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Determination of piroxicam and its major metabolites in the plasma, urine and bile of humans by high-performance liquid chromatography

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ABSTRACT

A simple and sensitive liquid chromatographic method with ultraviolet detection is described for the determination of the non-steroidal anti-inflammatory drug piroxicam and its major metabolites in human plasma, urine and bile. Separation of these components occurs on a reversed phase $C_{10}CN$ column with a mobile phase consisting of acetonitrile–water–sodium dihydrogenphosphate solution. The detection limit of the assay was 50 ng/ml with intra- and inter-assay coefficients of variation for piroxicam of the order of 2 and 5%, respectively. The assay linearity was good (typically $r = 0.9999$). This method can be readily utilised for clinical pharmacokinetic and mass-balance studies.

INTRODUCTION

The non-steroidal anti-inflammatory drug (NSAID) piroxicam is extensively metabolised by hepatic cytochrome P450 enzyme, principally to the hydroxyl metabolite. Hydroxylation occurs at the 5-position in the pyridine ring and the hydroxylated metabolite undergoes subsequent glucuronidation. Less than 5% of the parent drug is excreted in the urine, and, under steady state conditions, 75% of a dose is excreted as either 5-hydroxypiroxicam or 5-hydroxypiroxicam glucuronide in the urine and faeces [1]. Little is known of the biliary disposition of the drug in man.

Early studies on the pharmacokinetics of piroxicam employed a degradative fluorometric "wet" chemical analytical technique. This involved strong acid hydrolysis of piroxicam to generate 2-aminopyridine followed by fluorometric assessment of this product after purification. External standards were used to determine the concentration of drug present in the sample. This technique was not ideal as it lacked both sensitivity and selectivity. By using chromatographic techniques improved assay systems were developed. Chromatography could be performed on biological samples containing piroxicam without chemical modification of the drug. Furthermore, they were simpler analytically, more rapid and, as they incorporated internal standards, they were also more accurate. Table I lists the published chromatographic methods for the analysis of this drug (and in some instances 5-hydroxypiroxicam).

It was considered important to develop an assay system which would be flexible enough to cope with a variety of biological samples (plasma, urine and bile) with sufficient accuracy and precision to be able to detect quantities of both piroxicam and 5-hydroxypiroxicam at concentrations as low as 50 ng/ml in each of these body fluids. A high-performance liquid chromatographic (HPLC) system was envisaged for the

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TABLE I

PUBLISHED ASSAY METHODS FOR PIROXICAM QUANTITATION IN HUMAN BIOLOGICAL SAMPLES

Reference	Year	Column type	Plasma/ urine	Sample volume (ml)	Internal standard (I.S.)
Twomey *et al.* [2]	1980	HPLC (μ Bondapak CN)	P	0.1	Isoxicam (10 μg)
Riedel and Laufen [3]	1983	HPTLC	P/U (and tissue)	0.5	Piretanide (0.5 μg)
Fraser and Woodbury [4]	1983	LC (LiChrosorb RP-8)	P	1.0	Naproxen (100 μg)
Dixon *et al.* [5]	1984	HPLC (LiChrosorb RP-18)	P	1.0	Tenoxicam (100 μg)
Tsai *et al.* [6]	1985	HPLC (LiChrosorb RP-18)	P/U	0.5 (plasma) 2.0 (urine)	Indomethacin (10 μg)
Richardson *et al.* [7]	1986	HPLC (μBondapak CN for plasma) (μBondapak C_{18} for urine)	P/U	0.5 (plasma) 1.0 (urine)	Isoxicam (10 μg)
Macek and Vacha [8]	1987	HPLC (Separon Six CN)	P	0.2	–
Boudinot and Ibrahim [9]	1988	HPLC (Econosphere)	P	1	6-Methylpiroxicam (500 μg)
Gillilan *et al.* [10]	1989	HPLC (Spherisorb C_{18})	P	0.5	Naproxen (10 μg)
Lapicque *et al.* [11]	1989	HPLC (Nova Pak C_{18})	P	0.2	(Benzoyl-4-phenyl)-2-butyric acid (0.1–5 μg)
Saeed and Becher [12]	1991	HPLC (Supelcosil LC18 DB)	P	0.02	–

reasons described. From the point of view of simplicity, use of a single column to enable analysis of piroxicam and 5-hydroxypiroxicam in all biological samples, without sacrificing accuracy, was desired. None of the published assays met these conditions for both plasma and urine and no published reports were available for the analysis of this drug and/or metabolite in bile.

