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A simple bioanalytical assay for determination of loxoprofen in human plasma: Application to a pharmacokinetic study

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ABSTRACT

A simple, rapid, sensitive and accurate HPLC-UV detection method for the quantification of loxoprofen sodium (Lox) in human plasma has been developed and validated. Diclofenac potassium (Dic) was employed as internal standard (I.S). The analytes were chromatographically separated on Agilent eclipse ODS C18 column (150 x4.6 mm, 5µm) with mobile phase consisting of phosphate buffer pH 2.5: acetonitrile (55:45, v/v). Detection was performed with UV detection at 320 nm.

Calibration curves for Lox in plasma was linear over concentration ranges of 0.06-10.00 µg /ml with inter-assay coefficients of variations (CV %) less than 10 % in the plasma and with correlation coefficient of 0.999. The average recovery was 90.00 %. The LLOD and LLOQ are 0.04 and 0.06 µg / ml, respectively. This method was utilized successfully for the analysis of plasma samples following oral administration of Lox (60 mg) in 6 healthy male human volunteers under fasting conditions. The results obtained indicated that this method is suitable for pharmacokinetics and bioavailability studies.

KEYWORDS

Loxoprofen sodium;
Diclofenac potassium;
HPLC;
Human plasma and
Pharmacokinetic.

INTRODUCTION

Loxoprofen sodium is sodium (±) alpha-Methyl-4-(2-oxocyclopentyl) methyl benzene acetic acid, Figure 1[1]. It is non-steroidal anti-inflammatory agent that has marked analgesic and antipyretic activities and relatively weak gastrointestinal ulcerogenicity[2]. However, the mechanism of action of Lox is inhibition of prostaglandin biosynthesis by its action on cyclooxygenase. Lox itself is not the major in vivo inhibitor of prostaglandin synthesis. After oral administration, Lox is absorbed as the free acid rather than the sodium salt from the gastrointestinal tract, which causes only weak irritation of the gastric mucosa, and is potentially inhibit prostaglandin biosynthesis[3-5]. Several HPLC methods have been reported for analyzing Lox in human plasma[6-13] and urine[6,7, 14,15]. In the previous methods prior to injection, sample pretreatment was required to remove protein[7,13] and/or coupling with a chiral reagent[6,14]. These pretreatment methods included precipitation by organic solvents, resulting in decreased efficiency.

In the present study, a sensitive and accurate alter-

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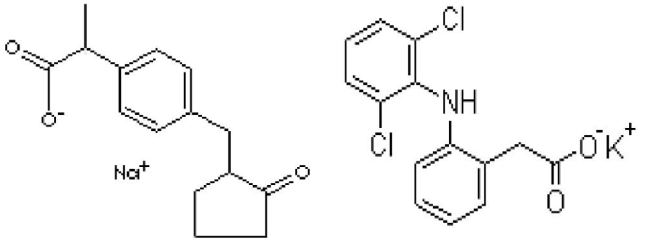
native HPLC method with UV detection for the determination of Lox in human plasma based on the commercially available internal standard Dic. This method was developed and validated as per FDA guidelines[16] and was successfully employed in the analysis of plasma samples following oral administration of Lox (60 mg) in healthy human volunteers.



Loxoprofen sodium Diclofenac potassium

Figure 1 : Chemical structures of loxoprofen sodium and diclofenac potassuim (IS)

EXPERIMENTAL

Chemicals and reagents

Lox (99.9%) was kindly supplied by SAJA, Pharmaceutical Co. (Saudi Arabia). Dic (99.6%) was kindly supplied by ADWIA Pharmaceutical Co. (Egypt). Roxonen tablets (60 mg/tablets as loxoprofen sodium) were kindly supplied by SAJA pharmaceuticals Co. (Saudi Arabia).

Sodium dihydrogen orthophosphate (E- Merck, Darmstadt, Germany) was of pure analytical grade, deionized water was obtained from a Milli-Q water purification system (Millipore, France), human plasma (supplied by VACSERA). Hydrochloric acid, orthophosphoric acid and dicloromethane (Labscan, Ireland) were of analytical grade and acetonitrile (E-Merck, Darmstadt, Germany) was of HPLC grade. The mobile phase was filtered through a Whatman, 0.45-μm pore size membrane filter prior to its use.

