Vortex-Assisted Inverted Dispersive Liquid-Liquid Microextraction of Naproxen from Human Plasma and Its Determination by High Performance Liquid Chromatography

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ABSTRACT: *Extraction and determination of naproxen from human plasma were performed using Vortex-Assisted Inverted Dispersive Liquid–Liquid MicroExtraction (VA−IDLLME) and High Performance Liquid Chromatography (HPLC). The parameters affecting extraction recovery such as type and volume of extraction and disperser solvents, pH of sample solution, stirring rate, extraction time and salt addition were optimized. Optimal extraction conditions were: 100 µL dodecane as extraction solvent, 350 µL methanol as disperser solvent, pH of sample = 5, stirring rate: 500 rpm, with no effect of extraction time and no addition of NaCl to the sample solution. Under the optimal conditions a linear range of 1.0–1000.0 ng/mL (R^2 = 0.9993) was obtained. Limit of detection, the extraction recovery and preconcentration factor were 0.32 ng/mL, 87% and 108 respectively.*

KEYWORDS: *Naproxen; Vortex-assisted dispersive liquid−liquid microextraction; HPLC; Plasma.*

INTRODUCTION

Naproxen (Nap) [6-methoxy-methyl-2-naphthalene acetic acid] (Fig. 1) belongs to an important group of Non−Steroidal Anti−Inflammatory Drugs (NSAIDs). Naproxen commonly used for the reduction of moderate to severe pains, fever and inflammation. High doses of naproxen (over 1000 mg) cause the body's clotting system, causing severe gastrointestinal problems including stomach bleeding. Naproxen is often preferred to other types of NSAIDs (e.g. aspirin) because of its better absorption following oral administration and fever adverse effects [1-3]. Thus, determination of naproxen in human plasma is important for health. The analytical methods such as capillary electrophoresis [4], colometry [5], thin layer chromatography [6], liquid chromatography with fluorescence detection [7], High Performance Liquid Chromatography−Mass Spectrometry (HPLC−MS) [8], HPLC−chemiluminescence [9] and liquid chromatography with UltraViolet (UV) detection [10] reported for determination of naproxen in blood human plasma. Extraction techniques such as liquid phase microextraction [11] and solid phase microextraction [12] were used for preconcentration of naproxen from human plasma. These methods are expensive, time−consuming and dangerous to health due to high volume of potentially toxic solvents used [13]. In 2006, Assadi and co−workers applied Dispersive

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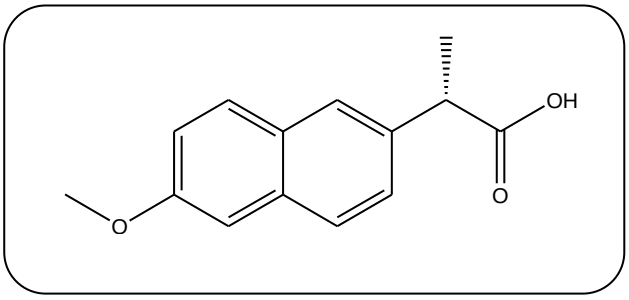

Fig. 1: Chemical structure of naproxen.

