Hiroshima J. Med. Sci.
Vol.43, No.3, 81~86, September, 1994
HIJM 43-9

Biochemical Studies of Inherited Diseases Related to Abnormal Cholesterol Metabolism. II: Absence of Unusual C_{28} and C_{29} Bile Acid Homologs in Bile and Urine of Sitosterolemia

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ABSTRACT

Bile acids, bile alcohols and sterols excreted in bile and urine from a patient with sitosterolemia were studied. Glycine- and taurine-conjugated cholic acid, deoxycholic acid and chenodeoxycholic acid were identified as the major constituents of both the bile and urine. Lesser amounts of unconjugated cholic acid and 3α, 7α, 12α, 24-tetrahydroxy-5β-cholestan-26-oic acid were found in the bile, but cholic acid was the only unconjugated bile acid in the urine. Relatively high proportions of campesterol and sitosterol compared to cholesterol were excreted in the bile, while cholesterol was the only sterol detected in the urine. Bile alcohols were not detected in the bile, but the following bile alcohols were excreted in the urine as glucurono-conjugates: 5β-cholestane-3α,7α,12α,25-tetrol; 27-nor-5β-cholestane-3α,7α,12α,24,25-pentol; 5β-cholestane-3α,7α,12α,23,25-pentol; 5β-cholestane-3α,7α,12α,24,25-pentol; 5β-cholestane-3α,7α,12α,25,26-pentol. In neither the bile nor urine, were C_{28} and C_{29} bile acid homologs detected. Thus, the main route for the excretion of plant sterols in sitosterolemia is thought to be secretion into the bile as neutral sterols.

Key words: *Sitosterolemia, Sitosterol, Campesterol, Bile acid, Bile alcohol*

It is well known that human C_{28} and C_{29} sterols (Fig. 1) are not synthesized endogenously and that less than 5% of the daily intake is absorbed[15]. Kritchevsky et al have shown that ergosterol was oxidized by mitochondrial preparations from rat and mouse livers and that one of the oxidation products shows a chromatographic behavior close to that of 3α,7α,12α-trihydroxy-5β-cholestan-26-oic acid[11]. It has been also shown that monkeys fed sitosterol excreted 3β-hydroxy-24-ethylcholest-5-en-26-oic acid, 3α-hydroxy-24-ethyl-5β-cholestan-26-oic acid and 3α,7α-hydroxy-24-ethyl-5β-cholestan-26-oic acid in feces (Fig. 2)[12]. These findings indicate that mammalian systems can metabolize C_{28} and C_{29} sterols into C_{28} and C_{29} bile acid homologs.

Sitosterolemia is a rare inherited lipid storage disease which shows tendon xanthomas, premature atherosclerosis and increased amounts of serum plant sterols[2,16]. Unusually increased intestinal absorption and sluggish turnover of plant sterols are suspected to cause this disease. In order to obtain information for the metabolism of C_{28} and C_{29} sterols in humans, especially to ascertain whether unusual bile acids and bile alcohols carrying carbon skeletons of C_{28} and C_{29} sterols would be produced in sitosterolemia, we examined bile acids, bile alcohols and sterols in the bile and urine of a patient with sitosterolemia.

MATERIALS AND METHODS

Reference Steroids

Cholesterol, campesterol, sitosterol, cholic acid, deoxycholic acid and chenodeoxycholic acid were commercial products. 3α,7α,12α,24-Tetrahydroxy-5β-cholestan-26-oic acid (TEHCA)[17], 5β-cholestane-3α,7α,12α,25-tetrol (25-tetrol)[10], 27-nor-5β-cholestane-3α,7α,12α,24,25-pentol (27-nor-24,25-pentol)[13], 5β-cholestane-3α,7α,12α,23,25-pentol (23,25-pentol)[9], 5β-cholestane-3α,7α,12α,24,25-pentol (24,25-pentol)[8], 5β-cholestane-3α,7α,12α,25,26-pentol (25,26-pentol)[8] were synthesized or isolated from natural sources according to the methods reported previously.

