Isolation and Characterization of Major Phase I and II Metabolites of Ibuprofen

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INTRODUCTION

A number of metabolites of the non-steroidal anti-inflammatory drug ibuprofen, 2-(4-isobutylphenyl)propionic acid, have previously (1,2) been identified in urine and plasma samples obtained from humans after oral intake of ibuprofen. The two major metabolites were identified as the 2-[4-(2-hydroxy-2-methylpropyl)phenyl]propionic acid (2-hydroxy ibuprofen) and the 2-[4-(2-carboxypropyl)phenyl]propionic acid (carboxy ibuprofen) (2–6). 1-hydroxy ibuprofen and 3-hydroxy ibuprofen were found to be present in the urine in very small concentrations (2,3). The amount of 3-hydroxy ibuprofen and its glucuronic acid conjugate present in urine and plasma has not previously been quantified. An overview of the metabolic pattern of ibuprofen is shown in Figure 1.

A major metabolic pathway of xenobiotics containing a carboxylic acid group is conjugation with glucuronic acid, to yield acyl glucuronides. In recent years, an increasing interest has emerged towards drugs excreted in the form of acyl glucuronides, which potentially may react with proteins *in vivo* and give rise to allergic reactions (7,8). By directly coupling HPLC with NMR, the glucuronides of ibuprofen and the 2-hydroxy ibuprofen have been identified to be β-1-*O*-acyl glucuronides (9,10).

In the present study 2-hydroxy ibuprofen, carboxy ibuprofen and 3-hydroxy ibuprofen as well as the glucuronic acid conjugates of ibuprofen, 1-hydroxy ibuprofen, 2-hydroxy ibuprofen, 3-hydroxy ibuprofen and carboxy ibuprofen were isolated by preparative solid phase extraction followed by preparative HPLC. The substances were identified by one and two-dimensional ^{1}H-NMR experiments and were further characterized quantitatively in order to be used as reference substances. The glucuronic acid conjugates of the 1-hydroxy ibuprofen and the 3-hydroxy ibuprofen were isolated and identified for the first time. It was found that in humans all the known phase I metabolites of ibuprofen form β-1-*O*-acyl glucuronides at the carboxylic group of the propionic acid side chain.

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MATERIALS AND METHODS

Reagents

2-(4-isobutylphenyl)propionic acid (ibuprofen) and the metabolites 2-[4-(2-hydroxy-2-methylpropyl)phenyl]propionic acid (2-hydroxy ibuprofen) and 2-[4-(2-carboxypropyl)phenyl] propionic acid (carboxy ibuprofen) were kindly donated by Knoll Pharmaceuticals (Nottingham, UK), and Ipren® tablets (200 mg ibuprofen) were purchased form Nycomed DAK (Roskilde, Denmark).

Cetyl trimethylammonium (CTMA) hydroxide was purchased from Eastman Kodak (Rochester, USA). Acetic acid and 3-trimethyl-silyl-1-propane sulfonate (TSP) were purchased from Riedel-De-Haën (Seelze, Germany). Acetonitrile (HPLC grade) was obtained from Lab-Scan (Dublin, Ireland). Potassium dihydrogen phosphate was obtained from Merck (Darmstadt, Germany), and β-glucuronidase (*Escherichia coli*) (200 U/ml) was purchased from Boehringer (Mannheim, Germany). All other reagents were of analytical grade.

EQUIPMENT

Preparative Solid Phase Extraction Chromatography

A 1000 ml glass column (200 mm × 80 mm I.D.) was used for the preparative column chromatography, packed with XAD-2 resin (particle size 0.3–1 mm) obtained from Serva Feinbiochemica (Heidelberg, Germany). An Ismatec mp-4 peristaltic pump (Zurich, Switzerland) was used to generate a constant flow rate of 1.5 ml/min.

Preparative Chromatography

The preparative HPLC-system, consisted of a Perkin Elmer 250 LC pump (Norwalk, Connecticut, USA), a Rheodyne 7161 injector (Cotati, California, USA) with a 1.2 ml loop and a Kontron 430 variable-wavelength UV-detector (Zürich, Switzerland) operated at 220 nm. Chromatograms were recorded on a Shimadzu, C-R5A, Chromatopac integrator (Kyoto, Japan).

