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STUDIES ON STEROIDS

CCXXVIII.* TRACE ANALYSIS OF BILE ACIDS BY GAS CHROMATOGRAPHY–MASS SPECTROMETRY WITH NEGATIVE ION CHEMICAL IONIZATION DETECTION

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SUMMARY

A suitable derivatization method for the trace analysis of bile acids by gas chromatography (GC) in combination with negative ion chemical ionization (NICI) mass spectrometry is described. Of various derivatives for the carboxyl group, the pentafluorobenzyl (PFB) ester provided the highest value of the ratio of the negative to the positive ion current. A characteristic carboxylate anion $[M - 181]^-$ was produced as the most abundant ion by the loss of the PFB group in NICI. The PFB esters were further derivatized to the dimethylethylsilyl (DMES) ethers, whereby lithocholic acid, deoxycholic acid, chenodeoxycholic acid, ursodeoxycholic acid and cholic acid were distinctly separated by GC on a cross-linked methyl silicone fused-silica capillary column. The detection limit for the PFB-DMES derivatives of dihydroxylated bile acids was 2 fg when the fragment ion was monitored at m/z 563 in the NICI mode using isobutane as a reagent gas.

INTRODUCTION

Bile acids are synthesized from cholesterol in the liver and assist the lipolysis and absorption of fats by the formation of mixed micelles in the intestinal lumen. In recent years, considerable attention has been directed to the biodynamics of bile acids in man in connection with the diagnosis of cerebro-hepato-renal syndrome, and the etiology of gastric and colon cancer. Therefore, a reliable method is urgently needed for the trace analysis of bile acids in tissue.

Among various methods, gas–liquid chromatography (GC) in combination with mass spectrometry (MS) is well recognized as a powerful tool for the profile

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analysis of bile acids in biological materials. The electron impact (EI) ionization mode is widely used but still unsatisfactory as regards the sensitivity for the determination of bile acids in tissue. Recently, negative ion chemical ionization (NICI) MS has been applied to the analysis of catecholamines[1,2] and prostaglandins[3,4], providing much higher sensitivity than EI-MS.

The present paper describes the development of a suitable derivatization method for the trace analysis of bile acids by means of GC–NICI-MS.

EXPERIMENTAL

Materials

Bile acids and stearic acid were obtained from Sigma (St. Louis, MO, U.S.A.) and Tokyo Kasei Kogyo (Tokyo, Japan), respectively. After repeated recrystallization from the appropriate solvents, their purities were checked by GC, thin-layer chromatography (TLC) and high-performance liquid chromatography. All chemicals employed were of analytical reagent grade and were purified by distillation or recrystallization prior to use.

Gas chromatography–mass spectrometry

Capillary GC–MS was carried out using a VG Analytical MM12030 quadrupole mass spectrometer equipped with a Hewlett-Packard HP 5790A gas chromatograph. Methane, isobutane, ammonia and nitrous oxide were used as reagent gases. Cross-linked methyl silicone fused-silica capillary columns (12.5 m × 0.2 mm I.D., 25 m × 0.3 mm I.D.; Hewlett-Packard) were inserted into the ion source through the direct inlet. The carrier gas was helium at a linear velocity of 45 cm/s. The test samples were injected through a Van den Berg solventless injector with an inlet pressure of 0.4–0.7 kg/cm^2. The injection port, column oven and ion source were kept at 230–300, 215–285 and 240–270°C, respectively. The ionization energy was 30 eV for the EI mode and 70 eV for the chemical ionization (CI) mode[7]. The emission current was 100 μA for the EI mode and 400 μA for the CI mode.

Derivatization of stearic acid and bile acids

Methyl (Me) and ethyl (Et) esters. Stearic acid and bile acids were treated with diazomethane, hydrochloric acid–methanol or hydrochloric acid–ethanol in the usual manner.

Hexafluoroisopropyl (HFIP) and trichloroethyl (TCE) esters. To a solution of stearic acid in trifluoroacetic anhydride–chloroform was added 1,1,1,3,3,3-hexafluoro-2-propanol or 2,2,2-trichloroethanol, and the whole was stirred at room temperature. HFIP ester: colourless needles, m.p. 32–33°C. TCE ester; colourless needles, m.p. 31–32°C.

p-Nitrophenyl (PNP) and N-succinimidyl (NSI) esters. To a solution of stearic acid and N,N′-dicyclohexylcarbodiimide in dioxane was added *p*-nitrophenol or N-hydroxysuccinimide, and the whole was stirred at room temperature. PNP ester: colourless needles, m.p. 69–70°C. NSI ester: colourless needles, m.p. 76–77°C.

