7β,12β-DIHYDROXY-5β-CHOLAN-24-OIC ACID AS AN INTERNAL STANDARD FOR QUANTITATIVE DETERMINATION OF BILE ACIDS BY GAS CHROMATOGRAPHY

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Received 4-12-82

ABSTRACT

In order to find an artificial internal standard compound for quantitative determination of bile acids by gas chromatography, 7α,12α-, 7α,12β-, 7β,12α- and 7β,12β-dihydroxy-5β-cholan-24-oic acids were chemically synthesized with cholic acid (1) as the first starting material. The gas chromatographic retention time of 7β,12β-dihydroxy-5β-cholan-24-oic acid (ββ-isomer) was more different from that of natural bile acids than the other isomers. Moreover, ββ-isomer was extracted in the same fraction as the bile acids from urine, and no urinary substance had the same retention time as ββ-isomer. No artifact was produced from ββ-isomer during the analysis procedure. It was concluded that the ββ-isomer is an internal standard compound with certain advantages for the quantitative determination of bile acids in urine by gas chromatography, irrespective of the recovery rate during the analysis procedure.

INTRODUCTION

Standard compounds are indispensable for measuring bile acids in biological samples such as bile, urine, blood and feces by gas chromatography. Internal standard compounds are added to the samples to be assayed prior to the extraction of bile acids. Very few reports have established what compounds should be adopted as internal standards. Some investigators have reported the use of hyodeoxycholic acid and hyocholic acid in quantitative analysis of bile acids in human biological samples (2,3). However, these acids were found in samples from healthy subjects, neonates and some patients (4,5, 6, 7, 8) and thus may be present in the samples to be assayed. Some workers even

state only that ''bile acids were measured by gas chromatography'' with no mention of the use of any standard compound.

This paper describes the chemical synthesis of 7,12-dihydroxy-5β-cholan-24-oic acids and examines whether 7,12-dihydroxy-5β-cholan-24-oic acids can be used as an internal standard compound for measuring bile acids in urine by gas chromatography.

MATERIALS AND METHODS

Syntheses of various 5β-cholan-24-oic acids

Melting points are uncorrected.

1) 7α,12α-Dihydroxy-5β-cholan-24-oic acid

7α,12α-Dihydroxy-5β-cholan-24-oic acid (5.0g) was synthesized from methyl cholate 3,7,12-triacetate according to the method of Nakada (9). mp. 204-206°C. Yield 2.2 g. [mp. 207-208°C (9)]. The melting point of the methyl ester was 155-156°C. [mp. 155-156°C (9)].

2) 7β,12α-Dihydroxy-5β-cholan-24-oic acid

7α,12α-Dihydroxy-5β-cholan-24-oic acid (5 g) in acetone-water containing $NaHCO_3$ was oxidized with N-bromo succinimide (3.7g) in the dark, by the same method reported for the oxidation of the 7α-hydroxy group of cholic acid (10). The reaction mixture was acidified with dilute HCl, and the precipitate of the oxidation product was obtained. Recrystallizations of the product from acetone-n-hexane gave 7-oxo-12α-hydroxy-5β-cholan-24-oic acid of mp 171-173°C. The 7-oxo acid (2g) was reduced by refluxing with metal Na (4g) in 40 ml of n-propyl alcohol until the Na had dissolved (11). The reaction mixture was cooled at room temperature and water was added. Next, it was evaporated under reduced pressure to remove the organic solvent, and acidified to pH 3. The precipitate, which was a mixture of 7α,12α- and 7β,12α-dihydroxy-5β-cholan-24-oic acids, was filtered. We found that 7β,12α-dihydroxy-5β-cholan-24-oic acid was separated from the precipitate using alkaline solution, which hardly dissolved 7α,12α-dihydroxy-5β-cholan-24-oic acid at low temperature. Therefore, the precipitate which was added into 5 ml of 5% KOH was warmed on a water-bath and then was cooled at 2-3°C. The solution was separated from an insoluble substance and acidified with dilute HCl. Recrystallizations of the product from methanol and then from ethyl acetate gave 7β,12α-dihydroxy-5β-cholan-24-oic acid of mp 180-181°C. Yield 460 mg.

