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Capillary gas chromatographic separation of bile acid acyl glycosides without thermal decomposition and isomerization

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Abstract

A direct method for the capillary gas chromatographic (cGC) separation of the acyl glycosides of bile acids was successfully attained. The free acyl glycosides were derivatized to their complete trifluoroacetyl (TFA) derivatives with *N*-methyl-bis(trifluoroacetamide). The highly volatile TFA derivatives were chromatographed on a short-length (10 m), narrow-bore (0.1 mm) capillary column coated with a thin film (0.1 μm) of 5% phenyl polysilphenylene-siloxane at a column temperature below 280 °C. Each exhibited a single, well-separated peak of the theoretical shape without any accompanying peaks due to the thermal decomposition and isomerization. The bile acid 24α-glucosides were always eluted faster than the corresponding 24β-glucosides, which eluted before the corresponding 24β-galactosides. The method could be usefully applied to biosynthetic and metabolic studies of bile acid acyl glycosides in biological materials.

Keywords: Capillary gas chromatography; Narrow-bore short capillary column; Glycosidic conjugates of bile acids; Bile acid acyl glycosides; TFA derivatives

1. Introduction

Abnormalities in cholesterol biosynthesis or metabolism often reflect in the proportions, concentrations and conjugation forms of bile acids in various human tissues, and, therefore, the qualitative and quantitative determinations of bile acids are important in the diagnosis of hepatobiliary diseases. Naturally occurring glycosidic conjugates of bile acids usually imply hydroxy-linked glycosides, which are bonded by *O*-glycosidic linkage between the hydroxy group in the 5β-steroid moiety and the C-1′ anomeric hydroxy group in β-D-pyranoses. Several groups of workers have recently reported the occurrence of a new type of bile acid glycosides in biological materials of human and rat [1–4]. The novel conjugates, termed acyl, ester or carboxyl-linked glycosides of bile acids, are formed by esterification between the C-24 carboxyl group in bile acids and the C-1′ anomeric hydroxy group in pyranoses.

In a previous paper, we have reported a direct capillary gas chromatographic (cGC) analysis of the bile acid acyl glycosides without the need for a deconjugation step [5]. The method involved the pre-column trimethylsilylation (TMS) of the acyl glycosides, followed by the cGC determination on a short capillary column (7 m) chemically coated with non-polar stationary phase, measuring under isothermal conditions at 300 °C. Although the procedure provided well-resolved peaks of each glycosidic conjugate with theoretical peak shape, it partially caused the thermal decomposition and isomerization during the cGC determination. We herein report an improved method for the direct cGC determination and separation of a series of bile acid acyl glycosides.

Abbreviations: cGC, capillary gas chromatography; MBTFA, *N*-methyl-bis(trifluoroacetamide); GC–EI-MS, gas chromatography–electron ionization mass spectrometer

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2. Experimental

2.1. Materials and reagents

All the α- and β-anomers of the ester glucopyranosides and galactopyranosides of bile acids examined in this study were from our laboratory collection, including new and natural glycosidic conjugates recently synthesized in these laboratories [6]. The acylating agent, *N*-methyl-bis(trifluoroacetamide) (MBTFA), was obtained from Tokyo Kasei Kogyo (Tokyo, Japan). A mixture of C_{20}–C_{38} *n*-alkanes and C_{44} were obtained from GL Science (Tokyo, Japan). All other reagents and solvents used were of analytical reagent grade.

2.2. Derivatization of samples

To ca. 0.5 mg of conjugated bile acid sample in a vial flask 250 μl of dry pyridine and then 250 μl of MBTFA were added successively. The vial was capped and heated at 40 °C for 60 min with occasional shaking. An aliquot of the resulting trifluoroacetylated solution (0.1–0.2 μl) was injected directly into the gas chromatograph with C_{44} as an internal standard. The resulting TFA derivative was stable for at least a week in a refrigerator.