Instruments and chromatographic conditions

The HPLC system (Waters, Milford, USA) equipped with pump controlled by Waters 610 controller, Waters 717 autosampler injector, Waters 486 variable wavelength UV detector. For the data acquisition and integration Waters millennium software operated by Pentium III (450 MHz) processor (Compaq, UK) was used. The analytical column employed was Agilent eclipse ODS C_{18} column (150 x4.6 mm, 5μm). The mobile phase was comprised of phosphate buffer (pH adjusted to 2.5 by orthophosphoric acid): acetonitrile (55:45, v/v). The mobile phase was prepared freshly and was filtered before use. All separations were performed isocratically at a flow rate of 1.5 ml/min and column condition was maintained at ambient temperature. Peaks were monitored by UV detector adjusted at 320nm.

Standard solutions and calibration curves

Stock solutions of Lox and Dic (I.S) were prepared separately in acetonitrile at concentration of 1.00 mg/ml and 100.00 μg /ml, respectively. Evaluations of the assay were performed by six point calibration curves at the nominal concentration range 0.06-10.00 μg/ml of Lox which made by dilution of aliquots of the stock solution of Lox with human plasma and each one containing the internal standard at a concentration of 2μg/ml. All stock solutions were stored at 4 ºC and all prepared plasma samples were stored at –80 ºC until analysis.

Sample preparation

To one ml of plasma spiked with the drug and internal standard, 0.5 ml of 2 M hydrochloric acid were added and after vortexed, 7 ml dichloromethane were added. The tube was capped and the contents were vortexed for 1 min and centrifuged for 10 min at 4000 rpm. The upper aqueous layer was discarded and 5 ml of the organic layer was removed to a conical glass tube and evaporated to dryness at 40 ±1ºC. The dry residue was dissolved in 200μl of mobile phase and the supernatants were transferred to glass vials and inserted in the autosampler rack. 150 μl of the supernatants were injected into HPLC system.

Method validation

The method validation assays were carried out according to the United States Food and Drug Administration (FDA) bioanalytical method validation guidance[16].

(a) Selectivity

Selectivity was evaluated by extracting drug-free plasma samples from a pool of plasma. The absence of interfering peaks at the same retention time of Lox or Dic (I.S) was considered as evidence for selectivity.

(b) Accuracy, precision and recovery

The intra-day precision of the assay was assessed by calculating the coefficients of variation (CV %) of samples in six replicates on the same day. The inter-day precision was determined through the analysis of the samples on three consecutive days. Accuracy was determined by comparing the calculated concentrations to known concentrations with calibration curves.

The recovery of an analyte in an assay is the detector response obtained from an amount of the analyte added to and extracted from the biological matrix, compared to the detector response obtained for the true concentration of the pure authentic standard

(c) Calibration/standard curve

(A) Linearity

Calibration curves were prepared by determining the best-fit of peak area ratios (peak area of analyte/ peak area of internal standard) versus concentration, and fitted to the linearity equation Y = bC + a and the regression coefficient was calculated.

The linearity of the calibration curve (18 replicates for each concentration) for Lox was assessed in the range 0.06- 10.00 µg/ml in plasma samples.

(B) Lower limit of detection (LLOD) and lower limit of quantification (LLOQ)

LLOD is a parameter that provides the lowest concentration of analyte in a sample that can be detected, but not quantified, under the stated experimental conditions. LLOQ is defined as the lowest concentration of analyte that can be determined with acceptable precision and accuracy under the stated experimental conditions.

LLOD and LLOQ were determined as the concentrations with a signal-to-noise ratio of 3:1 and 10:1, respectively, by comparing test results from samples with known concentrations of analyte to blank samples. Each concentration standard should meet the following acceptable criteria: a precision of 20% and accuracy of 80-120%. The LLOD and LLOQ were 0.04 µg /ml and 0.06 µg/ml, respectively for Lox in 18 standard calibration curves.

(d) Stability

The stability of Lox in plasma was studied under a variety of storage and handling conditions using a set of plasma samples containing 0.20, 1.00 and 10.00 µg/ml Lox. Short-term stability indicated reliable stability behavior under the experimental conditions of the regular runs. It was assessed by analyzing six aliquots each of low, medium and high concentration samples that were thawed at room temperature and kept at this temperature for 6 h. Post-preparation stability was measured by re-analyzing the extracted low, medium and high concentration plasma samples kept under the auto-sampler conditions for 24 h. Freeze-thaw stability (at –80°C in plasma) was checked through three freeze-and-thaw cycles. Six aliquots at each of the low, medium and high concentrations were stored at –80°C for 24 h and thawed unassisted at room temperature. When completely thawed, the samples were refrozen for 24 h under the same conditions. The freeze-thaw cycles were repeated three times, and then the samples analyzed on the third cycle. The long-term stability was determined by analyzing six aliquots of each of the low, medium and high concentrations stored at –80°C for 6 weeks.

