Hollow Fiber Liquid Phase Microextraction Method Combined with High-Performance Liquid Chromatography for Simultaneous Separation and Determination of Ultra-Trace Amounts of Naproxen and Nabumetone in Cow Milk, Water, and Biological Samples

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Abstract A simple and highly sensitive method based on hollow fiber liquid phase microextraction combined with high-performance liquid chromatography and fluorescence detection has been developed for simultaneous separation, preconcentration, and determination of naproxen and nabumetone from water, wastewater, milk, and biological samples. Parameters affecting the microextraction efficiency were evaluated and optimized. Under optimum conditions (extractant (14 µL of 1-undecanol), sample pH (3.0), extraction time (20 min), stirring rate (600 rpm), temperature (45 °C), potassium chloride concentration (4.0 %) and sample volume (9 mL)), the limits of detection based on (S/N = 3) were 1.3 ng L^{-1} for naproxen and 2.9 ng L^{-1} for nabumetone. The intra- and inter-assay relative standard deviations for naproxen and nabumetone were in the ranges of 3.2–6.1 % and 6.5–9.5 %, respectively. The calibration curves were linear in concentration ranges of 4.0–300.0 ng L^{-1} and 9.0–300.0 ng L^{-1} for naproxen and nabumetone, respectively, with good coefficient of determination ($r^2 > 0.999$). The method was successfully applied to the determination of naproxen and nabumetone in cow milk, water, wastewater, human plasma, and urine samples.

Keywords Naproxen · Nabumetone · Hollow fiber liquid phase microextraction · HPLC

Introduction

Naproxen (NAP) [(S)-2-(6-methoxy-2-naphthyl) propionic acid] and nabumetone (NAB) [4-(6-methoxy-2-naphthyl)-butan-2-one] are two non-steroidal anti-inflammatory drugs (NSAIDs) (Mikami et al. 2000) widely used by humans for the treatment of a variety of pain-related diseases (Kobylinska et al. 2003). They are also used for food-producing animals in conjunction with antibiotics to reduce pain, prevent inflammation, and treat respiratory diseases (Gallo et al. 2010; Alshana et al. 2013). Several studies have also shown that prolonged consumption of NSAIDs by humans can reduce the risk of the development of Alzheimer's disease and tend to lengthen the human life through the reduction of pain (Breitner et al. 1995). Besides, when administered to cattle, they improve the quality of the product such as the production of pale meat and the reduction of edible fat (Gallo et al. 2008). However, consumption of NSAIDs can have some side effects such as gastrointestinal bleeding, intestinal ulceration, aplastic anemia, and inhibition of platelet aggregation. New research has also shown that long-term use of some NSAIDs brings about the development of kidney tumors in rats as well as liver tumors in mice (Hu et al. 2012). The widespread use of these drugs causes a risk of spreading their residual traces in the water and food chain posing a potential risk to human health. Thus, close evaluation and monitoring of the traces of these drugs in water and food samples is an important task.

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Several analytical methods including capillary electrophoresis (Phillips and Wellner 2006), spectrophotometry (Keyhanian et al. 2014), spectrofluorimetry (Murillo Pulgarín et al. 2012), ultra-high-performance supercritical fluid chromatography (Ji et al. 2014), gas chromatography (GC) (Yilmaz et al. 2014), and high-performance liquid chromatography (HPLC) (Costi et al. 2008) have been applied in order to determine the naproxen and nabumetone in matrices such as pharmaceuticals formulations and environmental, biological, and food samples. Among these methods, high-performance liquid chromatography has widely been used for the separation and determination of several types of these drugs in real samples as a powerful separation technique coupled with ultra-violet (Costi et al. 2008), electrochemical (Adhoum et al. 2003), mass spectrometry (Aresta et al. 2006), or fluorescence detector (Santos et al. 2005). However, due to the low concentration of drugs and complexity of the matrices of the real samples, a separation and preconcentration step is essential prior to the HPLC analysis.

The conventional sample preparation techniques are based on liquid–liquid extraction (LLE) (Gallo et al. 2010) and solid phase extraction (SPE) (Gallo et al. 2008). These techniques have suitable enrichment factors, high reproducibility, and high sample capacity. However, they have some limitations such as tedious procedure and being labor intensive besides being time consuming. Moreover, the traditional LLE method uses large amounts of toxic solvents which are often hazardous to environment as well as to the operator, and the SPE is a time-consuming, multistage process (Pereira et al. 2009). To solve these problems, research has been oriented toward the development of efficient, economical, and miniaturized green analytical sample extraction methods (Asadi et al. 2015a). In this regard, the liquid phase microextraction (LPME) techniques have received a growing amount of attention due to their simplicity, ease of operation, low consumption of organic solvent, low cost, and the possibility of obtaining a high enrichment factor (Dadfarnia and Haji Shabani 2014). Liquid phase microextraction (LPME), first introduced by Jeannot and Cantwell in 1996 (Jeannot and Cantwell 1996), is the miniaturized implementation of the conventional LLE in which the amount of organic solvent was greatly reduced. Up to now, several modes of LPME have been developed including single-drop microextraction (SDME) (Sarafraz-Yazdi et al. 2012b), headspace liquid phase microextraction (Enteshari et al. 2014), dispersive liquid–liquid microextraction (DLLME) (Hossien-poor-Zaryabi et al. 2014; Zhang and Xu 2014), solidified floating organic drop microextraction (SFODME) (Bordagaray et al. 2014; Asadi et al. 2015b), and hollow fiber liquid phase microextraction (HF-LPME) (Yamini et al. 2015).