2.3. Capillary gas chromatography

Capillary Gas chromatography was performed with a Shimadzu (Kyoto, Japan) model GC-2010 gas chromatograph equipped with a flame ionization detector and a Chromatopak C-R7A plus data-processing system. It was fitted with a BPX5 fused silica capillary column (10 m × 0.1 mm i.d.) (SGE Japan, Yokohama, Japan) coated with a thin film (0.1 μm) of 5% phenyl polysilphenylene-siloxane and operated under the following conditions: carrier gas (helium) flow-rate, 36 ml/min; average gas velocity, 40 cm/min, 0.38 ml/min; purge gas flow rate, 3 ml/min; make-up gas flow rate, 60 ml/min; split ratio, 85:1; injector temperature, 280 °C; detector temperature, 280 °C; column oven temperature, either isothermal, 265 °C or programmed, raised from 240 to 255 °C at 1 °C/min and then raised to 280 °C at 3 °C/min.

2.4. Gas chromatography–mass spectrometry

Gas chromatography combined with electron ionization mass spectrometer (GC–EI-MS) was performed with a Jeol (Tokyo, Japan) model JMC-Automass 150 quadrupole mass spectrometer interfaced with a Hewlett-Packard model 5890 series III gas chromatograph. A BPX5 capillary column was inserted into the ion source and operated under the following conditions: ionization energy, 70 eV; emission current, 300 μA; mass range, 4–1000 u; injection port, column oven and ion source were kept at 270, 270 and 180 °C, respectively.

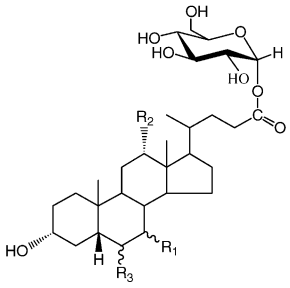
3. Results and discussion

Fig. 1 shows the structures of 1′-*O*-α and 1′-*O*-β acyl glycosides of bile acids (**1**–**17**) examined in this study. Chemical properties of acyl glycosides such as facile hydrolysis, intramolecular rearrangement or transesterification [3,7–11] and thermal decomposition or isomerization [5] have been reported by many groups of workers.

The chemical instabilities and/or activities of bile acid acyl glycosides may hamper their direct cGC, GC–EI-MS, and high-performance liquid chromatographic analyses without prior deconjugation of the *O*-glycosidic linkage. However, the deconjugation has some drawbacks, since information about the type, site and amount of conjugation is lost. Also incomplete hydrolysis may lead to artefact formation. Alternatively, the avoidance of the deconjugation provides direct information about the glycosidic conjugated form by cGC. Therefore, the development of a reliable method for the direct cGC analysis without need for prior deconjugation is urgently required.

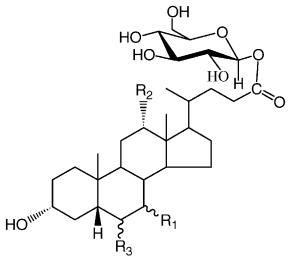
The cGC thermal decomposition of bile acid acyl glycosides is significant at column temperature in excess of 280 °C [5]. A combination of a pre-column derivatization yielding more volatile analytes and a capillary column with short analysis time as well as higher resolution would appear an appropriate strategy. Exploratory experiments revealed that the pre-column derivatizations such as a trialkylsilylation (e.g., TMS ether) and acetylation, which are commonly used for bile acid analysis [11–14], are inadequate because of insufficient volatility of these derivatives at column temperature below 280 °C. The problem may be solved by perfluoroacetylation of the substrates [15,16]. Of the various perfluoroacylating reagents examined (e.g., pentafluoropropionyl imidazole and heptafluorobutyryl imidazole), MBTFA was found to be the reagent of choice [16–19]. With MBTFA, all the compounds **1**–**17** were completely derivatized under mild conditions and neither excess MBTFA nor by-products of the derivatization interfered chromatographically with the peaks of the desired products under the cGC conditions employed. The TFA derivatives of the various bile acid acyl glycosides show significantly higher volatility and better chromatographic resolution than the corresponding TMS derivatives.

In cGC analysis of unconjugated bile acids, the commonly employed capillary column is a conventional-bore open tubular column with a length of 25–50 m and an internal diameter of 0.25–0.32 mm; the inner wall is bonded with non-polar or less-polar liquid phase with the film thickness of 0.25–0.35 μm [11–14]. Attempt to measure the TFA derivatives by using the type of a conventional middle-bore capillary column was unsuitable and resulted in the formation and accumulation of unidentified peaks in the chromatograms measured at the column temperature of above 280 °C. The use of a short-length (10 m), wide-bore (i.d., 0.53 mm) capillary column was also unsatisfactory.



