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Simultaneous quantitative determination of the major phase I and II metabolites of ibuprofen in biological fluids by high-performance liquid chromatography on dynamically modified silica

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Abstract

Ibuprofen has previously, after ingestion by man, been demonstrated to yield four major phase I metabolites, which are excreted in the urine partly as glucuronic acid conjugates. However, in previous investigations the quantitative determinations of the conjugates were performed by indirect methods. The purpose of the present investigation was to develop a high-performance liquid chromatographic (HPLC) system for the simultaneous determination of the major phase I and II metabolites of ibuprofen in biological fluids. The separation was performed using bare silica dynamically modified with *N*-cetyl-*N*,*N*,*N*-trimethylammonium hydroxide ions contained in the mobile phase. The separation of the metabolites of ibuprofen is greatly improved with this system compared to other published reversed-phase HPLC systems intended for the same purpose. The method developed makes it possible to simultaneously determine the intact glucuronic acid conjugates of ibuprofen as well as its phase I metabolites in human urine. In a study involving four healthy volunteers, a total recovery in urine of the dose given was found to be 58–86% within 8 h. This may be compared to an average of 67% earlier reported in the literature. © 1997 Elsevier Science B.V.

Keywords: Ibuprofen

1. Introduction

Ibuprofen [2-(4-isobutylphenyl)propionic acid] is a non-steroidal anti-inflammatory drug, used in the treatment of rheumatoid arthritis and other rheumatic conditions [1–3]. Pharmacokinetic studies (in rats, rabbits and dogs) using radioactive tracers have shown that about 66% of the drug is excreted in the urine whereas about 34% is excreted in the faeces (biliary excretion) [4]. Recovery studies revealed that 60% of the dose given was excreted within the first 24 h, the proportion increasing to between 80 and 100% after three to five days [4].

Oxidative metabolism is the major route for biotransformation of ibuprofen, and four oxidative metabolites have been identified in urine and plasma samples obtained from humans after oral intake of ibuprofen (Fig. 1). In humans, the parent drug, as well as the metabolites, are found to be conjugated with glucuronic acid [5–7], and glucuronidation has

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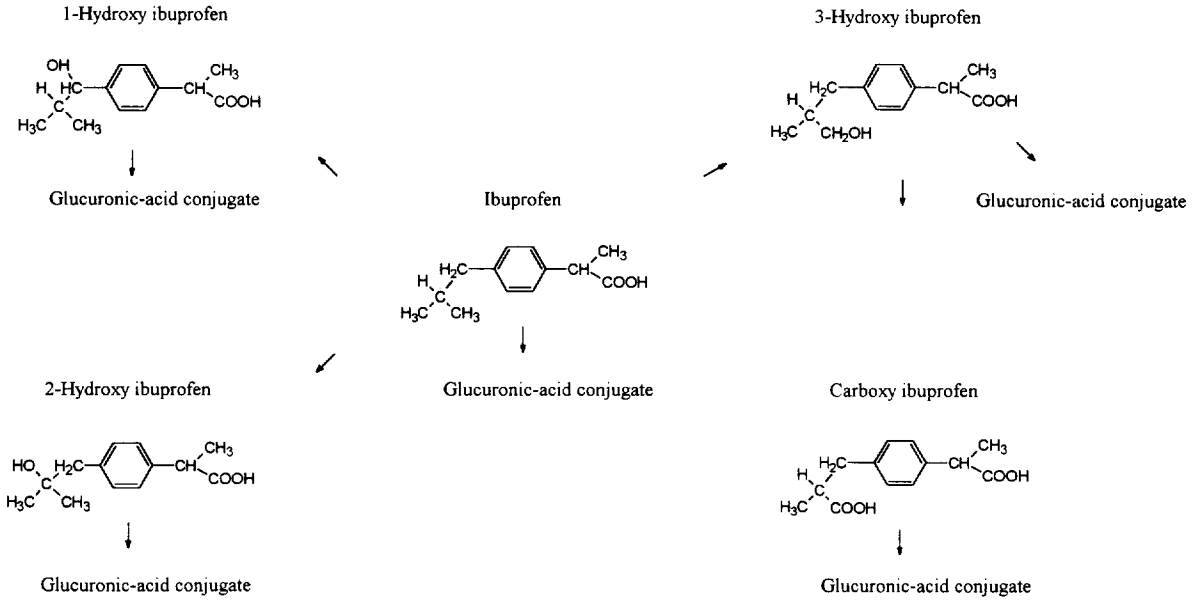

