ULTRASOUND-ASSISTED EMULSIFICATION MICROEXTRACTION COUPLED WITH HPLC-DAD FOR THE SIMULTANEOUS DETERMINATION TRACE LEVELS OF ASPIRIN AND DICLOFENAC IN HUMAN URINE SAMPLES

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Abstract

In this research, a simple, selectivity, and environmentally friendly method based on ultrasound-assisted emulsification microextraction (USAEME) was proposed for simultaneous determination of trace levels of aspirin and diclofenac in urine samples. High performance liquid chromatography with diode-array detection (HPLC-DAD) was employed to analysis the extraxtants. Coupling the USAEME method with HPLC-DAD was presented for the first time in this study. Several analytical parameters affecting the USAEME method on the recovery extraction were investigated and optimized. The experimental conditions, including pH of sample solution, type of extraction solvent, temperature and time of ultrasound, centrifugation time and ionic strength were considered and optimized. Under the optimal conditions, relative standard deviations (RSD) of the analysis less than 9% (n= 3) and detection limit were determined as 2.29 and 3.05 ng mL⁻¹ for aspirin and diclofenac respectively. Recoveries of both in human urine and synthesis samples were in the ranges of 75–89%. USAEME - HPLC-DAD was successfully applied for the simultaneous determination of trace levels of aspirin and diclofenac in human urine and synthesis samples.

Keywords: Ultrasound-assisted emulsification-microextraction, Aspirin, Diclofenac, Human urine sample, HPLC-DAD.

Introduction

Aspirin and diclofenac are nonsteroidal anti-inflammatory drugs (NSAIDs). They have been widely used to treat non-inflammatory conditions such as fever and a variety of conditions that cause pain and inflammation [1]. Beside of their widely use, there are unwanted side effects such as indigestion, ulcers and bleeding parts of the gastrointestinal tract along
with kidney, liver and heart problems [2]. Therefore, monitoring NSAID drug concentrations in human urines are considered an important issue in pharmacokinetic and medicine studies for improving the toxicological management of long-term NSAID therapy [3]. Several chromatographic methods have been reported for determination of NSAIDs in various matrices, such as capillary electrophoresis (CE) [4-5], high-performance thin-layer chromatography (HPTLC) [6], high-performance liquid chromatography [7-8] and gas chromatography (GC) [9-10]. Ultrasound-assisted emulsification microextraction method (USAEME) was reported by Regueiro et al. (2008) [11] as an effective technique among the microextraction methods USAEME as a dispersive liquid–liquid microextraction (DLLME) method, USAEME has several advantages including simplicity of operation, rapidity, high recovery, low consumption of organic solvents, simplicity of experiment, and low cost [12]. In USAEME, a microvolume of water-immiscible extraction solvent is dispersed into sample aqueous solution by ultrasound-assisted emulsification without using any dispersive solvent. The consequence is a very efficient and fast analyte extraction. After mass transfer, the two phases can be readily separated by centrifugation. In this way, USAEME can be employed as a simple and efficient extraction and preconcentration procedure for organic compounds in aqueous samples [13]. This study was aimed to investigate and validate a HPLC method for simultaneous determination of aspirin and diclofenac in urine samples. USAEME is used to extract and preconcentrate of analytes from urine samples. Goodness of the proposed method was validated by applying it to analysis the drugs in in human urine and synthesis samples.

**Experimental**

**Materials and Reagents**

The analytical-reagent grade of the drugs (>99%) was purchased from Sigma Aldrich (Steinheim, Germany). The stock solutions (1000 ng mL⁻¹) were prepared by dissolving appropriate amount of each drug in methanol. The working solutions were prepared by diluting of the stock solutions with methanol. Deionized water prodused by Milli-Q system (Millipore, Bedford, MA, USA). Methanol (HPLC-grade) was purchased from Merck (Darmstadt, Germany). All of the standard solutions were stored at 4°C and brought to ambient temperature just prior to use. Throughout the experimental runs, all the solvents, calibration, and samples were filtered through 0.45 μm nylon filter membranes (Varian, USA).

**Instrumentation**: The chromatography measurements were carried out by a KNAUER HPLC system equipped with a micro vacuum degasser, HPLC column (C₁₈ 250 mm×4.6mm, 5μm), and UV-Vis diode array detector set to recorde
absorbance at 254 nm. The pH was measured using a pH meter (Metrohm 827, Switzerland) combined with a glass electrode. A 320R Hettich centrifuge (Germany) and a digital 10P ultrasonic bath (Sonorex, Germany) were also used.

