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Hollow fiber liquid-phase microextraction combined with high performance liquid chromatography for the determination of trace mitiglinide in biological fluids

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ABSTRACT

A hollow fiber liquid phase microextraction (HF-LPME) in conjunction with reversed phase HPLC–UV method was developed for the extraction and determination of trace amounts of the antidiabetic drug, mitiglinide (MIT) in biological fluids. The drug was extracted from 10 mL aqueous sample (donor phase (DP)) into an organic phase impregnated in the pores of hollow fiber, followed by the back extraction into a second aqueous solution (acceptor phase (AP)) located in the lumen of the hollow fiber. Parameters influencing the extraction efficiency including the kind of organic solvent, composition of DP and AP, extraction time, stirring rate and salt addition were investigated and optimized. Under the optimized extraction conditions, high enrichment factors (210-fold), good linearity (5–1000 ng mL^{-1}) and detection limit lower than 1.38 ng mL^{-1} were achieved. Recoveries of spiked samples were in the range (88.3–96.3%) and (92.0–99.3%) for urine and plasma samples, respectively. The percent relative standard deviation ($n = 9$) for the extraction and determination of three concentration levels (100, 400 and 800 ng mL^{-1}) of MIT were less than 10.6% and 13.6% for urine and plasma samples, respectively. The developed method is simple, sensitive and has been successfully applied to the analysis of MIT in biological fluids.

1. Introduction

It is estimated that currently there are about 285 million diabetics worldwide, and the number is expected to increase to 439 million by 2030 [1,2]. Due to the alarming increase, the World Health Organization has declared diabetes mellitus as a global epidemic and 14 November as World Diabetes Day [3]. Diabetes is incurable but manageable. Many new drugs are currently in the developmental stage for the treatment of diabetes. One such drug is mitiglinide (MIT), (2S)-benzyl-4-(cisperhydroisoindol-2-yl) butyric acid (Fig. 1). It is a novel glinide class compound with fast-onset of action as insulinotropic agent [4]. It has been used in clinical studies as calcium hydrate form. Similar to other glinide group members, MIT acts by stimulating the secretion of insulin from pancreatic beta cells by closing the ATP-sensitive K^+ [K(ATP)] channels [5]. MIT and the other glinides such as, repaglinide and the phenylalanine derivative were found to share similar pharmacokinetic and pharmacodynamic properties [6], and it has been used either singly or in combination with metformin for the treatment of type II diabetes mellitus. The daily intake dosage of MIT ranges between 5 and 20 mg (three times) [7]. However, the pharmacokinetic properties of MIT are still not well understood. Therefore, the determination of MIT at physiological levels in biological fluids using suitable methods of analysis is of much interest.

High-performance liquid chromatography (HPLC) employing either ultraviolet (UV) [8] or mass spectrometry (MS) [9–11] detections and ultra high pressurized liquid chromatography with MS detection [12] methods have previously been reported for the determination of MIT in biological fluids [8,10,12] and to establish the pharmacokinetic properties of MIT [9,11]. These methods either lack sensitivity to monitor at the therapeutic levels (lower limit of quantification (LLOQ) (0.1 μg mL^{-1})) [8] or use expensive mass spectrometers [9–12]. Moreover, the long separation time involved (14 min) [8,10], is not suitable for the analysis of large batches of biological samples.

The drive for "green" methods to overcome the inherent problems of the conventional liquid–liquid extraction (LLE) has led to the development of numerous of solventless and microextraction techniques. Of these, the hollow-fiber liquid phase microextraction (HF-LPME) technique, originally proposed by Pedersen-Bjergaard and Rasmussen in 1999 [13] has gained prominence. The heart of method lies in the use of a porous polypropylene hollow fiber (HF) where an organic solvent (~4 μL) is usually used to fill its pores before the initiation of the extraction procedure. Experimental

