated.

In the experiment, different volumes of chloroform (30, 40, 50, 60, or 70 μL) were added to 5 mL sample solution containing 0.102 mg mL^{-1} CTAB. As shown in Fig. 2, the EFs increased with increasing extractant volume from 30 to 40 μL , and then decreased with increasing extractant volume from 40 to 70 μL . As a result, 40 μL chloroform was selected for subsequent work.

Effect of type and concentration of surfactant

The choice of surfactant is of great importance to achieving a satisfactory preconcentration and extraction of analytes. Surfactant, which serves as a disperser, could accelerate dispersion of the water-immiscible extraction solvent into the aqueous solution under ultrasound irradiation. After dispersion, the extraction solvent is dispersed as fine droplets in the sample solution, which favors mass transfer of the analytes from the aqueous phase to the organic phase. The effect of different surfactants (Tween 80, Triton X-100, SDS, CTAB) on the EFs is shown in Fig. 3. Among the surfactants investigated, SDS gave the lowest EFs for the analytes. Tween 80 and Triton X-100 gave comparable results for extraction of the analytes, and 0.102 mg mL^{-1} CTAB resulted in the highest EFs for both ketoconazole and econazole nitrate. This might be because ketoconazole and econazole nitrate are acidic and are most compatible with the cationic surfactant (CTAB). SDS is an anionic surfactant with greater hydrophilicity. Tween 80 and Triton-100 are polyoxyethylene-type nonionic surfactants. The higher hydrophilic-lipophilic balance (HLB) value means

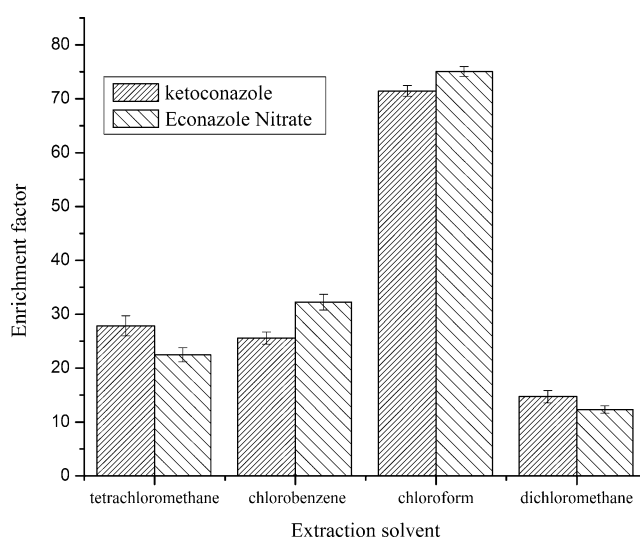


Fig. 1 Effect of extraction solvent (chloroform) on extraction efficiency. Extraction conditions: sample volume 5.0 mL, 0.102 mg mL^{-1} CTAB, room temperature, no salt addition, ultrasonic extraction time 2 min, spike level 200 $\mu\text{g L}^{-1}$. Error bars represent the standard deviation of the mean peak area for $n=3$ replicates

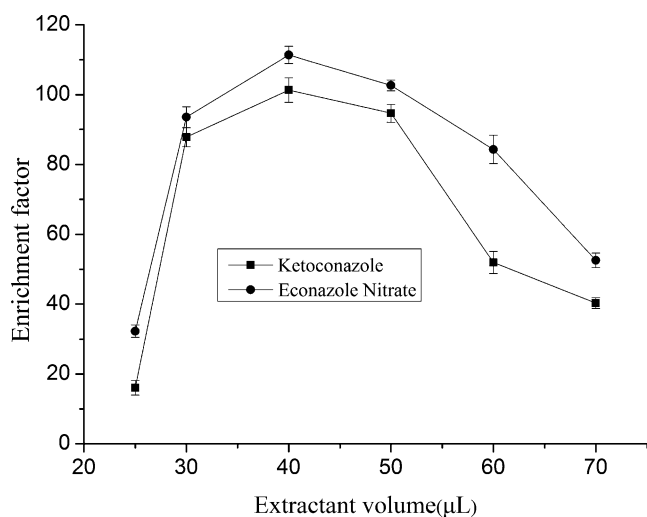


Fig. 2 Effect of extraction solvent (chloroform) volume on extraction efficiency. Extraction conditions: sample volume 5.0 mL, 0.102 mg mL⁻¹ CTAB, other conditions as for Fig. 1

greater hydrophilicity. When the HLB value of a surfactant is between 8 and 18, the surfactant can be used as a disperser. The HLBs of Tween 80, Triton X-100, CTAB, and SDS are 15.0, 13.4, 15.8 and 40, respectively. So, SDS is not suitable for use as disperser. On the basis of the experimental and analytical results, CTAB was chosen as surfactant for further studies.