Liquid-Liquid MicroExtraction (DLLME) successfully for the extraction and preconcentration of pesticides from water samples. DLLME is a mode of Liquid−Liquid Extraction (LLE) in smaller level, which in comparison with the LLE method, its consumption of solvent significantly lower, and the obtained preconcentration factor is much higher, with lower price, time independent and wider linear range. DLLME employs a mixture of a high density extraction solvent (water immiscible) and disperser solvent (water miscible polar disperser solvent). In DLLME, after a rapid injection of an appropriate mixture containing extraction and disperser solvents into the aqueous sample a cloudy state is formed. The contact area between the extraction solvent and the sample solution is very large. Thus the extraction equilibrium is achieved rapidly. After centrifugation, the extracted phase is settled at the bottom of the conical test tube [14]. In 2009, *Farajzadeh* and co−workers presented Inverted Dispersive Liquid–Liquid MicroExtraction (IDLLME) [15]. IDLLME is invert of DLLME, because in IDLLME the extraction solvent is lighter than water, so the separated extracted phase is collected at the top of the sample solution. In this work, Vortex−Assisted Inverted Dispersive Liquid–Liquid MicroExtraction (VA−IDLLME) method combined with HPLC was applied for the extraction and determination of naproxen in human plasma sample. Here the mixture of extraction and disperser solvents was injected into the sample solution and the mixture was shaken vigorously by a vortex agitator simultaneously. Vortex increases the contact area between the extraction solvent droplets and sample solution. Effect of eight experimental factors including type and volume of extraction and disperser solvents, pH of sample solution, stirring rate, extraction time and salt addition on extraction recovery were studied.

EXPERIMENTAL SECTION

Chemicals and stock solutions

Naproxen was purchased from Cipla pharmacy (Mumbai, India). HPLC grade (Methanol, acetonitrile and acetone), sodium hydroxide, hydrochloric acid, phosphoric acid and sodium chloride were obtained from Merck (Darmstadt, Germany). Xylene, n-hexan, toluene, dodecane, cyclohexane and 1-octanol were obtained from Aldrich (Milwaukee, WI, USA). Water used was double distilled deionized. Stock solution of naproxen (1.0 mg/L) was prepared in methanol and stored in the dark at 4°C. Working standard solutions were diluted with double distilled deionized water at concentration of 10.0 ng/mL when ever needed.

Instrumentation and operating condition

Chromatographic measurements were carried out using a HPLC system equipped with a series 10-LC pump, UV detector model LC-95 set at 270 nm and model 7725i manual injector with a 20 µL sample loop (Perkin-Elmer, Norwalk, CT, USA). Column used was C_{18} (250 × 4.6 mm, 10 µm particle size) from Dr. Maisch GmbH (Ammerbuch-Entringen, Germany). In order to select the composition of mobile phase, several mobile phases with different percents of methanol in water (40, 50, 60 and 70 ٪ v/v) were tested and the best mobile phase was 60 percent methanol based on peak shape, retention time and resolution at flow rate of 1.0 mL/min at room temperature. The pH of sample solutions were measuerd by a Jenway pH meter model 3030 (Leeds, UK). Sample shaking was done on a vortex agitator (Fisher Scientific, USA).

Vortex−assisted inverted dispersive liquid–liquid microextraction procedure

For VA−IDLLME, 10 mL of sample solution containing 10 ng/mL of naproxen was placed in a handmade centrifuge tube with narrow neck (~4 mm i.d.) which was specifically designed for ease of taking supernatant phase. A mixture of 100 µL dodecane (as extraction solvent) and 350 µL methanol (as disperser solvent) was rapidly injected into the sample solution using 1.0 mL syringe and mixed by vortex agitator at 500 rpm stirring rate to obtain a cloudy solution. The cloudy solution was centrifuged for 5 min at 3500 rpm and the extraction product (supernatant phase) collected

in the neck of the tube (about 80 ± 2 µL). This supernatant phase was injected in to the HPLC. For determination of naproxen all of the experiments were carried out in triplicates and the average of the results were reported.

Calculation of recovery and preconcentration factor

Extraction Recovery (ER %) and Preconcentration Factor (PF) for naproxen were calculated according to the following equations [16].

$$ER\% = \frac{n_{sup}}{n_o} \times 100 = \frac{C_{sup} v_{sup}}{C_o V_o} \times 100 \quad (1)$$

$$PF = \frac{C_{sup}}{C_o} \quad (2)$$

$$ER\% = PF \times \frac{v_{sup}}{V_o} \times 100 \quad (3)$$

Where n_o and n_{sup} are the number of moles of analyte in the initial aqueous sample and collected phase respectively. C_o, C_{sup}, V_0 and V_{sup} are the initial concentration of analyte in sample solution, concentration analyte in the supernatant phase, volume of aqueous sample and supernatant phase, respectively.

