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Short Communication

Capillary gas chromatographic analysis of oxo, oxo-hydroxy and unsaturated bile acid glycine conjugates

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ABSTRACT

A method for the direct gas chromatographic analysis without prior hydrolysis has been developed for a series of glycine-conjugated bile acids having oxo or multiple functional groups, including oxo and hydroxyl groups and double bonds. The glycine conjugates without and with the hydroxyl groups were derivatized to their ethyl or methyl esters and ethyl ester-trimethylsilyl or methyl ester-dimethylethylsilyl ethers, respectively, and chromatographed on an aluminum-clad, flexible, fused-silica capillary column coated with a thin film (0.1 μm) of chemically bonded and cross-linked methyl polysiloxane. The change of methylene unit value exerted by derivatization and glycination for each compound is discussed.

INTRODUCTION

In previous papers [1,2], we have reported a simultaneous gas chromatographic (GC) analysis of the unconjugates and glycine conjugates of a series of hydroxylated bile acid stereoisomers as their ethyl ester-trimethylsilyl (Et-TMS) and methyl ester-dimethylethylsilyl (Me-DMES) ether derivatives on an aluminum-clad, flexible, fused-silica capillary column coated with a thin film (0.1 μm) of chemically bonded with non-selective methyl polysiloxane. A simple method for the micro-scale (< 1 mg) preparation of glycine-conjugated bile acid esters as authentic specimens from the corresponding unconjugates was also developed.

Since glycine-conjugated bile acids containing oxo or multiple functional groups, including oxo and hydroxyl groups and double bonds, are also potential metabolites [3], we are extending our GC approach to include these compounds. Thus the availability of a variety of oxo, oxo-hydroxy and unsaturated bile acids having

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one to four substituents at positions C-3, C-4, C-6, C-7 and/or C-12 has prompted us to compare the GC behavior of the glycine conjugates and the corresponding unconjugates reported previously [4].

EXPERIMENTAL

Samples and reagents

Almost all of the oxo, oxo-hydroxy and unsaturated bile acids related to 5α- and 5β-cholanoic acids, which differ from one another in the number, position and configuration of substituents in the molecules, were taken from our laboratory collections.

The silylating reagents, hexamethyldisilazane and trimethylchlorosilane in anhydrous pyridine (TMS-HT) and dimethylethylsilylimidazole (DMESI), were obtained from Tokyo Kasei Kogyo (Tokyo, Japan). 1-Ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline (EEDQ), glycine methyl ester hydrochloride and glycine ethyl ester hydrochloride were obtained from Wako Pure Chemical Industries (Osaka, Japan). All solvents used were of analytical-reagent grade.

GC instrumentation and column

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 aluminum-clad, flexible, fused-silica capillary column (25 m × 0.25 mm I.D.) with a thin film (0.1 μm) of bonded and cross-linked methyl polysiloxane (equivalent to OV-101) and operated under the following conditions: carrier gas (helium) flow-rate, 1.5 ml/min; auxiliary gas flow-rate, 40 ml/min; splitting ratio, 1:50; injection temperature, 330°C; and column temperature, 300°C (isothermal). The column, HiCap CBPM1, was purchased from Shimadzu (Kyoto, Japan).

Derivatization procedures

The Et-TMS and Me-DMES ether derivatives of glycine-conjugated bile acids as authentic specimens were prepared in two steps from their corresponding unconjugates by a combination of glycination followed by silylation [2]. Initially, oxo, oxo-hydroxy and unsaturated C_{24} bile acid samples were converted into their glycine conjugate ethyl (Et) or methyl (Me) esters by treatment with EEDQ, glycine ethyl (or methyl) ester hydrochloride and triethylamine in acetonitrile. Each of the glycine conjugate esters containing hydroxyl groups was then converted to the Et-TMS and Me-DMES ether derivatives using TMS-HT and DMESI as silylating reagents, respectively. An aliquot of the derivatized sample solutions was injected into the GC system together with an internal standard.

