Journal of Chromatography, 239 (1982) 571–583
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROM. 14,690

CHARACTERIZATION OF BILE ACID METHYL ESTER ACETATE DERIVATIVES OF RAT BILE USING SOLVENTLESS GLASS CAPILLARY GAS CHROMATOGRAPHY AND ELECTRON IMPACT AND AMMONIA CHEMICAL IONIZATION MASS SPECTROMETRY

TAKESHI MURATA*, SEIJI TAKAHASHI, SHOZO OHNISHI and KIYOSHI HOSOI
Analytical Application Laboratory, Shimadzu Corporation, Nakagyo-ku, Kyoto 604 (Japan)
and
TOSHIAKI NAKASHIMA, YASUCHIKA BAN and KINYA KURIYAMA
Department of Pharmacology, Kyoto Prefectural University of Medicine, Kyoto (Japan)

SUMMARY

Methyl ester acetate derivatives of rat bile acids were analysed by a combined technique of solventless glass capillary gas chromatography and electron impact and ammonia chemical ionization mass spectrometry. Glass capillary columns ensure an excellent separating efficiency and effectiveness in detailed studies of many kinds of bile acid components. We found that the ammonia chemical ionization mass spectrometry gave $(M+NH_4)^+$ ions for all the methyl ester acetate derivatives of bile acids, and that these ions had intensities equal, or almost equal, to that of the base peak. This indicates that the technique is useful for the identification of unknown components, especially at trace levels. The results of electron impact and ammonia chemical ionization mass spectrometry of the major compound and the product obtained by catalytic hydrogenation of the compound indicated that this bile acid was a derivative of $\Delta\beta$-muricholic acid having a double bond in the side-chain. We also found that most components had one double bond in their side-chain.

INTRODUCTION

Bergström *et al.*[1], Eneroth and co-workers[2,3], Sjövall and co-workers[4,5] and Miyazaki and co-workers[6,7] have presented reports on the gas chromatography (GC)–mass spectrometry (MS) of bile acids. Using electron impact ionization mass spectrometry (EI-MS), they made detailed study of the method of derivatization and fragmentation and succeeded in obtaining useful information on the structures of bile acids.

Chemical ionization mass spectrometry (CI-MS)[8,9], which has the advantages that fewer fragment ions are produced and that the molecular ion intensities are higher, has been used for the analysis of bile acids, in addition to EI-MS.

Szczepanic *et al.*[10] analysed bile acid methyl ester derivatives by isobutane CI-MS, and found that only keto bile acids gave MH^+ ions, that monohydroxy, dihydroxy and trihydroxy bile acids gave no MH^+ ions, and that, although these bile acids

0021-9673/82/0000–0000/$02.75

gave $MH^+ - 60$, $MH^+ - 60 \times 2$, and $MH^+ - 60 \times 3$ ions (which are molecular ions without the acetic acid groups), these ions were not very useful for the qualitative analysis of multi-component samples.

Muschik *et al.*[11] analysed bile acid methyl esters by GC–CI (CH_4)-MS with methane as the reagent gas, and found that only keto bile acids gave MH^+ ions, and that all the other components gave fragment ions which were $(M \pm H)^+$ ions from which methanol, water or methane groups were lost.

Kuriyama and co-workers[12,13] analysed bile acid methyl ester acetate derivatives by GC–CI (NH_3)-MS, and found that the $(M + NH_4)^+$ ions which are the QM^+ ions were detected at intensities almost equal to that of the base peak. They succeeded in identifying an unknown component as β-muricholic acid having a double bond in its side-chain. A. Maquestion *et al.*[14] reported the formation of some cation to aliphatic and aromatic ketones using ammonia chemical ionization mass analyzed ion kinetic energy spectrometry, and Carroll *et al.*[15] also reported the adduct ion formation in ammonia chemical ionization mass spectrometry.