RESULTS AND DISCUSSION

To obtain high extraction recovery and good precision for extraction of naproxen from human plasma, various experimental parameters which influence the extraction recovery of VA−IDLLME procedure were investigated. These parameters were type of extraction and disperser solvents as well as their volume, pH of sample solution, stirring rate, extraction time and salt addition. These parameters were optimized using one-variable-at-a time method.

Selection of the extraction and disperser solvents

A major factor for obtaining a good extraction recovery for VA−IDLLME of naproxen, is selection of an appropriate extraction and disperser solvents. The extraction solvent has to meet two properties: having less density than water and low solubility in water. Accordingly, xylene, n-hexan, dodecane, toluene, 1-octanol and cyclohexane were tested. The miscibility of disperser solvent with extraction solvent and aqueous phase (sample solution) is a key factor. Acetonitrile, acetone and methanol were examined as the disperser solvent in the extraction of naproxen. To obtain a high recovery factor, all combinations of 250 µL of different extraction solvents were examined with 400µL different disperser solvents. Results in Fig. 2 indicate that the maximum extraction recovery (about 84%) is achieved by applying dodecane and methanol as extraction and disperser solvents, respectively. Therefore, dodecane-methanol combination was selected for subsequent experiments**.**

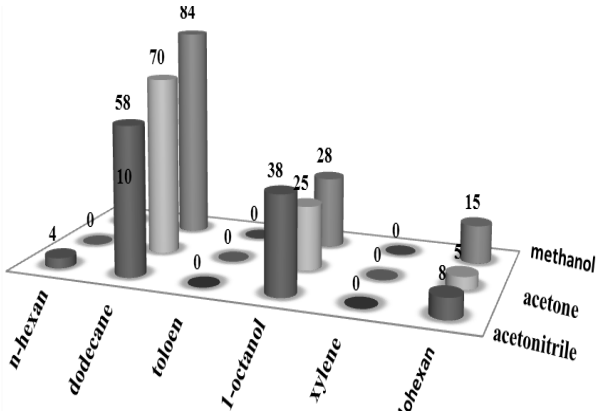

Fig. 2: Effect of different types of extraction and disperser solvents on the extraction recovery of naproxen. Extraction conditions: aqueous sample volume, 10 mL (10 ng/mL of naproxen); extraction solvent volume, 250 µL; disperser solvent volume, 400 µL.

Effect of extraction solvent volume on the extraction recovery

To examine effect of the extraction solvent volume, experiments involving different volumes of dodecane were used for extraction of naproxen from standard sample solution (10.0 ng/mL) with 400 µL methanol as disperser solvent. At the extraction solvent volumes lower than 50 µL, no supernatant organic phase was formed at the top of aqueous phase and considering solvent saving and environment protection, more volumes of extraction solvent than 350 µL were not been evaluated. So the effect of extraction solvent volume was studied in the range of 50−350 µL. Table 1 shows the supernatant phase volume, extraction recovery and preconcentration factor with the extraction solvent volume. Results in this table show that, 100 µL of the extraction solvent, produced the highest recovery and

Table 1: Effect of extraction solvent (dodecane) volume on supernatant phase volume, ER (%) and PF.

Extraction solvent volume (μL)	Supernatant phase volume (μL)	ER (%)	PF
50	35	61	18.4
90	70	78	89.3
100	80	84	105.0
150	120	75	62.5
200	180	68	38.0
250	225	63	29.0
300	279	63	23.8
350	335	61	18.2

Extraction conditions: disperser solvent (methanol) volume, 350 μL.
Concentration of sample: 10.0 ng/mL.

preconcentration factor. Thus this volume was selected as an optimum volume for the extraction solvent.

