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Hollow fiber liquid-phase microextraction for the determination of nimesulide in human plasma and its application to a pharmacokinetic study

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A hollow fiber liquid-phase microextraction (HF-LPME) method in combination with HPLC-UV for the determination of nimesulide in human plasma was developed and validated. A small volume of dihexyl ether contained within a polypropylene hollow fiber was used for the extraction of nimesulide from acidified plasma solutions. Factors affecting the extraction efficiency were optimized and discussed. With HPLC-UV as the end analysis technique, the procedure was validated for nimesulide in the concentration range of 50–5000 ng/mL. The intra- and inter-assay precisions were less than 9.1%, and accuracy was within 3.2%. The lower limit of quantification (LLOQ) was 50 ng/mL. Enrichment factor from 144-fold to 156-fold was achieved at three quality control (QC) concentrations. The mean extraction recovery was greater than 41.2%. This method was successfully applied for the evaluation of pharmacokinetics of nimesulide after single oral doses of 100 mg nimesulide to six healthy Chinese volunteers.

1. Introduction

Nimesulide (4-nitro-2-phenoxymethane sulfonanilide, Fig. 1) is an NSAID used in the treatment of various inflammatory conditions (Jaworowicz et al. 1999). It is weakly acidic (pKa 6.5) and acts through specific inhibition of cyclooxygenase-2 (COX-2) thus being associated with less gastrointestinal side effects (Hutt et al. 2001; Hoyo-Vadillo et al. 2003; Guptan et al. 1999).

Several methods have been reported for the determination of nimesulide in pharmacokinetic and bioavailability studies. Most procedures used liquid–liquid extraction (LLE) (Jin et al. 2002; Wu et al. 2000; Zhang et al. 2004) and solid-phase extraction (SPE) techniques (Ferrario and Bianchi 2003) combined with (HPLC) or liquid chromatography with tandem mass spectrometric (LC-MS/MS) detection (Barrientos-Astigarraga et al. 2001). The conventional pretreatment techniques generally suffer from some essential limitations. LLE requires the use of large amounts of solvent which is often hazardous and results in the production of toxic laboratory waste. Besides, LLE can produce emulsions and often involves evaporation and reconstitution steps before injection into the chromatographic system. SPE requires time-consuming extraction processing and often introduces contaminant into the sample extracts.

Recently, some new techniques, such as solid-phase microextraction (SPME) and liquid-phase microextraction (LPME) have been developed for sample preparation. Pedersen-Bjergaard and Rasmussen introduced hollow fiber based liquid-phase microextraction (HF-LPME) in 1999. This technique is based on the use of a single, disposable, porous hollow fiber made of polypropylene. In three phase HF-LPME, the ionizable analytes are extracted from the sample (aqueous phase) in their uncharged form, through a thin layer of organic phase immobilized within the pores of the porous hollow fiber and into the acceptor solution (aqueous phase) in the lumen of the hollow fiber (Payán et al. 2009; Wen et al. 2004; Lin and Huang et al. 2008).

The hollow fiber liquid-phase microextration technique provides efficient pre-concentration due to the use of very small volumes of extraction phase. The small pore size of the hollow fiber prevents large molecules and particles in the sample solution from entering the acceptor phase thus yielding very clean extracts and excellent detection sensitivity. The acceptor phase could be injected into the HPLC system directly (Kataoka 2010; Sobhi et al. 2007; Wu and Hu et al. 2009). The disposable nature of the hollow fiber totally eliminates the possibility of sample carryover and ensures reproducibility. An additional advantage of HF-LPME is its tolerance to a wide pH range which is limited in SPE or SPME (Nerín et al. 2009; Rodriguez-Lafuente et al. 2009).

Applications of HF-LPME in biological samples, such as the extraction of drugs from urine, hair and plasma were reported (Esrafili et al. 2007; Emıdio et al. 2010). However, these studies were generally related to basic drugs, e.g., clenbuterol, amphetamines, benzodiazepines and so on. Payán et al. (2009) developed a HF-LPME method for the determination of ibuprofen, diclofenac and salicylic acid in human urine. As plasma is more complex than urine and some endogenous organic compounds in plasma possess properties similar to those of the acidic drugs, development of new pretreatment technique in determination of acidic drugs in plasma is meaningful.