Extraction and Fractionation of Steroids from Bile and Urine

Bile and urine were collected from a patient with sitosterolemia (female, 51 years) and stored at –20°C until analysis. The bile sample (1.0 ml)

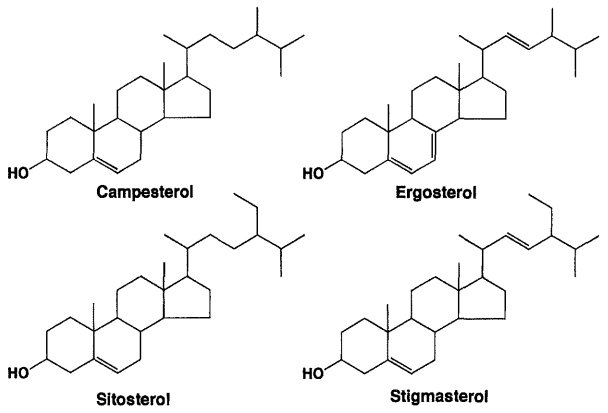

Fig. 1. C_{28} and C_{29} Sterols

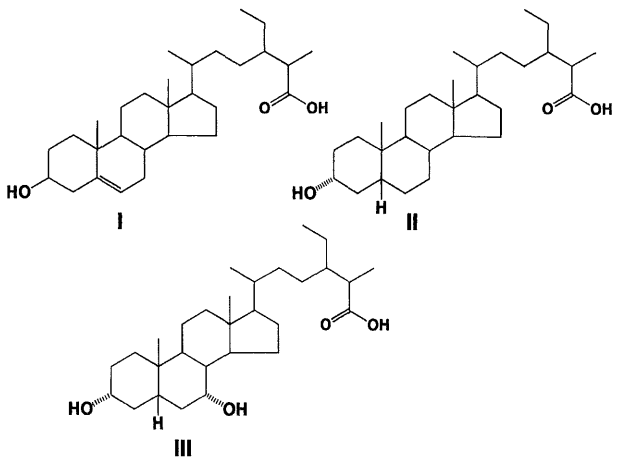

Fig. 2. C_{29} Steroidal Acids Excreted in Feces of a Monkey Fed Sitosterol.
I, 3β-Hydroxy-24-ethylcholest-5-en-26-oic acid;
II, 3α-hydroxy-24-ethyl-5β-cholestan-26-oic acid;
III, 3α,7α-dihydroxy-24-ethyl-5β-cholestan-26-oic acid

was extracted with ten volumes of ethanol at room temperature. The ethanolic extract was concentrated to dryness under a reduced pressure to leave a residue. The urine sample (260 ml) was passed through a Sep-pak C_{18} cartridge (Waters). The cartridge was washed with 10 ml of water and then eluted with 10 ml of methanol. The methanolic eluate was concentrated to dryness. The residue from each of the bile and urine samples was dissolved in 90 % ethanol and the solution was passed through a column of piperidinohydroxypropyl Sephadex LH-20 (PHP-LH-20, 4 ml)[7]. The column was eluted successively with 12 ml of 90 % ethanol, 40 ml of 0.1 M acetic acid in 90 % ethanol, 40 ml of 0.2 M formic acid in 90 % ethanol, and 40 ml of 1 % ammonium carbonate in 70 % ethanol, to give sterols (S-fraction), unconjugated bile acids (U-fraction), glycine-conjugated bile acids and glucurono-conjugated bile alcohols (G-fraction) and taurine-conjugated bile acids (T-fraction), respectively.

Hydrolysis of Conjugated Steroids

The sample containing glucurono-conjugated bile alcohols was hydrolyzed enzymatically with 500 U of β-glucuronidase (EC 3.2.31, Sigma Chemical Co., Type H-1) at 37°C in 0.1 M phosphate buffer at pH 6.8. After a 48 h incubation, the incubation mixture was applied to a Sep-pak C_{18} cartridge and the cartridge was eluted with 5 ml of methanol. The methanolic 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 (4 ml). The column was eluted with 12 ml of 90 % ethanol to give deconjugated bile alcohols. The sample containing glycine- or taurine-conjugated bile acids was subjected to hydrolysis at 120°C for 3 h in 5 ml of 2.5 N KOH. After dilution with water and acidification with diluted HCl, the hydrolysate was extracted with ether. The ethereal extract was washed with water and evaporated to dryness to give a residue containing deconjugated bile acids.

Gas-liquid chromatography (GLC)

The samples, as their trimethylsilyl (TMS) ether derivatives, or their methyl ester-TMS ether derivatives, were run on a capillary column (15m×0.32mm i.d.) coated with DB-1HT (J & W Scientific) and a glass column (2m×3mm i.d.) packed with 3% Poly I-110 on 80/100 mesh Gas Chrom Q. The TMS ether derivatives were prepared as described previously[13]. Quantitation was accomplished by comparing GLC peak area of the biological sample to that of the external reference compound. Measurements of peak areas were accomplished with an automatic integrator.

