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Determination of diclofenac in plasma and urine by capillary gas chromatography–mass spectrometry with possible simultaneous determination of deuterium-labelled diclofenac

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ABSTRACT

A specific and sensitive method for the determination of diclofenac at concentrations down to *ca.* 1 ng/ml, the limit of detection being 100 pg/ml, in human plasma and urine by gas chromatography–mass spectrometry with $^{2}H_{4}$-labelled diclofenac as internal standard is described. The method is also suitable for the simultaneous assay of these two compounds when both are present in samples of human plasma or urine. In this case, 5-chlorodiclofenac is used as internal standard. After toluene extraction from plasma or without extraction for urine, the method involves the formation of a dimethylindolinone derivative by extractive alkylation. The technique was applied to determine low plasma concentrations and urinary excretion of labelled and unlabelled diclofenac after percutaneous applications of Voltaren Emulgel to humans applied simultaneously under occlusive dressing as deuterated diclofenac sodium, and without occlusive dressing as unlabelled diclofenac sodium.

INTRODUCTION

Diclofenac sodium is a non-steroidal antiinflammatory drug used in the treatment of rheumatic diseases. Several assay procedures for biological fluids are now available, based on gas chromatography [1,2], gas chromatography–mass spectrometry (GC–MS) [3,4] and high-performance liquid chromatography [5–7]. A compilation has been recently published [8]. Only GC–MS methods [3,4] are suitable for the measurement of the very low plasma concentrations (less than 10 ng/ml) of diclofenac achieved after percutaneous application of diclofenac. A method using GC with negative-ion chemical ionization MS with [$^{18}O_2$]diclofenac as internal standard has been recently published [4] for the quantitative measurement of diclofenac in human plasma at femtomole levels. But validation data showing the precision and accuracy were not detailed. A signal-to-noise ratio of 4 was considered appropriate for the lowest detectable amount of 2 pg/ml.

The simultaneous administration of a drug and the same molecule labelled with a stable isotope is used in pharmacokinetic studies to avoid intra-individual

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variability. The simultaneous determination of the unlabelled and labelled drug is performed by GC–MS.

The method described in this paper is based on the GC assays already published for unchanged diclofenac and its known metabolites [1,2], which were adapted to a gas chromatograph with mass-selective detection (MSD).

Diclofenac sodium labelled with deuterium (D_4-diclofenac) has been synthesized [9] and used as an internal standard for the determination of diclofenac (D_0-diclofenac) in human plasma and urine. Also, both compounds were administered simultaneously in studies based on the stable isotope labelling technique, using 5-chlorodiclofenac as internal standard, for the simultaneous determination of D_0- and D_4-diclofenac in plasma and urine.

EXPERIMENTAL

Chemicals and reagents

Diclofenac sodium and the internal standard, 5-chlorodiclofenac, were supplied by Ciba-Geigy (Basle, Switzerland).

Diclofenac sodium labelled with deuterium in the phenylacetic ring was prepared from [$^2H_5$]bromobenzene in our laboratories according to a reaction scheme derived from that described by Stierlin *et al.* [9]. The isotopic composition of labelled diclofenac was 36.4% D_4-, 36.2% D_3-, 19.6% D_2-, 6.6% D_1- and 1.1% D_0-diclofenac.

The solvents and reagents used were all of analytical grade: toluene, dichloromethane and hexane (Pestipur, SDS, Peypin, France); 0.05 *M* tetrahexylammonium hydrogensulphate solution (Fluka 87303, Fluka, Buchs, Switzerland), prepared in 0.1 *M* sodium hydroxide; iodomethane (Fluka 67692); 5 *M* phosphoric acid, prepared from 85% phosphoric acid (Merck 573, Merck, Darmstadt, Germany).

Equipment

A Hewlett-Packard 5890A gas chromatograph equipped with a capillary inlet system and an HP 7673A automatic sampler was used. The column was a 12.5 m × 0.2 mm fused-silica capillary column coated with cross-linked methyl silicone (Hewlett-Packard 19091A, option 101), the film thickness was 0.33 μm. The carrier gas was helium with an inlet pressure of 62 kPa (9 p.s.i.g.). Splitless injection was used with a 0.30-min splitless period. The injection temperature was 270°C. The column was initially at 150°C for 0.5 min, and the temperature was then raised at 50°C/min to 230°C. A Hewlett-Packard 5970B mass-selective detector was interfaced with the 5890A gas chromatograph, with the capillary column inserted directly into the ion source. The invariable electron energy was 70 eV, and the voltage of electron multiplier was 1800 V. The GC–MS interface was maintained at 280°C.

The MS was calibrated with the Autotune program at the beginning of each

day using perfluorotributylamine (PFTBA). The MS was turned on from 2 to 5 min after injection. The selected ions, monitored with a dwell time of 10 ms corresponding to the various dimethylindolinone derivatives, were m/z 305 for D_0-diclofenac, m/z 309 for D_4-diclofenac and m/z 339 for 5-chlorodiclofenac.

Calibration and validation samples

For the assay of both D_0- and D_4-diclofenac, 5-chlorodiclofenac was used as internal standard. D_4-Diclofenac was the internal standard for the assay of D_0-diclofenac.

Plasma. Aliquots of working solutions and a constant amount of internal standard (313 pmol per 50 μl of 5-chlorodiclofenac or 165 pmol per 50 μl of D_4-diclofenac) in methanol were evaporated before the addition of 1 ml of human plasma to produce reference samples in the concentration range 3.4–340 n*M*.

Urine. Aliquots of working solutions and a constant amount of internal standard (319 pmol per 50 μl of 5-chlorodiclofenac or 165 pmol per 50 μl of D_4-diclofenac) in methanol were evaporated to dryness before the addition of 1 ml of human urine to produce reference samples in the concentration range 3.4–1700 n*M*. The amounts of compounds were different in plasma and urine owing to different weighings for the calibration.

