# Identification of unconjugated bile acids in human bile

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**Abstract** Unconjugated bile acids in the bile of healthy and diseased humans were determined qualitatively and quantitatively by means of gas–liquid chromatography and gas–liquid chromatography–mass spectrometry, after their isolation by ion-exchange chromatography. In a healthy person and three patients with cholelithiasis, unconjugated bile acids comprised 0.1–0.4% of total biliary bile acids. The bile acid composition of the unconjugated fraction was quite different from that of the glycine- or taurine-conjugate fraction, in that it contained a relatively large proportion of unusual bile acids including $C_{23}$ and $C_{27}$ bile acids. In two patients with cerebrotendinous xanthomatosis, $C_{22}$ and $C_{23}$ bile acids were the major constituents of the biliary unconjugated bile acids, and comprised about 0.8% of total bile acids; no detectable amounts of $C_{27}$ bile acids were found in their bile. The analysis of biliary unconjugated bile acids may be useful for the diagnosis of metabolic diseases concerning bile acids, particularly the accumulation or disappearance of unusual bile acids.—**Matoba, N., M. Une, and T. Hoshita.** Identification of unconjugated bile acids in human bile. *J. Lipid Res.* 1986. **27:** 1154–1162.

**Supplementary key words** gas–liquid chromatography–mass spectrometry • ion-exchange chromatography • $C_{22}$ bile acid • $C_{23}$ bile acid • $C_{27}$ bile acid

Although unconjugated bile acids are found in considerable amounts in biles of some frogs (1–3), salamander (4), and turtle (5), bile acids occurring in mammalian bile are almost exclusively conjugated with taurine and glycine (6). Thus, insufficient attention has been paid to the presence of unconjugated bile acids in human bile. The aim of the present study was to find out whether unconjugated bile acids occur in bile of healthy or diseased humans.

## MATERIALS AND METHODS

### Subjects

Six subjects were included in this study. Diagnoses, which are shown in **Table 1,** were based on clinical findings, laparotomy, or pathological examinations of resected specimens. Informed consent was obtained from each subject participating in this study. Patients C and D were treated with ursodeoxycholic acid preoperatively for about 2 weeks. The gallbladder bile of three patients with cholelithiasis was obtained by needle aspiration from the gallbladder during the laparotomy. In the other cases bile samples were collected by the use of a duodenal catheter. These samples were stored immediately at −20°C until analysis to avoid the bacterial deconjugation of conjugated bile acids.

### Analysis of bile acids

Each bile sample (about 10 ml) was extracted with 10 volumes of ethanol at 4°C, and the extract was filtered. The filtrate was evaporated to dryness under reduced pressure to leave crude bile salts as a solid (0.5–1.5 g). The solid was dissolved in 10 ml of 90% ethanol and applied to a column (15 cm × 3 cm i.d.) of piperidinohydroxypropyl Sephadex LH-20 (7). After washing with 300 ml of 90% ethanol, the column was eluted successively with 800 ml of 0.1 M acetic acid in 90% ethanol, 800 ml of 0.2 M formic acid in 90% ethanol, and 800 ml of 1% ammonium carbonate in 70% ethanol, to give fractions containing unconjugated bile acids, glycine-conjugated bile acids, and taurine-conjugated bile acids, respectively.

The unconjugated bile acid fraction was taken to dryness. The resulting residue was analyzed by gas–liquid chromatography (GLC) and gas–liquid chromatography–mass spectrometry (GLC–MS) after preparation of the methyl ester–trimethylsilyl (TMS) ether derivatives (1).

GLC was performed on a Shimadzu GC-6A gas chromatograph equipped with a flame ionization detector. The columns used were a glass column (2 m × 3 mm i.d.) packed with 3% OV-17, 3% Poly I-110, or 2% OV-1 on 80/100 mesh Gas Chrom Q, and a WCOT glass capillary column (25 m × 0.25 mm i.d.) coated with OV-1. All the

Abbreviations: CTX, cerebrotendinous xanthomatosis; GLC, gas–liquid chromatography; GLC–MS, gas–liquid chromatography–mass spectrometry; TMS, trimethylsilyl; RRT, relative retention time; PMR, proton nuclear magnetic resonance; THCA, 3α,7α,12α-trihydroxy-5β-cholestan-26-oic acid; $\Delta^{24}$-THCA, 3α,7α,12α-trihydroxy-5β-cholest-24-en-26-oic acid; 24-TeHCA, 3α,7α,12α,24-tetrahydroxy-5β-cholestan-26-oic acid; 26-TeHCA, 3α,7α,12α,26-tetrahydroxy-5β-cholestan-27-oic acid.

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TABLE 1. Bile acid concentration in human bile

| Patient | Age | Sex | Diagnosis | Bile Acids[a] U | G | T | Total |
|---|---|---|---|---|---|---|---|
| | | | | *mg/ml* | | | |
| A | 67 | M | Healthy | 0.03 | 14.07 | 5.39 | 19.49 |
| B | 55 | F | Cholelithiasis | 0.07 | 51.30 | 15.16 | 66.53 |
| C[b] | 40 | M | Cholelithiasis | 0.15 | 37.14 | 4.04 | 41.33 |
| D[b] | 60 | M | Cholelithiasis | 0.02 | 20.60 | 2.85 | 23.47 |
| E | 43 | M | CTX[c] | 0.03 | 1.13 | 2.62 | 3.78 |
| F | 43 | M | CTX[c] | 0.01 | 0.68 | 0.54 | 1.23 |

[a] U, unconjugated bile acids; G, glycine-conjugated bile acids; T, taurine-conjugated bile acids.
[b] Two patients were treated with ursodeoxycholic acid.
[c] CTX, cerebrotendinous xanthomatosis.

retention times listed in the text are given relative to the TMS ether of methyl cholate (relative retention time (RRT) = 1.00). Quantitation was accomplished by comparing GLC area on the capillary OV-1 column and the OV-17 or Poly I-110 packed column of the biological samples with that of the external standard, the TMS ether of methyl cholate. Measurements of peak areas were accomplished with a Shimadzu E1A automatic integrator.