Gas-liquid chromatography-mass spectrometry (GC-MS)

GC-MS was carried out on a Shimadzu GCMS-QP 1000 gas chromatograph-mass spectrometer equipped with a data processing system. The following conditions were used : column, DB-1HT (15m×0.32mm i.d.) ; column temperature, 200–260°C at a rate of 3°C/min ; ion source temperature, 250°C ; ionizing energy, 70 ev ; and trap current, 60μA.

RESULTS

The bile sample obtained from a patient with sitosterolemia was examined for bile acids, bile alcohols and sterols. The bile sample was subjected to ion-exchange chromatography using PHP-LH-20 to give the S-fraction, U-fraction, G-fraction and T-fraction (Fig. 3).

An aliquot of the S-fraction was concentrated to dryness and the residue was derivatized into the TMS ethers and analyzed by GLC and GC-MS. A

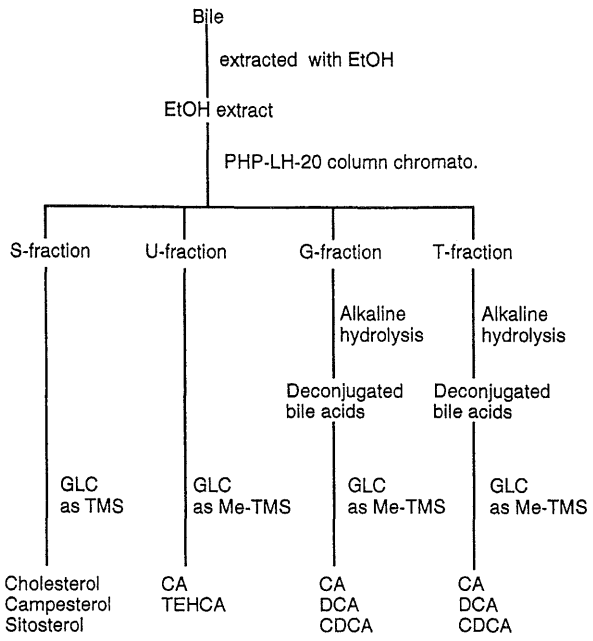

Fig. 3. Extraction, Fractionation and Identification of Biliary Steroids
CA, cholic acid; DCA, deoxycholic acid; CDCA, chenodeoxycholic acid; TEHCA, 3α,7α,12α,24-tetrahydroxy-5β-cholestan-26-oic acid.

typical gas-liquid chromatogram of the TMS ether derivatives of the biliary neutral compounds is shown in Fig. 4. There were three sterol peaks,1, 2, and 3. These sterols were identified as cholesterol (56 % of the total biliary sterols), campesterol (24 %), and sitosterol (20 %) , respectively, by comparing their mass spectra and chromatographic behaviors with those of the TMS ether derivatives of authentic compounds. The concentration of the total biliary sterols estimated by GLC was 22 μg / ml of the bile.

The U-fraction was concentrated to dryness and the residue was converted into the methyl ester-TMS ether derivatives and analyzed by GLC and GC-MS. A typical gas-liquid chromatogram of the methyl ester-TMS ether derivatives of the biliary unconjugated bile acids is shown in Fig. 5. Two bile acid peaks, 4 and 5, were seen. The GLC behaviors and mass spectra of the major peak 4 (75 % of the total unconjugated bile acids in the bile) and the minor peak 5 (25 %) were identical with those of the methyl ester-TMS ether derivatives of authentic cholic acid and TEHCA, respectively. The concentration of the total unconjugated bile acids in the bile estimated by GLC was 9.2 μg / ml of the bile.

