Journal of Hepatology 1997; **27**: 1029–1040
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Munksgaard · Copenhagen

Journal of Hepatology
ISSN 0168-8278

Effects of ursodeoxycholic acid on conjugated bile acids and progesterone metabolites in serum and urine of patients with intrahepatic cholestasis of pregnancy

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Background/Aims and Methods: **The mechanism(s) behind the effects of ursodeoxycholic acid on serum steroid sulphate profiles in patients with intrahepatic cholestasis of pregnancy is not clear. Conjugated progesterone metabolites and bile acids have therefore been analysed in serum and urine of patients with intrahepatic cholestasis of pregnancy before and during treatment with ursodeoxycholic acid using chromatographic and mass spectrometric methods.**

Results: **The concentration of glycine-/taurine-conjugated bile acids decreased from 8.9±3 μmol/l (mean± SEM) before treatment to 1.8±0.6 μmol/l during treatment with ursodeoxycholic acid. The total bile acid excretion in urine decreased from 56±14 to 32±5.6 μmol/g creatinine. The proportion of cholic acid in serum and urine, and of 1β-, 2β- and 6α-hydroxylated cholic acids in urine decreased markedly during ursodeoxycholic acid while the percentages of 3α,12α-dihydroxy-3-oxo-4-cholenoic acid and chenodeoxycholic acid were unchanged. The levels in serum and excretion in urine of sulphated steroids decreased during ursodeoxycholic acid, by 45–49% for disulphates and 33–35% for monosulphates. The ratios of 3α- to 3β-hydroxysteroid disulphates were lowered by ursodeoxycholic acid from 1.1 (mean) to 0.68 in serum, and from 1.2 to 0.70 in urine. The corresponding ratios for monosulphates before and during ursodeoxycholic acid were 6.9 and 4.5, respectively, in serum, and 21 and 5.2, respectively, in urine. The major monosulphates in urine, dominated by 5α-pregnane-3α, 20α-diol, were also conjugated with *N*-acetylglucosamine. The excretion of these double conjugates decreased from 27±8.4 to 15±5.3 μmol/g creatinine during ursodeoxycholic acid. In contrast to sulphated steroids, the concentrations of glucuronides were unchanged in serum and their excretion in urine tended to increase during ursodeoxycholic acid. The metabolism of ursodeoxycholic acid was similar to that described in nonpregnant subjects. In addition to metabolites hydroxylated in the 1β-, 5β-, 6α/β and 22-positions, a 4-hydroxy-ursodeoxycholic acid was tentatively identified. This occurred predominantly as a double conjugate with glycine/taurine and glucuronic acid, as did other 4-hydroxylated bile acids of probable foetal origin.**

Conclusions: **The results are compatible with the contention that ursodeoxycholic acid stimulates the biliary excretion of sulphated progesterone metabolites, particularly those with a 3α-hydroxy-5α(H) configuration and disulphates. The effect(s) appears to be independent of the stimulation of bile acid secretion. An effect of ursodeoxycholic acid on the reductive metabolism of progesterone cannot be excluded.**

Key words: **Bile acids; Chromatography; Glucuronides; Intrahepatic cholestasis of pregnancy; Mass spectrometry; *N*-acetylglucosaminides; Progesterone metabolites; Serum; Sulphates; Urine; Ursodeoxycholic acid.**

THE LEVELS and patterns of conjugated bile acids and steroids in serum and urine are markedly different from normal in patients with intrahepatic cholestasis of pregnancy (ICP) (1–9). Oral administration of ursodeoxycholic acid (UDCA) improves the clinical condition (10) and reduces the concentrations of bile acids and sulphated steroids in serum towards normal values (11). The mechanism(s) behind the effects on the steroids is not clear. In order to gain further information on this point, we have performed comprehensive analy-

Received 9 May; revised 1 July; accepted 2 July 1997

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ses of steroids and bile acids in both serum and urine of patients with ICP before and during oral administration of UDCA. The results described in this paper support the contention that UDCA or some of its metabolites improves the biliary secretion of sulphated steroids with a 3α-hydroxy-5α(H) configuration.

Materials and Methods

Patients

The study was performed on seven patients with early onset of pruritus, five with ICP and two with uncertain diagnosis. They were hospitalised and treated with UDCA, 1 g per day (12–16 mg · kg^{-1} · day^{-1}) until delivery. The clinical observations at the time of collection of serum and urine samples are given in Table 1. Patients 1 to 5 were part of a double-blind placebo-controlled study of the clinical effects of UDCA in this disease (12), and they fulfilled several pre-established criteria: pruritus had appeared between the 21st and 32nd week of pregnancy without any other possible explanation besides cholestasis of pregnancy; simultaneously, serum alanine and/or aspartate aminotransferase were above the upper limits observed in normal pregnancies, and serum total bile salts were also abnormally high, estimated by an enzymatic method using a commercially available kit (Merckotest®). Patients 6 and 7 also had pruritus of pregnancy of early onset, with similar severity, but in them serum levels of aminotransferases were normal; they received an identical treatment with UDCA, but in an open fashion.

In all these patients, the remaining pruritus disappeared and liver function tests normalised during the week following delivery.

TABLE 1

Clinical data and liver function tests in the seven patients before and during the administration of UDCA

Parameter[a]	Patients[b]						
	1	2	3	4	5	6	7
Gestational weeks	30	34	34	35	34	27	35
Days of treatment	14	21	21	28	21	14	14
Pruritus severity (0–4)	3	3	3	3	3	3	3
Treated[c]	1	0	0	1	1	2	3
Bilirubin, total (mg/dl;<1.1)	0.9	1.3	0.6	0.6	0.7	0.2	0.3
Treated[c]	0.6	0.7	0.2	0.2	0.2	0.5	0.1
S-ALAT (U/l;<40)	112	278	205	120	104	7	14
Treated[c]	24	66	16	19	15	7	8
S-ALP (U/l;<117)	190	167	207	271	183	59	141
Treated[c]	170	179	120	293	131	60	146
Cholesterol, total (mg/dl;<200)	186	268	229	352	280	268	265
Treated[c]	132	262	237	362	205	262	234

[a] S-ALAT: serum alanine aminotransferase; S-ALP: serum total alkaline phosphatases. In parentheses, units of measurement followed by the upper limit acceptable in fasting blood samples obtained from nonpregnant healthy women in the comparable age range.

[b] Patients nos. 1–5 with ICP participated in the double-blind, placebo-controlled study; patients nos. 6 and 7 with pruritus were given UDCA openly.

[c] Values obtained after treatment with UDCA or placebo for the period indicated.

Blood and urine samples

Fasting morning serum samples and 12-h overnight urine collection samples were obtained on the same day, before and after 2 to 4 weeks of UDCA administration. They were frozen immediately at −20°C until analysis.