**Extraction procedure:** The real samples in this study were collected from human urine samples orthopedic patient volunteers at Taleghani medical center (Abadan, Iran) and then stored at 5-8°C until analysis (female, age 25 ± 4.5 years; and male, age 21 ± 7.0 years). Human urine samples were prepared using the USAEME method. Aliquots of 1 mL human urine sample were alkanized with 200 µL (NaOH 1 mol L⁻¹) for the hydrolysis of acyl glucuronic acid conjugates and then neutralized with 200 µL (HCl 1 mol L⁻¹). The samples were placed in centrifuge glass vials and their ionic strength and pH were adjusted to the optimum level (NaCl, 2% (w/v); pH 5.0). Then, 100 µL of 1-octanol was injected into the sample solution. The vial was immersed in an ultrasonic, sonicated for 5 min, and shaken manually. A cloudy solution was centrifuged for 4 min at 5000 rpm in order to disrupt the emulsions and separate both phases. After centrifugation extraction, the organic phase on the bottom of the tube was collected with a Hamilton microsyringe. Finally, 20 µL of the obtained mixture was injected into the separation system. Scheme of the USAEME procedure is shown in Fig. 1.

**Fig.1. Schematic representation of the ultrasound-assisted emulsification microextraction (USAEME) procedure for urine sample preparation.**

**Results and Discussion**

In order to establish a sensitive and simple analytical method for the simultaneous analysis of selected NSAIDs, all affecting experimental variables were investigated and optimized. These variables were pH, type and volume of extraction solvent, time and temperature of ultrasound, conditions of centrifuging, ionic strength were studied and optimized.
Optimization of chromatographical condition

The goal of this study is to HPLC determination of aspirin and diclofenac after extraction and preconcentration by USAEME. Two variables including type of mobile phase and column oven temperature were optimized with the hope to find an experimental conditions in which highly resolved chromatograms can be recorded for both analytes. Mixtures of acetonitrile/water, acetonitrile/methanol/water, and methanol/water with different pH values were checked. The best symmetry of the peak shapes was found in the mobile phase containing methanol and water with pH value of 3. Formic acid was used to adjust pH of the mobile phase in all experiments. It was found that during the chromatographic analysis increasing the ratio of water to methanol caused to elute efficiently the analytes from the column. Effect of column oven temperature was also studied in the range of 20-30 °C with the selected mobile phase and flow rate of 1.0 mL min⁻¹. According to the results, temperature of 25 °C was found to be optimal and used in the subsequent analysis. It should be mentioned that changing the flow rate of mobile phase did not affect on the chromatographic peaks. Scheme of the gradient used in the HPLC analysis are presented in Table 1.

Table 1. Scheme of the gradient used in the HPLC analysis.

<table>
<thead>
<tr>
<th>Time(min)</th>
<th>%H₂O (pH 3.0)</th>
<th>%MeOH</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>30</td>
<td>70</td>
</tr>
<tr>
<td>2</td>
<td>35</td>
<td>65</td>
</tr>
<tr>
<td>7</td>
<td>33</td>
<td>67</td>
</tr>
<tr>
<td>10</td>
<td>30</td>
<td>70</td>
</tr>
</tbody>
</table>

Optimization of the extraction parameters

The extraction efficiency of USAEME method depends on some important analytical parameters, which should be investigated in detail. Effects of various analytical parameters including pH of the sample solution, type and volume of extraction solvent, centrifugation time, ultrasound extraction time and temperature, and ionic strength were investigated and optimized using one-at-a time method.

pH of sample solution

pH of the sample solution is one of the factors studied in this work. It was found from literature that pH affects the extraction recovery. Effect of pH on the recoveries of drugs, phosphate buffers in the range of 3.0 - 7.0 were investigated. According to the obtained results, it can be concluded that recoveries of drugs were increased when the sample pH was
decreased to 5.0. This is due to the fact that at low pH, the considered drugs were not in ionic form in solution. The results are shown in Fig. 2. Finally, pH of 5.0 was chosen as the optimum pH sample solution for the following experiments.