Sample preparation

Plasma. Plasma samples were extracted with toluene prior to extractive alkylation. A 50-μl volume of internal standard solution (313 pmol per 50 μl of 5-chlorodiclofenac or 165 pmol per 50 μl of D_4-diclofenac) was placed in a 10-ml glass tube and evaporated to dryness, then 1 ml of plasma, 1 ml of 5 *M* H_3PO_4 and 5 ml of toluene were added. The mixture was shaken for 15 min at 300 rpm and centrifuged at 1600 g for 4 min. The organic phase was transferred to a 10-ml conical glass tube and evaporated to dryness under a stream of nitrogen at 40°C.

Urine. Direct extractive alkylation was used with urine for the assay of free (unconjugated) diclofenac. For total diclofenac (free + conjugated), prior alkaline hydrolysis of conjugates was carried out: the mixture of 1 ml of urine, 30 mg of ascorbic acid and 5 *M* NaOH was left at 75°C for 30 min.

Extractive alkylation

The same conditions for extractive alkylation were applied to both the dry residue obtained from plasma, with 3 ml of 5 *M* NaOH added, and urine after alkaline hydrolysis. A 100-μl volume of 0.05 *M* tetrahexylammonium hydrogensulphate and 40 μl of iodomethane were added. After mixing, the tube was left at room temperature for 1 min. Then, 3 ml of dichloromethane were added and the mixture was shaken for 30 min at 150 rpm. After centrifugation at 1600 *g* for 2 min, the aqueous phase was discarded. The organic phase was transferred to a conical tube and evaporated to dryness under a nitrogen stream at 40°C. Then, 1 ml of hexane and 2 ml of water were added to the dry residue followed by shaking

for 10 min at 200 rpm. After centrifugation at 1600 *g* for 2 min, the tube was placed in dry-ice to freeze the aqueous phase. The organic phase was transferred to a small conical tube and evaporated to dryness. The residue was dissolved in 20 μl of toluene, and 2 μl were injected into the gas chromatograph.

Correction of the data for isotopic contributions

Owing to isotopic contributions, the measurements at *m/z* 305 and 309 were not specific for D_0-diclofenac and D_4-diclofenac, respectively.

To estimate these contributions, two solutions containing either D_0-diclofenac or D_4-diclofenac at a concentration of *ca.* 1.4 μ*M* were processed as described for the extractive alkylation. After separate injection of each derivative, the recorded heights at *m/z* 305 and 309 were used for the estimation of the isotopic contributions. The measured peak heights were corrected in accordance with a method adapted from published procedures [10–12]. The mathematical treatment was based on the assumption that variable amounts of the compounds do not result in distortions of the mass spectra, and the fact that the signal measured at a given mass is the sum of the signals from all the molecular species at the considered mass.

Human study

A healthy subject was treated simultaneously with 2.5 g of Voltaren Emulgel containing 1% diclofenac sodium without occlusive dressing and 1.25 g of Voltaren Emulgel containing 1% deuterated diclofenac sodium applied 10 h with occlusive dressing.

Blood samples were collected before and 2, 4, 6, 12 and 24 h after application. Plasma was separated by centrifugation and stored at −20°C until analysis.

Urine was collected at the following time intervals: 0–6, 6–12, 12–24 and 24–48 h after application. The volume was measured and an aliquot was stored at −20°C.

RESULTS AND DISCUSSION

The dimethylindolinone derivatives, formed by extractive alkylation as previously described [1,2], of D_0-diclofenac, D_4-diclofenac and the internal standard were prepared. With tetrahexylammonium as the counter ion, the compounds were extracted from the aqueous phase as their ion pairs into iodomethane, in which dimethylation of the α-carbon atom of the phenylacetic acid moiety occurred. With the hydroxylated metabolites, methylation of the hydroxy groups also occurred.

Mass spectra and isotopic contributions

Electron-impact spectra of the various derivatives are shown in Fig. 1.

