Development And Validation Of High Performance Liquid Chromatographic Method For The Determination Of Etoricoxib In Human Plasma

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ABSTRACT

A rapid, specific and accurate high performance liquid chromatographic method for the determination of Etoricoxib in human plasma using Diclofenac as internal standard was developed and validated by UV detection. The extraction process involved a liquid-liquid extraction using a 70:30 % v/v mixture of t-butyl methyl ether and Dichloromethane. Both Etoricoxib and the internal standard were eluted under isocratic mode. The mobile phase composed a mixture of 70:30 % v/v Methanol and 0.1% ortho phosphoric acid at a flow rate of 0.8 mL/minute. The wavelength of detection is 287 nm. The method showed good linearity in the range of 60–3920 ng/mL. Matrix effects were not observed.

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INTRODUCTION

Etoricoxib (ETO), 5-chloro-6'-methyl-3-[4-(methylsulfonyl)phenyl]-2,3'-bipyridine [Figure 1], is an non-steroidal anti-inflammatory drug (NSAID) categorized as a highly selective COX-2 inhibitor. Etoricoxib is licensed for treatment of osteoarthritis, rheumatoid arthritis, psoriatic arthritis, ankylosing spondylitis, chronic low back pain, acute pain and gout. Compared with traditional NSAIDs, Etoricoxib is less likely to cause gastro-intestinal adverse affects. A cumulative analysis of 10 randomized controlled clinical trials involving about 6000 patients found confirmed upper gastro-intestinal events among 0.95 % of patients treated with Etoricoxib compared with 2.2 % treated with traditional NSAIDs. The NICE guideline on the usage of etoricoxib mentions that Etoricoxib must not be used in patients with uncontrolled hypertension and blood pressure should be monitored. Etoricoxib is available as 60 – 120 mg once daily tablets. Etoricoxib is metabolized extensively via oxidation of 6-methyl hydroxylation and 1-N-oxidation. These metabolites are excreted largely in the urine. A high fat meal decreased the rate of absorption without affecting the extent of absorption of etoricoxib; therefore, etoricoxib can be dosed irrespective of food.

Figure 1: Structure of Etoricoxib and Diclofenac

Several methods have been employed for the estimation of ETO alone and combination with other drugs by UV and RP-HPLC methods. Specific and sensitive methods based on mass spectrometry methods were reported earlier. Earlier reports on HPLC based bioanalytical estimation of Etoricoxib resulted in lesser sensitivity, and high noise in the base line indicating a need to develop a more efficient, sensitive, simple and rapid method in human plasma. We therefore focused on to achieve the optimum chromatographic conditions for the determination of ETO using Diclofenac (DIC) as internal standard. To access the reproducibility and wide applicability of the developed method, it was validated as per FDA guidelines.
MATERIALS AND METHODS

Solvents and Chemicals

Etoricoxib (purity 98.00 % w/w) was used as received from Ramdev chemicals Pvt Ltd. Diclofenac (DIC) (used as Internal Standard, Purity 99.0 % w/w) is purchased Laboratories Ltd. HPLC grade Methanol, Ortho phosphoric acid was purchased from Merck Ltd (Mumbai, India). Deionized water was processed through a Milli-Q water purification system (Millipore, USA). All other chemicals and reagents were of analytical grade.

Chromatographic System

The Chromatographic system consisted of a Shimadzu Class VP Binary pump LC-10ATvp, SIL-10ADvp Auto sampler, CTO-10Avp Column Temperature Oven, SPD-10Avp UV-Visible Detector. All the components of the system are controlled using SCL-10Avp System Controller. Data acquisition was done using LC Solutions software. The detector is set at a wavelength of 287 nm. Chromatographic separations were accomplished using a Phenomenex C\textsubscript{18}, 5 μm, 150 mm×4.6 mm column. The mobile phase consists of a mixture of 70 parts of Methanol and 30 parts of 0.1 % ortho phosphoric acid. The mixture was filtered through 0.22 μm membrane (Millipore, Bedford, MA, USA) under vacuum, and then degassed by flushing with nitrogen for 5 min. The mobile phase was pumped isocratically at a flow rate of 0.8 ml/min during analysis, at ambient temperature. The rinsing solution consists of a mixture of 50: 50 % v/v of methanol: HPLC Grade Water.