Various column packings, such as OV-1, OV-17, OV-225, QF-1, AN-600 and SP-525, were tried in this analysis, but none gave satisfactory separations. Although glass capillary columns ensure for better separations, there have been few reports on their application in GC–MS analysis. Among the few examples of the reports are the following: Yanagisawa *et al.*[16] carried out trace quantitative analyses of bile acids in human liver tissue by the selected ion monitoring method, using an SE-30 wall-coated open tubular (WCOT) column (25 m × 0.35 mm I.D.); Barnes[17] used an SP-225 WCOT column (30 m × 0.2 mm I.D.) for studying the permethylation of bile acids.

We reported[12,13] that, in GC–CI (NH_3)-MS analysis of bile acid methyl ester acetate derivatives, all the bile acids gave $(M + NH_4)^+$ ions. Our recent experiments showed that the solventless glass capillary GC-CI (NH_3)-MS method is effective for the identification of so far unknown peaks and minor peaks.

EXPERIMENTAL

Materials

The authentic bile acids and cholylglycine hydrolase (*Clostridium welchii* acetone powder, type IV) were purchased from Sigma (St. Louis, MO, U.S.A.). Amberlite XAD-2 and Amberlyst A-15 (H^+) resins were supplied by Rohm and Haas (Philadelphia, PA, U.S.A.). The XAD-2 resin was treated as follows prior to use. A suspension of resin in 3–5 volumes of methanol containing 0.5% hydrochloric acid was heated for 1 h under gentle refluxing, then washed successively with methanol and water. The column used was a WCOT type (20 m × 0.36 mm I.D.), coated with SE-30 (LKB, Stockholm, Sweden).

Preparation of bile

Male Wistar rats (200–250 g) were fasted for 24 h before the analysis, then anaesthetized with pentobarbital (50 mg/kg, i.p.). Bile samples were collected for 30 min after inserting a bile duct cannula. The collected samples were stored at −15°C until the chemical treatments for GC–MS analysis.

The collected bile sample was added dropwise to 50 ml of ethanol and agitated in an ultrasonic bath, then the solution was heated under refluxing for 10 min. After

cooling to room temperature, the insoluble materials were filtered off and the filtrate was concentrated under reduced pressure. A solution of the residue in 50 ml of 72% ethanol was passed through a column of Amberlyst A-15 (H^+) (50 × 16 mm I.D.) followed by the elution with 10 ml of 72% ethanol. The effluent from this column was concentrated *in vacuo* and the residue was dissolved in 50 ml of 90% ethanol.

A sample containing unconjugated and glycine- and taurine-conjugated bile acids was dissolved in 8 ml of 0.2 M acetate buffer (pH 5.6) and then hydrolysed with cholylglycine hydrolase[18]. After acidifying and extracting with diethyl ether, the unconjugated and hydrolysed bile acids were methylated with diazomethane and then acetylated by heating with 2 ml of acetic anhydride at 140°C for 4 h[19]. Each methylated and acetylated sample was evaporated to dryness and the residue was dissolved in 0.2 ml of acetone.

Catalytic hydrogenation of the methyl ester acetate of bile acids was performed at room temperature in 5 ml of 90% ethanol using 3 mg of platinum oxide.

Gas chromatograph–mass spectrometer

GC–EI/CI-MS analyses were performed on LKB-2091 and Shimadzu AUTO GCMS-6020 instruments equipped with an EI/CI dual ion source using an all-glass, solventless injector constructed according to Van den Berg and Cox[20], mounted in the heated injector block of the column oven.

Open-tubular glass capillary columns (20 m × 0.36 mm I.D.) coated with SE-30, were held in aluminium column holders. The column temperature was programmed from 250 to 290°C at 2°C/min and kept isothermal at 290°C until the last peak had been recorded.

The mass spectrometric conditions for EI-MS were as follows: the ion source temperature was held at 310°C; the mass spectra were all obtained at an electron energy of 20 eV, an emission current of 60 μA and an accelerating voltage of 3.5 kV. In the CI-MS the ion source temperature was held at 230°C, the mass spectra were all obtained at an electron energy of 500 eV, an emission current of 500 μA and of an accelerating voltage of 3.5 kV. Ammonia was used as the reagent gas and the pressure in the ion source was adjusted to 0.9 Torr.

