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Determination of indomethacin, its metabolites and their glucuronides in human plasma and urine by means of direct gradient high-performance liquid chromatographic analysis

Preliminary pharmacokinetics and effect of probenecid

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ABSTRACT

Indomethacin is metabolized in humans by O-demethylation, and by acyl glucuronidation to the 1-O-glucuronide. Indomethacin, its metabolite O-desmethylindomethacin (DMI) and their conjugates can be measured directly by gradient high-performance liquid chromatographic analysis without enzymic deglucuronidation. The glucuronide conjugates were isolated by preparative HPLC from human urine samples. In plasma only indomethacin was present. No isoglucuronides were present in acidic urine of the volunteer. The possible metabolite deschlorobenzoylindomethacin (DBI) was not detectable in urine. Calibration curves were constructed by enzymic deconjugation of samples containing different concentrations of isolated indomethacin acyl glucuronide, DMI acyl glucuronide and DMI ether glucuronide. The limit of quantitation of indomethacin in plasma is 0.060 μg/ml. The limits of quantitation in urine are: indomethacin 0.053 μg/ml, DMI 0.065 μg/ml, DMI acyl glucuronide 0.065 μg/ml and DMI ether glucuronide 0.254 μg/ml. A pharmacokinetic profile of indomethacin is shown, and some preliminary pharmacokinetic parameters of indomethacin obtained from one human volunteer are given. Probenecid inhibits the formation of both the ether and the acyl glucuronide of DMI.

INTRODUCTION

Indomethacin is a non-steroidal anti-inflammatory drug (NSAID) used in painful and inflammatory rheumatic and non-rheumatic conditions, and for the closure of patent ductus arteriosus in premature infants. The anti-inflammatory effects are related to the inhibition of cyclooxygenase and consequent decrease in prostaglandin concentrations in various fluids and tissues. Inhibition of prostaglandin synthesis may result in gastrointestinal microbleeding and in a reduction in kidney function [1].

In humans indomethacin is metabolized by phase I metabolism to 6-O-desmethylindomethacin (DMI) and deschlorobenzoylindomethacin

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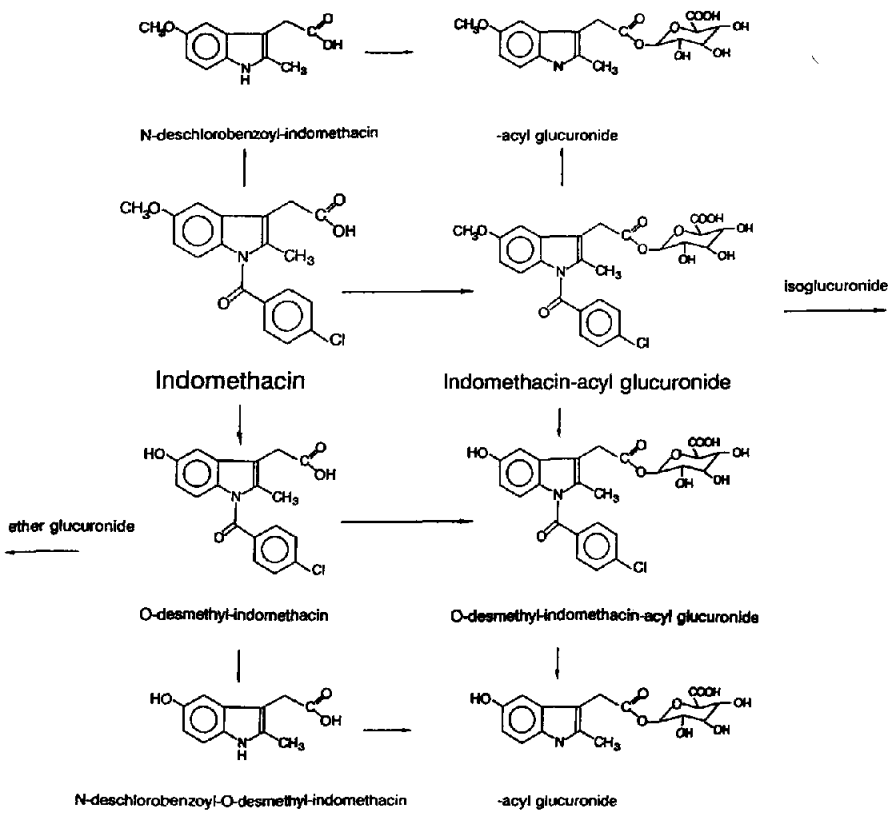

Fig. 1. Structures of indomethacin and its possible metabolites.