Preparation of Standard Solutions

A stock solution of Etoricoxib is prepared in methanol such that the final concentration is approximately 2.0 mg/mL. Stock solution of Diclofenac (approx 1 mg/mL) is prepared in HPLC Grade methanol. The solutions were stored at 4°C and they were stable for at least two weeks. Aqueous stock dilution of Etoricoxib is prepared in diluent solution (mixture of 50: 50 % v/v of methanol: HPLC Grade water).

Sample Preparation

Aqueous stock dilutions were prepared initially. 0.5 ml of each aqueous stock dilution is transferred into a 10 mL volumetric flask. The final volume is made up with screened drug-free K\textsubscript{2}EDTA human plasma and mixed gently for 15 minutes to achieve the desired concentration of calibration curve standards. The final calibration standard concentrations are 0.0 (Blank; no Etoricoxib added), 60, 120, 250, 980, 1720, 2940, 3430 and 3920.60 ng/ml. Each of these standard solutions was distributed in disposable polypropylene micro centrifuge tubes (2.0 ml, eppendorf) in volume of 0.7 ml and stored at -70°C until analysis. Similarly quality control samples were prepared in plasma such that the final concentrations were 70, 210, 2060 and 3190 ng/ml respectively and labeled as Lower limit of quantification (LLOQ), Low quality control (LQC), median quality control (MQC) and high quality control (HQC) respectively.

The extraction of the plasma samples involved Liquid-Liquid Extraction process. For processing, the stored spiked samples were withdrawn from the freezer and allowed to thaw at room temperature. An aliquot of 500 μL is then transferred to pre-labeled 2.0 mL polypropylene centrifuge tubes. 25 μL of internal standard dilution (100 μg/mL) is then added and mixed. 1.2 mL of extraction solvent is then added to extract the drug and internal standard. The samples are then kept on a reciprocating shaker and allowed to mix for 20 minutes. Samples are then centrifuged at 5000 rpm for 5 minutes at 4°C. 1 mL of the supernatant is then transferred into
prelabelled polypropylene tubes and allowed to evaporate to dryness under nitrogen at constant temperature of 40°C. The dried residue is then dissolved in 200 µL of mobile phase and transferred into shell vials and containing vial inserts for analysis. 20 µL of the samples is then injected into the system for analysis. The autosampler temperature is maintained at 4°C throughout the analysis. The column temperature oven is maintained at ambient temperature.

**Validation of quantitative HPLC method**

The quantitative HPLC-UV method was validated to determine selectivity, calibration range, accuracy and precision, limit of detection (LOD), limit of quantitation, % recovery, freeze–thaw, and auto sampler stability. The initial assay was fully validated for etoricoxib analysis in human plasma according to FDA guidelines.

**Selectivity**

The selectivity of the method was evaluated by analyzing six independent drug-free K2EDTA human plasma samples with reference to potential interferences from endogenous and environmental constituents.

**Calibration curve**

Calibration curves were generated to confirm the relationship between the peak area ratios and the concentration of ETO in the standard samples. Fresh calibration standards were extracted and assayed as described above on three different days and in duplicate. Calibration curves for ETO were represented by the plots of the peak-area ratio (ETO/DIC) versus the nominal concentration of the ETO in calibration standards. The regression line was generated using 1/concentration² factor as the mathematical model of best fit. ETO concentrations in QC samples, recovery, and stability samples were calculated from the resulting area ratio and the regression equation of the calibration curve.

**Accuracy and precision**

Intra-day accuracy and precision were evaluated by analysis of QCs at four levels (LLOQ, LQC, MQC and HQC; n = 6 at each level) on the same day. Inter-day precision and the accuracy were determined by analyzing four QC levels on 3 separate days (n = 6 at each level) along with three separate standard curves done in duplicates.

The accuracy of an analytical method describes how close the mean test results obtained by the method are to the nominal concentration of the analyte. Accuracy was calculated by the following equation, expressed as a percentage:

\[
\text{Accuracy} \% = \frac{\text{mean observed concentration}}{\text{nominal concentration}} \times 100
\]

The precision was expressed by co-efficient of variation (CV). The CV % indicates the variability around the mean in relation to the size of the mean, and is defined as:

\[
\text{CV} \% = \frac{\text{standard deviation}}{\text{mean observed concentration}} \times 100
\]
Stability Studies

Autosampler, and freeze–thaw stability of ETO was determined at low, medium and high QC concentrations. To determine the impact of freeze–thaw cycles on ETO concentration, samples were allowed to undergo 3 freeze (−70°C) thaw (room temperature) cycles. Following sample treatment/storage conditions, the ETO concentrations were analyzed in triplicates and compared to the control sample that had been stored at −70°C. Autosampler stability of extracted samples was determined by comparing ETO concentration in freshly prepared samples and samples kept in autosampler at 4°C for 24 h.

