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Extraction of trace amounts of pioglitazone as an anti-diabetic drug with hollow fiber liquid phase microextraction and determination by high-performance liquid chromatography-ultraviolet detection in biological fluids

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ABSTRACT

The applicability of hollow fiber liquid phase microextraction (HF-LPME) for extraction and preconcentration of trace amounts of pioglitazone (PGL) as an anti-diabetic drug in biological fluids, prior to determination by high-performance liquid chromatography (HPLC), was evaluated. In this technique, the target drug was extracted into di-*n*-hexyl ether immobilized in the wall pores of a porous hollow fiber from 10 mL of the aqueous sample (source phase, SP) with pH 8.0, and then back extracted into the receiving phase (RP) with pH 2.2 located in the lumen of the hollow fiber. The extraction occurred due to a pH gradient between the two sides of the hollow fiber. After extracting for a prescribed time, 24 μL of the RP solution was taken back into the syringe and injected directly into a HPLC instrument for quantification. The Taguchi orthogonal array (OAD) experimental design with an OA_{16} (4^5) matrix was employed to optimize the HF-LPME conditions. Different factors affecting the HF-LPME efficiency such as the nature of organic solvent used to impregnate the membrane, pH of the SP and RP, stirring speed, extraction time and ionic strength were studied and optimized. Under the optimum conditions (di-*n*-hexyl ether as membrane impregnation solvent, pHs of the SP and RP equal to 8.0 and 2.2, respectively, extraction time of 30 min, stirring speed of 500 rpm and 10% (w/v) NaCl for adjusting the ionic strength), preconcentration factor of 180, linear dynamic range (LDR) of 2.5–250 $\mu g\,L^{-1}$ with good correlation of determination ($r^2 > 0.998$) and limit of detection (LOD) of 1.0 $\mu g\,L^{-1}$ were obtained for the target drug. The percent relative intra-day and inter-day standard deviations (RSDs%) based on five replicate determinations were 4.7 and 15%, respectively. Once LPME was optimized, the performance of the proposed technique was evaluated for the determination of PGL in different types of biological fluids such as plasma and urine samples. The results showed that the proposed HF-LPME method could be successfully applied to determine trace amounts of PGL in biological samples.

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1. Introduction

Pioglitazone ([(±)-5-[[4-[2-(5-ethyl-2-pyridinyl)ethoxy] phenyl]methyl]-2,4-] thiazolidinedione) hydrochloride (PGL) (Fig. 1) is an oral anti-hyperglycemic agent that acts primarily by increasing insulin sensitivity in target tissues. It is used both as monotherapy and in combination with sulfonylurea or insulin in the management of type 2 diabetes mellitus (non-insulin-dependent diabetes mellitus, NIDDM) [1–3]. Pharmacological studies indicate that PGL improves sensitivity to insulin in muscle and adipose tissues and inhibits hepatic gluconeogenesis. Therapeutic concentration range of PGL in plasma is 34–2000 $\mu g\,L^{-1}$ [4]. Several liquid chromatography and capillary electrophoresis methods have been reported in the literature for quantitative determination of PGL and its metabolites in biological fluids [5–13]. In the reported methods, the sample preparation techniques are based on either liquid–liquid extraction (LLE) or solid-phase extraction (SPE) [5–13]. Although these techniques provide adequate analyte enrichment, high reproducibility and high sample capacity [14], they have many disadvantages as they are tedious, labor intensive and time consuming. LLE, in particular, requires the use of large amounts of high-purity solvents, which are often hazardous and result in the production of toxic laboratory waste. Both LLE and SPE require solvent evaporation in order to preconcentrate the analytes. During the evaporation step, loss and/or deterioration of target analytes has been reported [15]. In response to the problems with traditional sample preparation techniques, solid-phase microextraction (SPME) as a solvent-free

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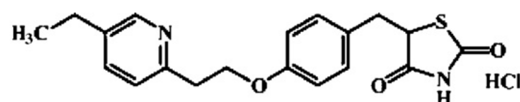

Fig. 1. Chemical structure and log K_{ow} value of pioglitazone.