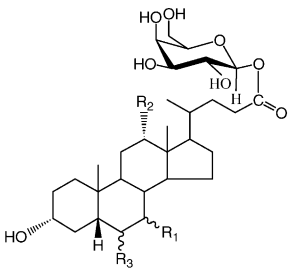
α-anomer

	R_1	R_2	R_3	Name	Abbreviation
1	H	H	H	1-*O*-(24-lithocholyl)-α-D-glucopyranose	LC-24α-Glc
2	α-OH	H	H	1-*O*-(24-chenodeoxycholyl)-α-D-glucopyranose	CDC-24α-Glc
3	β-OH	H	H	1-*O*-(24-ursodeoxycholyl)-α-D-glucopyranose	UDC-24α-Glc
4	H	OH	H	1-*O*-(24-deoxycholyl)-α-D-glucopyranose	DC-24α-Glc
5	H	H	α-OH	1-*O*-(24-hyodeoxycholyl)-α-D-glucopyranose	HDC-24α-Glc
6	α-OH	OH	H	1-*O*-(24-cholyl)-α-D-glucopyranose	C-24α-Glc



β-anomer

	R_1	R_2	R_3	Name	Abbreviation
7	H	H	H	1-*O*-(24-lithocholyl)-β-D-glucopyranose	LC-24β-Glc
8	α-OH	H	H	1-*O*-(24-chenodeoxycholyl)-β-D-glucopyranose	CDC-24β-Glc
9	β-OH	H	H	1-*O*-(24-ursodeoxycholyl)-β-D-glucopyranose	UDC-24β-Glc
10	H	OH	H	1-*O*-(24-deoxycholyl)-β-D-glucopyranose	DC-24β-Glc
11	H	H	α-OH	1-*O*-(24-hyodeoxycholyl)-β-D-glucopyranose	HDC-24β-Glc
12	α-OH	OH	H	1-*O*-(24-cholyl)-β-D-glucopyranose	C-24β-Glc



β-anomer

	R_1	R_2	R_3	Name	Abbreviation
13	H	H	H	1-*O*-(24-lithocholyl)-β-D-galactopyranose	LC-24β-Gal
14	α-OH	H	H	1-*O*-(24-chenodeoxycholyl)-β-D-galactopyranose	CDC-24β-Gal
15	β-OH	H	H	1-*O*-(24-ursodeoxycholyl)-β-D-galactopyranose	UDC-24β-Gal
16	H	OH	H	1-*O*-(24-deoxycholyl)-β-D-galactopyranose	DC-24β-Gal
17	α-OH	OH	H	1-*O*-(24-cholyl)-β-D-galactopyranose	C-24β-Gal

Fig. 1. Structures of the acyl glycosides of bile acids examined.

However, the difficulty was overcome by using a short-length (10 m), narrow-bore (i.d., 0.1 mm) BPX5 capillary column coated with thin film (thickness, 0.1 μm) of non-polar 5% phenyl polysilphenylene-siloxane. Using column temperature between 250 and 270 °C, each TFA derivative afforded a single symmetrical peak without artefact peaks due to the thermal decomposition and/or isomerization during the cGC determination. The TFA derivatives, therefore, proved to be well suited for use with the BPX5 capillary column, shortening the analysis time and lowering the column oven temperature. When the TFA derivatives were measured under isothermal cGC conditions at 260 °C, peak overlapping of some isomeric pairs was observed. However, the temperature programming from 240 to 280 °C resulted in improved resolution (see Section 2 for more details).

Table 1 shows the retention data for compounds **1**–**17**, which were expressed as the retention indices (*I*). The *I* values were calculated by the cubic expression of approximate polynominal expression method using C_{20}–C_{38} *n*-alkanes [20]. As expected, the *I* (ca. 32.6–34.3) for the TFA derivatives were much smaller than those reported for the corresponding TMS derivatives (*I*, ca. 43.3–44.8) [5]. The $\Delta[I]_{\beta-\alpha}$ values, which are defined as the difference in *I* values between the α-anomers (**1**–**6**) and the corresponding β-anomers (**7**–**12**), are also listed in the table. Similarly, the $\Delta[I]_{Glc-Gal}$ values are the differences in *I* values between the bile acid acyl glucosides (**7**–**12**) and the corresponding galactosides (**13**–**17**).