3) 7β,12β-Dihydroxy-5β-cholan-24-oic acid

First, a partial acetylation at 7-position of 7β,12α-dihydroxy-5β-cholan-24-oic acid (3g) was carried out in benzene (30 ml) using 7.5 ml of pyridine and 7.5 ml of acetic anhydride, by the same method reported

for the preparation of methyl cholate 3,7-diacetate (12). Water was poured into the mixture. The benzene layer was separated, washed with water and the solvent was evaporated to dryness. Without further purification, the residue was dissolved in acetone and oxidized with the CrO_3-H_2SO_4 mixture of Bladon et al.(13). The product, 7β-acetoxy-12-oxo-5β-cholan-24-oic acid, was recrystallized from methanol. mp. 192-195°C. Reduction of the 7β-acetoxy-12-oxo-5β-cholan-24-oic acid was carried out as described above for the preparation of 7β,12α-dihydroxy-5β-cholan-24-oic acid. Since the product was contaminated with 7β,12α-dihydroxy-5β-cholan-24-oic acid, 7β,12β-dihydroxy-5β-cholan-24-oic acid was separated from it by fractional crystallization using methanol-water. Recrystallizations from ethyl acetate gave 7β,12β-dihydroxy-5β-cholan-24-oic acid of mp 183-185°C. Yield 300 mg.

4) 7α,12β-Dihydroxy-5β-cholan-24-oic acid

Application of partial acetylation and oxidation described above for the preparation of 7β,12β-dihydroxy-5β-cholan-24-oic acid led to transformation of methyl 7α,12α-dihydroxy-5β-cholan-24-oate (1 g) into methyl 7α-acetoxy-12-oxo-5β-cholan-24-oate (mp. 167-169°C) after conversion into methyl 7α-acetoxy-12α-hydroxy-5β-cholan-24-oate (mp. 134-137°C). The 12-oxo acid was transformed into methyl 7α,12β-dihydroxy-5β-cholan-24-oate by treatment with metal Na (0.6 g) in 6 ml of n-propyl alcohol. The product recrystallized from ethyl acetate had a mp of 185-187°C. Yield 310 mg.

5) 7,12-Dioxo-5β-cholan-24-oic acid

7α,12α-Dihydroxy-5β-cholan-24-oic acid (0.5g) in acetone was oxidized with the CrO_3-H_2SO_4 mixture of Bladon et al.(13). mp. 178-179°C. Yield 350 mg.

Gas chromatography and gas chromatography-mass spectrometry

The gas chromatograph (Model GC-4BPF, Shimazu Co.) used was equipped with a flame ionization detector. A 1% OV-1 (2 m) column was used at 250°C with N_2 as the carrier gas.

The gas chromatography-mass spectrometer used, JMS D-300 (Japan Electon Optics Laboratory Co. Ltd. Tokyo), had a 1% OV-1 (1 m x 2 mm) column which was maintained at 250°C.

The bile acid derivatives subjected to gas chromatography and gas chromatography-mass spectrometry were the methyl ester of the acetate prepared by the method of Roovers et al.(14) and the methyl ester of the dimethylethylsilyl (DMES) ether treated at room temperature for 45 min with DMES imidazole after having been synthesized by the method of Miyazaki et al. (15).

Extraction of bile acids

Bile acids in 30 ml of aqueous sample acidified to pH 4 were absorbed on Amberlite XAD-2 column (10 x 150 mm, Rohm and Hass Co.), extracted with 50 ml of ethanol containing 0.5 ml of 25% ammonia and after removal of the solvent, solvolyzed in 25 ml of an acidified methanol-acetone (1 : 9 v/v) mixture at 37°C for 16 hr. Hydrolysis of the bile acids was carried out in 5 ml of 2 N NaOH solution at 130°C for 4 hr in a sealed glass tube. Neutral substances were removed from the hydrolyzate with n-hexane, then the aqueous layer was acidified with HCl and free bile acids were extracted with ether (4, 16, 17).

