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GAS CHROMATOGRAPHY OF DIMETHYLALKYLSILYL ETHER DERIVATIVES OF BILE ACID METHYL ESTERS

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SUMMARY**

The gas chromatographic separation of the DMAS ether derivatives of ten bile acid methyl esters, namely DMES, DMnPS and DMiPS ethers, have been studied by use of an open tubular glass capillary column, coated with SE-30. The DMAS ether derivatives were eluted in sequence according to the number of hydroxyl groups except for 12-KCDCA, and separated. This contrasts with the poorly resolved gas chromatographic peaks produced by the TMS ether derivatives. The DMES ether derivatives were resolved with baseline separation in 30 min, but those of HCA and 12-KCDCA had similar methylene unit values.

INTRODUCTION**

The attention in bile acid research has now turned toward the relative amounts of the metabolites in bile acid transformations in order to elucidate their physiological role and differences in the metabolic profiles in health and disease.

Gas chromatographic (GC) separation of bile acids has been investigated exclusively with several kinds of derivatives and columns packed with various station-

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** Abbreviations: CA = cholic acid; CDCA = chenodeoxycholic acid; DCA = deoxycholic acid; DMAS = dimethylalkylsilyl; DMES = dimethylethylsilyl; DMESI = DMES-imidazole; DMiPS = dimethylisopropylsilyl; DMiPSI = DMiPS-imidazole; DMnPS = dimethyl-*n*-propylsilyl; DMnPSI = DMnPS-imidazole; FID = flame ionization detector; GC = gas chromatography; HCA = hyocholic acid; HDCA = hyodeoxycholic acid; 12-KCDCA = 12-ketochenodeoxycholic acid; LCA = lithocholic acid; α-MCA = α-muricholic acid; β-MCA = β-muricholic acid; MU = methylene unit; MS = mass spectrometry; SCTAS = sterically crowded trialkylsilyl; TAS = trialkylsilyl; TMS = trimethylsilyl; TMSI = trimethylsilylimidazole; UDCA = ursodeoxycholic acid.

ary liquid phases[1–8]. Use of glass capillary columns has provided profile analysis of biologically important substances, such as steroids[9,10] and prostaglandins[11,12].

Capillary GC separation of bile acids has been reported by Laatikainen and Hesso[13] and Karlaganis and Paumgartner[14,15]. However, these methods seem to be unsuitable for the profiles of bile acids due to incomplete separation. The combination of methyl ester–TMS ether derivatives and glass capillary columns, coated with PEG-20M, compels the bile acids to emerge in reverse order of the number of hydroxyl groups in the molecule[14,15]. When this technique was applied to the analysis of a metabolic profile, difficulties arose in the quantitation of LCA, a minor component in human bile, because its peak became too broad owing to the longer retention time in comparison with the di- and trihydroxy bile acids.

We have recently reported the baseline separation of the DMES ether derivatives of five bile acid methyl esters and cholesterol by use of a glass capillary column, coated with SE-30[16].

Use of DMAS, TAS and SCTAS ether derivatives of hydroxylated steroids has provided better separation of some complex mixtures than the corresponding TMS ether derivatives[5,17–23]. This paper deals with the capillary GC behaviour of the TMS, DMES, DMnPS and DMiPS ether derivatives of bile acid methyl esters on SE-30 as the stationary liquid phase.

EXPERIMENTAL

Gas chromatography

A Shimadzu GC-7AG gas chromatograph equipped with a FID and a solventless injector was employed. The capiliary column (25 m × 0.35 mm I.D.) was coated with SE-30 (LKB, Stockholm, Sweden). The temperature of column oven was maintained at 270°; those of the injection port and detector were 300°. The flow-rate of carrier gas (helium) was 1.5 ml/min. Peak areas were calculated by use of a digital integrator (Shimaduzu Chromatopac C-RIA).

Samples and reagents

LCA, DCA, CDCA, UDCA and CA were purchased from Tokyo Kasei Kogyo (Tokyo, Japan), 12-KCDCA, HDCA, HCA, α-MCA and β-MCA from Steraloids (Wilton, N.H., U.S.A.) and [24-^{14}C]CDCA from Daiichi (Tokyo, Japan).

