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Note

High-performance liquid chromatographic method for the determination of diclofenac and its hydroxy metabolites in human plasma and urine

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Diclofenac sodium, 2-[(2,6-dichlorophenyl)amino]benzeneacetic acid sodium salt, like indomethacin, ibuprofen and naproxen, is a phenylacetic acid derivative and belongs to the group of the non-steroidal anti-inflammatory agents. Several methods have been developed for the determination of diclofenac and its metabolites in biological fluids, including high-performance liquid chromatography (HPLC) [1–4], gas chromatography (GC) [5–8] and even isolation with ^{14}C-labelled diclofenac [9,10]. However, most of these were developed for the measurement of diclofenac itself, although some include the measurement of one or two principal metabolites, i.e. 4′-hydroxydiclofenac and 5-hydroxydiclofenac [1,7].

Two studies reported the analysis of diclofenac and its four known metabolites in urine [6,7] and one in plasma [8] by means of GC with electron-

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capture detection. So far no HPLC analysis for diclofenac and its four metabolites has been reported.

This paper describes an HPLC method with UV detection for the determination of diclofenac and metabolites in human plasma and urine.

EXPERIMENTAL

Chemicals

Diclofenac sodium and its metabolites 3′-hydroxydiclofenac, 4′-hydroxydiclofenac, 5-hydroxydiclofenac and 4′,5-dihydroxydiclofenac were a gift from Ciba-Geigy (Basel, Switzerland). Methanol, methyl *tert.*-butyl ether, acetonitrile, tetrahydrofuran and water were all of HPLC grade (Fisons, Loughborough, U.K.). Methanol was the solvent for the standard stock solutions.

For the construction of the calibration curves in plasma the standard stock solution was diluted with water and used in drug-free human plasma with concentrations in the range 0–3 μg/ml.

For the construction of the calibration curves in urine the standard stock solution was diluted with drug-free human urine to obtain standard samples in the range 0–200 μg/ml.

Apparatus

The Waters chromatographic system consisted of a Model 510 solvent-delivery system and a Lambda-Max Model 481 UV detector connected to a 10-mV BBC SE-120 recorder. A Rheodyne Model 7125 injection valve with a 50-μl injection loop was used.

Chromatography

The analytical column (250 mm × 4.6 mm I.D., 5 μm particle size) was prepacked with Hypersil 5-ODS (Chrompack, Middelburg, The Netherlands). A stainless-steel precolumn (100 mm × 2.0 mm I.D.) packed with pellicular reversed-phase material (Chrompack) was used as guard column. The column temperature was 30°C.

The mobile phase was acetonitrile–methanol–tetrahydrofuran–water (15:10:3:72, v/v) in which 4.48 g of disodium hydrogenphosphate dihydrate and 2.28 g of potassium dihydrogenphosphate were dissolved. This mixture was filtered through a Millipore 0.5-μm filter, Type FH (Milford, MA, U.S.A.). The flow-rate was 1.5 ml/min. Detection was effected at 282 nm.

Sampling handling procedures

Free concentrations in plasma. A 150-μl volume of phosphate buffer (pH 4.0) and 2 ml of methyl *tert.*-butyl ether were added to 100 μl of plasma. The mixture was vortex-mixed for 60 s and then centrifuged at 2600 *g* for 5 min. The organic layer was transferred to a glass tube, which contained 200 μl of

sodium bicarbonate. The mixture was vortex-mixed for 60 s and then centrifuged at 2600 *g* for 5 min. The organic layer was discarded. Then 1 ml of the phosphate buffer and 2 ml of methyl *tert.*-butyl ether were added to the aqueous layer. The mixture was vortex-mixed for 60 s and then centrifuged at 2600 *g* for 5 min. Thereafter the organic layer was transferred to a clean glass tube and evaporated to dryness under a gentle stream of nitrogen in a heating block at 50 °C. The residue was dissolved in 200 µl of the mobile phase, and 50 µl were injected onto the column.

