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Simultaneous analysis and retention behavior of the glucuronide, glucoside, and N-acetylglucosaminide conjugates of bile acids in conventional and inclusion high-performance liquid chromatographic methods

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Abstract

Simultaneous analysis and structure–retention correlation were examined for various glycosidic-conjugated bile acids in reversed-phase high-performance liquid chromatography (HPLC). The substrates examined included the β-glucuronide, β-glucoside, and β-N-acetylglucosaminide conjugates of C_{24} bile acids related to lithocholic, hyodeoxycholic, chenodeoxycholic, ursodeoxycholic, deoxycholic, hyocholic, and cholic acids having an O-β-D-glycosidic linkage at the position C-3, C-6, or C-7 in the 5β-steroid nucleus. The bile acids as their fluorescence-sensitive C_{24} pyrenacyl ester derivatives were measured on a C_{18} reversed-phase column, eluting with methanol–water mixtures in the absence and presence of methyl-β-cyclodextrin (Me-β-CD). Changes in the retentions of these compounds depended significantly not only on the Me-β-CD concentration but rather on the methanol content, implying the possible formation of solute–Me-β-CD–methanol ternary complexes by hydrogen-bonding interactions. The combined use of conventional and inclusion HPLC retention data reported herewith provided important information for characterizing the structures of bile acid glycosides as well as inclusion complexes.

Keywords: Inclusion complexes; Complexation; Mobile phase composition; Bile acids; Glucuronides; Glucosides; Acetylglucosamides; Glycosidic bile acids

1. Introduction

In recent years, considerable interest has been directed to the site of origin, pathways of biosynthesis and metabolism, physiological significance, and excretory routes of the glycosidic conjugates of bile acids in humans in connection with hepatobiliary diseases. At present, the three variants of glycosidic conjugation are known in bile acid metabolism in humans: those include glucuronidation [1–3], glucosidation [4–6], and N-acetylglucosaminidation [6–8]. Hence, a number of the O-β-D-glucuronide-(GlcA), O-β-D-glucoside- (Glc), and O-β-D-N-acetylglucosaminide-(GlcNAc)-conjugated bile acids have recently been isolated and identified as novel glycosidic conjugates in biological materials such as

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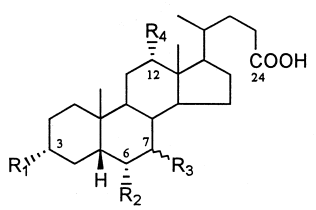
urine, serum, plasma or bile of normal humans and patients with liver diseases. The concurrent occurrence of these glycosylated bile acids is also of keen current interest in biological and metabolic studies in connection with liver diseases.

High-performance liquid chromatography (HPLC) with a C_{18} reversed-phase column is the most powerful and reliable method for the profile analysis of such conjugated bile acids, which are comparatively polar, less volatile, and thermolabile compounds. In addition, direct HPLC analysis of individual glycosidic-conjugated bile acids without the need for prior group fractionation and deconjugation may provide important information concerning the type and site of glycosidic conjugations. Therefore, the reversed-phase HPLC separation and behavior have been reported separately for each homologous series of the 3-GlcA [9,10], 3-Glc [11,12], and 3-GlcNAc [13,14] conjugates of five prominent bile acids, lithocholic (LCA), chenodeoxycholic (CDCA), ursodeoxycholic (UDCA), deoxycholic (DCA), and cholic (CA) acids. However, simultaneous HPLC analysis and comparative retention behavior of the three groups of the glycosidic conjugates, which co-exist in the same biological material, has not yet been examined.

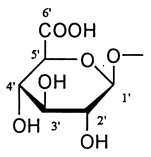
On the other hand, the recent successful use of α-, β- or γ-cyclodextrin (CD) and its derivatives as a mobile phase additive in inclusion HPLC [15–17] for the separation and characterization of some common bile acids prompted us to apply this technique with a CD in the analysis of bile acid glycosides.