Recovery

Recovery was determined by comparing the area under the curve (AUC) of extracted QC samples (LQC, MQC and HQC) with direct injection of extracted blank plasma spiked with the same nominal concentration of ETO as in the QC samples. This should highlight any loss in signal due to the extraction process. IS recovery was determined for a single concentration of 200 µg/mL.

Data analysis

HPLC data acquisition and processing was performed by Shimadzu LC Solutions Ver 1.23 SP 1 software. Standard curves for quantitation of ETO were constructed using a 1/concentration^2 weighted linear regression of the peak area ratio versus ETO concentration. Unknown and QC sample concentrations were back-calculated from the standard curves.

RESULTS AND DISCUSSION

Method Development

The HPLC procedure was optimized with a view to develop a sensitive and reproducible method for the determination of ETO in Human Plasma. Since both etoricoxib and internal standard are highly non-polar [13, 14, 15] we employed the usage of liquid-liquid extraction process with a mixture of 70 parts of t-butyl methyl ether and 30 parts of dichloromethane. To get a better response the pH of the mobile phase is set to the acidic side. During our observation, a pH value around 7.4 resulted in better peak shape for the internal standard while that of the drug is not acceptable. Also, alkaline mobile phase characteristics causes deterioration of the bonded phase in the column due to alkaline hydrolysis of end-capped silica [16, 17]. Compared to acid catalyzed hydrolysis, the hydrolysis of end-capped silica in alkaline conditions is usually very rapid. Therefore experiments were performed using potassium dihydrogen phosphate in a limited pH range of 3.0 to pH 5.5. The response was checked at the detector using a connector (without the column). A pH value of 3.0 ± 0.1 gave maximum response for the analyte at 287 nm. A similar response was observed with the usage of 0.1 % orthophosphoric acid. Therefore the final mobile phase consisted of 70: 30 % v/v methanol and 0.1 % orthophosphoric acid. The run time of analysis is higher when a longer reverse phase column (150 X 4.6 mm id) is used. The resolution between the peaks was decreased and peaks were not acceptable peak shape when the experiment is performed using a shorter column (50 X 4.6 mm id). However better resolution, less tailing and high theoretical plates are obtained with a Phenomenex column C18 150 X 4.6 cm 5 µm column.

The flow rate of the method is 0.8 ml/min. The column temperature is maintained at ambient. At the reported flow rate, peak shape was acceptable, however increasing or decreasing the flow rate increased the tailing factor and resulting in poor peak shape and decreased resolution between the drug and internal standard.
There was no interference in the drug and internal standard, from the extracted blank. The peak symmetry were found to be good when the mobile phase composition of 70:30 v/v methanol and 0.1 % ortho phosphoric acid leading to better resolution of the drug and internal standard. Increasing the organic portion of the mobile phase caused DIC to elute early. A mobile phase containing aqueous portion less than 60 % led to very late elution and very poor peak shape for internal standard. The peaks were also broad with unacceptable asymmetry factor.

Extraction methods were initially attempted using Protein precipitation technique. The use of organic solvents as reagents for protein precipitation was described in (25). Precipitation technique was adopted using Acetonitrile and or Methanol. Initial experiments of protein precipitation were done using 1: 3 ratio of plasma : Organic solvents. The recovery of the ETO is poor while that of the internal standard is relatively unchanged as compared with liquid-liquid extraction.

Since the noise effects in solid phase extraction (SPE) method are similar to that of liquid-liquid extraction, we have done the final analysis using liquid-liquid extraction (LLE). SPE methods although render a neat sample for final analysis, polar interferences do enter into the final sample during reconstitution. SPE is further expensive as compared to LLE technique.

Various solvents such as Ethyl acetate, Diethyl Ether, 100 % t-butyl methyl ether and combinations of t-butyl methyl ether and Dichloromethane were used for extraction. The recovery of ETO and internal standard was poor when Ethyl acetate or Diethyl ether was used individually. The highest recovery from the plasma samples is obtained with a 70:30 % v/v of t-butyl methyl ether : Dichloromethane.

