Development and Validation of A High-Performance Liquid Chromatographic Method with Ultraviolet Detection for Quantitation of Lornoxicam in Human Plasma: Application to Bioequivalence Study

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ABSTRACT

A simple, rapid and feasible high-performance liquid chromatographic method with ultraviolet detection has been developed and validated according to the FDA guidelines for the quantitation of lornoxicam in human plasma. Sample was prepared by simple liquid-liquid extraction. The chromatographic separation was carried out in a Hypersil BDS, C18 column (250 mm × 4.6 mm; 5 µm particle size). The mobile phase was a mixture of 10 mmol phosphate buffer (pH 7.0) and acetonitrile (55:45, v/v) at a flow rate of 1.0 mL/min. The UV detection was set at 290 nm. The method was specific and sensitive with lower limit of quantification (LLOQ) of 25 ng/mL. The accuracy and precision values obtained from six different sets of three quality control (QC) samples along with LLOQ analyzed in separate occasions ranged from 95.06%-99.82% and 1.014%-3.384%, respectively. The extraction recovery of lornoxicam in plasma samples at three QC samples along with LLOQ was above 94.67%. In stability tests, lornoxicam in human plasma was stable during storage and assay procedure. The developed and validated method was successfully applied to quantitative determination of lornoxicam in plasma for the bioequivalence study in 12 healthy human volunteers following a single dose of lornoxicam 16 mg sustained release tablet.

Key words: Lornoxicam; High performance liquid chromatography; Plasma analysis; Pharmacokinetics; Bioequivalence study.

INTRODUCTION

Lornoxicam (LOR), is a novel non-steroidal anti-inflammatory drug (NSAID) with analgesic as well as antpyretic properties. It works by blocking the action of cyclooxygenase (COX) enzyme. It is used for the treatment of various types of pain, especially resulting from inflammatory diseases of the joints, osteoarthritis, sciatica, and other inflammations.

In the present study, a method has been developed and validated for determination of LOR in human plasma by HPLC with UV detection for a bioequivalence study of 16 mg sustained release (SR) dose of two LOR tablets in 12 healthy volunteers following oral administration.

MATERIALS AND METHODS

Chemicals and reagents

LOR was obtained from DM Pharma (Himachal Pradesh, India) and rofecoxib (internal standard; IS) from Apex Drugs and Intermediates Ltd. (Hyderabad, India). Ethyl acetate of HPLC-grade was obtained from Merck (Mumbai, India). HPLC-grade water was generated from a Milli-Q gradient system.

Instrumentation and chromatographic conditions

HPLC apparatus consisted of a Knauer (Germany) Model, 1000 HPLC pump, 2500 variable wave length UV detector, Rhodyne injector and Clarity lite software. Chromatographic separation was performed isocratically at room temperature using a Hypersil BDS, C18 column (250 mm × 4.6 mm, 5 µm particle size) with guard column. Mobile phase consisted of a mixture of 10 mmol phosphate buffer of pH 6.0 and acetonitrile (55:45, v/v) at a flow rate of 1.0 mL/min. The UV detection was set at 290 nm.

Preparation of standard and quality control samples

Stock solution of LOR and IS was prepared by dissolving accurate amount of standard compound to obtain concentration of 100 µg/mL. Working solutions of LOR and IS were prepared from stock solution by adding appropriate volume of mobile phase. A eight-point calibration curve (CC) was prepared by spiking required volume of working solution of LOR and IS into blank plasma to obtain final concentrations of 25, 50, 100, 200, 300, 500, 1000 and 1500 ng/mL for LOR and 500 ng/mL for IS. All stock solutions and standard working solutions were stored in polypropylene vials at –20 °C freezer.

Three levels of QC samples in plasma were 75 ng/mL (low quality control i.e. LQC), 750 ng/mL (medium quality control i.e. MQC), and 1200 (high quality control i.e. HQC) whereas 25 ng/mL was taken as lower limit of quantitation (LLOQ) for LOR. QC samples were stored in polypropylene vials at –20 °C.

