

# Analysis of Conjugated Bile Acids in Bile by High-Pressure Liquid Chromatography II. Clinical Application in Bile of Patients with Gallstones

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## Introduction

Bile acids in human bile have been studied by many workers using various analytical methods since the middle of the nineteenth century<sup>3,13,14,17,28,45,50,62,69,73,83,86,88,96,101,104,109</sup>. Of relevance to the etiology of gallstones, a decrease in the pool of bile acids<sup>108</sup>) and some changes in biliary bile acid composition, such as a decreased ratio of trihydroxy to dihydroxy bile acids<sup>14,69,88</sup>) and a decreased ratio of glycine to taurine conjugates<sup>9</sup>), have been noted in cases of cholelithiasis. ADMIRAND and SMALL described the physicochemical properties of bile acids in the formation of cholesterol gallstone in man<sup>1</sup>). The lithogenicity of hepatic and gallbladder bile has been discussed in relation to the pathogenesis of cholesterol gallstones<sup>32,39,98,103</sup>). Moreover, chenodeoxycholic acid and its  $7\beta$ -epimer, ursodeoxycholic acid, have recently been used to dissolve cholesterol gallstone<sup>15,68</sup>), and their effectiveness in many cases has been recognized.

In view of the important roles of bile acids in cholelithiasis, it is essential to know the bile acid composition of human bile in relation to both the pathogenesis and the medical treatment of gallstones. In past studies bile acids were determined by colorimetry<sup>13,91</sup>), or paper<sup>14,45,88</sup>), thin-layer<sup>38,50,74</sup>) or gas-liquid chromatography<sup>62,69,101,108</sup>). These are unsatisfactory for the direct differential determination of each bile acid occurring in human bile or detecting the state of conjugation of the bile acids with either taurine or glycine. Furthermore, there are few reports on the bile acid composition in the bile of patients with bilirubin stones<sup>45</sup>), because of the high incidence of cholesterol stones and the low incidence of bilirubin stones in Europeans and Americans<sup>33,34</sup>).

Recently the author and co-workers developed the analysis of conjugated bile acids by high-pressure liquid chromatography<sup>56,57,58,59</sup>). Using this advanced analytical method, we measured conjugated bile acids in the gallbladder bile of patients with and without gallstones (cholesterol, bilirubin and black stones), and in that of those treated with chenodeoxycholic or ursodeoxycholic acid. Other biliary lipids (cholesterol and phospholipids) were also determined

Key words: Gallstone, Conjugated bile acid, Chenodeoxycholic acid (CDCA), Ursodeoxycholic acid (UDCA), High-pressure liquid chromatography.

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by enzymatic methods<sup>4,37</sup>).

### Materials and Methods

Patients with gallstones were divided into three groups according to the gross appearance and chemical composition of their gallstones<sup>64,65,99</sup>. The first group consisted of 21 patients with cholesterol stones (more than 70% cholesterol), the second group of 14 patients with bilirubin stones (more than 30% bilirubin), and the third group of five patients with black stones which were grossly black and amorphous and had a large amount of black residue on chemical analysis. Eight control patients undergoing laparotomy for gastric cancer had no known hepatic or biliary tract disease. Eight other patients with cholesterol gallstones were treated preoperatively with chenodeoxycholic acid (Eisai Co., Tokyo, Japan), 400 mg per day, for five to 28 days and six patients were treated preoperatively with ursodeoxycholic acid (Tokyo Tanabe Co., Tokyo, Japan), 600 mg per day, for five to 17 days. These patients had well functioning gallbladders and normal liver function tests. All these patients were fed ordinary diets preoperatively, and preoperative administration of antibiotics and drugs affecting lipid metabolism or liver function was strictly avoided (except for cheno- or ursodeoxycholic acid in the experimental treatment group).

Samples of gallbladder bile were collected by direct puncture of the gallbladder cavity immediately after the peritoneal cavity was opened and kept frozen at  $-20^{\circ}\text{C}$  until analysis.

Biliary lipid extraction was performed by SHIODA's method<sup>86</sup>. One or two ml of bile was dropped into 10 times its volume of FOLCH solution and swirled in a vortex-mixer. The solution was filtered through filter paper with suction, and the filter paper was washed with small quantities of FOLCH solution. Distilled water equal to 0.2 times the volume of the filtrates was added, and the mixture was shaken and allowed to separate into two layers. The top layer was removed, and the bottom layer was washed again with distilled water. The second top layer was combined with the first top layer, which contained the conjugated bile acids. Cholesterol and phospholipids were extracted into the bottom layer. Each layer was evaporated to dryness under nitrogen stream.

Extracts of conjugated bile acids were redissolved in methanol equivalent to the original volume of bile, and 10 or 5  $\mu\text{l}$  aliquots were analyzed by a high-pressure liquid chromatography method developed by the author and co-workers<sup>56,57,58,59</sup>. The chromatographic conditions were as follows: column, 8.0 mm (I.D.)  $\times$  300 mm packed with Lichrosorb RP 18 (5  $\mu\text{m}$ ) obtained from Merck (Darmstadt, Germany); mobile phase, methanol/water (75:25, v/v) acidified to pH 2 with phosphoric acid; flow rate, 2.0 ml/min; detection, UV 210 nm; apparatus, Hitachi (Tokyo, Japan) model 635 HPLC instrument.

Total cholesterol in the bile was determined by an enzymatic method<sup>4</sup>) with a TC-Kit K (Nihon Shoji Co., Tokyo, Japan) followed by redissolution in *n*-propanol.

The extracts of phospholipids were suspended in distilled water by ultrasonic agitation, and the enzymatic method<sup>37</sup>) with a PL-Kit K (Nihon Shoji Co., Tokyo, Japan) was used to calculate phospholipids in the bile. When the concentration of cholesterol or phospholipids was high,

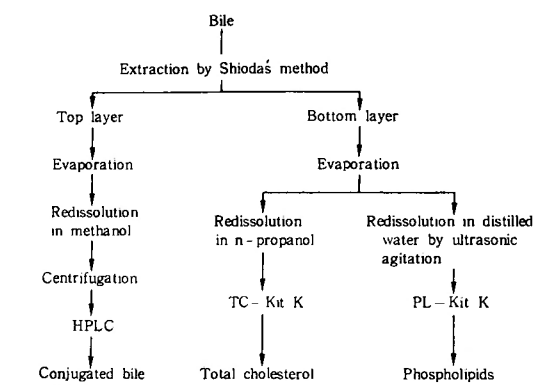


Fig. 1. Diagram of analytical procedures of biliary lipids.

the samples were diluted in *n*-propanol or distilled water. These procedures are shown in Fig. 1.

As a lithogenic index, THOMAS's index<sup>103)</sup> was calculated according to the limits of cholesterol saturation defined by ADMIRAND et al.<sup>1)</sup>, and by HOLZBACH et al.<sup>39)</sup> and HEGARDT et al.<sup>32)</sup>.

## Results

### I) *Bile of patients with and without gallstones*

The absolute concentrations of biliary lipids (total bile acids, cholesterol and phospholipids) are shown in Tables 1-4.

The mean values of total bile acids were  $200.0 \pm 45.9 \mu\text{mol/ml}$  in the control group,  $78.5 \pm 42.3 \mu\text{mol/ml}$  in the cholesterol stone group,  $25.8 \pm 11.8 \mu\text{mol/ml}$  in the bilirubin stone group and  $115.3 \pm 77.6 \mu\text{mol/ml}$  in the black stone group. The mean values of cholesterol were  $20.3 \pm 11.2 \mu\text{mol/ml}$  in the control group,  $8.6 \pm 5.1 \mu\text{mol/ml}$  in the cholesterol stone group,  $3.1 \pm 1.6 \mu\text{mol/ml}$  in the bilirubin stone group and  $6.3 \pm 5.1 \mu\text{mol/ml}$  in the black stone group. The absolute concentrations of both total bile acids and cholesterol were significantly lower in all groups with gallstones than in the controls. The mean values of phospholipids were  $49.2 \pm 19.0 \mu\text{mol/ml}$  in the control group,  $24.6 \pm 15.1 \mu\text{mol/ml}$  in the cholesterol stone group,  $8.7 \pm 4.7 \mu\text{mol/ml}$  in the bilirubin stone group and  $27.1 \pm 24.9 \mu\text{mol/ml}$  in the black stone group. The absolute concentrations of phospholipids in the cholesterol and bilirubin stone group were also significantly lower than in the control group. These are shown in Fig. 2. Thus, the patients with gallstones had lower concentrations of biliary lipids than the patients without gallstones. In the bilirubin stone group, the biliary lipids were especially low.

The molar percentages of total bile acids, cholesterol and phospholipids were not, however, significantly different among the groups, and THOMAS's indices were also not significantly different among the groups. These results are plotted on the triangular coordinates of ADMIRAND and SMALL as shown in Figs. 3-6. In most of the patients with and without gallstones they were in the metastable-labile zone, and in some of the patients with gallstones they were in the micellar zone.

The mean values of the absolute concentrations of taurine and glycine conjugates of each