These techniques have been subjected to several reviews (Dadfarnia and Haji Shabani 2010).

HF-LPME was introduced by Pedersen-Bjergaard and Rasmussen (1999). It provides excellent sample cleanup in addition to the abovementioned advantages. In this mode of LPME, a small volume of an extraction solvent is protected by porous of hollow fiber made of polypropylene which prevents the loss of the extractant during the extraction process and facilitates the extraction process by stirring the sample at a high rate. Thus, the lipophilic analytes are separated from the sample matrix and are transferred into the extractant phase hold within the hollow fiber micropores. Then, the extractant phase containing the analyte is withdrawn by using a microsyringe and is directly introduced to a proper instrument for quantification. The HF-LPME has been used for cleaning up, separation, and preconcentration of various drugs and pollutants from different biological (Pan et al. 2015), dietary (Wu and Hu 2009), and environmental samples (Tao et al. 2009) followed by their determination with a suitable analytical instrument. However, according to the literature survey, there are a few reports on the application of DLLME (Alshana et al. 2013) or DLLME-SDME (Sarafraz-yazdi et al. 2012b) in the extraction of NAP or NAB, and there is only one report for the simultaneous extraction of NAP and NAB by DLLME (Ji et al. 2014).

In this work a two-phase HF-LPME method was developed for simultaneous extraction of NAP and NAB from various matrices for the first time. Furthermore, as these drugs are nicely fluorescent due to the presence of two conjugated aromatic rings in their structures, the developed HF-LPME was combined with HPLC by means of a fluorescence detector for the quantification of the analytes. The overall combination provided very low detection limits making the quantification of trace amounts of NAP and NAB in the complex matrix possible. Thus, the developed HF-LPME-HPLC method is compatible with the modern complicated and expensive instruments like LC-MS which are not available in most laboratories.

Experimental

Chemicals and Reagents

Naproxen and nabumetone (analytical standard) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Methanol (HPLC grade) and water (HPLC grade), potassium dihydrogen phosphate, ortho phosphoric acid, trichloroacetic acid, potassium chloride, 1-decanol, 2-undecanol, 1-dodecanol, 1-octanol, and 1-undecanol were purchased from Merck Company (Darmstadt, Germany). The hollow fiber polypropylene membranes, Q3/2 Accurel PP (200-μm-thick

wall, 600-μm inner diameter, and 0.2-μm average pore size), were supplied from Membrana (Wuppertal, Germany).

The phosphate buffer (pH = 3.0, 1.5 mol L^{-1}) was prepared by dissolving 204.129 g of potassium dihydrogen phosphate in 900 mL of deionized water. Then, the pH of the solution was adjusted to 3.0 with hydrochloric acid and was diluted to 1000 mL with deionized water.

The stock standard solutions of NAP and NAB were prepared through the accurate dissolving of a weighed amount of each reference compound in methanol in order to yield a 1000-mg L^{-1} drug concentration. These solutions were then adequately diluted with HPLC grade water to obtain the corresponding working solutions. Stock solutions were stored in refrigerator at −4 °C away from light. All the solutions were stored in clean polypropylene (Nalgene, Lima, OH, USA) containers.

Instrumentation

Chromatographic separation was performed by a Knauer HPLC system (Berlin, Germany) equipped with a LC-pump 1000, 10 μL sample loop, and a fluorescence detector RF-10A$_{XL}$ (Shimadzu, Japan) operating within the excitation and emission wavelengths of 230 and 356 nm, respectively. A personal computer equipped with a ChromGate program for LC was used to process the chromatographic data. The analytes were separated on Nucleosil-C$_{18}$ column (250 mm × 4.6 mm, 5 μm). Gradient separation for NAP and NAB was carried out by using methanol and water as the A and B solvents, respectively. The percentage of the methanol varied during the chromatographic run to give the following values at the specified times: 40 % A at 0–3 min (flow rate of 0.9 mL min^{-1}), linear gradient from 40–100 % A at 3–6 (flow rate of 0.9 mL min^{-1}), 100 % A at 6–13 min (linear flow rate of 0.9–2.0 mL min^{-1}, and then linear gradient from 100–40 % A in 1 min (linear flow rate of 2.0−0.9 mL min^{-1}), and 40 % A for 5 min at a flow rate of 0.9 mL min^{-1} to equilibrate the column. The column temperature was set at 40 °C.