Analytical Chromatography

A Waters (Milford, MA, USA) liquid chromatographic system consisting of a Model 6000 A pump, a 715 Ultra WISP autoinjector, a 490E programmable multiwavelength detector was used and data were collected using Maxima 820 software. A Shimadzu (Kyoto, Japan) CTO-6A column oven was used to set the column temperature at 40°C.

ISOLATION AND PURIFICATION OF METABOLITES

Column Chromatography

Urine samples obtained 0–8 hours after intake of 600 mg ibuprofen were collected from 5 male and 5 female normal healthy subjects. Immediately after collection, the urine was adjusted to pH 2 with about 5 ml 2 M hydrochloric acid to each 100 ml urine and the urine samples were finally pooled.

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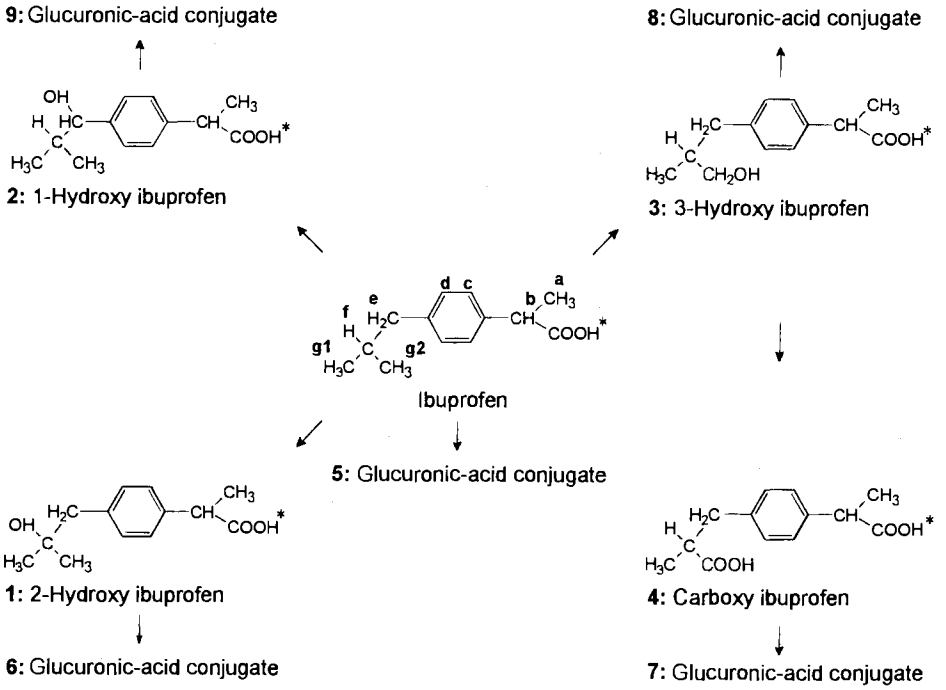

Fig. 1. Metabolic pattern of ibuprofen. The asterix indicates the site of glucuronidation.

Preparative solid phase extraction (SPE) chromatography was used to remove very polar as well as insoluble compounds from the urine. A stepwise elution procedure was employed in order to obtain selective recovery of the metabolites of ibuprofen in separate fractions. The column containing the XAD-2 resin was first conditioned with 3.0 L methanol followed by 3.0 L 1% acetic acid. 2.5 L of the pooled urine was applied to the column at a constant flow of 1.5 ml/min. After washing the column with an eluent consisting of 10% methanol in 1% acetic acid, the metabolites were eluted with methanol-water mixtures starting with 20% methanol progressing through 40%, 60% and 80% methanol, and finally an eluent consisting of 100% methanol was applied. 1250 ml of each eluent was applied, and a corresponding volume of eluate was collected in each fraction. In order to prevent acyl migration of the glucuronides during the chromatographic procedure, all the eluents contained 1% acetic acid.

The methanol of the collected fractions was partly evaporated using a rotary evaporator at 30°C, and residual water was removed by freeze drying. The fractions were then reconstituted in mobile phase A (see below), for further purification by preparative HPLC.

