J. Biochem. 119, 725-730 (1996)

Analysis of Bile Acids in Urine Specimens from Healthy Humans: Determination of Several Bile Acids with β-Hydroxyl and Carbonyl Groups

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Received for publication, October 23, 1995

Urinary bile acids of 39 healthy male undergraduates were analyzed by capillary gas chromatography and capillary gas chromatography-mass spectrometry. 3α-Hydroxy-12-oxo-5β-cholanoic acid, 3α,12β-dihydroxy-5β-cholanoic acid, 3β,7α-dihydroxy-5β-cholanoic acid, and 3α,7α,12β-trihydroxy-5β-cholanoic acid, in addition to known bile acids, were identified and then quantified. The major part of the urinary bile acids was occupied by secondary bile acids. Every 7β-hydroxylated bile acid species was found in more than 80% of the subjects. The bile acid detected in the largest amount was 3α-hydroxy-12-oxo-5β-cholanoic acid. The metabolites of cholic acid were quantitatively more predominant than those of chenodeoxycholic acid. These results indicate that bile acids with β-hydroxyl and carbonyl groups at the C-3,7 and/or 12 positions are usual bile acids usually found in the urine of healthy humans. It is concluded that the occurrence of these bile acids is an effect of the intestinal bacterial flora and living conditions.

Key words: 3α,12β-dihydroxy-5β-cholanoic acid, healthy humans, 3α-hydroxy-12-oxo-5β-cholanoic acid, 3α,7α,12β-trihydroxy-5β-cholanoic acid, urine specimens.

Recently, we reported that large amounts of 7β-hydroxylated bile acids belonging to one category of unusual bile acids in healthy humans were present in a urine specimen from a healthy and young volunteer (*1*). In that study, the question arose of whether or not the excretion of 7β-hydroxylated bile acids might be exceptional for a healthy person. To date, there have been few reported control data as to urinary bile acids in healthy humans (*2*). The purposes of this experiment were to answer the above question, and to clarify the average composition of urinary bile acids in healthy humans by analyzing the bile acids in urine specimens from 39 healthy male undergraduates.

EXPERIMENTAL PROCEDURES

Subjects and Specimens—The subjects were 39 male healthy undergraduates (mean age, 23 years). The subjects all ate in the same dining hall for undergraduates at least once a day. No subjects had ingested either bile acid-drugs or therapeutic drugs for at least six months before the collection of urine. The urine specimens were collected once a day (at 3:00 p.m. in most subjects) and kept frozen until analysis.

Materials—Authentic bile acids and internal standards for gas chromatography were those kept in this laboratory (*1, 3, 4*).

Amberlite XAD-2 was purchased from Rohm and Hass (Philadelphia, PA, USA), and dimethylethylsilylimidazole from Tokyo Kasei Kogyo (Tokyo). Other chemicals were of analytical reagent grade, and all organic solvents were distilled before use. Authentic 3β,7α-dihydroxy-5β-cholanoic acid was kindly supplied by Dr. M. Tohma (Health Sciences University of Hokkaido, Hokkaido).

Synthesis of 3α-Hydroxy-12-Oxo-5β-Cholanoic Acid, 3α,12β-Dihydroxy-5β-Cholanoic Acid, and 3α,7α,12β-Trihydroxy-5β-Cholanoic Acid—3α-Hydroxy-12-oxo-5β-cholanoic acid, 3α,12β-dihydroxy-5β-cholanoic acid, and 3α,7α,12β-trihydroxy-5β-cholanoic acid were essentially synthesized by classical methods (*5, 6*) with some modifications. Melting points were uncorrected.

3α-Hydroxy-12-oxo-5β-cholanoic acid: Deoxycholic acid was partially acetylated with a mixture of acetic anhydride and pyridine at room temperature for 2 h, yielding 3α-acetoxy-12α-hydroxy-5β-cholanoic acid. The compound obtained was oxidized with a CrO_3-H_2SO_4 solution and then hydrolyzed with a 70% methanolic solution containing 5% NaOH, yielding 3α-hydroxy-12-oxo-5β-cholanoic acid. 3α-Hydroxy-12-oxo-5β-cholanoic acid with mp. 158-160°C was obtained by recrystallization from a methanol-water solution [mp. 160-161°C (*5*)].

