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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 Morphine (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

accumulation 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.

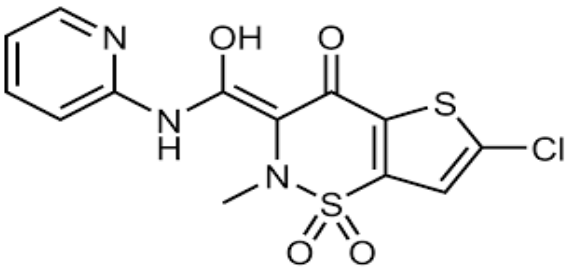

Figure 1: Structure of Lornoxicam

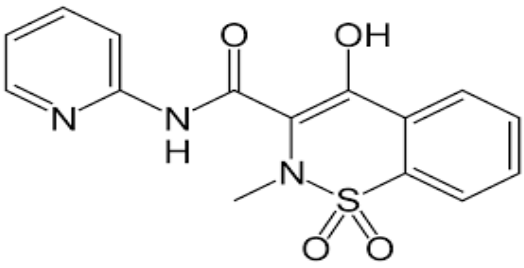

Figure 2: Structure of Piroxicam

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^{0}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 vertexed 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^{o}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.

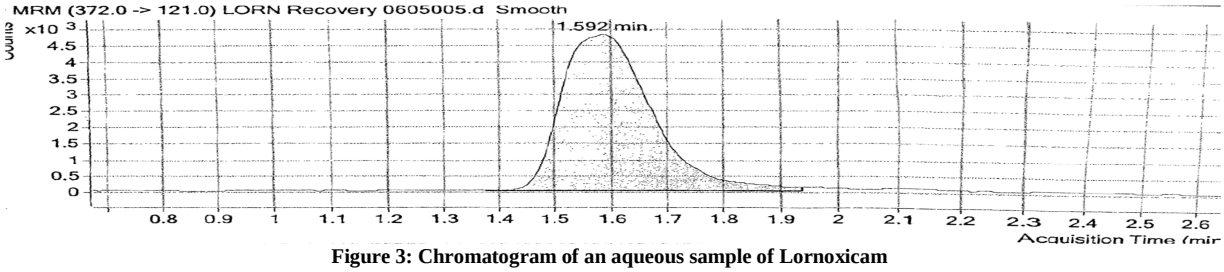

Figure 3: Chromatogram of an aqueous sample of Lornoxicam

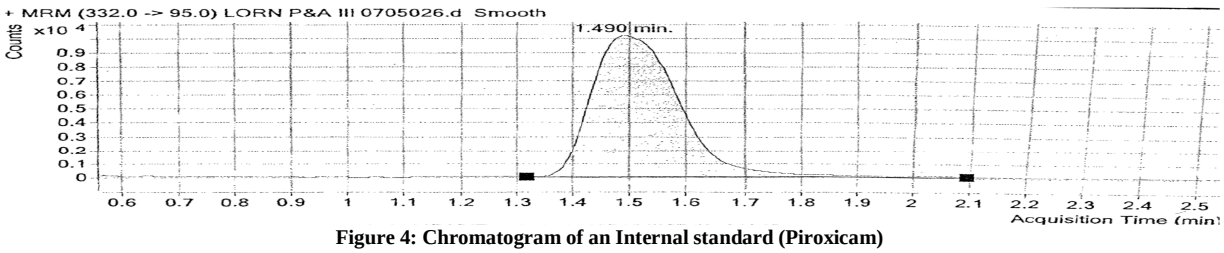

Figure 4: Chromatogram of an Internal standard (Piroxicam)

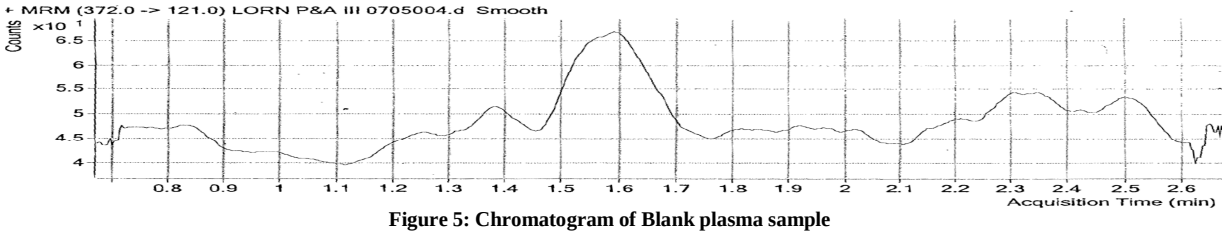

Figure 5: Chromatogram of Blank plasma sample

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.

