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Studies on steroids

CCLIII☆. Capillary gas chromatographic behaviour of diethylhydrogensilyl–diethylsilylene derivatives of stereoisomeric bile acids

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ABSTRACT

The capillary gas chromatographic behaviour of diethylhydrogensilyl (DEHS) ethers and/or diethylsilylene (DES) derivatives of fifty bile acids including 4- and 6-hydroxylated compounds is described. The methylene unit (MU) values of methyl and pentafluorobenzyl esters of bile acids were determined as their trimethylsilyl (TMS), dimethylethylsilyl (DMES) ethers and DEHS–DES derivatives. The differences in methylene unit values between the corresponding TMS ethers and DMES ethers or DEHS–DES derivatives were used for estimating the number and stereochemistry of hydroxyl groups on the steroid nucleus. On treatment with the silylating agent N,O-bis (diethylhydrogensilyl)trifluoroacetamide, bile acids possessing isolated hydroxyl in addition to diaxial *trans*-glycol groups were easily converted into the DEHS ehters, whereas those having a vicinal glycol group except for the diaxial group were converted into cyclic DES derivatives. The mass spectrometric properties obtained with negative-ion chemical ionization detection are discussed.

INTRODUCTION

Bile acids are the major biotransformation products from cholesterol and assist the lipolysis and absorption of fats by the formation of mixed micelles in the intestinal lumen. Unusual bile acids having a vicinal glycol structure at C-3,4 or C-6,7 have recently been found in patients with liver diseases and in newborn infants and foetuses [1–3]. Accordingly, the development of a reliable method for the determination of these unusual bile acids in biological materials in connection with the diagnosis of hepatobiliary diseases is urgently required.

Gas chromatography (GC) is a powerful tool for the profile analysis of bile acids in biological specimens. The trimethylsilyl (TMS) [4,5] and dimethylethylsilyl (DMES) ethers [6–8] have been extensively used as derivatives suitable for the GC determination of bile acids. However, no satisfactory derivatization of bile acids including unusual ones is at present available for their complete separation. Also, diacetoxydimethylsilane has been shown to be suitable for the derivatization of biological substances with a vicinal glycol moiety [9]. Unfortu-

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nately, this derivatizatization procedure has a disadvantage regarding the stability of the resulting dimethylsiliconide [10]. Although alkyl boronic acids are commonly used for the formation of stable cyclic boronates from the 1,2- and 1,3-glycols, this method requires successive derivatization of isolated hydroxyl groups [11].

In order to overcome these problems, a new silylating agent, N,O-bis(diethylhydrogensilyl)trifluoroacetamide (DEHS-BSTFA), has recently been developed and applied to the separation and determination of steroids and prostaglandins in biological fluids [12–14]. This novel reagent reacts readily with an isolated hydroxyl group to form the diethylhydrogensilyl (DEHS) ether and with a vicinal glycol to form the cyclic diethylsilylene (DES) derivative simultaneously. This paper deals with the capillary GC behaviour of DEHS ether and/or DES derivatives of bile acids having hydroxyl group(s) at C-3, -4, -6, -7 and/or -12.

EXPERIMENTAL

Gas chromatography

A Model GC-15A gas chromatograph (Shimadzu, Kyoto, Japan) equipped with a flame ionization detector and a Van den Berg solventless injector was employed. A cross-linked methylsilicone fused-silica capillary column (25 m × 0.3 mm I.D.) (Hewlett-Packard, Avondale, PA, USA) was used. The temperature of the column oven was maintained at 240–290°C and those of the injection heating block and detector were kept at 280°C and 290°C, respectively. The carrier gas was nitrogen at a linear velocity of 40 cm/s.

Gas chromatography–mass spectrometry (GC–MS)

Capillary GC–MS was carried out using a Model MM12030 quadrupole mass spectrometer (VG Analytical, Manchester, UK) interfaced to an HP 5790A gas chromatograph (Hewlett-Packard) with a Van den Berg solventless injector. Isobutane was used as a reagent gas. A cross-linked 5% phenylmethylsilicone fused-silica capillary column (20 m × 0.3 mm I.D.) (J &W Scientific, Folsom, CA, USA) was inserted into the ion source through the direct inlet. The carrier gas was helium at a linear velocity of 65 cm/s. The injection port, column oven and ion source were kept at 280, 260–290 and 270°C, respectively. The ionization energy was 70 eV and the emission current was 400 μA.