process was developed based on partition equilibrium of the analytes in an aqueous samples and a polymer coating on a fused-silica fiber that has been successfully used for extracting different compounds [16–18]. However, SPME fibers are fragile and relatively expensive. They tend to degrade with multiple usages. To facilitate automation and effective reduction of the consumption of organic solvents in sample preparation, the miniaturized LLE or liquid phase microextraction (LPME) was introduced in 1996 [19]. LPME can be classified as two phase and three phase techniques [20–28] and may be performed as hollow fiber or droplet based modes. In two-phase LPME, the analytes are extracted from an aqueous sample matrix into an organic acceptor phase and, after the extraction, the extracted organic phase is directly injected into a gas chromatograph (GC) for analysis. Three-phase LPME was previously developed to extract ionizable and charged compounds from aqueous samples [28–30]. In single drop based three-phase LPME, the analytes are first extracted from an aqueous sample matrix into an organic phase immiscible with water and located on top of the aqueous phase, followed by back-extraction into a separate microdrop of aqueous phase suspended from the tip of a microsyringe and penetrating into the organic phase [24]. In recent years, an alternative concept of LPME has been introduced based on the use of single, low-cost, disposable and porous hollow fibers made of polypropylene to support the organic phase in the pores of the wall while holding the second aqueous phase in the lumen of the fiber [31–38]. This mode of hollow fiber liquid phase microextraction (HF-LPME) is limited to the basic or acidic analytes with ionizable functionalities. After extraction, the acceptor solution may be directly injected into high-performance liquid chromatography (HPLC) or capillary electrophoresis (CE) without further treatment. Due to the high ratio of the source phase (SP) to the receiving phase (RP) volume, very high preconcentration factors can be obtained. An important advantage of three-phase LPME is an excellent clean-up that enables the extraction of drugs and metabolites from biological matrixes and pollutants from the environmental samples with simultaneous clean-up of the extracts [31–43].

Orthogonal array design (OAD) is a type of fractional factorial design [44–46] that has proved to be a cost-effective optimization strategy. OAD is used to assign factors to a series of experimental combinations, whose results can then be analyzed using a common mathematical procedure. The theory and methodology of OAD, as a chemometric method for the optimization of analytical procedures, has been described in detail elsewhere [47–50]. In this way, it allows for the identification of key factors that are highly effective on the performance of the characteristic value. Analysis of variance (ANOVA) is employed for estimating the main significant factors and two-way interaction factors after the OAD procedure has been conducted [47–59].

Based on our knowledge, no LPME experiences have been published previously on the extraction of PGL from biological samples. Therefore, in the present study, HF-LPME combination with HPLC/UV was applied for the extraction and preconcentration of PGL in aqueous samples. Moreover, mixed-level OAD procedure with OA_{16} (4^5) matrix (Taguchi Method) was applied to study the factors influencing HF-LPME efficiency. Analysis of variance (ANOVA) was employed for estimating the main significant factors and their percentage contributions. The optimized conditions were then applied for the analysis of PGL in different aqueous and biological samples.

2. Experimental

2.1. Chemicals

Pioglitazone was kindly donated by the Department of Medical Sciences of Tehran University (Tehran, Iran). HPLC-grade acetonitrile and methanol were purchased from Aldrich (Milwaukee, WI, USA). Di-*n*-hexyl ether was purchased from the International Laboratory (USA). The ultrapure water used was purified on Aqua Max-Ultra Youngling ultrapure water purification system (Dongangu, South Korea). All the other chemicals used were of reagent grade or the highest purity available. The plastic and glassware used for the experiments were previously soaked in nitric acid (0.1 M) for 24 h and rinsed carefully with the ultrapure water.

2.2. Apparatus

Chromatographic separations were carried out on a Varian HPLC equipped with a 9012 HPLC pump (Mulgrave, Victoria, Australia), a six-port Valco HPLC valve (Houston, USA) equipped with a 20 μL sample loop and a Varian 9050 UV–vis detector. Chromatographic data were recorded and analyzed using Chromana software (version 3.6.4). The separations were carried out on an ODS-3 column (150 mm × 4.0 mm, with 3 μm particle size) from MZ-Analysentechnik GmbH (Mainz, Germany). A mixture of 50 mM ammonium acetate (pH 4.6) and acetonitrile (20/80, v/v) with a flow rate of 0.7 mL min^{-1} was used as the mobile phase. The injection volume was 20 μL for all the standards and the samples, and the detection was performed at the wavelength of 270 nm. All of the pH measurements were performed with a WTW Inolab pH meter (Weilheim, Germany). All of the extractions were carried out using a Q3/2 Accurel polypropylene hollow fiber membrane from Membrana (Wuppertal, Germany) with a 0.2 μm pore size, 600 μm internal diameter and 200 μm wall thickness.