RESULTS AND DISCUSSION

Table I shows the retention data of 65 glycine-conjugated bile acids observed for the two classes of derivatization products on a HiCap CBPM1 column. Retention data were expressed as the relative retention time (RRT) and methylene unit (MU) values: RRT values were expressed relative to an appropriate derivative of glycodeoxycholic acid (GDCA: Et-TMS, 7.12 min; Me-DMES, 8.95 min) and MU values were

TABLE I

RRT AND MU VALUES OF THE Et-TMS AND Me-DMES ETHER DERIVATIVES OF OXO, OXO-HYDROXY AND UNSATURATED BILE ACID GLYCINE CONJUGATES[a]

Position and configuration of substituents	Et-TMS		Me-DMES		$\Delta[U_m]_{M-E}$
	RRT	MU	RRT	MU	
Monooxo[b]					
3-Oxo	1.05	39.12	0.69	38.50	−0.62
3-Oxo (5α)	1.14	39.56	0.76	39.00	−0.56
6-Oxo	0.94	38.62	0.63	38.02	−0.60
6-Oxo (5α)	1.06	39.17	0.71	38.60	−0.57
7-Oxo	0.89	38.33	0.59	37.73	−0.60
7-Oxo (5α)	1.01	38.99	0.68	38.41	−0.58
12-Oxo	0.84	38.09	0.56	37.49	−0.60
12-Oxo (5α)	0.93	38.58	0.62	37.98	−0.60
Dioxo[b]					
3,6-Dioxo	1.76	41.68	1.17	41.15	−0.53
3,6-Dioxo (5α)	1.76	41.68	1.17	41.15	−0.53
3,7-Dioxo	1.44	40.73	0.96	40.14	−0.59
3,7-Dioxo (5α)	1.66	41.42	1.10	40.86	−0.56
3,12-Dioxo	1.44	40.73	0.96	40.14	−0.59
3,12-Dioxo (5α)	1.62	41.29	1.07	40.72	−0.57
7,12-Dioxo	1.16	39.68	0.78	39.15	−0.53
7,12-Dioxo (5α)	1.37	40.47	0.91	39.90	−0.57
Trioxo[b]					
3,7,12-Trioxo	1.84	41.91	1.22	41.34	−0.57
3,7,12-Trioxo (5α)	2.17	42.73	1.45	42.18	−0.55
Monooxo-monohydroxy					
3-Oxo-6α-OH	1.39	40.57	1.17	41.15	0.58
3-Oxo-6β-OH	1.28	40.17	1.06	40.66	0.49
3-Oxo-7α-OH	1.28	40.17	1.04	40.56	0.39
3-Oxo-7α-OH (5α)	1.17	39.72	0.97	40.22	0.50
3-Oxo-7β-OH	1.75	41.70	1.43	42.09	0.39
3-Oxo-7β-OH (5α)	1.53	41.03	1.28	41.61	0.58
3-Oxo-12α-OH	1.16	39.68	0.92	39.97	0.29
3-Oxo-12α-OH (5α)	1.25	40.04	1.00	40.41	0.37
3-Oxo-12β-OH (5α)	1.25	40.04	1.00	40.41	0.37
6-Oxo-3β-OH (5α)	1.85	41.95	1.61	42.69	0.74
7-Oxo-3α-OH	1.38	40.53	1.15	41.08	0.55
12-Oxo-3α-OH	1.34	40.37	1.11	40.91	0.54
12-Oxo-3β-OH	1.34	40.37	1.15	41.08	0.71
12-Oxo-7α-OH (5α)	1.00	38.96	0.82	39.40	0.44
12-Oxo-7β-OH	1.15	39.62	0.96	40.14	0.52
12-Oxo-7β-OH (5α)	1.28	40.17	1.06	40.66	0.49
Monooxo-dihydroxy					
3-Oxo-7α,12α-$(OH)_2$	1.34	40.37	1.37	41.90	1.53
3-Oxo-7α,12α-$(OH)_2$(5α)	1.22	39.89	1.25	41.47	1.58
6-Oxo-3α,7β-$(OH)_2$ (5α)	1.46	40.80	1.48	42.30	1.50
7-Oxo-3α,6α-$(OH)_2$	1.38	40.53	1.43	42.09	1.56
7-Oxo-3α,12α-$(OH)_2$	1.49	40.89	1.47	42.27	1.38
12-Oxo-3α,7α-$(OH)_2$	1.57	41.15	1.59	42.61	1.46

(Continued on p. 454)

TABLE I (*continued*)

