Appearance of atypical 3α,6β,7β,12α-tetrahydroxy-5β-cholan-24-oic acid in spgp knockout mice

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Abstract Bile formation and its canalicular secretion are essential functions of the mammalian liver. The *sister-of-p-glycoprotein (spgp)* gene was shown to encode the canalicular bile salt export protein, and mutations in *spgp* gene were identified as the cause of progressive familial intrahepatic cholestasis type 2. However, target inactivation of *spgp* gene in mice results in nonprogressive but persistent cholestasis and causes the secretion of unexpectedly large amounts of unknown tetrahydroxylated bile acid in the bile. The present study confirms the identity of this tetrahydroxylated bile acid as 3α,6β,7β,12α-tetrahydroxy-5β-cholan-24-oic acid. The data further show that in serum, liver, and urine of the spgp knockout mice, there is a significant increase in the concentration of total bile salts containing a large amount of tetrahydroxy-5β-cholan-24-oic acid. The increase in total bile acids was associated with up-regulation of the mRNA of cholesterol 7α-hydroxylase in male mice only. It is suggested that the lower severity of the cholestasis in the spgp knockout mice may be due to the synthesis of 3α,6β,7β,12α-tetrahydroxy-5β-cholan-24-oic acid, which neutralizes in part the toxic effect of bile acids accumulated in the liver.—Perwaiz, S., D. Forrest, D. Mignault, B. Tuchweber, M. J. Phillip, R. Wang, V. Ling, and I. M Yousef. **Appearance of atypical 3α,6β,7β,12α-tetrahydroxy-5β-cholan-24-oic acid in spgp knockout mice.** ***J. Lipid Res.*** **2003.** 44: **494–502.**

Supplementary key words bile salt • cholestasis • canalicular membrane • knockout mice • gas chromatography mass spectrometry • electrospray tandem mass spectrometry • sister-of-p-glycoprotein

Bile acids are synthesized from cholesterol in the liver and secreted into the bile duct via an active process in the canaliculus. This process is mediated by an ATP-dependent system, sister of p-glycoprotein (spgp), which is also known as bile salt-exporting pump (BSEP) (1–5). A defect in the human SPGP gene has been suggested to be the basis for type 2 progressive familial intrahepatic cholestasis (PFIC2) (6). Recently, the mouse spgp was cloned and Spgp knockout mice were generated (7). In these mice, the target disruption of the Spgp gene showed reduction in biliary excretion of bile salts and impaired bile flow; however, mutant mice did not display the expected progressive cholestatic phenotype seen in human PFIC2 (7). It is suggested that alteration in bile acid metabolism, especially the production of a tetrahydroxylated bile acid, may alleviate the deleterious effects of possible accumulation of high bile salts in the liver of Spgp mutated mice. Therefore, the objective of this study is to characterize the biliary, liver, plasma, and urinary bile acids in the Spgp mutated mice. The data confirm for the first time the structure of the tetrahydroxylated bile acid as 3α,6β,7β,12α-tetrahydroxy-5β-cholan-24-oic acid, and further show a significant increase in the concentration of total bile salts in liver, plasma, and urine of the mutant mice that contain a large amounts of this tetrahydroxylated bile acid. Despite the increase in the total bile acids in the liver of both male and female spgp knockout mice, the expression of the mRNA for cholesterol 7α-hydroxylase was significantly up-regulated in the male mice but not affected in the females. These data suggest that the expression of cholesterol 7α-hydroxylase could not explain the changes in the bile acids pool in the spgp knockout mice.

MATERIALS AND METHODS

Reagents

Glycine- and taurine-conjugated cholic acid, chenodeoxycholic acid, deoxycholic acid, lithocholic acid, 5β-cholanic acid,

Manuscript received 2 October 2002 and in revised form 5 November 2002.

Published, JLR Papers in Press, December 1, 2002.
DOI 10.1194/jlr.M200394-JLR200

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TABLE 1. Distribution of bile acids (%) in the gallbladder bile of spgp knockout mice

	Male (Bile)			Female (Bile)		
Bile Acid	+/+	+/−	−/−	+/+	+/−	−/−
G-mono	0.01 ± 0.01	ND	0.01 ± 0.00	ND	ND	0.01 ± 0.01
G-di	0.01 ± 0.00	0.01 ± 0.00	0.02 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00
G-tri	0.14 ± 0.02[a]	0.15 ± 0.03[a]	0.05 ± 0.01	0.09 ± 0.02[a]	0.08 ± 0.00[a]	0.07 ± 0.08
G-tetra	ND	ND	ND	ND	ND	ND
T-mono	0.12 ± 0.05	0.06 ± 0.01[a]	0.08 ± 0.02	0.08 ± 0.02	0.17 ± 0.03[a]	0.07 ± 0.02
T-di	11.00 ± 1.70	8.00 ± 1.20	4.50 ± 0.70	11.00 ± 1.50	9.50 ± 0.70	2.70 ± 2.90
T-tri	83.00 ± 2.60	87.00 ± 1.30[a]	61.00 ± 0.70	84.00 ± 1.20	84.00 ± 0.70[a]	49.00 ± 20.00
T-tetra	5.00 ± 1.00	4.00 ± 0.40	34.00 ± 0.00	5.00 ± 0.70	6.50 ± 0.70	48.00 ± 23.00

ND, not detectable. Values shown are concentrations (percent) of glycine (G) and taurine (T) conjugated mono-, di-, tri-, and tetrahydroxylated bile acids. Bile acids were extracted from 20 μl of bile. Each value represents the average of three individual determinations for six animals (means ± SD).