Informed consent was obtained from all the patients and permission was granted from their attending obstetricians and the local ethical committee.

Analytical procedures

A detailed description of the analytical method has been published (13). A solid-phase method was used to extract bile acids and steroids from 10 ml urine and 1–4 ml serum. Unconjugated bile acids and different groups of conjugated bile acids and steroids were then separated by anion exchange chromatography. The groups of conjugated compounds were qualitatively analysed by fast bombardment mass spectrometry (FABMS). After hydrolysis and solvolysis, the liberated bile acids and neutral steroids were purified by passage through the anion exchanger. A separate procedure was employed to analyse glucuronides of aminoacyl amidated bile acids (GlcA-G/T) in the urine of patient nos. 1 and 2 during the treatment with UDCA. The urine extract was separated on the same anion exchange column (13). However, elution was performed first with 0.4 M formic acid in 95% methanol and then with 0.5 M acetic acid-potassium hydroxide in 72% aqueous methanol, apparent pH 10, to obtain the bile acids doubly conjugated with glucuronic acid and amino acid. Following neutralisation and solid-phase extraction, the double conjugates were hydrolysed with cholylglycine hydrolase (13). The hydrolysate was extracted and glucuronides were isolated by ion exchange chromatography. After washing with 0.1M acetic acid in 95% methanol, the glucuronidated bile acids were eluted with 0.4 M formic acid in 95% methanol. They were then hydrolysed with *Helix pomatia* digestive juice (13). The liberated unconjugated bile acids were isolated by ion exchange chromatography (13).

The steroids and bile acids were analysed by gas-liquid chromatography, following derivatisation (13). Bile acids were methylated and the methyl esters and the steroids were converted into trimethylsilyl ethers (13). *n*-Dotriacontane (C_{32}) and *n*-hexatriacontane

(C_{36}) were added as internal standards prior to derivatisation. A GLC temperature programme was used to permit detection of compounds with retention indices (RI) between 2300 and 4500, i.e. covering a range from C_{18} steroids to sugar conjugates of C_{21} steroids. The method of derivatisation excluded several glucocorticoid metabolites since emphasis was put on the analysis of progesterone metabolites. The identification of steroids and bile acids was based on mass spectra and retention times, as compared with those of reference compounds or data from the literature. Quantification was based on comparison of peak areas given by the individual compounds with the peak area given by the internal standards. Bile acids with a 7β-hydroxy group are known to occur in forms conjugated with *N*-acetylglucosamine. The derivatives of these conjugates have been previously analysed after removal of the glycine/taurine moiety. However, the batches of cholylglycine hydrolase used in the present study were unable to hydrolyse these double conjugates. Therefore, the occurrence of *N*-acetylglucosaminides of UDCA in urine during treatment was evaluated from the FAB spectra of the urine extracts and the fractions from the anion exchanger. The relative intensities of ions at *m/z* 651 and 701, corresponding to *N*-acetylglucosaminides of glycine- and taurine-conjugated dihydroxycholanoic acids, were monitored.

TABLE 2

The effects of treatment with UDCA on the bile acids in five patients (nos. 1–5) with ICP. The values include potential metabolites of UDCA but not UDCA itself or its conjugates with *N*-acetylglucosamine

Mode of conjugation[a]	Before UDCA mean±SEM		During UDCA mean±SEM		Change[b] %
Serum	μmol/l		μmol/l		
G/T	8.9±3		1.8±0.6		−80 (4)
Urine	μmol[c]	%[d]	μmol[c]	%[d]	
U	3.6±1.4	6.4	1.8±0.3	5.6	−50 (3)
G/T	22±5.4	39	9.7±2.0	30	−56 (5)
S,S-G/T	28±7.3	50	11±3.7	34	−61 (5)
GlcA	1.8±1.0	3.2	4.1±1.4	13	+128
S-GlcA	0.21±0.1	0.4	0.13±0.1	0.4	−38 (3)
GlcA-G/T[e]	n.d.		6.6; 3.2	15	
Total (urine)	56±14		32±5.6		

[a] U: unconjugated; G/T: aminoacyl amidated; S,S-G/T: sulphated and both sulphated and aminoacyl amidated; GlcA: glucuronidated; S-GlcA: both sulphated and glucuronidated; GlcA-G/T: both glucuronidated and aminoacyl amidated. n.d.: not determined.

[b] Number of patients showing a decrease given in parentheses.

[c] μmol/g creatinine.

[d] Percentage of the total urinary bile acids analysed, excluding UDCA.

[e] The data are from patients nos. 1 and 2.

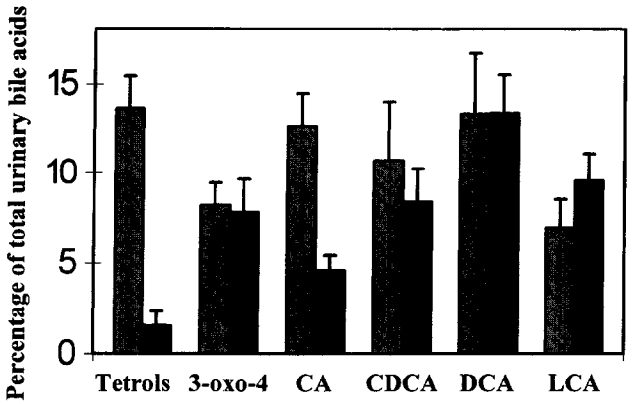

Fig. 1. Effects of UDCA administration on major urinary bile acids in the five patients (nos. 1–5) with ICP. The results are expressed as mean±SEM; the hatched bars represent data before treatment, the filled bars data during treatment. Tetrols: the sum of tetrahydroxy bile acids; 3-oxo-4: 3α,12α-dihydroxy-3-oxo-4-cholenoic acid; CA: cholic; CDCA: chenodeoxycholic; DCA: deoxycholic; LCA: lithocholic acids.

Results

Bile acids

The levels of conjugated bile acids in *serum* decreased in patients nos. 2–4 during treatment with UDCA. Patients nos. 1 and 5 had normal concentrations of endogenous bile acids in serum: 2.1 and 2.9 μmol/l, respectively, before, and 4.1 and 2 μmol, respectively, during the administration of UDCA. UDCA was present in the serum of all patients during the treatment, at concentrations of 0.1–4.8 μmol/l. The mean ratios of cholic:chenodeoxycholic:deoxycholic acids in patients nos. 2–5 were 2.6:1:0.2 before treatment, as compared to 1:1:0.9 in normal pregnancy (1), and 4:1:0.1 in more advanced ICP (1). These ratios were 0.7:1:0.2 in patient no. 1. During administration of UDCA, the mean ratios were 0.5:1:0.3 in the five patients.

