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Determination of tramadol in human plasma and urine samples using liquid phase microextraction with back extraction combined with high performance liquid chromatography

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Abstract

Liquid phase microextraction by back extraction (LPME–BE) combined with high performance liquid chromatography (HPLC)-fluorescence detection was developed for the determination of tramadol in human plasma. Tramadol was extracted from 2 mL of basic sample solution (donor phase) with pH 11.5 through a micro liter-size organic solvent phase (100 μL *n*-octane) for 25 min and finally into a 3.5 μL acidic aqueous acceptor microdrop with pH 2.5 suspended in the organic phase from the tip of a HPLC microsyringe needle for 15 min with the stirring rate of 1250 rpm. After extraction for a period of time, the microdrop was taken back into the syringe and injected into HPLC. Effected the experimental parameters such as the nature of the extracting solvent and its volume, sample temperature, stirring rate, volume of the acceptor phase, pH and extraction time on LPME–BE efficiency was investigated. At the optimized condition, enrichment factor of 366 and detection limit (LOD) of 0.12 μg L^{-1} were obtained. The calibration curve was linear ($r = 0.999$) in the concentration range of 0.3–130 μg L^{-1}. Within-day relative standard deviation RSD (S/N = 3) and between-day RSD were 3.16% and 6.29%, respectively. The method was successfully applied to determine the concentration of tramadol in the plasma and urine samples and satisfactory results were obtained.

Keywords: Liquid phase microextraction with back extraction; Tramadol; HPLC; Urine

1. Introduction

Tramadol hydrochloride ((±) *trans*-2-[(dimethylamino) methyl]-1-(3-methoxyphenyl)-cyclohexanol) is a synthetic, centrally acting, analgesic agent (Fig. 1), used for the relief of moderate to chronic pain and has no clinically relevant cardiovascular or respiratory depressant activity. Furthermore, it does not have a prostaglandin inhibitory effect. The racemate is successfully used for the treatment of severe postoperative or cancer pains. The dosage of tramadol should be adjusted to the intensity of pain and to the response of an individual patient [1,2]. (Its therapeutic plasma concentration is in the range of 100–300 ng L^{-1}. Tramadol is rapidly and almost completely absorbed after oral administration but its absolute bioavailability is only 65–70% due to first-pass metabolism. Approximately 10–30% of the parent drug is excreted unmetabolised in the urine [3,4].

Determination of tramadol in biological samples involves an initial sample pretreatment step for isolation of target analytes, using liquid–liquid extraction (LLE) [5–10] and solid phase extraction (SPE) [11,12] techniques, prior to high performance liquid chromatography (HPLC). But these techniques have many disadvantages as they are tedious, labor intensive, time consuming and very prone to loss of analyte.

LLE in particular requires the use of large amount of high purity solvents, which are often hazardous and result in the production of toxic laboratory waste. LLE and also SPE require solvent evaporation in order to preconcentrate the analyte [13]. Recently, other extraction techniques such as solid phase microextraction (SPME), liquid phase microextraction (LPME), liquid phase microextraction with back extraction

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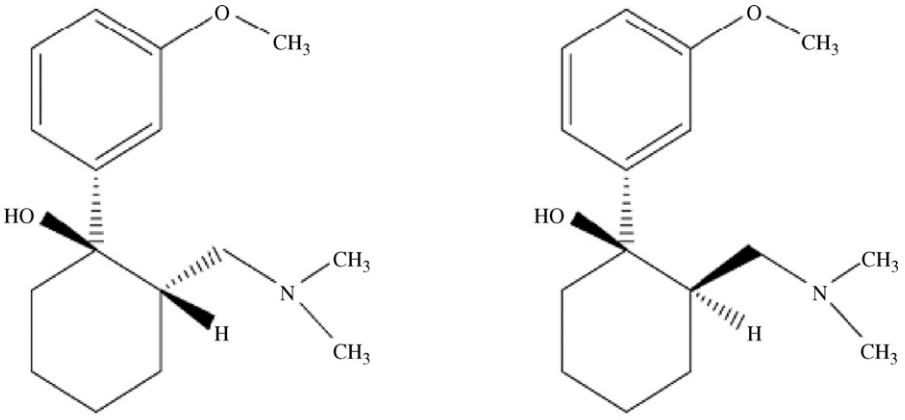

Fig. 1. Chemical structure of *cis*-tramadol and *trans*-tramadol.

