# Identification of 3β,7β-dihydroxy-5β-cholan-24-oic acid in serum from patients treated with ursodeoxycholic acid

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**Abstract** An unknown bile acid was found by gas–liquid chromatography in the serum of patients who were administered ursodeoxycholic acid for the treatment of cholesterol gallstones. Identification of the chemical structure of the unknown bile acid was performed by the use of gas–liquid chromatography–mass spectrometry. Mass spectrum analysis of the methyl ester trimethylsilyl ether of the bile acid showed explicitly that this is dihydroxy-5β-cholanoic acid, since peaks at m/e 460 and 370 characteristic of methyl ester trimethylsilyl ether of a dihydroxy bile acid were clearly exhibited. Sites of the two hydroxyl groups on the steroid nucleus were determined to be at the 3- and 7-positions by conversion of the bile acid to the corresponding dioxo-cholanoic acid and by comparison of the gas–liquid chromatographic behavior with those of authentic dioxo bile acids. Four authentic 3,7-dihydroxy-5β-cholan-24-oic acids were chemically synthesized and retention times and mass spectra of their methyl ester trimethylsilyl ether derivatives compared precisely with that of the unknown bile acid. The results indicate that the unknown bile acid is 3β,7β-dihydroxy-5β-cholan-24-oic acid. Preliminary experiments suggest that 3β,7β-dihydroxy-5β-cholan-24-oic acid is absent as amino acid-conjugated forms in serum. It is also suggested that the bile acid is excreted into urine but not into bile—**Maeda, M., H. Ōhama, H. Takeda, M. Yabe, M. Nambu, and T. Namihisa.** Identification of 3β,7β-dihydroxy-5β-cholan-24-oic acid in serum from patients treated with ursodeoxycholic acid. *J. Lipid Res.* 1984. **25:** 14–26.

**Supplementary key words** serum bile acids • gas–liquid chromatography • gas–liquid chromatography–mass spectrometry

Ursodeoxycholic acid (3α,7β-dihydroxy-5β-cholan-24-oic acid, UDCA) was first reported to dissolve cholesterol gallstones in man by Makino et al. (1, 2) and Nakagawa et al. (3). Clinical application of UDCA has been studied in many laboratories and serum and biliary levels of the bile acid have been determined (2, 4–8). However, precise analysis of serum bile acids in connection with the metabolic conversion of UDCA has scarcely been done in man. We administered UDCA to patients with radiolucent gallstones and determined serum and biliary levels of bile acids, and we found an unknown peak on the chromatogram when serum bile acids were analyzed by gas–liquid chromatography (GLC) as methyl ester trimethylsilyl (TMS) ether derivatives (9). The unknown peak was characteristic of serum from the patient who received UDCA.

The present report deals with the confirmation of the chemical structure of the unknown compound found in serum by GLC, and contains preliminary information on the metabolism and distribution of the compound in man.

## MATERIALS AND METHODS

### Bile acids

Extra-pure UDCA was obtained from Tokyo Tanabe Co., Ltd. (Tokyo, Japan). The purity of UDCA was checked by thin-layer chromatography (TLC) using Silica Gel G (E. Merck, Darmstadt, see below) and GLC (0.2% Poly I-110, see below). UDCA was calculated to be 99.8% pure as judged by GLC. Chenodeoxycholic acid (3α,7α-dihydroxy-5β-cholan-24-oic acid, CDCA) was obtained from Tokyo Tanabe Co., Ltd. and was demonstrated to be as pure as 99.0% when analyzed by GLC. Cholic acid, deoxycholic acid, lithocholic acid, and 5β-cholan-24-oic acid were purchased from Applied Science Laboratories, Inc. (State College, PA).

3β,7β-Dihydroxy-5β-cholan-24-oic acid was prepared as follows. 3-Oxo-7β-hydroxy-5β-cholan-24-oic acid pre-

Abbreviations: UDCA, 3α,7β-dihydroxy-5β-cholan-24-oic acid; CDCA, 3α,7α-dihydroxy-5β-cholan-24-oic acid; GLC, gas–liquid chromatography; TLC, thin-layer chromatography; MS, mass spectrometry; GLC–MS, gas–liquid chromatography–mass spectrometry; TMS, trimethylsilyl; RRT, relative retention time; $^1$H-NMR, proton magnetic resonance.

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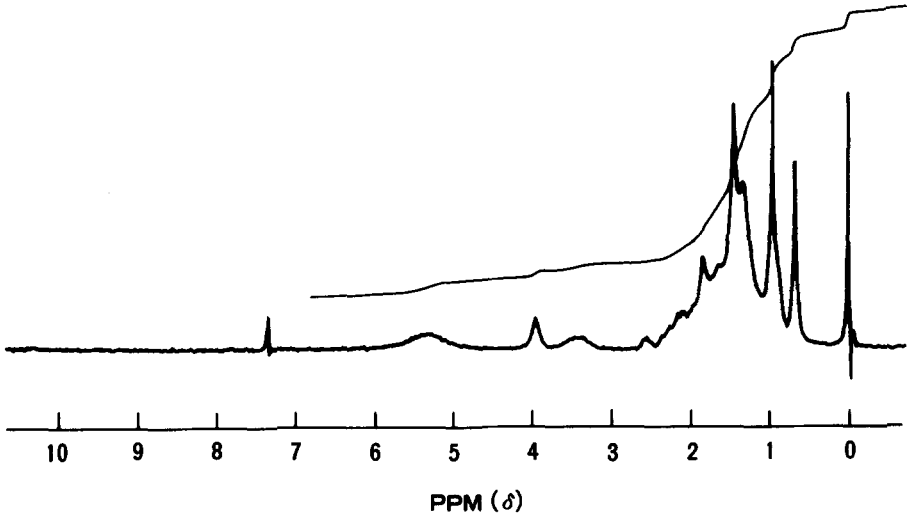


**Fig. 1.** Proton magnetic resonance spectrum of 3$\beta$,7$\beta$-dihydroxy-5$\beta$-cholan-24-oic acid in $CDCl_3$ and DMSO-$d_6$, 8:1 (v/v). Concentration, 120 mg/ml; internal standard, tetramethylsilane.