Five groups of bile acids in *urine* were analysed, differing by the mode of conjugation: unconjugated (U), aminoacyl amidated (G/T), glucuronidated (GlcA), sulphated and both sulphated and aminoacyl amidated (S,S-G/T), and both glucuronidated and sulphated (S-GlcA). In addition, bile acids doubly conjugated with glucuronic acid and amino acid (GlcA-G/T) were analysed in patients nos. 1 and 2 during treatment with UDCA, when FAB spectra indicated the appearance of substantial amounts of such conjugates.

A total of 45 bile acids was found in the urine of the patients before administration of UDCA. They were identified or partly characterised as described in previous studies (13,15). The mean urinary excretion of total bile acids in each group of conjugates in the five

patients is shown in Table 2 (UDCA is not included in these values). As seen in this Table, bile acids in the S,S-G/T and G/T groups constituted the largest fraction. Their percentage decreased during the administration of UDCA when the total bile acid excretion decreased (except in patient no.1 whose total bile acid excretion (but not the composition) in urine remained the same before and during UDCA).

The fraction of glucuronidated bile acids, containing mainly 6-hydroxylated bile acids before administration of UDCA, increased in all patients during administration of UDCA. This was due to the appearance of putative metabolites of UDCA in this fraction. Peaks of bile acids conjugated with both glucuronic acid and amino acid also appeared in the FAB spectra of urine collected during treatment with UDCA. This group of conjugates was therefore analysed by GC/MS of samples from two patients (nos. 1 and 2) during treatment. It constituted a mean of 15% of the total bile acids in these patients (UDCA not included).

The relative composition of bile acids in urine changed towards normal during treatment with UDCA (Fig. 1). The proportions of cholic acid and particularly of its 1β, 2β, 5β- (tentative identification) and 6α-hydroxylated products were markedly reduced in all five patients during administration of UDCA. Eight additional bile acids (not including conjugates with *N*-acetylglucosamine) appeared, which were assumed to be metabolites of UDCA (see below). The percentage of 7α, 12α-dihydroxy-3-oxo-4-cholenoic acid remained essentially unchanged, as did those of the common bile acids in the sulphated fraction, chenodeoxycholic, deoxycholic and lithocholic acids. However, the absolute excretion of these acids decreased, contributing to the marked reduction of urinary bile acid secretion seen in Table 2. The excretion of lithocholic acid in urine decreased from 4.6±1.7 μmol/g creatinine (mean±SEM) before UDCA to 2.8±0.8 during UDCA. The three 3-hydroxyandrostane-17β-carboxylic acid isomers previously identified in urine of patients with ICP (15) were only found in trace amounts in the present patients; probably these patients were in an earlier stage of ICP.

TABLE 3

The effects of treatment with UDCA upon the progesterone metabolites in five patients (nos. 1–5) with ICP

Mode of conjugation	Before UDCA mean±SEM		During UDCA mean±SEM		Change[a] %
Serum	μmol/l	%[b]	μmol/l	%[b]	
Monosulphates	23±8	50	15±7	51	−35 (5)
Disulphates	20±8	43	11±5	38	−45 (5)
Glucuronides	2.8±0.9	6	3±0.7	10	+7
Total	46±15		29±11		
Urine	μmol[c]	%	μmol[c]	%	
Monosulphates[d]	39±10	13	26±6	9	−33 (5)
Disulphates[d]	35±7	12	18±2	6	−49 (5)
Glucuronides[d]	224±41	75	249±33	85	+11
Total	298±38		293±34		

[a] Number of patients showing a decrease given in parentheses.
[b] Percentage of all the steroids analysed.
[c] μmol/g creatinine.
[d] The predominant part but not all the steroids analysed are progesterone metabolites.

TABLE 4

Ratios (mean (range)) of sulphated 3α- to 3β-hydroxysteroids in serum and urine of five patients (nos. 1–5) with ICP before and during UDCA

Steroids	ICP		Healthy[a]
	Before UDCA	During UDCA	
Disulphates			
In serum	1.1 (0.83–1.7)	0.68 (0.32–1.0)	0.5 (0.3–0.7)
In urine	1.2 (0.91–1.7)[b]	0.70 (0.42–1.3)[b]	0.9 (1.9–2.1)[b]
Monosulphates			
In serum	6.9 (3.5–10)	4.5 (2.3–7.6)	2.3 (2.1–2.6)
In urine	21 (8.9–33)[c]	5.2 (3.4–6.3)[c]	2.0 (1.9–2.1)[c]

[a] The values are from three healthy pregnant women at 36–38 weeks of gestation in a former study (15).
[b] The sulphates of the following steroids were included in the calculations: 5α(β)-pregnane-3α(β), 20α-diols, 5α-pregnane-3α(β), 21-diol-20-one, and 5α-pregnane-3α, 20α, 21-triol.
[c] All sulphated *N*-acetylglucosaminides were included in the calculation.

Progesterone metabolites

Three groups of progesterone metabolites in serum and urine were analysed, differing with respect to their mode of conjugation, i.e., monosulphates, disulphates and glucuronides. Eleven progesterone metabolites in serum and 44 steroids in urine, not all being metabolites of progesterone, were analysed (15). Sulphated steroids predominated in serum and glucuronides in urine, both before and during administration of UDCA. The effects of UDCA are shown in Tables 3 and 4 and Fig. 2. In general, the levels and patterns of sulphated steroids changed towards normal in patients nos. 1–5. The concentrations in serum and excretion in urine of total mono- and disulphates decreased to similar extents, the decrease of disulphates being slightly larger, 45–49% than that of monosulphates, 33–35%. In contrast, the levels of glucuronides in serum were not affected and their excretion in urine tended to increase (Table 3).

Confirming the results of a previous study (11), the pattern of sulphated steroids in plasma changed in a characteristic way during administration of UDCA. Thus, the levels of mono- and disulphated 5α/β-pregnane-3α, 20α-diols and 5α-pregnane-3α, 20α, 21-triol decreased markedly, while 5α-pregnane-3β, 20α-diol

