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Rapid analysis of piroxicam level in microsample of human plasma by fully validated HPLC assay

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ABSTRACT

A rapid, simple HPLC assay for piroxicam measurement in human plasma was developed and validated. 50 µl of 24% perchloric acid and 0.2 ml acetonitrile were mixed with 0.1 ml plasma sample, and the supernatant was injected directly into 4.6 × 150 mm, XTerra® RP_{18}, 5µm steel column at room temperature (RT). The mobile phase, 0.2% triflouroacetic acid, and acetonitrile (70:30, v:v), was delivered at 1.2 ml/min with a run time of 6 min. Doxycycline (internal standard, IS) and piroxicam were detected using Waters 996 photodiode array detector set at 339 nm IS at 3.7 and 4.8 min, respectively. The response was linear over the range of 0.2-20µg/ml, and the intra- and inter-run coefficient of variations were ≤ 5.2% and ≤ 6.7%, respectively. Extraction recovery and intra- and inter-run bias were ≥ 86% (mean 93%), ≤ 9%, and ≤ 11%, respectively. Piroxicam was stable in plasma for 24 hours at RT (≥ 96%), 8 weeks at -20 ^{0}C (≥ 100%), and after 3 cycles of freeze at -20^{0}C and thaw at RT (≥ 94%). In processed samples, piroxicam was stable for 24 hours at RT (≥100%) and 48 hours at -20^{0}C (≥100%). Stock solution of piroxicam (1 mg/ml in methanol) was stable for 48 hours at RT and 8 weeks at -20^{0}C (100%).

KEYWORDS

Piroxicam;
Doxycycline;
HPLC;
Validation;
Stability.

INTRODUCTION

Piroxicam, 2H-1,2-Benzothiazine-3-carboxamide, 4-hydroxy-2-methyl-N-2-pyridinyl-, 1,1-dioxide (CAS number: 36322-90-4) is an oxicam, non-steroidal anti-inflammatory drug with analgesic and antipyretic properties[1]. It is well absorbed from the gastrointestinal tract, metabolized in the liver by hydroxylation and conjugation with glucuronic acid, extensively (99%) bound to plasma proteins, and it has a long plasma half-life of approximately 50 hours[1]. After long-term daily use of 10 - 30 mg piroxicam, plasma drug concentrations ranged from 2.4 to 11.7 µg/ml[1].

Several methods have been described for the determination of piroxicam level in plasma[2-27], most of them involve HPLC with UV detection[2-20]. The majority of the HPLC-UV methods require liquid-liquid extraction with consecutive evaporation[2-11], some employ solid-phase extraction[12-14], and some are based on protein precipitation[15-20]. An HPLC method with electrochemical detection involved multiple steps of liquid-liquid extraction[21]. A number of liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) methods were also reported[22-27]. Some of

these methods suffered from low recovery[21,25], required long chromatographic run time[19,21], were not validated using human plasma[9,11,23,24,27], required column switching[12], or did not address stability[7-9,12,16,18,26], a requirement for evaluating a large number of samples in pharmacokinetic and bioequivalence studies.

The objectives of the work described here were to 1) establish a simple, fully validated piroxicam HPLC assay in human plasma with a run time and quantitation limit suitable for bioequivalence studies, and 2) determine the stability of piroxicam under various clinical laboratory conditions.

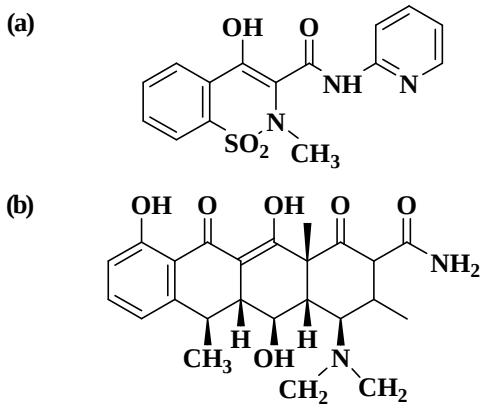

Figure 1 : (a) Structure of piroxicam (C_{15}-H_{13}-N_3-O_4-S). (b) Structure of doxycycline, the internal standard (C_{22}-H_{24}-N_2-O_8)