2.3. Standard solutions and real samples

The stock standard solution of PGL (400 μg mL^{-1}) was prepared by dissolving its hydrochloride salt in acetonitrile. All of the working standard solutions were freshly prepared by proper dilution of the stock standard solution with acetonitrile or ultrapure water to the required concentration. The concentration of the drug in the preliminary experiments was 500 μg L^{-1}. All of the standard solutions were stored at 4 °C and re-prepared every month. Tap water sample was collected freshly from our laboratory (Tarbiat Modares University, Tehran, Iran) and the human urine sample was obtained from a healthy volunteer (Tehran, Iran). The drug free plasma sample was obtained from the Iranian Blood Transfusion Organization (Tehran, Iran). The pH of the real samples was adjusted at 8.0 by dropwise addition of 0.1 M NaOH solution. Before extraction of the drug, the plasma and urine samples were diluted to 1:4 and 1:1 with ultrapure water, respectively. The working standards for real sample analysis were prepared by spiking the target drug in the water and biological samples.

2.4. Extraction procedure

The extraction was performed according to the following steps: (1) before use, the hollow fiber was ultrasonically cleaned in acetone for several minutes in order to remove any contaminants and then the solvent was allowed to evaporate completely; (2) a 10 mL aliquot of the sample solution was added to a 12 mL sample vial containing a 4 mm × 14 mm magnetic stirrer bar; (3) the sample

vial was placed on an IKA multi-station magnetic stirrer (Staufen, Germany); (4) 24 μL of the RP (pH 2.2) was withdrawn using a 25 μL microsyringe (Hamilton, Bondaduz, Switzerland) and then its needle was inserted into the hollow fiber (8.8 cm length); (5) the hollow fiber was immersed in the organic solvent (di-*n*-hexyl ether) for 10 s in order to impregnate the solvent into the pores of the fiber wall. Then it was inserted into the water for 10 s to wash the extra organic solvent from the surface of the hollow fiber; (6) the RP in the syringe was completely injected into the hollow fiber; (7) the end of the hollow fiber was sealed by a piece of aluminum foil; (8) the fiber was bent to a U-shape and introduced into the sample vial; (9) a piece of Parafilm was used to cover the sample vial in order to prevent or reduce evaporation of the organic solvent and the sample vial was then stirred for a prescribed period of time; (10) at the end of the extraction time, the hollow fiber was removed from the sample vial and its closed end was opened; (11) the extract was withdrawn into the syringe and the hollow fiber was discarded, and finally (12) the extract (24 μL) was directly injected into the HPLC loop. A fresh hollow fiber was used for each extraction to decrease the memory effect.

3. Results and discussion

3.1. Organic solvent selection

The selection of extraction solvent is of major importance in HL-LPME in order to obtain efficient analyte preconcentration, good sensitivity, precision and selectivity in the extraction of the target compounds. The selected organic solvent has to satisfy the following requirements: (1) the organic solvent should be easily immobilized within the pores of the fiber; (2) it should have high selectivity for the analyte and low tendency to extract the interferences existing in the SP; (3) it should be immiscible with water to avoid dissolution and be nonvolatile to prevent solvent loss during the extraction; (4) solvents with low viscosity are preferred due to large diffusion coefficient of the analytes; and finally (5) the solvent should have low toxicity. On the basis of these considerations, six organic solvents including 1-octanol, benzyl alcohol, *n*-dodecane, 1-dodecanol, 1-undecanol and di-*n*-hexyl ether were evaluated as the membrane solvents. The evaluations were accomplished with the extraction of 500 μg L^{-1} of the drug from 10 mL of the aqueous solution (pH 8.5) to the RP (pH 1.5) for 30 min stirred at 800 rpm. The experiments indicated that the extraction efficiency was significantly different for the respective solvents. Among the six types of organic solvents tested, di-*n*-hexyl ether showed the best extraction efficiency in term of analyte peak area while benzyl alcohol showed the lowest efficiency. Thus, the di-*n*-hexyl ether was used in further experiments taking into account not only its good extraction efficiency but also the low solvent loss compared to the other organic solvents tested, as well as the ability to be easily immobilized in the pores of the fiber within few seconds.