In this paper, we examined the simultaneous analysis and the structure–retention correlation of various glycosidic-conjugated bile acids (**8**–**28**) (Fig. 1) in conventional and inclusion HPLCs and compared the behaviors with those of the corresponding unconjugated ones (**1**–**7**). The bile acid glycosides examined are classified into the GlcA (**8**–**15**), Glc (**16**–**23**), and GlcNAc (**24**–**28**) conjugates of LCA, CDCA, UDCA, DCA, CA, hyodeoxycholic acid (HDCA), and hyocholic acid (HCA) having an O-β-D-glycosidic linkage at a different position of C-3, C-6 or C-7 in the 5β-steroid nucleus. These compounds were analyzed as fluorescence (FL)-sensitive C-24 pyrenacyl ester derivatives on a C_{18} reversed-phase column, eluting with methanol–water mixtures



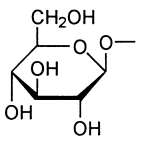
Unconjugates

	R_1	R_2	R_3	R_4
1: LCA	OH	H	H	H
2: HDCA	OH	OH	H	H
3: CDCA	OH	H	α-OH	H
4: UDCA	OH	H	β-OH	H
5: DCA	OH	H	H	OH
6: HCA	OH	OH	α-OH	H
7: CA	OH	H	α-OH	OH



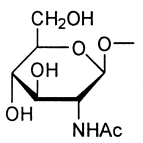
Glucuronide (GlcA)

	R_1	R_2	R_3	R_4
8: LCA 3-GlcA	GlcA	H	H	H
9: HDCA 3-GlcA	GlcA	OH	H	H
10: HDCA 6-GlcA	OH	GlcA	H	H
11: CDCA 3-GlcA	GlcA	H	α-OH	H
12: UDCA 3-GlcA	GlcA	H	β-OH	H
13: DCA 3-GlcA	GlcA	H	H	OH
14: HCA 3-GlcA	GlcA	OH	α-OH	H
15: CA 3-GlcA	GlcA	H	α-OH	OH



Glucoside (Glc)

	R_1	R_2	R_3	R_4
16: LCA 3-Glc	Glc	H	H	H
17: HDCA 3-Glc	Glc	OH	H	H
18: HDCA 6-Glc	OH	Glc	H	H
19: CDCA 3-Glc	Glc	H	α-OH	H
20: UDCA 3-Glc	Glc	H	β-OH	H
21: DCA 3-Glc	Glc	H	H	OH
22: HCA 3-Glc	Glc	OH	α-OH	H
23: CA 3-Glc	Glc	H	α-OH	OH



N-Acetylglucosaminide (GlcNAc)

	R_1	R_2	R_3	R_4
24: HDCA 3-GlcNAc	GlcNAc	OH	H	H
25: HDCA 6-GlcNAc	OH	GlcNAc	H	H
26: UDCA 3-GlcNAc	GlcNAc	H	β-OH	H
27: UDCA 7-GlcNAc	OH	H	GlcNAc	H
28: HCA 3-GlcNAc	GlcNAc	OH	α-OH	H

Fig. 1. Structures of nonglycosylated and glycosylated bile acids examined.

in the absence and presence of methyl β-cyclodextrin (Me-β-CD) as a mobile phase additive.

2. Experimental

2.1. Materials and reagents

Almost all of the 3- and 6-GlcA (**8**–**15**), 3- and 6-Glc (**16**–**23**), and 3-, 6- and 7-GlcNAc (**24**–**28**) conjugates of bile acids used in this study were prepared in these laboratories [18–20]. These compounds were of satisfactory purity according to thin-layer chromatographic (TLC) and HPLC analyses. The FL-prelabeling reagent, 1-bromoacetylpyrene, was obtained from Wako (Tokyo, Japan). Me-β-CD [heptakis-(2,6-di-D-methyl)-β-cyclodextrin] was available from Funakoshi (Tokyo, Japan). Prepacked silica cartridges, Sep-Pak Light (sorbent mass, 120 mg), for solid-phase extraction were purchased from Waters Assoc. (Milford, MA, USA). Normal-phase TLC plates precoated with silica gel 60 F_{254} and reversed-phase TLC plates precoated with C_{18}-bonded silica gel RP-18 F_{254S}, each of 0.2 mm layer thickness, 7 μm particle size and 10×10 cm, were obtained from Merck (Darmstadt, Germany). For the separation of the free bile acid glycosides, the following developing solvents were used: normal-phase TLC, chloroform–methanol–acetic acid (24:6:3, v/v/v); reversed-phase TLC, methanol–water (80:20, v/v).

