Journal of Chromatography, 305 (1984) 145—151
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 1888

HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC ANALYSIS OF ISOXICAM IN HUMAN PLASMA AND URINE

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(First received March 16th, 1983; revised manuscript received August 12th, 1983)

SUMMARY

A sensitive, selective, and rapid high-performance liquid chromatographic procedure was developed for the determination of isoxicam in human plasma and urine. Acidified plasma or urine were extracted with toluene. Portions of the organic extract were evaporated to dryness, the residue dissolved in tetrahydrofuran (plasma) or acetonitrile (urine) and chromatographed on a μBondapak C_{18} column preceded by a 4—5 cm × 2 mm I.D. column packed with Corasil C_{18}. Quantitation was obtained by UV spectrometry at 320 nm. Linearity in plasma ranged from 0.2 to 10 μg/ml. Recoveries from plasma samples seeded with 1.8, 4 and 8 μg/ml isoxicam were 1.86 ± 0.077, 4.10 ± 0.107 and 8.43 ± 0.154 μg/ml with relative standard deviations of 3.3%, 2.5% and 5.4%, respectively. The linearity in urine ranged from 0.125 to 2 μg/ml. The precision of the method was 3.3—9.0% relative standard deviation over the linear range.

INTRODUCTION

Isoxicam (Fig. 1) is one of the oxicams [1], a class of non-steroidal anti-inflammatory drugs (NSAID) that is structurally distinct from other classes of NSAID. The drug is sparingly soluble in water (2 μg/ml), has a pK_a of 3.65, shows good partitioning in an octanol—pH 4 acetate system (log P = 1.716), and is completely extracted into octanol from 0.1 M hydrochloric acid solution. Isoxicam is a potent long-acting anti-inflammatory agent, highly effective in relieving the signs and symptoms of rheumatoid arthritis and degenerative joint disease. The pharmacology, pharmacokinetics, and clinical efficacy of isoxicam have been recently summarized [2].

In the early stages of development a fluorometric assay procedure was used for the analysis of the drug in biological fluids. Although the method was simple and rapid it lacked specificity. The present report describes an assay for

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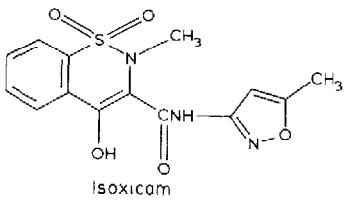

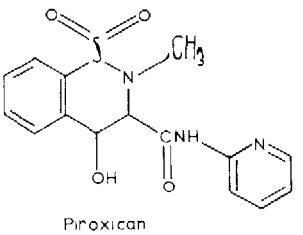

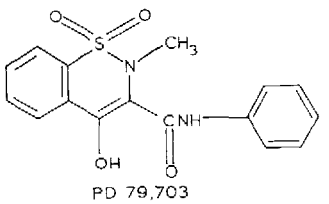

Fig. 1. Chemical structures of isoxicam [4-hydroxy-2-methyl-N-(5-methyl-3-isoxazolyl)-2H-1,2-benzothiazine-3-carboxamide 1,1-dioxide], piroxicam [4-hydroxy-2-methyl-N-2-pyridinyl-2H-1,2-benzothiazine-3-carboxamide 1,1-dioxide], and PD 79,703 [4-hydroxy-2-methyl-2H-1,2-benzothiazine-3-carboxanilide 1,1-dioxide].

isoxicam in human plasma and urine based on reversed-phase high-performance liquid chromatography (HPLC). The method has been validated for human biological fluids and it was found to be rapid, precise, and of sufficient sensitivity for its intended use.