3.2. Experimental design and data analysis

Using the Taguchi method an orthogonal array design (OAD) with an OA_{16} (4^5) matrix, the effects of the five factors including pH of the source and receiving phases, stirring speed, extraction time and ionic strength in four levels were evaluated. Sixteen experiments were performed in order to estimate the best conditions for the HF-LPME of PGL. For increasing the precision of the optimization process, each trial was repeated (n = 32). The factors and their respected levels are reported in Table 1. Direct observation analysis was statistically employed to estimate the importance of a given factor. The mean value of each response for the corresponding factors at each level was calculated according to the assignment of the experiment. The mean values of each factor at different levels reveal how the extraction efficiency will change when the level of that factor changes. Fig. 2 shows the extraction efficiency of the studied factors as a function of the levels.

Table 1
OA_{16} (4^5) experimental design for the optimization of HF-LPME of pioglitazone.

Trail no.	A	B	C	D	E
1	2.2	6.0	200	10	0
2	2.2	8.0	500	30	10
3	2.2	10.0	800	45	20
4	2.2	12.0	1000	60	30
5	2.6	6.0	500	45	30
6	2.6	8.0	200	60	20
7	2.6	10.0	1000	10	10
8	2.6	12.0	800	30	0
9	3.0	6.0	800	60	10
10	3.0	8.0	1000	45	0
11	3.0	10.0	200	30	30
12	3.0	12.0	500	10	20
13	3.4	6.0	1000	30	20
14	3.4	8.0	800	10	30
15	3.4	10.0	500	60	0
16	3.4	12.0	200	45	10

A (pH of the receiving phase), B (pH of the source phase), C (stirring speed, rpm), D (extraction time, min), E (salt%, w/v).

ANOVA results for the calculated models are shown in Table 2. According to the methods given [43–46,54], the sum of squares (SS) for different variables were calculated and the results are reported (Table 2). The error estimation of the experiments was calculated. The SS of error is obtained by subtracting all the SS of the items from the total SS [54]. The ANOVA results showed that all factors were statistically significant at $P < 0.05$. Furthermore, from the percentage contribution (Table 2), it can be deduced that the most important factor contributing to the extraction efficiency was factor B (pH of the source phase, 47.2%). Further experiments were performed under the optimum conditions and the results showed that under the optimum conditions obtained from the OA_{16} (4^5) matrix, the recoveries were similar to the optimum performance calculated using the following expression:

$$A_{\rm opt} = \frac{T}{N} + \left(\overline{\rm pH_S} - \frac{T}{N}\right) + \left(\overline{\rm pH_R} - \frac{T}{N}\right) + \left(\overline{\rm ss} - \frac{T}{N}\right) + \left(\bar{t} - \frac{T}{N}\right) + \left(\overline{s\%} - \frac{T}{N}\right)$$

where T is the grand total of all results, N is the total number of the results, $A_{\rm opt}$ is the performance under the optimum conditions,

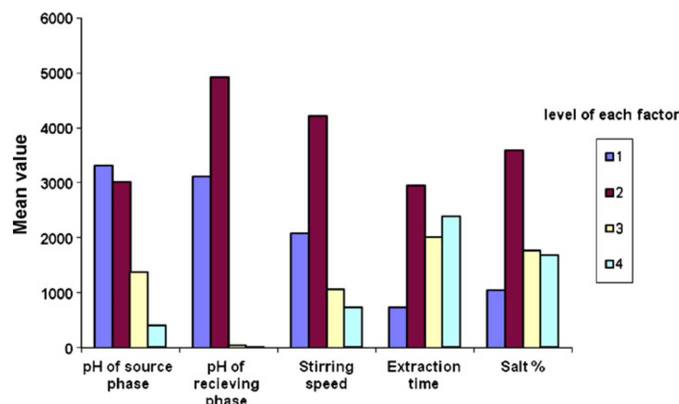

Fig. 2. The effect of pH of the source phase (SP) and receiving phase (RP), stirring speed, extraction time and percent of salt on the HF-LPME of piglitazone. Levels of the parameters are: SP pH: 2.2, 2.6, 3.0 and 3.4; RP pH: 6, 8, 10 and 12; stirring speed: 200, 500, 800 and 1000 rpm; extraction time: 10, 30, 45 and 60 min; salt%: 0, 10, 20 and 30% (w/v) of NaCl.

