Isoursodeoxycholic acid: metabolism and therapeutic effects in primary biliary cirrhosis[1]

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Abstract Significant amounts of ursodeoxycholic acid (UDCA) used for the treatment of patients with primary biliary cirrhosis (PBC) become epimerized at C-3 to isoUDCA. We investigated the metabolism of isoUDCA and a possible pharmacologic effect in five patients (51.4 ± 5.8 years old; 3 females, 2 males) with PBC and persistent elevations of γ-glutamyl transpeptidase (γ-GT) and alkaline phosphatase despite treatment with UDCA for more than one year. Serum samples were analyzed for bile acid metabolites and surrogate markers of cholestasis in 4-week intervals after 1 g/d UDCA, wash-out, 0.5 g/d isoUDCA, 0.75 g/d isoUDCA, 0.75 g/d UDCA, and two further periods with 1 g/d UDCA. Bile acids in urine were analyzed after wash-out, 0.5 and 0.75 g/d isoUDCA, and 0.75 and 1 g/d UDCA. During wash-out, AST, AP, and γ-GT rose significantly ($P < 0.05$) but reversed to previous levels during the first isoUDCA period, with 0.5 g/d only. No further improvements were observed after increasing the dose of isoUDCA or switching back to UDCA. In serum, the relative amounts of isoUDCA and UDCA were 8.1 ± 7.4% and 16.2 ± 6.4% during 0.5 g/d isoUDCA, 6.2 ± 2.5% and 45.0 ± 4.1% during 0.75 g/d isoUDCA, and 0.5–3% and 56.4–60.0%, respectively, during UDCA. In urine, UDCA was the predominant bile acid both during isoUDCA and UDCA medications. The similar serum enrichment and urinary excretion of UDCA during administration of either isoUDCA or UDCA together with low concentrations of the intermediate of isomerization, 3-dehydro-UDCA, indicate a first-pass epimerization of isoUDCA to UDCA in the liver. Approximately 25% of serum isoUDCA and 10% of serum UDCA were conjugated with either glucuronic acid or *N*-acetylglucosamine, indicating hepatic formation and systemic secretion of glycosidic conjugates. In PBC patients, isoUDCA becomes isomerized to UDCA and has similar effects on surrogate markers of cholestasis. Thus, isoUDCA has pro-drug characteristics.—Marschall, H-U., U. Broomé, C. Einarsson, G. Alvelius, H. G. Thomas, and S. Matern. **Isoursodeoxycholic acid: metabolism and therapeutic effects in primary biliary cirrhosis. *J. Lipid Res.* 2001.** 42: **735–742.**

Supplementary key words ursodeoxycholic acid • bile acid metabolis • *N*-acetylglucosaminidation • glucosidation • glucuronidation • gas chromatography-mass spectrometry • electrospray mass spectrometry

Ursodeoxycholic acid (UDCA) improves clinical and biochemical indices in a variety of cholestatic liver diseases (1). UDCA is now considered as the first line treatment option for patients with primary biliary cirrhosis (PBC), since the results from the combined analysis of the three largest randomized clinical trials of UDCA in PBC indicate that UDCA improves survival free of liver transplantation (2). The validity of this finding has, however, recently been questioned (3). The mechanisms of action of UDCA are still under debate. There is evidence that the hydrophilic UDCA protects against injury of bile ducts by hydrophobic bile acids and stimulates the excretion of these and other potentially hepatotoxic compounds (1).

One of the major metabolites of orally administered UDCA is the 3β-hydroxy epimer, isoursodeoxycholic acid (isoUDCA) (4–10), mostly likely formed in the intestine by bacterial enzymes (8). IsoUDCA and UDCA have very similar hydrophilicity (11) and are excreted in urine mainly as 7β-*N*-acetylglucosamine conjugates (11–16). We found isoUDCA in vitro to be even more cytoprotective than UDCA against ethanol-induced cell injuries in HepG2 cells (15). We assumed from the chemical and cytroprotective properties that isoUDCA could be pharmacologically active in cholestatic liver disease. After our previous study of the metabolism of isoUDCA in healthy humans (15), we now present the data of a pilot study with isoUDCA in patients with PBC. We investigated whether the metabolism of isoUDCA might be altered in cholestasis and whether isoUDCA would have a pharmacodynamic effect comparable to UDCA.

Abbreviations: CA, cholic acid; CDCA, chenodeoxycholic acid; LCA, lithocholic adid; GLC, gas-liquid chromatography; GC-MS, gas chromatography-mass spectrometry; ES-MS, electrospray-mass spectrometry; Glc, glucose; GlcA, glucuronic acid; GlcNAc, *N*-acetylglucosamine; isoUDCA, isoursodeoxycholic acid; UDCA, ursodeoxycholic acid.

[1] Preliminary data of this study were presented (and published in abstract forms) at the EASL meeting, April 1999, Naples, Italy (*J. Hepatol.* 1999. **30: Suppl. 1**, 144) and at the DDW, May 1999, Orlando, Florida, USA (*Gastroenterology.* 1999. **116:** G0086).