GLC–MS was carried out on a Shimadzu GCMS-QP 1000 gas chromatograph–mass spectrometer. The following operating conditions were employed: column, OV-1 (10 m × 0.25 mm i.d.); column temperature, 230–260°C at a rate of 2°C/min; injection port temperature, 280°C; separator temperature, 250°C; ion source temperature, 250°C; flow rate of helium carrier gas, 40 ml/min; ionizing voltage, 70 eV; ionizing current, 60 μA.

Glycine- and taurine-conjugated bile acids were hydrolyzed with 2.5 N KOH solution at 130°C for 3 hr to give deconjugated bile acids which were analyzed in the same manner as described for the unconjugated bile acids.

### Reference compounds

Cholic acid (3α,7α,12α-trihydroxy-5β-cholan-24-oic acid) and deoxycholic acid (3α,12α-dihydroxy-5β-cholan-24-oic acid) were commercial products (Sigma Chemical Co., St. Louis, MO). Chenodeoxycholic acid (3α,7α-dihydroxy-5β-cholan-24-oic acid) and ursodeoxycholic acid (3α,7β-dihydroxy-5β-cholan-24-oic acid) were supplied from Tokyo Tanabe Pharmaceutical Co. (Tokyo, Japan). Bisnorcholic acid (3α,7α,12α-trihydroxy-22,23-dinor-5β-cholan-24-oic acid) (8), allonorcholic acid (3α,7α,12α-trihydroxy-23-nor-5α-cholan-24-oic acid) (9), norchenodeoxycholic acid (3α,7α-dihydroxy-23-nor-5β-cholan-24-oic acid) (10), norcholic acid (3α,7α,12α-trihydroxy-23-nor-5β-cholan-24-oic acid) (8), allocholic acid (3α,7α,12α-trihydroxy-5α-cholan-24-oic acid) (4, 11), 7-epicholic acid (3α,7β,12α-trihydroxy-5β-cholan-24-oic acid) (12), 7-ketodeoxycholic acid (3α,12α-dihydroxy-7-oxo-5β-cholan-24-oic acid) (13), 22-dehydrocholic acid (3α,7α,12α-trihydroxy-5β-chol-22-en-24-oic acid) (14), 3α,7α,12α-trihydroxy-5β-cholestan-26-oic acid (THCA) (15), 3α,7α,12α-trihydroxy-5β-cholest-24-en-26-oic acid ($\Delta^{24}$-THCA) (16), 3α,7α,12α,24-tetrahydroxy-5β-cholestan-26-oic acid (24-TeHCA) (16), and 3α,7α,12α,26-tetrahydroxy-5β-cholestan-27-oic acid (26-TeHCA) (1) were prepared according to the procedures as described previously.

### Synthesis of norursodeoxycholic acid

A solution of methyl ursodeoxycholate (8 g) dissolved in 100 ml of anhydrous benzene was added to an anhydrous ethereal solution containing a Grignard reagent prepared from methyl iodide (53 ml) and magnesium (10 g) (8). The reaction mixture was refluxed for 1.5 hr. After addition of 5% $H_2SO_4$, the solution was extracted with ethyl acetate (200 ml × 3). The extract was washed successively with water, 2% $NaHCO_3$, and 3% $Na_2S_2O_3$, and concentrated to dryness under a reduced pressure. The residue was chromatographed on a column of 80 g of silica gel 60 (Merck). Elution with ethyl acetate and crystallization from ethyl acetate gave crystals (4.1 g) of 24-nor-5β-cholestane-3α,7β,25-triol, mp: 154–155°C; PMR (pyridine-$d_5$) δ ppm: 0.74 (s, 3H, 18–$CH_3$), 0.98 (s, 3H, 19–$CH_3$), 1.05 (d, J = 6Hz, 3H, 21–$CH_3$), 1.43 (s, 6H, 26–$CH_3$, 27–$CH_3$), 3.60–4.00 (m, 2H, 3β–H, 7α–H).

A solution of 24-nor-5β-cholestane-3α,7β,25-triol (3 g) dissolved in a mixture of acetic anhydride (10 ml) and pyridine (3 ml) was refluxed for 2 hr. After evaporation of the solvents, the residue was dissolved in acetic acid and to the solution of the acetylated material was added a solution of 2 g of $CrO_3$ in 5 ml of water and 10 ml of acetic acid. The reaction mixture was heated at 70°C for 2 hr and diluted with 20 volumes of water. The resulting precipitate was extracted with ether. The extract was shaken with 2% NaOH. The alkaline extract was heated at 100°C for 2 hr. The solution was acidified with *d*-HCl

and extracted with ether. The ether extract was washed with water and dried over anhydrous $Na_2SO_4$. The solvent was evaporated to give crude crystals. Recrystallization from acetone–methanol gave crystals (0.9 g) of norursodeoxycholic acid (3$\alpha$,7$\beta$-dihydroxy-23-nor-5$\beta$-cholan-24-oic acid), mp: 235–237°C; PMR (pyridine-$d_5$) $\delta$ ppm: 0.72 (s, 3H, 18–$CH_3$), 0.94 (s, 3H, 19–$CH_3$), 1.21 (d, J = 6Hz, 3H, 21–$CH_3$), 3.50–3.90 (m, 2H, 3$\beta$–H, 7$\alpha$–H).