Effect of disperser solvent volume on the extraction recovery

To obtain appropriate volume of disperser solvent, several extractions were carried out by changing the volume of methanol in the range of 100–600 μL. Results in Fig. 3 show that by increasing the volume of methanol up to 350 μL, the extraction recovery increased and decreased after this volume. The lower extraction recovery at volume of methanol less than 350 μL can be attributed to the fact that, cloudy state was not well formed and the extraction solvent (dodecane) could not be well dispersed among aqueous solution in the form of very little droplets. So for the following experiments, 350 μL methanol was used as optimal disperser solvent volume.

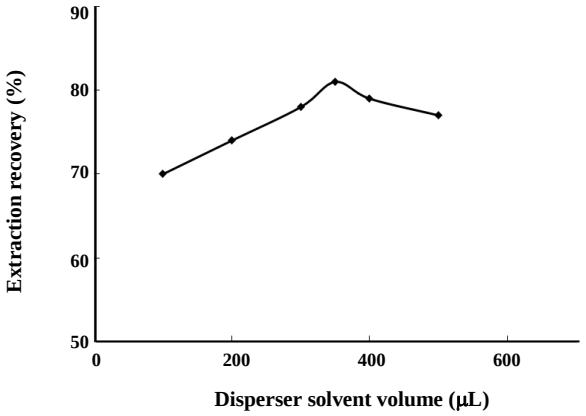

Fig. 3: Effect of disperser solvent (methanol) volume on the extraction recovery of naproxen Extraction conditions: extraction solvent (dodecane) volume, 100 μL. Other conditions as Fig. 2.

Effect of sample pH on the extraction recovery

In IDLLME pH of the sample solution is a key factor for extraction of acidic and basic compounds. To obtain high extraction recovery for acidic compounds, the sample solution was acidified (by addition of 2 M formic acid) to deionize the analyte molecules and consequently increase their transfer from the sample solution into the extraction solvent. Thus, pH of sample solution should be adjusted to make neutral molecular forms of the analytes prior the microextraction step. For this purpose, effect of pH of the sample solution on the extraction recovery of naproxen was investigated in the range of 3–8. Values of pH higher than 8.0 were not examined, because naproxen is a weak acid (pK_a = 4.2), and can be ionized at basic pH. Fig. 4 shows that, the highest extraction recovery is obtained at pH = 5 and subsequent experiments were performed at this pH.

Effect of stirring rate on the extraction recovery

In VA-IDLLME, stirring the sample solution during injection the mixture of extraction and disperser solvents makes a stable cloudy solution and accelerates the mass transfer of analytes from the sample solution to the extraction solvent for obtaining a good extraction recovery. The effect of stirring rate on the extraction of naproxen was studied in the range of 0–1000 rpm.

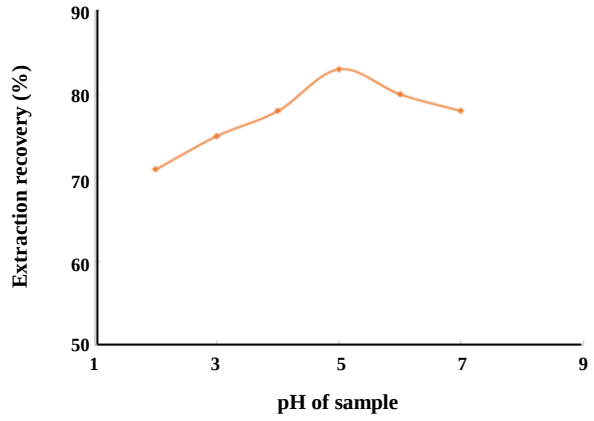

Fig. 4: Effect of the sample solution pH on the extraction recovery of naproxen. Extraction conditions: disperser solvent (methanol) volume, 350 µL. Other conditions as Fig. 3.