The concentration of the surfactant is another important conditions that could affect extraction efficiency. The experimental results showed that if the concentration of CTAB was higher than 0.202 mg mL⁻¹ (its critical micelle

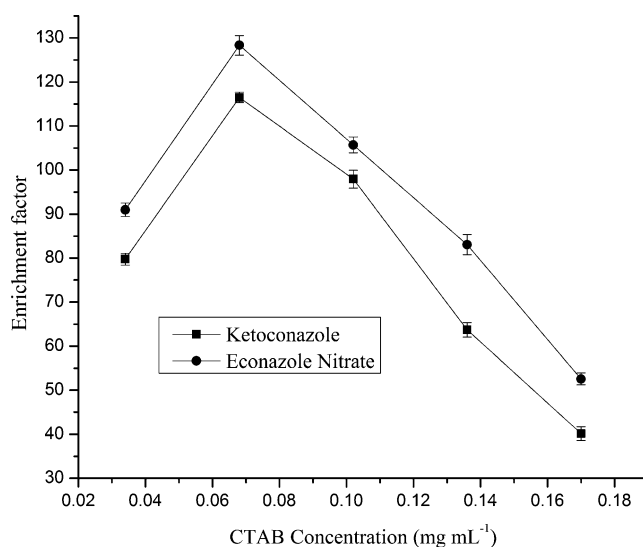


Fig. 4 Effect of the amount of surfactant on extraction efficiency. Extraction conditions: sample volume 5.0 mL, extraction solvent volume 40 μL, other conditions as for Fig. 1

concentration is 0.337 mg mL⁻¹ [27]), almost no phase separation occurred after centrifugation and no settled droplet appeared. This result occurred because the surface tension between the aqueous and organic phases decreased with increasing surfactant concentration, and the extraction droplet was soluble in the sample solution. Thus, the effect of CTAB was investigated at concentrations of 0.034, 0.068, 0.102, 0.136, and 0.17 mg mL⁻¹. As shown in Fig. 4, the EFs increased with increasing CTAB concentration from 0.034 to 0.068 mg mL⁻¹ then decreased with increasing CTAB concentration from 0.068 to 0.17 mg mL⁻¹. As a

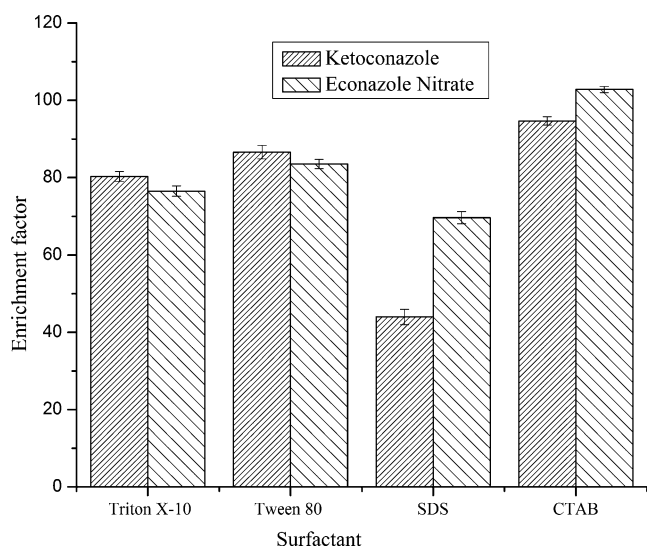


Fig. 3 Effect of surfactant on extraction efficiency. Extraction conditions: sample volume 5.0 mL, extraction solvent volume 50 μL, surfactant (CTAB, SDS, Tween 80, TX-100) volume 300 μL, other conditions as for Fig. 1

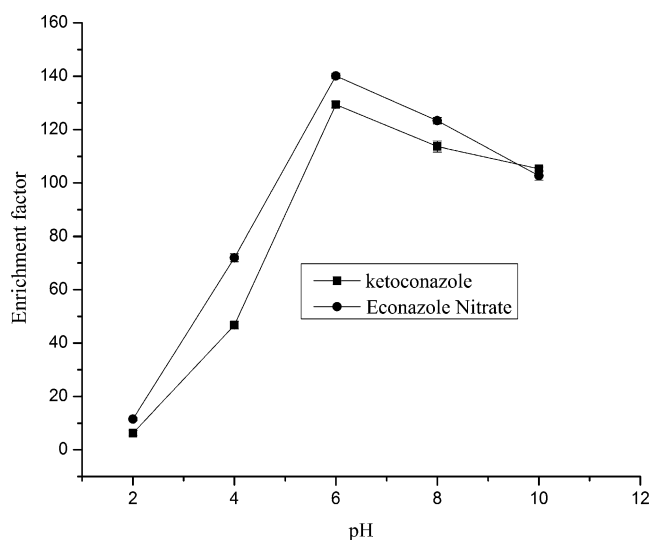


Fig. 5 Effect of pH on extraction efficiency. Extraction conditions: sample volume 5.0 mL, extraction solvent volume 40 μL, 0.068 mg mL⁻¹ CTAB, other conditions as for Fig. 1