Since preliminary TLC analysis revealed that

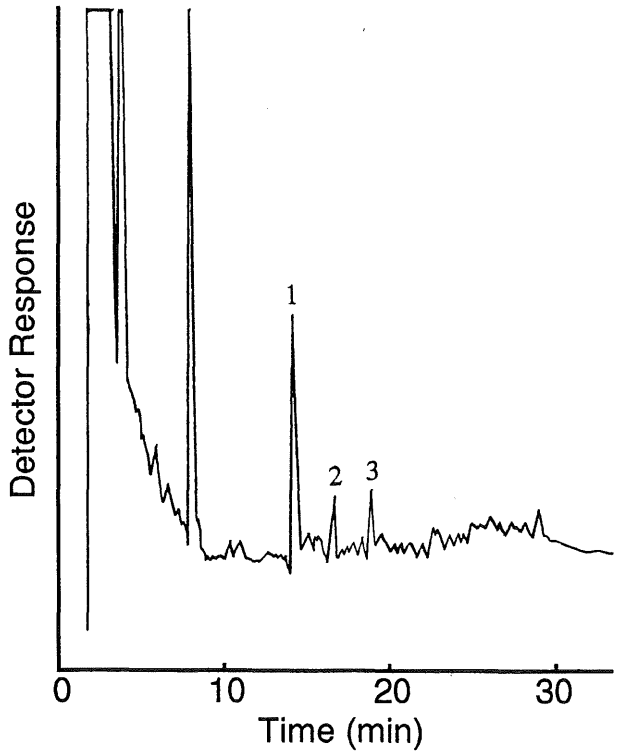

Fig. 4. Gas-Liquid Chromatogram of the TMS Ether Derivatives of the Biliary Neutral Compounds (S-Fraction).
Capillary column DB-1HT (15m × 0.32 mm i.d.) was employed; column temperature, 200–260°C at a rate of 3°C/min. The peaks 1, 2 and 3 were identified as cholesterol, campesterol and sitosterol, respectively.

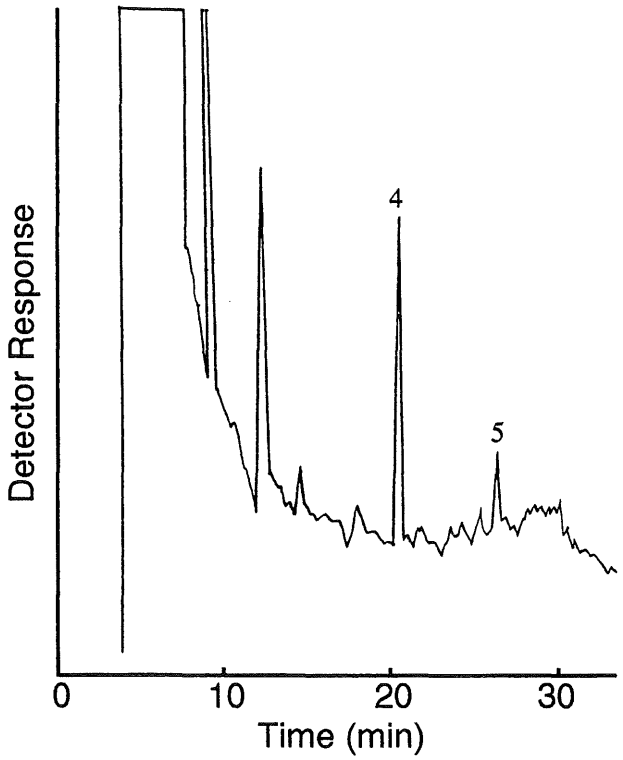

Fig. 5. Gas-Liquid Chromatogram of the Methyl Ester-TMS Ether Derivatives of the Biliary Unconjugated Bile Acids (U-Fraction).
Conditions as in Fig. 1. The peaks 4 and 5 were identified as cholic acid and 3α,7α,12α,24-tetrahydroxy-5β-cholestan- 26-oic acid, respectively.