3α,12β-Dihydroxy-5β-cholanoic acid: 3α-Hydroxy-12-oxo-5β-cholanoic acid dissolved in dioxane was reduced with borohydrate. The reduced compound was a mixture of 3α,12α- and 3α,12β-dihydroxy-5β-cholanoic acids. 3α,12β-Dihydroxy-5β-cholanoic acid was separated from the mixture using diethyl ether, which scarcely dissolves 3α,12α-dihydroxy-5β-cholanoic acid at room temperature

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The trivial names used are: Lithocholic acid, 3α-hydroxy-5β-cholanoic acid; deoxycholic acid, 3α,12α-dihydroxy-5β-cholanoic acid; chenodeoxycholic acid, 3α,7α-dihydroxy-5β-cholanoic acid; norcholic acid, 3α,7α,12α-trihydroxy-5β-norcholan-23-oic acid; ursodeoxycholic acid, 3α,7β-dihydroxy-5β-cholanoic acid; cholic acid, 3α,7α,12α-trihydroxy-5β-cholanoic acid.

(6). The 3α,12β-dihydroxy-5β-cholanoic acid recrystallized from ethyl acetate had mp. of 175-178°C [mp. 176-177°C (6) and 172.5-173.6°C (7, 8)].

3α,7α,12β-Trihydroxy-5β-cholanoic acid: Cholic acid was partially acetylated by the same procedure as for the above bile acid producing 3α,7α-diacetoxy-12α-hydroxy-5β-cholanoic acid. The compound obtained was oxidized with a CrO_3-H_2SO_4 solution and then reduced with metal sodium in propanol. To obtain 3α,7α,12β-trihydroxy-5β-cholanoic acid (mp. 194-195°C) [mp. 200.0-201.5°C (8, 9)], the large amount of cholic acid contaminating the above reduced compound was removed by repeated recrystallization from ethyl acetate. This bile acid gave a peak differing from that of cholic acid on capillary gas chromatography. The melting point of 3α,7α,12β-trihydroxy-5β-cholanoic acid mixed with pure cholic acid was decreased.

Methods—Extraction of bile acids from urine: Prior to extraction of bile acids, 7β,12β-dihydroxy-5β-cholanoic acid (ββ, 5.3 μg) and glyco-7β,12α-dihydroxy-5β-cholanoic acid (G-βα, 5.1 μg as free form) were added to 5 ml of urine as internal standards (1, 3, 4). Bile acids in the urine were extracted essentially as reported previously (1). Briefly, 5 ml of urine adjusted to pH 10 was applied on an Amberlite XAD-2 column, followed by washing with distilled water. Bile acids were extracted with ethanol containing 0.25% ammonia. After solvolysis with 5 ml of an acetone-methanol mixture (9 : 1, v/v) containing 2 drops of 6 N HCl at 37°C for 16 h, and hydrolysis with 2.5 ml of 2 N NaOH for 4 h in an autoclave at 1.2 kg/cm² and 120°C, bile acids were extracted from the acidified solution with diethyl ether.

Derivatization: Extracted bile acids were converted into methylester dimethylethylsilylether (Me-DMES) derivatives. Briefly, bile acids were methylated with diazomethane, and then the methyl esters were left in 70 μl of dimethylethylsilylimidazole in an air-tight vessel at room temperature overnight (1, 3, 4).

Capillary Column Gas Chromatography (Capillary GC) and Capillary Gas Chromatography-Mass Spectrometry (Capillary GC/MS)—A gas chromatograph (Model GC-14A; Shimadzu, Kyoto) equipped with a Hicap CBP-1 capillary column (25 m×0.25 mm I.D.; Shimadzu, Kyoto) was used.