**Table 1.** Biliary lipid and conjugated bile acid composition in patients without gallstones.

Case	BA. $\mu$ mol/ml (%)	Chol. $\mu$ mol/ml (%)	PL. $\mu$ mol/ml (%)	T.I. (1) (2)	Tauro. $\mu$ mol/ml	Glyco. $\mu$ mol/ml	G/T ratio	TCA $\mu$ mol/ml (%)	TCDC $\mu$ mol/ml (%)	TDCA $\mu$ mol/ml (%)	TUDCA $\mu$ mol/ml (%)	GCA $\mu$ mol/ml (%)	GCDCA $\mu$ mol/ml (%)	GDCA $\mu$ mol/ml (%)	GUDCA $\mu$ mol/ml (%)
1	274.4 (71.1)	36.8 (9.5)	74.9 (19.4)	0.97 1.52	195.2	79.2	0.4	113.5 (41.4)	55.8 (20.3)	25.9 (9.4)	— (—)	36.4 (13.3)	29.8 (10.9)	13.0 (4.7)	— (—)
2	211.4 (70.0)	28.6 (9.5)	62.0 (20.5)	0.95 1.45	3.5	207.9	59.4	3.5 (1.7)	— (—)	— (—)	— (—)	56.1 (26.5)	67.5 (31.9)	79.3 (37.5)	5.0 (2.4)
3	216.2 (68.4)	30.7 (9.7)	69.1 (21.9)	0.97 1.42	39.5	176.7	4.5	16.1 (7.4)	23.4 (10.8)	— (—)	— (—)	54.9 (25.4)	68.1 (31.5)	50.0 (23.1)	3.7 (1.7)
4	115.0 (79.4)	7.0 (4.8)	22.8 (15.7)	0.52 0.93	26.2	88.8	3.4	12.4 (10.8)	13.8 (12.0)	— (—)	— (—)	30.3 (26.3)	48.0 (41.7)	10.5 (9.1)	— (—)
5	179.9 (70.5)	21.3 (8.4)	53.8 (21.1)	0.84 1.27	40.8	139.1	3.4	25.5 (14.2)	11.5 (6.4)	— (—)	3.8 (2.1)	81.6 (45.4)	55.1 (30.6)	2.4 (1.3)	— (—)
6	172.9 (83.9)	5.7 (2.8)	27.4 (13.3)	0.31 0.60	22.1	150.8	6.8	7.8 (4.5)	14.3 (8.3)	— (—)	— (—)	62.4 (36.1)	56.0 (32.4)	28.4 (16.4)	4.0 (2.3)
7	218.6 (78.9)	16.5 (6.0)	42.1 (20.4)	0.64 1.16	22.7	195.9	8.6	8.8 (4.0)	13.8 (6.3)	— (—)	— (—)	67.3 (30.8)	101.4 (46.4)	21.8 (10.0)	5.3 (2.4)
8	211.3 (78.6)	16.0 (6.0)	41.5 (15.4)	0.64 1.15	73.8	137.5	1.9	41.8 (19.8)	32.0 (15.2)	— (—)	— (—)	52.3 (24.7)	58.9 (27.8)	26.3 (12.5)	— (—)
Mean $\pm$ S.D.	200.0 $\pm$ 45.9 (75.1) $\pm$ 5.7	20.3 $\pm$ 11.2 (7.1) $\pm$ 2.6	49.2 $\pm$ 19.0 (17.8) $\pm$ 3.3	0.73 $\pm$ 0.24 1.19 $\pm$ 0.31	53.0 $\pm$ 61.0	147.0 $\pm$ 46.5	11.0 $\pm$ 19.7	28.7 $\pm$ 36.4 (13.0) $\pm$ 12.9	20.6 $\pm$ 17.0 (9.9) $\pm$ 6.2	3.2 $\pm$ 9.1 (1.2) $\pm$ 3.3	0.5 $\pm$ 1.3 (0.3) $\pm$ 0.7	55.2 $\pm$ 16.4 (28.6) $\pm$ 9.4	60.6 $\pm$ 20.5 (31.7) $\pm$ 10.5	28.9 $\pm$ 24.9 (14.3) $\pm$ 11.5	2.3 $\pm$ 2.5 (1.1) $\pm$ 1.2

Abbreviations: BA., bile acids; Chol., cholesterol; PL., phospholipids; T.I., Thomas's index according to the limits of cholesterol saturation defined by Admirand et al. (1), and by Holzbach et al. (2); Tauro., taurine conjugated bile acids; Glyco., glycine conjugated bile acids; G/T ratio, glycine to taurine conjugates ratio; TCA, taurocholic acid; TCDC, taurochenodeoxycholic acid; TDCA, taurodeoxycholic acid; TUDCA, tauroursodeoxycholic acid; GCA, glycocholic acid; GCDCA, glycochenodeoxycholic acid; GDCA, glycodeoxycholic acid; GUDCA, glyoursodeoxycholic acid.

Table 2. Biliary lipid and conjugated bile acid composition in patients with cholesterol stones.

Case	BA. $\mu$ mol/ml (%)	Chol. $\mu$ mol/ml (%)	PL. $\mu$ mol/ml (%)	T.I. (1) (2)	Tauro. $\mu$ mol/ml	Glyco. $\mu$ mol/ml	G/T ratio	TCA $\mu$ mol/ml (%)	TCDCa $\mu$ mol/ml (%)	TDCA $\mu$ mol/ml (%)	TUDCA $\mu$ mol/ml (%)	GCA $\mu$ mol/ml (%)	GCDCA $\mu$ mol/ml (%)	GDCA $\mu$ mol/ml (%)	GUDCA $\mu$ mol/ml (%)
1	114.3 (80.2)	9.3 ( 6.5)	18.9 (13.3)	0.73 1.38	11.9	102.4	8.6	1.9 ( 1.7)	8.0 ( 7.0)	— ( — )	2.0 ( 1.7)	23.5 (20.6)	70.1 (61.3)	— ( — )	8.8 ( 7.7)
2	119.5 (77.0)	11.4 ( 7.3)	24.3 (15.7)	0.78 1.39	26.4	93.1	3.5	9.0 ( 7.5)	8.7 ( 7.3)	7.9 ( 6.6)	0.9 ( 0.8)	20.5 (17.2)	45.0 (37.7)	23.2 (19.4)	4.3 ( 3.6)
3	43.3 (74.3)	3.9 ( 6.7)	11.1 (19.0)	0.68 1.11	16.5	26.9	1.6	6.5 (15.0)	6.2 (14.3)	2.0 ( 4.6)	1.8 ( 4.2)	8.4 (19.3)	13.3 (30.7)	5.2 (11.9)	— ( — )
4	78.7 (68.7)	11.2 ( 9.8)	24.6 (21.5)	0.98 1.45	12.6	61.1	4.8	4.1 ( 5.6)	4.7 ( 6.4)	2.0 ( 2.7)	1.8 ( 2.4)	14.0 (19.0)	24.0 (32.5)	22.2 (30.1)	1.0 ( 1.4)
5	25.0 (66.0)	3.2 ( 8.4)	9.7 (25.6)	0.84 1.12	9.7	15.3	1.6	3.9 (15.6)	4.5 (18.0)	1.3 ( 5.2)	— ( — )	5.4 (21.6)	6.0 (24.0)	3.6 (14.4)	0.3 ( 1.2)
6	134.1 (68.2)	13.2 ( 6.7)	49.3 (25.1)	0.67 0.91	17.5	116.6	6.7	6.5 ( 4.8)	10.2 ( 7.6)	0.6 ( 0.4)	— ( — )	28.3 (21.1)	63.1 (47.7)	25.2 (18.8)	— ( — )
7	66.0 (70.5)	8.2 ( 8.8)	19.4 (20.7)	0.88 1.34	13.3	52.7	4.0	4.6 ( 7.0)	5.7 ( 8.6)	— ( — )	3.0 ( 4.5)	16.0 (24.2)	26.8 (40.6)	8.1 (12.3)	1.8 ( 2.7)
8	54.4 (63.7)	7.5 ( 8.8)	23.5 (27.5)	0.88 1.12	4.4	50.0	11.4	0.4 ( 0.7)	1.1 ( 2.0)	2.0 ( 3.7)	0.9 ( 1.7)	5.4 ( 9.9)	24.9 (45.8)	18.0 (33.1)	1.7 ( 3.1)
9	21.8 (72.7)	3.1 (10.3)	5.1 (17.0)	1.07 1.80	5.5	16.3	3.0	2.1 ( 9.6)	1.2 ( 5.5)	2.2 (10.1)	— ( — )	4.1 (18.8)	4.7 (21.6)	7.5 (34.4)	— ( — )
10	19.0 (59.9)	3.4 (10.7)	9.3 (29.4)	1.10 1.31	4.1	14.9	3.7	2.2 (11.8)	1.3 ( 6.7)	— ( — )	0.6 ( 3.1)	5.5 (29.1)	7.9 (41.5)	0.4 ( 2.0)	1.1 ( 5.9)
11	29.3 (40.6)	8.4 (11.7)	34.4 (47.7)	2.56 2.59	13.2	16.1	1.2	7.4 (25.4)	5.8 (19.7)	— ( — )	— ( — )	4.8 (16.5)	9.0 (30.6)	2.3 ( 7.8)	— ( — )
12	106.8 (88.2)	3.3 ( 2.7)	11.0 ( 9.1)	0.35 0.71	24.4	82.4	3.4	10.4 ( 9.7)	11.7 (11.0)	— ( — )	2.3 ( 2.2)	33.7 (31.5)	47.3 (44.0)	— ( — )	1.7 ( 1.6)
13	129.2 (60.9)	19.4 ( 6.2)	63.5 (29.9)	0.94 1.12	48.8	80.4	1.7	12.5 ( 9.5)	11.6 ( 9.0)	2.2 ( 1.7)	22.6 (17.5)	32.0 (24.8)	39.4 (30.6)	8.7 ( 6.9)	— ( — )
14	105.2 (68.2)	13.2 ( 8.6)	35.8 (23.2)	0.85 1.21	20.5	84.7	4.1	12.2 (11.6)	8.4 ( 7.9)	— ( — )	— ( — )	35.9 (34.2)	45.5 (43.2)	3.2 ( 3.1)	— ( — )
15	22.5 (67.4)	1.9 ( 5.7)	9.0 (26.9)	0.57 0.75	4.9	17.5	3.6	2.1 ( 9.3)	2.8 (12.6)	— ( — )	— ( — )	6.1 (27.1)	8.9 (39.6)	2.5 (11.4)	— ( — )

16	92.8 (62.6)	17.5 (11.8)	38.0 (25.6)	1.18 1.53	13.5	79.3	5.9	5.2 ( 5.6)	5.6 ( 6.1)	2.7 ( 2.9)	— ( —)	23.2 (25.0)	33.3 (35.8)	21.7 (23.4)	1.1 ( 1.2)
17	56.5 (67.5)	9.3 (11.1)	17.8 (21.3)	1.11 1.64	8.7	47.8	5.5	4.4 ( 7.8)	4.3 ( 7.6)	— ( —)	— ( —)	14.3 (25.3)	14.1 (24.9)	19.4 (34.4)	— ( —)
18	76.7 (78.4)	5.6 ( 5.7)	15.5 (15.8)	0.61 1.08	15.8	60.9	3.9	— ( —)	7.1 ( 9.3)	8.7 (11.3)	— ( —)	12.5 (16.3)	21.0 (27.3)	25.4 (33.1)	2.0 ( 2.7)
19	163.6 (73.1)	16.1 ( 7.2)	44.0 (19.7)	0.73 1.16	28.0	135.6	4.8	11.5 ( 7.0)	12.4 ( 7.6)	4.1 ( 2.5)	— ( —)	39.9 (24.4)	40.3 (24.6)	50.1 (30.6)	5.3 ( 3.2)
20	99.2 (76.9)	6.4 ( 5.0)	23.4 (18.1)	0.51 0.86	15.8	83.4	5.3	6.8 ( 6.8)	8.0 ( 8.1)	1.0 ( 1.0)	— ( —)	31.6 (31.8)	31.9 (32.1)	20.0 (20.2)	— ( —)
21	90.2 (72.0)	5.8 ( 4.6)	29.3 (23.4)	0.46 0.67	21.6	68.6	3.2	12.9 (14.3)	8.7 ( 9.6)	— ( —)	— ( —)	38.1 (42.2)	30.5 (33.9)	— ( —)	— ( —)
Mean	78.5	8.6	24.6	0.88	16.1	62.2	4.3	6.0	6.6	1.8	1.7	21.0	26.7	11.5	3.0
+S.D.	±42.3 ( 69.4) (±9.5)	±5.1 ( 8.0) (±2.5)	±15.1 ( 22.7) (±7.9)	±0.45 1.25 ±0.43	±10.3	±35.6	±2.4	±4.1 ( 8.9) (±5.7)	±3.4 ( 9.1) (±4.1)	±2.5 ( 2.8) (±3.4)	±4.9 ( 1.8) (±3.9)	±15.6 ( 25.6) (±9.5)	±17.4 ( 32.5) (±12.0)	±12.7 ( 15.6) (±13.1)	±6.3 ( 3.7) (±7.5)