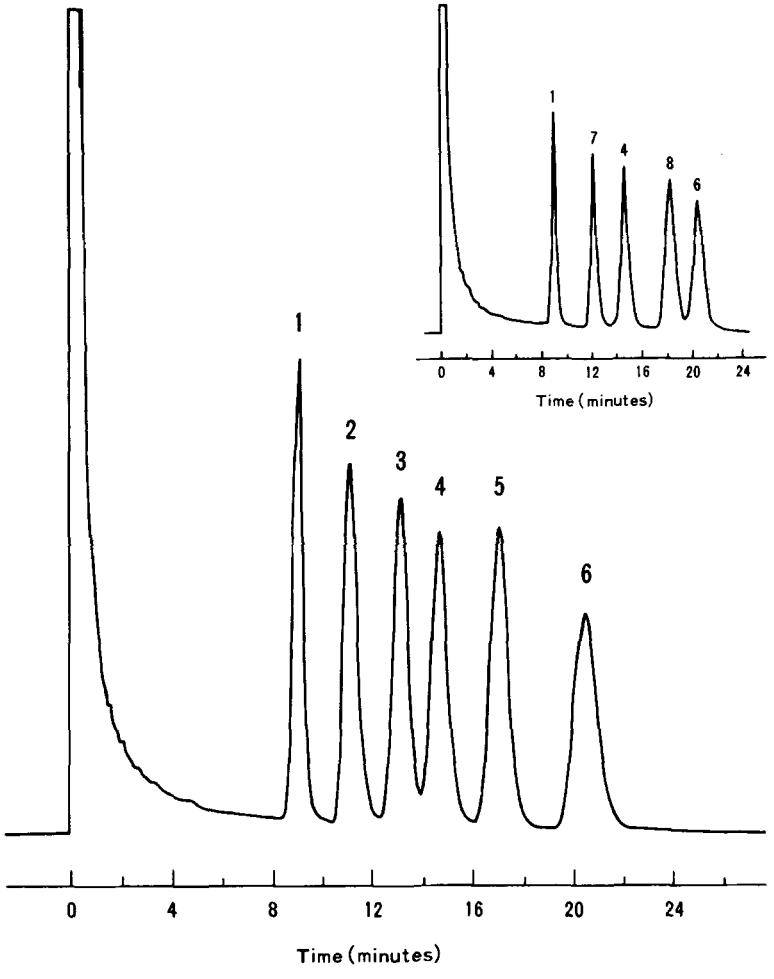


**Fig. 2.** Gas–liquid chromatogram of methyl ester TMS ether derivatives of bile acids. 1) 5$\beta$-Cholan-24-oic acid (internal standard); 2) cholic acid; 3) deoxycholic acid; 4) CDCA; 5) lithocholic acid; 6) UDCA. Inset: gas–liquid chromatogram of methyl ester TMS ether derivatives of authentic 3,7-dihydroxy-5$\beta$-cholan-24-oic acid; 7) 3$\beta$,7$\alpha$-dihydroxy-5$\beta$-cholan-24-oic acid; 8) 3$\beta$,7$\beta$-dihydroxy-5$\beta$-cholan-24-oic acid.

pared by oxidation of UDCA with sodium hypochlorite was dissolved in an alcohol and reduced with sodium. Lower alkyl alcohols were available for this reaction as the solvent. The resulting products were methylated by a conventional procedure. After methyl ursodeoxycholate was predominantly removed by crystallization from 80% aqueous methanol, addition of a large quantity of water to the mother liquor gave a 1:1 mixture of both esters of UDCA and the desired compound as crystals. The mixture was chromatographed on a column of aluminum oxide 90 (E. Merck) with chloroform–carbon tetrachloride 5:6 (v/v). The fraction containing methyl 3$\beta$,7$\beta$-dihydroxy-5$\beta$-cholan-24-oate was subjected to another chromatography on a column of aluminum oxide 90 with benzene–ethyl acetate 65:35 (v/v). After repeating the final step several times, the eluate was evaporated to dryness under reduced pressure and the residue was crystallized from 80% aqueous methanol. The ester was saponified by a conventional method. Crystallization from water gave needles of 3$\beta$,7$\beta$-dihydroxy-5$\beta$-cholan-24-oic acid, mp 184.5°–185.5°C, $[\alpha]_D^{20°}$ + 57.4° (c, 0.5; ethanol). Elemental analysis showed: $C_{24}H_{40}O_4$; calculated: C 73.4, H 10.3; found: C 73.2, H 10.5. The product gave a single spot ($R_f$ value, 0.30) on the TLC plate (see below) and gave a single peak when analyzed by the GLC (see below).

The configuration of hydroxyl residues at C-3 and C-7 carbons of steroid nucleus was confirmed by a proton magnetic resonance ($^1$H-NMR) spectroscopy. The spectrum was obtained using JNM-PMX 60 NMR spectrometer (JEOL, Ltd., Tokyo, Japan) at 25°C. Bhattacharyya and Bankawala (10) described that in UDCA, which is the epimer at C-3 position of this bile acid, the C-3 ($\beta$) and C-7 ($\alpha$) methine protons are both in the axial position and cause a broad signal at approximately 3.45 ppm. On the other hand, the C-7 ($\beta$) skeletal proton in CDCA, the epimer of UDCA at the C-7 position, gives rise to a rather sharp signal at 3.78 ppm, because of equatorial configuration. In this report, the NMR spectrum was interpreted

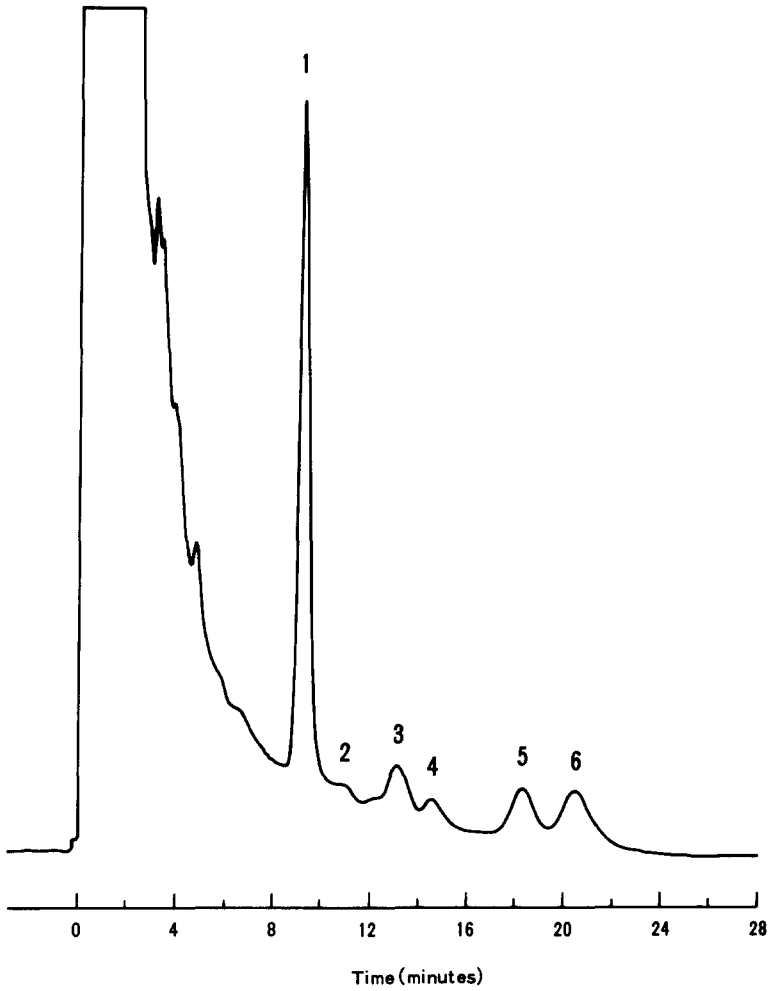


**Fig. 3.** Gas–liquid chromatogram of serum bile acids from the patient K.W. 1) 5$\beta$-Cholan-24-oic acid (internal standard); 2) cholic acid; 3) deoxycholic acid; 4) CDCA; X) unknown bile acid; 6) UDCA.

based on the assignment of NMR signals by Bhattacharyya and Bankawala (10). The NMR spectrum of the synthesized bile acid exhibited two separate signals with broad and sharp peaks at 3.40 and 3.93 ppm, respectively (**Fig. 1**). The C-3 ($\alpha$) and C-7 ($\alpha$) methine protons are in equatorial and axial positions, respectively, in the case of this compound. The C-7 methine proton must cause a broad peak whose chemical shift is essentially the same as that of UDCA, because of its axial position. Therefore, the broad signal observed at 3.40 ppm in Fig. 1 was assigned to be the C-7 skeletal proton. On the other hand, the C-3 methine proton is expected to be shifted to lower field and to cause a relatively sharp signal because of the equatorial configuration. The rather sharp signal at 3.93 ppm in Fig. 1 substantiated this presumption. From these results, this compound was identified to be 3$\beta$,7$\beta$-dihydroxy-5$\beta$-cholan-24-oic acid.