Mass spectra were obtained with a HP 5890A GC/HP 5970B MSD System (Hewlett Packard Instrument, Downers Grove, IL, USA). The gas chromatographic column used was a DB-1 capillary one (30 m×0.25 mm I.D.; J & W Scientific, Folsom, CA, USA). The column was programmed to operate at 70°C for 1 min, followed by a temperature increase from 70 to 270°C at 30°C/min, and subsequent operation at 280°C for 1 h. Helium of high purity was used as the carrier gas. The ion source temperature was 280°C, and the ionization voltage was 70 eV.

Qualitative and Quantitative Determination of Bile Acids—The bile acids in specimens were analyzed by capillary GC and capillary GC/MS, and identified on the basis of agreement of the relative retention times (RRT values) and mass spectra between the peaks for the urine specimens and the peaks for authentic bile acids.

Quantitative data for bile acids in specimens were obtained with a computerized data system (Model C-R4A Chromatopac; Shimadzu, Kyoto) connected with a gas chromatograph (Model GC-14A), as reported previously (1, 3).

In the present study, the sum of 13 identified bile acids was estimated as the amount of total bile acids.

RESULTS

Identification of Bile Acids—The bile acids in the urine specimens from the 39 healthy male subjects were analyzed by capillary GC and capillary GC/MS. A gas chromatogram of Me-DMES derivatives of an extract of a specimen in addition to one of authentic bile acids are shown in Fig. 1. The gas chromatogram shows that this specimen contained 18 components in the detectable region for bile acids. The gas chromatograms for 9 subjects included all the peaks detected in the specimen from any subject after analysis for all 39 subjects.

First, the presence of these 9 known bile acids was confirmed in each specimen from the agreement of the RRT values (Table I) and mass spectra (not shown), as follows; peak 1 is lithocholic acid; peak 3, 3β-hydroxychol-5-enoic acid; peak 6, deoxycholic acid; peak 7, chenodeoxycholic acid; peak 9, norcholic acid; peak 10, ursodeoxycholic acid; peak 11, 3β,7β-dihydroxy-5β-cholanoic acid; peak 14, cholic acid; and peak 15, 3α,7β,12α-trihydroxy-5β-cholanoic acid. Furthermore, four other bile acids (corresponding to peaks 4, 5, 8, and 13) were identified, as described below.

3α,12β-Dihydroxy-5β-cholanoic acid: Peak 4 exhibited a RRT value of 1.04 as to ββ, and main ions at *m/z* 255 (base ion), 370, 445, and 549 in its mass spectrum. The RRT value and mass spectrum of peak 4 completely coincided with those of the Me-DMES derivative of 3α,12β-dihydroxy-5β-cholanoic acid, which was synthesized according to the procedure described under "*Methods.*"

3α-Hydroxy-12-oxo-5β-cholanoic acid: Peak 8 exhibited a RRT value of 1.15, and main ions at *m/z* 231 (base ion), 271, 368, 386, and 490 in its mass spectrum. Both the RRT value and mass spectrum of the peak coincided with those of the Me-DMES derivative of authentic 3α-hydroxy-12-oxo-5β-cholanoic acid synthesized in the present study.

3β,7α-Dihydroxy-5β-cholanoic acid: Peak 5 exhibited a RRT value of 1.07, and the mass spectrum of peak 5 completely coincided with that of the Me-DMES derivative of 3β,7α-dihydroxy-5β-cholanoic acid, which was supplied by Dr. M. Tohma.

3α,7α,12β-Trihydroxy-5β-cholanoic acid: Peak 13 exhibited a RRT value of 1.29, which coincided with that of synthesized 3α,7α,12β-trihydroxy-5β-cholanoic acid. The mass spectrum of this material (Fig. 2) was also similar to that of the Me-DMES derivative of 3α,7α,12β-trihydroxy-5β-cholanoic acid synthesized in the present study.

Quantitative Composition of Bile Acids—As mentioned above, 13 species of bile acid were identified in the specimens from the subjects studied, and were quantified. The results of analysis of the urinary bile acids from the 39 subjects are summarized in Table II.

