Bioanalytical method for lornoxicam determination in human plasma by using piroxicam as internal standard by LC-MS/MS

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ABSTRACT

High Performance Liquid Chromatographic tandem mass spectrometric method for the estimation of Lornoxicam in human plasma has been developed and validated using Piroxicam as internal standard. Sample preparation process was accomplished by protein precipitation technique. The processed sample was chromatographed and analyzed on Hypurity advance, 50×4.6mm, 5 µm column using mobile phase [0.3% formic acid in water and 0.3% formic acid in Acetonitrile (50:50% v/v)] and diluent as 50% methanol in water. Lornoxicam were chromatographed and analyzed by MS Detector. The analytical method described is valid the determination of Lornoxicam (over a range of 21.51 ng/ml to 1276.61 ng/ml) using Piroxicam as internal standard in human plasma. Signal from the detector were captured in a computer and processed using Mass Hunter software.

Key words: Lornoxicam, Piroxicam, internal standard, LC/MS/MS and validation etc.

INTRODUCTION

Lornoxicam ((3E)-6-chloro-3-[hydroxy(pyridin-2-ylamino) methyl ene]-2-methyl-2,3-dihydro-4H-thieno[2,3-e][1,2]thiazin-4-one 1,1-dioxide) [1] is a non-steroidal anti-inflammatory drug (NSAID). Lornoxicam is a compound in the same chemical class as Piroxicam, Meloxicam and Tenoxicam, with potent anti-inflammatory, antipyretic and analgesic activity. Lornoxicam (chlortenoxicam), is a new nonsteroidal anti-inflammatory drug (NSAID) of oxicam class. It is distinguished from established oxicams by a relatively short elimination half-life [2]. Lornoxicam inhibits the COX-1/COX-2 system, the production of interleukin-6, and the inducible NO synthase [3]. It may be applied by the intramuscular or intravenous route; its bioavailability after oral application is approximately 90%. Although its elimination half-life is only about four hours, the duration of effect is approximately eight hours, analogous to other acidic antipyretic analgesics. The analgesic potency of Lornoxicam is remarkable. In doses of 16mg (i.m.) its analgesic effect is comparable with that of 20mg Morphone (i.m.) or 50mg Tramadol (i.v.) [4].

Lornoxicam readily penetrates into synovial fluid. Lornoxicam synovial fluid: plasma AUC ratio is 0.5 after administration of 4mg twice daily [5]. In elderly patients the clearance of Lornoxicam is reduced by about 30% to 40%; thus the half-life is somewhat longer. Even in the presence of impaired kidney and liver function, no major differences in pharmacokinetics have been observed. On account of its short half-life, no accumulation is likely to occur even in cases of repeated administration – in contrast to NSAID with a longer half-life. Like other Oxicams and Diclofenac, Lornoxicam is metabolised via Cytochrome P450 (CYP-2C9). Due to a genetic polymorphism some individuals may metabolise slowly and therefore have elevated levels of Lornoxicam. Lornoxicam's potency of effect on the two COX isoenzymes in vitro is similar to that of Diclofenac and about two powers of ten stronger than that of Tenoxicam. Lornoxicam is an active substance from the group of acidic anti-pyretic analgesics. The
accretion of acidic analgesics in the inflamed tissue is considered to be a significant aspect of their anti-
inflammatory effect. In cases of painful inflammatory reactions, the capillaries in the inflamed tissue are damaged
and plasma proteins along with bound pharmaceutical substances are discharged into the extra vascular space. On
account of the reduced pH value in inflamed tissue, analgesic acids are able to move from the extracellular space
and enter the cells more easily. This also explains why the duration of action of acidic substances is generally longer
than one would expect in consideration of their plasma half-life [7]. The inflamed tissue probably behaves like a
deep compartment whose filling and depletion adjust to the plasma concentrations with substantial delay. Like all
other NSAID Lornoxicam's mechanism of action is based on the inhibition of Cyclo-oxygenase (COX); an almost
equivalent inhibition of COX-1 and COX-2 is achieved [4]. Only limited methods have been reported in the HPLC
and GC. The objective of the work was to develop and validate LC-MS/MS method for quantification in Human
Plasma. The method shows more sensitive limit of detection and Limit of Quantification is very less to the previous
reported methods.

EXPERIMENTAL SECTION

Chemicals and reagents
Lornoxicam with purity 98.87% w/w (Figure 1) and Piroxicam as internal standard with purity 99.24% w/w (Figure
2) working standards were used. HPLC grade Acetronitrile and Methanol were manufactured by Qualigens Fine
Chemicals. Formic acid AR grade manufactured by S.D. Fine chemicals, Human Plasma (CPD), Plastic container
(Polypropylene), Isopropyl alcohol (HPLC Grade) and Water HPLC grade from Milli-Q RO system was used
throughout the analysis.