Position and configuration of substituents	Et-TMS		Me-DMES		$\Delta[U_m]_{M-E}$
	RRT	MU	RRT	MU	
12-Oxo-3α,7α-$(OH)_2$ (5α)	1.54	41.05	1.61	42.69	1.64
12-Oxo-3α,7β-$(OH)_2$	1.57	41.15	1.59	42.61	1.46
12-Oxo-3α,7β-$(OH)_2$ (5α)	1.61	41.28	1.66	42.85	1.57
12-Oxo-3β,7α-$(OH)_2$	1.39	40.55	1.42	42.06	1.51
12-Oxo-3β,7α-$(OH)_2$ (5α)	1.54	41.05	1.61	42.69	1.64
12-Oxo-3β,7β-$(OH)_2$	1.57	41.15	1.65	42.79	1.64
12-Oxo-3β,7β-$(OH)_2$ (5α)	2.00	42.32	2.08	43.96	1.64
Monooxo-trihydroxy					
6-Oxo-3α,7β,12α-$(OH)_3$ (5α)	1.39	40.56	1.69	42.92	2.36
Dioxo-monohydroxy					
7,12-Dioxo-3α-OH	1.82	41.85	1.53	42.45	0.60
7,12-Dioxo-3β-OH	1.77	41.72	1.53	42.45	0.73
Unsaturated					
3-Oxo-$\Delta^{4,6}$	1.38	40.53	0.93	40.05	−0.48
3-Oxo-7α-OH-Δ^4	1.41	40.64	1.15	41.08	0.44
3-Oxo-12α-OH-Δ^4	1.45	40.77	1.15	41.08	0.31
3-Oxo-7α,12α-$(OH)_2$-Δ^4	1.53	41.03	1.50	42.35	1.32
12-Oxo-3α-OH-$\Delta^{9(11)}$	1.33	40.33	1.12	40.91	0.58
3β-OH-Δ^5	1.16	39.68	0.98	40.34	0.66
3α-OH-Δ^5	0.87	38.28	0.74	38.85	0.57
3α-OH-Δ^7	0.93	38.62	0.79	39.17	0.55
3α-OH-$\Delta^{5(6)}$	1.00	38.96	0.83	39.46	0.50
3α-OH-$\Delta^{9(11)}$	0.85	38.15	0.72	38.71	0.56
3α-OH-Δ^{11}	0.91	38.48	0.75	39.08	0.60
7α,12α-$(OH)_2$-Δ^5	0.80	37.83	0.82	39.40	1.57
3α,12α-$(OH)_2$-Δ^5	0.99	38.88	0.99	40.35	1.47
3α,12α-$(OH)_2$-Δ^7	0.93	38.70	0.94	40.11	1.41
3α,12α-$(OH)_2$-$\Delta^{8(14)}$	0.91	38.48	0.91	39.90	1.42

[a] RRT values were expressed relative to the Et-TMS or Me-DMES ether derivatives of GDCA. The designation 5α in parentheses refers to "*allo*" (*trans* 5α-H) compounds.

[b] RRT and MU values of these compounds correspond to the Et or Me ester derivatives of the glycine conjugates.

determined relative to *n*-alkane. The $\Delta[U_m]_{M-E}$ values [5], which are defined as the differences in the MU values between analogous Me-DMES and Et-TMS ethers, are also listed in the table.

Under the derivatization conditions, the GC peaks arising from bile acids containing hydroxyl groups are assigned to the corresponding glycine-conjugated Et-TMS or Me-DMES ethers, whereas those from oxo bile acids, which are not susceptible to silylation, correspond to the glycine-conjugated Et or Me esters. However, both the ester and ether derivatives afforded sharp and symmetrical peaks on this column, with brief analysis times (within 13 min for the Et esters and Et-TMS ethers, and within 17 min for the Me esters and Me-DMES ethers). Fig. 1 illustrates the distinct separation of a mixture of oxo and oxo-hydroxy bile acid glycine conjugate isomers.

