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Determination of Non-Steroidal Anti-Inflammatory Drugs in Human Urine Sample using HPLC/UV and Three Phase Hollow Fiber-Liquid Phase Microextraction (HF-LPME)

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Three phase hollow fiber-liquid phase microextraction (HF-LPME), which is faster, simpler and uses a more environmentally friendly sample-preparation technique, was developed for the analysis of Non-Steroidal Anti-Inflammatory Drugs (NSAIDs) in human urine. For the effective simultaneous extraction/concentration of NSAIDs by three phase HF-LPME, parameters (such as extraction organic solvent, pH of donor/acceptor phase, stirring speed, salting-out effect, sample temperature, and extraction time) which influence the extraction efficiency were optimized. NSAIDs were extracted and concentrated from 4 mL of aqueous solution at pH 3 (donor phase) into dihexyl ether immobilized in the wall pores of a porous hollow fiber, and then extracted into the acceptor phase at pH 13 located in the lumen of the hollow fiber. After the extraction, 5 μL of the acceptor phase was directly injected into the HPLC/UV system. Simultaneous chromatographic separation of seven NSAIDs was achieved on an Eclipse XDB-C18 (4.6 mm i.d. × 150 mm length, 5 μm particle size) column using isocratic elution with 0.1% formic acid and methanol (30:70) at a HPLC-UV/Vis system. Under optimized conditions (extraction solvent, dihexyl ether; pH_{donor}, 3; $pH_{acceptor}$, 13; stirring speed, 1500 rpm; NaCl salt, 10%; sample temperature, 60 °C; and extraction time, 45 min), enrichment factors (EF) were between 59 and 260. The limit of detection (LOD) and limit of quantitation (LOQ) in the spiked urine matrix were in the concentration range of 5-15 ng/mL and 15-45 ng/mL, respectively. The relative recovery and precision obtained were between 58 and 136% and below 15.7% RSD, respectively. The calibration curve was linear within the range of 0.015-0.96 ng/mL with the square of the correlation coefficient being more than 0.997. The established method can be used to analyse of NSAIDs of low concentration (ng/mL) in urine.

Key Words : Non-Steroidal Anti-Inflammatory Drugs (NSAIDs), Hollow fiber liquid phase microextraction (HF-LPME), Urine, HPLC-UV

Introduction

A simple, effective, precise, and accurate analytical method for Non-steroidal anti-inflammatory drugs (NSAIDs) detection in urine for use in pharmacokinetics, forensic toxicology, clinical and therapeutic drug monitoring, and doping analysis is needed. NSAIDs are among the most commonly prescribed agents worldwide to treat a variety of pain-related conditions, including arthritis and other rheumatic diseases.[1]

Several analytical methods have been reported for the analysis of NSAIDs from biological fluids, but most of these publications are based on liquid-liquid extraction (LLE), solid-phase extraction (SPE) and other methods for the sample preparation.[2-7] LLE methods are time consuming for sample preparation and require a large sample volume and specialized apparatus. In addition, the organic solvent used may affect human health and contaminate the environment. SPE requires longer sample preparation times and expensive expendable solid phases.[8-10]

To overcome the drawbacks of the LLE and SPE methods which are required large volume of organic solvent, longer sample preparation time and expensive experimental units, hollow fiber liquid phase microextraction (HF-LPME) methods were used in this paper. HF-LPME not only allows an efficient clean-up procedure, but also produces a high degree of pre-concentration.[11-13] Additionally, because very little solvent are used, there is minimal exposure to toxic organic solvent and the method is environmentally friendly during the sample preparation process.[14] Hollow fiber is a single use, low-cost and disposable polypropylene material. In addition, the small pore size prevents large molecules and particles present in the sample from entering the acceptor phase.[9,15] Also, because of the excellent clean-up efficiency, it is good for the environmental and suitable for biological samples which may have complex matrices.[16,17]

