

Gas chromatographic separation of bile acid 3-glucosides and 3-glucuronides without prior deconjugation on a stainless-steel capillary column

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First received 21 June 1994; revised manuscript received 5 September 1994

Abstract

A method for the gas chromatographic (GC) separation of the 3-glucoside and 3-glucuronide conjugates of bile acids without the necessity for a hydrolytic step is described. The bile acid glycosides were derivatized to their complete methyl ester trimethylsilyl (Me-TMS) or methyl ester dimethylethylsilyl (Me-DMES) ether derivatives, which in turn were chromatographed on an inert and thermostable stainless-steel capillary column, Ultra ALLOY-1 (HT), coated with a thin film (0.15 μm) of chemically bonded and cross-linked dimethylsiloxane. They exhibited a single peak of the theoretical shape without any accompanying peaks due to thermal decomposition, even at oven temperatures of 320–330°C. Excellent GC separation of isomeric bile acid glycosides was achieved by the combined use of suitable derivatives and column. This method, which does not need the prior deconjugation of the glycosidic moiety, could be usefully applied to biosynthetic and metabolic studies of bile acids in biological materials.

1. Introduction

In recent years, considerable attention has been directed to the biosynthesis and metabolism of bile acids in connection with hepatobiliary diseases. Particular interest has been focused on the physiological significance of the glycosidic conjugates of bile acids. Bile acid glucuronides, one of the earliest known glycosidic conjugates, have been reported to exist in various human biological fluids [1–4]. In addition to bile acid glucuronides, analogous glucosides [5–8] and N-acetylglucosaminides [9,10] have recently been

identified as novel bile acid conjugates in human urine.

Gas chromatography (GC) is a powerful tool for the profile analysis of bile acids in biological specimens. Prior to GC analysis, glycoside-conjugated bile acids and amidated bile acids with glycine or taurine and/or sulphated bile acids are conventionally hydrolysed to their unconjugated forms, which are then converted into suitable volatile derivatives, because they are comparatively polar and lack volatility [11]. However, deconjugation has several drawbacks, because it loses information about the type and site of conjugation and has the possibility of producing artifacts by inefficient hydrolysis.

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Therefore, a reliable method for the direct GC determination of these conjugated bile acids without need for prior deconjugation is urgently required.

We have been investigating the direct GC analysis of conjugated bile acids without the necessity for a hydrolytic step, and previous papers have demonstrated that GC of glycine- [11–14] and glucoside-conjugated [6,8] bile acids is possible. In addition, the recent successful use of a new type of a metal capillary column, Ultra ALLOY, for the separation of alcohols, amines and triglycerides prompted us to examine this column in the analysis of bile acid conjugates [15]. It has been reported that the performance of this column is superior to those of fused-silica and aluminium-clad fused-silica capillary columns with respect to inertness, thermal stability and mechanical strength under high temperature and vibration.

In continuation of an ongoing programme of GC analyses of conjugated bile acids, we report here a method for the direct GC determination of 3-glucoside and 3-glucuronide conjugates of bile acids without prior deconjugation. For the purpose of comparison, the corresponding unconjugated and glycine-conjugated bile acids were also analysed under the same GC conditions.

2. Experimental

2.1. Materials and reagents

The following abbreviations of compounds are used: LCA = lithocholic acid (1); CDCA = chenodeoxycholic acid (2); UDCA = ursodeoxycholic acid (3); DCA = deoxycholic acid (4); CA = cholic acid (5); glyco-LCA = glycolithocholic acid (6); glyco-CDCA = glycochenodeoxycholic acid (7); glyco-UDCA = glycoursodeoxycholic acid (8); glyco-DCA = glycodeoxycholic acid (9); glyco-CA = glycocholic acid (10); LCA 3-Glc. = lithocholic acid 3-glucoside (11); CDCA 3-Glc. = chenodeoxycholic acid 3-glucoside (12); UDCA 3-Glc. = ursodeoxycholic acid 3-glucoside (13); DCA 3-Glc. = deoxycholic acid 3-glucoside (14);

CA 3-Glc. = cholic acid 3-glucoside (15); LCA 3-GlcA. = lithocholic acid 3-glucuronide (16); CDCA 3-GlcA. = chenodeoxycholic acid 3-glucuronide (17); UDCA 3-GlcA. = ursodeoxycholic acid 3-glucuronide (18); DCA 3-GlcA. = deoxycholic acid 3-glucuronide (19); CA 3-GlcA. = cholic acid 3-glucuronide (20).

