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Three-phase hollow fiber microextraction based on two immiscible organic solvents for determination of tricyclic antidepressant drugs: Comparison with conventional three-phase hollow fiber microextraction

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ABSTRACT

The aim of this research was to compare the extraction efficiencies of two modes of three-phase hollow fiber microextraction (HF-LLLME) based on aqueous and organic acceptor phases for analysis of tricyclic antidepressant (TCA) drugs. High-performance liquid chromatography with photodiode array detection (HPLC-DAD) was applied for determination of the drugs. In order to examine the ability of the new concept of HF-LLLME based on organic acceptor solvent in comparison with aqueous acceptor phase to extract the analytes, four TCAs were selected. The effect of different extraction conditions (i.e., type of acceptor phase, hollow fiber length, ionic strength, stirring rate, and extraction time) on the extraction efficiency of the TCAs was investigated and optimized using central composite design (CCD) as a powerful tool. Both methods were characterized by good linearity and high repeatability, but HF-LLLME with organic acceptor provided higher extraction efficiency and thus lower limits of detection (LODs). Calibration curves were linear ($r^2 > 0.996$) in the range of 0.2–200 $\mu g\,L^{-1}$. LODs for all the TCAs ranged from 0.08 to 0.2 $\mu g\,L^{-1}$ using HPLC-DAD. Also an improvement in sensitivity of several orders of magnitude was achieved using single-ion monitoring GC–MS analyses (0.04 $\mu g\,L^{-1}$) due to compatibility of this technique with GC instrument. The applicability of the proposed HF-LLLME/GC–MS and HPLC-DAD methods was demonstrated by analyzing the drugs in spiked urine and plasma samples. The obtained recoveries of the drugs in the range of 87.9–109.2% indicated the excellent capability of the developed method for extraction of TCAs from complex matrices.

1. Introduction

Tricyclic antidepressants (TCAs) are widely used for the treatment of a variety of depressive states and other psychiatric disorders [1]. These drugs are also frequently encountered in emergency toxicology screening, drug abuse testing, and forensic medical examinations [2–6]. Therapeutic drug measurement for antidepressant agents in biofluids is important for quality assurance in preparations and for obtaining optimum therapeutic concentrations, while minimizing the risk of overdose and adverse effects [7,8]. For example, the therapeutic concentration range for most tricyclic antidepressants is approximately 100–300 $\mu g\,L^{-1}$, while toxic effects can occur when plasma concentrations exceed 500 $\mu g\,L^{-1}$ [9]. Therefore, methods for determining the concentration of tricyclic antidepressants in human samples are required for diagnosis and effective treatment of intoxication and for forensic purposes. Several methods have been reported for the extraction of tricyclic antidepressants from human body fluids including liquid–liquid extraction (LLE) [10–13], solid-phase extraction (SPE) [14,15], and solid-phase microextraction (SPME) [16,17], before their analysis by gas chromatography (GC) [14,17], gas chromatography–mass spectrometry (GC–MS) [10,11], high-performance liquid chromatography (HPLC) [18,19], HPLC–MS [12], HPLC–MS–MS [13,20], and capillary electrophoresis (CE) [16].

Sample preparation is an essential step in the analytical procedure [21]. Especially biological samples, such as human plasma and urine, often need a pre-treatment in terms of analyte enrichment, clean-up and matrix separation. In the last years, the so-called microextraction techniques have become popular as sample preparation techniques [22]. Microextraction techniques require very small amounts of solvent (μL range) and sample (mL range) and work mostly in equilibrium mode. In three-phase hollow fiber based liquid phase microextraction methods (HF-LLLME), the hollow fiber membrane is used to separate the three liquid phases. The analytes of interest are extracted from aqueous samples, through a thin layer of organic solvent (several microlitres) immobilized within the pores of a porous hollow fiber, and into an acceptor

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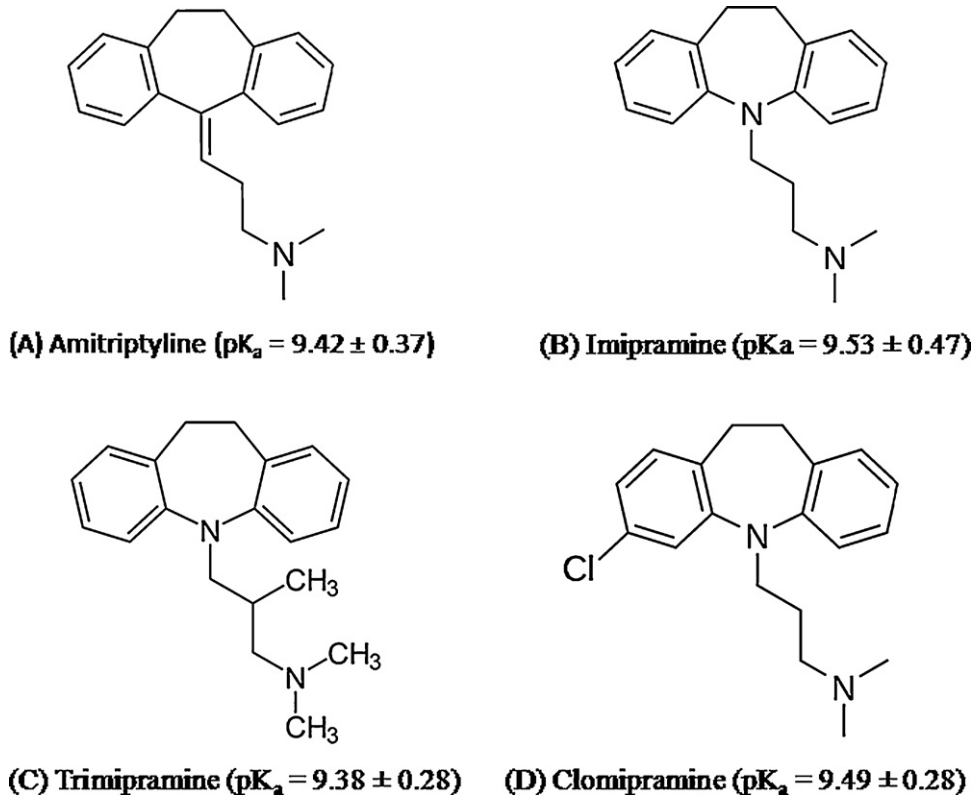