Fig. 1. Metabolic pathway of ibuprofen.

in all cases taken place at the carboxy group in the propionic acid side chain. The two major metabolites 2-hydroxyibuprofen and carboxyibuprofen and their glucuronic acid conjugates were found to account for approximately 58% of the given dose of ibuprofen, whereas the two minor metabolites 1-hydroxyibuprofen and 3-hydroxyibuprofen and their glucuronic acid conjugates were found to be present in the urine in only very small concentrations [8–13].

A number of chromatographic methods have been reported for the quantitative determination of ibuprofen, 2-hydroxyibuprofen and carboxyibuprofen, using reversed-phase HPLC [10–14]. Some of these papers describe methods for the indirect determination of the glucuronic acid conjugates. This is performed as a measurement of the difference in concentration of the parent drug or phase I metabolite before and after chemical or enzymatic hydrolysis of the conjugates. The determination of the conjugates has in most instances been performed indirectly because of lack of reference compounds or lack of a sufficiently selective separation method.

In this paper, we present an HPLC method for the simultaneous determination of the major metabolites of ibuprofen as well as the glucuronic acid conjugates of ibuprofen and its major phase I metabolites in biological fluids. The separation is performed using bare silica dynamically modified with *N*-cetyl-*N*,*N*,*N*-trimethylammonium (CTMA) ions dissolved in the mobile phase. The retention and the separation of the metabolites of ibuprofen is greatly improved (separation of all phase I and II metabolites in a relatively short time) with this system compared to the earlier published HPLC systems used in studies of the metabolism of ibuprofen [10–14].

2. Experimental

2.1. Chemicals

2-(4-Isobutylphenyl)propionic acid (ibuprofen) and the metabolites 2-[4-(2-hydroxy-2-methylpropyl)phenyl]propionic acid (2-hydroxyibuprofen) and 2-[4-(2-carboxypropyl)phenyl] propionic acid (carboxyibuprofen) were kindly donated by Knoll Pharmaceuticals (Nottingham, UK), and Ipren tablets (200 mg ibuprofen) were purchased from Nycomed DAK (Roskilde, Denmark). The β-1-*O*-acyl glucuronic acid conjugates of ibuprofen (Ibu-glcU), 2-hydroxyibuprofen (Ibu-2-OH-glcU) and of carbox-

yibuprofen (Ibu-COOH-glcU) were isolated and purified as previously described [5].

N-Cetyl-*N*,*N*,*N*-trimethylammonium (CTMA) hydroxide (25% in methanol) was purchased from Eastman Kodak (Rochester, MN, USA). Acetic acid was purchased from Riedel-De-Haën (Seelze, Germany) and acetonitrile (HPLC grade) was obtained from Lab-Scan (Dublin, Ireland). Potassium dihydrogen phosphate was obtained from Merck (Darmstadt, Germany). All other reagents were of analytical grade.

2.2. Apparatus

A Kontron (Tegimenta, Switzerland) liquid chromatographic system consisting of a Model 420 pump, a Model 460 autosampler, a column oven Model 480 and a plotter Model 800 was used. A Shimadzu (Kyoto, Japan) Model SPD-6A UV detector was used and data was collected using Kontron Data system 450 software.

2.3. Sample preparation

Urine samples were prepared for HPLC analysis using solid-phase extraction on C_{18} bonded cartridges containing 100 mg of sorbent (Bond Elut, Varian, CA, USA). The cartridges were activated prior to use, by washing with 2×1 ml of acetonitrile followed by 2×1 ml of 1% acetic acid. Urine (1000 μl) was then applied to the activated cartridges which was then washed with 500 μl of acetonitrile–1% acetic acid (10:90) followed by elution with 500 μl of acetonitrile. The eluate was evaporated to dryness using nitrogen and after reconstitution with 500 μl of the mobile phase a 20-μl aliquot was injected onto the chromatographic column. If necessary, the reconstituted eluate was diluted (1:10) with water in order to fit the range of the calibration curve.

