Identification of bile alcohols in rat bile

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Abstract Bile alcohols in rat bile were analyzed by gas–liquid chromatography–mass spectrometry. Six bile alcohols were newly identified as minor constituents in addition to 5β-cholestane-3α,7α,12α,26-tetrol, major bile alcohol of rat bile. The bile alcohols newly identified were 27-nor-5β-cholestane-3α,7α,12α, 24, 25-pentol, 5α-cholestane-3α,7α,12α,26-tetrol, 5β-cholestane-3α,7α,12α, 24,26-pentol, 5α-cholestane-3α,7α,12α,25,26-pentol, 5β-cholestane-3α,7α,12α,25,26-pentol, and 5β-cholestane-3α,6β,7β,25,26-pentol. The biliary bile alcohols of the rat occurred mainly as the sulfuric acid esters and, in lesser amounts, as glucuronoconjugated and unconjugated forms. The amount of total bile alcohols was about 27.9 nmol in 1 ml of bile. —**Hiraoka, T., T. Kohda, D. Kosaka, T. Yamauchi, K. Kihira, K. Kuramoto, T. Hoshita, and G. Kajiyama.** Identification of bile alcohols in rat bile. *J. Lipid Res.* 1989. **30:** 1889–1893.

Supplementary key words β-trichechol

Bile alcohols are known to be major bile constituents of lower vertebrates such as amphibians and some fishes (1). In mammals, bile alcohols have been considered to be intermediates in the pathway for the formation of bile acids from cholesterol and not to be found in considerable amounts under normal conditions (2, 3). However, it has been found that increased amounts of bile alcohols have been observed in serum, urine, and bile (4–10) in patients with cerebrotendinous xanthomatosis, a rare inherited lipid storage disease, and in patients with liver dysfunction, such as acute hepatitis or cirrhosis. Furthermore, it has been recently shown that the West Indian manatee, *Trichechus manatus latirostris*, contains bile alcohol sulfates as major biliary constituents (11). In connection with studies to clarify the mechanism of the biosynthesis of bile alcohols from cholesterol in mammals, we examined bile alcohols in rat bile using 25-homo-5β-cholan- 3α,7α,12α,25-tetrol as an internal standard by gas–liquid chromatography–mass spectrometry.

MATERIALS AND METHODS

General

Gas–liquid chromatography (GLC) was performed on a Shimadzu model GC-14A gas chromatograph equipped with a flame ionization detector and Van den Berg's solventless injector. The column used was 25 m × 0.35 mm i.d., coated with OV-1 or OV-17 with temperature program of 260 to 290°C at 2°C/min.

Gas–liquid chromatography–mass spectrometry (GLC–MS) was carried out on a Shimadzu model GCMS-QP-1000 equipped with a data processing system. Operating conditions were as follows: columns and column temperature, same conditions as for GLC; ion source temperature, 250°C; ionizing energy, 70 eV; and trap current, 60 μA.

Reference compounds

27-Nor-5β-cholestane-3α,7α,12α,24,25-pentol (12), 5β-cholestane-3α,7α,12α,26-tetrol (13), 5β-cholestane-3α,7α,12α,24,26-pentol (14), 5α-cholestane-3α,7α,12α,26-tetrol (15), 5α-cholestane-3α,7α,12α,25,26-pentol (5α-bufol) (15), and 5β-cholestante-3α,7α,12α,25,26-pentol (5β-bufol) (16) were synthesized or isolated from natural sources as described previously.

Internal standard

25-Homo-5β-cholane-3α,7α,12α,25-tetrol was used as an internal standard for quantitation of biliary bile alcohols by GLC. It was prepared as described previously (17) with mp 194–195°C (reported 189°C); PMR (CD_3OD): 0.71 (3H, s, 18-H_3), 0.91 (3H, s, 19-H_3), 0.99 (3H, d, J = 6Hz, 21-H_3), 3.30 (1H, m, 3β-H), 3.52 (2H, t, J = 6Hz, 24-H_2OH), 3.78 (1H, m, 7β-H), 3.94 (1H, m, 12β-H); MS (as TMS ether): 516 (M–90, 14%, 426 (M–90 × 2, 47%), 343 (M–side chain–90 × 2, 43%), 253 (M–side chain–90 × 3, 100%).