TMSI was purchased from Tokyo Kasei Kogyo. DMESI, DMnPSI and DMiPSI were prepared as described previously[24,25]. Hydrogen chloride methanol solution (5%, w/v) was prepared by introducing dry hydrogen chloride gas into methanol.

Derivatization

To each of 0.1–0.2 mg bile acids, 1 ml of 5% (w/v) hydrogen chloride methanol solution was added and allowed to stand for 30 min at room temperature. The solution was then evaporated to dryness under reduced pressure. The dried residue was treated with 100 μl of TMSI, DMESI, DMnPSI or DMiPSI, and was chromatographed over Sephadex LH-20 with 2 ml of chloroform–*n*-hexane–methanol (10:10:1)[26]. The eluate was evaporated, and the residue dissolved in *n*-hexane–pyridine

(95:5) and subjected to glass capillary GC. A mixture of 0.1 ml each of bile acids was treated in the same manner.

Sample preparation

To 0.1 ml of hepatic bile, 1.0 ml of 5α-cholestane–ethanol solution (50 μg/ml) was added as an internal standard and mixed well. After addition of 30 ml of ethanol and 1.0 ml of 0.1 *N* sodium hydroxide solution, the sample was shaken well by hand and further dispersed by a 10-min treatment with ultrasound, allowed to stand for 1 h at room temperature and then centrifuged for 10 min at *ca.* 1500 *g*. The supernatant was evaporated under reduced pressure, and the residue subjected to solvolysis according to the method described by Palmer[27]. After solvolysis the reaction mixture was evaporated under reduced pressure and dissolved in 2 ml of 1.25 *N* sodium hydroxide solution. Hydrolysis was performed in a sealed tube at 120° for 7 h.[28]. After acidifying with 3 *N* hydrochloric acid, the deconjugated bile acids were extracted three times with 10 ml of ethyl acetate. The ethyl acetate phase was washed with 10 ml of saturated sodium chloride solution and evaporated under reduced pressure. The dried residue was derivatized as described above.

RESULTS AND DISCUSSION

Bile acids (LCA, DCA, CDCA, HDCA, UDCA, 12-KCDCA, CA, HCA, α-MCA and β-MCA) were converted into the TMS, DMES, DMnPS and DMiPS ether derivatives of their methyl esters, and the derivatives were used for investigation of their gas chromatographic properties.

In order to avoid a side-reaction on esterification, the bile acids were treated with 5% HCl–methanol solution[3]. This esterification step at room temperature was shown to proceed quantitatively by utilizing [24-^{14}C]CDCA. Each of the bile acid methyl esters was readily silylated with TMSI, DMESI, DMnPSI and DMiPSI at room temperature and gave a single, well-shaped GC peak, suggesting that the two-step derivatization proceeds smoothly and quantitatively.

Each of the products obtained by this derivatization method was confirmed by GC–mass spectrometry (MS) to be the silyl ether derivative of the respective bile acid methyl ester. The mass spectra of DMES ether derivatives are summarized in Table I. In contrast to the corresponding TMS ether derivatives, the DMES ether derivatives have a tendency to yield characteristic fragment ions with abundant intensity in the high mass region.

The methylene unit (MU) values of the DMES, DMnPS and DMiPS ether derivatives were increased in comparison with those of the corresponding TMS ether derivatives, and this was multiplied by increasing the number of the hydroxyl groups in the molecule. Table II lists the MU values of the TMS, DMES, DMnPS and DMiPS ether derivatives of bile acid methyl esters on a glass capillary column, coated with SE-30.

The DMES, DMnPS and DMiPS ether derivatives of bile acid methyl esters were eluted in sequence according to the number of hydroxyl groups in the molecule, whereas in the case of the corresponding TMS ether derivatives the elution order of UDCA and CA was interchanged when SE-30 was used.

The DMES, DMnPS and DMiPS ether derivatives not only enabled bile acids

TABLE I

MS DATA OF THE DMES ETHER DERIVATIVES OF BILE ACID METHYL ESTERS

Mass spectra were obtained under the conditions described under Experimental. Under other ions, the five most intense ions above *m/z* 100 (other than those previously specified and those solely attributable to isotope peaks) are cited.