2.2. High-performance liquid chromatography

The apparatus used in this work consisted of Hitachi (Tokyo, Japan) L-6200 and L-6000 pumps equipped with an L-7610 degasser and an L-1050 FL spectrophotometer. A Capcell Pak C_{18} UG 120 column (250×4.6 mm I.D.; particle size, 5 μm) (Shiseido; Tokyo, Japan) was used at a temperature of 35°C. The effluents from the column were monitored with the fluorophotometer by using an excitation wavelength of 370 nm and an emission wavelength of 440 nm.

Methanol–water mixtures without and with Me-β-CD (3 and 5 m*M*) as a mobile phase additive were used as the eluents in conventional and inclusion HPLC, respectively, at a flow-rate of 1.0 ml/min. In order to elevate the reproducibility of the retention data, a gradient elution system was utilized to adjust the desired solvent composition and Me-β-CD concentration under an isocratic or gradient-programmed condition. The methanol content of 90, 84 or 80% (v/v) aqueous eluent was employed under an isocratic condition. The gradient-programmed condition used was as follows: 80% aqueous methanol for 30 min by an isocratic condition and then gradually raised to 100% methanol for an additional 50 min by a linear gradient-programmed condition.

2.3. Derivatization of free bile acids to the C-24 pyrenacyl esters

Each of the free nonglycosylated and glycosylated bile acid sample (ca. 200 μg) was converted into the 24-pyrenacyl ester derivatives by a procedure reported by Kamada et al. [21], using 1-bromoacetylpyrene as a FL-prelabeling reagent. After the reaction, the derivatized product was diluted with benzene (ca. 5 ml) and passed through a preconditioned Sep-Pak silica cartridge, in order to remove the excess prelabeling reagent and by-products, which often interfere with early-emerging bile acid peaks. The cartridge was washed successively with benzene (5 ml) and benzene-ethyl acetate (4:1, v/v; 5 ml), and the desired bile acid pyrenacyl ester was eluted with methanol–acetonitrile (1:9, v/v; 10 ml). An aliquot of the sample solution was injected into the HPLC system.

3. Results and discussion

3.1. Preliminary study

Twenty-one bile acid glycosides (**8**–**28**) possessing a glucuronic acid, glucose or N-acetylglucosamine moiety at the position C-3, C-6 or C-7 in the 5β-steroid nucleus were used to study the HPLC separation and retention behavior. For the purpose of comparison, the respective parent unconjugates (**1**–**7**) were also examined under identical HPLC conditions. The four groups of the unconjugated and conjugated bile acids were analyzed as their C-24 pyrenacyl ester derivatives, because of their easy preparation, high FL sensitivity, and

excellent properties in both conventional [21] and inclusion HPLC [17]. All of these bile acids were quantitatively converted into the corresponding pyrenacyl esters under mild conditions according to a procedure described in the literature [21].

A preliminary work on the TLC mobility of nonderivatized bile acid glycosides having an identical aglycone moiety on normal and C_{18} reversed-phase plates showed that the polarity of GlcA conjugates is much stronger than the corresponding Glc and GlcNAc analogs. However, when the GlcA conjugates were subjected to the reversed-phase HPLC on a Capcell Pak C_{18} column after esterification with 1-bromoacetylpyrene, the derivatized GlcA conjugates were eluted much more slowly than anticipated for the C-24 monoesters indicating a significant decrease of the polarities. The anomalous behavior is well explained as follows: in the nonderivatized GlcA conjugates, the pyrenacyl esterification of carboxyl groups occurs not only at C-24 in the aglycone moiety, but also at C-6′ in the sugar moiety, thus forming the corresponding dipyrenacyl ester derivatives (see below).

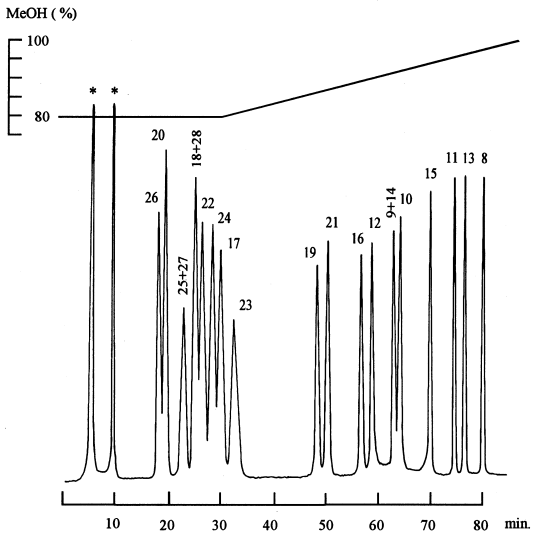


Fig. 2. Simultaneous HPLC separation of a mixture of GlcA-, Glc- and GlcNAc-conjugated bile acids as their pyrenacyl ester derivatives. Conditions: column, Capcell Pak C_{18}; detection, FL; mobile phase, methanol–water (80:20, v/v) for 30 min under an isocratic condition and then gradually 100% methanol for 55 min under linear gradient conditions. Peak numbers refer to Fig. 1; *= reagent and/or by-products.

