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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC DETERMINATION OF DICLOFENAC AND ITS MONOHYDROXYLATED METABOLITES IN BIOLOGICAL FLUIDS

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SUMMARY

Sensitive and selective high-performance liquid chromatographic assays for diclofenac and its monohydroxylated metabolites in biological fluids are described. Using ultraviolet detection at 282 nm, diclofenac is assayed in plasma at concentrations down to 10 ng/ml; total (free + conjugated) diclofenac and its monohydroxylated metabolites (the sum of 3'- + 4'-hydroxydiclofenac and 5-hydroxydiclofenac) are assayed in urine after chemical hydrolysis at concentrations down to 200 ng/ml. The applicability of the described assays is shown.

INTRODUCTION

Diclofenac sodium (Voltaren®) is a non-steroidal anti-inflammatory drug used in the treatment of rheumatic diseases. Several methods for its assay in plasma have been reported including thin-layer chromatography (TLC) [1], high-performance liquid chromatography (HPLC) [2—4] and gas chromatography (GC) [5—8].

The GC methods are based on the formation of an indolinone [5], a methyl ester [7,8], or an acetylated [6] derivative. Those involving electron-capture detection [5,7,8] are extremely sensitive; however, they require an extensive sample clean-up (three-step extraction) before derivatization.

The TLC method [1] as well as two of the HPLC methods reported [2,3] lack the sensitivity required for pharmacokinetic investigations.

Chan et al. [4] recently described a simple and sensitive HPLC method, with single-step extraction and direct UV detection without derivatization. The overall recovery in the range 5—1000 ng/ml was 94.8 ± 7.8%, indicating good precision and accuracy. However, the data reported suggest that the repro-

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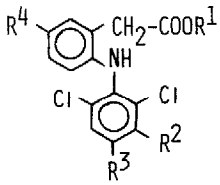
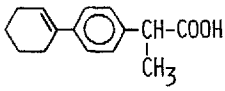
Diclofenac and its known metabolites



Compound		R^1	R^2	R^3	R^4	24-h Renal elimination in percentage of dose (free + conjugated)
Diclofenac sodium	D	Na	H	H	H	5–10
3'-Hydroxydiclofenac	3'-OH-D	H	OH	H	H	<5
4'-Hydroxydiclofenac	4'-OH-D	H	H	OH	H	20–30
5-Hydroxydiclofenac	5-OH-D	H	H	H	OH	5–10
4',5-Dihydroxydiclofenac	4',5-OH-D	H	H	OH	OH	5–10

Internal standards

Assay I: 2-(*p*-cyclohexen-1'-yl-phenyl)propionic acid (I)



Assay II: 4'-hydroxy-5-chlorodiclofenac (II)

R^1=R^2=H; R^3=OH; R^4=Cl

Fig. 1. Chemical structures of diclofenac, its known metabolites and internal standards.

ducibility at low levels was not fully satisfactory. The coefficient of variation of the peak height ratio was 20% for 5 ng/ml (three replicates) and 21.7% for 10 ng/ml (four replicates). The recovery for samples spiked with 9 ng/ml ranged from 67% to 144%.

A similar HPLC method, with better precision and accuracy, was developed for the assay of unchanged diclofenac in plasma (Assay I).

Diclofenac sodium is extensively metabolized in man: 35—65% of an oral dose is excreted in urine as conjugates of the parent drug, mono- and dihydroxylated metabolites [9]. The unconjugated compounds account for less than 1% [10]. 3'-, 4'-, and 5-hydroxydiclofenac (3'-, 4'- and 5-OH-D) and 4',5-dihydroxydiclofenac (4',5-OH-D) (see Fig. 1 for chemical structures) have been identified in human urine as conjugates [11]. They account, respectively, for less than 5%, 20—30%, 5—10% and 5—10% of the administered dose [9].

A method based on extractive alkylation and GC has been described for the determination of the total (free + conjugated) monohydroxylated metabolites in urine [12]. However, the derivatives of the three compounds are not resolved on the column and are measured as a single peak.

A procedure based on extractive alkylation, GC with capillary column and electron-capture detection is the only method available for the simultaneous and specific assay in urine of the parent drug and its four mono- and dihydroxylated metabolites [13].

This paper describes an HPLC method for the assay of diclofenac and its monohydroxylated metabolites in urine after chemical hydrolysis (Assay II). It is selective for diclofenac and 5-hydroxydiclofenac. The sum of 3'- and

4′-OH-D is measured as a single compound since they have identical retention times. The dihydroxylated metabolite cannot be assayed because of interference.