The molecular ions at *m/z* 305, 309 and 339 were selected for quantitative

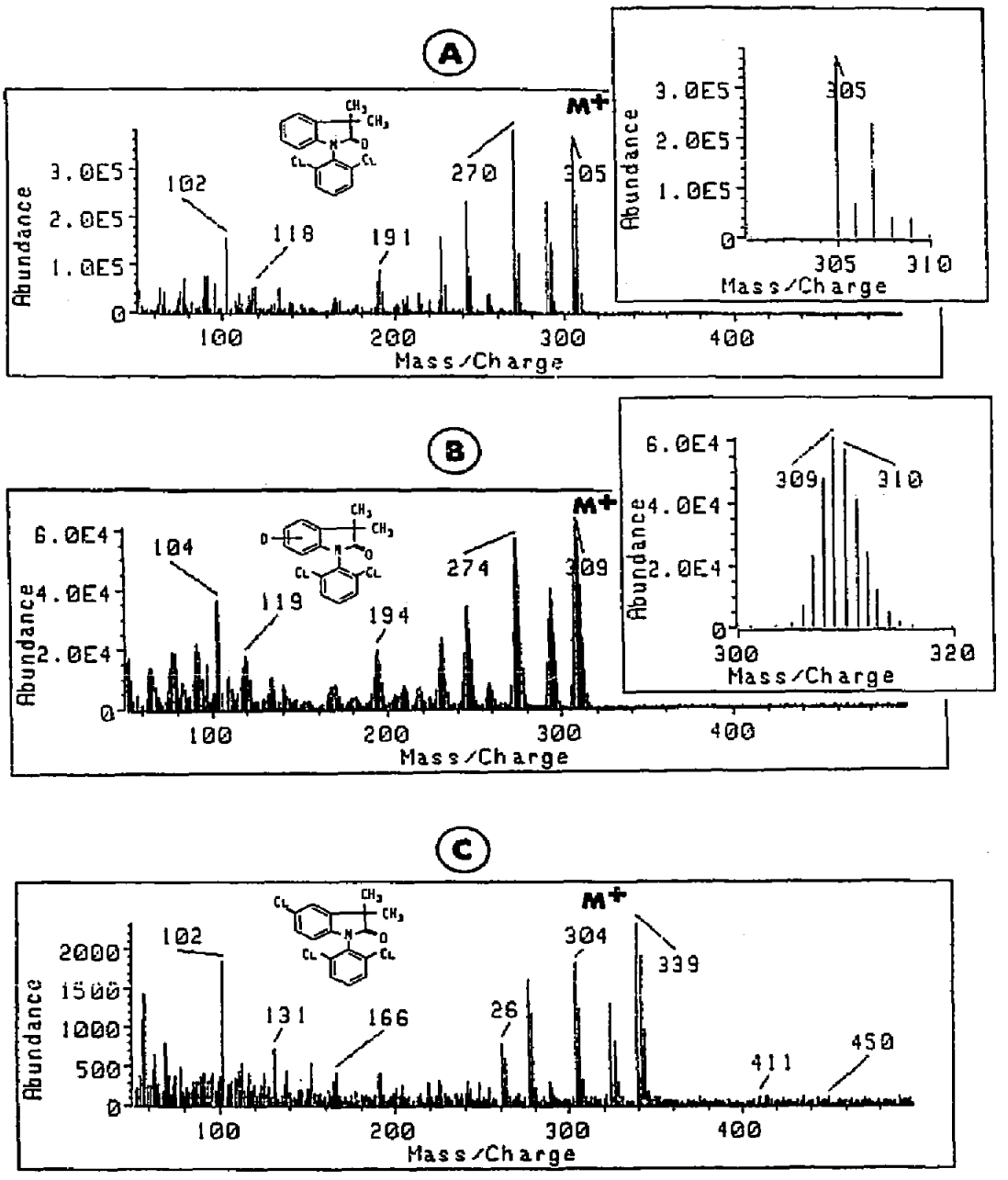

Fig. 1. Mass spectra of the dimethylindolinone derivatives of D_0-diclofenac (A), D_4-diclofenac (B) and 5-chlorodiclofenac (C).

measurements in the selected-ion monitoring mode. As shown in Fig. 1, D_0-diclofenac and D_4-diclofenac interfered with each other when measured at *m/z* 305 and 309. The isotopic contributions were estimated from the heights recorded at the two *m/z* values after injection of the derivative of each compound. The values of these isotopic contributions were slightly variable from day to day. They were measured daily.

Plasma and urine interferences

The extracts of blank human plasma and urine showed a clean baseline at *m/z* 305, 309 and 339; typical selected-ion current profiles are shown in Figs. 2 and 3.