2.4. Analytical chromatography

In the chromatographic system, a saturation column (150×4.6 mm I.D.) dry packed with LiChroprep Si 60 15–25-μm particles obtained from Merck was installed between the pump and the injection device. The final analytical column was a Knauer column (40×4.6 mm I.D.) packed with Hypersil (3 μm) obtained from Shandon Scientific (Cheshire, UK). For development of the analytical method, a Knauer column (120×4.6 mm I.D.) packed with Polygosil 60–5 (5 μm) obtained from Macherey-Nagel (Düren, Germany) was used. 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) with 1.5 m*M* CTMA–hydroxide added. Initially the flow-rate was 1 ml/min, after 8 min of the chromatographic runtime it was raised to 2.2 ml/min. The UV detector was operated at 220 nm.

2.5. Validation procedures

The method developed was validated with respect to specificity by looking for interfering substances in the urine matrix. Linearity and range were tested using calibration standards of Ibu-2-OH, Ibu-COOH and the isolated and purified glucuronic acid conjugates Ibu-glcU, Ibu-2-OH-glcU and Ibu-COOH-glcU. Repeatability and accuracy were investigated at three different concentration levels (n=6). The accuracy was determined using a standard addition procedure.

3. Results and discussion

3.1. Chromatography

The HPLC separation methods for ibuprofen and its major oxidative metabolites previously described in the literature are all based on reversed-phase chromatography performed on chemically bonded phases [10–14]. However, these systems were not well suited for simultaneous separation of the metabolites and their conjugates, due to large differences in polarity between the phase I and II metabolites. The dynamically modified silica approach has earlier proved to be a valuable separation method in the analysis of biological samples, especially for the simultaneous determination of parent drug, metabolites and conjugates [15–18], with large differences in polarity. The retention in systems based on

dynamically modified silica are mainly controlled by four factors: the chain length of the quaternary ammonium compound, type and amount of organic modifier, ionic strength and pH of the buffer [19–21]. In this technique, bare silica is dynamically modified with a long-chain quaternary ammonium compound (CTMA), resulting in a reversed-phase HPLC system [22]. The presence of CTMA ions results in the formation of hydrophobic ion-pairs with anions, which are subsequently separated by reversed-phase chromatography. This system is therefore very suitable for the analysis of the intact glucuronides as well as the anionic phase I metabolites.

Detection of the analytes was performed by UV absorption at 220 nm in order to achieve sufficient low limits of quantitation. Often CTMA bromide ions have been used as the quartenary ammonium reagent [20]. However, bromide ions absorb UV light at 220 nm, thus CTMA hydroxide was chosen as the reagent for the separation of the metabolites of ibuprofen.

The chromatographic system was optimized with respect to the amount of organic modifier in the mobile phase. In the present study acetonitrile was used as the organic modifier. When the amount of acetonitrile in the eluent was increased above 23% (v/v), the separation of some of the metabolites was lost (Fig. 2). The final eluent was chosen to contain 18% (v/v) of acetonitrile. Lower concentrations of acetonitrile were not used as the retention of the Ibu-glcU would then be unnecessarily long.

It has previously been demonstrated [23] that reversed-phase HPLC-systems based on the dynamically modified silica approach exhibit a high degree of reproducibility of selectivity independent of the brand of silica used. Thus, it is possible to decrease the time of analysis by changing the brand of silica to a silica with a lower surface area. This will result in less stationary phase and thus shorter retention