EXPERIMENTAL

Reagents and materials

The following chemicals were used in all analyses: acetic acid, diethyl ether, hydrochloric acid, orthophosphoric acid, sodium dihydrogenphosphate (Pronalys AR, May and Baker; Manchester, UK); acetonitrile (HPLC Far UV, Fisons, Loughborough, UK); citric acid, sodium acetate (AnalaR, FSA, Loughborough, UK); disodium phosphate, sodium hydroxide (AnalaR, BDH, Poole, UK); β-glucuronidase enzyme (Bovine Type B1, Sigma, Poole, UK).

Drugs

Piroxicam, 4-hydroxy-2-methyl-N-(2-pyridyl)-2H-1,2-benzothiazine-3-carboxamide 1,1-diox-

Extraction agent	Injection volume (μl)	Detection	Recovery (%)	Linear range (mg/l)
Diethyl ether	100	UV (365 nm)	I.S. 74 Piroxicam, 77	0.5–20
Dichloromethane	50	Fluorescence (excitation 366 nm)	I.S. 85 Piroxicam, 95	0.1–15
Chloroform	10	UV (330 nm)	I.S. 85 Piroxicam, 88	0.5–30
Dichloromethane	20	UV (361 nm)	I.S. 81 Piroxicam, 81	0.2–20
Diethyl ether	50	UV (365 nm)	5-Hydroxypiroxicam, 65 Piroxicam, 70	0.5–25
Diethyl ether	50	UV (365 nm)	I.S. 90	
		Plasma	5-Hydroxypiroxicam, 90 Piroxicam, 85	0.05–5
		Urine	5-Hydroxypiroxicam 84 Piroxicam, 86	0.1–5
– (precipitation)	50	UV (360 nm)	–	0.27–10.8
Methylene chloride	5–200	UV (360 nm)	Piroxicam, 91	0.002–50
Diethyl ether	100	UV (360 nm)	Piroxicam, 100	0.25–5
Diethyl ether, chloroform, ethyl acetate	200	UV (254, 370 nm)	Piroxicam, 98	0.1–80
Bond Elut C_2 (solid phase)	–	UV (331 nm)	Piroxicam, 95–97	1–25

ide, was a gift from Pfizer (Sandwich, UK) and 5-hydroxypiroxicam was a gift from Pfizer (Groton, CT, USA). Isoxicam, 4-hydroxy-2-methyl-N-(5-methyl-3-oxazolyl)-2H-1,2-benzothiazine-3-carboxamide 1,1-dioxide, was a gift from Warner-Lambert/Parke-Davis (Pontypool, UK).

Extraction procedure in plasma

To 0.5 ml of plasma, in a 12-ml heavy duty glass centrifuge tube (manufactured by Glass Blowing Department, Glasgow University) were added 1 ml of 0.01 *M* sodium hydroxide (containing piroxicam and 5-hydroxypiroxicam if spiking samples in order to obtain calibration line), 0.1 ml of internal standard solution (0.1 mg/ml isoxicam in 0.01 *M* sodium hydroxide), 0.25 ml of 1 *M* hydrochloric acid and 5 ml of diethyl ether. The tubes were stoppered and shaken for 6 min at 240 rpm using an orbital shaker (Gallenkamp, Loughborough, UK) and centrifuged at 900 *g* for 10 min at 4°C (MSE Mistral 2L, Loughborough, UK). The ether phase

was transferred to a Pyrex tube (Corning, Halstead, UK) and evaporated to dryness (37°C) under nitrogen (Techne Dri-block DB3, Duxford, UK). The residue was reconstituted in 250 μl of acetonitrile-water (50:50) and vortex-mixed for 60 s (SMI multi-tube vortexer 2601, Emeryville, CA, USA) immediately prior to injection (50 μl) on to the column.

