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Note

Gas chromatographic determination of indomethacin and its O-desmethylated metabolite in human plasma and urine

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Indomethacin (I) is one of the most widely used non-steroidal anti-inflammatory agents [1–3]. However, its precise pharmacokinetic parameters are still poorly known and recently they have been shown to be largely influenced by the administration time [4—6].

In order to determine if in humans these circadian changes in the pharmacokinetics of I would be related to an alteration in its biotransformation rate, it was necessary to measure simultaneously plasma and urine concentrations of I and of its major metabolite, O-desmethylindomethacin (DMI) [7, 8].

Several methods have already been described for the measurement of I in biological fluids including spectrofluorimetry [9, 10], gas chromatography (GC) [11—16], GC—mass spectrometry [17], high-performance liquid chromatography (HPLC) [18—24], thin-layer chromatography [25, 26] and radioisotopic dilution [7]. Most of them were devoted to the determination of I only. Recently, three HPLC methods [19—21] were developed which allow the quantification of I and its three metabolites, DMI, deschlorobenzoylindomethacin (DBI) and O-desmethyldeschlorobenzoylindomethacin (DMBI). However, these methods exhibited some drawbacks due to interferences of endogenous compounds in plasma and urine [19], or to the complexity of the whole procedure [21], or to the need of the use of an expensive gradient analysis system. Since the metabolic studies of I in man [7, 8] showed that demethylation followed by deacylation is the major pathway while direct deacylation of I is a minor one, it seems that DMI concentration could be an accurate index of the biotransformation rate of I in man.

Therefore we thought it useful to develop an easy to use GC method with electron-capture detection which could allow the measurement of I and DMI

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with a high sensitivity and specificity in plasma as well as in urine. The application of this technique to the pharmacokinetic study of I, given as a single oral dose, is shown.

EXPERIMENTAL

Standards and reagents

I and DMI were supplied by Merck Sharp and Dohme—Chibret Laboratories (Paris, France). Penfluridol [(chloro-4-α,α,α-trifluoromethyl-*m*-tolyl)-4-bis(*p*-fluorophenyl)-4,4-butyl-1-piperidinol-4] used as internal standard, was a generous gift of Janssen Pharmaceutica (Beerse, Belgium).

Acetone, diethyl ether and hexane (Merck, Darmstadt, F.R.G.) were distilled before use. Diazomethane as a methylating agent was extemporaneously generated from N-methyl-N-nitroso-*p*-toluenesulfonamide (Merck).

Diethyl ether and a mixture of diethyl ether—hexane (50:50) were used as extraction solvents for plasma and urine, respectively.

The stock solutions of I, DMI and internal standard were prepared in acetone (100 μg/ml) and were found to be stable for at least six months at +4°C. Working solutions were prepared weekly by diluting the stock solutions in acetone.

Gas chromatographic conditions

Analyses were performed under isothermal conditions on a Hewlett-Packard Model 5710 A gas chromatograph equipped with a ^{63}Ni linear electron-capture detector. The glass column (1 m × 2 mm I.D.) was packed with 3% OV-17 (Pierce, Rockford, IL, U.S.A.) on Chromosorb W HP (80—100 mesh) (Pierce), and conditioned for 2 h at 270°C (argon—methane, 90:10, carrier gas flow-rate, 10 ml/min), 4 h at 320°C (no gas flow) and 24 h at 270°C (carrier gas flow-rate, 30 ml/min). The column temperature was 260°C, injection port and detector temperature 300°C and the carrier gas (argon—methane, 90:10) flow-rate was at 60 ml/min.

Sample preparation

Extraction procedure. In a glass-stoppered centrifuge tube 0.2 ml of internal standard solution (10 μg/ml) was introduced. The solvent was evaporated to dryness under a gentle stream of nitrogen in a 37°C water-bath, then 1 ml of plasma or urine, 1 ml of 0.1 *N* hydrochloric acid and 8 ml of extraction solvent were added. The mixture was shaken for 15 min on a rotating mixer (60 rpm) and then centrifuged at 4000 rpm (1800 *g*) for 15 min at 4°C. A 7-ml volume of the organic layer was transferred into a conical test-tube and the solvent was evaporated to dryness under the conditions already described.