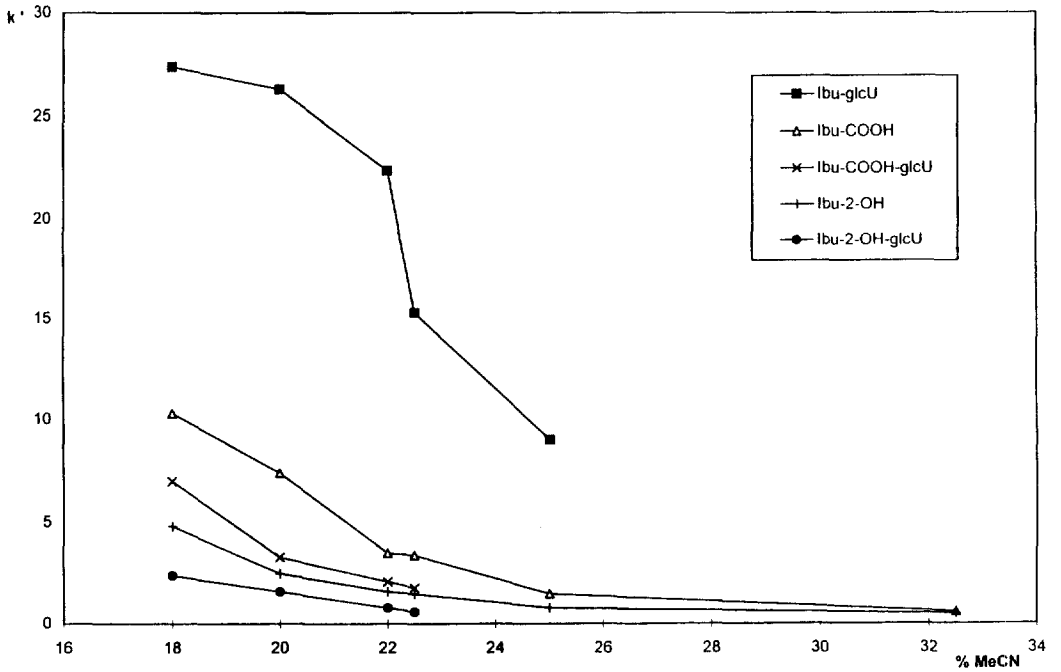

Fig. 2. Relationship between the concentration of acetonitrile and the capacity factor (k') for Ibu-glcU (■), Ibu-2-OH (+), Ibu-2-OH-glcU (●), Ibu-COOH (△) and Ibu-COOH-glcU (×). Chromatographic conditions: saturation column (150×4.6 mm I.D.), dry packed with LiChroprep Si 60 (15–25 μm) placed between pump and injector. Analytical column, Knauer column (120×4.6 mm I.D.), slurry packed with Polygosil 60 (5-μm), temperature set at 40°C; eluent, acetonitrile–0.2 *M* potassium phosphate (pH 7.4)–water [(18–32.5):15:(52.5–67), v/v/v], with 1.5 m*M* CTMA–hydroxide 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.

Table 1
Analysis of drug-free human urine spiked with the major phase I and II metabolites of ibuprofen

Sample	Added (μg/ml)	Found (μg/ml)	Recovery (%)	R.S.D. (n=6) (%)
Ibu-glcU	23.2	21.3	85.2	3.0
	46.4	4.1	88.5	6.6
	232.3	210.2	90.5	1.5
Ibu-2-OH	27.8	28.6	102.9	2.2
	55.6	55.0	98.7	1.5
	278.6	275.0	98.7	3.8
Ibu-2-OH-glcU	26.7	26.8	100.4	5.3
	53.4	52.4	98.1	1.2
	267.1	252.7	94.6	4.3
Ibu-COOH	29.1	25.3	86.8	1.8
	58.2	55.4	95.2	2.1
	291.3	283.5	97.3	4.4
Ibu-COOH-glcU	27.2	26.8	98.4	2.9
	54.4	55.7	102.4	4.7
	272.4	242.1	88.9	3.4

times but will keep the selectivity of the system unchanged. The time of analysis was further decreased by use of a shorter column. The reduction of the number of theoretical plates due to the shorter column was partly compensated by use of a packing material with a smaller particle size.

3.2. Assay validation

The detection limits for the phase I metabolites of ibuprofen and the glucuronic acid conjugates of ibuprofen and the two phase I metabolites in urine were determined to be the following: for Ibu-2-OH-glcU, Ibu-2-OH and Ibu-COOH-glcU it was 1 μg/ml, for Ibu-COOH it was 5 μg/ml and for Ibu-glcU it was 12 μg/ml. This was estimated as three times the standard deviation (δ) of the peak-to-peak noise ($N_{p\to p}$), where $\delta = N_{p\to p}/5$. The limits of quantitation were for Ibu-2-OH-glcU and Ibu-2-OH were 4 μg/ml, it was 5 μg/ml for Ibu-COOH-glcU, 17 μg/ml for Ibu-COOH and 23.2 μg/ml for Ibu-glcU.

The calibration curves for Ibu-2-OH (5–300 μg/ml), Ibu-2-OH-glcU (5–300 μg/ml), Ibu-COOH (25–300 μg/ml), Ibu-COOH-glcU (10–300 μg/ml) and Ibu-glcU (25–400 μg/ml) were all linear within the concentration ranges specified (r=0.997).