Materials

Cholic, chenodeoxycholic, deoxycholic and lithocholic acids were purchased from Sigma (St. Louis, MO, USA) and ursodeoxycholic acid was kindly donated by Tokyo Tanabe (Tokyo, Japan). Other stereoisomeric bile acids were prepared in these laboratories by methods reported previously [15–17]. Dimethylethylsilylimidazole was supplied by Tokyo Kasei Kogyo (Tokyo, Japan) and DEHS-BSTFA, supplied by Tokyo Kasei Kogyo, was synthesized by a known method [12]. All chemicals employed were of analytical-reagent grade and purified by distillation prior to use.

Derivatization of bile acids

The pentafluorobenzyl (PFB) esters were prepared by treating bile acids with 5% (v/v) PFB bromide in acetonitrile (60 μl) and diisopropylethylamine (10 μl) at 37°C for 45 min. The reaction mixture was treated on a Sep-Pak C_{18} cartrdige (Waters–Millipore, Milford, MA, USA) and the PFB esters were eluted with ethanol–acetonitrile (1:1) [18]. The methyl esters of bile acids were prepared by treatment with hydrochloric acid–methanol or diazomethane–diethyl ether–methanol in the usual manner [4].

The bile acid esters thus obtained were derivatized to the corresponding TMS, DMES and DEHS–DES derivatives with hexamethyldisilazane–trimethylchlorosilane in pyridine at 60°C for 90 min, DMES-imidazole in 1% pyridine in hexane at 60°C for 60 min and DEHS-BSTFA in pyridine for 60 min at room temperature, respectively. The reaction product exhibited a single peak of the theoretical shape.

RESULTS AND DISCUSSION

Gas chromatographic behaviour of bile acids with hydroxyl groups at C-3, -7 and -12

Fifty bile acids were used for investigating the GC behaviour of DEHS ether and/or DES derivatives as well as dimethylethylsilyl (DMES) ethers, which have been widely utilized in the GC analysis of biologically active compounds [6–8].

Initially, methylene unit (MU) values of DMES

and DEHS ethers of bile acids with isolated hydroxyl group(s) at C-3, -7 and -12 were determined and the results obtained are given in Table I. The $\Delta[Um]_{DMES}$ and $\Delta[Um]_{DEHS}$ values [19], which are defined as the differences in MU values between TMS ethers and DMES or DEHS ethers, are also listed. All bile acids were readily converted into TMS, DMES and DEHS ethers under the mild condition and the resulting derivatives exhibited a single peak of the theoretical shape. The DEHS ethers gave larger MU values than the corresponding DMES ethers. The $\Delta[Um]_{DEHS}$ values of methyl esters were 1.80 ± 0.26 for six monohydroxylated, 3.32 ± 0.24 for twelve dihydroxylated and 4.84 ± 0.25 for eight trihydroxylated bile acids, whereas those of PFB esters were 1.69 ± 0.32, 3.30 ± 0.25 and 4.74 ± 0.22, respectively.

The relationships between hydroxyl group number and $\Delta[Um]$ values of DMES and DEHS ethers are shown in Fig. 1. When $\Delta[Um]_{DEHS}$ values of the methyl and PFB esters were plotted against hydroxyl group number, good linearities, defined as $y = 1.514x + 0.294$ ($r = 0.979$) and $y = 1.517x + 0.217$ ($r = 0.976$), were observed. The data indicate that the presence of one hydroxyl group exerts consistently an increment of 1.5 units in the $\Delta[Um]_{DEHS}$ value. Regression lines expressed as $y = 0.940x + 0.347$ ($r = 0.979$) and $y = 0.985x + 0.187$ ($r = 0.977$) were obtained for DMES ether-methyl ester and -PFB ester derivatives, respectively. These results are in good agreement with earlier findings on hydroxysteroids [6], implying that the number of hydroxyl groups on the steroid nucleus of bile acids may possibly be deduced from the regression equation.