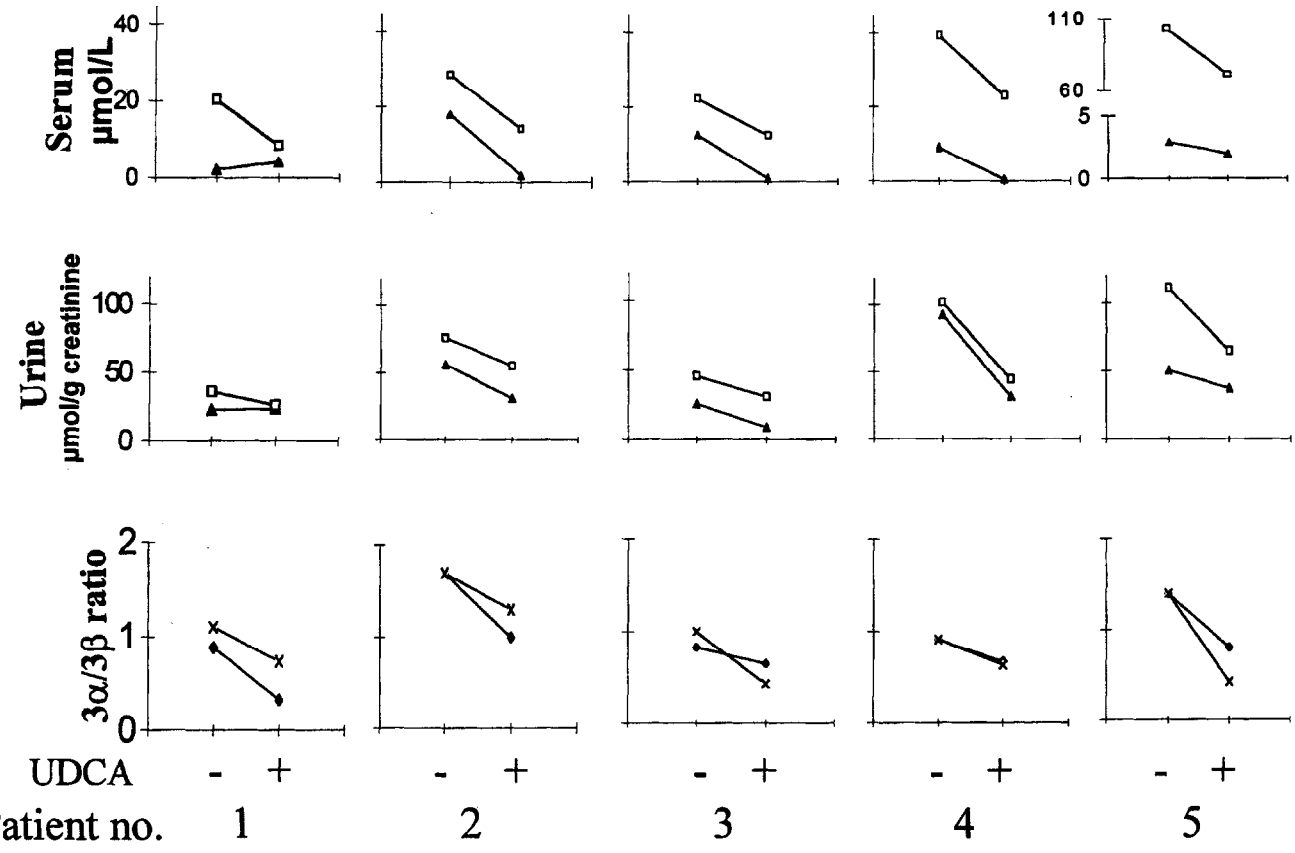

Fig. 2. Total concentration in serum (upper panels) and urinary excretion (middle panels) of sulphated steroids and bile acids, and ratio of 3α- to 3β-hydroxysteroid disulphates (lower panels) in patients nos. 1–5 before (−) and during (+) administration of UDCA. Open squares, sulphated steroids; filled triangles, bile acids; crosses, disulphated steroids in urine; filled diamonds, disulphated steroids in serum.

changed less or not at all. Pregnanolones were also affected to a smaller extent.

In urine, the major sulphated progesterone metabolites are double conjugates with sulfuric acid and *N*-acetylglucosamine, 5α-pregnane-3α, 20α-diol being the predominant steroid in this fraction both in normal pregnancy and in ICP (15,16). The urinary excretion of these double conjugates decreased markedly in all patients during administration of UDCA, the mean excretion being 27±8.4 and 15±5.3 µmol/g creatinine, respectively, before and during UDCA.

Two steroids in the monosulphate fraction from urine changed in opposite directions in all five patients. 5-Pregnene-3β, 15β, 17α, 20α-tetrol (17,18), which is not a progesterone metabolite, increased, while that of 5α-pregnane-3β, 16α, 20β, 21-tetrol, which might be a progesterone metabolite, decreased during administration of UDCA.

The predominant steroids in the disulphate fraction from urine were 5α/β-pregnane-3α/β, 20α-diols, 5α-pregnane-3α/β,21-diol-20-ones and 5α-pregnane-3α,20α,21-triol. During treatment with UDCA, the isomers with 3α-hydroxy-5α/β(H) configuration decreased more than those with a 3β-hydroxy-5α(H) configuration, 64% and 31%, respectively. The latter showed a slight increase in two individuals, reflecting the progress of the pregnancy (20).

As a result of the isomer-selective changes, the 3α-/3β-hydroxysteroid ratios, calculated for the major mono- and disulphated progesterone metabolites mentioned above, decreased during administration of UDCA (Table 4). The ratios for disulphated steroids were similar in serum and urine both before and during UDCA, while the ratio for monosulphates in urine, predominantly conjugated also with *N*-acetylglucosamine, was much higher before and during UDCA than that for monosulphates in serum.

The glucuronidated steroids in urine were dominated by the expected progesterone metabolites, 5α/β-pregnane-3α,20α-diol, 3α-hydroxy-5β/α-pregnan-20-one, 3,16-dihydroxy-5β/α-pregnan-20-ones and 5α-pregnane-3α,16α,20α-triol, and the oestrogen estriol. Preg-

nanolone and pregnanediol isomers with a 3β, 5α(H) configuration and 5α-pregnane-3α,20α,21-triol were not present in glucuronidated form. The patterns of progesterone metabolites were similar before and during administration of UDCA, and ratios between the predominant 5β(H) steroids and 5α(H) steroids did not change.

Estriol constituted 13±2.8% (mean±SEM) and 15±0.8%, respectively, of the total glucuronidated steroids analysed before and during treatment with UDCA. Its excretion was 30±5.4 μmol/g creatinine before treatment and 37±7.1 during treatment. While this difference is not statistically significant, a slight increase in the estriol excretion during treatment was observed in all patients. This may reflect the progress of the pregnancy or a restoration of the enterohepatic circulation of estriol conjugates (21–24).

Bile acids and progesterone metabolites in patients 6 and 7

The results of the analyses of serum samples from these patients are summarised in Table 5. Patient no. 6 had elevated levels of bile acids, but a normal bile acid composition and a normal pattern of sulphated steroids before treatment. The bile acid concentrations were decreased during administration of UDCA, while the steroid sulphate pattern remained largely unchanged. Patient no.7 had normal serum bile acids, the concentrations of which were increased during treatment with UDCA. The 3α-/3β-hydroxysteroid ratio was normal and not affected by the treatment. The percentage of monosulphated steroids was unusually high in this patient, and increased rather than decreased (20) with progressing pregnancy.