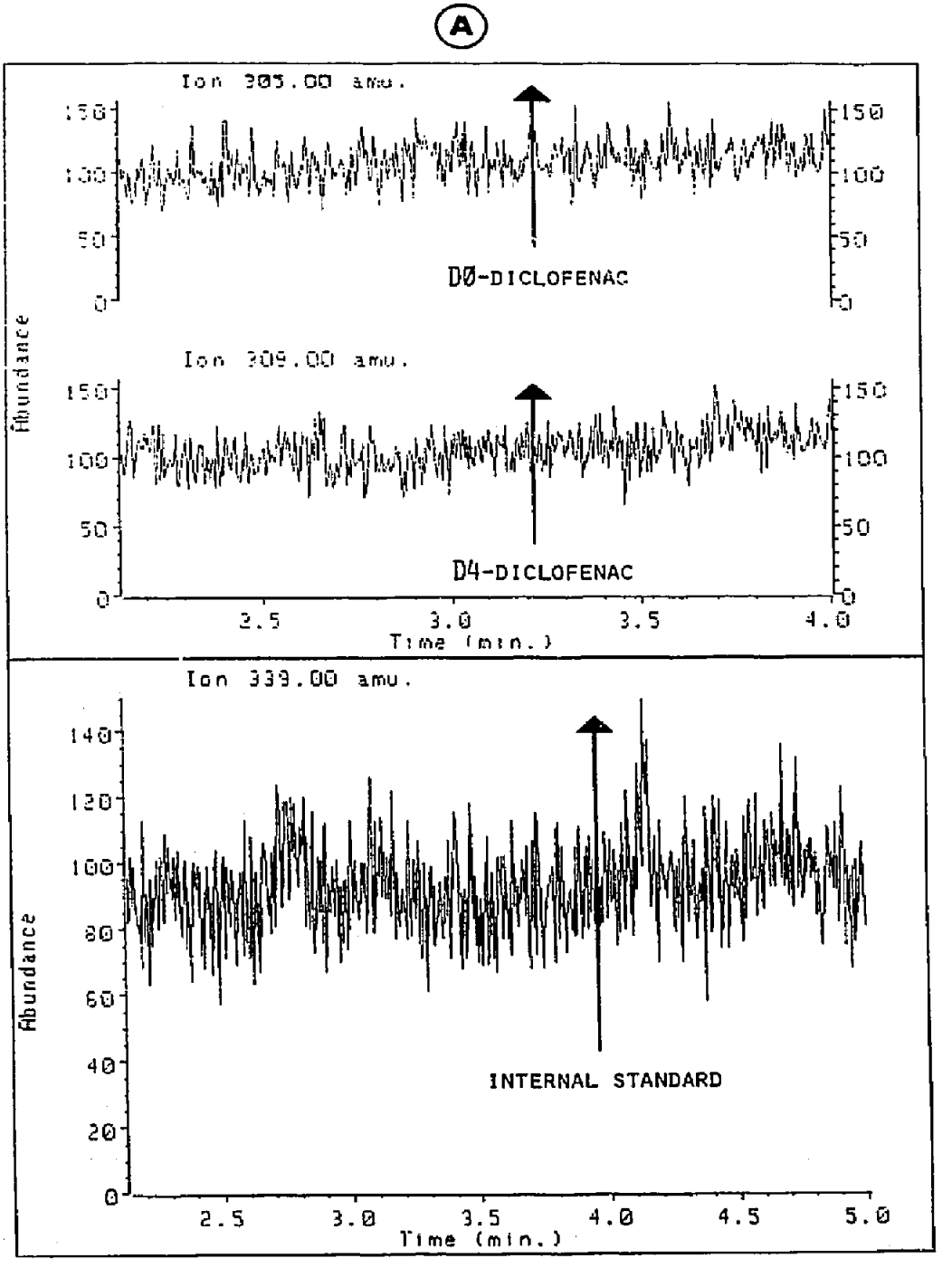

Fig. 2.

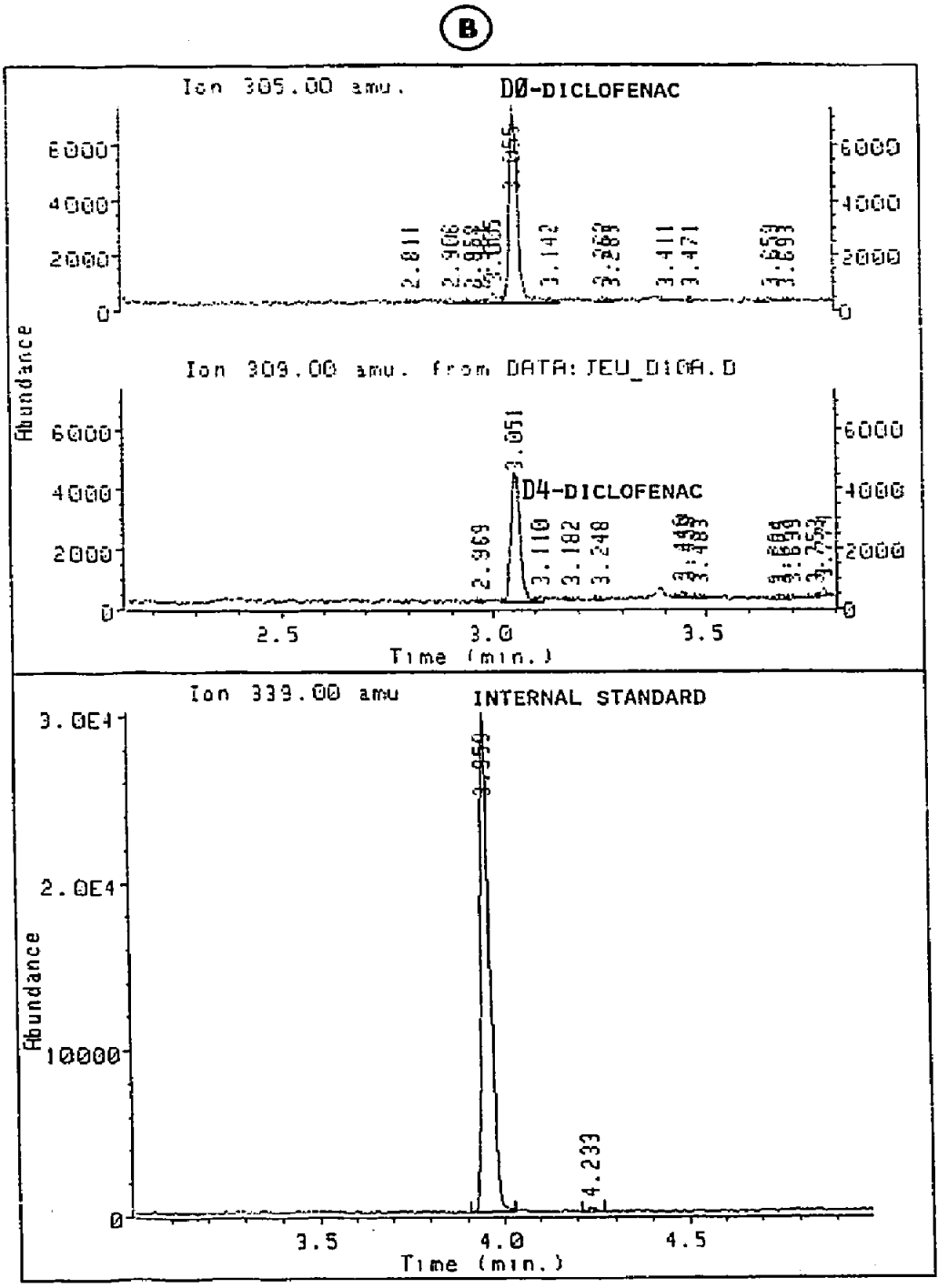

Fig. 2. Selected-ion current profiles of (A) an extract of 1 ml of blank human plasma and (B) the same plasma spiked with 32 pmol of D_0-diclofenac, 33.5 pmol of D_4-diclofenac and 313 pmol of 5-chloro-diclofenac as internal standard.

Calibration curves

Quantification was based on the peak-height (after correction of isotopic contributions) ratio of the compound and of the internal standard. Calibration curves were obtained by plotting the peak-height ratio *versus* the concentration of the compound. Their equation was calculated by weighted linear regression with a weighting factor of $1/(\text{concentration})^2$. A calibration curve was prepared on each day of analysis.