In the case of NSAIDs which are acidic drugs, adjusting the pH to acidic values before SPE to obtain higher retention efficiency leads to the formation of some colloidal precipitation which makes it difficult to perform SPE in a reasonable loading time and makes it necessary to filter the extract before SPE.[9] Especially, the three-phase HF-LPME can provide a great enrichment and sample clean-up, reducing or eliminating potential problems from matrix components.[18-20] Even though a few publications have reported on the application of 2- or 3-phase LPME method to analysis of NSAIDs, there are not papers that include mefenamic acid and tol-

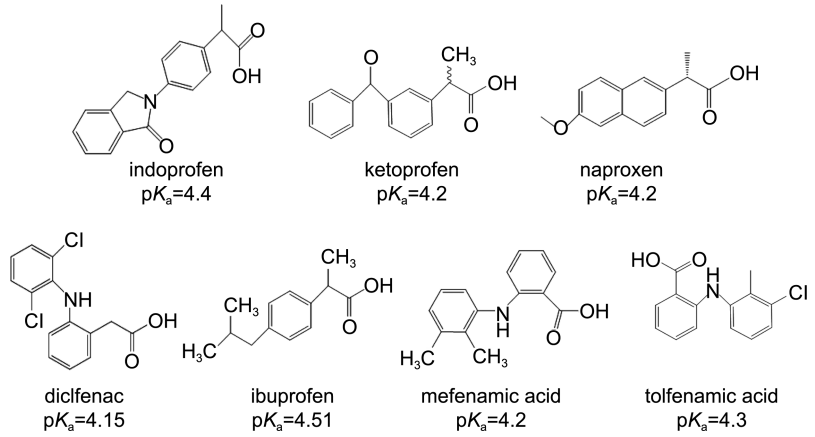

Figure 1. Chemical structures and pK_a of the NSAIDs.

fenamic acid, three or four NSAIDs only were determined simultaneous and the limit of quantitation (LOQ) was poor.[11,21,22]

The aim of this paper are to apply the three-phase U-shape HF-LPME as a extraction/concentration/clean-up device for the simultaneous analysis of seven NSAIDs (indoprofen, ketoprofen, naproxen, diclofenac, ibuprofen, mefenamic acid, and tolfenamic acid) (Figure 1) from a urine sample. The extracted samples were directly analyzed by a HPLC/UV-Vis system. To obtain optimized conditions, the parameters (extraction solvent, pH of donor and acceptor phase, stirring rate, salt-out effect, temperature, and extraction time) influencing the extraction/concentration were investigated.

Experimentals

Reagents and Apparatus. Indoprofen, ketoprofen, naproxen, diclofenac, ibuprofen, mefenamic acid, and tolfenamic acid, which are above 99% purity, were purchased from Sigma-Aldrich (St Louis, MO, USA), and the individual working standard solutions were prepared by dissolving in methanol (500 μg/mL) and stored in a refrigerator. Before use, these standard solutions were diluted with methanol. Hydrochloric acid and sodium hydroxide were supplied from Daejung Co. (Gyeonggi, Korea). 1-Octanol and n-amyl alcohol were purchased from Junsei Co. (Tokyo, Japan), and dihexyl ether and octyl ether were obtained from TCI (Tokyo, Japan).

The micro-syringe (50 μL) for sample injection was obtained from ILS Co. (Stutzerbach, Germany). The Q3/2 Accurel polypropylene hollow fiber membranes (600 μm i.d., 200 μm thickness, and 0.2 μm pore size)[11,23-27] were supplied by Membrana GmbH (Wuppertal, Germany). Ultra-pure reagent water purified by a Milli-Q system (Millipore, Bedford, MA, USA) was used.

An Agilent 1050 series HPLC system (Palo Alto, CA, USA) equipped with a Rheodyne injector was connected to a UV-Vis detector. The analytical column was an Eclipse XDB-C18 (4.6 mm i.d. × 150 mm length, 5 μm particle size). 0.1% formic acid (pH 2.6) and methanol (30:70) were used with a flow rate of 1.0 mL/min with the isocratic system. The injection volume was 5 μL, and the measured wavelength was set at 230 and 280 nm.

