

Miniaturized Hollow Fiber Liquid Phase Microextraction Method Followed by High-Performance Liquid Chromatography for the Extraction and Determination of Diclofenac in Biological Samples

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Abstract

Diclofenac sodium (DIC) is a nonsteroidal anti-inflammatory drug (NSAID) taken to reduce inflammation and as an analgesic reducing pain in certain conditions.

Diclofenac sodium has a dissociation constant (pKa) of 4.0 ± 0.2 at 25°C in water. In this study, hollow fiber liquid phase microextraction (HF-LPME) coupled with high-performance liquid chromatography (HPLC) with UV detection was applied to preconcentration and determination of Diclofenac sodium in biological fluids. (DIC) was extracted into an organic extracting solvent (*n*-octanol) that was immobilized in the wall pores of a porous hollow fiber from 7/5 mL of aqueous sample (donor phase) and was back extracted into 10 µL of acceptor phase.

Parameters affecting the extraction process including donor phase PH, acceptor phase PH, type of extraction solvent, stirring rate, extraction time and also the salt addition were studied and optimized. Under the optimized conditions donor phase PH=3, acceptor phase PH=11.7, extraction solvent *n*-octanol, stirring rate =750 rpm, extraction time=27 min and without the addition of salt, enrichment factors up to 170 were achieved and the relative standard deviation (R.S.D %) of the method was in the range of 2-3%. The method was successfully applied to determine the concentration of Diclofenac sodium in the biological samples and satisfactory results were obtained.

Keywords: Diclofenac, Liquid-phase microextraction, HPLC, biological samples

Introduction

Diclofenac (DIC) is the most frequently administered non-steroidal analgesic, antipyretic and anti-inflammatory drug (NSAID) with properties mainly used for the treatment of the rheumatic diseases. It is produced and used in a great annual increasing volumes. In recent years, it has been found as environmental contaminants in sewage, surface, ground and drinking water samples [1,2,5,9]. Several methods have been described for the quantification of the NSAIDs in biological samples, based on different extraction procedures and various analytical techniques such as high-performance liquid chromatography (HPLC) [3,4], capillary electrophoresis [6], and spectrofluorimetry [7]. Pharmaceutical residues are usually present in biological samples at trace levels; therefore, a pre-concentration step is generally required for determination of them. The most common sample preparation technique is solid phase micro extraction (SPME). when SPME is coupled with HPLC, an elaborate SPME–HPLC interface device has to be used for the solvent desorption. Because of these problems, another miniaturized sample preparation method, i.e., liquid-phase microextraction (LPME) was emerged for overcoming. In LPME, only a small

amount of the extracting solvent (microliter) is needed for concentrating of the analytes from the aqueous and biological samples. In recent studies, several micro extraction methods were used for the separation of diclofenac in real samples (water, urine and plasma) such as single drop micro extraction (SDME), and solid phase micro extraction (SPME) [8].

In the present study, the HPLC method combined with prior HF-LPME was developed for the separation and pre concentration of diclofenac from biological

samples. In this purpose, the micro porous hydrophobic hollow fiber was used to separate the aqueous donor sample solution and the aqueous acceptor phase. All the HF-LPME and HPLC parameters have been optimized in order to propose a rapid, simply and sensitive determination of that drug as pollutants in biological samples. This method can be compared with the other microextraction methods which were mentioned above. The obtained data show good advantages of the proposed method. For example, it presents lower limit of detection values, higher enrichment factor and also it requires lower extraction time. The aim of this work was to develop a highly simple, sensitive and effective method for the determination of (DIC) which can easily be applicable to plasma. DIC was extracted from plasma and urine by HF-LPME and various parameters on extraction were optimized and determined by HPLC-UV.

Materials and methods

Standards and Reagents

Diclofenac sodium salt was donated from food and drug organization laboratory (Tehran, Iran). n-octanol, methanol (HPLC grade) were purchased from Merck (Darmstadt, Germany) and were used without further purification. Also, Sodium hydroxide, hydrochloric acid, and sodium chloride were obtained from Merck. A stock solution of 100 mg L⁻¹ of each analyte was prepared in methanol. Standard sample solutions were provided daily at different concentrations by diluting the stock standard solutions with distilled water. The PP polypropylene microporous hollow-fiber membrane (200 µm wall thickness, 600µm inner diameter, 0.2µm pore size, and 75% porosity) was obtained from Membrana(Wuppertal, Germany).