(LPME–BE) have successfully been developed for sample preparation [14–16]. SPME needs only a small volume of sample to achieve high preconcentration, however, when coupled to HPLC, a solvent desorption step is required to recover all the absorbed analytes and to avoid carry-over in the experiment. Owing to these reasons, most current applications of SPME are limited to nonpolar or slightly polar compounds [14]. In LPME method, only one drop of organic solvent is used to extract the compounds from water samples. It is usually more suitable as a sample preparation step for subsequent GC analysis, and it is always used for nonpolar compounds [17]. LLE and SPE methods in conjunction with HPLC have been reported for extraction and determination of tramadol in biological fluids [5,12]. LLE can produce emulsion, and large amounts of organic solvents are often needed to extract analytes [5,6]; SPE techniques often introduce artifacts in the sample extracts and can be lengthy, with a series of stages including washing, conditioning, eluting and drying of the process [12].

LPME–BE is a sample preparation technique that has been utilized by Ma and Cantwell in 1997 as a means of preconcentration and purification for ionizable analytes without the need for both solvent evaporation and analyte desorption steps [18,19]. In this method, extraction of analytes occurs via using three liquid phases: (1) sample solution (donor phase) where, pH is adjusted to deionize the compounds, (2) the organic membrane phase which is layered over the donor phase and (3) the receiving aqueous phase layered over the organic phase, the pH of which is adjusted to ionize the analyte. By using the stirring, ionizable compounds are extracted into the organic solvent and then back extracted into the receiving phase, which can be directly analysed. The LPME–BE technique after some modifications was applied for extraction and determination of methamphetamine and amphetamine [15], phenylacetic acid and phenyl propionic acid [20] and phenols [18] in biological or environmental samples. The aim of the present study was to investigate the feasibility of LPME–BE to extract and determine tramadol in biological samples. Various parameters including type of organic solvent, composition of acceptor and donor phases, extraction times in each step and stirring rate on the extraction efficiency were investigated and optimized. Under optimized conditions, the method was applied to determine concentration of tramadol in blood plasma and urine samples.

2. Experimental

2.1. Reagents

The pure substances of *trans*-tramadol and *cis*-tramadol were kindly gifted by Grünenthal (Stolberg, Germany). Methanol, *n*-octane and water were of HPLC grade from Merck (Darmstadt, Germany). Phosphoric acid and potassium hydroxide were of analytical reagent grade from Merck and were used without further purification.

2.2. Apparatus

The extraction and injection procedures were carried out using 10 μL flat-cut Hamilton HPLC syringe (Hamilton Bonadzu AG, Switzerland) and 3 mL extraction vial from Supelco (Bellefonte, PA, USA). Stirring of the solution was carried out with a Heidolph MR 3001 K magnetic stirrer (Schwabach, Germany) and a 7 mm × 1.5 mm magnetic stirring bar. A water bath was used to control the sample temperature and an aluminum foil was used to cover the glass vial to prevent the evaporation of the organic solvent. A WellChrom HPLC instrument from Knauer Company (Berlin, Germany) was used to separate and analyse the drug. The chromatographic system was composed of a gradient HPLC K-1001 pump, a fluorescence RF-10AXL detector (excitation wavelength of 200 nm and emission wavelengths of 301 nm) and an online K-5020 degasser. A Rheodyne model 7725i injector with a 20 μL loop was used to inject the samples. The data were acquired and processed by ChromGate Chromatography Software from Knauer Company. Chromatographic separation was achieved on a Chromolith^{TM} Performance RP-18e, 100 mm × 4.6 mm column (Merck, Darmstadt, Germany) protected by a Chromolith^{TM} Guard Cartridge RP-18e 5 mm × 4.6 mm. A mixture of methanol and water adjusted to pH 2.5 by phosphoric acid (19:81, v/v) in isocratic mode at the flow rate of 2 mL min^{-1} was used as a mobile phase.