Benzyl (BZ), p-nitrobenzyl (PNB) and pentafluorobenzyl (PFB) esters. To a solution of stearic acid in triethylamine–acetonitrile was added benzyl bromide, *p*-nitrobenzyl bromide or pentafluorobenzyl bromide, and the whole was stirred at

room temperature. BZ ester; colourless plates, m.p. 44–45°C. PNB ester: colourless needles, m.p. 55–56°C. PFB ester: colourless plates, m.p. 58–59°C.

The formation of esters was monitored by TLC and their structures were characterized by nuclear magnetic resonance spectroscopy.

Trimethylsilyl (TMS) and dimethylethylsilyl (DMES) ethers. Bile acid PFB esters obtainable by the method described above were treated with hexamethyldisilazane–trimethylchlorosilane in pyridine at 60°C for 1 h or with dimethylethylsilylimidazole in pyridine–hexane at 60°C for 1 h.

RESULTS AND DISCUSSION

An initial effort was directed to the development of suitable derivatization for the carboxyl group which facilitates efficient electron capture and the formation of stable anions. For this purpose, three types of stearic acid esters, *i.e.*, alkyl esters (Me, Et, HFIP and TCE esters), active esters (PNP and NSI esters) and benzyl esters (BZ, PNB and PFB esters) were prepared as model compounds (Fig. 1). The HFIP and PFB esters are known to be highly responsive to an electron-capture detector, whereas the active esters are used as key compounds forming the amide bond.

The typical EI mass spectra of each group are illustrated in Fig. 2. The Me ester exhibited relatively intense fragment ions at *m/z* 255, 143 and 87 formed by fission of the carbon chain of the stearic acid moiety, and a base peak at *m/z* 74 formed by the McLafferty rearrangement. A carbonium cation due to the loss of the alcohol moiety was also observed at *m/z* 267 as a base peak for the PNP ester. On the other hand, the PFB ester provided a prominent fragment ion at *m/z* 181 which is inherent to the PFB group. Other stearates exhibited similar fragmentation patterns depending on the type of esters. The fragment ions involving the ester moiety in the alkyl esters, the carbonium cation in the active esters and benzyl cations in the benzyl esters would be formed preferentially in the EI mode. These data implied that the benzyl esters may provide abundant carboxylate anions in the NICI mode.

The base peaks formed from various esters of stearic acid in the positive ion (PI) and NICI modes using isobutane as a reagent gas are listed in Table I. The alkyl esters gave intense quasi-molecular ions in both CI modes. The behaviour of the active esters and benzyl esters is of particular interest. As for the active esters, elimination of the alcohol group produced a carbonium cation in the PICI mode. On the other hand, the benzyl esters afforded a carbonium cation and a carboxylate anion as base peaks in the PICI and NICI modes, respectively. On the basis of these results,

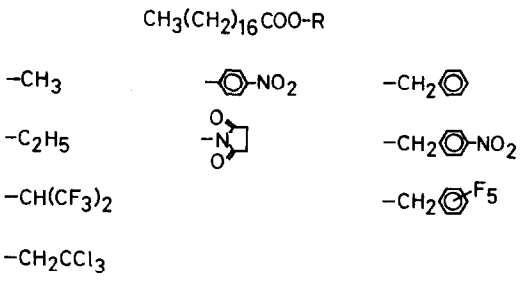


Fig. 1. Structures of various esters of stearic acid.