RESULTS AND DISCUSSION

7α,12β-, 7β,12α- and 7β,12β-Dihydroxy-5β-cholan-24-oic acids except 7α,12α-dihydroxy-5β-cholan-24-oic acid (9) were newly synthesized with cholic acid as the first material. As shown in Table 1A, the gas chromatographic retention times of the four synthesized compounds differed and coincided with those of the four products obtained from the reduction of 7,12-dioxo-5β-cholan-24-oic acid (100 mg) by Na (50 mg) in 0.5 ml of n-propyl alcohol. Table 2 shows the important fragment ions of the four compounds, as their free form, their methyl ester acetate derivative and their methyl ester DMES derivative. An ion of m/e 255, which is characteristic of dihydroxy-5β-cholan-24-oic acid (18), was observed in all mass spectra, and M^+ (578) appeared only in that of the methyl ester DMES derivative. The four synthesized compounds gave the same fragment ions when they were recorded their spectra as the same derivative. But, the principal differences in the mass spectra among the four compounds were: 1) In the mass spectrum of the methyl ester acetate derivative, the base peak was at an ion of m/e 370 when the hydroxy groups at the 7- and 12-positions were of the same configuration, as in 7α,12α- and 7β,12β-dihydroxy-5β-cholan-24-oic acids, whereas it was at an ion of m/e 255 when the hydroxy groups were of opposite configuration, as in 7α,12β- and 7β,12α-dihydroxy-5β-cholan-24-oic acids. 2) In the mass spectra of the free form, the relative intensity of m/e 273 ($M-2H_2O$-sidechain) of the 7β-hydroxy acid type among the four compounds was clearly predominant over that of the 7α-hydroxy acid type. These results indicate that the four compounds synthesized chemically

Table 1 Retention times and relative retention times of the artificial bile acids and some natural bile acids on the 1% OV-1 column.

	Methyl ester acetate deriv.	Methyl ester DMES deriv.
A)	min sec	min sec
7α,12α-dihydroxy-5β-cholan-24-oic acid	11. 32 (0.71)	16. 20 (0.8)
7α,12β-dihydroxy-5β-cholan-24-oic acid	14. 17 (0.88)	15. 31 (0.76)
7β,12α-dihydroxy-5β-cholan-24-oic acid	15. 25 (0.95)	18. 47 (0.92)
7β,12β-dihydroxy-5β-cholan-24-oic acid	16. 14 (1.00)	20. 25 (1.00)
the product derived from 7,12-dioxo-5β-cholan-24-oic acid by reduction with Na	11. 35 14. 17 15. 25 16. 14	16. 20 15. 31 18. 46 20. 25
B)		
lithocholic acid	13. 29 (0.83)	15. 07 (0.74)
3β-hydroxy-Δ^5-cholen-24-oic acid	15. 14 (0.94)	18. 35 (0.91)
deoxycholic acid	17. 14 (1.06)	22. 40 (1.11)
chenodeoxycholic acid	20. 08 (1.24)	24. 43 (1.21)
ursodeoxycholic acid	26. 22 (1.62)	28. 11 (1.38)
cholic acid	22. 25 (1.38)	38. 55 (1.66)
hyocholic acid	30. 52 (1.90)	40. 01 (1.96)

Numbers in parentheses indicate the relative retention times (7β,12β-dihydroxy-5β-cholan-24-oic acid = 1.00).

Table 2 Important mass fragment ion and relative intensity of the four synthesized compounds as their free form, their methyl ester acetate derivative and their methyl ester DMES derivative.