2.3. Procedure

Standard solutions containing 100 μg mL^{-1} of tramadol and 100 μg mL^{-1} *cis*-tramadol were prepared in HPLC grade water and stored in a refrigerator (4 °C) and brought to ambient temperature just prior to use. Working solutions were prepared daily by dilution of standard solutions prior to use. The pH of the aqueous sample solutions containing varying amounts of tramadol and 10 μg L^{-1} of *cis*-tramadol (internal standard) were adjusted at pH 11.5 by drop wise addition of 0.1 M of KOH. Then 2 mL of the sample solution (donor phase) which containing 10 μg L^{-1} of *cis*-tramadol (internal standard) was placed in a 3 mL clear glass vial and a magnetic stirring bar (7 mm) was placed into the sample solution to provide sufficient stirring during the extraction. Then, 100 μL of *n*-octane was poured onto the top of the solution to form the solvent layer. The vial was covered with aluminum foil to prevent the evaporation of the organic phase and a thermometer was used to control the temperature. Then, the vial was placed into the water bath located on a heater-magnetic stirrer in order to control the temperature of the solution. After the uptake of 3.5 μL of the acceptor phase, the aluminum foil was pierced by the needle of the syringe and was located near the organic phase. The preextraction equilibrium between donor phase and organic phase was achieved in 20 min. Then, the plunger of the syringe depressed completely to suspend a microdrop of the acceptor phase (3.5 μL) at the needle top which exposed it to the organic solvent phase. After back extraction for 15 min extraction for a prescribed time, the syringe plunger was withdrawn and the microdrop was retracted into the microsyringe and injected into the HPLC for the analysis. Finally, all the quantifications made in this study were based on the relative peak area of the analyte to the internal standard (*cis*-tramadol) from the average of the five replicate measurements.

3. Results and discussion

In this study, the parameters related to LPME–BE were optimized using a univariate method for simplifying the optimization procedure.

3.1. Selection of organic phase solvent and optimization of its volume

The type of solvent used in LPME–BE is very important to achieve satisfactory analyte preconcentration. The organic phase serves to separate the aqueous acceptor phase from the aqueous donor phase. Three factors were considered during the solvent selection: (1) the solvent should be immiscible with both the acceptor and the donor phases; (2) the solvent should have lower density than that of water; (3) in the organic phase, the solubility of drugs in their natural forms should be higher than in the donor solution, but the affinity for ionic forms must be much lower than in the acceptor phase [15]. In this study, six solvents were investigated and their extraction ability was evaluated. The extraction efficiency generally decreased with increase in the solvent polarity, because of high solubility of the analyte in such a solvent. On the other hand, with a decrease in the solvent polarity, the analyte could easily be extracted back into the acceptor phase. Among them, as shown in Fig. 2 the maximum transport efficiency was obtained in the presence of *n*-octane. Then, *n*-octane was selected as the most suitable organic solvent.

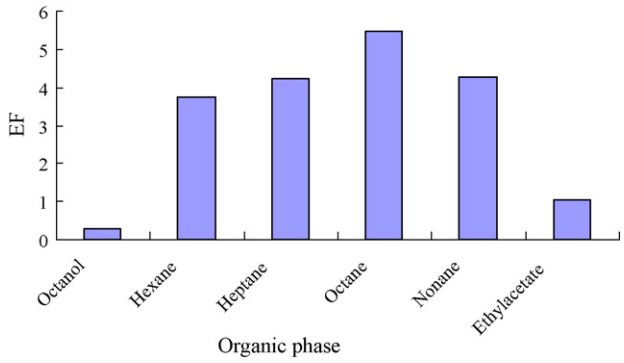

Fig. 2. The effect of type of organic solvent on LPME–BE efficiency. The results are based on the average of five replicate measurements. In these experiments, 2-mL sample solution containing 100 μg L^{-1} of tramadol at pH 12 was extracted using 200 μL of solvent for 20 min with the stirring rate of 800 rpm, and back extracted into a 3 μL drop of H_3PO_4/NAH_2PO_4 (0.01 M) adjusted at pH 3 suspended in the organic phase for 5 min.

In three phase LPME, the effect of different parameters on the extraction recovery can be written as [21]:

$$R = \frac{100 n_{eq,a}}{C_i V_d} = \frac{100 K_{a/d} V_a}{K_{a/d} V_a + K_{org/d} V_{org} + V_d}$$

Where, $n_{eq,a}$ is the amount of the analyte present in the acceptor phase at equilibrium; C_i is the initial concentration of the analyte in the donor phase; V_d, V_{org} and V_a are the volumes of the sample (donor phase), organic and acceptor phase, respectively. Also, $K_{a/d}$, and $K_{org/d}$ are the partition coefficient between the acceptor phase and donor phase as well as between the organic phase and donor phase. According to the equation, since the organic phase can act as a receiving medium to increase the recovery rate, the organic phase volume should be reduced.