Correlation between bile acids and steroid sulphates

The concentrations in serum and the urinary excretion of bile acids and steroid sulphates did not change in parallel in all patients with ICP (Fig. 2). Furthermore, the excretion of total bile acids or steroid sulphates in urine was not always a reflection of the levels of the compounds in serum (Fig. 2). Patients nos.1 and 5 were particularly interesting. Their serum bile acids were low, as in normal pregnancy, while the urinary excretion was increased. The cholestasis (Table 1) appeared to be best reflected in the elevated levels of sulphated steroids and in the increased ratio of 3α- to 3β-hydroxysteroids.

Metabolism of UDCA in patients with ICP

The FAB spectra of urine extracts from the patients with ICP showed intense peaks at *m/z* 624 and 640 during administration of UDCA. These are consistent with glucuronidated glycine conjugates of di- and trihydroxycholanoates, respectively. Corresponding peaks of taurine conjugates at *m/z* 674 and 690 also appeared during the treatment, and peaks corresponding to sulphated dihydroxycholanoates with and without glycine or taurine conjugation also increased. Peaks of *N*-acetylglucosaminides of glycine (*m/z* 651) or taurine (*m/z* 701) conjugates of dihydroxycholanoates were also observed, but were much less intense than those of the glucuronides. As mentioned in Materials and Methods, the cholylglycine hydrolase failed to hydrolyse the double conjugates with *N*-acetylglucosamine and they were not further studied.

The aminoacyl amidated bile acids and the double conjugates with glucuronic acid and glycine or taurine were hydrolysed and the liberated bile acids were derivatised and analysed by GC/MS. Figure 3 shows the total ion current chromatograms of the bile acids in the G/T and GlcA-G/T fractions (Table 2) from urine of patient 2 during treatment with UDCA. Peaks 12, 15–17, 20, 22 and 24 were assumed to represent metabolites of UDCA.

TABLE 5

Concentration, μmol/l, of bile acids and sulphated steroids in the serum of patients nos. 6 and 7 before and during treatment with UDCA

Compounds[a]	Patient no. 6				Patient no. 7			
	Before treatment		During treatment		Before treatment		During treatment	
	μmol/l	ratio	μmol/l	ratio	μmol/l	ratio	μmol/l	ratio
CA+CDCA+DCA	9.3		1.4		1.6		9.2	
CA:CDCA:DCA		0.7:1:0.8		1:1:0.7		1:1:0.7		0.8:1:0.5
UDCA	0		4.1		0		6.9	
Total MoS	9.2		7.5		19		13	
Total DiS	3.2		7.6		3.9		1.8	
3α/3β (MoS)		2		1.9		2.2		2
3α/3β (DiS)		0.37		0.49		0.34		0.39

[a] CA: cholic acid; CDCA: chenodeoxycholic acid; DCA: deoxycholic acid; UDCA: ursodeoxycholic acid; MoS: monosulphated steroids; DiS: disulphated steroids; 3α/3β: 3α-/3β-hydroxysteroid ratio.

