INTRODUCTION

Diclofenac sodium is a non-steroidal anti-inflammatory drug (NSAID) with analgesic, anti-inflammatory and antipyretic properties. These properties are primarily achieved by its ability to block the enzyme cyclooxygenase in the arachidonic acid cascade and to decrease prostanooid synthesis, but also by an additional direct effect on hyperalgesia due to the functional downregulation effect of sensitized peripheral pain receptors\(^1,2\). The efficacy of diclofenac is equal to that of many newer NSAIDs,
and it has a fast onset and long-duration analgesic action. Compared to other NSAIDs, diclofenac is well tolerated and rarely produces gastrointestinal ulcerations or other serious side effects. Thus, diclofenac can be considered as one of a few NSAIDs of first choice used in the treatment of acute and chronic painful and inflammatory conditions. In ophthalmology, the well-tolerated anti-inflammatory drug (NSAID) diclofenac sodium is used for perioperative applications in cataract surgery to maintain mydriasis, for prophylaxis, and for treatment of postoperative symptoms of inflammation.Diclofenac sodium eye-drops are used in ophthalmology to decrease intraoperative miosis and to reduce the breakdown of the blood-aqueous barrier, thus its penetration. Several methods for the determination of diclofenac in biological fluids have been reported. Recently, gas chromatographic methods have been replaced by liquid chromatographic methods. High performance liquid chromatography (HPLC) with ultraviolet detection is the most popular method for quantification of diclofenac. Nevertheless, these methods have some shortcomings, like a lack of sensitivity and poor specificity, and therefore they are liable to interference from diclofenac metabolites or other endogenous compounds. Ultraviolet detection of diclofenac in synovial fluids is described after involving n-hexane liquid-liquid extraction step. The prediction of diclofenac penetration to subretinal and aqueous humor fluids has a clinical importance. HPLC method for diclofenac determination in aqueous humor using fluorescence and ECD has been described; however, the former requires ETFE reactor on-line post column sample photoderivatization and the latter hydrophilic polyoxyethylene polymer-coated octyl (C₈) column. In addition, they utilize external calibration and do not cover diclofenac determination in subretinal fluid, which has an important eye layer for drug penetration in ophthalmology. In this paper, we describe a simple and specific method for quantitative determination of diclofenac in subretinal and aqueous humor samples.

**MATERIALS AND METHOD**

**Chemicals and reagents**

Diclofenac sodium was donated by Novartis İlaç San. A.Ş., and quinine, the internal standard (IS), was obtained from Sigma. HPLC grade acetonitrile was purchased from Lab-Scan (Dublin, Ireland), and glacial acetic acid and triethylamine from J.T. Baker (Deventer, Holland).

**Equipment**

Chromatography was performed by a Shimadzu liquid chromatograph equipped with a pump (LC-10AT VP), a controller (SCL-10A VP) connected to a computer using a software (Class-VP 5.03), an autosampler (SIL-10AD VP) and electrochemical detector (Decade). pH of the solutions was measured by a pH meter (Mettler Toledo MA 235).

**Chromatographic conditions**

The separation was performed on a reversed phase Nucleosil 100-5 C₁₈ (250 x 4.6 mm, 5 µm particle size) column. The mobile phase consisted of acetonitrile (containing 0.065% triethylamine) and 1.65% glacial acetic acid (50:50, v/v) (pH: 4.30). The mobile phase was prepared daily, filtered, sonicated before use and delivered to the system at a flow rate of 1.0 ml/min at room temperatures (20-22°C). The working potential was set to 1000 mV at electrochemical detector. Glassy carbon working electrode was used against the Ag/AgCl reference electrode.

**Standard and sample preparation**

**Standard solutions**

Initial stock solutions of diclofenac and quinine (as IS) (1000 µg/ml) were prepared by dissolving 5 mg of each compound in 5 ml of methanol. Intermediate standard solutions (10 and 1 µg/ml) were diluted from the primary stock solution using mobile phase. Stock and intermediate solutions were stored at 4°C and remained stable for at least six months.
standard solutions (20, 50, 100, 200, 400, 600, 800 and 1000 ng/ml) were prepared by appropriate dilution from intermediate solutions by mobile phase.

**Sample extraction**

Aqueous humor or subretinal fluid sample (100 µl) was spiked with 250 ng/ml of IS and 500 µl of acetonitrile added, then mixed on a vortex-mixer vigorously for 90 s and centrifuged at 3000 rpm for 20 min. Upper phase was decanted and evaporated to the dryness under nitrogen flow at 40°C. The residue was reconstituted in 100 µl of mobile phase and 50 µl was injected to the HPLC system.

