

Development and validation of liquid chromatographic method for quantitative determination of Loxoprofen in mobile phase and in human plasma

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Abstract: A Simple, sensitive and accurate high-performance liquid chromatographic (HPLC) method for effective and specific analysis of Loxoprofen (LXP) in the mobile phase and human plasma was developed. Effective chromatographic separation was attained on a Mediterranean Sea C18 column (250×4.6mm, 5μm) with mobile phase containing acetonitrile and 0.01 M NaH₂PO₄ buffer (55: 45) by adjusting pH 6.5 with sodium dihydrogen phosphate buffer at a flow rate of 1ml/min. Calibration ranges from 0.1ppm to 10 ppm with a coefficient of relation value (R²=0.999) by using a linear regression method and lower limit of quantification was 0.1ppm. The current method showed inter-day and intra-day accuracy and precision within the range of ±10%. % RSD was found to be less than 5 %. Analytical recovery was more than 90% which confirmed the reliability of current method. The proposed method was found appropriate for assessment of LXP in pharmacokinetic and bioequivalence study.

Keywords: Loxoprofen sodium, validation, HPLC, mobile phase, human plasma.

INTRODUCTION

Loxoprofen sodium (±) -2-{4-(2-oxocyclopentamethyl) phenyl} propionic acid is non-steroidal anti-inflammatory (NSAID) drug having marked analgesic, anti-inflammatory and antipyretic activities (Loya *et al.*, 2011, Kashif *et al.*, 2013). LXP contain two chiral centres and composed of four enantiomers which are marketed in racemic mixture form. LXP mechanism of action is inhibition of prostaglandin biosynthesis by its effect on cyclooxygenase enzyme (Kang *et al.*, 2011, Jhee *et al.*, 2007). After oral administration, absorbed as a free acid form in GIT and then converts into active trans-alcohol metabolites (*trans*-OH)(Cho *et al.*, 2006). The active isomer has 2S, 1R, 2'S configuration which is major cause of prostaglandin biosynthesis inhibition (Kang *et al.*, 2011, Cho *et al.*, 2006).

Literature has shown using different quantification methods contains online column switching technique, solid phase extraction, protein salting out by precipitation agent and liquid-liquid extraction (Kanazawa *et al.*, 2002, Choo *et al.*, 2001, Naganuma and Kawahara, 1990, Hirai *et al.*, 1997). Previously quantification of Loxoprofen and its active metabolites in urine and blood sample have been done through HPLC -UV detector or with a fluorescence detector (Choo *et al.*, 2001, Naganuma and Kawahara, 1990, Nanthakumar *et al.*, 2016). In above methods, total

run time was quite long and preparation of sample was significantly complex (Cho *et al.*, 2006). Therefore extensive tedious process involves in fluorescence coupling and such coupling agents are not frequently available in the routine assay (Murakami *et al.*, 2008).

The present study was aimed to develop a simple, precise, sensitive and validated method for determination of LXP using HPLC-UV detection with short retention time for daily routine estimation in human plasma after oral administration of LXP. This method can be applied for therapeutic drug monitoring, pharmacokinetics and pharmacodynamics studies.

MATERIALS AND METHODS

Chemicals

LXP standard was donated by Hilton pharma Ltd (Karachi, Pakistan). Ketoprofen was donated by Shrooq Pharmaceuticals (Lahore, Pakistan). Sodium dihydrogen phosphate, analytical grade sodium hydroxide, acetonitrile, were purchased from Merck (Germany). Double distilled water was used during research study.

Instruments and chromatographic condition

The analysis was performed through High-Performance Liquid Chromatography Pump, LC- 10 VP (Shimadzu, Japan) with UV detector (SPD-10A Shimadzu, Japan). Digital ultrasonic sonicator (Clifton, Nickel-Electro Ltd, England), the Analytical column was Mediterranean Sea

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C18 column (250×4.6mm, 5µm, Teknokroma, Spain). Mobile phase composed of acetonitrile and water mixture (40:60) by adjusting pH 6.5 with sodium dihydrogen phosphate at flow rate 1ml/min. Quantitative determination of LXP in samples were carried out by UV detector at 220 nm wavelength. Filtration of samples was carried out through 0.45-micron Millipore filter. The volume of injection was 10ul for assay and 100ul for plasma.