3$\beta$,7$\alpha$-Dihydroxy-5$\beta$-cholan-24-oic acid was prepared according to the method of Danielsson et al. (11); mp 196°C (reported, 193°C), $[\alpha]_D^{23°}$ + 7.3° (c, 2.0; ethanol) (reported, $[\alpha]_D^{23°}$ + 8.5°, c, 1.30; ethanol). 3-Oxo-5$\beta$-cholan-24-oic acid, 3,6-dioxo-5$\beta$-cholan-24-oic acid, 3,7-dioxo-5$\beta$-cholan-24-oic acid, and 3,12-dioxo-5$\beta$-cholan-24-oic acid were purchased from Steraloids Inc. (Wilton, NH). Each of these oxo bile acids gave a single peak by GLC (0.5% OV-25, see below).

### Reagents

Poly I-110 and Silicon OV-25 were obtained from Applied Science Laboratories, Inc. Chromosorb W-HP (80–100 mesh) obtained from Gasukuro Kogyo Co., Ltd. (Tokyo, Japan) gave the best chromatographic separation among the supports purchased from several companies. Amberlite XAD-2 was from Rohm and Haas (Philadelphia, PA) and Sephadex LH-20 was from Pharmacia (Uppsala, Sweden). Solvents and all other reagents were analytical grade and used without further purification.

### Human serum and bile specimens

Blood samples were obtained from twelve patients with cholesterol gallstones and from four healthy subjects with normal liver function as proved by clinical laboratory

TABLE 1. Analysis of bile acids in serum and bile from patients with cholesterol gallstones

| Specimens | Patients | Sex | Age | Duration of Treatment with UDCA | Bile Acids | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | CA | DCA | CDCA | LCA | Unknown | UDCA |
| | | | *yr* | *months* | | | *μg/ml* | | | |
| Serum | | | | | | | | | | |
| Not treated | T.M. | F | 49 | | 1.02 | 0.61 | 1.32 | nd-1[a] | nd-1 | 0.28 |
| | F.H. | M | 38 | | 0.13 | 0.43 | 0.70 | nd-1 | nd-1 | 0.16 |
| | K.O. | F | 46 | | 0.17 | 0.28 | 0.80 | nd-1 | nd-1 | 0.14 |
| | S.O. | M | 50 | | 0.31 | 1.01 | 1.72 | nd-1 | nd-1 | 0.20 |
| | T.Y. | M | 45 | | tr[b] | 0.48 | 0.08 | nd-1 | nd-1 | nd-1 |
| | H.S. | F | 66 | | 0.08 | 0.69 | 0.56 | nd-1 | nd-1 | tr |
| Mean ± SD | | | | | 0.29 ± 0.37 | 0.58 ± 0.25 | 0.86 ± 0.58 | | | 0.20 ± 0.06 |
| Treated | A.K. | F | 35 | 15.0 | 0.20 | 0.60 | 1.00 | nd-1 | 1.70 | 6.34 |
| | M.N. | F | 41 | 8.5 | 0.60 | 0.80 | 2.80 | nd-1 | 1.00 | 14.60 |
| | T.M.* | F | 49 | 22.0 | 0.12 | 2.48 | 0.58 | tr | 0.17 | 0.62 |
| | K.W.** | F | 51 | 20.0 | tr | 0.37 | 0.19 | nd-1 | 0.59 | 0.64 |
| | T.F.** | F | 58 | 10.0 | tr | nd-1 | 0.34 | nd-1 | 0.89 | 0.70 |
| | H.S.** | F | 33 | 6.0 | tr | 0.58 | 0.24 | nd-1 | 0.29 | 0.56 |
| | F.H.* | M | 38 | 13.5 | 1.17 | 0.15 | 3.68 | nd-1 | 0.64 | 5.46 |
| | K.O.* | F | 46 | 11.5 | 0.28 | 0.34 | 1.45 | tr | 0.49 | 3.01 |
| | M.K. | M | 37 | 0.2 | 0.20 | 0.13 | 0.56 | nd-1 | 2.79 | 2.43 |
| Mean ± SD | | | | | 0.42 ± 0.40 | 0.68 ± 0.76 | 1.20 ± 1.24 | | 0.95 ± 0.82 | 3.82 ± 4.59 |
| | | | | | | | *mg/ml* | | | |
| Bile | T.M. | F | 49 | 22.0 | 5.19 | 8.18 | 5.35 | 0.41 | nd-2[c] | 1.09 |
| | K.W. | F | 51 | 20.0 | 4.43 | 4.84 | 5.10 | 0.58 | nd-2 | 4.62 |
| | T.F. | F | 58 | 10.0 | 3.35 | 5.69 | 5.67 | 0.92 | nd-2 | 5.27 |
| | H.S. | F | 33 | 6.0 | 0.81 | 0.81 | 0.55 | 0.07 | nd-2 | 0.29 |
| Mean ± SD | | | | | 3.45 ± 1.91 | 4.88 ± 3.06 | 4.17 ± 2.42 | 0.50 ± 0.35 | | 2.82 ± 2.49 |

[a] Not detected, identification limit: 0.03 μg/ml.
[b] Trace, less than 0.05 μg/ml.
[c] Not detected, identification limit: 0.01 μg/ml.
* Blood samples were obtained before and after treatment with UDCA. ** Biliary bile acids were also determined. Abbreviations: UDCA, ursodeoxycholic acid; CA, cholic acid; DCA, deoxycholic acid; CDCA, chenodeoxycholic acid; LCA, lithocholic acid.

tests. The samples were collected in the morning after an overnight fast. Oral cholecystography demonstrated that all patients had radiolucent gallstones. UDCA (600 mg/day) was given orally to nine patients over a 6-month period. Four healthy subjects who consented to the experiment were administered 200 mg of UDCA, 3 times daily, for 10 days. Blood samples were collected from the subjects before administration of the drug and on the 7th and 10th days of the experimental period. Bile was collected from four patients by aspiration from the duodenum after administration of magnesium sulfate. Serum and bile specimens were stored at −20°C prior to analysis.

### Extraction of bile acids

The bile specimen was mixed with 20 volumes of ethanol and the precipitates were removed. Neutral lipids were removed by repetitive extraction with *n*-hexane under alkaline conditions. The alkaline hydrolysis was carried out at 120°C for 4 hr in a Teflon tube. After extraction of bile acids with diethyl ether, a known amount of 5β-cholan-24-oic acid was added as an internal standard.

Extraction of bile acids from serum and urine with Amberlite XAD-2 column and separation of sulfate from nonsulfate fraction by using Sephadex LH-20 were performed substantially by the method of Makino et al. (12). Solvolysis of the sulfated bile acids was carried out according to the method of Parmentier and Eyssen (13). Removal of neutral lipids and hydrolysis of amino acid conjugates were carried out by the same methods as described above.

### Gas–liquid chromatography

Bile acids were converted into methyl ester TMS ether derivatives according to the methods of Makita and Wells (14) and analyzed on a glass column (2.0 m × 3 mm) packed with 0.2% Poly I-110 on Chromosorb W-HP. All GLC analyses were carried out on a Hitachi Model 163 gas chromatograph (Hitachi, Ltd., Tokyo, Japan) equipped with a flame ionization detector. The column temperature was maintained at 225°C and the flow rate of helium was fixed at 60 ml/min. Under these conditions, peaks of ordinarily occurring bile acids in human serum and bile and peaks of all four epimers of 3,7-dihydroxy-5β-cholan-24-oic acid were well resolved (**Fig. 2**).

Dioxo bile acids were methylated (14) and chromatographed on a column (2.0 m × 3 mm) of 0.5% OV-25 on Chromosorb W-HP. The temperature of the column was 265°C and the flow rate of helium was 60 ml/min.

