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Note

High-performance liquid chromatographic assay for piroxicam in human plasma

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Piroxicam is a non-steroidal anti-inflammatory drug that is used in the treatment of rheumatoid arthritis and osteoarthritis [1,2]. It has become apparent that accurate characterization of the pharmacokinetic profile of a drug requires drug quantitation methodology that is highly sensitive. Several high-performance liquid chromatographic (HPLC) techniques have been developed for piroxicam determinations in plasma [3–8]. The limit of quantitation of piroxicam for these assays ranges from 50 to 500 ng/ml.

This report describes a rapid and highly sensitive (2 ng/ml) HPLC method for the assay of piroxicam in human plasma.

EXPERIMENTAL

Chemicals and reagents

Piroxicam was purchased from Sigma (St. Louis, MO, U.S.A.); 6′-methylpiroxicam and 5′-hydroxypiroxicam were obtained from Pfizer (Groton, CT, U.S.A.); methanol and methylene chloride, HPLC grade, were purchased from J.T. Baker (Phillipsburg, NJ, U.S.A.). All other chemicals were of analytical grade and obtained from J.T. Baker.

Preparation of standards

Stock solutions of 1 mg/ml piroxicam and internal standard (6′-methylpiroxicam) were prepared in 0.04 *M* phosphate buffer, pH 8.0 and stored at 4°C. Appropriate dilutions of these solutions were made with water to produce working solutions containing 100, 10, 1 and 0.1 μg/ml for piroxicam and 10 μg/ml for

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internal standard. Plasma calibration standards containing 2–50 000 ng/ml piroxicam were prepared by diluting the working solutions with blank plasma; the final volume of all plasma calibration standards was 1.0 ml.

Extraction procedure

Samples of plasma (1 ml) were placed in 20 mm × 150 mm glass culture tubes with screw caps (PTFE-lined); 50 µl of internal standard (500 ng), 250 µl of 1 *M* phosphate buffer, pH 2, and 10 ml of methylene chloride were added. The tubes were capped and shaken for 15 min at low speed and then centrifuged at 2000 *g* for 5 min. The aqueous layer was removed by aspiration and the methylene chloride was transferred to a 16 mm × 100 mm disposable glass culture tube and evaporated to dryness at 30 °C under a stream of nitrogen gas. The extraction residue was reconstituted with 300 µl of mobile phase (0.04 *M* phosphate buffer, pH 8–methanol, 60:40, v/v) and transferred to a disposable 300-µl polypropylene HPLC injection tube.

Chromatography

The HPLC system consisted of a Model M45 solvent delivery system, a Model 710B WISP autoinjector, a Model 481 variable-wavelength UV detector (Waters Assoc., Milford, MA, U.S.A.) and a Model 3390A reporting integrator (Hewlett-Packard, Avondale, PA, U.S.A.). An Econosphere ODS column (25 cm × 4.6 mm I.D., 5 µm particle size) (Alltech Assoc., Deerfield, IL, U.S.A.) protected with a 40 mm × 4.6 mm guard column packed with pellicular C_{18} (Alltech Assoc.) was used to separate the compounds. The mobile phase was 0.04 *M* phosphate buffer, pH 8.0–methanol (60:40, v/v).

Aliquots (5–200 µl) of reconstituted sample extracts were injected into the HPLC system and eluted with mobile phase at a flow-rate of 1.2 ml/min. The eluates were monitored at 360 nm with the detector range setting fixed at 0.005 a.u.f.s.

Quantitation

Concentrations of piroxicam in unknown samples were determined from the slope of calibration plots of the peak-area ratio of piroxicam/internal standard versus the calibration standard piroxicam concentrations. A weighted ($1/y$) least-squares regression was fitted to the entire range of standards. Reciprocal peak-area ratios were found to be acceptable as weighting factors for generating a normal distribution of weighted residuals.

Assay specifications

The extraction recovery of piroxicam was assessed at various piroxicam concentrations covering the range of the calibration standards (2–50 000 ng/ml). The recovery of internal standard was assessed at the concentration (500 ng/ml) at which it is used in the assay. Three plasma samples (1 ml) containing piroxicam and internal standard were extracted and injected. Three injections of the same amount of compound in mobile phase were directly injected. The peak areas

of the compounds were measured and the percentage recovery was calculated from 100×peak area, extract/mean peak area, direct injection.

The precision of the assay was determined by assay of ten samples prepared by spiking blank plasma with piroxicam. Assay reproducibility was assessed at 10, 100, 1000 and 10 000 ng/ml piroxicam.

