

Simultaneous Determination of Piroxicam and 5-hydroxypiroxicam: HPLC/UV Method Development, Validation and Application for Pharmacokinetic Evaluation in Pakistani Population

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Summary: Aim of the present study was to develop and validate a rapid, selective, sensitive and economical reverse phase high performance liquid chromatography (RP-HPLC) method for quantification of Piroxicam, 5-hydroxypiroxicam (metabolite) and internal standard (meloxicam) in human plasma and evaluation of pharmacokinetics of piroxicam in Pakistani population, using the developed RP-HPLC/UV method. Piroxicam, 5- hydroxypiroxicam and internal standard (I.S) were separated using a CNW C18 RP (250 mm × 4.6 mm, 5 µm) column as a stationary phase and mixture of acetonitrile and aqueous solution of trifluoro acetic acid (0.05%) in the ratio of 62:38 was used as a mobile phase. The flow of mobile phase was adjusted at the rate of 1 mL/min under the ambient temperature and eluents were studied at 353 nm. The analysis time was 8 min. The spiked and real plasma samples were processed using acetonitrile and diethyl ether as protein precipitating agent and extraction solvent, respectively.

Blood samples were collected from all volunteers at different time intervals *i.e.*, 0.5, 1, 1.5, 2, 2.5, 3, 4, 6, 8, 24, 48, 72 and 96 hr after taking piroxicam 20 mg oral dose. Various pharmacokinetic parameters of piroxicam were determined like AUC₀₋₉₆ hr (104074.2029 ± 21782.84582 ng-h/mL), clearance (2.362155 ± 0.532145 mL/min), volume of distribution (8.858337 ± 1.549941 L) and elimination half-life (55.89 ± 10.39 hr), after analyzing the human plasma samples..

Key words: Piroxicam, 5- hydroxypiroxicam, RP- HPLC/UV, Validation, Human plasma.

Introduction

Piroxicam (PX) (4-hydroxy-2-methyl-3-(pyrid-2-yl-carbamoyl)-2H-1, 2-benzothiazine 1,1-dioxide) is a non steroidal anti inflammatory drug [1], belonging to oxycam class of drug and is prototype of this group [2]. Commonly prescribed for treatment of osteoarthritis, rheumatoid arthritis and other acute musculoskeletal disorders [3-4]. Maximum plasma concentration of piroxicam (1.50 – 2.50 µg/mL) is achieved within 2 – 4 hr, after administration of single oral dose (20 mg) [1]. Piroxicam is 99 % bound to plasma protein, having long elimination half life (50 hr) and is extensively metabolized by CYP2C9 (isoenzymes of cytochrom P450) into 5-hydroxypiroxicam (4-hydroxy-N-(5-hydroxy-2-pyridyl)-2-methyl-2H-1,2-benzothiazine-3-carboxamide 1,1-dioxide) [5]. Chemical structures of Piroxicam, 5-hydroxypiroxicam and meloxicam (I.S) are shown in Fig. 1.

Various RP-HPLC/UV methods have been reported for analysis of Piroxicam alone in biological samples using various detectors [6, 7]. The methods reported for quantification of Piroxicam and 5-hydroxypiroxicam simultaneously in biological fluids are associated with various drawbacks like low sensitivity, inappropriate validation, tedious drug extraction procedures from plasma and complex mobile phase composition (use of buffers) [8-10]. In

some methods sample were directly injected to HPLC system without following extraction procedures, which gave poor resolution of peaks [11]. The developed method is simple, validated, sensitive, accurate, reliable, and selective for the determination of piroxicam, 5- hydroxypiroxicam and meloxicam (I.S). The developed method was applied to determine pharmacokinetics of piroxicam for the first time in Pakistani population and also used for *in-vivo* quantification of pharmacokinetic herb- drug interaction (PK- HDIs) studies of Piroxicam.

Experimental

Chemicals and reagents

Piroxicam and meloxicam was kind gift from medicraft pharmaceuticals, Peshawar, Pakistan, 5-hydroxypiroxicam was purchased from Witega Laboratories GmbH, Germany (Batch no: 241525). The HPLC grade chemicals (acetonitrile, methanol, trifluoro acetic acid, diethyl ether) were purchased from Sigma Aldrich (Oslo, Norway). Purified distilled water was prepared by Millipore distillation apparatus (Milford, USA). Stock solutions (100 µg/mL) of the studied analytes (Piroxicam, 5-hydroxypiroxicam and IS) were prepared in methanol and stored at -20 ± 2 °C.