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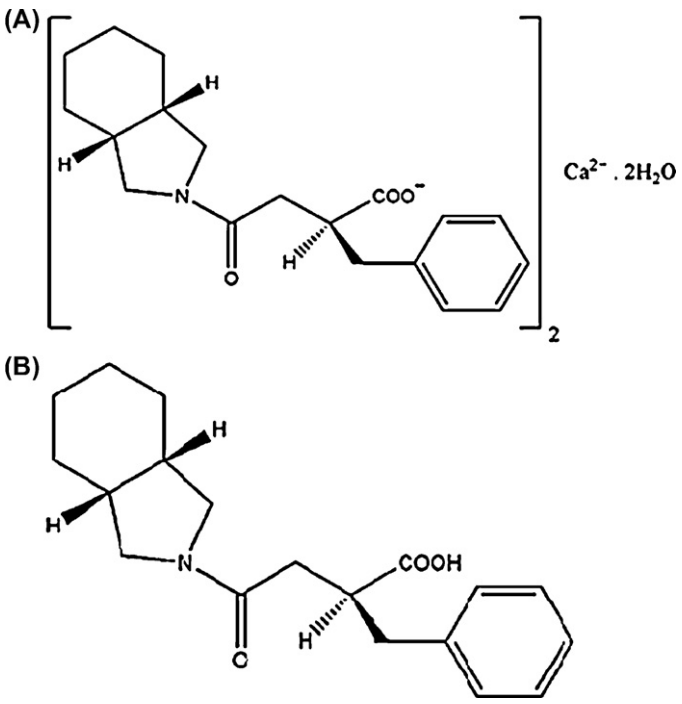

Fig. 1. Chemical structures of mitiglinide (A) the state of administration, (B) the state of detection; log P = 2.73.

conditions are adjusted so that the analyte is transferred through the organic phase of the HF pores to the acceptor solution which is situated within the lumen of the HF. In cases, where a large number of samples need to be analyzed, HF-LPME method could be more economical compared to the solid phase extraction for the same equivalent number of samples [14]. The technique has found numerous applications, especially for pharmaceutical and environmental samples [15].

In this study, a HF-LPME combined with HPLC–UV was applied for the extraction and determination of MIT in biological samples. As MIT is acidic (pK_a = 4.37) [16] so a three-phase HF-LPME method was developed using an acidic donor phase (DP) and a basic solution as acceptor phase (AP) which can be directly injected into the HPLC system. All the HF-LPME parameters have been optimized in order to propose a sensitive and simple method for the determination of MIT in biological fluids.

2. Experimental

2.1. Chemicals and reagents

Mitiglinide calcium hydrate reference standard (100.0% purity) was kindly donated by Hikma Pharmaceuticals (Amman, Jordan). HPLC-grade methanol (≥99.96%) was purchased from Fisher Scientific (Milwaukee, USA). Potassium dihydrogen phosphate (98–102%), orthophosphoric acid (37%) and hydrochloric acid (37%) were purchased from Merck (Darmstadt, Germany). 1-Heptanol (≥99.9%) and 1-octanol (≥99.5%) were purchased from Fluka (Buchs, Switzerland). *n*-Decane (99.0%) and *n*-octane (97.0%) were from Acros Organics (Geel, Belgium). Sodium hydroxide (≥98.0%), dihexyl ether (97.0%), *n*-tridecane (99.8%), 2-hexanol (≥99.0%), *n*-hexadecane (99.0%), 1-nonanol (99%) and diethylether (98%) were purchased from Sigma–Aldrich (St. Louis, USA). Ultrapure water (resistivity, 18.2 MΩ cm^{-1}) was produced by a Milli-Q system (Millipore, USA), and was used throughout for the preparation of solutions.

2.2. Materials

Q3/2 Accurel polypropylene HF membrane (600 μm inner diameter, 200 μm wall thickness and 0.2 μm pore size) was purchased from Membrana GmbH (Wuppertal, Germany). 6 cm of the HF was cut. The HF was discarded after single use. A 25 μL Hamilton microsyringe (model 702SNR) with a blunt needle tip was used to introduce the AP and support the HF. The syringe with the attached HF was clamped to a retort stand during the extraction. A hot plate stirrer (model GLHPS-G) purchased from Global Lab (South Korea) was used for stirring during the extraction. An Orion pH meter model EA 940 (Cambridge, USA) was used for pH measurements. Centrifuge (model 2100) was purchased from Kubota (Tokyo, Japan).