Preparative HPLC

A gradient system was employed in order to purify all phase I and phase II metabolites within one chromatographic system. The preparative HPLC column (250 mm × 16 mm I.D.) was packed with RoGel RP 10 μm particles obtained from Chemie Uetikon, (Uetikon, Switzerland).

The eluents were of the following compositions: Mobile phase A consisted of acetonitrile—water (20:80; v/v). Mobile phase B consisted of 100% acetonitrile. 1 ml glacial acetic acid was added to each litre of eluent. The flow rate of the mobile phase was 8 ml/min. Each chromatographic run was performed in the following manner: the column was equilibrated with mobile phase A for 10 minutes. Initially isocratic elution with mobile phase A was used; after 5 minutes the acetonitrile content was increased to 35% in 20 minutes and further to 65% in 10 minutes using linear gradients.

Identification of Metabolites by ^{1}H-NMR Spectroscopy

The purified metabolites and glucuronic acid conjugates of ibuprofen were identified by ^{1}H NMR spectroscopy. The solvent used was D_2O. The NMR data were acquired using a Bruker AMX-400 NMR spectrometer (Rheinstetten, Germany). One- and two-dimensional ^{1}H-NMR spectra were obtained at 400.14 MHz. Free induction decays (FIDs) were collected into 16 K computer data points with a spectral width of 5050.5 Hz, 90° pulses were used with an acquisition time of 2.58 sec. and the spectra were acquired by accumulation of 64 scans. Prior to Fourier transformation an exponential apodisation function was applied to the FID corresponding to a line broadening of 0.3 Hz. Two dimensional ^{1}H-NMR COSY-experiments were performed, in order to verify the structures of the 3-hydroxy ibuprofen and the carboxy ibuprofen-glucuronide. The parameters for the COSY experiments were as follows: The number of scans per increment was 16, the spectral width was 5881.62 Hz, and 512 increments were performed in the F1 dimension. The FIDs were collected into 1 K computer data points. The relaxation delay between successive pulses was 1.5 sec.

Analytical Chromatography

In the chromatographic system a saturation column (150 × 4.6 mm I.D.) dry packed with LiChroprep Si 60 (15–25 μm) was installed between the pump and the injection device in order to saturate the mobile phase with silica. The analytical column was a Knauer column (40 × 4.6 mm I.D.) packed

with Hypersil Si 60 (3 μm) obtained from Shandon Scientific, (Cheshire, UK). The temperature of the columns was set at 40°C. The final mobile phase developed for the assay of the phase I and II metabolites of ibuprofen consisted of acetonitrile - 0.2 M potassium phosphate (pH 7.4)—water (18:15:67; v/v/v) with 1.5 mM CTMA added. From 0–8 minutes the flow-rate was 1 ml/min, after 8 minutes run it was raised to 2.2 ml/min. The UV-detector was operated at 220 nm.

Purity of the Isolated Metabolites

Purity of the final products were determined by quantitative cleavage with β-glucuronidase followed by HPLC analysis of the parent drug towards a reference standard. The reference standards available were ibuprofen, 2-hydroxy ibuprofen and carboxy ibuprofen, which were used to determine the purity of the glucuronic acid conjugate of ibuprofen, 2-hydroxy ibuprofen, carboxy ibuprofen and their glucuronic acid conjugates.

Cleavage of the glucuronides with β-glucuronidase was performed as follows: 5 μl β-glucuronidase were added to 200 μl of the sample (pH 7.4) and the mixture was incubated at 37°C. After 24 hours 400 μl methanol were added, the sample was centrifuged for 12 min (5000 g), and 20 μl were injected into the analytical HPLC-system. The purity of the glucuronic acid conjugates of 1-hydroxy ibuprofen and 3-hydroxy ibuprofen was estimated by ^{1}H-NMR, with TSP as internal standard.