Bile acid	*MW*	*Relative intensity (%)*			
		$[M]^+$	$[M-15]^+$	$[M-29]^+$	*Other ions*
LCA	476	1.0	5.9	100.0	372(71) 341(37) 323(54) 215(57) 161(25)
DCA	578	0.4	5.5	100.0	370(11) 255(55) 208(10) 147(7) 161(6)
CDCA	578	0.3	0.3	4.4	445(65) 371(100) 163(25) 161(25) 149(6)
UDCA	578	0.6	6.2	100.0	475(18) 446(13) 440(12) 369(41) 339(11)
HDCA	578	1.3	0.4	10.2	371(100) 209(18) 175(18) 161(40) 135(30)
CA	680	0.2	5.2	100.0	547(8) 473(7) 369(11) 337(7) 253(20)
HCA	680	0.3	—	0.6	547(14) 369(100) 337(11) 161(14) 159(17)
α-MCA	680	0.4	0.2	0.6	547(37) 472(100) 369(97) 209(25) 159(27)
β-MCA	680	0.2	0.7	6.7	547(70) 369(100) 313(48) 209(57) 159(18)
12-KCDCA	592	9.6	3.3	17.2	488(100) 384(51) 367(90) 243(49) 229(52)

to be divided into the three distinct groups of mono-, di- and trihydroxylated bile acids, but also greatly improved the separation of individual bile acids in comparison with the corresponding TMS ether derivatives.

Table II also shows the $\Delta[Um]$ value[24], which is defined as the difference of the MU values between the TMS and the DMES, DMnPS, or DMiPS ether derivatives of a bile acid methyl ester. $\Delta[Um]$ increases in the order of $\Delta[Um]_E < \Delta[Um]_{nP} < \Delta[Um]_{iP}$. Among these silyl ether derivatives, the largest change in MU values was observed between the TMS and DMES ether derivatives ($\Delta[Um]_E$). The $\Delta[Um]_{nP}$ and $\Delta[Um]_{iP}$ values were found to be close to each other. The averages and standard deviations of $\Delta[Um]_E$, $\Delta[Um]_{nP}$ and $\Delta[Um]_{iP}$ were 2.21 ± 0.08, 3.30 ± 0.13 and

TABLE II

GC DATA OF TMS, DMES, DMnPS AND DMiPS ETHER DERIVATIVES OF BILE ACID METHYL ESTERS

Methylene unit values were determined at 270°. $\Delta[Um]$ value is defined as the difference of methylene unit values between TMS and DMES, DMnPS or DMiPS ether derivatives (see text).

Bile acid	*Methylene unit value*				$\Delta[Um]_E$	$\Delta[Um]_{nP}$	$\Delta[Um]_{iP}$
	TMS	*DMES*	*DMnPS*	*DMiPS*			
Lithocholic acid	31.18	32.37	33.04	33.14	1.19	1.86	1.96
Deoxycholic acid	31.83	33.93	35.00	35.28	2.10	3.17	3.45
Chenodeoxycholic acid	32.05	34.23	35.32	35.65	2.18	3.27	3.60
Ursodeoxycholic acid	32.46	34.70	35.76	36.10	2.24	3.30	3.64
Hyodeoxycholic acid	32.24	34.45	35.76	36.02	2.31	3.52	3.78
12-Ketochenodeoxycholic acid	33.94	36.17	37.20	37.57	2.23	3.26	3.63
Cholic acid	32.24	35.54	37.18	37.93	3.30	4.94	5.69
Hyocholic acid	33.03	36.11	37.54	37.46	3.08	4.51	4.43
α-Muricholic acid	32.12	35.25	36.75	37.37	3.13	4.63	5.21
β-Muricholic acid	33.16	36.29	37.73	38.47	3.13	4.57	5.31

3.62 ± 0.12 for dihydroxylated bile acids ($n = 5$), and 3.16 ± 0.10, 4.66 ± 0.19 and 5.16 ± 0.53 for trihydroxylated bile acids ($n = 4$). A linear relationship exists between the $\Delta[Um]$ and the number of hydroxyl groups in the bile acid molecule. The average $\Delta[Um]_E$ was related to the number of hydroxyl groups in the molecule, as reported previously[24]. The $\Delta[Um]_E$ value may thus be useful for estimating the number of the hydroxyl groups in an unknown bile acid by GC.