Table 2
ANOVA results for the optimization of HF-LPME of pioglitazone.

Factor	DOF[a]	Sum of squares	Variance	F-ratio[b]	Pure sum of squares	PC%[c]
pH of the receiving phase	3	45,436,830	15,145,610	154.937	45,143,572	15.25
pH of the source phase	3	1,40,094,714	46,698,238	477.717	139,801,456	47.22
Stirring speed	3	58,920,513	19,640,171	200.916	58,627,254	19.80
Extraction time	3	21,431,480	7,143,826	73.080	21,138,221	7.14
Salt%	3	28,609,117	9,536,372	97.555	28,315,858	9.56
Error	16	1,564,045	97,752			1.03
Total	31	296,056,700				100

[a] Degrees of freedom.
[b] $F_{\text{critical}\,(3,\,16;\,0.05)} = 3.24$.
[c] Percent contribution.

$\overline{\text{pH}_\text{S}}$, $\overline{\text{pH}_\text{R}}$, $\overline{\text{ss}}$, $\bar{t}$ and $\overline{s\%}$ are the average performances of the pH of the source and receiving phases, stirring speed, extraction time and salt% at those optimum levels, respectively. Based on the above equation, under the optimum conditions the performance is estimated using only the significant factors (all the factors in this study) [42].

Under the optimum conditions, the confidence interval (C.I.) of the performance is calculated using the following expression:

$$\text{C.I.} = \pm\sqrt{\frac{F(1, n_2) \times V_\text{e}}{N_\text{e}}}$$

where $F(1, n_2)$ is the F-value from the F-table at a required confidence level at the degrees of freedom (DOFs) of 1 and of error, n_2; V_e is the variance of error term (from ANOVA) and N_e is the effective number of replications. Taguchi method predicted that the result at the optimum conditions would be in the range of 10,531–11,244 (based on the peak area). The average of the results of experiments under the optimum conditions ($n = 5$) was $11{,}212 \pm 530$. These results showed that Taguchi optimization method can be a rapid and safe method for the optimization of three-phase HF-LPME of PGL.

3.2.1. Influence of SP and RP pHs

The pH of the SP and RP phases plays an important role in the three-phase LPME. In this method, the ionizable analytes should be in their neutral form in the SP so that they can be transferred into the organic phase while in the RP, they should exist as their ionized form and, therefore, they cannot be back extracted into the organic phase. In the case of basic analytes, the SP should be sufficiently basic to maintain the neutrality of the analytes and consequently reduce their solubility within the SP. Also, the RP should be acidic in order to promote the dissolution of the basic analytes. Accordingly, to investigate the effect of the pH of both SP and RP, the pH values in the SP and RP solutions were varied from 6.0 to 12.0 and 2.2 to 3.4, respectively. The pH adjustments were performed by the addition of NaOH or HCl solutions. As seen in Fig. 2, the extraction efficiency increased as the pH increased from 6.0 to 8.0, whereas it strongly decreased as the pH increased above this level. In the case of RP, as shown in Fig. 2, the higher extraction efficiency was obtained at the lower pH values. Therefore, the pHs of 8.0 and 2.2 were selected for the SP and RP, respectively.

3.2.2. Influence of extraction time

There are two liquid–liquid interfaces in HF-LPME (i.e. source phase–organic phase, organic phase–receiving phase), thus it is supposed that the solute molecules need long time to pass through these interfaces. LPME is not an exhaustive extraction technique, thus maximum sensitivity is attained at equilibrium conditions. On the other hand, complete equilibrium need not be attained for accurate and precise analysis [60,61]. However, to increase the precision and sensitivity of the LPME method, it is necessary to select an exposure time that guarantees the equilibrium between the phases. Therefore, extraction time is another important factor to be considered. To study the effect of extraction time on extraction efficiency, the experiments were performed at various extraction times in the range of 10–60 min (Table 1). As shown in Fig. 2, the extraction efficiencies increased rapidly by increasing the extraction time up to 30 min and then remained relatively constant. Thus the exposure time of 30 min was selected for the subsequent experiments. Although, the extraction time was relatively long, but by applying a multi-stirrer, many samples could be extracted simultaneously.

