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Three-phase hollow fiber liquid-phase microextraction based on two immiscible organic solvents for determination of tramadol in urine and plasma samples

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ABSTRACT

Recently, the new concept of three-phase liquid microextraction was introduced based on applying two immiscible organic solvents in lumen and wall pores of hollow fiber. In the present work, this novel microextraction technique combined with gas chromatography–mass spectrometry has been developed for determination of tramadol, an analgesic agent, in plasma and urine samples. A systematic investigation of the proposed method was applied to find optimal extraction conditions and evaluate the interaction effects between the factors by designing experiments according to the methodology of Box–Behnken response surface design. Analysis of variance (ANOVA) revealed that the important factors contributing to extraction efficiency are extraction time, stirring rate and hollow fiber length. Under the optimum conditions, the developed method provided a preconcentration factor of 546, good repeatability (RSD % = 6.4), and good linearity ($r^2 = 0.995$) for spiked plasma and urine real samples. The linear dynamic range from 0.1 to 400 $\mu g\,L^{-1}$ and limit of detection (LOD) of 0.08 $\mu g\,L^{-1}$ were obtained under selected ion monitoring mode. The results demonstrated that three-phase hollow fiber microextraction based on two immiscible solvents is a simple and accurate technique with very good preconcentration factor and clean-up for extraction of tramadol from biological samples.

1. Introduction

Tramadol hydrochloride, (1RS, 2RS)-2-[(dimethylamino) methyl]-1-(3-methoxyphenyl) cyclohexanol HCl, is a centrally acting opioid analgesic in widespread clinical use throughout the world [1]. Tramadol like other narcotics used for the treatment of pain, may be abused. Its therapeutic plasma concentration is in the range of 100–300 $\mu g\,L^{-1}$ [2].

Several analytical methods such as gas chromatography (GC) coupled to nitrogen selective or even mass-spectrometry detection [3], capillary electrophoresis [4] and high performance liquid chromatography (HPLC) with electrochemical [5], mass spectrometry [6] or fluorescence detectors [7] have been introduced for separation and determination of tramadol. Usually, an initial sample preparation step is essential for isolation and preconcentration of tramadol in biological samples prior to its final analysis. Generally, liquid–liquid extraction (LLE) [8] and solid-phase extraction [9] are widely used as sample preparation step to extract tramadol from biological samples. Recent research activities are oriented toward the development of efficient, economical, and miniaturized sample preparation methods. As a result, solid-phase microextraction (SPME) [10] and liquid-phase microextraction (LPME) [11,12] have been developed to extract tramadol from various matrices. SPME is a simple and solventless extraction technique that has proved to be a powerful alternative to traditional extraction techniques. However, SPME fibers are fragile and relatively expensive and tend to degrade with repeated use. LPME is an emerging technique based on the use of a small amounts of organic solvents to extract analytes from aqueous matrices. Subsequently, Pedersen-Bjergaard and Rasmussen developed hollow fiber-protected LPME based on the principle of a supported liquid membrane (SLM) which is an improved type of LPME [13].

The aim of this study was to develop and evaluate an alternative approach to three-phase hollow fiber liquid-phase microextraction (HF-LPME) based on using two immiscible organic solvents for extraction of tramadol from plasma and urine samples. This technique can be a complement to traditional HF-LPME previously presented by Pedersen-Bjergaard and Rasmussen [14]. In this technique, the three phases involved are donor aqueous solution, a very small volume of organic solvent immobilized in the pores of the hollow fiber (*n*-dodecane), and a small volume of another organic solvent inside the lumen of hollow fiber (acetonitrile or methanol). This methodology is compatible with most of analytical instruments such as GC, HPLC, CE and electrothermal atomic absorption spectrometry (ET-AAS) because of superfine chromatographic behaviors of acetonitrile and methanol as well as their sufficient volatility to avoid the interference effect during analysis by ET-AAS.

