Capillary Gas Chromatographic Determination of C₂₇-Bile Acids in Biological Samples and Its Application to the Urine of a Patient with Zellweger Syndrome

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Gas chromatographic determination of C₂₇-bile acids was investigated for the study of bile acid biosynthesis and related congenital disorders. The intermediate C₂₇-bile acids in the β -oxidation step and related compounds were conveniently synthesized from the corresponding C₂₄-bile acids as standard samples. The separation of methyl ester-trimethylsilyl ether (Me-TMS) and methyl ester-dimethylethylsilyl ether (Me-DMES) derivatives of the C₂₇-bile acids was carried out by capillary gas chromatography. Then methylene units of the above derivatives were measured. The Me-DMES derivatives were more efficient for the separation of C₂₇-bile acids than the Me-TMS derivatives. The calibration curves for the Me-DMES derivatives of C₂₇-bile acids showed good linearities over the range of 5–200 ng; the detection limits were ca. 0.5 ng (S/N=5). The relative recoveries from biological samples were 93–105% for all C₂₇-bile acids using 24-nor-chenodeoxycholic acid as an internal standard. An application to the diagnosis of Zellweger syndrome is also discussed.

Keywords C₂₇-Bile acid, gas chromatography, methylene unit, bile acid biosynthesis, Zellweger syndrome

Primary bile acids (3 α ,7 α ,12 α -trihydroxy- and 3 α ,7 α -dihydroxy-5 β -cholan-24-oic acids, CA and CDCA) are synthesized from cholesterol through C₂₇-bile acid intermediates, such as 3 α ,7 α ,12 α -trihydroxy- and 3 α ,7 α -dihydroxy-5 β -cholestan-26-oic acids (THCA and DHCA) by the side chain cleavage (β -oxidation) in mammalian livers.^{1–3}

Recently, we demonstrated that fetal bile acids, such as 1 β -, 6 α -, and 19-hydroxylated cholic acids, were the major bile acids in early life.^{4–6} The biosynthetic pathway of these fetal bile acids has not been clearly established. Sjövall *et al.* reported the production of 1 β -, 6 α - and 19-hydroxylated bile acids by incubating secondary bile acids (3 α ,12 α -dihydroxy- and 3 α -hydroxy-5 β -cholan-24-oic acids, DCA and LCA) with fetal liver homogenates.^{7,8} However, the hydroxylation at 1 β -, 6 α - and 19-positions of primary bile acids (CA and CDCA) is not known. Hoshita *et al.* tentatively identified 1 β ,3 α ,7 α ,12 α - and 3 α ,6 α ,7 α ,12 α -tetrahydroxy-5 β -cholestan-26-oic acids (THCA-1 β -ol and THCA-6 α -ol) in the urine of an infant with Zellweger syndrome⁹, which is a congenital disorder of peroxisomal enzyme deficiency.^{10,11} This suggested that the hydroxylation at 1 β - or 6 α -position occurred before the side chain

degradation step; these hydroxylated C₂₇-bile acids are therefore considered to be precursors of the fetal C₂₄-bile acids.

In order to clarify the biosynthetic pathway of fetal bile acids, the quantitative analysis of intermediate C₂₇-bile acids is required. Gas chromatography coupled with mass spectrometry (GC/MS) is considered to be one of the most suitable tools for the quantitative determination of C₂₇-bile acids, so the gas chromatographic separation of C₂₇-bile acids will be essential to establish for the analysis by GC/MS. The gas chromatographic separation of C₂₇-bile acids have been reported^{9,12}, but no systematic analysis of various C₂₇-bile acids including their β -oxidation intermediates (24-unsaturated and 24-hydroxylated C₂₇-bile acids) by gas chromatography has appeared. The aim of this work is to investigate the gas chromatographic separation of various C₂₇-bile acids, to establish the quantitative analytical method for the study of bile acid biosynthesis, and its application to the clinical study of Zellweger syndrome.

Experimental

Synthesis of the standard C₂₇-bile acids

All solvents were used after distillation. C₂₄-Bile

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acids were either purchased from Sigma Chemical Co., (St. Louis, MO, USA) or synthesized in our laboratory. C₂₇-Bile acids were synthesized in our laboratory by a standard procedure (Fig. 1). Elemental analyses and melting points are summarized in Table 1 and other spectral data supported their structures.