Animals

Adult male Wistar rats weighing 250–300 g were placed in individual cages in an air-conditioned room (25 ± 1°C, 50–60% humidity). They were maintained on laboratory chow (Type MF, Oriental Yeast Co., Tokyo, Japan) and

Abbreviations: GLC, gas–liquid chromatography; MS, mass spectrometry; TMS, trimethylsilyl.

water ad libitum for 7 days prior to use. There was no significant weight change in the animals during the experiment.

Analysis of bile alcohols in bile

Bile was collected by drainage of six male Wistar rats within 3 hr. The collected bile was diluted with ten volumes of 0.1 M NaOH and stirred for 30 min at 64°C in a water bath. After the addition of 10 μg of 25-homo-5β-cholane-3α,7α,12α,25-tetrol as an internal standard for unconjugated bile alcohols, the mixture was passed through Bond Elut C18 cartridges (Analytichem. Co.).

The column was eluted with 10 ml of methanol. The eluant was evaporated to dryness and the resulting residue was hydrolyzed enzymatically with 50 units of cholylglycine hydrolase (EC 3.5.1.24; Sigma Chemical Co.). After a 24-h incubation period, the mixture was passed through a Bond Elut C18 cartridge. The methanol eluate was evaporated to dryness, and the resulting residue was dissolved in 5 ml of 90% ethanol and passed through a column of piperidinohydroxypropyl Sephadex LH-20 (PHP-LH-20) (2 ml) (18). The column was eluted successively with 30 ml of 90% ethanol, 50 ml of 0.1 M acetic acid in 90% ethanol, 30 ml of 0.2 M formic acid in 90% ethanol, and 30 ml of 1% ammonium carbonate in 70% ethanol to give unconjugated bile alcohols, unconjugated and deconjugated bile acids, glucuronoconjugated bile alcohols, and sulfated bile alcohols along with sulfated bile acids, respectively. After the addition of 10 μg of the internal standard as described above, the glucuronoconjugated bile alcohol fraction was hydrolyzed enzymatically with 5000 units of β-glucuronidase (EC 3.2.31; Sigma Chemical Co., Type H-1) in 0.1 M sodium phosphate buffer (10 ml, pH 5.0). After a 48-h incubation period, the mixture was extracted on a Bond Elut C18 cartridge. The methanol eluate was evaporated to dryness, and the resulting residue was dissolved in 5 ml of 90% ethanol and passed through a column of PHP-LH-20 (2 ml). The eluant and an additional wash with 20 ml of 90% ethanol were collected and evaporated to give a fraction of deglucuronidated bile alcohols. To the sulfated bile alcohol fraction, 10 μg of the internal standard was added and the mixture was evaporated to dryness. The residue was dissolved in water and the pH was adjusted to pH 1 by adding dilute HCl; 200 mg of NaCl and 5 ml of ethyl acetate were then added. The mixture was incubated at 37°C for 48 h. After dilution with 15 ml of 10% Na_2CO_3 solution, the mixture was extracted with ethyl acetate. The organic layer was washed with water and evaporated to dryness. The residue was then hydrolyzed with 5% methanolic KOH at 60°C for 2 h. Methanol was removed under a N_2 stream and replaced with water. The solution was adjusted to pH 7 with 2 N HCl and passed through a Bond Elut C18 cartridge. The methanol eluate was evaporated and replaced with 90% of ethanol and applied to a column of PHP-LH-20 (2 ml). Elution with 20 ml of 90% ethanol gave solvolyzed bile alcohols. The unconjugated, deglucuronidated, and solvolyzed bile alcohol fractions were evaporated under reduced pressure. Each resulting residue was treated with pyridine–hexamethyldisilazane–trimethylchlorosilane 5:2:1 (by vol) for 2 h at 60°C. The resulting trimethylsilyl (TMS) ether derivatives were analyzed by GLC and GLC–MS.