3.2. Conventional HPLC behavior of glycosidic-conjugated bile acids

Based on the above preliminary work on TLC, our initial effort was directed to the optimization of conditions for simultaneous HPLC analysis of the three variants of GlcA-, Glc- and GlcNAc-conjugated bile acids as their pyrenacyl ester derivatives, which have a wide range of polarities according to the number, position, and configuration of hydroxyl groups attached to the aglycone and sugar moieties. All the attempts of simultaneous analysis of these compounds under isocratic HPLC conditions using various solvent systems and columns afforded unsatisfactory results. However, the combined use of isocratic and linear gradient-programmed conditions as described in the Section 2 proved to be well-suited for the simultaneous separation of the compounds.

Fig. 2 illustrates a clean separation of a mixture of the pyrenacyl ester derivatives of the 21 GlcA, Glc and GlcNAc conjugates (**8**–**28**) measured on a Capcell Pak C_{18} column, eluting with 80% aqueous methanol to 100% methanol. As can be seen, the three variants of analogous bile acid 3-glycosides having the same aglycone moiety were generally eluted in the order of GlcNAc, Glc, and GlcA conjugates: e.g., UDCA 3-GlcNAc (**26**), UDCA 3-Glc (**20**), and UDCA 3-GlcA (**12**). In addition, the mobility of individual bile acid glycosides within each homologous series possessing the same sugar moiety was essentially influenced only by the structure of aglycone moieties in the substrates, emerging in the order of the 3-glycosides of UDCA, HCA, HDCA, CA, CDCA, DCA, and then LCA. This elution order of 3-glycosylated bile acids is completely compatible with that observed for the corresponding nonglycosylated ones (**1**–**7**). The result strongly suggests that the mobility of glycosylated bile acids in conventional HPLC depends exclusively on the difference in their affinities with the C_{18} stationary phase, in analogy with the corresponding unconjugates [22].

A comparison of the mobility of 3-glycosides with that of the corresponding 6- (or 7-) analogs also revealed that the elution order of each positional isomer depends on the structure of sugar moieties. For example, HDCA 6-Glc (**18**) is eluted faster than

the 3-isomer (**17**), while the reverse relationship is observed for HDCA 3- and 6-GlcA pairs (**9** vs. **10**). Furthermore, biologically important UDCA 7-GlcNAc (**27**) [8] moves more slowly than the corresponding 3-isomer (**26**). Under the HPLC conditions used, only three pairs, HDCA 6-GlcNAc (**25**) vs. UDCA 7-GlcNAc (**27**), HDCA 6-Glc (**18**) vs. HCA 3-GlcNAc (**28**), and HDCA 3-GlcA (**9**) vs. HCA 3-GlcA (**14**), overlapped each other.

In order to clarify quantitatively the effects of sugar moieties on retentions, the pyrenacyl ester derivatives of the nonglycosylated and glycosylated bile acids (**1–28**) examined were grouped into the two classes of compounds depending upon their HPLC mobilities, and each class of compounds was measured under isocratic HPLC conditions, eluting with methanol–water mixtures (80:20 and 90:10, v/v) without and with Me-β-CD (3 and 5 m*M*) as a mobile phase additive. Unconjugated HDCA (**2**) and its three 3-glycosidic conjugates (**9**, **17**, and **24**) were also measured by eluting 84% aqueous methanol without and with Me-β-CD. The conventional and inclusion HPLC data for each compound are compiled in Table 1. The capacity factors obtained from the conventional and inclusion HPLCs were expressed as k_1' and k_2' values, respectively. The rk_1' (or rk_2') values are defined as the ratios of the k_1' (or k_2') values of glycosidic conjugates relative to those of the corresponding unconjugates, representing a measure of the contribution on retentions due to the introduction of a sugar moiety into the respective parent 5β-cholanoates.