RESULTS AND DISCUSSION

NMR-Results

The isolated metabolites of ibuprofen, and their glucuronic acid conjugates were identified by ^{1}H-NMR spectroscopy to be 2-[4-(2-hydroxy-2-methylpropyl)phenyl]propionic acid (2-hydroxy ibuprofen), 2-[4-(3-hydroxy-2-methylpropyl)phenyl]-propionic acid (3-hydroxy ibuprofen), 2-[4-(2-carboxypropyl)-phenyl]propionic acid (carboxy ibuprofen), 2-(4-isobutyl-phenyl)propionic acid-acyl glucuronide (ibuprofen-β-1-*O*-acyl glucuronide), 2-[4-(1-hydroxy-2-methylpropyl)phenyl]propionic acid-acyl glucuronide (1-hydroxy ibuprofen-β-1-*O*-acylglucuronide), 2-[4-(2-hydroxy-2-methylpropyl)phenyl]propionic acid-acyl glucuronide (2-hydroxy ibuprofen-β-1-*O*-acylglucuronide), 2-[4-(3-hydroxy-2-methylpropyl)phenyl]propionic acid-acylglucuronide (3-hydroxy ibuprofen-β-1-*O*-acylglucuronide) and 2-[4-(2-carboxypropyl)phenyl]propionic acid-acyl glucuronide (carboxy ibuprofen-β-1-*O*-acyl glucuronide). The structures of these metabolites are shown in Figure 1. The 1-hydroxy ibuprofen was not isolated from urine in the present investigations. In order to obtain 1-hydroxy ibuprofen as a reference compound for development of the chromatographic method, cleavage of the 1-hydroxy ibuprofen-β-1-*O*-acylglucuronide with β-glucuronidase was performed.

Table I. NMR Assignment of the Isolated Metabolites

Metabolites:		a:−CH_3	b:−CH	c:−CH	d:−CH	e:−CH_2	f:−CH	g1:−CH_3	g2:−CH_3	1′β
IBU(*)	δ:	1.32	3.56	7.05	6.92	2.28	1.69	0.71	0.71	—
	M:	d	q	d	d	d	m	d	d	—
	J:	7.1	7.1	7.8 1	7.8	7.0		6.8	6.8	—
IBU-glcU	δ:	1.63	4.07	7.43	7.37	2.61	1.95	0.98	0.98	5.67
	M:	d	q	d	d	d	m	d	d	d
Metabolite 5	J:	7.2	7.2	7.9	7.9	7.0		6.9	6.9	8.2
1-OH-glcU	δ:	1.47	4.01	7.27	7.27	4.29	1.90	0.62	0.92	5.55
	M:	d	q	s	s	d	m	d	d	d
Metabolite 9	J:	6.7	6.9			7.4	7.4	7.1	6.9	7.9
2-OH-IBU	δ:	1.49	3.86	7.33	7.28	2.82	—	1.23	1.23	—
	M:	d	q	d	d	s	—	s	s	—
Metabolite 1	J:	7.1	7.1	8.1	8.1		—			—
2-OH-glcU	δ:	1.38	3.90	7.15	7.15	2.67	—	1.09	1.09	5.56
	M:	d	q	s	s	s	—	s	s	d
Metabolite 6	J:	7.4	7.4				—			7.9
3-OH-IBU	δ:	1.45	3.82	7.30	7.22	2.85	1.75	1.12	0.61	—
	M:	d	q	d	d	d	m	d	dd	—
Metabolite 3	J:	7.1	7.1	8.3	8.3	7.35		6.9	6.9	—
3-OH-glcU	δ:	1.47	3.85	7.34	7.28	2.43	2.90	1.18	1.55	5.63
	M:	d	q	d	d	d	m	d	d	d
Metabolite 8	J:	7.2	7.2	8.0	8.0	5.3		7.1	6.9	7.8
COOH-IBU	δ:	1.48	3.84	7.31	7.27	2.98	2.89	1.22	—	—
	M:	d	q	d	d	m	d	d	—	—
Metabolite 4	J:	7.2	7.2	8.2	8.2			6.6	—	—
COOH-glcU	δ:	1.53	3.96	7.30	7.27	2.94	2.85	1.20	—	5.48
	M:	d	q	d	d	m	m	d	—	d
Metabolite 7	J:	7.4	7.4	6.9	6.9			6.4	—	7.9

Note: a, b, c, d, e, f, g1, and g2 refer to the protons as indicated in Figure 1 on the structure of ibuprofen. δ = Chemical shift in ppm, M = Multiplicity, J = Coupling constant, s = singlet, d = doublet, dd = doublet of doublets, q = quartet and m = multiplet. (*)Solvent = $CDCl_3$.