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MATERIALS AND METHODS

Study drugs

IsoUDCA was synthesized from UDCA (a gift of Dr. Falk, Freiburg, Germany) as described in (15). Purity was >99% as estimated by elemental analysis, thin-layer chromatography, high performance liquid chromatography, gas-liquid chromatography (GLC), and gas chromatography-mass spectrometry (GC-MS). IsoUDCA contained approximately 0.2% of UDCA. The study drug was administered in 250-mg capsules identical to those used for prescribed UDCA (Ursofalk®, Dr. Falk).

Patients and study protocol

Five patients, 51.4 ± 5.8 years old; 72.8 ± 7.1 kg of body weight, 3 females and 2 males, positive for antimitochondrial antibodies type M2 (AMA-M2), with biopsy-proven, stage I-III PBC, were enrolled in an open-label pilot study with isoUDCA. They featured persistent elevations in serum concentrations of γ-GT (γ-glutamyl transpeptidase) and alkaline phosphatase (ALP), at least five times and two times the upper limit of normal, respectively, despite treatment with UDCA, 15–20 mg/kg/d, for more than one year. Last screening biochemistry was obtained 4–6 weeks before the start of study. The study consisted of seven periods of 4 weeks each: 1 g/d UDCA, wash-out, 0.5 g/d isoUDCA, 0.75 g/d isoUDCA (10.3 ± 1.1 mg/kg/d), 0.75 g/d UDCA, 1 g/d UDCA, and 1 g/d UDCA. The appropriate amount of isoUDCA or Ursofalk® capsules was administered in two daily doses. No further medication was given.

The patients were evaluated every fourth week in the liver outpatient center. They were asked to report side effects and to estimate the degree of fatigue or itching on a 10-point scale. The patients collected 24-h urine samples on the last day of the wash-out and the following four treatment periods. At each visit, blood was drawn for bile acid analysis and standard laboratory tests in the fasting state without medication, at 8:00 AM.

Informed consent was obtained by all patients studied. The study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki and was approved by the local ethics committee.

Analytical methods

Laboratory tests. Haemoglobin, blood cell counts, and serum concentrations of glucose, renal function tests, and liver tests such as bilirubin, alanine transferase (ALT), asparagine transferase (AST), γ-GT, and ALP were determined by routine methods.

Bile acid analysis

Extraction, purification, and hydrolysis. A comprehensive bile acid analysis including all possible conjugates and metabolites was performed with pooled aliquots of serum and urine samples from wash-out and the following four treatment periods. The analytical procedure previously used for bile acids from healthy subjects (10, 11, 15) was adjusted for the analysis of serum and urine samples from cholestatic patients as described below. Chemicals were obtained from Sigma-Aldrich (St. Louis, MO), solvents from Merck (Darmstadt, Germany), if not otherwise indicated. Sep-Pak C_{18} cartridges (Waters, Milford, MA) were used for the quantitative extraction of bile acids from 0.1% aliquots of 24-h urine and from 1 ml of serum, the latter diluted 10-fold with 0.5 mol/l triethylammonium sulfate, pH 7.4, and heating for 10 min to 65°C to diminish protein adsorption. Separation into different groups of conjugates was performed using anion-exchange chromatography on Lipidex-DEAP (Packard-Becker, Groningen, The Netherlands) after the addition of cholyl [^{14}C]glycine (Amersham, Buckinghamshire, UK) as internal standard for the monitoring of recovery and group separation. The following fractions were eluted from Lipidex-DEAP (bed size 10 cm × 4 mm) with the buffers in parenthesis, all in 70% ethanol: #1 (7.5 ml 0.1 M acetic acid), unconjugated bile acids and nonamidated bile acid glucosides (Glc) and *N*-acetylglucosaminides (GlcNAc); #2 (10 ml 0.3 M ammonium acetate, pH 6.6), glycine- or taurine-conjugated bile acids (gly-/tau-) and glycine- or taurine-conjugated bile acid glucosides and *N*-acetylglucosaminides; #3 (5 ml 0.25 M formic acid), nonamidated and glycine- or taurine-conjugated bile acid glucuronides (GlcA); and #4 (5 ml 0.5 M ammonium acetate, pH 9.6), nonamidated and glycine- or taurine-conjugated bile acid mono- and disulfates (S, diS). Glycine and taurine conjugates in fractions #2–#4 were hydrolyzed with cholylglycine hydrolase (CGH) for 64 h at 37°C. Bile acid sulfates were then solvolyzed with trifluoroacetic acid in water-free tetrahydrofuran for 1 h at 45°C. Aliquots of samples before and after separation on Lipidex-DEAP were analyzed by electrospray mass-spectrometry (ES-MS) to obtain information about occurrence, successful separation, and hydrolysis of bile acid conjugates.

For the quantification of bile acids in individual serum and urine samples, a shortened purification of SepPak-C_{18} extracts on Lipidex-DEAP was used. After removal of cholesterol and other neutral steroids with 10 ml 72% ethanol, all bile acids were eluted with 10 ml 0.5 M ammonium sulfate, pH 9.6, in the same solvent. The elutes were diluted ten-fold with water, extracted with SepPak C_{18}, recovered with 5 ml methanol and evaporated under a stream of nitrogen. CGH hydrolysis for sixty-four hours followed by overnight solvolysis was performed. ES-MS was used to confirm complete cleavage of all conjugates.