	7α,12α-dihydroxy-5β-cholan-24-oic acid	7α,12β-dihydroxy-5β-cholan-24-oic acid	7β,12α-dihydroxy-5β-cholan-24-oic acid	7β,12β-dihydroxy-5β-cholan-24-oic acid
	m/e	m/e	m/e	m/e
free form	374 (3.2)	374 (1.3)	374 (3.6)	374 (3.3)
	356 (72.5)	356 (32.4)	356 (24.5)	356 (66.6)
	273 (8.8)	273 (23.5)	273 (77.5)	273 (84.3)
	255 (100.0)	255 (100.0)	255 (100.0)	255 (100.0)
methyl ester acetate deriv.	430 (1.7)	430 (2.6)	430 (0.6)	430 (0.8)
	370 (100.0)	370 (63.4)	370 (70.3)	370 (100.0)
	255 (86.1)	255 (100.0)	255 (100.0)	255 (99.3)
methyl ester DMES deriv.	578 (0.2)	578 (0.6)	578 (0.2)	578 (0.2)
	563 (3.1)	563 (2.9)	563 (4.0)	563 (3.6)
	549 (100.0)	549 (100.0)	549 (100.0)	549 (100.0)
	370 (29.7)	370 (14.9)	370 (34.7)	370 (19.8)
	255 (33.7)	255 (26.7)	255 (56.4)	255 (17.8)

Numbers in parentheses indicate the relative intensity of ion observed.
m/e 374, M-18; 356, M-2x18; 273, M-side chain-18; 430, M-AcOH; 370, M-2xAcOH in methyl ester acetate deriv. and M-2xDMESOH in methyl ester DMES deriv.; 578, M^+; 563, M-15; 549, M-29; 255, M-side chain-2x18 in free form, M-side chain-2xAcOH in methyl ester acetate deriv. and M-side chain-2xDMESOH in methyl ester DMES deriv.

are isomers of 7,12-dihydroxy-5β-cholan-24-oic acid.

Table 1B shows the relative retention time (RRT) value as well as the retention time of some natural bile acids as methyl ester acetate derivative and methyl ester DMES derivative when the OV-1 column was used (7β,12β-dihydroxy-5β-cholan-24-oic acid = 1.00). In both derivatives, the RRT value of the ββ-isomer is located between that of lithocholic acid which appears first and that of hyocholic acid which appears last and also differs from that of natural bile acids. Especially, the methyl ester DMES derivative is better than the methyl ester acetate derivative in the difference of the RRT value. The gas chromatogram of some natural bile acids, including ββ-isomer, as their methyl ester DMES derivative is shown in Fig. 1.

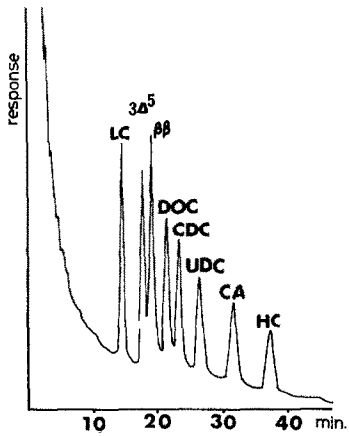

Fig. 1 Gas chromatogram of methyl ester DMES derivative of some natural bile acids including ββ-isomer. Column used was 1% OV-1.
LC, lithocholic acid; $3\Delta^5$, 3β-hydroxy-5-cholen-24-oic acid; ββ, 7β,12β-dihydroxy-5β-cholan-24-oic acid; DOC, deoxycholic acid; CDC, chenodeoxycholic acid; UDC, ursodeoxycholic acid; CA, cholic acid; HC, hyocholic acid.