### Gas–liquid chromatography–mass spectrometry (GLC–MS) and mass chromatography

Analysis by GLC–MS was carried out on a JMS D-300 mass spectrometer (JEOL, Ltd., Tokyo, Japan) coupled to a Model 163 gas chromatograph (Hitachi, Ltd.) and

TABLE 2. Analysis of bile acids in human serum after successive administration of UDCA (600 mg/day) to healthy subjects

| Bile Acids | Time[a] | Subjects (Age/Sex) M.Y. (38/M) | K.K. (38/F) | H.T. (30/M) | H.O. (41/M) | Mean ± SD |
|---|---|---|---|---|---|---|
| | *days* | | *μg/ml* | | | |
| CA | 0 | 0.16 | 0.14 | 0.09 | 0.11 | 0.13 ± 0.03 |
| | 7 | 0.07 | tr | 0.09 | 0.26 | 0.14 ± 0.10 |
| | 10 | 0.09 | 0.11 | 0.09 | 0.19 | 0.12 ± 0.05 |
| DCA | 0 | 0.63 | 1.03 | 1.54 | 1.35 | 1.14 ± 0.40 |
| | 7 | 0.22 | 0.42 | 0.15 | 0.43 | 0.31 ± 0.14 |
| | 10 | 0.17 | 0.58 | 0.08 | 0.44 | 0.32 ± 0.23 |
| CDCA | 0 | 0.48 | 0.45 | 0.24 | 0.20 | 0.34 ± 0.14 |
| | 7 | 0.24 | 0.30 | 0.56 | 0.68 | 0.45 ± 0.21 |
| | 10 | 0.26 | 0.96 | 0.69 | 0.68 | 0.65 ± 0.29 |
| LCA | 0 | 0.12 | 0.10 | 0.15 | nd | 0.12 ± 0.03 |
| | 7 | nd | nd | nd | nd | nd |
| | 10 | nd | nd | nd | nd | nd |
| Unknown | 0 | tr | nd | nd | nd | nd |
| | 7 | 0.47 | 1.09 | 0.56 | 0.43 | 0.64 ± 0.31 |
| | 10 | 0.63 | 1.55 | 0.14 | 1.03 | 0.84 ± 0.60 |
| UDCA | 0 | 0.28 | 0.20 | 0.15 | 0.14 | 0.19 ± 0.06 |
| | 7 | 1.00 | 1.80 | 1.38 | 2.61 | 1.70 ± 0.69 |
| | 10 | 1.29 | 3.83 | 1.76 | 3.20 | 2.52 ± 1.19 |

[a] Days after administration of UDCA; 0, immediately before administration.
tr, Trace, less than 0.05 μg/ml; nd, not detected; abbreviations, see Table 1.

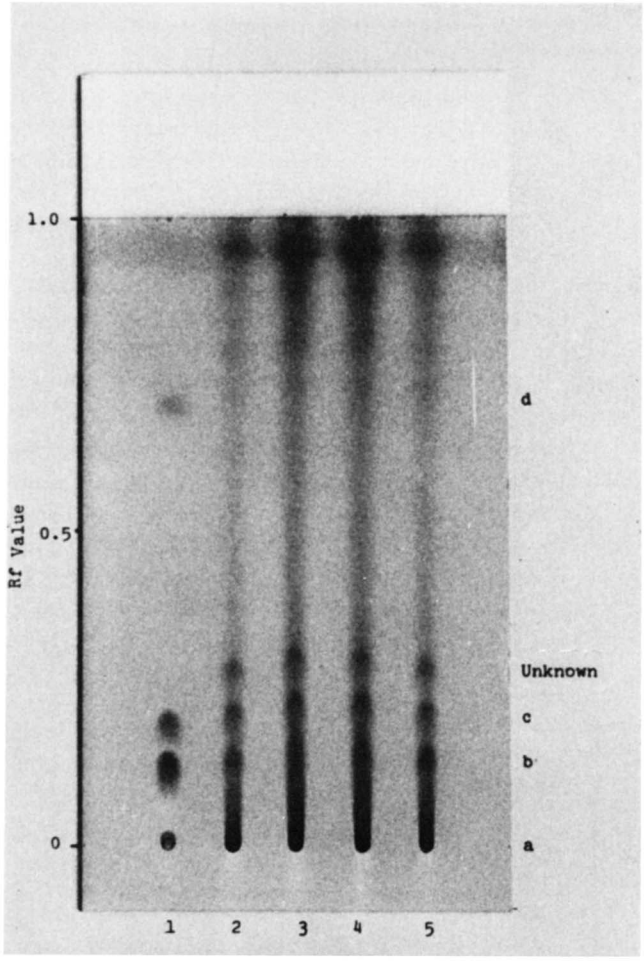


**Fig. 4.** Thin-layer chromatogram of bile acids isolated from sera of the healthy subjects. 1) Authentic bile acids, a, b, c, and d are spots of cholic acid, deoxycholic acid plus CDCA, UDCA, and lithocholic acid, respectively; 2) bile acids isolated from subject M.Y.; 3) from subject K.K.; 4) from subject H.T.; 5) from subject H.O.

equipped with a JMA-2000 disk mass data acquisition system (JEOL, Ltd.). The energy of the bombarding electrons was 20 eV. The temperatures of the ion source and the molecular separator were 175° and 300°C, respectively.

### Thin-layer chromatography (TLC)

TLC was carried out using precoated plates of Silica Gel G (E. Merck) in a solvent system of diethyl ether–acetic acid 50:0.1 (v/v). Plates were developed three times with the same solvent system. Bile acids were detected by spraying the plate with 10% phosphomolybdic acid in ethanol and heating at 105°C.

### Conversion of the hydroxy bile acid into the oxo derivative

The extract containing about 2 $\mu$g of the unknown bile acid was separated on a TLC plate (20 × 20 cm) with the solvent system mentioned above. The bile acid was repeatedly extracted from the silica gel with ethyl acetate and the solvent was removed by evaporation. The recovery of the bile acid was 95% or more. The residue was dissolved in 0.2 ml of acetone and subjected to oxidation with chromium trioxide according to the method described by Djerassi, Engle, and Bowers (15).

## RESULTS

### Analysis of bile acids from patients

GLC analyses of serum and biliary bile acids were carried out on nine and four patients, respectively, who had been treated with UDCA (600 mg, daily). In the present study, solvolysis of the sulfates was not performed unless otherwise mentioned. **Fig. 3** shows the chromatogram of serum bile acids. An unknown compound with an RRT of 1.96 (relative to methyl 5$\beta$-cholan-24-oate) was observed between peaks of lithocholic acid (RRT 1.89) and UDCA (RRT 2.22). The results of serum and biliary bile acid analyses are listed in **Table 1.** In serum from every treated patient we found the unknown peak, while we failed to detect it in sera from six patients who did not receive UDCA. Further, we could not detect any amount of the compound in bile, suggesting that the compound was not excreted into bile. The appearance of the unknown peak was attended by that of UDCA in every assay of serum bile acids. However, there was not any corre-

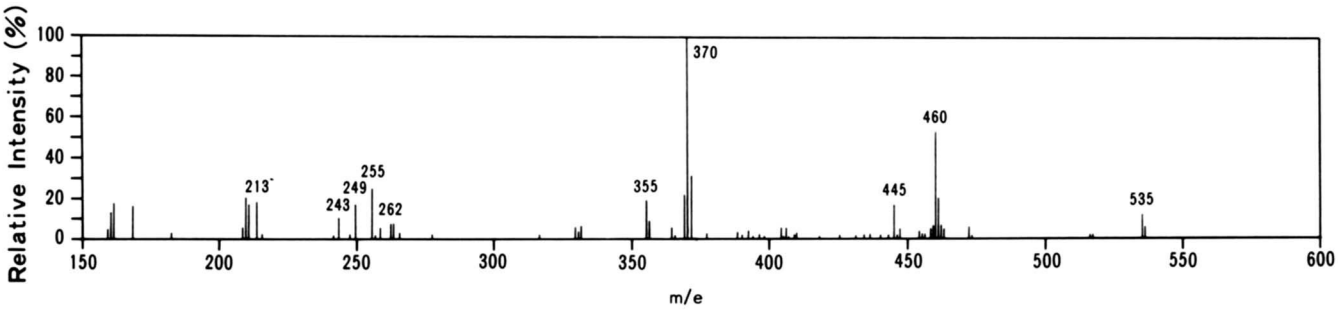


**Fig. 5.** Mass spectrum of methyl ester TMS ether derivative of the unknown bile acid isolated from the patient K.W.

lation between the levels of the compound and of the individual bile acid in serum.