Comparing the differences in MU values between pairs of isomers, the CDCA–UDCA and α-MCA–β-MCA separations were enhanced in the order of the DMiPS < TMS < DMES < DMnPS and TMS < DMES < DMnPS < DMiPS ether derivatives, respectively. However, drastic separation of isomers was not produced by increasing the carbon number in the silyl groups. The DMnPS and DMiPS ether derivatives may be unsuitable for complete separation of the minor bile acids on SE-30, as seen in the MU values of CDCA and HDCA.

Among the derivatives tested, the DMES ether derivatives of bile acid methyl esters exhibited the best separation on a 25-m SE-30 glass capillary column. Fig. 1 shows the separation of the DMES ether derivatives of bile acid methyl esters. The DMES ether derivatives gave sharp, symmetrical peaks in the elution order LCA, DCA, CDCA, HDCA, UDCA, α-MCA, CA, HCA, 12-KCDCA and β-MCA. The DMES ether derivatives of these bile acid methyl esters were completely separated within 30 min, but the HCA and 12-KCDCA derivatives gave similar MU values.

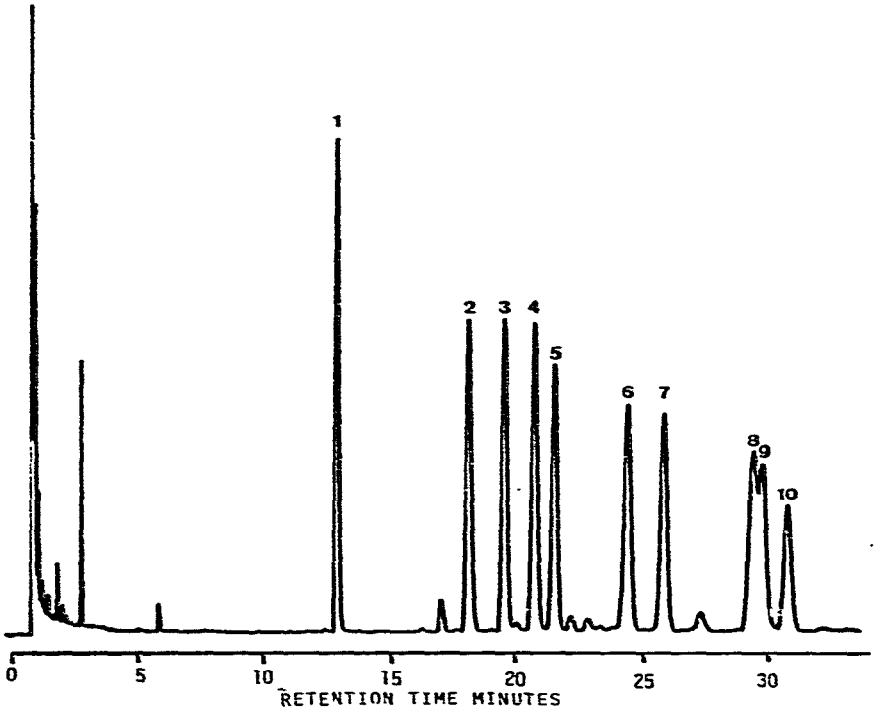

Fig. 1. Capillary gas chromatogram of the DMES ether derivatives of bile acid methyl esters. A mixture of 100 ng each of the authentic bile acids was injected. Peaks: 1 = LCA; 2 = DCA; 3 = CDCA; 4 = HDCA; 5 = UDCA; 6 = α-MCA; 7 = CA; 8 = HCA; 9 = 12-KCDCA; 10 = β-MCA.