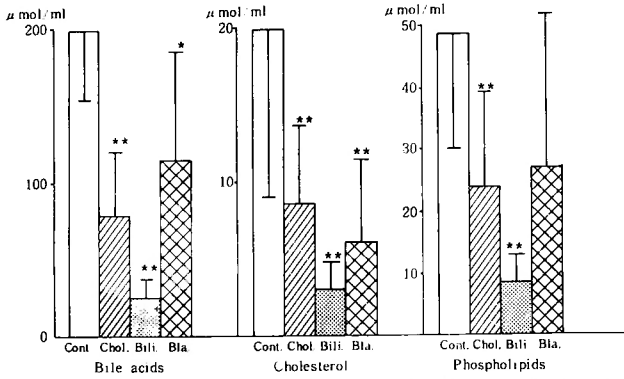
Table 3. Biliary lipid and conjugated bile acid composition in patients with bilirubin stones.

Case	BA. μ mol/ml (%)	Chol. μ mol/ml (%)	PL. μ mol/ml (%)	T.I. (1) (2)	Tauro. μ mol/ml	Glyco. μ mol/ml	G/T ratio	TCA μ mol/ml (%)	TCDC μ mol/ml (%)	TDCA μ mol/ml (%)	TUDCA μ mol/ml (%)	GCA μ mol/ml (%)	GCDCA μ mol/ml (%)	GDCA μ mol/ml (%)	GUDCA μ mol/ml (%)
1	19.4 (70.3)	2.1 ( 7.6)	6.1 (22.1)	0.76 1.12	5.8	13.6	2.3	2.4 (12.5)	3.4 (17.4)	— ( —)	— ( —)	2.7 (13.7)	9.2 (47.6)	— ( —)	1.7 ( 8.8)
2	25.7 (71.8)	2.3 ( 6.4)	7.8 (21.8)	0.64 0.97	4.5	21.2	4.7	0.5 ( 2.0)	2.7 (10.5)	1.3 ( 5.1)	— ( —)	3.4 (13.2)	10.6 (41.2)	6.7 (26.1)	0.5 ( 2.0)
3	11.7 (55.7)	2.3 (11.0)	7.0 (33.3)	1.19 1.30	6.6	5.1	0.8	4.8 (41.0)	1.8 (15.4)	— ( —)	— ( —)	2.7 (23.1)	2.4 (20.5)	— ( —)	— ( —)
4	34.2 (71.0)	4.2 ( 8.7)	9.8 (20.3)	0.88 1.35	12.1	22.1	1.8	4.9 (14.4)	6.0 (17.7)	— ( —)	1.1 ( 3.1)	8.7 (25.3)	10.7 (31.4)	2.8 ( 8.1)	— ( —)
5	46.0 (70.4)	5.2 ( 8.0)	14.1 (21.6)	0.80 1.19	19.7	26.3	1.3	11.1 (24.1)	7.3 (15.9)	1.3 ( 2.8)	— ( —)	10.8 (23.5)	15.5 (33.7)	— ( —)	— ( —)
6	10.1 (66.9)	1.6 (10.6)	3.4 (22.5)	1.05 1.51	8.7	1.4	0.2	6.7 (66.3)	2.0 (19.8)	— ( —)	— ( —)	1.1 (10.9)	0.3 ( 3.0)	— ( —)	— ( —)
7	26.6 (70.7)	3.2 ( 8.5)	7.8 (20.8)	0.86 1.30	7.4	19.2	2.6	2.0 ( 7.5)	3.7 (13.9)	1.7 ( 6.4)	— ( —)	2.8 (10.5)	10.2 (38.3)	6.2 (23.3)	— ( —)
8	28.8 (57.7)	6.7 (13.4)	14.4 (28.9)	1.38 1.63	9.6	19.3	2.0	4.0 (13.8)	4.2 (14.6)	1.4 ( 4.8)	— ( —)	7.9 (27.4)	7.5 (25.9)	3.9 (13.5)	— ( —)
9	41.2 (63.9)	4.1 ( 6.3)	19.2 (29.8)	0.64 0.79	23.2	18.0	0.8	13.9 (33.6)	8.4 (20.4)	— ( —)	1.0 ( 2.4)	7.8 (19.0)	10.1 (24.6)	— ( —)	— ( —)
10	21.4 (70.6)	2.2 ( 7.3)	6.7 (22.1)	0.73 1.07	6.0	15.4	2.5	3.0 (14.3)	1.7 ( 7.7)	1.4 ( 6.3)	— ( —)	5.7 (26.8)	5.2 (24.4)	4.4 (20.5)	— ( —)
11	21.7 (77.5)	1.6 ( 5.7)	4.7 (16.8)	0.60 1.04	0.5	21.2	41.6	— ( —)	0.5 ( 2.3)	— ( —)	— ( —)	4.5 (20.5)	12.9 (59.4)	— ( —)	3.8 (17.7)
12	44.0 (72.4)	4.2 ( 6.9)	12.6 (20.7)	0.70 1.07	4.2	39.9	9.6	1.2 ( 2.7)	3.0 ( 6.7)	— ( —)	— ( —)	11.9 (27.1)	7.3 (16.5)	20.7 (47.0)	— ( —)
13	17.5 (70.9)	2.2 ( 8.9)	5.0 (20.2)	0.90 1.38	3.2	14.2	4.4	1.3 ( 7.7)	1.9 (10.9)	— ( —)	— ( —)	5.8 (33.3)	8.4 (48.2)	— ( —)	— ( —)
14	13.4 (76.1)	1.0 ( 5.7)	3.2 (18.2)	0.59 0.98	8.6	4.8	0.6	6.1 (45.6)	2.5 (18.6)	— ( —)	— ( —)	3.1 (23.4)	1.7 (12.4)	— ( —)	— ( —)
Mean	25.8	3.1	8.7	0.84	8.6	17.3	5.4	4.4	3.5	0.5	0.2	5.6	8.0	3.2	0.4
±S.D.	±11.8	±1.6	±4.7	±0.23	±6.2	±9.8	±10.9	±4.0	±2.3	±0.7	±0.4	±3.3	±4.3	±5.6	±1.1
	( 69.0)	( 8.2)	( 22.8)	1.19				( 20.4)	( 13.7)	( 1.8)	( 0.4)	( 21.3)	( 30.5)	( 9.9)	( 2.0)
	(±6.2)	(±2.2)	(±4.6)	±0.23				(±19.4)	(±5.4)	(±2.7)	(±1.0)	(±7.0)	(±15.4)	(±14.5)	(±5.1)

**Table 4.** Biliary lipid and conjugated bile acid composition in patients with black stones.

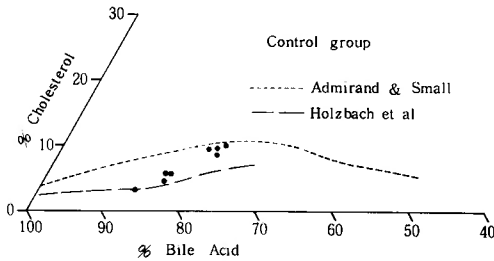
Case	BA. $\mu$ mol/ml (%)	Chol. $\mu$ mol/ml (%)	PL. $\mu$ mol/ml (%)	T.I. (1) (2)	Tauro. $\mu$ mol/ml	Glyco. $\mu$ mol/ml	G/T ratio	TCA $\mu$ mol/ml (%)	TCDC $\mu$ mol/ml (%)	TDCA $\mu$ mol/ml (%)	TUDCA $\mu$ mol/ml (%)	GCA $\mu$ mol/ml (%)	GCDC $\mu$ mol/ml (%)	GDCA $\mu$ mol/ml (%)	GUDCA $\mu$ mol/ml (%)
1	179.1 (87.9)	2.4 (1.2)	22.6 (10.9)	0.14 0.28	23.4	155.7	6.7	6.0 (3.4)	11.2 (6.3)	1.4 (0.8)	4.8 (2.7)	32.6 (18.2)	96.4 (53.8)	7.5 (4.2)	19.2 (10.7)
2	34.8 (72.0)	3.5 (7.2)	10.0 (20.7)	0.73 1.12	3.2	31.6	9.9	1.5 (4.3)	1.7 (4.9)	— (—)	— (—)	10.8 (31.0)	11.3 (32.5)	9.5 (27.3)	— (—)
3	202.9 (71.8)	11.2 (4.0)	68.5 (24.2)	0.39 0.56	45.6	157.3	3.4	10.5 (5.2)	32.0 (15.8)	— (—)	3.1 (1.5)	37.9 (18.7)	107.8 (53.1)	— (—)	11.6 (5.7)
4	116.2 (74.0)	12.4 (7.9)	28.5 (18.1)	0.81 1.34	13.6	102.6	7.6	6.2 (5.4)	2.6 (2.2)	4.8 (4.1)	— (—)	22.8 (19.6)	42.6 (36.7)	34.0 (29.2)	3.2 (2.8)
5	41.0 (83.7)	2.0 (4.1)	6.0 (12.2)	0.47 0.92	14.2	26.8	1.9	7.1 (17.3)	7.1 (17.3)	— (—)	— (—)	12.2 (29.8)	14.6 (35.6)	— (—)	— (—)
Mean	115.3	6.3	27.1	0.51	20.0	94.8	5.9	6.3	10.9	1.2	1.6	23.3	54.5	10.2	6.8
$\pm$ S.D.	$\pm 77.6$	$\pm 5.1$	$\pm 24.9$	$\pm 0.27$	$\pm 16.0$	$\pm 63.8$	$\pm 3.2$	$\pm 3.2$	$\pm 12.4$	$\pm 2.1$	$\pm 2.3$	$\pm 12.1$	$\pm 45.2$	$\pm 14.0$	$\pm 8.4$
	(77.9) ( $\pm 7.4$ )	(4.9) ( $\pm 2.7$ )	(17.3) ( $\pm 5.6$ )	0.84 $\pm 0.43$				(7.1) ( $\pm 5.8$ )	(9.3) ( $\pm 6.8$ )	(1.0) ( $\pm 1.8$ )	(0.8) ( $\pm 1.2$ )	(23.5) ( $\pm 6.4$ )	(42.3) ( $\pm 10.3$ )	(12.1) ( $\pm 14.8$ )	(3.8) ( $\pm 4.5$ )



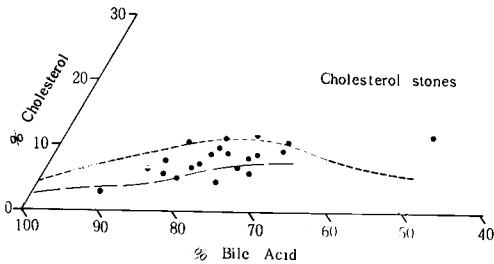


**Fig. 2.** Biliary lipid composition in patients with and without gallstones (Mean ± S.D.).  
 \*; p < 0.05, \*\*; p < 0.01  
 Abbreviations: Cont., patients without gallstones; Chol., patients with cholesterol stones; Bili., patients with bilirubin stones; Bla., patients with black stones.