The aim of this work was to establish an alternative extraction method for nimesulide applicable to plasma samples. The simplicity of the HF-LPME could be an effective way to obtain enough sensitivity. The procedure allows the HPLC-UV determination of nimesulide in plasma with a quantification limit of 50 ng/mL. It was validated for precision, linearity, and accuracy. The feasibility of the HF-LPME method was demonstrated by

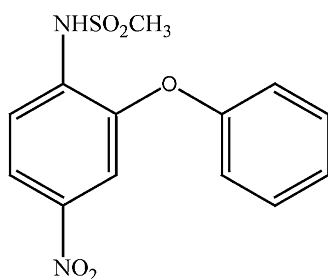


Fig. 1: Chemical structure of nimesulide

analysis of nimesulide in human plasma in a pharmacokinetic study.

2. Investigations, results and discussion

2.1. Selection of hollow fiber

Two different commercially available hollow fibers, polyvinylidene fluoride hollow fiber membrane and polypropylene hollow fiber membrane were considered for this work. As described in Section 3.4, both of these fibers were cut into 5.5 cm and pretreated. Extractions were performed using dihexyl ether as the extraction solvent from 1 μg/mL plasma samples at room temperature (25 °C). The stirring rate was set at 300 rpm for 30 min. HCl (10 mM) and NaOH (10 mM) solutions were used as the donor phase and acceptor phase, respectively. Polypropylene hollow fiber membrane exhibited much higher extraction efficiency than the polyvinylidene fluoride fiber and was selected. The fiber is far less expensive than the polymer coating of SPME so that could be discarded after each extraction. This eliminates the possibility of sample carryover and ensures reproducibility.

2.2. Organic solvent selection

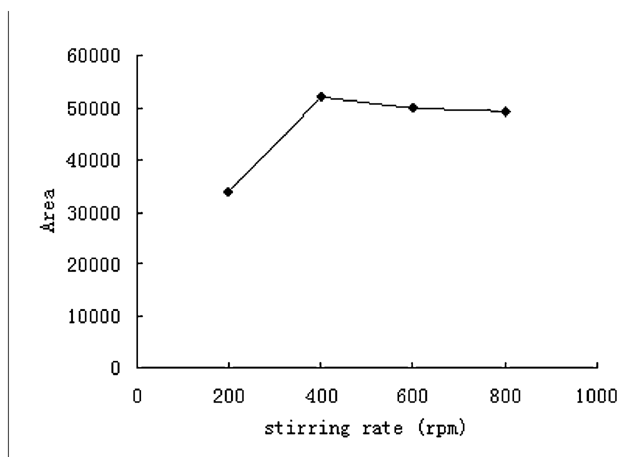
The selection of organic solvent is of significant importance in HF-LPME in order to obtain efficient enrichment, good sensitivity and selectivity. In general, the chosen organic solvent must present good affinity for the analyte of interest, be immiscible with both the acceptor and donor phase, be easily immobilized within the pores of the fiber and of low volatility to prevent solvent loss during the extraction (Tahmasebi et al. 2009). During method development, six organic solvents including octanol, dibutyl phthalate, hexamethylene, toluene, benzyl alcohol and dihexyl ether were evaluated. Dihexyl ether was selected for its good extraction efficiency. Conventional LLE for nimesulide required several milliliters of tolune, dichloromethane or chloroform, while only microliters of dihexyl ether were used in this HF-LPME procedure. The hazards produced by the toxic solvents were reduced significantly. Besides, the formation of emulsions could also be avoided. And the dramatic improvement in concentration was meaningful.

2.3. Effect of stirring speed

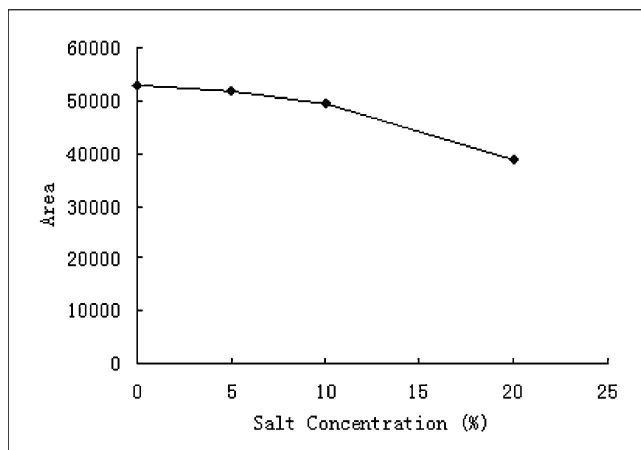
The stirring speed during the extraction affects the rate of equilibrium and the physical stability of the organic solvent layer on the outside of the hollow fiber. Four different stirring rates (200, 400, 600 and 800 rpm) were tested. No significant increase was observed (Fig. 2A) with further increase of the stirring rate. As a result, the stirring speed was set at 400 rpm.

