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SIMULTANEOUS DETERMINATION OF DICLOFENAC SODIUM AND ITS HYDROXY METABOLITES BY CAPILLARY COLUMN GAS CHROMATOGRAPHY WITH ELECTRON-CAPTURE DETECTION

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SUMMARY

In humans, diclofenac sodium (Voltaren®), a potent anti-inflammatory agent, is metabolized to a large extent by hydroxylation and conjugation to glucuronides and sulfates. This paper describes a procedure whereby unchanged diclofenac as well as all known hydroxylated metabolites (3′-hydroxy-, 4′-hydroxy-, 5-hydroxy- and 4′,5-dihydroxy-diclofenac) are determined quantitatively in the same biological sample. The procedure is based on extractive alkylation and gas chromatography with capillary columns and electron capture detection. The method has been applied to analyse urine samples of volunteers treated with single doses of diclofenac sodium.

INTRODUCTION

Diclofenac sodium, a potent anti-inflammatory agent, is extensively metabolized in humans. About 60–70% of an oral dose of diclofenac sodium is excreted in the urine as conjugates of mono- or di-hydroxylated metabolites[1,2]. In view of the fact that some of the metabolites, in their free form, also possess anti-inflammatory activity[3] the simultaneous measurement of all known metabolites is of special interest.

Conjugates of the following compounds have been identified[4] in human urine (Fig. 1): diclofenac, 3′-hydroxydiclofenac, 4′-hydroxydiclofenac, 5-hydroxydiclofenac and 4′,5-dihydroxydiclofenac. Quantitative determinations of these compounds, after hydrolysis, have been performed with radioactively labelled material using thin-layer chromatography.

Gas chromatography (GC) was first used for the quantitative determination of unchanged diclofenac by Geiger *et al.*[5]. A method based on extractive alkylation and GC was described by Schweizer *et al.*[6] for the determination of the total monohydroxylated metabolites. This paper describes a GC procedure based on extractive alkylation and simultaneous determination of diclofenac and all known hydroxylated metabolites using a capillary column.

After alkaline hydrolysis of the conjugates, 4′-hydroxy-5-chlorodiclofenac is added as internal standard. The compounds are then extracted from urine as ion pairs (tetrahexylammonium as counter ion) into dichloromethane containing iodometh-

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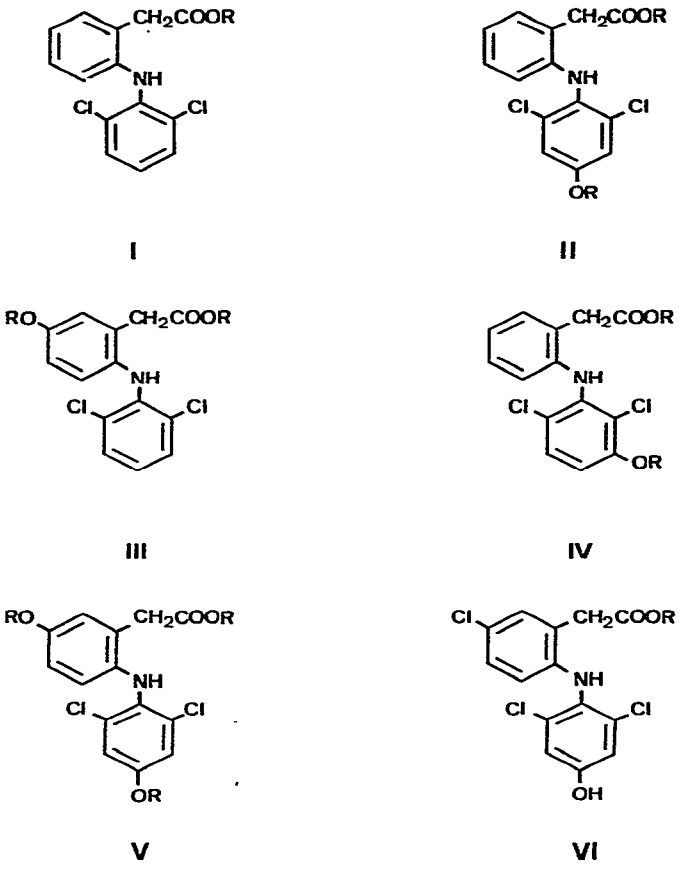