**Method validation**

The method was validated according to International Conference on Harmonization (ICH) Guideline for validation of analytical procedures. The validation parameters were linearity, accuracy, precision, limit of quantitation (LOQ), limit of detection (LOD) and selectivity. To evaluate the performance of the developed method, the acceptance criteria in chromatography for validation parameters were considered. It was not possible to take adequate drug-free human eye layer samples for the validation study because of ethical restrictions. Hence, validation studies were performed using the standard samples prepared in mobile phase.

**Linearity**

For calibration curves, the peak area ratios of diclofenac to the IS were plotted versus diclofenac concentrations. Linearity was evaluated by standard solutions containing 20, 50, 100, 200, 400, 600, 800 and 1000 ng/ml (n=8) of diclofenac in mobile phase. A calibration curve was prepared at each analysis day for seven days, and the regression equation and correlation coefficient were determined.

**Accuracy and precision**

To evaluate accuracy and precision, standard solutions at three concentration levels (50, 200 and 800 ng/ml) in the calibration range were analyzed. To determine the intra-day and inter-day accuracy and precision, samples were independently prepared and analyzed six times in the same day and on six different days. Precision and accuracy were calculated as relative standard deviation (RSD %) and percentage deviation of obtained concentration from added concentration (bias %), respectively.

**Sensitivity (LOD and LOQ)**

The limits of detection and quantitation were determined as the sensitivity of the developed method. The LOD was decided as the diclofenac concentration giving a ratio of signal to noise equal to three (S/N=3). The LOQ was the lowest diclofenac concentration of calibration curve with RSD value of < 15%.

**Selectivity**

The selectivity was described as the ability of the method to resolve diclofenac from baseline and other peaks. The specificity was determined for the HPLC method in order to verify that diclofenac does not interfere with any of the matrices composing the eye layers. For these studies, the chromatograms of aqueous humor and subretinal fluid and their drug-free sample solutions were compared.

**Recovery**

In order to establish the reliability and suitability of the proposed methods, recovery experiments were carried out. For absolute recovery, known amounts of diclofenac (200 ng/ml) and IS (250 ng/ml) were added to water, and the mixtures were analyzed by the proposed method (n=6). For relative recovery, drug-free samples were spiked with 400 ng/ml of diclofenac and 250 ng/ml of IS, and the mixtures were analyzed by the proposed method (n=3).

**Application of developed method to the eye samples**

The eye samples, aqueous humor and subretinal fluids, were obtained from patients administered Voltaren® (0.1% diclofenac sodium) eye drops before
retina decollement surgery. Patients were instilled three times with 15 min intervals one night before surgery and four times with 15 min intervals on the day of surgery. Aqueous humor and subretinal fluids were collected during surgery, stored at –25°C till analysis and protected from light. The analysis of the samples was performed by the method developed and validated in this study.

The local ethical committee of Ankara Hospital approved this study, and written informed consent was taken from all patients accepted into the study.

RESULTS AND DISCUSSION

Chromatographic conditions and parameters

Chromatographic conditions were optimized by changing the ratio of acetonitrile, flow rate, pH and working potential. The best resolution with the best peak shape and low retention time were obtained by a mobile phase consisting of acetonitrile (containing 0.065% triethylamine) and 1.65% glacial acetic acid (50:50, v/v) (pH: 4.30) at a flow rate of 1.0 ml/min, and the working potential was set to 1000 mV at electrochemical detector. Under the described chromatographic conditions, diclofenac and quinine (IS) were well separated from baseline and each other (Figs. 1, 2). Retention times for diclofenac and IS were 14.74±0.02 (RSD=0.99%, n=10) min and 3.86±0.02 (RSD=1.08%, n=10) min, respectively. The relative retention time of diclofenac/quinine was 3.81±0.02 (RSD=1.05%, n=10). Inter-day and intra-day variations of diclofenac and quinine (IS) retention times were less than 1.08%. For both compounds, sharp and symmetric peaks were obtained with a good baseline resolution (Rs: 15.89) and minimal tailing (symmetry factors: 0.94 and 0.98, respectively), thus facilitating the accurate measurement of the peak area.

