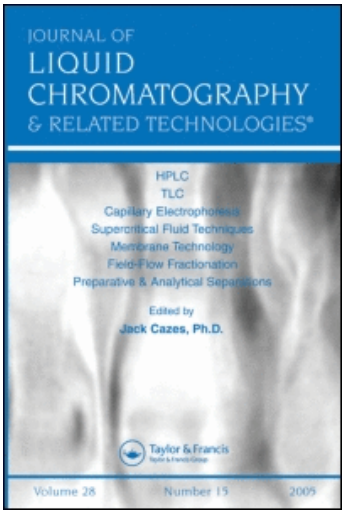

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A SIMPLE AND SENSITIVE HPLC ASSAY FOR PIROXICAM IN PLASMA AND ITS APPLICATION TO BIOAVAILABILITY STUDY

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ABSTRACT

A rapid, simple and selective method for the determination of piroxicam in human plasma is described. The sample was prepared by direct plasma protein precipitation. Isoxicam was used as the internal standard. For sample analysis, 100 µl of the internal standard (isoxicam 500 µg/ml) and 50 µl of 60% w/v trichloroacetic acid were added to the 500 µl of plasma. After brief vortex and centrifugation, the clear supernatant (70 µl) was injected onto the HPLC column. Chromatographic separation and quantitation was performed by reversed-phase HPLC using a 5 µm CN (Spherisorb) microbore column. The mobile phase consisted of an acetronitrile-water mixture (6 : 94, v/v) containing 10 mM Na_2HPO_4, adjusted to pH 2. The eluant was monitored at 363 nm. The lower limit of sensitivity for piroxicam is 0.05 µg/ml (i.e., 50 ng/ml). The standard curve was linear over the concentration range of 0.05 to 10 µg/ml. The intra- and inter-assay coefficients of variation were less than 8%. Applicability of the method is demonstrated by a bioavailability study in 18 healthy volunteers who received a single oral dose of 30 mg piroxicam.

INTRODUCTION

Piroxicam is one of the most commonly prescribed non-steroidal anti-inflammatory drugs for the treatment of rheumatoid arthritis, osteoarthritis and other

inflammatory disorders. Many reports have described the determination of piroxicam in plasma by reversed-phase liquid chromatography (1-7), however, these methods required an organic solvent extraction. Such extraction of plasma samples prior to chromatographic separation is a time consuming and tedious exercise. The present report describes a sensitive and rapid HPLC assay for quantitation of piroxicam using small volume plasma samples. The method involves a simple plasma protein precipitation procedure requiring no extraction steps.

MATERIALS AND METHODS

Reagents and Chemicals

All chemicals were of analytical grade. Piroxicam was kindly supplied by Pfizer Laboratories Ltd. (Auckland, New Zealand). Isoxicam, the internal standard, was a gift from Dr D G Ferry of HRC Toxicology Research Unit, University of Otago. HPLC-grade acetonitrile, sodium acetate and trichloroacetic acid were obtained from BDH Ltd (Poole, UK). Water was double glass distilled and MilliQ® filtered.

Standard Solutions and Internal Standard

A stock solution containing 1 mg/ml piroxicam was prepared in methanol. The internal standard solution of isoxicam (500 μg/ml) was also prepared in methanol. The solutions were prepared freshly on each day of analysis. Plasma standard solutions of piroxicam for the calibration curves were prepared by appropriate dilution of the stock piroxicam solutions with drug-free plasma so that concentrations of 0.05, 0.1, 0.5, 1, 2.5, 5 and 10 μg/ml were obtained.

Analysis Procedure

A protein precipitation technique was used in the preparation of plasma standards and samples. To 500 μl of plasma sample or standard in a 1.5 ml plastic Eppendorf microcentrifuge tube, 100 μl of the internal standard solution (500 μg/ml isoxicam) and 50 μl of perchloric acid (60% w/v) were added. The contents were then vortexed for 10 seconds and centrifuged at 1500 g for 5 minutes. The clear

supernatant (370 µl) was then mixed with 50 µl of 5 M sodium acetate. The resultant solution was transferred to a small plastic vial in the autoinjector and 70 µl of this solution was injected onto the HPLC column. The concentrations of piroxicam in samples were determined from calibration plots of the chromatographic peak height ratios (piroxicam/internal standard) versus plasma piroxicam concentration.