Detection and chromatography

Fig. 3 shows the typical chromatograms of a blank human plasma sample (A), a zero blank sample with DIC (B), LLOQ sample extracted using DIC as internal standard and (D) with a sample containing ULOQ sample extracted using DIC as internal standard indicating the specificity of the method. The retention times for ETO and IS were 2.7 and 10.69 min, respectively.

Method validation

Selectivity

The method was found to have high selectivity for the analytes; since no interfering peaks from endogenous compounds were observed at the retention time for ETO in any of the six independent blank plasma extracts evaluated (Fig. 2A).

Figure 2: Chromatograms of
(A) Extracted Blank Sample
(B) Zero Blank Containing Diclofenac as Internal Standard
(C) Etoricoxib containing Diclofenac as Internal Standard at LLOQ level.
(D) Etoricoxib containing Diclofenac as Internal Standard at ULOQ level.
Calibration curves

A system suitability exercise is performed before the initiation of the validation. A system is assumed to be suitable for analysis if and only if the % CV for the retention times of ETO and internal standards is less than 2 %. The results are tabulated in Table 1. Calibration curves for ETO in human plasma were fitted by weighted 1/concentration^2 quadratic regression, with the r^2 values of >0.99 for all curves generated during the validation. The calibration curve accuracy for plasma is presented in Table 2 demonstrating that measured concentration is within ±15% of the actual concentration point (20% for the lowest point on the standard curve, the LLOQ). Results were calculated using peak area ratios. A representative calibration curve showing the regression equation and r^2 value is depicted in Figure – 3.

Table 1  
System Suitability Study

<table>
<thead>
<tr>
<th></th>
<th>Diclofenac Internal Standard (200 µg/mL)</th>
<th>Etoricoxib (2500.00 ng/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Retention Time (min)</td>
<td>Peak Area</td>
</tr>
<tr>
<td>Mean (n = 6)</td>
<td>10.68 (n = 6)</td>
<td>566536.8</td>
</tr>
<tr>
<td>S. D.</td>
<td>0.02</td>
<td>18513.25</td>
</tr>
<tr>
<td>% CV</td>
<td>0.20</td>
<td>3.27</td>
</tr>
</tbody>
</table>

Figure 3: Calibration Curve of Etoricoxib (Curve – 1)
Table 2: Results of regression analysis of the linearity data

<table>
<thead>
<tr>
<th>Linearity parameters</th>
<th>Mean ± SD (n = 3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Slope</td>
<td>0.00038</td>
</tr>
<tr>
<td>Intercept</td>
<td>0.006863</td>
</tr>
<tr>
<td>Correlation coefficient ($r^2$)</td>
<td>0.9989</td>
</tr>
</tbody>
</table>

**Accuracy and precision**

A detailed summary of the intra-day and inter-day precision and accuracy data generated for the assay validation is presented in Table 3. Inter-assay variability was expressed as the accuracy and precision of the mean QC concentrations (LLOQ, LQC, MQC, and HQC) of three separate assays. Intra-assay variability was determined as the accuracy and precision of the six individual QC concentrations within one assay. The inter- and intra-assay accuracy and precision was <5% for all QC concentrations, which was within the general assay acceptability criteria for QC samples according to FDA guidelines [12].

**Limit of detection and limit of quantification**

LOD is defined as the lowest concentration that produced a peak distinguishable from background noise (minimum ratio of 3:1). The approximate LOD was 30ng/mL. The LLOQ has been accepted as the lowest points on the standard curve with a relative standard deviation of less than 20% and signal to noise ratio of 5:1. Results at lowest concentration studies (60ng/mL) met the criteria for the LLOQ (Table 3). The method was found to be sensitive for the determination of RIF in human plasma samples. The ULOQ has been accepted as the highest points on the standard curve with a relative standard deviation of less than 15% [24].