TABLE III

RECOVERY OF TAURINE-CONJUGATED BILE ACIDS ADDED TO HUMAN HEPATIC BILE

Bile acid	*Added (μg/0.1 ml)*	*Recovered (μg/0.1 ml)*			*Recovery (%)*			*Mean ± S.D. (%)*
LCA	0.62	0.51	0.53	0.51	81.6	85.7	82.3	83.2 ± 2.19
DCA	6.41	5.53	5.77	5.68	86.3	90.0	88.6	88.3 ± 1.87
CDCA	91.08	80.24	83.07	80.84	88.1	91.2	88.8	90.3 ± 2.23
UDCA	6.66	5.52	5.95	5.70	82.9	89.3	85.6	85.9 ± 3.21
CA	120.85	109.00	113.84	107.52	90.2	94.2	89.0	91.1 ± 2.72

A combination of the present DMES ether derivatives of bile acid methyl esters and glass capillary GC was applied to the quantitation of bile acids in the extracts from human hepatic bile. A linear relationship was found between the peak areas and amounts of bile acids in the range of 10–250 ng when cholestane was used as an internal standard. The detection limit of LCA was found to be 500 pg, with a signal-to-noise ratio of 5:1.

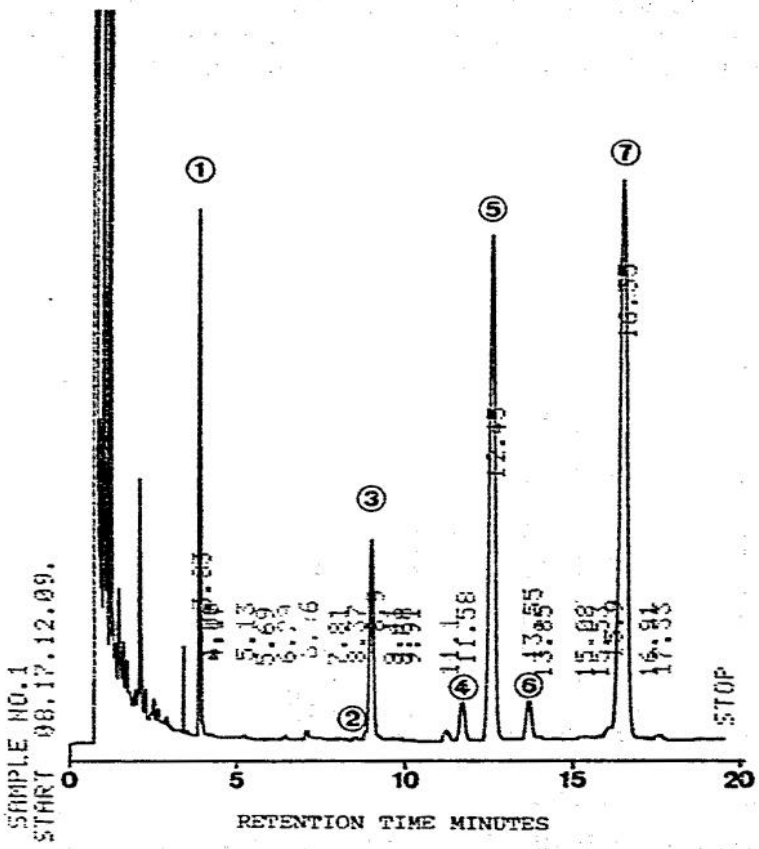

Fig. 2. Capillary gas chromatogram of the DMES ether derivatives of bile acid methyl esters in an extract from human hepatic bile. Peaks: 1 = 5α-cholestane (internal standard); 2 = LCA, 3 = cholesterol; 4 = DCA; 5 = CDCA; 6 = UDCA; 7 = CA.

Taurine-conjugated bile acids were added to the hepatic bile, the amount of each being half of that of the endogenous bile acids. Table III shows the analytical data and recovery of taurine-conjugated bile acids. All bile acids added were recovered in the range of 83–91%, and there was no statistically significant difference between different bile acids and between different concentrations.

Fig. 2 shows a typical gas chromatogram, obtained from an extract of 0.1 ml of human hepatic bile, containing an internal standard of cholestane. The peaks corresponding to CDCA and CA were calculated to represent *ca.* 150 and 200 ng, respectively.

The present method may be useful for the study of the bile acid metabolism and for the quantitation of bile acid such as LCA, which has been claimed to be one of the sources of hepatitis. Details of the application of this method to biological fluids will be discussed elsewhere.

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