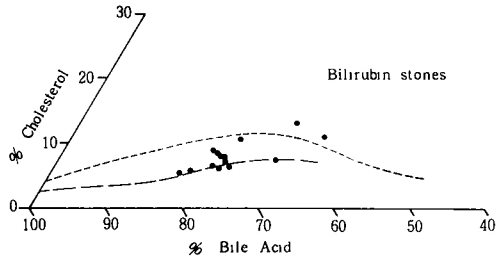
group are shown in Fig. 7. The taurine conjugates were significantly low in the cholesterol and bilirubin stone groups (mean ± S.D., 16.1 ± 10.3 μmol/ml and 8.6 ± 6.2 μmol/ml, respectively). The taurine conjugates of the black stone group were less than those of the control group, but not significantly so (mean ± S.D., 20.6 ± 16.0 μmol/ml vs. 53.0 ± 61.0 μmol/ml). The mean values of the glycine conjugates were 147.0 ± 46.5 μmol/ml in the control group, 62.2 ± 35.6 μmol/ml in the cholesterol stone group, 17.3 ± 9.8 μmol/ml in the bilirubin stone group and 94.8 ± 63.8 μmol/ml in the black stone group. This decrease was significant in the cholesterol and bilirubin stone groups, and the difference between the bilirubin group and the control group was greater



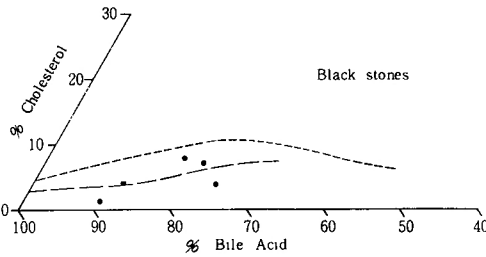
**Fig. 3.** Relative concentrations plotted on a triangular coordinate of biliary lipids in patients without gallstones.



**Fig. 4.** Relative concentrations of biliary lipids plotted on a triangular coordinate in patients with cholesterol stones.



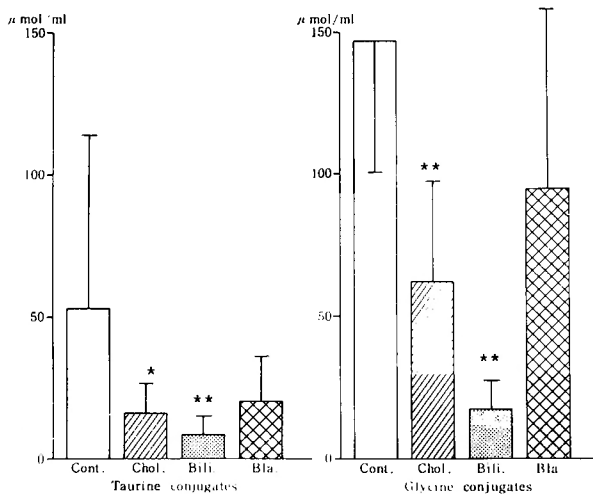
**Fig. 5.** Relative concentrations of biliary lipids plotted on a triangular coordinate in patients with bilirubin stones.



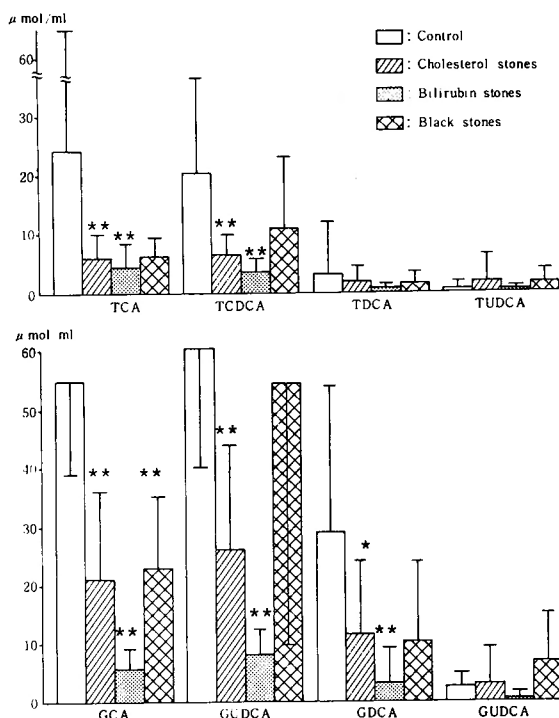
**Fig. 6.** Relative concentrations of biliary lipids plotted on a triangular coordinate in patients with black stones.

than that between the cholesterol group and the control group. On the contrary, there was no significant difference between the black stone group and the control group.

The mean values of individual conjugated bile acids are shown in Fig. 8. In both the cholesterol and bilirubin stone groups, the absolute concentrations of taurocholic, taurochenodeoxycholic, glycocholic, glycochenodeoxycholic and glycodeoxycholic acid were lower than in the control group, whereas no significant differences of conjugated bile acids, except for



**Fig. 7.** Taurine and glycine conjugates in patients with and without gallstones (Mean  $\pm$  S.D.). \*;  $p < 0.05$ , \*\*;  $p < 0.01$



**Fig. 8.** Conjugated bile acid concentrations in patients with and without gallstones (Mean  $\pm$  S.D.). \*;  $p < 0.05$ , \*\*;  $p < 0.01$

glycocholic acid, were found between the black stone and the control group. Moreover, the decreases of glycocholic and glycochenodeoxycholic acids were greater in the bilirubin stone group than in the cholesterol stone group.

Figs. 9-12 show the chromatograms of conjugated bile acids analyzed by high-pressure liquid chromatography in cases of cholesterol, bilirubin and black stones and in controls.

## II) *Bile of patients treated with chenodeoxycholic acid or ursodeoxycholic acid.*

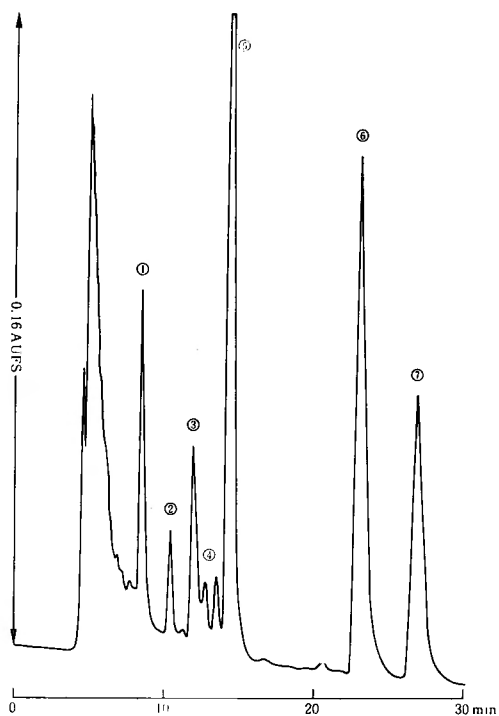
The levels of biliary lipids and conjugated bile acids in patients treated with chenodeoxycholic acid, 400 mg per day, are listed in Table 5.

The mean values of total bile acids increased significantly during chenodeoxycholic acid administration (mean  $\pm$  S.D.,  $143.3 \pm 74.4 \mu\text{mol/ml}$  vs.  $78.5 \pm 42.3 \mu\text{mol/ml}$ ), but the other biliary lipids showed no significant change (Fig. 13).

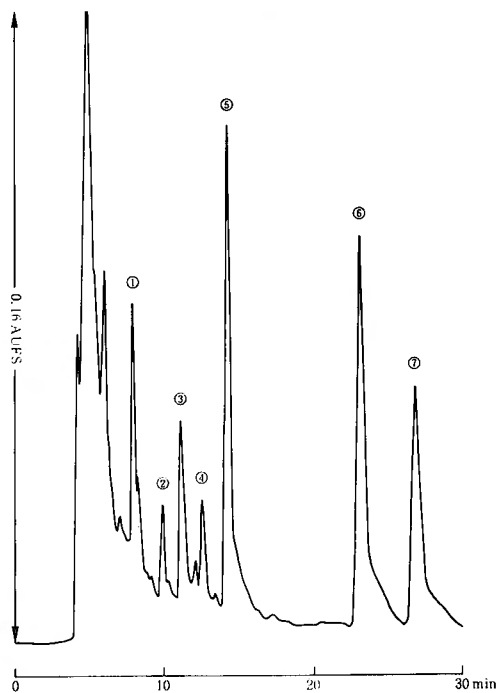
Although the relative concentrations of total bile acids and phospholipids were not changed by chenodeoxycholic acid treatment, the molar percentage of cholesterol was significantly lower than in the untreated cholesterol stone group (Fig. 14: mean  $\pm$  S.D.,  $5.3 \pm 1.4\%$  vs.  $8.0 \pm 2.5\%$ ).

THOMAS's indices according to the limits of cholesterol saturation defined by HOLZBACH et al. and HEGARDT et al. decreased significantly from  $1.25 \pm 0.43$  to  $0.85 \pm 0.20$ . These results are plotted on the triangular coordinates of ADMIRAND and SMALL, as shown in Fig. 15.