## RESULTS

Unconjugated bile acids occurring in the bile of healthy or diseased humans were isolated by means of ion-exchange chromatography and analyzed as the methyl ester–TMS ether derivatives by GLC and GLC–MS. Glycine- and taurine-conjugated bile acids were also analyzed after hydrolysis by the same procedures for the unconjugated bile acids.

Total amounts of unconjugated and conjugated bile acids in the bile of six subjects are shown in Table 1 with their diagnoses. In a healthy person and three patients with cholelithiasis, the mean amount of total bile acids in bile was about 38 mg/ml. The mean percentage of unconjugated bile acids was about 0.2% of the total biliary bile acids. On the other hand, the total amounts of biliary bile acids in two patients with cerebrotendinous xanthomatosis (CTX) were about 1 and 4 mg/ml of bile, a much lower concentration than observed for the other subjects. Nevertheless, the amounts of unconjugated bile acids in the bile of the CTX patients were 0.01 and 0.03 mg/ml of bile, about equal to those of the other subjects. Thus, the mean ratio of unconjugated bile acids to total biliary bile acids in the CTX patients was about 4-fold higher than the mean ratio for the other subjects.

**Fig. 1** shows representative GLC profiles of the unconjugated bile acids. GLC and GLC–MS analyses revealed that there were at least 19 different unconjugated bile acids, which were tentatively named bile acids 1–19 in order of increasing retention times on OV-1 column (**Table 2**). As seen in Table 2, it was impossible to separate all of these bile acids by the use of only an OV-1 column because some pairs of bile acids had the same RRTs. However, these pairs could be separated using OV-17 or Poly I-110 columns. Mass fragment ions of these bile acids as the methyl ester–TMS ether derivatives are shown in **Table 3, Table 4,** and **Table 5.**

The mass spectrum of the methyl ester–TMS ether derivative of bile acid 1 was very similar to that of the corresponding derivative of cholic acid (bile acid 9) except that the high field fragments containing the entire side chain in the former differed by 28 mass units from those in the latter. The base peak at m/z 253 and the peak at m/z 343 were seen in both spectra. These ions represent

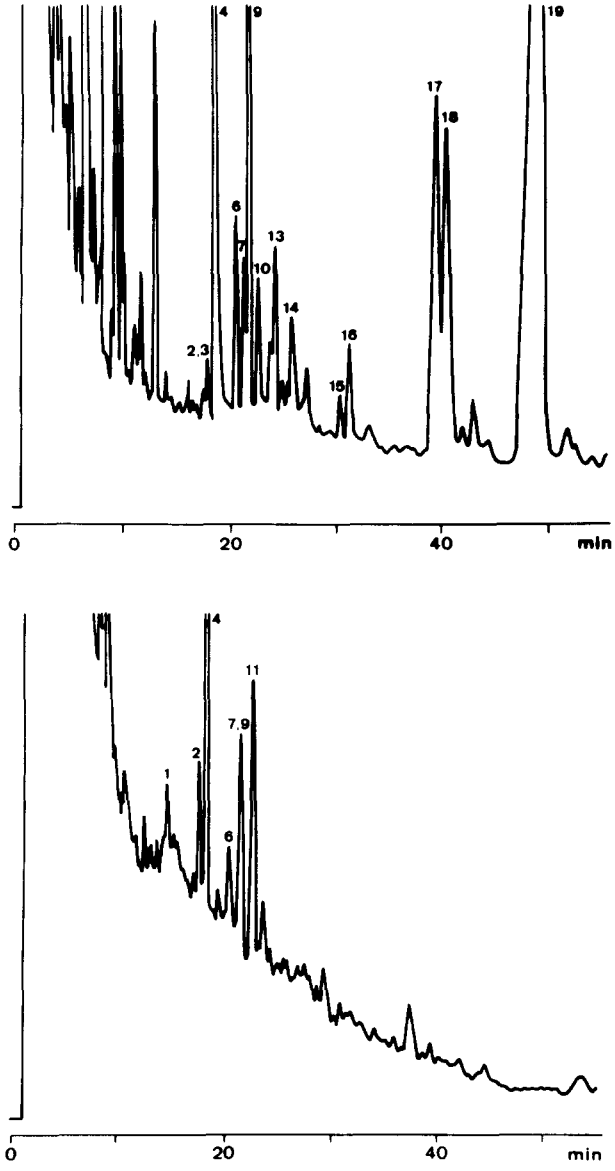


**Fig. 1.** Gas chromatograms of methyl ester-TMS ether derivatives of the biliary unconjugated bile acids obtained from patient B (top) and patient E (bottom). Capillary column OV-1 (25 m × 0.25 mm i.d.) was employed; column temperature, 240–280°C at a rate of 2°C/min. Each number of the peak corresponds to that of a bile acid shown in Table 2.

loss of the entire side chain plus three and two nuclear TMS groups, respectively. These data indicated that bile acid 1 is a lower homologue of cholic acid. This structural assignment was confirmed by direct comparison with the authentic sample of bisnorcholic acid. The RRTs on GLC and mass spectrum of authentic bisnorcholic acid were completely identical with those of the natural bile acid 1.