Extraction procedure in urine and bile

The extraction procedure for unconjugated piroxicam and 5-hydroxypiroxicam in the urine and bile was identical to that used in plasma, with the addition of one extra stage. After collection of the ether phase, a back-extraction was performed to purify the sample. Citric acid–phosphate buffer (pH 4.9) (2 ml) was added to the diethyl ether in a clean centrifuge tube. This was again shaken and centrifuged before collecting the ether phase. The diethyl ether was then evaporated to dryness and the residue reconstituted as before.

For the measurement of 5-hydroxypiroxicam glucuronide, 5 ml of urine or bile were adjusted to pH 5.0 with an equal volume of 0.1 *M* acetate buffer. This was incubated for 24 h in a water bath at 37°C, in the presence of 500 I.U. bovine liver β-glucuronidase. A 1-ml volume of this mixture was then acidified by 0.25 ml of 1.0 *M* hydrochloric acid after addition of internal standard (0.1 ml of 0.1 mg/ml isoxicam in 0.01 *M* sodium hydroxide). Extraction was performed as per the unconjugated 5-hydroxypiroxicam.

In all instances, residues were reconstituted in 250 μl of acetonitrile-water (50:50) and vortex-mixed for 60 s (SMI multi-tube vortexer 2601) prior to injection (50 μl) on to the column.

Reversed-phase chromatography

Instrumentation included a Waters (Milford, MA, USA) WISP 710B autosampler, a Waters M45 pump, a fixed-wavelength UV detector (Shimadzu SPD6A, Kyoto, Japan) and an integrator (Shimadzu CR3A Chromatopack). For simultaneous quantitation of piroxicam and 5-hydroxypiroxicam in plasma, urine and bile a Techsil C_{10} CN column (20 cm × 3.9 mm I.D., 10 μm particle size, HPLC Technology, Macclesfield, UK) was used. For the analysis of plasma and urine the mobile phase consisted of 50 m*M* sodium dihydrogenphosphate in acetonitrile–water (22:78) with a final pH of 3.5. For the analysis of bile the mobile phase was similar except that the acetonitrile concentration was 10%.

The flow-rate in all instances was 2.5 ml/min and the eluate was monitored at 365 nm. A guard column using Techsil C_{10} CN was employed in all instances and replaced after each batch run (48 samples). Analysis was performed at ambient room temperature.

Spiked samples were in each batch run to cover the concentration range 0.05–20 mg/l. These were used to construct a calibration line for that particular batch run. In addition, 0.5 mg/l standard samples were dispersed throughout the patient samples in order to evaluate assay performance during the analysis.

Pharmacokinetic study

The patient studied was a 72-year-old female with common bile duct stones and a history of obstructive jaundice and pyrexia. The study was performed five days after insertion of a nasobiliary drain at a time when her liver function tests had normalised.

As a single dose of piroxicam, 20 mg, would produce levels of 5-hydroxypiroxicam in plasma approaching the assay detection limits, the patient received 30 mg of piroxicam daily for two days prior to the study so that detectable levels of metabolites might be obtained. After an overnight fast, 20 mg of piroxicam was given at time zero. Blood samples (5 ml) were obtained at 0, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 12 and 24 h. Total urine output was collected for the 24-h period in addition to total bile output which was collected in aliquots from -2 to 0, 0 to 2, 2 to 4, 4 to 6, 6 to 8, 8 to 10, 10 to 12, and 12 to 24 h.

Plasma samples were assayed for both piroxicam and 5-hydroxypiroxicam. Urine and bile were assayed for piroxicam, 5-hydroxypiroxicam and 5-hydroxypiroxicam glucuronide.

Linear/logarithmic trapezoidal areas under the curve (AUCs) were calculated for both piroxicam

TABLE II

EXTRACTION RECOVERIES OF PIROXICAM (P) AND 5-HYDROXYPIROXICAM (5-OHP) FOR PLASMA, URINE AND BILE SAMPLES

Concentration (mg/l)	Recovery (mean ± S.D., n = 3) (%)					
	Plasma		Urine		Bile	
	P	5-OHP	P	5-OHP	P	5-OHP
0.05	93 ± 9	100 ± 15	100 ± 13	100 ± 3	100 ± 8	100 ± 8
0.1	88 ± 6	92 ± 6	100 ± 14	107 ± 4	87 ± 4	96 ± 5
0.5	93 ± 3	96 ± 4	94 ± 9	93 ± 4	88 ± 4	88 ± 3
5	94 ± 2	91 ± 2	99 ± 2	95 ± 4	89 ± 5	100 ± 2[a]
20	99 ± 1	90 ± 1	101 ± 2	90 ± 4	93 ± 3	88 ± 2[a]

[a] n = 2.