Gas chromatographic behaviour of bile acids with a vicinal glycol

The MU and $\Delta[Um]$ values of 4- and 6-hydroxylated bile acids were then determined (Table II). The $\Delta[Um]$ values of DMES ethers obtained from the data were 2.42 ± 0.24 (methyl ester) and 2.27 ± 0.15 (PFB ester) for seven dihydroxylated, 3.08 ± 0.18 (methyl ester) and 3.00 ± 0.18 (PFB ester) for thirteen trihydroxylated and 3.98 ± 0.21 (methyl esters) and 3.89 ± 0.20 (PFB ester) for four tetrahydroxylated bile acids, being in good agreement with those of bile acids listed in Table I [dihydroxylated, 2.23 ± 0.12 (methyl ester), 2.20 ± 0.14 (PFB ester); trihydroxylated, 3.16 ± 0.20 (methyl ester), 3.11 ± 0.16 (PFB ester)].

On the other hand, bile acid derivatives obtained with DEHS-BSTFA exhibited different chromatographic behaviour. Bile acids possessing isolated hydroxyl groups and/or a diaxial *trans*-glycol group at C-3,4 or C-6,7 provided almost identical $\Delta[Um]$ values with those of bile acids described above. These results indicate that all hydroxyl groups on the steroid nucleus would be derivatized into DEHS ethers. The correlation of MU values between DMES ethers of bile acids and corresponding DEHS ethers is expressed as the regression line A ($y = 1.072x - 1.412$, $r = 0.993$, $n = 68$) in Fig. 2. The $\Delta[Um]$ values obtained from bile acids possess-

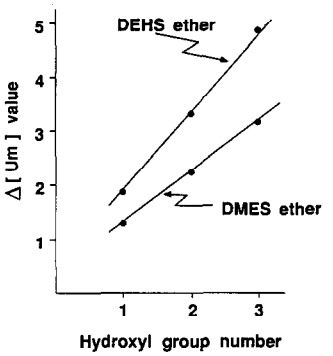

Fig. 1. Correlations between hydroxyl group number and $\Delta[Um]$ values of DMES or DEHS ethers for bile acid methyl esters with isolated hydroxyl group(s).

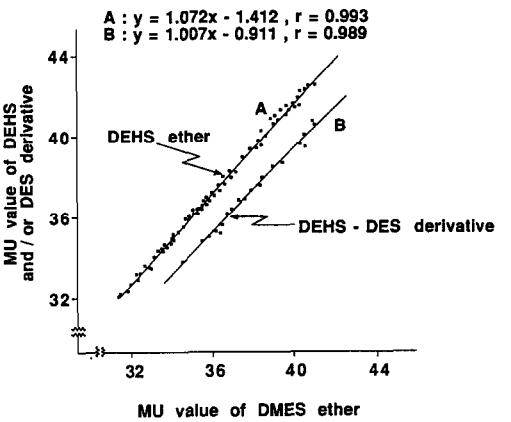

Fig. 2. Correlations in MU values between the corresponding DMES ethers and DEHS ethers or DEHS–DES derivatives.

TABLE I

METHYLENE UNIT AND Δ[Um] VALUES OF BILE ACIDS WITH ISOLATED HYDROXYL GROUP(S)