Bile acids 2, 3, and 4 were identified as allonorcholic acid, norchenodeoxycholic acid, and norcholic acid, respectively, by direct comparison of the RRTs on GLC

TABLE 2. Relative retention times of bile acids detected in unconjugated fraction of human bile

| No. | Bile Acids | RRT[a] 3% OV-1[b] | 2% OV-17[c] | 3% Poly I-110[d] |
|---|---|---|---|---|
| 1 | Bisnorcholic acid | 0.54 | 0.50 | 0.47 |
| 2 | Allonorcholic acid | 0.70 | 0.65 | 0.64 |
| 3 | Norchenodeoxycholic acid | 0.70 | 0.80 | 1.02 |
| 4 | Norcholic acid | 0.76 | 0.75 | 0.74 |
| 5 | Norursodeoxycholic acid | 0.78 | 0.90 | 0.74 |
| 6 | Deoxycholic acid | 0.91 | 1.09 | 1.32 |
| 7 | Chenodeoxycholic acid | 0.96 | 1.11 | 1.47 |
| 8 | Allocholic acid | 0.96 | 0.88 | 0.88 |
| 9 | Cholic acid | 1.00 | 1.00 | 1.00 |
| 10 | 22-Dehydrocholic acid | 1.06 | 1.09 | 1.17 |
| 11 | 7-Ketonordeoxycholic acid | 1.06 | 1.58 | 1.91 |
| 12 | Ursodeoxycholic acid | 1.10 | 1.24 | 1.91 |
| 13 | 7-Epicholic acid | 1.18 | 1.24 | 1.22 |
| 14 | 7-Ketodeoxycholic acid | 1.46 | 2.19 | 2.64 |
| 15 | THCA | 1.76 | 1.69 | 1.64 |
| 16 | (24Z)-$\Delta^{24}$-THCA | 1.84 | 1.91 | 1.83 |
| 17 | (24ξ,25S)-24-TeHCA | 2.66 | 2.30 | 1.80 |
| 18 | (24ξ,25R)-24-TeHCA | 2.66 | 2.30 | 1.80 |
| 19 | 26-TeHCA | 3.39 | 3.12 | 2.55 |

[a] Bile acids were chromatographed as their methyl ester-TMS ether derivatives and the values are represented relative to methyl cholate-TMS ether.
[b] Packed column. Column temperature, 250°C. Retention time of methyl cholate-TMS ether was 13.52 min.
[c] Packed column. Column temperature, 260°C. Retention time of methyl cholate-TMS ether was 14.79 min.
[d] Packed column. Column temperature, 250°C. Retention time of methyl cholate-TMS ether was 16.52 min.

and mass spectra of the methyl ester–TMS ether derivatives of the naturally occurring bile acids with the authentic compounds.

The mass fragmentation pattern of the methyl ester–TMS ether derivative of bile acid 5 was very similar to that of the corresponding derivative of ursodeoxycholic

TABLE 3. Relative abundances of important fragment ions of $C_{22}$ and $C_{23}$ bile acids found in human bile as methyl ester-TMS ethers[a]

| | Compound | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | 1 | | 2 | | 4 | 3 | | 5 | 11 | |
| Fragment Ion | m/z | % Int.[b] | m/z | % Int. | % Int. | m/z | % Int. | % Int. | m/z | % Int. |
| M | 610 | | 624 | | | 536 | | | 550 | |
| M–15 | 595 | 26.1 | 609 | 7.3 | 3.8 | 521 | | | 535 | 27.9 |
| M–90 | 520 | | 534 | | | 446 | | 97.4 | 460 | 21.9 |
| M–(90 + 15) | 505 | 11.7 | 519 | | | 431 | 2.7 | 23.0 | 445 | |
| M–(90 + 18) | | | | | | | | | 442 | 11.8 |
| M–(90 + 31) | 489 | | 503 | | | 415 | 4.3 | 19.2 | 429 | |
| M–(2 × 90) | 430 | 89.1 | 444 | 100.0 | 62.5 | 356 | 100.0 | 100.0 | 370 | 20.1 |
| M–(2 × 90 + 15) | 415 | 37.8 | 429 | 5.5 | 16.2 | 341 | 23.9 | 59.7 | 355 | 16.8 |
| M–(90 + side chain) | | | | | | | | | 359 | 40.6 |
| M–(2 × 90 + 18) | | | | | | | | | 352 | 42.1 |
| M–(90 + 18 + side chain) | | | | | | | | | 341 | 71.3 |
| M–(2 × 90 + 31) | 399 | 11.4 | 413 | 6.1 | 7.5 | 325 | 9.3 | 5.6 | 339 | 13.8 |
| M–(2 × 90 + 18 + 15) | | | | | | | | | 337 | 14.2 |
| M–(3 × 90) | 340 | 48.8 | 354 | 10.8 | 100.0 | | | | | |
| M–(2 × 90 + side chain) | 343 | 13.7 | 343 | 38.7 | 35.8 | 255 | 12.4 | 26.8 | 269 | 52.4 |
| M–(3 × 90 + 15) | 325 | 10.2 | 339 | 4.8 | 27.0 | | | | | |
| M–(3 × 90 + side chain) | 253 | 100.0 | 253 | 18.8 | 77.7 | | | | | |
| M–(2 × 90 + 18 + side chain) | | | | | | | | | 251 | 100.0 |

[a] These compounds were identified as follows: 1, bisnorcholic acid; 2, allonorcholic acid; 4, norcholic acid; 3, norchenodeoxycholic acid; 5, norursodeoxycholic acid; 11, 7-ketonordeoxycholic acid.
[b] % Int., percent intensity.