Extraction and Preconcentration Procedure. The HF-LPME configuration is shown in Figure 2. Two holes in the cap septum of the 4 mL sample vial were pierced using a 10 μL micro-syringe needle. A plunger of 10 μL micro-syringe was cut into 2 cm and the cut plunger was pierced into one hole of the prepared cap septum. After the hollow fibers were cut into 4 cm sections, the pieces were ultrasonically washed with acetone for 15 min to remove any contaminants and dried in drying oven. Each hollow fiber was used once to prevent memory effects. The needle of a 50 μL micro-syringe which was filled with acceptor phase solvent was used to introduce the acceptor phase into the lumen of one side of the prepared hollow fiber. The connected hollow fiber was immersed in organic solvent for 10 seconds to

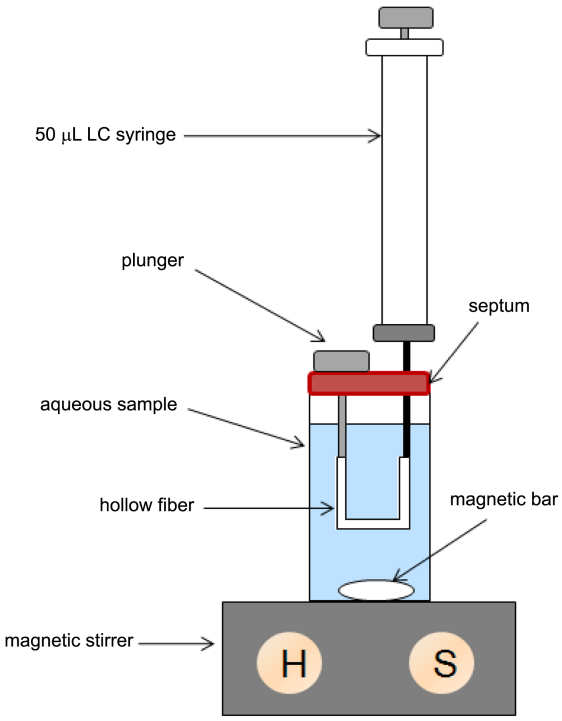

Figure 2. Schematic illustration of the HF-LPME configuration.

impregnate the pores of the hollow fiber membrane. At this time, the pores of the hollow fiber were filled with the extraction organic solvent. The acceptor phase, which was loaded in a 50 μL micro-syringe, was introduced into the lumen of the hollow fiber with a slow push of the plunger. At this time, the organic solvent came out the lumen of hollow fiber by the acceptor phase solvent. The other end of lumen of hollow fiber was connected with the cut plunger, which was pierced into the vial cap septum. Finally, the hollow fiber was bent (in to a U-shape) and immersed into the 4 mL sample vial, which was filled with an aqueous sample. The vial cap mounted with the hollow fiber system was screwed on before the extraction. After the extraction, the hollow fiber system was withdrawn from the sample vial and the cut plunger was removed from the end of the hollow fiber using tweezers. 5 μL of acceptor phase was withdrawn into the micro-syringe and injected into the HPLC/UV-Vis.

Results and Discussion

Optimization of Extraction/pre-concentration for Three Phase HF-LPME. To achieve effective extraction and concentration of NSAIDs from spiked water samples by three phases HF-LPME, the parameters influencing the extraction/pre-concentration must be optimized. The following parameters were investigated: organic solvent, which is supported in the pores of the hollow fiber membrane; pH of the acceptor and donor phases; stirring speed; salting-out effect; extraction temperature; extraction time. For the optimization experiment, a sample (5 μg/mL) spiked with seven standard NSAIDs was used. The effects of the parameters were investigated by the "one variable at a time" method and the extraction efficiency was calculated by the looking at the peak area obtained by HPLC.

The Extraction Solvent. The extraction solvent in three phases HF-LPME refers to the organic solvent that is impregnated into the pores of hollow fiber. As the hollow fiber is a hydrophobic membrane, the pores of the fiber can be filled with organic solvent, but the aqueous solution is repelled. The extraction solvent, which is immobilized in the pores of the hollow fiber, acts as a barrier that analytes can be penetrate into another aqueous phase (acceptor phase) via a fiber membrane from the original aqueous sample (donor phase). A crucial step in this method's optimization is the selection of the most suitable organic solvent to be employed. The organic solvent must have low solubility in water, low volatility, and high solubility with analytes.[25-27] By taking into account the previous conditions mentioned, several water-immiscible solvents differing in polarity and water solubility were investigated (Table 1). The tested organic solvents were 1-octanol, *n*-amyl alcohol, dihexyl ether, and octyl ether. The parameters of three phases HF-LPME system except the organic solvent were as follows: donor phase (aqueous sample solution), pH 3; acceptor phase, pH 13; stirring speed, 300 rpm; temperature of sample, 25 °C; extraction time, 45 min; no salting-out reagent.