2.3. Instrumentation

A Hitachi LC-6200 intelligent pump (Tokyo, Japan) was used to deliver the mobile phase to the analytical column (GraceSmart RP 18 Column 150 mm × 4.6 mm, 5 μm) and was purchased from Grace Davison Discovery Sciences (IL, USA). Sample injection was performed via a Rheodyne 7125 injection value (Cotati, California, USA) with a 5 μL loop. Detection was achieved by a Hewlett-Packand 1050 UV detector (Waldbronn, Germany) at 210 nm. A powerchrom data acquisition was purchased from eDAQ (Denistone East, Australia) and was performed with powerchrom V2.6.7 software.

2.4. Standard solutions and real samples

A stock solution of MIT (1000 μg mL^{-1} as mitiglinide calcium hydrate) was prepared by dissolving the proper amount in methanol and stored at 4 °C. Working standard solutions were prepared daily by appropriate dilution of the stock solution with water to the desired concentration. Human urine sample was obtained from a healthy student volunteer. Drug free plasma sample was obtained from the Centre for Drug Research, Universiti Sains Malaysia, Penang, Malaysia. The pH of the real samples was adjusted to 1.5 using 1.0 M HCl solution.

2.5. Preparation of hollow fiber and extraction procedure

The HF was cut into segments (6 cm) which approximately accommodated 15 μL of the AP. The HF segments were washed with acetone to remove any contaminants and it was allowed to evaporate completely. A 10 mL of the sample solution (pH 1.5, adjusted with 1.0 M HCl) containing 500 ng mL^{-1} of MIT was placed in a 12 mL vial. A magnetic stir bar (5 mm × 15 mm) was placed in the solution. A Hamilton micro syringe (25 μL) was used to introduce the AP into the HF. 15 μL of the basic receiving phase (0.1 M NaOH) was withdrawn into the micro syringe and was inserted into the lumen of the HF. The fiber was placed in the organic phase (1-octanol) for 10 s and any excess of the organic phase was carefully removed by washing the outside of the HF with ultrapure water for 5 s. Subsequently, 15 μL of the receiving phase was injected into the lumen of the HF and tip needles were used to connect the HF ends.

The fiber was formed as U-shape and it was immersed into the sample solution. The extraction was performed at room temperature and the sample was stirred at 300 rpm during the extraction (45 min). After the extraction, the HF was removed from the sample vial, and one end of the HF was opened. A 5 μL of AP was retracted into the micro syringe and finally injected into the HPLC system for subsequent analysis.

2.6. Minimizing protein binding in plasma

Glinides in general are known to be favorably bound to plasma proteins, especially albumin [17]. Thus, in order to eliminate this problem, a miniaturized liquid–liquid extraction (MLLE) pretreatment steps as outlined in the work of Cai et al. [12] was used. These pretreatment steps were conducted as follows: (i) undiluted plasma (0.2 mL) was spiked at the desired concentration levels of the drug, (ii) methanol (50 μL) and 1.0 M HCl (200 μL) were added to the spiked sample. The sample was vortexed for 30 s and extracted with 3 mL of diethyl ether by shaking for 10 min, (iii) the mixture was centrifuged at 3500 rpm for 10 min. The upper organic layer was allowed to dry at 40 °C under a gentle stream of nitrogen. The dried residue was then reconstituted in 10 mL with methanol:water (0.2:9.8, v/v) with pH adjusted to 1.5 using 1.0 M HCl solution. Finally, the HF-LPME extraction procedure was conducted under the optimum conditions.

2.7. Minimizing matrix effect in urine

In order to decrease the matrix effects (e.g., albumins, sugars, urea, etc.) in the urine samples, the following steps were used: (i) the urine sample was spiked with the drug at the desired concentration levels and diluted 1:4 with water. (ii) pH of the diluted sample was adjusted to 1.5 using 1.0 M HCl solution. The drug was then extracted under the optimum conditions mentioned previously.