2.4. Effect of ionic strength

The addition of neutral salt is often used to decrease the solubility of the analyte in the sample through the salting-out effect



(A)



(B)

Fig. 2: Influence of stirring rate on extraction efficiency (A). Conditions: Nimesulide, 1 μg/mL; organic solvent, dihexyl ether; HCl in donor phase, 10 mM; NaOH in acceptor phase, 10 mM; extraction time, 30 min; room temperature. Influence of salt concentration on extraction efficiency (B). Conditions: Nimesulide, 1 μg/mL; organic solvent, dihexyl ether; HCl in donor phase, 10 mM; NaOH in acceptor phase, 10 mM; extraction time, 30 min; stirring rate, 300 rpm; room temperature

and therefore improve the extraction performance (Shrivas and Wu 2007). In our study, the effect of ionic strength on extraction of nimesulide was determined by adding sodium chloride (NaCl) (0, 5%, 10% and 20%) into spiked plasma sample. The result (Fig. 2 B) suggested that the peak area of nimesulide was decreased with the increase NaCl concentration. We also observed that addition of salt could increase the density of the aqueous sample which may have in a produce negative influence on the passive diffusion of analyte from the sample phase to the organic phase. Therefore, no salt was added in the sample solution.

2.5. Orthogonal array design

Orthogonal array design (OAD) was used to estimate the composition of the donor phase and the acceptor phase as well as the extraction time in the HF-LPME of nimesulide. A L_9 ($_3{}^4$) orthogonal form (Table 1) with a blank column was applied, and the data were analyzed by direct observation analysis and variance analysis. Nimesulide is an acidic drug; therefore, the sample was adjusted to acidic pH with HCl so that the analyte was in its uncharged form and easily extracted into the organic phase. aqueous NaOH was employed as the acceptor phase to ionize the drug. The charged ions produced have a high solubility in the aqueous phase, facilitating a continuous

Table 1: Orthogonal array $L_9(3^4)$ and experimental results

Experimental No.	A (HCl Concentration)[a]	B (NaOH Concentration)[b]	C (Extraction Time)[c]	D (Blank)	Results[d]
1	1	1	1	1	6928
2	1	2	2	2	54978
3	1	3	3	3	35553
4	2	1	2	3	5058
5	2	2	3	1	61213
6	2	3	1	2	26686
7	3	1	3	2	7796
8	3	2	1	3	29786
9	3	3	2	1	28476
K_1	32486	6594	21133	32205	
K_2	30986	48659	29504	29820	
K_3	22019	30238	348454	23466	
R	10467	42065	13720	87400	

Conditions: Nimesulide, 1 μg/mL; organic solvent, dihexyl ether; stirring rate, 400 rpm; room temperature.
[a] 1 = 2 mM, 2 = 10 mM, 3 = 50 mM.
[b] 1 = 5 mM, 2 = 20 mM, 3 = 100 mM.
[c] 1 = 15 min, 2 = 30 min, 3 = 45 min.
[d] The average results of two experiments.

pumping of nimesulide into the basic solution until an equilibrium was reached. The levels employed in the OAD design were selected during preliminary experiments considering the limitations of the experimental system. HCl concentrations of 2, 10 and 50 mM as well as NaOH concentrations of 5, 20 and 100 mM were evaluated. Stirring times of 15, 30 and 45 min were also tested. The plasma was prepared containing 1 μg/mL of nimesulide. The extraction was carried out under room temperature with dihexyl ether as the extraction solvent. The stirring rate was set at 400 rpm. Each trial was repeated ($n = 18$) to obtain suitable precision.