Almost all of the unconjugated bile acids (1–5) and their corresponding glycine (6–10), glucoside (11–15) and glucuronide (16–20) conjugates were from our laboratory collection, which include new and natural bile acid 3-glucosides [16] and 3-glucuronides [17] recently synthesized in these laboratories. UDCA 3-GlcA. (18) was kindly supplied by Tokyo Tanabe (Tokyo, Japan).

The silylating reagents, N-trimethylsilylimidazole (TMSI) and N,N-dimethylethylsilylimidazole (DMESI), were obtained from Tokyo Kasei Kogyo (Tokyo, Japan). All solvents used were of analytical-reagent grade.

2.2. Derivatization

Ethereal diazomethane was prepared with a Wheaton generator using N-methyl-N'-nitro-N-nitrosoguanidine as methyl donor. The diazomethane solution (0.5 ml) was added to a solution of the bile acid glycoside (ca.0.5 mg) in methanol (50 μ l) in a reaction vial, and the mixture was allowed to stand at room temperature for 1 h. After the excess reagent and solvent had been evaporated under a stream of nitrogen, silylating reagent (50 μ l, TMSI or DMESI) was added to the residue and the mixture was heated at 60°C for 1 h in a Reacti-Therm (Pierce, Rockford, IL, USA). An aliquot of the derivatized sample solutions [methyl ester trimethylsilyl (Me-TMS) ether or methyl ester dimethylethylsilyl (Me-DMES) ether] diluted with benzene (200 μ l) was injected into the GC system together with internal standards (C₃₂ and C₄₄).

2.3. Gas chromatography

A Shimadzu GC-14A gas chromatograph equipped with a flame ionization detector and data processing system (Shimadzu Chromatopac

C-R6A) was used. It was fitted with an Ultra ALLOY-1 (HT) stainless-steel capillary column (30 m × 0.25 mm I.D.) coated with a thin film (0.15 μm) of chemically bonded and cross-linked dimethylsiloxane (equivalent to 400-HT) and operated under the following conditions: carrier gas (helium) flow-rate, 0.7 ml/min; purge gas flow-rate, 20 ml/min; make-up gas flow-rate, 60 ml/min; injection method, split (1:50) or splitless; injector temperature, 330°C; detector temperature, 350°C; column temperature, either isothermal, 320°C for Me-TMS ethers or 330°C for Me-DMES ethers, or programmed, raised from 280 to 290°C at 4°C/min, held for 7 min, and then raised to 330°C at 2°C/min for Me-TMS ethers, or raised from 260 to 300°C at 2°C/min, held for 3 min, and then raised to 330°C at 2°C/min for Me-DMES ethers. The metal capillary column was purchased from Frontier Lab. (Koriyama, Japan).

3. Results and discussion

The structures of bile acid 3-glucosides [LCA 3-Glc. (11), CDCA 3-Glc. (12), UDCA 3-Glc. (13), DCA 3-Glc. (14) and CA 3-Glc. (15)] and 3-glucuronides [LCA 3-GlcA. (16), CDCA 3-GlcA. (17), UDCA 3-GlcA. (18), DCA 3-GlcA. (19) and CA 3-GlcA.(20)] examined in this study are shown in Fig. 1. For the purpose of comparison, the GC behaviour of the corresponding unconjugated (1–5) and glycine-conjugated (6–10) bile acids was also studied.