3. Results and discussion

Chromatographic conditions were initially optimized in order to choose a suitable mobile phase for the determination of MIT. Various mobile phase compositions consisting of methanol (50–80%) and phosphate buffer solution (50–20%) at different pH (3.5–5.0) were tested. To avoid matrix interferences from the biological fluids samples, a mixture of 40% of phosphate buffer solution at pH 4 (containing 20 mM of potassium dihydrogen phosphate and 1% orthophosphoric acid) and 60% methanol was selected as mobile phase at flow rate of 1 mL min^{-1}. Under the selected conditions, the retention time of the drug was less than 6 min, which is faster than the previously reported methods (14 min) [8,10]. Moreover, the present method is considered more environmental friendly (use methanol) compared to the previous reported methods which involved acetonitrile as mobile phase [8,10,18].

3.1. Selection of the organic extraction solvent

Organic solvents play an important role in the extraction efficiency and the analyte pre-concentration. Generally, the organic solvent must be non-volatile, immiscible with both the acceptor and donor (DP) phases, easily immobilized in the HF and the solubility of analytes in this organic solvent should be lower but higher than that of the AP and DP, respectively. This causes the analyte to migrate from the DP through the pores of the HF, and finally to the AP.

Nine organic solvents (1-octanol, 1-nonanol, 1-heptanol, dihexyl ether, 2-hexanol, *n*-hexadecane, *n*-tridecane, *n*-octane and *n*-decane) with different viscosities, volatilities and partition coefficients were examined in this work. The tested extracting solvents were: All of these solvents were easily immobilized in the pores of the HF. As shown in Fig. 2, 1-octanol produced the highest enrichment factor, thus, it was selected as the organic solvent for further studies.

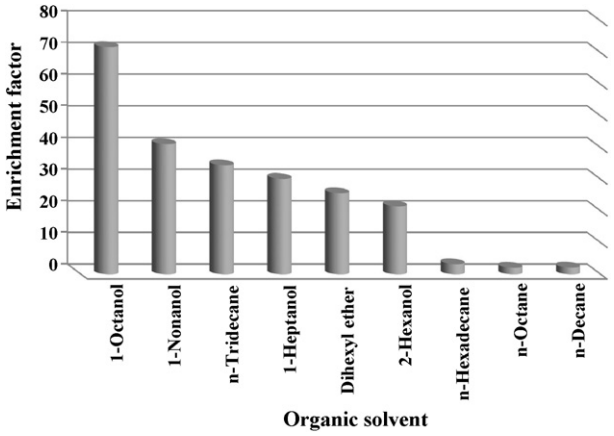

Fig. 2. Effect of organic solvents on the enrichment factor ($n = 3$). Experimental conditions: donor phase volume, 10 mL (pH, 1.5); acceptor phase, 15 μL (0.1 M NaOH); extraction time, 15 min; stirring speed, 300 rpm; and concentration level, 500 ng mL^{-1}.

3.2. Effect of donor phase pH

pH of DP and AP are very important parameters which affect the extraction efficiency in three phase LPME. The pH of the DP should be adjusted to maintain the analytes in the deionize form, while the AP should be adjusted to ionize them. The difference in pH between the DP and AP is one of the major parameters which can promote the transfer of analytes from the DP to the immobilized organic solvent and further to the AP. Therefore, pH values for the DP and AP are usually selected slightly less or higher than the pK_a value. Based on the pK_a value of MIT (4.37) [16], it is fully ionized at pH values 2–3 units higher than the pK_a value. Thus, it should be adjusted to acidic conditions (2–3 units less than the pK_a value) to maintain neutrality and allow the drug to migrate to the immobilized organic solvent (1-octanol) in the fiber pores. Therefore, the DP was adjusted to acidic using 1.0 M HCl solution to different pH values ranging between 0.7 and 3.0 (Fig. 3A).