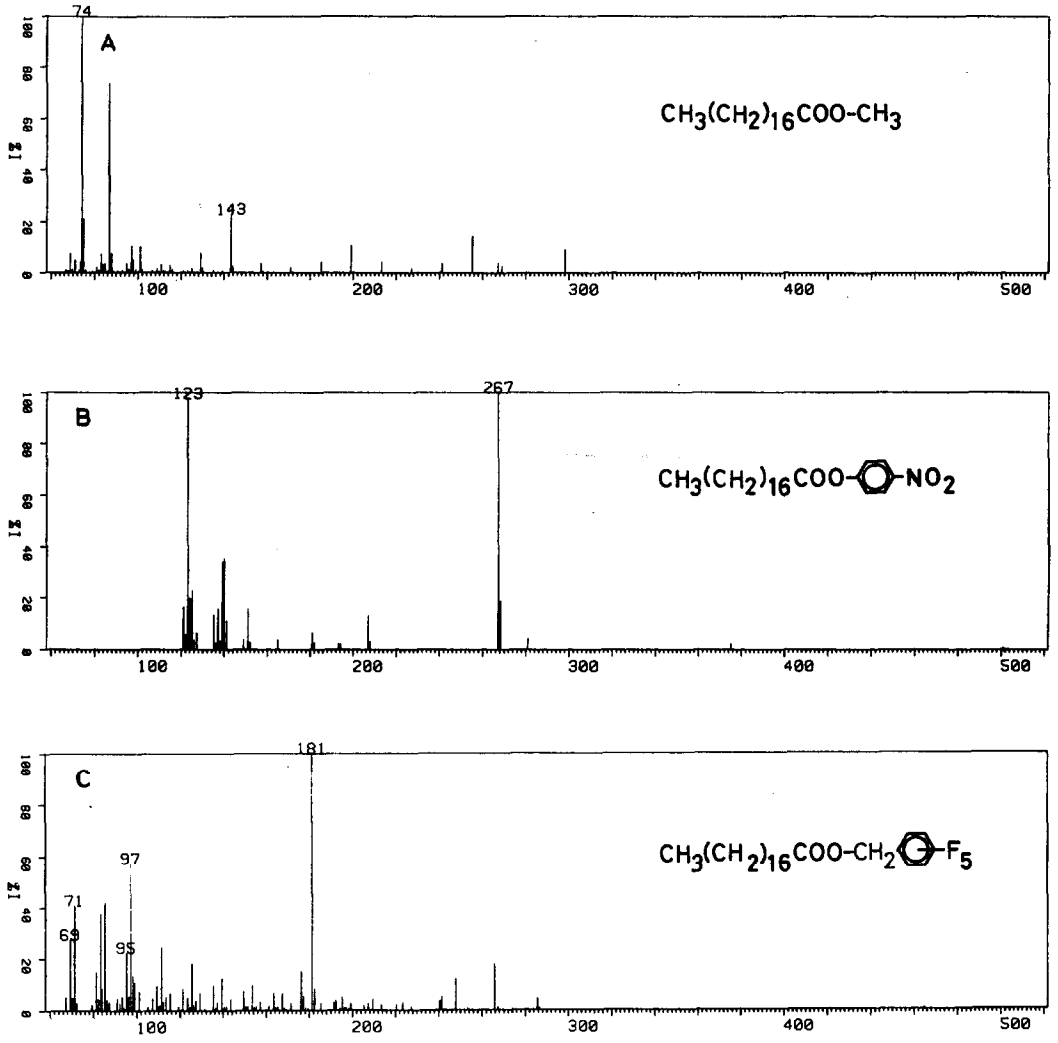


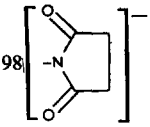
Fig. 2. EI mass spectra of Me (A), PNP (B) and PFB (C) esters of stearic acid. Ionization energy: 30 eV.

the PICI and NICI MS of the active esters and benzyl esters appear to be complementary. The ratios of the negative to the positive ion currents (N/P) of various stearates obtained by CI using isobutane were then determined (Table II). These data strongly indicated that the benzyl groups having high electron affinity could enhance the formation of anions in the NICI mode.

It is well known that negative ions can be produced by the capture of low energy electrons generated by the passage of an electron beam through an inert moderating gas such as methane, isobutane or ammonia, or by reaction with an ion like hydroxide which is readily formed by electron bombardment of a mixture of nitrous

TABLE I

BASE PEAKS FORMED FROM VARIOUS ESTERS OF STEARIC ACID BY PICI AND NICI

Compound R	*PICI*		*NICI*	
	Ion	Σ_{60} *(%)*	*Ion*	Σ_{60} *(%)*
Me	299 $[M + H]^+$	100	297 $[M - H]^-$	100
Et	313 $[M + H]^+$	100	311 $[M - H]^-$	100
NSI	267 $[CH_3(CH_2)_{16}CO]^+$	100	98 	57
BZ	91 $[CH_2C_6H_5]^+$	45	283 $[CH_3(CH_2)_{16}COO]^-$	48
PFB	181 $[CH_2C_6F_5]^+$	57	283 $[CH_3(CH_2)_{16}COO]^-$	100

oxide and methane. The former type is referred to electron-capture NICI, while the latter is called reactant ion NICI[5]. The effect of the reagent gas on NICI mass spectral fragmentation was therefore investigated. The results obtained are collected in Table III. The carboxylate anion from the Me and PNP esters, and the benzyloxy anion from the PFB ester formed by nucleophilic attack of the reactant ion, that is hydroxide, have not been observed before.

The results implied that introduction of the PFB group into the carboxyl function followed by electron-capture NICI using isobutane as a reagent gas would be most favourable for the formation of characteristic carboxylate anions. Accordingly, selected-ion monitoring (SIM) was carried out to determine the detection limit for stearic acid under these conditions. The stearate was monitored by its characteristic negative ion at m/z 283, the detection limit being estimated to be 20 fg at a signal-to-noise ratio of 10 as illustrated in Fig. 3.