The precision and accuracy for the stability samples must be within ≤15 and ± 15%, respectively, of their nominal concentrations.

Clinical protocol

This method was applied in the analysis of plasma samples after the administration of a single dose of one tablet of Lox 60 mg (Roxonen tablets) to healthy male volunteers. The study protocol was approved by the Ethical committee of bioavailability Studies (NODCAR). The age of 6 volunteers ranged from 29 to 38 years, and subjects had a body weight ranging from 58 and 71kg. All subjects gave their written informed consent. The study was conducted in accordance with the provisions of the Declaration of Helsinki. After an overnight fast period volunteers received a single dose of one tablet of Lox 60 mg (Roxonen tablets) with 200 ml of water. Blood samples (3 ml) from a suitable antecubital vein were collected into heparin-containing tubes immediately before dose (0.0) and at 0.166, 0.33, 0.5, 0.75, 1.0, 1.5, 2.0, 2.5, 3.0, 4.0, 6.0, 8.0, and 12.0 h after the administration of the drug. The blood samples were centrifuged at 3000 rpm for 5 min at room temperature and the plasma was removed and stored at –80ºC until assayed for its Lox content. All samples from a single volunteer were analyzed in

the same run in order to avoid inter-assay variations.

Pharmacokinetic analysis

Pharmacokinetics parameters from the human plasma samples were calculated by a noncompartmental statistics model using MINITAB® software Release 13.1.

Blood samples were taken for a period of 3 to 5 times the terminal elimination half-life ($t_{1/2}$) and it was considered as the area under the concentration time curve (AUC) ratio higher than 80% as per FDA guidelines[17,18].

The first-order terminal elimination rate constant (K_{el}) was estimated by linear regression from the points describing the elimination phase on a log-linear plot. The maximum observed plasma concentration (C_{max}) and the time taken to achieve this maximum level (T_{max}) were obtained directly from the curves. The areas under the curve for Lox plasma concentration versus time for 0-12h (AUC_{0-12}h) were calculated by applying the linear trapezoidal method. The extrapolation of this area to infinity (AUC_{0-inf}) was done by adding the value C_{12}/K_{el} to the calculated AUC_{0-12} where C_{12} is the Lox plasma concentration at 12h and K_{el} is the first-order terminal elimination rate constant.

RESULTS AND DISCUSSION

There are several published methods for determination of Lox in biological fluid[6-15]. Some of these methods require high retention times[7-9], or using special chiral column for stereospecific chromatographic analysis[8]. Other characterized with more complicated extraction procedures such as solid-phase extraction were also reported[8,15]. Nevertheless, solid-phase extraction procedures are requiring solid-phase extraction cartridges, increasing the cost of the analysis. Recent study reported the determination of Lox using LC-MS-MS detection[10], but mass spectrometers are expensive and not readily available. The present study describes a simple, sensitive, accurate and reproducible HPLC method for the determination of Lox in human plasma. This method has several advantages over the previously reported methods. Sample preparation is simpler while others, the sample pretreatment was required to remove protein[7,9,13], use of special pretreatment column[9], coupling with a chiral reagent[6,14] or require mixture of reagents for sample preparation[11-12]. These pretreatment methods included precipitation by organic solvents, resulting in decreased efficiency. Also the very low quantification limit obtained with a UV detector in the present work makes this method particularly useful for pharmacokinetic studies and allowed us to avoid using fluorimetric and LC/MS detection, which require more expensive equipments.