The four groups of the bile acids (1–20) were examined in a study of the two classes of derivatives and the column chosen. The free unconjugated and conjugated bile acids were converted into their methyl esters in both the sugar and steroidal side-chain moieties with ethereal diazomethane. The resulting bile acid methyl esters were then derivatized to their complete Me-TMS and Me-DMES ethers using TMSI and DMESI, respectively, as silylating agents of hydroxyl groups. All four groups of the bile acids including the glucoside and glucuronide conjugates were readily converted into the corresponding methyl ester silyl ethers under the mild conditions described under Experimental.

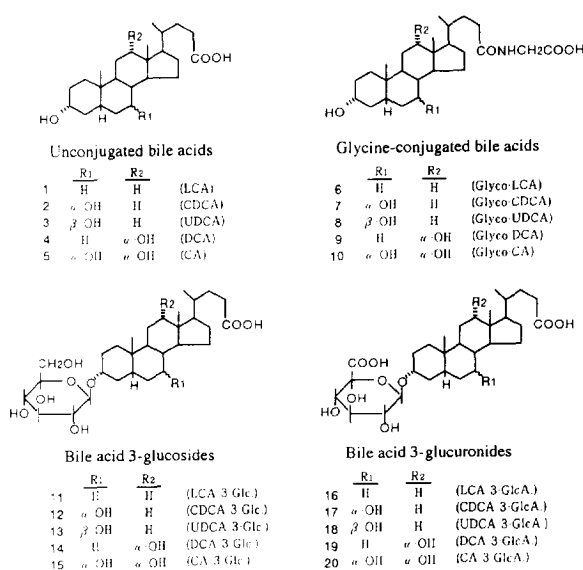


Fig. 1. Structures of compounds 1–20.

When the Me-TMS and Me-DMES ether derivatives of the bile acid glycosides were chromatographed on a stainless-steel capillary column of Ultra ALLOY-1 (HT), coated with a thin film (0.15 μm) of chemically bonded and cross-linked dimethylsiloxane, they exhibited a single peak of the theoretical shape without any accompanying peaks due to thermal decomposition in the column, even at oven temperatures of 320–330°C. In addition, the retention data (see below) for UDCA 3-Glc. (13), DCA 3-Glc. (14) and CA 3-Glc. (15) agreed well with those reported by Marschall and co-workers [6,8], demonstrating the thermal stability of these compounds. This result has considerable advantages; the avoidance of a hydrolytic step for the glycosidic linkages would prevent inefficient hydrolysis and provides direct information about the site of conjugation by GC. Further, the use of a thermostable metal capillary column has an advantage over a fused-silica column [8]; the analysis time for the same compounds is much shortened at relatively high temperatures, suggesting that the successful analysis of more polar and high-boiling compounds is possible.

The combined use of suitable derivatives and a column giving a sharp GC peak also proved to

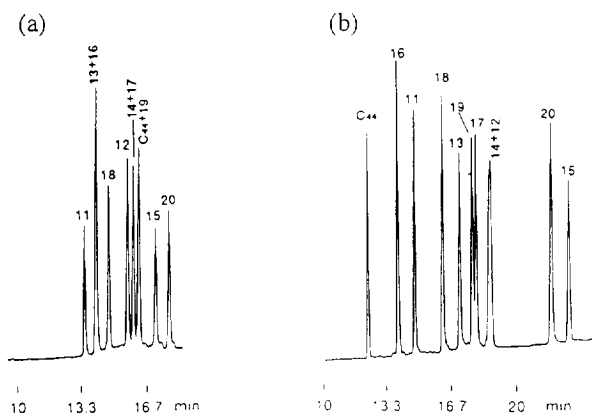


Fig. 2. Capillary GC of a mixture of the ten glucoside and glucuronide conjugates of bile acids as their (a) Me-TMS and (b) Me-DMES ether derivatives. GC conditions, isothermal (see text). Peaks: 11 = LCA 3-Glc.; 12 = CDCA 3-Glc.; 13 = UDCA 3-Glc.; 14 = DCA 3-Glc.; 15 = CA 3-Glc.; 16 = LCA 3-GlcA.; 17 = CDCA 3-GlcA.; 18 = UDCA 3-GlcA.; 19 = DCA 3-GlcA.; 20 = CA 3-GlcA.