Chromatographic Conditions

The HPLC system consisted of an LKB 2150 pump (LKB, Stockholm, Sweden) connected to a Waters 712B (Milford, MA, USA) with samples stored at 4°C until injection. The detector used was a Linear UVis 200 spectrophotometer (Linear Instruments Corp., Nevada, USA) operated at 363 nm. The chromatographic response was recorded by a Shimadzu R3A integrator (Tokyo, Japan). A microbore HPLC column (2 mm I.D. x 10 cm) packed with a reversed-phase CN material, 5 µm CN Spherisorb (Shandon, London, UK) was used. Analysis of the samples of piroxicam was performed using a mobile phase consisting of an acetonitrile-water mixture (6 : 94, v/v) containing 10 mM Na_2HPO_4 adjusted to pH 2 with orthophosphoric acid. The flow rate of the mobile phase was 0.5 ml/min (back pressure approximately 90 bars). Chromatographic separations were performed at room temperature.

Recovery

The assay recovery was determined at 0.1 and 5 µg/ml for piroxicam and 77 µg/ml for the internal standard (isoxicam). The peak areas from six extracted plasma samples and six direct injections of the same amount of drug in mobile phase were compared. The assay recovery of each compound was estimated using the following equation:

$$\text{Recovery} = \frac{\text{mean peak area (extracted drug)}}{\text{mean peak area (direct injection)}} \times 100\%$$

Bioavailability Study

Eighteen healthy male volunteers between the ages of 18 and 35 were recruited for the randomized two way crossover study. The study was approved by the local ethical committee. Informed consent was obtained from all subjects before the study. The investigation was designed to evaluate the bioavailability of a new formulation of piroxicam 10 mg dispersible tablets (Pacific Pharmaceuticals Ltd,

Auckland, New Zealand) relative to that of the currently marketed product, Feldene-D 10 mg tablets (Pfizer Laboratories Ltd., New Zealand).

Subjects ingested 30 mg piroxicam (3 tablets) of either formulation. Blood samples were withdrawn at intervals following drug administration for up to 144 hours (i.e., 6 days). Blood, collected in heparinized evacuated tubes, was centrifuged and plasma was stored at -70°C until analysis.

Pharmacokinetic parameters including the maximum plasma piroxicam concentration (Cmax), time to achieve the maximum concentration (tmax), elimination half-life ($t_{1/2}$) and the area under the plasma concentration-time curve (AUC) were derived by standard procedures (8). The relative bioavailability (Fr) of the new product, Piroxicam dispersible tablets (Pacific Pharmaceuticals Ltd., New Zealand) was determined from the AUC from time 0 to infinity as follows:

$$Fr = \frac{AUC_{Pacific}}{AUC_{Feldene\text{-}D}}$$

The comparison of the piroxicam pharmacokinetic parameters of the two dosage forms was performed using 2-way ANOVA and paired Student *t*-test.

RESULTS AND DISCUSSION

A mobile phase of an acetonitrile-water mixture (6 : 94, v/v) containing 10 mM Na_2HPO_4 adjusted to pH 2 was chosen as it provides a good resolution between piroxicam and the internal standard. The reversed-phase 5 μm CN (Spherisorb) microbore column provided reasonable retention times for the compounds of interest. The retention times for piroxicam and the internal standard (isoxicam) were 3.8 and 7.4 minutes (Figure 1).

Three chromatograms, one obtained from blank plasma (i.e., drug-free plasma), a second from blank plasma spiked with piroxicam (0.05 μg/ml) and a third from plasma taken from a subject after ingestion of piroxicam are presented in Figure 1. Under these chromatographic conditions, there was no endogenous interference from plasma. Blank plasma samples from 20 subjects were analysed and no plasma endogenous peaks co-eluting with the internal standard and piroxicam were detected.

