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Note

Simultaneous determination of diclofenac sodium and its metabolites in plasma by capillary column gas chromatography with electron-capture detection

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The anti-inflammatory drug diclofenac sodium is extensively metabolized in humans. Oxidation reactions at various positions of the molecule are predominant and the primary metabolites are conjugated subsequently. Besides the parent drug (I), the following compounds have been identified in human urine [1]: 3′-hydroxydiclofenac (IV), 4′-hydroxydiclofenac (II), 5-hydroxydiclofenac (III) and 4′,5-dihydroxydiclofenac (V); and in human and baboon plasma [2]: 3′-hydroxy-4′-methoxydiclofenac (VI) (Fig. 1). In view of the fact that some of these metabolites, in their free form, also possess anti-inflammatory activity [3], the simultaneous measurement of all known metabolites is of special interest.

Gas chromatography (GC) for the quantitative determination of unchanged diclofenac and the total monohydroxylated metabolites has been described by Schweizer et al. [4]. Godbillon et al. [5] have published a high-performance liquid chromatographic method for the determination of diclofenac and its monohydroxylated metabolites, but 3′- and 4′-hydroxydiclofenac were not separated and 4′,5-dihydroxydiclofenac could not be measured.

The present method is based on the assay published earlier for urine samples [6]. Owing to unknown reasons, direct extractive alkylation with plasma does not give satisfactory results. Thus, an extraction step was added to the procedure.

After the internal standard (4′-hydroxy-5-chlorodiclofenac, VII) has been added, the compounds are extracted from plasma at pH 5. Methylation of the hydroxyl groups with iodomethane and tetrahexylammonium hydrogen sulphate under alkaline conditions occurs almost instantaneously, as well as dimethylation of the α-carbon of the phenylacetic acid moiety (Fig. 2). The resulting deriv-

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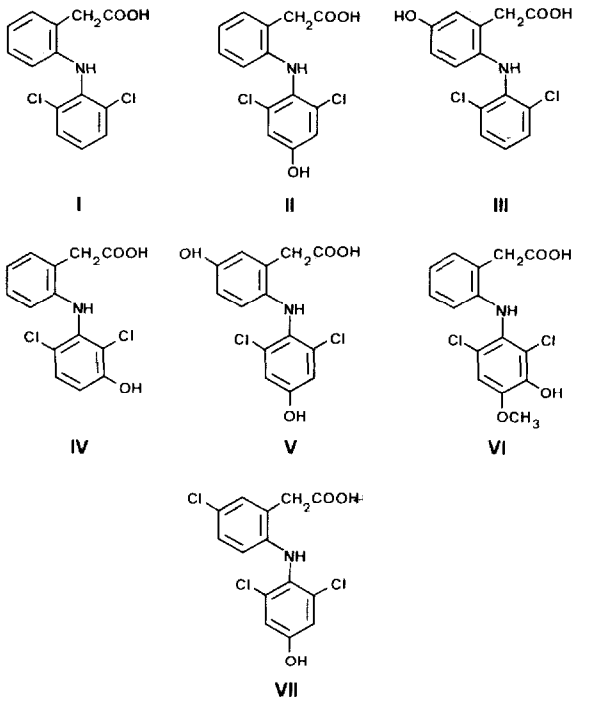

Fig. 1. Structures of diclofenac, its metabolites and the internal standard. I = Diclofenac; II = 4′-hydroxydiclofenac; III = 5-hydroxydiclofenac; IV = 3′-hydroxydiclofenac; V = 4′,5-dihydroxydiclofenac; VI = 3′-hydroxy-4′-methoxydiclofenac; VII = 4′-hydroxy-5-chlorodiclofenac.

atives are stable for at least 48 h at 4 °C. The use of a 25-m glass capillary column coated with Carbowax 40M allows the separation and quantitative determination of all six compounds and the internal standard. This method has been applied to plasma samples of a baboon and a human volunteer after an oral dose of diclofenac sodium (Voltaren®).