It was found that the extraction efficiency and enrichment factor increased as the pH value increased from 0.7 to 1.5, but decreased abruptly thereafter. pH 1.5 was subsequently utilized in the remaining studies. The low extraction efficiency at pH less than 1.5 is due to the presence of the amide group, which is delocalized at low pH value to form partial double bond between (N–C) and carbonyl (resonance) [19]. Thus, carbonyl can act as hydrogen acceptor, which increase the solubility of MIT in the DP and reduce its migration to the organic solvent (Fig. 4) [20].

3.3. Effect of the acceptor phase concentration

The extraction of acidic substances requires a highly basic AP to ensure that the extracted analyte is in the ionized form, and does not diffuse back into the organic solvent. The carboxyl group in MIT is deprotonated under basic conditions and is converted to the ionized form [21]. Therefore various concentrations of NaOH (0.02–0.2 mol L^{-1}) were studied. Enrichment factors were found to increase as the concentration of NaOH increased up to 0.1 M (Fig. 3B), due to the lack of the basic environment at low concentration levels. Therefore, 0.1 mol L^{-1} NaOH was used as the AP.

3.4. Effect of stirring speed

The extraction can be accelerated by stirring the donor solution, thereby reducing the time required to attain thermodynamic equilibrium [22]. In this work, the effect of stirring speeds (200–500 rpm) on the extraction efficiency was investigated. The