5β-Cholanoic acid	TMS MU	DMES		DEHS	
		MU	Δ[Um][a]	MU	Δ[Um][a]
Methyl ester					
3α	30.95	32.39	1.44	32.98	2.03
3β	30.88	32.31	1.43	32.97	2.09
7α	29.46	30.74	1.28	31.12	1.66
7β	30.32	31.43	1.11	32.17	1.85
12α	29.29	30.44	1.15	30.68	1.39
12β	29.10	30.38	1.28	30.90	1.80
3α,7α	31.88	34.23	2.35	35.27	3.39
3α,7β	32.43	34.65	2.22	35.86	3.43
3β, 7α	31.49	33.76	2.27	34.93	3.44
3β,7β	32.41	34.76	2.35	36.06	3.63
3α,12α	31.59	33.78	2.19	34.82	3.23
3α,12β	31.50	33.62	2.12	34.88	3.38
3β,12α	31.58	33.74	2.16	34.73	3.15
3β,12β	31.65	33.94	2.29	35.32	3.67
7α,12α	30.09	32.51	2.42	33.05	2.96
7α,12β	30.02	32.29	2.27	33.30	3.28
7β,12α	31.04	33.02	1.98	33.90	2.86
7β,12β	31.20	33.39	2.19	34.59	3.39
3α,7α,12α	32.00	35.39	3.39	36.94	4.94
3α,7α,12β	32.18	35.08	2.90	36.78	4.60
3α,7β,12α	32.67	35.62	2.95	37.20	4.53
3α,7β,12β	32.79	35.86	3.07	37.63	4.84
3β,7α,12α	31.76	35.14	3.38	36.76	5.00
3β,7α,12β	31.92	35.14	3.22	36.81	4.89
3β,7β,12α	32.67	35.70	3.03	37.30	4.63
3β,7β,12β	33.01	36.38	3.37	38.29	5.28
PFB ester					
3α	36.32	37.51	1.19	38.19	1.87
3β	36.36	37.66	1.30	38.38	2.02
7α	34.73	35.76	1.03	36.38	1.65
7β	35.35	36.70	1.35	37.25	1.90
12α	34.58	35.45	0.87	35.72	1.14
12β	34.25	35.24	0.99	35.82	1.57
3α,7α	36.93	39.14	2.21	40.26	3.33
3α,7β	37.39	39.48	2.09	40.84	3.45
3β,7α	36.56	38.91	2.35	39.90	3.34
3β,7β	37.40	39.85	2.45	41.16	3.76
3α,12α	36.71	38.80	2.09	39.72	3.01
3α,12β	36.36	38.46	2.10	39.74	3.38
3β,12α	36.70	38.91	2.21	39.86	3.16
3β,12β	36.60	39.01	2.41	40.13	3.53
7α,12α	35.19	37.52	2.33	38.49	3.30
7α,12β	35.03	37.12	2.09	37.98	2.95
7β,12α	35.86	37.88	2.02	38.79	2.93
7β,12β	35.86	38.02	2.16	39.23	3.37
3α,7α,12α	36.98	40.40	3.42	41.80	4.82
3α,7α,12β	36.72	39.80	3.08	41.36	4.64
3α,7β,12α	37.50	40.40	2.90	41.88	4.38
3α,7β,12β	37.27	40.30	3.03	42.08	4.81
3β,7α,12α	36.67	39.88	3.21	41.52	4.85
3β,7α,12β	36.65	39.73	3.08	41.42	4.77
3β,7β,12α	37.47	40.44	2.97	41.98	4.51
3β,7β,12β	37.69	40.84	3.15	42.80	5.11

[a] Differences in MU values between the corresponding TMS ethers and DMES or DEHS ethers.