EXPERIMENTAL

Chemicals

Tetrahydrofuran (THF) and acetonitrile, distilled in glass grade, were purchased from Burdick and Jackson Labs. (Muskegon, MI, U.S.A.). Toluene and glacial acetic acid, both reagent grade, were obtained from MCB (Norwood, OH, U.S.A.). Citric acid was dissolved and diluted to 0.2 *M* and hydrochloric acid was diluted to 1 *M* with distilled water, respectively. Both were reagent grade. 1-Heptanesulfonic acid sodium salt was purchased from Eastman Kodak (Rochester, NY, U.S.A.). Isoxicam, piroxicam (internal standard in plasma) and PD 79,703 (internal standard in urine) were used as received from Warner-Lambert/Parke-Davis (Ann Arbor, MI, U.S.A.). The structures of all the compounds are shown in Fig. 1.

Apparatus

An Altex Model 110 pump (Berkeley, CA, U.S.A.) was used for solvent delivery and a Varian Varichrom detector (Palo Alto, CA, U.S.A.) was used for

quantitation. Peak heights were measured with a Shimadzu Chromatopac C-RIA (Kyoto, Japan) and injection was done via a WISP 710B automatic injector, Waters Assoc. (Milford, MA, U.S.A.).

Chromatography

The column was a 300 × 3.9 mm I.D. stainless-steel μBondapak C_{18} (10 μm) obtained from Waters Assoc. A guard column 40 mm × 2 mm I.D. packed with Corasil C_{18} was used. The mobile phase for isoxicam in plasma consisted of 45% THF, 54% water, 1% glacial acetic acid, and 0.005 *M* 1-heptanesulfonic acid. The mobile phase for isoxicam in urine was 50% acetonitrile, 49% water, 1% glacial acetic acid, and 0.005 *M* 1-heptanesulfonic acid. The flow-rate was set at 1.5 ml/min; the wavelength setting was 320 nm.

Sample preparation

Plasma. To 0.5 ml plasma in a 5-ml test tube with a ground-glass stopper were added 0.5 ml water, 0.1 ml isoxicam standard in THF (0.1 ml THF in unknowns), 0.25 ml of 0.2 *M* citric acid and 1 ml internal standard in toluene (2 μg/ml piroxicam). The tube was capped tightly and shaken for 10 min at moderate speed on a variable-speed shaker. After centrifugation for 5 min at 2000 *g*, 0.6 ml of the toluene layer was transferred into a 5-ml conical tube and evaporated to dryness under a stream of clean air, the tube being in a water bath maintained at 67°C. The residue was then dissolved in 0.3 ml of THF and transferred into a limited-volume insert tube. A 30-μl aliquot was injected via the WISP automatic injector.

Urine. To 1 ml of urine 0.1 ml isoxicam standard in THF (0.1 ml THF in unknowns), 0.1 ml of 1 *M* hydrochloric acid and 3 ml of PD 79,703 internal standard in toluene (0.6 μg/ml) were added. The tube was capped tightly and shaken for 10 min on a heavy-duty shaker. Following centrifugation, 2 ml of the toluene layer were transferred into a conical tube and processed as for plasma.

RESULTS

Recovery

Recovery of isoxicam and piroxicam added to plasma was 98% and 90%, respectively. Recovery of isoxicam and PD 79,703 added to urine was 98% and 93%, respectively.

Linearity

A straight line was obtained by plotting μg/ml isoxicam in plasma or urine against the peak height ratio of isoxicam over the internal standard; these data are listed in Table I. The relationship was linear in the range of 0.1—5 μg/ml in plasma and 0.125–2 μg/ml in urine.

Precision

Bulk control standards A, B and C were prepared by adding known amounts of isoxicam to blank plasma at levels of 1.8, 4 and 8 μg/ml, respectively. The precision of the assay was determined by analyzing triplicates of each of these

TABLE I

MEANS, STANDARD DEVIATIONS AND RELATIVE STANDARD DEVIATIONS OF PEAK HEIGHT RATIOS OF ISOXICAM TO INTERNAL STANDARD IN HUMAN PLASMA AND URINE