The average peak area for nimesulide was termed as the response value. Direct observation analysis (Table 1) was employed to estimate the importance of each given factor. The main factor contributing to the extraction efficiency was the factor B (NaOH concentration) followed by factor C (extraction time) and lastly, factor A (HCl concentration). In the observation analysis, the best combination of levels of the tested factors was $A_1B_2C_3$, i.e., 2 mM HCl in the donor phase, 20 mM NaOH in the acceptor phase and the extraction time of 45 min.

The ANOVA result obtained from SPSS software (Table 2) exhibits that the Sig. value of NaOH concentration is below 0.05, suggesting a significant effect of NaOH concentration on the extraction. On the other hand, the Sig. values of the other two factors are higher than the critical value, implying that the two factors are not statistically significant. Consequently, the NaOH concentration must be carefully controlled, while with respect to HCl concentration and time, the extraction procedure can be considered a robust extraction procedure.

Considering the extraction of the internal standard (IS), diclofenac sodium, as well as the experiment efficiency, the final HF-LPME procedure was fixed as described in Section 3.4 (10 mM HCl in the donor phase, 20 mM NaOH in the acceptor phase and the extraction time of 30 min). A verification test was carried out in triplicate. The result indicated that the final procedure provided adequate extraction recovery for both nimesulide and the IS. By using OAD, the optimization could be effective and simple.

2.6. *Method validation*

The validation included evaluation of the selectivity, lower limit of quantification (LLOQ), linearity of calibration curves, precision and accuracy, stability and recovery. The validation results are summarized as follows.

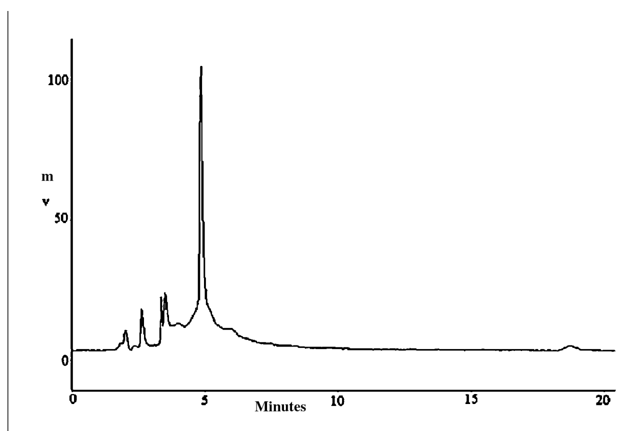
No endogenous interference with nimesulide or IS was detected in six different sources of blank plasma. The typical chromatograms of a blank plasma sample (A), blank plasma sample spiked with nimesulide and IS (B) and a plasma sample from a healthy volunteer 2 h after oral administration of nimesulide (C) are shown in Fig. 3.

Previously described analytical methods for the determination of nimesulide in biological samples based on HPLC separation with UV detection often have the LLOQ higher than 100 ng/mL. An SPE-HPLC-UV method (Ferrario and Bianchi, 2003) obtained a LLOQ of 25 ng/mL for nimesulide in rat plasma, but the operation was tedious and SPE

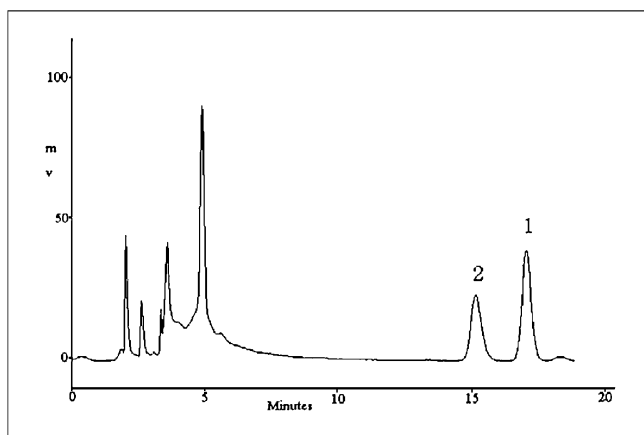
Table 2: Results of variance analysis

Source	Sum of squares	df	Mean square	F	Sig.
Corrected model	3.147×10^{9}[a]	6	5.245×10^{8}	8.566	0.108
	7.309×10^{9}	1	7.309×10^{9}	119.369	0.008
A	1.922×10^{8}	2	9.610×10^{7}	1.57	0.389
B	2.668×10^{9}	2	1.334×10^{9}	21.786	0.044
C	2.869×10^{8}	2	1.435×10^{8}	2.343	0.299
Error	1.225×10^{8}	2	6.123×10^{7}	8.566	0.108
Total	1.058×10^{10}	9			
Corrected total	3.269×10^{9}	8			