[a] $P < 0.05$ male (+/+, +/−, −/−) versus female (+/+, +/−,−/−) spgp group.

and 3α,12α,diol 7-one 5β-cholanic acid were obtained from Calbiochem, San Diego, CA, and were of at least 98% purity. Muricholic acid was purchased form Steraloid Inc, Wilton, NH. Two pure reference compounds of tetrahydroxylated bile acids, namely 3α,6β,7β,12α-tetrahydroxy-5β-cholan-24-oic acid and 1β, 3α,7α,12α-tetrahydroxy-5β-cholan-24-oic acids, were gifts from Prof. Taiju Kuramoto, Institute of Pharmaceutical Sciences, Hiroshima University School of Medicine, 1-2-3 Kasumi, Minami-Ku, Hiroshima, 734, Japan. Octadecyl (C_{18}) Baker bond (R) extraction column was obtained from J. J. Baker, Inc. All other solvents and chemicals used were of either HPLC grade or of known analytical purity obtained from Sigma-Aldrich Chemical Co. Re-

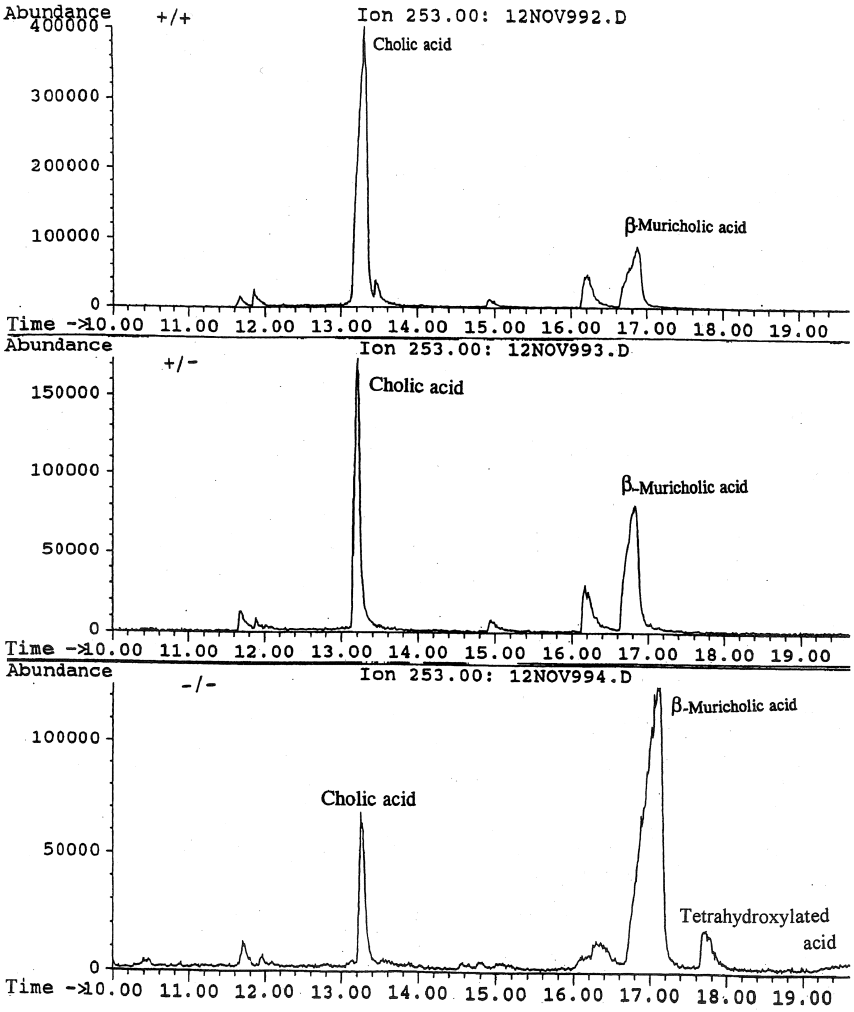

Fig. 1. Shows the gas chromatography-mass spectrometry (GC-MS) profile containing only ions of *m/z* 253, which is indicative of the trihydroxylated bile acids in the bile obtained from wild-type (+/+), heterozygous (+/−), and homozygous (−/−) spgp knockout mice obtained by GC-MS.

agents for the Northern blotting of cholesterol 7α-hydroxylase mRNA were purchased from Invitrogen Canada, Inc, Burlington, ON; Amersham Canada, Ltd., Oakville, ON; and Qiagen Canada Inc., Mississauga ON.

Samples

Gallbladder bile, serum, liver, and urine samples were obtained from homozygous, heterozygous, and the wild-type male and females of spgp knockout mice.

Extraction of bile acids

The methodology used for the determination of total and individual bile acids in bile, serum, and urine was similar to that described previously by this laboratory (8). One microgram of internal standard (3,12 diol 7-one 5β-cholanic acid) was added to each sample of serum (0.5 ml), urine (50–100 μl), and bile (20 μl), followed by the addition of 0.1 M sodium hydroxide (2 ml) to each sample, then heated at 64°C for 5–10 min. These samples were then subjected to a solid-phase extraction using a Bond-Elute C_{18} cartridge. The C_{18} cartridge was preconditioned prior to loading the samples with successive elution of 2 ml of chloroform-methanol (2:1, v/v), methanol, and HPLC water solutions. After loading the samples, the column was washed with 2 ml of HPLC water and n-hexane. The column was left for 10 min to remove any excess solvents. Bile acids were recovered from the cartridge by elution with methanol (5 ml). One part of the eluent was then evaporated to dryness under nitrogen. The residue was dissolved in 1 ml of acetonitrile-water (1:1, v/v) for the electrospray-tandem mass spectrometry (ES-MS/MS) analysis. The other portion was hydrolyzed and derivatized for gas chromatography mass-spectrometry (GC-MS) analysis. The bile acids from the liver tissues were extracted in a similar manner from the homogenate of 1 g of liver tissues in saline to which 10 μg of the internal standard was added.