Table 1. Properties of the extraction solvent

Solvent	Boiling Point (°C)	Melting point (°C)	Density (g/cm^3)	Solubility in water (% w/w)	Partition coefficient
n-Amyl alcohol	138	-78	0.811	2.2	1.348
Dihexyl ether	228	-3.9	0.8	0.0003	4.98
1-Octanol	194	-16	0.824	0.08	2.87
Octyl ether	286	-7.6	0.811	0.00003	6.94

As shown in Figure 3, although 1-octanol provided the highest peak areas for the indoprofen, ketoprofen and naproxen, mefenamic acid and tolfenamic acid were not measurable. As dihexyl ether provided even high peak areas for seven analytes, dihexyl ether was chosen as the extraction solvent.

pH of Donor Phase and Acceptor Phase. In the sample preparation step, high partition coefficients are very important, these can be achieved by optimization of extraction solvent and the pH of the aqueous sample. Adjustment of the pH can enhance partition coefficients, as dissociation equilibria are affected together with the solubility of the acidic/basic target analytes.[26] In a three phase HF-LPME system, this is possible by control the pH of the donor phase (aqueous sample) and acceptor phase. To extract acidic analytes, the pH of the donor aqueous solution must be adjusted in the acidic range so as to deionise the target compounds, reduce their solubility within the sample solution and ensure efficient transfer into the organic phase which is impregnated in the pores of the hollow fiber.[29,30] In order to transfer the analytes into the acceptor phase through the organic phase without back-extraction into the donor phase, the pH of the acceptor phase have to be adjusted into the basic range. This results in ionization of target analytes (acidic compounds) and ensures higher solubility of target compounds in the acceptor phase than into the organic phase.

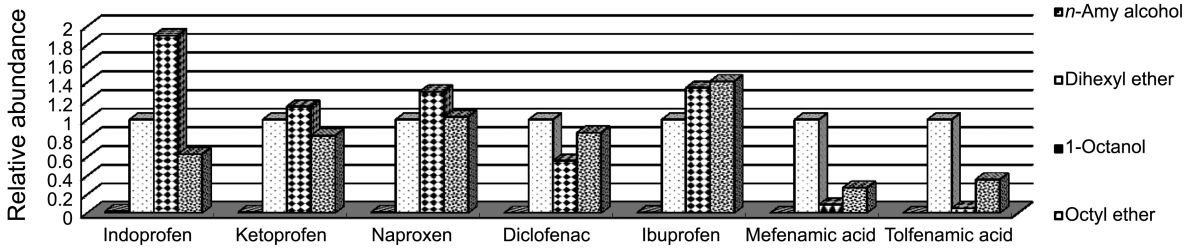

Figure 3. Effect of the extraction solvent on the HF-LPME yield. Extraction conditions: donor phase pH: 3; acceptor phase pH: 13; stirring rate: 300 rpm; addition of NaCl: 0%; temperature: 25 °C; and extraction time: 45 min.