Derivatization. To the dry plasma or urine extract, 2 ml of acetone were added and the formation of methyl esters of I and DMI was performed directly by passing a stream of diazomethane into the solution until the occurrence of a yellow coloration which indicated the presence of an excess of reagent [27]. The solvent was evaporated to dryness and the methylated extract was dissolved in 100—500 μl of acetone; 4 μl of this solution were injected into the gas chromatograph.

Standard curves

Using the analytical procedure described above, standard curves were obtained by running human biological samples spiked with I (25—5000 ng/ml and 100—5000 ng/ml for plasma and urine, respectively) and DMI (10—500 ng/ml and 100—5000 ng/ml for plasma and urine, respectively) and with internal standard at a fixed concentration of 2000 ng/ml. The ratios of the peak heights of I and DMI to internal standard were plotted against the concentrations of I and DMI, respectively.

RESULTS AND DISCUSSION

Typical chromatograms obtained from blank plasma and blank urine before and after spiking with known amounts of I and DMI, and a plasma from a patient having received 75 mg of I, 12 h before sampling, are shown in Fig. 1. The retention times were at 5.6, 8.2 and 12.6 min for I, DMI and internal standard, respectively.

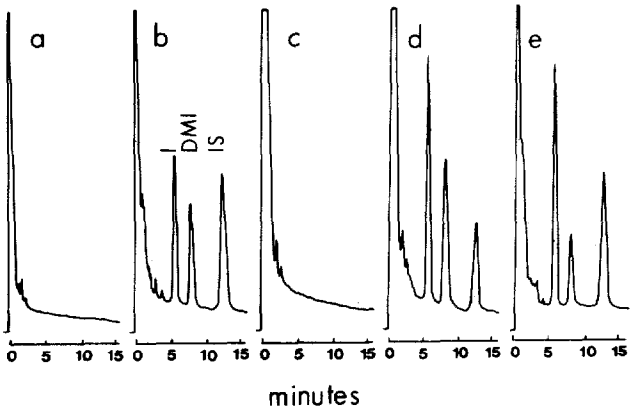

Fig. 1. Gas chromatograms of plasma and urine extracts: (a) control plasma; (b) plasma spiked with I (500 ng/ml), DMI (125 ng/ml) and internal standard (IS, 2000 ng/ml); (c) control urine; (d) urine spiked with I (500 ng/ml), DMI (500 ng/ml) and IS (2000 ng/ml); (e) plasma from a patient having received a single oral dose of 75 mg of I, 12 h before sampling.

No interferences from endogenous substances were observed. In addition, the possible interference of several drugs which are currently administered together with I, such as other non-steroidal anti-inflammatory agents (ketoprofen, naproxen, ibuprofen, phenylbutazone, diclofenac), analgesics (aspirin, paracetamol), tranquillizers (diazepam, lorazepam, oxazepam, nitrazepam) have been tested. These compounds did not exhibit any peak in the retention time range of the analysis, since most of them were not detectable with the electron-capture detector and the others appeared in the solvent peak. Compared to plasma, in the case of urine, instead of pure diethyl ether, a mixture of diethyl ether—hexane (50:50) was used as extraction solvent. This modification was necessary to reduce the front solvent chromatographic peak in order to quantify amounts of I and DMI smaller than 100

ng/ml. For this, 50% of hexane appeared sufficient to remove a large proportion of the interfering endogenous substances within the limits of the extraction capacity of the solvent.

The calibration graphs obtained after extraction of I and DMI from plasma and urine were found to be linear in the range of the concentrations studied. For plasma $Y = 0.00205X - 0.0184$ and $Y = 0.00131X - 0.0004$, and for urine $Y = 0.00122X - 0.0286$ and $Y = 0.00080X - 0.0037$ for I and DMI, respectively, with a regression coefficient $r^2 = 0.999$ in all cases.

The absolute sensitivity of the electron-capture detector was near 40 pg for I and 75 pg for DMI, which allowed the detection of plasma concentrations of 2 ng/ml and 4 ng/ml for I and DMI, respectively. In spite of the use of hexane in the extraction solvent, for urine the sensitivity limits were slightly higher than for plasma, being 10 ng/ml for both compounds.

The interassay reproducibility of the method was checked by analysing plasma and urine samples spiked with several concentrations of I and DMI. The results listed in Tables I and II show that the coefficients of variation do not exceed 7.0 and 4.9% for I and 9.2 and 6.1% for DMI in plasma and urine, respectively.