Fig. 1. Structures and calculated pK_a values of TCAs using the ACD/pK_a (ver. 1.20) software (Advanced Chemistry Development, Toronto, Canada).

solution inside the lumen of the hollow fiber. It appears that the hollow fiber decelerates the process of organic solvent dissolution into the bulk solution. The disposable nature of the hollow fiber totally eliminates the possibility of sample carryover and ensures reproducibility. In addition, the small pore size prevents from entering large molecules and particles present in the donor solution into the acceptor phase and, at the same time, most existing components in the solution do not enter the hollow fiber because of their very low solubility in the organic phase present in the pores, thus yielding very clean extracts. There are two modes of HF-LLLME. In the first mode introduced by Pedersen-Bjergaard and Rasmussen in 1999, the acceptor phase is an aqueous solution which is compatible with HPLC [23]. Another mode is based on using organic acceptor phase compatible with GC and HPLC [24]. Here, an organic solvent (*n*-dodecane) is immobilized in the pores of the hollow fiber, providing a supported liquid membrane, and the other organic solvent (acetonitrile or methanol) is filled in its lumen. The method has been successfully applied for the extraction of chlorophenols [24], polycyclic aromatic hydrocarbons [25] and azole antifungal drugs [26] from environmental and biological samples.

The aim of the present study was to systematically compare the extraction efficiency of TCAs from human urine and plasma samples using the two aforementioned modes of HF-LLLME, i.e., using aqueous and organic acceptor phases in the lumen of hollow fiber. The quantitative analyses were performed by use of HPLC-DAD and GC–MS. The design chosen to carry out optimization experiments was a central composite design (CCD) at five levels. Five operating factors were chosen as independent variables, namely, type of acceptor solvent, hollow fiber length, ionic strength, stirring rate and extraction time. With response surface methodology (RSM), the interactions of possible influencing parameters on TCAs extraction were evaluated with a limited number of planned experiments.

2. Experimental

2.1. Reagents and materials

The Q3/2 Accurel polypropylene hollow fiber was purchased from Membrana (Wuppertal, Germany). The inner diameter of the fiber was 600 μm, the thickness of its wall was 200 μm, and the pore size was 0.2 μm. HPLC-grade acetonitrile was bought from Caledon (Ontario, Canada). Analytical-grade *n*-dodecane and methanol were supplied by Merck (Darmesdat, Germany). Amitriptyline, imipramine, trimipramine and clomipramine were purchased from Razi Drug Company (Tehran, Iran). The chemical structures of these drugs are shown in Fig. 1. Each analyte was dissolved in HPLC-grade methanol to obtain a stock solution with a concentration of 1 mg mL^{-1} and stored at 4 °C. Mixtures of standard working solutions for extraction at different concentrations were prepared by dilution with water purified by a Milli-Q water purification system from Millipore Company (Bedford, MA, USA).

2.2. Instruments and apparatus

The HPLC system consisted of an Agilent (Wilmington, USA) 1200 Series quaternary pump, an injector equipped with a 20 μL sample loop, a 1200 Series diode array detector, and B.04.03 ChemStation software. An Agilent Zorbax Eclipse XDB – C18 150 mm × 4.6 mm i.d. column with particle sizes of 5 μm was used for separations. The mobile phase was acetonitrile and 50 mM potassium dihydrogen phosphate with pH 4.0 (40:60). The injection volume was 20 μL for all the samples and the detection was performed at the wavelength of 210 nm.

The gas chromatographic instrument comprised an Agilent (Wilmington, USA) 7890A GC coupled to an Agilent MSD 5975C quadrupole mass spectrometer. The GC was fitted with HP-5 MS capillary column (30 m × 0.25 mm i.d., 0.25 μm film thickness) from

Agilent J&W Scientific (Folsom, CA, USA). Helium (99.999%) was used as the carrier gas at 1.0 mL min^{-1}. The following temperature program was employed: 100 °C for 1 min, increased to 220 °C at 20 °C min^{-1}, and held for 6 min, finally increased to 280 °C at 30 °C min^{-1}, and held for 5 min. The MS quadrupole and the MS source temperatures were set at 150 and 230 °C, respectively. Data acquisition was performed in the full scan mode (m/z in the range of 50–550) to confirm the retention times of analytes and in selected ion monitoring (SIM) mode for quantitative determination of TCAs. A dwell time of 100 ms was used for each mass operated in SIM mode with high resolution. The filament delay time was set as 3 min. The monitored ions were 234 m/z (imipramine), 202 m/z (amitriptyline), 249 m/z (trimipramine), and 269 m/z (clomipramine). The injection volume to GC–MS instrument was 1 μL.

The 10 μL (model 701N), 25 μL (model 702N), and 50 μL (model 1705) syringes were obtained from Hamilton (Bonaduz, Switzerland). A magnetic stirrer/hot plate from Heidolph (Kelheim, Germany) was employed for stirring of the solutions.