The data processing system included a GCMS-PAC 500 FDG consisting of an OKITAC 4300C minicomputer with 16K core, a printer–plotter and a magnetic disk. The mass spectra were stored in a magnetic disk at 3-sec intervals and the mass chromatograms of desired m/z values were recorded.

RESULTS AND DISCUSSION

Gas chromatography–ammonia chemical ionization mass spectrometry

In the GC–EI-MS analysis of bile acids, keto bile acids were the only compounds that gave molecular ions; no other bile acids gave molecular ions in any derivative form. If isobutane of methane was used as the reagent gas in the CI-MS of bile acids, as used by Szczepanik *et al.* and Muschik *et al.*, QM^+ ions were given only by keto bile acids.

We found in analyses of sugars[21] and triglycerides[22] that, although QM^+ ions are not given when isobutane or methane is used as the reagent gas, the molecular ions are seen if ammonia is used. We applied this method to the analysis of bile acids.

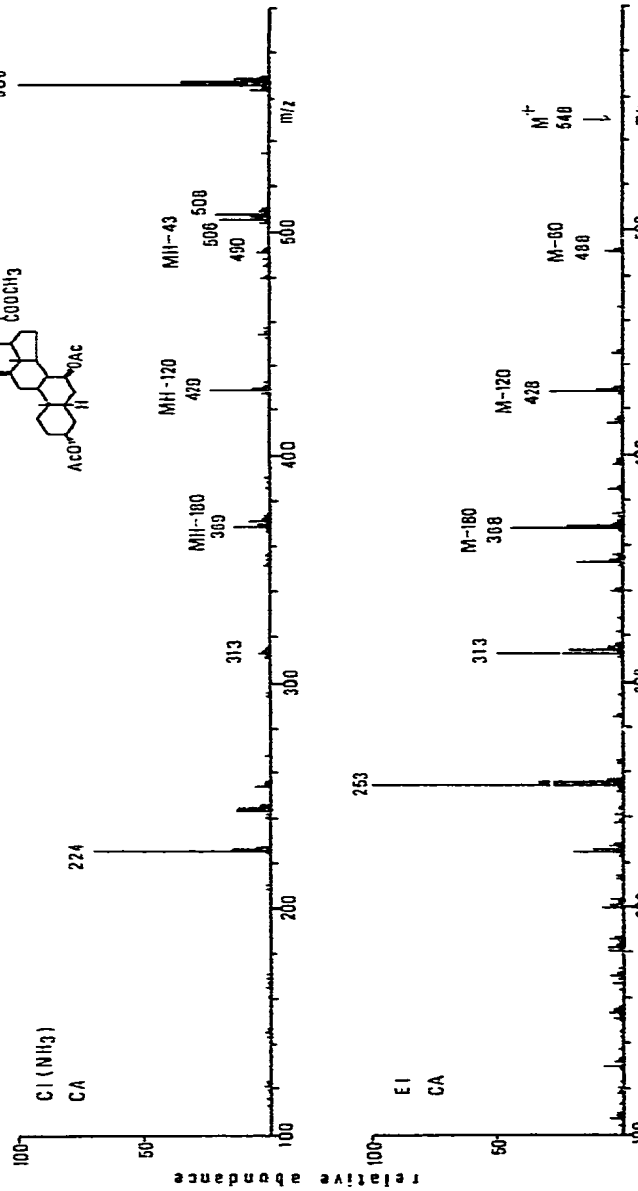

Fig. 1. CI(NH_3) and EI mass spectra of cholic acid methyl ester acetate. The $(M + NH_4)^+$ ion is recorded at *m/z* 566 as the base peak in the CI(NH_3) mass spectrum, but is not recorded in the EI mass spectrum.