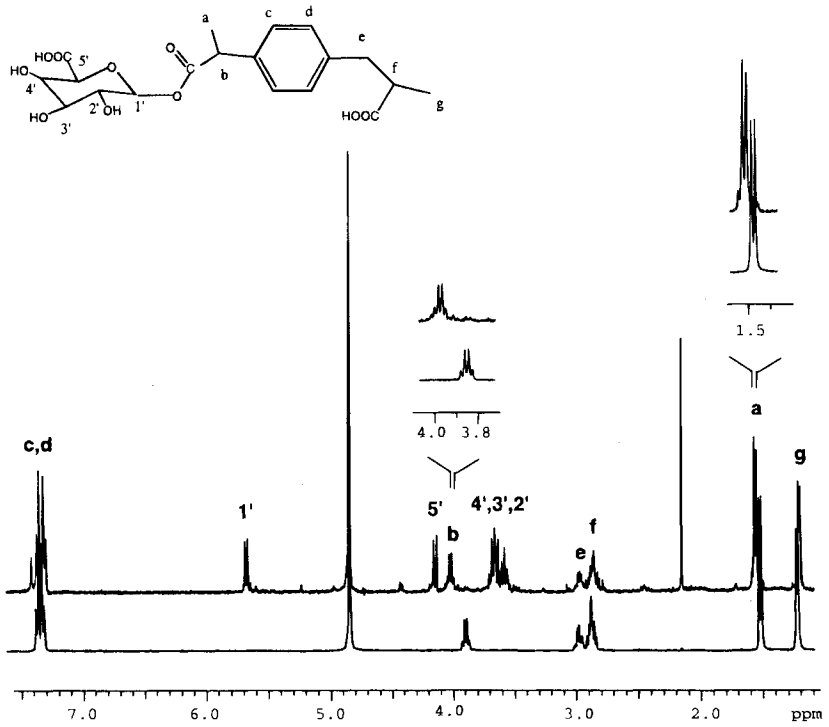

Fig. 2. ^{1}H-NMR spectra of carboxy ibuprofen and its glucuronic acid conjugate. ^{1}H 400 MHz NMR spectrum of the carboxy metabolite (lover spectrum) and its glucuronic acid conjugate (top spectrum). The expanded regions show the shifts observed for the resonances of the protons in the propionic acid side chain indicating that the site of glucuronidation is in this side chain.

The ^{1}H NMR assignments, as summarized in Table I, were based on the spin-spin coupling patterns and the coupling constants of the metabolites as well as the chemical shift values earlier found (11,12). 2D-COSY experiments were applied to the 3-hydroxy metabolite in order to verify the resonance of the g2-protons. These two protons are non equivalent because of the neighbouring chiral center and therefore they give rise to a double doublet.

Based on the appearance of a doublet at δ 5.5–5.7 that corresponds to the β-anomeric proton on the glucuronic acid ring, the glucuronic acid conjugates of the hydroxylated metabolites isolated were found to be conjugated at the carboxylic acid group of the propionic acid side chain and thus in the form of β-1-*O*-acylglucuronides. The carboxy metabolite was also found to be conjugated in the propionic acid side chain. This was based on the fact that significant shifts were observed for the resonances of the protons in this side chain as a consequence of glucuronidation (Figure 2). A shift of 0.12 ppm is observed for the b-proton and a shift of 0.05 ppm is observed for the a-proton; whereas the e, f and g-protons are not affected significant by glucuronidation. 2D-COSY-experiments were performed on the carboxy metabolite and its glucuronic acid conjugate in order to certify the resonance assignments.

Chromatography

Ibuprofen exhibit a chiral center in the propionic acid side chain, however, chiral separation was not the topic of the present study and was thus not attempted. A reversed-phase HPLC method for determination of ibuprofen, its major metabolites and their conjugates has been developed using bare silica dynamically modified with a long-chain quaternary ammonium compound (CTMA-hydroxide) (unpublished data). The resulting separation obtained for the phase I and II metabolites of ibuprofen is shown in Figure 3.