[a] R Squared = 0.963 (Adjusted R Squared = 0.850)



(A)



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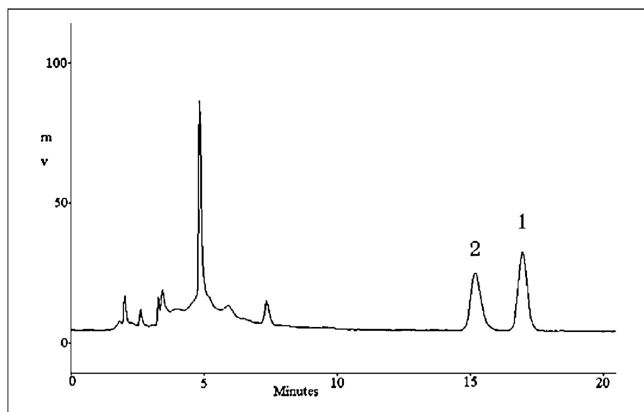

(C)

Fig. 3: Representative chromatograms of blank plasma (A); plasma spiked with nimesulide and IS. (B); plasma samples obtained 2 h after oral administration with 100 mg nimesulide (C) from a healthy volunteer. Peak 1: nimesulide; Peak 2: IS

cartridges were expensive. The LLOQ could reach 10 ng/mL using HPLC/MS/MS (Barrientos-Astigarraga et al. 2001), however, the instrument is not available in most routine laboratories, especially in developing countries. Under the optimal HF-LPME-HPLC-UV conditions, the LLOQ for nimesulide in human plasma was 50 ng/mL. The precision and accuracy at this concentration level were acceptable with RSD value of 6.5% and RE values from −6.5% to 9.1%. The simple and inexpensive method was sensitive enough to monitor the concentration of nimesulide for up to 24 h in most of the subjects.

Calibration curves ($n = 7$) with satisfactory linearity and precision were obtained for nimesulide over the concentration range of 50–5000 ng/mL. The typical calibration curve had a slope

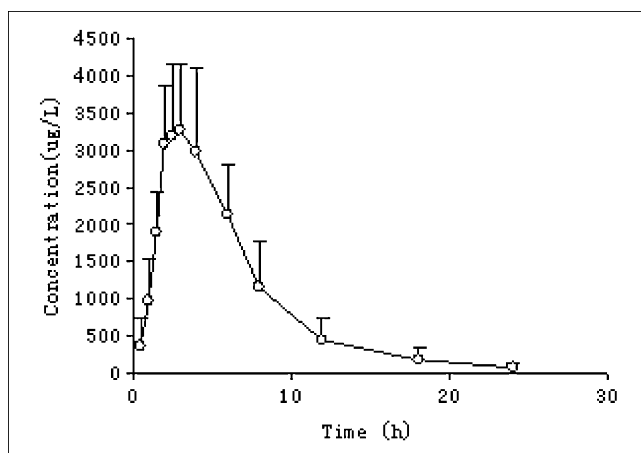

Fig. 4: Mean plasma concentration-time profile after oral administration of 100 mg nimesulide

of 1.341×10^{-3}, an intercept of 1.499×10^{-2} and correlation coefficient (r) of 0.9957.

The results of accuracies and precisions at three QC concentrations are presented in Table 3. All the data were within the defined acceptance criteria.

The RSD and RE for all stability samples were within 15%, suggesting that nimesulide was stable under the conditions tested.

Enrichment factors of about 144-fold to 156-fold were achieved for nimesulide at concentrations of 120, 1200 and 4000 ng/mL. The mean extraction recoveries were between 41.2%−44.6% at different QC concentrations of nimesulide. The results are also listed in Table 3.

2.7. Application

The validated HF-LPME method was applied to determine the plasma concentration of nimesulide in a pharmacokinetic study. The small sample volume (0.2 mL) made this technique suitable for clinical research. The mean plasma concentration-time curve of nimesulide in six healthy volunteers is shown in Fig. 4. Pharmacokinetic parameters (Table 4) were calculated using non-compartment model by Drug and Statistics (DAS, version 2.0). These parameters obtained were similar to those reported in previous papers (Bernareggi 1998).