### Analysis of bile acids in serum from healthy subjects

The results of serum bile acid analyses suggested that the appearance of the unknown compound was related to the administration of UDCA. To prove the relationship, the drug was administered to four healthy subjects at the dose of 600 mg a day for 10 days. The results of serum bile acid analyses are summarized in **Table 2.** Despite the appearance of the trace amount of UDCA, the unknown compound was scarcely found in sera before dosing. The compound evidently occurred in all cases after the administration of the drug and the serum level increased upon the successive administration. The unknown compound was also detectable by TLC giving an $R_f$ value of 0.30 with sufficient separation from other bile acids (**Fig. 4**).

### Determination of chemical structure of the unknown compound

**Fig. 5** shows a mass spectrum of the unknown compound obtained by GLC–MS as methyl ester TMS ether derivative. The spectrum showed peaks at m/e 535 (M-15), 460 (M-90), 355 [M-(2 × 90 + 15)], 262 (7-CD-20-24), 255 [M-(2 × 90 + 115)], 249 (CD-20-24), 243, 228 [M-(2 × 90 + 142)], 213 [M-(2 × 90 + 157)], and 201 [M-(2 × 90 + 115 + 54)] with the base peak at m/e 370 [M-(2 × 90)]. Since it was reported from many laboratories that all these ions are characteristic of TMS derivatives of methyl dihydroxycholanoates, e.g., CDCA (16, 17), the unknown compound must be a homolog of a dihydroxy bile acid. The molecular ion peak of a methyl ester TMS ether derivative of a dihydroxy bile acid, m/e 550, was not shown in the spectrum. The peaks at m/e 262, 249, and 243 which were reported to be characteristic of 3,7-bis(trimethylsiloxy) structure in a dihydroxy-5β-

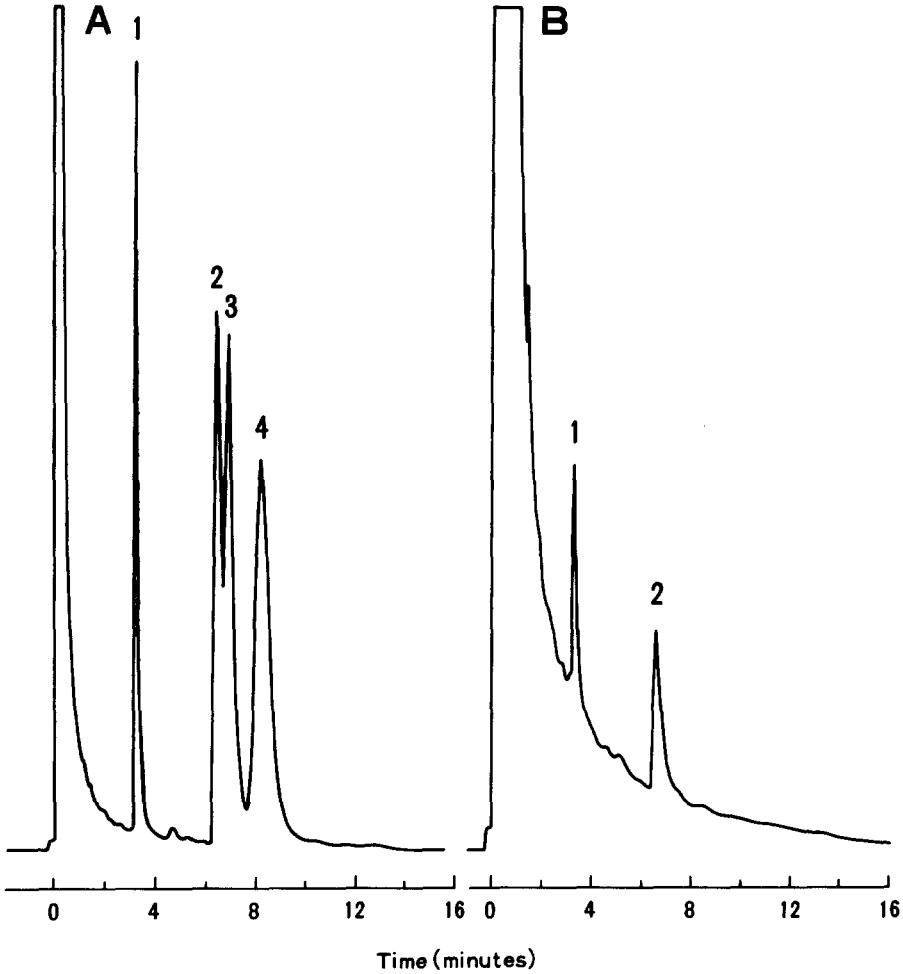


**Fig. 6.** Gas–liquid chromatographic analysis of methyl dioxo-5β-cholan-24-oate. A, Authentic methyl dioxo-5β-cholan-24-oates: 1) 3-oxo-5β-cholan-24-oate (internal standard); 2) 3,7-dioxo-5β-cholan-24-oate; 3) 3,12-dioxo-5β-cholan-24-oate; 4) 3,6-dioxo-5β-cholan-24-oate. B, Methyl ester of oxidized unknown bile acid from the patient A.K.; 1) 3-oxo-5β-cholan-24-oate (internal standard); 2) unknown bile acid.

cholanoate (16, 17) were evident. Particularly, the peak at m/e 243 is a specific ion for 3,7-bis-(trimethylsiloxy) moiety and the structure of the ion and the mechanism of fragmentation were suggested by Sjövall, Eneroth, and Ryhage (16). These results suggested that the unknown bile acid is 3,7-dihydroxy-5β-cholan-24-oic acid.

For further confirmation of the positions of the two hydroxyl groups on the steroid nucleus, the compound was isolated by TLC and led to the corresponding dioxo bile acid by oxidation. The resulting dioxo bile acid was identified by comparing the gas chromatograms (**Fig. 6**) and mass spectra (**Fig. 7**) with those of authentic dioxo-5β-cholan-24-oic acids.

The oxidized compound resulting from the unknown material had an RRT of 1.98 (relative to methyl 3-oxo-5β-cholan-24-oate) and this was identical to that of methyl 3,7-dioxo-5β-cholan-24-oate. The peak having the RRT of 2.54 was assigned to be methyl 3,6-dioxo-5β-cholan-24-oate and several other compounds were probably included in this peak. These were presumed to be degradation products of 3,6-dioxo-5β-cholan-24-oic acid formed in the process of the analytical procedure. The epimeric methyl 3,6-dioxo-cholan-24-oates were not separated from each other by GLC under the conditions employed. In the mass spectrum of methyl 3,6-dioxo-5β-cholan-24-oate, unidentified ion peaks were observed, e.g., m/e 400. These peaks probably represented degradation products. The mass spectrum of the dioxo-cholanoate resulting from the unknown compound was identical to that of methyl 3,7-dioxo-5β-cholan-24-oate among the authentic dioxo bile acids as shown in Fig. 7. From these results also we conclude that the unknown compound is a 3,7-dihydroxy-5β-cholan-24-oic acid.