Table 1 Linear ranges, limits of detection, and precision of the method

Analyte	Linear range ($\mu\text{g L}^{-1}$)	R	LOD ^a ($\mu\text{g L}^{-1}$)	LOQ ^b ($\mu\text{g L}^{-1}$)	RSD ^c (%) ($n=5$)
Ketoconazole	4–5000	0.9996	1.1	3.7	5.2
Econazole Nitrate	8–5000	0.9989	2.3	7.8	7.8

^a LOD, limit of detection ($S/N=3$)

^b LOQ, limit of quantification ($S/N=10$)

^c RSD, relative standard deviation

result, the most suitable surfactant concentration was 0.068 mg mL^{-1} , which was chosen for further studies.

Effect of pH

Sample pH value is important in the transfer of the target analytes into the organic phase in many LPME methods. Because of the weak alkalinity of azoles. (ketoconazole $pK_{a1}=2.9$, $pK_{a2}=6.5$, [1] and econazole ($pK_a=6.6$, [28]), and the importance of the effect of pH on their extraction, the effect of pH was investigated in the range 2–10. As can be seen in Fig. 5, the best extraction efficiency was obtained at pH 6. Enrichment factors increased as the pH was increased from 1 to 6, but decreased slightly from pH 6 to 10. It seems that both neutral and ionized azoles were efficiently extracted into the organic phase. Extraction of neutral azoles into the organic phase is reasonable, because of the normal interactions, but the extraction of deprotonated charged species looks like an interesting phenomenon which might be a result of ion-pair formation between cationic surfactant and deprotonated analytes. pH 6 was therefore chosen for subsequent studies.

Salt effect

In general, addition of sodium chloride to an aqueous solution increases its ionic strength, which reduces the solubility of the analytes in the sample solution and improves EF values. Therefore, the sodium chloride concentration was varied between 0 and 10% (w/v), and

the effect on the EF was observed. The results indicated that EFs decreased as the salt concentration increased from 0 to 10%. This might be because addition of salt led to the reduction of the solubility of the extraction solvent in the sample solution and hence reduced the EFs. Thus, no salt was added in this method.

Effect of extraction temperature and ultrasonic extraction time

Temperature can affect both mass transfer and emulsification, thus affecting extraction efficiency. Different temperatures ranging from 25 to 45°C were studied to determine the effect of extraction temperature. No significant differences were found among the results in this range of temperatures, possibly because emulsification and equilibrium of mass transfer were easily achieved under the combined effect of the ultrasound waves and surfactant. For convenience, the extraction procedure was performed at room temperature ($25 \pm 2^\circ\text{C}$).

Table 2 Recovery and reproducibility of the method

Analyte	Real ($\mu\text{g L}^{-1}$)	Added ($\mu\text{g L}^{-1}$)	RSD (%) ($n=3$)	Recovery (%)
Ketoconazole	5.10	10	3.9	80
	5.02	40	6.2	89
Econazole Nitrate	ND ^a	10	5.5	97
	ND ^a	40	6.4	75

^a ND, not detected

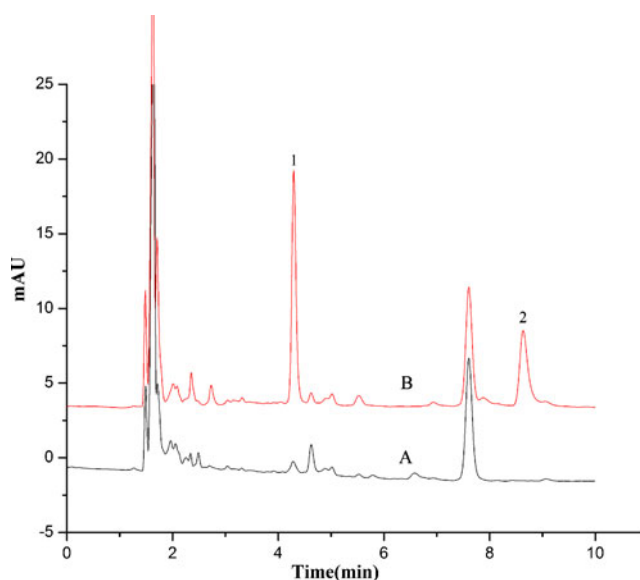


Fig. 6 Chromatograms obtained from blank serum (a) and from blood spiked with $40 \mu\text{g L}^{-1}$ ketoconazole and econazole nitrate (b)

Table 3 Results from analysis of ketoconazole and econazole in serum

Subject	Ketoconazole ($\mu\text{g L}^{-1}$)	Econazole nitrate ($\mu\text{g L}^{-1}$)
Treatment ($n=10$)	5.6–16.4	9.8–17.6
Control ($n=5$)	4.3–7.6	ND ^a

^a ND, not detected

The ultrasound extraction time is important in emulsification and mass transfer. The ultrasound extraction time was defined as the time between addition of the extraction solvent (chloroform) and the end of sonication. The effect of ultrasound extraction time on the EFs was examined by varying the ultrasound time from 0 to 10 min. The EFs increased with increasing ultrasound time in the range 0–2 min and remained almost constant after 2 min. Therefore, 2 min was chosen as the most appropriate ultrasound extraction time.