In Conclusion, a three-phase HF-LPME method combined with HPLC-UV was developed to detect nimesulide in human plasma. With little organic solvent consumption, the procedure is environmentally friendly and provides significant improvement in sensitivity. It integrates isolation, purification and enrichment into one single step and the extract can be directly injected into the HPLC system. Validation data exhibited acceptable repeatability, accuracy and linearity. This method could be an alternative procedure for the quantification of nimesulide in human plasma, and it was successfully applied in a pharmacokinetic study of nimesulide in healthy Chinese volunteers after oral administration.

3. Experimental

3.1. Chemicals and reagents

Nimesulide as well as diclofenac sodium (the internal standard, IS) was purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Nimesulide dispersible tablets were from Beijing Yongzheng Pharmacy Co., Ltd (Beijing, China). HPLC grade acetonitrile was obtained from Sinopharm Chemical Reagent Co., Ltd. Water was doubly distilled in the laboratory. All other reagents were analytical grade. Blank plasma was obtained from the Blood Supply Center (Shenyang, China). The Accurel Q3/2 polypropylene hollow fiber

Table 3: Precision, accuracy, enrichment factor and extraction recovery of nimesulide in plasma samples ($n = 5$)

	Concentration (ng/mL)	Intra-assay precision *RSD* (%)	Inter-assay precision *RSD*(%)	Accuracy *RE*(%)	Enrichment Factor (Mean ± S.D.) (fold)	Extraction Recovery (Mean ± S.D.) (%)
Nimesulide	120	7.6	7.3	3.2	144 ± 15	41.2 ± 4.4
	1200	8.9	6.3	1.2	156 ± 18	44.6 ± 5.1
	4000	9.1	8.3	0.2	147 ± 17	41.9 ± 5.0
IS	1000				158 ± 17	45.1 ± 4.7

membrane (600 μm I.D., 200 μm wall thickness, 0.2 μm pore size) was purchased from Membrana (Wuppertal, Germany). Polyvinylidene fluoride hollow fiber membrane (600 μm I.D., 200 μm wall thickness, 0.2 μm pore size) was from Tianjin Motimo Membrane Technology Co., Ltd (Tianjin, China).

3.2. HPLC system

The chromatographic system consisted of a Shimadzu (Tokyo, Japan) LC-10ATvp pump and a SPD-10Avp UV–Vis detector. All separations were performed on a Diamonsil C_{18} column (200 mm × 4.6 mm, 5 μm) at 30 °C. The mobile phase was acetonitrile–20 mM potassium dihydrogen phosphate (47:53, v/v) with a flow rate of 1.0 mL/min. UV detection was accomplished at 300 nm. The injection volume was 10 μL for all samples analyzed.

3.3. Preparation of standard solutions and quality control samples

A stock solution of nimesulide (100 μg/mL) was prepared from solid powder and dissolved with acetonitrile. This stock solution was further diluted with acetonitrile-water (50:50, v/v) to give a series of working standards with concentrations of 0.5, 1.0, 2.0, 5.0, 10, 20 and 50 μg/mL. A 10 μg/mL solution of the IS was also prepared by diluting a 100 μg/mL stock solution of diclofenac sodium with acetonitrile-water (50:50, v/v). The quality control (QC) solutions were prepared at concentrations of 1.2, 12 and 40 μg/mL in the same way. All the solutions were kept at 4 °C and were brought to room temperature before use.

Both calibration standard samples and QC samples were prepared by spiking 200 μL blank plasma with 20 μL working solution during validation and the pharmacokinetic study. Calibration samples were made at concentrations ranged from 50 to 5000 ng/mL. QC samples were prepared at the concentrations of 120, 1200 and 4000 ng/mL.

3.4. HF-LPME procedure

The whole fiber was cut into small segments with the length of 5.5 cm. Before using, each hollow fiber was ultrasonically cleaned for 3 min in acetone to eliminate any possible contaminant in the fiber. It was then removed from acetone and the solvent was allowed to evaporate completely. Each piece was used only once to avoid the carryover effect. A 25 μL syringe (Hamilton, Reno, USA) was employed to introduce the acceptor solution into the lumen of the hollow fiber, and the other syringe was used to inject the extracted analyte into the HPLC loop.