Fig. 1. Structures of diclofenac and its metabolites and of the internal standard: I = diclofenac; II = 4′-hydroxydiclofenac; III = 5-hydroxydiclofenac; IV = 3′-hydroxydiclofenac; V = 4′,5-dihydroxydiclofenac; VI = 4′-hydroxy-5-chlorodiclofenac. In the free compounds R = H. In the conjugated compounds R = glucuronic acid or sulfate residue.

ane. Methylation of the hydroxyl groups occurs almost instantaneously, as well as dimethylation of the α-carbon of the phenylacetic acid moiety (Fig. 2). The resulting derivatives are stable for at least 48 h at +4°C and can be measured quantitatively by GC. However, separation of the derivatives of 3′-, 4′- and 5-hydroxydiclofenac

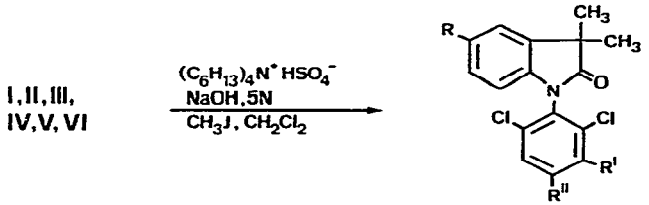

Fig. 2. Formation of the dimethyl indolinone derivatives by extractive alkylation: I, R = H, R′ = H, R″ = H; II, R = H, R′ = H, R″ = OCH_3; III, R = OCH_3, R′ = H, R″ = H; IV, R = H, R′ = OCH_3, R″ = H; V, R = OCH_3, R′ = H, R″ = OCH_3; VI, R = Cl, R′ = H, R″ = OCH_3.

cannot be achieved with packed columns. The use of a 30-m glass capillary column coated with Carbowax 40M allows separation of all five compounds and the internal standard. This method has been applied to urine samples of human volunteers after either i.m. or p.o. dosages of diclofenac sodium (Voltaren®).

EXPERIMENTAL

Reagents

All standard solutions (diclofenac sodium, metabolites and internal standard) were prepared daily in 0.1 *N* NaOH with 20 mg of ascorbic acid per 10 ml added as an anti-oxidant. Dichloromethane and hexane, laboratory grade, were distilled before use. Tetrahexylammonium hydrogen sulfate (Labkemi, Stockholm, Sweden) was dissolved in 0.1 *N* NaOH (0.05 *M*). Iodomethane (Fluka, Buchs, Switzerland) and ascorbic acid (Merck, Darmstadt, G.F.R.) were used as supplied.

Procedure

To 10–300 μl of urine (sample volume depending on concentration of metabolites) are added *ca.* 30 mg of ascorbic acid and 3 ml of 5 *N* NaOH. The mixture is left in a waterbath at 75°C for 30 min. After cooling to room temperature, 0.1 ml of internal standard (4.0 μg/ml), 50 μl of tetrahexylammonium hydrogen sulfate solution, and 20 μl iodomethane are added and the mixture is left at room temperature for 1 min. Then 3 ml dichloromethane are added and the mixture is shaken for 30 min at 150 rpm (mechanical rotary shaker). After brief centrifugation, the organic phase is removed and evaporated to dryness under a stream of nitrogen at 40°C. Then 0.5 ml of hexane and 2 ml of water are added to the dry residue, followed by shaking for 10 min at 150 rpm. After brief centrifugation the tubes are placed in dry-ice for 5 min. The organic phase is removed and aliquots of 0.2 μl are injected into the gas chromatograph.