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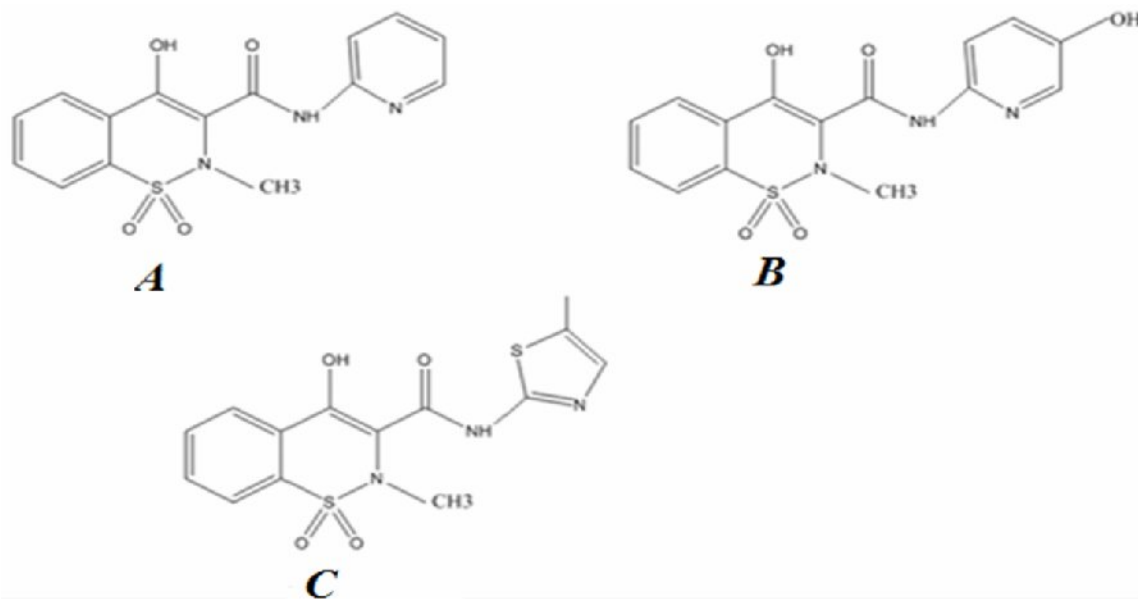


Fig-1: Structures; A) Piroxicam, B) 5-hydroxyproxicam, C) meloxicam (IS).

Instrumentation

RP- HPLC/UV (Perkin Elmer series 200 system, Norwalk, USA) was used for chromatographic analysis equipped with an auto sampler (sample injecting system), pump, peltier column oven, online vacuum degasser, and ultra violet visible detector (series-200). The data acquisition was performed on Perkin Elmer Total chrome workstation software (version 6.3.1) linked with the HPLC -system through net work chromatography interface (NCI: 900). The analytical column CNW C18 RP (250 mm \times 4.6 mm, 5 μ m) was used for quantification of analytes, which was protected by a Perkin Elmer pre-column guard cartridge C18 (30 mm \times 4.6 mm, 10 μ m). A Shimadzu (AX 200) electronic balance and temperature controlled centrifuge machine (model: k-2080, Centurion, UK) were used during the study.

Chromatographic conditions

Piroxicam, 5- hydroxyproxicam and meloxicam (IS) were analyzed using CNW C18 RP (250 mm \times 4.6 mm, 5 μ m) column protected by a perkin elmer pre- column guard cartridge C18 (30 mm \times 4.6 mm, 10 μ m) at ambient temperature. The mobile phase was composed of acetonitrile and 0.05% v/v, aqueous solution of trifluoro acetic acid (0.05%) in the ratio of 62:38 (v/v). The samples were injected at flow rate of 1 mL/min.

Sample Preparation

Stock Solutions Preparation

Stock solutions (100 μ g/mL) of the studied analytes (Piroxicam, 5-hydroxyproxicam and I.S) were prepared in methanol and stored at -20 ± 2 $^{\circ}$ C. Further dilutions from stock solutions were prepared with mobile phase, while I.S concentration (500 ng/mL) was kept constant in all dilutions.

Liquid- liquid extraction

Spiked Plasma Samples

Blank plasma samples were thawed at room temperature and then spiked with different concentrations of Piroxicam and 5-hydroxyproxicam, while the internal standard concentration was kept constant (500 ng/mL) in all spiked plasma samples and vortexed for 2 min. Then acetonitrile (300 μ L) was added as proteins precipitating agent. After protein precipitation, the supernatant was collected in an eppendorf tube; volume was made up with diethyl ether (extraction solvent) up to 1.5 mL, vortexed and centrifuged at 2500 x g at 4 $^{\circ}$ C for 10 min. The clear supernatant was collected in a glass tube. The solvent was evaporated under flush of nitrogen till dryness at 40 $^{\circ}$ C and residue were reconstituted with mobile phase up to 1 mL, then again vortexed and centrifuged. The clear sample was transferred to autosampler vial and 20 μ L was injected into the HPLC- system.

Real plasma samples

The method was applied for *in vivo* pharmacokinetics evaluation of Piroxicam (20 mg) in healthy Pakistani human volunteer.

In-vivo evaluation of the analytes was carried out in 30 male human volunteers, aged in the range of 19 – 24 years. The study was approved (application number; 01/EC-14/Pharm) by the Committee for Ethics in Research (Department of Pharmacy, University of Peshawar). All the volunteers signed the written consent form and were instructed to avoid use of medicines for at least one week, prior to the study. Each volunteer received the Piroxicam capsule (20 mg) orally, with a glass of water (200 mL).