3.2.3. Influence of stirring speed

Like other microextraction techniques, fast stirring of the sample could be employed in HF-LPME as well to enhance the extraction efficiency, since stirring permits the continuous exposure of the extraction surface to the fresh aqueous sample [62]. The thickness of the boundary layer in the interface between the sample solution and the hollow fiber is controlled by the level of sample stirring, and strong stirring reduces the thickness as well as the resistance towards the mass transport from SP to RP [63]. Stirring also reduces the time required to reach thermodynamic equilibrium and induces convection in the membrane phase. Furthermore, high stirring speeds generate some problems such as production of air bubbles on the surface of the hollow fiber and promotion of solvent evaporation. So the experiment becomes difficult to control and precision is poorer. In this work, different stirring speeds ranging from 0 to 1000 rpm were tested to determine their effects on the extraction efficiency of the drug. As shown in Fig. 2, higher extraction efficiencies were obtained when the solution was stirred at 500 rpm, so it was chosen as the optimum stirring speed.

3.2.4. Influence of ionic strength

The salting-out effect has been used extensively in different extraction methods and it has been reported that addition of salt can increase the extraction efficiency of LLE method [64]. But some contradictory results have been reported as well [65,66]. In this work, the effect of salt addition on the extraction efficiency of the drug using HF-LPME method was examined by adding of NaCl to the aqueous samples in the range of 0–30% (w/v). The ANOVA results showed that the salt concentration up to 10% increased the extraction efficiency of the analyte, but further addition of the salt decreased its extraction efficiency. This effect could be due to the increasing interactions between the analyte and salt and also the increasing viscosity of the sample solution with the increasing of salt concentration. Such interactions would tend to restrict the movement of the drug from the SP to the membrane solvent. Thus, according to Fig. 2, salt concentration of 10% (w/v) was selected as the optimum concentration for the subsequent experiments.

Table 3
Figures of merit of the proposed HF-LPME of pioglitazone.[a].

LOD	1.0
R^2	>0.998
Regression equation	$A = 21{,}042C + 598.2$
LDR	2.5–250
PF	180
ER%	43.2
RSD (intra-day, $n = 5$)	4.7
RSD (inter-day, $n = 5$)	15

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

3.3. Method validation

3.3.1. Analytical performance

To evaluate the practical applicability of the proposed extraction method, a number of performance parameters as the figures of merit such as linearity, limit of detection (LOD), preconcentration factor (PF), repeatability, reproducibility and percent of extraction were evaluated for extraction of the drug from the aqueous solutions under the optimum conditions and were tabulated in Table 3. Linear dynamic range was evaluated by plotting the calibration curve based on the peak areas versus the concentration of the drug using seven concentration levels over a range of 2.5–250 $\mu g\,L^{-1}$. For each level, three replicate extractions were performed under the optimum conditions. The analyte exhibited good linearity with the correlation of determination of $r^2 > 0.998$, in the studied range. Based on the signal-to-noise ratio of 3 (S/N = 3), LOD of 1.0 $\mu g\,L^{-1}$ was obtained.

Under the optimum conditions, the preconcentration factor was 180 and the extraction recovery (ER) of 43.2% was obtained. ER% was calculated based on the following equation:

$$ER\% = \left(\frac{C_{RP} \times V_{RP}}{C_0 \times V_{SP}}\right) \times 100$$

where C_{RP} and C_0 are the concentration of analyte in the receiving phase and the initial concentration of analyte in the source phase, respectively and V_{RP} and V_{SP} are the volumes of the receiving and the source phases, respectively.

Table 4
Determination of pioglitazone in different spiked samples.

Sample	Pioglitazone			
	C_{added} ($\mu g\,L^{-1}$)	C_{found} ($\mu g\,L^{-1}$)	RSD[a]	Recovery (%)
Tap water	5	5.35	5.2	107
Urine	10	9.7	6.5	97
Plasma	25	22.75	4.3	91

[a] For three replicate extractions.