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2. Experimental

2.1. Reagents and apparatus

The pure substance of *trans*-tramadol was purchased from Grünenthal (Stolberg, Germany). HPLC grade acetonitrile was bought from Caledon (Ontario, Canada). Analytical grade *n*-dodecane and methanol were supplied by Merck (Darmstadt, Germany). Ultrapure water was provided by a Milli-Q water purification system from Millipore Company (Bedford, MA, USA).

Models 701N-10 μL and 702N-25 μL syringes were purchased from Hamilton (Bonaduz, Switzerland). The Q3/2 Accurel polypropylene hollow fiber was bought from Membrana GmbH (Wuppertal, Germany). The inner diameter of the hollow fiber was 600 μm, the thickness of the wall was 200 μm, and the pore size was 0.2 μm. A magnetic stirrer/hot plate from Heidolph (Kelheim, Germany) was employed for stirring of the solutions. A centrifuge from Sepand Azma (Tehran, Iran) was used for phase separation. The pH of the solutions was determined and adjusted using a model WTW pH meter (Inolab, Germany) equipped with a combined glass-calomel electrode.

2.2. Instrumentation

The gas chromatographic system comprised an Agilent (Centerville Road, Wilmington, USA) series 7890A GC coupled to an Agilent MSD 5973C 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 2 min, increased to 280 °C at 20 °C min^{-1}, and held for 5 min. The MS quadrupole and the MS source temperatures were set at 150 and 230 °C, respectively. Electron impact (EI) mass spectra were obtained at acceleration energy of 70 eV. Also, 1 μL aliquot of extract was autoinjected by Agilent series 7683 automatic liquid sampler (Centerville Road, Wilmington, USA) in the splitless mode. Data acquisition was performed in the full scan mode (*m*/*z* in the range of 50–500) to confirm the retention times of analytes and in selected ion monitoring (SIM) mode for quantification, and *m*/*z* 58 was extracted for quantitative determination of tramadol.

2.3. Extraction procedure

A 1000 mg L^{-1} stock solution of tramadol was prepared in methanol and standard working solutions were prepared by spiking the stock solution in pure water. The sample solution was filled into a 20 mL vial which was placed on a magnetic stirrer plate to provide efficient stirring during the extractions. A 25 μL HPLC syringe and a conventional medical syringe needle were inserted through the silicon septum; one served to introduce the acceptor solution into the hollow fiber prior to extraction and to collect this solution after extraction, while the second needle was utilized to support the hollow fiber. The ends of the two needles were connected to a 10 cm piece of hollow fiber.

Extractions were performed according to the following scheme: a new 10 cm length of hollow fiber was placed between the two needle ends and subsequently dipped for a 5 s period into the organic solvent (*n*-dodecane) used for impregnation; the latter procedure served to fill the pores of the hollow fiber with the organic solvent. After impregnation, 25 μL of organic acceptor solution (acetonitrile) was injected into the hollow fiber with a micro syringe, and subsequently the fiber was placed in the sample solution. During extraction, the solution was stirred at 1000 rpm. After extraction, the acceptor solution was flushed into a micro-vial located inside the autoinjector and finally, 1 μL aliquot of the extract was autoinjected into the GC–MS instrument for analysis.

2.4. Experimental design approach

To fully understand the way in which three-phase HF-LPME experimental parameters affect the extraction, individual factors must be considered along with nonlinear effects and interaction terms. The chemometric approach relies on a rational experimental design, which allows the simultaneous variation of all experimental factors, saving time and materials. The Box–Behnken design (BBD) is probably the most widely used experimental design for fitting a second-order response surface. This cubic design is characterized by set of points lying at the midpoint of each edge of a multidimensional cube and center point replicates whereas the 'missing corners' help the experimenter to avoid the combined factor extremes. This property prevents a potential loss of data in those cases [15].