The blood samples (≈ 3 mL) were collected at specified time intervals (0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 4.0, 6.0, 8.0, 12, 24, 48, 72 and 96 hr) following oral administration of piroxicam. Samples were collected in ethylene diamine tetraacetic acid (EDTA) tubes and then centrifuged at $2500 \times g$ 4°C for 10 min. The plasma samples (real plasma samples) were stored at -20°C till further use. The plasma samples were deproteinated and extracted, as discussed in Section 2.3.2.1.

Blood concentration of piroxicam was quantified in plasma samples, collected from volunteers at different time intervals. Various pharmacokinetics parameters C_{max} , t_{max} , area under curve (AUC), clearance (Cl), volume of distribution (Vd) and half life ($t_{1/2}$) were determined applying MS office excel and PK Summit[®] software.

Optimization of chromatographic conditions

Various chromatographic parameters like stationary phase, composition of mobile phase, column oven temperature, wave length, flow rate, and injection volumes were optimized.

Selection of stationary phase

Different types of analytical columns like Athena C-18 WP (250 mm \times 4.6 mm, 5 μm), Supelco Discovery HS C18 RP column (150 mm \times 4.6 mm, 5 μm) and CNW C18 RP (250 mm \times 4.6 mm, 5 μm) were studied in terms of analytes retention time, selectivity, peaks resolution, column efficiency and peaks tailing factor. The column gave best result was selected.

Selection of mobile phase

Organic solvents (acetonitrile and methanol) in combination with aqueous TFA solution (0.025 % and 0.05 %) or purified water (pH adjusted to 3 with phosphoric acid) were used as mobile phases. The mobile phase that gave better peaks resolution and shorter analysis time was selected.

Flow rate

Mobile phase was pumped at different flow rates in the range of 0.8 – 1.5 mL/min. The one gave better results was selected.

Detector wavelength

Various wavelengths ranges from 330 to 370 nm were assessed for simultaneous quantification of piroxicam, 5- hydroxypiroxicam and I.S, wavelength that gave better peak resolution and sensitivity was selected.

Column Oven Temperature

Various column oven temperatures in the range of $25 - 35^{\circ}\text{C}$ were studied. The one gave better results in term of peak resolution, sensitivity and retention time was selected.

Suitable internal standard

Various internal standards (Naproxen sodium, tenoxicam, diclofenac sodium, and meloxicam) were evaluated for percent recovery, sensitivity and compatibility with PX and 5- HP. The one gave better result was selected.

Injection Volume

Various injection volumes were studied in the range of 10 – 50 μL .

Validation of the method

The developed method was validated according to standard guidelines with respect to linearity, accuracy, specificity/selectivity, precision, sensitivity sample stability and robustness. The main purpose of validation was to develop a reliable and reproducible RP- HPLC/UV method for quantification of piroxicam, 5- hydroxypiroxicam and meloxicam (I.S) and analysis of pharmacokinetics of piroxicam in human plasma.

Linearity

Different dilution from stock solution were prepared with the mobile phase to obtain the different concentrations in the range of 20 – 2500 ng/mL. The linearity of the method was determined by constructing calibration curves for analytes both in spiked plasma and standard solution samples, by plotting response ratios (ratios of peak areas of analytes to I.S) as a function of concentration (ranges from 20 – 2,500 and 22- 2,500 ng/mL for PX and 5-HP, respectively). Regression equation was used for calculation of Slope (a), intercept (b) and correlation coefficient (r^2).

Precision

The precision of the method was evaluated in terms of repeatability (injection repeatability and analysis repeatability) and intermediate precision (intra-day and inter-day reproducibility). Injection repeatability was evaluated by injecting spiked plasma samples ($n = 10$) containing both analytes (1500 ng/mL) into HPLC system. While, analysis repeatability was evaluated by injecting spiked plasma samples ($n = 6$) of both analytes (1000 ng/mL) and IS (500 ng/mL) and results are depicted on bases of percent recovery.

Intermediate precision was determined by analyzing spiked plasma samples of analytes (100 ng/mL, 500 ng/mL and 1500 ng/mL) at regular intervals (at 8h intervals) on day first (interaday) and daily for one week (inter-day). The results of percent recovery were expressed as mean \pm SD and covariance (%RSD).

Selectivity

Selectivity/ specificity was determined by the complete separation of analytes in solution (mobile phase), spiked plasma and real plasma samples.

Accuracy

The accuracy was determined by calculating percent recoveries of plasma samples spiked with PX, 5HP and IS, at three nominal concentrations (LLOQ concentration, 500 and 1000 ng/mL) while, the IS concentration was kept constant (500 ng/mL). The spiked plasma samples were extracted and injected 20 μ L into HPLC system ($n= 6$). The following equation was used to calculate percent recovery;

$$\text{Percent Recovery} = \frac{X}{Y} \times 100 \quad \text{----- Eq-1}$$

where

X is the response ratio of the analyte with reference to the IS in plasma samples;
Y is the response ratio of the analyte with reference to the IS in the mobile phase

The equation used for calculating amount recovered from plasma samples was given below;

$$C = \left(\frac{A}{B}\right) \times \left(\frac{X}{Y}\right) \times C_s \times F_d \quad \text{-----Eq-2}$$

Where

A and B = Analyte peak areas in plasma samples and 1:1 mixture, respectively
X and Y = Internal standard peak areas in 1:1 mixture and plasma samples
 C_s = the analyte concentration in 1:1 mixture
F_d = Dilution factor

Standard deviation (SD) and covariance (%RSD) were calculated for all samples.