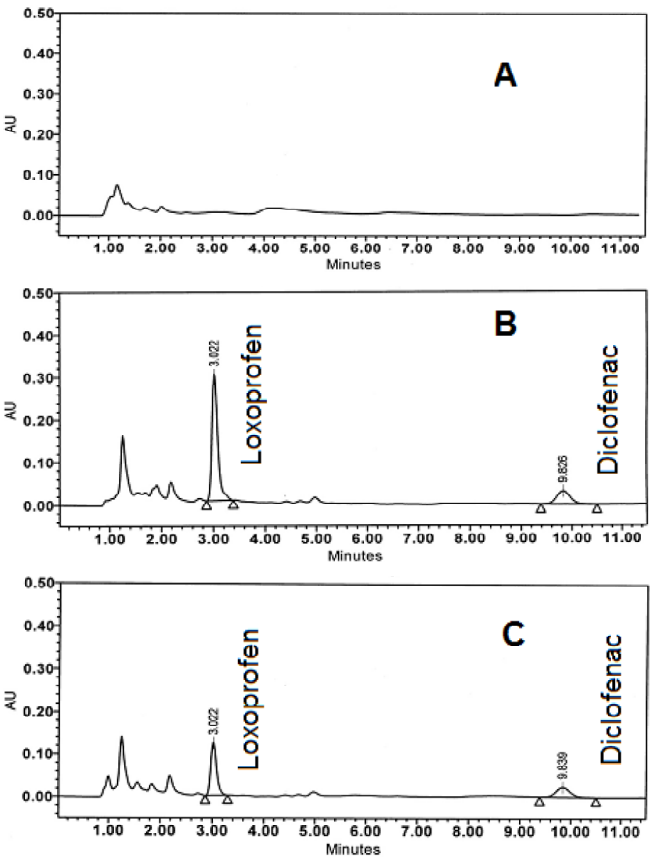


Figure 2 : Chromatograms of (A) blank human plasma, (B) blank human plasma spiked with loxoprofen sodium (10.00 µg/ml) and diclofenac potassium (2.00 µg/ml) and (C) plasma sample obtained from volunteer 45 minutes after oral administration of roxonen tablet (60 mg/tablet as loxoprofen sodium).

Selectivity

Different ratios of the mobile phase were tried and this ratio of phosphate buffer pH 2.5: acetonitrile (55:45, v/v) was the suitable one. Also different types of C18 columns were tried and the best one was Agilent eclipse. Under the chromatographic conditions described previously, Lox and Dic (I.S) peaks well resolved. Figure 2 shows typical chromatograms of blank plasma in comparison to spiked plasma samples

with Lox and Dic and plasma sample obtained at 45 minutes from volunteer who received single oral dose. The retention times of Lox and the Dic was 3.022 min and 9.839 min, respectively. No endogenous compounds appear to interfere with their peaks in blank plasma. The baseline was relatively free from drift.

Accuracy, precision and recovery

The intra- and inter-day accuracy and precision (CV %) results of Lox in human plasma were summarized in TABLE 1 and 2, respectively.

TABLE 1 : Precision and accuracy of standard calibration curves of loxoprofen (Intra- day) in human plasma. (n=6)

Theoretical Concentration (µg/ml)	Mean Found Concentration (µg/ml)	SD	CV %	Accuracy %
0.060	0.065	0.003	4.615	108.333
0.080	0.080	0.003	3.750	100.000
0.100	0.107	0.004	3.738	107.000
0.200	0.198	0.014	7.071	99.000
0.500	0.499	0.049	9.820	99.800
1.000	0.989	0.086	8.711	98.900
5.000	5.100	0.282	5.536	102.000
10.000	9.800	0.513	5.235	98.000

TABLE 2 : Precision and accuracy of standard calibration curves of loxoprofen (Inter- day) in human plasma. (n=18)

Theoretical Concentration (µg/ml)	Mean Concentration Found (µg/ml)	SD	CV %	Accuracy %
0.060	0.066	0.002	3.030	110.000
0.080	0.083	0.005	6.024	103.750
0.100	0.102	0.007	6.863	102.000
0.200	0.191	0.014	7.330	95.500
0.500	0.461	0.043	9.328	92.200
1.000	0.937	0.084	9.000	93.700
5.000	5.168	0.353	6.827	103.360
10.000	9.939	0.607	6.107	99.390

Intra-day precision and the accuracy were determined with six determinations per concentration in the same day with the range of 3.738%-9.820% and 98.000 – 108.333, respectively. Inter-day precision and the accuracy calculated from the results of the assay of six calibration curves in three different days with the range of 3.030%-9.328% and 92.200 – 110.000.

The extraction recovery determined for Lox was shown to be consistent, precise and reproducible. The mean recovery was 90.00% which is an acceptable for the routine measurement of Lox.