Cholestan-26-oic acids (3_{a-k})

C₂₄-Aldehydes (2_{a-k}) were prepared from the corresponding C₂₄-bile acids (1_{a-k}) by formylation with formic acid¹³, reduction with sodium borohydride after their conversion into mixed anhydrides with ethyl chloroformate¹⁴, followed by Swern oxidation (62–75%). These aldehydes were sufficiently pure for the following reaction steps. An equimolar amount of ethyl 1-(tri-phenylphosphoranylidene)propionate was added to a solution of the above aldehyde (4–5 mmol) in 1,2-dichloroethane (20–30 ml), and the solution was heated to reflux for 30 min. The reaction mixture was concentrated, and the residual oil was subjected to SiO₂ column chromatography (30–40 g) eluted with benzene/EtOAc (10:1–20:1, v/v) to give 24-unsaturated esters (84–92%). Then a suspended solution of the above ester (each 1 mmol) and 10% Pd/C (300 mg) in EtOAc (20 ml) were stirred under H₂ atmosphere for 4–12 h. After the usual work up, the product was treated with 2 M KOH in MeOH (25 ml) at 70°C for 1–3 h and concentrated *in vacuo*. The residue was acidified with 2 M HCl and the precipitates were recrystallized from an appropriate solvent to give cholestanic acid (3_{a-k}), 70–85%) as a diastereomixture at C-25.

Cholest-24-en-26-oic acids (4_{a-k})

The above unsaturated ester (*ca.* 1.5 mmol) was hydrolyzed with potassium *t*-butoxide (2 g) in *t*-butanol (8 ml) containing 5% of H₂O at 80°C for 0.5–1 h. After evaporation of the solvent, the residue was dissolved in H₂O and acidified with 2 M HCl. The precipitates were collected and recrystallized from an appropriate solvent to give cholest-24-en-26-oic acid (4_{a-k}, 65–72%). The compounds 4_{a-k} are all 24E-isomers.

24-Hydroxycholestan-26-oic acids (5_{a-k})

A solution of the aldehyde (2_{a-k}, 5–10 mmol), ethyl 2-bromopropionate (10–20 mmol) and Zn (2–4 g) in dry benzene (20–30 ml) containing a trace amount of I₂, was refluxed for 0.5–1 h. The reaction mixture was acidified with 10% H₂SO₄ and extracted with ether (50 ml×2). The combined organic layer was washed with H₂O and dried (Na₂SO₄). The product was refluxed with 2 M KOH in MeOH (50 ml) for 2–3 h. After concentration *in vacuo*, the reaction mixture was acidified with 2 M HCl and extracted with EtOAc (50 ml×3). The extract was washed with H₂O, dried (MgSO₄) and evaporated. The crude product was purified by SiO₂ column chromatography (30–40 g) eluted with CHCl₃/MeOH (8:1–15:1) to give 24-hydroxycholestan-26-oic acid (5_{a-k}, 55–80%) as a diastereomixture.

Apparatus and chromatographic conditions

Gas chromatography was performed with a Shimadzu GC 14A gas chromatograph equipped with a flame ionization detector (Shimadzu Co., Kyoto, Japan). A fused silica capillary column bonded with methyl silicone (Ulbon HR-1T, 25 m×0.25 mm i.d., Shinwa Co., Kyoto, Japan) was used as the gas chromatographic column. The carrier gas was helium at a linear velocity of 45 cm/s. The column oven program was 230°C (3 min hold) followed by temperature ramps at 10°C/min to 270°C, and then 2°C/min to 310°C.

Derivatization into the Me-DMES and Me-TMS derivatives

A mixture of 100 µl of standard solution (250 µg/10 ml in MeOH) and 100 µl of 24-nor-chenodeoxycholic acid solution (250 µg/10 ml in MeOH) was treated with diazomethane-ether for 5 min at room temperature, and then evaporated. Dimethylethylsilylimidazole (30 µl, Tokyo Kasei Kogyo Co., Tokyo, Japan) was added and warmed at 60°C for 45 min. The reaction mixture was diluted with hexane/EtOAc (0.5 ml, 3:1, v/v), and subjected to SiO₂ column (30×8 mm i.d.) eluted with hexane/EtOAc (5 ml, 3:1, v/v). After evaporation of the solvent, the residue was dissolved in hexane (50 µl) and 1 µl was used for gas chromatographic analysis. The Me-TMS derivatives were also prepared by the same procedure using trimethylsilylimidazole (30 µl, Tokyo Kasei Kogyo Co.) at 45°C in place of dimethylethylsilylimidazole.

Calibration curves

A mixture of 100 µl of each standard solution (25, 50, 100, 250, 500, 750 and 1000 µg/10 ml in MeOH) and 100 µl of 24-nor-chenodeoxycholic acid solution (250 µg/10 ml in MeOH) was treated as above. The calibration curves were constructed by plotting the peak area ratios of C₂₇-bile acids for 24-nor-chenodeoxycholic acid as an internal standard.