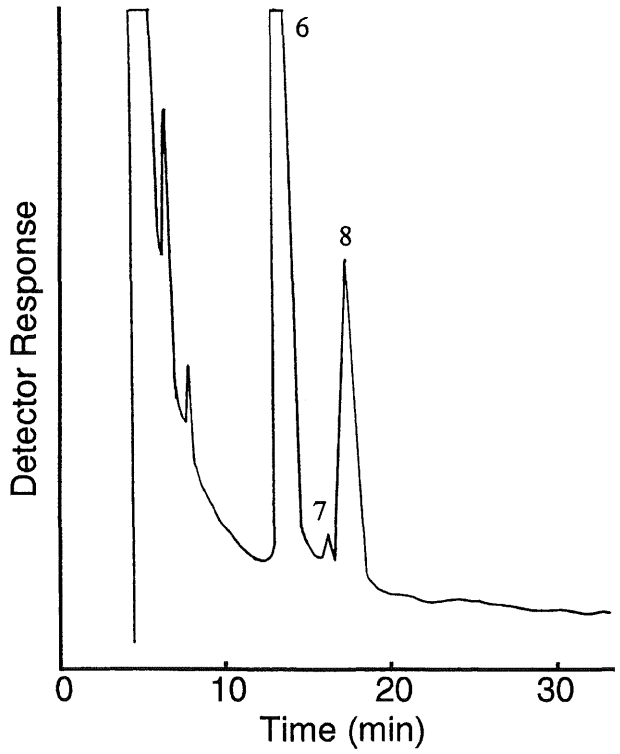

Fig. 6. Gas-Liquid Chromatogram of the Methyl Ester-TMS Ether Derivatives of the Biliary Glycine-Conjugated Bile Acids.
Glass column Poly I-110 (2m×3mm i.d.) was employed; column temperature, 240°C. The peaks 6, 7 and 8 were identified as cholic acid, deoxycholic acid and chenodeoxycholic acid, respectively.

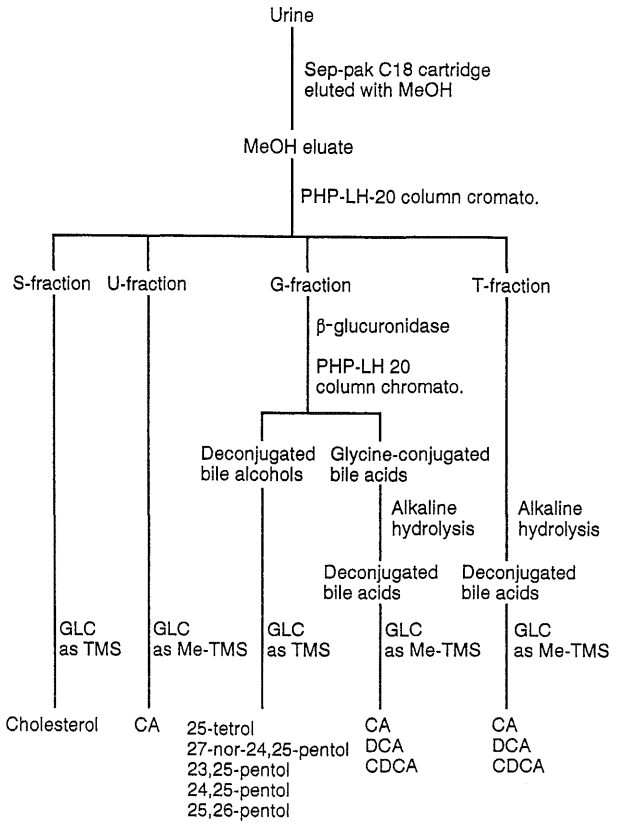

Fig. 7. Extraction, Fractionation and Identification of Urinary Steroids.
CA, cholic acid; DCA, deoxycholic acid; CDCA, chenodeoxycholic acid;
25-tetrol, 5β-cholestane-3α,7α,12α,25-tetrol;
27-nor-24,25-pentol, 27-nor-5β-cholestane-3α,7α,12α, 24,25-pentol;
23,25-pentol, 5β-cholestane-3α,7α,12α,23,25-pentol;
24,25-pentol, 5β-cholestane-3α,7α,12α,24,25-pentol;
25,26-pentol, 5β-cholestane-3α,7α,12α,25,26-pentol.

the biliary G-fraction contained only glycine-conjugated bile acids, the material eluted in the G-fraction was hydrolyzed with 2.5 N potassium hydroxide without the β-glucuronidase treatment. The resulting deconjugated bile acids were analysed by GLC and GC-MS as the methyl ester-TMS ether derivatives. A typical gas-liquid chromatogram is shown in Fig. 6. Three bile acid peaks 6, 7 and 8 were seen. These bile acids were identified as cholic acid (75 % of the total glycine-conjugated bile acids in the bile), deoxycholic acid (3 %), and chenodeoxycholic acid (22 %), respectively, by comparing their chromatographic behaviors and mass spectra with those of the methyl ester-TMS ether derivatives of authentic compounds. The concentration of the total glycine-conjugated bile acids in the bile estimated by GLC was 2.2 mg / ml of the bile.

The biliary T-fraction was treated and analyzed in the same manner as described for the biliary G-fraction. GLC and GC-MS analysis revealed that the T-fraction contained three bile acids, cholic acid (80 % of the total taurine-conjugated bile acids in the bile), deoxycholic acid (2 %), and chenodeoxycholic acid (18 %). No other bile acid was detected in this fraction. The concentration of the total taurine-conjugated bile acids in the bile estimated by GLC was 0.25 mg / ml of the bile.