Quantification of bile alcohols

Quantification was accomplished on GLC by the comparison of the peak areas of bile alcohols with those of known amounts of the internal standard, 25-homo-5β-cholane-3α,7α,12α,25-tetrol.

RESULTS AND DISCUSSION

Bile alcohols present in the bile samples obtained by drainage of six male Wistar rats were separated into unconjugated, glucuronoconjugated, and sulfated bile alcohol fractions by PHP-LH-20 column chromatography. After deconjugation and derivatization, bile alcohols of each fraction were analyzed by GLC and GLC–MS.

As shown in **Fig. 1**, GLC analysis revealed at least seven bile alcohols and their mass spectral data are listed in **Table 1**. Bile alcohols 1 through 6 were identified as 5α-choles-

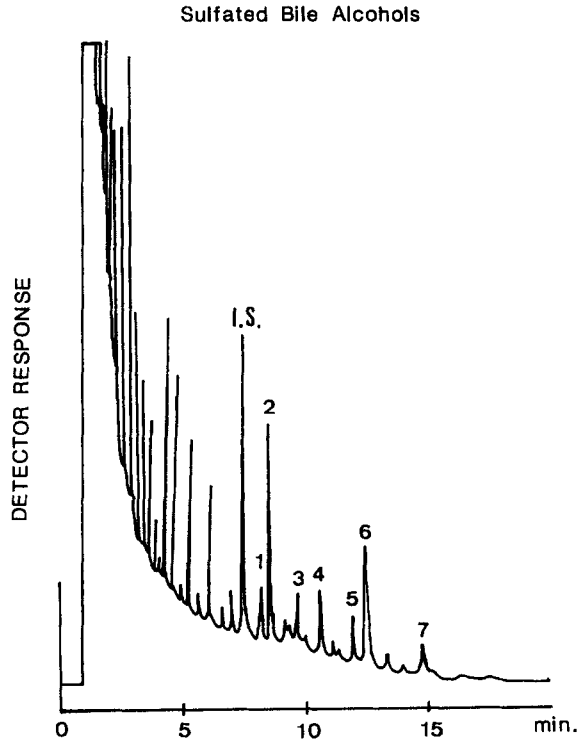

Fig. 1. Gas chromatogram of TMS ether derivatives of bile alcohols found in sulfated fraction of rat bile. Each bile alcohol was identified as follows: I. S. (internal standard), 25-homo-5β-cholestane-3α,7α,12α,25-tetrol; peak 1, 5α-cholestane-3α,7α,12α,26-tetrol; peak 2, 5β-cholestane-3α,7α,12α,26-tetrol; peak 3, 27-nor-5β-cholestane-3α,7α,12α,24,25-pentol; peak 4, 5β-cholestane-3α,7α,12α,24,26-pentol; peak 5, 5α-cholestane-3α,7α,12α,25,26-pentol (5α-bufol); peak 6, 5β-cholestane-3α,7α,12α,25,26-pentol (5β-bufol); peak 7, 5β-cholestane-3α,6β,7β,25,26-pentol.

tane-3α,7α,12α,26-tetrol, 5β-cholestane-3α,7α,12α,26-tetrol, 27-nor-5β-cholestane-3α,7α,12α,24,25-pentol,5β-cholestane-3α,7α,12α,24,26-pentol, 5α-bufol, and 5β-bufol, respectively, by the comparison of the retention times on GLC and their mass spectra with those of the TMS ethers of authentic samples.