The mean values of taurine and glycine conjugates were  $30.9 \pm 30.7 \mu\text{mol/ml}$  and  $112.8 \pm$



**Fig. 9.** High-pressure liquid chromatogram of a patient without gallstones (Case 6).  
 ① TCA ② GUDCA ③ TCDCA ④ TDCA ⑤ GCA ⑥ GCDCA ⑦ GDCA



**Fig. 10.** High-pressure liquid chromatogram of a patient with cholesterol stones (Case 16).  
 ① TCA ② GUDCA ③ TCDCA ④ TDCA ⑤ GCA ⑥ GCDCA ⑦ GDCA

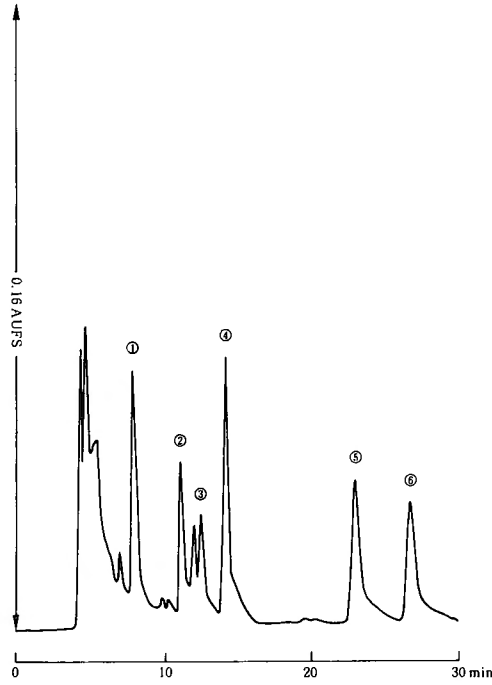


Fig. 11. High-pressure liquid chromatogram of a patient with bilirubin stones (Case 10).  
 ① TCA ② TCDCA ③ TDCA ④ GCA ⑤ GCDCA ⑥ GDCA

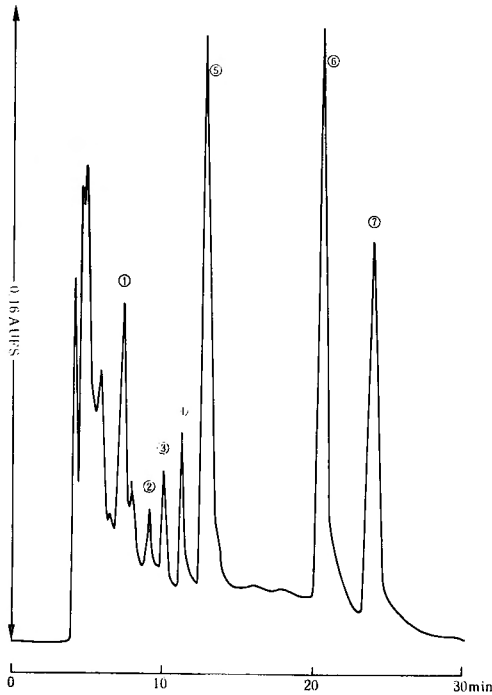
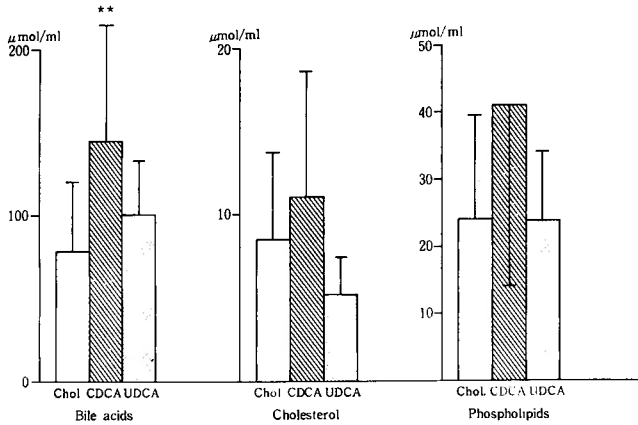


Fig. 12. High-pressure liquid chromatogram of a patient with black stones (Case 4).  
 ① TCA ② GUDCA ③ TCDCA ④ TDCA ⑤ GCA ⑥ GCDCA ⑦ GDCA

**Table 5.** Biliary lipid and conjugated bile acid composition in patients treated with chenodeoxycholic acid.

Case	Days administered.	BA. $\mu$ mol/ml (%)	Chol. $\mu$ mol/ml (%)	PL. $\mu$ mol/ml (%)	T.I. (1) (2)	Tauro. $\mu$ mol/ml	Glyco. $\mu$ mol/ml	G/T ratio	TCA $\mu$ mol/ml (%)	TCDCA $\mu$ mol/ml (%)	TDCA $\mu$ mol/ml (%)	TUDCA $\mu$ mol/ml (%)	GCA $\mu$ mol/ml (%)	GCDCA $\mu$ mol/ml (%)	GDCA $\mu$ mol/ml (%)	GUDCA $\mu$ mol/ml (%)
1	10	206.3 (86.3)	9.6 (4.0)	23.1 (9.7)	0.50 1.01	97.9	108.4	1.1	1.9 (0.9)	32.4 (15.7)	— (—)	63.6 (30.8)	5.8 (2.8)	101.4 (49.1)	1.2 (0.6)	— (—)
2	10	96.4 (79.8)	4.5 (4.2)	17.4 (16.1)	0.48 0.81	14.8	71.6	4.8	0.5 (0.6)	7.4 (8.6)	— (—)	— (—)	3.3 (3.8)	67.5 (78.1)	— (—)	0.8 (0.9)
3	28	69.0 (77.5)	3.7 (4.2)	16.3 (18.3)	0.43 0.72	13.7	55.3	4.0	2.5 (3.6)	10.0 (14.5)	— (—)	1.1 (1.7)	13.5 (19.5)	40.0 (57.5)	0.2 (0.3)	2.0 (2.8)
4	10	167.1 (72.2)	12.9 (5.6)	51.3 (22.2)	0.56 0.83	38.3	128.8	3.4	4.3 (2.6)	23.1 (13.8)	10.9 (6.5)	— (—)	5.3 (3.2)	95.3 (57.0)	23.4 (14.0)	4.8 (2.9)
5	17	264.0 (70.4)	25.9 (6.9)	85.1 (22.7)	0.69 1.01	29.7	234.3	7.9	— (—)	24.2 (9.2)	5.4 (2.0)	— (—)	— (—)	219.0 (82.9)	3.2 (1.2)	12.1 (4.6)
6	5	160.1 (66.2)	10.6 (4.4)	71.2 (29.4)	0.44 0.55	43.4	116.7	2.7	7.3 (4.5)	17.0 (10.6)	19.2 (12.0)	— (—)	— (—)	107.3 (67.0)	2.8 (1.8)	6.6 (4.1)
7	8	155.9 (71.3)	17.0 (7.8)	45.7 (20.9)	0.78 1.19	12.2	143.7	11.8	— (—)	12.2 (7.8)	— (—)	— (—)	6.3 (4.0)	131.6 (84.4)	5.8 (3.7)	— (—)
8	14	47.5 (69.2)	3.6 (5.2)	17.5 (25.5)	0.52 0.71	4.3	43.2	10.1	— (—)	3.6 (7.6)	— (—)	0.7 (1.5)	0.9 (1.9)	35.0 (73.7)	1.2 (2.5)	6.1 (12.8)
Mean $\pm$ S.D.	12.8 $\pm$ 7.1	143.3 $\pm$ 74.4 (73.9) ( $\pm$ 6.4)	11.0 $\pm$ 7.7 (5.3) ( $\pm$ 1.4)	41.0 $\pm$ 26.8 (20.8) ( $\pm$ 5.9)	0.55 $\pm$ 0.12 0.85 $\pm$ 0.20	30.9 $\pm$ 30.7	112.8 $\pm$ 60.7	5.7 $\pm$ 3.8	2.1 $\pm$ 2.6 (1.5) ( $\pm$ 1.8)	16.2 $\pm$ 9.7 (11.1) ( $\pm$ 3.2)	4.4 $\pm$ 7.2 (2.6) ( $\pm$ 4.4)	8.2 $\pm$ 22.4 (4.2) ( $\pm$ 10.8)	4.4 $\pm$ 4.5 (4.4) ( $\pm$ 6.3)	99.6 $\pm$ 58.7 (69.6) ( $\pm$ 14.0)	4.7 $\pm$ 7.8 (3.0) ( $\pm$ 4.6)	4.1 $\pm$ 4.2 (3.5) ( $\pm$ 4.1)



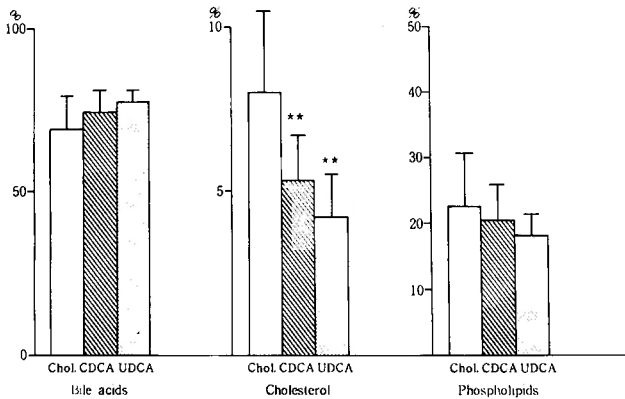
**Fig. 13.** Absolute concentrations of biliary lipids of patients treated with CDCA or UDCA (mean  $\pm$  S.D.). \*\* $p < 0.01$   
Abbreviations: Chol., patients with cholesterol stones; CDCA, patients treated with chenodeoxycholic acid, 400 mg/day; UDCA, patients treated with ursodeoxycholic acid, 600 mg/day.

60.7  $\mu\text{mol/ml}$ , respectively, significantly higher than in the untreated cholesterol stone group (Fig. 17).

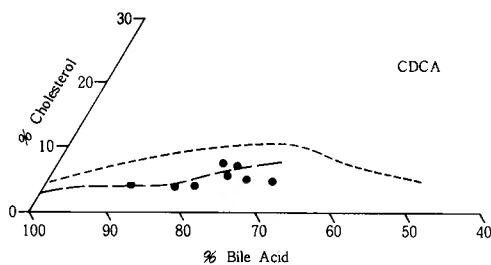
Of the conjugated bile acids, tauro- and glycochenodeoxycholic acid increased markedly, while tauro- and glycocholic acid decreased markedly during treatment with chenodeoxycholic acid (Fig. 18).