Sensitivity

Sensitivity of the method was determined on bases of lower limit of detection (LLOD) and lower limit of quantification (LLOQ) of studied analytes. HPLC software was used to for determination of Signal-to-noise ratio (S/N), taking $S/N \approx 3$ as LLOD and $S/N \approx 10$ as LLOQ.

Sample stability

Stability of the samples was determined by keeping analytes at various storage conditions (room temperature, 4°C, -20°C) for various durations (24 hrs, 48 hrs, one week and one month).

Stability and percent loss was determined by following equation;

$$\% \text{Stability} = \frac{S_t}{S_0} \times 100 \quad \text{----- Eq-3}$$

$$\% \text{Loss} = \frac{S_0 - S_t}{S_0} \times 100 \quad \text{----- Eq-4}$$

where, S_t = the stability of analytes at time t,
 S_0 = the stability at time zero

Robustness

The robustness of the method were determined by minor deliberate changes in different experimental parameters and studied their effects on retention time, peaks resolution, peak area and height. Changes were made in chromatographic conditions like column temperature (25 ± 5 °C), flow rate (1 ± 0.2 mL/min), acetonitrile proportion in mobile phase ($62 \pm 2\%$) and detector wavelength (353 ± 5 nm).

Statistical evaluation of the data

Statistical parameters such as mean (X), standard deviation (SD) and co-variance (%RSD) were calculated by applying Microsoft office Excell .

In- vivo application of the developed HPLC method

The present work is part of the evaluation pharmacokinetic herbs- drug interactions study of piroxicam in Biopharmaceutics Laboratory, Department of Pharmacy, University of Peshawar, Pakistan.

Results and Discussion

Piroxicam and 5-hydroxyiroxicam were simultaneously analyzed using meloxicam as internal standard in spiked plasma sample and the method was validated and optimized then used for evaluation of Piroxicam pharmacokinetics for the first time in Pakistani population. The present method is rapid, sensitive, and economical, while all of the analytes were eluted within 8 min. Various experimental parameters and chromatographic conditions of the developed method were optimized and validated according to ICH guidelines [12- 13].

Optimization of experimental parameters and analytical conditions

Different organic solvents like methanol, acetonitrile and ethanol, alone and in combinations, were used for protein precipitation. However, better results with respect to peaks resolution, peak shape and percent recovery were obtained with acetonitrile when used three times of plasma volume.

Dichloromethane, n-hexane, diethyl ether and mobile phase (combination of acetonitrile and 0.05 % TFA solution; 62:38, v/v) were evaluated for extraction of piroxicam, 5-hydroxyiroxicam and IS from plasma samples. Best recovery was achieved with diethyl ether. Table-1 shows comparative

recoveries of the studied analytes with different extraction solvents.

Different types of analytical columns like Athena C-18 WP (250 mm \times 4.6 mm, 5 μ m), Supelco Discovery HS C18 RP column (150 mm \times 4.6 mm, 5 μ m) and CNW C18 RP (250 mm \times 4.6 mm, 5 μ m) were evaluated for separation of the analytes. The CNW C18 RP (250 mm \times 4.6 mm, 5 μ m) column was selected on the basis of better peak shape, retention time, low tailing factor, better peak symmetry, good resolution and shorter analysis time.

Various mobile phases consisting of methanol- purified water (pH 2.5 – 3), methanol–acetonitrile (50:50 v/v), methanol- 0.05 % TFA solution, acetonitrile and 0.025 % TFA solution and acetonitrile– 0.05 % TFA solution (68:32, v/v) were studied as the mobile phases. Comparatively to methanol, better results (peak shape and resolution) were obtained with acetonitrile (Table-2), therefore acetonitrile and 0.05 % aqueous solution of TFA (0.05 %) in 62:38 ratios was selected as a mobile phase. It was observed that when acetonitrile proportion in mobile phase was increased the retention time of all the analytes were decreased. Various wavelengths ranges from 330 to 370 nm were assessed for simultaneous quantification of piroxicam, 5- hydroxyiroxicam and IS. Best result was achieved at 353 nm with respect to peak resolution and sensitivity.

Flow rate exhibited significant effects on retention time, peak area and peak height of analytes. Flow rate of 1 mL/min showed shorter retention time with optimum peak characteristics. The effects of column oven temperature over peak characteristics and retention time of all the analytes were studied in the range of 25 – 40°C. No significant changes were observed by varying temperature while keeping other chromatographic conditions constant, therefore further analysis was carried out at ambient (25°C) temperature.