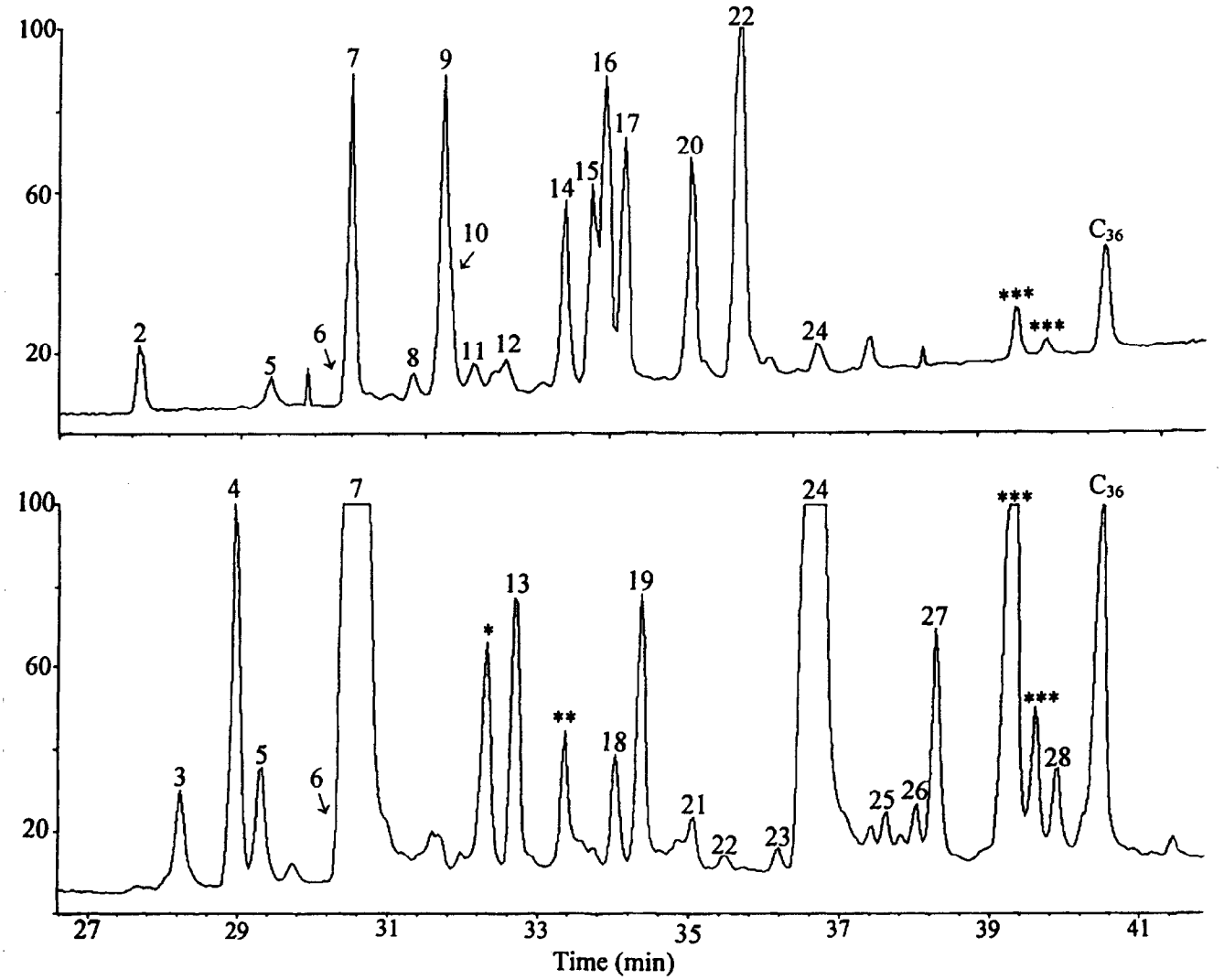

*Fig. 3. Total ion current chromatograms obtained in the GC/MS analyses of bile acids in the G/T (top) and GlcA-G/T (bottom) fractions (Table 2) isolated from urine of patient no. 2 with ICP during administration of UDCA. The numbered peaks correspond to the following derivatised bile acids: 2. 3α,12β-dihydroxy-5β-cholanoic; 3. deoxycholic; 4. chenodeoxycholic; 5. cholic; 6. iso-UDCA; 7. UDCA; 8. trihydroxycholanoic; 9. 3α-hydroxy-12-oxo-5β-cholanoic; 10. 1β,3α,12α-trihydroxy-5β-cholanoic; 11. 3α,6α,7α,12α-tetrahydroxy-5β-cholanoic; 12. 3α,6β,7β-trihydroxy-5β-cholanoic; 13. 3α,4β-dihydroxy-5β-cholanoic; 14: 3α,12α-dihydroxy-3-oxo-4-cholenoic; 15. 3α,7β,22-trihydroxy-5β-cholanoic; 16. 3α,5β,7β-trihydroxy-5β-cholanoic; 17. trihydroxycholanoic; 18. 3α,4β,7α-trihydroxy-5β-cholanoic; 19. 3α,4β,12α-trihydroxy-5β-cholanoic; 20. 1β,3α,7β-trihydroxy-5β-cholanoic; 21.trihydroxycholanoic; 22. 3α,6α,7β-trihydroxy-5β-cholanoic; 23. 3α,4β,7α,12α-tetrahydroxy-5β-cholanoic; 24. 3α,4ξ,7β-trihydroxy-5ξ-cholanoic; 25. trihydroxycholanoic; 26. trihydroxycholanoic; 27. ethyl ester of 3α,4ξ,7β-trihydroxy-5β-cholanoic; 28. isomer of 3α,4ξ,7β-trihydroxy-5β-cholanoic acid; *. ethyl ester of UDCA; **. TMS ester of UDCA; ***. Contaminants.*

The metabolites were identified, some tentatively, as shown in Table 6. As expected, a 6α-hydroxylated non-amidated UDCA was a major metabolite in the glucuronide fraction. Mass spectra taken at peaks 16 (from G/T fraction) and 24 (from GlcA-G/T fraction) are shown in Fig. 4. The spectrum of peak 16 showed a molecular ion at *m/z* 566 (very low intensity) with losses of CH_3 (*m/z* 551) and one and two trimethylsilanols (*m/z* 476 and 386). The loss of water to give *m/z* 458 and 368 indicated the presence of a hydroxyl group that had not been derivatised under the conditions used. This suggested a tertiary hydroxyl group, and the peak at *m/z* 259 indicated that this could be located at C-5. Thus, the ion at *m/z* 243, normally present in 3,7–ditrimethylsiloxycholanoates, was absent from the spectrum and possibly replaced by *m/z* 259 (i.e.

TABLE 6

The urinary excretion of UDCA and its hydroxylated products in the five patients (nos. 1–5) with ICP during administration of UDCA

No.[b]	Bile acid	Urinary excretion (μmol/g-creatinine, mean±SEM)[a]					
		RI[c]	U	G/T	S,S-G/T	GlcA	GlcA-G/T[d]
1	nor-ursodexoycholate	3120	–	–	–	0.2±0.1	–
5	ursodeoxycholate	3250	0.2±0.1	2.8±1.4	26±11	0.6±0.2	3.8; 1.9
12	3α,6β,7β-trihydroxy-5β-cholanoate	3321	–	0.3±0.1	–	–	–
15	3α,7β,22-trihydroxy-5β-cholanoate	3362	–	0.7±0.2	–	–	–
16	3α,5β,7β-trihydroxy-5β-cholanoate	3367	–	1.5±0.4	–	–	–
17	Trihydroxycholanoate	3376	–	0.4±0.2	–	–	–
20	1β,3α,7β-trihydroxy-5β-cholanoate	3407	–	0.6±0.2	–	0.2±0.1	–
22	3α,6α,7β-trihydroxy-5β-cholanoate	3428	–	0.8±0.5	–	0.9±0.5	0.12; 0.05
24	3α,4ξ,7β-trihydroxy-5β-cholanoate	3464	–	0.08±0.03	–	–	3; 1.4

[a] "–": not detected. The bile acids are: U=unconjugated; G/T=aminoacyl amidated; S,S-G/T=both amidated and non-amidated and sulphated; GlcA=glucuronidated; GlcA-G/T: both glucuronidated and aminoacyl amidated.

[b] The numbers are the same as in Fig. 4.

[c] Retention indices on the methyl silicone column.

[d] The data are from patients nos. 1 and 2.

243+16). Derivatisation with trimethylsilylimidazole under more vigorous conditions gave a molecular ion at *m/z* 638 (low intensity) and *m/z* 259 shifted to *m/z* 331, corresponding to trimethylsilylation of the tertiary hydroxyl group. Since the spectrum of the fully derivatised compound resembled that suggested by Koopman et al. to represent a derivative of 5-hydroxyursodeoxycholate and since a trimethylsilyl ether derivative of 5-hydroxylated norursodeoxycholate gave a peak at *m/z* 259, we identify the metabolite giving peak 16 as 3α,5β,7β-trihydroxycholanoic acid.

Peak 24 represented a major metabolite of UDCA in the GlcA-G/T fractions from patients nos. 1 and 2. It gave a mass spectrum similar to one published by Koopman et al. (26), who tentatively identified this metabolite as 3α,7β,21-trihydroxy-5β-cholanoic acid. However, our interpretation of the spectrum is different. The ABCD-ring fragment is clearly at *m/z* 253, indicating that the three hydroxyl groups are in the ring system, and there is a well-defined peak of 20% relative intensity at *m/z* 115, showing that the side chain does not contain any substituent (28). The peaks at *m/z* 507, 419 and 329 (base peak) indicate pronounced loss of carbons 1–3 with the trimethylsiloxy group, as seen in spectra of trimethylsilyl derivatives of bile acids with a 3,4-dihydroxy structure . The intense rearrangement ion at *m/z* 147 and the peak at *m/z* 129 further support this structure. Finally, the loss of 89 mass units to give the peak at *m/z* 369 supports the presence of vicinal trimethylsiloxy groups. Thus, we propose that a major metabolite of UDCA in pregnancy is 4-hydroxylated. It is of interest that four 4-hydroxylated bile acids, previously identified in urine from pregnant women not given UDCA, were also present in the GlcA-G/T fraction (peaks nos. 13, 18, 19 and 23, Fig. 3).