Extraction Procedure

The pH and ionic strength of 9 mL of standard or the sample solution were adjusted to 3.0 and 4.0 % (w/v) using phosphate buffer (pH = 3.0) and KCl, respectively. The mixture was transferred to a 12-mL sample vial containing a 8 mm × 4 mm magnetic stirring bar. The hollow fiber was cut up into 4-cm length pieces and sonicated in acetone for 5 min to remove any possible contaminants from the fiber. Thereafter, the acetone was allowed to evaporate completely. Fourteen microliters of the extractant (1-undecanol) was drawn into the 25-μL microsyringe, and its needle was inserted into the lumen of the hollow fiber while the other end of the fiber was attached to a medical syringe needle.

The fiber was bent forming a U-shape. Then, it was immersed in the extraction solvent for about 5 s to impregnate the pores with the solvent and then in water for 5 s to wash the extra organic solution off the surface of the fiber. Afterward, the solvent was passed through the fiber by means of the microsyringe and the U-shaped fiber was impregnated with the solvent dipped into the sample solution. The vial was placed on the hotplate stirrer stirring at 600 rpm for 20 min at 45 °C. At the end of the extraction period, the fiber was removed from the donor solution and was carefully dried with a piece of paper to avoid traces of water in the extract. The acceptor phase was drawn into the microsyringe, and 10 μL of it was directly injected into the HPLC system. A fresh hollow fiber was used in order to decrease the memory effect in each extraction procedure.

Sample Preparation

Milk Sample

Bottled cow milk was purchased from a local market (Mashhad, Iran). Nine milliliters of a milk sample was transferred to a 15-mL vial; 4 mL of 3 % (w/v) trichloroacetic acid was added to it and vortexed for 30 s. To gain the complete precipitation of proteins, the mixture was left for 15 min and centrifuged at 4000 rpm for 10 min (Campillo et al. 2013). The supernatant was filtered through a 0.45-μm Millipore filter and was evaporated to about 9 mL under nitrogen stream (at 50 °C). Finally, the pH and the ionic strength were adjusted to 3 and 4 % (w/v) using phosphate buffer (pH = 3.0) and KCl, respectively. It was then vigorously vortexed for 1 min and treated according to the given procedure.

Water and Wastewater Sample

Tap water was collected from a Bijan chemistry laboratory (Mashhad, Iran), packed water was purchased from a local
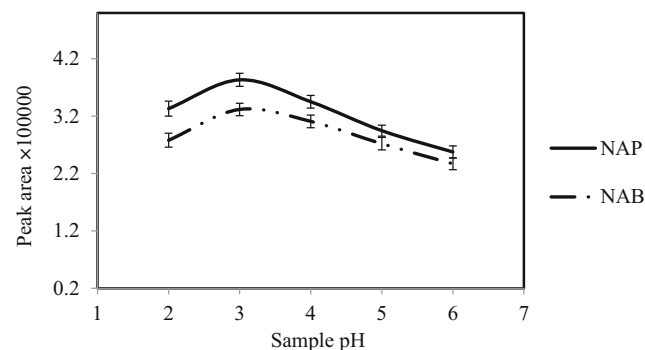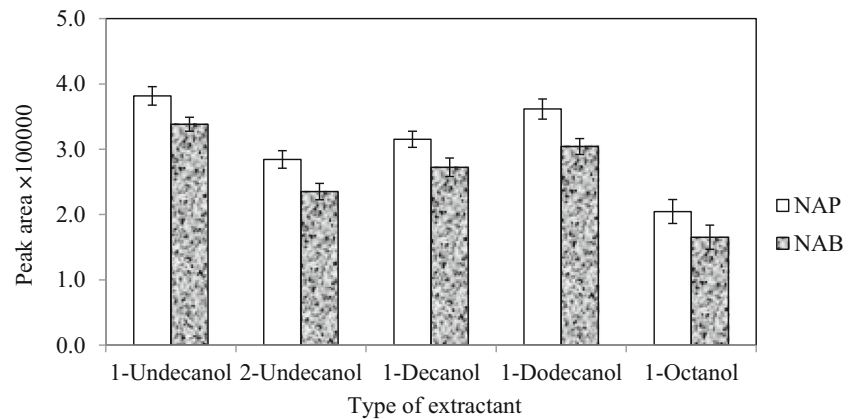

Fig. 1 Effect of the sample pH on the extraction. Conditions: concentrations of NAP and NAB, 100 and 200 ng L^{-1}, respectively; sample volume, 11 mL; temperature, 25 °C; extraction solvent, 14 μL of 1-undecanol; extraction time, 30 min; and stirring rate, 600 rpm

market (Mashhad, Iran), and wastewater samples were collected from the influent of wastewater treatment plant from Mashhad (Iran) and conveyed to the laboratory in plastic bottles. Just before the extraction, the sample was filtered through a 0.45-µm Millipore filter. Then, 9 mL of it was treated according to the extraction procedure.