TABLE 4. Relative abundances of important fragment ions of $C_{24}$ bile acids found in human bile as methyl ester-TMS ether[a]

| | Compound | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | 9 | | 8 | 13 | 10 | | 6 | 7 | 12 | 14 | |
| Fragment Ion | m/z | % Int.[b] | % Int. | % Int. | m/z | % Int. | m/z | % Int. | % Int. | % Int. | m/z | % Int. |
| M | 638 | | | | 636 | 5.3 | 550 | | | | 564 | |
| M–15 | 623 | 0.8 | 10.6 | 5.0 | 621 | | 535 | 2.9 | | 4.0 | 549 | 5.8 |
| M–90 | 548 | 0.6 | 6.4 | 6.3 | 546 | | 460 | 2.3 | 2.7 | 100.0 | 474 | 20.0 |
| M–(90 + 15) | 533 | 0.6 | 10.5 | 5.5 | 531 | | 445 | 1.7 | 2.3 | 4.2 | 459 | 3.1 |
| M–(90 + 18) | 530 | | | | 528 | | | | | | 456 | 9.2 |
| M–(90 + 31) | 517 | | 4.7 | | 515 | | 429 | 3.1 | 1.3 | 3.5 | 443 | 2.3 |
| M–(2 × 90) | 458 | 30.2 | 100.0 | 21.8 | 456 | 24.2 | 370 | 35.9 | 100.0 | 94.3 | 384 | 9.9 |
| M–(2 × 90 + 15) | 443 | 5.3 | 21.3 | 5.6 | 441 | | 355 | 16.9 | 24.5 | 59.5 | 369 | 5.5 |
| M–(2 × 90 + 18) | | | | | | | | | | | 366 | 54.4 |
| M–(90 + side chain) | | | | | | | 345 | 26.4 | | 6.3 | 359 | 53.9 |
| M–(2 × 90 + 18 + 15) | | | | | | | | | | | 351 | 20.8 |
| M–(2 × 90 + 31) | 427 | 0.9 | 4.3 | 8.6 | 425 | | 339 | 3.0 | 4.3 | 11.4 | 353 | 7.8 |
| M–(90 + 18 + side chain) | | | | | | | | | | | 341 | 100.0 |
| M–(3 × 90) | 368 | 71.5 | 29.8 | 64.1 | 366 | 88.4 | | | | | | |
| M–(3 × 90 + 15) | 353 | 19.9 | 23.4 | 14.5 | 351 | 14.9 | | | | | | |
| M–(2 × 90 + side chain) | 343 | 44.2 | 66.0 | 65.5 | 343 | 70.8 | 255 | 100.0 | 30.9 | 38.4 | 269 | 33.4 |
| M–(3 × 90 + side chain) | 253 | 100.0 | 55.3 | 100.0 | 253 | 100.0 | | | | | | |
| M–(2 × 90 + 18 + side chain) | | | | | | | | | | | 251 | 47.0 |

[a] These compounds were identified as follows: 9, cholic acid; 8, allocholic acid; 13, 7-epicholic acid; 10, 22-dehydrocholic acid; 6, deoxycholic acid; 7, chenodeoxycholic acid; 12, ursodeoxycholic acid; 14, 7-ketodeoxycholic acid.
[b] % Int., percent intensity.

acid (bile acid 12), except that the high field fragments containing the entire side chain in the former differed by 14 mass units from the corresponding fragments from the latter. The results suggested that bile acid 5 is a lower homologue of ursodeoxycholic acid. Thus, norursodeoxycholic acid was prepared from ursodeoxycholic acid by Barbier-Wieland degradation. The synthetic $C_{23}$ bile acid had GLC and GLC–MS parameters identical to those of the natural bile acid 5.

The mass spectrum of the methyl ester–TMS ether derivative of bile acid 11 closely resembled that of the corresponding derivative of 7-ketodeoxycholic acid (bile acid 14). The base peak at m/z 251 [M − (side chain + 90 × 2 + 18)], and the peaks at m/z 359 [M − (side chain