Bile acid analysis by ES-MS/MS

A Hewlett Packard LC system (HPLC series 1100) equipped with automatic sample injector and was connected to a Quattro electrospray tandem mass spectrometer (Micromass). The LC system was used only for injecting the samples and was operated isocratically at flow rate of 10 μl/min. Liquid nitrogen was used as nebulizer and argon as collision gas. Negative ion mass spectra

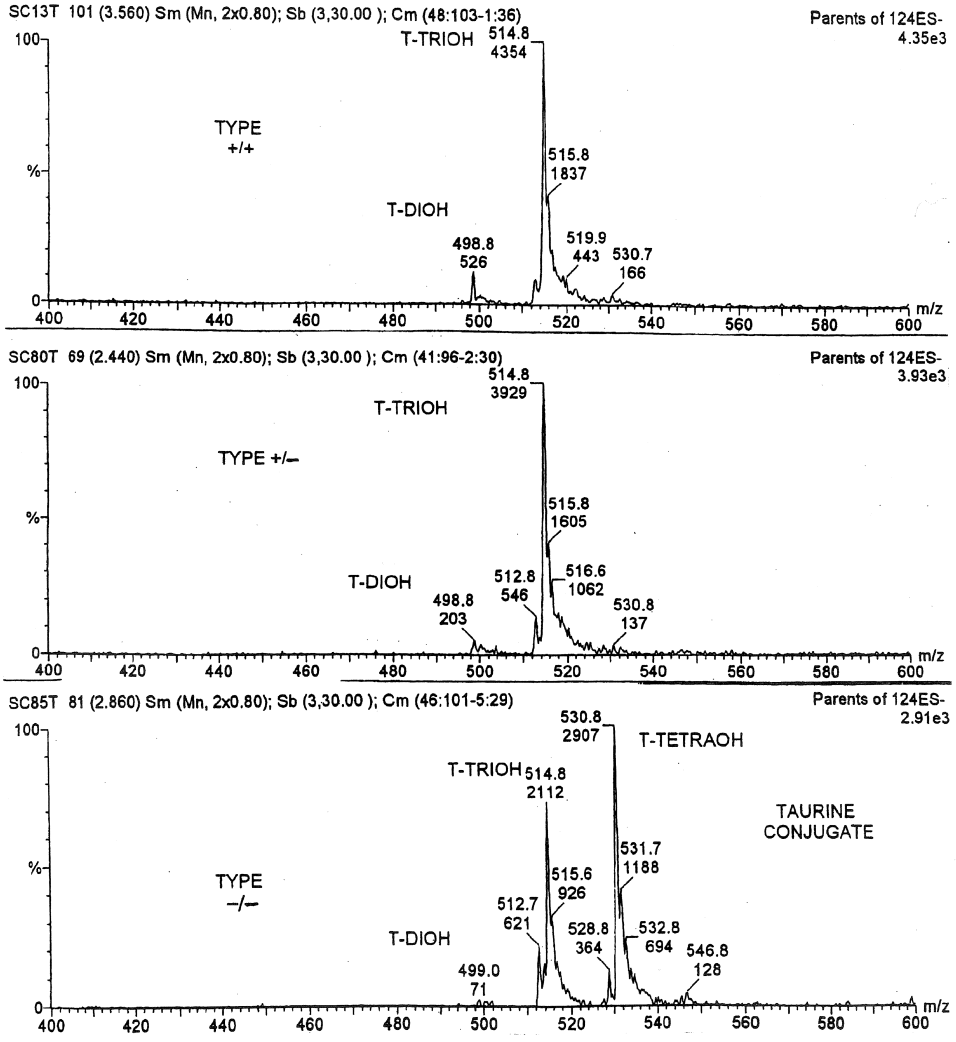

Fig. 2. ES-MS/MS spectra (negative ion mode) obtained from gallbladder bile of wild-type (+/+), heterozygous (+/−), and homozygous (−/−) of spgp knockout mice. Bile acids were extracted from 20 μl of bile. The figure shows the presence of mainly taurine conjugated bile acids in bile. The molecular ions $[M-H]^{-1}$ at *m/z* 498.8, 514.8, and 530.8 correspond to taurine conjugates of di-, tri-, and tetrahydroxylated bile acids, respectively. The other ions present in the different spectra do not correspond to bile acids.