Fig. 2 shows the typical chromatograms of mixtures of the bile acid acyl glucosides as their TFA derivatives measured on a BPX5 column under temperature-programmed (240–280 °C) cGC conditions. In the chromatograms, a

Table 1
I values of the TFA derivatives of bile acid acyl glycosides

No.	Abbreviation	I[a]	$\Delta[I]_{\beta-\alpha}$[b]	$\Delta[I]_{Glc-Gal}$[c]
1	LC-24α-Glc	33.48	0.56	
2	CDC-24α-Glc	33.23	0.58	
3	UDC-24α-Glc	33.41	0.50	
4	DC-24α-Glc	32.55	0.31	
5	HDC-24α-Glc	33.23	0.58	
6	C-24α-Glc	32.77	0.21	
7	LC-24β-Glc	34.04		
8	CDC-24β-Glc	33.81		
9	UDC-24β-Glc	33.91		
10	DC-24β-Glc	32.86		
11	HDC-24β-Glc	33.81		
12	C-24β-Glc	32.98		
13	LC-24β-Gal	34.29		−0.25
14	CDC-24β-Gal	33.98		−0.17
15	UDC-24β-Gal	34.20		−0.29
16	DC-24β-Gal	33.19		−0.33
17	C-24β-Gal	33.28		−0.30

[a] *I* values were calculated by a polynomial expression method [20].
[b] Differences in the *I* values between corresponding 24β- and 24α-anomeric glucosides.
[c] Differences in the *I* values between corresponding 24β-glucosides and 24β-galactosides.

slight fronting was observed for the peaks arising from the compounds having a 7-hydrpxy group, probably suggesting that they are more unstable than compounds having other OH positions: the problem could possibly be solved by lowering the column temperature and switching carrier gas to hydrogen with its higher linear velocity. A mixture of the five 24α-glucosides was well separated on the column, emerging in the order of DC-24α-Glc (**4**), C-24α-Glc (**6**), CDC-24α-Glc (**2**), UDC-24α-Glc (**3**) and LC-24α-Glc (**1**). Analogously, mixtures of the acyl 24β-glucosides and 24β-galactosides also exhibited clean separation and identical elution order: DC-24β-Glc (**10**) < C-24β-Glc (**12**) < CDC-24β-Glc (**8**) < UDC-24β-Glc (**9**) < LC-24β-Glc (**7**); DC-24β-Gal (**16**) < C-24β-Gal (**17**) < CDC-24β-Gal (**14**) < UDC-24β-Gal (**15**) < LC-24β-Gal (**13**).

The positive $\Delta[I]_{\beta-\alpha}$ values show that the α-anomers (**1**–**6**) of the bile acid acyl glucosides are always eluted faster than the corresponding β-ones (**7**–**12**) (Fig. 3). The elution order for the TFA derivatives of the anomeric pairs is in accord with that reported for respective monosaccharides [18]. On the other hand, the negative $\Delta[I]_{Glc-Gal}$ values imply that the acyl 24β-galactosides (**13**–**17**) are always eluted more slowly than the corresponding 24β-glucosides (**7**–**12**).

Above the elution orders observed for the TFA derivatives of bile acid acyl glycosides much differed from those reported for the corresponding TMS derivatives [5], probably suggesting that the low retention in the TFA derivatives has to do with the poor interaction (e.g., solubility) between the fluorine atoms and the stationary phase. Thus, the retention times of the TMS derivatives in both the acyl 24α- and 24β-glucosides increased in the order, LC-, DC-, UDC- = CDC- and C-, essentially according to the number of hydroxy groups present in the aglycone moieties; in addition, the TMS derivatives of the β-anomers always eluted before the corresponding α-ones.