Recovery studies of the major phase I and II

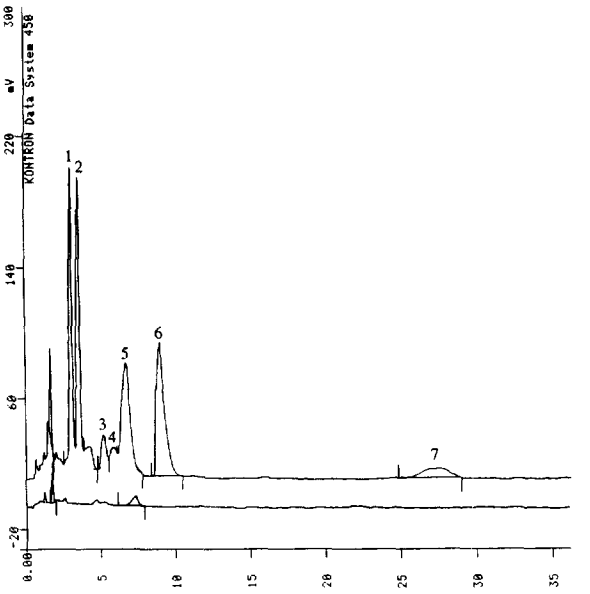

Fig. 3. Chromatograms of a urine sample from a volunteer in a period of 2–4 h after oral intake of 600 mg of ibuprofen (upper chromatogram) and a blank urine sample from the same volunteer before intake of ibuprofen (lower chromatogram). Peak indentification: (1) Ibu-2-OH-glcU, (2) Ibu-2-OH, (3) Ibu-1-OH-glcU, (4) Ibu-1-OH, (5) Ibu-COOH-glcU, (6) Ibu-COOH, (7) Ibu-glcU. Analytical column, Knauer column (40×4.6 mm I.D.), packed with Hypersil (3 μm), eluent, acetonitrile–0.2 *M* potassium phosphate (pH 7.4)–water (18:15:67, v/v/v) with 1.5 m*M* CTMA-hydroxide added. Other chromatographic conditions were as described in Fig. 2.

metabolites of ibuprofen in human urine were done by adding known amounts of the reference compounds to drug-free urine at three different concentrations ($n=6$). The recoveries for all solutes were found to be 85.2–102.9% (Table 1). The intra-assay relative standard deviations (R.S.D.s) are sufficiently low (1.2–6.6%) for the method to be used for routine analysis.