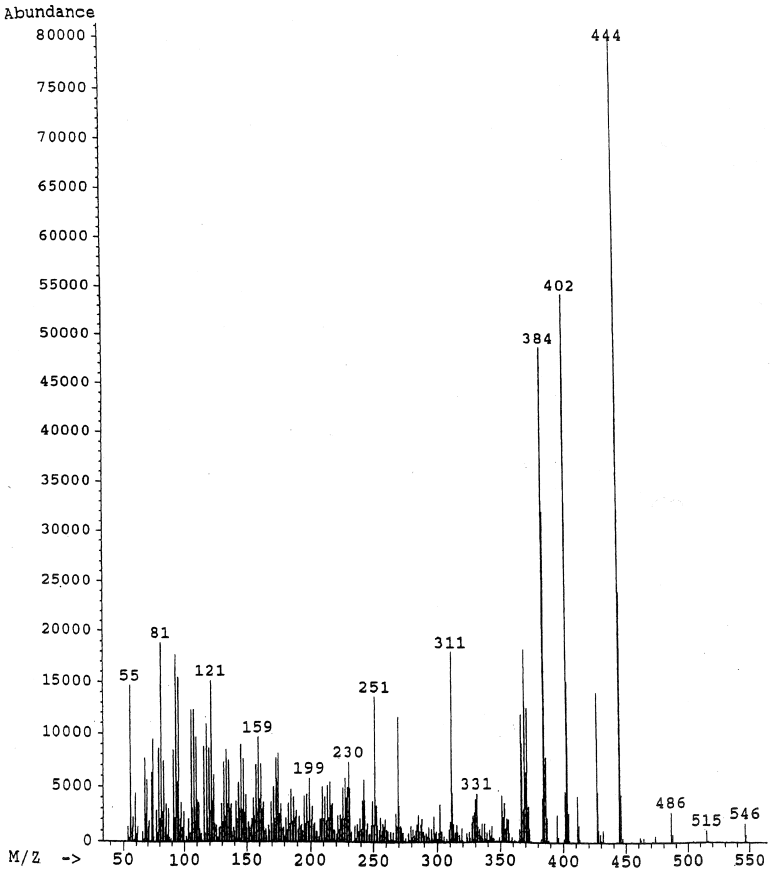

Fig. 3. Mass spectra of the methyl ester acetate derivatives of tetrahydroxylated bile acid present in the gallbladder bile, serum, and liver of the spgp knockout mice obtained by GC-MS.

of the elutes were recorded in the mass range of 300–800 amu. Multiple reaction-monitoring mode was used for the identification and quantification of bile acids. In this mode, both the MS1 and MS2 are used together, but in static mode, MS1 is set to transmit only the parent mass of interest, which is fragmented in the collision cell. Specific fragment ions obtained are selected and analyzed in the second spectrometer (MS2). To obtain consistent fragmentation for MS2, the collision offset voltage and collision energy were optimized to 25V and 50V, respectively. The molecular and daughter ions selected for the mass spectrometers MS1 and MS2 for each glycine-conjugated bile acid (tetra-, tri-, di-, and monohydroxylated bile acids) were: *m/z* 480.6 and 74; 464.6 and 74; 448.6 and 74; and 432.6 and 74 respectively; for taurine-conjugated bile acids (tetra-, tri-, di-, and monohydroxylated) were: *m/z* 530.8 and 124; 514.8 and 124; 498.8 and 124; and 482.6 and 124, respectively; and for the internal standard (3,12 diol 7-one 5β-cholanic acid) were *m/z* 405.6 and 123. Quantification was made using Micromass Mass lynx 3.0 software. Standard bile acids were processed and analyzed in a similar manner as described above.

Bile acid analysis by GC/MS

The methodology used for determination of total and individual bile acids was similar to that previously described (8). In this method, 5β-cholanic acid (100 μg) was added as internal standard to each sample (gallbladder bile, serum, liver, and urine tissue) and then extracted by octadecyl (C_{18}) cartridge as described before in the preparation of the samples for ES/MS/MS analysis. The extracted bile acids were then evaporated to dryness under nitrogen, and hydrolyzed in 2.5 N NaOH at 150–160°C overnight. Bile acids were then extracted methylated and acetylated. Identification and quantification of the bile acids were achieved by GC/MS using a Hewlett-Packard 5890 gas chromatography equipped with a Hewlett-Packard 5971A mass selective detector employing the selected ion-monitoring mode. In this method, the selected ions for the different bile acids were for 3α,6β,7β,12α-tetrahydroxy-5β-cholan-24-oic acid (*m/z* 444 and 384), cholic acid (*m/z* 253 and 368), chenodeoxycholic acid (*m/z* 255 and 370), deoxycholic acid (*m/z* 255 and 370), lithocholic acid, (*m/z* 257 and 372), and 5β-cholanic acid, (*m/z* 217 and 374). Quantification was carried out using a correction factor obtained by using 5β-cholanic acid as internal standard. Bile acid standards were processed and analyzed in a similar manner as samples.

Northern blotting cholesterol 7α-hydroxylase

Total RNA was isolated from frozen mouse liver using the TRIzol extraction (Invitrogen). Using the Qiagen Oligotex midi mRNA extraction kit, mRNA was isolated from total RNA. The mRNA sample was diluted 1:4 with denaturing buffer (500 μl formamide, 180 μl formaldehyde, 100 μl 10× MOPs buffer (0.4 M MOPS, pH 7.0, 0.1 M sodium acetate, 0.01 M EDTA) to a total volume of 12 μl and heated for 15 min at 55–60°C. After being heated, 3 μl loading buffer (50% v/v) glycerol (1 mM EDTA, pH 8.0, 0.25% w/v), bromophenol blue, and 0.25% (w/v) xylene cyanol FF were added to each sample. After electrophoresis, RNA was transferred to a nylon membrane (Hybond-N^+, Amersham). DNA probe template of cholesterol 7α-hydroxylase (748 bp, nt 675–1423 in NM_007824) was PCR amplified and purified using the Quiex II kit (Qiagen). The probe was labeled using Random Priming (Invitrogen), and labeled probe was purified using a SephaDex-G50 column. The blots were hybridized at 65°C.