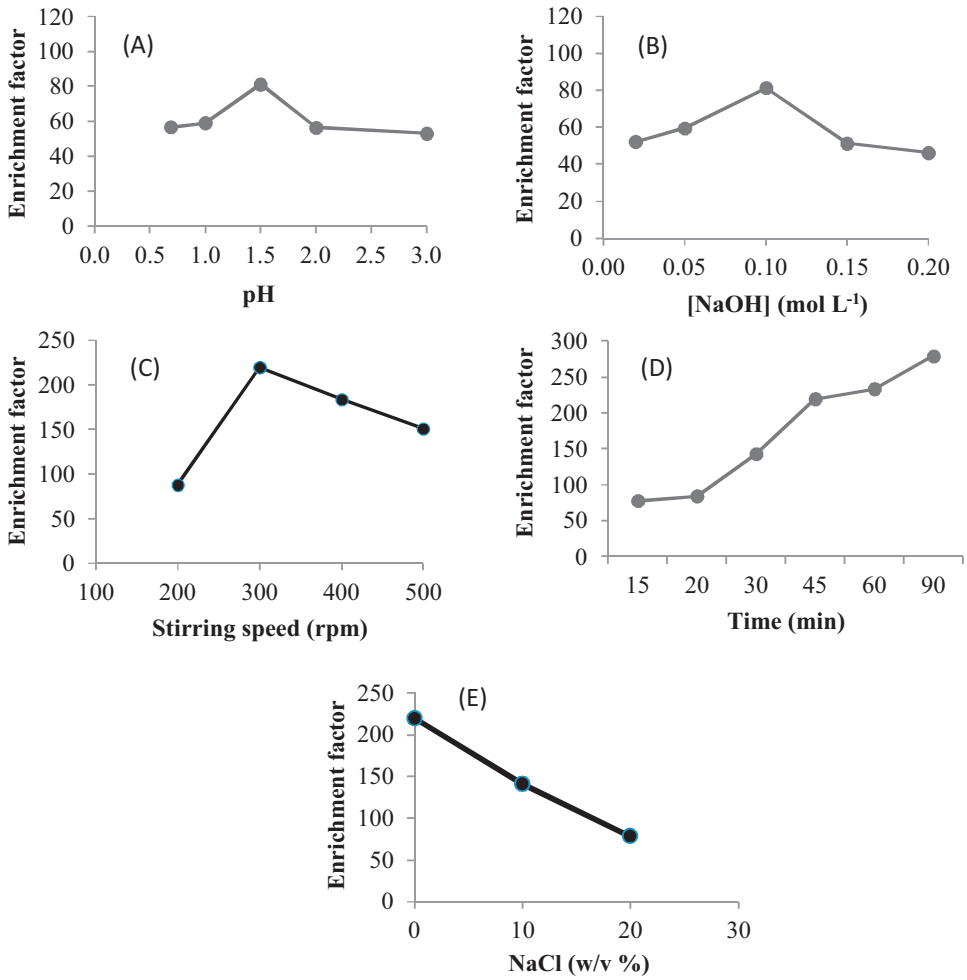

Fig. 3. Influence of different parameters on enrichment factor: (A) pH of the donor phase, (B) concentration of acceptor phase (NaOH), (C) stirring speed, (D) extraction time and (E) salt addition.

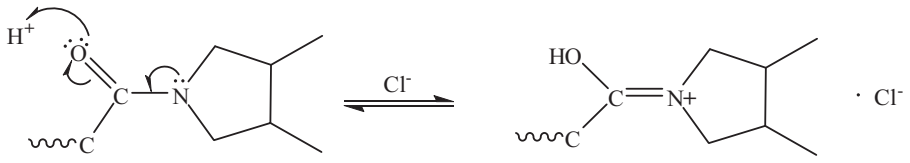

Fig. 4. Propose mechanism where the carbonyl on the amide acts as hydrogen acceptor, resulting in poor extractions under highly acidic conditions.

mass transfer process and partitioning of the analytes into the organic solvent were enhanced by increasing the stirring speed up to 300 rpm (Fig. 3C). Increasing the stirring speed over 300 rpm resulted in decreased extraction efficiency. This is due to the formation of vortex in the sample solution, resulting in rotation of the fiber rather than stirring due to the centrifugal force. Therefore, 300 rpm was selected for the rest of the studies.

3.5. Effect of extraction time

Since LPME is a time-dependent process, therefore sufficient time is needed to permit partitioning of the analyte between the phases. The rate of analyte diffusion through the pores of a HF also influences the time necessary for the extraction. The effect of extraction time was examined over 15, 20, 30, 45, 60 and 90 min (Fig. 3D). The enrichment factors were found to increase by increasing the exposure time from 15 to 90 min. The long extraction time required to reach equilibrium, especially for high viscosity sample, is undesirable because it will allow the organic solvent to dissolve into the aqueous phase [23,24]. For practical reasons, it is preferable to perform the extraction under non-equilibrium conditions. Therefore, an exposure time of 45 min was selected.

Table 1
Results for the determination of mitiglinide (MIT) in spiked samples subjected to the HF-LPME and analyzed using HPLC.

	C_{added} (ng mL^{-1})	C_{found} (ng mL^{-1})	Recovery ± SD (%)	% RSD[a]
Urine	100	88.3	88.3 ± 4.74	5.37
	400	385	96.3 ± 10.0	10.4
	800	743	92.9 ± 9.85	10.6
Plasma	100	99.3	99.3 ± 7.91	7.97
	400	372	93.0 ± 11.3	12.2
	800	736	92.0 ± 12.5	13.6

[a] $n = 9$.