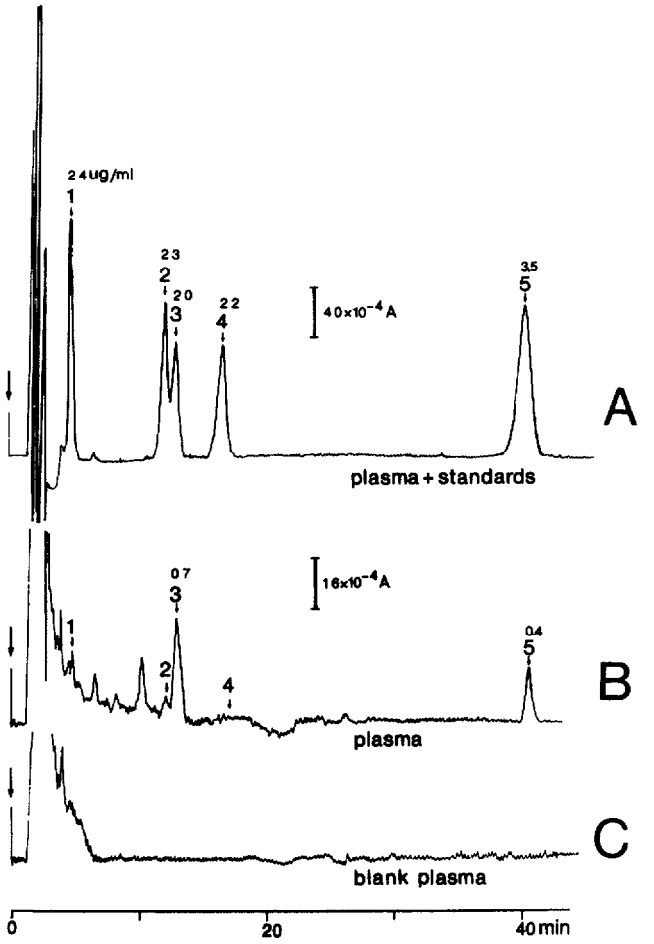

Fig. 1. Chromatograms of diclofenac and its four metabolites in plasma. (A) Various concentrations (as indicated) of standards etc. were added to blank plasma. (B) The plasma of a volunteer who received a rectal dose of 100 mg of diclofenac. (C) Drug-free plasma. Peaks: 1 = 4′,5-dihydroxydiclofenac; 2 = 3′-hydroxydiclofenac; 3 = 4′-hydroxydiclofenac; 4 = 5-hydroxydiclofenac; 5 = diclofenac.

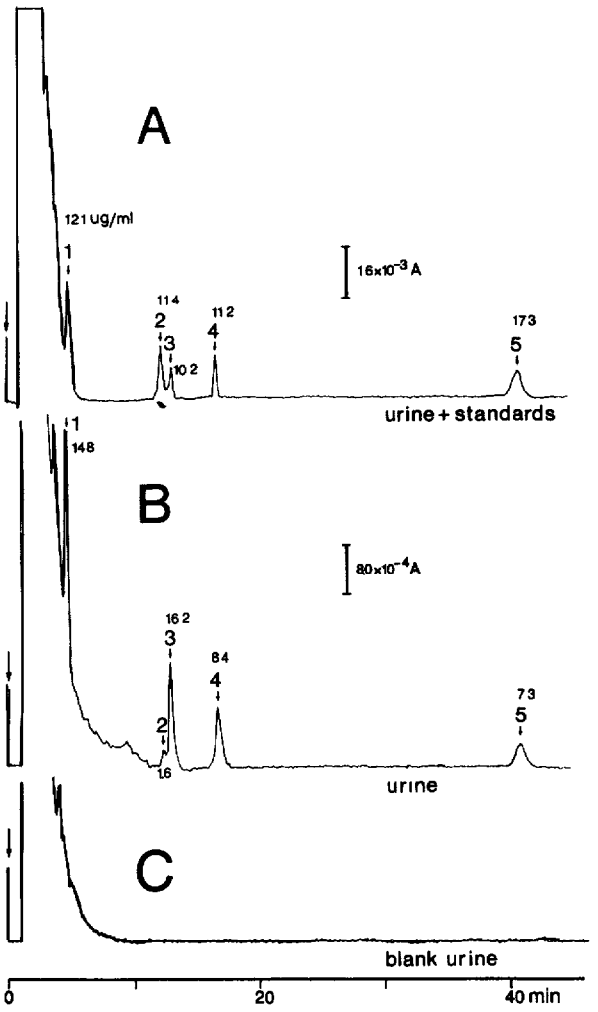

Fig. 2. Chromatograms of diclofenac and its four metabolites in urine. (A) Various concentrations (as indicated) of standards were added to blank urine. (B) The urine of a volunteer who received a rectal dose of 100 mg of diclofenac. (C) Drug-free urine. Peaks as in Fig. 1.