The biliary lipid and conjugated bile acid levels in patients treated with ursodeoxycholic acid, 600 mg per day, are listed in Table 6.

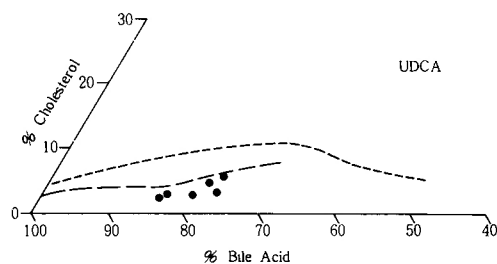
The absolute concentrations of biliary lipids remained unchanged but the molar percentage of cholesterol fell from  $8.0 \pm 2.5\%$  to  $4.2 \pm 1.3\%$  (Fig. 14). The mean values of THOMAS's indices were  $0.43 \pm 0.13$  according to ADMIRAND et al. and  $0.72 \pm 0.19$  according to HOLZBACH et al. and HEGARDT et al., significantly lower than in the untreated cholesterol stone group, and



**Fig. 14.** Relative concentrations of biliary lipids of patients treated with CDCA or UDCA (mean  $\pm$  S.D.). \*\* $p < 0.01$



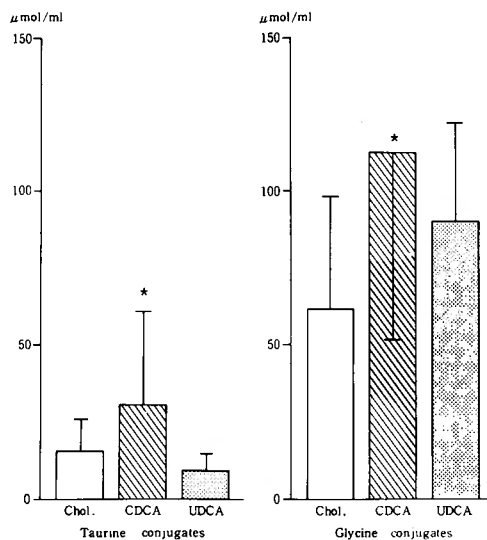
**Fig. 15.** Relative concentrations of biliary lipids plotted on a triangular coordinate in patients treated with CDCA.



**Fig. 16.** Relative concentrations of biliary lipids plotted on a triangular coordinate in patients treated with UDCA.

much lower than in the chenodeoxycholic acid treated group. Plotted on the triangular coordinates of ADMIRAND and SMALL, the results were all in the micellar zone, as shown in Fig. 16.

Of the conjugated bile acids, glycooursodeoxycholic acid was greatly increased (mean  $\pm$  S.D.,  $46.3 \pm 17.9 \mu\text{mol/ml}$ ), but the others were not changed by ursodeoxycholic acid treatment (Fig. 18).

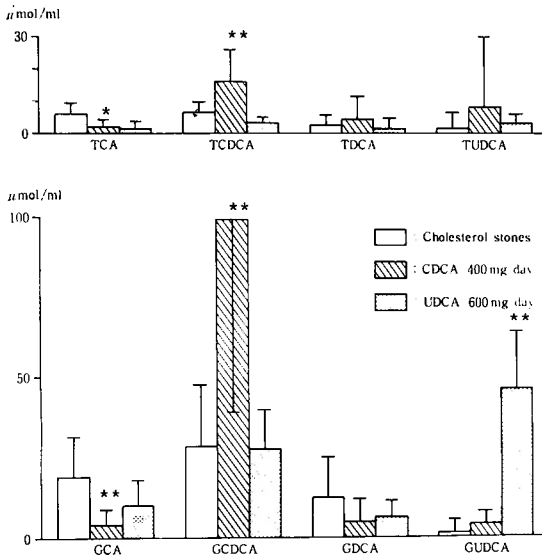


**Fig. 17.** Taurine and glycine conjugates of patients treated with CDCA or UDCA (mean  $\pm$  S.D.). \*;  $p < 0.05$

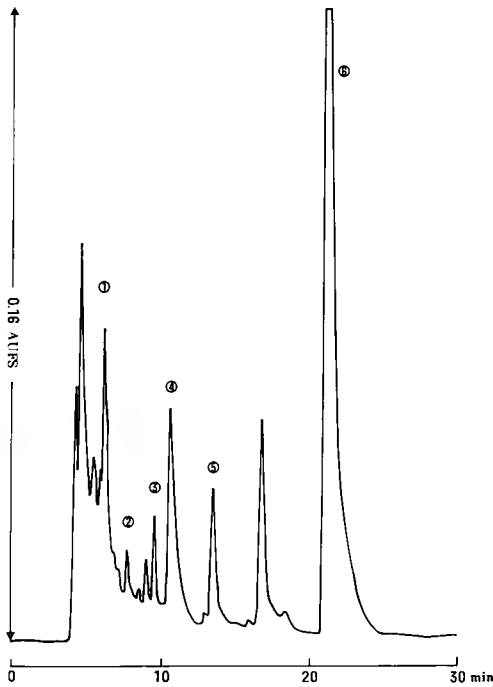


Table 6. Biliary lipid and conjugated bile acid composition in patients treated with ursodeoxycholic acid.

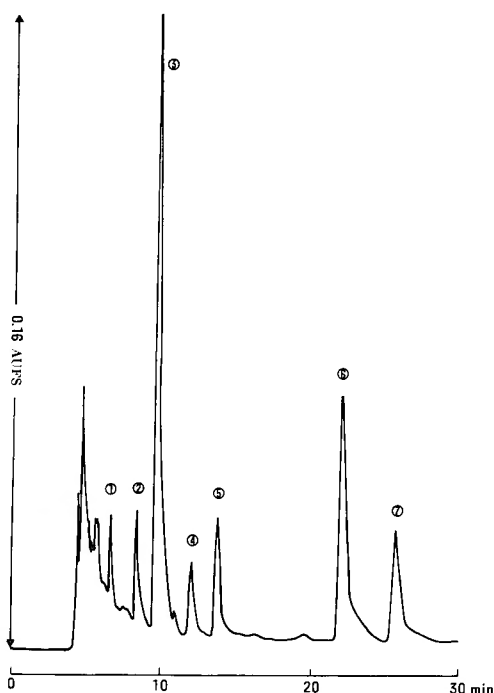
Case	Days administered.	BA. $\mu$ mol/ml (%)	Chol. $\mu$ mol/ml (%)	PL. $\mu$ mol/ml (%)	T.I. (1) (2)	Tauro. $\mu$ mol/ml	Glyco. $\mu$ mol/ml	G/T ratio	TCA $\mu$ mol/ml (%)	TCDC $\mu$ mol/ml (%)	TDCA $\mu$ mol/ml (%)	TUDCA $\mu$ mol/ml (%)	GCA $\mu$ mol/ml (%)	GDCA $\mu$ mol/ml (%)	GDCA $\mu$ mol/ml (%)	GUDCA $\mu$ mol/ml (%)
1	5	112.9 (80.4)	5.5 (3.9)	22.0 (15.7)	0.42 0.76	7.2	105.7	14.7	— (—)	3.2 (2.8)	2.6 (2.3)	1.4 (1.2)	6.8 (6.0)	26.8 (23.8)	13.1 (11.6)	59.0 (52.3)
2	10	108.6 (82.3)	4.0 (3.0)	19.3 (14.6)	0.33 0.62	5.6	103.0	18.4	— (—)	— (—)	— (—)	5.6 (5.2)	5.2 (4.8)	38.6 (35.5)	9.8 (9.0)	49.4 (45.5)
3	17	91.0 (77.9)	3.7 (3.2)	22.1 (18.9)	0.33 0.54	17.3	73.7	4.3	1.6 (1.8)	3.2 (3.5)	7.0 (7.7)	5.5 (6.0)	2.4 (2.6)	12.7 (14.0)	9.5 (10.5)	49.1 (54.0)
4	5	140.6 (75.3)	6.3 (3.4)	39.9 (21.3)	0.34 0.53	10.5	130.1	12.3	3.6 (2.5)	4.6 (3.3)	— (—)	2.4 (1.7)	22.1 (15.7)	41.4 (29.4)	2.5 (1.8)	64.0 (45.5)
5	6	106.5 (73.4)	8.9 (6.1)	29.7 (20.5)	0.62 0.97	13.4	93.1	6.9	4.2 (3.9)	5.4 (5.1)	— (—)	3.8 (3.6)	17.6 (16.5)	32.4 (30.4)	— (—)	43.1 (40.5)
6	10	39.4 (75.6)	2.8 (5.4)	9.9 (19.0)	0.55 0.90	3.0	36.4	12.1	1.2 (3.0)	1.2 (3.0)	— (—)	0.6 (1.5)	7.9 (20.1)	15.3 (38.8)	— (—)	13.2 (33.5)
Mean $\pm$ S.D.	8.8 $\pm$ 4.6	99.8 $\pm$ 33.7 (77.5) $\pm$ 3.4)	5.2 $\pm$ 2.2 (4.2) $\pm$ 1.3)	23.8 $\pm$ 10.1 (18.3) $\pm$ 2.7)	0.43 $\pm$ 0.13 0.72 $\pm$ 0.19)	9.5 $\pm$ 5.3	90.3 $\pm$ 32.1	11.5 $\pm$ 5.1	1.8 $\pm$ 1.8 (1.9) $\pm$ 1.6)	2.9 $\pm$ 2.0 (3.0) $\pm$ 1.7)	1.6 $\pm$ 2.8 (1.7) $\pm$ 3.1)	3.2 $\pm$ 2.1 (3.2) $\pm$ 2.0)	10.3 $\pm$ 7.7 (11.0) $\pm$ 7.3)	27.9 $\pm$ 11.9 (28.7) $\pm$ 8.9)	5.8 $\pm$ 5.7 (5.5) $\pm$ 5.4)	46.3 $\pm$ 17.9 (45.2) $\pm$ 7.6)



**Fig. 18.** Conjugated bile acid concentrations of patients treated with CDCA or UDCA (mean  $\pm$  S.D.). \*;  $p < 0.05$ , \*\*;  $p < 0.01$



**Fig. 19.** High-pressure liquid chromatogram of a patient treated with CDCA, 400 mg/day (Case 3).  
 ① TUDCA ② TCA ③ GUDCA ④ TCDC A ⑤ GCA  
 ⑥ GDC A



**Fig. 20.** High-pressure liquid chromatogram of a patient treated with UDCA. 600 mg/day (Case 4).

① TUDCA ② TCA ③ GUDCA ④ TCDCA ⑤ GCA ⑥ GCDCA ⑦ GDCA

The glycine to taurine conjugates ratio was greatly elevated from  $4.3 \pm 2.4$  to  $11.5 \pm 5.1$  by ursodeoxycholic acid, but not by chenodeoxycholic acid treatment.