Sample preparation from biological samples for gas chromatographic analysis

A mixture of incubating solution of C₂₇-bile acid with rat liver homogenate and 24-nor-chenodeoxycholic acid (1 µg) as an internal standard was diluted with EtOH. The supernatant obtained by centrifuging at 3000 rpm for 10 min was concentrated *in vacuo*. The residue was heated with 2 M NaOH in MeOH/H₂O (1:1, v/v, 3 ml) at 80°C for 16 h. The mixture was concentrated and acidified with 6 M HCl, and extracted with EtOAc (5 ml). After washing with H₂O (1 ml×2) and evaporation, the residue was fractionized using piperidinohydroxypropyl Sephadex LH-20 (PHP-LH20) column (35×8 mm i.d.) by washing with 90% EtOH (3 ml) and eluting with 0.1 M acetic acid in 90% EtOH (5 ml).¹⁵ The bile acid fraction was evaporated and the residue was derivatized for gas chromatographic analysis as described above.

Compound 2 (R = OCHO)				
	R ₁	R ₂	R ₃	R ₄
a	H	H	H	H
b*	H	H	H	H
c	H	OCHO	H	H
d	OCHO	H	H	H
e	H	H	α -OCHO	H
f	OCHO	OCHO	H	H
g	H	OCHO	β -OCHO	H
h	OCHO	H	α -OCHO	H
i	OCHO	OCHO	β -OCHO	H
j	OCHO	OCHO	α -OCHO	H
k	OCHO	OCHO	H	OCHO

* R = 3 β -OCHO, Δ^5

Compounds 1, 3, 4 and 5 (R = OH)				
	R ₁	R ₂	R ₃	R ₄
a	H	H	H	H
b**	H	H	H	H
c	H	OH	H	H
d	OH	H	H	H
e	H	H	α -OH	H
f	OH	OH	H	H
g	H	OH	β -OH	H
h	OH	H	α -OH	H
i	OH	OH	β -OH	H
j	OH	OH	α -OH	H
k	OH	OH	H	OH

** R = 3 β -OH, Δ^5

Fig. 1 Chemical structures and syntheses of C₂₇-bile acids.

Determination of C₂₇-bile acids in the urine of a patient with Zellweger syndrome

The bile acid fraction from urine (2 ml) of a patient with Zellweger syndrome was prepared according to the modification of a reported procedure.¹⁶ Urine (2 ml) with internal standard (1 μ g) was subjected to a Bond Elut C18 cartridge (Varian, Harbor City, CA, USA), washed with H₂O (2 ml) and eluted with 90% EtOH (3 ml). The bile acid fraction was hydrolyzed with 2 M NaOH (2 ml) at 80°C for 16 h. After acidification with 2 M HCl, the mixture was extracted with EtOAc (4 ml) and evaporated. The residue was purified with PHP-LH 20 column (35 $\times$ 8 mm i.d.) by washing with 90% EtOH (3 ml) and eluting with 0.1 M acetic acid in 90% EtOH (5 ml). The bile acid fraction was then derivatized and subjected to the analytical procedure described above.

Recovery

The relative recoveries were measured by two different concentrations using synthetic standard samples. The test sample was prepared by mixing known amounts of C₂₇-bile acids (0.5 μ g and 2.5 μ g) into a rat liver homogenate solution (0.5 ml) or a bile acid-free urine

(2 ml) with 24-nor-chenodeoxycholic acid (1 μ g). Each sample was subjected to the analytical procedure described above.

Results and Discussion

Gas chromatographic separation of C₂₇-bile acids

Figure 1 shows the various 5 β -cholestan-26-oic acids (3, 4 and 5) used in this study. These compounds were synthesized from the corresponding C₂₄-bile acids to evaluate the effect of number and position of hydroxyl groups on gas chromatographic separation. DHCA (3_a) and THCA (3_f) are precursors for CDCA (1_a) and CA (1_f). Tetrahydroxy C₂₇-bile acids (3_i, 3_j and 3_k) are considered to be precursors for fetal bile acids. The compound 3_h is thought to be a precursor for 3 β -hydroxy-5 β -cholen-24-oic acid (1_h), which was first found in the urine of a neonate with congenital biliary atresia.¹⁷ Other compounds (3_a, 3_c, 3_e, 3_g, 3_h) are selected for the biological study with rat liver. The compounds 4 and 5 are the biologically possible intermediates from the corresponding 5 β -cholestan-26-oic acids (3) in the β -oxidation step. Table 1 shows the results of elemental

Table 1 Melting points and elemental analyses of C₂₇-bile acids used in this experiment