Naproxen sodium, tenoxicam, diclofenac sodium, and meloxicam were evaluated to be used as an internal standard, among these meloxicam was preferred due to better resolution and percent recovery (90.27 ± 0.97 ; 1.06 %)

Validation of the developed method

Standard protocols [12, 13] were followed for validation of the developed method.

Table-1: Percent recovery of analytes and internal standard from plasma using various extraction solvents.

| Extraction Solvent | Percent Recovery (mean \pm SD; % RSD) | | |
|---|---|-------------------------|----------------------|
| | PX | 5-HP | IS |
| Dichloro methane | 79 \pm 1.21; 1.531 | 81 \pm 1.01; 1.246 | 85 \pm 1.45; 1.705 |
| Mobile phase* n- hexan | 53 \pm 1.18; 2.226 | 59 \pm 1.25; 2.118 | 40 \pm 1.05; 2.625 |
| | 71 \pm 1.22; 1.718 | 68 \pm 1.33; 1.955 | 66 \pm 1.14; 1.727 |
| Diethyl ether (combination with dichloro methan or n- hexane) | 80 \pm 0.89; 1.112 | 83 \pm 1.11; 1.337 | 75 \pm 1.02; 1.360 |
| Diethyl ether alone | 100.71 \pm 1.01; 1.002 | 95.14 \pm 0.99; 1.040 | 90 \pm 1.34; 1.488 |

*; Mobile phase consisted of acetonitrile and TFA solution (0.05%) in 62:38 by volume

Table-2: Effects of mobile phase on peak area, peaks resolution and retention time of Piroxicam and 5-hydroxy Piroxicam.

| Mobile Phase Composition | Peak area | | Retention time | | Resolution |
|-------------------------------------|------------------------------|---------------------------|---------------------------|-----------------------|------------|
| | Piroxicam (PX) | 5-Hydroxy Pyroxicam (SHP) | 5-Hydroxy Pyroxicam (SHP) | Pyroxicam (PX) | |
| Methanol- purified water (pH 2.5-3) | 48,821 \pm 1050; 2.15 | 32,450 \pm 1013; 3.10 | 5.94 \pm 0.04; 0.69 | 6.21 \pm 0.05; 0.83 | 1.52 |
| Methanol- Phosphate buffer (pH 2.5) | 80, 012 \pm 1416; 1.70 | 82, 371 \pm 1312; 1.59 | 4.81 \pm 0.03; 0.64 | 5.35 \pm 0.04; 0.74 | 1.60 |
| Methanol – 0.05% TFA (0.05%) | 58, 331 \pm 986; 1.69 | 65, 513 \pm 1021; 1.55 | 5.81 \pm 0.02; 0.34 | 6.33 \pm 0.03; 0.47 | 1.45 |
| Acetonitrile – TFA (0.025%) | 102,871 \pm 1133; 1.10 | 84, 122 \pm 1031; 1.22 | 4.35 \pm 0.02; 0.46 | 5.24 \pm 0.02; 0.40 | 2.81 |
| Acetonitrile – TFA (0.05%) | 110,412.391 \pm 1201; 1.08 | 87,409 \pm 1003; 1.14 | 4.50 \pm 0.02; 0.48 | 5.50 \pm 0.02; 0.39 | 3.54 |

Data is rounded off to two digits after decimal point
TFA; Tri flouro acetic acid

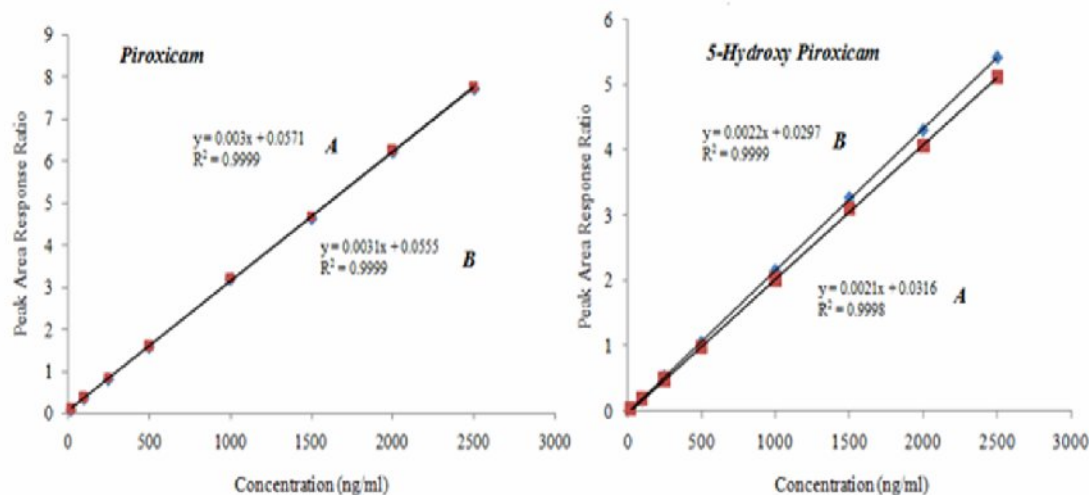


Fig. 2: Calibration curves of piroxicam and 5-hydroxypiroxicam in A) human plasma and B) mobile phase.