Chromatograms of conjugated bile acids analyzed by high-pressure liquid chromatography after treatment with chenodeoxycholic or ursodeoxycholic acid are shown in Figs. 19 and 20.

### Discussion

The earliest investigations of bile acids were reported in the middle of the nineteenth century<sup>104)</sup>. Since the application of PETTENKOFER's reaction<sup>76)</sup> to bile acid determination, various methods have been employed to analyze bile acids. In 1921, SCHMIDT et al. estimated the taurine conjugates and 'glycocol' from the nitrogen and sulfur content after hydrolysis of bile acids<sup>83)</sup>. A modification of PETTENKOFER's reaction and other color reactions were in wide use for bile acid determination in the first half of this century<sup>17,28,42,91)</sup>. These methods were, however, nonspecific and inaccurate, and measured only one or two bile acids or total bile acids without differentiating among them. Column chromatographic separation of bile acids was developed by BERGSTRÖM et al. in 1951<sup>8)</sup>, and AHRENS et al. described the separation of taurine and glycine conjugates and free bile acids with counter-current distribution in 1952<sup>3)</sup>. WOOTON et al. separated and determined the individual bile acids in human bile by a combination of column chromatography and infrared spectrometry in 1953<sup>109)</sup>. These procedures, however, required complicated sample preparation and identification.

Since GÄNSHIRT developed bile acid analysis by thin-layer chromatography in 1960<sup>35)</sup>, this simple method has been widely used for the separation and quantitative determination of bile acids in various biological materials<sup>21,38,74,106)</sup>. Although many solvent systems have been developed for the separation of free, taurine and glycine conjugates by many workers, the complete separation of isomeric dihydroxy bile acids was not possible, and rechromatography with a different solvent system is required for complete separation<sup>21,27,38)</sup>. In addition, this method includes complicated and time-consuming procedures for quantification, which are based on densitometric<sup>84)</sup>, fluorimetric<sup>74)</sup>, spectrophotometric<sup>25)</sup> or enzymatic<sup>106)</sup> determination after elution.

In 1960, VANDENHEUVEL et al.<sup>107)</sup> and RYHAGE et al.<sup>80)</sup> used gas-liquid chromatography for bile acid analysis. This accurate and specific analytical method has also been widely used in the quantitative determination of bile acids. The higher sensitivity of this method makes possible the micro quantification of bile acids in feces and serum<sup>29,55,82)</sup>. Furthermore, the recent development of combined gas-liquid chromatography and mass spectrometry has made it possible to elucidate the structure heretofore unknown bile acids in biological materials<sup>5,47,63,92)</sup>. However, this sophisticated method has the great disadvantage that the conjugated bile acids must be hydrolysed and changed to methylesters of free bile acids. These procedures lead to a loss of recovery and the formation of artifacts and take a long time.

In addition to the determination of individual bile acids by either thin-layer or gas-liquid chromatography, total bile acids can be assayed easily by the enzymatic method with 3 $\alpha$ -hydroxysteroid dehydrogenase developed by TALALAY<sup>93)</sup> and IWATA et al.<sup>44)</sup>. This method was improved in sensitivity by spectrofluorimetric determination<sup>66)</sup> and proved to be more specific with the use of purified 3 $\alpha$ -hydroxysteroid dehydrogenase<sup>22)</sup>. In addition to 3 $\alpha$ -hydroxysteroid dehydrogenase, 7 $\alpha$ -<sup>31,51)</sup> and 12 $\alpha$ -hydroxysteroid dehydrogenase<sup>52)</sup> are also effective in the determination of bile acids, and combination of these enzymatic methods have made possible the indirect measurement of individual bile acids in bile<sup>59)</sup>. However, the state of conjugation in biological samples cannot be detected by this method.

Since 1976 there have been some attempts to apply high-pressure liquid chromatography to the analysis of conjugated bile acids or their esters<sup>46,72,75,85)</sup>. Early reports on this analytical method indicated limited success in regard to the time required for analysis and the separation of dihydroxy bile acids and the simultaneous resolution of glycine and taurine conjugates. However, complicated preparations of the biological samples, such as esterification<sup>46,72)</sup> or group separation of bile acids<sup>26)</sup>, were required for analysis. Recently, the present author and co-workers reported the simple and rapid analysis of conjugated bile acids in bile using reversed phase high-pressure liquid chromatography without hydrolysis or esterification of the bile samples<sup>56,57,58,59)</sup>. With this method, ten conjugated bile acids occurring in human bile can be analyzed directly and simultaneously in less than 40 minutes. Although the possible contamination of the fraction of tauroursodeoxycholic acid with impurities contained in bile was described in the previous report<sup>59)</sup>, further study indicated that this contamination might be attributed to the degradation products of the extracted sample in methanol and the lowered capacity of the column used. When the sample is tested just after extraction, tauroursodeoxy-

cholic acid can be analyzed directly as shown in Figs. 19 and 20. More recently the combination of high-pressure liquid chromatography and the enzymatic method with 3 $\alpha$ -hydroxysteroid dehydrogenase has made possible the estimation of serum bile acids<sup>6)</sup>. This method is, however, costly and cannot completely separate all the bile acids in serum.

In this study, gallstones located in the gallbladder were classified as cholesterol stones (including pure cholesterol, combination and mixed stones), bilirubin stones and black stones from their gross appearance and chemical composition analyzed by the method of MUKAIHARA<sup>64,65)</sup>. CHEN et al. have classified gallstones in Chinese patients into the same three groups and have reported a good correlation between gross appearance and chemical composition<sup>12)</sup>. In recent years in Japan cholesterol stones have been found increasingly often, presumably because of the westernization of dietary habits<sup>67,71,94)</sup>. However, bilirubin stones are still more common and black stones fewer in Japan than in the United States<sup>34)</sup>. Studies of biliary lipids in patients with cholesterol gallstones have been numerous<sup>9,14,62,69,88,101,108)</sup>, but those in patients with bilirubin<sup>36,45)</sup> and black gallstones<sup>50,105)</sup> are rare. The results of this present study confirm the differences of biliary lipid and conjugated bile acid composition in gallbladder bile of patients with different types of gallstones and suggest the etiology of the gallstones.

The lower concentrations of biliary lipids in patients with gallstones in this study are in good agreement with the results of other investigators<sup>14,36,69)</sup>. Especially in patients with bilirubin stones, all biliary lipids were greatly decreased, presumably because of bacterial infection; i.e. bacterial degradation of conjugated bile acids and hydrolysis of phospholipids by enzymes of bacterial origin. This possible cause is confirmed by bacteriological examinations; cultures were positive in 11 of 13 cases of bilirubin stones (84.6%), three of 16 cases of cholesterol stones (18.8%) and none of the cases of black stones.

There were, however, no significant differences in the relative concentrations of biliary lipids among the varieties of gallstones. THOMAS's indices<sup>103)</sup> were also not different between those with and those without gallstones. Plotting on the triangular coordinates of ADMIRAND and SMALL<sup>1)</sup> showed that three cases of cholesterol and two cases of bilirubin stones were in the supersaturated zone, and others with and without gallstones were in the metastable-labile zone. Non-lithogenic bile in patients with cholesterol stones may be due to the ethnic difference of cholesterol saturation in the Japanese<sup>70)</sup>. HO et al. also reported lower cholesterol saturation in the Chinese<sup>36)</sup>. The chemical composition of bilirubin and black stones suggests that the etiology of these stones is different from that of cholesterol stones<sup>12,65)</sup>, and the cholesterol desaturated bile of cases of bilirubin or black stones observed in this study supports this hypothesis.

The analysis of conjugated bile acids by high-pressure liquid chromatography showed that patients with gallstones other than black stones had lower concentrations of both taurine and glycine conjugates than those without gallstones, which was attributed to decreased levels of taurocholic, taurochenodeoxycholic, glycocholic, glycochenodeoxycholic and glycodeoxycholic acids. The absolute concentration of each conjugated bile acid in our patients with cholesterol stones was almost the same as that reported by DAM<sup>14)</sup> but higher than that reported by SJÖVALL<sup>88)</sup> who used paper chromatography, while the level of each bile acid was similar to that

reported by VLAHCEVIC et al.<sup>108</sup>), but somewhat different from the findings of MACDOUGAL et al.<sup>62</sup>) and NAKAYAMA et al.<sup>69</sup>) in their analysis by gas-liquid chromatography with no consideration of conjugation.

The decrease of glycine conjugated bile acids in the bilirubin stone group was much greater than in the cholesterol stone group. The author predicts that one of the factors affecting this conjugation with glycine may be the diet. The nutritional research on Japanese patients with cholelithiasis showed a marked difference in protein intake between patients with cholesterol and with bilirubin stones; i.e. a lower intake of animal protein, which contains more glycine than plant protein in general, in the latter<sup>48,97</sup>). Although SJÖVALL et al. reported that oral glycine administration did not alter the conjugation of bile acids in gallbladder bile<sup>87</sup>), such dietary habits of patients with bilirubin stones for a long term (patients with bilirubin stones are older than those with cholesterol stones<sup>33,34</sup>) may affect the conjugation of bile acids with glycine. In addition, in vitro observation of bile acid conjugation with taurine or glycine in human liver homogenates revealed a decrease of glycine conjugation in liver diseases<sup>19,20</sup>). The patients with bilirubin stones, although they showed no abnormal liver function test results in this study, may have already had some liver damage not detectable by routine laboratory examinations, and the disease entity of bilirubin stones may be more serious and complicated than that of cholesterol stones. Furthermore, Ho et al. reported the inhibitory effect of glycochenodeoxycholic acid on  $\beta$ -glucuronidase activity in rats<sup>35</sup>), which has been thought to be the initiating factor in the formation of bilirubin stones<sup>54</sup>), and the observed decrease of glycochenodeoxycholic acid in cases of bilirubin stones might lead to the precipitation of free bilirubin preferentially in the bile in these cases.

In the present study, there were no cases of lithocholic acid conjugated with taurine or glycine, whereas in some of the patients with or without gallstones ursodeoxycholic acid was conjugated with taurine or glycine.