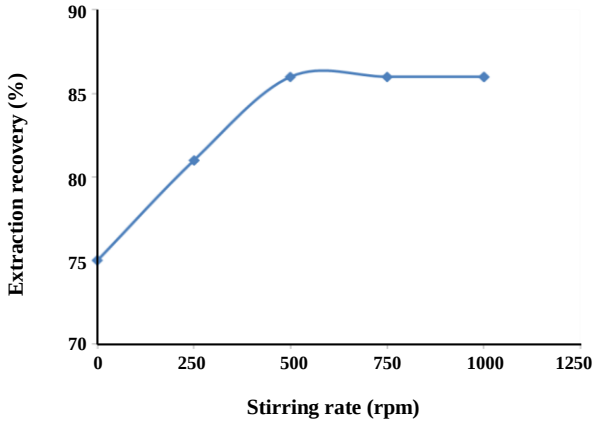

Fig. 5: Effect of stirring rate on the extraction recovery of naproxen. Extraction conditions: pH of sample= 5. Other conditions as Fig. 4.

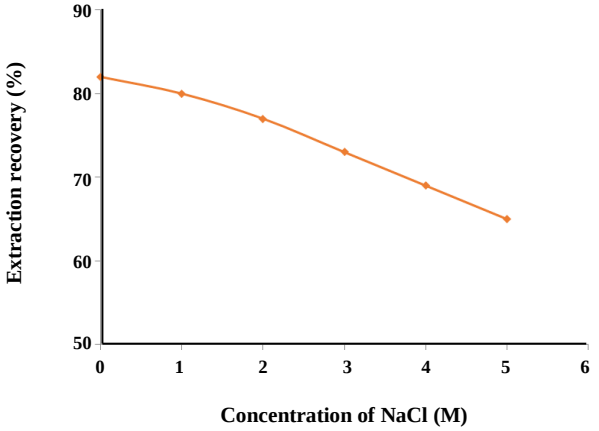

Fig. 6: Effect of salt addition on extraction recovery of naproxen. Extraction conditions: as Fig. 5.

Extraction recovery was increased by increasing stirring rate up to 500 rpm and after that; the variations of extraction recovery versus the stirring rate are not significant. Thus, all the extraction experiments were performed at 500 rpm stirring rate (Fig. 5).

Effect of extraction time on the extraction recovery

In VA−IDLLME, extraction time is defined as the gap time between injection of the mixture of extraction and disperser solvents into the aqueous sample and start of centrifugation. In this work, effect of the extraction time was examined from 0 to 30 min. The obtained results showed that the variations of extraction recovery with the extraction time are not significant (Fig. 6). This observation can be explained by the fact that after injection the cloudy solution was formed rapidly, and the contact area between extraction solvent and aqueous phase is very large. Thereby, migration of the analyte from aqueous phase to the extraction solvent is very fast, and extraction time has no effect on extraction recovery.

Effect of salt concentration on the extraction recovery

Some researchers reported that the addition of salt to the sample solution had beneficial for the extraction recovery in microextraction procedures [17, 18]. It is well known that the addition of salt to the aqueous sample is usually made to improve the extraction of analytes when LLE is used, since the increase in the ionic strength brings a reduction on the solubility of the analytes in the water solution and causes the transfer of molecules into extraction solvent [19]. In this study, the effect of salt addition on the extraction recovery was investigated in the sample solution of naproxen adjusted with different concentrations from zero to 5.0 *M* of NaCl (Fig. 7). Results showed that the recovery decreased by increasing NaCl concentration. This observation can be explained by the fact that in the presence of salt, interaction may take place between the analyte and the salt [20].