The configuration of hydroxyl residues at 3- and 7-positions was determined by precise comparison of the mass spectrum of the unknown compound with those of four authentic 3,7-dihydroxy-5β-cholan-24-oic acids. Four combinations of α- and β-configurations on a steroid nucleus are possible for the two hydroxyl residues. The RRTs of these four epimers are listed in **Table 3.** The unknown bile acid showed the same chromatographic behavior as that of 3β,7β-epimer (Table 3). The mass

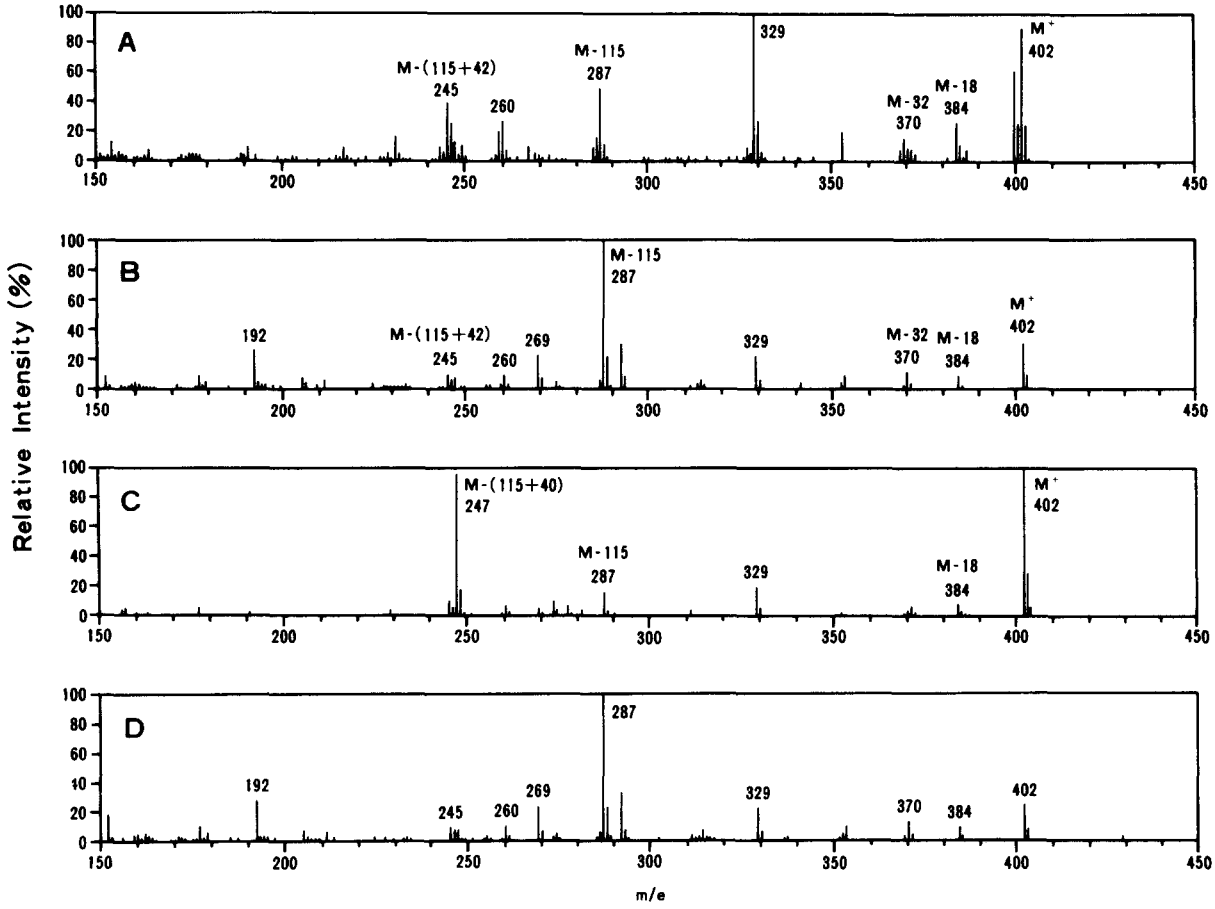


**Fig. 7.** Mass spectra of methyl 3,6-, 3,7-, and 3,12-dioxo-5β-cholan-24-oates and methyl ester of the oxidized unknown bile acid isolated from the healthy subject H.O. A, Methyl 3,6-dioxo-5β-cholan-24-oate; B, methyl 3,7-dioxo-5β-cholan-24-oate; C, methyl 3,12-dioxo-5β-cholan-24-oate; D, methyl ester of oxidized unknown bile acid.

TABLE 3. Gas–liquid chromatographic and mass spectrometric analyses of TMS ethers of methyl 3,7-dihydroxy-5β-cholan-24-oates and unknown bile acid methyl ester in serum from patients with cholesterol gallstones and healthy subjects

| | | Relative Intensities[c] | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | m/e | | | | | | | | | | |
| Bile Acids[a] | RRT[b] | 550 | 535 | 460 | 370 | 355 | 262 | 255 | 249 | 243 | 228 | 213 |
| 3β,7α-diol | 1.32 | | 1 | 175 | 1000 | 332 | 217 | 314 | 148 | 36 | 56 | 194 |
| 3α,7α-diol | 1.59 | | | 14 | 1000 | 170 | 199 | 335 | 145 | 41 | 78 | 156 |
| 3β,7β-diol | 1.96 | | 49 | 550 | 1000 | 342 | 131 | 484 | 200 | 69 | 71 | 250 |
| 3α,7β-diol | 2.22 | 7 | 92 | 1000 | 671 | 225 | 67 | 532 | 117 | 180 | 123 | 248 |
| Unknown from patients | | | | | | | | | | | | |
| A.K. | 1.96 | | 139 | 606 | 1000 | 317 | 117 | 304 | 177 | 183 | 18 | 276 |
| T.M. | 1.96 | | 222 | 597 | 1000 | | | 288 | 160 | 49 | | 486 |
| M.S. | 1.95 | | 125 | 534 | 1000 | 199 | 77 | 260 | 177 | 113 | 13 | 190 |
| T.F. | 1.95 | | 46 | 603 | 1000 | 238 | 98 | 358 | 169 | 169 | 26 | 241 |
| H.S. | 1.96 | | 120 | 599 | 1000 | 297 | 134 | 303 | 209 | 228 | 59 | 271 |
| Mean | 1.96 | | 130 | 588 | 1000 | 263 | 107 | 303 | 178 | 148 | 29 | 293 |
| | | | (63) | (30) | | (54) | (25) | (36) | (19) | (69) | (21) | (113) |
| Unknown from subjects | | | | | | | | | | | | |
| M.Y. | 1.97 | | 51 | 519 | 1000 | 358 | 118 | 520 | 221 | 72 | 53 | 258 |
| K.K. | 1.96 | | 52 | 538 | 1000 | 337 | 134 | 450 | 203 | 81 | 48 | 220 |
| H.T. | 1.95 | | 52 | 547 | 1000 | 579 | 121 | 493 | 170 | 91 | 50 | 420 |
| H.O. | 1.95 | | 52 | 537 | 1000 | 326 | 125 | 449 | 209 | 80 | 51 | 197 |
| Mean | 1.96 | | 52 | 535 | 1000 | 400 | 125 | 478 | 201 | 81 | 51 | 274 |
| | | | (0.5) | (12) | | (120) | (7) | (35) | (22) | (8) | (2) | (101) |

[a] Abbreviations: 3β,7α-diol, 3β,7α-dihydroxy-5β-cholan-24-oic acid; 3α,7α-diol, CDCA; 3β,7β-diol, 3β,7β-dihydroxy-5β-cholan-24-oic acid; 3α,7β-diol, UDCA.

[b] RRT, relative retention time, 5β-cholan-24-oic acid was used as an internal standard.