**Selection of extraction solvent**

Selection of suitable extraction solvent is the most important analytical parameter in USAEME methods [11]. The extracting solvent has to meet some properties such as lower density than that of water, low solubility in water, and high extraction capability of the target drugs. Different solvents including 1-decanol, n-hexane, 1-octanol, and n-decane were investigated. Among them, 1-octanol was chosen as the best extraction solvent because it had higher recoveries in comparison with the others. To obtain the highest extraction efficiency, volume of the solvent had to be optimized. To do so, volume of 1-octanol was changed in the range of 10.0 to 120.0 µL. From the results, shown in Fig. 3, it was found that 100.0 µL of extraction solvent was sufficient to recover both drugs. This volume was used throughout the experiments.

**Effect of time and temperature of ultrasound**

Exposing time to ultrasound radiation might affect extraction efficiency due to its own affecting on both emulsification and mass transfer process. This time was examined in the range of 0-7 min. Maximum recoveries were obtained after
ultrasonication for 5 min and no improvement was achieved by further ultrasonication. This foundation can be explained base on the fact that ultrasound could generate the emulsion quickly and make rapidly a very large contact surface area between the extraction phase and the aqueous phase. Therefore, 5 min was found to be the optimum time. The results are shown in Fig. 4.a. Temperature affects organic solvent solubility in water, distribution coefficients as well as the emulsification phenomenon. The effect of temperature on the recoveries was evaluated over different temperatures ranging from 20 °C - 35 °C. The results are shown in Fig. 4.b. It is clear from the results shown in this figure that emulsification at ambient temperature helps to reach higher extraction recoveries. Therefore, this temperature was taken in the extraction step. At lower temperature, extraction recoveries decreased due to decrease in mass transfer phenomenon. At higher temperature, distribution coefficients (K_D) of drugs were changed in the direction of decreasing in the recoveries.

Fig 4(a) Effect of ultrasound extraction time on the recoveries of drugs. Conditions: sample solution, 10 mL of 50 (ng mL⁻¹) of each drugs; pH sample solution: 5.0; volume and type of extracting solvent: 1-octanol, 100.0 µL(a).

Fig.4.b. Effect of ultrasound temperature on the extraction efficiency. Conditions: sample solution, 10 mL of 50 (ng mL⁻¹) of each drugs; pH sample solution: 5.0; volume and type of extracting solvent: 1-octanol, 100.0 µL; ultrasonication extraction time: 5 min (b).
Effect of centrifugation time

Centrifugation was required to break down the emulsion and accelerate the phase-separation process. This also affects the volume of organic phase and, so, the drug concentrations. Centrifugation time was investigated in the range of 0-6 min, whereas centrifuging rate was kept at 5000 rpm. Therefore, 4 min and 5000 rpm were selected as the optimum centrifugation time and rate. The results are as shown in Fig. 5.

![Fig 5](image_url)

**Fig 5** Effect of centrifugation extraction time on the recoveries of drugs. Conditions: sample solution, 10 mL of 50 (ng mL\(^{-1}\)) of each drugs; pH sample solution: 5.0; volume and type of extracting solvent: 1-octanol, 100.0 µL; ultrasonication extraction time: 5 min, ultrasound temperature 25 °C.

Ionic strength

Effect of ionic strength varies in different extraction methods and, therefore, it should be study. Influence of ionic strength was investigated by adding different amounts of NaNO\(_3\), NaCl, and KH\(_2\)PO\(_4\) 0–10% (w/v) to the aqueous drugs solution to be extracted. The results in Fig. 6 show that solubility of the drugs in aqueous phases was decreased in the present of salt, improving their transfer from water to organic layers. The best extraction recoveries were obtained when the aqueous solution was set to 2% with respect of NaCl. It should be noted that addition of salt increases the volume of organic phase after the centrifuge. Therefore, NaCl 2% (w/v) was used in the subsequent experiments.
Effect of ionic strength on the recoveries of drugs. Conditions: sample solution, 10 mL of 50 (ng mL\(^{-1}\)) of each drug; pH sample solution: 5.0; volume and type of extracting solvent: 1-octanol, 100.0 µL; ultrasound extraction time: 5 min; centrifugation time: 5000 rpm and 4 min.

**Analytical features of proposed method**

Under the optimal conditions, analytical features of the proposed method including limit of detection (LOD), dynamic range, enrichment factor (EF), limit of quantification (LOQ), and relative standard deviations (RSD) were investigated. Results are listed in Table 2. A good linear relationship is displayed between the corresponding peak areas and the concentrations of the both drugs based on the correlation coefficients (\(r^2 \geq 0.9972\)).