Statistical analysis

All values represent mean and standard deviation of six or more animals in each group. Differences between the groups

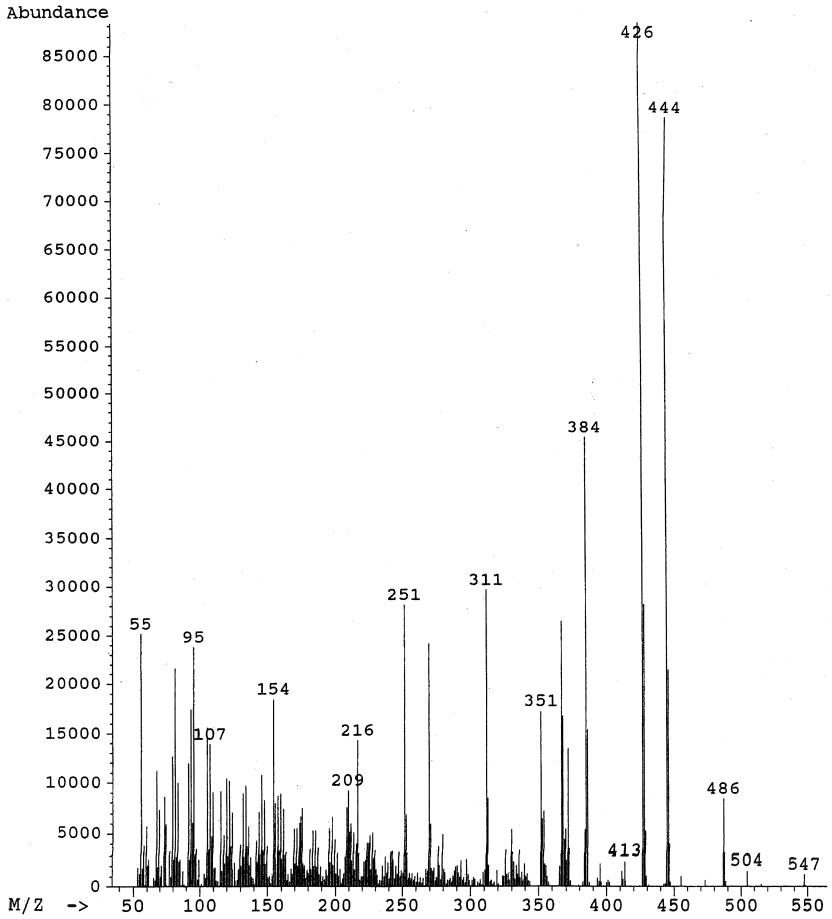

Fig. 4. Mass spectra of the methyl ester acetate derivatives of authentic standard of 3α,6β,7β,12α-tetrahydroxy-5β-cholan-24-oic acid obtained by GC-MS.

were analyzed by an ANOVA test. $P < 0.05$ was considered significant.

RESULTS

Gallbladder bile

In the wild-type, heterozygous, and the spgp$^{-/-}$ type mice, the gallbladder bile salts were mainly conjugated with taurine and very little was conjugated with glycine (0.1%–0.2%) (**Table 1**). In addition, Table 1 also shows that in the wild-type and heterozygous mice, more than 80% of the gallbladder bile salts were trihydroxylated bile salt in both the sexes. The GC-MS analysis showed that cholic acid accounts for 75% of the trihydroxylated bile acids and the rest is distributed between α, β, and φ muricholic acids, with β muricholic being the major bile acid (**Fig. 1**). There was no difference in the gallbladder bile salt profile obtained between the male and female of the wild-type and heterozygous animals. In the spgp$^{-/-}$ type, the biliary bile acid profile changed as the trihydroxy bile salts contribution was reduced to 61% in males and 49% in

TABLE 2. Distribution of bile acids (%) in the liver homogenate of spgp knockout mice

	Male (Liver)			Female (Liver)		
Bile Acid	+/+	+/−	−/−	+/+	+/−	−/−
T-mono	ND	ND	ND	ND	ND	ND
T-di	9.41 ± 2.11	6.94 ± 2.00[a]	3.91 ± 0.75[a]	8.25 ± 0.75	1.67 ± 2.36[a]	0.51 ± 0.61[a]
T-tri	85.63 ± 2.87[a]	80.46 ± 10.91[a]	65.54 ± 3.09[a]	78.14 ± 2.44[a]	50.38 ± 2.37[a]	41.83 ± 12.60[a]
T-tetra	5.23 ± 4.56[a]	6.95 ± 5.21[a]	30.54 ± 3.40[a]	13.78 ± 1.44[a]	47.93 ± 4.74[a]	57.64 ± 12.70[a]
Total (μmole/g liver)	0.14 ± 0.07	0.26 ± 0.25	1.19 ± 0.26	0.17 ± 0.08	0.35 ± 0.06	2.11 ± 0.61

Table 2 depicts the bile acid profile (%) in the liver homogenate of spgp knockout mice. Bile acids were extracted from 1 g of liver tissue. Values shown are concentrations (percent) of taurine (T) conjugated mono-, di-, tri-, and tetrahydroxylated bile acids. Each value represents average of three individual determinations for six animals (means ± SD).

[a] $P < 0.05$ male (+/+, +/−, −/−) versus female (+/+, +/−,−/−) spgp group.