In 9 of the 39 subjects, all 13 identified bile acids were detected, irrespective of the excreted amounts. Only cholic acid was detected in all of the specimens, while 9 other bile acids, *i.e.* excluding 3β,7α-dihydroxy-5β-cholanoic acid, chenodeoxycholic acid, and 3α,7α,12β-trihydroxy-5β-cholanoic acid, were detected in more than 70% of the total

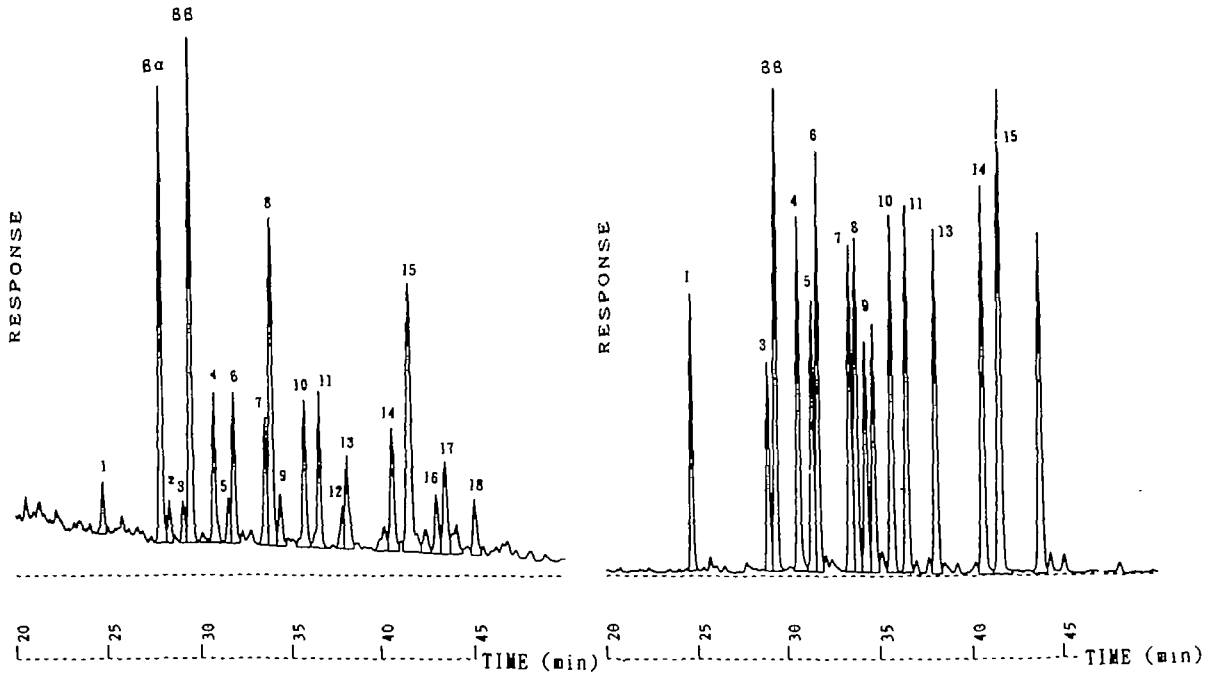

Fig. 1. **Gas chromatogram on a Hicap CBP-1 capillary column of the methyl ester dimethylethylsilyl ether derivatives of an extract of a urine specimen (left), and those of authentic bile acids (right).** 1, lithocholic acid; 3, 3β-hydroxychol-5-enoic acid; 4, 3α,12β-dihydroxy-5β-cholanoic acid; 5, 3β,7α-dihydroxy-5β-cholanoic acid; 6, deoxycholic acid; 7, chenodeoxycholic acid; 8, 3α-hydroxy-12-oxo-5β-cholanoic acid; 9, norcholic acid; 10, ursodeoxycholic acid; 11, 3β,7β-dihydroxy-5β-cholanoic acid; 13, 3α,7α,12β-trihydroxy-5β-cholanoic acid; 14, cholic acid; 15, 3α,7β,12α-trihydroxy-5β-cholanoic acid; $\beta\beta$, 7β,12β-dihydroxy-5β-cholanoic acid; $\beta\alpha$, 7β,12α-dihydroxy-5β-cholanoic acid; 19, hyodeoxycholic acid; and 20, hyocholic acid.

