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Note

High-performance thin-layer chromatographic assay for the routine determination of piroxicam in plasma, urine and tissue

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For monitoring the non-steroidal anti-inflammatory agent piroxicam in plasma and serum after administration of therapeutic doses, assays have been described that use spectrophotometric [1, 2] and fluorimetric [1, 3] wet chemistry quantitation and high-performance liquid chromatography (HPLC) with UV detection [4, 5]. Whereas the spectrophotometric method is lacking in both selectivity and sensitivity, the improvement of the wet chemical fluorimetric assay resulted in a limit of detection of 0.2 μg/ml, which was sufficient for first-stage pharmacokinetic investigations. However, the latter method is based on the measurement of 2-aminopyridine, which is formed by the acidic cleavage of piroxicam [1]. As 2-aminopyridine may also be generated by the acidic hydrolysis of some metabolites of piroxicam [6], the selectivity of this assay is questionable. A further disadvantage from a practical point of view is the lack of internal standardization. This drawback also impairs the practical value of HPLC, which was used for pharmacokinetic investigations in animals and man [4].

None of the published methods was described as being applicable to biological materials other than plasma and serum. This paper describes the use of high-performance thin-layer chromatography (HPTLC) for the quantitation of piroxicam in plasma, urine and tissue after administration of therapeutic doses. The assay is based on fluorodensitometric quantitation with internal standardization.

EXPERIMENTAL

Standard and reagents

Piroxicam (Lot No. 503/2148) and six metabolites of piroxicam were

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supplied by Pfizer (Groton, CT, U.S.A.). As the internal standard, piretanide (HOE-118, OP.30) [4-phenoxy-3-(1-pyrrolidinyl)-5-sulphamoylbenzoic acid] was used (a gift from Hoechst, Frankfurt, G.F.R.).

All reagents were of analytical-reagent grade, obtained from Merck (Darmstadt, G.F.R.), and were used without further purification. Plasma was obtained from blood samples to which heparin (25 I.E. per 10 ml of blood) was added before centrifugation at 800 *g* for 10 min. Tissue was frozen with liquid nitrogen and mechanically homogenized with a dismembrator (Braun, Melsungen, G.F.R.).

Extraction procedure

In a 10-ml round-bottomed centrifuge tube, 0.5 ml of plasma was spiked with 0.5 μg of the internal standard (50 μl of a 10 μg/ml solution of piretanide in methanol), 100 μl of 1 *N* hydrochloric acid and 8 ml of dichloromethane were added and the mixture was agitated vigorously with a helix-shaped stirrer for 1.5 min. The tube was then centrifuged for 4 min at 2500 *g*, the aqueous phase was discarded and the organic layer was evaporated to dryness in a water-bath at 40°C under a stream of nitrogen. The dried extracts can be kept at 4°C for at least 2 days without degradation. In our laboratory, the described extraction was fully automated. We used a programmable ASA modular system (Ismatec, Zürich, Switzerland), which represents a sort of assembly line: by means of a central transport module with a capacity of 160 sample tubes the samples are moved along special modules which carry out the various necessary steps such as pipetting, stirring, centrifuging and separation and transfer of phases. The system allows the extraction of at least 320 samples during a working day. The last step of drying the extracts was achieved by simultaneous evaporation of 60 samples, so this did not diminish the total number of samples per day.

Extracts of urine were obtained by the same procedure as described for plasma.

For the extraction of piroxicam from tissue, 0.5 g of the homogenized sample was vortexed together with 0.5 ml of chloroform containing the internal standard and 1.75 ml of 1 *N* sodium hydroxide solution. The mixture was centrifuged for 15 min at 2000 *g* and to the separated aqueous layer 100 μl of 15% hydrochloric acid were added. This solution was transferred to an Extrelut column (Merck). Piroxicam and the standard were eluted with 6 ml of diethyl ether and the organic solution was evaporated to dryness under a stream of nitrogen.