As expected in the conventional HPLC, the k_1' values for HDCA (**2**) and its 3-glycosides (**9**, **17**, and **24**) decreased dramatically by an increasing concentration of methanol as an organic mobile phase modifier [22]. However, changes in the eluent composition scarcely affected the rk_1' values for the same compound, giving a nearly constant value. For example, the rk_1' values for HDCA 3-Glc (**17**) obtained by using 80 and 84% aqueous methanol as the eluents are 0.61 and 0.60, respectively. Nevertheless, the contribution of an identical sugar moiety (e.g., 3-GlcA) on retentions varied widely from compound to compound. Apparently, the introduction of a sugar moiety into the parent 5β-cholanoates affect the original interaction with the liquid phase in currently unpredictable ways.

It is also evident that the rk_1' values for the pyrenacyl ester derivatives of all the Glc and GlcNAc conjugates are always less than 1.0 (0.43~0.63), whereas those of the GlcA analogs are more than 1.0 (1.96~3.73). Hence, introduction of glucose and N-acetylglucosamine moieties in the 5β-steroid nucleus increases the polarities, but considerable decreases in the polarity of the GlcA conjugates support the coesterification at C-6′ in the glucuronic acid moiety as mentioned above. Since the rk_1' values are characteristic for the individual GlcA-, Glc- and GlcNAc-conjugated bile acids, they are useful for determining and estimating the structure of these bile acid glycosides.

3.3. Inclusion HPLC behavior of glycosidic-conjugated bile acids

Addition of Me-β-CD into the solvent systems in the conventional HPLC mentioned above resulted in significant decreases in the retentions (see k_2' values in Table 1) of all the glycosylated bile acids as well as their unconjugates. The magnitude of the decreases was influenced by both the structural features of bile acid glycosides and the measuring conditions [15,17]. As a result, the elution order of individual compounds in inclusion HPLC with Me-β-CD differed much from that observed in the corresponding HPLC without Me-β-CD. For example, 3-GlcA conjugates were eluted on a Capcell Pak C_{18} column in the following orders (Fig. 3): UDCA (**12**), HCA (**14**), HDCA (**9**), CA (**15**), CDCA (**11**), DCA (**13**), and LCA (**8**), eluted with a methanol–water mixture of 90:10 (v/v); **12**, **9**, **14**, **8**≈**11**≈**15**, and **13**, eluted with the same eluent in the presence of 3 m*M* Me-β-CD; **12**, **9**, **14**, **8**, **11**, **15**, and **13**, eluted with the same eluent in the presence of 5 m*M* Me-β-CD.

Decreases in the retentions induced by Me-β-CD are ascribed to the formation of inclusion complexes, probably due to intermolecular hydrogen-bonding interactions between oxygen-containing functions of a guest bile acid and hydroxyl groups attached at the rims of a Me-β-CD cavity [23]. In particular, solute molecules, which fit well into the Me-β-CD cavity, form relatively stable inclusion complexes, which result in larger reductions of the k_2' values, compared to the corresponding k_1' values (see below).

Examination of the k_2' values for the same com-

Table 1
Conventional and inclusion HPLC data for the glycosidic conjugates of bile acids