be well suited for the separation of isomeric glycosides. Fig. 2 shows typical chromatograms of a mixture of the ten bile acid 3-glycosides and 3-glucuronides measured under isothermal GC conditions at 320 or 330°C. As can be seen, the five glycosides were completely resolved as their Me-TMS ethers on the metal capillary column, emerging in the order LCA 3-Glc. (11), UDCA 3-Glc. (13), CDCA 3-Glc. (12), DCA 3-Glc. (14), CA 3-Glc. (15). The corresponding glucuronides on this column exhibited the same elution order: LCA 3-GlcA. (16), UDCA 3-GlcA. (18), CDCA 3-GlcA. (17), DCA 3-GlcA. (19), CA 3-GlcA. (20). (Two variants of the glycosidic conjugate pairs, 13 vs. 16 and 14 vs. 17, overlap under the isothermal GC conditions examined.) The Me-DMES ether derivatives of each of the two groups of the glycosidic conjugates followed a similar elution order, except for the two isomeric pairs 12 vs. 14 and 17 vs. 19, which differed from those observed for the corresponding Me-TMS ethers: 11, 13, 12 = 14, 15; 16, 18, 19, 17, 20.

Table 1 shows the retention data of all twenty unconjugated and conjugated bile acids observed for the two classes of derivatization products on an Ultra ALLOY-1 (HT) column. Retention

data were expressed as the relative retention time (RRT) and methylene unit (MU) values; RRT was expressed relative to an appropriate derivative of DCA 3-GlcA. (19) and MU was determined using C_{30} – C_{50} *n*-alkanes [18]. The MU values were calculated by the cubic expression of approximate polynomial expression methods (see below). The $\Delta[Um]_{D-T}$ values [19], which were defined as the differences in the MU values between the Me-DMES and Me-TMS ethers for the same compound on this column, are also listed in Table 1.

As expected, the Me-DMES ether derivatives always gave longer retention times than the corresponding Me-TMS ethers, owing to the heavier ethyl groups. In order to clarify further the general features of the four groups of bile acids, the correlation of MU values between the Me-TMS and Me-DMES ether derivatives for the same compound was expressed graphically. As shown in Fig. 3, the plots afforded a regression line with good linearity, expressed as $y = 0.837x + 4.05$ ($r = 0.991$, $n = 20$). The correlation implies that if either the MU value of the Me-TMS or Me-DMES ether of an unknown bile acid is known, the three groups of bile acids (unconjugates and glycine and glycoside conjugates) can be easily characterized by applying the regression line.

Our previous GC studies on various unconjugated [19–21] and glycine-conjugated [13,14] bile acids as their ester silyl ether derivatives revealed that in general the addition of hydroxyl groups in the steroid molecules produces nearly consistent increases in the $\Delta[Um]_{D-T}$ values. On the basis of the finding, it is deduced from the $\Delta[Um]_{D-T}$ data shown in Table 1 that the $\Delta[Um]_{D-T}$ values for mono-, di- and trihydroxylated bile acid 3-glycosides are approximately 3.1, 4.0 and 5.1 and those for 3-glucuronides are approximately 2.3, 3.4 and 4.4, respectively. The corresponding values for unconjugated and glycine conjugated compounds are in good agreement with those reported in previous papers [13,19]. As the increment of the above units is essentially independent of the other structural characteristics, the determination of the $\Delta[Um]_{D-T}$ value for an unknown bile acid 3-glycoside affords a useful method not only for

Table 1

RRT and MU values of the Me-TMS and Me-DMES ether derivatives of unconjugated compounds and glycine, glucoside and glucuronide conjugates of bile acids