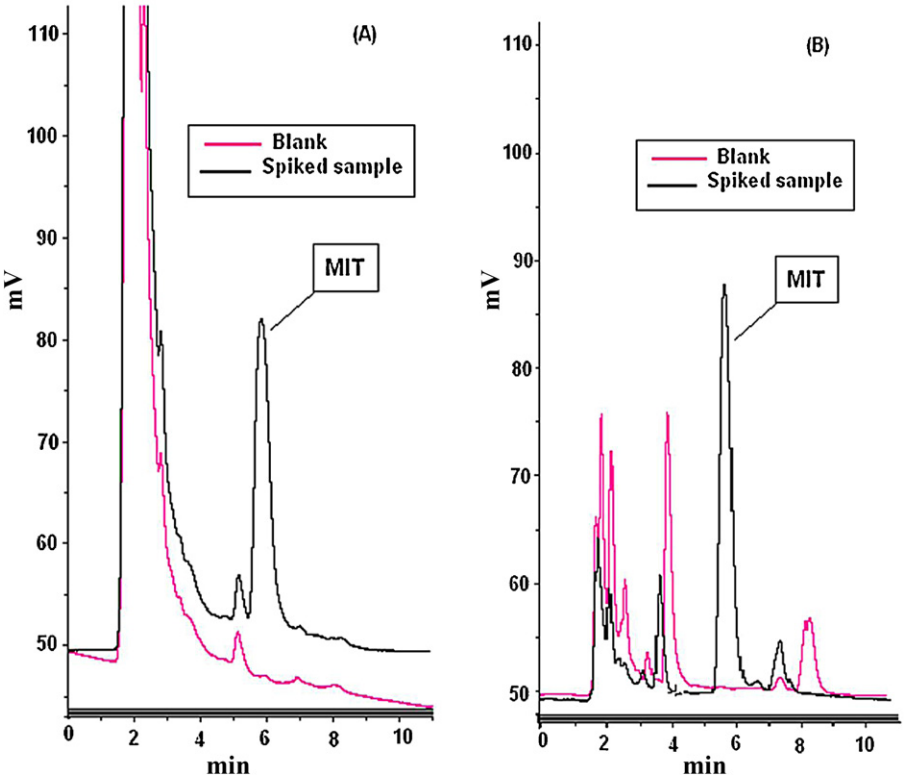

Fig. 5. Typical chromatogram of (A) spiked urine sample, (B) spiked plasma sample after undergoing LPME. Spiked concentration; 800 ng mL^{-1} of mitiglinide.

3.6. Effect of salt addition

Addition of salt to the sample can enhance the extraction efficiency which can decrease the solubility of analyte in the aqueous solution [25,26]. The effect of salt addition on enrichment factors was examined by adding sodium chloride to aqueous samples at the concentration levels of 0–20% (w/v). A marked decrease in extraction efficiency was observed as the concentration of the salt increase (Fig. 3E). This is due to the restricted analyte movement from the DP to the membrane solvent resulting from the increase of viscosity of solution. In addition a high salt concentration modifies the physical properties of the diffusion film, thus no salt was added in the subsequent experiments.

3.7. Optimized extraction procedure

The adopted LPME conditions were: 1-octanol as extracting solvent; 0.1 M NaOH as AP; DP, 10 mL (pH 1.5); stirring speed, 300 rpm; extraction time, 45 min at room temperature; without the addition of salt. Under these conditions, extraction recovery (ER) of 31.5% and an enrichment factor (EF) of 210 were achieved. The enrichment factor and extraction recovery of MIT were calculated by the following equations [27,28]:

$$\mathrm{EF} = \frac{C_{\mathrm{AP,final}}}{C_{\mathrm{DP,initial}}}$$

$$\mathrm{ER}(\%) = \mathrm{EF} \times \left(\frac{V_{\mathrm{AP}}}{V_{\mathrm{DP}}}\right) \times 100$$

where $C_{\mathrm{AP,final}}$ and $C_{\mathrm{DP,initial}}$ are the final and initial concentrations of the drug in the AP and the DP, respectively. $C_{\mathrm{AP,final}}$ of the extracted drug was calculated from the calibration curve. V_{AP} and V_{DP} are the volumes of the acceptor and donor phases, respectively.

3.8. Method validation

The proposed method was validated for linearity, limits of detection (LOD) and quantitation (LOQ) and repeatability. Calibration curve was plotted over the concentration range of 5–1000 ng mL^{-1}. For each level, three replicate extractions were performed under the adopted conditions. Regression equation and correlation coefficient were: $y = 1.1707x - 6.6290$ ($r^2 = 0.9975$). LOD and LOQ were calculated as the amount of the injected sample to yield a signal-to-noise ratio of 3 and 10, respectively. The obtained LOD and LOQ were 1.38 and 4.21 ng mL^{-1}, respectively. Repeatability was done by nine repetitive determinations of three different concentration levels (10, 100, 400 and 800 ng mL^{-1}). The results expressed as relative standard deviation (RSD%) were: 9.74%, 8.89%, 6.48% and 5.76%, respectively. The obtained results comply with the Food and Drug Administration recommendations [29].

