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Letter to the Editor

Rapid analysis of piroxicam in dog, rat and human plasma by high-performance liquid chromatography

Sir,

Piroxicam, 4-hydroxy-2-methyl-N-(2-pyridyl)-2H-1,2-benzothiazine-3-carboxamide 1,1-dioxide, a non-steroidal anti-inflammatory agent of the benzothiazine family, has demonstrated efficacy in man in the treatment of rheumatoid arthritis and other inflammatory disorders. Consequently, a number of assays have been developed for piroxicam and applied to investigations concerned with its disposition following administration to man [1–6]. However, few methods are available for plasma analysis of species other than man [7]. As animal species are frequently used as models for drug interaction studies prior to human studies, it is advantageous to utilize analytical methods with inter-species applicability. Such methods require sensitivity, resolution of parent drug and metabolite from interfering endogenous substances, and low volume sample size required by frequent sampling of small animals. This report describes a sensitive and rapid high-performance liquid chromatographic (HPLC) assay for quantitation of piroxicam in small-volume plasma samples obtained from rat, dog and human, and has demonstrated accuracy, precision and stability when used for the analyses of 600 canine and rat samples obtained from drug interaction studies.

EXPERIMENTAL

Reagents and standards

Piroxicam and naproxen, used as the internal standard, were obtained from Sigma (St. Louis, MO, U.S.A.). The metabolite 5′-hydroxypiroxicam was kindly provided by Pfizer (Groton, CT, U.S.A.). All solvents and reagents were HPLC analytical grade. Stock solutions of piroxicam and naproxen (1 mg/ml) were prepared in methanol and stable for at least one month at 4°C. Heparinized plasma standards were prepared in the concentration ranges 0.5–10.0 μg/ml (animal) and 0.25–5.0 μg/ml (human) using appropriate dilutions of the piroxicam sec-

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ondary solution (200 μg/ml in methanol). The internal standard concentrations were 20 μg/ml (animal) and 100 μg/ml (human) in 2% methanol.

Chromatography

The HPLC system was equipped with an absorbance detector (Kratos Model 773 or 783, Ramsey, NJ, U.S.A.). Small animal effluent was monitored at 330 nm (0.005 a.u.f.s.), whereas human sample effluent was monitored at 360 nm (0.01 a.u.f.s.) and 330 nm (0.02 a.u.f.s.) for piroxicam and naproxen, respectively. Separations were performed on a Spherisorb C_8 column (15 cm × 4.6 mm I.D., cut to 5 cm length, 5 μm particle size, Chromanetics, Williamstown, NJ, U.S.A.) with a mobile phase of acetonitrile–tetrahydrofuran–methanol–2% (v/v) acetic acid (pH 2.5) (5:18:5:72, v/v) operating at ambient temperature and at a flow-rate of 1.5 ml/min.

Sample preparation

For animal analysis, 0.1 ml of plasma standard or sample, 0.1 ml of 1 *M* hydrochloric acid, 0.1 ml of internal standard (20 μg/ml), and 6.0 ml of methyl *tert.*-butyl ether were pipetted into a 15-ml PTFE-lined screw-cap tube. After vigorous vortexing (1 min), samples were centrifuged (2 min, 1200 *g*) and the ether layer was transferred for evaporation under reduced pressure at 35°C. The resulting residue was immediately reconstituted with 1.0 ml of 2% (v/v) methanol and fully dissolved by a 2-min vortexing. A 100-μl volume was then injected onto the column; a 24-h stability of this solution in glass tubes was demonstrated. For the analysis of human samples, 0.5 ml of plasma, 0.2 ml of hydrochloric acid, 0.1 ml of internal standard (100 μg/ml) and 8 ml of diethyl ether were vortexed and centrifuged. Following evaporation of the ether extract, the residue was dissolved in 2.0 ml of 2% methanol, and 100 μl were injected on column.

RESULTS AND DISCUSSION

Typical chromatograms from pre- and post-dose extracted rat, dog and human plasma samples (Fig. 1) demonstrate the lack of interfering substances and baseline resolution of the peaks. The retention times for piroxicam, 5'-hydroxypiroxicam and naproxen are 2.2, 4.9 and 8 min, respectively. As seen from these comparative chromatograms, interferences from compounds in human plasma were generally less than those of dog and rat.