Comparison of the parameters obtained in this study with those reported in the literature is given in Table 3. It is obvious that figures of merit of the proposed procedure are comparable or better than the others reported for aspirin and diclofenac determination.

Chromatograms of solutions containing each drug and mixture of both under the optimal conditions are shown in Fig. 7.

**Table 2. Statistical parameters and figures of merit for determination of analytes in samples by applying USAEME method.**

<table>
<thead>
<tr>
<th>Drugs</th>
<th>LOD (ng mL(^{-1}))</th>
<th>Dynamic range (ng mL(^{-1}))</th>
<th>EF*</th>
<th>LOQ (ng mL(^{-1}))</th>
<th>RSD (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aspirin</td>
<td>2.29</td>
<td>5-950</td>
<td>116</td>
<td>4.73</td>
<td>8.61</td>
</tr>
<tr>
<td>Diclofenac</td>
<td>3.05</td>
<td>2-800</td>
<td>2010</td>
<td>5.61</td>
<td>7.05</td>
</tr>
</tbody>
</table>

*Average Enrichment factor

**Table 3. Comparison of this study and the reported different methods for the determination of aspirin and diclofenac.**

<table>
<thead>
<tr>
<th>Method</th>
<th>Appratus</th>
<th>LOD ngmL(^{-1})</th>
<th>LOQ ngmL(^{-1})</th>
<th>RSD %</th>
<th>EF</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>HF-LPME</td>
<td>HPLC–DAD</td>
<td>12.3*,52.9**</td>
<td>-</td>
<td>1.2*–</td>
<td>70*-</td>
<td>12</td>
</tr>
<tr>
<td>SPE</td>
<td>HPLC</td>
<td>7*</td>
<td>20*</td>
<td>1.3**</td>
<td>1060*</td>
<td>13</td>
</tr>
<tr>
<td>DLLME-SFO</td>
<td>HPLC</td>
<td>3.4*–5.2**</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>14</td>
</tr>
<tr>
<td>USAEME</td>
<td>HPLC-DAD</td>
<td>2.29*–3.05**</td>
<td>4.7*,–5.6*</td>
<td>7-8.8</td>
<td>116*-</td>
<td>2010**</td>
</tr>
</tbody>
</table>
Fig 7 Chromatograms two-dimensional chromatograms of the extracted aspirin (500 ppb) and diclofenac (700 ppb) in samples.

Application of the proposed method to real samples

To evaluate performance of the proposed method, determination of aspirin and diclofenac in human urine samples was carried out under the optimized conditions. The results are collected in Table 4. Recoveries in human urine (R1-R3) and synthesis urine (R4-R6) samples were in the ranges of 75–89%. The recoveries demonstrated that the matrixes have negligible effect on the quantification of these compounds and the method is accurate within the desired range. The obtained results revealed ability of the proposed method for the determination of aspirin and diclofenac in urine samples.

Table 4. Added and found aspirin and diclofenac concentrations human urine (R1-R3) and synthesis urine (R4-R6) samples.

<table>
<thead>
<tr>
<th>Samples</th>
<th>Added</th>
<th>Aspirin</th>
<th>Diclofenac</th>
</tr>
</thead>
<tbody>
<tr>
<td>R1*</td>
<td>Added</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Found</td>
<td>37.9</td>
<td>25.0</td>
</tr>
<tr>
<td></td>
<td>Recovery%</td>
<td>89.73</td>
<td>75.90</td>
</tr>
<tr>
<td>R2*</td>
<td>Added</td>
<td>10.0</td>
<td>5.0</td>
</tr>
<tr>
<td></td>
<td>Found</td>
<td>34.01</td>
<td>22.77</td>
</tr>
<tr>
<td></td>
<td>Recovery%</td>
<td>89.73</td>
<td>75.90</td>
</tr>
<tr>
<td>R3*</td>
<td>Added</td>
<td>200.0</td>
<td>200.0</td>
</tr>
<tr>
<td></td>
<td>Found</td>
<td>210.66</td>
<td>199.2</td>
</tr>
<tr>
<td></td>
<td>Recovery%</td>
<td>88.54</td>
<td>88.53</td>
</tr>
</tbody>
</table>
**Conclusions**

A new method has been proposed for the simultaneous determination trace levels of aspirin and diclofenac in synthetic and human urine samples using HPLC-DAD after optimization by USAEME. The proposed method has advantages such as; simplicity of operation, low consumption of organic solvents, good reproducibility and gives a precise, highly sensitive and selective procedure with good LODs.

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**References**


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