The mass spectrum of the TMS ether derivative of the bile alcohol 7 resembled that of the corresponding derivative of 5β-bufol with respect to a series of ions at *m/z* 619, 529, 439, 349 and an ion at *m/z* 219. It is well known that the series of the ions in the spectrum of the TMS ether of 5β-bufol arises by the scission of the bond between C-25 and C-26 and the successive losses of TMSOH groups (19). The ion at *m/z* 219 is ascribed to the side chain (C-25 to C-27) fragment arising by the scission of the bond between C-24 and C-25 (3). The presence of these fragment ions suggests that the bile alcohol 7 is an isomer of 5β-bufol having two hydroxyl groups at C-25 and C-26. The mass spectrum of the TMS ether of bile alcohol 7 revealed intense ions at *m/z* 195 and 285, which were not seen in the spectrum of the TMS ether of 5β-bufol. The ions at *m/z* 195 and 285 are known to be characteristic of methyl ester-TMS ether derivatives of 3α,6β,7β-trihydroxy-5β-cholanoic acid and 3α,6α,7β-trihydroxy-5β-cholanoic acid (20). 3α,6β,7β-Trihydroxy-5β-cholanoic acid is known to be one of the major bile acids of rat bile (21). On the basis of the mass spectral data and biological consideration, it is conceivable that bile alcohol 7 is 5β-cholestane-3α,6β,7β,25,26-pentol possessing the nuclear structure of 3α,6β,7β-trihydroxy-5β-cholanoic acid and the side chain structure of 5β-bufol. Recent studies have shown that the novel bile alcohols, as their sulfuric acid esters, are major constituents of the bile of the West Indian manatee (11). The major bile alcohol of the marine mammals was identified as 5β-cholestane-3α,6β,7α,25,26-pentol (named as α-trichechol) and one of the minor bile alcohols was tentatively identified as 5β-cholestane-3α,6β,7β,25,26-pentol (named as β-trichechol). The mass spectrum of the TMS ether of the bile alcohol 7 was identical with that reported for the TMS ether of β-trichechol (11).

Profiles of bile alcohol in the conjugated modes are summarized in **Table 2**. In the present study 25-homo-5β-cholane-3α,7α,12α,25-tetrol was used as an internal standard. It is desirable that internal standard should have the same behavior as the compounds to be analyzed, but should not interfere with the analysis. Since 25-homo-5β-cholane-3α,7α,12α,25-tetrol itself is a bile alcohol that does not overlap with any other bile alcohol to be analyzed on GLC, this bile alcohol is superior to the other internal standards used previously such as 5α-cholestane and 5β-bufol (11, 22). The mean excretion of total bile alcohol was 27.91 nmol/ml of fistula bile (range 10.27–54.67). The bile alcohols were excreted into bile mainly as sulfated forms and in lesser amounts as glucuronoconjugated and unconjugated forms. Approximately 69% of total biliary bile alcohols was found in the sulfated fraction and 22% and 10% of total biliary bile alcohols were found in the glucuronoconjugated and unconjugated bile alcohol fractions, respectively. The percentages of each conjugation form in the total biliary alco-

TABLE 1. Chromatographic and mass spectra data of TMS ethers of seven bile alcohols isolated from rat bile

Peak Number[a]	RRT and GLC[b]	Important Fragment Ions *m/z* (Relative Intensity, %)								
1	1.26	253 (30)	343 (100)	347 (28)	365 (5)	439 (4)	454 (14)	529 (5)	544 (68)	634 (2)
2	1.33	253 (100)	343 (43)	365 (7)	439 (7)	454 (63)	529 (4)	544 (26)	634 (1)	
3	1.49	117 (63)	129 (69)	253 (11)	321 (100)	343 (26)	411 (79)	501 (53)	591 (12)	681 (4)
4	1.63	129 (57)	131 (77)	253 (45)	321 (100)	343 (22)	411 (71)	501 (57)	591 (13)	
5	1.85	219 (77)	251 (39)	253 (31)	343 (55)	349 (76)	439 (59)	529 (100)	619 (19)	632 (13)
6	1.95	219 (79)	253 (66)	281 (41)	343 (21)	349 (74)	439 (62)	529 (100)	619 (18)	632 (11)
7	2.28	195 (100) 542 (4)	219 (33) 619 (9)	285 (76) 632 (3)	341 (4)	349 (6)	363 (1)	439 (11)	452 (2)	529 (45)

[a]Number of each bile alcohol is the same as shown in Fig. 1.
[b]RRTs are given relative to TMS ether of 25-homo-5β-cholane-3α,7α,12α,25-tetrol (capillary column coated with OV-1).