To optimize the organic solvent's volume, the volume of *n*-octane varied in the range of 100–300 μL. As shown in Fig. 3, the overall transport efficiency increases by decreasing of *n*-octane volume. The peak obtained for the drug using 100 μL of the organic phase was almost five times higher than those obtained using 300 μL of it. Volumes less than 100 μL were not considered, because at small organic solvent membrane volumes, mixing of the acceptor and donor phases was occurred. In the subsequent experiments, 100 μL of organic membrane was considered.

3.2. Volume of the acceptor phase

The influence of acceptor phase's volume containing 0.01 M of H_3PO_4/NaH_2PO_4 buffer, on the transport efficiency was studied in the volume range of 2–3.5 μL and the results are shown in Fig. 3. The absolute peak area was increased by increasing of the acceptor drop size. In the presence of larger drops, the interface area between the organic phase and the drop increases and consequently a higher amount of the analyte was extrac-

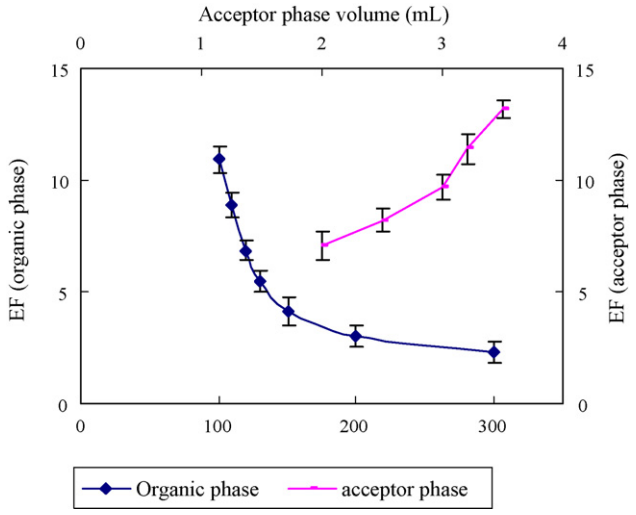

Fig. 3. Effect of the organic phase and acceptor phases' volumes on LPME–BE efficiency. The results are based on the average of five replicate measurements. In these experiments, 2-mL sample solution containing 100 μg L^{-1} of tramadol at pH 12 was extracted using *n*-octane for 20 min with the stirring rate of 800 rpm, and back extracted in to a drop of H_3PO_4/NAH_2PO_4 (0.01 M) adjusted at pH 3 suspended in the organic phase for 5 min.

ted into the drop in a given time. Accordingly, utilizing larger volumes of microdrop improves extraction efficiency whereas reduces enrichment factor. Thus, a 3.5 μL acceptor drop, which was also the largest stable drop in the 100 μL organic layer, was selected in the rest of the study.

3.3. Preextraction time (from donor phase to organic phase)

The extraction of tramadol from water sample into the organic phase is a pretty slow procedure because solute molecules need time to pass through the interface between the donor and acceptor phases and this procedure depends on liquid–liquid equilibrium. Extraction time was investigated ranging from 12 to 35 min. As Fig. 4 shows, the HPLC peak area increased up to the extraction time of 25 min. After this time, the response was almost constant and no significant increase was obtained because of the achievement of preextraction equilibrium between the donor and organic phases. On the basis of these results, the preextraction time of 25 min was applied in the subsequent experiments.

3.4. Back extraction time

The proposed LPME method is not an exhaustive extraction technique, thus maximum sensitivity is obtained at equilibrium conditions. The effect of back extraction time on the transport efficiency was investigated in 5–20 min time period. As shown in Fig. 4, in a constant preextraction time of 25 min, the HPLC peak area of tramadol increased up to 15 min, after which the peak area was constant. Thus 15 min was chosen as optimal back extraction time in subsequent experiments.