TABLE II

METHYLENE UNIT AND Δ[Um] VALUES OF 4- AND 6-HYDROXYLATED BILE ACIDS

5β-Cholanoic acid	TMS MU	DMES		DEHS and/or DES	
		MU	Δ[Um][a]	MU	Δ[Um][a]
Methyl ester					
3α,4β	33.17	35.34	2.17	34.85	1.68
3β,4α	31.22	33.57	2.35	34.97	3.75
3β,4β	32.66	34.97	2.31	34.51	1.85
3α,4β,7α	33.60	36.72	3.12	35.96	2.36
3α,4β,12α	33.74	37.01	3.27	35.43	1.69
3β,4β,7α	33.92	36.76	2.84	35.65	1.73
3β,4α,12α	32.49	35.67	3.18	36.86	4.37
3β,4β,12α	32.88	36.00	3.12	36.02	3.14
3α,4β,7α,12α	34.48	38.45	3.97	37.62	3.14
3β,4β,7α,12α	34.24	38.00	3.76	37.35	3.11
3α,6α	32.17	34.49	2.32	35.86	3.69
3α,6β	31.98	34.38	2.40	35.80	3.82
3β,6α	32.28	34.76	2.48	36.14	3.86
3β,6β	31.51	34.42	2.91	35.80	4.29
3α,6α,7α	32.94	36.02	3.08	37.31	4.37
3α,6α,7β	34.21	37.00	2.79	36.62	2.41
3α,6β,7α	32.08	35.21	3.13	36.82	4.74
3α,6β,7β	33.11	36.16	3.05	37.14	4.03
3β,6α,7α	32.81	35.92	3.11	36.54	3.73
3β,6α,7β	34.44	37.20	2.76	36.35	1.91
3β,6β,7α	31.70	34.94	3.24	36.54	4.84
3β,6β,7β	33.17	36.47	3.30	37.14	3.97
3α,6α,7α,12α	32.92	37.18	4.26	38.64	5.72
3α,6β,7β,12α	32.79	36.70	3.91	38.54	5.75
PFB ester					
3α,4β	38.42	40.56	2.14	40.10	1.68
3β,4α	36.47	38.65	2.18	40.48	4.01
3β,4β	38.14	40.28	2.14	39.76	1.62
3α,4β,7α	38.70	41.64	2.94	41.12	2.42
3α,4β,12α	38.91	41.98	3.02	40.42	1.51
3β,4β,7α	38.96	41.66	2.70	40.76	1.80
3β,4α,12α	37.45	40.66	3.21	41.96	4.51
3β,4β,12α	37.86	41.00	3.14	41.16	3.30
3α,4β,7α,12α	39.39	43.18	3.79	42.66	3.27
3β,4β,7α,12α	38.98	42.66	3.68	42.30	3.32
3α,6α	37.24	39.46	2.22	40.94	3.70
3α,6β	37.08	39.32	2.24	40.86	3.78
3β,6α	37.45	39.98	2.53	41.40	3.95
3β,6β	37.02	39.46	2.44	41.02	4.00
3α,6α,7α	37.82	40.88	3.06	42.38	4.56
3α,6α,7β	39.10	41.96	2.86	41.54	2.44
3α,6β,7α	36.91	39.96	3.05	41.66	4.75
3α,6β,7β	38.20	41.02	2.82	42.08	3.88
3β,6α,7α	37.74	40.92	3.18	41.68	3.94
3β,6α,7β	39.35	42.06	2.71	41.40	2.05
3β,6β,7α	36.63	39.72	3.09	41.38	4.75
3β,6β,7β	38.16	41.40	3.24	42.26	4.10
3α,6α,7α,12α	37.74	41.88	4.14	43.42	5.68
3α,6β,7β,12α	37.34	41.30	3.96	43.32	5.98

[a] Differences in MU values between the corresponding TMS ethers and DMES ethers or DEHS–DES derivatives.