Instrumentation and Chromatographic Conditions
The chromatography was performed on an ACQUITYTM UPLC system (Waters Corp, USA) with cooling auto
sampler and column oven enabling temperature control of the analytical column. Hypurity Advance, 50×4.6mm, 5
µm column was employed. The column temperature was maintained at 45 °C and chromatographic separations
were achieved with isocratic elution using a mobile phase composed of [0.3% formic acid in water and 0.3% formic acid
in Acetonitrile (50:50% v/v)]. The flow rate was set at 0.5 ml/min, run time was 3.00 minutes and retention time
for Lornoxicam (Figure 3) and Piroxicam (Figure 4) were 1.5 min and 1.5 min respectively. The auto sampler was
conditioned at 4°C and the injection volume was 10µl using Auto sampler mode for sample injection.

Compound Setting
Ion source ESI Positive mode, Ion spray voltage at 5.0 kV, temperature at 350 °C, curtain gas at 8 L/min, nebulizer
gas at 50 (psi). The Parent mass for Lornoxicam (372) m/z and Piroxicam (332) m/z , Product mass Lornoxicam
(121) m/z and Piroxicam (95) m/z, and collision energies were 26 eV and 24 eV respectively.

Preparation of Standard Stock Solution (w/v) for Lornoxicam and Piroxicam
10.76 mg of Lornoxicam working standard weighed and transferred into 10 ml volumetric flask methanol was
added, added 0.01 % tri-ethyl amine in water to dissolve working standard. The volume made up to mark with
methanol with concentration 1063841.20 ng/ml and for internal standard 10.55 mg of Piroxicam working standard
weighed and transferred into 10 ml volumetric flask, methanol was added to dissolve working standard. The volume
made up to mark with methanol with concentration 1046980.0 ng/ml for Piroxicam calculated on the basis of
molecular weight and purity. Stocks were Stored in refrigerator at 2-8°C.
Plasma sample Extraction Procedure
Thawed samples were vortexed to ensure complete mixing of contents. 0.200 ml aliquot of sample pipette out in eppendorf and 0.050 ml of IS (2000 ng/ml) was added and vortexed the content for 30 seconds. 0.75 ml of Acetonitrile was added. The content was vortexed for 10 min and centrifuged for 10 min at 14000 rpm. Supernatant layers were transferred into individual auto sampler vials for injection.

Validation Parameters
The Method was validated in accordance with FDA Guidelines [6]. Blank screening (Figure 5), Selectivity, Linearity, Accuracy, Precision, Recovery, Stability (Freeze thaw, bench top, long term, Auto sampler, stock solution at RT and RF) Dilution integrity, Matrix effect and Ruggedness were performed. Each batch of spiked plasma samples includes one complete calibration curve (consisting of one blank plasma, one blank plasma with internal standard and eight different non-zero concentration samples, six replicates quality control samples includes six replicate quality control samples LLOQ, LQC, MQC and HQC), except Bench top, freeze thaw stability, long term stability, Auto sampler stability and dilution integrity in which four replicate quality control samples were used. Quality control samples for Lornoxicam prepared by using CPD as an anticoagulant and stored at a temperature below -20°C (deep freezer), protected from light with concentrations taken as LLOQ (21.56ng/ml), LQC (59.07ng/ml), MQC (628.45ng/ml) and HQC (1122.22ng/ml).

Standardization and calculation
The chromatographic data were acquired and processed using computer based Mass Hunter software. The best-fit lines using weighting factor (1/concentration) linear least square regression analysis were obtained by peak area ratio of Lornoxicam to its internal standard (Piroxicam). The concentration of Lornoxicam in plasma samples were calculated using linear regression parameters by corresponding calibration curve.

RESULTS AND DISCUSSION

Chromatography
Representative chromatograms containing solvent reference mix solution, blank plasma, blank plasma with internal standard and quality control samples LLOQ (Figure 7), LQC (Figure 8), MQC (Figure 9) and HQC (Figure 10) and a calibration curve of representative regression analysis for Lornoxicam.
Selectivity
Six lots of plasma with CPD anticoagulant were evaluated and none showed significant interfering peaks at the retention time of Lornoxicam and Piroxicam (IS).

Linearity
The linearity of Lornoxicam was determined by weighted least square regression analysis of standard plot associated with eight point standard curve (Figure 6). The calibration was shown to be linear from 21.51ng/ml to 1276.61ng/ml for Lornoxicam. Best-fit calibration lines of chromatographic response versus concentration were determined by weighted least square regression analysis with weighting factor of 1/concentration. The coefficient of correlation ($r^2$) was consistently greater than or equal to 0.99 during the course of validation, which are within limits.