The urine sample obtained from a patient with sitosterolemia was also fractionated into the S-, U-, G- and T-fractions by the same procedure as described for the bile sample (Fig. 7).

GLC and GC-MS analysis of the urinary S-fraction as the TMS ether derivatives revealed that cholesterol (0.14 μg / ml of the urine) was the only steroid detected in this fraction.

GLC and GC-MS analysis of the urinary unconjugated bile acids (U-fraction) as the methyl-ester-TMS ether derivatives revealed that cholic acid (51 μg / ml of the urine) was the only detectable bile acid of this fraction.

Preliminary TLC analysis revealed that the urinary G-fraction contained both glycine-conjugated bile acids and glucurono-conjugated bile alcohols. The G-fraction was concentrated to dryness and the resulting residue was treated with β-glucuro-

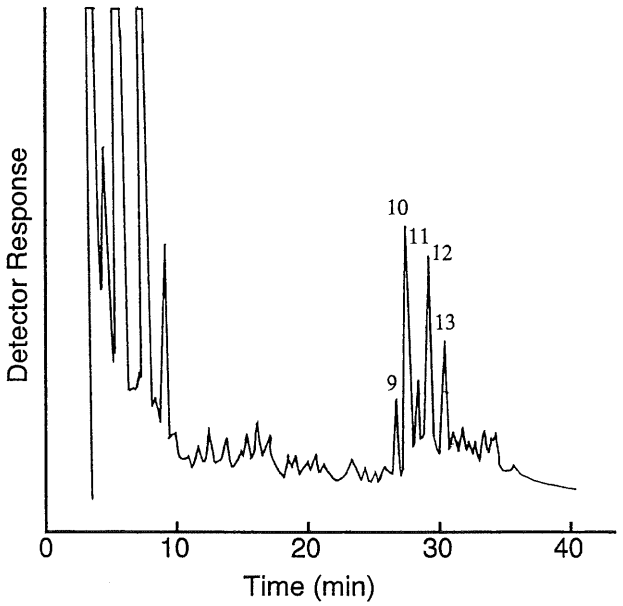

Fig. 8. Gas-Liquid Chromatogram of the TMS Ether Derivatives of the Urinary Glucurono-Conjugated Bile Alcohols.
Conditions as in Fig. 1. The peaks 9–13 were identified as 5β-cholestane-3α,7α,12α,25-tetrol, 27-nor-5β-cholestane-3α,7α,12α,24,25-pentol, 5β-cholestane-3α,7α,12α,23,25-pentol, 5β-cholestane-3α,7α,12α,24,25-pentol, 5β-cholestane-3α,7α,12α,25,26-pentol, respectively.

nidase as described in the section on Materials and Methods. The deconjugated bile alcohols were obtained in the ethanol eluate from the PHP-LH-20 column. The PHP-LH-20 column was further eluted with 0.2 M formic acid in 90 % ethanol to obtain glycine-conjugated bile acids, which were hydrolyzed with alkali to give deconjugated bile acids.

The deconjugated bile alcohols were analyzed by GLC and GC-MS as the TMS ether derivatives. A typical gas-liquid chromatogram is shown in Fig. 8. Five bile alcohol peaks 9–13 were seen. These bile alcohols were identified as 25-tetrol (10 % of the total bile alcohols in the urine), 27-nor-24,25-pentol (29 %), 23,25-pentol (12 %), 24,25-pentol (35 %) and 25,26-pentol (14 %), respectively, by comparing their chromatographic behaviors and mass spectra with those of the TMS ether derivatives of authentic bile alcohols. The concentration of the total bile alcohols in the urine estimated by GLC was 0.72 μg / ml of the urine.

GLC and GC-MS analysis revealed that the deconjugated bile acids obtained from the urinary G-fraction consisted of three bile acids, cholic acid (91 % of the total glycine-conjugated bile acids in the urine), deoxycholic acid (4 %) and chenodeoxycholic acid (5 %). The concentration of the total glycine-conjugated bile acids estimated by GLC was 0.69 μg / ml of the urine.