Thin-layer chromatography

The extraction residues were taken up with 50 μl of dichloromethane and 500 nl of the solution were applied to a pre-washed (methanol—25% ammonia solution, 9:1), pre-coated silica gel 60 HPTLC plate (10 × 20 cm) using a Camag-Nanomat sample applicator (Camag, Muttenz, Switzerland). During routine analysis, 35 spots, of which 9 were calibration samples, were placed at opposite sides of the plate. Chromatography was performed in a Camag HPTLC linear developing chamber, in which both sides of the plate were developed simultaneously with chloroform–acetic acid—methanol (18.2:1.0:0.8)

after saturating the chamber for 15 min. The developed plate was dried in a desiccator under vacuum and then dipped into a 10% (v/v) solution of paraffin oil in pentane to enhance the intensity of the fluorescence signals [7, 8]. The air-dried plate was scanned fluorimetrically with a TLC Scanner (Camag) with a 400-nm edge-filter at an excitation wavelength of 366 nm (Hg lamp). The scanner was controlled by a computer (HP 9826) allowing an automatic, peak-maximum-adjusted scan of one plate within 75 min. Simultaneously, the chromatographic peaks were integrated by a computer program. The ratio of the piroxicam to piretanide peak areas was used to determine piroxicam.

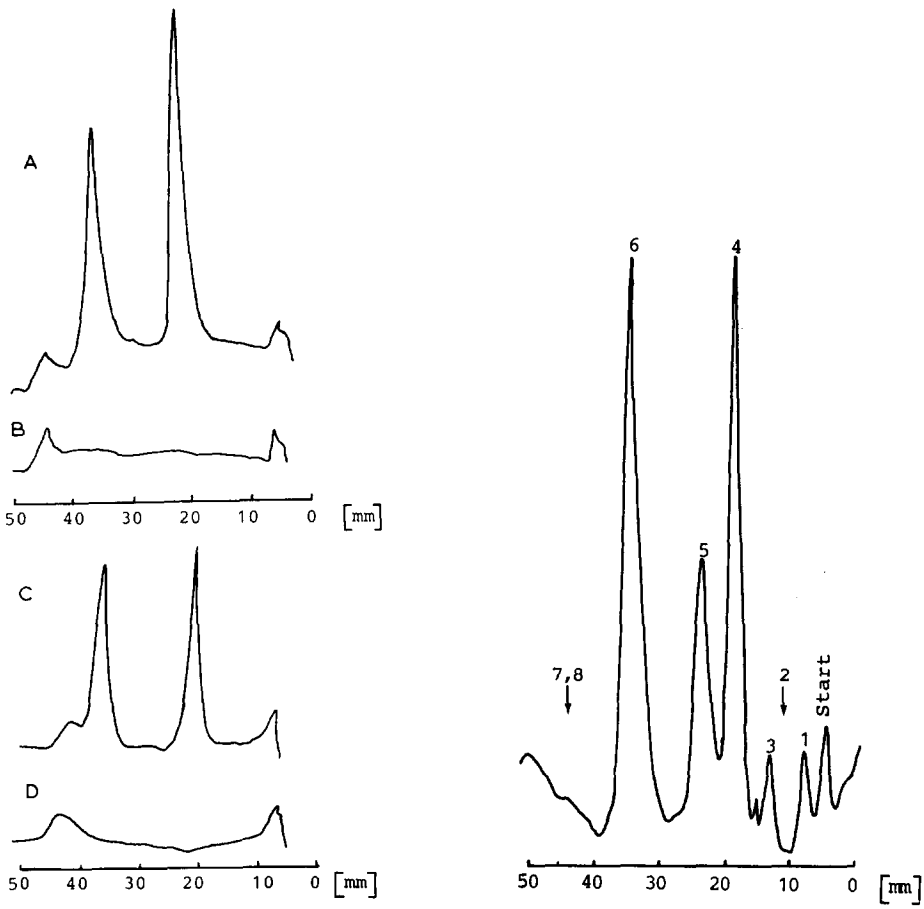

Fig. 1. Thin-layer chromatograms of processed plasma samples. A, Plasma spiked with equal amounts of piroxicam and piretanide; B, blank plasma; C, tissue from a patient under piroxicam medication; D, blank tissue.