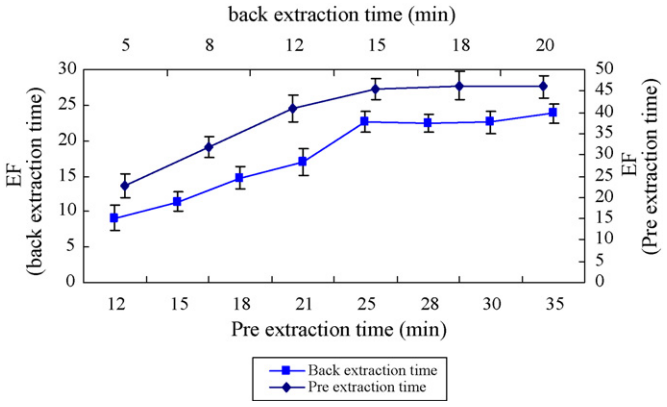

Fig. 4. Effect of pre-extraction and back extraction times on LPME–BE efficiency. The results are based on the average of five replicate measurements. In these experiments, 2-mL sample solution containing 100 μg L^{-1} of tramadol at pH 12 was extracted using 100 μL of *n*-octane with the stirring rate of 800 rpm, and back extracted into a 3.5-μL drop of H_3PO_4/NAH_2PO_4 (0.01 M) adjusted at pH 3 suspended in the organic phase.

3.5. Donor phase and acceptor drop pH

Donor phase's pH has very important role in the extraction efficiency. The drug was deionized under basic conditions in donor phase and its solubility in the sample solution was reduced. Thus the drug was extracted into the organic phase. On the other hand at acidic conditions the drug was protonated and it was entered into the receiving phase. For complete ionization of the acidic analytes, pH of the sample solution should be 2 units greater from the pK_a value of the analyte. Since the drug is ionizable (pK_a = 9.45), the donor solution should be sufficiently basic to change the drug to its neutral form. In this condition the solubility of the drug in the donor phase reduces and its tendency to organic membrane increases.

In this study donor phase was made basic (pH 10–12.5) using KOH (0.1 M) and the effect of donor phase's pH on the transport efficiency was investigated. As shown in Fig. 5 the maximum

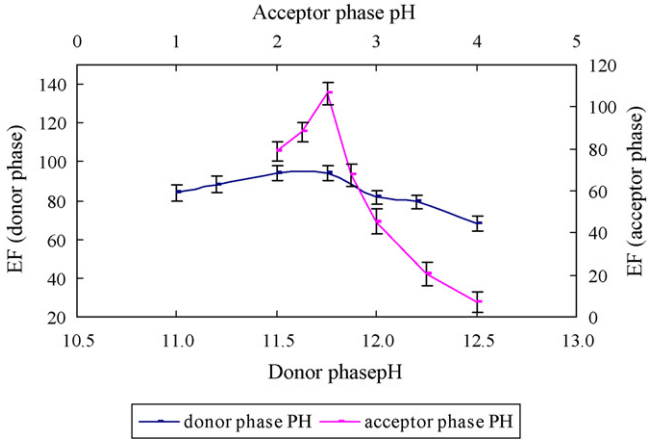

Fig. 5. Effect of the donor and acceptor phases' pH on LPME–BE efficiency. The results are based on the average of five replicate measurements. In these experiments, 2-mL sample solution containing 100 μg L^{-1} of tramadol was extracted using 100 μL of *n*-octane for 25 min with the stirring rate of 800 rpm, and back extracted into a 3.5-μL drop of H_3PO_4/NAH_2PO_4 (0.01 M) suspended in the organic phase for 15 min.

peak area was obtained at pH 11.5. At higher pH values, the extraction efficiency decreased may be due to an increase in the viscosity of the donor solution and less mass transfer of the analyte into the organic phase.

Thus the pH of donor phase was adjusted at 11.5 in further studies.

Also, the acceptor phase should be acidic in order to ionize analyte, therefore the effect of acceptor phase's pH on the transport efficiency was investigated in the range of 2–4 using H_3PO_4/NaH_2PO_4 (0.01 M) buffer. As shown in Fig. 5 the maximum peak area was obtained at pH 2.5. At lower pH values, the extraction efficiency decreased due to a decrease in the diffusion coefficient of the analyte molecules from the organic phase into the acceptor drop because of an increase in the viscosity of the acceptor phase. Thus the pH of phase was adjusted at 2.5 in further experiments.