MATERIALS AND METHODS

Chemicals

Diclofenac sodium, its metabolites and the internal standards [2-(*p*-cyclohexen-1′-yl-phenyl)propionic acid (I) for Assay I and 4′-hydroxy-5-chlorodiclofenac (II) for Assay II] were supplied by Ciba-Geigy (Basle, Switzerland) (see Fig. 1 for chemical structures).

Acetonitrile for spectroscopy (Uvasol from E. Merck, Darmstadt, F.R.G.) was used. All the other solvents and reagents were of analytical grade quality. Diisopropyl ether was purified just before use by passing it through a column filled with basic alumina (Woelm, Eschwege, F.R.G.).

Assay I — unchanged diclofenac in plasma

Chromatography. The chromatography was performed on a Hewlett-Packard instrument, Model 1084 B, equipped with a variable-wavelength detector set at 282 nm and a variable-volume injection system. The separation was achieved with a prepacked Merck column (25 cm × 4 mm I.D., LiChrosorb RP-8 10-μm packing). A precolumn (5 cm × 4.7 mm I.D.) filled with Co-Pell ODS 30—38 μm (Whatman, Clifton, NJ, U.S.A.) was used to protect the analytical column. The degassed mobile phase, methanol—pH 7 phosphate buffer (Titrisol from Merck) (60:40, v/v), was used at a flow-rate of 1.3 ml/min. The mobile phase and the columns were at room temperature.

The peak heights were measured manually, the peak areas were given by the integrator—recorder of the Hewlett-Packard 1084 B instrument (79850 A LC terminal).

Sample preparation. To 1 ml of plasma in a 10-ml glass tube were added 100 μl of the solution of the internal standard I (added amount 1.25 μg). The sample was acidified with 2 ml of 0.83 *M* phosphoric acid and 4 ml of hexane—isopropyl alcohol (90:10) were added. The tubes were capped, shaken for 10 min on a rotating shaker (Infors) at 250 rpm and then centrifuged at 1500 *g* for 10 min. The aqueous phase was frozen, the organic layer transferred into a 10-ml conical glass tube and evaporated to dryness under a nitrogen stream at room temperature. Just prior the analysis, the residue was redissolved in 300 μl of mobile phase. After shaking for 15 sec on a vortex mixer, 70 μl were injected onto the column.

Calibration curves. Calibration samples were prepared by adding 100 μl of reference solutions of diclofenac sodium and 100 μl of the solution of the internal standard I (all prepared in water from methanolic stock solutions) to 1 ml of blank plasma. The amounts corresponded to plasma concentrations of diclofenac sodium ranging from 10 ng/ml to 3 μg/ml.

The calibration curves were obtained by plotting versus the concentrations, either the peak height ratio diclofenac sodium/internal standard for the concentration range 10—250 ng/ml or the peak area ratio for the range 0.25—3 μg/ml. Their equations were calculated by the least-squares method using linear

regression. For routine analysis, a calibration curve must be established every ten days.

Assay II — unchanged drug and monohydroxylated metabolites in urine

Chromatography. The equipment was that used in Assay I.

The separation was achieved with a column (25 cm × 4.7 mm I.D.) filled with Nucleosil C_{18}, 10 μm particle size (Macherey Nagel, Düren, F.R.G.) using the balanced-density slurry packing technique. The slurry, made of 3.5 g of Nucleosil C_{18} (10 μm particle size) dispersed in a mixture of 17 ml methanol and 4 ml of 10^{-2} *M* sodium acetate solution, was forced into the column with methanol under a pressure of 500 bars. The degassed mobile phase methanol—acetonitrile—pH 7 buffer (30:17:53, v/v/v) was used at a flow-rate of 1.3 ml/min. The mobile phase and the column were at room temperature.

Sample preparation. A 100-μl volume of the methanolic solution of internal standard II was introduced into a 10-ml disposable glass ampoule (added amount 250 ng). Methanol was evaporated under a nitrogen stream. Then 250 μl of urine, about 100 mg of ascorbic acid and 250 μl of 5 *M* sodium hydroxide were introduced into the ampoule. After shaking for a few seconds on a vortex mixer, the ampoule was capped and heated at 75°C for 1 h. After cooling, 1.2 ml of 1 *M* hydrochloric acid were added, followed by 1 ml of pH 7 buffer and 4 ml of peroxide-free diisopropyl ether. The ampoule was closed with a polyethylene stopper, shaken for 15 min on a rotating shaker at 250 rpm and centrifuged at 1500 *g* for 5 min.