Apparatus And Chromatographic Conditions

Chromatographic separations were carried out on a Youngling HPLC equipped with a YL 9110 quaternary pump HPLC, a 10 µL sample loop and a YL 9120 UV-VIS detector. Chromatographic data were recorded and analyzed using a Youngling Autochro-3000. The chromatographic separation was carried out at room temperature (about 25 °C) on a C18 column (150 mm × 4.6 mm, with 5 µm particle size) with a C18guard column (4.0 mm × 10 mm, 5 µm) from Teknokroma (Barcelona, Spain). The degassed mobile phase consisting of methanol- potassium hydrogen phosphate (pH 2.5) (70:30, v/v) was delivered by a Quaternary pump at 0.9 mL min⁻¹. The UV detection wavelength was set at 254 nm. All of the pH measurements were performed with a GPHR 1400A pH meter (Germany). An MR Hei-Standard magnetic stirrer by Heidolph Company (Germany) and syringe (25 µL) model 702 NR from Hamilton (Bonaduz, Switzerland) were also used. The ultrapure water was obtained from a MilliQ Ultrapure water purification system (Millipore, Bedford, MA, USA). All extractions were carried out using a Q3/2 Accrual polypropylene hollow fiber membrane from Membrana (Wuppertal, Germany) with a 0.2 µm pore size, 600 µm internal diameter and 200 µm wall thickness.

Extraction procedure

In the present work, we used hollow fiber which was cut into segments with a length of 4.5 cm with the internal volumes of 10 μ L. These segments were placed for 5 min in acetone and put in an ultrasonic bath for 10 min to remove any contaminants. 7.5 mL of the aqueous Diclofenac solution as a donor phase (DP) with exact concentration was transferred into a 30 mL glass vial containing a magnetic stirring bar; the vial was then put on a magnetic stirrer. 15 μ L of the acceptor phase (AP) was injected by a 25 microliter Hamilton microsyringe into the cleaned hollow fiber and the fiber was submerged in the organic solution for 10 s and then into the reagent water for 3 s so that the extra organic solution would be washed from the outer surface of the fiber. After that, the fiber was bent into a U-shape and was submerged in the glass vial of the sample solution for different lengths of time. The vial was covered with ParaFilm and stirred for a prescribed time period. At the end of the extraction time, the hollow fiber was removed from the sample solution, and the acceptor phase was withdrawn into the syringe. Finally, 10 μ L of the acceptor phase was injected directly into the HPLC system for analyzing the Diclofenac.

Results and discussion

Optimization Method

To obtain the optimal extraction efficiency, various parameters that potentially affect plasma and urine sample extraction was studied which can be discussed respectively.

Selection of the organic Solvent

Four organic solvents with different viscosities and volatilities have been examined in this work.

These extracting solvents were: isobutyl methyl ketone, Dodecan-1-ol, *n*-octanol, and *n*-octan. All of these solvents were easily immobilized in the pores of the hollow fiber. Among of them, the extraction efficiencies of isobutyl methyl ketone, Dodecan-1-ol and *n*-octan were not desirable. Therefore, *n*-octanol was selected as the organic solvent for further studies due to the highest analytes enrichment among the others.[16,17]

The pH of donor and acceptor phases

The difference in pH between the donor and acceptor phase is one of the major parameters which can be a promoting factor for transferring the analytes from donor to acceptor phase. The pH of the donor phase should be adjusted to demonize the analytes and the acceptor phase adjusted to ionize them. Since, the target compounds are weak acids {diclofenac (2-[(2,6-dichlorophenyl) amino] -benzene acetic acid) ($pK_a = 4.0 \pm 0.2$)}, it is unionized in acidic, as well as neutral solutions. So, the pH of the sample solution is a very Premier factor. In this research, (pH= 3) was used as a donor phase. On the other hand, the extraction efficiency was more depended on the pH of the acceptor solution, which should be basic enough to ionize these weak acidic analytes by accepting of a proton from them. The results show that by increasing the NaOH concentrations in the aqueous

acceptor solution, the enriching of the analytes are improved. Therefore, (pH= 11.7) was selected as the pH of acceptor phase .the pH of acceptor phase is a very important factor. [13].