Method validation

Linearity, repeatability, LODs, and LOQs

Under the optimum conditions, working solutions containing the two azole pharmaceuticals at seven different concentrations were extracted and analyzed to construct calibration curves. The calibration data obtained are listed in Table 1. Linear calibration plots were obtained in the range 4 to 5000 $\mu\text{g L}^{-1}$ for ketoconazole and 8 to 5000 $\mu\text{g L}^{-1}$ for econazole nitrate. The limits of detection (LODs), calculated as three times the signal-to-noise ratio (S/N), were 1.1 and 2.3 $\mu\text{g L}^{-1}$ for ketoconazole and econazole nitrate, respectively. The limits of quantification (LOQs, $S/N=10$) were 3.7 and 7.8 $\mu\text{g L}^{-1}$ for ketoconazole and econazole nitrate, respectively. When the repeatability

of the method was measured by performing five parallel experiments, relative standard deviations (RSDs) ranged from 5.2% to 7.8 %. Recoveries of ketoconazole and econazole nitrate spiked at two concentrations (10 $\mu\text{g L}^{-1}$, 40 $\mu\text{g L}^{-1}$) in plasma were calculated; the results are shown in Table 2. Typical chromatograms obtained from blank and spiked blood samples treated by UESA-DLLME are illustrated in Fig. 6.

Application to real samples

UESA-DLLME was used to determine ketoconazole and econazole nitrate in human blood (control samples and treated samples). Blood samples from ten patients treated with the two pharmaceuticals and five control samples were analyzed. The results summarized in Table 3 indicate that the concentrations of ketoconazole and econazole nitrate detected in the blood of patients ranged from 5.6 to 16.4 $\mu\text{g L}^{-1}$ and from 9.8 to 17.6 $\mu\text{g L}^{-1}$, respectively. However, ketoconazole concentrations in normal people are in the range 4.3 to 7.6 $\mu\text{g L}^{-1}$, and econazole nitrate cannot be detected. This implies that ketoconazole is retained more easily than econazole nitrate by human blood.

Comparison with other methods

In Table 4 the performance of the UESA-DLLME method is compared with that of other methods reported for determination of azoles. The linearity of the UESA-DLLME method was comparable, LODs were much lower, and enrichment factors for the analytes were higher. Moreover, less organic extraction solvent is required in the proposed method. In addition, the results showed that surfactant as disperser was a favorable factor for extraction efficiency. So, UESA-DLLME was proved to be suitable for determination of ketoconazole and econazole nitrate in human blood.

Table 4 Comparison of the proposed method with other reported methods for extraction and determination of ketoconazole and econazole nitrate

Analyte	Method	Linearity ($\mu\text{g mL}^{-1}$)	LOD ($\mu\text{g mL}^{-1}$)	Extraction solvent volume	EF ^a	Ref.
Ketoconazole	Solid-phase extraction (SPE)	0.05–40	0.02	1 mL	–	[7]
Econazole nitrate	Online solid phase extraction (SPE)	–	–	8.1 mL	–	[16]
Ketoconazole	Liquid phase microextraction (LPME)	0.015–10	0.005	60 μL	–	[8]
Econazole nitrate	Liquid-liquid extraction (LLE)	0.2–0.5	0.04	5 mL	–	[14]
Ketoconazole	UESA-DLLME	0.005–5	0.001	40 μL	129	Proposed method
Econazole nitrate		0.005–5	0.002	40 μL	140	

^a EF, enrichment factor

Conclusions

In the work discussed in this paper, a simple UESA-DLLME method combined with HPLC–DAD was developed for rapid determination of ketoconazole and econazole nitrate in complex blood samples. An ultrasound-enhanced surfactant-assisted microextraction process was used to shorten the extraction time and accelerate dispersion of the extraction solvent into the sample solution, which led to enhanced extraction efficiency. The experimental results indicated that the method could provide low LOD, a good linear range, and good enrichment factors within a very short time. In addition, recovery of the analytes spiked at two levels was >80%. Therefore, the method proved to be a useful tool for rapid determination of the two azoles in clinical pharmaceutical analysis.

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