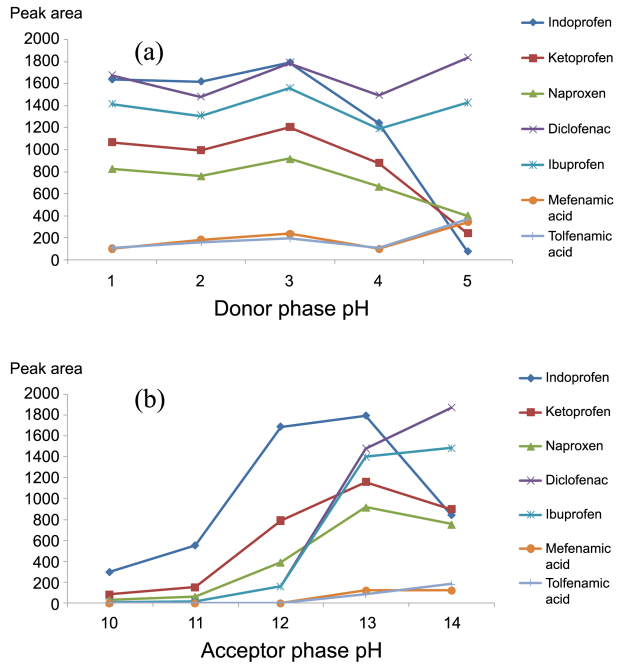

Figure 4. Effect of (a) the donor phase pH at acceptor phase pH 13 and (b) the acceptor phase pH at donor phase pH 3 on the HF-LPME yield. Extraction conditions: extraction solvent: dihexyl ether; stirring rate: 300 rpm; addition of NaCl: 0%; temperature: 25 °C; and extraction time: 45 min.

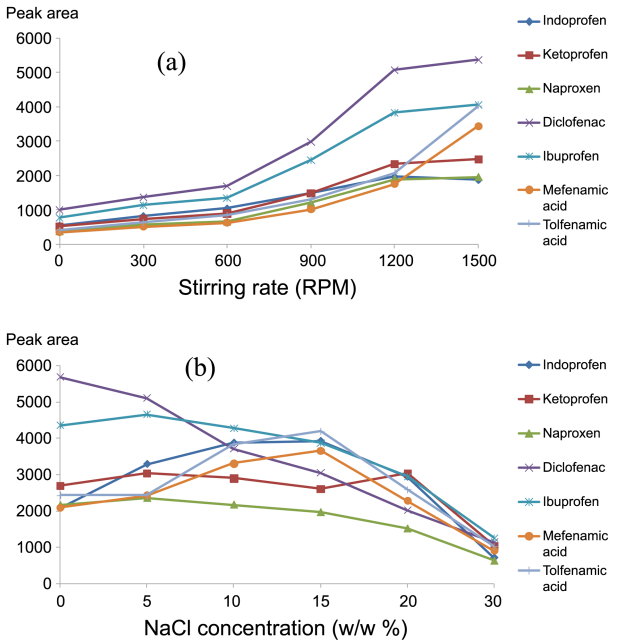

Figure 5. Effect of (a) the stirring rate at no NaCl addition and (b) addition of NaCl concentration at stirring rate 1500 rpm on the HF-LPME yield. Extraction conditions: extraction solvent: dihexyl ether; donor phase pH: 3; acceptor phase pH: 13; temperature: 25 °C; and extraction time: 45 min.

The pK_a range of the seven targeted NSAIDs is between 4.15 and 4.51. Therefore, the pH of the donor phase and acceptor were investigated in the range of acidic (pH 1-5) and basic range (pH 10-14), respectively. The donor phase was investigated in at pHs of 1, 2, 3, 4, and 5, adjusted using diluted hydrochloric acid. The parameters of the three phase HF-LPME system except the pH of the donor phase were as follow: extraction solvent, dihexyl ether; acceptor phase, pH 13; stirring speed, 300 rpm; temperature of sample, 25 °C; extraction time, 45 min; no salting-out reagent.

From Figure 4(a), it can be seen that pH 1-3 show little differences in their peaks, and pH 4 and 5 resulted in lower peak intensity. At pH 3, the extraction efficiency was slightly increased, so pH 3 was chosen as the optimum value for the donor phase.

For the study of the effect of the acceptor phase pH on the extraction efficiency, several pHs in the basic range were investigated. The adjustment of the pH was performed using sodium hydroxide solution. The parameters of three phase HF-LPME system except the pH of acceptor phase were as follows: extraction solvent, dihexyl ether; donor phase, pH 3; stirring speed, 300 rpm; temperature of sample, 25 °C; extraction time, 45 min; no salting-out reagent.