Human Plasma and Urine Samples

Preparation of human plasma: The protein content of plasma sample provided by a voluntary was precipitated by the addition of 4 mL of acetonitrile to 2 mL of the plasma sample. The mixture was stirred for 10 min at 1200 rpm and centrifuged for 15 min at 5000 rpm (Adlnasab et al. 2010). Thereafter, the acetonitrile content of the mixture was evaporated under a gentle nitrogen stream at 80 °C. After the residual volume (about 2 mL) was transferred to a sample vial, it was diluted to 9 mL, its pH and ionic strength were adjusted to 3.0 and 4.0 % (*w/v*) using phosphate buffer solution, and KCl, respectively, and it was vortex-mixed for 30 s. The resultant sample was then subject to the extraction procedure.

Preparation of urine sample: The urine sample was frozen after sampling, and it was thawed at room temperature just

prior to the extraction. It was then centrifuged for 15 min at 5000 rpm and filtered through a 0.45-µm Millipore filter (Amiri Pebdani et al. 2015). Finally, 2 mL of it was diluted to 9 mL using phosphate buffer; the ionic strength was adjusted and was then subject to the given procedure.

Results and Discussion

In order to obtain the best conditions for the separation and preconcentration of NAP and NAB by HF-LPME method, various parameters affecting the microextraction system were considered and optimized. All the experiments were replicated three times, and the average and standard deviations were calculated.

Effect of pH

The pH is an important factor for the extraction of analytes from the sample solution. The pH of the sample must be adjusted so that the analytes can mainly be in their neutral form in order to increase their affinity for the organic phase. The effect of the sample pH on the extraction of NAP and NAB by
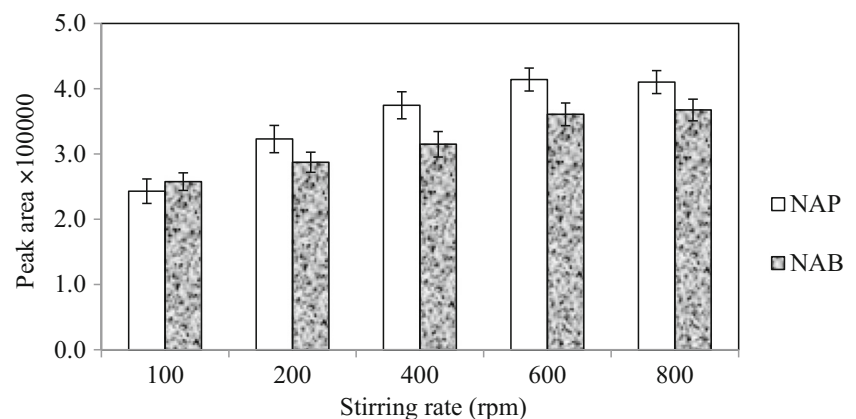
HF-LPME method was investigated by varying the pH in the range of 2.0–6.0, under other constant experimental conditions. The pH was adjusted by a proper phosphate buffer. The results (Fig. 1) indicated that the extraction efficiency of NAP and NAB is slightly increased by increasing the pH from 2.0 to 3.0 and then it decreases by a further increase in pH. Consequently, the pH of 3.0 was chosen as the optimum pH for further experiments.

Effect of Nature and Volume of Extractant

In LPME method, extractant solvent as the acceptor phase of the analytes must satisfy several criteria. It should be of low toxicity, low water solubility, high affinity for analytes, high boiling point in order to prevent its evaporating during extraction, as well as good chromatographic behavior. According to these requirements, the effect of several extracting solvents, including 1-undecanol, 2-undecanol, 1-decanol, 1-dodecanol, and 1-octanol, on the extraction efficiency was examined. The results (Fig. 2) revealed that 1-undecanol and 1-dodecanol displayed higher analytical signal for NAP and NAB. This observation can be related to the polarity of the solvent. Thus, a decrease in solvent polarity causes an increase in its extraction efficiency. So, 1-octanal with higher polarity has the lowest extraction efficiency. In the present study, 1-undecanol was selected as the extracting solvent due to its low vapor pressure, stability, sensitivity, low water solubility, as well as its lower price.

The evaluation of the preconcentration capability of the HF-LPME system is an important aspect of the method development. A decrease in the ratio of the volume of the extractant phase to the aqueous phase will increase the preconcentration factor, but it may reduce the extraction efficiency. The effect of the volume of extractant on the extraction process was studied by performing several experiments and using 14, 20, 25, and 30 μL of 1-undecanol in 4.0, 5.5, 7.0, and 8.5 cm of hollow fiber, respectively, while the other experimental conditions were kept constant. According to the results, the analytical signals of NAP and NAB were increased proportional to the decrease in the volume of extractant. The volume less than 14 μL was not examined because the collection of 10 μL of solvent for injection to HPLC was difficult. Thus, 14 μL of 1-undecanol was selected as the optimum organic phase volume for this method.