Table 3: Intra and Inter day accuracy and precision of HPLC assay

<table>
<thead>
<tr>
<th>Nominal Concentration in ng/mL (QC ID)</th>
<th>70.00 (LLOQ QC)</th>
<th>210.00 (LQC)</th>
<th>2060.00 (MQC)</th>
<th>3190.00 (HQC)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DAY 1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean</td>
<td>70.81</td>
<td>210.84</td>
<td>2134.45</td>
<td>3314.60</td>
</tr>
<tr>
<td>S.D.</td>
<td>1.18</td>
<td>4.08</td>
<td>53.74</td>
<td>173.48</td>
</tr>
<tr>
<td>% CV</td>
<td>1.67</td>
<td>1.93</td>
<td>2.51</td>
<td>5.23</td>
</tr>
<tr>
<td>DAY 2</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean</td>
<td>71.17</td>
<td>211.98</td>
<td>2146.24</td>
<td>3334.24</td>
</tr>
<tr>
<td>S.D.</td>
<td>1.63</td>
<td>7.60</td>
<td>90.50</td>
<td>231.21</td>
</tr>
<tr>
<td>% CV</td>
<td>2.28</td>
<td>3.62</td>
<td>4.22</td>
<td>6.93</td>
</tr>
<tr>
<td>DAY 3</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean</td>
<td>70.65</td>
<td>210.44</td>
<td>2130.58</td>
<td>3309.61</td>
</tr>
<tr>
<td>S.D.</td>
<td>1.30</td>
<td>6.79</td>
<td>81.37</td>
<td>216.48</td>
</tr>
<tr>
<td>% CV</td>
<td>1.85</td>
<td>3.23</td>
<td>3.81</td>
<td>6.54</td>
</tr>
</tbody>
</table>

*Each mean value is the result of triplicate analysis*
**Carryover test**

A critical issue with the analysis of many drugs is their tendency to get absorbed by reversed phase octa-decyl-based chromatographic packing materials, resulting in the carryover effect. However in this analysis no quantifiable carryover effect was obtained when a series of blank (plasma) solutions were injected immediately following the highest calibration standard.

**Stability studies**

The results of short-term, long term and freeze–thaw stability are presented in Table 4. Determination of ETO stability following three freeze–thaw cycles showed that for all QC samples there was a minor change in the ETO concentration.

**Table 4: Short term, long term and freeze thaw stability of ETO**

<table>
<thead>
<tr>
<th>Nominal Concentration (ng/mL)</th>
<th>210.00 (LQC)</th>
<th>3190.00 (HQC)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Short-term stability</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(4 Days)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean Accuracy (%)</td>
<td>102.54</td>
<td>101.23</td>
</tr>
<tr>
<td>S.D.</td>
<td>2.31</td>
<td>3.68</td>
</tr>
<tr>
<td>% CV</td>
<td>2.25</td>
<td>3.60</td>
</tr>
<tr>
<td><strong>Long-term stability</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(12 Days)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean Accuracy (%)</td>
<td>98.54</td>
<td>103.51</td>
</tr>
<tr>
<td>S.D.</td>
<td>1.28</td>
<td>4.12</td>
</tr>
<tr>
<td>% CV</td>
<td>1.29</td>
<td>3.90</td>
</tr>
<tr>
<td><strong>Freeze – Thaw stability</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(3 Cycles)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean Accuracy (%)</td>
<td>105.63</td>
<td>98.76</td>
</tr>
<tr>
<td>S.D.</td>
<td>5.42</td>
<td>1.72</td>
</tr>
<tr>
<td>% CV</td>
<td>5.12</td>
<td>1.74</td>
</tr>
</tbody>
</table>

*Each mean value is the result of triplicate analysis*

**Recovery**

Percentage recovery of ETO was measured by dividing the peak area values of extracted QC samples with direct injection of solution containing the same nominal concentration of compounds as the QC samples in extracted blank plasma. The mean recovery of ETO from plasma spiked samples of ETO at LQC, MQC and HQC levels was 85.15 %, 89.94 % and 79.54 % respectively. The overall recovery is 84.7 % with a %
Coefficient of variation of 8.68 %, respectively. IS recovery at 100 µg/mL of DIC was 62.69 % with a % Coefficient of variation of 3.65%.

CONCLUSION

A HPLC method was developed and validated for the determination of ETO in human plasma. The extraction process was a single-step liquid–liquid extraction procedure employing the use of 70:30 % v/v of t-butyl methyl ether and dichloromethane. LLE method is usually devoid of polar interferences thus rendering the sample clean for final analysis. The noise is usually absent or at minimum as compared to precipitation or SPE techniques. This assay requires only a small volume of plasma (500 µL). There is no carryover effect. Due to the LLE method of extraction, baseline noise is minimal. Matrix effects are not observed. In conclusion, method validation following FDA guideline indicated that the developed method had high sensitivity with an LLOQ of 60 ng/mL, acceptable recovery, reliability, specificity and excellent efficiency with a total running time of 12 min per sample, which is important for large batches of samples. Thus this method can be suitable for pharmacokinetic, bioavailability or bioequivalence studies of ETO in human subjects. This method has been successfully applied to analyze ETO concentrations in human plasma.

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