Linearity

Linearity of the method was determined from calibration curves of spiked plasma samples and standard solution of the studied analytes. Calibration curves (Fig. 2) were constructed for Piroxicam and 5-hydroxypiroxicam over concentration range of 20 – 2500 and 22 – 2500 ng/mL, respectively. The correlation co-efficient (r) and the regression analysis of studied analytes (Table-3) showed good correlation between the drug concentrations and instrumental response within the studied concentration range.

Table-3: Calibration range, linearity and sensitivity of the method.

| Parameters | Analytes | |
|--------------------------------------|------------------------|------------------------|
| | Piroxicam | 5-Hydroxypiroxicam |
| Linearity | 20 – 2500 ng/mL | 22 – 2500 ng/mL |
| Standard mixture | | |
| Regression equation | $y = 0.0031x + 0.0555$ | $y = 0.0022x + 0.0297$ |
| Correlation co efficient | 0.9999 | 0.9999 |
| Spiked plasma samples | | |
| Regression equation | $y = 0.003x + 0.0571$ | $y = 0.0021x + 0.0316$ |
| Correlation co efficient | 0.9999 | 0.9998 |
| Sensitivity | | |
| Limit of detection (LOD) | 6 ng/mL | 7 ng/mL |
| Lower limit of quantification (LLOQ) | 20 ng/mL | 22 ng/mL |

Table-4: Recovery and precision of the method.

| Parameter | Analytes | |
|---------------------------------------|------------------------------------|-----------------------------------|
| | Piroxicam | 5-Hydroxypiroxicam |
| Accuracy (percent recovery) | | |
| Spiked conc.* (at LLOQ level) (n = 6) | ^a (100.85 ± 0.96); 0.95 | ^a (95.64 ± 1.01); 1.06 |
| Spiked conc. (500 ng/mL) (n= 6) | ^a (99.98 ± 1.31); 1.31 | ^a (94.95 ± 0.92); 0.97 |
| Spiked conc. (1000 ng/mL) (n = 6) | ^a (101.32 ± 1.04); 1.03 | ^a (94.83 ± 1.16); 1.22 |
| Precision | | |
| <i>Injection repeatability</i> | | |
| Spiked conc. (1500 ng/mL) (n= 10) | ^b 154552.36; 1.25 | ^b 136315; 1.12 |
| Spiked conc. (1500 ng/mL) (n = 10) | ^c 5.50; 0.02 | ^c 4.50; 0.02 |
| <i>Analysis repeatability</i> | | |
| Spiked conc. (1000 ng/mL) (n = 6) | ^d 1006.69; 0.37 | ^d 950.98; 0.49 |
| <i>Intermediate precision</i> | | |
| <i>Intraday reproducibility</i> | | |
| Spiked conc. (100 ng/mL) (n= 6) | ^d 100.49 ± 1.21; 1.20 | ^d 95.17 ± 1.03; 1.08 |
| Spiked conc. (500 ng/mL) (n= 6) | ^d 499.61 ± 0.92; 0.18 | ^d 477.78 ± 1.95; 0.41 |
| Spiked conc. (1500 ng/mL) (n = 6) | ^d 1500.81 ± 1.75; 0.12 | ^d 1431.86 ± 2.13; 0.15 |
| <i>Inter day reproducibility</i> | | |
| Spiked conc. (100 ng/mL) (n= 6) | ^a 100.14 ± 0.87; 0.87 | ^a 94.87 ± 1.33; 1.40 |
| Spiked conc. (500 ng/mL) (n= 6) | ^a 498.94 ± 1.02; 0.20 | ^a 473.96 ± 1.63; 0.34 |
| Spiked conc. (1500 ng/mL) (n = 6) | ^a 1499.98 ± 1.29; 0.09 | ^a 1430.43 ± 1.51; 0.11 |

Results are presented as (mean); %RSD. Data is rounded off to two digits after decimal point. *: concentration of both the analytes was equal to their LOQ i.e. 20 ng/mL for piroxicam and 22 ng/mL for 5-hydroxypiroxicam, a: percent recovery; b: peak area; c: retention time; d: quantity recovered

Accuracy and recovery

Accuracy was determined on the basis of percent recovery at 20, 500 and 1000 ng/mL and 22, 500, 1000 ng/mL concentrations for PX and 5-HP, respectively. The results are presented in Table- 4.

Precision of the method

The repeatability (injection repeatability and analysis repeatability) and intermediate precision (intraday and inter-days precision) of the suggested method indicated complete harmony of the results as shown in Table-4.

Selectivity/specificity

The injection of the blank plasma samples did not show any endogenous interfering peak. The Peaks of the studied analytes were completely resolved, as shown in Fig. 3, indicating suitability of the method for analysis of the analytes in pharmaceutical and human plasma.

Sensitivity of the method

Lower limit of detection and quantification for analytes were very good, indicating better sensitivity of the method than the previously reported methods. The values are given in Table-3, while Fig. 4 shows chromatogram of LLOD and LLOQ of both the analytes.