By increasing the pH from 10 to 14, the extraction efficiency of the targeted NSAIDs was increased, and the extraction efficiencies of ibuprofen, ketoprofen and naproxen at pH 14 were decreased (Figure 4(b)). The enhanced extraction efficiency can be explained by the acidic characteristics of the NSAIDs (pK_a 4.15-4.51) which are deprotonated and more soluble in the basic aqueous sample.[11] According to the obtained results, the optimized pHs of the donor and acceptor phases were selected to be 3 and 13, respectively.

Stirring Speed. The stirring speed is one of the major parameters influencing the extraction efficiency and extraction time. The magnetic stirrer used was coated with Teflon to prevent contamination of the aqueous sample.

The parameters, except the stirring speed, were as follows: extraction solvent, dihexyl ether; donor phase, pH 3; acceptor phase, pH 13; temperature of sample, 25 °C; extraction time, 45 min; no salting-out reagent. The influence of the stirring speed was investigated between 0 and 1500 rpm.

As shown Figure 5(a), the extraction efficiencies were increased with increasing stirring speed. The maximum response was obtained at 1500 rpm, which was the highest speed possible with the stirrer used. The extraction efficiency could be enhanced further by increasing the stirring speed of the aqueous sample, thereby reducing the time required to attain thermodynamic equilibrium by facilitating mass transfer,[27] and by increasing the diffusion of analytes through the interfacial layer of the hollow fiber. Although the equilibration time is inversely related to stirring speed, excessive agitation may adversely affect the extraction efficiency and precision by damaging the surface of the hollow fiber and through the vaporization of extraction organic solvent.[31]

Salting-out Effects. Generally, the addition of salt into aqueous phase increases the ion strength and reduces the solubility of analytes in the aqueous solution, resulting in the enhanced partition of analytes in the extraction organic solvent.

The optimization experiments on the ion strength were carried out by dissolving solid sodium chloride into an

aqueous donor phase at 0, 5, 10, 15, 20, and 30% (w/v). The parameters of the three phase HF-LPME system were as follows: extraction solvent, dihexyl ether; acceptor phase, pH 13; donor phase, 3; stirring speed, 300 rpm; temperature of sample, 25 °C; extraction time, 45 min.

As shown in Figure 5(b), some analytes were had their highest extraction efficiency at 0% (no salt) and 5%, but the optimized condition was achieved at 10% of NaCl. Reduction of extraction efficiency was observed above 15% NaCl. The salting-out effect has been discussed widely and, some contradictory results have been reported. The addition of salt can change the physicochemical properties of the extraction film, thus reducing the diffusion rates of the analytes into the organic solvent.[31]

Temperature. At elevated temperatures, the physical advantages such as high diffusion, low viscosity and low surface tension of solvent are achieved. Also, at higher temperature, the vapor pressure of compounds is increased and thermal desorption from matrices could increase which can improve the extraction efficiency.[11] However, there may be adverse effects such as the decrease of the partition coefficient with the increase of the temperature.

The investigation for the optimal temperature was performed in a temperature range from 25 to 80 °C. The parameters of the three phase HF-LPME system were as follows: extraction solvent, dihexyl ether; acceptor phase, pH 13; donor phase, 3; stirring speed, 1500 rpm; extraction time, 45 min; NaCl, 10%.

As shown in Figure 6(a), the extraction efficiency increased when the temperature was increased from 25 to 60 °C, and then decreased as the temperature moved toward 80 °C. This