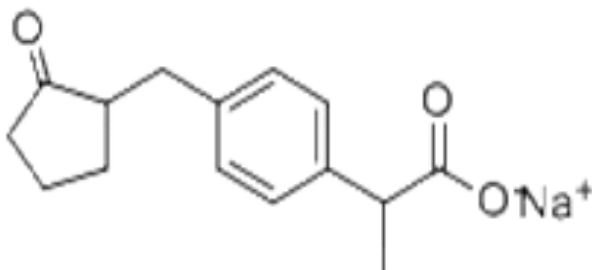


Fig. 1: Chemical structure of Loxoprofen sodium

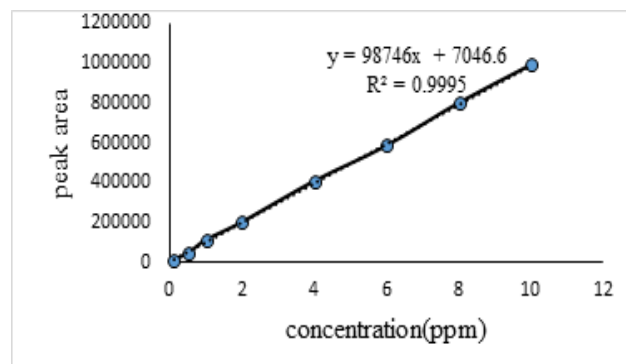


Fig. 2: Linearity curve of different concentrations of LXP in mobile phase

Preparation of standard solution

LXP and Ketoprofen working standard solutions were prepared by dissolving drug 1mg/ml in methanol. Stock solution was diluted serially to prepare concentrations of 10, 8, 6, 4, 2, 0.5 and 0.1ppm.

Preparation of plasma samples

Plasma samples were prepared by subsequent dilutions of stock solution followed by the addition of 20ul internal standard (Ketoprofen) and Acetonitrile in the ratio of 1:2. The mixture was then vortex mixed (whirl mixer, England) for 3 min and 500ul supernatant as collected. 500ul NaH₂PO₄ buffer was added in samples at the final stage. Supernatant solution was collected and filtered through 0.45µm membrane filter. 100ul of supernatant solution was manually injected at wavelength 220 nm with flow rate 1ml/min.

Method validation

For assay validation, recovery, linearity, precision and accuracy, LOD, LLOQ, specificity and selectivity and stability were established under guidelines international

conference on harmonization of pharmaceuticals for human use (ICH) (Hanif *et al.*, 2018).

Preparation of calibration curve

To calculate linear relation between API concentration range and detector, response linearity curve was used (Hanif *et al.*, 2016). Calibration curve of LXP was prepared by taking 10ppm to 0.1ppm in both mobile phase and plasma and coefficient of correlation (R^2) was determined by fitting in a linear equation.