(DBI), and all compounds can be conjugated by phase II metabolism into indomethacin acyl glucuronide (1-O-glucuronide), O-desmethylindomethacin acyl glucuronide (1-O-glucuronide), O-desmethylindomethacin ether glucuronide and deschlorobenzoylindomethacin acyl glucuronide, as shown in Fig. 1 [2,3]. The possible presence of isoglucuronide (4-O-glucuronide) in the urine must be anticipated as the result of isomerization of the unstable acyl glucuronides at blood pH (7.4) or in the gut via the enterohepatic recirculation [4].

Indomethacin concentration analysis in plasma and urine utilizes radioimmunoassay [3,5–7], radioisotopic dilution [5,8], fluorimetric methods [3,7], anion-exchange chromatography [8], GC methods [9–11], TLC methods [12,13] and mass fragmentography [14,15]. HPLC analysis of indomethacin and DMI is now the most commonly used method of analysis, however, the conjugates are determined after enzymatic or alkaline hydrolysis [16–20]. Direct HPLC analysis of other NSAIDs such as naproxen [4], carprofen [21], zomepirac [22] and the salicylic acid derivative diflunisal and their acyl glucuronide and sulphate conjugates has been reported [23,24]. A direct isocratic HPLC analysis of indomethacin and its acyl glucuronide was reported [25], but an analysis that enables the measurement of all metabolites is still lacking.

Acyl glucuronides are unstable in alkaline media (pH > 7.0). Therefore urine must be kept acidic at pH 5.0 (inside the body) in order to prevent hydrolysis of eventual formed acyl glucuronides [26].

The aims of this investigation were (a) to develop a simple and direct gradient HPLC analysis of the glucuronide conjugates of indomethacin and its metabolites DMI and DBI in plasma and urine and (b) to study the human pharmacokinet-

ics of indomethacin and the effect of probenecid in pilot experiments.

EXPERIMENTAL

Chemicals

Indomethacin and its metabolites, DMI and DBI, were obtained from Merck, Sharp and Dohme (Haarlem, Netherlands). Indocid (25-mg tablets) and probenecid (Benemid, 500-mg tablets) were obtained from the hospital pharmacy. All other reagents were of p.a. quality and obtained from Merck (Darmstadt, Germany).

Indomethacin acyl glucuronide, DMI acyl glucuronide and DBI ether glucuronide were identified in human urine after intake of 25 mg of Indocid and isolated after intake of 100 mg of Indocid. β-Glucuronidases were obtained from Sigma (St. Louis, MO, USA).

Gradient HPLC analysis

The HPLC system consisted of a Spectra Physics SP 8775 autosampler (Spectra Physics, Eindhoven, Netherlands), a Spectra Physics SP 8800 ternary HPLC pump, a Kratos Spectroflow 757 UV detector (Separations, Hendrik Ido Ambacht, Netherlands) and a Spectra Physics SP 4290 integrator. The column was Cp Spherisorb ODS 5 μm, 250 mm x 4.6 mm I.D. (Chrompack, Bergen op Zoom, Netherlands) with a guard column, 75 mm × 2.1 mm I.D., packed with pellicular reversed phase (Chrompack Cat. No. 28653). The mobile phase was a mixture of acetonitrile and orthophosphoric acid (5 g/l water). At $t = 0$, the mobile phase consisted of acetonitrile–phosphoric acid (80:20, v/v). During the following 30 min the mobile phase changed linearly until it attained a composition of acetonitrile–phosphoric acid (60:40, v/v). At 35 min ($t = 35$) the mobile phase was changed within 5 min to the initial composition, followed by equilibration over 2 min. The flow-rate was 1.2 ml/min. UV detection was achieved at 254 nm.

The capacity factors of indomethacin, O-desmethylindomethacin with their acyl and ether glucuronides are given in Table I.

Sample treatment

Plasma samples (100 μl) were deproteinized with 0.4 ml of 0.20 *M* perchloric acid, centrifuged at 3000 *g*, and 20 μl of the supernatant were injected onto the column. During the first day of the human experiments, 100 μl of plasma sample were processed immediately upon receipt in order to detect the presence of acyl glucuronides of indomethacin, DMI or DBI. Urine samples were diluted 1:1 with water and 20 μl were injected onto the column.

TABLE I

RETENTION TIMES AND CAPACITY FACTORS OF INDOMETHACIN, ITS METABOLITES AND CONJUGATES

t_0 = 2.17 min. Acyl glucuronide = 1-O-glucuronide; $-CH_3$ = group contribution of the CH_3 group; acyl/aglycon, ether/aglycon = group contribution of the glucuronide group; –CB = contribution of the chlorobenzoyl group to the chromatographic process.