TABLE 2. Bile alcohols excreted in rat bile

Bile Alcohol	Unconjugated	Glucuronoconjugated	Sulfated	Total
		nmol of bile alcohol excreted per ml of bile		
1	ND	0.19(0-0.72:2)	1.15(0-2.28:4)	1.35(0-2.76:5)
2	1.90(0.24-7.04:6)	4.54(4.09-5.94:6)	8.77(0.41-36.01:6)	15.22(6.83-38.53:6)
3	0.05(0-0.24:1)	ND	1.08(0-5.45:2)	1.12(0-5.69:2)
4	0.07(0-0.30:2)	0.60(0-2.38:2)	2.82(0-5.51:4)	3.50(0-6.25:5)
5	0.19(0-1.00:2)	ND	0.74(0-1.83:3)	0.93(0-1.83:4)
6	0.39(0-1.32:5)	0.60(0-2.78:2)	3.61(1.00-7.34:6)	4.61(2.31-7.34:6)
7	0.09(0-0.23:3)	0.16(0-0.90:1)	0.93(0-3.31:4)	1.18(0.12-3.31:6)
Total	2.69(0.31-7.19)	6.02(4.00-9.83)	18.99(1.41-49.43)	27.91(10.27-54.67)

Data are from six rats. Figures in parentheses indicate range and incidence of each bile alcohol in each fraction of the six rats. The number after the colon indicates the number of rats in which the particular bile alcohol form was found. Number of each bile alcohol is the same as shown in Fig. 1.

hols of rat were very similar to those of human gall bladder bile (3). Sulfation of bile alcohols seems to produce a form of bile alcohol that is easier to excrete into rat bile as well as into human bile, since bile alcohol sulfates are the most polar of the three forms. This seems to explain the reason for the predominance of sulfate forms in the biliary bile alcohols.

Until the present study, only 5β-cholestane-3α,7α,12α,26-tetrol had been identified from rat bile in the monosulfated form (23). The present study demonstrates the presence of six additional minor bile alcohols in the unconjugated and glucuronoconjugated bile alcohol fractions, as well as the sulfated bile alcohol fraction. Bile alcohol profiles of the three fractions were not very different from one another, and the major bile alcohol of all the fractions was 5β-cholestane-3α,7α,12α,26-tetrol. Five of seven bile alcohols observed in this study have a 3α,7α,12α-trihydroxylated steroid nucleus and a hydroxyl group at C-26 in the side chain. These bile alcohols seem to be biosynthesized through the pathway involving the transformation of cholesterol to 5α- or 5β-cholestane-3α,7α,12α,26-tetrols via 5α- or 5β-cholestane-3α,7α,12α-triols. The coexistence of 5α- and 5β-cholestane-3α,7α,12α,26-tetrols and 5α- and 5β-bufol suggests that the latter compounds are formed by the direct 25-hydroxylation of the former. Bile alcohol 3, 27-nor-5β-cholestane-3α,7α,12α,24,25-pentol, is the major bile alcohol excreted in human urine (22) and a minor component of human bile (3). However, the biosynthetic pathway of this bile alcohol is still not defined. Bile alcohol 7, which was tentatively identified as 5β-cholestane-3α,6β,7β,25,26-pentol, should be derived from 5β-cholestane-3α,7α-diol, an obligate precursor of chenodeoxycholic acid. The conversion of 5β-cholestane-3α,7α-diol to bile alcohol 7 includes the hydroxylations to the bufol-type side chain and also transformation to the β-muricholic acid-type steroid nucleus. However, the sequence of the side chain conversion and steroid nucleus conversion could not be clarified in the present study. It has been thought that the modification of the hydroxyl groups of the steroid nucleus occurs after the side chain cleavage (24). The presence of 5β-cholestane-3α,6β,7β,25,26-pentol showed that the modification of the steroid nucleus occurred even in C_{27} bile alcohols. This suggests that β-muricholic acid could be biosynthesized directly from the bile alcohol that has a 3α,6β,7β-trihydroxy steroid nucleus similar to the other primary bile acids.

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