3.6. Stirring rate and temperature

Agitation of the sample is routinely applied to accelerate the extraction kinetics. Increasing the stirring rate of the donor phase enhances extraction as the diffusion of the analyte through the organic phase is facilitated and also improves the repeatability of the extraction method [16]. The effect of stirring rate on the transport efficiency was investigated by stirring the donor phase with rate of 400–1250 rpm during pre and back extraction. As shown in Fig. 6 peak area increased as the stirring rate increased and the maximum peak area was obtained at the stirring rate of 1250 rpm. Thus the stirring rate of 1250 rpm was chosen as the optimum rate in further experiments.

Temperature of the donor phase affects the extraction kinetics and at higher temperatures, diffusion coefficient of the analyte increases and the equilibrium time reduces.

The effect of temperature on transport efficiency was investigated at the range of 30–70 °C. Based on Fig. 6, as the temperature increased up to 70 °C, the peak area increased and the maximum peak area was obtained at temperature of 70 °C. Although working at higher temperatures induces better extraction efficiency it causes both dissolution of the drop in the organic phase and instability at the drop while being recovered back to the syringe. Due to these problems, further experiments were done at 50 °C.

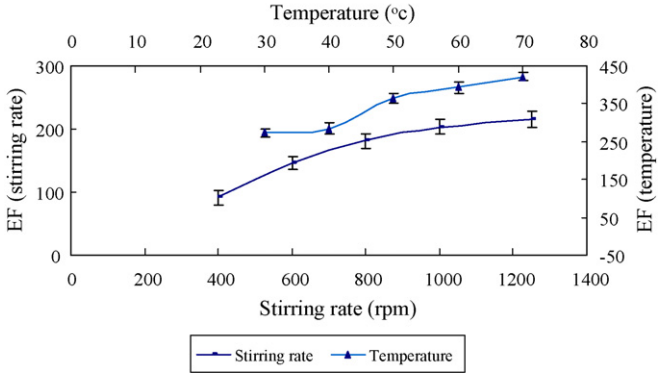

Fig. 6. Effect of stirring rate and temperature on LPME–BE efficiency. The results are based on the average of three replicate measurements. In these experiments, 2-mL sample solution containing 100 μg L^{-1} of tramadol at pH 11.5 was extracted using 100 μL of *n*-octane for 25 min, and back extracted into a 3.5-μL drop of H_3PO_4/NAH_2PO_4 (0.01 M) adjusted at pH 2.5 suspended in the organic phase for 15 min.

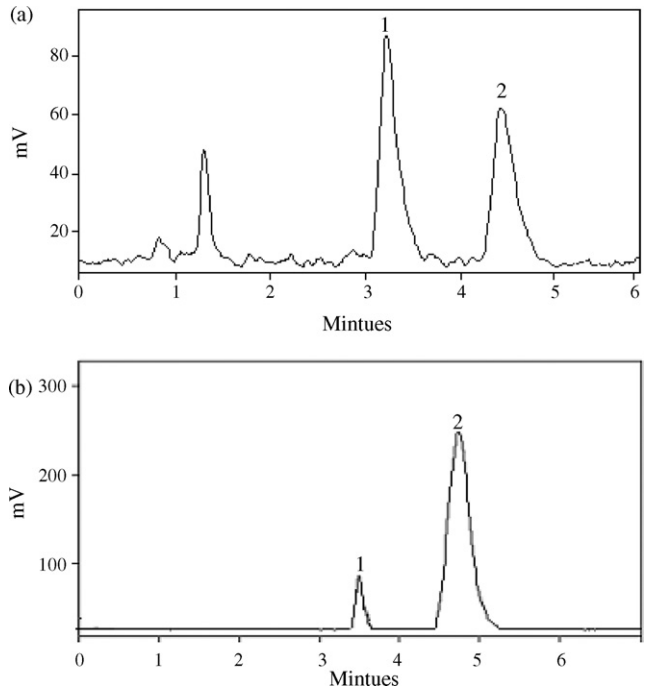

Fig. 7. (a) Chromatogram of the urine samples spiked with 5 ng mL^{-1} of tramadol and 10 ng mL^{-1} of *cis*-tramadol and (b) Chromatogram of the plasma sample spiked with 20 ng mL^{-1} of tramadol and 10 ng mL^{-1} of *cis*-tramadol. (1) c*is*-tramadol and (2) tramadol.