2.3. Extraction procedure

For the first step, the sample solution was held in a 20 mL reagent bottle. A hollow fiber was cut manually into segments of 8 cm, and then ultrasonically cleaned in acetone for 1 min to remove any contaminations and dried in air. A 25 μL HPLC syringe was used to introduce 25 μL of acceptor solution into the hollow fiber. For the second step, 25 μL of the acceptor phase was withdrawn into the micro-syringe and its needle was inserted into the lumen of the hollow fiber. In order to support the hollow fiber, its other end was sealed with a conventional medical syringe needle. The U-shaped assembly was immersed in an organic solvent (*n*-dodecane) for 10 s in order to impregnate wall pores of the fiber with the organic solvent. It was next placed into water for 5 s in order to wash the extra organic solvent from the surface of the hollow fiber. The connected hollow fiber to both syringe needle ends that were pierced through the silicon septum in the screw cap, was immersed into the sample and then the acceptor phase (methanol) was injected carefully and completely into the lumen of the hollow fiber. During extraction, the solution was stirred at 1000 rpm. After extraction, the fiber (with the attached syringes) was taken out of the solution and the analytes-enriched acceptor phase was withdrawn into the micro-syringe and subsequently injected into the HPLC instrument.

2.4. Statistical analysis

Statistical analysis was carried out by Expert Design analysis software for Windows, version 8.0, which comprises a number of "procedures": graphical, statistical, reporting, processing, and tabulating procedures that enable simple and rapid data evaluation.

2.5. Preparation of real samples

Urine collected from a volunteer served as the real sample, which was stored at 4 °C prior to use. Frozen plasma samples were obtained from the Iranian Blood Transfusion Organization (Tehran, Iran), and stored at −20 °C. Prior to use, the plasma sample was allowed to thaw at room temperature. Aliquots (5 mL) of both types of samples were put into the centrifuge tubes, to which small amount of trichloroacetic acid was added to remove the matrix effects. The supernatant sample solutions were placed in 20 mL sample vials and adjusted to pH 12; then, ultrapure water was added to complete the volume.

Table 1
Independent variables and their levels used in the central composite design.

Preliminary experiments					
pH of donor solution	6	9	10	12	13
HCl concentration in acceptor phase (mol L^{-1})	0.1	0.01	10^{-3}	10^{-4}	10^{-5}
CCD variables					
Numerical variable	−2	−1	0	+1	+2
A – Hollow fiber length	4	6	8	10	12
B – Stirring rate	200	400	600	800	1000
C – Ionic strength	0	1	2	3	4
D – Extraction time	10	20	30	40	50
Categorical variable					
E – Acceptor solvent	Acetonitrile		Water		Methanol

3. Results and discussion

3.1. HF-LLLME optimization through experimental design

The traditional optimization procedure, i.e. varying "one variable at-a-time", is a strategy "based on experience, educated guesswork and luck" [27] that does not guarantee the attainment of a true optimum of the extraction conditions [27–30]. Conversely, the chemometric approach relies on a rational experimental design, which allows the simultaneous variation of all experimental factors, saving time and materials [30]. The central composite design (CCD) is probably the most widely used experimental design for fitting a second-order response surface [31,32]. This design has the additional capability to intrinsically confirm the results and estimate the experimental error, using orthogonal designs and built-in "hidden replication" [27]. A CCD was employed in order to derive optimal conditions for extraction of TCAs using HF-LLLME technique. In total, 81 experiments were carried out to optimize the influence of five variables: type of acceptor solvent, hollow fiber length, stirring rate, ionic strength, and extraction time. Table 1 presents the levels of the actual experimental variables that were tested and their correspondence with the coded variables of the CCD. The aims of the CCD strategy were: (i) to maximize the extraction efficiency of the selected drugs; (ii) to determine which variables have a higher impact on extraction recovery; (iii) to give an insight on the robustness of the method close to the optimum conditions; and (iv) show interactions between variables.

Preliminary experiments for the selection of the best solvent to immobilize in the wall pores of the hollow fiber dictated the preference for *n*-dodecane. This evaluation took in consideration the requisites of compatibility with the fiber so as to form a very thin organic membrane film, immiscibility with water and acceptor phase (water, methanol, and acetonitrile), promotion of analyte diffusion from the source phase into the acceptor phase through the pores of the hollow fiber, and also non-volatility to prevent solvent loss during the extraction. With regard to composition of donor and acceptor solutions, the adjustment of the pH value of both phases is essential to achieve high distribution ratio and preconcentration factor. As the target compounds are basic, the pH of the donor solution was adjusted in the proper basic range to neutralize the compounds and reduce their solubility in the sample solution. To ensure efficient analyte transfer into the acceptor phase, the pH of the acceptor solution was adjusted to be in the acidic range. This leads to ionization of the analytes and prevents them from re-entering the organic phase. For the donor phase, the pH of sample solution was varied between 6 and 13 mol L^{-1}. As illustrated in Fig. 2a, the extraction recoveries for TCAs increased with increasing pH. According to the pK_a values of TCAs (pK_a = 9.38–9.53), pH of the solution 12 with 10^{-2} mol L^{-1} NaOH (equal to pK_a + 2) would be sufficiently basic to neutralize the analytes. Therefore, pH 12 was selected for the rest of the work. For the acceptor phase (methanol), the HCl concentration was varied in the range of 0.1–10^{-5} mol L^{-1}.