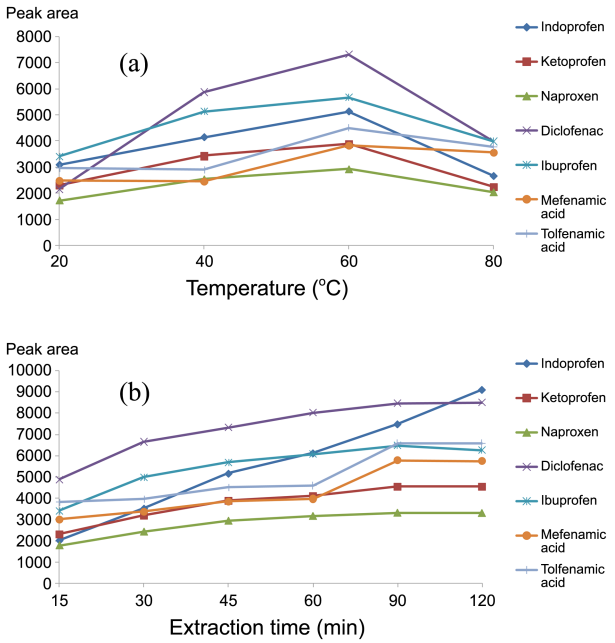

Figure 6. Effect of (a) the temperature at extraction time 45 min and (b) extraction time at temperature 60 °C on the HF-LPME yield. Other conditions: extraction solvent: dihexyl ether; donor phase pH: 3; acceptor phase pH: 13; stirring rate: 1500 rpm; and addition of NaCl: 10%.

Table 2. Optimized HF-LPME conditions

Parameters	Conditions
Hollow fiber's length	4 cm
Sample volume	4 mL
Injection volume	5 μL
Extraction solvent	dihexyl ether
Donor phase pH	3
Acceptor phase pH	13
Stirring rate	1500 rpm
Salting-out	10% (NaCl)
Temperature	60 °C
Extraction time	45 min

can be explained by partial dissolution of the extraction solvent into the aqueous phase.[32] Thus, an extraction temperature of 60 °C was selected as the optimal temperature.

Extraction Time. The mass transfer between phases depends on the extraction. Experiments were performed to investigate the influence of the extraction time (15, 30, 45, 60, 90, and 120 min). The parameters of three phase HF-LPME system except the extraction time were as followings: extraction solvent, dihexyl ether; acceptor phase, pH 13; donor phase, 3; stirring speed, 1500 rpm; temperature of sample, 60 °C; NaCl, 10%.

The extraction efficiency was increased until an extraction time of 45 min, after which considerable increases were not observed as extraction time increased except for indoprofen, tolfenamic acid and mefenamic acid (Figure 6(b)). Although longer extraction times result in enhanced extraction efficiency, it is not always practical to apply extended extraction times.[26] Therefore, to maximize the merits of HF-LPME, the extraction time, which is not too much longer than chromatographic time, was selected to be 45 min.

Method Validation. After optimization of the parameters influencing the extraction efficiency, method performance was evaluated for a spiked urine sample at the optimum condition as shown Table 2.

The limit of detection (LOD) was assumed from the HPLC/UV-Vis analysis of the standard NSAIDs solution, and the seven spiked urine samples at a concentration within three times of the assumed LODs were analyzed according to the optimized method. The standard deviation (σ) was calculated from the analytical results from seven samples. Another spiked urine sample, which was within five times concentration of the assumed LODs, was also analyzed. The calibration curve was obtained from this sample. The slope (m) of the calibration curves was used in the calculation of LODs and LOQs. The LODs and LOQs were calculated from 3 σ/m and 10 σ/m, respectively. After calculating the LODs and LOQs, three sets of spiked samples were prepared in blank urine, and analyzed. The LODs and LOQs must satisfy the conditions that the signal to noise (S/N) is above 3 and within 20% RSD, respectively.

The established method showed the LODs and LOQs to be in the concentration range of 5-15 ng/mL and 1545 ng/mL from spiked urine sample, respectively. The LODs from

Table 3. EF, LOD, LOQ, accuracy and precision for NSAIDs in urine

Compounds	EF (Enrichment factor)	LOD (μg/L)	LOQ (μg/L)	Concentration (μg/L)	Accuracy (recovery %)	RSD (%) (n=3)
Indoprofen	59	6	18	18	136	9.9
				40	99	8.6
				80	92	8.8
				160	102	3.6
				320	98	6.9
				640	101	4.6
Ketoprofen	214	5	15	15	86	15.7
				30	93	5.7
				60	102	7.8
				120	102	4.5
				240	101	4.4
				480	100	2.7
Naproxen	225	10	30	30	58	14.1
				60	98	6.5
				120	107	4.6
				240	102	3.5
				480	101	2.1
				960	97	1.3
Diclofenac	236	7	21	21	68	10.5
				30	100	4.3
				60	96	6.8
				120	104	3.5
				240	106	4.1
				480	99	2.2
Ibuprofen	260	15	45	45	78	8.2
				90	102	6.2
				180	110	8.5
				360	98	5.9
				720	93	3.1
				1440	109	2.7
Mefenamic acid	206	12	36	36	93	8.9
				60	103	5.3
				120	99	6.7
				240	101	7
				480	101	3.1
				960	100	2.5
Tolfenamic acid	202	10	30	30	99	15.2
				60	91	7.5
				120	97	4.4
				240	95	5
				480	107	3.8
				960	99	2.5