3.7. Quantitative analysis

Calibration curve was obtained using spiked solution of tramadol at the concentration range of 0.3–130 μg L^{-1} (10 μg L^{-1} of *cis*-tramadol as internal standard). The regression equation and the correlation coefficient were $A = 0.0131C$ (μg L^{-1}) + 0.0879 and $R^2 = 0.9992$, respectively. The proposed LPME–BE method revealed good RSD% values (3.16% and 6.29% for within and between-day RSDs, respectively). The limit of detection (LOD) based on a signal-to-noise ratio of three was 0.12 μg L^{-1}. In order to calculate the preconcentration factor of the analyte, five replicate extractions were performed at optimal conditions from an aqueous

Table 1
The results of real sample analysis at optimum conditions

Sample	Analyte Tramadol			
	C_{added} (μg L^{-1})	C_{found} (μg L^{-1})	RSD[a] (%)	*E* (%)
Plasma	5	4.7	3.7	−4.6
	20	20.5	5.4	2.7
Urine	5	4.8	4.5	−3.0
	20	19.2	7.8	−4.9

[a] Mean of five replicate measurements.

Table 2
Comparison of figures of merit of the present method with other reported methods for determination of tramadol

Method	RSD (%)		LOD ($\mu g\,L^{-1}$)	[a]DLR ($\mu g\,L^{-1}$)	Ref.
	Within-day	Between-day			
Proposed	3.16	6.29	0.12	0.3–130	–
SPE	0.51	18.32	50	50–3500	[12]
HS–SPME–GC–MS	4.8	7.8	0.2	1–100	[22]
Liquid extraction GC–MS	1.66	3.45	5	5–640	[23]
Liquid extraction HPLC	2.5	9.7	2.5	5–500	[5]

[a] Dynamic linear range.

solution containing $20\,\mu g\,L^{-1}$ of the drug and $10\,\mu g\,L^{-1}$ of *cis*-tramadol. The enrichment factor was calculated as the ratio of the final concentration of analyte in the microdrop and its concentration in the original solution. The standard solutions of tramadol were prepared in the acceptor phase and the calibration curve was drown at the concentration range of $0.2–10\,mg\,L^{-1}$ with five replicated direct injections. The actual concentration of tramadol in the acceptor phase was calculated from the calibration curve and the preconcentration factor of 366 was obtained (total recovery of 64%).

3.8. Real sample analysis

The applicability of the extraction method to real samples was examined by extraction and determination of tramadol in the blood plasma and urine samples (Fig. 7a and b). The blood plasma samples were obtained from the Faculty of Pharmacy (Tehran University) and the urine samples were obtained from the Clinic of Taleghani Hospital (Tehran, Iran). The blood plasma samples were diluted five times using doubly distilled water and the urine samples were used without any dilution. The pH of the plasma and urine samples was adjusted at 11.5 by dropwise addition of 6 M KOH. Table 1 shows that the results of five replicated analysis of each sample using the proposed method and the amount added are in satisfactory. Percent errors for determination of tramadol concentration in different samples are located at the range of 2.7–4.9. Figures of merit of the present method were compared with those of other methods to determine tramadol (Table 2). The current data was in accordance with alternative methods such as SPE–HPLC [12], SPME–GC–MS [22] and LLE–GC–MS [23] with an important difference that the proposed method uses minimum level of the organic solvent, and since a fresh portion of the solvent was used for each extraction, thus, there was no memory effect. Also in comparison with traditional methods, the method needs only a HPLC syringe to extract tramadol from different samples. Also only small amount of sample volume is consumed for each determination which is compatible with the biological samples such as blood.

4. Conclusion

The present work demonstrated that LPME–BE combined with HPLC can be utilized for the extraction and determination of tramadol in biological samples. Preconcentration factors as large as 366 were obtained in the present research using LPME–BE. Comparison of this method with the others such as HS–SPME, SPE and LLE demonstrated that LPME–BE is simple, inexpensive and with low contaminant of organic solvent. Further it enjoys good linearity, high analytical precision, easy handling, low detection limit and high enrichment factor.

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