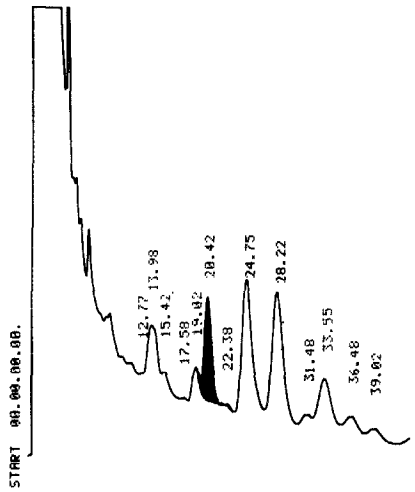

Fig. 2 Gas chromatogram of methyl ester DMES derivative of the extract from urine of a patient with hepatitis. Shaded peak, ββ-isomer; solid line + shade, the extract from urine of the patient when ββ-isomer was added to the urine, number, retention time; column, 1% OV-1.

Although the ββ-isomer is not a natural compound, it has the structure of a C-24 bile acid and thus would be extracted in the same fraction as the urinary bile acids. When bile acids in urine, to which the ββ-isomer had been added, were extracted and assayed as the methyl ester DMES derivative by gas chromatography (1% OV-1 column), the independent peak of the ββ-isomer was detected together with the peaks of other bile acids from the urine on the gas chromatogram. When the ββ-isomer was not added, no peak appeared at its position (Fig. 2). Also, no artifact from the ββ-isomer was produced during the analysis procedure. These findings suggest that we can regard the area of the ββ-peak on the gas chromatogram as representing the amount of ββ-isomer

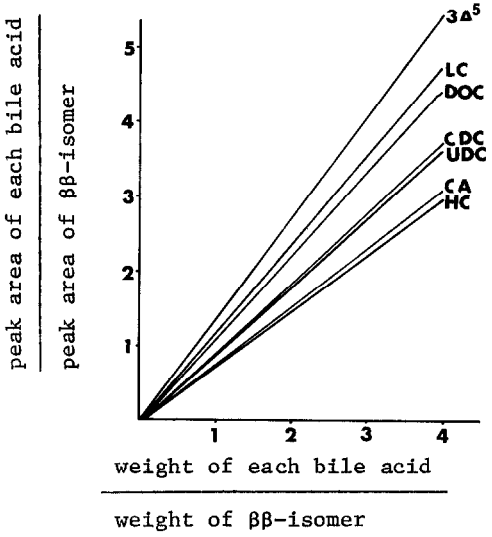

Fig. 3 Calibration curves of bile acids for quantitative determination.
For explanation, see legent for Fig. 4.

added to the urine, irrespective of the recovery rate in our technical procedure.

The calibration curves for the bile acids for quantitative determination by gas chromatography gave in a linear relationship between the peak area ratios and the weight ratios of each standard bile acid to the ββ-isomer (Fig. 3). Therefore, the absolute amount of each bile acid in the volume of the urine assayed can be calculated by the following formula.

$$W = A \times B$$

where W = absolute amount of each bile acid in the volume of the urine assayed.

A = weight ratio of each bile acid to the ββ-isomer obtained by applying the ratio of the peak area of each urinary bile acid to the additional ββ-isomer to the calibration curve.

B = amount of ββ-isomer added to the urine.

We concluded that the ββ-isomer is a suitable internal compound with more advantages than any other bile acids reported previously (2, 3, 19) for the quantitative determination of bile acids by gas chromatography, especially with chromatography of the methyl ester DMES derivative using the 1% OV-1 gas chromatographic column.

ACKNOWLEDGEMENTS

We gratefully acknowledge Professor K. Shimizu (Institute of Steroid Research, Tottori University School of Medicine) for his continuous advice and hearty encouragement throughout this investigation. We would like to thank Mrs. T. Hara for operating the gas chromatography-mass spectrometer.

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   Chenodeoxycholic acid, 3α,7α-dihydroxy-5β-cholan-24-oic acid.
   Ursodeoxycholic acid, 3α,7β-dihydroxy-5β-cholan-24-oic acid.
   Hyodeoxycholic acid, 3α,6α-dihydroxy-5β-cholan-24-oic acid.
   Cholic acid, 3α,7α,12α-trihydroxy-5β-cholan-24-oic acid.
   Hyocholic acid, 3α,6α,7α-trihydroxy-5β-cholan-24-oic acid.
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