Since individual bile acids have similar chemical structures, gas chromatographic resolution must be carefully considered and hence a suitable derivatization is important. The separation of bile acids was undertaken by means of capillary GC using a cross-linked methyl silicone fused-silica column (25 m × 0.3 mm I.D.). In-

TABLE II

RATIOS OF NEGATIVE TO POSITIVE ION CURRENTS (N/P) FOR VARIOUS ESTERS OF STEARIC ACID OBTAINED BY CI USING ISOBUTANE

Compound R	*N/P*	*Base peak in NICI (m/z)*
Me	0.03	297
Et	0.01	311
HFIP	1	167
TCE	1	283
PNP	0.3	138
NSI	1	98
BZ	100	283
PNB	30	283
PFB	1000	283

TABLE III

NICI MASS SPECTRAL DATA FOR VARIOUS ESTERS OF STEARIC ACID

Compound R	*Reagent gas*	*Relative intensity (%)*		
		$[M - H]^-$	$[CH_3(CH_2)_{16}COO]^-$	$[OR]^-$
Me	Isobutane	100		
	Methane–nitrous oxide	100	10	
PNP	Isobutane	10		100
	Methane–nitrous oxide		35	100
PFB	Isobutane		100	
	Methane–nitrous oxide		100	5

itially, bile acid PFB esters having hydroxyl groups at C-3, C-7 and C-12 were derivatized to the trimethylsilyl (TMS) ethers. The retention times relative to lithocholic acid (LCA) are listed in Table IV. The resolution of chenodeoxycholic acid (CDCA) and cholic acid (CA) was still unsatisfactory. It is sufficiently substantiated that displacement of a larger group for a methyl group in TMS improves the GC separation on a non-polar stationary phase, depending on how many hydroxyl groups are present[6,7]. Therefore, the PFB esters of bile acids were derivatized to the DMES ethers. From the data in Table IV, the retention increases with increasing number of hydroxyl groups, providing excellent separation of common bile acids.

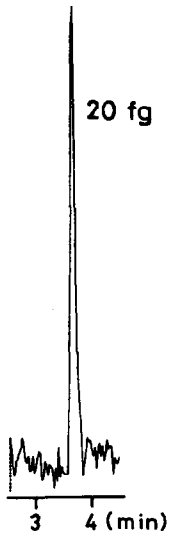


Fig. 3. Selected-ion chromatogram of stearic acid PFB ester at *m/z* 283.

TABLE IV

RETENTION TIMES OF PFB ESTER-TMS ETHERS AND -DMES ETHERS OF BILE ACIDS RELATIVE TO LITHOCHOLIC ACID (LCA)

5β-Cholanoic acid	*Relative retention time*	
	PFB-TMS	*PFB-DMES*
3α-OH (LCA)	1.000	1.000
3β-OH	0.998	1.026
7α-OH	0.746	0.742
7β-OH	0.847	0.846
12α-OH	0.724	0.705
12β-OH	0.682	0.679
3α,7α-$(OH)_2$ (CDCA)	1.149	1.366
3α,7β-$(OH)_2$ (UDCA)	1.235	1.449
3α,12α-$(OH)_2$ (DCA)	1.096	1.283
3α,12β-$(OH)_2$	1.009	1.195
7α,12α-$(OH)_2$	0.814	0.993
7α,12β-$(OH)_2$	0.773	0.913
7β,12α-$(OH)_2$	0.926	1.055
7β,12β-$(OH)_2$	0.921	1.093
3β,7α-$(OH)_2$	1.090	1.400
3β,7β-$(OH)_2$	1.246	1.516
3β,12α-$(OH)_2$	1.084	1.285
3β,12β-$(OH)_2$	1.065	1.310
3α,7α,12α-$(OH)_3$ (CA)	1.158	1.691
3α,7α,12β-$(OH)_3$	1.092	1.523
3α,7β,12α-$(OH)_3$	1.270	1.693
3α,7β,12β-$(OH)_3$	1.232	1.732
3β,7α,12α-$(OH)_3$	1.071	1.561
3β,7α,12β-$(OH)_3$	1.070	1.549
3β,7β,12α-$(OH)_3$	1.261	1.703
3β,7β,12β-$(OH)_3$	1.306	1.929