Compound	t_R (min)	k'	Acyl/aglycon	Ether/aglycon	$-CH_3$	–CB
DBI ether glucuronide[a]	8.20[a]	2.78[a]				
DBI acyl glucuronide[a]	8.83[a]	3.07[a]				
DBI	11.33	4.15				0.35
DMI ether glucuronide	15.53	6.06		0.67		
DMI acyl glucuronide	16.83	6.65	0.74		0.79	
Indomethacin acyl glucuronide	20.74	8.43	0.72			
O-Desmethylindomethacin	22.05	9.02			0.77	
Indomethacin	28.13	11.79				

[a] Calculated values, not present in human urine.

Isolation of the acyl glucuronides

The peaks in the chromatogram that were assumed to be metabolites of indomethacin were isolated by means of preparative HPLC. The preparative Gilson HPLC consisted of a Gilson 302 sample pump (Gilson, Meyvis, Bergen op Zoom, Netherlands), two 305 Gilson gradient pumps, an 811 B Dynamic mixer, a Kratos 757 UV detector (Separations), an LKB 2211 Superrac (LKB, Woerden, Netherlands) and a BD7 recorder (Kipp & Zonen, Delft, Netherlands). The column was a C_8 8-μm, 250 mm × 10 mm I.D., Rainin Dynamax 60-Å column (Meyvis).

The mobile phase consisted of 1% acetic acid in water–acetonitrile (80:20, v/v) for 1 min at the start and thereafter changed linearly over 15 min to 65:35 (v/v). The retention time of indomethacin acyl glucuronide was 14 min, of DMI acyl glucuronide 6.7 min and of DMI ether glucuronide 5.8 min.

Concentration of the trapped sample was carried out using a IKA rotavapor (Janke and Kunkel, Staufen, Germany) equipped with a Trivac vacuum pump (Leybold-Heraeus, Woerden, Netherlands).

Deconjugation of the acyl glucuronides

Deglucuronidation was carried out with 200 μl of human urine containing indomethacin and DMI acyl and ether glucuronides, 100 μl of β-glucuronidase and 200 μl of 0.2 *M* disodium hydrogenphosphate–potassium dihydrogenphosphate buffer at 37°C for 2 h.

Four different β-glucuronidase enzymes (A–D) were tested.

(A) β-Glucuronidase type B1, 100 000 U/ml (bovine liver, Sigma, Cat. No. G-0251), and phosphate buffer pH 5.0.

(B) β-Glucuronidase type H2, 107 200 U/ml (*Helix pomatia*, Sigma, Cat. No. G-0876), and phosphate buffer pH 5.0.

(C) β-Glucuronidase type LII, 100 000 U/ml (lyophilized powder from limpets *Patella vulgata*, Sigma, Cat. No. G-8132), and phosphate buffer pH 3.8.

(D) β-Glucuronidase type VIIA, 20 000 U/ml (*Escherichia coli*, Sigma, Cat. No. G-7646), and phosphate buffer pH 6.8.

Calibration curves

Samples containing different concentrations of indomethacin acyl glucuronide isolated from human urine by preparative HPLC were deconjugated by enzyme D. The increase in the concentration of indomethacin (aglycon) represented the concentration of the conjugate indomethacin acyl glucuronide. A calibration curve was constructed with the help of the following formula:

$$[\text{I-gluc}] = d[\text{I}] \times M_{\text{I-gluc}}/M_{\text{I}}$$

were $d[\text{I}]$ is the difference in concentration of indomethacin before and after deconjugation and M is relative molecular mass (all r 0.999). Calibration curves for isolated O-desmethylindomethacin ether and acyl glucuronides were constructed in a similar way.

The calibration curve for indomethacin in plasma was constructed by spiking blank human plasma samples with known concentrations of the compound ($r > 0.9998$) (Table II). Calibration curves for indomethacin and DMI in urine were constructed by spiking blank urine samples with known concentrations of the compounds ($r > 0.995$) (Table II).

Stability

The stability of indomethacin acyl glucuronide and DMI acyl-, and ether glucuronides in urine/water was tested as follows: Three samples of 2 ml of urine were brought to pH 5.0, 7.4 and 8.0 and incubated at 37°C for 24 h. At regular time intervals (1–2 h) a 100-μl sample was taken, and the reaction stopped with 900 μl of 0.01 *M* phosphoric acid. From this mixture, 20 μl were injected onto the column.

The stability of indomethacin and DMI acyl and ether glucuronides in the autosampler in water and 0.01 *M* phosphoric acid was tested over 24 h. Samples were taken every 0.5 h and injected onto the column.