EXPERIMENTAL

Reagents

Solutions of diclofenac sodium, 4′-hydroxydiclofenac, 5-hydroxydiclofenac, 3′-hydroxydiclofenac, 3′-hydroxy-4′-methoxydiclofenac, 4′,5-dihydroxydiclofenac and 4′-hydroxy-5-dichlorodiclofenac (all supplied by Ciba-Geigy, Basle, Swit-

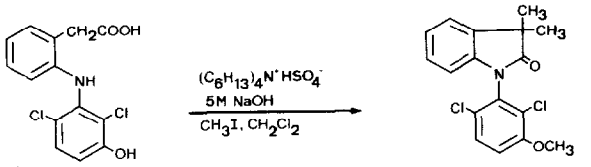

Fig. 2. Formation of the dimethyl indolinone derivative of 3′-hydroxydiclofenac (IV) by strongly alkaline alkylation. All the other compounds were derivatized analogously.

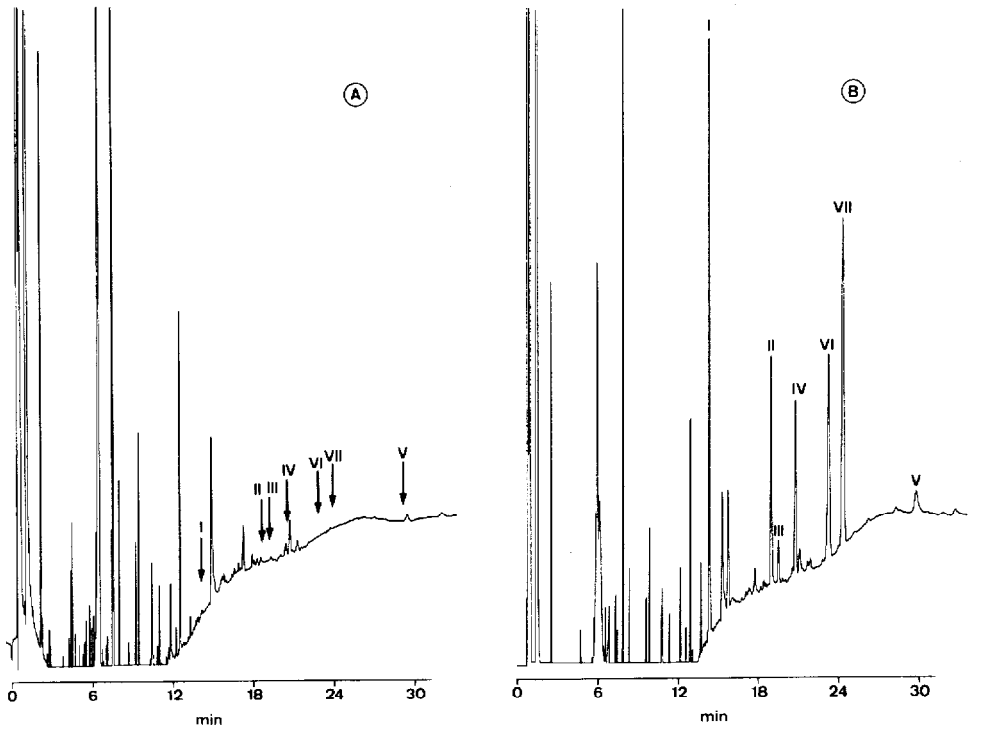

Fig. 3. Chromatograms of plasma extracts. (A) Blank plasma (1 ml); (B) plasma from a volunteer (1 ml) collected 6 h after a single oral dose of 100 mg of a slow release formulation of diclofenac sodium. The sample contains 1.21 nmol of I, 0.65 nmol of II, 0.15 nmol of III, 0.34 nmol of IV, 0.32 nmol of VI, 1.44 nmol of VII and 0.15 nmol of V. Injected aliquot: 1/500th.