Derivatization. For GLC and GC-MS analysis, bile acids were converted to volatile methyl ester trimethylsilyl (TMS) ether derivatives. Because the use of the carcinogenic diazomethane became restricted in the European Community, we tested alternative methods for methylation. The following bile acids were used as substrates: LCA, UDCA, CA (all from Sigma-Aldrich), enzymatically prepared 6α-GlcA-HDCA (17), 3α-Glc-CDCA and 7β-GlcNAc-isoUDCA (18), and chemically synthesized 3α-Glc-LCA, 3α-GlcNAc-UDCA, and 7β-GlcNAc-UDCA, kindly donated by Prof. Iida, Nihon University, Fukushima, Japan.

Between 1 and 50 μg of the reference compounds were dissolved in 100 μl methanol and methylated by adding a) freshly prepared diazomethane in 400 μl diethylether (11), b) 25 μl trimethylsilyldiazomethane in 400 μl toluene as described for the methylation of dansyl amino acids (19), and c) 1.1 μl concentrated hydrochloric acid in 70 μl dimethoxypropane (Merck). Mixtures a) and b) were incubated for 15 min at room temperature, mixture c) for 30 min at 55°C, evaporated under a stream of nitrogen and converted to TMS esters.

GC-MS analysis showed that the procedures a) and b) yielded exactly the same derivatives with all compounds. Procedure c), however, was suitable for the derivatization of LCA, UDCA, and CA only. Glycosidic conjugates were completely cleaved by the acidic procedure c), and only the derivatives of unconjugated bile acids were seen. Thus, for the present study, all derivatives were prepared according to procedure b).

GLC and GC-MS. GLC conditions have previously been described (10, 11, 15). Methylester-TMS ether derivatives of solvolyzed and hydrolyzed compounds were separated isothermically at 280°C on a nonpolar-fused silica capillary columns coated with 100% cross-linked methyl silicone (HP-1, Hewlett-Packard, Wiesbaden, Germany) and on polar-fused silica capillary column with various amounts of vinyl and phenyl groups (Unicoat UC-1625, KSV Chemicals, Helsinki, Finland) for the separation of the derivatives of UDCA and isoUDCA. Bile acids were identified by comparisons with authentic compounds and

retention indices. They were quantified from peak areas as related to that given by the internal standard 5α-cholestane, corrected for peak response factors for derivatized mono-, di-, and trihydroxy bile acids.

For GC-MS, derivatives were automatically injected in 1 μl hexane at 180°C in splitless mode. The temperature was taken to 220°C at 20°C/min and then to 315°C at 4°C/min. Both a nonpolar 100% methyl silicone column (DB-1, J & W Scientific, Folsom, CA) and a polar methyl silicone column containing 5% phenyl (DB-5, J & W) were used.

Glycosidic bile conjugates (Glc, GlcA, and GlcNAc) were, after CGH hydrolysis in case of amidated glycosides, analyzed as methylester TMS ether derivatives on the nonpolar columns. For the elution of these compounds, GLC temperature was taken to at 300°C. They were identified and quantified in relation to enzymatically (17, 18) or chemically synthesized reference compounds, the latter kindly donated by Prof. Iida. The retention indices for the derivatized glycosidic conjugates of UDCA and isoUDCA relative to *n*-alkanes with 40, 44, and 46 carbon atoms, respectively, were 4298 (Glc-UDCA), 4316 (GlcA-UDCA), 4389 (GlcNAc-UDCA), and 4433 (GlcNAc-isoUDCA).

Instruments. GLC was performed on a Hewlett-Packard HP 6890 ChemStation® instrument. For GC-MS, a HP 2890 GC instrument connected to a Nermaq R10-10H quadrupol mass spectrometer with an electron-impact ion source was used (70 eV bombardment, scan range *m/z* 70–700). ES-MS was performed on a Micromass Quattro 1 (Micromass, Manchester, UK) mass spectrometer. Samples in 50% aqueous methanol were injected into the ion source in a stream of the same solvents at a flow of 10 μl/min. Data were collected for 2 min with 10 sec per scan between *m/z* 200–800. The instrument calibration was used. ES-MS was routinely performed with aliquots of native and hydrolyzed fractions from Lipidex-DEAP to verify separation and hydrolysis of bile acid conjugates. For this purpose, ES-MS has widely replaced fast-atom bombardment ionization mass-spectrometry (FAB-MS) in our laboratory. The results and their interpretations are equivalent to those previously described for FAB-MS (10, 11).

Statistics. Data are reported as means ± SD (standard deviation). A two-tailed Student's *t*-test for paired data was used for analysis; $P < 0.05$ was considered statistically significant.

RESULTS

Effect of agents on symptoms

IsoUDCA was well tolerated by all patients. The scores for fatigue, at entry 1–2 in 4 patients and 7 in 1 patient, did not go up or down for more than one point during the whole study period. The scores for pruritus were 1–2 in 4 patients at entry and remained unchanged. One male patient reported an itching score of 3 at entry. Pruritus deteriorated to a peak score of 7 during the fifth study period and improved gradually to 4 at the end of the study.