To confirm the structures of bile acid acyl glycosides responsible for cGC peaks GC–EI-MS spectra were acquired for some representative compounds as shown in Table 2. Major fragment ions and fragmentation patterns for the TFA

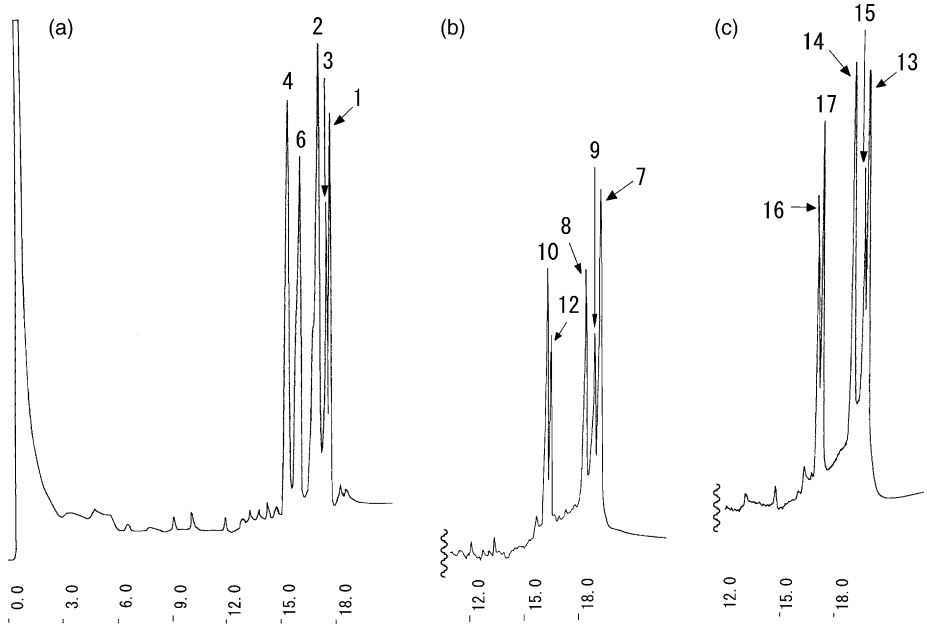


Fig. 2. Typical chromatograms of the mixtures of the (a) 24α-glucosides, (b) 24β-glucosides and (c) 24β-galactosides of bile acids; column temperature, 240–280 °C (see Section 2).

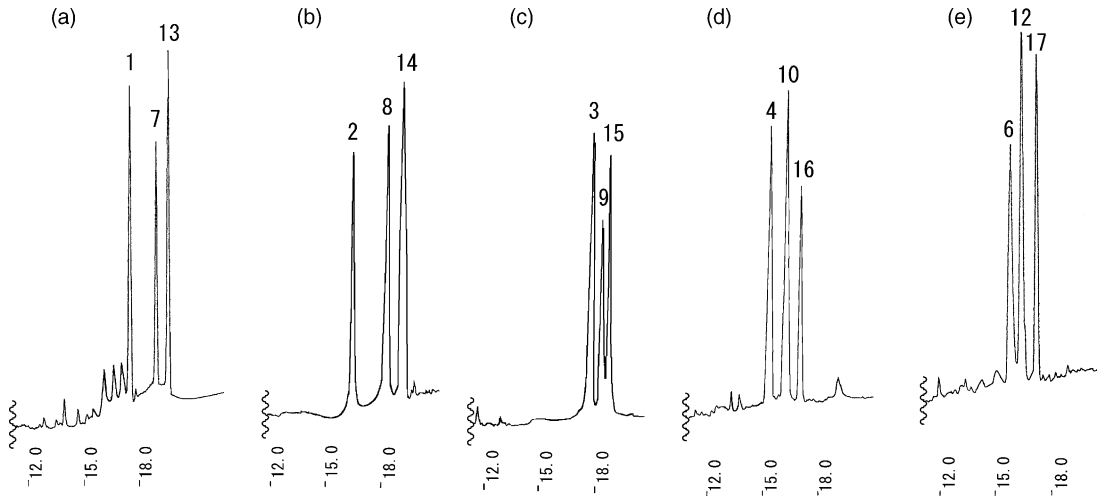


Fig. 3. cGC separation of the anomeric and isomeric mixtures of (a) LC-, (b) CDC-, (c) UDC-, (d) DC-, and (e) C-24-glycosides; column temperature, 240–280 °C (see Section 2).