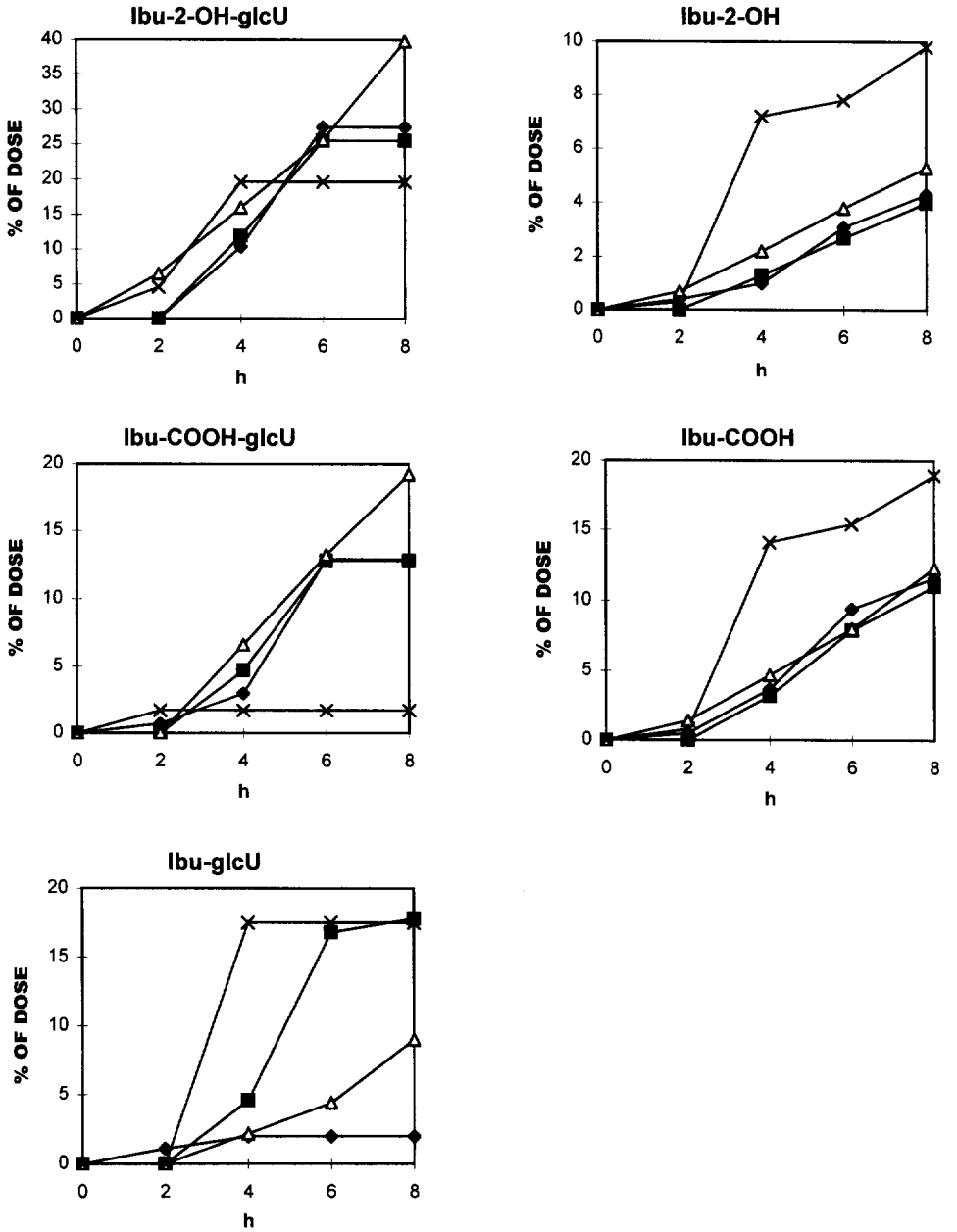

Fig. 4. Cumulated urinary excretion (% of dose) of the major phase I and II metabolites of ibuprofen, collected every 2 h in the time intervals 0–8 h after oral administration of 600 mg ibuprofen. Person identification: A=♦, B=■, C=△ and D=X.

Table 2
Cumulated urinary excretion (% of dose) within 8 h of the major phase I and II metabolites of ibuprofen, after oral administration of 600 mg ibuprofen to four volunteers (A, B, C and D)

Compound	A	B	C	D
Ibu-glcU	2.0	17.8	9.0	17.5
Ibu-2-OH	4.3	4.0	5.3	9.8
Ibu-2-OH-glcU	27.4	25.5	39.8	19.6
Ibu-COOH	11.6	11.0	12.3	18.9
Ibu-COOH-glcU	12.9	12.8	19.2	1.6
Total	58.2	71.1	85.6	67.4

3.3. Applications

The developed method was used for the analysis of the in vivo metabolism of ibuprofen in humans. Four healthy volunteers (A, B, C and D) were each given 600 mg of ibuprofen (Ipren) orally. Urine samples obtained 0–8 h after intake of ibuprofen, were collected in intervals of 2 h. Immediately after collection, the urine was adjusted to pH 2 with 5 ml of 2 *M* hydrochloric acid to each 100 ml of urine. Three metabolites and four conjugates were identified in the urine: Ibu-1-OH, Ibu-2-OH, Ibu-COOH, Ibu-1-OH-glcU, Ibu-2-OH-glcU, Ibu-COOH-glcU and Ibu-glcU. Earlier studies have shown that less than 1% of ibuprofen is excreted in its unchanged form [8–13], and it was therefore not determined in the present study. Furthermore, it should be noted that the possible diastereomers of Ibu-COOH and Ibu-COOH-glcU were not separated by the method decribed. An example of a chromatogram of a urine sample from one of the volunteers is shown along with a blank urine sample in Fig. 3.

The totally excreted amount of the given dose in the interval 0–8 h was 58.2–85.6% (Table 2). This is comparable to an average of 67% as earlier reported [4,10,12–14]. Some differences between the metabolic profile from the four volunteers especially with respect to excretion of glucuronidated compounds were observed (Fig. 4).

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