TABLE 3. Distribution of bile acids (%) in the serum of spgp knockout mice

	Male (Serum)			Female (Serum)		
Bile Acid	+/+	+/−	−/−	+/+	+/−	−/−
G-mono	ND	ND	ND	ND	ND	ND
G-di	17.88 ± 7.38	16.35 ± 11.43	10.30 ± 9.55	21.41 ± 22.96	27.46 ± 11.97	7.85 ± 10.84
G-tri	18.65 ± 5.98	16.06 ± 5.33[a]	10.15 ± 6.64	16.76 ± 5.86	22.95 ± 5.91	4.82 ± 6.77
G-tetra	ND	ND	ND	ND	ND	ND
T-mono	ND	ND	ND	ND	ND	ND
T-di	18.36 ± 1.31	20.36 ± 3.60	13.34 ± 3.40[a]	23.29 ± 3.64	15.63 ± 1.10	4.20 ± 4.24[a]
T-tri	44.91 ± 15.67	47.14 ± 13.91	44.61 ± 13.38	48.85 ± 13.28	32.69 ± 12.39	28.42 ± 16.09
T-tetra	ND	ND	21.53 ± 15.69[a]	ND	1.22 ± 2.73	54.65 ± 26.49[a]
Total (uM)	6.18 ± 2.22	5.84 ± 3.30[a]	25.29 ± 28.00[a]	7.20 ± 4.74	17.41 ± 4.13[a]	92.36 ± 51.58[a]

Table 3 represents the bile acid profile (%) in the serum of spgp knockout mice. Bile acids were extracted from 0.5 ml of serum. Values shown are concentrations (percent) of glycine (G) and taurine (T) conjugated mono-, di-, tri-, and tetrahydroxylated bile acids. Each value represents average of three individual determinations for six animals (means ± SD).

[a] $P < 0.05$ male (+/+, +/−, −/−) versus female (+/+, +/−, −/−) spgp group.

females ($P < 0.5$), and the β-muricholic acid became the prominent trihydroxy bile acid (Fig. 1). The ES-MS/MS spectra show new ions (**Fig. 2**) with molecular weight of 530 corresponding to the molecular weight of the tauro-tetra-hydroxylated bile salt. The spectra obtained by GC-MS (**Fig. 3**) confirm that this compound is tetrahydroxylated bile acid, as evidenced by the presence of an ion at *m/z* 251, 384, and 444, characteristic of the tetrahydroxylated bile acids (**Fig. 4**). This new bile acid accounted for 34% in males and 48% in females ($P < 0.5$) (See Table 1).

Liver bile acids

Similar to gallbladder bile acids, all the bile acid in the liver tissue was conjugated with taurine (**Table 2**). The bile acid concentration (μmole/g of liver tissue) was significantly (four to eight times) higher in the spgp$^{-/-}$ mice than in both heterozygote and wild type in both sexes. Similar to gallbladder bile, trihydroxylated bile acid was the major bile acid in wild-type mice and was significantly reduced to 66% in males and 42% in females of the spgp$^{-/-}$ mice. Although taurocholic acid was the major bile acid in the wild-type and heterozygous mice, β-muricholic acid was the major trihydroxy bile acid in the spgp$^{-/-}$ mice. Tetrahydroxylated bile acids accounted for 31% in males and 57% in females of the spgp$^{-/-}$ mice (Table 2).

Serum bile acids

The pattern of conjugation of the serum bile acids was different from both the gallbladder bile and the liver bile acids. In the wild-type and heterozygous mice, the glycine conjugates accounted for from 25% to 50% of the total bile acids in both the sexes. The percent of glycine-conjugated bile acids was reduced to 10–20% in the spgp$^{-/-}$ mice. Taurine-conjugated bile acids accounted for 79% and 87% in males and females of spgp$^{-/-}$ mice, respectively (**Table 3**). The total bile acids concentrations were significantly higher in the spgp$^{-/-}$ mice as compared with wild type and heterozygous in both the sexes, however, the increase was much higher in females as compared with males (Table 3). With regard to bile acid profile, there was an increase in the percent contribution of dihydroxylated bile acids in the wild type and heterozygous types as compared with the profile in the gallbladder bile and the liver tissue. In the spgp mice, there was a reduction in the percentage contribution of dihydroxy and trihydroxy bile acids that could be accounted for by an increase in the proportion of tetrahydroxylated bile acids.

TABLE 4. Urinary bile acids (%) in spgp knockout mice

	Male (Urine)			Female (Urine)		
Bile Acid	+/+	+/−	−/−	+/+	+/−	−/−
G-mono	ND	ND	ND	ND	ND	ND
G-di	32.15 ± 20.12	51.81 ± 45.12	0.14 ± 1.18	20.12 ± 14.86	25.11 ± 27.65	1.42 ± 2.16
G-tri	25.58 ± 18.73	24.99 ± 17.67	0.01 ± 1.28	25.77 ± 16.65	25.15 ± 16.83	1.16 ± 1.19
G-tetra	11.13 ± 6.68	6.24 ± 7.81	0.05 ± 2.33	14.07 ± 5.98	11.33 ± 5.11	0.74 ± 2.23
T-mono	ND	ND	ND	ND	ND	ND
T-di	9.30 ± 6.78	5.95 ± 6.68	0.25 ± 3.17	11.30 ± 6.22	11.12 ± 4.81	1.29 ± 2.86
T-tri	12.69 ± 7.54	6.98 ± 6.17	61.01 ± 12.77[a]	16.30 ± 5.12	15.09 ± 5.85	10.88 ± 10.17[a]
T-tetra	9.17 ± 6.38	4.84 ± 6.23	38.36 ± 11.92[a]	12.91 ± 6.56	12.24 ± 3.92	84.55 ± 15.35[a]
Total (uM)	91.00 ± 53.24	110.20 ± 105.53[a]	1139.00 ± 700.00[a]	39.80 ± 37.05	66.50 ± 45.00[a]	302.50 ± 319.33[a]

Values shown are concentrations (percent) of glycine (G) and taurine (T) conjugated mono-, di-, tri-, and tetrahydroxylated bile acids. Each value represents average of three individual determinations for six animals (means ± SD).