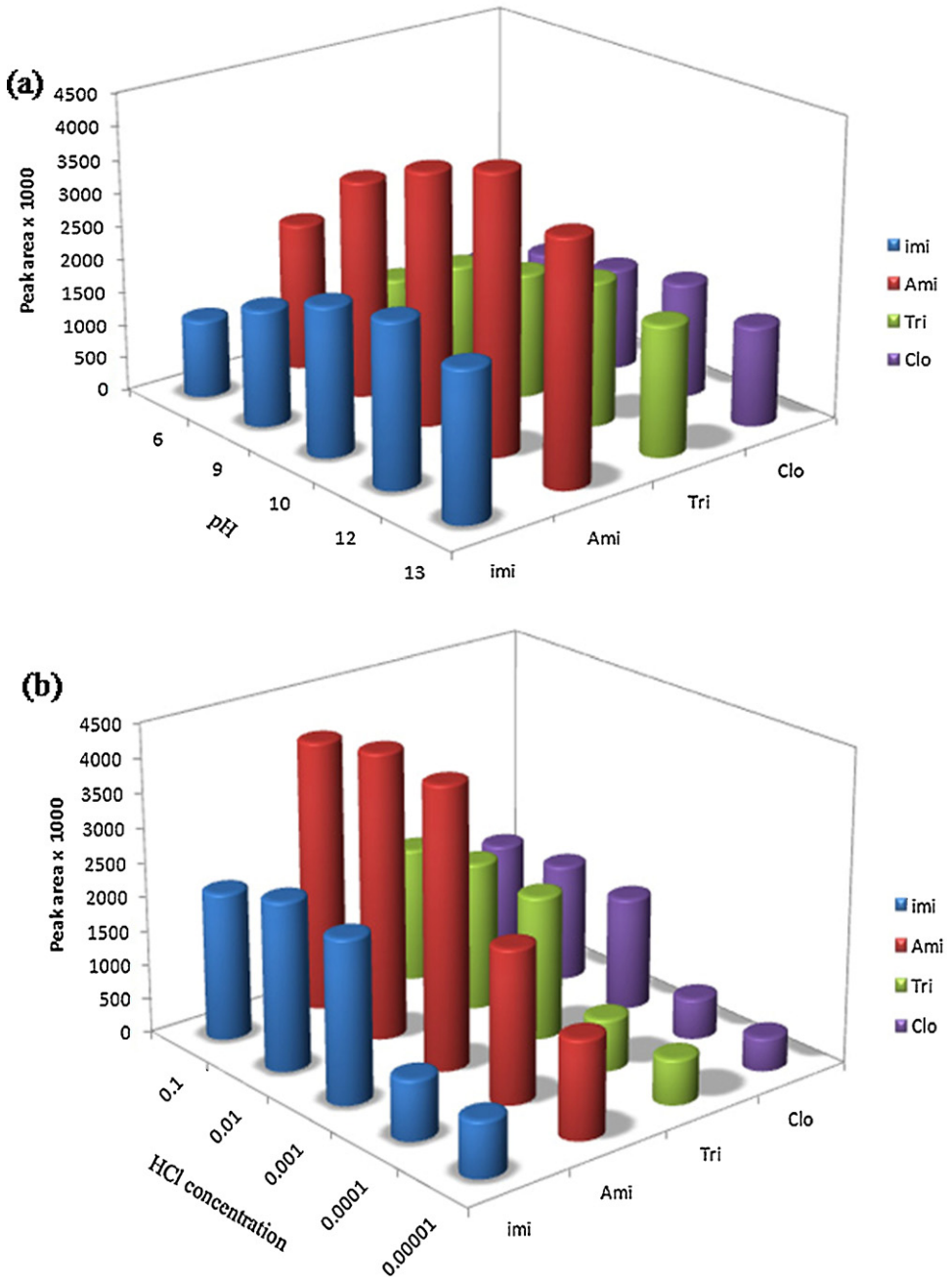

Fig. 2. Effect of the pH of (a) the source and (b) acceptor phases on the transport efficiency of drugs. *Conditions*: (a) 0.01 mol L^{-1} of HCl, stirring speed of 1000 rpm, t = 30 min; (b) 0.01 mol L^{-1} of NaOH, stirring speed of 1000 rpm, t = 30 min.

The peak areas of all the analytes increased with increasing the HCl concentration from 0.1 to 10^{-2} mol L^{-1}; after that, as the HCl concentration was decreased below 10^{-2} mol L^{-1}, the extraction recoveries were dramatically reduced. Thus, on the basis of the results in Fig. 2b, 10^{-2} M HCl was selected as the acceptor solution in the further experiments. Similar results were obtained for conventional HF-LLLME.

After performing some preliminary experiments, optimization of the extraction conditions by the proposed HF-LLLME method was conducted using a central composite design. The design consisted of three distinct sets of experimental runs: a factorial design in the factors studied, each having two levels; a set of center points, i.e. experimental runs whose values for each factor are the medians of the values used in the factorial portion, and is often replicated in order to improve the precision of the experiment; a set of axial points, i.e. experimental runs identical to the center points except for one factor, which will take on values both below and above the median of the two factorial levels, and typically both outside their range. All factors are varied in this way. The second-order polynomial equation correlating the response function with the independent variables could be established using the data provided by CCD.

$$Y = \beta_0 + \sum_{j=1}^{k} \beta_j x_j + \sum_{j=1}^{k} \beta_{jj} x_j^2 + \sum_{i<}^{} \sum_{j=2}^{k} \beta_{ij} x_i x_j$$

where Y represents the dependent variables, X_j stands for the independent variable, β_0 is the intercept term, β_j, β_{jj} and β_{ij} denote the linear, quadratic and interactive coefficients, respectively.

Table 2
A summary ANOVA table for obtained experimental responses.