Bile acid (Position of OH)	5 β -Cholestan-26-oic acid (3) ^a			5 β -Cholestan-24-en-26-oic acid (4) ^a			24-Hydroxy-5 β -cholestanic acid (5) ^a		
	mp/°C	Anal. (C; H)		mp/°C	Anal. (C; H)		mp/°C	Anal. (C; H)	
		Calcd., %	Found, %		Calcd., %	Found, %		Calcd., %	Found, %
a (3 α)	156–160 ^b	77.46; 11.08	77.28; 11.15	170–172	77.83; 10.65	77.81; 10.66	161–162	74.61; 10.67	74.44; 10.93
b (3 β , Δ^5)	180–182 ^b	77.83; 10.65	77.60; 10.72	235.5–237	78.21; 10.21	78.10; 10.30	189–190	74.95; 10.25	74.76; 10.13
c (3 α , 12 α)	139–142 ^b	74.61; 10.67	74.56; 10.67	160–162	74.95; 10.25	74.79; 10.29	98–100	71.96; 10.29	72.11; 10.44
d (3 α , 7 α)	111–114 ^b	74.61; 10.67	74.49; 10.53	168–171 ^b	74.95; 10.25	74.96; 10.23	143–146 ^b	71.96; 10.29	71.78; 10.35
e (3 α , 6 α)	216–217	74.61; 10.67	74.59; 10.68	222–223	74.95; 10.25	74.94; 10.28	217–218	71.96; 10.29	72.04; 10.27
f (3 α , 7 α , 12 α)	184–186 ^b	71.96; 10.29	71.94; 10.25	176–178 ^b	72.28; 9.89	72.16; 9.76	178–181 ^b	69.49; 9.94	69.38; 9.90
g (3 α , 6 β , 12 α)	128–130	71.96; 10.29	71.68; 10.16	143–145	72.28; 9.89	72.13; 9.88	139–140	69.49; 9.94	69.39; 9.80
h (3 α , 6 α , 7 α)	202–204	71.96; 10.29	71.89; 10.20	183–184	72.28; 9.89	71.99; 9.89	230–232	69.49; 9.94	69.28; 10.00
i (3 α , 6 β , 7 α , 12 α)	145–147	69.49; 9.94	69.41; 9.86	162–163.5	69.79; 9.55	69.68; 9.44	156–157	65.96; 9.64 ^c	65.84; 9.40
j (3 α , 6 α , 7 α , 12 α)	266–268 ^b	69.49; 9.94	69.53; 9.73	146–148	69.79; 9.55	70.01; 9.43	150–152	65.96; 9.64 ^c	65.76; 9.42
k (1 β , 3 α , 7 α , 12 α)	228–230	69.49; 9.94	69.40; 9.75	230–232	69.79; 9.55	69.77; 9.51	231–232	65.96; 9.64 ^c	66.23; 9.59

a. The carboxylic acids were obtained as a mixture of diastereoisomers, however, these isomers were not separable by gas chromatography and the ratio of these isomers did not interfere with the gas chromatographic analysis. b. The synthesis of these compounds have been already reported (see refs. 22–30). c. Calculated for C₂₇H₄₂O₇·1/2H₂O.

analyses of the above synthesized compounds for the confirmation of their purities (melting points were also shown).

These synthesized compounds were used as standard samples for the development of gas chromatographic analysis. Initially, derivatization of C₂₇-bile acids was attempted using two kinds of silylating reagents. The standard samples were first treated with diazomethane, and the methyl esters were then treated with trimethylsilylimidazole or dimethylethylsilylimidazole to give the Me-TMS or Me-DMES derivatives. The chromatographic separation of Me-TMS and Me-DMES derivatives of C₂₄-bile acids are well investigated.^{18–20} The DMES derivatives are more stable for hydrolysis than TMS derivatives. However, there has been no report on the application of Me-DMES derivatives for gas chromatographic separation of C₂₇-bile acids. We investigated the separation of the above derivatives of C₂₇-bile acids by capillary gas chromatography. The methylene units (MU)²¹ of these derivatives are summarized in Table 2 and Table 3 (C₂₄-bile acids are also shown). In the cases of the Me-TMS derivatives (Table 2), the number of hydroxyl groups on the steroid nucleus affected the MU. The MU values of C₂₇-bile acids seemed to have a slight correlation with those of corresponding C₂₄-bile acids. The ratio of the increased MU values of 5 β -cholestanic acids to the corresponding values of C₂₄-bile acids ranged from 1.2–2.3. These irregularities cause some difficulty about predicting the retention time of C₂₇-bile acid from that of a corresponding C₂₄-bile acid. In the series of C₂₇-bile acids, the insertion of a double bond (Δ^4) led to an increase of MU of approximately 1 unit, except for 3i (1.9 unit). The increased MU values by a 24-hydroxyl group ranged from 1.5 to 2.6. These results indicated that the addition of a hydroxyl group into the side chain may cause a different effect to each C₂₇-bile acid. It is also thought that complex separation modes, such as distribution and adsorption, may play a role in the separation of Me-TMS derivatives of bile acids. It was also noted that the MU values of the Me-TMS derivatives of individual bile acids had a relatively narrow range (32–37).