Fig. 2. Thin-layer chromatogram of processed plasma samples spiked with piroxicam (6), piretanide (4) and the piroxicam metabolites 4-hydroxy-2-methyl-2H-1,2-benzothiazine-3-carboxylic acid 1,1-dioxide (1), 1,2-benzisothiazol-3(2H)-one 1,1-dioxide (2)*, 6-methyl-6H-7-oxopyrido[1,2-*a*]pyrimido[5,4-*c*]-1,2-benzothiazine 5,5-dioxide (3), N-(5'-hydroxy-2'-pyridyl)-4-hydroxy-2-methyl-2H-1,2-benzothiazine-3-carboxamide 1,1-dioxide (5), 2H-1,2-benzothiazine-4(3H)-one 1,1-dioxide (7)* and 2-methyl-1,2-benzisothiazol-3(2H)-one 1,1-dioxide (8)* (* = no fluorescence signal observed).

RESULTS AND DISCUSSION

Fig. 1 shows typical chromatograms of human plasma and tissue extracts. Piroxicam has an R_F value of 0.65 and piretanide appears at R_F 0.39. Neither the piroxicam nor the internal standard spots are subject to interference from co-extracted endogenous compounds, which is demonstrated by the corresponding blank chromatograms. The assay is specific for metabolites of piroxicam. The chromatogram of a plasma sample spiked with six metabolites before extraction (see Fig. 2) shows sufficient separation from piroxicam and internal standard.

As piroxicam is used in long-term therapy, other drugs might be taken simultaneously with piroxicam treatment by rheumatic patients. Therefore, we tested the interference of this assay with other frequently prescribed drugs. The results are shown in Table I. The piroxicam assay described here is specific for the listed drugs with the exceptions of tinidazole and sulphamethoxypyridazine.

TABLE I

THIN-LAYER CHROMATOGRAPHIC BEHAVIOUR OF SOME COMMON DRUGS

Drug	R_F	Drug	R_F
Piroxicam	0.65	Tinidazole	0.39
Piretanide	0.39	Caffeine	0.45*
Cefoperazone	0.00*	Nordiazepam	0.55
Cimetidine	0.00*	Indomethacin	0.56
Codeine	0.00	Naproxen	0.56
Doxycycline	0.00	Diclofenac	0.57*
Prazosin	0.04	Tolbutamide	0.57*
Metoclopramide	0.05	Flufenaminic acid	0.58
Doxepin	0.11*	Prazepam	0.58
Propranolol	0.13	Diazepam	0.59
Sulphamethoxypyridazine	0.38		

*No fluorescence signal observed.

TABLE II

ANALYTICAL VALUES AND REGRESSION LINES OF FIVE DIFFERENT CALIBRATION GRAPHS

Amount of piroxicam added (μg/ml)	Parameter	Calibration graph No.				
		1	2	3	4	5
0.5	Ratio of peak areas	0.334	0.300	0.363	0.275	0.302
1	(piroxicam:piretanide)	0.723	0.592	0.775	0.622	0.703
2		1.543	1.222	1.538	1.639	2.018
4		2.754	2.439	3.110	2.876	3.086
	Slope	0.689	0.613	0.782	0.750	0.804
	Intercept	0.046	−0.011	−0.021	−0.054	0.020
	Correlation coefficient	0.997	0.999	0.999	0.994	0.979

Calibration graphs were calculated by linear regression of the ratio of the peak area of piroxicam to that of the internal standard as a function of piroxicam concentration. The calibration graphs were linear over the range 0.1–15 μg/ml with correlation coefficients > 0.98. Table II shows the measured values for five calibration graphs that were established on different plates and in different weeks. The variance of the calibration slope is attributed mainly to different concentrations of the internal standard solutions.