Solvent composition		k_1'	rk_1'	k_2'		rk_2'		k_2'/k_1' [a]	
Compound				3 m*M* Me-β-CD	5 m*M* Me-β-CD	3 m*M* Me-β-CD	5 m*M* Me-β-CD	3 m*M* Me-β-CD	5 m*M* Me-β-CD
Methanol–water (80:20, v/v)									
HDCA	(2)	7.36	1.00	2.01	1.42	1.00	1.00	0.27	0.19
3-Glc	(17)	4.48	0.61	1.46	0.79	0.73	0.56	0.33 (0.06)	0.18 (−0.01)
6-Glc	(18)	3.60	0.49	1.46	0.79	0.73	0.56	0.41 (0.14)	0.22 (−0.03)
3-GlcNAc	(24)	4.18	0.57	1.98	1.09	0.99	0.77	0.47 (0.20)	0.26 (0.07)
6-GlcNAc	(25)	3.22	0.43	1.46	0.79	0.73	0.56	0.45 (0.18)	0.25 (0.06)
UDCA	(4)	5.33	1.00	1.78	1.42	1.00	1.00	0.33	0.27
3-Glc	(20)	2.58	0.49	1.23	0.65	0.69	0.46	0.48 (0.15)	0.25 (−0.02)
3-GlcNAc	(26)	2.32	0.44	1.23	0.65	0.69	0.46	0.53 (0.20)	0.28 (0.01)
7-GlcNAc	(27)	3.22	0.60	1.46	0.79	0.83	0.56	0.45 (0.12)	0.25 (−0.02)
HCA	(6)	6.37	1.00	2.66	2.03	1.00	1.00	0.42	0.32
3-Glc	(22)	3.84	0.60	1.98	1.09	0.74	0.54	0.52 (0.10)	0.28 (−0.04)
3-GlcNAc	(28)	3.60	0.57	1.98	1.09	0.74	0.54	0.55 (0.13)	0.30 (−0.02)
CA	(7)	7.79	1.00	5.51	4.62	1.00	1.00	0.71	0.59
3-Glc	(23)	4.90	0.63	4.32	2.76	0.78	0.60	0.88 (0.17)	0.56 (−0.03)
Methanol–water (90:10, v/v)									
LCA	(1)	5.18	1.00	2.29	1.28	1.00	1.00	0.44	0.25
3-GlcA	(8)	13.82	2.67	3.64	2.72	1.59	2.13	0.26 (−0.18)	0.20 (−0.05)
3-Glc	(16)	2.80	0.54	0.74	0.57	0.32	0.45	0.26 (−0.18)	0.20 (−0.05)
HDCA	(2)	1.35	1.00	0.77	0.39	1.00	1.00	0.57	0.29
3-GlcA	(9)	3.21	2.38	1.21	0.89	1.57	2.28	0.38 (−0.19)	0.28 (−0.01)
6-GlcA	(10)	3.55	2.63	1.93	1.64	2.51	4.21	0.54 (−0.03)	0.46 (0.17)
CDCA	(3)	2.65	1.00	1.83	1.18	1.00	1.00	0.69	0.45
3-GlcA	(11)	7.95	3.00	3.64	3.08	1.99	2.61	0.46 (−0.23)	0.39 (−0.06)
3-Glc	(19)	1.65	0.62	0.74	0.57	0.40	0.48	0.45 (−0.24)	0.35 (−0.10)
UDCA	(4)	1.02	1.00	0.62	0.39	1.00	1.00	0.61	0.38
3-GlcA	(12)	2.00	1.96	0.90	0.76	1.45	1.95	0.45 (−0.16)	0.38 (0.00)
DCA	(5)	2.87	1.00	2.53	2.02	1.00	1.00	0.88	0.70
3-GlcA	(13)	9.70	3.38	6.49	6.32	2.57	3.13	0.67 (−0.21)	0.65 (−0.05)
3-Glc	(21)	1.78	0.62	1.21	1.13	0.48	0.56	0.68 (−0.20)	0.63 (−0.07)
HCA	(6)	1.21	1.00	0.88	0.56	1.00	1.00	0.73	0.46
3-GlcA	(14)	3.00	2.48	1.61	1.35	1.83	2.41	0.54 (−0.19)	0.45 (−0.01)
CA	(7)	1.38	1.00	1.27	1.03	1.00	1.00	0.92	0.75
3-GlcA	(15)	5.15	3.73	3.64	3.59	2.87	3.49	0.71 (−0.21)	0.70 (−0.05)
Methanol–water (84:16, v/v)									
HDCA	(2)	2.75	1.00	1.13	0.89	1.00	1.00		
3-GlcA	(9)	6.61	2.40	2.68	2.19	2.37	2.46		
3-Glc	(17)	1.66	0.60	0.58	0.44	0.51	0.49		
3-GlcNAc	(24)	1.54	0.56	0.80	0.64	0.71	0.72		

[a] Values in parentheses refer to the differences in the k_2'/k_1' values between glycosidic conjugates and the corresponding unconjugates.

pound further revealed that they are very sensitive not only to the Me-β-CD concentration, but also to the solvent composition in the aqueous organic mobile phases. For instance, the k_2' values observed for HDCA 3-GlcA (9) were as follows: 1.21 and 0.89 for 90% aqueous methanol containing 3 and 5 m*M* Me-β-CD, respectively; 2.68 and 2.19 for 84% aqueous methanol containing 3 and 5 m*M* Me-β-CD, respectively. A similar result was also found for the k_2' values for unconjugated HDCA (2) and its 3-Glc