Stability Studies

Piroxicam and 5 hydroxypiroxicam were stable during the freeze/thaw cycles for 48 hr. The short term stability study showed that spiked plasma and standard solution samples of PX, 5HP and IS were stable for 48 hr under ambient condition. Long term stability studies showed that the standard solutions and plasma samples were stable for at least three months when stored at 4°C, and -20°C. The percent degradation of Piroxicam, 5 hydroxypiroxicam and IS in plasma samples stored at

-4°C (0.125, 0.56 and 0.74 % loss, respectively) was comparatively higher than the samples stored at -20°C (0.02, 0.03 and 0.13 % loss, respectively), therefore -20°C is recommended storage temperature for plasma samples.

Robustness

Minor deliberate changes in experimental parameters had no significant effects on peaks retention times and other peak parameters of the studied analytes . The method was robust for minor changes in column oven temperature (25 ± 2°C), flow rate (1 ± 0.2 mL/min) and detector wavelength (353 ± 5 nm).

Pharmacokinetics of piroxicam in Pakistani population

Blood samples were collected from volunteers (n= 30) before, 0.5, 1, 1.5, 2, 2.5, 3, 4, 6, 8, 24, 48, 72 and 96 hr after administration of piroxicam 20 mg oral dose [6, 23, 24]. The blood samples were processed according to the procedure discussed in section 2.3.1.1. The developed method was used for quantification of drug content of each plasma sample and then pharmacokinetic parameters of piroxicam were calculated applying PK Summit[®] software. Plasma concentration versus time curve of piroxicam is shown in Fig. 5. Various pharmacokinetic parameters were calculated for piroxicam, as presented in Table-5. The pharmacokinetic parameters determined in this study are in close agreement with previously reported studies [5, 14-15].

Table-5: Various pharmacokinetic parameters of piroxicam in healthy human Pakistani volunteers.

| Pharmacokinetic parameter | Mean ± SD (no = 30) |
|---------------------------------|----------------------|
| C _{max} (µg/mL) | 1965.60 ± 301.94 |
| t _{max} (hr) | 1.95 ± 0.44 |
| t _{1/2} | 54.76 ± 9.37 |
| AUC (0, 96) (ng-hr/mL) | 73714.09 ± 15715.75 |
| AUC (0, ∞) (ng-hr/mL) | 104074.20 ± 21782.84 |
| Clearance (Cl) (mL/min) | 2.36 ± 0.53 |
| Volume of distribution (Vd) (L) | 8.86 ± 1.54 |

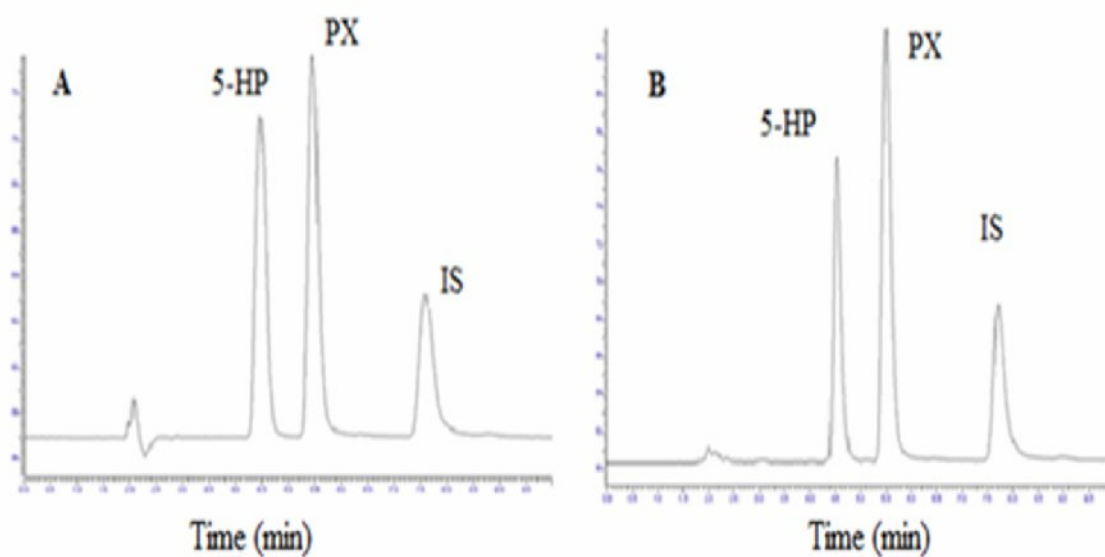


Fig. 3: Representative chromatograms of PX, 5HP and internal standard in standard solution (A) (Pharmaceutical Solution) and spiked Plasma Sample (B).