Effect of Salt

An increase in the ionic strength of the sample solution may reduce the solubility of the organic analytes in the aqueous phase through the salting out effect. To investigate the effect of the ionic strength on the extraction of NAP and NAB by the designed HF-LPME method, various experiments were conducted with different concentrations of potassium chloride
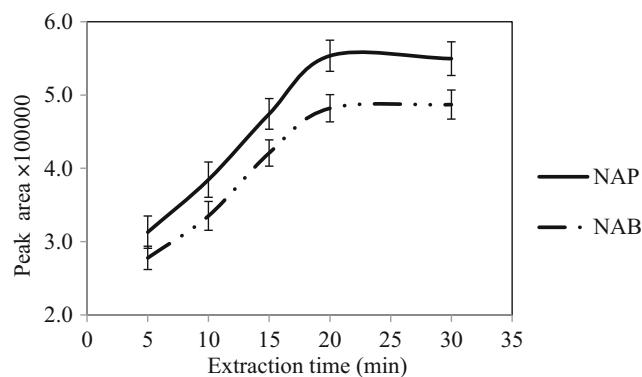

Fig. 4 Effect of extraction time on the extraction. Conditions: sample pH, 3.0; concentrations of NAP and NAB, 100 and 200 ng L^{-1}, respectively; sample volume, 11 mL; temperature, 45 °C; extraction solvent, 1-undecanol (14 μL); stirring rate, 600 rpm; and potassium chloride, 4.0 % *w/v*

(0.0–6.0 %, *w/v*). The results showed that the peak areas of NAP and NAB were increased with an increase in the salt concentration from 0.0 to 4.0 % *w/v*, and then, no significant changes were observed at the higher potassium chloride concentration. Thus, 4.0 % *w/v* was chosen as the optimum concentration of potassium chloride.

Effect of Stirring Rate

In LPME techniques, agitation of the sample solution facilitates the mass transfer process and reduces the time required to reach the equilibrium between the sample solution and the organic phase. Among different modes of LPME, HF-LPME can tolerate higher stirring rate because the acceptor phases are protected within the hollow fiber. For this purpose, several experiments were carried out by varying the stirring rate in the range of 100–800 rpm. As shown in the results (Fig. 3), the analytical signals were increased with the increase of the
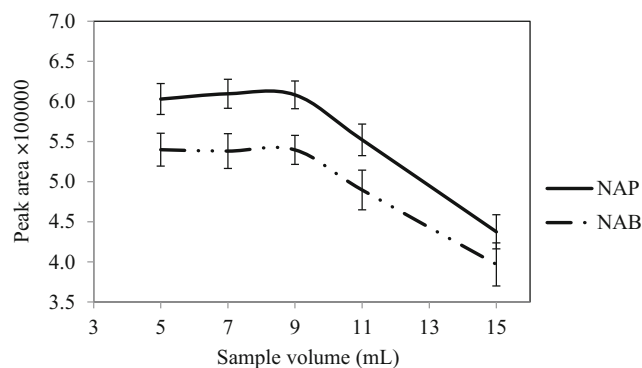

Fig. 5 Effect of sample volume on the extraction. Conditions: sample pH, 3.0; concentrations amounts of NAP and NAB, 1.1 and 2.2 ng, respectively; temperature, 45 °C; extraction solvent, 1-undecanol (14 μL); extraction time, 20 min; stirring rate, 600 rpm; and potassium chloride, 4.0 % *w/v*

Table 1 Figures of merit of the proposed method (HF-LPME-HPLC/FLD)

Analyte	LDR (ng L^{-1})	Linear equation	r^2	LOD (ng L^{-1})	EF	ER%
NAP	4.0–300.0	$y = 4974x + 3209$	0.999	1.3	609	94.7
NAB	9.0–300.0	$y = 2203x + 2022$	0.999	2.9	610	94.9

LDR linear dynamic range, *LOD* limit of detection, *EF* enhancement factor, *ER* extraction recovery, r^2 coefficient of determination

stirring rate from 100 to 600 rpm and then remained constant with a further increase in it up to 800 rpm. Thus, 600 rpm was selected as the optimum stirring rate.

Effect of Temperature

Temperature has a significant effect on the extraction process in most of the LPME. A temperature raise tended to facilitate the mass transfer of the analyte from the sample solution to the organic phase and to increase the extraction efficiency in a constant extraction time (Bagheri et al. 2004). The effect of the sample solution temperature on the extraction efficiency

was studied in the range of 25–55 °C, while the other experimental parameters were kept constant. The results revealed that the analytical signals of NAP and NAB increase by an increase in temperature from 25 to 45 °C and then remain constant up to 55 °C. Therefore, the sample vial was held at 45 °C as the optimum extraction temperature.