The aqueous phase was frozen. The organic layer was transferred into a 10-ml glass ampoule and 500 μl of a potassium carbonate solution (20 g/l) were added. The ampoule was closed, shaken for 5 min at 300 rpm and centrifuged for 3 min. The aqueous phase was frozen and the organic layer discarded. About 100 mg of ascorbic acid, 1 ml of pH 6 buffer (citric acid—sodium hydroxide; Titrisol from Merck, four-fold concentrated), 100 μl of 1 *M* hydrochloric acid and 4 ml of peroxide-free diisopropyl ether were added. After shaking for 5 min at 300 rpm and centrifugation for 3 min, the aqueous phase was frozen. The organic layer was transferred into a 5-ml disposable glass ampoule and taken to dryness under a nitrogen stream at 37°C. The dry residue was taken up into 300 μl of methanol—pH 4 buffer (sodium citrate—hydrochloric acid, according to Sörensen) mixture (50:50). After shaking for 15 sec on a vortex mixer, 100 μl were injected onto the column. The samples must be injected as soon as possible after preparation since degradation of 5-OH-D was observed.

Calibration curves. Calibration solutions of diclofenac sodium, 4'- and 5-OH-D as well as the solution of the internal standard II were prepared in methanol. Ascorbic acid was added to the 5-OH-D solution to prevent oxidation.

Calibration samples were prepared by introducing aliquots of the methanolic solutions of each compound into an ampoule. The solvent was evaporated and 250 μl of blank urine were added.

The range of the calibration curves for each compound was 0.20—20 μg/ml. A log—log plot of the peak area or peak height ratio (compound/internal standard) versus concentration was used. Near the limit of quantitation, a curve with the peak height ratio was used.

A calibration curve remains valid over one week for 4′- and 5-OH-D, and over about one month for diclofenac.

RESULTS AND DISCUSSION

Assay I

Plasma interference. Diclofenac and the internal standard I were well separated from the plasma components with UV detection at 282 nm (Fig. 2). More interferences were observed at 215—220 nm, as proposed by Chan et al. [4].

Linearity. A linear relationship was obtained in the range 10—250 ng/ml with the peak height ratios, in the range 0.25—3 μg/ml with the peak area ratio (correlation coefficients higher than 0.9990).

Accuracy, precision and reproducibility. Table I shows that concentrations of diclofenac down to 10 ng/ml can be accurately and precisely measured using 1-ml plasma samples. This limit corresponds to a peak height of about 0.5 cm.

Selectivity. The known metabolites (Fig. 1) do not interfere in the assay of diclofenac. Their retention times were 3.12, 3.24, 3.29 and 3.80 min, respectively, for 3′-OH-D, 4′-OH-D, 4′,5-OH-D and 5-OH-D, compared to 5.8 min for diclofenac and 7.2 min for the internal standard I.

The retention times of other anti-inflammatory drugs, relative to that of diclofenac, were 1.04 min for indomethacin, 0.54 min for ketoprofen, and 0.40 min for piroxicam.

Stability. Diclofenac sodium and internal standard I solutions were stable over one month at 5°C. The dry residue remains stable for one night when stored as such in the refrigerator. Once dissolved in the mobile phase, the solution must be injected on the day of preparation.

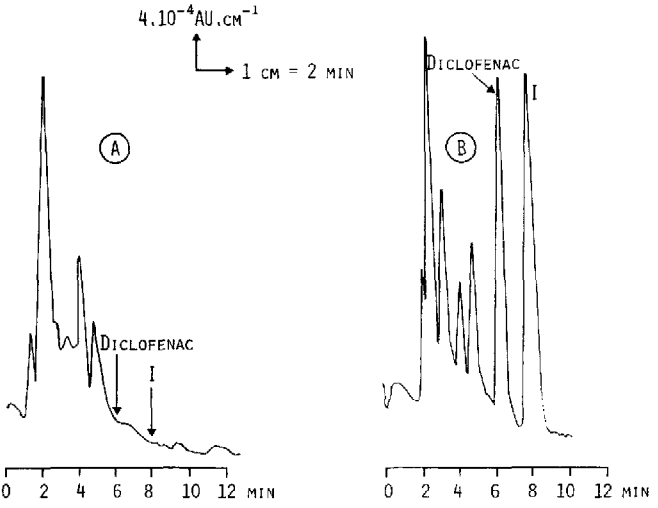


Fig. 2. Chromatograms corresponding to extracts of (A) a 1-ml sample of blank human plasma and (B) a 1-ml sample of human plasma spiked with 200 ng/ml diclofenac sodium and 1.25 μg of internal standard (I).