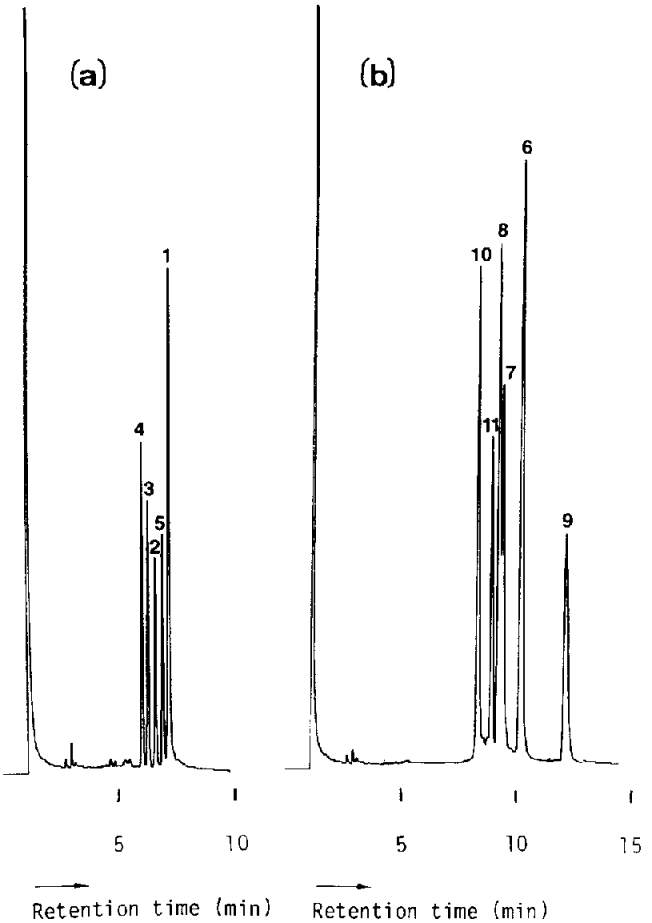

Fig. 1. Capillary GC of a mixture of (a) oxo and (b) oxo-hydroxy 5β-bile acid glycine conjugate isomers as their Et ester and Me-DMES ether derivatives. Peak identification and position of substituents: 1 = 3-oxo; 2 = 6-oxo; 3 = 7-oxo; 4 = 12-oxo; 5 = Et-TMS ether of GDCA; 6 = 3-oxo-6α-hydroxy; 7 = 3-oxo-6β-hydroxy; 8 = 3-oxo-7α-hydroxy; 9 = 3-oxo-7β-hydroxy; 10 = 3-oxo-12α-hydroxy; and 11 = Me-DMES ether of GDCA.

As shown in Fig. 2, plots of the MU values of glycine conjugate Et-TMS ethers (or Et estes) *versus* those of the corresponding Me-DMES ethers (or Me esters) afforded three regression lines, a, b and c, with a similar slope of 1 and good linearity depending upon the number of hydroxyl groups in the molecules. Line a (Fig. 2), expressed as $y = 1.03x + 0.38$ ($r = 0.998$, $n = 17$), consists of all of the mono-, di- and trioxo compounds without a hydroxyl group. The straight line reflects the fact that these compounds show nearly consistent negative $\Delta[U_m]_{M-E}$ values (*ca.* -0.57). On the other hand, regression line b, expressed as $y = 1.03x - 0.67$ ($r = 0.994$, $n = 28$), was obtained from the data for compounds with a hydroxyl group (*e.g.* mono-oxo-monohydroxy and dioxo-monohydroxy), indicating that these compounds have consistent postive $\Delta[U_m]_{M-E}$ values of *ca.* 0.50. Further, compounds possessing two

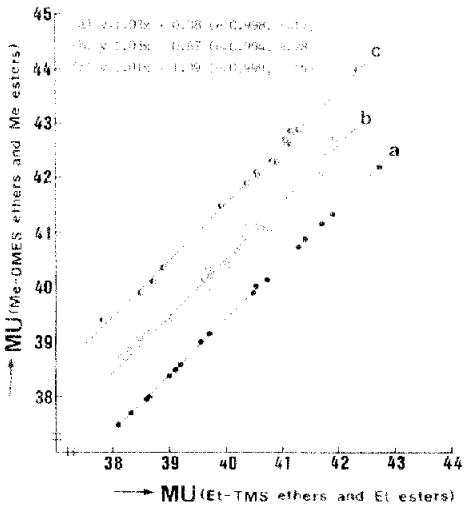

Fig. 2. Relationship between the MU values of Me-DMES and Et-TMS ether (or Me and Et ester) derivatives of glycine-conjugated bile acids without (a) or with one (b) or two (c) hydroxyl groups.

hydroxyl groups (*e.g.* monooxo-dihydroxy) belong to the remaining regression line c, defined as $y = 1.01x - 1.09$ ($r = 0.999$, $n = 19$), implying that the retention times of the Me-DMES ethers are much longer (*ca.* 1.53 in the $\Delta[U_{m|M-1}$ values) than those of the corresponding Et-TMS ethers. These significant correlations are, therefore, useful for characterizing each of the three types of compounds.