Effect of Extraction Time

The extraction time is defined as the time the sample is stirred after the insertion of the hollow fiber into the sample solution. The effect of the extraction time was examined by varying the

Table 2 Precision, accuracy, and recovery of the developed method for determination of NAP and NAB in various drug-free samples

		Matrix	Analyte					
			NAP (ng L^{-1})			NAB (ng L^{-1})		
			10	50	200	10	50	200
Precision (RSD%)	Inter-assay ($n = 3$)	Water	8.3	7.5	8.0	9.3	7.5	7.0
		Waste water	8.7	8.2	7.4	9.1	8.7	8.4
		Cow milk	9.1	8.7	9.4	9.5	9.0	9.2
		Urine	8.2	7.8	6.5	8.0	8.7	7.3
	Intra-assay ($n = 5$)	Human plasma	9.4	9.3	8.9	9.5	9.3	8.7
		Water	4.7	5.2	3.2	4.7	4.2	4.1
		Waste water	5.1	4.5	4.3	5.1	4.5	4.9
		Cow milk	5.5	5.5	5.5	5.5	5.0	5.4
		Urine	5.0	4.7	4.6	5.0	4.7	4.2
		Human plasma	6.1	5.7	6.0	6.1	5.6	5.0
Accuracy (Error%)	Inter-assay ($n = 3$)	Water	+3.2	+3.9	−5.2	+5.2	−3.2	+3.1
		Waste water	−2.8	−4.2	−3.6	+4.1	−5.5	−2.8
		Cow milk	−5.3	+3.2	+3.9	−5.2	+4.8	−3.5
		Urine	+4.4	+4.4	−3.7	−4.0	−3.7	−2.2
		Human plasma	−3.7	−5.2	+4.5	−3.9	+4.5	+3.7
	Intra-assay ($n = 5$)	Water	−2.8	−1.7	+2.2	+1.8	−2.2	+0.2
		Waste water	+0.6	−2.2	−1.8	−1.8	−2.8	+2.5
		Cow milk	−1.8	+3.2	−1.4	−3.1	+3.8	−2.4
		Urine	+2.7	+1.4	−1.7	+2.7	−2.3	−1.2
		Human plasma	−2.4	−1.2	+0.6	−2.4	+2.4	−2.1
Recovery (%)		Water	97.2	98.3	102.2	101.8	97.8	100.2
		Waste water	100.6	97.8	98.2	98.2	97.2	102.5
		Cow milk	98.2	103.2	98.6	96.9	103.8	97.6
		Urine	102.7	101.4	98.3	102.7	97.7	98.8
		Human plasma	97.6	98.8	100.6	97.6	102.4	97.9

stirring time in the range of 5–30 min. The results (Fig. 4) showed that an increase in the extraction time up to 20 min causes the augmentation of the analytical signals, and then, basically, the system reaches the steady state and no dramatic increase in peak areas is observed upon a further increase in extraction time. Therefore, in order to achieve the high extraction efficiency and a good speed, an extraction time of 20 min was chosen as the best extraction time.

Effect of Sample Volume

In method development, demonstrating of the capability of the system for the enrichment of analytes from a large sample volume is an important task. The sample volume affects the efficiency of the convection; thus, it influences the extraction efficiency (Shariati-Feizabadi et al. 2003). To explore the possibility of the extraction of trace amounts of analytes from the large sample volume, the developed HF-LPME was performed on five different volumes of the sample (5, 7, 9, 11, and 15 mL) containing fixed amounts of analytes (1.1 ng NAP and 2.2 ng NAB). The results (Fig. 5) indicated that the

analytical responses were constant up to the sample volume of 9 mL, and then, the extraction efficiency decreased by a further increase in sample volume due to the insufficient convection at a fix stirring rate. Thus, a sample volume of 9 mL was selected for further studies.

Analytical Performance

The performance characteristic of the proposed extraction method was investigated according to the recommendations made by the Food and Drug Administration (FDA). The extraction recoveries, enhancement factors, regression equations, coefficient of determination (r^2), limits of detection, and the linear dynamic ranges were investigated under the optimum conditions. The accuracy was determined by analyzing samples in triplicate containing known amounts of the NAP and NAB at three concentration levels in the linear dynamic range of analytes. Precision of the method was validated by intra- and inter-assays and by five and three replicate measurements at three concentrations (10, 50, and 200 ng L^{-1}). The

Table 3 Determination of NAP and NAB ($n = 3$)