Effect of stirring rate

In this work, different stirring speeds ranging from 250 to 1000 rpm were tested to determine their effects on the extraction efficiency of the drug. The results showed that extraction efficiency was improved by increasing the stirring rate to 750rpm.However, very high stirring rates (up to 750) would lead to the production of excessive air bubbles and lose solvent that could affect the precision. Therefore, 750 rpm was chosen as a suitable stirring rate for future experiments.

Effect of extraction time

The impact of extraction time on extraction efficiency was studied in the range of 20–40 min. The amount of the extracted. At the extraction times higher than 45 min, the extraction efficiency reduced. This could be due to solvent evaporation and bubble formation on the fiber wall... Therefore, in order to obtain high sensitivity, the extraction time of 27 min was chosen as the optimum time for the subsequent experiments.[18,19]

Influence of temperature

In this step, extraction temperatures in the range of 20-45° C were investigated. The results show that increasing the temperature up to 40° C causes reduce extraction efficiency. On the one hand, the solvent loss may occur due to increased solubility of the organic solvent at higher temperatures, which causes to decline the extraction efficiency, the optimum extraction temperature of 40° C was selected for further experiments.

The effect of volume ratio of donor phase to acceptor phase

Pre-concentration factor (PF) in three phase HF-LPME was calculated with $PF = V_d R / V_a \cdot 100$ where V_d is the volume of the donor phase and V_a is the volume of the acceptor phase and R is the extraction efficiency. Thus, in this method, the optimization of the volume of DP and AP is very important on the extraction efficiency. In this study, the volume of the acceptor phase was kept constant (20 μ L) and the volume of the donor phase was changed and its effect on extraction rate was investigated . 15 ml of DP was selected as the optimum volume for subsequent experiments.

Quantitative considerations

Under optimal extraction conditions, enrichment factors, repeatability, the linearity and the limits of detection were determined by utilizing standard solutions of analyte in plasma and urine. By plotting peak areas versus concentrations of the analyte in the sample solution, calibration curves were obtained which showed that correlation coefficient (r) were above 0.9971. The limits of detection (LOD), limits of quantitation (LOQs), enrichment factors and other analytical data are summarized in Table 2.

Volume of DP (mL)	T (°C)	Time (min)	stirring rate (rpm)	PH of AP	PH of DP	Solvent
15	40	27	750	11.7	3	n-octanol

Analysis of real samples

Under the optimized conditions, the developed HF-LPME-HPLC technique was applied to preconcentration and determination of diclofenac in plasma and urine samples. The pH of the real samples was adjusted to 3 by adding of 0.1M NaOH to plasma and H_3PO_4 to urine solution.

Prior to the spiking and extraction procedure of the target drug, the plasma samples were diluted 1:1 with water. Figure 1. is the typical chromatograms for the spiked plasma sample obtained by HF-LPME-HPLC and Figure 2. is the typical chromatograms for the urine sample obtained by HF-LPME-HPLC. The present method was also compared with other studies in terms of method of extraction, validation, and precision. As can be deduced, the method is quite comparable to those mentioned in Table 3. As can be seen, the LODs of this method are comparable with those obtained in the previous studies and even lower than those reported in the literature. In addition, due to the simplicity and low cost of the extraction device, the hollow fiber can be discarded after each extraction to avoid carryover and cross-contamination. (Table 3, Table 4)

PF	LOD (ng mL ⁻¹)	LOQ (ng mL ⁻¹)	R ²	DLR (ng mL ⁻¹)	%RSD Intra-day	%RSD Inter-day
178	2.8	8.4	0.99	50-2000	2.7	3.8

Table 3. The results of diclofenac analysis in plasma sample in optimum condition of HF-LPME (n=5)				
Sample	C _{Added} (ng mL ⁻¹)	C _{Found} (ng mL ⁻¹)	RSD%	Recovery (%)
Plasma	3	3.1	3.6	103
	10	9.7	3.1	97