Analysis of the experimental design data and calculation of predicted responses were carried out using Design Expert software (version 8.0, Stat-Ease, Inc., Minneapolis, USA). This software was used for ANOVA analysis of the obtained experimental data. The significance of each term in the equation is to estimate the goodness of fit in each case. The analysis of variance tables were generated and the effect and regression coefficients of individual linear, quadratic and interaction terms were determined. The *p*-values less than 0.05 were considered to be statistically significant.

3. Results and discussion

In order to obtain the optimum conditions for three-phase HF-LPME of tramadol from the urine and plasma samples, two optimization strategies were applied. For preliminary optimization stage, the effects of three parameters including pH of donor phase, as well as types of organic membrane and organic acceptor solvents were evaluated using one variable at a time method. Other effective parameters including hollow fiber length, stirring rate, ionic strength and extraction time were studied using a Box–Behnken response surface design. Each of the variables had levels set at three coded levels: −1, 0 and +1, and 29 experiments were necessary in total which were randomly performed to nullify the effect of extraneous or nuisance variables. Five replicates were carried out in the central point in order to give an estimate of the experimental variance. The variables considered and the levels studied are shown in Table 1.

3.1. Finding the optimum extraction conditions

3.1.1. Two immiscible organic phases as organic membrane and acceptor phase

The selection of types of the two immiscible organic solvents, one immobilized in the pores of the hollow fiber and the other filled in the lumen of fiber, is critical in three-phase HF-LPME. The organic membrane solvent serves to separate aqueous solution and organic acceptor phase. It should be compatible with the fiber so as to fill the pores of the fiber wall and to create a suitable

Table 1
Independent variables and their levels used in the response surface design.

Variable	Coded	Level		
		−1	0	+1
Stirring rate/rpm	A	200	600	1000
Hollow fiber length/cm	B	4	7	10
Ionic strength/mol L^{-1}	C	0	2	4
Extraction time/min	D	5	22.5	40

Table 2
A summary ANOVA table for obtained experimental responses.

Source	Sum of squares	Df	Mean square	*F*-ratio	*p*-Value
A: Stirring rate	2.24636	1	2.24636	9.83	0.0046
B: HF length	1.93512	1	1.93512	8.47	0.0079
C: Salt addition	0.0122	1	0.0122	0.05	0.8194
D: Time	13.8491	1	13.8491	60.58	<0.0001
BC	2.29917	1	2.29917	10.06	0.0043
Total error	5.25771	23	0.228596		
Total (corr.)	25.5997	28			

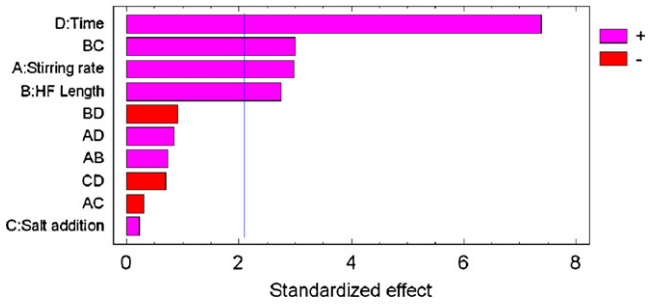

Fig. 1. Pareto chart of main effects obtained from Box–Behnken design. The vertical line defines the 95% confidence interval.

medium for extraction. It also must be immiscible with water and acceptor phase and stable enough over the extraction time. In this system, the target analytes are extracted from the aqueous sample into the organic membrane based on diffusion, in which extraction is promoted by high partition coefficients, and then, easily back-extracted into organic acceptor phase by concentration gradient between both of organic solvents. On the other hand, the organic acceptor phase must have low solubility in *n*-dodecane and effectively remain in lumen of the fiber during the extraction period without leakage to organic membrane and solvent loss due to evaporation. It should also have excellent chromatographic behavior so that it can be easily separated from analyte peaks in comparison with solvents commonly applied in two-phase HF-LPME.