[c] Intensities of fragment ions were normalized against base peak. Standard deviation is shown in parentheses.

spectra of these four isomeric bile acids are shown in **Fig. 8** and compared with the unknown. The characteristic peaks and their relative intensities are also listed in Table 3. There were no essential differences between the spectra of the compounds isolated from the sera of the patients and those of healthy subjects. The base peaks were observed at m/e 370 with the unknown and the authentic 3,7-dihydroxy bile acids except for UDCA which has a base peak at m/e 460. Both the unknown bile acid and the 3β,7β-epimer exhibited the next most intense peak at m/e 460 to the base peak. On the other hand, the peak at m/e 460 was weak in the spectra of 3α,7α- and 3β,7α-epimers. From the close similarity between the mass spectra of the unknown and 3β,7β-epimer (Fig. 8), as well as the agreement between RRTs of these two bile acids, we conclude that the unknown bile acid is 3β,7β-dihydroxy-5β-cholan-24-oic acid.

## Conjugation

*Serum bile acid.* To determine the ratio between free and amino acid-conjugated bile acid in serum, the analysis was carried out excluding the deconjugation procedure, and the serum level (free) of each individual bile acid was compared with the level (free plus amino acid-conjugated bile acid) determined by the complete method mentioned in Materials and Methods. **Table 4** shows that the ratios of the free to the free plus conjugated 3β,7β-epimers obtained for all healthy subjects were around 1.0, while the ratios for other bile acids varied from 0.36 to 1.00. This result indicates that the 3β,7β-epimer occurs extensively in blood as an unconjugated form. Further, we failed to demonstrate the occurrence of the sulfate of 3β,7β-epimer in serum using the method which included Sephadex LH-20 column chromatography and solvolysis. Since the sulfate fraction from the Sephadex LH-20 column was contaminated with a small amount (about 5%) of the nonsulfate fraction, it was difficult to confirm whether a small GLC peak at the 3β,7β-epimer position was due to the sulfuric acid ester of the 3β,7β-epimer or to a nonsulfated epimer contaminant.

*Urine bile acid.* To establish the metabolic sulfation of 3β,7β-epimer, we analyzed the urine specimen from a UDCA-administered patient. The mass chromatogram demonstrated (**Fig. 9**) that the 3β,7β-epimer was unequivocally identified by the characteristic ions at m/e 370 and 460 in both the sulfate and nonsulfate fractions.

## DISCUSSION

The unknown compound observed in sera from healthy subjects and patients with cholesterol gallstones, who received UDCA, was identified as 3β,7β-dihydroxy-5β-cho-

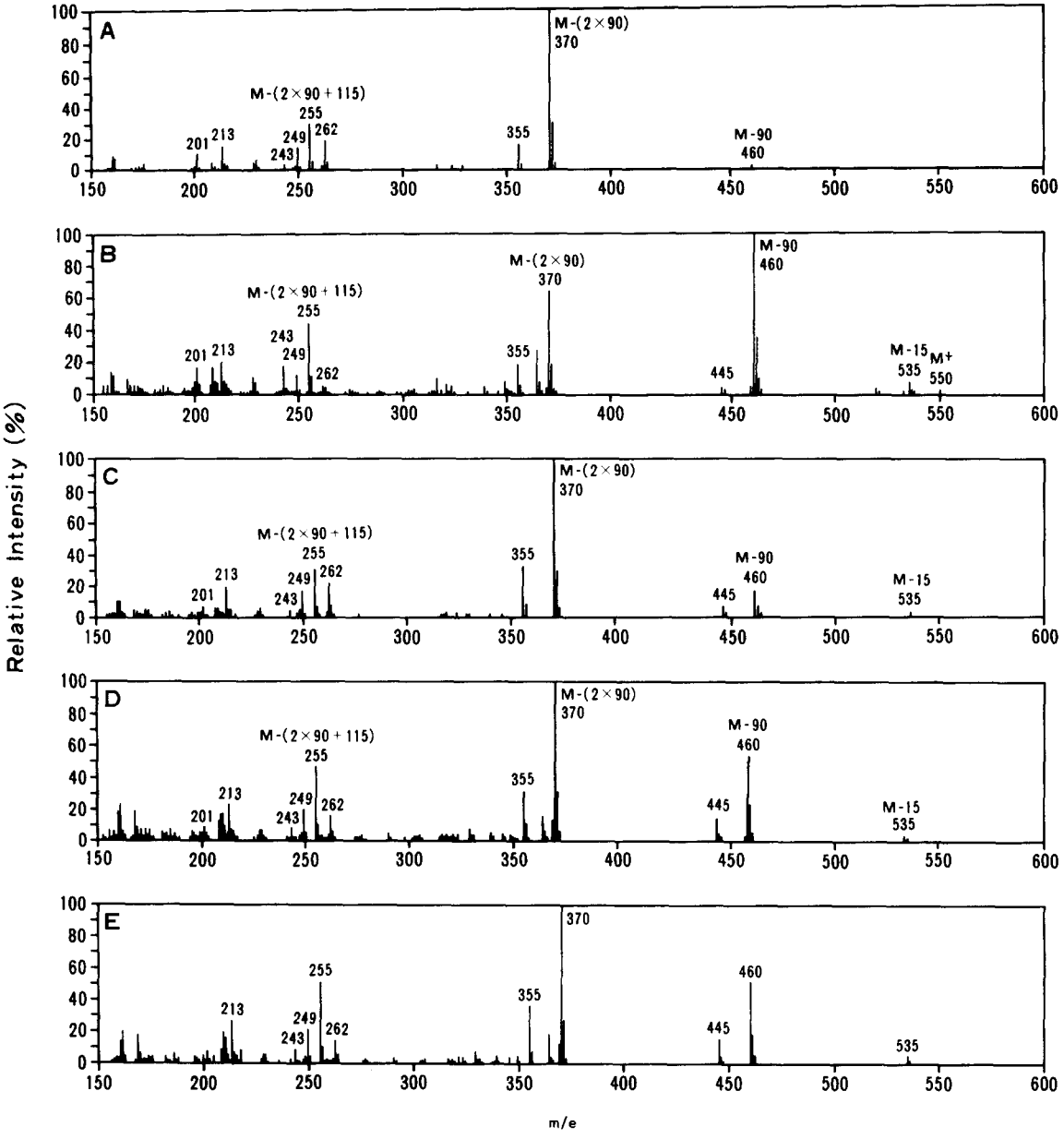


**Fig. 8.** Mass spectra of methyl ester TMS ether derivatives of authentic 3,7-dihydroxy-5$\beta$-cholan-24-oic acid and the unknown bile acid isolated from the healthy subject H.O. A, 3$\alpha$,7$\alpha$-; B, 3$\alpha$,7$\beta$-; C, 3$\beta$,7$\alpha$-; D, 3$\beta$,7$\beta$-; E, unknown bile acid.

lan-24-oic acid by GLC–MS. Nevertheless, the possibility that the unknown compound is an analog of a 3,7-dihydroxy-5$\alpha$-cholan-24-oic acid still remains (16–18). However, we excluded the possibility that the unknown is a 5$\alpha$-analog of 3,7-dihydroxy bile acids by the following considerations. Elliott (17) reported that the mass spectrum of 3,7-dioxo-5$\beta$-cholan-24-oic acid was characteristically different from that of the 5$\alpha$-epimer; in the case of methyl 3,7-dioxo-5$\beta$-cholanoate, the base peak appears at m/e 287 and the peak of the molecular ion (m/e 402) is the next intense peak. On the other hand, the 5$\alpha$-epimer exhibits the base peak at m/e 192 and the ions at m/e 287 and 402 are much less intense. Our results, obtained with the oxidation product of the unknown bile acid, were consistent with the spectrum of the 5$\beta$-epimer cited in the review of Sjövall et al. (16). Consequently, we conclude that the unknown bile acid found in human serum is a 3$\beta$,7$\beta$-epimer.