Type	No.	Compound	Me-TMS		Me-DMES		$\Delta[U_m]_{D-T}^a$
			RRT	MU	RRT	MU	
Unconjugated	1	LCA	0.18	31.51	0.22	32.45	0.94
	2	CDCA	0.208	32.37	0.29	34.31	1.94
	3	UDCA	0.22	32.70	0.31	34.69	1.99
	4	DCA	0.20	32.15	0.28	33.97	1.82
	5	CA	0.214	32.55	0.35	35.60	3.05
Glycine conjugates	6	Glyco-LCA	0.45	37.60	0.50	38.74	1.14
	7	Glyco-CDCA	0.51	38.44	0.60	40.51	2.07
	8	Glyco-UDCA	0.54	38.82	0.62	40.86	2.04
	9	Glyco-DCA	0.49	38.09	0.58	40.06	1.97
	10	Glyco-CA	0.51	38.44	0.68	41.71	3.27
Glucoside conjugates	11	LCA 3-Glc.	0.83	42.45	0.83	45.58	3.13
	12	CDCA 3-Glc.	0.97	43.61	1.05	47.59	3.98
	13	UDCA 3-Glc.	0.87	42.78	0.96	46.85	4.07
	14	DCA 3-Glc.	0.98	43.75	1.05	47.59	3.84
	15	CA 3-Glc.	1.05	44.29	1.22	49.40	5.11
Glucuronide conjugates	16	LCA 3-GlcA.	0.87	42.78	0.79	45.08	2.30
	17	CDCA 3-GlcA.	0.98	43.75	1.01	47.26	3.51
	18	UDCA 3-GlcA.	0.91	43.12	0.92	46.40	3.28
	19	DCA 3-GlcA.	1.00	43.89	1.00	47.16	3.27
	20	CA 3-GlcA.	1.10	44.60	1.23	49.01	4.41

RRT was expressed relative to the methyl ester silyl ether derivatives of DCA 3-GlcA. (19). MU was calculated by a polynomial method (see text).

^a Differences in the MU values between Me-DMES and Me-TMS ether derivatives for the same compound.

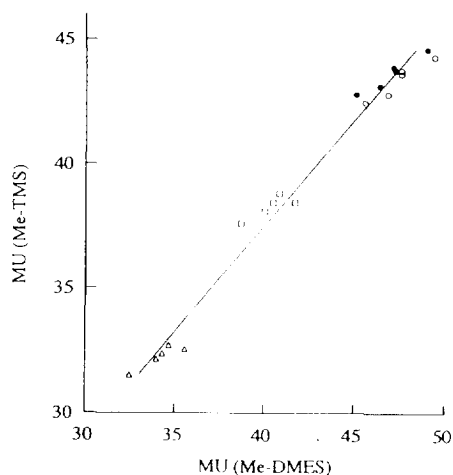


Fig. 3. Correlation in the MU values between the corresponding Me-TMS and Me-DMES ether derivatives. Δ = Unconjugated compounds; \bullet = glucuronide conjugates; \circ = glucoside conjugates; \square = glycine conjugates, $y = 0.837x + 4.05$ ($r = 0.991$, $n = 20$).

estimating the number of hydroxyl groups in the aglycone moiety, but also for differentiating the two variants of sugar moieties.

In Table 2, the differences in the MU values

Table 2

$\Delta[U_m]_{Glc. \text{ GlcA.}}$ values^a observed for 3-glycoside conjugates of bile acids

3-Glycoside conjugate of	Me-TMS	Me-DMES
LCA	-0.33	0.50
CDCA	-0.14	0.33
UDCA	-0.34	0.45
DCA	-0.14	0.43
CA	-0.31	0.39

^a Differences in the MU values between analogous bile acid 3-glycoside and 3-glucuronide for the same derivative.