On the contrary, regularity in the alternation of MU values of the Me-DMES derivatives was clearly observed (Table 3). The MU values of C₂₄-bile acids represented the numbers of hydroxyl groups on the steroid nucleus, in which the addition of a hydroxyl group increased the MU by about 1.5–2 MU, as reported by Iida *et al.*²⁰ The MU values were about 32 for the monohydroxy bile acid (1a), 34 for the dihydroxy bile acids (1c–e), 35–36 for the trihydroxy bile acids (1f–h) and 36–37 for the tetrahydroxy bile acids (1i–k). The position of a hydroxyl group seemed to affect the MU values in the order of 12 α <7 α <6 β <6 α <1 β . The Δ^5 -double bond may also increase about 1 MU as in the case of 1b. The elution order of C₂₇-bile acids (3,4,5) was in good agreement with that of the corresponding C₂₄-bile acids. All MU values of the cholestanic acids were about 2.3 MU more than those of the corresponding C₂₄-bile acids, which

Table 2 Methylene units (MU) of methyl ester-trimethylsilyl ether derivatives

Bile acid (Position of OH)	Cholanoic acid (1)		Cholestan-26-oic acid (3)		Cholest-24-en-26-oic acid (4)		24-Hydroxycholestanic acid (5)	
	MU	ΔMU^a (1-3)	MU		MU	ΔMU^a (4-3)	MU	ΔMU^a (5-3)
a 3 α	31.6	-1.8	33.4		34.5	1.1	36.0	2.6
b 3 β , Δ^5	32.3	-2.0	34.3		35.4	1.1	36.0	1.7
c 3 α , 12 α	32.3	-2.3	34.6		35.6	1.0	36.5	1.9
d 3 α , 7 α	32.6	-1.4	34.0		35.1	1.1	35.8	1.8
e 3 α , 6 α	32.7	-1.7	34.4		35.5	1.1	35.9	1.5
f 3 α , 7 α , 12 α	32.6	-1.7	34.3		35.5	1.2	35.8	1.5
g 3 α , 6 β , 12 α	33.1	-1.2	34.3		35.3	1.0	36.4	2.1
h 3 α , 6 α , 7 α	33.6	-1.7	35.3		36.4	1.1	36.8	1.5
i 3 α , 6 β , 7 α , 12 α	32.8	-1.6	34.4		36.3	1.9	36.7	2.3
j 3 α , 6 α , 7 α , 12 α	33.6	-1.2	34.8		35.8	1.0	36.5	1.7
k 1 β , 3 α , 7 α , 12 α	33.7	-1.6	35.3		36.4	1.1	37.0	1.7

a. Difference of the methylene units from the corresponding cholestan-26-oic acid.

Table 3 Methylene units (MU) of methyl ester-dimethylethylsilyl ether derivatives

Bile acid (Position of OH)	Cholanoic acid (1)		Cholestan-26-oic acid (3)		Cholest-24-en-26-oic acid (4)		24-Hydroxycholestanic acid (5)	
	MU	ΔMU^a (1-3)	MU		MU	ΔMU^a (4-3)	MU	ΔMU^a (5-3)
a 3 α	32.3	-2.3	34.6		35.7	1.1	38.3	3.7
b 3 β , Δ^5	33.1	-2.4	35.5		36.5	1.0	38.9	3.4
c 3 α , 12 α	33.8	-2.4	36.2		37.2	1.0	39.1	2.9
d 3 α , 7 α	34.2	-2.3	36.5		37.7	1.2	39.4	2.9
e 3 α , 6 α	34.4	-2.3	36.7		37.9	1.2	39.8	3.1
f 3 α , 7 α , 12 α	35.4	-2.3	37.7		38.8	1.1	40.4	2.7
g 3 α , 6 β , 12 α	35.7	-2.2	37.9		39.0	1.1	40.6	2.7
h 3 α , 6 α , 7 α	36.0	-2.3	38.3		39.5	1.2	41.0	2.7
i 3 α , 6 β , 7 α , 12 α	36.3	-2.2	38.6		39.8	1.2	41.2	2.6
j 3 α , 6 α , 7 α , 12 α	37.1	-2.3	39.4		40.5	1.1	41.9	2.5
k 1 β , 3 α , 7 α , 12 α	37.4	-2.2	39.6		40.7	1.1	42.1	2.4

a. Difference of the methylene units from the corresponding cholestan-26-oic acid.

indicated that the elongation (3 carbon units) of the side chain caused the MU values to increase constantly. The introduction of a double bond in the side chain increased the MU value by about 1 unit. The above increase in the MU value was also observed in the case of Me-TMS derivatives (Table 2), although the elution order did not agree with that of the corresponding cholestanic acids. The elution order of the Me-DMES derivatives of cholest-24-enoic acids, however, was the same as that of the corresponding cholestanic acids. The introduction of a hydroxyl group at the C-24 position of the mono-hydroxycholestanic acids (3_{a-h}) increased the MU values by about 3.5. In case of di-, tri- and tetrahydroxycholestanic acids (3_{c-e}, 3_{f-h} and 3_{i-k}), the increased values were about 3, 2.7 and 2.5, respectively. The elution order were not changed. The MU values of the Me-DMES derivatives of bile acids ranged from 32 to 42. This range is two times wider than that of the Me-TMS derivatives.