Concn. (μg/ml)	Mean peak height ratio	S.D.	R.S.D. (%)
Plasma (n = 10)			
0.2	0.035	0.004	10.8
1.0	0.190	0.010	5.26
2.0	0.400	0.013	3.21
3.0	0.610	0.024	4.02
6.0	1.23	0.058	4.76
10.0	2.10	0.093	4.45
Urine (n = 8)			
0.125	0.114	0.010	9.05
0.25	0.239	0.008	3.38
0.5	0.489	0 023	4.79
1.0	0.984	0.033	3 31
2.0	1.966	0.103	5.22

TABLE II

CONCENTRATION OF SEEDED CONTROL SAMPLES OF HUMAN PLASMA ASSAYED DURING A THREE-DAY PERIOD

Day	Concn. (μg/ml)		
	1.8	4.0	8.0
1	1.88	4.20	8.50
	2.04	4.16	8.68
	1.86	4.14	8.44
2	1.86	4.16	8.34
	1.82	3.96	8.38
	1.78	4.24	8.24
3	1.82	3.94	8.58
	1.85	4.02	8.52
	1.79	4.07	8.22
Mean	1.86	4.10	8.43
S.D.	0.077	0.107	0.154
R.S.D. (%)	4.14	2.60	1.82
Difference from theory (%)	3.33	2.50	5.38

TABLE III

BACK-CALCULATED VALUES OF STANDARD CURVES OF ISOXICAM IN HUMAN PLASMA

Day	Concn. (μg/ml)						
	0	0.4	1.0	2.0	5.0	8.0	10.0
1	0	0.40	1.04	2.04	5.24	8.80	10.44
	0	0.44	0.96	1.97	4.82	8.22	9.20
	0	0.40	0.98	2.02	4.92	8.02	9.66
2	0	0.38	0.86	1.94	4.94	8.02	9.90
	0	0.36	0.90	1.90	5.34	8.10	9.82
	0	0.36	0.88	1.70	4.90	8.10	8.68
3	0	0.38	0.92	2.22	5.56	7.98	9.74
	0	0.38	0.92	2.00	4.90	8.16	10.56
	0	0.36	0.92	1.92	5.02	7.98	9.56
Mean		0 38	0.93	1.97	5 07	8.15	9 73
S D.		0.026	0.055	0.138	0 251	0.256	0.575
R.S.D. (%)		6.77	5.89	7.00	4.95	3.14	5.91
Difference from theory (%)		5.0	7.00	1.50	1.40	1.88	2.70

TABLE IV

BACK-CALCULATED VALUES OF STANDARDS OF ISOXICAM IN HUMAN URINE

	Concn. (μg/ml)					
	0	0.125	0 25	0.5	1 0	2.0
	0	0.11	0.24	0.48	0.99	2.16
	0	0.11	0.24	0.48	1.05	1.88
	0	0.10	0.24	0.52	0.94	1.94
	0	0.11	0.23	0.48	1.02	2.12
	0	0.12	0.26	0 49	0.99	1.93
	0	0.13	0.24	0.50	1.00	1.94
	0	0.11	0.24	0.54	0.97	2.03
	0	0.13	0.24	0.48	1.01	1.93
Mean		0.12	0.24	0.50	1.00	2.00
S.D.		0.011	0.008	0.023	0.033	0.101
R.S.D. (%)		9.3	3.46	4.56	3.30	5.80
Difference from theory (%)		4.0	4.0	0.0	0.0	0.0

controls along with triplicate standard curves on three consecutive days. Relative standard deviations (R.S.D., Table II) of the controls over the three days ranged from 4.1% at the lowest level to 1.8% at the highest level. Percent difference from theory was 4.3, 2.50 and 5.4 for A, B and C, respectively. Concentrations of the standard curves were back-calculated from the slope for the day and the values are listed in Table III. Relative standard deviations ranged from 6.8% at 0.4 μg/ml to 3.1% at 8 μg/ml. Difference from theory ranged from 7.0% to 1.4%.

Blank urine was spiked with 0. 0.125, 0.25, 0.5, 1 and 2 μg/ml isoxicam. Eight replicates at each level were carried through the assay. The results (Table IV) show a back-calculated difference from theory ranging from 0 to 4%. The relative standard deviation ranged from 9.3 to 3.3%.