the spiked urine samples were as follows: indoprofen, 6 ng/mL; ketoprofen, 5 ng/mL; naproxen, 10 ng/mL; diclofenac, 5 ng/mL; ibuprofen, 15 ng/mL; mefenamic acid, 12 ng/mL; tolfenamic acid, 10 ng/mL. The LOQs were as follows: indoprofen, 18 ng/mL; ketoprofen, 15 ng/mL; naproxen, 30 ng/mL; diclofenac, 21 ng/mL; ibuprofen: 45 ng/mL; mefenamic acid, 36 ng/mL; tolfenamic acid, 30 ng/mL (Table 3).

The LODs and LOQs by the developed method were similar or good compared to previous published results.[2-5]

As shown in Table 3, the precision, expressed in terms of RSD values, was between 1.3 and 15.7% for the NSAIDs. The accuracy, expressed as a relative recovery, was between 58 and 136 RSD % .

As shown in Table 4, the 6-point (in triplicate) calibration curves were obtained from the spiked urine samples using a least-square linear regression analysis of the standard mix-

Table 4. Working range, linear equation and R^2 for NSAIDs in urine

Compounds	Working range (μg/mL)	Linear equation	R^2
Indoprofen	0.018-0.64	y=0.800x−9.948	0.999
Ketoprofen	0.015-0.48	y=0.484x+1.339	0.999
Naproxen	0.030-0.96	y=0.419x+1.257	0.999
Diclofenac	0.021-0.48	y=1.348x−3.0161	0.998
Ibuprofen	0.045-1.44	y=0.925x−5.737	0.997
Mefenamic acid	0.036-0.96	y=1.054x−8.269	0.999
Tolfenamic acid	0.03-0.96	y=1.0634x−5.9065	0.9978

ture of the NSAIDs depending on the LOQs of each compounds. Each analyte exhibited good linearity with correlation coefficients ($r^2 > 0.997$).

The enrichment factors (EFs) were calculated according to the following equation: $EF=\frac{C_{a,f}}{C_{d,i}}=\frac{n_{a,final}\times V_d}{n_{d,initial}\times V_a}=\frac{R}{100}\times\frac{V_d}{V_a}$, where $C_{d,i}$ is the initial concentration of the analyte in the donor phase and $C_{a,f}$ is the final concentration of the analyte. V_d and V_a represent the donor volume and the acceptor volume, respectively. R is the recovery of the extraction given as a percentage.[33] As shown in Table 3, the enrichment factors (EFs) were between 59 and 260.

Conclusion

A simple and effective three phase HF-LPME method for the simultaneous extraction/concentration/clean-up of seven NSAIDs from a urine sample was established. The optimized parameters influencing the extraction efficiency were as follows: extraction solvent, dihexyl ether; acceptor phase, pH 13; donor phase, 3; stirring speed, 300 rpm; temperature of sample, 60 °C; NaCl, 10%; extraction time, 45 min. With the developed method it is possible to analyze upto a few ng/mL concentration range in the urine sample. The precision and accuracy were below 15.7 RSD% and 58-136%, respectively. The LODs and LOQs were 5-15 ng/mL and 15-45 ng/mL, respectively. The enrichment factors (EF) were between 59 and 260. The established three phase HF-LPME and HPLC/UV-Vis method for the simultaneous analysis of seven NSAIDs in urine samples can be used in the fields of pharmacokinetics, forensic toxicology, clinical and therapeutic drug monitoring, and doping analysis.

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