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Note

Determination of total monohydroxylated metabolites of diclofenac in urine by electron-capture gas–liquid chromatography

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The anti-inflammatory agent diclofenac sodium is extensively metabolised in man[1]. Stierlin and co-workers[2,3] have reported that up to 60% of the dose excreted appears in urine, less than 1% of which is eliminated unchanged. In man, phenolic metabolites are formed (Fig. 1) which are mainly conjugated to glucuronide and sulphate esters[3]. The purpose of this investigation was to establish a simple gas–liquid chromatographic method to quantitate the two major metabolites, 4-hydroxy- and 5'-hydroxy-diclofenac.

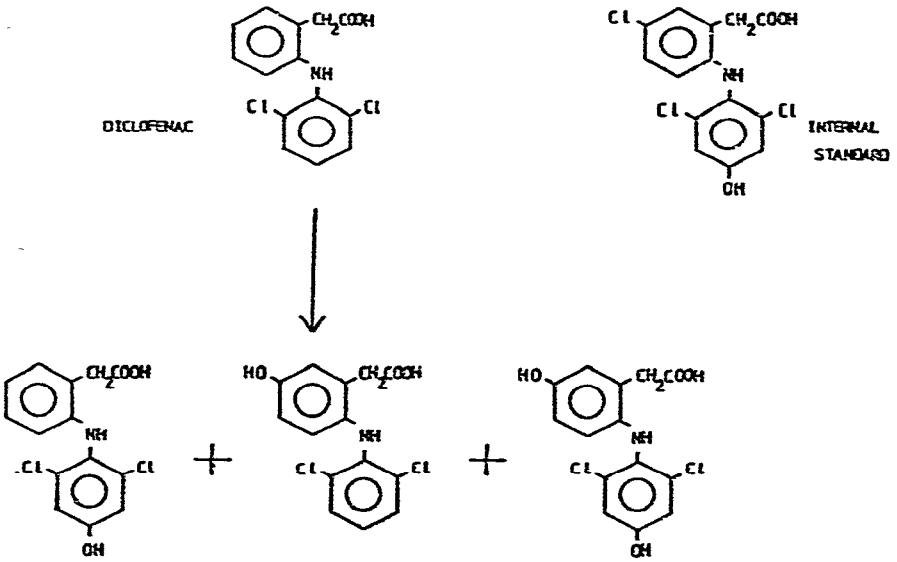

Fig. 1. Structure of diclofenac and its phenolic metabolites and of the internal standard used in their determination.

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MATERIALS AND METHODS

Diethyl ether and *n*-hexane, standard laboratory grade, were redistilled before use, and dichloromethane (Distol® grade; Fisons, Loughborough, Great Britain) was used without further purification. For extraction, ether and dichloromethane were mixed (3:1; v/v). Sodium acetate buffer (1.0 *M*, pH 5.0), tetrabutylammonium hydrogen sulphate (0.05 *M*) and sodium hydroxide (2.5 *M*) were used. Iodomethane (Sigma London, Poole, Great Britain) and ascorbic acid (Fisons) were used as supplied. All reagents were of analytical grade.

Standard solutions were prepared in distilled water as follows: 4-hydroxy-diclofenac (GP 47 766), 0.2 μg/ml; standard solution of 5'-hydroxy-diclofenac (GP 47 852), 0.2 μg/ml; and internal standard, CGP 7406, 0.8 μg/ml. Metabolites and internal standard were supplied by Ciba-Geigy (Basle, Switzerland). Metabolite solutions were prepared daily and internal standard solutions every two weeks.

Extraction of free metabolites from urine

A 1-ml volume of urine, diluted if necessary with 10 ml distilled water, and 0.5 ml of internal standard solution were acidified with 3 ml of sodium acetate buffer and 100 mg of ascorbic acid added as an anti-oxidant. A 3.5-ml volume of the extraction solvent was added and the mixture shaken mechanically for 20 min at 90 strokes/min, then centrifuged at 300 *g* for 10 min.

Methylation

The organic layer was removed and evaporated to dryness at 45°C by a gentle stream of nitrogen. The residue was dissolved in 2 ml sodium hydroxide to which 0.2 ml tetrabutyl ammonium hydrogen sulphate solution was added. To this, 0.05 ml of iodomethane in 3.5 ml dichloromethane was added. The mixture was shaken at room temperature for 20 min then centrifuged as before. After centrifugation, the dichloromethane layer was removed and evaporated to dryness. The residue was re-dissolved in 0.2 ml hexane and 3 μl injected into the gas-liquid chromatograph. The structure of the derivatives formed may be seen in Fig. 2.

Procedure for conjugates

Both diclofenac and its hydroxylated metabolites are excreted in urine in free and conjugated forms[3]. These conjugates may be hydrolysed by treatment with alkali and the total measured. A 400-mg amount of sodium hydroxide was added to 3 ml of urine which was then incubated at 75°C for 60 min. This hydrolysed urine was submitted to the extraction and derivatisation procedures described for the free metabolites.

Gas chromatography

Chromatography of the derivatives was performed on a Hewlett-Packard 5710A gas chromatograph equipped with an electron-capture detector (^{63}Ni, 15 mCi). A 2 m × 3 mm I.D. glass column was used packed with 3% OV-17 on Gas-Chrom Q, 80–100 mesh (Field, Richmond, Great Britain). The oven temperature was 245°C, the injection port 250°C and detector 300°C. The flow-rate of the carrier gas (argon–methane, 90:10) was maintained at 60 ml/min.