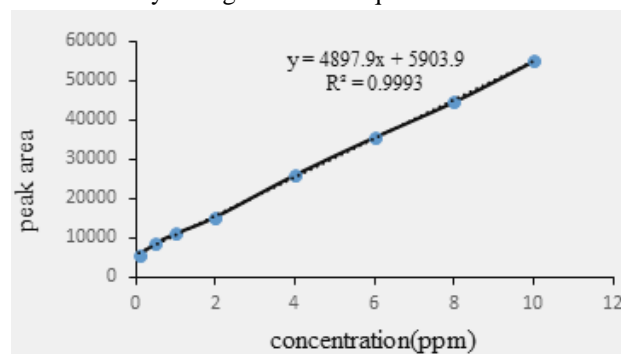


Fig. 3: Linearity curve of different concentrations of LXP in plasma

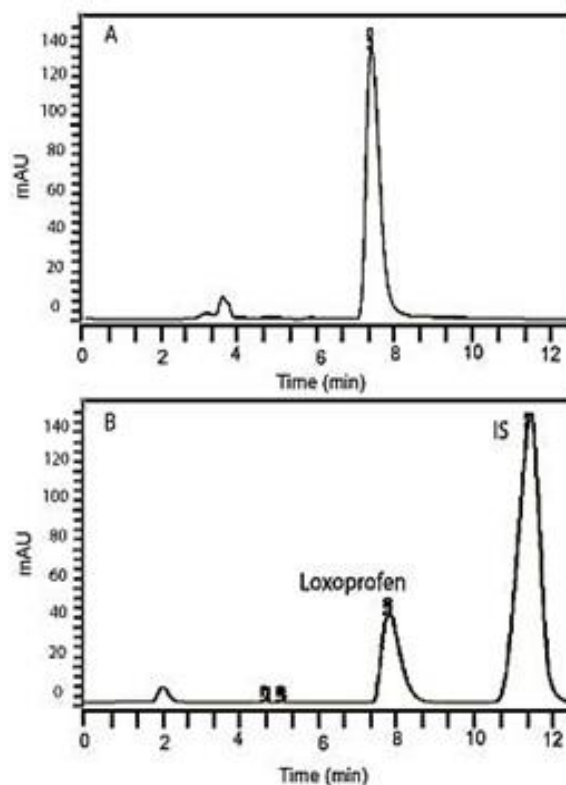


Fig. 4: Representative HPLC chromatograms of (A) LXP in mobile phase (B) LXP and internal standard (Ketoprofen) in mobile phase

Recovery

The extraction recovery was determined by comparing peak area of extracted drug sample with unextracted pure

drug solution (Addo *et al.*, 2015). LXP recovery from human plasma was determined by injecting four concentrations 0.1, 2, 6 & 10 ppm in plasma (n=5) and compared with the standard solution of respective concentration.

Accuracy and precision

Accuracy and precision of method were evaluated for interday and intraday, four concentrations of 0.1, 2, 6 & 10 ppm in a set of five replicate were analyzed on three consecutive days. Intra and interday accuracy should be less than 15%. Similarly, precision was expressed as relative standard deviation (RSD) which should be an acceptable limit of 15% as required by U.S. Food and Drug Administration guidelines. Relative standard deviation (% RSD) was determined by following formula (Hanif *et al.*, 2018),