Gas chromatography

In this study a Pye GCV gas chromatograph, equipped with a ^{63}Ni electron-capture detector, was used. The column was 30 m × 0.3 mm I.D. soft glass capillary, layered with $BaCO_3$ according to Grob and Grob[7], and coated with Carbowax 40M. Splitless injection technique was used, the flow-rate of helium through the column being 2 ml/min and that of the purge gas nitrogen 30 ml/min. The temperatures were: injector, 200°C; detector, 350°C; column, 200°C for 1 min, then increasing by 30°C/min up to 230°C.

Under these conditions the retention times are as follows: diclofenac derivative, 4.0 min; 4′-hydroxydiclofenac derivative, 6.8 min; 5-hydroxydiclofenac derivative, 7.2 min; 3′-hydroxydiclofenac derivative, 8.1 min; internal standard derivative, 10.4 min; 4′,5-dihydroxydiclofenac derivative, 14.3 min.

Chromatograms of a blank urine sample and of a urine sample spiked with 400 ng of each compound are shown in Fig. 3.

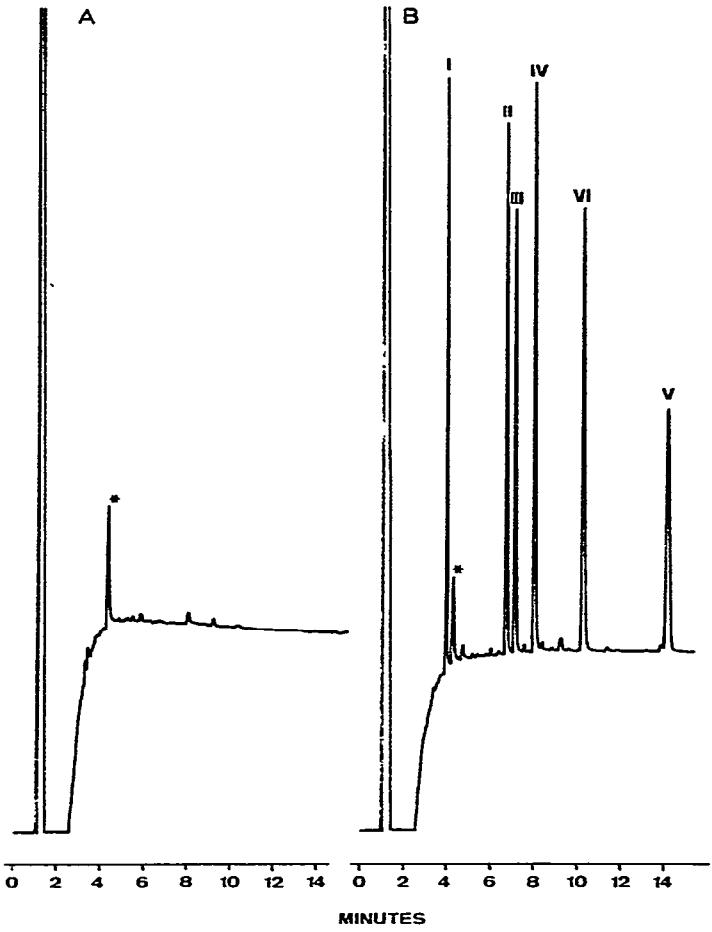

Fig. 3. Typical chromatograms of (A) extract of a blank urine sample (50 μl) and (B) extract of a urine sample spiked with 400 ng of each compound. Injected aliquot, 1/2500th. (The peak marked with an asterisk is an unknown constituent of urine.)

RESULTS AND DISCUSSION

Hydrolysis

Hydrolysis of the conjugated diclofenac and the metabolites was found to be optimal with 3 ml of 5 *N* NaOH at 75°C. The hydrolysed metabolites and diclofenac itself are stable under these conditions, provided ascorbic acid is added at the beginning.

Extractive alkylation

Extractive alkylation at strongly alkaline pH cyclizes diclofenac and the metabolites to their respective dimethyl indolinones. The aromatic hydroxyl groups are converted into methoxyl derivatives, and the protons of the α-carbon in the phenylacetic acid moiety are replaced by methyl groups. The conditions used are based on

the procedure of Schweizer *et al.*[6] with slight modifications. The structures of the derivatives have been verified by mass spectrometry.