Source	Sum of squares	Df	Mean square	*F*-Ratio	*p*-Value
A: HF length	0.0037	1	0.0037	0.0752	0.7848
B: Stirring rate	6.3722	1	6.3722	127.9408	<0.0001
C: Ionic strength	3.7569	1	3.7569	75.4307	<0.0001
D: Time	3.7434	1	3.7434	75.1596	<0.0001
E: Acceptor solvent	2.5655	2	1.2827	25.7550	<0.0001
AB	0.3112	1	0.3112	6.2490	0.0148
A^2	0.9429	1	0.9429	18.9312	<0.0001
B^2	1.1140	1	1.1140	22.3671	<0.0001
C^2	0.4103	1	0.4103	8.2385	0.0055
D^2	0.4941	1	0.4941	9.9201	0.0024
Residual	3.5432	69	0.0514		
Lack-of-fit	3.4109	63	0.054	2.4564	0.1284
Pure error	0.1322	6	0.0220		
Total (corr.)	21.8988	80			

The experiments were carried out according to the CCD and the data was computed as the sum of normalized responses (mmol of target analytes in the extractant) to assign an equal weight to all TCAs. An analysis of the results from the ANOVA is presented in Table 2. Using a Fisher's statistical test (*F*-test), ANOVA determined which of the factors significantly affect the response variables in the study. The significance and the magnitude of the effect estimates of each variable as well as all the possible linear and quadratic interactions among the response variables were determined. The effect estimate of each variable represents the improvement in the response variable, which is expected as the value of the variable is changed from low to high. Effects with a significance value less than 95%, i.e. effects with a *p*-value higher than 0.05, were discarded and pooled into the error term (often called residual error) and a new analysis of variance was performed for the reduced model. Note that the *p*-value represents a decreasing index of the reliability of a result. The lack-of-fit test is useful to determine whether the selected model is adequate to describe the observed data or whether a more complicated model should be used. The test is performed by comparing the variability of the current model residuals with the variability between observations at replicate setting of the factors. Since the *p*-value for lack-of-fit in the ANOVA table was greater than 0.05, the model appears to be adequate at a 95% confidence level.

It was observed that stirring rate (*B*) and extraction time (*D*) have a strong positive linear effect. The volume of acceptor phase was studied using various hollow fiber lengths (*A*) that showed no significant and positive linear effect on the extraction efficiency. There was also negative quadratic effect of hollow fiber length, indicating that the response value increases with the increase in hollow fiber length, then it reaches a maximum, and finally decreases at higher lengths of the hollow fiber. It is known that the volume of the acceptor phase should be large enough to promote analyte transport to the acceptor phase. Generally, an increase in acceptor volume enhances the extraction efficiency as much as it does not lead to dilution of extractant. For stirring rate, the extraction can be accelerated by stirring the aqueous sample because of the decreased thickness of the Nernst diffusion layer as well as the continuous exposure of the extraction surface to fresh aqueous sample. However, the extraction recovery raised as the stirring rate of sample solution increased in the range of 200–1000 rpm. The higher rates were not evaluated because of generation of excessive air bubbles that adhered to the surface of the hollow fiber, making the experiments difficult to control, and thus leading to poor reproducibility. For extraction time, the amounts of extracted analytes were increased dramatically by increasing exposure time from 10 to 40 min and thereafter the curves became flat. On the other hand, if the extraction time is long, solvent loss and formation of air bubbles may occur, which would decrease the extraction efficiency.

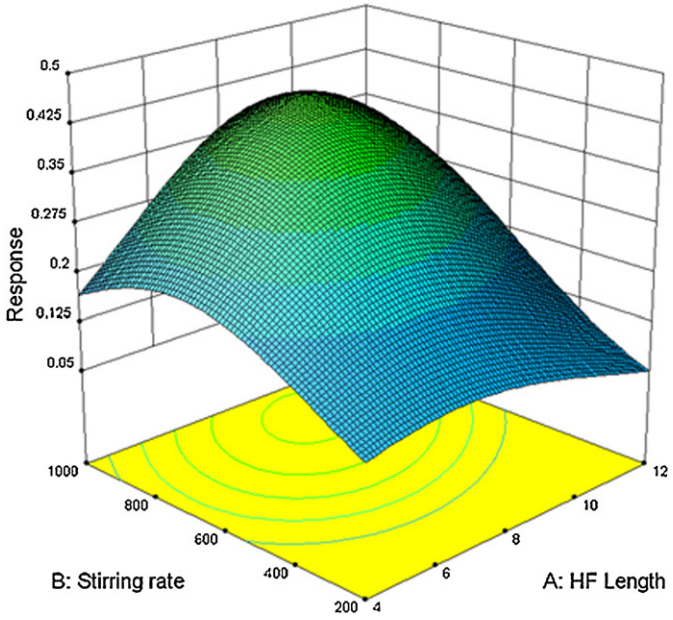

Fig. 3. Response surface from central composite design.

Addition of NaCl to the sample solution may have several effects on LPME as it may change the activity coefficients of the analytes in the aqueous phase and, in this way, improve the extraction efficiency. Nevertheless, the change in the physical properties of the Nernst diffusion film could negatively influence the kinetics of the process and, consequently, the extraction efficiency. The results exhibited that salt addition has significant negative linear effect on the response in the range of 0–4 mol L^{-1}. This may be due to the increase in viscosity that in turn decreases the mass transfer of the analyte to the organic membrane solvent.

It is interesting to note that the type of acceptor solvent has prominent effect on the extraction efficiency of TCAs. This phenomenon may be due to formation of a thinner Nernst diffusion layer between organic acceptor (methanol or acetonitrile) and *n*-dodecane than aqueous acceptor and *n*-dodecane because of possessing less hydrophilicity properties in comparison with water. Therefore, the mass transfer of analytes from organic membrane to immiscible organic acceptor phase was higher than that to the aqueous acceptor. The results were shown that the maximum extraction efficiency was obtained using methanol as acceptor phase. As demonstrated in Table 2, the magnitude of the significant effects on extraction efficiency of TCAs increased in the following order:

acceptor solvent type < extraction time < ionic strength < stirring rate

Significant interactions were noted between stirring rate and hollow fiber length. The effect of stirring rate–hollow fiber length interaction can be observed in Fig. 3. In general, at low to high stirring rates, the extraction efficiency of TCAs increased and then decreased when increasing the length of hollow fiber. Nevertheless, the influence of stirring rate is large when its values vary between low to high. According to the overall results of optimization study, the following experimental conditions were chosen: acceptor solvent: methanol; stirring rate: 1000 rpm; hollow fiber length: 8 cm; and extraction time: 40 min, without addition of salt. Also, the model generated by multiple nonlinear regression has the coefficient of determination of $R^2 = 0.9147$, that indicates a high degree

Table 3
Performance of HF-LLLME based on two immiscible organic solvents (Org) and aqueous acceptor phase (Aqua).