There have been a few reports that free bile acids can be detected in the bile in gallbladders with cholecystitis<sup>13,45,73</sup>), while some investigators have found no free bile acids in human gallbladders with gallstones<sup>14,88</sup>). Although free bile acids cannot be determined by our high-pressure liquid chromatographic analysis, the amount of free bile acids is probably negligible, since there are no significant differences between the amounts of total bile acids determined by the enzymatic method with 3 $\alpha$ -hydroxysteroid dehydrogenase and by our high-pressure liquid chromatography.

DANZINGER et al. reported the effectiveness of chenodeoxycholic acid in dissolving cholesterol gallstones in 1972<sup>15</sup>), and NAKAGAWA et al. reported the same effect of ursodeoxycholic acid which had been administered as a choleric agent in Japan<sup>68</sup>). The effects of these bile acids on bile acid composition in bile have been investigated by many workers<sup>7,11,16,23,24,43,61,79,89,90,93,100,102</sup>). However, only one of these reports compares the conjugated bile acid composition after the administration of these two bile acids<sup>90</sup>). Its results are similar to those of this study.

In this study patients were given a daily dose of 400 mg of chenodeoxycholic acid or 600 mg of ursodeoxycholic acid orally for five to 28 days (average, 11.1 days). These patients were

neither markedly obese nor emaciated (average body weight, 52.3 kg).

A previous study showed that the bile acid composition in bile was altered by chenodeoxycholic acid treatment for four days<sup>43)</sup>. Indeed, chenodeoxycholic or ursodeoxycholic acid administration for five days had already altered the bile acid composition in bile in this study, and there was no relationship between length of treatment and bile acid composition.

Chenodeoxycholic acid administration increased the total amount of conjugated bile acids, due to the increase of both taurine and glycine conjugates, but did not alter the amounts of cholesterol and phospholipids, while ursodeoxycholic acid treatment did not alter the amounts of any of the biliary lipids. However, the molar percentage of cholesterol and THOMAS'S indices<sup>103)</sup> according to the limits of cholesterol saturation defined by HOLZBACH et al.<sup>39)</sup> and HEGARDT et al.<sup>32)</sup> were found to be reduced by the oral administration of either bile acid, and the decrease of THOMAS'S indices by ursodeoxycholic acid administration was greater than that by chenodeoxycholic acid administration. These data confirm the previous reports on cholesterol saturation of bile after administration of chenodeoxycholic or ursodeoxycholic acid<sup>7,16,23,61,78,89,90,102)</sup>. The oral administration of chenodeoxycholic acid reduces the activity of HMG CoA reductase<sup>2,11,49)</sup>, the rate limiting enzyme of hepatic cholesterol synthesis, and the biliary secretion of cholesterol<sup>18,49)</sup>. MATON et al. reported the same effect of ursodeoxycholic acid on the activity of HMG CoA reductase<sup>61)</sup>, whereas CARULLI et al. reported that short-term administration of ursodeoxycholic acid (one week) increased the activity of this enzyme<sup>11)</sup>. In addition, the absorption of cholesterol was found to be inhibited by the oral administration of chenodeoxycholic acid or ursodeoxycholic acid<sup>11,77)</sup>. This diminished hepatic cholesterologenesis and reduced absorption of cholesterol may be the cause of lowered saturation of cholesterol in the present study. Although it has been shown that ursodeoxycholic acid desaturates biliary cholesterol more than does chenodeoxycholic acid<sup>61,89,90)</sup>, the cholesterol solubility of ursodeoxycholic acid solution *in vitro* is lower than that of chenodeoxycholic acid<sup>40,41)</sup>, and CAREY et al. have provided the correction factor of the saturation index in ursodeoxycholic acid rich bile<sup>10)</sup>. However, in this study THOMAS'S indices were not corrected, since the exact number of the correction factor could not be utilized because of the higher ratio of glycine to taurine conjugates (average,  $11.5 \pm 5.1$ ).

The increase of taurine and glycine conjugated chenodeoxycholic acid and the decrease of taurine and glycine conjugated cholic acid during chenodeoxycholic acid administration are in general agreement with previous results determined by gas-liquid chromatography<sup>11,16,23)</sup>, which did not mention conjugation with taurine or glycine. DANZINGER et al. speculated that chenodeoxycholic acid might inhibit cholic acid synthesis by negative feed back<sup>16)</sup>. However, in previous reports chenodeoxycholic acid administration did not affect the activity of 7 $\alpha$ -hydroxylase<sup>11,49)</sup>, which is the enzyme regulating the rate of hepatic synthesis of bile acids from cholesterol, and there is no direct proof that either primary bile acid suppresses the synthesis of the other in man.

There are a few reports that the oral administration of large amounts of chenodeoxycholic acid increases the concentration of ursodeoxycholic acid in bile<sup>23,24,81)</sup>, and in this study one patient showed an increase in tauroursodeoxycholic acid after chenodeoxycholic acid admini-

stration (Case 1). However, the average level of conjugated ursodeoxycholic acid was not increased by the oral administration of chenodeoxycholic acid, 400 mg per day, in this study. In some of patients, a conversion of chenodeoxycholic acid to ursodeoxycholic acid via 7-keto-lithocholic acid probably occurs<sup>81</sup>).

Both taurine and glycine conjugated chenodeoxycholic acids were increased by chenodeoxycholic acid administration, whereas only glycine conjugated ursodeoxycholic acid was increased by ursodeoxycholic acid administration accompanied by marked elevation of the glycine to taurine conjugates ratio. This finding is somewhat different from that of STIEHL et al. as determined by thin-layer chromatography<sup>90</sup>). This suggests that ursodeoxycholic acid is more likely to be conjugated with glycine than taurine. Although HABER et al. reported the *in vitro* study of bile acid conjugation with taurine and glycine<sup>30</sup>), there is no strong evidence of different kinetics of conjugation with taurine or glycine between these two bile acids.

In this study, the clinical effects of these two bile acids on gallstone dissolution could not be compared because they were given for only a short time. However, our *in vitro* study, with the rotating disk method<sup>78</sup>), of cholesterol dissolution in bile samples obtained from these subjects treated with chenodeoxycholic or ursodeoxycholic acid showed the greater effectiveness of chenodeoxycholic acid<sup>33,60</sup>); this is in agreement with the recent report of IGIMI et al.<sup>41</sup>).

The oral administration of chenodeoxycholic acid or ursodeoxycholic acid had no side effects such as diarrhea or liver function disorders. The 400 mg per day dose of chenodeoxycholic acid is sufficient for gallstone dissolution without any side effects in Japanese patients.

### Conclusions

Biliary cholesterol and phospholipids were determined by enzymatic method, and conjugated bile acids were measured by high-pressure liquid chromatography in a clinical application of our method in patients with and without gallstones and in patients treated with chenodeoxycholic acid or ursodeoxycholic acid.

1) The amounts of total bile acids and cholesterol were lower than normal in the bile of patients with gallstones. In those with cholesterol stones and bilirubin stones, the amount of phospholipids in the bile was also decreased. These biliary lipid concentrations were lowest in patients with bilirubin stones.

2) The relative concentrations of biliary lipids and the lithogenic indices did not differ in patients with and without gallstones.

3) The absolute concentrations of taurocholic, taurochenodeoxycholic, glycocholic, glycochenodeoxycholic and glycodeoxycholic acids were significantly lower in patients with gallstones, except for black stones, than in those without gallstones. Especially in the patients with bilirubin stones, glycocholic and glycochenodeoxycholic acids were markedly decreased.

4) The oral administration of chenodeoxycholic acid, 400 mg per day, resulted in an increase of total bile acids and a decrease of the molar percentage of cholesterol. The lithogenic indices were also lowered. Of the conjugated bile acids, tauro- and glycochenodeoxycholic acids were increased, while tauro- and glycocholic acids were decreased.



5) The oral administration of ursodeoxycholic acid, 600 mg per day, also lowered the molar percentage of cholesterol and the lithogenic indices. Of the conjugated bile acids, glycourso-deoxycholic acid was increased with a marked elevation of the glycine to taurine conjugates ratio, but the others were not altered significantly.

These data suggest that different etiological factors should be considered for each type of gallstone (cholesterol, bilirubin and black stone). The differences in the effectiveness and mechanisms of gallstone dissolution by chenodeoxycholic acid and ursodeoxycholic acid are probably due to increased glycochenodeoxycholic acid or glycourso-deoxycholic acid.

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## 和文抄録

高速液体クロマトグラフィによる胆汁中抱合型胆汁酸の分析  
第Ⅱ編：胆石症患者における胆汁分析への臨床応用

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胆石症患者を胆石の種類により、コレステロール系石、ビリルビン系石および黒色石の3群に分類し、胃癌患者を対照とし、その胆汁脂質を分析し比較した。また、胆石溶解剤としてケノデオキシコール酸 (CDCA) 1日 400 mg、ウルソデオキシコール酸 (UDCA) 1日 600 mg を術前投与し、それらの胆汁脂質に及ぼす影響を検討した。胆汁中の抱合型胆汁酸は、著者らの考案した高速液体クロマトグラフィにより分析し、胆汁中コレステロールおよびリン脂質は、酵素法により測定した。

その結果は以下の如くである。

1) 胆石症患者では、対照に比し、胆汁中総胆汁酸量および、コレステロール量は低下し、コレステロール系石群およびビリルビン系石群では、更にリン脂質濃度も低下していた。これら胆汁脂質濃度の低下は、ビリルビン系石群において最も著明であった。

2) 胆汁脂質の相対濃度は、有石群および無石群の間に差は認められず、催石指数としての Thomas 指数も、各群間において差は認められなかった。

3) 黒色石を除く有石群では、対照に比しタウロコール酸、タウロケノデオキシコール酸、グリココール

酸、グリコケノデオキシコール酸およびグリコデオキシコール酸が減少し、特にビリルビン系石群では、グリココール酸およびグリコケノデオキシコール酸の減少が著明であった。

4) CDCA 400 mg/日投与により、総胆汁酸量は増加し、コレステロールの相対濃度は低下し、催石指数も低下した。抱合型胆汁酸では、タウロおよびグリコケノデオキシコール酸が増加し、タウロおよびグリココール酸は減少した。

5) UDCA 600 mg/日投与により、コレステロールの相対濃度および催石指数は低下した。抱合型胆汁酸では、グリコウルソデオキシコール酸のみ増加し、G/T 比は著明に上昇した。その他の抱合型胆汁酸には、変化が認められなかった。

以上の結果より、コレステロール系石、ビリルビン系石および黒色石の成因には、各々異った生成機序が考えられ、CDCA および UDCA による胆石溶解効果の差、あるいは、それらの胆石溶解機序の差は、グリコケノデオキシコール酸およびグリコウルソデオキシコール酸の増加によるものであろうと推測される。