Purity of Isolated Compounds

The analytical chromatographic method developed was used to verify the purity of the isolated and purified metabolites. Purity of the final products were estimated by quantitative cleavage with β-glucuronidase followed by HPLC analysis of the parent drug towards a reference standard. The results found showed the purity of the glucuronic acid conjugate of ibuprofen to be 85%, the purity of the 2-hydroxy ibuprofen was 92% and its glucuronic acid conjugate was found to be 88% pure. The carboxy ibuprofen was estimated to be 89% pure and its glucuronic acid conjugate was found to be 90% pure. Large amounts of these five major phase I and II metabolites were isolated, from about 60 mg of the 2-hydroxy ibuprofen to about 400 mg of the ibuprofen-glucuronide. Standards of the 1-hydroxy ibuprofen and the 3-hydroxy ibuprofen are not available, so the purity of 1-hydroxy ibuprofen-glcU, 3-hydroxy ibuprofen and its glucuronic acid conjugate was therefore estimated by ^{1}H-NMR. With TSP as internal standard the glucuronic acid conjugate of 1-hydroxy ibuprofen was estimated to be 48% pure. The 3-hydroxy ibuprofen was estimated to be 58% pure and its glucuronic acid conjugate was found to be only 33% pure. Only about 10 mg of each of these three minor metabolites were isolated and purified, but the purity was adequate to identify the structures by ^{1}H-NMR experiments.

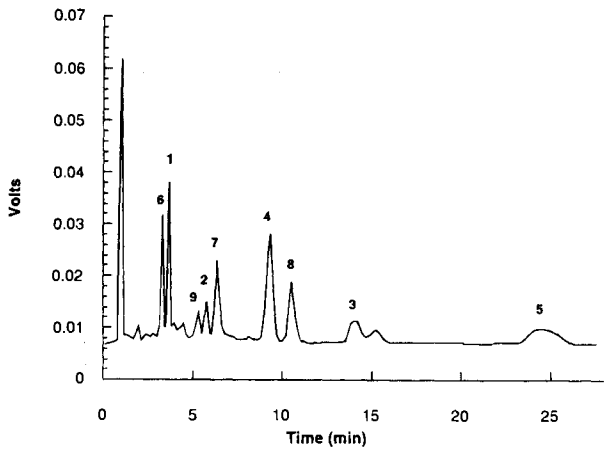

Fig. 3. Chromatogram of a standard mixture of phase I and II metabolites of ibuprofen. The numbers in the chromatogram refer to the metabolites as indicated in Figure 1. Peak 1, 2, 6 and 9 each correspond to 200 ng on column and peak 3, 4, 5, 7 and 8 each correspond to 500 ng on column. Retention times (min) for the metabolites are 2-hydroxy ibuprofen-glucuronide (3.22), 2-hydroxy ibuprofen (3.63), 1-hydroxy ibuprofen-glucuronide (5.30), 1-hydroxy ibuprofen (5.95), carboxy ibuprofen-glucuronide (6.64), carboxy ibuprofen (9.63), 3-hydroxy ibuprofen-glucuronide (10.96), 3-hydroxy ibuprofen (14.46), ibuprofen-glucuronide (24.67). Chromatographic conditions: saturation column (150 × 4.6 mm I.D.), dry packed with LiChroprep Si 60 (15-25 μm) and placed between pump and injector. Analytical column, Knauer column (40 × 4.6 mm I.D.), slurry packed with Hypersil Si 60 (3 μm), temperature set at 40°C. Eluent, acetonitrile—0.2 *M* potassium phosphate (pH 7.4)—water (18:15:67; v/v/v/) with 1.5 m*M* CTMA added. The flow rate was initially 1.0 ml/min, after 8 min it was increased to 2.2 ml/min. UV detection at 220 nm.

CONCLUSIONS

Three phase I and five phase II metabolites of ibuprofen were isolated in larger amounts from human urine in order to be used as reference substances. The advantage of the described procedure compared to chemical synthesis is primarily that several metabolites may be isolated from the same sample of urine and secondly chemical synthesis of acylglucuronides is still not an easy task to do in larger scale. Two of the phase II metabolites isolated were identified for the first time and it was found that all known phase I metabolites of ibuprofen form β-1-O-acylglucuronides at the carboxylic group in the propionic acid side chain.

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