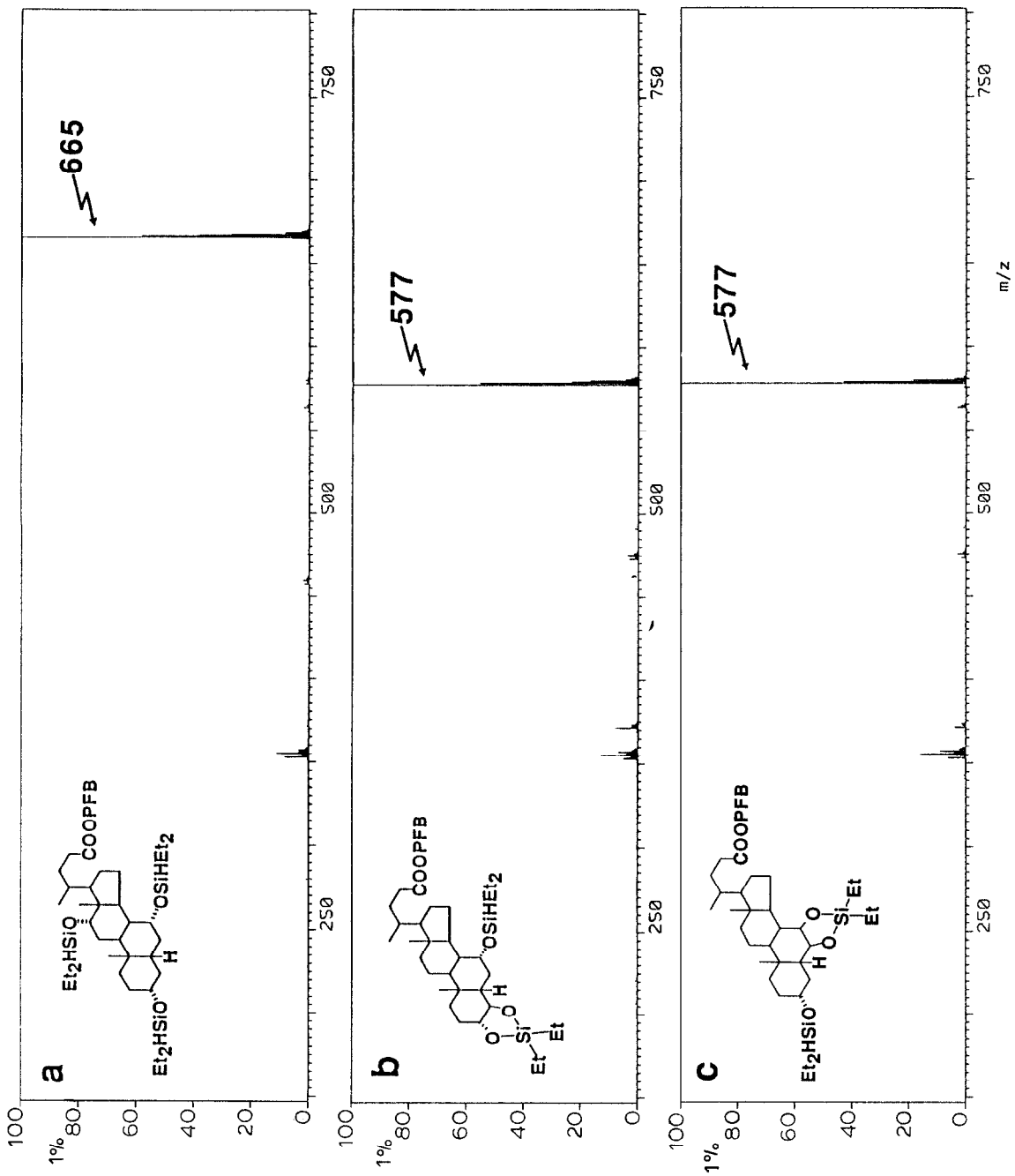

Fig. 3. Isobutane NICI mass spectra of trihydroxylated bile acids. (a) DMES ether of 3α, 7α, 12α-trihydroxy-5β-cholanoic acid (cholic acid) (b) DEHS–DES derivative of 3α,4β.7α-trihydroxy-5β-cholanoic acid; (c) DEHS–DES derivative of 3α,6β.7β-trihydroxy-5β-cholanoic acid. Et = Ethyl.

ing a diequatorial *trans*-glycol or axial–equatorial *cis*-glycol structure at C-3,4 and a diequatorial *trans*-glycol structure at C-6,7 were much smaller than those of DEHS ethers obtained from bile acids with isolated hydroxyl groups. Moreover, the MU values of these bile acid derivatives were smaller than those of the corresponding DMES ethers, as shown in the regression line B ($y = 1.007x - 0.911$, $r = 0.989$, $n = 20$), indicating the formation of cyclic DES derivatives. It is of interest that the Δ[Um] values of 6-hydroxylated bile acids having a *cis*-glycol of axial–equatorial nature (or *vice versa*), which are easily transformed into cyclic boronates with alkylboronic acids [20], were much larger than those of DES derivatives of bile acids mentioned above, but smaller than those of DEHS derivatives of bile acids having isolated hydroxyl groups.

It has been demonstrated that the introduction of a PFB group into the carboxyl function would be most favourable for the formation of a characteristic carboxylate anion, $[M-PFB]^-$, in the negative-ion chemical ionization (NICI) mode [6,18]. Therefore, GC–MS with NICI detection was utilized for the structural characterization of these derivatives. As illustrated in Fig. 3a, DEHS derivatives exhibited carboxylate anions at appropriate mass number (*m/z*: monohydroxylated, 461; dihydroxylated, 563; trihydroxylated, 665; tetrahydroxylated, 767), reflecting the persilylated structure. On the other hand, the DES derivatives gave intense negative ions, $[M-PFB]^-$, with a decrement of 88 mass units from those of the corresponding DEHS derivatives (Fig. 3b). As illustrated in Fig. 3c, the mass spectrum of the reaction product from 3α, 6β, 7β-trihydroxylated bile acid with DEHS-BSTFA indicates the formation of the DES derivative. The stereoisomeric 6-hydroxylated bile acids with a *cis*-glycol exhibited the same mass spectrometric properties, indicating the formation of the C-6,7 DES derivatives.