Based on all above considerations and previous studies [14,16], *n*-dodecane was selected as the organic membrane solvent while acetonitrile and methanol were evaluated in preliminary experiments as the acceptor organic solvents. Both of the solvents have all above mentioned characteristic requirements and on the basis of the obtained results, variations of extraction recovery using these solvents were not remarkable. Thus, acetonitrile was selected as acceptor solvent in the further experiments.

3.1.2. Adjustment of pH

The drug is basic and thus pH of the donor solution was adjusted in the proper basic range to neutralize the compound and reduce its solubility in the sample solution. The pH of donor phase was studied in the range of 8–12. The peak area increases when the pH value of sample solution is increased from 8.0 to 11.0, but is then decreased by further increase of pH. The observation may be attributed to the relationship between pK_a of tramadol and pH of the sample solution. It is well known that the pH of solution should generally be 2 units higher than its pK_a to completely deionize a basic compound (the pK_a's of tramadol is 9.4) to form the neutral molecule. Hence, at pH 11 the highest extraction efficiency was obtained for the drug and that pH was chosen as the optimum value.

3.1.3. Optimization by Box–Behnken design

In the next step, optimization by a response surface modeling (RSM, selected Box–Behnken) design was carried out. Effects of the four key factors including hollow fiber length, stirring rate, ionic strength, and extraction time were studied for optimization of extraction process. The experimental limits for these parameters were as follows: stirring rate within the range 200–1000 rpm, hollow fiber length between 4 and 10 cm, ionic strength of 0–4 mol L^{-1}, and extraction time from 5 to 40 min. Analysis of variance (ANOVA) was used to estimate the significance of the main effects and interactions (Table 2). The sum of squares is the information used to estimate the *F*-ratios (considering the respective mean square effect and the mean square error). The *p*-value indicates when the effect of each factor is statistically significant ($p < 0.05$). A Pareto chart of effects is shown in Fig. 1. In this chart, the bar lengths are proportional to the absolute value of the estimated main effects. Fig. 1 also includes a vertical line corresponding to the 95% confidence interval. An effect, which exceeds this reference line, may be considered significant as regards the response. A positive value for the estimated effect indicates an increase in the response if the variable increases to its high level. A negative value indicates that a better response is obtained at low levels of the variable. For the interactions, a positive value indicates that the response will increase if both variables change to the same level, low or high. A negative value indicates an increase in the response if the variables change in opposite directions (one variable increases to a high level and the other decreases to a low level).

In this study, the factors which had significant effects on the signal were extraction time, stirring rate and hollow fiber length. The interaction between ionic strength and hollow fiber length also had some significant effect. The sign of the main effects showed that the response would be improved on passing a given factor (extraction time, stirring rate and hollow fiber length) from the low to the high level.

In this work, the influence of acceptor phase volume on the extraction efficiencies was studied using various hollow fiber lengths. It is known that in the HF-LPME methods, 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. On the other hand, ionic strength modification is a very useful way to enhance the extraction efficiency due to salting out effect. Commonly, addition of salt can decrease the solubility of analytes in the aqueous sample and enhance their partitioning into the organic phase. However, change of physical properties of the Nernst diffusion film can reduce the diffusion rate of analytes into the organic phase, therefore decreasing the extraction efficiency of the organic phase within a period of time. Taking into account the interaction between these variables, i.e. hollow fiber length and ionic strength, the observed effect is positive; that is, by employing higher hollow fiber length, mass transfer of the analytes is favored through employing high sodium chloride concentration.

As a result of this study, the optimum working conditions to obtain the highest response were selected as hollow fiber length, 10 cm; stirring rate, 1000 rpm; ionic strength, 4 mol L^{-1}; and extraction time, 40 min.