Sample preparation

For calibration standards, an aliquot of 0.45 mL of plasma spiked with 0.05 mL of working standard solution of LOR was taken in a 10 mL stopper test tube. To this 0.05 mL of 10 µg/mL IS was added and mixed properly by vortexing. 5 mL ethyl acetate was added and hand mixed for 15 min followed by centrifugation at 5000 r.p.m for 20 min. 4 mL of organic layer was separated and evaporate to dryness at 40 °C under N2 atmosphere. The residue are reconstituted in 400 µL of mobile phase and 50 µL of it injected into the HPLC system.

Method validation

The method was validated to meet the acceptance criteria of industrial guidance for bioanalytical method validation.

Specificity and selectivity

Specificity of the method was determined by analyzing twelve different blank human plasma samples from human volunteers, to demonstrate lack of chromatographic interference from endogenous plasma components at the retention time of LOR and IS.

Limit of detection (LOD) and lower limit of quantitation (LLOQ)

Limit of detection (LOD) and lower limit of quantitation (LLOQ) were determined from the ratio of peak signal and baseline noise level (S/N) as three and five folds respectively.

Linearity

Linearity of calibration curve for LOR was assessed over the range of 25-1500 ng/mL by subjecting plasma samples to the sample preparation procedure followed by injecting it onto HPLC system. Plasma calibration curve was prepared by taking area ratio of LOR to IS as Y-axis and concentration of LOR (ng/mL) as X-axis. Results were fitted to linear regression analysis. Six replicates of calibration curve were prepared taking each concentration for five times.

Accuracy and precision

Within-run and between-run precision and accuracy was determined by analyz-
ing six replicates at three QC levels (LQC, MQC and HQC) and LLOQ on four different days. The criteria for acceptability of the data included accuracy within ±15% deviation (SD) from the nominal values and a precision of within ±15% relative standard deviation (RSD), except for LLOQ, where it should not exceed ±20% of SD.\(^{[5]}\)

**Extraction recovery**
In order to calculate recovery, representative blank plasma were mixed with standard solutions of LOR at LLOQ, LQC, MQC and HQC concentrations and extracted as described. Then, IS was added. In addition, blank plasma was also extracted. Blank plasma extracts were mixed with standard solutions of LOR at LLOQ along with three QC samples (n = 6). The extraction recovery was calculated by the formula:

\[
\text{Recovery} = \left( \frac{\text{Mean peak area of non-extracted analyte mixed with blank matrix extracted IS}}{\text{W} \times \text{transfered fraction}} \right) \times 100
\]

(W: transferred fraction, 5/4)

**Stability**
Stability of LOR in the plasma after 8 h exposure in bench top was determined at LQC and HQC samples in six replicates. Freezer stability of LOR in plasma was assessed by analyzing above two concentrations stored at -20 °C for at least 30 days. Stability of LOR in plasma following repeated three freeze-thaw cycles (stored at -20 °C between cycles) was assessed using QC samples spiked with LOR. Samples were processed as described as above. Samples were considered stable if assay values were within the acceptable limits of accuracy (i.e. ±15% SD) and precision (i.e. ±15% RSD).\(^{[5]}\)

**Bioequivalence study**
The validated method was successfully used for determination of LOR in plasma samples of a bioequivalence study of Lornoxicam 16 mg SR tablet as test and reference product.

**Study design**
Twelve healthy volunteers aged between 18 and 45 years (25.50 ± 3.85 years) with body mass index between 18 and 24 (20.33 ± 1.57) took part in the study which was only initiated after the protocol and subject information forms had been approved from the Drugs Control General of India (DCGI), New Delhi, India and Institutional Ethical Committee (IEC) of Jadavpur University, Kolkata, India. The study was in compliance with Good Clinical Practice (GCP) and Declaration of Helsinki. It was randomized, single dose, fasting, two-period, two-sequence crossover study with one week wash out period.\(^{[5]}\)

**Drug administration and sample collection**
A total of 15 blood samples were collected at 0 h (before drug administration) and at 0.5, 0.75, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, 6.0, 8.0, 12.0, 24.0 and 36.0 h (after drug administration) in the test tubes with EDTA-K$_2$. Samples were considered stable if assay values were within the acceptable limits of accuracy (i.e. ±15% SD) and precision (i.e. ±15% RSD).\(^{[5]}\)