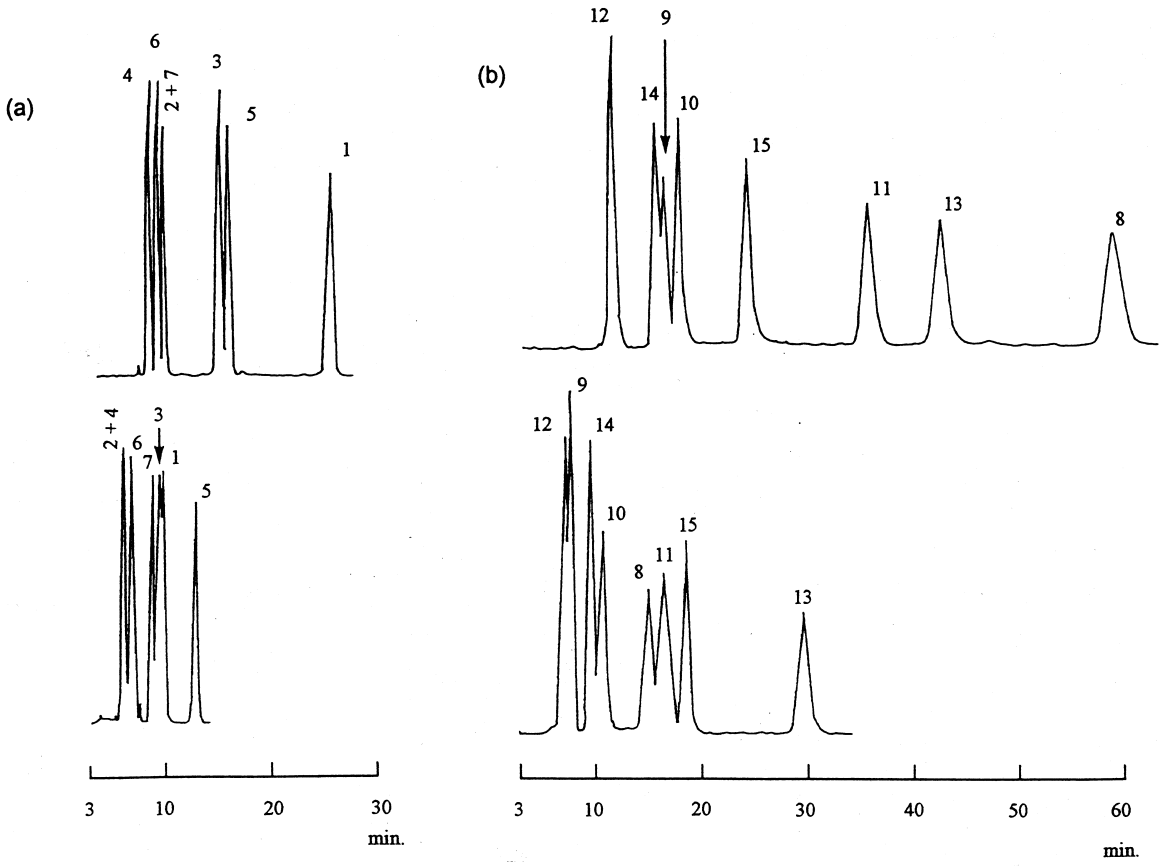


Fig. 3. Changes in the HPLC chromatograms of a mixture of (a) unconjugated and (b) GlcA-conjugated bile acids in the absence (upper) or presence (lower) of 5 m*M* Me-β-CD in a mobile phase. Conditions: column, Capcell Pak C_{18}; detection, FL; mobile phase, methanol–water (90:10, v/v) under isocratic conditions. Peak numbers refer to Fig. 1.

(17) and 3-GlcNAc (24) conjugates. Thus the retentions decrease sharply with increasing concentrations of Me-β-CD and/or organic solvent modifier (i.e., methanol) in the mobile phases.

In contrast to the nearly constant rk_1' values observed for the same compound, the corresponding rk_2' values differed alot from each other in two different concentrations of methanol or Me-β-CD in eluents examined, probably owing to the combined effect of Me-β-CD and methanol. For example, the rk_2' values of 0.73 and 0.51 were obtained for HDCA 3-Glc (17), when it was eluted with 80 and 84% aqueous methanol containing 3 m*M* Me-β-CD, respectively. In addition, any bile acid glycosides, measured with 80% aqueous methanol, always showed smaller rk_2' values in the presence of 5 m*M* Me-β-CD than in 3 m*M*; the reverse was true for the remaining glycosides, which were measured with 90% aqueous methanol in the presence of 3 and 5 m*M* Me-β-CD. Particular attention should therefore be paid for both the methanol and Me-β-CD concentrations in a mobile phase, when the structure of an unknown bile acid glycoside is characterized from the inclusion HPLC.