3.2. Extraction efficiency and distribution ratio

On the basis of the experiments discussed above, optimal three-phase HF-LPME of tramadol was yielded by utilizing a porous hollow fiber impregnated with *n*-dodecane, an acceptor phase of acetonitrile, and a donor solution with pH = 11. In this case, tramadol was typically enriched by a factor of 546 from aqueous samples. This corresponded to a 68.3% extraction recovery. With V_{ac} = 25 μL, V_d = 20 mL and initial analyte concentration of 100 μg L^{-1}, the value of 3.2 was obtained for logarithm of distribu-

Table 3
Comparison of the proposed method with other methods applied for the extraction and determination of tramadol.

Method	Sample matrix	LDR ($\mu g\,L^{-1}$)	LOD ($\mu g\,L^{-1}$)	RSD %	PF	Ref.
SPE/HPLC	Plasma	50–3500	50	18.32	–	[9]
HS-SPME/GC–MS	Plasma	1–100	0.2	7.8	–	[3]
LPME-BE/HPLC	Plasma & urine	0.3–130	0.12	6.29	366	[17]
LLE/HPLC	Plasma	5–500	2.5	9.7	–	[8]
LLE/GC–MS	Plasma	5–640	5	3.45	–	[18]
New three-phase HF-LPME/GC–MS	Plasma & urine	0.1–400	0.08	6.4	546	Proposed method

Table 4
Results obtained from analysis of real samples.

Sample	Add ($\mu g\,L^{-1}$)	Concentration before spiking ($\mu g\,L^{-1}$)	Concentration after spiking ($\mu g\,L^{-1}$)	RSD %	Relative recovery (%)[a]
Urine 1	20.0	21.6	40.3	7.5	93.5
Urine 2	20.0	34.8	53.8	6.3	95.0
Plasma 1	20.0	Non-detected	17.5	7.4	87.5
Plasma 2	20.0	Non-detected	18.1	7.9	90.5

Relative recovery (RR) was acquired from the following equation: $RR = \frac{C_{found} - C_{real}}{C_{added}} \times 100$ where C_{found}, C_{real}, and C_{added} are the concentration of analyte after addition of known amount of standard into the real sample, the concentration of analyte in real sample, and the concentration of known amount of standard which was spiked into the real sample, respectively.

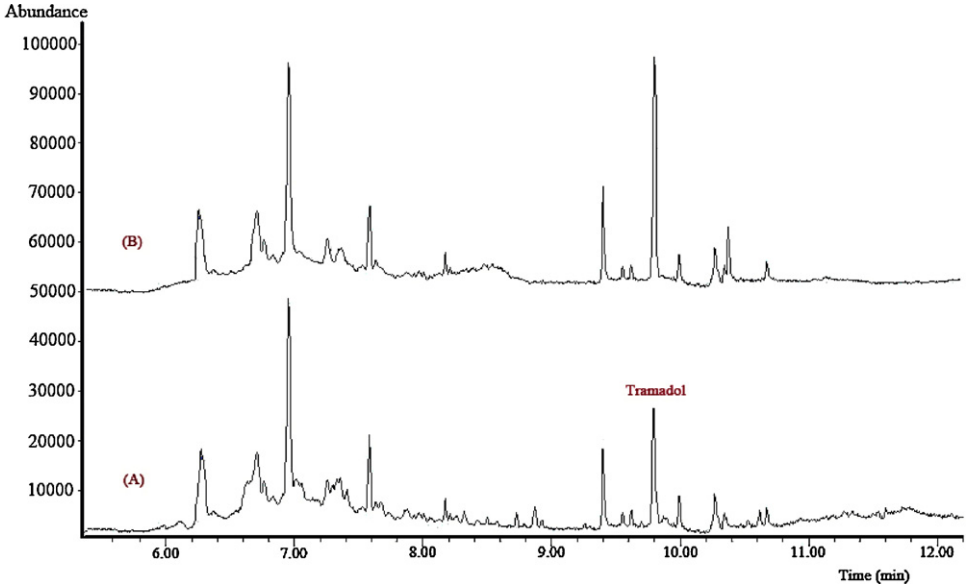

Fig. 2. Chromatograms of tramadol after extraction from the urine sample using three-phase HF-LPME combined with GC–MS: (A) urine sample before spiking; (B) the spiked urine sample at concentration level of 20 $\mu g\,L^{-1}$.