The urinary T-fraction was treated and analyzed in the same manner as described for the biliary T-fraction. Cholic acid (82 % of the total taurine-conjugated bile acids in the urine), deoxycholic acid (8 %) and chenodeoxycholic acid (9 %) were identified. The concentration of the total taurine-conjugated bile acids in the urine estimated by GLC was 0.07 μg / ml of the urine.

DISCUSSION

The present study demonstrates that the bile acid and bile alcohol profiles in the bile and urine of a patient with sitosterolemia are the same as those in the bile and urine of healthy humans. Postulated biosynthetic pathways for the formation of C_{27} bile acids and C_{27} bile alcohols in the patient with sitosterolemia are shown in Fig. 9. Although we postulated the presence of unusual bile acids and bile alcohols, such as compounds carrying carbon skeletons of C_{28} and C_{29} sterols, such compounds could not be found in the bile and urine of the patient with sitosterolemia.

Salen et al have reported that in man about 20

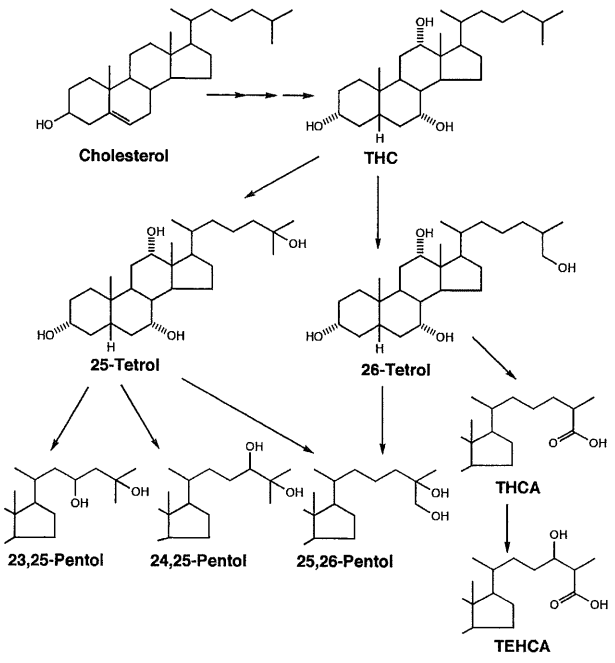

Fig. 9. Postulated Biosynthetic Pathways for the Formation of C_{27} Bile Acids and C_{27} Bile Alcohols in the Patient with Sitosterolemia.
THC, 5β-Cholestane-3α,7α,12α-triol;
25-Tetrol, 5β-Cholestane-3α,7α,12α,25-tetol;
26-Tetrol, 5β-Cholestane-3α,7α,12α,26-tetol;
23,25-Pentol, 5β-Cholestane-3α,7α,12α,23,25-pentol;
24,25-Pentol, 5β-Cholestane-3α,7α,12α,24,25-pentol;
25,26-Pentol, 5β-Cholestane-3α,7α,12α,25,26-pentol;
THCA, 3α,7α,12α-trihydroxy-5β-cholestan-26-oic acid;
TEHCA, 3α,7α,12α,24-tetrahydroxy-5β-cholestan-26-oic acid.

% of the sitosterol absorbed from the intestine was converted to cholic acid and chenodeoxycholic acid, and the remainder was excreted in bile as free sterol[15]. The present study demonstrated the biliary excretion of relatively high proportions of plant sterols as compared with cholesterol in the patient with sitosterolemia. This result, in agreement with the results of the previous investigation by Salen et al , indicates that in sitosterolemia a large part of the absorbed plant sterols are excreted in the bile without modification, though a small part would be converted to the primary C_{24} bile acids.

Mechanism for the conversion of C_{28} and C_{29} sterols to C_{24} bile acids is still unknown. A possible mechanism includes the dealkylation of plant sterols to provide C_{27} sterols, which are then degraded into C_{24} bile acids. Several species of insects are known to dealkylate plant sterols to form cholesterol[5].

A possible reason for the absence of C_{28} and C_{29} bile acid homologs in sitosterolemia is that sitosterol is a poor substrate for cholesterol 7α-hydroxylase, the rate-limiting enzyme for bile acid biosynthesis[1,3,4].

Dayal et al have found substantial amounts of bile alcohols in the urine and feces of a patient with sitosterolemia[6]. The present study confirmed the presence of bile alcohols in the urine of the sitosterolemia patient. The increased formation and excretion of bile alcohols suggests an abnormal cholesterol metabolism in sitosterolemia. The depression of HMG-CoA reductase activity in sitosterolemia was also reported[14].