The absolute analytical recovery of piroxicam from plasma was 69 ± 3% (S.D.) at 0.1 μg/ml and 71 ± 4% at 5 μg/ml of piroxicam (n = 6). The recovery of the

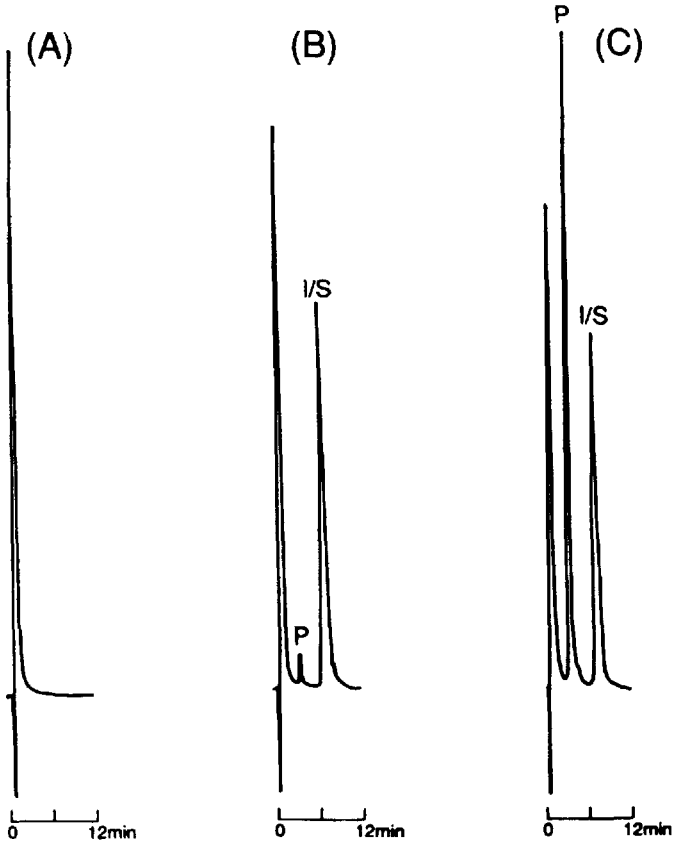

FIGURE 1

Typical chromatograms of extracts of human plasma: (A) blank plasma; (B) plasma spiked with 0.05 μg/ml piroxicam; and (C) plasma with 1.4 μg/ml piroxicam from a healthy subject 4 hours after an oral dose of 30 mg piroxicam.

Peaks: P = Piroxicam; I/S = internal standard (isoxicam).

TABLE 1

Within-day Reproducibility and Precision of the Assay for Piroxicam in Plasma

Spiked Concentration (μg/ml)	n	Observed Concentration[1] (μg/ml)	C.V. (%)	Accuracy[2] (%)
0.05	6	0.057 ± 0.004	7.0	114
0.1	5	0.102 ± 0.007	6.0	102
2.5	5	2.40 ± 0.088	3.7	96
5	5	4.65 ± 0.16	3.4	93

[1] Results given are mean ± S.D.

[2] Accuracy (%) $= \frac{\text{observed concentration}}{\text{spiked concentration}} \times 100$

internal standard, isoxicam was also good at the concentration used with a recovery of 77 ± 2% (n = 6).

The calibration curve for piroxicam was linear over the concentration range of 0.05 to 10 μg/ml with the square of correlation coefficient (r^2) greater than 0.99. The typical linear relationship for the calibration curve can be expressed by the equation : $y = 1.2945x$, where y is the peak height ratio and x is the plasma piroxicam concentration. The intercepts (a) in all calibration curves were found to be statistically insignificant ($p > 0.1$) and were thus not included for the calculations. The day-to-day coefficient of variation (C.V.) of the slope of the calibration curves of piroxicam was 6.3% (n = 5).

The within-day reproducibility and accuracy of the piroxicam assay in plasma is shown in Table 1. The intra-assay (within-day) coefficients of variation were less than 8% at all concentrations studied. The inter-assay precision (between days) of the assay for piroxicam was reasonable with a coefficient of variation less than 10% at piroxicam concentrations of 0.1 μg/ml and 5 μg/ml. The accuracy of the assay was greater than 93% for all the evaluated concentrations of piroxicam (Table 1).

Repeated determinations of the piroxicam concentration in the subject samples were also carried out to assess the accuracy of the analytical method. Random plasma samples from a bioavailability study were selected and analysed for piroxicam (2 samples/subject, 18 subjects). The repeated determinations were performed on different days, usually 7-10 days after the original determination.