Analytical performance of the VA−IDLLME−HPLC for determination of naproxen

To evaluate the analytical performance of the VA−IDLLME technique, figures of merit of this method including limit of detection (LOD), linear range, extraction recovery and preconcentration factor were investigated for extraction of naproxen from standard

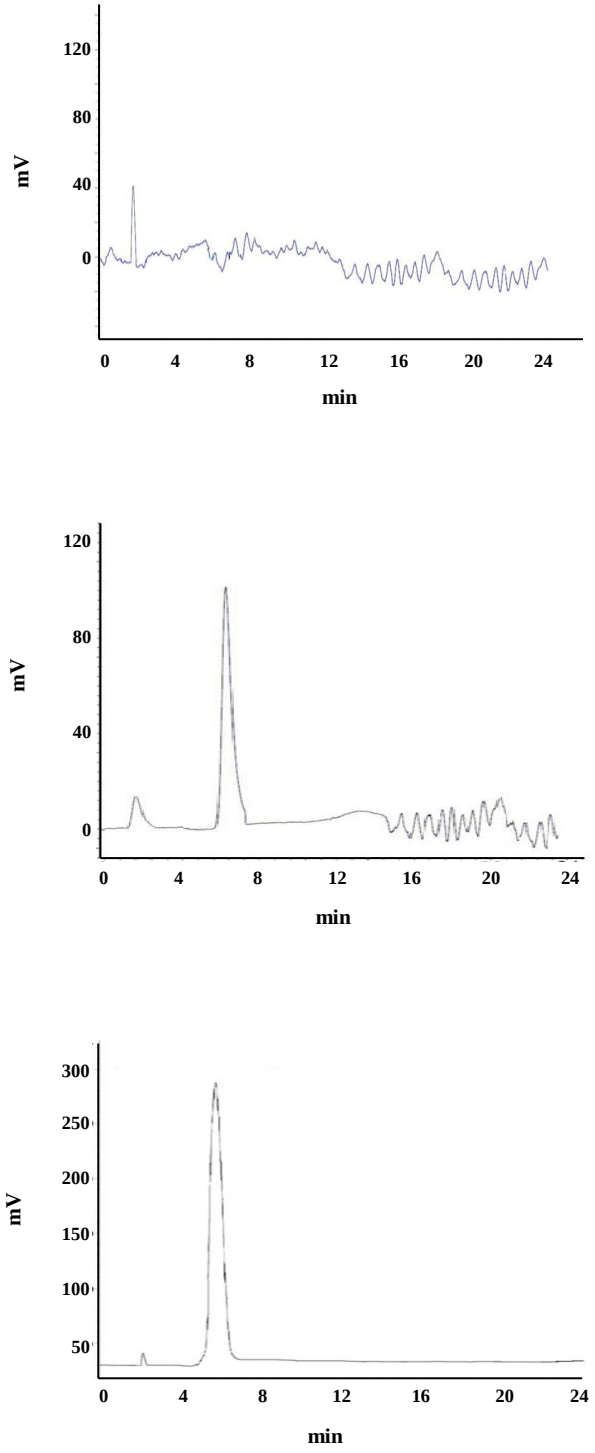

Fig. 7: Chromatograms of naproxen (Nap) after extraction at optimum conditions. (a) blank plasma sample (b) patient plasma sample and (c) spiked patient plasma sample with naproxen (50 ng/mL). Mobile phase: methanol: water (60: 40 v/v); flow rate: 1.0 mL/min; column: C_{18} (250 × 4.6 mm, 10µm); λ =270 nm.

aqueous solutions under the optimal conditions (100 µL dodecane as extraction solvent, 350µL methanol as disperser solvent, pH of sample = 5, stirring rate: 500 rpm, with zero extraction time and no addition of NaCl). The LOD (0.32 ng/mL) was calculated based on $3S_b/m$ where S_b is the standard deviation of blank and is equal to Peak to Peak noise when only mobile phase was passing through the column for 45 min and m is the slope of calibration curve. Linear range was 1.0 to 1000 ng/mL with determination coefficient of $R^2 = 0.9993$ using peak area. The extraction recovery and preconcentration factor were 87% and 108 respectively.