**Pharmacokinetic analysis**
The following pharmacokinetic parameters were directly determined by standard non-compartmental method. Both maximum plasma concentration (C$_{\text{max}}$) and time to peak plasma concentration (t$_{\text{max}}$) were obtained directly from the analytical data. The elimination half-life (t$_{1/2}$) was calculated as 0.693/K$_{\text{el}}$, where K$_{\text{el}}$ was the apparent elimination rate constant. K$_{\text{el}}$ was, in turn, calculated as the slope of the linear regression line of natural log-transformed plasma concentrations. The last seven quantifiable levels were used to determine K$_{\text{el}}$. The area under the plasma concentration-time curve (AUC$_{\text{0-t}}$) was calculated from the measured levels, from time zero to the time of last quantifiable level, by the linear trapezoidal rule. AUC$_{\text{0-t}}$ was calculated according to following formula:

\[
\text{AUC}_{\text{0-t}} = \text{AUC}_{\text{0-\alpha}} + \frac{C_{\text{t}}}{K_{\text{el}}} 
\]

where C$_{\text{t}}$ is the last quantifiable plasma level.\(^{[7]}\)

**Statistical analysis**
For each subject, descriptive statistics were used to summarize the estimated pharmacokinetic parameters. For the purpose of bioequivalence analysis AUC$_{\text{0-\alpha}}$, AUC$_{\text{0-t}}$, C$_{\text{max}}$ and C$_{\text{t}}$ values were considered primary variables. Their log-transformed data were analyzed by an analysis of variance (ANOVA), including treatment, period and subject. Test product was considered bioequivalent to reference product if 90% confidence interval (CI) for the ratio between each parameter fell within the predetermined equivalence range of 80–120%.\(^{[7]}\)

**RESULTS AND DISCUSSION**

**Specificity and selectivity**
No significant interfering peak from endogenous compounds is observed at the retention times of LOR and IS which were 5.91 min and 10.15 min respectively (Figure 1). The total chromatographic run time was 12 min.

![Figure 1. Chromatogram showing peak of (A) lornoxicam and rofecoxib, (B) lornoxicam and omeprazole and (C) lornoxicam and no valdecoxib during IS selection in the final mobile phase composition at 230 nm.](image)

**Limit of detection (LOD) and lower limit of quantitation (LLOQ)**
LOD and LLOQ were 15 ng/mL and 25 ng/mL, respectively for this method.

**Linearity**
The linearity of the calibration curve was evaluated by calculating the regression coefficient (R$^2$) values. The standard curves of LOR in human plasma were linear with a reliable reproducibility over the ranges of 25 to 1500 ng/mL and the regression coefficients (R$^2$) were over 0.9998 from each standard curve of six separate runs.

**Accuracy and precision**
The accuracy of this bioanalytical method for within-run and between-run was from 95.41% to 99.82% and from 95.06% to 99.20%, respectively. The within-run and between-run precision ranged from 1.210% to 3.384% and from 1.014% to 3.096% respectively. The accuracy and precision data of three QC and LLOQ samples are presented in Table 1. The assay results demonstrated that the values were within acceptable limit as well as a high degree of accuracy and precision.

**Table 1. Precision and accuracy data for lornoxicam in human plasma.**

<table>
<thead>
<tr>
<th>Concentration (ng/mL)</th>
<th>Concentration found (ng/mL)</th>
<th>SD</th>
<th>CV (%)</th>
<th>Accuracy</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Within-run</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>25</td>
<td>23.852</td>
<td>1.292</td>
<td>5.419</td>
<td>95.41</td>
<td>6</td>
</tr>
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<td>75</td>
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<td>4.784</td>
<td>3.195</td>
<td>99.82</td>
<td>6</td>
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<tr>
<td>750</td>
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<td>8.972</td>
<td>1.210</td>
<td>98.84</td>
<td>6</td>
</tr>
<tr>
<td>1200</td>
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<td>21.46</td>
<td>1.814</td>
<td>98.59</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>Between-run</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>25</td>
<td>23.765</td>
<td>1.449</td>
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<tr>
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<td>98.658</td>
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</tr>
<tr>
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<td>7.543</td>
<td>1.014</td>
<td>99.207</td>
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</tr>
<tr>
<td>1200</td>
<td>1183.800</td>
<td>16.882</td>
<td>1.426</td>
<td>98.850</td>
<td>24</td>
</tr>
</tbody>
</table>
SD: Standard deviation;
\[
\text{Coefficient of Variation (CV; Precision) } = \frac{SD}{\text{Mean}} \times 100
\]