Extraction was performed in the following steps: 0.2 mL plasma sample spiked with 0.02 mL IS solution was transferred into a 6 mL glass vial containing a 10 mm × 4 mm magnetic stirring bar; aqueous hydrochloric acid solution (HCl) (10 mM) was used to acidify the sample and the total volume was about 5 mL; the fiber was exposed to the organic solvent (dihexyl ether) for 10 s to impregnate its pores with organic phase; about 20 μL of the receiving phase (20 mM NaOH) was injected into the lumen of the hollow fiber by the inlet microlitre syringe; both ends of the hollow fiber were heat-sealed; the fiber was bent into U-shape and was submerged in the sample solution; the vial was placed on a 85–2A magnetic stirrer from Jintan Ronghua Instrument Manufacture Co., Ltd (Jintan, China); the vial was covered and the extraction was accomplished by constant agitation (400 rpm) at ambient temperature (25 °C) for 30 min; at the end of the extraction, the hollow fiber was removed from the sample solution, and its closed ends were cut and the receiving phase was withdrawn into the syringe; finally an aliquot of the receiving phase (10 μL) was introduced into the LC system. Three samples were processed at the same time.

Table 4: Pharmacokinetic parameters of nimesulide in human plasma

Parameter		Value (Mean ± SD)
$AUC_{(0\text{-}t)}$	(μg h/L)	22695 ± 5276
$AUC_{(0\text{-}\infty)}$	(μg h/L)	23198 ± 5390
$MRT_{(0\text{-}t)}$	(h)	5.7 ± 1.3
$MRT_{(0\text{-}\infty)}$	(h)	6.2 ± 1.3
T_{max}	(h)	3.3 ± 0.8
$t_{1/2}z$	(h)	3.7 ± 1.0
C_{max}	(μg/L)	4077 ± 561
CLz/F	(L/h)	4.5 ± 1.1
Vz/F	(L)	23.6 ± 7.2

3.5. Method validation

The procedures and criteria used for validation of the method were based on the FDA guidance document on bioanalytical method validation. The specificity was evaluated by processing and analyzing blank plasma obtained from six different sources. Decreasing concentrations of nimesulide were injected into the analytical system to determine the LLOQ with a signal-to-noise ratio of at least 10:1.

Duplicate calibration curves were analyzed each run. For each standard calibration curve, the peak area ratio of nimesulide to IS was calculated and plotted against normal nimesulide concentrations. The curves were fitted by least squares linear regression with $1/x^2$ weighing. Concentrations of nimesulide in samples were calculated from the calibration curves.

Precision and accuracy of the method were determined from QC samples (120, 1200 and 4000 ng/mL). These samples were extracted in five replicates on three consecutive days. Assay precision was calculated by ANOVA through Statistical Product and Service Solutions (SPSS 16.0, SPSS Inc., Chicago, USA). The intra- and inter-day precisions (RSD) were required to be below 15%, and the accuracy (RE) to be within ± 15%.

Stability of nimesulide in plasma was evaluated after long-term (frozen at −20 °C for 45 d), short-term (room temperature for 24 h) storage and after three freeze thaw cycles. For post-preparative stability, the processed QC samples were stored at 4 °C for 12 h and tested. The concentration of nimesulide was obtained by freshly prepared calibration curve.

Enrichment factor was determined by comparing the response obtained in processed QC samples with those of the solution standards in mobile phase at the same concentrations. Extraction recovery was calculated by multiply the enrichment factor by the corresponding volume ratio of donor phase to acceptor phase.

3.6. Application

The validated method was employed in a clinical pharmacokinetic study of nimesulide. Six healthy Chinese male subjects were enrolled in the study. The clinical trial protocol was approved by the Independent Ethics Committee (IEC) in accordance with the Declaration of Helsinki and all the volunteers gave written informed consent. Blood samples were obtained before dosing (time 0) and at 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 4.0, 6.0, 8.0, 12, 18 and 24 h after administration of 100 mg (Hoyo-Vadillo et al., 2003) nimesulide dispersible tablet. Plasma samples were obtained by centrifugation at 3000 rpm for 10 min and were frozen at −20 °C until analysis.

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