Analysis of naproxen from plasma sample using VA−IDLLME

Blank plasma sample was provided by a healthy student volunteer. Real plasma sample was from the same student after administration of an oral dose (500 mg) of naproxen after 4 hours. In order to eliminate the protein binding of the drug in plasma (greater than 99%) [21], the pretreatment was performed as outlined in the work of *Tahmasebi et al.* [22]. In order to eliminate protein binding of naproxen, 3 mL of methanol was added to 2 mL of the plasma and the resulting mixture was strongly vortexed for 10 min. The mixture was placed in an ice bath for 10 min, and centrifuged at 3500 rpm for 10 min. The supernatant was transferred into a 10 mL volumetric flask and diluted to the mark with double distilled deionized water after adjusting pH at 4.5 using 2 M formic acid solution and the extraction procedure was done under the optimized conditions. The chromatograms of VA−IDLLME extracts from blank plasma sample, patient plasma sample and spiked patient plasma sample are shown in Fig. 8. The concentration of naproxen in patient plasma sample was 341 ng/ mL using standard addition method.

CONCLUSIONS

The extraction and determination of naproxen in aqueous sample solution and human plasma were performed using VA−IDLLME and HPLC. Results showed that VA−IDLLME is an effective method for extraction and preconcentration of naproxen from the biological sample. This method presented a high extraction recovery and preconcentration factor, while enabled efficient sample clean-up. VA−IDLLME

provides a simple, inexpensive, easy to use and benign to the environment method for extraction of naproxen from plasma samples. One of the best advantages of VA-IDLLME in comparison with DLLME is the elimination of protein precipitation and centrifugation steps before the extraction process because in IDLLME the extraction phase is accumulated at the top of the sample solution after centrifugation [14].

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REFERENCES

[1] Todd P.A., & Clissold S.P., Naproxen. *Drugs*, **40**(1): 91-137 (1990).

[2] Boynton C.S., Dick C.F., Mayor G.H., NSAIDs: An Overview, *The Journal of Clinical Pharmacology*, **28**(6): 512-517 (1988).

[3] Trelle S., Reichenbach S., Wandel S., Hildebrand P., Tschannen B., Villiger P.M., Jüni P., Cardiovascular Safety of Non-Steroidal Anti-Inflammatory Drugs: Network Meta-Analysis. *Bmj*, *342* (2011).

[4] Soini H., Novotny M.V., Riekkola M.L., Determination of Naproxen in Serum by Capillary Electrophoresis with Ultraviolet Absorbance and Laser-Induced Fluorescence Detection, *Journal of Microcolumn Separations*, **4**(4): 313-318 (1992).

[5] Warner-Schmidt J.L., Vanover K.E., Chen E.Y., Marshall J.J., Greengard P., Anti-Depressant Effects of Selective Serotonin Reuptake Inhibitors (SSRIs) are Attenuated by Anti-Inflammatory Drugs in Mice and Humans, *Biological Psychiatry*, **68**: 528-535 (2010).

[6] Bebawy L.I., El-Kousy N.M., Simultaneous Determination of Some Multicomponent Dosage Forms by Quantitative Thin Layer Chromatography Densitometric Method, *Journal of Pharmaceutical and Biomedical Analysis*, **20**(4): 663-670 (1999).

[7] Kayiran S., Pehlivan S.B., Çelebier M., Ünlu N., Determination of Naproxen Sodium from Poly (Lactide-co-Glycolide) Corneal Scaffolds, *Turkish Journal of Pharmaceutical Sciences*, **7**(1): 57-68 (2010).

[8] Hsu Y.H., Liou Y.B., Lee J.A., Chen C.Y., Wu A.B., Assay of Naproxen by High-Performance Liquid Chromatography and Identification of Its Photoproducts by LC-ESI MS, *Biomedical Chromatography*, **20**(8): 787-793 (2006).

[9] Sun Y., Zhang Z., Xi Z., Shi Z., Determination of Naproxen in Human Urine by High Performance Liquid Chromatography with Direct Electro Generated Chemiluminescence Detection, *Talanta*, **79**(3): 676-680 (2009).

[10] Aresta A., Palmisano F., Zambonin C.G., Determination of Naproxen in Human Urine by Solid-Phase Microextraction Coupled to Liquid Chromatography, *Journal of Pharmaceutical and Biomedical Analysis*, **39**(3): 643-647 (2005).