3.9. Recovery

The method was successfully used for the analysis of MIT in human urine samples. However, the analysis of MIT in plasma samples resulted in low recoveries due to the strong binding between MIT and proteins in the plasma. Therefore, a pre-treatment step was conducted using a miniaturized liquid–liquid extraction (MLLE). Combination of the two extraction methods resulted in clean extracts with sufficient sensitivity for the trace determination of MIT in plasma samples. Three different concentration levels (100, 400 and 800 ng mL^{-1}) were examined for accuracy, each concentration was prepared thrice and each preparation was extracted

Table 2
Comparison of the developed method with other reported methods for the determination of mitiglinide (MIT).

Instrument	Sample preparation	Type of sample	Linearity range (ng mL^{-1})	LOD (ng mL^{-1})	LOQ (ng mL^{-1})	(%RSD)	Rec. (%)	Ref.
LC[a]–MS/MS[b]	MLLE[f]	Human plasma	0.5–4000	–	0.5	<15 (n = 3)	72.8–73.8	[9]
LC–ESI-MS[c]	MLLE	Urine	5–1000	2	5	<10 (n = 5)	85–115	[10]
UPLC[d]–MS/MS	MLLE	Human plasma	1.1–5400	–	1.080	<15 (n = 5)	75.8–83.2	[12]
HPLC–UV[e]	MLLE	Rat plasma	100–20,000	–	100	<15 (n = 5)	98.6–97.4	[8]
LC–ESI-MS	MLLE	Human plasma	2–7965.5	1	–	<15 (n = 6)	85–115	[11]
LC–ESI-MS	SPE[g]	Human plasma	10–1600	–	10	<12 (n = 5)	80.8–90.1	[18]
HPLC–UV	MLLE/HF-LPME[h]	Plasma	5–1000	1.4	4.2	<13.6 (n = 9)	92.0–99.3	Current
	HF-LPME	Urine				<10.6 (n = 9)	88.3–96.3	work

[a] Liquid chromatography.
[b] Mass spectrometry.
[c] Electrospray ionization mass-spectrometry.
[d] Ultra-pressure liquid chromatography.
[e] High performance liquid chromatography coupled with UV detector.
[f] Miniaturized liquid–liquid extraction.
[g] Solid-phase extraction.
[h] Hollow-fiber liquid-phase microextraction.

three times. Acceptable accuracy and precision for urine and plasma samples was observed (Table 1). Representative chromatograms of a spiked plasma and urine samples are shown in Fig. 5.

3.10. Comparison of the proposed method with other reported methods

Table 2 compares the figures of merit of the proposed method and the previously reported methods for the analysis of MIT. The proposed method is clearly sensitive (lower LOQ) compared to an earlier work using SPE coupled with LC–ESI-MS [18] and MLLE coupled with HPLC–UV [8], but comparable to the MLLE coupled with LC–ESI-MS [10] method. The repeatability and recovery of the proposed method is better than those obtained from the other methods [9–12]. To the best of our knowledge this is the first paper that describes the analysis of MIT in biological fluids using microextraction method.

4. Conclusions

A new analytical method suitable for the trace analysis of MIT in biological samples has been successfully developed. It integrates the "green" features of the HF-LPME sample pre-treatment method (minimum consumption of organic solvent, simple, cheap) and a rapid HPLC separation (~6 min). The good enrichment enables the method to reach low sensitivities (quantitation limit, 4.2 ng mL^{-1}), seriously rivaling to that of the more expensive equipments such as the LC–ESI-MS [10,18]. The good enrichments (enrichment factor, 210) also open up the possibility of more widespread determination of trace MIT in biological fluids. The method can be used directly for the analysis of urine samples but a simple MLLE step is necessary to reduce binding of the drug to the sample matrix for plasma samples. Additional advantages of the developed method are the clean chromatograms, largely due to the effective blockage of macromolecules being extracted that is afforded by the pores of the HF.

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