zerland) were prepared daily in 0.1 *M* sodium hydroxide with 20 mg of ascorbic acid per 10 ml added as an anti-oxidant. Dichloromethane, diethyl ether and hexane, laboratory grade, were distilled before use. Acetate buffer pH 5.12 (1.795 mol sodium acetate and 0.825 mol acetic acid per litre). Tetrahexylammonium hydrogen sulphate (Labkemi, Stockholm, Sweden) was dissolved in 0.1 *M* sodium hydroxide (0.05 *M*). Iodomethane (Fluka, Buchs, Switzerland) and ascorbic acid (Merck, Darmstadt, F.R.G.) were used as supplied.

Extraction

First 0.1–2 ml of plasma, 0.2 ml of a 20% aqueous solution of ascorbic acid and 0.1 ml of the internal standard (1.40 nmol) were acidified with 1 ml of acetate buffer (resulting pH 4.7–5.0) and shaken with 5 ml of a mixture of diethyl ether–dichloromethane (2:1) for 15 min at 150 rpm on a mechanical rotary shaker (Infors). After brief centrifugation (2 min at 2500 *g*), the organic phase was transferred into a clean tube and evaporated to dryness under a stream of nitrogen.

Alkylation

The residue was dissolved in 3 ml of 5 *M* sodium hydroxide, and 100 μl of tetrahexylammonium hydrogen sulphate solution and 40 μl of iodomethane were

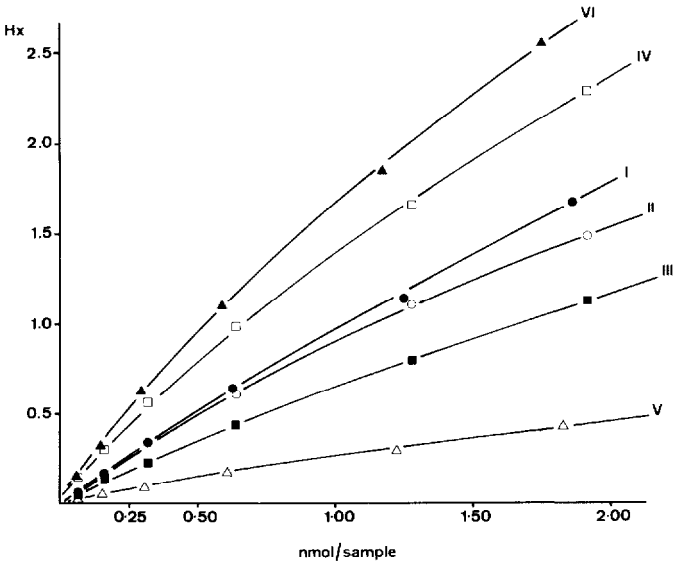

Fig. 4. Calibration curves for the entire analytical procedure. Hx = peak height of derivative (I, II, III, IV, V or VI)/peak height of internal standard (VII).

added, mixed and left at room temperature for 2 min. Then 3 ml of dichloromethane were added and the mixture was shaken for 30 min at 150 rpm on a mechanical rotary shaker. After brief centrifugation (2 min at 2500 *g*), the organic phase was removed and evaporated to dryness under a stream of nitrogen at 40 °C. Then 0.3 ml of hexane and 1.5 ml of water were added to the dry residue, followed by shaking for 10 min at 150 rpm. After centrifugation (2 min at 2500 *g*), the tubes were placed in dry-ice for 5 min. The organic phase was removed and aliquots of 1 µl were analysed by GC.