TABLE I

ACCURACY, PRECISION AND REPRODUCIBILITY OF ASSAY I

Plasma samples were spiked with diclofenac sodium and assayed within a day or on several days

Added (ng/ml)	Found, mean (ng/ml)	*n*	C.V. (%)	Mean recovery* (%)
Within-day reproducibility				
10	10.5**	12	8.5	105
15	15.9**	8	5.9	106
20	20.4	6	5.8	102
1498	1510	6	3.7	101
Day-to-day reproducibility				
20	18.8***	9§	6.7	95
200	202***	10§	3.3	101
1498	1472***	9§	7.2	98

*Mean of the individual recovery values.
**Spiked samples prepared with plasma from six volunteers.
***Spiked samples prepared with plasma from three volunteers.
§One or two samples per day over six days.

Application. Fig. 3 displays the mean plasma concentration—time curve of diclofenac in six subjects after administration of a single 75-mg dose as suppository.

Assay II

Urine interferences. Diclofenac and monohydroxylated metabolites were well separated from the endogenous components extracted from urine after chemical hydrolysis (Fig. 4).

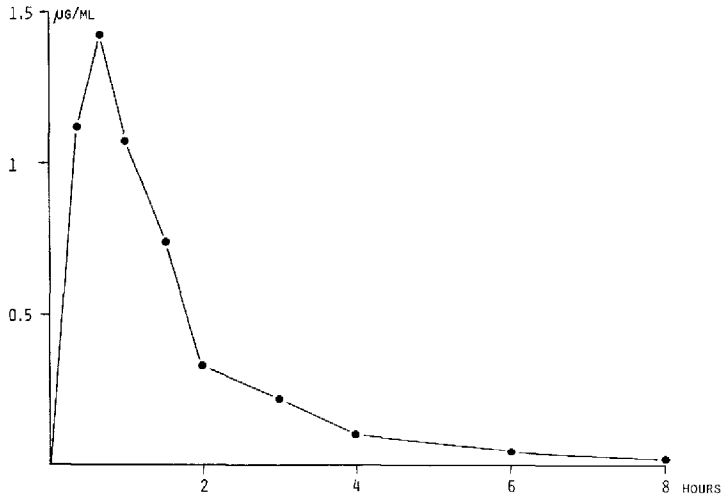


Fig. 3. Plasma concentrations of diclofenac after rectal administration of a 75-mg suppository (each point is the mean of six subjects).

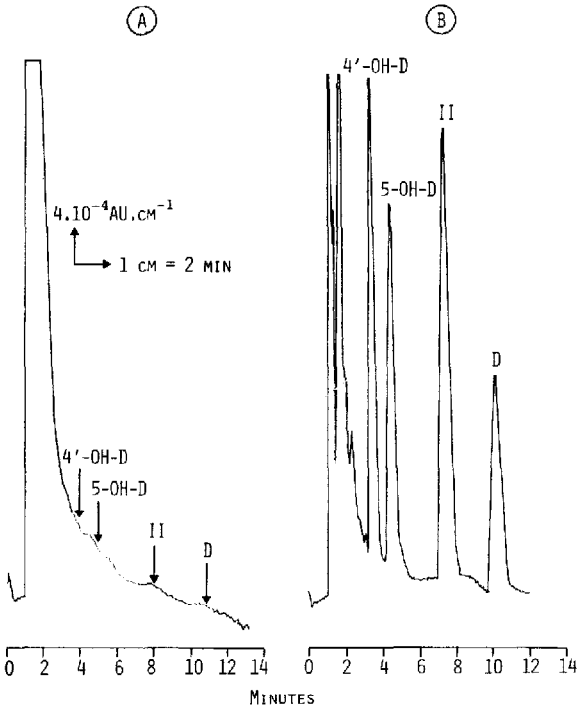


Fig. 4. Assay II. Typical chromatograms of diclofenac and its metabolites extracted from urine after chemical hydrolysis: (A) Urine blank; (B) urine spiked with 1 μg/ml diclofenac (D), 4'-hydroxydiclofenac (4'-OH-D) and 5-hydroxydiclofenac (5-OH-D) and 250 ng of internal standard (II).