TABLE V

EI MASS SPECTRAL DATA FOR PFB ESTER-TMS ETHERS AND -DMES ETHERS OF BILE ACIDS

Bile acid		*Relative intensity (%)*				
		M − Me or M − Et	*M − $(TMSOH)_n$* or M − $(DMESOH)_n$*	*m/z 257 255 253*	*m/z 215 213 211*	*m/z 181*
LCA	TMS			14.8	100	36.4
	DMES	4.7	5.6	10.6	43.6	100
CDCA	TME			51.1	65.6	100
	DMES		15.1	22.1	10.5	100
UDCA	TMS			61.7	48.9	100
	DMES	18.2	9.1	16.4	14.6	100
DCA	TMS			100	9.4	36.5
	DMES	9.3		83.7	11.6	100
CA	TMS			100	17.0	67.4
	DMES	11.4	23.8	59.0	15.2	100

* n = Number of hydroxyl groups.

TABLE VI

NICI MASS SPECTRAL DATA FOR PFB ESTER-TMS ETHERS AND -DMES ETHERS OF BILE ACIDS

Bile acid		*Relative intensity (%)*		
		M − 181	*M − 181 − (TMS)$_n$**	*M − 181 − (TMSOH)$_n$ or M − 181 − (DMESOH)$_n$*
LCA	TMS	100	5.3	3.7
	DMES	100		12.6
CDCA	TMS		100	8.1
	DMES	100		21.0
UDCA	TMS	100		2.9
	DMES	100		14.2
DCA	TMS		100	2.9
	DMES	100		5.4
CA	TMS		100	2.2
	DMES	100		14.0

* n = Number of hydroxyl groups.

The EI and NICI mass spectral data for the TMS and DMES derivatives of bile acid PFB esters are collected in Tables V and VI. It has been reported that the EI mass spectra of the DMES derivatives of various steroids are characterized by inherent ions $[M - 29]^+$ with high relative intensities. The DMES derivatives of bile acid PFB esters, however, gave only low intensity ions in the high mass region, and base peaks assignable to the steroid nucleus or PFB group in the low mass region in the EI mode. On the other hand, in the NICI mode the DMES derivatives exhibited the characteristic negative ions $[M - 181]^-$ as base peaks formed by elimination of the PFB group (Fig. 4).

A typical selected-ion chromatogram of PFB-DMES derivatives of dihydroxylated bile acids, deoxycholic acid (DCA), CDCA and ursodeoxycholic acid (UDCA), monitored at m/z 563 in the NICI mode, is shown in Fig. 5. Approximately 2 fg of each bile acid could be detected at a signal-to-noise ratio of 10.

The combined use of the PFB-DMES derivative and capillary GC–NICI-MS

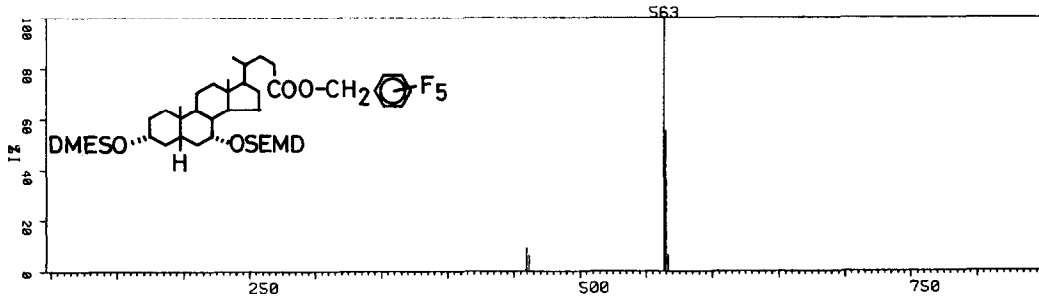


Fig. 4. Isobutane NICI mass spectrum of the PFB ester-DMES ether of CDCA.

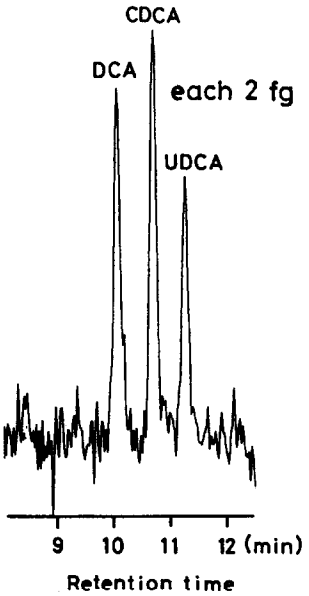


Fig. 5. Selected-ion chromatogram of PFB ester-DMES ethers of DCA, CDCA and UDCA at *m/z* 563.

would be useful and serve for the trace analysis of bile acids by SIM. We are now applying the method described to the determination of bile acids in liver tissue and the results will be reported elsewhere.

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