We found that in the CI (NH_3)-MS analysis of bile acid methylester acetate derivatives, all of the keto bile acids, dihydroxy bile acids and trihydroxy bile acids gave $(M+NH_4)^+$ ions. However, if the bile acids were derivatized into methyl ester trimethylsilylated derivatives, none of them gave $(M+NH_4)^+$ ions even when ammonia was used as the reagent gas.

Figs. 1 and 2 show the CI(NH_3) and EI-MS spectra of cholic acid methyl ester derivatives (CA) and β-murucholic acid methyl ester acetate (β-MCA). These mass spectra are presumed that the high intensity of the $(M + NH_4)^+$ ions is attributable to the bonding of the basic NH_4^+ ion in the molecule. This intramolecular bonding occurs in CA at the 3α-OAc, 7α-OAc and 12α-OAc, and in β-MCA of 3α-OAc, 6β-OAc and 7β-OAc positions. The $-COOCH_3$ group hardly combines with NH_4^+ ion in the molecule. This can be seen from the CI(NH_3)-MS spectra of the methyl ester trimethylsilylated derivatives. In these derivatives, the only position where intramolecular bonding with NH_4^+ ion can possibly occur is the $-COOCH_3$ group, but in fact this group does not give any $(M+NH_4)^+$ ion. It is known that $(M+NH_4)^+$ ions are seen in the CI(NH_3)-MS spectra of fatty acid methyl esters. The fact that the $-COOCH_3$ group of some compounds gives $(M+NH_4)^+$ ions and that of other compounds does not may be explained by the influence of the stereoscopic conformation of the molecules concerned.

In the CI(NH_3) mass spectrum, the $(M+NH_4)^+$ ion is recorded at *m/z* 566 as the base peak. In the EI-mass spectrum, the peak of the largest mass number is the M−60 (CH_3COOH) peak recorded at a low intensity at *m/z* 488. The CI(NH_3) mass spectrum always has a peak at *m/z* 224, probably given by the steroid skeleton. The EI mass spectra give more information on chemical structures. In Figs. 1 and 2, for example, CA and β-MCA, which are both trihydroxy bile acids, can be easily differentiated in the EI mass spectrum, while they are hardly differentiated in the CI(NH_3) mass spectrum.

Some components cannot be separated by either a packed column or a capillary column. Fig. 3 shows the CI(NH_3) and the EI mass spectra of hyodeoxycholic acid methyl ester acetate (HDCA) and 7-ketodeoxycholic acid methyl ester acetate (7-keto-DCA), which cannot be separated even with a capillary column. In the EI mass spectrum, these two compounds, which are recorded as main peaks, or peaks as intense as the main peaks, on the chromatogram can be identified from the fragment ion peaks, but they cannot be identified from the fragment peaks if they are not so intense on the chromatogram and, moreover, are overlapped by intenser peaks. In the CI(NH_3) mass spectrum, the QM^+ ions of HDCA and 7-keto-DCA are recorded at *m/z* 508 and 522, respectively. These two peaks show that dihydroxy and keto bile acids coexist. The EI mass spectrum is informative about chemical structure only when the information about molecular weight is given. The three ions at *m/z* 444 (M−60), *m/z* 426 (M−60−18) and *m/z* 384 (M−120−18), which are fragment ions of 7-keto-DCA, cannot be distinguished from the fragment ions of *m/z* 444 (M-60-42), *m/z* 426 (M−120) and *m/z* 384 (M−180−42) of trihydroxy bile acids, which are given when one double bond exists in the molecules.

Fig. 4 shows the CI(NH_3) and EI mass spectra of the main peak of Δβ-muricholic acid methyl ester acetate. In the CI(NH_3) mass spectrum, the QM^+ ion was recorded at *m/z* 564. In the spectra of CA and β-MCA shown in Figs. 1 and 2, the QM^+ ions were recorded at *m/z* 566. Compared with these spectra, that in Fig. 4 gives