Linearity. A good linear relationship was obtained in the range 200 ng/ml to 20 μg/ml for each compound (correlation coefficients higher than 0.9990).

Selectivity. The relative retention times were respectively 1, 1.74, 1.78, 2.25, 3.90 and 5.29 for 4',5-OH-D, 4'-, 3'- and 5-OH-D, 4'-hydroxy-5-chlorodiclofenac (II) and diclofenac. 3'- and 4'-OH-D give a single peak.

According to Riess et al. [9], the latter accounts for 20—30% of the total ^{14}C-radioactivity recovered in urine after oral administration whereas the former represents less than 5%. Therefore, the interference of 3'-OH-D in the assay of 4'-OH-D is within 15—20%.

The dihydroxylated metabolite, although well separated, could not be assayed because of interferences by endogenous components.

Accuracy, precision and reproducibility. 3'- and 4'-OH-D were not resolved and gave a single peak. For this reason, the accuracy and precision were assessed with samples spiked only with one of these two compounds, namely 4'-OH-D. Spiked samples prepared with urines from five volunteers by adding the unconjugated compounds were analysed. The results (Table II) show that diclofenac and its monohydroxylated metabolites can be precisely and accurately assayed in urine after chemical hydrolysis at concentrations down to 200 ng/ml.

The reproducibility was also assessed on samples collected after oral administration of diclofenac sodium, and analysed twice at a five-month interval. The

TABLE II

ACCURACY, PRECISION AND REPRODUCIBILITY OF ASSAY II

Urine samples were spiked with unconjugated compounds and assayed within a day.

Compound	Added (ng/ml)	Found, mean (ng/ml)	n	C.V. (%)	Mean recovery* (%)
Diclofenac	200	219	8	14.2	109
	500	486	4	5.6	97
	4000	4023	9	4.8	100
4'-Hydroxydiclofenac	200	184	8	6.7	93
	500	474	4	4.4	95
	4000	3884	8	3.5	97
5-Hydroxydiclofenac	200	200	8	9.3	100
	500	496	4	4.4	100
	4000	4025	7	8.8	101

*Mean of the individual recovery values.

slope of the regression line for second (HPLC 2) versus first (HPLC 1) analysis for the three compounds combined was 1.01 (r = 0.9988 for 35 values).

Stability. The methanolic solutions of diclofenac, 4'-OH-D and the internal standard II are stable for one month, that of 5-OH-D for only ten days (with ascorbic acid). Degradation of 5-OH-D was observed when the sample preparation was done in glass tubes used repeatedly instead of disposable glass ampoules.

Urine samples appeared stable for five months at −20°C. Three freezing—thawing cycles during this period had no influence on the stability.

Application. Urinary excretions in the study mentioned above (Fig. 5) demonstrate the applicability of Assay II. Recoveries are in good agreement with literature values [9,13].

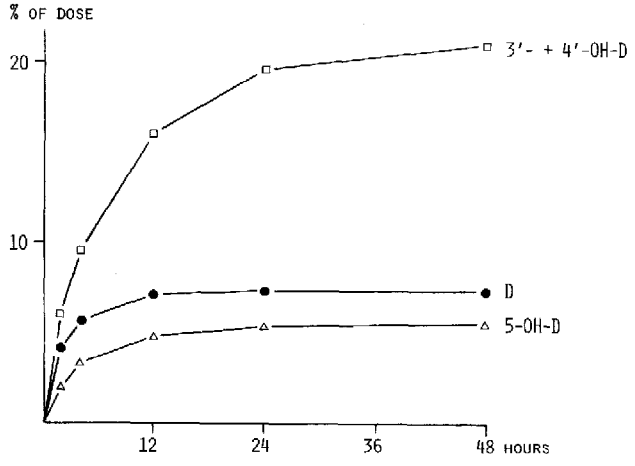


Fig. 5. Cumulative excretion of total (free + conjugated) diclofenac and its monohydroxylated metabolites in urine after a 75-mg dose given as a suppository (each point is the mean of six subjects). For abbreviations, see legend to Fig. 4.

CONCLUSION

Although 3′- and 4′-OH-D cannot be separated and 4′,5-OH-D cannot be measured as in the capillary GC method [13], the HPLC method described for diclofenac and its monohydroxylated metabolites in urine appears valuable and very useful for bioavailability evaluations. The HPLC technique developed for the assay of unchanged diclofenac in plasma is suited to the analysis of large numbers of samples.

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