Biochemical Studies of Inherited Diseases Related to Abnormal Cholesterol Metabolism. II: Absence of Unusual C₂₈ and C₂₉ Bile Acid Homologs in Bile and Urine of Sitosterolemia

Akira OHSHIMA¹, Mizuho Une² and Takahiko HOSHITA*²

¹ Department of Surgery 1, Kyushu University School of Medicine, Maidashi, 3-1-1, Higashi-ku, Fukuoka, 812, Japan
² Institute of Pharmaceutical Sciences, Hiroshima University School of Medicine, Kasumi, 1-2-3, Minami-ku, Hiroshima, 734, Japan

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, 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 C₂₈ and C₂₉ 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 C₂₈ and C₂₉ sterols (Fig. 1) are not synthesized endogenously and that less than 5% of the daily intake is absorbed². 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 acid¹⁵. It has been also shown that monkeys fed sitosterol excreted 3β-hydroxy-24-ethyl cholesterol-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 C₂₈ and C₂₉ sterols into C₂₈ and C₂₉ bile acid homologs. Sitosterolemia is a rare inherited lipid storage disease which shows tendon xanthomas, premature atherosclerosis and increased amounts of serum plant sterols. 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 C₂₈ and C₂₉ sterols in humans, especially to ascertain whether unusual bile acids and bile alcohols carrying carbon skeletons of C₂₈ and C₂₉ 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), 5β-cholestane-3α, 7α, 12α, 25-tetrol (25-tetrol), 27-nor-5β-cholestone-3α, 7α, 12α, 24, 25-pentol (27-nor-24, 25-pentol), 5β-cholestane-3α, 7α, 12α, 23, 25-pentol (23, 25-pentol), 5β-cholestane-3α, 7α, 12α, 24, 25-pentol (24, 25-pentol), 5β-cholestane-3α, 7α, 12α, 25, 26-pentol (25, 26-pentol), 5β-cholestane-3α, 7α, 12α, 25, 26-pentol (25, 26-pentol) 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)
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 C18 cartridge (Waters). The cartridge was washed with 10 ml of water and then eluted with 10 ml of methanol. The methanolic eluate was evaporated to dryness and the resulting residue was dissolved in 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 x 0.32mm i.d.) coated with DB-1HT (J & W Scientific) and a glass column (2m x 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. 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 x 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
Absence of Abnormal Bile Acids in Sitosterolemia

---

**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.

---

**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.
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-
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 C27 bile acids and C27 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 C28 and C29 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
% 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. The present study demonstrated the biliary excretion of relatively high proportions was converted to cholic acid and chenodeoxycholic acid, and the remainder was excreted in bile as

Mechanism for the conversion of C28 and C29 sterols to C24 bile acids is still unknown. A possible mechanism includes the dealkylation of plant sterols to provide cholesterol biosynthesis. A reasonable mechanism for the conversion of C28 and C29 bile acid homologs in sitosterolemia is that sitosterol is a poor substrate for cholesterol 7α-hydroxylase, the rate-limiting enzyme for bile acid biosynthesis. Bhattacharayya et al. have found substantial amounts of bile acids in the urine and feces of a patient with sitosterolemia. The present study confirmed the presence of bile acids in the urine of the sitosterolemia patient. The increased formation and excretion of bile acids suggests an abnormal cholesterol metabolism in sitosterolemia. The depression of HMG-CoA reductase activity in sitosterolemia was also reported.

In conclusion, we could not find C28 and C29 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