TABLE I

REPRODUCIBILITY OF I AND DMI MEASUREMENT IN HUMAN PLASMA

Amount added (ng/ml)		Mean of ten assays (ng/ml, ± S.D.)		Coefficient of variation (%)	
I	DMI	I	DMI	I	DMI
25	10	22.4 ± 1.5	9.2 ± 0.9	6.5	9.2
100	25	100.8 ± 7.0	25.3 ± 1.5	7.0	6.1
500	50	494.9 ± 22.5	49.7 ± 2.5	4.5	5.1
1000	100	1004.2 ± 36.8	98.9 ± 5.9	3.7	6.0
2000	250	2004.7 ± 81.0	250.1 ± 11.7	4.0	4.7
5000	500	4999.8 ± 122.6	501.2 ± 17.8	2.5	3.5

TABLE II

REPRODUCIBILITY OF I AND DMI MEASUREMENT IN HUMAN URINE

Amount added (ng/ml)		Mean of six assays (ng/ml, ± S.D.)		Coefficient of variation (%)	
I	DMI	I	DMI	I	DMI
100	100	100.2 ± 4.1	105.0 ± 5.1	4.1	4.8
250	250	245.8 ± 10.1	259.3 ± 9.8	4.1	3.8
500	500	508.5 ± 10.3	498.2 ± 30.2	2.0	6.1
1000	1000	1004.2 ± 49.2	973.8 ± 14.6	4.9	1.5
2000	2000	2012.5 ± 26.9	2005.2 ± 46.1	1.3	2.3
5000	5000	5012.5 ± 52.2	5001.0 ± 86.6	1.0	1.7

Compared to HPLC methods, the derivatization step during GC methods could be considered a drawback in terms of reproducibility and time consumption. However, through the classical methylation procedure used, it appeared that the esterification of the studied carboxylic acids was complete, rapid (only a few minutes are required for the treatment of each sample) and reproducible as confirmed by the low coefficients of variation given above.

In addition, the sensitivity obtained with the electron-capture detector is ten times higher than that of the HPLC methods. Another advantage of the described GC method is the minimal plasma or urine background, even when very low concentrations of I and DMI have to be detected

Fig. 2 illustrates the time course of plasma levels of the two compounds observed in a patient after a single oral dose of 75 mg of a slow-release form of I. These curves clearly indicate that the analytical method described above is suitable for a pharmacokinetic study of I and DMI.

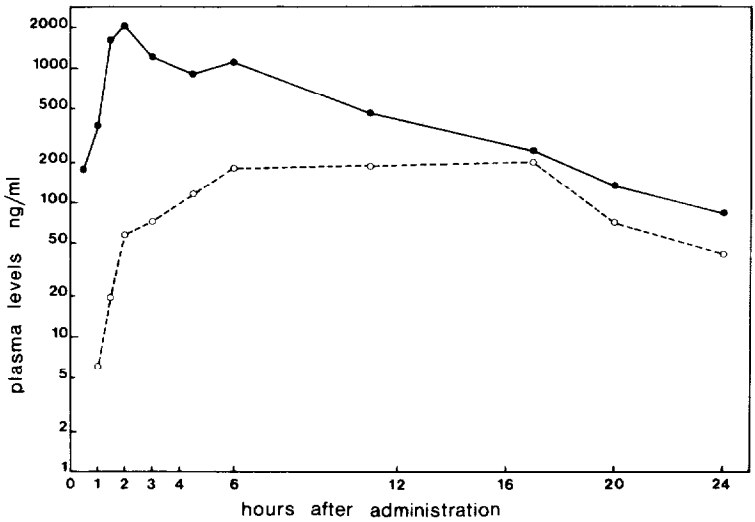

Fig. 2. I (•) and DMI (○) plasma concentration—time curves in a patient having received at 8 p.m. a single oral dose (75 mg) of a slow-release form of I.

CONCLUSIONS

The method described is simple, specific, reliable and highly sensitive. The time of analysis needed for a batch of twelve to fifteen samples is about 6 h. The stability of the chromatographic system is remarkably high since no change could be observed after routine analysis of 1000 samples within a period of one year.

The sensitivity and the accuracy of the method appear suitable to follow plasma and urine concentrations of I and DMI after a single therapeutic dose of I.

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