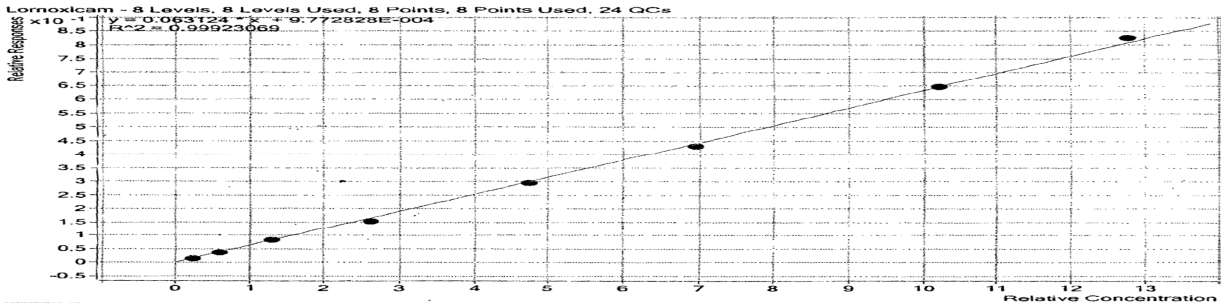

Figure 6: Regression analysis of calibration curve

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.

S. No	Parameters	Acceptance Criteria	Results (%)
1	Within Batch Accuracy	Nominal concentration : 85-115 %	105.3-109.1
2	Between Batch Accuracy	Nominal concentration : 85-115 %	105.3-107.8
3	Within Batch Precision	% CV : 15%	3.7-10.3
4	Between Batch Precision	% CV : 15%	3.8-9.0
5	Recovery of Analyte	% CV : 20%	9.1
6	Recovery of internal standard	% CV : 20%	1.0

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% and1.0% which are within limits (Refer Table 1).