[11] Pedersen-Bjergaard S., Rasmussen K.E., Liquid Phase Microextraction and Capillary Electrophoresis of Acidic Drugs, *Electrophoresis*, **21**(3): 579-585 (2000).

[12] Mallett D.N., Dayal S., Dear G.J., Pateman A.J., The Determination of Naproxen in Plasma by Mixed-Mode Liquid Chromatography–Tandem Mass Spectrometry Following Ion Exchange Solid Phase Microextraction, *Journal of Pharmaceutical and Biomedical Analysis*, **41**: 510-516 (2006).

[13] Mehta P., Sharma C.S., Nikam D., Ranawat M.S., Development and Validation of Related Substances Method by HPLC for Analysis of Naproxen in Naproxen Tablet Formulations, *International Journal of Pharmaceutical Sciences and Drug Research;* **4**(1): 63-69 (2012).

[14] Ranjbari E., Biparva P., Hadjmohammadi M.R., Utilization of Inverted Dispersive Liquid-Liquid Microextraction Followed by HPLC-UV as a Sensitive and Efficient Method for the Extraction and Determination of Quercetin in Honey and Biological Samples, *Talanta.*, **89**: 117-123 (2012).

[15] Farajzadeh M.A., Seyedi S.E., Shalamzari M.S., Bamorowat M., Dispersive Liquid-Liquid Microextraction Using Extraction Solvent Lighter Than Water, *Journal of Separation Science*, **32**: 3191-3200 (2009).

[16] SarafrazYazdi A., Es'haghi Z., Surfactant Enhanced Liquid-Phase Microextraction of Basic Drugs of Abuse in Hair Combined with High Performance Liquid Chromatography, *Journal of Chromatography A.*, **1094**: 1-8 (2005).

[17] D. Nagaraju S.D., Huang, Determination of Triazine Herbicides in Aqueous Samples by Dispersive Liquid-Liquid Microextraction with Gas Chromatography-Ion Trap Mass Spectrometry, *Journal of Chromatography A.*, **1161**: 89-97 (2007).

[18] Mohammadi A., Yamini Y., Alizadeh N., Dodecylsulfate-Doped Polypyrrole Film Prepared by Electrochemical Fiber Coating Technique for Headspace Solid-Phase Microextraction of Polycyclic Aromatic Hydrocarbons, *Journal of Chromatography A.*, **1063**: 1-8 (2005).

[19] Shrivas K., Patel D.K., Quantitative Determination of Nicotinic Acid in Micro Liter Volume of Urine Sample by Drop-to-Drop Solvent Microextraction Coupled to Matrix Assisted Laser Desorption/ionization Mass Spectrometry, *Spectrochimica Acta Part A: Molecular and Biomolecular Spectroscopy*. **78**: 253-257 (2011).

[20] Ebrahimzadeh H., Yamini Y., Abedi Firozjaei H., Kamarei F., Tavassoli N., Tavassoli N., Rouini M.R., Hollow Fiber-Based Liquid Phase Microextraction Combined Withhigh-Performance Liquid Chromatography for the Analysis of Gabapentin in Biological Samples, *J. Analytica Chim Acta*, **665**: 221-226 (2010).

[21] Van Der Merwe H.M., De Kock M.J., Reversed-Phase High-Performance Liquid Chromatographic Method for the Determination of Warfarin from Biological Fluids in the Low Nanogram Range, *Journal of Chromatography B: Biomedical Sciences and Applications*, **378**: 254-260 (1986).

[22] Tahmasebi E., Yamini Y., Saleh A., Extraction of Trace Amounts of Pioglitazone as an Anti-Diabetic Drug with Hollow Fiber Liquid Phase Microextraction and Determination by High-Performance Liquid Chromatography-Ultraviolet Detection in Biological Fluids, *Journal of Chromatography B.*, **877**: 1923-1929 (2009).