Isomerization of the acyl glucuronides

Isolated indomethacin acyl glucuronide and DMI acyl glucuronide were subjected to hydrolysis and isomerization in a phosphate buffer of pH 5.0, 7.4 and 8.0 during 24 h at 37°C. The

TABLE II

CALIBRATION CURVES OF INDOMETHACIN AND ITS METABOLITES

Compound	Equation[a]	r
Plasma		
Indomethacin	$y = 5.74x - 2.96$	0.9998
Urine (concentration range: quantitation limit to 100 μg/ml)		
Indomethacin	$y = 5.08x + 1.582$	0.9999
Indomethacin acyl glucuronide	$y = 3.69x - 4.383$	0.9989
O-Desmethylindomethacin	$y = 4.268x + 1.425$	0.9999
O-Desmethylindomethacin acyl glucuronide	$y = 2.463x + 0.782$	0.9995
O-Desmethylindomethacin ether glucuronide	$y = 2.380x + 0.235$	0.9998
N-Desbenzoylindomethacin	$y = 1.032x - 0.496$	0.9998

[a] Peak height y (integration units) and concentration x (μg/ml).

formation of isoglucuronides was followed by taking and analysing a sample every hour.

Limits of quantitation

The limits of detection in water and quantitation of indomethacin, its metabolite and glucuronide conjugates in plasma and urine were determined at a signal-to-noise ratio of 3, and are shown in Table III.

Subjects

One human subject (I, male, 50 years, 92 kg) took on two different occasions 25 and 100 mg of indomethacin orally (Indocid). On a third occasion 1 g of probenecid (Benemid, MSD, Haarlem, Netherlands) was taken after an overnight fast, followed 1 h later by intake of 25 mg of indomethacin. The study had the approval of the hospital ethics committee and informed consent was obtained from the volunteer.

Sampling

Blood samples were drawn at regular time intervals after administration over two days by means of fingertip puncture with Monolet lancets (Monoject, St. Louis, MO, USA). After centrifu-

TABLE III

LIMITS OF DETECTION AND QUANTITATION OF INDOMETHACIN AND ITS METABOLITES

Detection limit in water; quantitation limit in the biological matrix.

Compound	Detection limit (μg/ml)	Quantitation limit (μg/ml)
Plasma		
Indomethacin	0.0053	0.060
Urine		
Indomethacin	0.0053	0.146
Indomethacin acyl glucuronide	0.120	0.238
O-Desmethylindomethacin	0.065	0.138
O-Desmethylindomethacin acyl glucuronide	0.112	0.240
O-Desmethylindomethacin ether glucuronide	0.120	0.254

gation plasma samples were stored at −20°C pending analysis. Urine was collected upon spontaneous voiding. The total time of sample collection was 70 h [seven times the expected half-life ($t_{1/2}$) of 10 h]. Urinary pH was kept acidic (pH 5.0–5.5) by the oral intake of 1 g of ammonium chloride q.i.d (Ammonchlor, Südmedica, Munich, Germany). Four urine samples of 5 ml of each void were immediately stored at −20°C pending analysis.

Pharmacokinetics

The pharmacokinetic parameters were calculated using the MediWare computer package [27].

RESULTS AND DISCUSSION

Chromatograms of a human urine sample after oral administration of 100 mg of indomethacin before and after β-glucuronidase treatment are shown in Fig. 2. The human sample shows the presence of the acyl glucuronides of indomethacin and DMI, and the ether glucuronide of DMI. Neither DBI with its acyl glucuronide or deschlorobenzoyldesmethylindomethacin with its acyl or ether glucuronide was present in the urine. The anticipated retention times of the last compounds can be calculated from the group contributions of each moiety of the indomethacin molecule to the retention behaviour. In plasma only indomethacin could be detected; no isoglucuronides of indomethacin and DMI were observed. Table I shows the retention times (t_R), capacity factors (k') of drug and metabolites and group contributions to the retention behaviour. When no mass spectrometric identification of the glucuronides is possible, the group contribution, for example of the glucuronide group to the retention behaviour of the indomethacin skeleton, gives valuable information about similarities in molecular structure. Table II shows the equations of the calibration curves and Table III the detection and quantitation limits of indomethacin and its metabolites.

It is shown in Fig. 3 that the acyl glucuronides of both indomethacin and its metabolite DMI