$$\% \text{ RSD} = \frac{\text{SD}}{\text{Mean}} \times 100$$

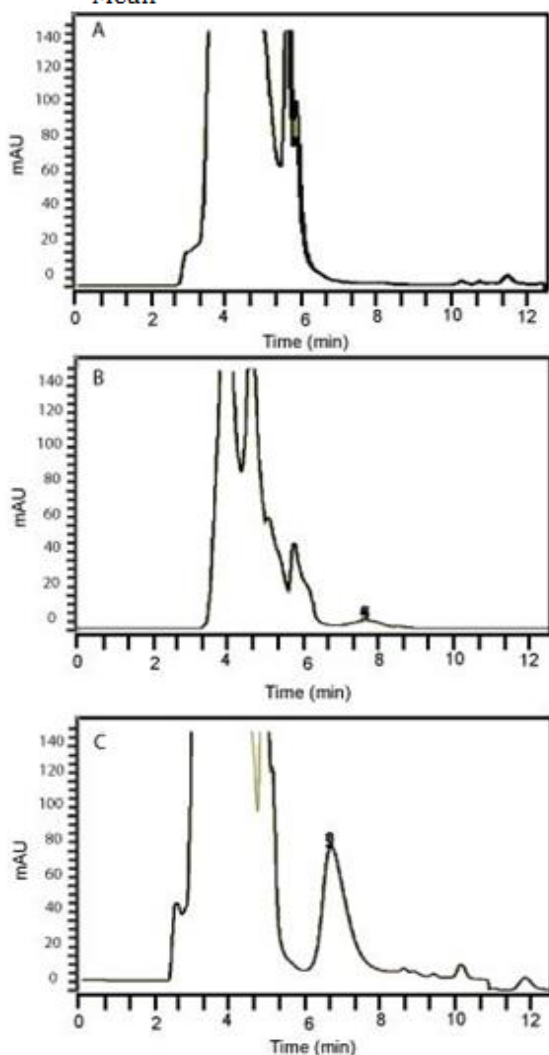


Fig. 5: Representative HPLC chromatograms of (A) blank plasma (B) LLOQ sample in plasma (C) 6 ppm LXP sample in plasma.

Limit of detection (LOD)

Limit of detection was calculated by determination of signal to noise ratio from injections of different concentrations. LOD is basically comparison of the low concentration of an analyte sample with blank sample. The adequate concentration of analyte must be required to exhibit an analytical signal that can distinguish reliably from baseline noise. The ratio can be considered as acceptable for limit of detection (Guideline, 2005).

Lower limit of quantification (LLOQ)

Lower concentration of 10:1 signal to noise ratio was established at which analyte was reliably quantified which yield a precision of <20% coefficient of variation and an accuracy between 80 and 120% of theoretical value (Kang *et al.*, 2011). Multiple concentrations were prepared and injected in HPLC column for determination of lower limit of quantification and lower limit of detection, i.e. 1.0, 0.5, 0.1 and 0.05 ppm. Subsequently, standard deviation, % accuracy and % RSD were calculated.

Specificity and selectivity

The method specificity was used to differentiate between components in sample and analyte. Interference of endogenous substances was estimated by drug-free plasma, plasma spiked with LXP for specificity. Selectivity was determined at Lower limit of quantification (0.1 ppm) (Health and Services, 2001).

Stability

Short and long-term stability of two LXP samples of concentration (0.1 ppm and 10 ppm) were evaluated for fresh and stored frozen samples at -20°C for one month (Health and Services, 2001). Stability of plasma samples was evaluated by freeze-thaw cycle method with five aliquots of each high and low concentration. Compounds were considered stable if assay variation in results were less than 10%.

RESULTS

Calibration curve was established by plotting peak area versus concentration that ranges from 0.1 ppm to 10 ppm which determined $R^2=0.999$ in both mobile phase and plasma (fig. 1&2). Four concentrations of 0.1, 2, 6 and 10 ppm concentrations were applied and their analytical recoveries were 97, 92, 96 and 99% respectively for LXP in mobile phase (table 1). Similar concentration of LXP was applied in human plasma and their recoveries were found to be 93, 90, 94 and 95%, respectively (table 2). fig. 3 & 4 illustrates chromatogram for mobile phase, drug and internal standard in mobile phase, blank plasma and blank plasma spiked with LXP.

The retention time of LXP in mobile phase and plasma were 7.7 ± 0.05 minutes (fig. 4) and 7.4 ± 0.05 minutes (fig. 5), respectively. Replicates of four concentrations 0.1, 2, 6

Table 1: Back-calculation of selected concentration of LXP in mobile phase

Concentration (ppm)	LXP in mobile phase	
	Found (ppm) Mean \pm SD (n=3)	Recovery (%)
0.1	0.097 \pm 0.001	97
2	1.85 \pm 0.06	92.5
6	5.76 \pm 0.26	96
10	9.9 \pm 0.03	99

Table 2: Back-calculation of selected concentration of LXP in plasma

Concentration (ppm)	LXP in plasma	
	Found (ppm) Mean \pm SD (n=3)	Recovery (%)
0.1	0.093 \pm 0.02	93
2	1.88 \pm 0.08	90
6	5.66 \pm 0.41	94
10	9.5 \pm 0.10	95