Liver tests

The male patient (No. 4) with deteriorating itching developed jaundice with an increase in bilirubin that started

TABLE 1. Serum bilirubin and serum and urine bile acids measured at the end of the indicated study periods

Patient	UDCA 1 g/d	Wash-out	IsoUDCA 0.5 g/d	IsoUDCA 0.75 g/d	UDCA 0.75 g/d	UDCA 1 g/d	UDCA 1 g/d
Serum bilirubin, *μmol/l* (normal, <26 μmol/l)							
1	9	11	8	10	8	6	11
2	12	13	18	16	9	12	13
3	16	20	24	19	25	18	18
4	29	48	90	113	132	100	69
5	52	64	62	62	65	61	66
Mean	23.6	31.2	40.4	44.0	47.8	39.4	35.4
SD	2.4	19.8	28.5	34.8	40.6	32.9	25.7
Median	12.0	20.0	24.0	19.0	25.0	18.0	18.0
Serum bile acids, *μmol/l* (normal, <10 μmol/l)							
1	47	33	50	28	104	66	84
2	30	125	102	45	87	87	77
3	83	69	27	76	106	157	116
4	130	172	219	366	357	366	186
5	257	141	152	316	327	349	344
Mean	109.4	109.4	110.0	166.2	196.2	205.0	161.4
SD	67.3	45.6	60.4	139.8	116.6	122.0	82.9
Median	83.0	125.0	102.0	76.0	106.0	157.0	116.0
Urine bile acids, *μmol/24 h* (normal, <10 μmol/24 h)							
1		65	160	250	175	190	
2		190	225	510	205	295	
3		55	275	575	215	105	
4		250	840	830	400	330	
5		260	1,260	780	770	665	
Mean		164.0	552.0	589.0	353.0	317.0	
SD		83.2	398.4	172.8	185.6	144.4	
Median		190.0	275.0	575.0	215.0	295.0	

Upper limits of normal for bile acids in serum and urine from (10, 15).

during the wash-out period already and peaked at 132 μmol/l, 12 weeks after wash-out, during 0.75 g/d UDCA. Bilirubin was 59 ± 7 μmol/l during the whole study period in the other male patient (No. 5) and normal (i.e., <26 μmol/l) in the 3 female patients (**Table 1**).

At the end of the wash-out period, all patients showed a marked increase in ALT ($P < 0.05$), AST, γ-GT ($P < 0.05$), and ALP ($P < 0.05$). **Figure 1** shows that this increase was completely reverted in all patients at the end of the first treatment period with 0.5 g/d isoUDCA. The percent reduction in ALT, AST, γ-GT, and ALP compared with wash-out was 45.7 ± 29.0%, 30.0 ± 10.2%, 41.4 ± 15.0%, and 32.4 ± 12.3%, respectively. Liver function tests did not change significantly during the following four treatment periods with 0.75 g/d of isoUDCA and 0.75–1 g/d of UDCA. In fact, beside the above-mentioned increase in bilirubin, all 5 patients featured liver function tests not more than ±15% different from those before the study under a medication of 1 g/d UDCA. All other routine laboratory tests remained normal during the whole study period.

Bile acids in serum

Total bile acid concentration at the end of the wash-out period was 108 ± 46 μmol/l (mean ± SD). As shown in Table 1, this was not significantly different from total bile concentrations after administration of 0.5 and 0.75 g/d isoUDCA (110 ± 60 and 166 ± 140 μmol/l) and 0.75 or 1 g/d UDCA (between 196 ± 117 and 205 ± 122 μmol/l). The wide scatter of total bile concentrations reflects the changes in serum bilirubin (Table 1). The three female patients Nos. 1, 2, and 3 with normal bilirubin values throughout the study period showed only minor changes in total serum bile acids. Total bile acids increased almost 3-fold, however, in the male patient No. 4, who developed itching and jaundice. Approximately the same high levels were found in the other male patient No. 5, who had a constant bilirubin elevation around 60 μmol/l.

Figure 2 shows the total amounts of major serum bile acids. UDCA was the predominant bile acid before wash-out, consisting of 56.9 ± 18.6% and, at the end of the administration, of 0.75 g/d of isoUDCA and 0.75–1 g/d of UDCA (between 45.0 ± 4.1% and 60.0 ± 15.8%). CA was the predominant bile acid at the end of wash-out (58.5 ± 11.6%) and 0.5 g/d isoUDCA treatment periods (35.7 ± 10.9%). IsoUDCA was not found after wash-out and increased to 8.1 ± 7.4% and 6.2 ± 2.5% at the end of 0.5 and 0.75 g/d isoUDCA treatment periods. At the end of 0.75 and 1 g/d UDCA treatment periods, the relative amount of isoUDCA in serum ranged from 1.3% to 2.4% of total bile acids. The intermediate of isoUDCA and UDCA isomerization, 3-dehydro-UDCA, was found in trace amounts only (<0.1 μmol/l), both during UDCA and isoUDCA medications. Other bile acids constituted less than 2%.