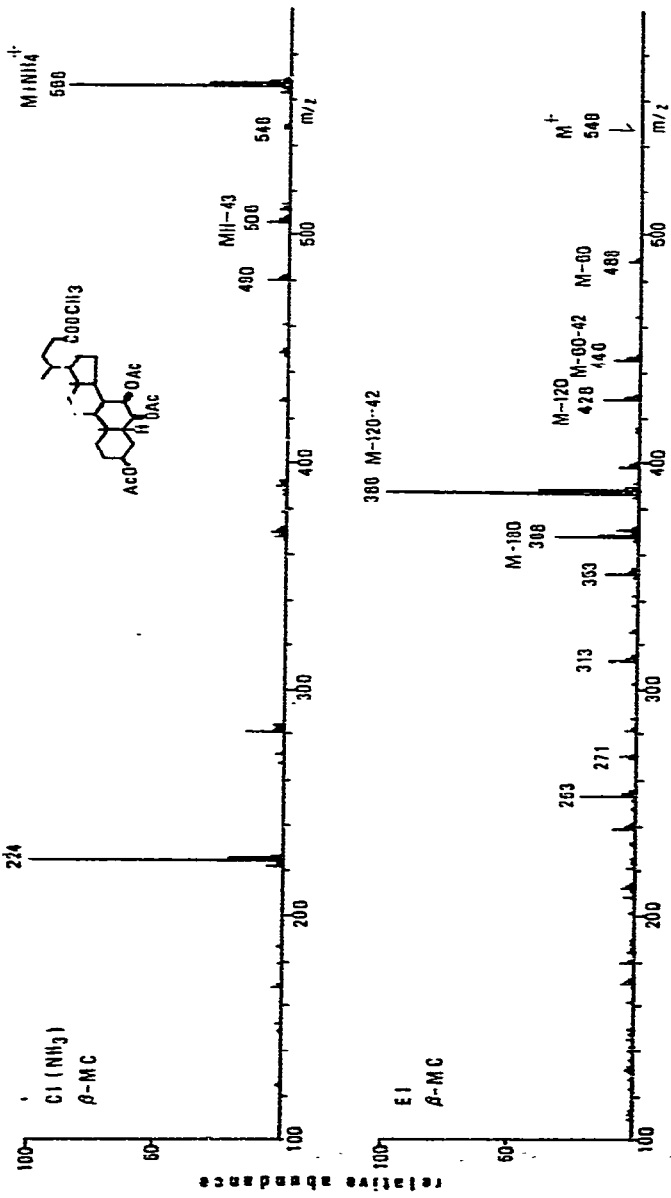

Fig. 2. CI(NH_3) and EI mass spectra of β-muricholic acid methyl acetate.