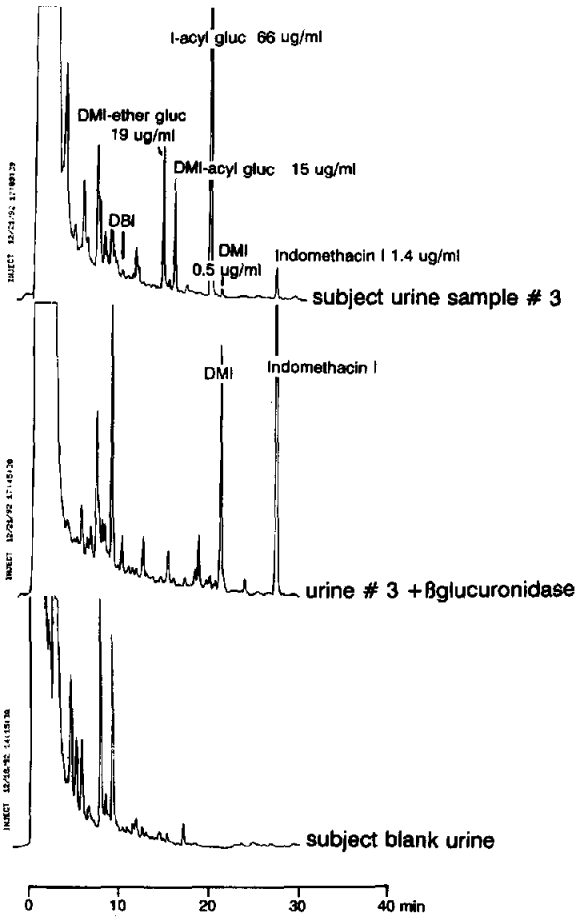

Fig. 2. Chromatograms of human urine containing indomethacin and its metabolites before and after β-glucuronidase treatment. UV detection at 254 nm.

were unstable in plasma at 37°C and pH 7.4. The chromatogram shows that in plasma only the isoglucuronide of the metabolite was formed, while indomethacin acyl glucuronide was hydrolysed.

The acyl glucuronides of indomethacin and DMI were stable at pH 5, and unstable at pH 7.4 and 8 (Fig. 4). Samples of pH 5 were stable in the autosampler of the HLPC system for 24 h.

In Fig. 5 the chromatogram and the rate of isomerization of isolated indomethacin acyl glucuronide (1-O-glucuronide) into indomethacin isoglucuronides (2-O-, 3-O- and 4-O-acylglucuronide) and indomethacin are shown. This reaction does not proceed at pH 5. It was assumed that 4-O-glucuronide had the shortest retention time, and that 2-O- and 3-O-acylglucuronide

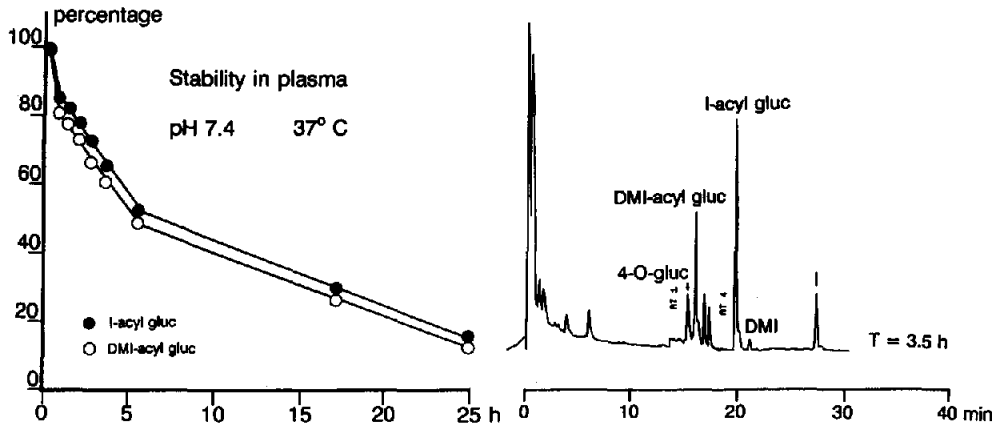

Fig. 3. Stability of the acyl glucuronides of indomethacin (I-acyl gluc; solid dots) and its metabolite DMI (DMI-acyl gluc; open dots) in plasma at 37°C and pH 7.4 (left). The right panel shows the chromatogram of the plasma sample at t = 3.5 h.

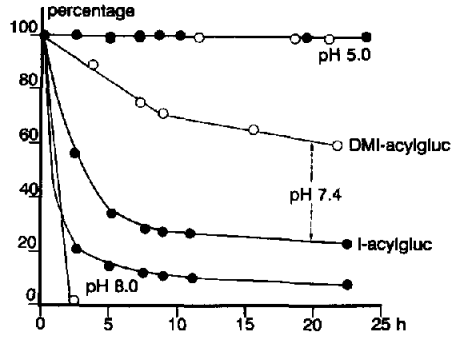

Fig. 4. Stability of indomethacin acyl glucuronide (solid dots) and DMI acyl glucuronide (open dots) at different pH values in urine at 37°C.

could not be separated. The isoacylglucuronides were glucuronidase-resistant. Indomethacin isoglucuronide (4-O-glucuronide) was not present in the human urine. The position of the glucuronide group (2-O-, 3-O- and 4-O) of the isoglucuronides has to be identified by LC–mass spectrometry. Similar data for the isomerization and hydrolysis of isolated DMI acyl glucuronide are shown in Fig. 6. The three isoglucuronides are almost separated. No isoglucuronides were present in human urine.