Gas chromatography

A Carlo-Erba Fractovap Model 4160 chromatograph with on-column injection system was employed. The detection system was a ^{63}Ni (10 mCi) electron-capture detector, used in the constant-current mode (voltage 50 V, pulse width 5 µs). The column was a 25 m × 0.3 mm I.D. soft-glass capillary, coated with barium carbonate according to Grob and Grob [7], and statically coated with Carbowax 40M (film thickness 0.15 µm). The detector temperature was 300 °C. The column was initially held at 65 °C for 1 min and then its temperature was increased fast by 30 °C min^{-1} to 190 °C and then to a final temperature of 240 °C at a rate of 10 °C min^{-1}. The carrier gas was hydrogen at a flow-rate (measured at 220 °C) of 2.0 cm^3 min^{-1}, and the make-up gas was nitrogen at a flow-rate of 30 cm^3 min^{-1}.

Under these conditions the retention times of the derivatives were as follows: diclofenac (I), 14.4 min; 4′-hydroxydiclofenac (II), 18.8 min; 5-hydroxydiclofenac (III), 19.3 min; 3′-hydroxydiclofenac (IV), 20.6 min; 3′-hydroxy-4′-methoxydiclofenac (VI), 22.7 min; internal standard (VII), 23.7 min; 4′,5-dihydroxydiclofenac (V), 28.8 min.

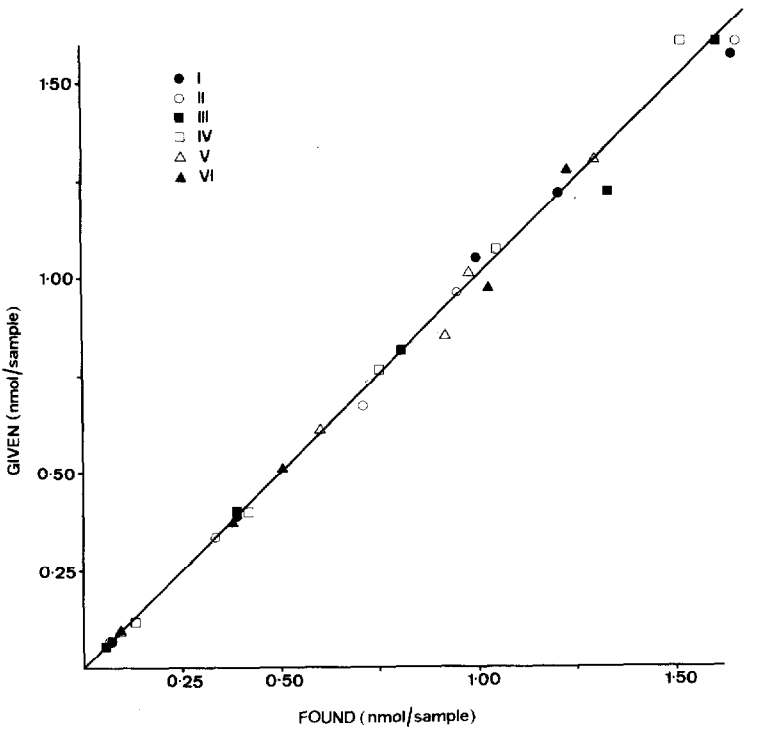

Fig. 5. Correlation of given and found concentrations of diclofenac and its metabolites in spiked plasma samples. The straight line represents given equal to found.

Chromatograms of a blank plasma and plasma from a volunteer collected 6 h after a single oral dose of 100 mg of a slow release formulation of diclofenac sodium are shown in Fig. 3.

RESULTS AND DISCUSSION

Alkylation

Alkylation at strongly alkaline pH cyclizes diclofenac and the metabolites to their respective dimethylindolinones. The aromatic hydroxyl groups are converted into methoxy 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 described earlier [6] with slight modifications. The structures of the derivatives have been verified by mass spectrometry.