Sensitivity
The limit of Quantitation was 21.56 ng/ml for Lornoxicam. The between batch precision and accuracy at LLOQ concentration for Lornoxicam using internal standard ratio method was 6.2 % and 106.9 %, respectively.

Accuracy
The accuracy of the assay was defined as the absolute value of calculated mean values of the quality control samples to their respective nominal values, expressed as percentage.

Within batch accuracy
The within batch accuracy using internal standard area ratio method ranged from 105.3 % to 109.1 % for Lornoxicam, which are within limits (Refer Table 1).

Between batch accuracy
The between batch accuracy using internal standard area ratio method ranged from 105.3 % to 107.8 % for Lornoxicam, which are within limits (Refer Table 1).

Precision
The precision of the assay was measured by the percent coefficient of variation over the concentration range of LLOQ, LQC, MQC and HQC quality control samples of Lornoxicam.

Within Batch Precision
The within batch precision using internal standard area ratio method ranged from 3.7 % to 10.3 % for Lornoxicam, which are within limits (Refer Table 1).
Between Batch Precision
Between Batch Precision using internal standard area ratio method ranged from 3.8% to 9.0% for Lornoxicam which are within limits (Refer Table 1).

Table 1: Results of Accuracy, Precision and Recovery.

<table>
<thead>
<tr>
<th>S. No</th>
<th>Parameters</th>
<th>Acceptance Criteria</th>
<th>Results (%)</th>
</tr>
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<tbody>
<tr>
<td>1</td>
<td>Within Batch Accuracy</td>
<td>Nominal concentration :</td>
<td>105.3-109.1</td>
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<td></td>
<td>85-115 %</td>
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</tr>
<tr>
<td>2</td>
<td>Between Batch Accuracy</td>
<td>Nominal concentration :</td>
<td>105.3-107.8</td>
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<tr>
<td></td>
<td></td>
<td>85-115 %</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>Within Batch Precision</td>
<td>% CV : 15%</td>
<td>3.7-10.3</td>
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<tr>
<td>4</td>
<td>Between Batch Precision</td>
<td>% CV : 15%</td>
<td>3.8-9.0</td>
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<tr>
<td>5</td>
<td>Recovery of Analyte</td>
<td>% CV : 20%</td>
<td>9.1</td>
</tr>
<tr>
<td>6</td>
<td>Recovery of internal standard</td>
<td>% CV : 20%</td>
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</table>

Recovery of Analyte
The percentage recovery of Lornoxicam was determined by measuring the peak area response of spiked (extracted) quality control samples (LQC, MQC and HQC) against the peak area response of aqueous (Unextracted) quality control samples (LQC, MQC and HQC) of equivalent concentrations. The percent recovery of Lornoxicam at LQC, MQC and HQC quality control samples were 62.37%, 70.97%, 74.61% respectively. The % CV for recovery of inter-quality control samples for Lornoxicam was 9.1%, which is within limits (Refer Table 1). The % mean of recovery was 69.32%, which are within limits.

Recovery of Internal Standard
The percentage recovery of Piroxicam (IS) was determined by measuring the peak area response of spiked (extracted) quality control samples (MQC) against the peak area response of aqueous (un-extracted) IS dilution of same concentration. The mean percentage recovery and % CV for recovery were 95.19% and 1.0% which are within limits (Refer Table 1).
Freeze thaw stability
The stability of the spiked plasma samples was determined during three freeze-thaw cycles. Four replicates numbers of LQC and MQC samples (Stability samples) kept at -80°C and were analysed after third freeze thaw cycle against freshly spiked calibration curve standards and freshly spiked QC samples(comparison samples). The comparative stability ranged from 99.1% to 102.6 % for Lornoxicam, which are within limits (Refer Table 2).

Bench top stability
The bench top stability (Short term stability in matrix) determined by analyzing four replicates of low and high QC stability samples, which had been kept at room temperature for a designed time against the freshly spiked QC samples (comparison samples). The comparative stability ranged from 96.8% to 102.8% for Lornoxicam, which are within limits (Refer Table 2).

Auto sampler stability
The auto sampler stability (Post-processing stability) determined by analyzing four replicates of low and high QC stability samples, which had been processed and kept in Auto sampler and were analyzed after against freshly spiked calibration curve standards and freshly spiked QC samples (comparison samples). The comparative stability ranged from 95.0% to 102.0% for Lornoxicam, which are within limits (Refer Table 2).

Long term stability
For long term stability (Long term stability in matrix) four replicates of low and high QC stability samples were analyzed against freshly spiked calibration curve. The low and high QC samples were stored for 07 days in deep freezer (at below -20°C) with CPD as an anticoagulant in polypropylene tubes. The stability ranged from 100.6% to 101.6%, which are within limits (Refer Table 2).