Method validation

Linearity

The method was linear in the range of 20 - 1000 ng/ml diclofenac containing quinine as an IS at 250 ng/ml (Table 1, r = 0.9998±0.0003, n = 7). The equation of regression obtained was y = 12.07x + 0.02, and the mean determination coefficient of seven consecutive calibration curves was r² = 0.9996±0.0005.

Table 1. The analytical characteristics of proposed method (n = 7)

<table>
<thead>
<tr>
<th>HPLC-ECD</th>
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<tbody>
<tr>
<td>Regression equation</td>
<td>y = 12.07x + 0.02</td>
</tr>
<tr>
<td>Standard error of slope</td>
<td>0.109</td>
</tr>
<tr>
<td>Standard error of intercept</td>
<td>3.44 x 10⁻³</td>
</tr>
<tr>
<td>Correlation coefficient (r)</td>
<td>0.9998 ± 0.0003</td>
</tr>
<tr>
<td>Linearity range</td>
<td>20 - 1000 ng/ml</td>
</tr>
<tr>
<td>Number of data points</td>
<td>8</td>
</tr>
<tr>
<td>LOD</td>
<td>10 ng/ml</td>
</tr>
<tr>
<td>LOQ</td>
<td>20 ng/ml</td>
</tr>
</tbody>
</table>

y = bx + a; x: concentration, y: peak area ratio, b: slope, a: intercept
LOD: Limit of detection. LOQ: Limit of quantitation.

Accuracy and precision

The accuracy and precision were investigated at three concentration levels of diclofenac in the linear range with six independent replicates on the same day and on six consecutive days. The intra-day and inter-day bias values were found to be less than 1.72 and 1.14%, and the intra-day and inter-day RSD values were not higher than 2.19 and 1.94%, respectively (Table 2).

Table 2. Accuracy and precision assay data of diclofenac determination (n = 6)

<table>
<thead>
<tr>
<th>Added (ng/ml)</th>
<th>Intra - day</th>
<th>Inter - day</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Founda (ng/ml)</td>
<td>Precisionb RSD %</td>
</tr>
<tr>
<td>50.00</td>
<td>50.86 ± 0.09</td>
<td>2.19</td>
</tr>
<tr>
<td>200.00</td>
<td>200.27 ± 3.20</td>
<td>1.78</td>
</tr>
<tr>
<td>800.00</td>
<td>801.52 ± 1.42</td>
<td>0.20</td>
</tr>
</tbody>
</table>

aFound: x = mean ± standard error.
bPrecision: RSD % = Relative standard deviation %.
cAccuracy: Bias % = | (Founded - Added) | / Added | x 100.
Sensitivity

The LOD, defined as three times the level of the baseline noise, was 10 ng/ml for diclofenac, and the LOQ, defined as the lowest concentration at the calibration curve, was 20 ng/ml, with a RSD value of 7.11% (n = 7).

Selectivity

Diclofenac and IS were well resolved from baseline and each other with an acceptable resolution value much higher than 1.5 (Fig. 1a, 2a). The comparison of the chromatograms of drug-free and diclofenac-spiked aqueous humor samples demonstrated that there was no interfering peak from matrix (Fig. 1b, 1c). The same behavior was obtained from drug-free and diclofenac-spiked subretinal fluids, suggesting that there was also no interfering peak from matrix (Fig. 2b, 2c). These results indicated that the method presented in this study was selective and specific.

Recovery

While the relative recovery was 97.76±2.11% (n=3), the absolute recovery was found to be 99.51±1.26% (n=6). The relative recovery could not be repeated more than three times because of the lack of drug-free eye fluid due to ethical considerations.

Application to the eye samples

The eye samples, aqueous humor and subretinal fluids, were obtained from patients topically administered diclofenac before retina décollement surgery. The determination of diclofenac levels in these eye samples was successfully performed by the method presented in this study. The concentration in aqueous humor ranged from 5.84 to 10.49 ng/ml (n=5), while diclofenac concentration in subretinal fluid was 13.81 and 80.69 ng/ml (n=2).
CONCLUSION

In this study, a simple, fast, efficient and reliable HPLC-ECD method was developed and validated for the analysis of diclofenac in subretinal fluids and aqueous humor. The method presented in this study was selective enough using a conventional RP18 analytical column and applicable to subretinal and aqueous humor samples obtained from patients after simple protein denaturation with acetonitrile. Thus, the developed method is recommended for clinical investigations based on determination of diclofenac levels in subretinal and aqueous humor fluid after its topical administration in view of its high recovery, precision and accuracy.

REFERENCES