EXPERIMENTAL

Apparatus

The liquid chromatograph consisted of Waters Alliance 2690 Separations Module, a 4-μm (particle-size), 4.6 × 150 mm XTerra ® RP_{18}, 5-μm (particle-size) steel column, a Guard Pak pre-column module with Radial-Pak C_{18}, 5-μm insert, and Waters 996 photodiode array detector (Water Associates, Milford, MA, USA) set at 339 nm. Data were collected with a Pentium III computer using Millennium[32] Chromatography Manager Software (Water Associates, Milford, MA, USA).

Chemicals and reagents

Piroxicam (Figure 1-a), and the internal standard (IS) doxycycline (CAS number; 564-25-0) (Figure 1-b) were analytical grade and obtained from Sigma-Aldrich CO., St. Louis, MO, USA. Acetonitrile was purchased from Fisher Scientific (Fairlawn, NJ, USA) and perchloric acid (69-72%) was purchased from Fisher Scientific, Pittsburgh PA, Canada (both HPLC grade). Trifluoroacetic acid was purchased from Merk, Darmstadt, Germany. Water for HPLC was prepared by reverse osmosis and further purified by passing through a Millipore-Synergy UV obtained from Millipore Co. (Bedford, MA, USA).

Chromatographic conditions

The mobile phase consisted of 0.2 % triflouroacetic acid (pH = 1.7) and acetonitrile (70:30, v:v). It was filtered through a 0.22 μm size membrane filter (Millipore Co., Bedford, MA, USA), degassed, and delivered at 1.2 ml/min at room temperature. The autosampler was programmed to inject 100 μl into the chromatograph with a run time of 6 minutes.

Preparation of stock and working solutions

A 1 mg/ml piroxicam stock solution was made in methanol and used for stability studies and to prepare a 20 μg/ml working solution in plasma. The working solution was prepared weekly to construct calibration curve and quality control (QC) samples. A 20 μg/ml doxycycline working solution was prepared weekly in mobile phase from a 1 mg/ml stock solution in water.

Calibration standard/Quality control samples

Calibration standards were prepared by mixing appropriate volumes of piroxicam working solutions with blank human plasma to produce final concentrations of blank, zero (blank plasma spiked with IS only), 0.2, 0.4, 0.8, 1.6, 2, 4, 8, 16 and 20 μg/ml. Quality control (QC) samples were prepared by mixing appropriate volumes of piroxicam working solution in blank human plasma to produce final concentrations of 0.2, 0.6, 10, and 18 μg/ml. Samples were vortexed for 20 seconds, and aliquots of 0.1 ml of calibration standards QC samples were transferred into 1.5 ml eppendorf microcentrifuge tubes and stored at -20ºC.

Sample preparation

Aliquots of 0.1 ml of calibration standard or QC samples in microcentrifuge tubes were allowed to equilibrate to room temperature. To each tube, 100 μl of the 20 μg/ml IS working solution was added and vortexed for 10 seconds. After the addition of 50 μl of the 24%

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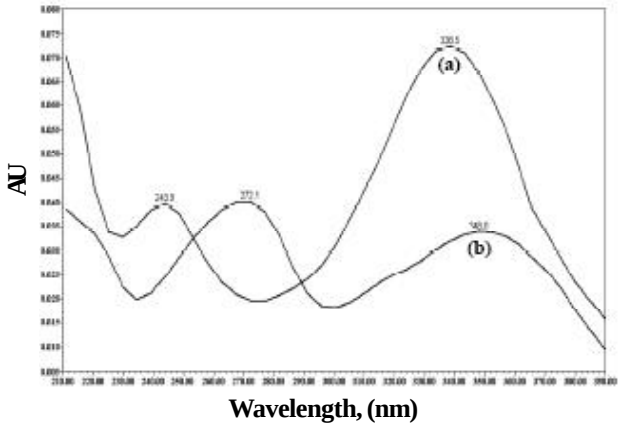