The overall precision of the method was evaluated by carrying out five replicate extractions and determination of the drug at a concentration level of 50 $\mu g\,L^{-1}$ during 1 day (intra-day precision) and five replicates over a period of 1 week (inter-day precision). The values of relative standard deviations (RSDs) for intra-day and inter-day precision were 4.7 and 15%, respectively.

3.3.2. Extraction of the pioglitazone from real samples

In order to investigate the practical utility of the proposed extraction method for the analysis of the drug in the real samples with complex matrices, the developed technique was applied for the analysis of the target analyte in the tap water sample. Prior to extraction, the tap water sample was spiked with the desired concentration levels of the drug. Then, the extraction was performed under the optimum extraction conditions. The characteristics of the samples and the analytical results are shown in Table 4. Based on these data, satisfactory results can be obtained using the proposed method.

The performance of the proposed procedure was also tested by extraction and determination of the drug in the plasma and urine samples. Due to the high protein bonding for the drug in the plasma (>99%), the extraction recoveries of the spiked plasma samples were low, so some pretreatments were required to eliminate the drug–protein interactions and also to release the drug from the plasma proteins. Therefore, to obtain higher analyte recoveries, the pretreatment was performed according to the following scheme: 2 mL of the plasma was spiked at the desired concentration levels of the drug. Then 3 mL of methanol was added and the obtained mixture was strongly vortexed for 10 min. After staying for 10 min on ice, followed by 10 min at ambient temperature, the mixture was centrifuged at 3500 rpm for 10 min. The supernatant was transferred into a 10 mL volumetric flask and diluted to the mark. Finally, pH of the mixture was adjusted to 8.0 and the extraction procedure was followed under the optimum conditions. As a result, the matrix effect was reduced and high extraction recoveries were obtained. In order to reduce the matrix effects in the urine sample, it was spiked with the drug at the desired concentrations and then diluted to 1:1 with ultrapure water. After dilution, pH of the sample was adjusted to 8.0. Then the drug was extracted under the optimum conditions. As Table 4 shows, the obtained results for the spiked urine and plasma samples indicate a reasonable agreement with the respected values. Table 4 shows the relative recoveries as 91 and 97% for the plasma and urine samples, respectively. RSDs for drug determination in the examined real samples based on three replicate measurements were 6.5 and 4.3% for the urine and plasma samples, respectively. Fig. 3 depicts the chromatograms of HF-LPME extracts from the non-spiked and the spiked urine and plasma samples with PGL under the optimum conditions (the spiked levels for the urine and plasma samples were 10 and 25 $\mu g\,L^{-1}$ of PGL, respec-

Table 5
Comparison of the proposed method with other developed methods to determine pioglitazone in aqueous solutions.

Extraction procedure	Instrumentation	LOD ($\mu g\,L^{-1}$)	LDR ($\mu g\,L^{-1}$)	RSD (%) (intra-day)	RSD (%) (inter-day)	Recovery (%)	Ref.
HF-LPME[a]	HPLC-UV[b]	1.0	2.5–250	4.7 ($n = 5$)	15 ($n = 5$)	91 (plasma)	Present work
LLE[c]	LC-MS[d]	<1.0	–	28.9 (urine) 18.8 (plasma)	29 (urine) 28 (plasma)	53.5 (urine) 44.4 (plasma)	[7]
SPE[e]	HPLC/UV	25	50–2000	2.0–7.8 ($n = 6$)	0.9–6.8 ($n = 6$)	96.6–106.3	[8]
–	HPLC/UV	0.1	0.1–100	4.57	3.80	92.37–106.50	[9]
–	MEKC[f]	–	–	2.89 ($n = 6$)	3.26 ($n = 3$)	101.4	[10]

[a] Hollow fiber liquid phase microextraction.
[b] High-performance liquid chromatography with UV detector.
[c] Liquid–liquid extraction.
[d] Liquid chromatography mass spectrometry.
[e] Solid-phase extraction.
[f] Micellar electrokinetic chromatography.