In conclusion, we could not find C_{28} and C_{29} bile acid homologs in the urine and bile of the patient with sitosterolemia. The main route for the excretion of plant sterols in this disease would be their secretion into the bile as neutral sterols.

(Received May 17, 1994)
(Accepted September 12, 1994)

REFERENCES

1. **Aringer, L. and Eneroth, P.** 1973. Studies on the formation of C_7-oxygenated cholesterol and β-sitosterol metabolites in cell-free preparations of rat liver. J. Lipid Res. **14:** 563–572.
2. **Bhattacharayya, A.K. and Connor, W.E.** 1974. β-Sitosterolemia and Xanthomatosis. A newly described lipid storage disease in two sisters. J. Clin. Invest. **53:** 1033–1043.
3. **Boberg, K.M., Akerlund, J.E. and Bjorkhem, I.** 1989. Effect of sitosterol on the rate-limiting enzymes in cholesterol synthesis and degradation. Lipids **24:** 9–12.
4. **Boberg, K.M., Einarsson, K. and Bjorkhem, I.** 1990. Apparent lack of conversion of sitosterol into C_{24}-bile acids in humans. J. Lipid Res. **31:** 1083–1088.
5. **Clayton, R.B.** 1964. The utilization of sterols in insects. J. Lipid Res. **5:** 3–19.
6. **Dayal, B., Tint, G.S., Toome, V., Batta, A.K., Shefer, S. and Salen, G.** 1985. Synthesis and structure of 26 (or 27-)-nor-5β-cholestane-3α,7α,12α,24S,25-pentol isolated from the urine and feces of a patient with sitosterolemia and xanthomatosis. J. Lipid. Res. **26:** 298–305.
7. **Goto, J., Hasegawa, M., Kato, H. and Nambara, T.** 1978. A new method for simultaneous determination of bile acids in human bile without hydrolysis. Clin. Chim. Acta **87:** 141–147.
8. **Hoshita, T.** 1962. Synthesis of 3α,7α,12α,25ξ,26- and 3α,7α,12α,24ξ,25-pentahydroxycoprostanes. J. Biochem. **52:** 176–179.
9. **Hoshita, T., Yasuhara, M., Kihira, K. and Kuramoto, T.** 1976. Identification of (23S)-5β-cholestane-3α,7α,12α,23,25-pentol in cerebrotendinous xanthomatosis. Steroids **27:** 657–664.
10. **Kihira, K., Kosaka, D., Une, M., Hiraoka, T., Kajiyama, G. and Hoshita, T.** 1987. Synthesis of deuterium labeled bile alcohols. J. Label. Comp. Radiopharm. **24:** 1421–1428.
11. **Kritchevsky, D., Staple, E. and Whitehouse, M.W.** 1961. Oxidation of ergosterol by rat and mouse liver mitochondria. Proc. Soc. Exptl. Biol. Med. **106:** 704–708.
12. **Kritchevsky, D., Davidson, L.M., Mosbach, E.H. and Cohen, B.I.** 1981. Identifical of acidic steroids in feces of monkeys fed β-sitosterol. Lipids **16:** 77–78.
13. **Kuwabara, M., Ushiroguti, T., Kihira, K., Kuramoto, T. and Hoshita, T.** 1984. Identification of bile alcohols in urine from healthy humans. J. Lipid Res. **25:** 361–368.
14. **Nguyen, L.B., Shefer, S., Salen, G., Ness, G.C., Tint, G.S., Zaki, F.G. and Rani, I.** 1990. A Molecular Defect in Hepatic Cholesterol Biosynthesis in Sitosterolemia with Xanthomatosis. J. Clin. Invest. **86:** 923–931.
15. **Salen, G., Ahrens, E.H.Jr. and Grundy, S.T.** 1970. Metabolism of β-Sitosterol in Man J. Clin. Invest. **49:** 952–967.
16. **Salen, G., Shefer, S., Nguyen, L., Ness, G.C., Tint, G.S. and Shore, V.** 1992. Sitosterolemia. J. Lipid Res. **33:** 945–955.
17. **Une, M., Nagai, F., Kihira, K., Kuramoto, T. and Hoshita, T.** 1983. Synthesis of four diastereoisomers at carbons 24 and 25 of 3α,7α,12α,24-tetrahydroxy-5β-cholestan-26-oic acid, intermediates of bile acid biosynthesis. J. Lipid Res. **24:** 924–929.