Within-day accuracy and precision

The within-day precision of the method was checked by determining six plasma and urine samples spiked with various amounts of either D_0-diclofenac (using

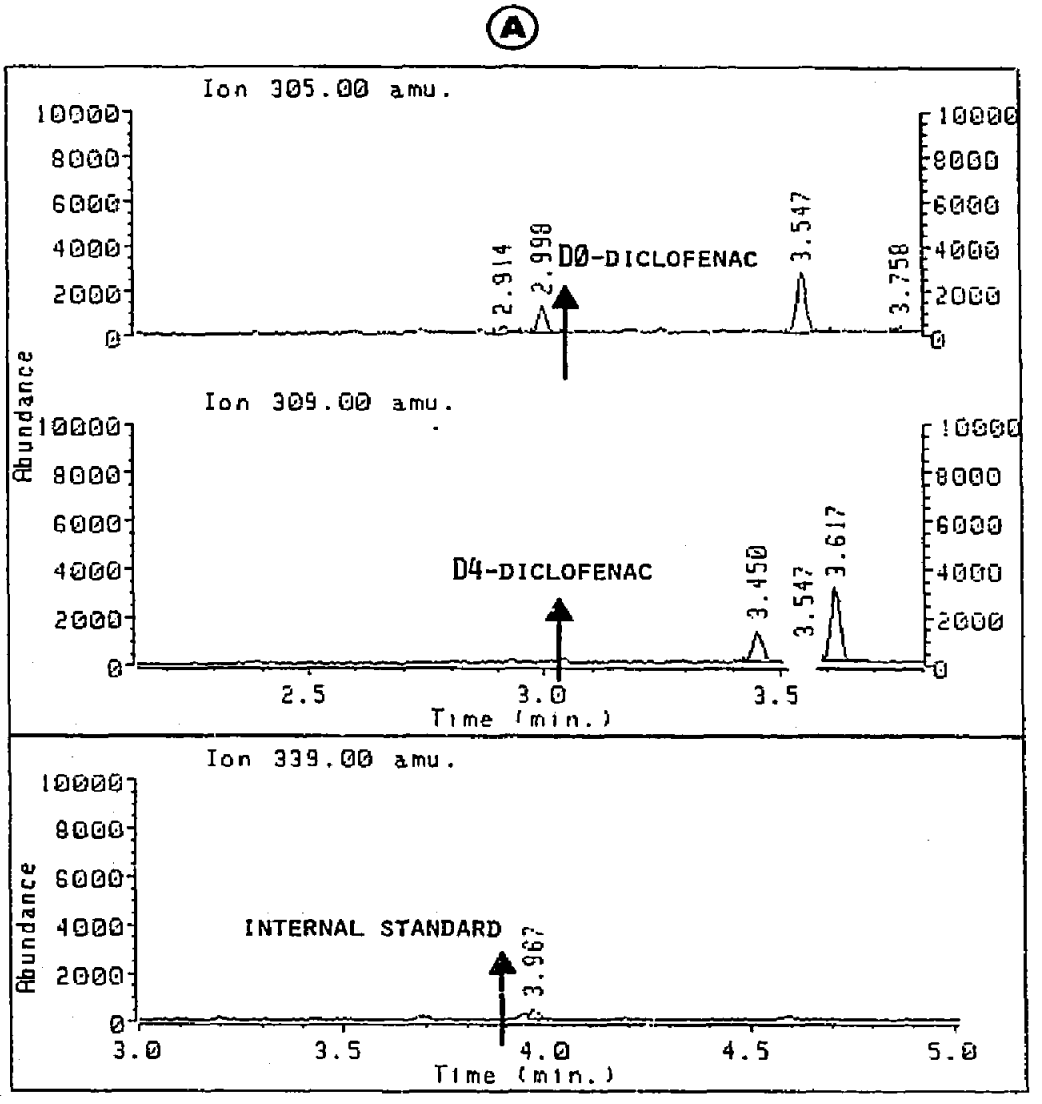

Fig. 3.