Figure 2 : PDA extracted ultraviolet spectra of (a) piroxicam, and (b) doxycycline (internal standard, IS)

perchloric acid and 0.2 ml of acetonitrile, the mixture was vortexed again for 1 min and then centrifuged for 5 min at 13200 rpm at room temperature. The supernatant organic layer was carefully transferred into the auto-sampler vials and 100 µl were injected into the HPLC system. The run time was 6 minutes.

Stability studies

Stability of piroxicam in plasma

Adequate numbers of aliquots of three QC samples (0.2, 0.6, and 18 µg/ml) were prepared. Five aliquots of each QC sample were analyzed immediately (baseline), the other aliquots were analyzed after being processed and stored at room temperature for 8 or 24 hours or at -20^{0}C for 48 hours (auto-sampler stability). Five aliquots of each QC sample were allowed to stand on the bench-top for 8 or 24 hours at room temperature before processing (counter stability), and five aliquots were stored at -20^{0}C for 2, 4, or 8 weeks before analysis (long term freezer stability). Finally, fifteen aliquots of each QC sample were stored at -20^{0}C for 24 hours. They were then left to completely thaw unassisted at room temperature before being returned to -20^{0}C for another 24 hours. The cycle was repeated three times (freeze-thaw stability).

Stock solutions stability

Five aliquots of the stock solutions of piroxicam or the IS were diluted to 10 µg/ml in mobile phase and analyzed at baseline, after storage for 48 hours at room temperature, or after storage at -20^{0}C for 8 weeks. Stability of the working solutions of piroxicam and IS, were evaluated up to 2 weeks at -20^{0}C.

Assay validation method

The procedures used for validation were as described in US food and drug administration (FDA) bioanalytical method validation guidance[28].

RESULTS

Optimization of chromatographic conditions

During analytical method development, different combinations of mobile phase at different pH were investigated at different flow rates to optimize separation of piroxicam and the internal standard. A mobile phase composed of 0.2 % triflouroacetic acid (pH = 1.7) and acetonitrile (70:30, v:v), was found best to achieve adequate separation of piroxicam from the IS, minimize background absorbance, and avoid peak tailing. Under the described conditions, the IS and piroxicam were resolved within a run time of 6 minutes, with a retention time of 3.7 and 4.8 minutes, respectively. In order to improve specificity and minimize interference from plasma or solvent system that may occur at lower wavelengths, we optimized the absorbance wavelength based on photodiode array extracted spectra (Figure 2). We performed the analysis at 339 nm.

Linearity

Linearity was determined in the range of 0.2 - 20 µg/ml using ten calibration curves. The data were analyzed by linear regression using the formula: Conc. = a + b (PAR), where Conc. is the concentration of piroxicam, a is the intercept, b is the slope, and PAR is the peak area of piroxicam divided by the peak area of the IS. The concentrations of the calibration standards of the ten calibration curves were back-calculated using the individual regression lines. Linearity studies (n=10) showed mean (SD) for R^2 of 0.9978 (0.0014), slope of 0.1314 (0.0140), and intercept of 0.0004 (0.0197). Figure 3 depicts an overlay of chromatograms of a representative standard curve.

Limit of detection

The limit of detection (LOD), defined as three times the baseline noise, was 0.1 µg/ml.

Specificity

To evaluate specificity, we screened eight frequently used medications (10 μg/ml in mobile phase) and six different batches of human plasma. All batches of blank plasma were free from interfering components. None of eight commonly used drugs co-eluted with piroxicam or the IS (TABLE 1).