The ethereal glucuronide of DMI is stable at

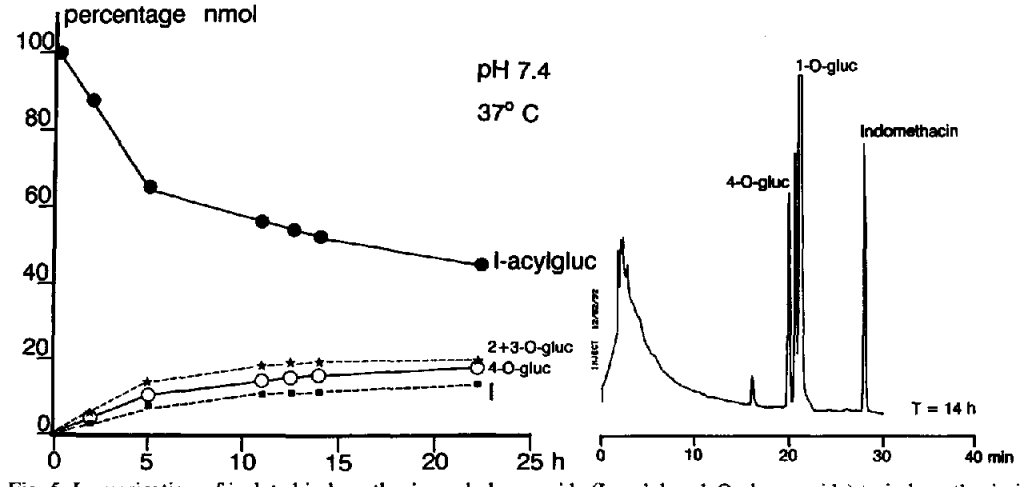

Fig. 5. Isomerization of isolated indomethacin acyl glucuronide (I-acylgluc, 1-O-glucuronide) to indomethacin isoglucuronides (2-O-, 3-O- and 4-O-glucuronide) and to indomethacin (I; left panel). The right panel shows the chromatogram of the reaction mixture at t = 14 h.

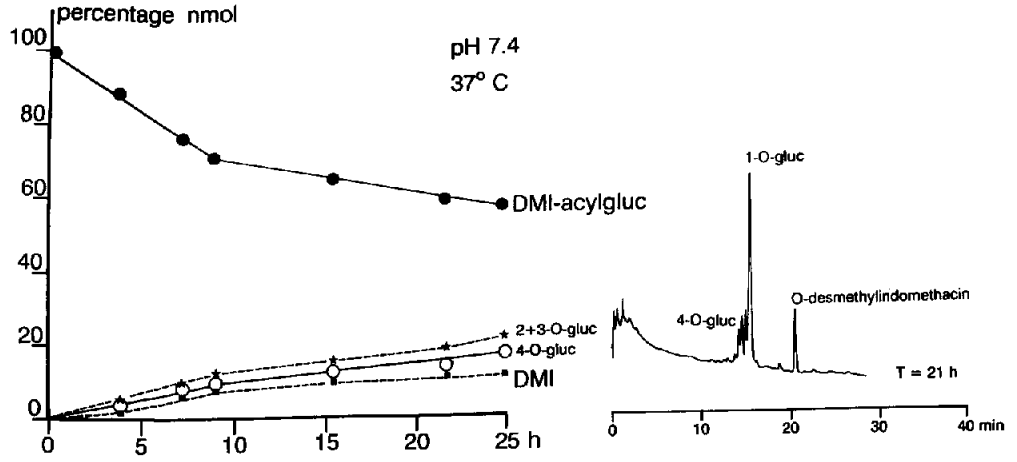

Fig. 6. Isomerization of isolated O-desmethylindomethacin acyl glucuronide (1-O-glucuronide) to isoglucuronides and to O-desmethylindomethacin (left panel). The right panel shows the chromatogram of the reaction mixture at t = 21 h.

pH 5.0 and at pH 7.4 (Fig. 7). The ethereal glucuronide of DMI was only present in urine. Ethereal glucuronides are present in more phenolic compounds such as salicylic acid [28], diflunisal [24], the 7-hydroxy metabolite of nalidixic acid [29], codeine [30] and morphine [31], hydroxysulphapyridine [32] and *p*-hydroxyphenytoin [33].

The absence of the isoglucuronide of indomethacin and of DMI in human urine, under the precautions of keeping the urine acidic *in vivo*, can be explained when it is assumed that both compounds are conjugated by the human kidney [7,25]. Similar behaviour was observed with probenecid [34,35] and nalidixic acid [29]. In contrast, the presence of isoglucuronides in urine was reported for naproxen and O-desmethylnaproxen [4].

The intra- and inter-day variations of indomethacin and its metabolites in plasma and urine are shown in Tables IV and V, respectively.