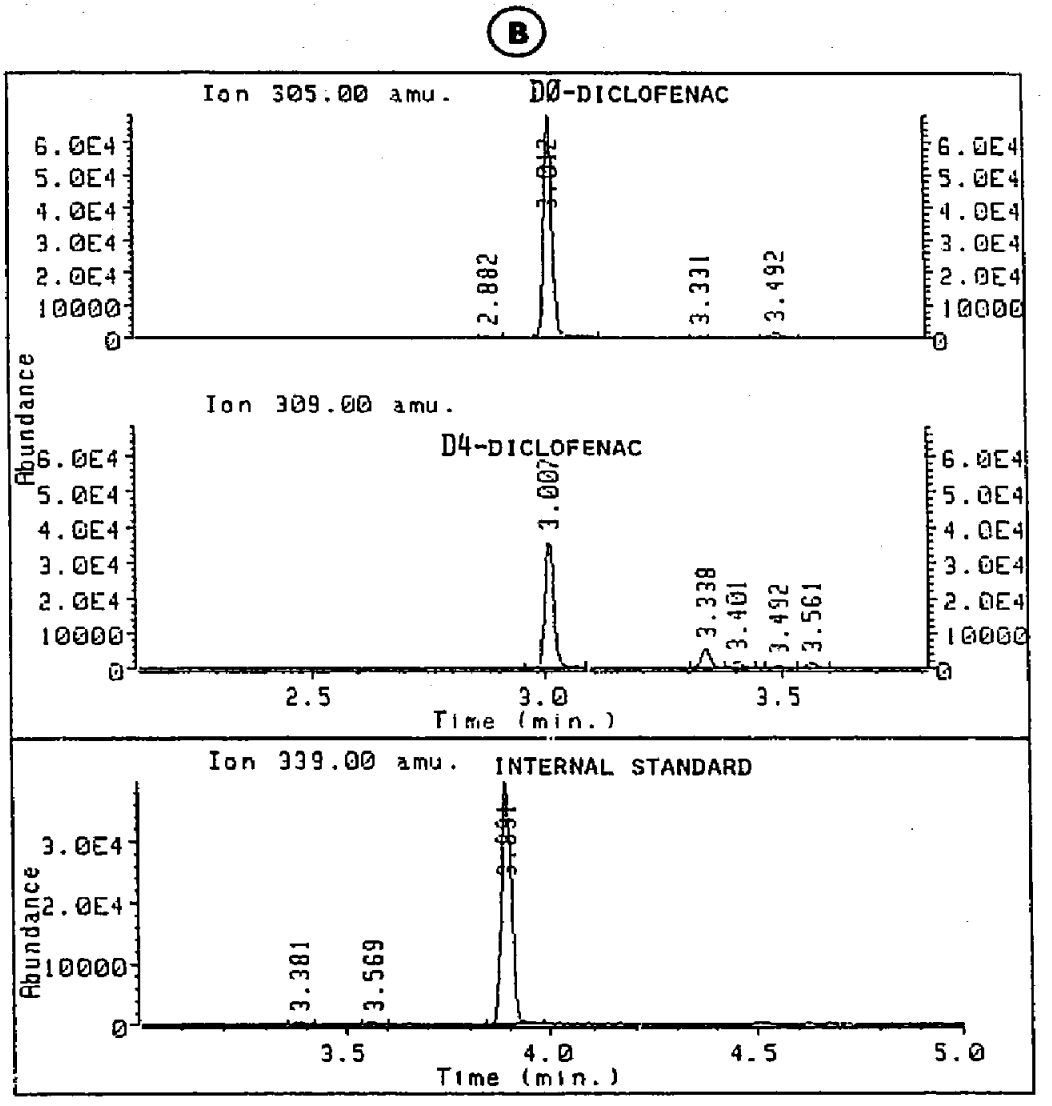

Fig. 3. Selected-ion current profiles of (A) an extract of 1 ml of blank human urine and (B) the same urine spiked with 346 pmol of D_0-diclofenac, 329 pmol of D_4-diclofenac and 313 pmol of 5-chlorodiclofenac as internal standard.

D_4-diclofenac as internal standard) or D_0- and D_4-diclofenac (using 5-chlorodiclofenac as internal standard).

The relative standard deviation (R.S.D.) was used as a measure for the precision. The relative difference between found and added amounts was a measure for the accuracy. The results obtained with the procedure described are given in Table I. A better reproducibility was obtained for D_0-diclofenac when D_4-diclofenac was used as internal standard. Indeed, the labelled drug used as internal standard perfectly compensates for losses occurring during the analytical procedure.

TABLE I

WITHIN-DAY PRECISION AND ACCURACY FOR D_0-DICLOFENAC, USING D_4-DICLOFENAC AS INTERNAL STANDARD, AND FOR D_0- AND D_4-DICLOFENAC USING 5-CHLORODICLOFENAC AS INTERNAL STANDARD, IN SPIKED PLASMA AND URINE SAMPLES

Compound[a]	Given (n*M*)	Mean found (*n* = 6) (n*M*)	R.S.D. (*n* = 6) (%)	Relative error (%)
Plasma				
D_0-Diclofenac	3.43	3.34	8.1	−2.6
(I.S. = D_4-D)	17.1	15.9	6.3	−7.0
	68.5	68.2	1.8	−0.4
	171	171	4.8	0
	240	251	5.7	+4.6
D_0-Diclofenac	3.30	3.25	16.8	−1.5
(I.S. = 5-ClD)	12.8	12.5	10	−2.3
	64.1	61.9	10.7	−3.4
	135	135	8.5	0
	231	245	4.3	+6.1
D_4-Diclofenac	3.32	3.23	21.2	−2.7
(I.S. = 5-ClD)	13.4	13.6	13.2	+1.5
	67.0	67.0	12.4	0
	133	138	9.8	+3.8
	232	252	3.8	+8.6
Urine				
D_0-Diclofenac	34.3	32.8	3.2	−4.4
(I.S. = D_4-D)	858	860	5.8	+0.2
D_0-Diclofenac	34.6	37.5	4.7	+8.4
(I.S. = 5-ClD)	864	848	7.5	−1.9
D_4-Diclofenac	32.9	36.0	8.5	+9.4
(I.S. = 5-ClD)	823	829	7.6	+0.7

[a] D_4-D = D_4-diclofenac; 5-ClD = 5-chlorodiclofenac.

Limit of quantitation

The limit of quantitation in plasma and urine was estimated at 3.4 n*M* (1 ng/ml) for D_0-diclofenac using D_4-diclofenac as internal standard with a coefficient of variation (C.V.) of 8.1% in plasma. It was estimated at 3.3 n*M* (*ca.* 1 ng/ml) for D_0- and D_4-diclofenac using 5-chlorodiclofenac as internal standard, with a C.V. of *ca.* 20% in plasma. A C.V. lower than 13% was otained for D_0- and D_4-diclofenac in plasma for a concentration of 13 n*M* (*ca.* 4 ng/ml).