between analogous bile acid glucosides and glucuronides [e.g., CA 3-Glc. (15) vs. CA 3-GlcA. (20)] for the same derivative are expressed in terms of $\Delta[Um]_{\text{Glc.}-\text{GlcA.}}$ values; a negative value denotes that the retention time of a glucoside conjugate is shorter than that of the corresponding glucuronide. The Me-TMS ether derivatives always show the negative $\Delta[Um]_{\text{Glc.}-\text{GlcA.}}$ values. On the other hand, the reverse relationship (positive $\Delta[Um]_{\text{Glc.}-\text{GlcA.}}$ values) was observed on changing from Me-TMS to Me-DMES ether derivatives. As the two glycosidic pairs differ from each other only in the number of hydroxyl groups present in the sugar moieties (four for glucosides and three for glucuronides), the above significant correlation can be reasonably explained as a result of the retarding effect on the retention times due to the DMES group and is therefore useful for identifying the structure of the sugar moieties.

Fig. 4 illustrates typical chromatograms for the simultaneous analysis of a mixture of fifteen unconjugated and glycine- and glucuronide-conjugated bile acids as their Me-TMS and Me-DMES ethers measured under temperature-programmed GC conditions (see Experimental). As can be seen, this column provided clean separa-

rations of the three groups of the bile acids with short analysis times (for example, 7 min for unconjugated compounds, 15 min for glycine conjugates and 30 min for glucuronide conjugates as their Me-TMS ethers). In particular, the Me-DMES ether derivatives provide an improved resolution not only of each of the three groups of unconjugated and conjugated bile acids but also of the individual isomers in each group. In addition, the general order of elution in each group of unconjugated and conjugated bile acids for the Me-DMES ethers was related to the number of hydroxyl groups in the aglycone moiety: mono-, di-, trihydroxylated.

The detection limits of these compounds were found to be in the range 3–5 ng (by the splitless injection method) with a signal-to-noise ratio of 5:1. These chromatographic responses may be much improved by measuring the GC-selected ion monitoring in GC-MS [22].

Table 3 shows the MU values [18] of bile acid 3-glycoside Me-DMES ethers obtained under two isothermal and temperature-programmed GC conditions and with two calculation methods (approximate linear and polynomial expressions). As a result, the coefficients of variation relative standard deviations (R.S.D.) of the

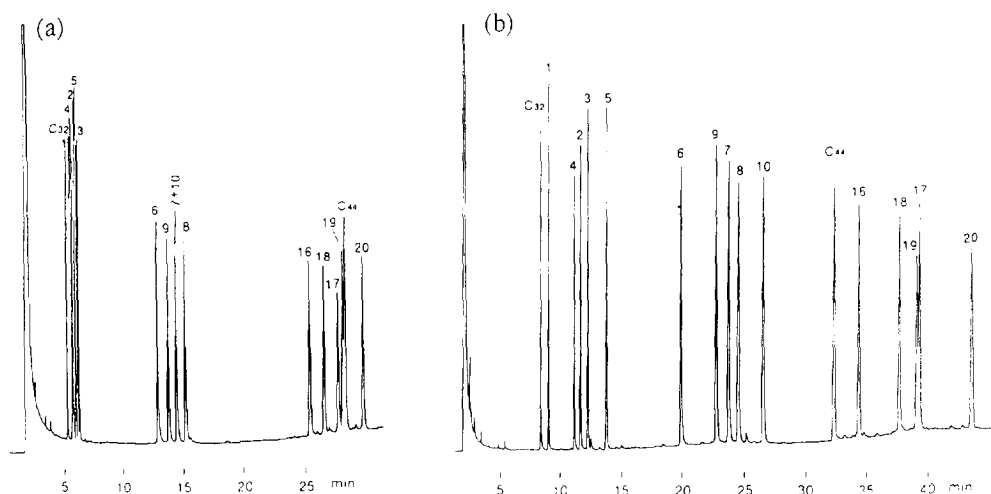


Fig. 4. Capillary GC (temperature programmed) of a mixture of the fifteen unconjugated compounds and glycine and glucuronide conjugates of bile acids as their (a) Me-TMS and (b) Me-DMES ether derivatives. Peaks: 1 = LCA; 2 = CDCA; 3 = UDCA; 4 = DCA; 5 = CA; 6 = glyco-LCA; 7 = glyco-CDCA; 8 = glyco-UDCA; 9 = glyco-DCA; 10 = glyco-CA; 11–20 as in Fig. 2.