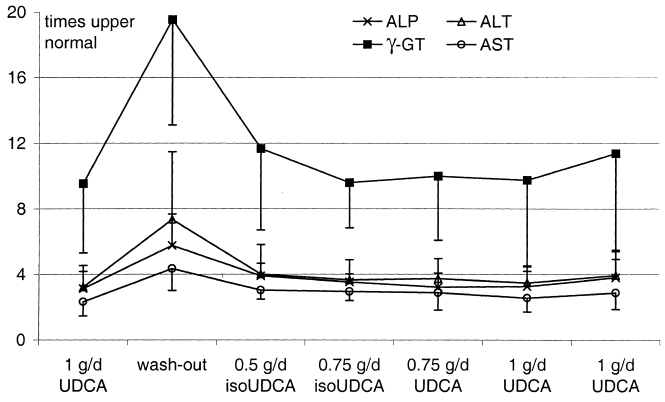

Fig. 1. Liver function tests. The values for γ-GT, alkaline phosphatase (ALP), and alanine transferase (ALT) are significantly higher after wash-out as compared with any treatment period.

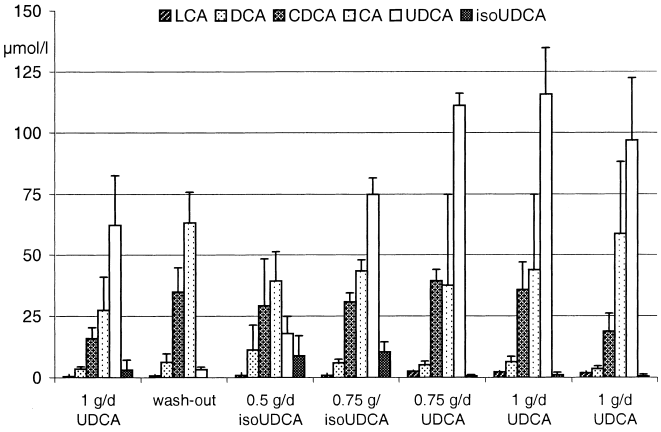

Fig. 2. Serum bile acids (mean ± SD).

Bile acids in urine

Total bile acid excretion rate during wash-out was 164 ± 83 μmol/24 h. This was significantly ($P < 0.05$) lower than after administration of 0.5 and 0.75 g/d isoUDCA (552 ± 398 and 589 ± 173 μmol/24 h) and 0.75 and 1 g/d UDCA (353 ± 186 and 317 ± 144 μmol/24 h). Total excretions rates during isoUDCA or UDCA medications did not differ significantly (Table 1). UDCA was the predominant bile acid at the end of isoUDCA and UDCA treatment periods (between 129 ± 81 and 207 ± 76 μmol/24 h), representing approximately 8–10% of administered bile acid (**Fig. 3**). UDCA and isoUDCA excretion rates after wash-out were 10 ± 3 and 11 ± 9 μmol/24 h. IsoUDCA excretion increased to 77 ± 49 and 102 ± 67 μmol/24 h after 0.5 and 0.75 g/d isoUDCA. This represents approximately 5–6% of the administered dose of isoUDCA. Substantial amounts of hydroxylation products of UDCA were found in urine, in particular, 1β, 3α,7β-, and 3α,7β,22-trihydroxy-5β-cholanoic acids after administration of UDCA. The intermediate of isoUDCA/UDCA conversion, 3-dehydro-UDCA, was found in amounts of 2–4%. These compounds are included in other bile acids, which constituted 5.5% of total bile acids after wash-out and between 3.3% and 16.7% after isoUDCA and UDCA treatment.

Conjugation patterns of isoUDCA and UDCA

ES-MS analysis of bile acid conjugates in pooled serum samples before group separation on Lipidex-DEAP gave

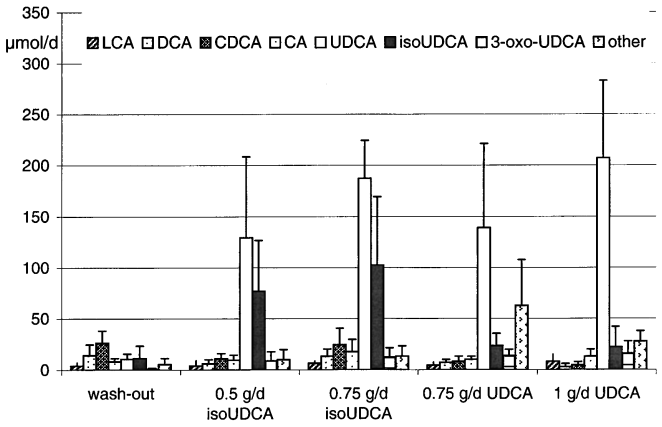

Fig. 3. Urine bile acids (mean ± SD).

the following results: After wash-out, deprotonated molecular ions were recorded at *m/z* 448, 464, 498, and 514 (percent intensity; 70:70:100:100), indicative for glycine- or taurine-conjugated di- or trihydroxy bile acids. The ion at *m/z* 448 was the most intense during treatment with isoUDCA or UDCA. Ions with intensities of 5–10% were at *m/z* 391, 471, 567, and 594, indicative for unconjugated or S-, GlcA-, and GlcNAc-conjugated diols and at *m/z* 528, 624, and 651, indicative for glycine- and S-, GlcA-, and GlcNAc-double conjugated diols. Low intense ions at *m/z* 674 and 701, indicative for taurine-amidated GlcA- and GlcNAc-double conjugated diols, were recorded as well. Almost exclusively amidated compounds were seen during the administration of UDCA. The spectrum from pooled serum after administration of 0.75 g/d isoUDCA is shown in **Fig. 4A**.