Limit of detection

The limit of detection of D_0-diclofenac using D_4-diclofenac as internal standard was estimated by processing plasma samples (1 ml) spiked with different

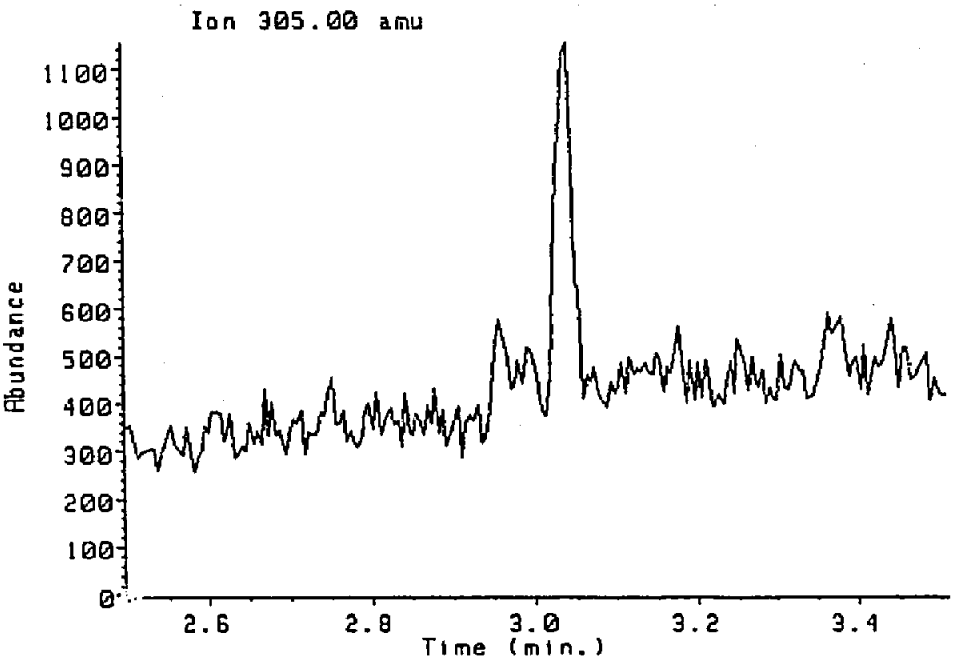

Fig. 4. Limit of detection: selected-ion current profile at m/z 305; extract from 1 ml of plasma spiked with 333 fmol of D_0-diclofenac and 168 pmol of D_4-diclofenac.

amounts of D_0-diclofenac from 133 to 3332 fmol and a constant amount of 168 pmol of internal standard, taking into account the isotopic contributions.

After correction of the data for isotopic contributions, the signal at m/z 305 from D_0-diclofenac was non-existent for a concentration of 133 pM D_0-diclofenac. This signal appeared at a concentration of 333 pM D_0-diclofenac.

A selected-ion current profile at m/z 305 obtained from 1 ml of human plasma spiked with 333 fmol (106 pg) of D_0-diclofenac and 168 pmol (54 ng) of D_4-diclofenac is shown in Fig. 4.

In plasma, the limit of detection was found to be 333 pM (106 pg/ml) of D_0-diclofenac, and a signal-to-noise ratio of 6 was found to be appropriate for the lowest detectable amount.

Selectivity

All known hydroxylated metabolites were derivatized and injected under the same conditions as D_0-diclofenac. They were clearly separated from diclofenac. 3′-Hydroxy-, 4′-hydroxy- and 5-hydroxydiclofenac showed the same molecular ion at m/z 335 with different retention times. 3′-Hydroxy-4′-methoxydiclofenac and 4′,5-dihydroxydiclofenac showed the same molecular ion at m/z 365 with different retention times.

Typical selected-ion current profiles from a mixture containing D_0-diclofenac and five metabolites are shown in Fig. 5.