Recovery

The extraction recovery of piroxicam was determined by dividing mean peak areas of five replicates of three quality control samples (0.2, 0.6, and 18 μg/ml) prepared in plasma (as described under sample preparation above), by mean peak areas of five replicates of equivalent concentrations prepared in the mobile phase. The recovery of the IS was determined similarly at a concentration of 20 μg/ml. The results of the extraction recovery studies of piroxicam and the IS are presented in TABLE 2. Recovery was = 86% (mean 93%) for piroxicam and 94% for the IS.

TABLE 1: Specificity of piroxicam assay

Drug name	Retention time
Piroxicam	4.8
Doxycycline (IS)	3.7
Aspirine	ND
Acetaminophen	ND
Ranitidine	ND
Nicotinic acid*	ND
Ascorbic acid*	ND
Caffeine*	ND
Diclofenac	ND
Omeprazole	ND

1 mg/ml solutions in methanol or water* were diluted in mobile phase to 10 μg/ml and 100 μl were injected.

TABLE 2 : Extraction recovery of piroxicam and doxycycline

Nominal Concentration (μg/ml)	Plasma		Mobile phase		** Recovery (%)
	*Mean peak area	SD	*Mean peak area	SD	
Piroxicam 0.2	9235	1056	10212	130	90
0.6	46823	1115	54777	1497	86
10	682113	15125	703906	645	97
18 Doxycycline	1263040	43408	1293554	1407	98
20	560059	20140	593774	4443	94

***Mean peak area of 5 replicates; ** Mean peak area of spiked plasma sample divided by mean peak area of spiked mobile phase sample × 100. SD, Standard deviation.**

Precision and bias

Precision was calculated as coefficient of variation (standard deviation divided by mean measured concentration × 100), and bias as the absolute value of (1 minus mean measured concentration divided by nominal concentration) × 100. The intra-run and inter-run precision and bias were determined by analyzing four QC samples: 0.2, 0.6, 10, and 18 μg/ml over three different days (TABLE 3). Intra-run precision and bias (n = 10) ranged from 2.0 % to 5.2 % and from 4 % to 9 %, respectively. The inter-run precision and bias (n = 20) ranged from 5.4% to 6.7% and from 2 % to 11 %, respectively.

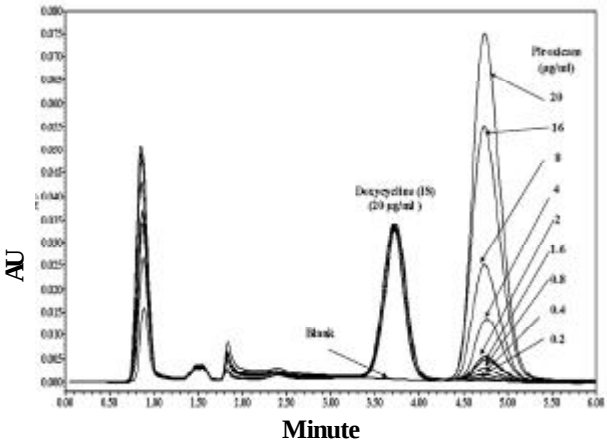

Figure 3 : Overlay of calibration curve chromatograms of piroxicam spiked with doxycycline (internal standard, IS)

TABLE 3 : Intra-run and inter-run accuracy and precision of piroxicam assay

Nominal concentration (μg/ml)	Intra-run (n=10)				Inter-run (n=20)			
	Mean measured concentration (μg/ml)	SD	Precision (CV*, %)	** Bias (%)	Mean measured concentration (μg/ml)	SD	Precision (CV*, %)	** Bias (%)
0.2	0.2126	0.0088	4.2	6	0.2047	0.0127	6.2	2
0.6	0.6545	0.0327	5.0	9	0.6641	0.0357	5.4	11
10	9.5650	0.4949	5.2	4	10.1276	0.6740	6.7	1
18	17.4000	0.3395	2.0	3	18.3320	0.9996	5.5	2

***Coefficient of variation (CV) = Standard Deviation (SD) divided by mean measured concentration × 100. **Bias = absolute value of 1 minus mean measured concentration divided by nominal concentration × 100**