Calibration graphs

Calibration graphs for the six compounds to be measured (I–VI) were prepared as follows: 500-μl aliquots of blank plasma samples were spiked with amounts between 0.058 and 1.922 nmol of all six compounds and with 1.40 nmol of internal standard per sample; the samples were then processed as described; the peak height of each derivative was divided by the peak height of the internal standard

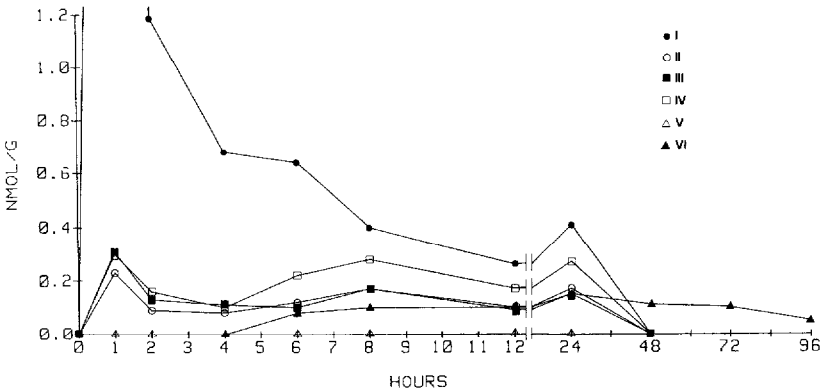

Fig. 6. Plasma concentration–time curves of diclofenac and its metabolites after an oral dose of 5 mg/kg diclofenac sodium (in form of a buffered solution) given to a baboon.

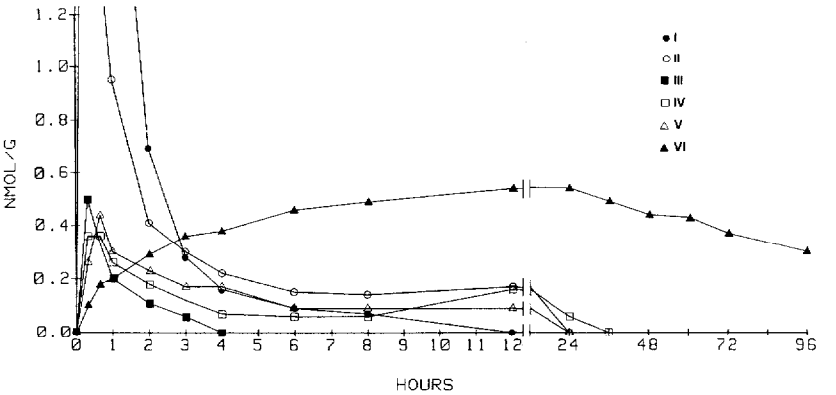

Fig. 7. Plasma concentration–time curves of diclofenac and its metabolites after an oral dose of 100 mg of diclofenac sodium (in form of a buffered solution) given to one healthy human volunteer.

and plotted against the initial concentration. The resulting calibration graphs are shown in Fig. 4.

Accuracy, precision and limit of quantitation

Accuracy and precision were evaluated by analysing spiked plasma samples. Five different samples were prepared, containing random amounts of diclofenac sodium and metabolites at concentrations between 0.064 and 1.60 nmol per sample. Each sample was analysed twice. The differences between the mean found and the initial concentrations were between −10% and +8.3% (Fig. 5).

The limit of quantitation was ca. 30 pmol/g plasma for diclofenac and the five metabolites. The relative standard deviation, at the 30 pmol/g level, was ca. ± 10%.

Application

Figs. 6 and 7 show the plasma concentration–time curves of diclofenac and the five metabolites in a baboon and in one healthy human volunteer after single oral administration of diclofenac sodium in form of a buffered solution. The elimination of unchanged diclofenac from plasma was faster in the human than in the baboon. The plasma concentration profile of metabolite II was similar to that of

diclofenac in the human, but considerably lower in the baboon. Metabolites III, IV and V in the human, as well as metabolites III and IV in the baboon, occurred in much lower concentrations than diclofenac. Metabolite V was not detected in the baboon. In both species the profiles of metabolite VI were characterized by a slow rise of the concentration followed by a rather slow decrease, resulting in a long-lasting concentration plateau.

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