[a] $P < 0.05$ male (+/+, +/−, −/−) versus female (+/+, +/−, −/−) spgp group.

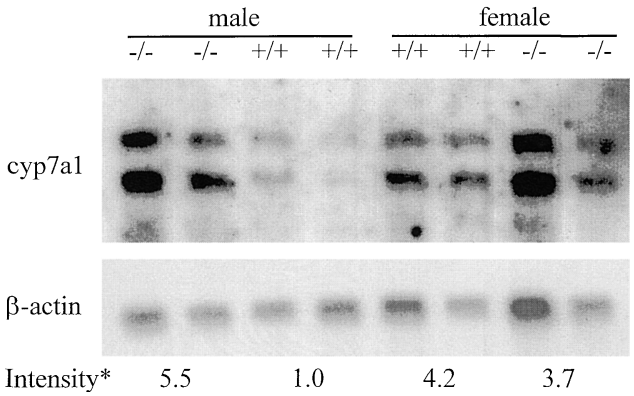

Fig. 5. Shows the expression of cholesterol 7α-hydroxylase mRNA in the liver of spgp$^{+/+}$ and spgp$^{-/-}$ mice. Data are the mean of two independent blots.

Urinary bile acids

The pattern of urinary bile acids was also different from the gallbladder and liver bile acid pattern, however, it was somewhat similar to serum bile acids. In the wild-type, the glycine conjugated bile acids accounted for 69% and 60% in the male and female mice, respectively (**Table 4**). The percentage of glycine-conjugated bile acids in heterozygote mice was similar (62%) in females but higher (83%) in males. However, in the spgp$^{-/-}$, the urinary bile acids were mainly conjugated with taurine (99.62% and 96.72% in the males and females, respectively). The contribution of the dihydroxy bile acids was 41.45%, 31.42%, and 57.76%, 36.23% in spgp$^{+/+}$ and spgp$^{+/-}$ males and females, respectively. However, in the spgp$^{-/-}$ the contribution of of the dihydroxy bile acids was less than 3%. The bile acids in the spgp$^{-/-}$ were composed mainly of the taurine-conjugated tri- and tetrahydroxylated bile acids (Table 4).

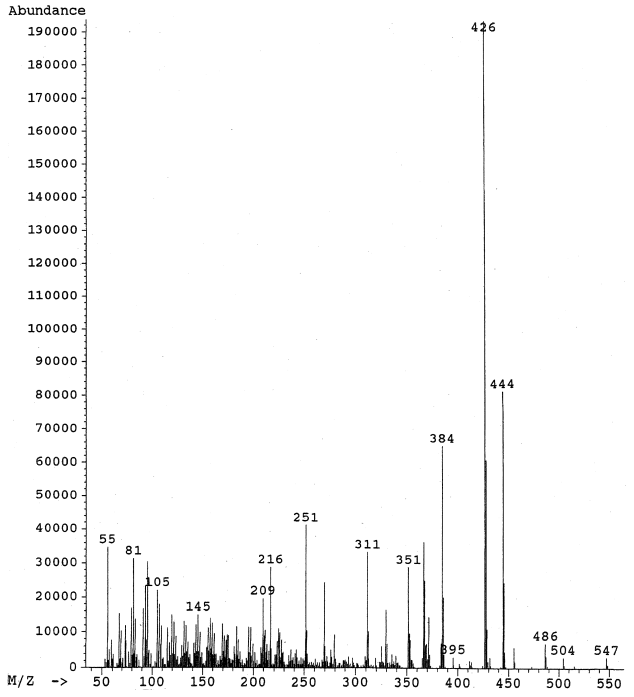

Fig. 6. Mass spectra of the methyl ester acetate derivatives of authentic standard of 1β,3α,7α,12α-tetrahydroxy-5β-cholan-24-oic acid obtained by GC-MS.

Expression of hepatic cholesterol 7-α hydroxylase mRNA

There was a 450% increase in the level of mRNA of cholesterol 7-α hydroxylase in the male species of spgp$^{-/-}$ mice in comparison to spgp$^{+/+}$; however, in the females, a 12% reduction in the expression of the mRNA of cholesterol 7-α hydroxylase was observed (**Fig. 5**).

DISCUSSION

This study represents the first detailed report about the bile acid distribution in gallbladder bile, liver tissues, serum, and urine of spgp knockout mice that lack the bile acid exporting pump in the canalicular membrane. The results show, as expected, a significant increase in liver, serum, and urinary bile acids in the spgp$^{-/-}$ mice, supporting the concept that disruption of the canalicular Bsep/Spgp leads to hyperchloremia associated with cholestasis (7). However, the most striking finding is the biliary secretion of newly identified tetrahydroxylated bile acid (3α,6β,7β,12α-tetrahydroxy-5β-cholan-24-oic acid) as well as its presence in liver tissue, serum, and urine of the spgp$^{-/-}$ mice. There was also a significant difference observed between bile acids profiles of the male and female spgp$^{-/-}$ mice, possibly indicating an interaction between the gene disruption and other sex-related factors (9).