Accuracy = \frac{\text{Mean assayed concentration - Nominal concentration}}{\text{Nominal concentration}} \times 100

**Extraction recovery**

Peak area ratios (LOR/IS) were used for the calculations of recovery. Recovery was not concentration dependent and extraction recovery of LOR from LLOQ, LQC, MQC and HQC samples were 94.67 ± 1.27, 96.76 ± 3.01, 98.59 ± 2.25, and 98.73 ± 3.25, respectively. Results were found to be satisfactory as these were consistent, precise and reproducible.

**Stability**

The predicted concentrations for LOR at LQC and HQC samples deviated within ±15% of the nominal concentrations in a battery of stability tests viz. bench top (8 h), repeated three freeze/thaw cycles and at –20°C for at least 30 days (Table 2). The results were found to be within the assay variability limits during the entire process.

| Table 2. Stability study data of lornoxicam in human plasma (n = 6). |
|-------------------------|-------------------------|-------------------------|-------------------------|
| Measured concentration of lornoxicam (ng/mL) | Quality control | Stability study | Mean | SD | %CV | Accuracy (%) |
| LQC | 8 hr. bench top | 147.666 | 2.294 | 1.553 | 99.079 |
| | 30 days at -20°C | 141.313 | 1.473 | 1.042 | 94.804 |
| | 3 freeze/thaw cycle | 145.167 | 2.739 | 1.887 | 97.380 |
| HQC | 8 hr. bench top | 1175.935 | 24.137 | 2.053 | 99.692 |
| | 30 days at -20°C | 1146.332 | 25.556 | 2.229 | 97.182 |
| | 3 freeze/thaw cycle | 1157.495 | 24.431 | 2.111 | 98.129 |

SD: Standard deviation;
\[
\text{Coefficient of Variation (CV; Precision) } = \frac{SD}{\text{Mean}} \times 100
\]

Accuracy = \frac{\text{Mean assayed concentration - Nominal concentration}}{\text{Nominal concentration}} \times 100

**Bioequivalence Study**

Values of main pharmacokinetic parameters and plasma concentration-time profile are shown in Table 3 and Figure 2, respectively. The limits of the 90% CIs for the ratios of Cₘₐₓ, AUC₀₋ₐ and AUC₀₋ₜ for their log-transformed data fell within 0.80 to 1.25 (Table 3). On the basis of comparison of the AUC₀₋ₜ, the relative bioavailability of test preparation was 99.33% to that of reference preparation and both the products were bioequivalent.

| Table 3. Pharmacokinetic parameters (Mean ± SD) after oral administration of 16 mg lornoxicam SR tablet of the test and reference formulations (n = 12). |
|-------------------------|-------------------------|-------------------------|-------------------------|
| Parameter | Test | Reference | 90% CI (Log-transformed data) |
| AUC₀₋ₜ (ng h/mL) | 5836.769 ± 624.230 | 5876.073 ± 741.189 | 0.98829 – 1.00158 |
| AUC₀₋ₜ (ng h/mL) | 6288.802 ± 586.666 | 6330.068 ± 718.970 | 0.98976 – 1.00014 |
| Cₘₐₓ (ng/mL) | 833.223 ± 103.946 | 872.956 ± 144.950 | 0.97758 – 1.00109 |
| Cₘₐₓ (h) | 2.042 ± 0.396 | 2.083 ± 0.417 | |
| Cₘₐₓ (h) | 0.130 ± 0.009 | 0.132 ± 0.010 | |
| T₀ (h) | 5.337 ± 0.365 | 5.289 ± 0.415 | |

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**REFERENCES**