Free concentrations in urine. A 150-μl volume of phosphate buffer (pH 4.0) and 4 ml of methyl *tert.*-butyl ether were added to 100 μl of urine. After vortex-mixing for 1 min and centrifuging for 5 min at 2600 *g* the organic layer was transferred to a glass tube which contained 200 μl of sodium bicarbonate. The mixture was vortex-mixed for 1 min and centrifuged. The organic layer was discarded, and 1 ml of the phosphate buffer and 4 ml of methyl *tert.*-butyl ether were added to the aqueous layer. The mixture was vortex-mixed and centri-

TABLE I

CAPACITY FACTORS AND DETECTION LIMITS OF DICLOFENAC AND ITS HYDROXY METABOLITES

Compound	Capacity factor (k')	Detection limit[a] (mg/l)	
		Plasma	Urine
4',5-Dihydroxydiclofenac	3.2	0.050	0.60
3'-Hydroxydiclofenac	9.5	0.060	1.00
4'-Hydroxydiclofenac	10.2	0.040	1.40
5-Hydroxydiclofenac	13.2	0.060	1.10
Diclofenac	33.8	0.020	2.50

[a]Signal-to-noise ratio = 3.

TABLE II

ACCURACY AND PRECISION OF THE DETERMINATION

Spiked human plasma and urine were used for four determinations at each concentration.

Compound	Concentration added (μg/ml)	Mean concentration found (μg/ml)	Accuracy (%)	Precision (R.S.D.) (%)
Plasma				
4'-Hydroxydiclofenac	0.13	0.14	101.2	1.6
	0.51	0.53	100.4	4.0
	2.04	1.99	99.0	2.6
Mean			100.2	2.7
Diclofenac	3.46	3.45	100.4	1.5
	0.87	0.83	95.4	4.1
	0.22	0.22	100.0	4.5
Mean			98.6	3.4
Urine				
4',5-Dihydroxydiclofenac	3.0	3.1	104.4	2.0
	6.1	5.9	94.7	4.1
	12.1	12.1	99.4	1.3
Mean			99.5	2.5
3'-Hydroxydiclofenac	2.9	2.9	98.3	2.0
	5.7	5.7	100.4	4.6
	11.4	11.4	100.0	2.9
Mean			99.6	3.2
4'-Hydroxydiclofenac	2.6	2.7	102.9	5.8
	5.1	5.0	98.1	2.3
	10.2	10.3	100.5	1.9
Mean			100.5	3.3
5-Hydroxydiclofenac	2.8	2.8	98.2	3.6
	5.6	5.8	104.0	5.1
	11.2	11.4	101.8	3.6
Mean			101.3	4.1
Diclofenac	4.3	4.4	101.2	5.9
	8.7	8.8	101.2	6.1
	17.3	17.4	100.4	3.1
Mean			100.9	5.0

fuged, and the organic layer was transferred to a clean glass tube and evaporated to dryness under a gentle stream of nitrogen in a heating block at 50°C. The residue was dissolved in 200 μl of the mobile phase, and 50 μl were injected onto the column.

Total concentrations in plasma and urine. Ascorbic acid (40 μg) and 100 μl of 5 *M* sodium hydroxide were added to 100 μl of urine or plasma. The mixture was vortex-mixed for 60 s and then heated at 75°C for 1 h. Thereafter, 600 μl of 1 *M* hydrochloric acid and 4 ml of methyl *tert.*-butyl ether were added. After vortex-mixing for 60 s the glass tube was centrifuged at 2600 *g* for 5 min. The organic layer was transferred to a clean glass tube and evaporated to dryness under a gentle stream of nitrogen in a heating block at 50°C. The residue was dissolved in 400 μl of the mobile phase and vortex-mixed for 30 s, and 50 μl of the obtained mixture were injected onto the column.

Testing of the analytical procedures

The accuracy, precision and linearity of the method were determined using spiked samples of human plasma and urine analysed at random. Total recovery of diclofenac and its metabolites was determined as the response from extracted standards relative to the response of standards dissolved in the mobile phase and injected directly onto the column.