The data obtained by the ES-MS/MS negative scan give the molecular weights minus one of the compounds present in the biological sample, and thus it is not possible to differentiate between the different mono-, di-, tri-, and tetrahydroxy bile acids. However, it is possible to quantify the different conjugated classes of these bile acids. The GC-MS, on the other hand, gives accurate identification and quantification of the different mono-, di-, tri-, and tetrahydroxy bile acids. However, during the preparation of the sample for the GC-MS analysis, the hydrolysis of the amino acid conjugates makes it impossible to obtain information about the state of conjugation of the different bile acids in the samples. Thus, combining both techniques is a powerful means of obtaining all the information for the identification and quantification of the bile salts.

The biliary bile acids in mice are composed mainly of taurine-conjugated cholic acid and muricholic acids, with very little glycine-conjugated bile acids or dihydroxylated bile acids (10). The data obtained in this study confirm this profile in the wild-type and heterozygous mice, indicating that any changes in bile acid profile in the spgp$^{-/-}$ type must be due to the disruption of the spgp gene. In the spgp$^{-/-}$ mice, there was an increase in the contribution of muricholic acids and the presence of newly previously unrecognized tetrahydroxylated bile acid (Fig. 1). The presence of the tetrahydroxylated bile acids (1β, 3α, 7α, 12α) has been reported before in intrahepatic cho-

lestasis of pregnancy (11). However, the structure of the 1β,3α,7α,12α tetrahydroxylated bile acid reported before and obtained from our reference compounds (**Fig. 6**) appears to differ from the structure obtained for the tetrahydroxy compound seen in this study (Fig. 3). Another two tetrahydroxylated bile acids were also reported in cholestasis (3α,4β,7α,12α and 3α,6β,7α,12α) (12–13). However, none of the characteristic ions of these two compounds were present in Fig. 3. The GC-MS spectrum of the methyl ester acetate derivatives of the tetrahydroxylated bile acid obtained in this study is similar to the spectra obtained from the 3α,6β,7β,12α-tetrahydroxy-5β-cholan-24-oic acid standard (Fig. 4). The spectra revealed an ion at *m/z* 251, indicating the presence of four hydroxyl groups on the ABCD steroid ring. This is further supported by the presence of different fragment ions at *m/z* 444 (M-[3*43+15]), 402 (M-[4*43+15]), and 384(M-[4*43+15+18]) observed by the sequential loss of $-CH_3$ CO, CH_3, and H_2O groups from the molecular ion (*m/z* 589). The presence of the four hydroxyl groups on the molecule is further confirmed by the ES-MS/MS analysis (Fig. 2), in which the molecular weight of these bile acids was 530, or 16 more than the molecular weight of the trihydroxy bile acids. To further support the identification of this tetrahydroxylated bile acid, the retention time of the methyl ester acetate derivatives of 3α,6β,7β,12α-tetrahydroxy-5β-cholan-24-oic acid was identical to the retention time of their corresponding authentic bile acid.

It is assumed that the polarity of the bile acids depends in part on the number of hydroxyl groups in the bile acids, and thus tetrahydroxylated bile acids could be more hydrophilic than trihydroxylated bile acids (14). This may be the reason that this mutant mouse is able to compensate in part for the toxicity of high liver and serum bile acids and could represent a major difference between the disruption of the spgp gene in mice and in humans. In humans, this disruption was associated with PFIC2; however, in these mice, this disruption leads to persistent but not progressive cholestasis (7).

The difference in the structure of the tetrahydroxylated bile acids reported in different human cholestasis and in the spgp$^{-/-}$ mice may indicate the difference in the activities of the hydroxylating enzyme induced by different cholestases. The structure of tetrahydroxylated compound (3α,6β,7β,12α-tetrahydroxy-5β-cholan-24-oic acid) reported in this study is in agreement with the bile acid synthesis in these mice. The high concentration of cholic acid in the wild type indicates an active 12α-hydroxylation. The absence of chenodeoxycholic acid and the presence of muricholic acid suggests an active 6β-hydroxylation of chenodeoxycholic acid or its intestinal metabolite, 3α,7-keto lithocholic acid in the liver of the wild type. Thus, it is possible that during the detoxification process induced by the presence of high bile acid concentration, 6β- and 12α-hydroxylation increase, causing the synthesis of 3α,6β,7β,12α-tetrahydroxy-5β-cholan-24-oic acid. It has been suggested that decreased hydrophobicity of the bile acids pool could result in up-regulation of cholesterol 7α-hydroxylase and influence bile acid synthesis (15). Our data confirm this suggestion in male$^{-/-}$ mice, only indicating further sex differences between the spgp males and females (Fig. 5).

In conclusion, analysis of the bile acid profile in the gallbladder bile, liver, serum, and urine confirm the identity of tetrahydroxylated bile acid previously found to be increased in the spgp knockout mouse. The data further suggest that the toxicity of the liver of the mutant Spgp mice caused by high concentration of bile acids resulting from the disruption of the BSEP may be counteracted in part by the production of the tetrahydroxylated bile acid. Furthermore, the biliary secretion of muricholic acid and tetrahydroxylated bile acids in the spgp knockout mice indicates the presence of another bile acid transporter or mechanism for the secretion of these bile acids in the spgp$^{-/-}$ mice (16–19).

This work was supported by a grant from the CIHR of Canada and Canadian Liver Foundation.

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