Calibration graphs

Calibration graphs for the five compounds to be measured (diclofenac, 3′-, 4′- and 5-hydroxy-, and 4′,5-dihydroxydiclofenac) were prepared as follows: blank urine samples (50 μl) were spiked with known amounts of all five compounds (30–800 ng per sample). The samples were then processed as described. The peak heights of each derivative was divided by the peak height of the internal standard and plotted against initial concentration. The resulting calibration graphs are shown in Fig. 4.

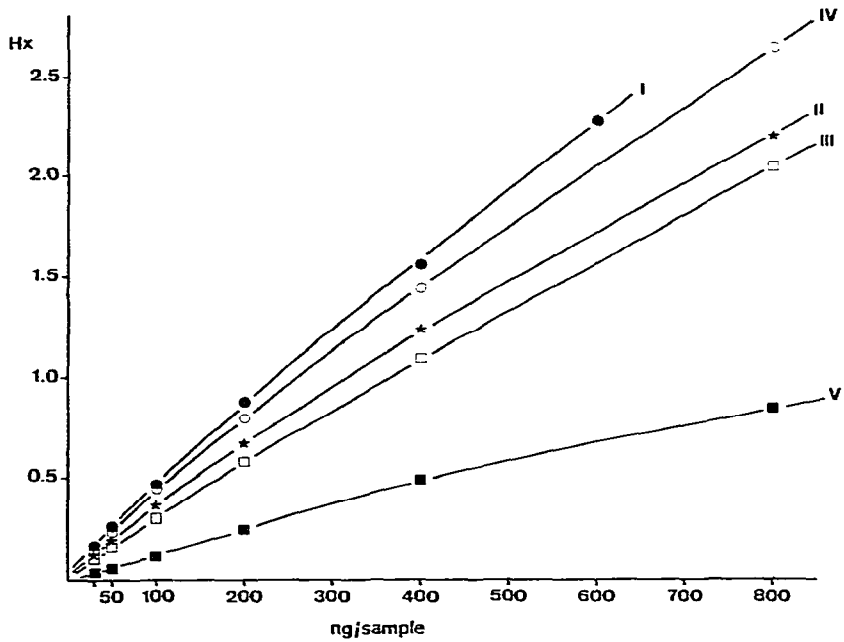

Fig. 4. Calibration curves for the entire analytical procedure.

$$H_x = \frac{\text{peak height of derivatives I, II, III, IV, V}}{\text{peak height of internal standard (VI)}}$$

Biological material, 50 μl of urine; amount of internal standard, 400 ng of compound VI; injected aliquot, 1/2500th.

Accuracy and precision

Accuracy and precision were evaluated by analysing spiked urine samples. Seven different samples were prepared, containing random compositions of diclofenac sodium and metabolites at concentrations between 30 and 800 ng per sample. Each sample was analysed twice. The coefficient of variation ranged from 0 to 14%. The differences between the mean found and the initial concentrations were between −7.8% and +14% (Fig. 5).