tion coefficient between the acceptor and the donor phases ($\log K$) for tramadol by utilizing Eq. (1). This value effectively explained the significant preconcentration obtained in the present work.

$$PF = \frac{1}{[1/K + V_{ac}/V_d]} \quad (1)$$

where V_d represents the volume of donor solution (sample) and V_{ac} stands for the volume of acceptor phase inside the hollow fiber.

3.3. Method validation

To evaluate the practical applicability of the proposed HF-LPME technique, repeatability, linearity, and detection limit were investigated by utilizing standard solutions of tramadol in water and biological samples. In the first experiment, five-replicate extraction and determination of tramadol (5 $\mu g\,L^{-1}$) were performed by HF-LPME/GC–MS and the relative standard deviations (RSDs) of 6.4, 7.5, and 7.9% were obtained for determination of analyte in water, urine and plasma matrices, respectively. In a subsequent experiment, linearity was evaluated within the range of 0.1–400 $\mu g\,L^{-1}$ where coefficient of determination (r^2) of 0.995 was obtained. Limit of detections (LODs) of tramadol studied in aforementioned sample matrices, calculated based on the signal to noise ratio of 3 (S/N = 3) under MS-SIM condition, were 0.08, 0.1, and 0.5 $\mu g\,L^{-1}$ in water, urine and plasma, respectively. The broad linear range combined with the low detection limit suggests a high-potential HF-LPME/GC–MS for monitoring tramadol in human urine and plasma samples. A comparison between the figures of merit of the proposed method and some of the published methods for extraction and determination of tramadol is summarized in Table 3. Clearly, the proposed method has a good sensitivity and precision with a suitable dynamic linear range in comparison with the other methods.

3.4. Preconcentration of tramadol in human urine and plasma

For further evaluation of the proposed three-phase HF-LPME, tramadol was preconcentrated from both human urine and plasma. After administration of tramadol capsules (50 mg) from Kimia Daru Pharmaceutical Co. (Tehran, Iran) to two volunteers, the urine

sampling was accomplished in the interval of 12–24 h. Drug-free human plasma was obtained from Iranian Blood Transfusion Organization (Tehran, Iran). Frozen human plasma samples were left on the bench to thaw naturally and were vortexed prior to use. Aliquots (5 mL) of both samples were spiked with 20 $\mu g\,L^{-1}$ tramadol and put into the centrifuge tubes, to which small amount of trichloroacetic acid was added to remove proteins from plasma. The resultant samples were placed in 20 mL sample vials and ultrapure water was added to the mark. Then, pH of the sample was adjusted to provide the optimum pH and submitted to HF-LPME as described previously. The relative recoveries of analyte from human urine and plasma were in the range of 93.6–95.2% and 87.5–90.7%, respectively, as shown in Table 4. In addition to excellent enrichment, a high sample clean-up potential was observed for HF-LPME regarding the biological samples. Fig. 2 shows the typical chromatograms of the extracted tramadol from urine before and after spiking with 20 $\mu g\,L^{-1}$ tramadol.

4. Conclusions

The proposed three-phase HF-LPME technique is attractive enough owing to its simplicity, sensitivity, selectivity, analytical precision, low consumption of organic solvent, low cost and short sample preparation time. Additionally, there are two major advantages for this technique: firstly, it is compatible with GC due to using organic acceptor solvent; secondly, in the present method, the used organic acceptor solvent is acetonitrile which has the best chromatographic behavior and could be easily separated from analyte peaks in comparison with applied solvents in two-phase HF-LPME. Finally, the advantages of hollow fiber-protected LPME allow its potential application as a sample preparation and clean-up technique for drug analysis from biological matrices.

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