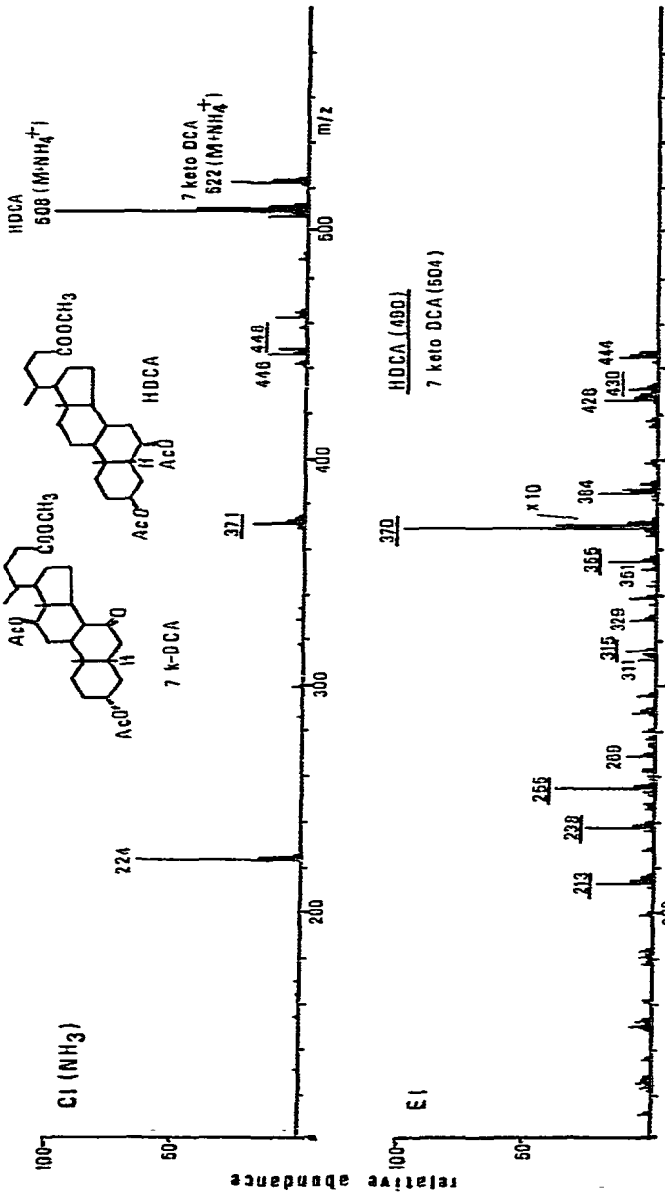

Fig. 3. CI(NH_3) and EI mass spectra of 7-ketodeoxycholic acid methyl ester acetate contained in hyodeoxycholic acid methyl acetate. In the CI(NH_3) mass spectrum the QM^+ ions are recorded at *m/z* 508 and 522, but in the EI spectrum these two peaks cannot be differentiated.