The complete separation of all 44 bile acids was

impossible under the conditions. However, these expanded values of MU in the Me-DMES derivatives are very useful for the determination of the expected intermediates from an individual C₂₇-bile acid as shown in Fig. 2, in which the C₂₇-intermediates (4_{d,f} and 5_{d,f}) and final products (1_{d,f}) of DHCA (3_d) and THCA (3_f) in β -oxidation are completely separated. Other 5 β -cholestan-26-oic acids and their corresponding intermediates were also completely separated. These results showed the possibility of establishing an analytical method for the biosynthetic study of bile acids.

Moreover, the Me-DMES derivatives may be useful in the determination of cholestanic acids in biological fluids, such as urine, serum and bile. These C₂₇-bile acids are not generally detectable by gas chromatography in a healthy human, although several cholestanic acids have been identified in the urine of patients with Zellweger syndrome.⁹ As shown in Fig. 3, cholestanic acids (3_{a-k}) could be separated, making it possible to carry out quantitative analysis of the cholestan-26-oic

Fig. 2 Standard gas chromatograms of $3\alpha,7\alpha$ -dihydroxy- and $3\alpha,7\alpha,12\alpha$ -trihydroxy- 5β -cholestan-26-oic acids and their possible intermediates as the Me-DMES derivatives. (a) 1: 1_d ; 2: 3_d ; 3: 4_d ; 4: 5_d , (b) 1: 1_r ; 2: 3_r ; 3: 4_r ; 4: 5_r (the chemical structures of the compounds are shown in Fig. 1).

Fig. 3 Gas chromatogram of standard 5β -cholestan-26-oic acids as the Me-DMES derivatives. 1: 3_a ; 2: 3_b ; 3: 3_c ; 4: 3_d ; 5: 3_e ; 6: 3_f ; 7: 3_g ; 8: 3_h ; 9: 3_i ; 10: 3_j ; 11: 3_k (the chemical structures of the compounds are shown in Fig. 1).

acids in the urine of such patients.

Quantitative analysis of C_{27} -bile acids

To establish an analytical method for determining of C_{27} -intermediates and C_{24} -bile acids, calibration curves for the Me-DMES derivatives of these bile acids were constructed by plotting peak area ratios of the bile acids for 24-nor-chenodeoxycholic acid as an internal standard. Calibration curves of all 44 bile acids showed good linearities in the range of 5–200 ng ($y=0.020 -$

$0.023x \pm 0.01 - 0.04$, $r=0.998 - 0.999$), and the detection limit was about 0.5 ng at $S/N=5$ for each bile acid. Since our study on the biosynthesis of bile acids requires the quantitative determination of the intermediates in biological samples, the relative recoveries of the bile acids from rat liver homogenate and bile acid-free urine were measured. Table 4 shows the results of the recoveries of MHCA (3_a), DHCA (3_d), THCA (3_i), THCA- 6α -ol (3_j)

Table 4 Relative recoveries from rat liver homogenates and urines^a

Compound	Recoveries (% , mean $\pm$ S.D.)			
	Rat liver homogenate ($n=5$) ^b		Urine ($n=4$) ^c	
	added (0.5 μ g)	added (2.5 μ g)	added (0.5 μ g)	added (2.5 μ g)
3_a (MHCA)	95.2 $\pm$ 6.9	94.8 $\pm$ 5.8	95.9 $\pm$ 7.1	96.8 $\pm$ 5.4
4_a (24E-MHCA)	104.1 $\pm$ 6.2	100.2 $\pm$ 4.5	103.1 $\pm$ 5.3	99.8 $\pm$ 3.2
5_a (24-OH-MHCA)	102.2 $\pm$ 5.8	97.4 $\pm$ 4.0	102.2 $\pm$ 6.6	98.4 $\pm$ 5.0
3_d (DHCA)	100.7 $\pm$ 4.7	99.6 $\pm$ 5.1	104.6 $\pm$ 5.5	101.4 $\pm$ 3.8
4_d (24E-DHCA)	104.3 $\pm$ 3.3	101.5 $\pm$ 2.6	100.3 $\pm$ 4.9	98.5 $\pm$ 2.1
5_d (24-OH-DHCA)	98.1 $\pm$ 4.2	97.4 $\pm$ 3.1	94.6 $\pm$ 5.5	95.2 $\pm$ 4.9
3_i (THCA)	103.6 $\pm$ 6.1	98.4 $\pm$ 1.8	93.4 $\pm$ 6.0	97.7 $\pm$ 2.1
4_i (24E-THCA)	102.1 $\pm$ 5.3	97.4 $\pm$ 2.6	104.8 $\pm$ 7.1	102.0 $\pm$ 3.0
5_i (24-OH-THCA)	101.8 $\pm$ 4.4	95.4 $\pm$ 3.6	99.0 $\pm$ 6.2	94.2 $\pm$ 5.1
3_j (THCA- 6α -ol)	99.3 $\pm$ 2.6	97.6 $\pm$ 3.2	100.3 $\pm$ 5.2	98.5 $\pm$ 4.1
4_j (24E-THCA- 6α -ol)	98.1 $\pm$ 4.3	98.6 $\pm$ 2.3	98.8 $\pm$ 5.1	96.9 $\pm$ 3.2
5_j (24-OH-THCA- 6α -ol)	100.2 $\pm$ 5.2	97.3 $\pm$ 2.6	99.2 $\pm$ 6.3	97.1 $\pm$ 3.8
3_k (THCA- 1β -ol)	100.5 $\pm$ 5.6	98.6 $\pm$ 3.2	101.3 $\pm$ 6.1	96.5 $\pm$ 4.4
4_k (24E-THCA- 1β -ol)	97.1 $\pm$ 4.7	96.6 $\pm$ 3.3	99.2 $\pm$ 5.2	97.6 $\pm$ 3.7
5_k (24-OH-THCA- 1β -ol)	96.2 $\pm$ 5.5	96.8 $\pm$ 1.9	96.9 $\pm$ 7.1	95.1 $\pm$ 4.6