The plasma concentration–time curve of indomethacin and renal excretion rate–time profiles

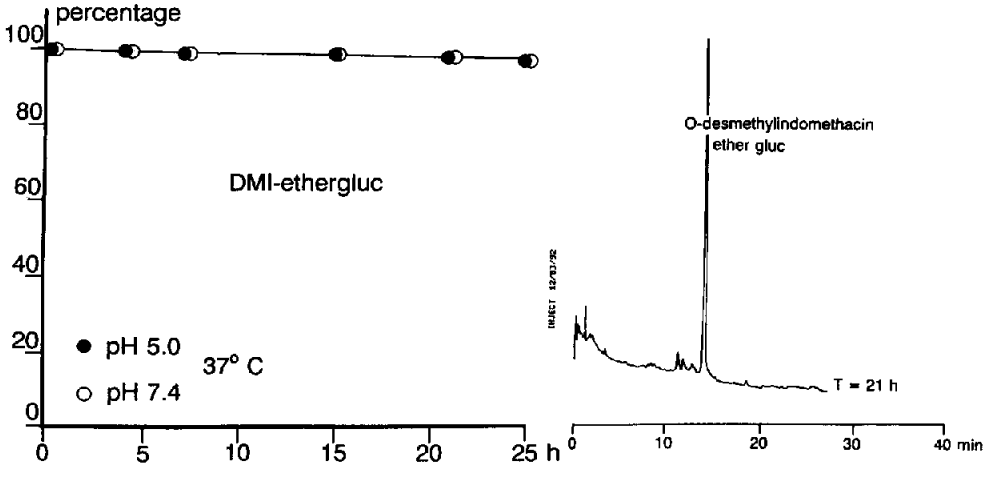

Fig. 7. Stability of isolated O-desmethylindomethacin ether glucuronide (DMI-ethergluc) at pH 5.0 (solid dots) and pH 7.4 (open dots). The right panel shows the chromatogram of the reaction at pH 7.4 and at t = 21 h.

TABLE IV
INTER-DAY AND INTRA-DAY COEFFICIENTS OF VARIATION OF SPIKED INDOMETHACIN IN HUMAN PLASMA (n = 4, *IN VITRO*)

Concentration (μg/ml)	C.V. (%)	
	Inter-day	Intra-day
5.74	0.84	0.84
2.17	1.75	1.35
0.63	4.44	1.50
0.27	3.58	1.82
0.06	10.7	4.08

of indomethacin acyl glucuronide and those of the metabolites DMI acyl and ether glucuronides in one male volunteer are shown in Fig. 8. No acyl glucuronides of indomethacin or DMI were detectable in plasma. Both acyl glucuronides are eliminated more rapidly than the ether glucuronide, as their renal excretion rate–time profiles run parallel to the indomethacin plasma concentration–time curve.

Some pharmacokinetic parameters of indomethacin in the human volunteer after the three pilot experiments are summarized in Table VI. The 25- and 100-mg doses gave similar yields of the metabolites. As no indomethacin acyl glucuronide is found in plasma, because of its presumed high renal clearance and hydrolysis in an alkaline medium (pH 7.4), the high concentration of indomethacin acyl glucuronide found in controlled acidic urine may therefore in part be formed by the kidney [7,25]. Previously the hypothesis was put forward that, if probenecid and a particular drug/compound were glucuronidated in the human kidney, probenecid must be able to interfere with or inhibit the glucuronidation of the concomitantly administered drug [36].

Probenecid co-medication totally inhibits the glucuronidation of the metabolite DMI, and 50% inhibits that of the parent drug (Table VI). Indomethacin itself shows low renal clearance values, owing to extensive tubular reabsorption [37,38]. Extreme tiredness was experienced as a side-effect with the 25- and 100-mg doses.

TABLE V
INTER-DAY AND INTRA-DAY COEFFICIENTS OF VARIATION OF INDOMETHACIN, ITS METABOLITE AND CONJUGATES IN HUMAN URINE (n = 4, *IN VIVO*)

Acyl glucuronide = 1-O-glucuronide.

Concentration (μg/ml)	C.V. (%)	
	Inter-day	Intra-day
Indomethacin		
1.5	3.2	2.4
1.3	4.6	1.4
0.92	2.3	2.0
0.15	3.4	3.2
Indomethacin acyl glucuronide		
66	1.4	0.2
50	0.8	0.2
24	2.1	2.6
5.5	4.8	0.6
0.2	4.5	3.5
O-Desmethylindomethacin		
0.5	3.7	2.7
0.3	3.3	3.5
0.2	6.6	4.9
0.1	5.4	4.2
O-Desmethylindomethacin acyl glucuronide		
16	4.0	2.2
11	2.0	1.6
7.1	3.0	1.1
1.9	2.0	0.8
0.50	1.7	1.7
O-Desmethylindomethacin ether glucuronide		
26	3.4	2.7
19	1.2	0.9
14	3.4	0.4
5.5	1.1	1.7
3.6	3.3	1.4
0.6	1.4	0.8

In conclusion, the analysis of indomethacin and its metabolites in human plasma and urine samples can easily be performed with gradient HPLC with UV detection. In plasma only the parent drug could be detected, while in urine the parent drug as well as metabolite and conjugates were present. The discrepancy between plasma and urine kinetics of the drug and its metabolites

TABLE VI

SOME PHARMACOKINETIC PARAMETERS OF INDOMETHACIN, ITS METABOLITE AND CONJUGATES IN HUMAN

Acyl glucuronide = 1-O-glucuronide; NP, not present.