The specificity of the assay was determined by comparing retention times of standards to those of samples and by comparing the sample peak-area ratio of piroxicam/internal standard at three wavelengths (300, 360 and 400 nm) to the ratio of the standards.

RESULTS AND DISCUSSION

Chromatograms of extracts of blank plasma, blank plasma spiked with piroxicam and internal standard and a plasma sample taken after a single oral dose of 20 mg piroxicam (Feldene, Pfizer) in a healthy male volunteer are shown in Fig. 1. No endogenous interfering peaks were visible in blank plasma. The two peaks are well separated with retention times of 5.9 and 8.4 min for piroxicam and

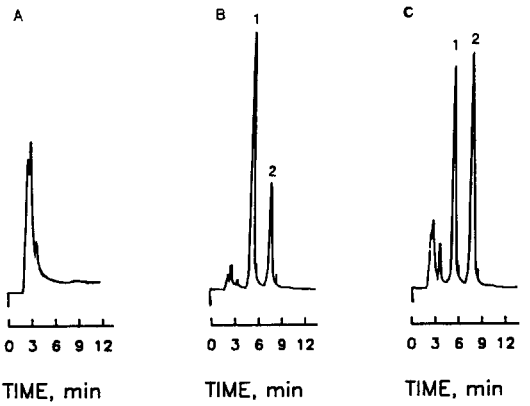

Fig. 1. Chromatograms of plasma extracts for (A) blank plasma, (B) blank plasma spiked with 2000 ng piroxicam (1) and 500 ng internal standard (2) and (C) plasma sample taken 120 h (437 ng/ml piroxicam) after a single oral dose of 20 mg piroxicam.

TABLE I

ASSAY PRECISION

Concentration (ng/ml)	Coefficient of variation (%)	
	Intra-day	Inter-day
10	5.5	7.8
100	2.1	2.0
1000	2.6	1.1
10 000	2.4	1.2

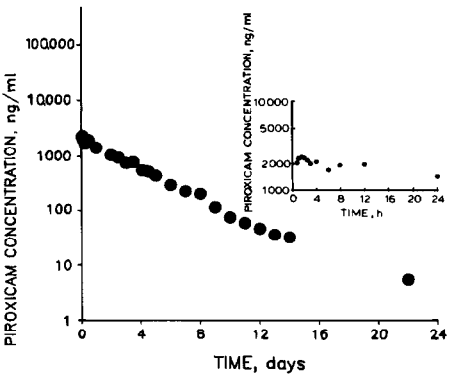

Fig. 2. Plasma concentrations of piroxicam after 20 mg oral piroxicam in a healthy volunteer. The inset shows plasma piroxicam concentrations up to 24 h.

internal standard, respectively. Injection of 5′-hydroxypiroxicam, the major metabolite of piroxicam [9], yielded a peak at 4.4 min which did not interfere with piroxicam. The baseline separation of 5′-hydroxypiroxicam from the solvent front and from piroxicam suggests that this piroxicam assay could be easily adapted for the determination of the metabolite as well. An additional assurance of specificity was provided by observing that the 300 to 360 nm and 400 to 360 nm peak-area ratios of standard and subject samples were the same.

The extraction recovery of piroxicam and internal standard was independent of concentration with a mean ($\pm$S.D.) recovery of 91 $\pm$ 6%. The sensitivity limit of the assay was 2 ng/ml when a signal-to-noise ratio of 3 was used as a criterion for a significant response. Calibration plots of peak-area ratio (piroxicam/internal standard) were linear over the range of 0–50 000 ng/ml and the precision of the assay was acceptable as demonstrated in Table I. The accuracy of the method, calculated by comparing the results from the precision study to the known piroxicam concentrations, was 97% at piroxicam concentrations equal to or greater than 100 ng/ml and 93% at lower drug concentrations.

A typical plasma concentration–time profile after 20 mg oral piroxicam (Feldene) in a normal volunteer obtained with this assay is presented in Fig. 2. These results are in good agreement with those obtained using other methods. However, whereas other methods allow for quantitation of piroxicam for up to ten days after a single 20-mg dose, plasma drug concentrations can be measured for more than three weeks utilizing this method.

In summary, the HPLC method presented here is precise, specific and very sensitive. The technique is relatively rapid and over 100 samples can be processed in one day. The method is sufficiently sensitive to quantitate plasma concentrations of piroxicam for over three weeks following administration of a single 20-mg dose.

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