TABLE III

TYPICAL CORRELATION COEFFICIENTS OF PIROXICAM (P) AND 5-HYDROXYPIROXICAM (5-OHP) FOR PLASMA, URINE AND BILE SAMPLES

Sample	Compound	r	Slope	Intercept
Plasma	P	0.9999	0.4633	$-4.7 \cdot 10^{-3}$
	5-OHP	0.9999	0.2362	$-1.1 \cdot 10^{-3}$
Urine	P	0.9999	0.4175	0.0199
	5-OHP	0.9999	0.2198	$9.1 \cdot 10^{-3}$
Bile	P	0.9997	0.6583	−0.0795
	5-OHP	0.9999	0.2674	$8.1 \cdot 10^{-3}$

and 5-hydroxypiroxicam in the plasma and for piroxicam and 5-hydroxypiroxicam (before and after glucuronidase incubation) in bile and urine.

RESULTS

Extraction recoveries

Table II lists the extraction recoveries consistently obtained in each biological sample. The addition of a purification step to both urine and bile samples improved the recovery of low concentration samples as the influence of interfering peaks was lessened. The results obtained are in agreement with the previously published values shown in Table I. The extraction recoveries obtained for isoxicam, the internal standard, were

TABLE IV

INTRA-ASSAY (n = 4) ACCURACY AND REPRODUCIBILITY OF PIROXICAM (P) AND 5-HYDROXYPIROXICAM (5-OHP) FOR PLASMA, URINE AND BILE SAMPLES

Concentration (mg/l)	Plasma				Urine				Bile			
	P		5-OHP		P		5-OHP		P		5-OHP	
	Mean (mg/l)	C.V. (%)	Mean (mg/l)	C.V. (%)	Mean (mg/l)	C.V. (%)	Mean (mg/l)	C.V. (%)	Mean (mg/l)	C.V. (%)	Mean (mg/l)	C.V. (%)
0.05	0.051	2.9	0.049	8.0	0.053	4.9	0.055	2.0	0.055	3.4	0.045	3.9
0.1	0.096	2.4	0.098	2.6	0.099	2.1	0.102	4.5	0.104	3.9	0.115	1.4
0.5	0.49	0.8	0.50	0.7	0.49	1.8	0.49	0.6	0.49	3.0	0.51	3.4
5	5.05	0.3	5.00	0.3	4.99	0.6	5.14	0.3	4.94	1.3	4.93	0.7
20	20.23	0.6	20.05	0.6	19.80	0.9	20.06	0.6	20.05	1.2	20.58	0.7

TABLE V

INTER-ASSAY (n = 6) ACCURACY AND REPRODUCIBILITY OF PIROXICAM (P) AND 5-HYDROXYPIROXICAM (5-OHP) FOR PLASMA, URINE AND BILE SAMPLES

Concentration (mg/l)	Plasma				Urine				Bile			
	P		5-OHP		P		5-OHP		P		5-OHP	
	Mean (mg/l)	C.V. (%)	Mean (mg/l)	C.V. (%)	Mean (mg/l)	C.V. (%)	Mean (mg/l)	C.V. (%)	Mean (mg/l)	C.V. (%)	Mean (mg/l)	C.V. (%)
0.05	0.049	5.4	0.067	19.1	0.051	3.3	0.057	2.0	0.079	24.0	0.074	21.2
0.1	0.096	6.8	0.100	2.9	0.099	11.6	0.102	3.4	0.107	6.7	0.089	3.0
0.5	0.50	1.5	0.49	4.7	0.50	3.2	0.50	6.3	0.49	8.6	0.48	8.6
5	5.00	1.5	5.00	1.4	4.99	2.7	4.99	1.9	5.00	8.1	4.95	7.6
20	19.99	2.8	19.99	2.5	19.97	2.5	19.96	1.8	20.08	9.9	20.02	9.3

of the order of 88, 94 and 90%, in plasma, urine and bile samples, respectively.