Sample	NAP				NAB			
	Added (ng L^{-1})	Found (ng L^{-1})	R (%)	Error (%)	Added (ng L^{-1})	Found (ng L^{-1})	R (%)	Error (%)
Tap water	–	–	–	–	–	–	–	
	20	19.4 ± 1.2	97.0	−3.0	20	19.4 ± 1.2	97.0	−3.0
	100	97.4 ± 5.1	97.4	−2.6	100	101.8 ± 3.9	101.8	+1.8
	200	203.8 ± 10.4	101.9	+1.9	200	194.2 ± 8.7	97.1	−2.9
Packed water	–				–	–	–	
	20	20.9 ± 1.0	104.5	+4.5	20	19.5 ± 1.2	97.5	−2.5
	100	95.7 ± 4.1	95.7	−4.3	100	98.1 ± 5.5	98.1	−1.9
	200	202.5 ± 10.4	101.2	+1.2	200	204.9 ± 9.9	102.4	+2.4
Waste water	–	23.1 ± 1.4	–		–	–	–	
	20	43.6 ± 2.1	102.5	+2.5	20	19.0 ± 1.2	95.0	−5.0
	100	118.9 ± 5.3	95.8	−4.2	100	101.2 ± 3.9	101.2	+1.2
	200	215.7 ± 10.2	96.3	−3.7	200	203.0 ± 8.7	101.5	+1.5
Cow milk	–	–	–		–	–	–	
	20	20.6 ± 1.0	103.0	+3.0	20	19.0 ± 0.8	95.0	−5.0
	100	96.5 ± 4.2	96.5	−3.5	100	102.5 ± 5.2	102.5	+2.5
	200	195.4 ± 8.0	97.7	−2.3	200	198.5 ± 8.7	99.2	−0.8
Human plasma	–	59.3 ± 3.4	–	–	–	–	–	–
	20	78.3 ± 4.7	95.0	−5.0	20	19.4 ± 1.1	97.0	−3.0
	100	161.4 ± 7.2	102.1	+2.1	100	96.4 ± 5.1	96.4	−3.6
	200	250.2 ± 12.0	95.4	−4.6	200	203.3 ± 7.9	101.6	+1.6
Urine	–	63.3 ± 4.0	–	–	–	–	–	–
	20	83.2 ± 4.1	99.5	−0.5	20	19.1 ± 1.2	95.5	−4.5
	100	159.7 ± 6.3	96.4	−3.6	100	102.0 ± 4.9	102.0	+1.2
	200	266.6 ± 11.2	101.6	+1.6	200	198.6 ± 8.1	99.3	−0.7

recovery (R%) and the accuracy (Error%) were determined according to the following equations:

$$R\% = \frac{C_{found} - C_{real}}{C_{added}} \times 100 \tag{1}$$

$$Error\% = R\% - 100 \tag{2}$$

where C_{found}, C_{real}, and C_{added} are the concentrations (ng L^{-1}) of the analytes after the addition of a known amount of standard to the real sample, the concentration of the analytes in real sample, and the concentration of the known amount of standard spiked with the real sample, respectively.

The enhancement factors (EFs) defined as the slope ratios of calibration curves after HF-LPME to that before the extraction were found to be 609 for NAP and 610 for NAB. The preconcentration factor defined as the ratio of sample volume to extraction solvent was found to be 643; the closeness of EF to PF indicates that the extraction is quantitative (>94.7 %). The extraction recovery (ER) was calculated through the following equation:

$$ER\% = EF \times \frac{V_o}{V_a} \times 100 \tag{3}$$

where V_o and V_a are the volumes of the organic phase and the sample, respectively (Zhou et al. 2014). The analytical performances of the developed method are summarized in Tables 1 and 2. Calibration curves were linear in the concentration ranges of 4.0–300.0 ng L^{-1} of NAP and 9.0–300.0 ng L^{-1} of NAB. The coefficient of determination (r^2) was 0.999 for NAP and NAB. The relative recoveries were in the range of 96.9–103.8 for NAP and NAB, The intra- and inter-assay relative standard deviations and the Error% of the developed method for extraction and determination of NAP and NAB in various samples were in the range of 3.2–6.1 %, 6.5–9.5 %, and (−5.5)–(+4.8)%, respectively. The limits of detection, based on a signal-to-noise ratio (S/N) of 3, were 1.3 ng L^{-1} for NAP and 2.9 ng L^{-1} for NAB.

Application

The applicability of the proposed extraction method (HF-LPME) in the extraction of NAP and NAB from samples with different matrices (human plasma, urine, tap water, packed water, wastewater, and milk) was studied by performing the extraction on unspiked and spiked samples at three different levels of analytes. The results of the analysis along with the recoveries have been summarized in Table 3 indicating that the recoveries are in the acceptable range of 95.0–104.5 %. The chromatograms of the unspiked and spiked samples (water, wastewater, cow milk, human plasma, and urine) after HF-LPME, under the optimum conditions are shown in Fig. 6. The chromatograms