Table 3: Intra-day and Inter-day accuracy and precision of LXP in plasma

Concentration (ppm)	Intra- day (n=5)			Inter- day (n=5)		
	Conc. Found (Mean \pm SD)	RSD (%)	Accuracy (%)	Conc. found (Mean \pm SD)	RSD (%)	Accuracy (%)
0.1	0.091 \pm 0.002	2.19	91.8	0.098 \pm 0.001	1.02	98
2	1.86 \pm 0.05	2.68	93	1.82 \pm 0.06	3.29	91
6	5.82 \pm 0.08	1.37	97	5.94 \pm 0.04	0.67	99
10	9.38 \pm 0.21	2.23	93.8	9.37 \pm 0.29	3.09	93.7

Table 4: Long-term stability of LXP sodium in plasma

Samples	Low concentration (0.1ppm)		High concentration (10ppm)	
	Fresh plasma	After 4 weeks at -20°C	Fresh plasma	After 4 weeks at -20°C
Mean \pm SD	0.098 \pm 0.001	0.092 \pm 0.004	9.9 \pm 0.064	9.12 \pm 0.40
RSD (%)	1.02	4.34	0.65	4.39
Accuracy (%)	98.6	92	99	91

and 10 ppm of LXP in plasma on the same day and three consecutive days were applied for determinations of accuracy and precision (table 1 & 2). Intra-day accuracy was explained in a range of 91.8, 93, 97 and 93.8% whereas inter-day accuracy of three consecutive days ranged from 98, 91, 99, 93.7%, respectively (table 3). Intra-day values (% RSD) precision results of 2.19, 2.68, 1.37 and 2.23% were determine for LXP in respective concentrations. Inter-day (% RSD) results were 1.02, 3.29, 0.67, 3.09%, respectively (table 3).

Percentage accuracy of 0.1ppm concentration of fresh and after four week samples were 98.6% and 92%, respectively (table 4) (Health and Services, 2001). The method showed excellent stability with % RSD 0.65-4.39%.

DISCUSSION

Different parameters like linearity, selectivity, recovery, accuracy, precision, sensitivity, LOD, LLOQ and stability were calculated effectively and was found in line with FDA guidelines. Analytical recovery of LXP from human plasma at four selected concentrations was found reliable

and reproducible. Makhija and Vavia also reported the similar finding of recovery linearity values of pseudoephedrine and cetirizine as model drug (Makhija and Vavia, 2001). Chromatogram confirmed that there was no interference of plasma components with LXP peaks. Accuracy and precision variation was satisfactory due to less than 5% variation. Similar results were described at different concentration of accuracy level of Loxoprofen sodium by HPLC method (Kashif *et al.*, 2013).

Relative standard deviation (% RSD) were less than 5% which indicated that the proposed method has satisfactory precision and accuracy for LXP. Stability studies elucidated that there was no effect on stability of LXP samples (0.1ppm and 10ppm) stored at -20°C for one month. The lower limit of quantification was 0.1ppm (fig. 5). Similar findings were observed by Jhee H *et al* after explaining Loxoprofen sodium method validation by HPLC method (Jhee *et al.*, 2007). These results recommended that plasma sample of LXP can be handled at normal laboratory condition without significant loss of compound. Hence, it was confirmed that this method has acceptability because of its convenience, simplicity, cost-

effectiveness and faster than already reported complex methods (Kanazawa *et al.*, 2002, Choo *et al.*, 2001).

CONCLUSION

The validation results proposed that the current method is accurate, specific, precise, linear and reproducible for estimation of LXP human plasma. LOD and LLOQ were observed linear with respect to fit (R^2) for LXP determination mobile phase and plasma. The proposed method shows the excellent stability of LXP with an accuracy of less than 10% and reagents are low-cost and readily available. Relatively short run time (10 min) allows rapid quantification of routine samples and quality control analysis.

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