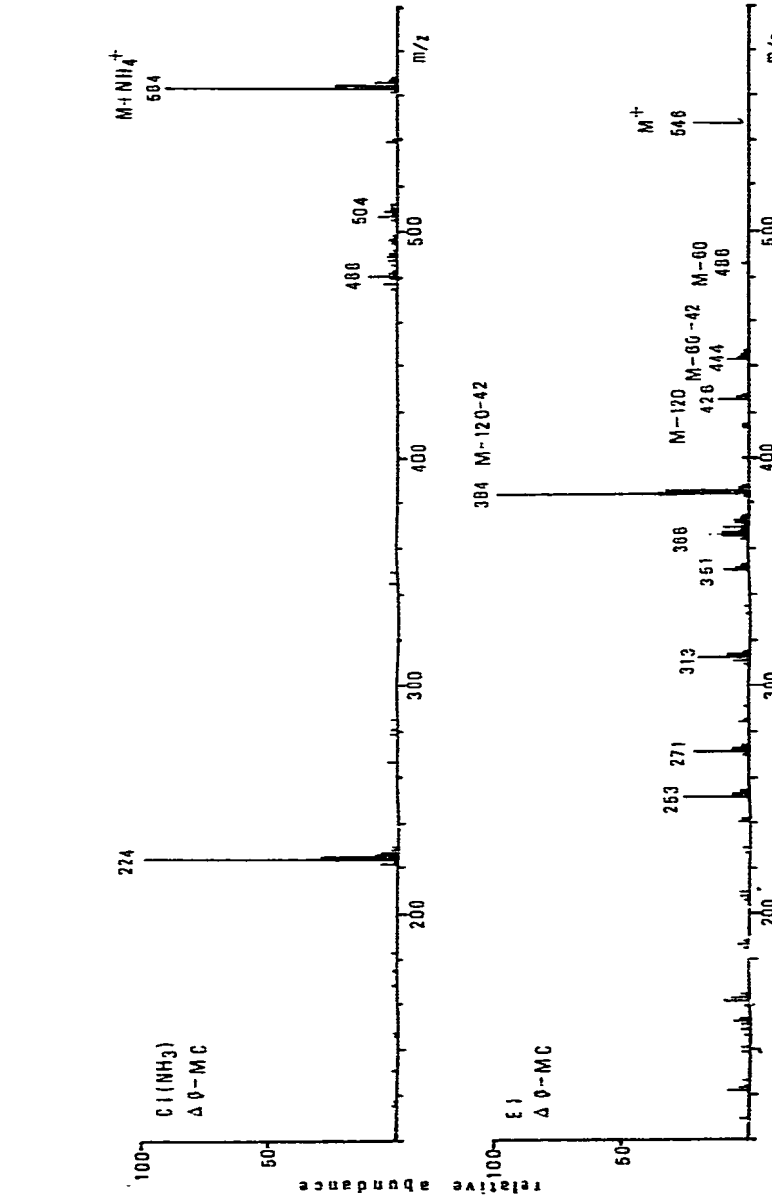

Fig. 4. CI(NH_3) and EI mass spectra of $\Delta\beta$-muricholic acid methyl ester acetate. The pattern is similar to that of β-MCA shown in Fig. 2, but the fragment ions M^+, M − 60, M − 60 − 42, M − 120, M − 120 − 42 and M − 180 and the ions below *m/z* 351 are recorded 2 a.m.u. lower.

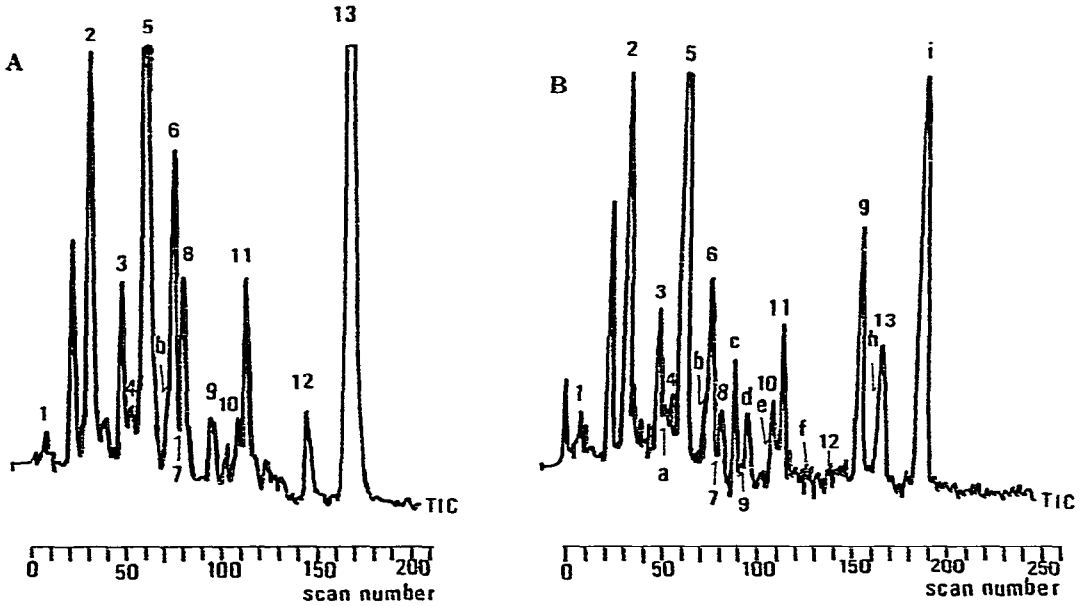

Fig. 5. Total ion chromatogram obtained by glass capillary column GC–EI-MS. (A) After catalytic hydrogenation; (B) before catalytic hydrogenation.

the QM^+ ion at a position lower by 2 a.m.u. This shows the presence of one double bond in the molecule. The combination of the $CI(NH_3)$ and EI mass spectra shows that this compound is $\Delta\beta$-MCA, which is β-MCA having a double bond in the molecule. Also, the data provided by the $CI(NH_3)$ mass spectrum on the QM^+ ion show that the molecules of dihydroxy and trihydroxy bile acids, as well as $\Delta\beta$-MCA, have double bonds in their molecules. The EI mass spectra were further studied to determine the positions of double bonds. Not only the molecular ion but also the fragment ions such as M − 60, M − 60 − 42, M − 120, M − 120 − 42 and M − 180 and the ion of *m/z* 351 of trihydroxy bile acids were recorded at positions 2 a.m.u. lower than the ions having no double bonds. In other words, the fragment ions produced in the process in which the hydroxy groups are cleaved off one by one are recorded at positions 2 a.m.u. lower. The *m/z* 313 and 253 ions produced when the side-chains are cleaved off are given by both compounds having double bonds and those having no double bonds (dihydroxy groups commonly give *m/z* 315 and 255 ions).