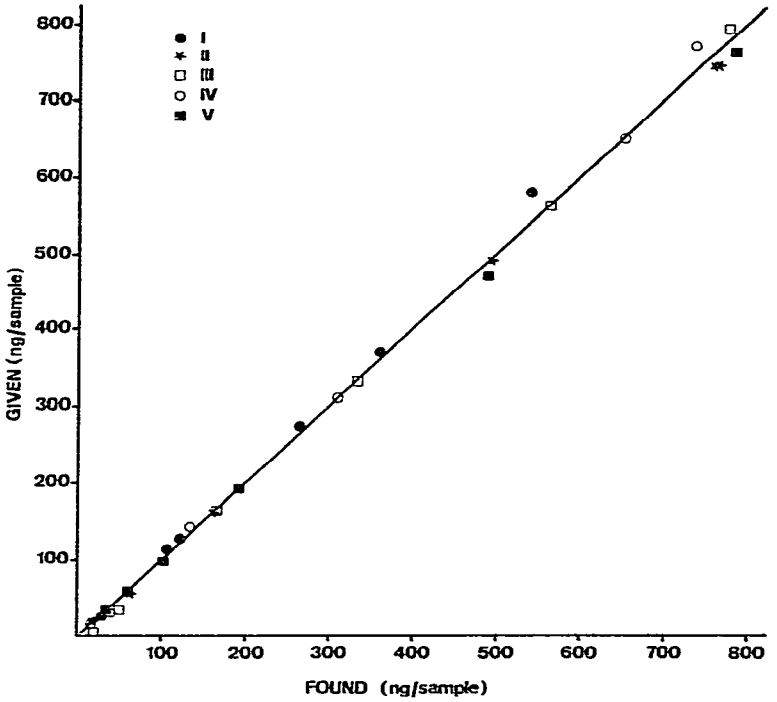

Fig. 5. Correlation of *given* and *found* concentrations of diclofenac and the hydroxy metabolites in spiked samples.

Cross-check with another method

A series of urine samples obtained from volunteers treated with single i.m. doses of 150 mg of Voltaren® was analysed for total (free and conjugated) diclofenac by the standard diclofenac assay, described by Geiger *et al.*[5], and by the new capillary

TABLE I

CONCENTRATIONS OF TOTAL (FREE AND CONJUGATED) DICLOFENAC IN URINE AS MEASURED BY THE ORIGINAL GC ASSAY (PACKED COLUMN) AND BY THE NEW (CAPILLARY COLUMN) ASSAY PROCEDURE, IN SAMPLES OBTAINED FROM HUMAN VOLUNTEERS AFTER INTRAMUSCULAR ADMINISTRATION OF VOLTAREN

Sample No.	*Original GC assay[5] for total diclofenac (μg/ml)*	*Extractive alkylation and capillary column procedure for total diclofenac (μg/ml)*
1	41.75	45.00
2	2.60	2.60
3	3.28	2.95
4	2.32	2.30
5	16.20	17.33
6	3.28	3.35
7	5.85	5.90
8	6.85	5.80

column method described here. The values of the new method deviate by between −15.3% and +7.8% from the values obtained by the original assay procedure (Table I).

Application

The applicability of this method was tested by analysing 24-hour urine samples of five healthy volunteers following treatment with single i.m. injections of 150 mg of Voltaren. The mean renal elimination of total (free and conjugated) diclofenac and its metabolites is shown in Table II.

TABLE II

MEAN RENAL ELIMINATION OF DICLOFENAC AND ITS METABOLITES

Total (free and conjugated) compound	*Renal elimination in 24 h (% of dose)*
Diclofenac	6.5 ± 2.9 ($\bar{x}$ ± S.D.)
4′-Hydroxydiclofenac	18.1 ± 8.7
5-Hydroxydiclofenac	8.2 ± 4.5
3′-Hydroxydiclofenac	1.4 ± 0.7
4′,5-Dihydroxydiclofenac	15.4 ± 4.7
Sum total	49.6

As can be seen the standard deviations are high, but only owing to the inter-individual variation in the group of five volunteers. The ratios of the specifically measured metabolites within each individual, however, appear to be fairly constant (Fig. 6).

One of these volunteers was further treated with a single oral dose of 50 mg of Voltaren. Urine was collected in fractions of 0–2, 2–4, 4–8 and 8–24 h. The renal elimination kinetics of free and conjugated diclofenac and its metabolites are shown in Fig. 7. The total recoveries (Table III) are in good agreement with those previously found[4] by use of radiolabelled diclofenac sodium.