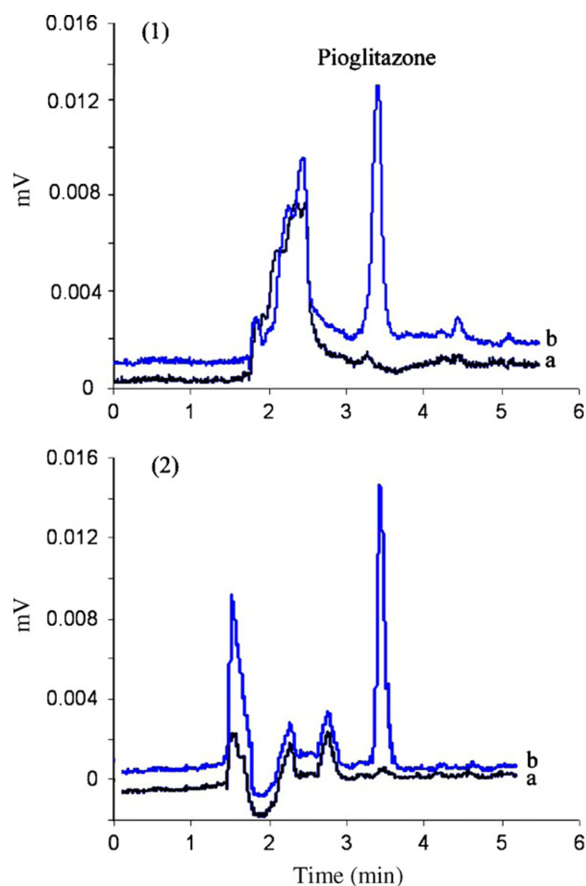

Fig. 3. The HF-LPME-HPLC/UV chromatograms of (1) the diluted urine sample (1:1) for (a) non-spiked and (b) 10 μg L^{-1} spiked of pioglitazone, and (2) the diluted plasma sample (1:4) for (a) non-spiked and (b) 25 μg L^{-1} spiked of pioglitazone under the optimum conditions (the source phase pH, 2.2; the receiving phase pH, 8.0; stirring speed, 500 rpm; 10% (W/V) of NaCl and extraction time, 30 min).

tively). The results indicated that the proposed method has a high clean-up power and that the biological matrices had no significant effect on the extraction efficiency of the method.

3.3.3. Comparison of the applied method with other reported methods

The present method was compared with the other methods in terms of validation and precision (Table 5). As can be deducted, the method is quite comparable to those mentioned in Table 5. The proposed HF-LPME method has some advantages in comparison with other extraction methods (LLE, SPE, etc.) as low consumption of organic solvents and reagents, simplicity and low cost of the extraction device, minimum carry-over and cross-contamination and producing a clean extracting phase for the analysis.

4. Conclusions

In the present study, the three-phase HF-LPME was successfully developed for the extraction and preconcentration of PGL in biological samples. Moreover, Taguchi method was efficiently employed to optimize the HF-LPME conditions for analyzing the target drug. A five-factor four-level design (OA_{16} (4^5)) was chosen to optimize the levels of the selected factors. The results showed that the Taguchi approach is a suitable method for the optimization of the HF-LPME conditions for the extraction of PGL from biological fluids.

The results further demonstrated that the proposed method has good precision, linearity and accuracy over the investigated concentration range. This method has several advantages over the other extraction methods: (1) the equipment needed is very simple and inexpensive, (2) in the proposed three-phase mode excellent clean-up has been observed, even from complicated urine and plasma samples (Fig. 3), (3) because of high obtained extraction recovery (43.2%), the proposed three-phase LPME provided very high preconcentration factor (180), since the ratio V_{SP}/V_{RP} is normally high (417), thus no further concentration of the extract is required before the final analysis, (4) after the extraction, the extract is directly injected into the HPLC, (5) due to the simplicity and the low cost of the extraction device, the hollow fiber can be discarded after each extraction to eliminate possible carry-over problems and cross-contaminations as compared to the SPME. This serves to maintain high reproducibility and repeatability of the method, (6) the volume of the organic phase is less than 30 μL, resulting in an extremely low consumption of organic solvent per extraction. Thus the present LPME method may therefore be utilized as a green chemistry approach to reduce the consumption of hazardous organic solvents in the chemical laboratory, and finally (7) although, the extraction time was relatively long (30 min), but by applying a multi-stirrer, many samples could be extracted simultaneously. In the present work six samples were extracted together using a multi stirrer, but much higher parallelization is feasible.

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