Parameter	Value		
Subject	I	I	I[a]
Gender	Male	Male	Male
Body weight (kg)	92	92	92
Dose (mg)	100	25	25[a]
C_{max} (μg/ml)	5.4	1.4	4.1
T_{max} (h)	1.0	0.8	1.7
$t_{1/2_\alpha}$ (h)			
Indomethacin	0.84	0.77	0.20
$t_{1/2_\beta}$ (h)			
Indomethacin	5	6	4.6
Indomethacin acyl glucuronide[b]	6	6	9
O-Desmethylindomethacin acyl glucuronide[b]	9	9	NP
O-Desmethylindomethacin ether glucuronide[b]	27	27	NP
MRT indomethacin (h)	5.2	7.3	7.1
Total body clearance (l/h)	4.45	3.96	1.53
Volume of distribution (l)	31.5	28.8	8.1
Percentage of the dose excreted (%)			
Indomethacin	0.76	4.2	1.7
O-Desmethylindomethacin	0.58	0.6	1.1
Indomethacin acyl glucuronide	32.1	37.0	18.7
O-Desmethylindomethacin acyl glucuronide	7.3	7.1	NP
O-Desmethylindomethacin ether glucuronide	29.8	36.0	NP
Total	70.5	85.6	20.5
Urinary pH kept acidic (between pH 5.0 and 5.5).			

[a] + 1000 mg probenecid.

[b] Derived from renal excretion rate–time profiles.

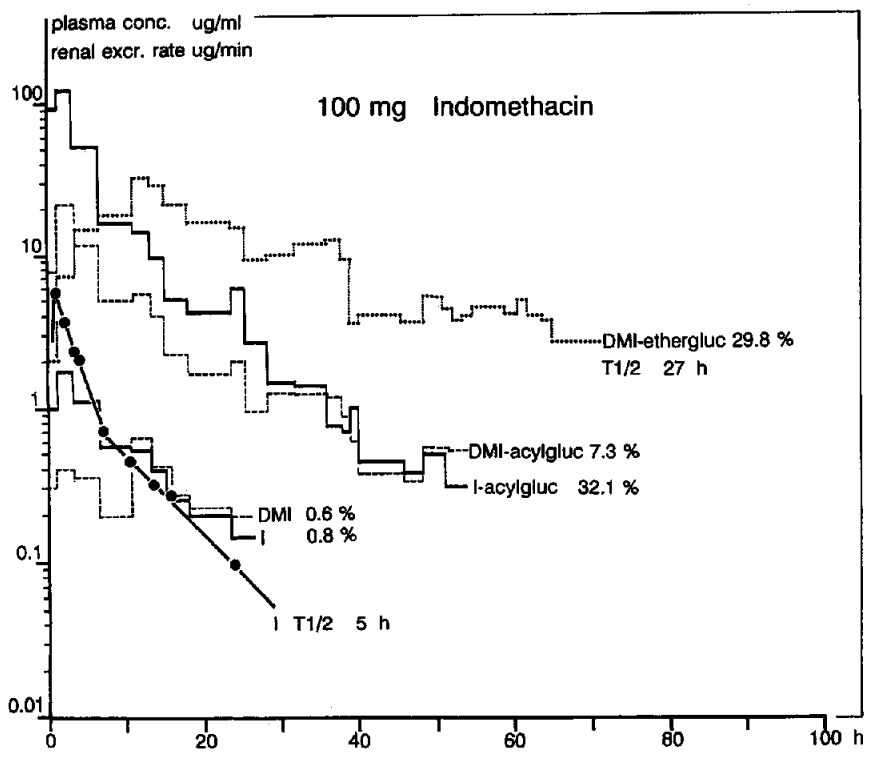

Fig. 8. Plasma concentration–time curve of indomethacin (I) and renal excretion rate–time profiles of indomethacin (I), O-desmethylindomethacin (DMI), indomethacin acyl glucuronide (I-acylgluc), DMI acyl glucuronide (DMI-acylgluc) and DMI ether glucuronide (DMI-ethergluc) in a human volunteer after an oral dose of 100 mg of indomethacin.

gives rise to the hypothesis that conjugation of these compounds may take place in the human kidney.

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