CONCLUSIONS

Isolated hydroxyl groups of bile acids were readily converted into DEHS ethers, while vicinal glycols except for that of diaxial nature were transformed into cyclic DES derivatives. The use of DEHS ether and/or DES derivatives in combination with the corresponding TMS or alkyldimethylsilyl ethers may provide valuable information for establishing the number and nature of hydroxyl groups on the steroid nucleus of bile acids. In addition, as the DES derivative has a much lower molecular weight than the corresponding DEHS ether, the combined use of the PFB–DEHS–DES derivative and capillary GC–MS with NICI detection would be more favourable for the trace analysis of unusual bile acids with a vicinal glycol group. Applications of the present derivatization method to the determination of 4- and 6-hydroxylated bile acids in biological fluids are being studied and the results will be reported elsewhere.

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REFERENCES

1 W. H. Elliot, in H. Danielsson and J. Sjövall (Editors), *Sterols and Bile Acids*, Elsevier, Amsterdam, 1985, p. 303.
2 R. Dumaswala, K. D. R. Setchell, L. Zimmer-Nechemias, T. Iida, J. Goto and T. Nambara, *J. Lipid Res.*, 30 (1989) 847.
3 M. Nakagawa and K. D. R. Setchell, *J. Lipid Res.*, 31 (1990) 1089.
4 P. Eneroth and J. Sjövall, in P. P. Nair and D. Kritchevsky (Editors), *The Bile Acids*, Vol. 1, Plenum Press, New York, 1971, p. 121.
5 J. M. Street and K. D. R. Setchell, *Biomed. Chromatogr.*, 2 (1988) 229.
6 H. Miyazaki, M. Ishibashi, M. Itoh and T. Nambara, *Biomed. Mass Spectrom.*, 4 (1977) 23.
7 J. Yanagisawa, M. Itoh, M. Ishibashi, H. Miyazaki and F. Nakayama, *Anal. Biochem.*, 104 (1980) 75.
8 J. Goto, K. Watanabe, H. Miura, T. Nambara and T. Iida, *J. Chromatogr.*, 388 (1987) 379.
9 T. A. Baillie, C. J. W. Brooks and B. S. Middleditch, *Anal. Chem.*, 44 (1972) 30.
10 R. W. Kelly, *J. Chromatogr.*, 43 (1969) 229.
11 C. J. W. Brooks and D. J. Harvey, *J. Chromatogr.*, 54 (1971) 193.
12 H. Miyazaki, M. Ishibashi, M. Itoh and K. Yamashita, *Biomed. Mass Spectrom.*, 11 (1984) 377.
13 M. Ishibashi, T. Irie and H. Miyazaki, *J. Chromatogr.*, 399 (1987) 197.
14 K. Yamashita, K. Watanabe, M. Ishibashi, M. Katori and H. Miyazaki, *J. Chromatogr.*, 424 (1988) 1.

15 T. Iida, T. Momose, T. Tamura, T. Matsumoto, F. C. Chang, J. Goto and T. Nambara, *J. Lipid Res.*, 30 (1989) 1267.
16 T. Iida, T. Momose, F. C. Chang, J. Goto and T. Nambara, *Chem. Pharm. Bull.*, 37 (1989) 3323.
17 T. Iida, I. Komatsubara, S. Yoda, J. Goto, T. Nambara and F. C. Chang, *Steroids*, 55 (1990) 530.
18 J. Goto, H. Miura, M. Inada, T. Nambara, T. Nagakura and H. Suzuki, *J. Chromatogr.*, 452 (1988) 119.
19 H. Miyazaki, M. Ishibashi, K. Yamashita and M. Katori, *Biomed. Mass Spectrom.*, 8 (1981) 521.
20 T. Iida, I. Komatsubara, F. C. Chang, J. Goto and T. Nambara, *J. Chromatogr.*, 537 (1991) 345.