With the routine procedure described here, drug levels of 100 ng/ml in plasma or urine are easily quantified. The limit of sensitivity (signal-to-noise ratio = 3:1) can be improved to 50 ng/ml by taking up the dried residue of the extract in only 20 μl of dichloromethane before it is applied to the thin-layer plate.

The recovery after the extraction from plasma was calculated using ten samples each spiked with 10 μg/ml of piroxicam or piretanide. For piroxicam a mean extraction rate of 94.8 ± 2.4% (S.D.) was obtained. Piretanide was extracted from plasma with an average recovery of 85.1 ± 3.4% (S.D.).

In the assay of tissue samples, piroxicam and piretanide were almost completely partitioned into the aqueous sodium hydroxide phase. The recovery from the off-column extraction was 79.5 ± 5.9% (S.D.) for piroxicam and 74.3 ± 4.2% (S.D.) for piretanide.

To test the reproducibility of the assay, spiked plasma samples containing 1, 2 and 5 μg/ml were run in parallel on the same thin-layer plate. The reproducibilities of the measured concentrations for eight samples each were 4.9, 3.8 and 3.1%, respectively.

As a check of the reliability of the analytical results during routine application of the method, spiked piroxicam plasma samples with concentrations

TABLE III

RELIABILITY OF MEASURED PIROXICAM CONCENTRATIONS

The amounts added were unknown to the analyst.

Amount of piroxicam (μg/ml)		Deviation (%)
Added	Found	
0.26	0.25	−3.8
0.28	0.29	3.6
0.42	0.40	−4.8
0.62	0.53	−14.5
0.71	0.81	14.1
1.04	0.94	−9.6
1.32	1.27	−3.8
1.77	1.65	−6.8
1.89	1.89	0
2.50	2.76	10.4
3.08	2.86	−7.1
3.53	3.76	6.5
4.80	4.26	−11.3
9.01	8.95	−0.7

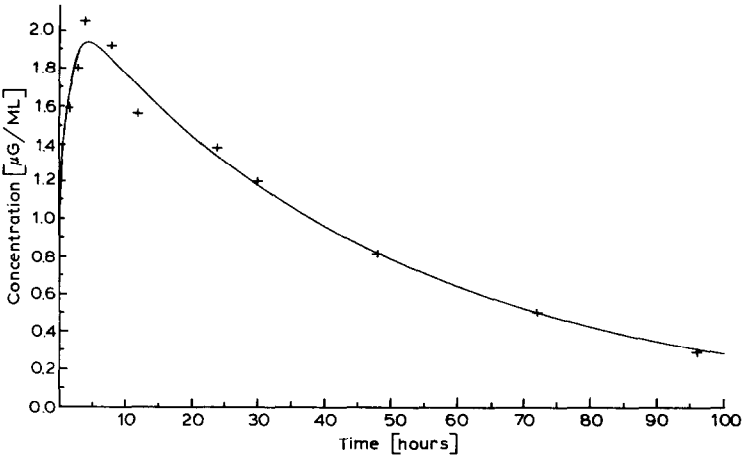

Fig. 3. Plasma concentrations in human subjects after oral administration of 20 mg of piroxicam.

unknown to the analyst were mixed with the original samples. Table III presents the results of assays performed on different days during a period of 6 months. The mean deviation of the measured values from the amounts of the piroxicam added to the test samples was 6.9%. The suitability of this assay procedure is demonstrated by the analysis of plasma samples from a human subject after a single oral dose of 20 mg of piroxicam (see Fig. 3). The half-life of elimination was 33.8 h.

The characteristics of our piroxicam assay make it well suited for routine work in clinical laboratories. Further, it proved to be compatible with extensive automation. With the equipment described, about 150 samples including standards can be handled in a working day, which corresponds to an average of 4.5 min per sample.

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