The ES-MS spectrum of pooled unseparated urine after wash-out was dominated by steroid hormone metabolites (at *m/z* 465, 495, 539, and 441, matching glucuronides of etiocholanolone, pregnanediol, tetrahydrocortisone, and tetrahydrocortisol, respectively). ES-MS analysis of pooled urine samples during isoUDCA and UDCA medications showed the same major ions as in serum, although in different intensities, as shown in Fig. 4B. After the administration of isoUDCA, ions at *m/z* 448 (diol-gly), 471 (diol-S), 528 (S-diol-gly), and 594, 651, and 701 (nonamidated, glycine-, or taurine-amidated GlcNAc-diol) emerged. Ions at *m/z* 471 and 594 were much less intense after UDCA medication when the spectra were dominated by the ions at *m/z* 448, 528, 624, 651, and 701 (percent of intensity: 100:70:20:50:30). A similar shift in intensities was seen in the spectra from pooled serum samples. Ions indicative for glucose conjugates were not seen.

GC-MS analysis of Lipidex-DEAP separated bile acid metabolites confirmed these conjugation patterns (**Fig. 5**): Serum isoUDCA was unconjugated (40–74%), conjugated with sulfate (9–26%), glucuronic acid (8–16%), or *N*-acetylglucosamine (9–22%). IsoUDCA was not amidated with glycine or taurine, in contrast to serum UDCA, of which the major part (75–81%) was amidated. Serum UDCA was also found as sulfate (8–14%), glucuronic acid (3–6%), or *N*-acetylglucosamine conjugate (4–7%). IsoUDCA was excreted in urine preferably as nonamidated *N*-acetylglucosamine (72–86%), whereas most of UDCA was found in the glycine-/taurine-amidated group as the double-conjugate GlcNAc-UDC-gly/tau (40–49%). Nonamidated GlcNAc-UDCA consisted 3–5% of total UDCA excretion. Amidated glucuronides and sulfates of UDCA consisted approximately 8% and 26%, respectively, of total UDCA excretion. Fig. 4B shows an intense deprotonated molecular ion at *m/z* 453 formally matching the sulfate of 3β-hydroxy Δ^5-cholenoic acid (S-3β-Δ^5). This peak was found with similar intensity in all ES-MS spectra from pooled urine samples. S-3β-Δ^5, however, consisted only 2% of total urinary bile acids after wash-out, and less than 0.5% during any treat-

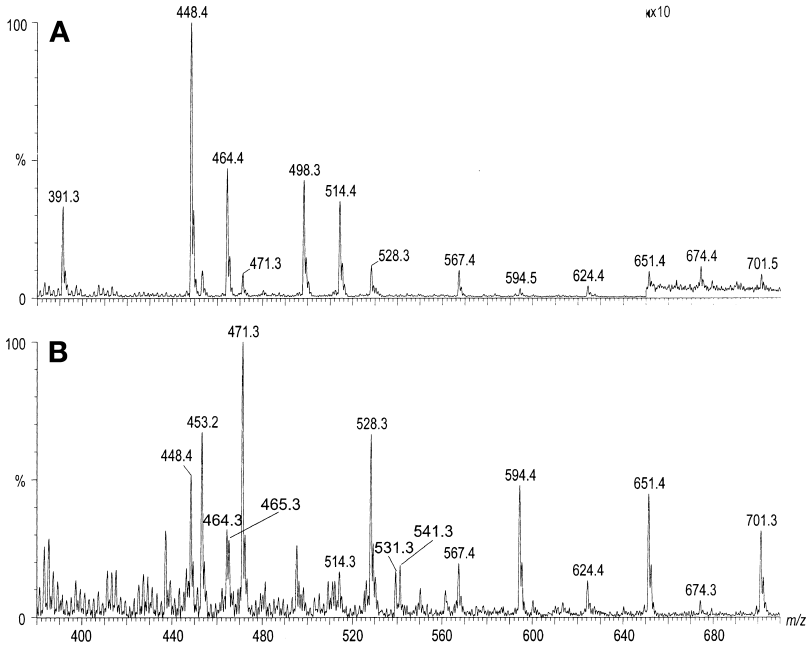

Fig. 4. Negative ion ES-MS of Sep-Pak C_{18} extracts of pooled serum (A) and urine (B) samples after administration of 0.75 g/d isoUDCA. *M/z* 594, 651, 701, GlcNAc-diol, GlcNAc-diol-gly, and GlcNAc-diol–tau; *m/z* 567, 624, 674, GlcA-diol, GlcA-diol-gly, and GlcA-diol-tau; *m/z* 471, 528, S-diol and S-diol-gly; *m/z* 407/391, 464/448, 514/498, triol/diol, triol/diol-gly, and triol/diol-tau. *M/z* 465, 495, 539, and 541, GlcA-conjugates of etiocholanolone, -pregnanediol, tetrahydrocortisone and tetrahydrocortisol; *m/z* 453 matching the sulfate of 3β-hydroxy Δ^5-cholenoic acid. ES-MS does not give quantitative information; sulfates give particularly intense ions.