Analyte	Linear range ($\mu g\,L^{-1}$)		r^2		LOD ($\mu g\,L^{-1}$)[a]		LOD ($\mu g\,L^{-1}$)[b]		*PF*		RSD% ($n=5$)		ER (%)	
	Org	Aqua	Org	Aqua	Org	Aqua	Org	Aqua	Org	Aqua	Org	Aqua	Org	Aqua
Imipramine	0.5–200	5–200	0.997	0.998	0.1	0.5	0.04	–	675.8	315	4.4	5.1	67.6	68.0
Amitriptyline	0.2–200	5–200	0.997	0.997	0.08	0.5	0.04	–	680.2	313	4.6	4.9	68.0	68.0
Trimipramine	0.5–200	5–200	0.998	0.997	0.1	0.5	0.04	–	690.5	291	6.3	5.8	69.1	65.6
Clomipramine	0.5–200	5–200	0.996	0.996	0.2	0.8	0.04	–	629.5	265	6.8	6.3	63.0	58.2

[a] The obtained LODs using HPLC-DAD.
[b] The obtained LODs using GC–MS.

Table 4
Comparison of the proposed method with other methods applied for the extraction and determination of TCAs.

Method	LOD ($\mu g\,L^{-1}$)	LDR ($\mu g\,L^{-1}$)	*PF*	RSD%	Ref.
SPE/LC–MS–MS	0.1–1	10–1000	–	<20	[33]
HF-LPME/HPLC-UV	0.5–0.7	5–500	298–315	12	[34]
In-tube SPME/LC–MS	0.06–2.95	1–500	3.5–7.5	<16.5	[35]
MAE/HPLC	10–50	40–2000	–	<4.34	[36]
SBSE/HPLC-UV	10–40	10–1000	–	<15	[37]
F-LLLME					
HPLC-DAD	0.08–0.2	0.2–200	630–690	<6.8	Present work
GC–MS	0.04				

of correlation between the response (mmol of target analytes in the extractant) and model.

3.2. Analytical performance

To evaluate the usefulness of HF-LLLME/HPLC-DAD for quantitative analysis of TCAs in biological matrices, the analytical performance of the proposed method was studied and validated in terms of linearity, limits of detection, and precision of the technique. Calibration curves were found to be linear in the range of 0.2–200 $\mu g\,L^{-1}$. Values for the coefficient of determination, r^2, were >0.996 for the four analytes. The method's limits of detection (LODs) were estimated by extraction of the drugs from spiked water samples with low concentration levels and injecting them into the instrument to give a signal to noise ratio of 3. Good detectability in the range of 0.08–0.2 $\mu g\,L^{-1}$ was obtained adopting the developed method. The repeatability of the peak areas was studied for five-replicate experiments by spiking ultrapure water with TCAs at a concentration level of 5 $\mu g\,L^{-1}$. The relative standard deviations (RSDs) for the TCAs were below 6.8%, indicating the good repeatability achieved by the procedure. Based on the optimum conditions, TCAs were typically preconcentrated by factors in the range of 630–690 from aqueous samples. These *PFs* corresponded to extraction recoveries (ERs) of 63–69% (Table 3). An improvement in detection limits of the methodology could be achieved using GC–MS analyses due to compatibility of this technique with gas chromatography instrument. All analyses were performed in SIM mode, thereby increasing the sensitivity for each analyte, as well as lowering the detection limits to ppt levels. The LODs calculated at a signal to noise ratio of 3 were 0.04 $\mu g\,L^{-1}$ for all of the TCAs. Also the figures of merit of the conventional HF-LLLME followed by HPLC-DAD method for extraction and determination of the drugs are tabulated in Table 3 for comparison. One can see by using the new HF-LLLME procedure better results were obtained in comparison with conventional HF-LLLME method.

A comparison between the figures of merit of the proposed method and some of the published methods for extraction and determination of TCAs was made, whose results are summarized in Table 4. Clearly, the proposed method has a good sensitivity, preconcentration factor, and precision with a suitable dynamic linear range, especially in comparison with HF-LLLME based on aqueous acceptor phase [34]. All of the results reveal that the developed method is not only a good sample preconcentration technique but also an excellent sample clean-up procedure that can be used for the ultra-trace analysis of TCAs in real samples.

3.3. Application of the proposed method to real samples

To further investigate the proposed method for real sample analysis, the urine sample containing the drugs was obtained from

Table 5
Results obtained from analysis of real samples.