Stock solution stability at room temperature
Stock solution stability at room temperature was performed by storing Lornoxicam and Piroxicam (IS) stock solutions at room temperature. The evaluation of stability was done by assaying six replicate injections of appropriately prepared dilutions of stored stock aliquot solutions of Lornoxicam, and Piroxicam (IS) against six replicate injection of appropriately prepared dilution from fresh stock solutions of Lornoxicam, and Piroxicam (IS).

Dilution Integrity
Dilution Integrity was determined by assaying four replicates QCs spiked approximately two times the concentration of ULOQ. Samples were diluted by an appropriate factor (two) prior to extraction, against freshly spiked calibration standard samples. The accuracy for two times diluted concentration was 107.1% and four times diluted concentration were 1.2%, and four times diluted concentration was 4.1% for Lornoxicam which are within limits (Refer Table 2).

Matrix effect
Matrix effect was calculated by spiking Analyte and IS at LQC and HQC levels into each of blank plasma extracts from six different batches of matrix respectively and analyzed in duplicate against six replicate injections of aqueous samples at low and high QCs samples. At low and QCs samples, the % coefficient of variation for matrix factor was found to be 11.77% and 7.20%, respectively for Lornoxicam which are within limits (Refer Table 2).
Table 2: Result of stability Dilution integrity, Matrix effect and Ruggedness

<table>
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<th>S. No</th>
<th>Parameters</th>
<th>Acceptance Criteria</th>
<th>Results (%)</th>
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<td>1</td>
<td>Freeze and thaw cycle at -80°C</td>
<td>Mean % change after 3 cycles ±15%</td>
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<td>2</td>
<td>Bench top stability</td>
<td>Mean % change after 10.14 h ±15%</td>
<td>96.8-102.8</td>
</tr>
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<td>3</td>
<td>Auto sampler stability</td>
<td>Mean % change after 50.04 h : 85.115%</td>
<td>95.0-102.0</td>
</tr>
<tr>
<td>4</td>
<td>Long term Stability</td>
<td>Mean % change after 7 Days : ±15%</td>
<td>100.6-101.6</td>
</tr>
<tr>
<td>5</td>
<td>Stock solution stability at room temp.</td>
<td>% Nominal concentration : ±15%</td>
<td>100.4 (Analyte) 99.8 (IS)</td>
</tr>
<tr>
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<td>Dilution integrity</td>
<td>Accuracy (% Nominal) : ±15%</td>
<td>107.1</td>
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<tr>
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<td></td>
<td>Precision(%CV) : ±15%</td>
<td>4.1</td>
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<td>7</td>
<td>Matrix effect</td>
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<td></td>
<td>HQC 7.20</td>
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<tr>
<td>8</td>
<td>Ruggedness</td>
<td>% Nominal : ±15%</td>
<td>105.0-109.6</td>
</tr>
<tr>
<td></td>
<td></td>
<td>% CV : ≤15%</td>
<td>3.3-7.0</td>
</tr>
</tbody>
</table>

**Ruggedness**

The ruggedness of the extraction procedure and chromatographic method was evaluated by analysis of a batch of six sets of quality control samples and a set of calibration standards using different column (same type) by another analyst. The within batch accuracy ranged from 105.0% to 109.6% for Lornoxicam. The within batch precision ranged from 3.3% to 7.0% for Lornoxicam. The results indicated that the batch met the acceptance criteria of linearity, precision and accuracy data of the quality control samples, which are within limits (Refer Table 2).

**Stock solution stability at refrigerated temperature (7.0 days)**

Stock solution stability at refrigerated temperature was performed by storing Lornoxicam and Piroxicam (IS) stock solutions at refrigerated temperature. The evaluation of stability was done by assaying six replicates injections of appropriately prepared dilution from stored aliquot solutions of Lornoxicam and Piroxicam (IS) against six replicate injection of appropriately prepared dilution from fresh stock solutions of Lornoxicam and Piroxicam (IS). The percent stability of the stock solution for Lornoxicam and Piroxicam (IS) was 106.0% and 93.8% respectively which are within limits.

**CONCLUSION**

The above analytical method described is valid for the determination of Lornoxicam (over a range of 21.51 ng/ml to 1276.61 ng/ml) using Piroxicam as internal standard in human plasma using a Hypurity Advance, 50×4.6mm, 5µm column. This method for quantification of Lornoxicam in human plasma is accurate, precise, rapid, and selective. It is a simple, practical, and economical alternative for studies of the bioavailability, bioequivalence, and pharmacokinetics of this drug in human plasma.

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**REFERENCES**