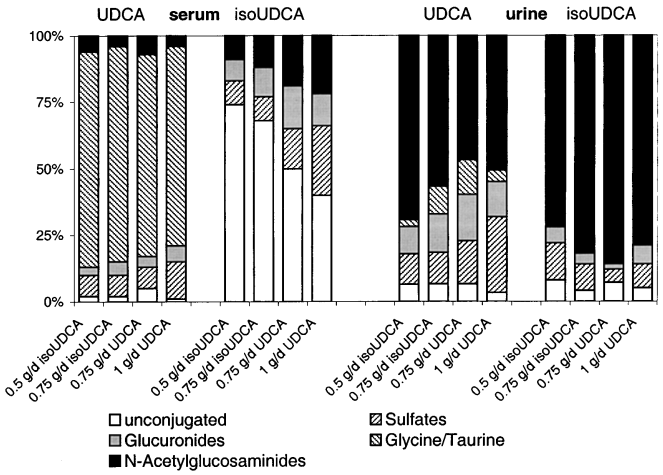

Fig. 5. Conjugation patterns of UDCA and isoUDCA. Determined from pooled serum and urine samples during 0.5 and 0.75 g/d isoUDCA, and during 0.75 and 1 g/d UDCA. Values for *N*-acetylglucosaminides, glucuronides, and sulfates are the sum for the respective nonamidated and amidated compounds.

ment period. Thus, the ion at *m/z* 453 may be derived from another urinary metabolite as well. Substantial qualitative or quantitative differences in serum or urine bile acid profiles depending on the clinical stage of PBC, i.e., normal or elevated serum bilirubin, were not observed.

DISCUSSION

The objective of this study was the metabolism and a possible pharmacodynamic effect of isoUDCA in comparison with UDCA in the same PBC patients. The main results are the following: 1) isoUDCA is almost completely converted to UDCA, and 2) isoUDCA has a positive impact of surrogate markers of cholestasis such as γ-GT and ALP. These effects may be caused by UDCA, which would give isoUDCA pro-drug characteristics.

We found that a 4-week treatment with 0.5 g/d isoUDCA was able to revert the elevation of ALT, AST, γ-GT, and ALP that occurred during the wash-out period in all patients. Increasing the dose of isoUDCA from 0.5 g/d to 0.75 g/d and then switching back to UDCA at 0.75–1 g/d did not further improve these parameters in the 5 patients studied.

IsoUDCA was well tolerated by all patients. Medication-related side effects were not reported. The increase in bilirubin and deterioration in itching in 1 patient started during wash-out and thus was interpreted as PBC-related. In fact, the same patient had had a similar episode two years earlier under UDCA medication. In long-term follow-up after finishing the study, he remained clinically stable with intermittent mild itching and bilirubin levels at approximately 50 μmol/l bilirubin.

Other hydrophilic bile acids have been tested for the treatment of PBC at doses of 0.75–0.9 g/d for 1 or 2 months, regimens considered sufficient for reaching steady-state conditions. The study with the 3α,7β,12α-trihydroxy bile acid ursocholic acid (UCA) and the even more hydrophilic tauroursocholic acid did not show any improvement of liver biochemistry, as compared with UDCA (20). The reason for the failure of UCA is, at least in part, the formation of DCA by 7β-dehydroxylation of UCA (20). The taurine conjugate of UDCA, TUDCA, seems to be better resorbed than UDCA but has otherwise the same effects on biochemical parameters as UDCA, at least in the short-time trial (21). The better resorption of TUDCA may be relevant at the start of the treatment. In long-term treatment, however, most of the UDCA pool will contain glycine- or taurine-conjugates that are preserved during the enterohepatic circulation. Liver tissue UDCA concentrations or enrichment seem to be the same in patients administered UDCA or TUDCA (22). The study with UCA (20) shows that hydrophilicity alone is not sufficient for a pharmaceutically active bile acid since this feature may change during metabolism. Thus, hydrophilicity remains a badly defined criterion (21). Rather, pharmacological effects seem to be related to the prevalence of an intact 7β-hydroxy group as in UDCA or isoUDCA, whatever the specific effects in the liver cell are.

In the patients studied, isoUDCA was only a minor compound during administration of UDCA. The relative amount of 1.3–2.5% of total bile acids is in the range reported by others for patients with PBC during treatment with UDCA (6–8). The relative amount of isoUDCA during administration of isoUDCA reached 6–8% of total serum bile acids. This is substantially lower than the 24.7% isoUDCA enrichment in serum that we determined in healthy subjects after 7 days administration of 0.75 g/d isoUDCA (15). A related finding in healthy subjects was reported for serum isoUDCA during administration of UDCA, where isoUDCA consisted 12.8% of total bile acids (8). The lower enrichment of isoUDCA in patients with PBC is probably due to impaired biliary secretion of bile acids in cholestatic liver disease.