TABLE 5. Relative abundances of important fragment ions of $C_{27}$ bile acids found in human bile as methyl ester-TMS ether[a]

| | Compound | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | 15 | | 16 | | 17 | | 18 | 19 |
| Fragment Ion | m/z | % Int.[b] | m/z | % Int. | m/z | % Int. | % Int. | % Int. |
| M | 680 | | 678 | | 768 | | | |
| M–15 | 665 | | 663 | | 753 | | | 0.5 |
| M–90 | 590 | | 588 | | 678 | | | 0.5 |
| M–(2 × 90) | 500 | 13.8 | 498 | 15.3 | 588 | 2.8 | 1.6 | 1.0 |
| M–($C_{22}$–$C_{27}$ + 90) | 461 | | 461 | | | | | |
| M–(3 × 90) | 410 | 44.8 | 408 | 76.3 | 498 | 18.7 | 23.8 | 20.1 |
| M–(4 × 90) | | | | | 408 | 16.7 | 8.0 | 1.0 |
| M–($C_{23}$–$C_{27}$ + 90) | 385 | | 385 | 5.4 | | | | |
| M–($C_{22}$–$C_{27}$ + 2 × 90) | 371 | 10.5 | 371 | 6.8 | | | | |
| M–(2 × 90 + side chain) | 343 | 65.9 | 343 | 57.1 | 343 | 100.0 | 100.0 | 100.0 |
| M–($C_{23}$–$C_{27}$ + 3 × 90) | 295 | 11.6 | 295 | 14.5 | | | | |
| M–($C_{25}$–$C_{27}$ + 4 × 90) | | | | | 321 | 25.3 | 25.6 | 1.0 |
| M–($C_{22}$–$C_{27}$ + 3 × 90) | 281 | 100.0 | 281 | 51.0 | 281 | 36.8 | 33.7 | 25.9 |
| M–(3 × 90 + side chain) | 253 | 98.7 | 253 | 100.0 | 253 | 95.4 | 85.9 | 89.6 |

[a] These compounds were identified as follows: 15, THCA; 16, (24Z)-$\Delta^{24}$-THCA; 17, (24ξ,25S)-24-TeHCA; 18, (24ξ,25R)-24-TeHCA; 19,26-TeHCA.
[b] % Int., percent intensity.

+ 90)], 341 [M − (side chain + 90 + 18)], and 269 [M − (side chain + 90×2)] were seen in both spectra. The only difference observed between the two spectra was that the high field fragments containing the entire side chain in the spectrum of bile acid 11 were low by 14 mass units compared with those in the spectrum of bile acid 14. These results strongly suggest, but do not prove, that bile acid 11 is a lower homologue of 7-ketodeoxycholic acid, namely, 7-ketonordeoxycholic acid (3$\alpha$,12$\alpha$-dihydroxy-7-oxo-23-nor-5$\beta$-cholan-24-oic acid).

The following $C_{24}$ bile acids were identified with certainty by direct comparison of their GLC–RRTs and mass spectra to those of reference compounds: bile acid 6, deoxycholic acid; bile acid 7, chenodeoxycholic acid; bile acid 8, allocholic acid; bile acid 9, cholic acid; bile acid 12, ursodeoxycholic acid; bile acid 13, 7-epicholic acid; bile acid 14, 7-ketodeoxycholic acid.

The mass spectrum of the methyl ester–TMS ether derivative of bile acid 10 closely resembled that of the corresponding derivative of cholic acid (bile acid 9), except that the high field fragments containing the entire side chain in bile acid 10 differed by two mass units from those of cholic acid. These results suggested that bile acid 10 is a derivative of cholic acid with a double bond in the side chain. This structural assignment was confirmed by direct comparison with the synthetic sample of 22-dehydrocholic acid. The RRTs on GLC and the mass spectrum of the synthetic bile acid were completely identical to those of the natural bile acid 10.

Bile acids 15, 16, 17, 18, and 19 were identified as THCA, (24Z)-$\Delta^{24}$-THCA, (24$\xi$,25S)-24-TeHCA, (24$\xi$,25R)-24-TeHCA, and 26-TeHCA, respectively, on the basis of the GLC properties and mass spectral data of the methyl ester–TMS ether derivatives of these bile acids which were identical with the data for the authentic compounds. Since diastereoisomers at C-24 of 24-TeHCA and at C-25 of THCA or 26-TeHCA cannot be separated under the gas–liquid chromatographic conditions that were used, the configurations at C-24 or C-25 of bile acids 15, 17, 18, and 19 remain to be determined. However, (24Z)-$\Delta^{24}$-THCA is separated from the 24E-isomer with shorter RRTs on GLC. Thus, the geometry of bile acid 16 was assigned with certainty as the 24Z form.

**Table 6** shows the approximate composition of unconjugated bile acids. In the CTX patients, more than 50% of the total unconjugated bile acids were $C_{23}$ bile acids and no $C_{27}$ bile acids were found in the bile. In the other subjects, $C_{23}$ bile acids and $C_{27}$ bile acids comprised up to about 5–15% and 23–68% of the total unconjugated bile acids, respectively.

Conjugated bile acid composition is shown in **Table 7.** Almost all of the conjugated bile acids were usual $C_{24}$ bile acids such as cholic acid, deoxycholic acid, and chenodeoxycholic acid. In the cases of two patients with cho-

TABLE 6. Unconjugated bile acid composition in human bile[a]