TABLE I. **The relative retention times of internal standards, authentic bile acids and peaks for urine specimens from the subjects.**

Bile acid[a]	RRT value	Peaks in Fig. 1
7β,12β-Dihydroxy-5β-cholanoic acid ($\beta\beta$)[b]	1.00	
7β,12α-Dihydroxy-5β-cholanoic acid ($\beta\alpha$)[b]		
Lithocholic acid	0.84	1
Unknown peak	0.94	2
3β-Hydroxychol-5-enoic acid	0.98	3
3α,12β-Dihydroxy-5β-cholanoic acid	1.04	4
3β,7α-Dihydroxy-5β-cholanoic acid	1.07	5
Deoxycholic acid	1.08	6
Chenodeoxycholic acid	1.14	7
3α-Hydroxy-12-oxo-5β-cholanoic acid	1.15	8
Norcholic acid	1.17	9
Hyodeoxycholic acid	1.18	
Ursodeoxycholic acid	1.21	10
3β,7β-Dihydroxy-5β-cholanoic acid	1.24	11
Unknown peak	1.28	12
3α,7α,12β-Trihydroxy-5β-cholanoic acid	1.29	13
α-Muricholic acid	1.32	
Cholic acid	1.38	14
3α,7β,12α-Trihydroxy-5β-cholanoic acid	1.42	15
Unknown peak	1.46	16
Unknown peak	1.47	17
Hyocholic acid	1.49	
Unknown peak	1.52	18
β-Muricholic acid	1.53	

[a]Derivative, methyl ester dimethylethylsilylether. [b]Internal standard compound. For gas chromatography conditions, see the text.

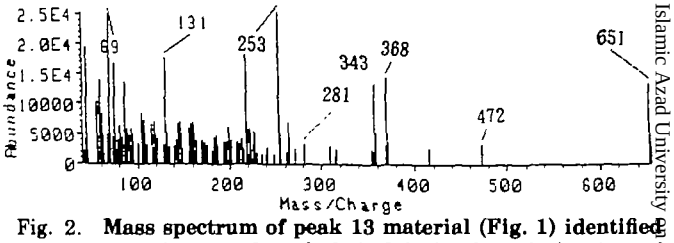

Fig. 2. **Mass spectrum of peak 13 material (Fig. 1) identified as the methyl ester dimethylethylsilyl ether derivative of 3α,7α,12β-trihydroxy-5β-cholanoic acid.**

subjects. 3α,7α,12β-Trihydroxy-5β-cholanoic acid was detected in 24 (62%) of the 39 subjects (mean, 1.00 μg/10 ml; max., 10.14 μg/10 ml, and min., 0 μg/10 ml), and 3β,7α-dihydroxy-5β-cholanoic acid in 56% of the subjects studied (mean, 0.55 μg/10 ml; max., 3.75 μg/10 ml, and min., 0 μg/10 ml). 3α-Hydroxy-12-oxo-5β-cholanoic acid was found in 95% of the total subjects and detected in the largest amount in the specimens on average (mean, 8.48 μg/10 ml, max., 68.17 μg/10 ml).

Bile acids with β-hydroxyl and carbonyl groups comprised 64.1% of the total bile acids, and some of them were found in all subjects. Notably, every 7β-hydroxylated bile acid species, which consist of ursodeoxycholic acid, 3β,7β-dihydroxy-5β-cholanoic acid and 3α,7β,12α-trihydroxy-5β-cholanoic acid, was found in more than 80% of the total subjects, and 7β-hydroxylated bile acids comprised 21.3% of the total bile acids.

Cholic acid and chenodeoxycholic acid, primary bile

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