The standard curves were generated by least-squares linear regression analysis of the peak-height ratio of piroxicam to naproxen versus the concentration. The curves were linear over a twenty-fold concentration range for all three species ($r^2 \geq 0.999$). The mean standard error of estimate, an estimate of the standard deviation about the computed line which quantitates assay precision, was 0.11, 0.14 and 0.09 for dog, rat and human, respectively. A further two- to five-fold increase of the quantitation range was successfully achieved by using sample dilution. Table I displays the accuracy and precision obtained from dilution studies. To evaluate the between-day reproducibility of the assay, plasma samples, spiked over the concentration range (0.25–50.0 μg/ml) and stored at −20°C, were ana-

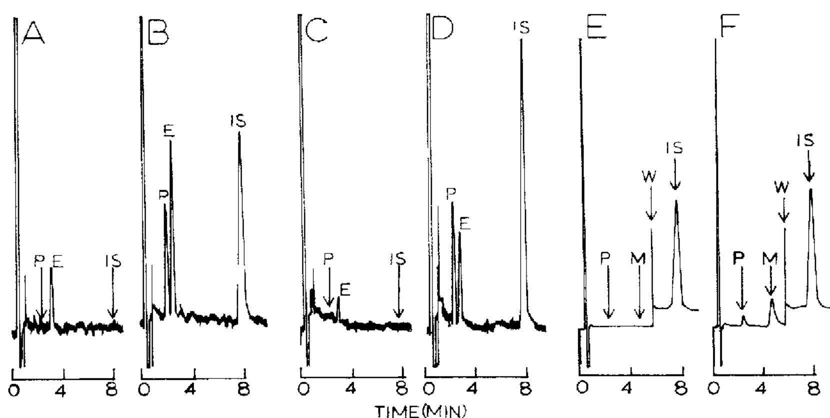

Fig 1 Chromatograms of extracted plasma samples. (A) Pre-dose canine plasma sample with endogenous substance (E); arrows mark retention times for piroxicam (P) and internal standard (IS); (B) 30 min post-dose sample (0.4 mg/kg dose); measured concentration of piroxicam is 1 5 μg/ml; (C) pre-dose rat plasma sample; (D) 90 min post-dose sample (0.5 mg/kg dose); measured concentration of piroxicam is 1.3 μg/ml; (E) blank human plasma with internal standard; wavelength change (W) from 360 to 330 nm; (F) extract from human plasma spiked to contain 0.25 μg/ml piroxicam (P), 1.0 μg/ml 5′-hydroxypiroxicam (M) and 100 μg/ml naproxen (IS)

TABLE I

INTER-ASSAY ACCURACY AND PRECISION OF DILUTION CONTROL AND QUALITY CONTROL SAMPLES FOR PIROXICAM IN DOG, RAT AND HUMAN PLASMA

Species	Nominal value (μg/ml)	Dilution factor	n	Mean found (μg/ml)	C.V. (%)	Recovery (%)
Dog	20.0	5	6	22.5	5.1	113.0
	0.5	–	17	0.45	13.3	90.0
	1.0	–	17	0.98	7.1	98.0
	3.0	–	18	3 2	4.4	106.0
	8.0	–	17	8.3	3.0	103.0
Rat	50.0	10	6	49.3	2.3	98.5
	0.5	–	10	0.50	13.4	104.0
	1.0	–	10	1.0	6 8	103.0
	3.0	–	10	3.1	3.5	104.0
	8 0	–	10	8.5	4 2	106.0
Human	20.0	5	6	21.1	9.7	105.4
	0.25	–	6	0.24	12 5	96.0
	0.6	–	6	0.61	6.6	102.0
	2.5	–	6	2.5	6.5	99.0
	4.0	–	6	3.9	6.4	98.0

lyzed over at least a six-day period. Table I presents the precision (coefficient of variation) and analytical recovery data obtained from assayed plasma from the three species. The extraction efficiency of piroxicam from these samples averaged greater than 86%. Freeze–thaw stability was evaluated by subjecting selected rat

and dog quality control samples to three freeze–thaw cycles; the coefficients of variation and analytical recovery were comparable to the single-thaw data.

Our laboratory has been unsuccessful in performing piroxicam analysis of rat and canine plasmas utilizing published methods optimized for human samples. The resolution of the major metabolite from endogenous plasma compounds was obtained by modifying a C_8 column to 5 cm in conjunction with tetrahydrofuran as the principle organic eluter to provide good resolution and preserve peak symmetry of all peaks. An apparent lack of stability of redissolved extract [6] was remedied by use of 2% methanol as the solvent; this provided at least a 24-h stability and permits unattended automated injection technique. While Tsai et al. [7] reported a suitable method for human and rabbit plasma analysis, a sample volume of 0.5 ml is unsatisfactory for serial sampling in rats; the present method provides the same sensitivity (0.5 μg/ml) using a 0.1-ml sample.

Piroxicam absorbs maximally at 339 or 358 nm, in dilute acid or in methanolic solutions, respectively [8]. However, naproxen does not absorb above 340 nm [1,9]. The use of 330 nm provides an acceptable compromise wavelength for the detection of both compounds, whereas 360 and 330 nm for piroxicam and 5′-hydroxypiroxicam, and naproxen, respectively, can be advantageously used with the more technically sophisticated instrumentation to provide maximum sensitivity for piroxicam with minor baseline disturbance.

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CHAU-HWEI JOKY FU

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