The above interesting behaviors of the k_2' and rk_2' values imply that methanol is essential for the formation and stabilization of inclusion complexes between solute and Me-β-CD. According to the previous finding, methanol can also enter the Me-β-CD cavity and form a weak inclusion complex [24]. Shimada, et al. [15,17] have previously pointed out that methanol competes with a solute for the Me-β-

CD cavity and a change in the proportion of methanol influences the solute interaction with the Me-β-CD. In more recent studies on inclusion HPLC and TLC, an additional interaction, other than the known solute-β-CD interaction, has been proven and the formation of ternary complexes comprised of solute–Me-β-CD–methanol has been proposed by Cserháti et al. [25,26]. On this basis, assuming that both the solute and methanol molecules are included inside the Me-β-CD cavity, hydrogen bondings between the solute, Me-β-CD, and methanol may occur competitively and/or connectively, as shown in Fig. 4. However, further study is necessary to obtain more precise information on the ternary complexes.

Table 1 also contains the k_2'/k_1' values for each compound, which reflect the decreasing rates of retentions induced by adding 3 or 5 m*M* Me-β-CD in mobile phases. As expected, all the compounds had always smaller k_2'/k_1' values in the presence of 5 m*M* Me-β-CD than in 3 m*M*, regardless of the solvent composition in mobile phases and the structure of substrates. This observation supports that an increase in the Me-β-CD concentration causes a decrease in the retention of the same substrate [15–17]. In addition, LCA (1) and its glycosidic conjugates (8 and 16) had the smallest k_2'/k_1' values within each group of analogous bile acids, suggesting the formation of the most stable inclusion complexes. On the other hand, glycosidic conjugates (13, 15, 21, and 23) of DCA and CA, which possess a 12α-hydroxyl group in the 5β-steroid nucleus, had much larger k_2'/k_1' values than the others. These behaviors of the glycosidic conjugates are consistent with those of the corresponding unconjugated bile acids (5 and 7) and their glycine and taurine conjugates reported previously [15].

Of further interest was that glycosylated bile acids and their corresponding nonglycosylated ones usually show similar k_2'/k_1' values, when they are measured with eluents containing 5 m*M* Me-β-CD. For instance, the k_2'/k_1' values for unconjugated UDCA (**4**) and its 3-GlcNAc conjugate (**26**) in the presence of 5 m*M* Me-β-CD are 0.27 and 0.28, respectively, while those in the presence of 3 m*M* Me-β-CD are 0.33 and 0.53, respectively. The correlation suggests that the presence of Me-β-CD more than 5 m*M* in mobile phases has no substantial difference in the decreasing rates of retentions between the two groups of the bile acids and that nearly maximal decreases in the retentions of these substrates are attained. The nature of the k_2'/k_1' values would therefore be an alternative tool for elucidating the structure of an unknown bile acid glycoside.

It is hoped that the method for the simultaneous determination and characterization of GlcA-, Glc- and GlcNAc-conjugated bile acids in conventional and inclusion HPLC may provide much more precise knowledge on the metabolic profile of bile acids. Applications of the present methods to human biological specimens are now being conducted in these laboratories.

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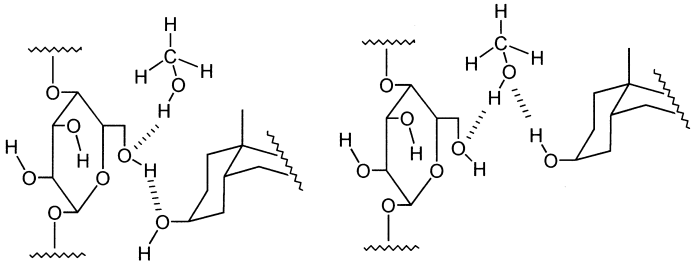

(a) Inclusion complex formed competitively (b) Inclusion complex formed connectively

Fig. 4. Conceptual illustration of possible inclusion complex formations of solute, Me-β-CD, and methanol by hydrogen bonding.

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