TABLE III

COMPARISON OF TWO METHODS OF DETERMINING TOTAL RECOVERY

Total (free and conjugated compound	*Renal elimination in 24 h (% of dose)*	
	GC	*Radiolabelling*
Diclofenac	5.2	5–10
4′-Hydroxydiclofenac	11.5	20–30
5-Hydroxydiclofenac	4.8	5–10
3′-Hydroxydiclofenac	1.8	less than 5
4′,5-Dihydroxydiclofenac	9.4	5–10

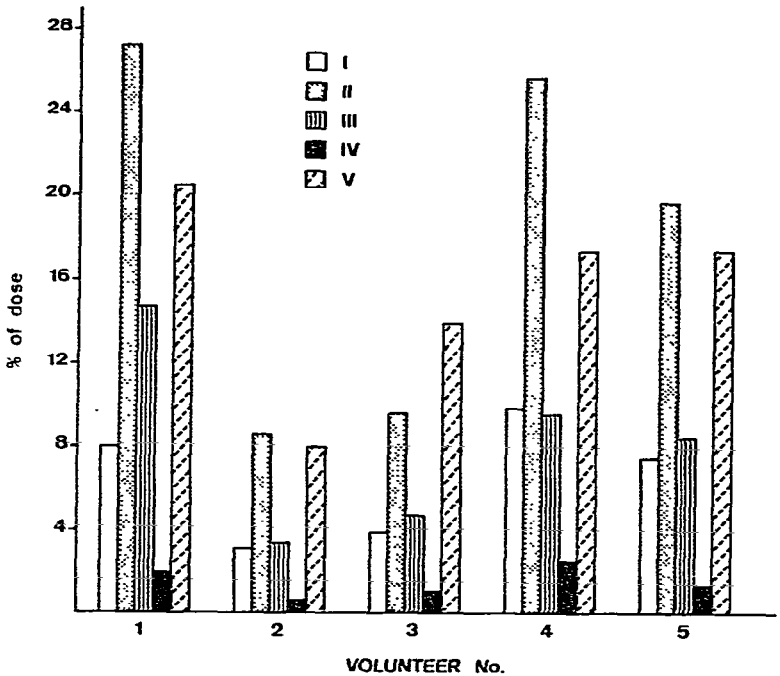

Fig. 6. Renal elimination of free and conjugated compounds I, II, III, IV and V (see Fig. 1) within 24 h after a single i.m. dose of 150 mg of Voltaren in five healthy volunteers.

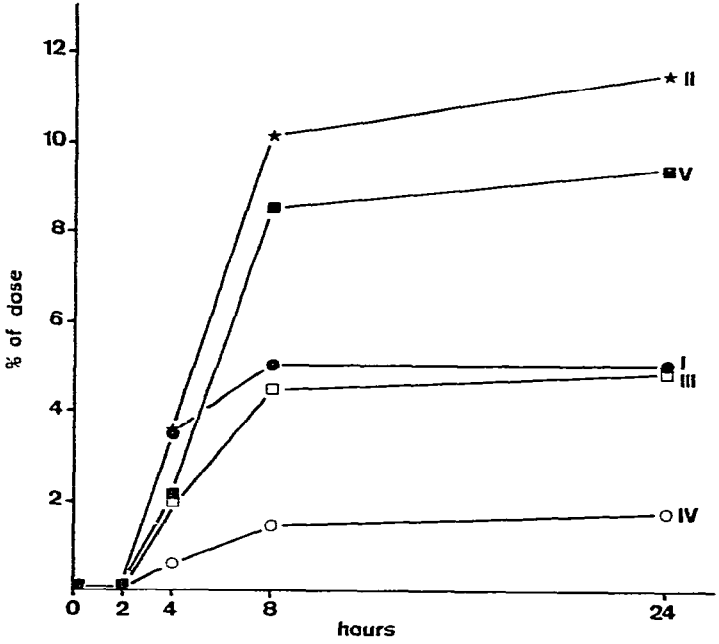

Fig. 7. Cumulative renal elimination of free and conjugated compounds I, II, III, IV and V (see Fig. 1) 2, 4, 8 and 24 h after a single oral dose of 50 mg of Voltaren in one healthy volunteer.

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