The muricholic acids (peaks 12 and 22), the 1β-hydroxylated (peak 20) and the 22-hydroxylated (peak 15 with a typical mass spectral peak at *m/z* 175) bile acids were identified based on literature data (26). The 3β isomer of UDCA, not separated from UDCA on the methyl silicone column, was identified by analysis on a polar column (Unicoat UC-1625 (14)). Its excretion in the G/T fraction constituted less than 10% of that of UDCA.

Semi-quantitative estimates of the urinary excretion of UDCA and its hydroxylated metabolites are given in Table 6. As seen in this Table, a large part of UDCA was excreted in sulphated form. However, the interindividual variation was very large. The metabolites of UDCA were found mainly in the G/T and GlcA-G/T fractions. The major metabolite in the former fraction in three patients was 5-hydroxy-UDCA, while the tentatively identified 4-hydroxy-UDCA was glucuronidated.

Discussion

The present study has demonstrated that treatment with UDCA effectively changes the levels and profiles of bile acids and progesterone metabolites, which are altered in serum and urine of patients with ICP, towards those seen in normal pregnancy.

Bile acids

The effects of UDCA on the bile acids are characterised by a significant reduction of the levels and percentage of aminoacyl amidated cholic acid in serum and urine and of cholic acid metabolites in urine hydroxylated in positions 1β, 2β, 5β and 6α (Fig. 1). In cholestasis, accumulated less polar bile acids are sulphated and excreted in urine (30) and sulphated gly-

cine and taurine conjugates of CDCA are major urinary bile acids in cholestasis (31–33). It is possible that the urinary excretion of less polar bile acids as sulphates leads to the increased ratio of cholic to chenodeoxycholic acid in serum of patients with ICP. The lower proportion of deoxycholic acid in ICP is explained by the impairment of the enterohepatic circulation of cholic acid. Administration of UDCA stimulates biliary excretion, which will reduce the concentrations of bile acids in the hepatocytes and thus the sulfation of less polar bile acids and the hydroxylation of cholic acid. UDCA participates in the enterohepatic circulation and is converted by intestinal bacteria into lithocholic acid (for review, see ref. (34)), which explains the slightly increased percentage of sulphated lithocholic acid in urine of patients with ICP treated with UDCA.

The 7α,12α-dihydroxy-3-oxo-4-cholenoic acid is a quantitatively important urinary bile acid in both healthy pregnant women and patients with ICP (15). We have suggested that the appearance of this acid in pregnant women may reflect a rate limitation in the reduction of the 4, 5-double bond in bile acid biosynthesis, due to the large load of progesterone (with a 3-oxo-Δ^4 structure) to be reduced in the liver in pregnancy (15). Its analogue without the 12α-hydroxyl group strongly inhibits the ATP-dependent canalicular transport of taurocholate (35), and is the probable cause of the cholestasis in patients with a primary (36) or secondary (37) deficiency of 5β-reductase. While treatment with UDCA reduced the urinary excretion of 7α,12α-dihydroxy-3-oxo-4-cholenoic acid, it did not change the relative proportion of this acid in urine. Therefore, UDCA may not affect bile acid biosynthesis

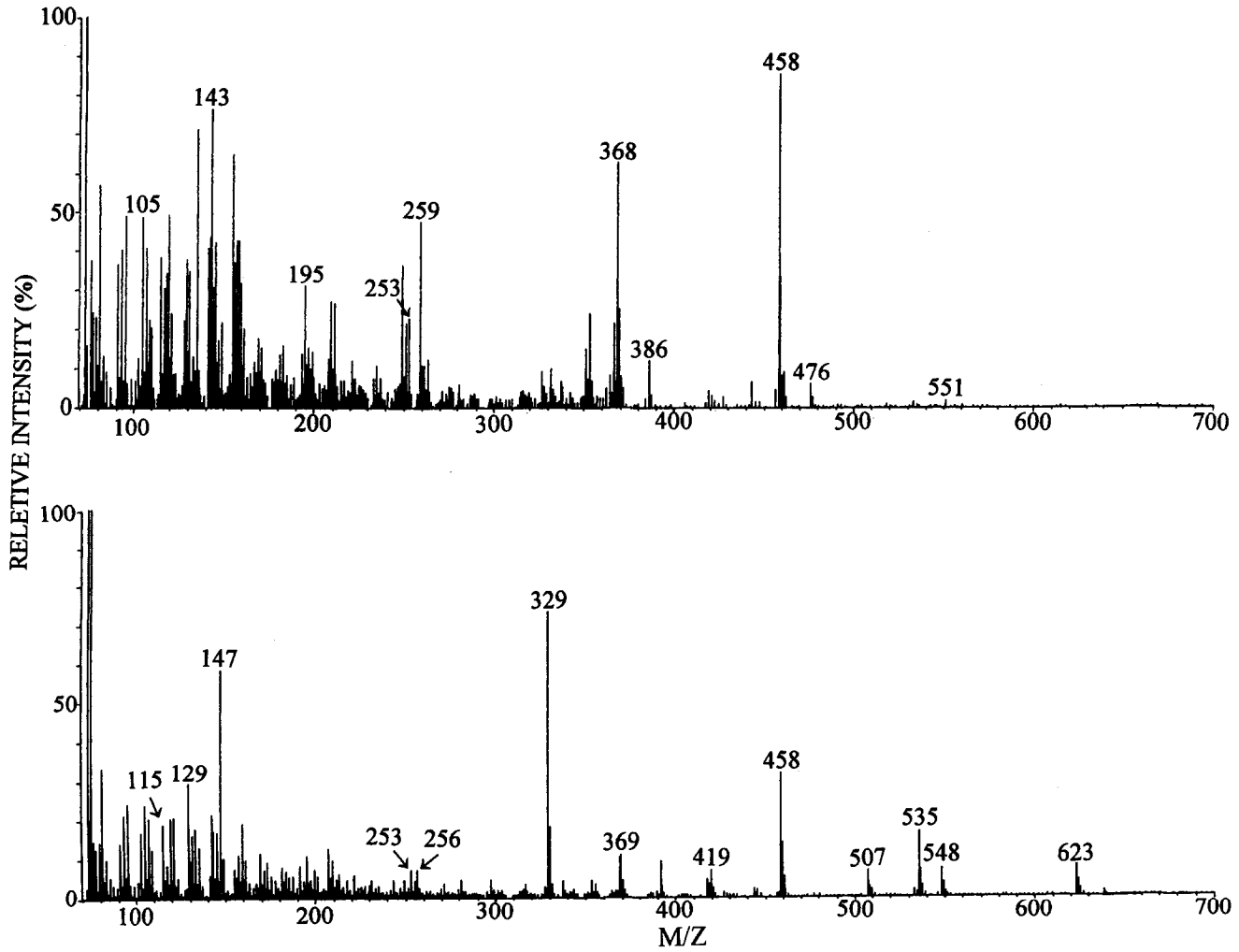

Fig. 4. Electron impact (70eV) ionisation mass spectra taken during elution of peaks 16 (upper spectrum) and 24 (lower spectrum) shown in Fig. 3.

at the step of 5β-reduction. The relief of cholestasis by UDCA has been suggested to be due to stimulation of vesicular exocytosis resulting in mobilisation of an increased number of transport proteins to the canalicular membrane (38–40). If this is the case in ICP, the effect is transient, since cholestasis reappeared when the administration of UDCA was discontinued (11). Although the formation of 7α, 12α-dihydroxy-3-oxo-4-cholenoic acid is not correlated to the appearance of ICP, it may be related to the mild physiological cholestasis in pregnancy (see a review in (41)), and the possibility exists that it may be an aetiological factor in some women due to genetic polymorphism of canalicular transport proteins.