Compound	Added concentration	Urine			Plasma		
		Found[a]	RR%[b]	RSD% ($n=3$)	Found[a]	RR%[b]	RSD% ($n=3$)
Imipramine	0	42.3_5	–	5.2	Not detected	–	–
	20	60.8_8	92.7	5.0	21.2_8	106.4	4.2
	40	78.4_3	90.2	4.6	37.2_4	93.1	3.5
Amitriptyline	0	Not detected	–	–	Not detected	–	–
	20	19.6_0	98.0	3.8	18.6_9	93.5	4.9
	40	43.6_8	109.2	4.5	37.6_0	94.0	4.6
Trimipramine	0	Not detected	–	–	Not detected	–	–
	20	20.5_1	102.6	5.5	19.1_2	95.6	5.8
	40	37.7_2	94.3	6.3	36.2_8	90.7	5.1
Clomipramine	0	Not detected	–	–	Not detected	–	–
	20	18.4_3	92.2	4.8	17.6_6	88.3	6.5
	40	35.7_6	89.4	5.9	35.1_6	87.9	7.2

[a] All concentrations are in $\mu g\,L^{-1}$.
[b] Relative recovery.

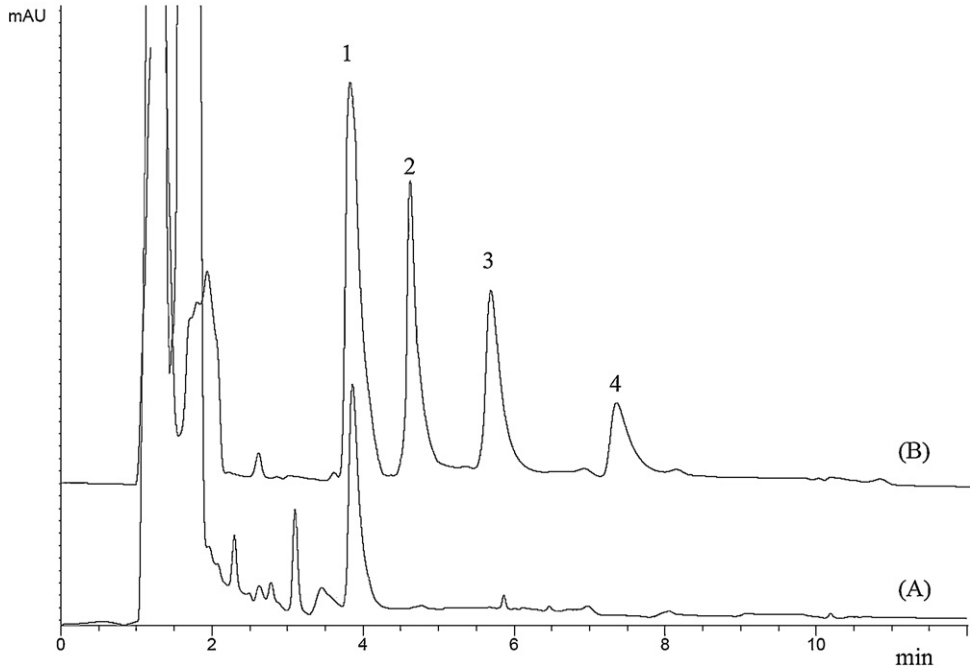

Fig. 4. Chromatograms of TCAs after extraction from the urine sample using HF-LLLME combined with HPLC-DAD: (1) imipramine, (2) amitriptyline, (3) trimipramine, and (4) clomipramine. (A) Urine sample before spiking, and (B) the spiked urine sample at concentration level of 40.0 $\mu g\,L^{-1}$.

a patient of the Mental Health Department (Tehran, Iran) and analyzed using HF-LLLME followed by HPLC-DAD. Also, drug-free human plasma was obtained from Iranian Blood Transfusion Organization (Tehran, Iran). In general, and for all the samples analyzed, the presence of TCAs was confirmed by GC–MS in MS-only mode using the peak area from the extracted ion chromatograms (EIC) of the base peak ion using a mass window of 0.1 Da. Table 5 summarizes the founded concentrations of TCAs in each of the real samples. The relative recoveries, which may present the matrix effect, were calculated by comparing the peak area ratios of TCAs