Table 3
MU values calculated by approximate linear and cubic expression methods

Compound	Linear expression		$\Delta[U_m]_{\text{Iso.}-\text{Prog.}}$ ^a	Cubic expression		$\Delta[U_m]_{\text{Iso.}-\text{Prog.}}$ ^a
	Isothermal ^b	Programmed ^c		Isothermal ^d	Programmed ^e	
LCA 3-Glc.	45.49	45.62	0.13	45.53	45.58	0.05
CDCA 3-Glc.	47.42	47.32	0.10	47.54	47.59	0.05
UDCA 3-Glc.	46.69	46.70	0.01	46.77	46.85	0.08
DCA 3-Glc.	47.42	47.32	0.10	47.54	47.59	0.05
CA 3-Glc.	49.20	48.78	0.42	49.40	49.40	0.00
LCA 3-GlcA.	45.02	45.18	0.16	45.04	45.08	0.04
CDCA 3-GlcA.	47.11	47.04	0.07	47.21	47.26	0.05
UDCA 3-GlcA.	46.26	46.32	0.06	46.32	46.40	0.08
DCA 3-GlcA.	47.01	46.96	0.05	47.11	47.16	0.05
CA 3-GlcA.	48.82	48.48	0.34	49.00	49.01	0.01

The samples were measured as Me-DMES ether derivatives.

^a Differences in the MU values between isothermal and temperature-programmed GC conditions.

^b $y = -2.66 + 0.76 \times 10^{-1}x$ ($n = 27$, R.S.D. = 0.50%).

^c $y = -2.48 + 0.74 \times 10^{-1}x$ ($n = 27$, R.S.D. = 1.11%).

^d $y = -3.15 + 1.08 \times 10^{-1}x - 0.67 \times 10^{-2}x^2 - 0.04 \times 10^{-4}x^3$ ($n = 27$, R.S.D. = 0.19%).

^e $y = -2.53 + 0.57 \times 10^{-1}x + 1.04 \times 10^{-2}x^2 - 0.14 \times 10^{-4}x^3$ ($n = 27$, R.S.D. = 0.13%).

linear and cubic expressions obtained were 0.50% (isothermal) and 1.11% (programmed) ($n = 27$) and 0.19% (isothermal) and 0.13% (programmed) ($n = 27$), respectively, indicating a higher reliability of the latter expression. It is also evident from Table 3 that in general the differences in the MU values between isothermal and temperature-programmed GC conditions by applying a cubic expression, defined as $\Delta[U_m]_{\text{Iso.}-\text{Prog.}}$ values, are much smaller than those obtained by applying a linear expression. Accordingly, the MU values of comparatively polar and high-boiling compounds such as bile acid glycosides may be calculated by using a polynomial equation.

In conclusion, the 3-glucoside and 3-glucuronide conjugates of bile acids have been successfully separated by GC without need for the prior deconjugation. The combined use of a suitable derivative and a stainless-steel capillary column, Ultra ALLOY-1 (HT), chemically bonded and cross-linked with dimethylsiloxane, provided excellent GC characteristics and separations of isomeric bile acid glycosides as their methyl ester silyl ether derivatives with relatively short analysis times. The retention data reported here provide an insight into the structural elucidation of these biologically important bile acid glycosides, and the method may have the ability to

determine simultaneously unconjugated and glycine-, glucoside- and glucuronide-conjugated bile acids in biological fluids without prior group separation and deconjugation.

Acknowledgements

We are grateful to Dr. C. Watanabe, Frontier Lab., for his helpful advice on the use of the stainless-steel capillary column. Thanks are also due to Tokyo Tanabe for the generous supply of ursodeoxycholic acid 3-glucuronide. This work was supported in part by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science and Culture of Japan.

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