a. One microgram of internal standard (24-nor-chenodeoxycholic acid) was added. b. Relative recoveries from the mixture of 10% rat liver homogenates (150 μ l, 800 $\times$ g supernatant) and 20 mM phosphate buffer (350 μ l, pH 7.6). c. Relative recoveries from bile acid free urine prepared by treatment with charcoal and passing through Bond Elut C18 cartridge column.

and THCA-1 β -ol (**3_k**) as typical mono-, di-, tri- and tetrahydroxy bile acids, and their corresponding intermediates (**4_{a,d,e,j,k}** and **5_{a,d,e,j,k}**) under two concentrations (0.5 μ g and 2.5 μ g in 0.5 ml of incubation mixture). The recoveries of other C₂₇-bile acids were all within 93%–104%.

Application to the determination of C₂₇-bile acids in the urine of a patient with Zellweger syndrome

The above method was applied to the quantitative determination of bile acids in biological samples. Figure 4 shows a gas chromatogram of the bile acid fraction from the urine of a patient with Zellweger syndrome, which is a congenital disorder owing to a lack of the liver's peroxisomal β -oxidation system.¹¹ THCA (**3_f**) was found as a major component (2.9 μ g/ml) together with 3 α ,7 α ,12 α ,24-tetrahydroxy-5 β -cholestan-26-oic acid (VA, **5_f**, 0.65 μ g/ml). Other C₂₇-bile acids were THCA-6 α -ol (**3_j**, 0.28 μ g/ml) and THCA-1 β -ol (**3_k**, 0.34 μ g/ml), which are likely to be precursors of fetal bile acids. The structures of these C₂₇-bile acids were confirmed by a comparison of their mass spectra with those of standard samples. The amount of CA (**1_f**, 0.70 μ g/ml) was decreased by the deficiency of peroxisomal enzymes. It is noted that (24Z)-3 α ,7 α ,12 α -trihydroxy-5 β -cholest-24-en-26-oic acid (24Z-THCA, 0.06 μ g/ml) was identified, though 24E-THCA (**4_f**), known as an intermediate for CA (**1_f**), was not identified. The structure of 24Z-THCA was elucidated by comparing the mass spectra with that of synthetic standard²² as shown in Fig. 5 and by the MU values (24Z-THCA: 37.9; 24E-THCA: 38.8). The presence of 24Z-THCA may reflect the lack of peroxisomal β -oxidation system for CA through an intermediate 24E-THCA.

Fig. 4 Gas chromatogram of the Me-DMES derivatives of bile acids from the urine of a patient with Zellweger syndrome. 1: internal standard; 2: **1_f**; 3: **3_f**; 4: (24Z)-3 α ,7 α ,12 α -trihydroxy-5 β -cholest-24-en-26-oic acid; 5: **3_j**; 6: **3_k**; 7: **5_f** (the chemical structures of the compounds are shown in Fig. 1).

In conclusion, a convenient analytical method of C₂₇-bile acids for the study of biosynthesis of bile acids has been developed by capillary gas chromatography. The present method was also applicable to the analysis of bile acids in urine of a patient with Zellweger syndrome and the C₂₇-bile acids (THCA, VA, THCA-1 β -ol, THCA-6 α -ol, 24Z-THCA) could be quantitatively determined. The presence of 1 β - and 6 α -hydroxylated C₂₇-bile acids in the

Fig. 5 Mass spectra of (24Z)-3 α ,7 α ,12 α -trihydroxy-5 β -cholest-24-en-26-oic acid as the Me-DMES derivatives. (a) synthetic standard, (b) identified in urine of a patient with Zellweger syndrome.

urine suggested that THCA (3r) might be hydroxylated before the side chain cleavage. However, the β -oxidation of these hydroxylated bile acids is not known yet. Further detailed investigation on the β -oxidation of these hydroxylated C₂₇-bile acids in rat livers using the present method will be reported elsewhere.