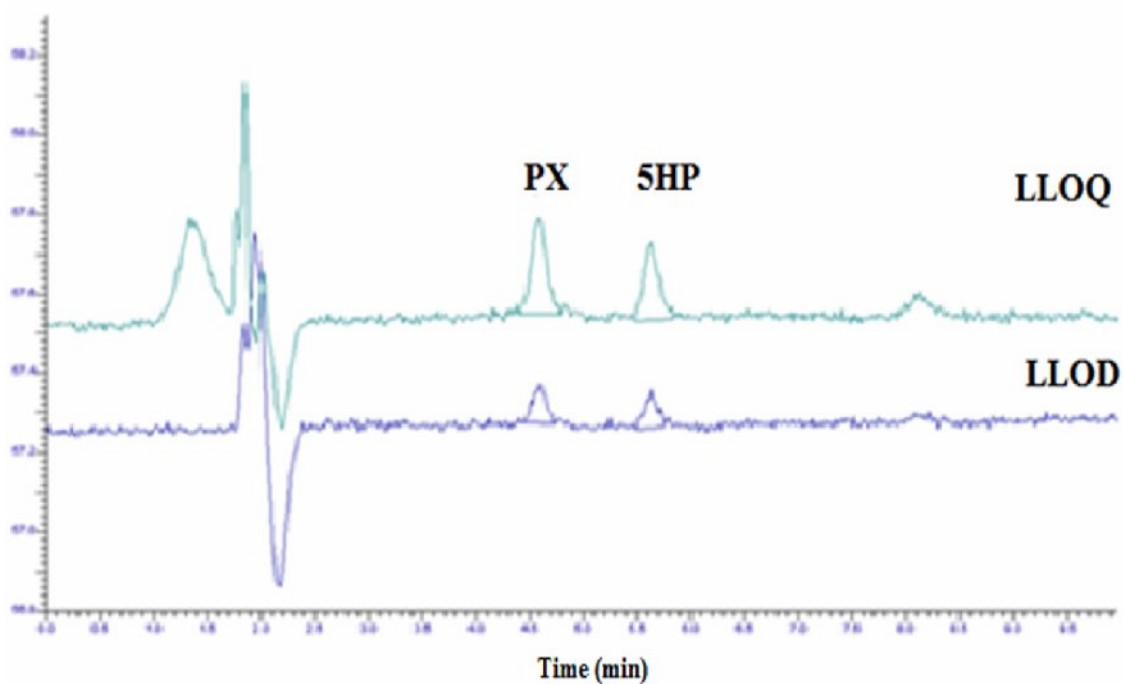


Fig-4: Chromtogram representing lower limit of detection (LLOD) and lower limit of quantification (LLOQ) of piroxicam (PX) and 5- hydroxypiroxicam (5HP).

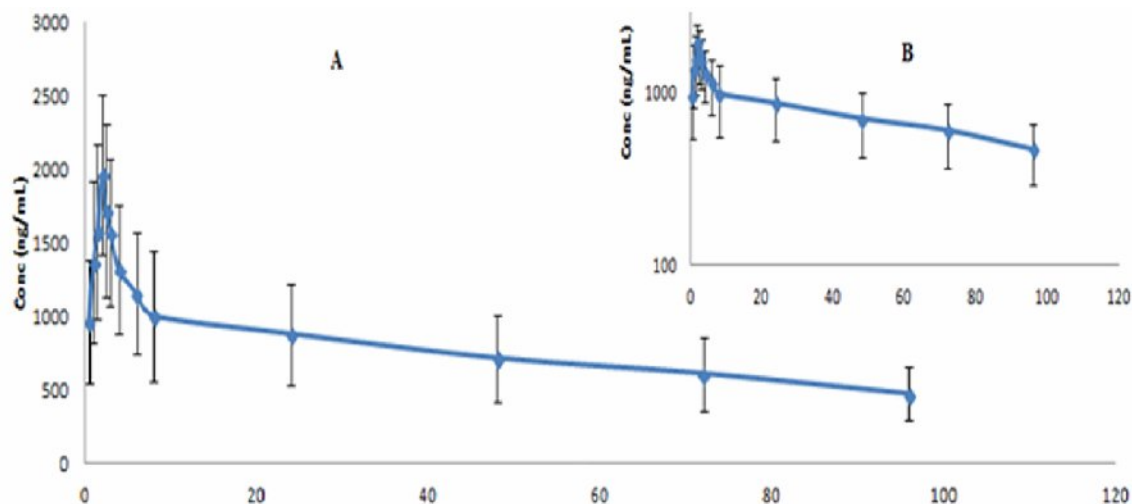


Fig. 5: Mean \pm SD Plasma Drug Concentration Vs Time of Healthy Human Volunteers (n = 30) following oral administration of Piroxicam capsule (20 mg), A: Normal plot and B: Semi-log scale.

Conclusion

A simple, rapid, sensitive, selective and robust method was developed and validated for quantification of piroxicam and 5-hydroxyproxicam in spiked human plasma, using isocratic RP-HPLC connected to UV detector. Various experimental conditions such as stationary phase, column oven temperature, composition of mobile phase, sample injection volume, and flow rate were validated and optimized following standard protocols. The developed method was comparatively more precise and sensitive than the reported HPLC/UV methods for simultaneous quantification of piroxicam and 5-hydroxyproxicam. This method was efficiently applied for the quantification of piroxicam in human volunteers for the first time in Pakistani population and PK-HDI study of piroxicam with herbal products.

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