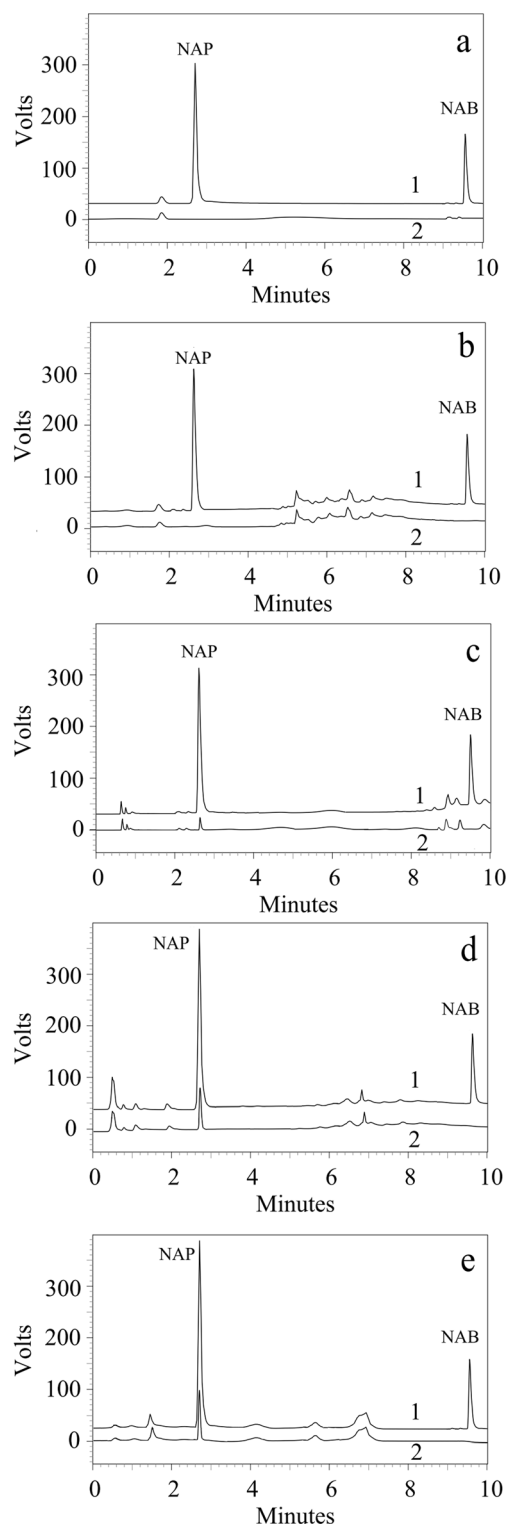were characterized by a symmetrical peak shape, and the retention time of the analytes was constant in

Fig. 6 Chromatograms of samples after HF-LPME under the optimum conditions. (**a** 1, **b** 1, **c** 1 **d** 1, and **e** 1) are water, milk, wastewater, human plasma, and urine samples, respectively, spiked with 200 ng L^{-1} of NAP and NAB; (**a** 2, **b** 2, **c** 2, **d** 2, and **e** 2) are water, milk, wastewater, human plasma, and urine unspiked samples, respectively

Table 4 Comparison of HF-LPME-HPLC/FLD with other microextraction methods for determination of NAP and NAB

Method	Sample	Analyte	LOD (ng L^{-1})	RSD%		EF and PF	Ref.
				Inter-assay	Intra-assay		
DLLME-FASS-CE	Milk	NAP	11,900	4.3	3.0	113 (PF)	Alshana et al. (2013)
US-IL-DLLME-UHPSFC	Water	NAP	620	<12.4	–	126 (PF)	Ji et al. (2014)
		NAB	1560			129	
DLLME-SDME-HPLC/UV	Wastewater	NAP	30	8.8	–	70 (PF)	Sarafraz-yazdi et al. (2012b)
SPME-GC/FID	Water	NAP	30	8.5	8.1	–	Sarafraz-Yazdi et al. (2012a)
M-MSPD-HPLC/UV	Wastewater	NAP	2000–10,000	1.0–4.0	1.0–2.1	–	Aguilar-Arteaga et al. (2010)
HF-LPME-HPLC/FLD	Milk, water, wastewater, urine, and plasma	NAP	1.3	6.5–9.4	3.2–6.1	609 (EF)	This work
		NAB	2.9	7.0–9.5	4.1–6.1	610	

LOD limit of detection, *RSD* relative standard deviation, *PF* preconcentration factor, *EF* enhancement factor, *FASS-CE* field-amplified sample stacking in capillary electrophoresis, *SPME* solid phase microextraction, *DLLME-SDME* dispersive liquid–liquid microextraction–single-drop microextraction, *M-MSPD* magnetic matrix solid phase dispersion, *HF-LPME* hollow fiber liquid phase microextraction, *US-IL-DLLME* ultrasound-assisted ionic liquid dispersive liquid–liquid microextraction, *UHPSFC* ultra-high-performance supercritical fluid chromatography

different matrices. Thus, the procedure is reliable and accurate for the determination of NAP and NAB in the sample type examined.

Comparison of the Developed Method with Other Microextraction Methods

The developed extraction method was compared with the other microextraction methods reported in the literature (Table 4). The results revealed that most of the extraction methods used for the extraction of these drugs are solid phase microextractions (Sarafraz-Yazdi et al. 2012a; Aguilar-Arteaga et al. 2010), and the HF-LPME benefits from a higher enhancement factor and consequently lower limits of detection for NAP and NAB. The method is also applicable to a wider range of sample matrices with relatively good standard deviations.

Conclusions

HF-LPME was combined with HPLC/fluorescence detection (FLD), and a very sensitive method was developed for simultaneous separation/preconcentration and determination of ultra-trace amounts of NAP and NAB in tap water, packed water, wastewater, cow milk, human urine, and plasma samples in this survey. This method has high enhancement factor, good precision, efficient recovery, and very low detection limit. Thus, the HF-LPME can be used as a simple, inexpensive, and efficient sample preparation technique whose combination with HPLC/FLD makes it possible to monitor the ultra-trace amounts of NAP and NAB in various matrices without any need for modern instruments like LC-MS.

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