Linearity

The assay linearity for drug and metabolite was determined by performing linear regression analysis on the plot of the peak-height ratios of either piroxicam or 5-hydroxypiroxicam over the internal standard *versus* concentration in the range 0.05–20 mg/l. Table III lists the correlation coefficients obtained in the assay of both piroxicam and 5-hydroxypiroxicam using known standards in the range 0.05–20 mg/l. Correlation coefficients obtained were typically 0.9999.

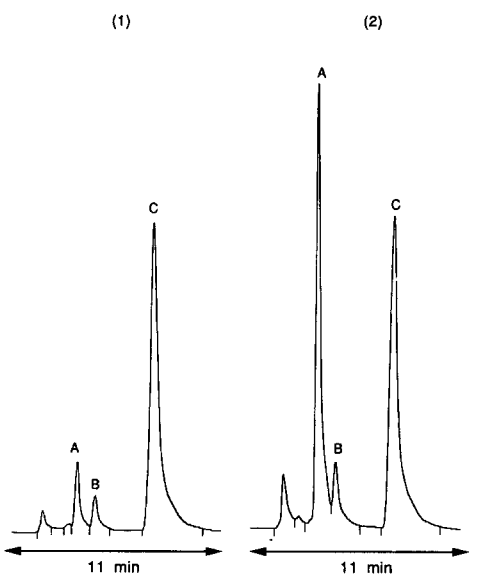

Fig. 1. Example of plasma chromatograms. (1) Plasma spiked with known concentrations of piroxicam and 5-hydroxypiroxicam (0.5 mg/l). (2) Patient sample corresponding to a piroxicam concentration of 3.26 mg/l and a 5-hydroxypiroxicam concentration of 0.89 mg/l. Peaks: A = piroxicam; B = 5-hydroxypiroxicam; C = isoxicam.

Assay precision

Bulk control standards were prepared by adding known amounts of piroxicam and 5-hydroxypiroxicam to blank plasma and urine. Pre-dose bile was collected from the individual and this was used as the control. Tables IV and V summarise the intra- and inter-assay coefficients of variation (C.V.) obtained over the range 0.05–20 mg/l, respectively. Only at the extreme end of the concentration range does the inter-assay variability approach 20%. For concentrations greater than 0.1 mg/l inter-assay variability is for most instances less than 10%.

Pharmacokinetic study

Fig. 1 shows an example of a typical plasma chromatogram. Figures 2 and 3 show an example of a chromatogram obtained both pre- and post-glucuronidase incubation in urine and bile, respectively.

Table VI lists the AUCs obtained for piroxicam, 5-hydroxypiroxicam and the total 5-hydroxypiroxicam (after glucuronidase incubation)

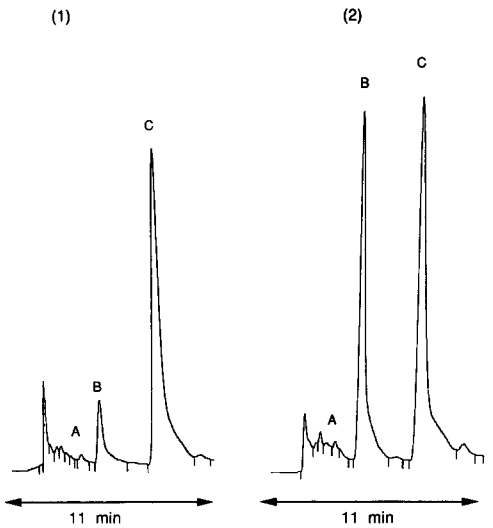

Fig. 2. Example of urine chromatograms. (1) Patient sample taken from urine pre-glucuronidase incubation (concentration of 5-hydroxypiroxicam is 0.90 mg/l). (2) Patient sample taken from urine post-glucuronidase incubation (concentration of 5-hydroxypiroxicam is 4.45 mg/l). Peaks: A = piroxicam; B = 5-hydroxypiroxicam; C = isoxicam.