Table 2
GC–EI-MS data for the TFA derivatives of bile acid acyl glycosides

No.	Major fragment ions above *m/z* 200 (relative intensity, %)[a]
1	471 (M-Glc, 1%), 433 (Glc-TFA, 1), 371 (M-Glc-S.C., 12), 357 (M-Glc-TFA, 14), 319 (Glc-2TFA, 3), 281 (64), 227 (M-Glc-TFA-2CH_3-S.C., 44), 205 (Glc-3TFA, 100)
4	469 (M-Glc-TFA, 1%), 433 (Glc-TFA, 3), 369 (M-Glc-TFA-S.C., 13), 355 (M-Glc-2TFA, 18), 319 (Glc-2TFA, 32), 281 (51), 255 (M-Glc-2TFA-S.C., 3), 225 (M-Glc-2TFA-2CH_3-S.C., 73), 205 (Glc-3TFA, 52)
6	565 (M-Glc-2CH_3-S.C., 1%), 467 (M-Glc-2TFA, 1), 433 (Glc-TFA, 4), 367 (M-Glc-2TFA-S.C., 53), 319 (Glc-2TFA, 14), 280 (44), 253 (M-Glc-3TFA-S.C., 78), 226 (M-Glc-3TFA-S.C.-part of ring D, 43), 223 (M-Glc-3TFA-2CH_3-S.C., 64), 205 (Glc-3TFA, 46)
7	471 (M-Glc, 1%), 433 (Glc-TFA, 1), 371 (M-Glc-S.C., 10), 357 (M-Glc-TFA, 17), 319 (Glc-2TFA, 3), 281 (64), 227 (M-Glc-TFA-2CH_3-S.C., 79), 205 (Glc-3TFA,54)
10	469 (M-Glc-TFA, 9%), 454 (M-Glc-TFA-CH_3, 11), 433 (Glc-TFA, 3), 369 (M-Glc-TFA-S.C., 93), 319 (Glc-2TFA, 32), 255 (M-Glc-2TFA-S.C., 18), 214 (36), 205 (Glc-3TFA, 14)
12	565 (M-Glc-2CH_3-S.C., 14%), 467 (M-Glc-2TFA, 18), 433 (Glc-TFA, 4), 367 (M-Glc-2TFA-S.C., 100), 319 (Glc-2TFA, 59), 253 (M-Glc-3TFA-S.C., 78), 226 (M-Glc-3TFA-S.C.-part of ring D, 47), 223 (M-Glc-3TFA-2CH_3-S.C., 14), 205 (Glc-3TFA, 15)
13	471 (M-Gal, 1%), 433 (Gal-TFA, 2), 371 (M-Gal-S.C., 10), 357 (M-Gal-TFA, 18), 319 (Gal-2TFA, 11), 281 (26), 227 (M-Gal-TFA-2CH_3-S.C., 56), 205 (Gal-3TFA,37)
16	469 (M-Gal-TFA, 6%), 454 (M-Gal-TFA-CH_3, 7), 433 (Gal-TFA, 4), 369 (M-Gal-TFA-S.C., 65), 319 (Gal-2TFA, 41), 281 (21), 255 (M-Gal-2TFA-S.C., 21), 205 (Gal-3TFA, 22)
17	565 (M-Gal-2CH_3-S.C., 21%), 467 (M-Gal-2TFA, 12), 433 (Gal-TFA, 8), 367 (M-Gal-2TFA-S.C., 60), 319 (Gal-2TFA, 81), 281 (30), 226 (M-Gal-3TFA-S.C.-part of ring D, 44), 223 (M-Gal-3TFA-2CH_3-S.C., 43), 205 (Gal-3TFA, 47)

[a] M, molecular ion; TFA, trifluoroacetic acid ($C_2HO_2F_3$, 4 u); S.C., side chain ($C_5H_8O_2$, 100 u); ring D, 42 u; part of ring D, 27 u; Glc and Gal, 4TFA derivative, 547 u.

derivatives of corresponding acyl 24α-glucosides (**1**, **4** and **6**), 24β-glucosides (**7**, **10** and **12**) and 24β-galactosides (**13**, **16** and **17**) were similar to one another.

In conclusion, bile acid acyl glycosides have been successfully analyzed by direct cGC without any accompanying by-product peaks due to the thermal decomposition and/or isomerization. The combined use of the TFA derivatization of the substrates with MBTFA and a narrow-bore, short capillary column chemically coated with thin-film of non-polar liquid phase was essential for their direct cGC determination. The method, therefore, may be helpful for studies on the metabolism and disposition of the acyl glycosides of bile acids in human biological fluids, and the details will be reported elsewhere in the near future.

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