Palmer and Bolt (19) reported that strong alkaline hydrolysis of bile acid 3-sulfates results in inversion of the 3-hydroxyl group into the epimer of the original compound, on the basis of their experimental results on lithocholic acid sulfate. The possibility that the unknown bile acid might be a by-product formed during the alkaline

TABLE 4. Ratios of free bile acid against free plus conjugated bile acid in serum from healthy subjects administered UDCA 600 mg per day for 7 days

| Bile Acids[a] | | Subjects M.Y. | K.K. | H.T. | H.O. | Mean ± SD |
|---|---|---|---|---|---|---|
| | | | | μg/ml | | |
| CA | F[b] | 0.07 | 0.03 | 0.04 | 0.20 | 0.09 ± 0.08 |
| | T[c] | 0.07 | 0.04 | 0.09 | 0.26 | 0.12 ± 0.10 |
| | F/T | 1.00 | 0.75 | 0.44 | 0.77 | 0.74 ± 0.23 |
| DCA | F | 0.13 | 0.40 | 0.09 | 0.37 | 0.25 ± 0.16 |
| | T | 0.22 | 0.42 | 0.15 | 0.43 | 0.31 ± 0.14 |
| | F/T | 0.59 | 0.95 | 0.60 | 0.86 | 0.75 ± 0.18 |
| CDCA | F | 0.10 | 0.13 | 0.30 | 0.60 | 0.28 ± 0.23 |
| | T | 0.24 | 0.30 | 0.56 | 0.68 | 0.45 ± 0.21 |
| | F/T | 0.42 | 0.43 | 0.54 | 0.88 | 0.57 ± 0.22 |
| LCA | F | | | | | |
| | T | | | | | |
| | F/T | | | | | |
| Unknown | F | 0.46 | 1.12 | 0.56 | 0.43 | 0.64 ± 0.32 |
| | T | 0.47 | 1.09 | 0.56 | 0.43 | 0.64 ± 0.31 |
| | F/T | 0.98 | 1.03 | 1.00 | 1.00 | 1.00 ± 0.02 |
| UDCA | F | 0.36 | 0.94 | 0.78 | 2.06 | 1.04 ± 0.73 |
| | T | 1.00 | 1.80 | 1.38 | 2.61 | 1.70 ± 0.69 |
| | F/T | 0.36 | 0.52 | 0.57 | 0.79 | 0.56 ± 0.18 |

[a] Abbreviations, see Table 1.
[b] F, Free bile acid.
[c] T, Free + conjugated bile acid.

hydrolysis of 3-sulfated UDCA should be considered. However, the serum levels of the unknown bile acid determined without the alkaline hydrolysis were quite consistent with those obtained by the complete analytical method, suggesting that the by-product was not formed during the hydrolysis. In addition, van Berge Henegou-

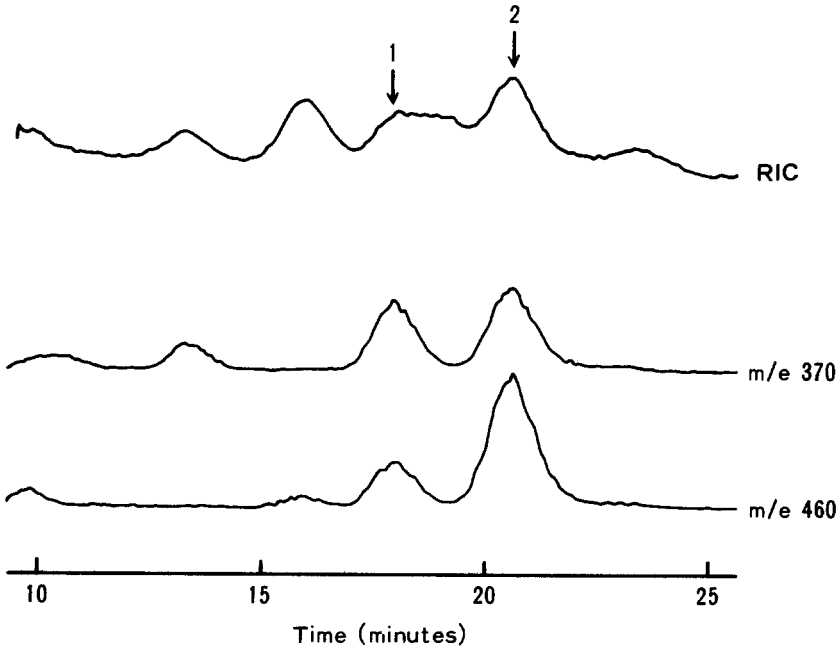


**Fig. 9.** Reconstructed ion chromatogram (RIC) and mass chromatogram of sulfate fraction of unknown bile acid in urine from the patient M.K. 1, Retention time corresponding to 3β,7β-dihydroxy-5β-cholan-24-oic acid; 2, retention time corresponding to UDCA.

wen et al. (20) studied the stability of the 3-sulfated hydroxyl group of bile acids under acidic conditions of solvolysis. They could obtain essentially complete recovery of the original bile acids from the process of solvolysis followed by extraction into ether. The results of van Berge Henegouwen et al. (20) suggested that the formation of by-products, such as an epimerized bile acid, did not occur under the conditions they used. Accordingly, we consider from these facts that the 3$\beta$,7$\beta$-epimer is not formed in the course of the analysis but occurs in human blood.

Although a 3$\beta$,7$\alpha$-epimer as well as UDCA and CDCA was reported in human feces, urine, and bile (21–23), a 3$\beta$,7$\beta$-epimer has not been reported as a naturally occurring bile acid. Whether a 3$\beta$,7$\beta$-epimer occurs naturally or is metabolically formed after administration of UDCA still remains an unresolved question. Eneroth et al. (21) and Almé et al. (22) reported that the formation of a 3$\beta$-epimer is a major process in the intestinal tract. Hirano, Masuda, and Oda (24) also suggested that the 3$\alpha$-hydroxyl group of bile acids is epimerized to a 3$\beta$-configuration by the human intestinal flora. One of the metabolic products of UDCA in the presence of intestinal flora is presumed to be the 3$\beta$-epimer as judged from the mass spectrum, which is nearly identical to that of the unknown compound. Since a considerable amount of 3$\beta$,7$\beta$-epimer was determined in the serum of subjects after subsequent administration of UDCA but not before, it is probable that the 3$\beta$,7$\beta$-epimer is metabolically induced by epimerization of the 3$\alpha$-hydroxyl group in the intestinal tract.

From the results obtained here, it is conceivable that most of the 3$\beta$,7$\beta$-epimer seems to occur as an unconjugated form in serum. All other dihydroxy bile acids were found in blood and bile conjugated with amino acids. The contradiction between these phenomena may be accounted for by a hypothesis that the enzymes that conjugate a bile acid with an amino acid have less affinity for the 3$\beta$-configuration of hydroxyl residue than for the 3$\alpha$-hydroxyl on the steroid nucleus. On the contrary, sulfotransferase is supposed to have a considerable affinity for the 3$\beta$-configuration, since an appreciable amount of 3$\beta$,7$\beta$-epimer was observed in urine.

The 3$\beta$,7$\beta$-epimer was not demonstrated in human bile in this experiment, suggesting that the bile acid was not excreted into bile. The fact that the bile acid was not conjugated with an amino acid is probably related to its absence in bile. O'Máille and Richards (25) reported from their kinetic study in dogs that unconjugated bile acids have a lesser rate of transportation from blood to bile compared to conjugated bile acids. The major excretion route of this bile acid is supposed to be via the urine.

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