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References

1. H. Danielsson, "The Bile Acids", Vol. 2, p. 1, Plenum Press, New York, 1973.
2. I. Björkhem, "Sterols and Bile Acids", p. 231, Elsevier Science Pub. B. V., Amsterdam, 1985.
3. D. W. Russell and K. D. R. Setchell, *Biochemistry*, **31**, 4737 (1992).
4. M. Tohma, R. Mahara, H. Takeshita, T. Kurosawa and S. Ikegawa, *Chem. Pharm. Bull.*, **34**, 2890 (1986).
5. T. Kurosawa, R. Mahara, H. Nittono and M. Tohma, *Chem. Pharm. Bull.*, **37**, 557 (1989).
6. T. Kurosawa, Y. Nomura, R. Mahara, T. Yoshimura, A. Kimura, S. Ikegawa and M. Tohma, *Chem. Pharm. Bull.*, **43**, 1551 (1995).
7. J. Gustafsson, S. Anderson and J. Sjövall, *Biol. Neonate*, **47**, 26 (1985).
8. J. Gustafsson, S. Anderson and J. Sjövall, *Pediatr. Res.*, **21**, 99 (1987).
9. M. Une, Y. Tazawa, K. Tada and T. Hoshita, *J. Biochem.*, **102**, 1525 (1987).
10. S. Goldfischer, C. Moore, A. Johnson, A. Spiro, M. Valsamis, H. Wisniewski, R. Ritch, W. Norton, I. Rapin and L. Gartner, *Science* [Washington, D.C.], **182**, 62 (1973).
11. B. F. Kase, I. Björkhem, P. Haga and J. I. Pedersen, *J. Clin. Invest.*, **75**, 427 (1985).
12. H. Eyssen, E. Eggermont, J. V. Eldere, J. Jaeken, G. Parmentier and G. Janssen, *Acta Paediatr. Scand.*, **74**, 539 (1985).
13. K. Tserng and P. D. Klein, *Steroids*, **29**, 635 (1977).
14. R. Somanathan and S. Krisans, *Steroids*, **43**, 651 (1984).
15. J. Goto, M. Hasegawa, H. Kato and T. Nambara, *Clin. Chim. Acta*, **87**, 141 (1978).
16. R. Mahara, H. Takeshita, T. Kurosawa, S. Ikegawa and M. Tohma, *Anal. Sci.*, **3**, 449 (1987).
17. I. Makino, J. Sjövall, A. Norman and B. Strandvik, *FEBS Lett.*, **15**, 161 (1971).
18. Y. Nishikawa, K. Yamashita, M. Ishibashi and H. Miyazaki, *Chem. Pharm. Bull.*, **26**, 2922 (1978).
19. A. Fukunaga, Y. Hatta, M. Ishibashi and H. Miyazaki, *J. Chromatogr.*, **190**, 339 (1980).
20. T. Iida, T. Momose, T. Tamura, T. Matsumoto, J. Goto, T. Nambara and F. C. Chang, *J. Chromatogr.*, **389**, 155 (1987).
21. E. C. Horning, M. G. Horning, N. Ikekawa, E. M. Chambaz, P. I. Jaakomaki and C. J. W. Brooks, *J. Gas Chromatogr.*, **5**, 283 (1967).
22. N. I. Mohammed, P. H. Patrick and W. H. Elliott, *Steroids*, **56**, 505 (1991).
23. R. J. Bridgwater, *Biochem. J.*, **64**, 593 (1956).
24. T. Briggs, *J. Org. Chem.*, **35**, 1431 (1970).
25. R. K. Varma, M. Koreeda, B. Yagen, K. Nakanishi and E. Caspi, *J. Org. Chem.*, **40**, 3680 (1975).
26. M. Une, N. Kisaka, M. Yoshii and T. Hoshita, *J. Biochem.*, **106**, 501 (1989).
27. M. Une, F. Nagai, K. Kihira, T. Kuramoto and T. Hoshita, *J. Lipid Res.*, **24**, 924 (1983).
28. Y. Inai, Y. Tanaka, S. Betsuki and T. Kazuno, *J. Biochem.*, **56**, 591 (1964).
29. M. Une, A. Inoue, T. Kurosawa, M. Tohma and T. Hoshita, *J. Lipid Res.*, **35**, 620 (1994).
30. T. Kurosawa, M. Sato, H. Nakano and M. Tohma, *Steroids*, **61**, 421 (1996).

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