Selectivity

No interfering peaks were visible in blank plasma at the retention times of isoxicam and piroxicam, 8.1 min and 4.1 min, respectively. Representative chromatograms are shown in Fig. 2. Chromatograms of blank urine and of urine to which isoxicam and internal standard were added as well as chromatograms of pre- and post-dose urine samples are reproduced in Fig. 3.

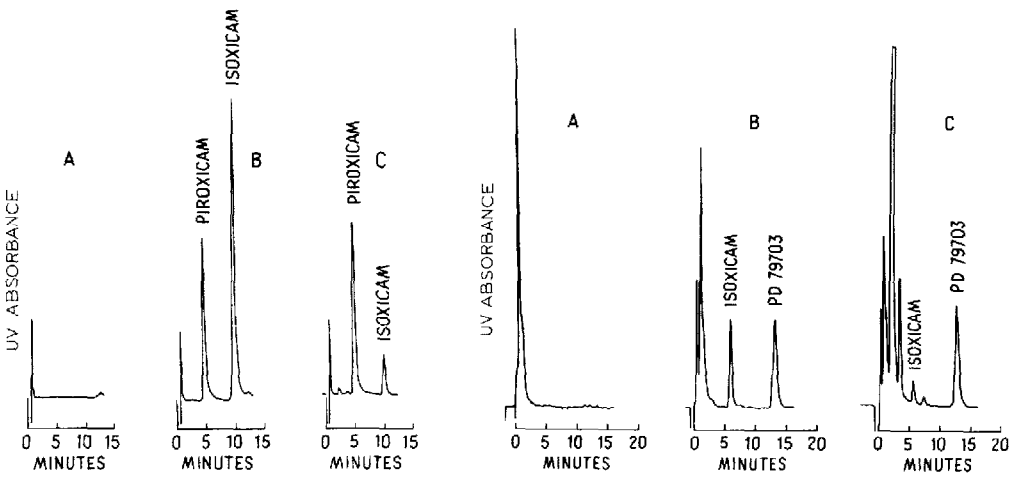

Fig. 2. Chromatograms of isoxicam in human plasma. A, Control plasma; B, plasma spiked with internal standard plus 8 μg/ml isoxicam; C, plasma from a subject 48 h post-dose, isoxicam concentration was found to be 1.2 μg/ml.

Fig. 3. Chromatograms of isoxicam in human urine. (A) Control urine; (B) urine spiked with 1 μg/ml isoxicam plus internal standard; (C) urine from a subject 48—72 h post-dose, isoxicam concentration was found to be 0.28 μg/ml.

Sensitivity

The minimum detectable amounts of isoxicam in plasma, based on three times the standard deviation of the lowest level of precision test was 0.12 μg/ml. That for urine was 0.07 μg/ml, based on twice the baseline noise.

DISCUSSION

Initially all plasma samples were assayed using piroxicam as internal standard. However, occasionally at higher doses, subjects receiving 400 mg, an isoxicam metabolite may interfere with the internal standard. An alternative internal standard, PD 79,703, with a longer retention time than piroxicam was employed where this occurred. PD 79,703 was the internal standard of choice in the case of urine where the metabolite was present in sufficient quantity to preclude the use of piroxicam. However, substitution of acetonitrile for

tetrahydrofuran in the mobile phase, can resolve the isoxicam metabolite from piroxicam. This would make piroxicam a suitable internal standard for the analysis of isoxicam in both plasma and urine. One other consideration, however, is that piroxicam has been on the market for some time and it is possible that patients may have access to both isoxicam and piroxicam.

The method as presented here has been routinely used in our laboratories for the analysis of isoxicam in human plasma and urine in clinical studies. It has proven to be a simple, straightforward procedure with a high degree of reliability, precision and accuracy. The method is sufficiently sensitive to be used in monitoring the levels of isoxicam in biological fluids of patients treated with the drug and in pharmacokinetic studies.

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