Calibration curves, linearity, LLOD and LLOQ

Six concentrations defined the calibration curves. A calibration curve was obtained by plotting the peak-area ratio against the concentration of Lox in plasma. The linearity of the calibration curves was verified from 0.06 -10.00 µg/ml and the corresponding regression equation was (y= 0.399x - 0.021, r =0.999), where y is the peak area ratio of Lox to Dic, x is the concentration of Lox (µg/ml) in plasma and r is the correlation coefficient. The LLOD and LLOQ were 0.04µg/ml and 0.06, respectively.

Stability

The stability tests of three QC samples were designed to cover anticipated conditions that clinical samples may experience. Stability data were summarized in TABLE 3. Briefly, three freeze-thaw cycles and ambient temperature storage of the freezed quality control samples up to 6 h prior to sample preparation appeared to have no effect on the quantification of analyte. Quality control samples stored in a freezer at –80°C remained stable for at least 6 weeks. For auto sampler stability, there was no observed effect on quantification for the extracted samples kept in auto sampler for 24 h at 4°C.

Application to pharmacokinetic study

In this study plasma concentrations were determined in six healthy volunteers, who received a single oral dosing of 60-mg tablet formulation. The derived pharmacokinetic parameters of 6 healthy volunteers are summarized in TABLE 4. The mean plasma concentration–

Time curve of Lox was shown in Figure 3. The pharmacokinetic parameters such as T_{max}, C_{max} and AUC are in good agreement with those found previously and no significant difference was observed between our pharmacokinetic data and results reported in the literature[7,9,13].

The present method proved to be reproducible and reliable based on the results of validation assessment. Also, sensitivity and selectivity of the method allowed us to apply it successfully for the routine analysis of biological samples in pharmacokinetic research on Lox.

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TABLE 3 : Data showing stability of loxoprofen in human plasma at different QC levels (n = 6).

	Accuracy (mean ± CV%)		
	0.20µg/ml	**1.00µg/ml**	**10.00µg/ml**
Short-term stability (6h, room temperature)	99.38±9.39	98.57±10.41	102.24±11.78
Freeze and thaw stability (3 cycles, –80 °C–room temperature)	96.41±5.58	95.25±5.33	99.30±8.53
Long-term stability (42 days, –80 °C)	99.73±6.43	88.73±5.98	98.80±7.95
Auto sampler stability (24 h, 4 °C)	97.23±3.45	96.11±4.68	99.75±5.13

TABLE 4 : Pharmacokinetic parameters required for assessment of loxoprofen bioavailability.

Parameters	Mean
C_{max} (µg/ml)	6.537 ± 3.939
t_{max} (h)	0.50
$AUC_{0\text{-}12}$ (µg.h/ml)	9.757 ± 4.649
$AUC_{0\text{-}inf}$ (µg.h/ml)	9.894 ± 4.509
$t_{1/2}$ (h)	2.334 ± 1.320
MRT (h)	2.291 ± 0.654
K_{el} (h)	0.191 ± 0.015

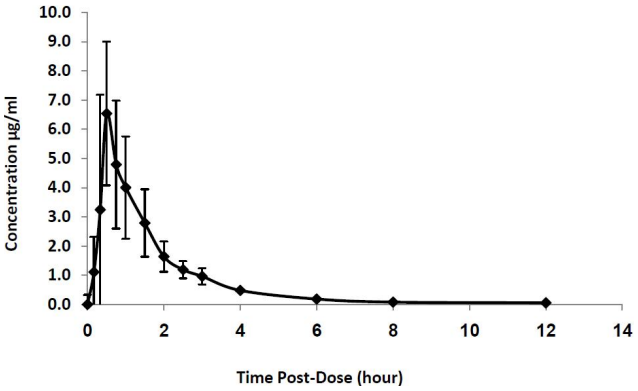

Figure 3 : Mean drug plasma concentration–time curve of loxoprofen from 6 volunteers after oral administration of roxonin tablets.

CONCLUSION

A simple, accurate and reliable HPLC method for the quantitation of Lox in human plasma has been developed and validated. The described method uses a fast and easy sample preparation, a commercially available internal standard and a simple HPLC system coupled with UV detection and no need for chiral reagent. The sensitivity of the assay is adequate for application in the study of clinical pharmacokinetic or bioequivalence test of Lox formulation in human.

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