| Bile Acid | Subject A | B | C[b] | D[b] | E[c] | F[c] |
|---|---|---|---|---|---|---|
| $C_{22}$ Bile acid | nd | nd | nd | nd | 2.3 | tr |
| Bisnorcholic acid | nd | nd | nd | nd | 2.3 | tr |
| $C_{23}$ Bile acids | 3.7 | 7.3 | 7.4 | 2.2 | 16.7 | 4.9 |
| Norchenodeoxycholic acid | tr | tr | 1.8 | 0.6 | nd | nd |
| Norursodeoxycholic acid | nd | nd | 1.0 | tr | nd | nd |
| Allonorcholic acid | tr | 0.5 | 0.1 | 0.3 | 1.2 | 0.6 |
| Norcholic acid | 3.7 | 6.8 | 4.5 | 1.3 | 10.5 | 3.6 |
| 7-Ketonordeoxycholic acid | nd | nd | nd | nd | 5.0 | 0.7 |
| $C_{24}$ Bile acids | 12.1 | 14.9 | 79.2 | 9.3 | 6.4 | 4.9 |
| Chenodeoxycholic acid | 2.7 | 1.3 | 14.2 | 3.1 | 2.1 | nd |
| Ursodeoxycholic acid | tr | 0.3 | 15.3 | 1.3 | nd | nd |
| Deoxycholic acid | 3.7 | 2.3 | 1.6 | nd | 1.5 | nd |
| Allocholic acid | nd | nd | nd | nd | nd | 0.1 |
| Cholic acid | 4.5 | 6.6 | 37.4 | 2.4 | 2.8 | 4.8 |
| 22-Dehydrocholic acid | nd | 0.5 | nd | nd | nd | nd |
| 7-Epicholic acid | 0.6 | 3.1 | 1.7 | 1.2 | nd | nd |
| 7-Ketodeoxycholic acid | 0.6 | 0.9 | 9.0 | 1.3 | nd | nd |
| $C_{27}$ Bile acids | 11.1 | 46.4 | 58.5 | 3.5 | nd | nd |
| THCA | 0.4 | 0.3 | 2.9 | 0.8 | nd | nd |
| (24Z)-$\Delta^{24}$-THCA | 0.6 | 1.5 | 3.6 | 0.4 | nd | nd |
| (24$\xi$,25S)-24-TeHCA | 1.4 | 6.4 | 2.9 | 0.5 | nd | nd |
| (24$\xi$,25R)-24-TeHCA | 1.3 | 5.8 | 2.6 | 0.4 | nd | nd |
| 26-TeHCA | 7.4 | 32.4 | 46.5 | 1.4 | nd | nd |

[a] The values are represented as $\mu$g/ml of bile; nd, not detected; tr, trace amount.
[b] Two patients were treated with ursodeoxycholic acid.
[c] CTX patients.

lelithiasis who had been treated with ursodeoxycholic acid, large amounts of the administered bile acid were also found as glycine- and taurine-conjugates. In contrast to the unconjugate fraction, 7-epicholic acid, 7-ketodeoxycholic acid, and bile acids having shorter or longer side chains were found in amounts less than 1% of total conjugated bile acids.

## DISCUSSION

Until recently, it was supposed that little or no unconjugated bile acids occurred in the bile of humans, at least in healthy subjects, since the normal liver conjugates all bile acids before their secretion into bile (6). The present study clearly confirmed the presence of unconjugated bile acids even in the bile of a healthy individual as well as in the bile of three patients with cholelithiasis and two patients with CTX.

The amount and the composition of unconjugated bile acids in the bile of three patients with cholelithiasis were similar to those of the healthy subject, except for the presence of relatively large amounts of ursodeoxycholic acid and its metabolites in two patients who had received the administration of ursodeoxycholic acid before the sampling of the bile. These results suggested that cholelithiasis does not affect the metabolic profile of bile acids, and the unconjugated bile acids found in the bile of the patients with cholelithiasis are not abnormal metabolites in this pathological condition. In each sample from these four subjects, the composition of unconjugated bile acids was quite different from that of conjugated bile acids due to the presence of large proportions of $C_{23}$ and $C_{27}$ bile acids. The occurrence of the high proportions of $C_{23}$ and $C_{27}$ bile acids in the unconjugate fraction may be explained as the result of the decreased ability of these unusual bile acids to be conjugated during their hepatic passage. It has been noticed by several workers (17–20) that bile acids with short or long side chains are poor substrates for the hepatic conjugating enzymes.

Although norcholic acid had been detected in urine from normal healthy humans (21), the present investigation confirms the occurrence of the $C_{23}$ bile acid in healthy human bile. Nakatomi et al. (20) demonstrated that norcholic acid administered into the rat intestine was quickly absorbed and efficiently secreted into the bile predominantly as the unconjugated form. They have, therefore, proposed that if norcholic acid is formed in human liver, a major part of the $C_{23}$ bile acid is secreted into the bile rather than urine. The present finding of biliary norcholic acid supports their proposal. The origin of norcholic acid and other $C_{23}$ bile acids is obscure. Possibly these $C_{23}$ bile acids may be formed from the corresponding $C_{24}$ bile acids by the shortening of the side chain by one carbon atom ($\alpha$-oxidation). This proposal receives support from the fact that norursodeoxycholic acid was present only in the bile of the patients who had received ursodeoxycholic acid.

Compared to the conjugated bile acid fractions, in which common $C_{24}$ bile acids, cholic acid, chenodeoxycholic acid, and deoxycholic acid predominated, and uncommon $C_{24}$ bile acids, 7-epicholic acid, and 7-ketodeoxycholic acid occurred in trace amounts, relatively large proportions of the latter acid were present in the unconjugate fraction. The difference in the compositions of $C_{24}$ bile acids between the conjugate and the unconjugate