As expected, glycine-conjugated bile acids are eluted much more slowly than the corresponding unconjugates, and the MU values observed for the glycine conjugate Me esters and Me-DMES ethers are in the range 37–44 (*cf.* 30–38 for the unconjugates [4]); 38–43 for the Et esters and Et-TMS ethers. For the purpose of com-

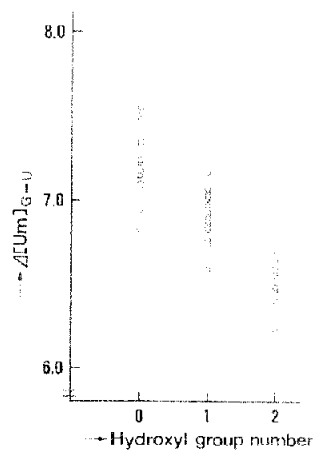

Fig. 3. Relationship of hydroxyl group number to $\Delta[U_{m|k,\ -1}$ value of oxo and oxo-hydroxy bile acids as their Me-DMES ether (or Me ester) derivatives.

parison, the differences in the MU values between the Me-DMES ethers (or Me esters) of analogous glycine-conjugated and unconjugated bile acids (determined on a HiCap CBPM1 column) [4], which are defined as $\Delta[U_m]_{G-U}$ values, were calculated. The result is expressed graphically in Fig. 3. In analogy with hydroxylated bile acid glycine conjugates reported previously [2], the $\Delta[U_m]_{G-U}$ values were found to depend on the number of hydroxyl substituents on the steroid nucleus, and the average values obtained were as follows: 7.3 for nineteen oxo (S.D. = 0.201), 6.9 for nineteen monooxo-monohydroxy and dioxo-monohydroxy (S.D. = 0.142) and 6.5 for fourteen monooxo-dihydroxy (S.D. = 0.128) compounds, as well as their unsaturated analogues.

The above generalization suggests that the elution order of each group of oxo and oxo-hydroxy bile acid glycine conjugate isomers is essentially identical and corresponds well with the order observed for the corresponding unconjugates [4] on this column. In fact, the positional isomers of the oxo bile acid glycine conjugates in both the 5α and 5β series are well separated as their Et and Me esters, emerging from the column in the order 12- < 7- < 6- < 3-ketones, 7,12- < 3,12- < 3,7- < 3,6-diketones, and then 3,7,12-triketones, precisely corresponding to the order found for their unconjugate esters [4]. In addition, the mono-, di- and triketones in the 5β series move faster than the corresponding ketones in the 5α series with the exception of the C-5 epimeric 3,6-diketones. Similar behavior was also observed for each of the two series of the monooxo-monohydroxy and monooxo-dihydroxy isomers.

The relative mobilities of individual analogues of the two derivatives were also in a similar order. However, some of recalcitrant pairs could be separated successfully by changing the derivatization from Et to Me esters or Et-TMS to Me-DMES ethers (or *vice versa*). For example, while epimeric pairs in the 5β series, 12-oxo-3β-hydroxy *versus* 7-oxo-3α-hydroxy and 7,12-dioxo-3α-hydroxy *versus* 7,12-dioxo-3β-hydroxy, completely overlap as the Me-DMES ethers, the two pairs are well resolved as the Et-TMS ethers. The reverse was true for the pairs 3-oxo-6β-hydroxy *versus* 3-oxo-7α-hydroxy and 12-oxo-3α-hydroxy *versus* 12-oxo-3β-hydroxy.

For the positional isomers of unsaturated 3α-hydroxy bile acid glycine conjugates, the following order of increasing retention was observed: $\Delta^{9(11)} < \Delta^{6} < \Delta^{11} < \Delta^{7} < \Delta^{8(14)}$. Interestingly, this elution order differs from that found for the analogous unsaturated 3α,12α-dihydroxy compounds: $\Delta^{8(14)} < \Delta^{7} < \Delta^{6}$.

The retention data reported here provide an insight into structural elucidation of these biologically important glycine-conjugated bile acids, and the method depends on the ability to measure simultaneously unconjugated and glycine-conjugated ketonic bile acids in biological fluids in a single profile without prior separation and hydrolysis.

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