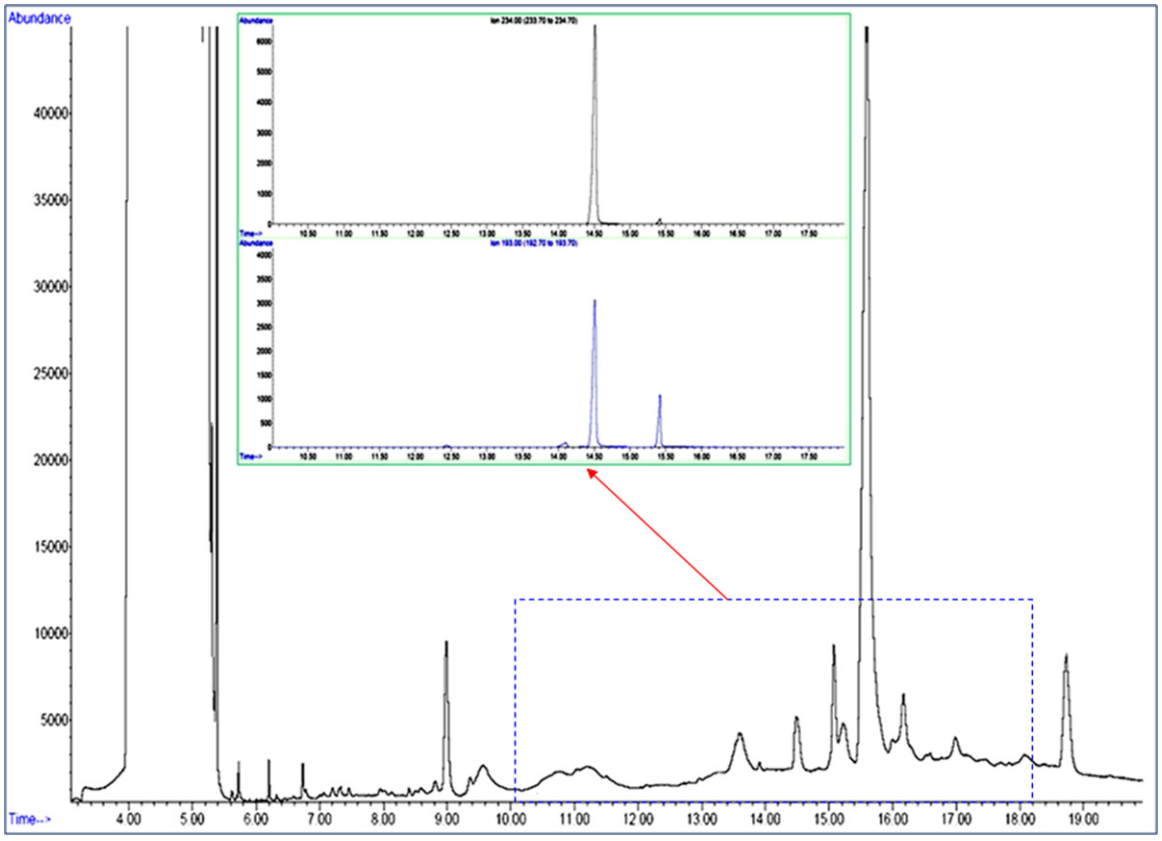

Fig. 5. Identification of imipramine using extracted ion chromatograms of 193 and 234 m/z from GC–MS chromatogram of TCAs after extraction of the urine sample.

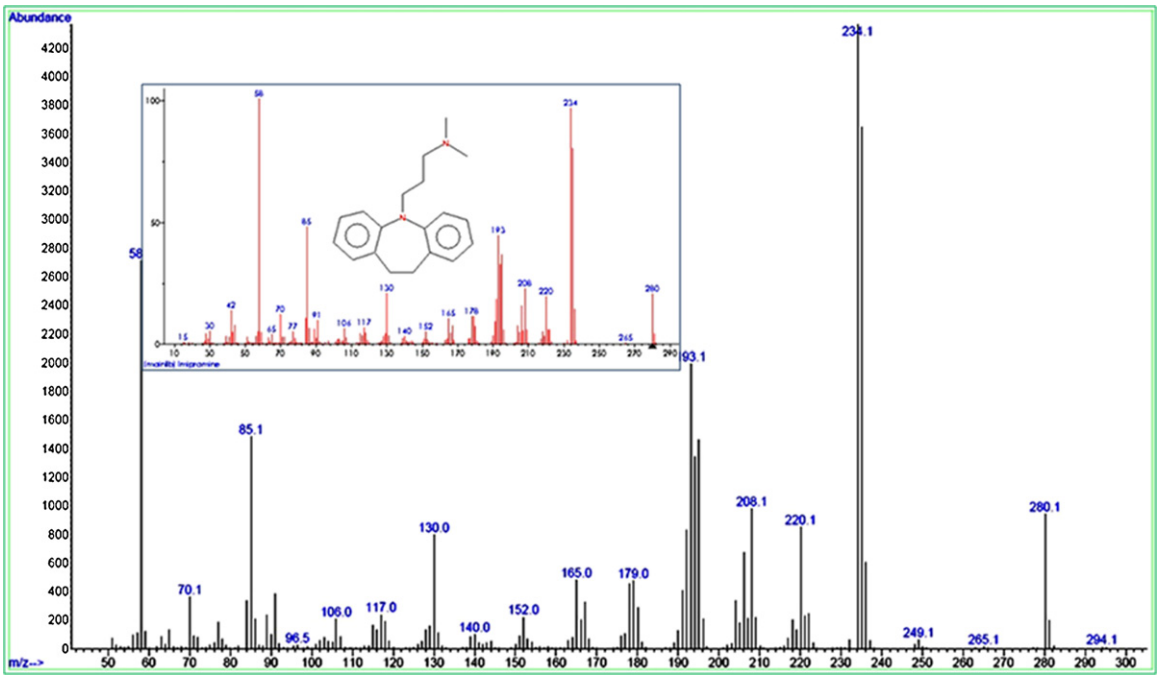

Fig. 6. Mass spectra of detected imipramine in urine sample. *Note*: Insert on the center of main figure represents reference mass spectra of imipramine.

from the spiked urine and plasma sample to those obtained from the working standard solutions at the same concentration. To eliminate possible matrix effects, small amount of trichloroacetic acid was added to the real samples and the quantitative determination of the TCAs was performed on the obtained supernatant solution after centrifugation. The relative recoveries of the method were in the range of 89.4–109.2% for urine and 87.9–94.0% for plasma samples, indicating the good performance of the presented method for determination of the TCAs in complex matrices. Fig. 4 shows the typical chromatograms of the extracted TCAs from urine sample before and after spiking with 40.0 $\mu g\,L^{-1}$ of TCAs. The findings were verified by GC–MS analysis based on extracting ion chromatograms from real samples. Fig. 5 illustrates the chromatogram for a urine sample, showing the presence of imipramine in the extracted ion chromatograms. Furthermore, the existence of imipramine in the urine sample was confirmed using GC–MS (Fig. 6).

4. Conclusions

The present study demonstrated the feasibility of using two immiscible organic solvents for HF-LLLME method in combination with HPLC-DAD and GC–MS for extraction of TCA drugs from plasma and urine samples. The data herein represents the higher efficiency of HF-LLLME based on two immiscible organic solvents compared to using an aqueous acceptor phase. Features of the method include its simplicity, desirable sensitivity, selectivity and analytical precision, low consumption of organic solvent, low cost, and short sample preparation time. The other compelling analytical feature of the method is its compatibility with GC instruments due to using organic acceptor solvent. Finally, the advantages of hollow fiber-protected LPME allow its potential application as a sample preparation and clean-up technique for drug analysis in biological samples.

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