TABLE 7. Conjugated bile acid composition in human bile[a]

| Bile Acid | Subject A | B | C[b] | D[b] | E[c] | F[c] |
|---|---|---|---|---|---|---|
| Glycine-conjugated | | | | | | |
| Lithocholic acid | 0.28 | | | | | |
| Chenodeoxycholic acid | 7.69 | 15.91 | 11.57 | 10.18 | 0.08 | 0.03 |
| Ursodeoxycholic acid | | | 11.25 | 2.94 | | |
| Deoxycholic acid | 5.57 | 16.71 | 1.92 | | 0.34 | |
| Allocholic acid | | | | | | 0.01 |
| Cholic acid | 0.52 | 18.67 | 12.39 | 7.48 | 0.71 | 0.64 |
| Taurine-conjugated | | | | | | |
| Lithocholic acid | 0.25 | 0.54 | | | | |
| Chenodeoxycholic acid | 3.07 | 4.79 | 1.50 | 2.38 | 0.22 | 0.03 |
| Ursodeoxycholic acid | | | 0.94 | 0.24 | | |
| Deoxycholic acid | 1.68 | 4.22 | 0.10 | | 0.98 | |
| Allocholic acid | | | | | | 0.02 |
| Cholic acid | 0.38 | 5.60 | 1.50 | 0.23 | 1.42 | 0.49 |

[a] The values are represented as mg/ml of bile. Only major components (more than 1% of total glycine- or taurine-conjugated bile acids) are listed.
[b] Two patients were treated with ursodeoxycholic acid.
[c] CTX patients.

fractions suggests that the hepatic conjugation of the uncommon $C_{24}$ bile acids is less efficient than that of the common $C_{24}$ bile acids.

22-Dehydrocholic acid, the $\Delta^{22}$-derivative of cholic acid, was found only in one subject. The derivative having a double bond in the side chain of $\beta$-muricholic acid has been detected in rat bile (22–24). The biosynthesis and metabolism of 22-dehydrocholic acid remain fully unknown, though the $\alpha,\beta$-unsaturated bile acid seems to be an intermediate in the pathway of the formation of bisnorcholic acid from cholic acid by $\beta$-oxidation.

According to the most accepted concept for the biosynthesis of cholic acid from cholesterol in human liver, elaboration of the cholesterol nucleus precedes the oxidative degradation of the side chain, forming 5$\beta$-cholestane-3$\alpha$,7$\alpha$,12$\alpha$-triol as an intermediate (25). The side chain degradation starts with a hydroxylation at C-26 of the triol followed by oxidation to yield THCA, which is then transformed into cholic acid by a process analogous to $\beta$-oxidation of fatty acids, involving the intermediary formation of $\Delta^{24}$-THCA and 24-TeHCA (15). The present study revealed the presence of a number of $C_{27}$ bile acids as the major constituents of the biliary unconjugated bile acid fraction in humans. These $C_{27}$ bile acids appear to represent intermediates or simple modification of intermediates in the 26-hydroxylation pathway for the biosynthesis of cholic acid from 5$\beta$-cholestane-3$\alpha$,7$\alpha$,12$\alpha$-triol.

The composition of unconjugated bile acids in the bile of the CTX patients was different from that of the other subjects in the absence of $C_{27}$ bile acids and the presence in higher proportions of bile acids having the short side chain. CTX is a rare inherited disease characterized biochemically by storage of cholestanol in most tissues (26), decreased synthesis of bile acids (27, 28), and accumulation of bile alcohols in bile, urine, and feces (27, 29–32). The metabolic defect in CTX is considered to be the lack of an enzyme that catalyzes the 26-hydroxylation of 5$\beta$-cholestane-3$\alpha$,7$\alpha$,12$\alpha$-triol (33). The lack of 26-hydroxylase activity in CTX patients was supported by the present study. Our CTX patients had no detectable amounts of $C_{27}$ bile acids in their bile.

In the CTX patients, norcholic acid was present as the most predominant bile acid in the unconjugate fraction of biliary bile acids. Because of the decreased production of bile acids in CTX, it is unlikely that cholic acid is an obligatory precursor of norcholic acid in CTX. As a possible route for the formation of norcholic acid in CTX, we postulate that there is degradation of bile alcohols that accumulate in this disease. In CTX, 5$\beta$-cholestane-3$\alpha$,7$\alpha$,12$\alpha$-triol, the substrate for 26-hydroxylase, would tend to accumulate in the liver cell, exposing it to the action of 22-, 23-, 24-, and 25-hydroxylases to result in the formation and accumulation of bile alcohols having one, two, and three hydroxyl groups in their side chain. The cleavage of the bond between C-23 and C-24 of 23-hydroxylated $C_{27}$ bile alcohols would directly lead to the formation of norcholic acid.

This investigation shows that bisnorcholic acid was found only in the bile of the CTX patients whose major bile constituents were bile alcohols, but absent in the bile of the other subjects whose major bile constituent was cholic acid. Monohydroxylated bisnorcholanoic acid derivatives were detected in human meconium (34) and serum (35). These compounds seem to be the microbial metabolites of the corresponding $C_{24}$ bile acids. However, it seems likely that in CTX patients bisnorcholic acid is formed by the degradation of 22-hydroxylated $C_{27}$ bile alcohols rather than cholic acid.

In the present study, only six bile samples from healthy or diseased persons were analyzed. Of course, more cases will have to be studied to evaluate clinical values for biliary unconjugated bile acids. The analysis restricted to biliary unconjugated bile acids for detecting the complete spectrum of unusual bile acids is relatively simple. Thus, this method may afford a useful alternative to the detailed analysis of biliary bile acids with all types of conjugation for the diagnosis of metabolic diseases concerning bile acids, particularly the accumulation or disappearance of unusual bile acids.

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