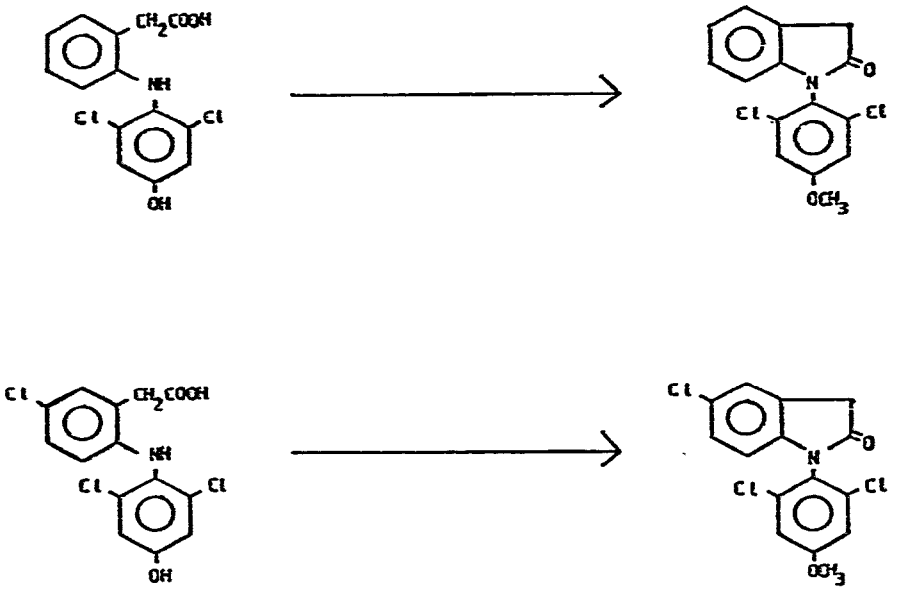

Fig. 2. Methylation of 4-hydroxy-diclofenac and the internal standard. 5'-Hydroxy-diclofenac undergoes a similar reaction.

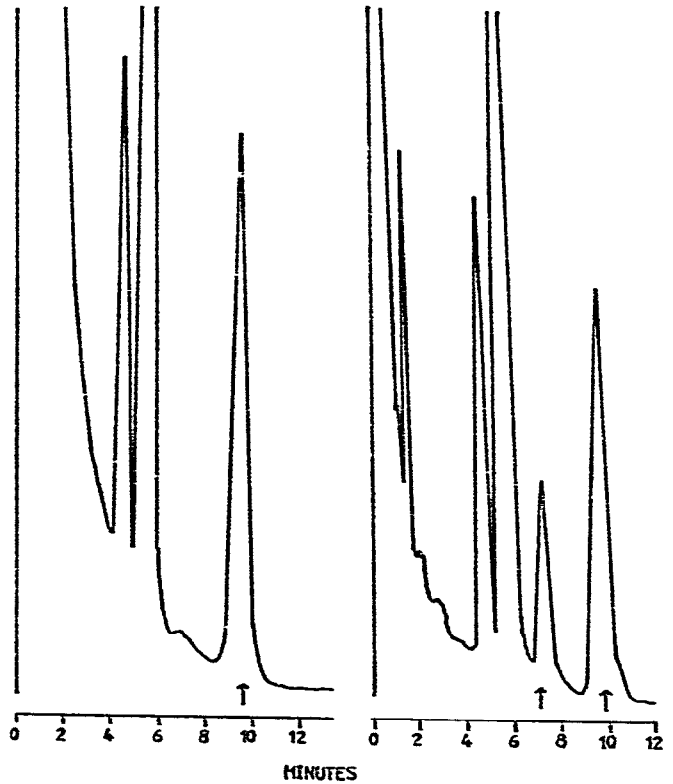

Fig. 3. Typical chromatograms obtained after injection of 400 ng/ml of internal standard only (left) and 200 ng/ml of metabolite with 400 ng/ml of internal standard (right).

RESULTS AND DISCUSSION

The two metabolites were not resolved and were measured as one peak. Quantitation was by measurement of peak height. Examples of typical chromatograms obtained can be seen in Fig. 3. Analysis of blank urine samples obtained from volunteers showed no substances interfering with the method of assay.

Known amounts of 4-hydroxy- and 5'-hydroxy-diclofenac were added to biological fluids and carried through the extraction and derivatization procedures. Peak heights of drug and internal standard were measured and calibration curves constructed. Calibration curves were linear within the range 0–500 ng/ml and metabolite levels could be determined down to 10 ng/ml.

The precision of the assay procedure was assessed from the coefficient of variation determined for five different concentrations of metabolite assayed on six independent occasions. The results may be seen in Table I. A coefficient of variation of 7% was obtained. The method has now been successfully used in a number of studies and no modifications have been necessary.

TABLE I

PRECISION OF THE ASSAY FOR TOTAL MONOHYDROXYLATED METABOLITES

Concentration (ng/ml)		*S.D.*	*C.V. (%)*
Actual	*Mean (n = 6)*		
100	98	5	5.1
200	205	11	5.3
300	295	13	4.3
400	405	26	6.4
500	498	18	3.7

NOTE ADDED IN PROOF

We have now established that the minor metabolite 3-hydroxy-diclofenac will also be measured by this method since its lactam derivative has the same retention time as the 4- and 5'-hydroxy derivatives under the conditions described.

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