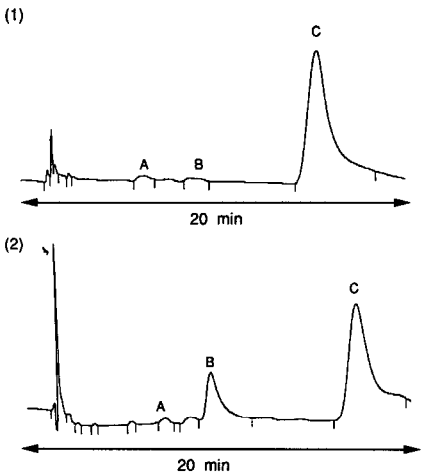

Fig. 3. Example of bile chromatograms. (1) Patient sample taken from bile pre-glucuronidase incubation (concentration of piroxicam is 0.11 mg/l and of 5-hydroxypiroxicam is 0.17 mg/l). (2) Patient sample taken from bile post-glucuronidase incubation (concentration of piroxicam is 0.11 mg/l and of 5-hydroxypiroxicam is 1.53 mg/l). Peaks: A = piroxicam; B = 5-hydroxypiroxicam; C = isoxicam.

in the plasma, urine and bile in this subject. The extensive metabolism of piroxicam is shown by the very small levels of the parent drug in either the urine or the bile. When 5-hydroxypiroxicam is considered, it is present in both the urine and plasma to a similar extent, with bile only contributing 15% of the total AUC value. However, when glucuronidated metabolite is considered, it can be seen that, similar to the situation found in the urine, the majority of 5-hydroxypiroxicam is glucuronidated. In the urine, approximately 20% of the total metabolite is unconjugated. In the bile this only accounts for 11%, indicating that the bile must be considered as a significant route of elimination for the metabolite in its conjugated form.

DISCUSSION

As has been previously outlined, the development of an HPLC assay system conferred advantages over other analytical techniques. However, problems were encountered in the development of this assay. Initially disposable plastic test tubes (not glass) were employed. It was discovered that adsorption of the drug and internal standard occurred into the plastic if left in contact with the plastic container walls. For this reason, disposable and heavy duty re-usable glassware were essential in this assay system.

Initially bile analysis was performed in a 22% acetonitrile mobile phase as discussed earlier. However, after glucuronidase incubation, interfering peaks were obtained which compromised

TABLE VI

PIROXICAM (P) AND 5-HYDROXYPIROXICAM (5-OHP) AUCs IN PLASMA, URINE AND BILE

Sample	AUC (mg/l/h)		
	P	5-OHP	
		Pre-incubation	Post-incubation
Plasma	113.76	12.97	–
Urine	1.39	16.56	81.36
Bile	2.52	4.77	42.28

the detection of small quantities of piroxicam and 5-hydroxypiroxicam. By decreasing the acetonitrile concentration to 10%, the retention times of these drugs relative to each other were increased and good separation was obtained. However, instead of tall, narrow peaks, short, broad peaks were obtained which the integrator, at very low concentrations, had difficulty in quantitating satisfactorily. Ratios of drug to internal standard peaks were always quantified in terms of heights and not areas as it was found that height was less variable. It also conferred the advantage that manual assessment of individual chromatograms could be performed at all stages which proved to be of particular benefit in the analysis of bile samples.

From the pharmacokinetic study it was observed that very little piroxicam enters the bile and biliary excretion, therefore cannot be considered an important route of elimination for piroxicam in this patient. However, 5-hydroxypiroxicam is present in twice the piroxicam concentration, in marked contrast to the situation in plasma, where piroxicam concentrations are approximately ten times the 5-hydroxypiroxicam concentrations. Furthermore, it would appear, at least in this subject, that 5-hydroxypiroxicam (as the glucuronide) is excreted into the bile in significant amounts. Piroxicam, in the form of glucuronides, was not detected in the bile.

In conclusion this assay provides a simple, rapid and sensitive method for the detection of piroxicam and its major metabolites in variety of biological fluids allowing the disposition of this drug to be investigated in greater detail than previously possible.

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