RESULTS AND DISCUSSION

Representative chromatograms of the determination of diclofenac and the metabolites in plasma and urine are shown in Figs. 1 and 2, respectively. The capacity factors and detection limits are given in Table I. These samples were obtained from six volunteers after rectal administration of 100 mg of diclofenac in a suppository. Drug-free samples gave no interfering peaks. The analytical procedure was found to be accurate and precise (Table II). The cali-

TABLE III

RECOVERY OF THE EXTRACTION PROCEDURE

$n=4$.

	Recovery (%)	
	Urine	Plasma
4′,5-Dihydroxydiclofenac	44.1 ± 3.8	46.2 ± 2.9[a]
3′-Hydroxydiclofenac	50.0 ± 4.2	53.3 ± 4.8[a]
4′-Hydroxydiclofenac	32.6 ± 4.1	52.9 ± 3.1
5-Hydroxydiclofenac	74.4 ± 5.0	64.7 ± 3.1[a]
Diclofenac	47.5 ± 3.9	50.1 ± 4.9

[a]Concentration in patient plasma below detection limit

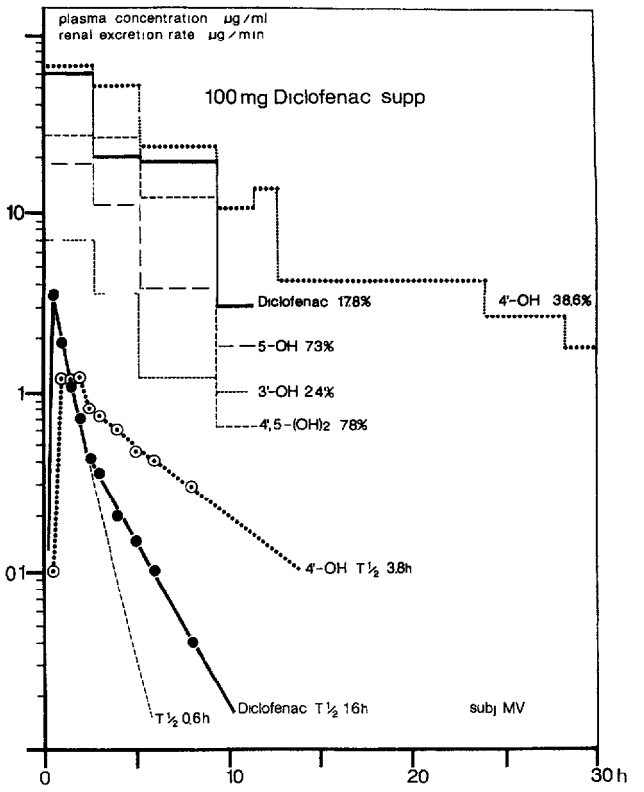

Fig. 3. Pharmacokinetics of diclofenac and its metabolites after a rectal dose of 100 mg of diclofenac to a volunteer.

bration curves (peak area versus concentration) were linear for both plasma and urine ($r=0.995$ and 0.997, respectively). Recoveries of the extraction in plasma and urine are shown in Table III.

After a single dose of 100 mg of diclofenac, only unconjugated diclofenac and 4′-hydroxydiclofenac were present in plasma. The concentrations of the other three metabolites (conjugated and unconjugated) in plasma were below the detection limit. This may be due to a slow formation process followed by rapid renal excretion. The plasma concentration of 3′-hydroxydiclofenac is much lower than that of the main metabolite 4′-hydroxydiclofenac. Even in the urine the 3′-hydroxy metabolite is a minor metabolite. The poor separation of 3′- and 4′-hydroxydiclofenac is of limited importance, since the 3′-hydroxy metabolite is only present in concentrations below the detection limit of 50 ng/ml [7]. Thus this method is not sensitive enough to measure the metabolites 3′-, 5- and 4′,5-hydroxydiclofenac in plasma. Fig. 3 shows the plasma concentration–time curves and the renal excretion rate–time profile of diclofenac and the metabolites in one volunteer after a rectal dose of 100 mg of diclofenac.

This method appeared to be useful to detect diclofenac and its metabolites in urine, and diclofenac and 4′-hydroxydiclofenac in plasma. As shown in Fig. 3, it can be used for pharmacokinetic studies in which the metabolites must be involved.

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