Approximately 15–20% of the administered dose of isoUDCA and UDCA (including UDCA formed from isoUDCA and minor metabolites), respectively, were excreted in urine. Thus, assuming steady-state conditions at the end of the respective treatment periods, more than 80% of the drug was eliminated in feces.

Total serum bile acids and, in particular, serum UDCA, did not differ significantly after administration of 0.75 g/d of isoUDCA or 0.75–1 g/d of UDCA. The bile acid profiles were in the range of those reported for patients with PBC during long-term treatment with UDCA (6, 7, 23, 24). This indicates a good intestinal absorption and an efficient hepatic uptake of isoUDCA, which is then converted to UDCA, both in the PBC patients studied and in healthy humans (15). This is also the case in rats, as determined by tracer experiments (25, 26). We recently defined the cytosolic liver enzyme catalyzing the first step in the epimerization of the hydroxyl group at C-3. It is alcohol dehydrogenase class I γγ (27). The abundance of this enzyme in human liver clearly facilitates the conversion despite of the rather low turnover rates (27). UDCA will then be excreted into bile as a glycine- (80%) or taurine- (20%) conjugate (20, 28, 29). Consistent with a complete

hepatic conversion of isoUDCA to UDCA, glycine or taurine conjugates of isoUDCA were not found in serum (this study; 8, 9). Whether amidation precedes or follows epimerization of isoUDCA remains speculative.

However, part of isoUDCA and UDCA, the latter mostly enterohepatic recirculated UDC-gly/tau, will not pass the liver cell from the sinusoid to the canaliculus. This is concluded from the following: Neither isoUDCA nor glycosidic conjugates of bile acids have been described in bile of patients during UDCA medication (8, 9, 21, 29). Furthermore, our FAB- or ES-MS screening of bile samples obtained endoscopically from 8 patients with cholestasis (3 patients with sclerosing cholangitis, 5 with bile duct stones) with or without UDCA medication did not give evidence for the biliary excretion of GlcA or GlcNAc conjugates (data not shown). However, we find these compounds in serum. Thus, although the activity of the conjugating enzymes is higher in kidney than in liver (11, 17), part of the glycosidic conjugates excreted in urine must be of hepatic origin, in particular, glycosidic conjugates of nonamidated isoUDCA and the double-conjugate GlcNAc-UDC-gly. One can speculate that these highly polar compounds after glycosidic conjugation are excreted from the liver cell at the sinusoidal (basolateral) membrane followed by rapid renal clearance thus preventing deterioration of cholestasis. It is noteworthy that GlcA and GlcNAc conjugates formed in the renal tubular cell would have to exit via the basolateral membrane as well.

Membrane proteins able to transport glycosidic compounds need to be defined as well as the importance of the specific metabolism of UDCA and isoUDCA, i.e., 7β-conjugation with *N*-acetylglucosamine. We found that 7β-*N*-acetylglucosamine conjugates are the major metabolites of isoUDCA and UDCA in urine of patients with PBC, confirming our previous studies (10, 15) and those of others (12) in healthy subjects and patients with PBC (13), cystic fibrosis (14), and cholestasis of pregnancy (16). FAB-MS (10, 14–16) or ES-MS (this study) was, with the exception of one study (21), particularly useful for the detection of GlcNAc-UDC-gly in urine. ES-MS monitoring of hydrolysis procedures also helped to manage an analytical problem that recently has been reported by others (14, 16). ES-MS spectra of fractions from Lipidex-DEAP confirmed the predominant ion during bile acid medication to be at *m/z* 651, consistent with a GlcNAc-diol-gly. ES-MS then revealed that the hydrolysis of this compound with CGH was substantially difficult, whereas sulfates and glucuronides were easily deamidated by the conventional overnight treatment. Cleavage of the amide bond in GlcNAc-UDC-gly was obtained after prolonged CGH hydrolysis only. This problem was not observed in our previous study (10) and seems to be related to different CGH preparations. Monitoring of CGH hydrolysis by the loss of the [^{14}C]glycine moiety from the [^{14}C]GCA label was not sufficient.

FAB- or ES-MS, however, does not give quantitative information on these conjugates. For the quantification by GLC or GC-MS, hydrolysis and derivatization must be performed that bears a substantial risk of systematic underestimation using common routines for these reactions. First, both strong acidic (this study) and alkaline conditions (12) cleave the sugar bond. Second, the amide bond may not be hydrolyzed sufficiently with currently available CGH batches. We now have provided hints to resolve these analytical problems.

In summary, we found isoUDCA to become epimerized to UDCA and to improve liver biochemistry in patients with PBC, which indicates pro-drug characteristics. Our data on the metabolism and clinical effects of isoUDCA may help to improve the efficacy of bile acid treatment for PBC (30). We suggest further investigations be conducted on the importance of an intact 7β-hydroxy group in therapeutically used bile acids.

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