Double conjugates of bile acids with glucuronic acid and glycine or taurine were analysed for the first time in pregnant women. It is of interest that a number of 4-hydroxylated bile acids were present in this conjugated form (Fig. 3). Such bile acids have been found in foetal bile (29,42), amniotic fluid (43) and in infant urine (44). Recently, they were found in urine of pregnant women and suggested to be of foetal origin (15), based on the above reports (29,42–44) and our failure to find any of them in the urine of healthy women during the menstrual cycle (unpublished results). If 4-hydroxylation is a unique feature of bile acid metabolism in early human life (29,42), analysis of urinary bile acids doubly conjugated with glucuronic acid and glycine or taurine may provide information about the condition of the foetus.

Steroids

The most striking change in progesterone metabolism in ICP is the increased concentration of sulphated metabolites, particularly those with a 3α-sulphoxy-5α(H) configuration, in maternal serum and urine (Table 3, (refs. 6,8,9,15)) as well as in cord plasma (45). Administration of UDCA induced a change of these steroids towards normal (Tables 3 and 4). In contrast, glucuronidated metabolites were not significantly affected, either by ICP (11,15) or by administration of UDCA (Table 3). Sulfated progesterone metabolites are excreted in bile (8,46) and are hydrolysed by intestinal bacteria and excreted as unconjugated steroids in faeces (7,23,47). It has been suggested that part of the unconjugated steroids is also reabsorbed and conjugated with glucuronic acid in the intestinal mucosa for final elimination in urine (48). This is supported by the findings that administration of antibiotics leads to a large increase of sulphated progesterone metabolites in faeces (7,23) and a decrease of glucuronidated metabolites in urine (49). Thus, part of the glucuronidated 5α-reduced metabolites in urine of healthy pregnant women may be formed from sulphated biliary metabolites. This pathway would be inhibited in cholestasis and stimulated by UDCA to produce the slight increase of glucuronides in urine during administration of UDCA (Table 3).

An important question for the understanding of the pathogenesis of ICP is whether the abnormal profile of sulphated progesterone metabolites characterised by an increased ratio of 3α- to 3β-hydroxylated steroids is primary or secondary to the cholestasis. Previous studies with deuterium-labelled steroid sulphates have clearly shown that the 3α- and 3β-hydroxysteroid sulphates are not interconverted (50,51). Therefore, the increased 3α-/3β-hydroxysteroid ratio is not due to the effect of ICP on an enterohepatic circulation (8). Consequently, it is either due to a relative increase in the formation of 3α-hydroxy isomers from progesterone, or is secondary to an impaired biliary excretion of these isomers (8).

The levels of total sulphated 3β-hydroxysteroid isomers in serum are higher than those of 3α isomers in late pregnancy (20), in spite of a lower production rate of 3β isomers (50,51). This may be due to the longer half-life of 5α-pregnane-3β, 20α-diol disulphate (50,51) and a slower biliary elimination of 3β isomers. Thus, the proportion of 3β-hydroxysteroid sulphates in bile (46) is lower than that in serum of healthy pregnant women. It is conceivable that the reduced bile flow in ICP could lead to an accumulation in serum and an increased urinary excretion of predominantly 3α-hydroxysteroid sulphates, i.e. the abnormal pattern in ICP would be secondary to cholestasis. However, this is not in agreement with the data for patients 6 and 7. They had elevated serum bile acids before (patient no. 6) or during (patient no. 7) treatment, reflecting a mild cholestasis, while the ratios of 3α- to 3β-hydroxysteroid sulphates were normal and not influenced by UDCA. Therefore, the alternative explanation that a change in the reductive metabolism of progesterone is a primary event in ICP cannot be excluded. This is also supported by the observation of abnormal steroid sulphate patterns in two patients prior to the development of ICP (6) and by the report that the abnormal steroid sulphate pattern characteristic of ICP is not seen in patients with cholestasis due to viral hepatitis (9). Further studies are needed to resolve the question whether the changes in steroid sulphates in ICP are primary or secondary, and whether UDCA can induce a recruitment of transport proteins to the canalicular membrane (40) for excretion of steroid sulphates.

Patients nos. 6 and 7 were initially thought to have ICP, but were excluded from the double-blind study because of normal values for serum alanine amino-

transferase. Our analyses showed that the ratios of sulphated 3α- to 3β-hydroxysteroids were normal. The pruritus in these patients is therefore unlikely to be due to ICP, and the results support the contention that the ratio of 3α- to 3β-hydroxysteroid sulphates in serum is a selective parameter for the diagnosis of ICP, especially in the early stage of the disease (6).

Metabolism of UDCA

The metabolism of UDCA has been extensively studied in health and disease. Conjugation with *N*-acetylglucosamine is a selective pathway for 7β-hydroxylated bile acids (14,52) and was also observed by FAB mass spectrometry of urine samples from the patients with ICP. UDCA has been found to be hydroxylated at carbon atoms 1, 5, 6, 12, 21, 22 and 23 in humans (26,53–56). We found metabolites hydroxylated in these positions, except for C-12, C-21 and C-23, which may have been minor components. Hydroxylation at C-5 of UDCA in humans has been reported in two studies (26,54), and 5β-hydroxylation of nor-UDCA and nor-CDCA occurs in rodents (27). Our data strongly support the 5-hydroxylation, which gave the predominant hydroxylated metabolite in the urine from three of the patients.

We have tentatively identified 4-hydroxy-UDCA as a double conjugate with glucuronic acid and glycine or taurine. As mentioned above, other 4-hydroxylated bile acids are also conjugated in this way. Since 4-hydroxylation has been suggested to be a unique feature of bile acid metabolism in early human life (29,42), it will be of interest to investigate whether the tentative 4-hydroxy-UDCA is a metabolite of UDCA in the foetus.

Acknowledgements

This work was supported by grants from the Swedish Medical Research Council (no. 03x-219), Karolinska Institutet and Fondecyt (Chile) (no. 1940420).

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