Table 4. The results of diclofenac analysis in urine sample in optimum condition of HF-LPME (n=5)				
Sample	C _{Added} (ng mL ⁻¹)	C _{Found} (ng mL ⁻¹)	RSD%	Recovery (%)
Plasma	3	3	3.9	100
	10	9.9	3.5	99

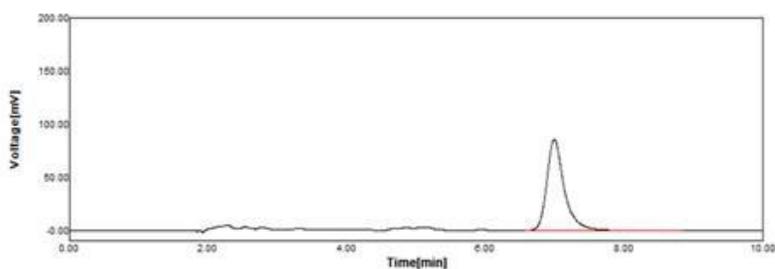


Figure 1. Chromatograms obtained after applying HF-LPME to plasma sample spiked with 3 ng mL⁻¹ of Diclofenac under the optimum conditions (pH of donor phase:3; pH the acceptor phase:11.7; stirring rate:750 rpm; extraction time:27 min; temperature:40 °C)

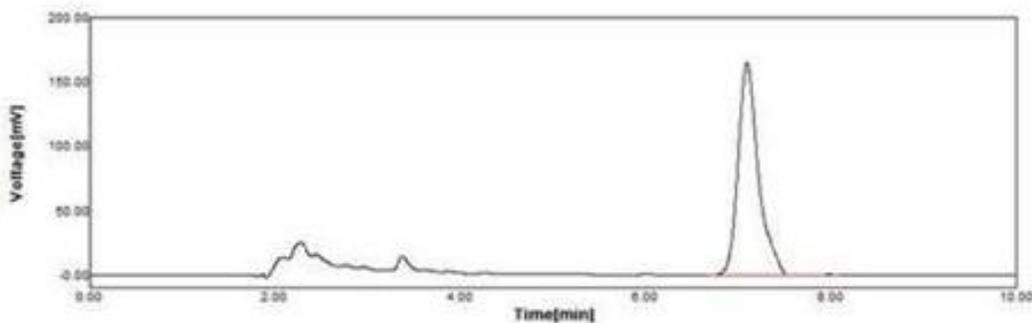


Figure2. Chromatograms obtained after applying HF-LPME to urine sample spiked with 3 ng mL⁻¹ of Diclofenac under the optimum conditions (pH of donor phase:3; pH the acceptor phase:11.7; stirring rate:750 rpm; extraction time:27 min; temperature:40 °C)

Table 5. Comparison of the proposed method with other developed methods for determination of Dic in biological samples .						
Instrumentation	Sample preparation	Sample	DLR ng mL ⁻¹	LOD ng mL ⁻¹	RSD%	Ref.
HPLC	LLE ²	water	500-2000000	100	7/2	15
HPLC/UV	S.P.E ¹	urine	-	280	3/56	12
HPLC-UV	-	urine	100-2000	12.03	-	14
LC-ESI-MS	HF-LPME ³	sewage	-	1-3.9	-	20
HPLC	CFHF-LPME	sewage	-	0.25	-	21
HPLC	-	urine	-	40	-	11
LC/MS	-	drugs Mixture	50-300	0.5-4	8-9	10
HF-LPME	HF-LPME	urine and plasma	50-2000	2.8	2.7	This work
¹ Solid-phase extraction ² Liquid-liquid extraction ³ Hollow fiber-liquid phase microextraction						

Conclusions

In the present study, a new method of liquid-phase microextraction, using a microporous polypropylene was developed for the extraction of diclofenac from urine and plasma samples. The extraction was carried out by using a volatile organic solvent which has a low viscosity that leads to the increase of mass transfer and extraction efficiency along with a decrease in the extraction time. This method is very practical and simple and the eluted analytes are directly determined by

HPLC. Using this technique, the analytes can be extracted from water samples quantitatively with a good linearity and repeatability.

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