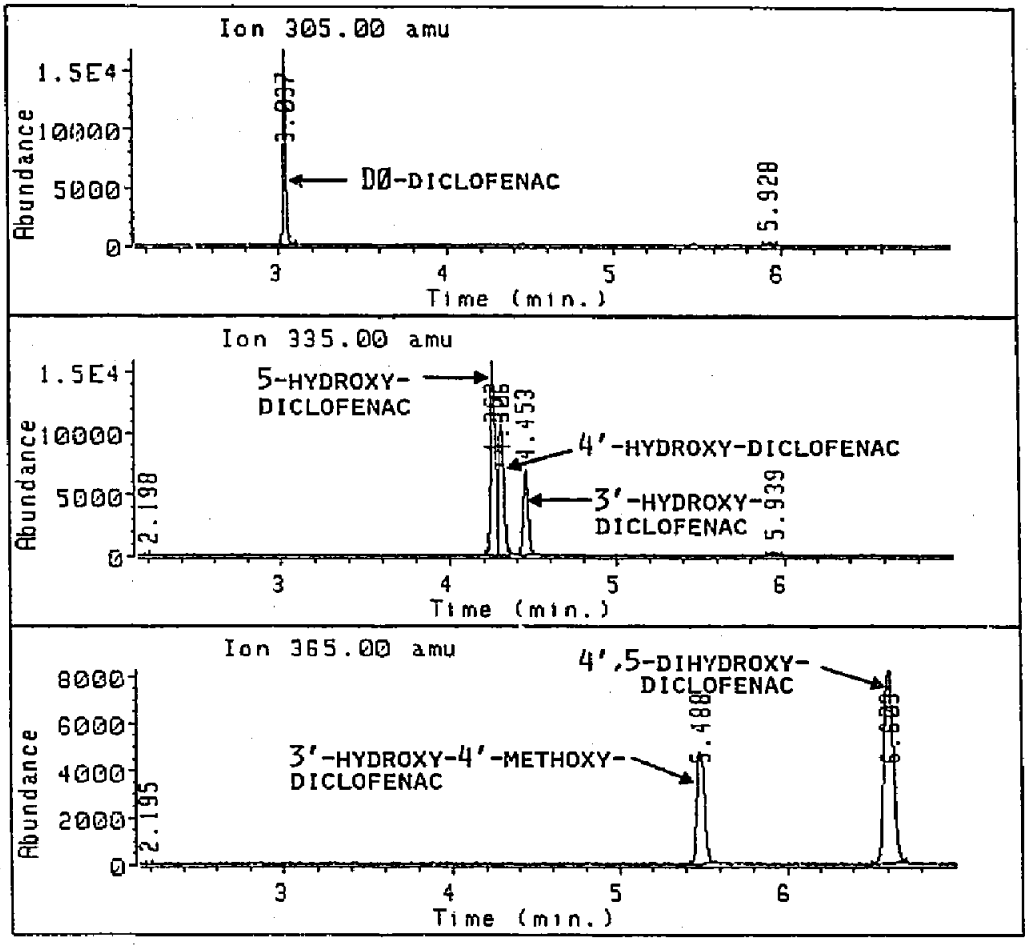

Fig. 5. Example of selected-ion current profile obtained from a mixture of the dimethylindolinone derivatives of D_0-diclofenac and its five metabolites.

Application

The present method was used to determine plasma concentrations of unchanged diclofenac (D_0- and D_4-) and the urinary excretion of total (free + conjugated) D_4- and D_0-diclofenac after application of Voltaren Emulgel applied simultaneously under occlusive dressing as deuterated diclofenac sodium and without occlusive dressing as unlabelled diclofenac sodium.

Fig. 6 shows the plasma concentration curves of unchanged D_0- and D_4-diclofenac and the cumulative urinary excretion of total (free + conjugated) D_0- and D_4-diclofenac.

These results show that application of Voltaren Emulgel under occlusive dressing leads to an increase in the amount of diclofenac absorbed.

CONCLUSION

The proposed technique permits the quantitative assay of D_0-diclofenac, using D_4-diclofenac as internal standard, in plasma and urine at concentrations down

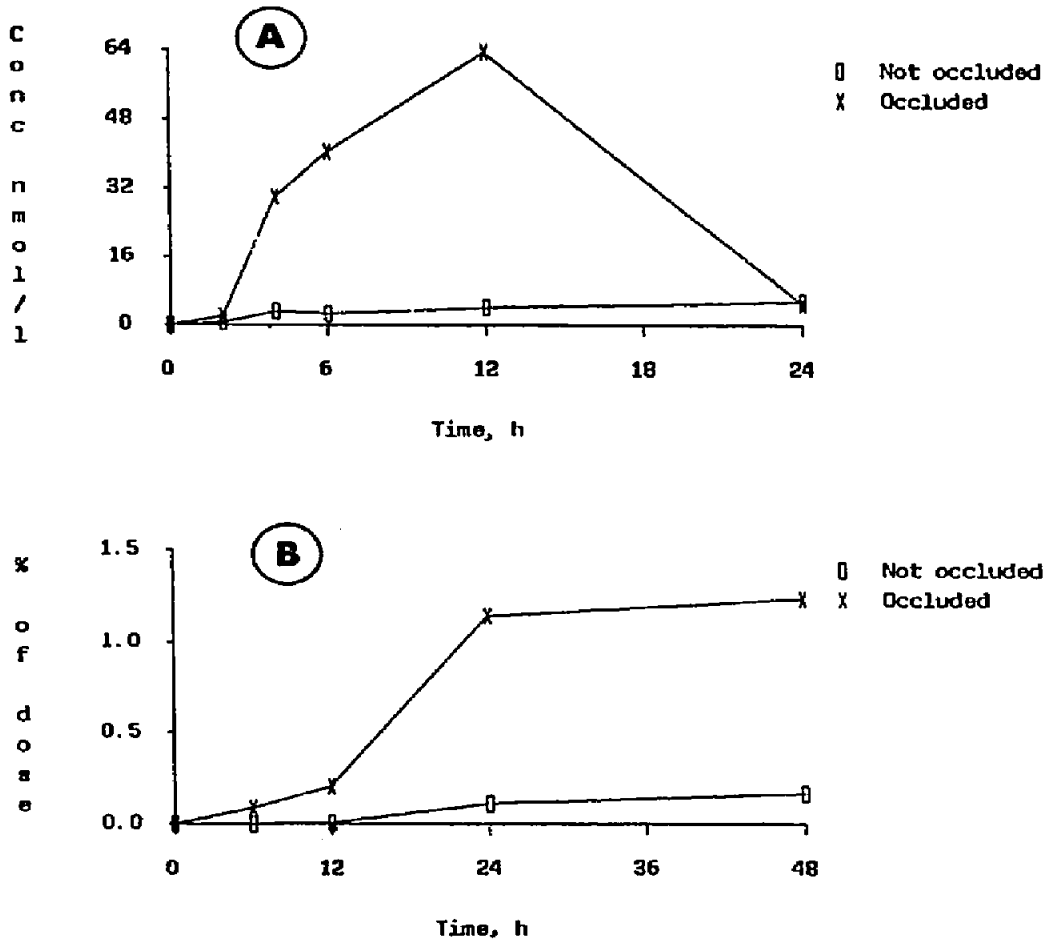

Fig. 6. Plasma concentrations (A) and cumulative urinary excretions (B) of D_0- (○) and D_4-diclofenac (×) in one healthy subject after simultaneous application of Voltaren Emulgel under occlusive dressing as deuterated diclofenac sodium and without occlusive dressing as unlabelled diclofenac sodium.

to 3.4 n*M*, with a limit of detection of 333 p*M*. The technique is also suitable for the simultaneous determination of D_0- and D_4-diclofenac, using 5-chlorodiclofenac as internal standard, for the performance of pharmacokinetic studies in which both compounds are administered simultaneously to the same individual by two different routes and/or as two different preparations.

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