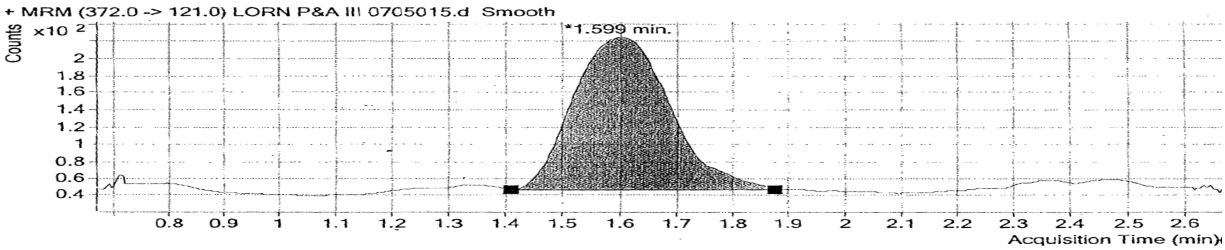

Figure 7: Chromatogram of LOQQ sample

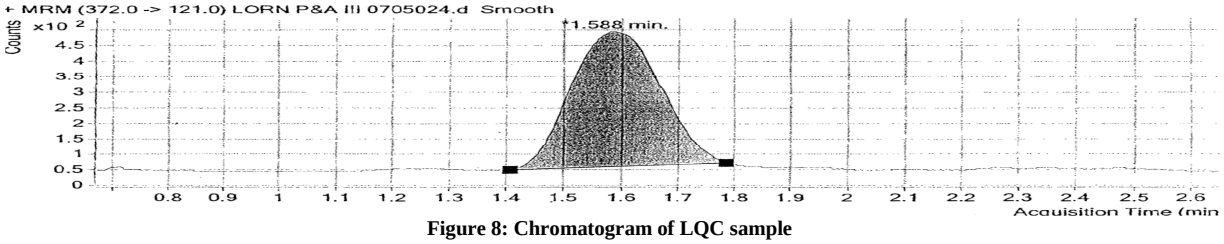

Figure 8: Chromatogram of LQC sample

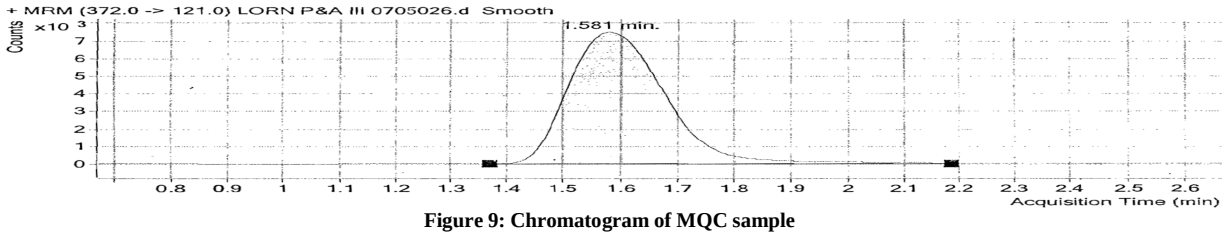

Figure 9: Chromatogram of MQC sample

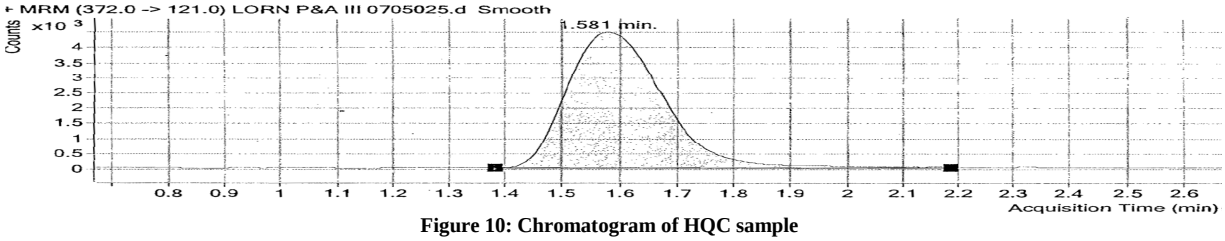

Figure 10: Chromatogram of HQC sample

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^{0}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^{0}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).
The stock solution of Lornoxicam and Piroxicam (IS) was found to be both Analyte and IS, when stored at room temperature. The percent stability of the stock solution stability was 100.4% and 99.8% respectively, which are within limits (Refer Table 2).

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

S. No	Parameters	Acceptance Criteria	Results (%)
1	Freeze and thaw cycle at -80^{0}C	Mean % change after 3 cycles ± 15%	99.1-102.6
2	Bench top stability	Mean % change after 10.14 h ± 15%	96.8-102.8
3	Auto sampler stability	Mean % change after 50.04 h : 85-115 %	95.0-102.0
4	Long term Stability	Mean % change after 7 Days : ± 15%	100.6-101.6
5	Stock solution stability at room temp.	% Nominal concentration : ± 15%	100.4 (Analyte) 99.8 (IS)
6	Dilution integrity	Accuracy (% Nominal) : ± 15% Precision(%CV) : ≤15%	107.1 4.1
7	Matrix effect	% CV ≤15%	LQC 11.77 HQC 7.20
8	Ruggedness	% Nominal : ± 15% %CV : ≤15%	105.0-109.6 3.3-7.0

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 from105.0% to 109.6% for Lornoxicam.The within batch precision ranged from3.3% to7.0% 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 the determination of Lornoxicam (over a range of 21.51ng/ml to 1276.61ng/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.

Acknowledgement

I avail this opportunity, with great pleasure and deep sense of gratitude, to express my thanks to my research fellows for providing enough patience, zeal and strength that enabled me to complete this work successfully.

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