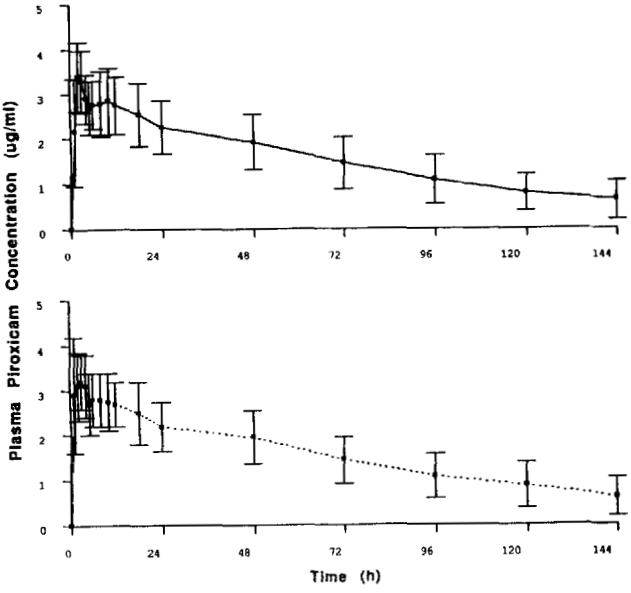

FIGURE 2

Mean plasma concentration of piroxicam versus time in 18 healthy subjects after an oral administration of 30 mg piroxicam. Subjects ingested either an oral dose of Feldene-D (Pfizer Lab. Ltd, New Zealand, *upper panel*) or a new formulation of piroxicam dispersible tablets (Pacific Pharm. Ltd., New Zealand, *lower panel*). The vertical bars represent the S.D. of the mean.

The results indicate a consistency in the piroxicam concentration between the original and the repeated determination with an accuracy of 101 ± 6.7% (n = 36), range 88 to 113%.

The reproducibility result at a concentration of 0.05 μg/ml gave mean and S.D. for the observed value of 0.057 ± 0.004 μg/ml with a C.V. of 7.0%. This C.V. is much lower than the generally accepted C.V. of 20% for minimum quantifiable concentration (MQC). A typical chromatogram of 0.05 μg/ml plasma piroxicam standard is presented in Figure 1 (B). Thus the MQC or the detection limit of sensitivity for this assay was assigned at 0.05 μg/ml.

TABLE 2

Pharmacokinetic Parameters of Piroxicam for the New Preparation, 10 mg Piroxicam Dispersible Tablets (Pacific Pharmaceuticals Ltd, New Zealand) and 10 mg Feldene-D Tablets (Pfizer Laboratories Ltd, New Zealand).

Parameter	10 mg Piroxicam Tablet		ANOVA
	Pacific	Feldene-D	
Cmax (μg/ml)	3.8 ± 1.1*	3.8 ± 1.0	$p > 0.05$
tmax (hr)	2.3 ± 1.0	2.2 ± 1.5	$p > 0.05$
$t_{1/2}$ (hr)	58.6 ± 28.0	57.3 ± 26.6	$p > 0.1$
AUC (μg hr/ml)	293 ± 149	290 ± 158	$p > 0.2$
Relative bioavailability (Fr)**	1.03 ± 0.15	-	-

* Data given are mean ± S.D. (n = 18)
**Fr = $AUC_{Pacific}/AUC_{Feldene\text{-}D}$

Plasma samples stored at -70°C for up to 4 months showed no signs of decomposition and practically the same concentration values were obtained (n = 6). This suggests that piroxicam is stable under these storage conditions for at least 4 months.

The applicability of the assay described was demonstrated by the analysis of plasma samples from 18 subjects receiving an oral dose (30 mg) of piroxicam in a bioavailability study. Mean plasma piroxicam concentration-time profiles following an oral administration of either 3 x 10 mg Piroxicam dispersible tablets or 3 x 10 mg Feldene-D tablets are shown in Figure 2. Table 2 lists the Cmax, tmax, $t_{1/2}$, AUC and relative bioavailability (Fr) of piroxicam resulting the oral administration of the two dosage forms. There was no difference in Cmax, tmax, $t_{1/2}$ and AUC between the two formulations ($p > 0.05$). These results indicate that the 10 mg Piroxicam dispersible tablets (Pacific Pharmaceuticals Ltd, New Zealand) are bioequivalent to 10 mg Feldene-D tablets (Pfizer). In this particular study, the plasma samples were collected up to 6 days after a single oral dose of 30 mg piroxicam. None of the samples had a concentration of piroxicam below the detection limit of 0.05 μg/ml.

In summary, a simple and sensitive HPLC procedure has been described for the quantitative analysis of piroxicam. This assay is a direct plasma protein precipitation so is less tedious and rapid. The method has been shown to be suitable for routine use in bioavailability and pharmacokinetic studies.

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