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TABLE 4: Stability of piroxicam in plasma samples and stock solution

Stability (%)										
	*Plasma samples								**Stock solution	
Nominal concentration (μg/ml)	Unextracted			Extracted		Freeze-thaw				
	8h RT	24 h RT	8 wks –20°C	24 h RT	48 h –20°C	One cycle	Two cycles	Three cycles	48 h RT	8 wks –20°C
0.2	100	100	100	100	100	100	100	99		
0.6	100	96	100	100	100	95	100	94	100	100
18	100	100	100	100	100	100	100	100		

Stability (%) = mean measured concentration (n=5) at the indicated time divided by mean measured concentration (n=5) at baseline x 100. *Spiked plasma samples were processed and analyzed immediately (baseline, data not shown), after 8 or 24 hours at room temperature (8 h RT and 24 h RT), after 8 weeks at - 20°C (8 wks -20°C), or processed and analyzed after 24 hours at room temperature (24 h RT) or 48 hours at -20°C (48 h -20°C); or after 1 to 3 cycles of freezing at -20°C and thawing at room temperature (freeze-thaw). ** Piroxicam, 1 mg/ml in methanol.

Stability

The stability of piroxicam under usual storage conditions of plasma and processed samples was investigated. The results are presented in TABLE 4. The data indicate that: 1) piroxicam in plasma is stable for at least 24 hours at room temperature and 8 weeks at -20°C, 2) in processed samples, piroxicam is stable for at least 24 hours at room temperature and 48 hours at -20°C, 3) piroxicam in plasma is stable after at least three cycles of freeze at -20°C and thaw at room temperature, and 4) piroxicam in methanol (1 mg/ml) is stable for at least 48 hours at room temperature and 8 weeks at -20°C. The IS in water (1 mg/ml) was also stable under the same conditions (106 % and 97 %, respectively). Further, the working solutions of piroxicam and the IS (20 μg/ml in plasma or mobile phase, respectively) were stable for at least 2 weeks at -20°C (109% and 95%, respectively).

Robustness

The robustness of the proposed method was evaluated by slightly altering the strength of the triflouroacetic acid and amount of acetonitrile in mobile phase. No significant effects were observed. Further, the chromatographic resolution and peak responses were stable over about 700 injections of processed plasma samples using one column.

DISCUSSION

We describe a rapid, simple, accurate, and precise HPLC assay for the determination of therapeutic levels of piroxicam in human plasma. The simplicity, rapidity, and requirement of smaller sample volume are the main advantages of the current assay.

It involves a simple precipitation step avoiding one or multiple steps liquid-liquid[2-11,21] or solid-phase[12-14] extractions with subsequent evaporation, and does not required column switching[12], a high column temperature[18,21], MS-MS[22-27]. A short run time of 6 minutes and a small plasma volume of 100 μl favorably compares to more than 10 minutes[19-21] and from 0.25 to 1 ml in previously reported assays, respectively[19-21]. Further, the recovery of piroxicam from plasma was ≥ 86% (mean 93%) compared to previously reported recovery of 64.3%[21] and 78.3%[25]. Further more. some of the other previously reported assays were not validated to measure piroxicam level in human plasma[9,11,23,24,27], or did not examine piroxicam stability[7-9,12, 16,18,26].

Dadashzadeh et al.[19] described piroxicam stability study in plasma maintained at -20°C for one month period. Using the current assay, we found that piroxicam is stable in plasma under various laboratory conditions, including 3 freeze thaw cycles, 48 hours at room temperature, and 8 weeks at -20°C, as well as, 24 hours at room temperature and 48 hours at -20°C after processing.

In summary, we describe a new, fully validated assay for the analysis of therapeutic piroxicam levels in 100 μl human plasma, utilizing a simple plasma precipitation technique. We also provide extensive data on piroxicam and IS stability. The performance characteristics of the assay together with the information on stability indicate that the assay is suitable for use in therapeutic drug monitoring and bioequivalence studies.

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