We can conclude from these facts that the double bond is present in the side-chains. We could not obtain the fragment ions that indicate the position of the double bond in the side-chains, but it is probable that it occurs at one of the positions between the 20th and 21st, 20th and 22nd and 22nd and 23rd positions.

Further, the sample was treated by catalytic hydrogenation, and analysed by $CI(NH_3)$-MS and EI-MS. None of the compounds that we presumed to have a double bond was detected.

Glass capillary column GC–electron impact ionization mass chromatography

As described earlier, the technique using $CI(NH_3)$-MS is effective for determining molecular weights but is not very effective for structural studies. We identified the

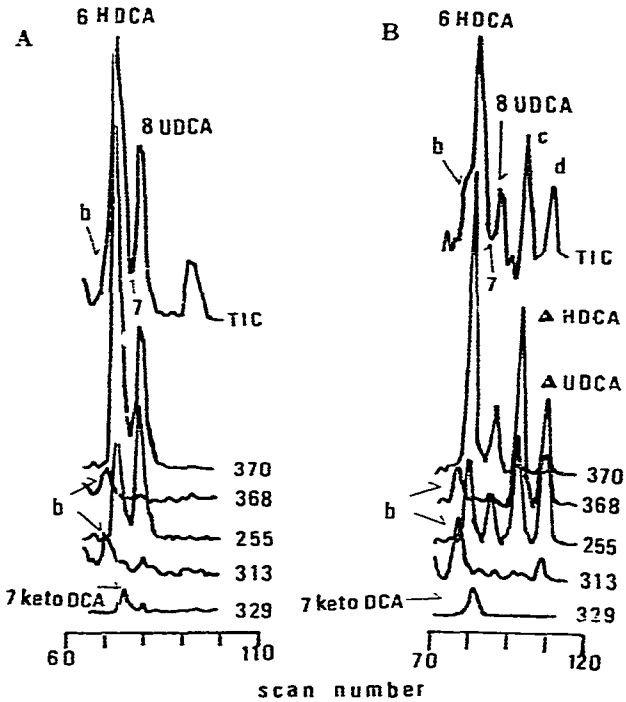

Fig. 6. Mass chromatograms from scans 60–110 (A) and from scans 70–120 (B). (A) is the MC of the *m/z* 370 ion, and gives the peaks for HDCA and UDCA. (B) is the MC of the *m/z* 370 and ions and gives the peaks for HDCA, UDCA, ΔHDCA and ΔUDCA. The MC of the *m/z* 255 ion formed when two acetic acid groups and side-chains are cleaved off is common to HDCA, UDCA, ΔHDCA and ΔUDCA. This shows the presence of double bond in the side-chains. The MC of the *m/z* 329 ion shows the presence of 7-keto-DCA. The ion at *m/z* 313 is not influenced by catalytic hydrogenation. The EI mass spectrum shows that it is trihydroxy bile acid.

components by electron impact ionization mass chromatography (MC). Fig. 5 shows the total ion chromatograms (TIC) of samples before (B) and after catalytic hydrogenation (A). Of the many components detected, those marked with numerals and letters were identified; those marked with numerals correspond to representative bile acid components, and those with letters were identified in our experiments.

The peaks a–h, except for b, in the chromatogram B are not seen in chromatogram A. This indicates that all of these peaks have double bonds.

Fig. 6 shows the data obtained by MC. The ion at *m/z* 370 ($M-CH_3COOH \times 2$) is seen on both chromatograms A and B as HDCA and UDCA. The ion at *m/z* 368 ($M-CH_3COOH \times 2$), lower than *m/z* 370 by 2 a.m.u., is seen only on chromatogram B. This shows that sample B contains ΔHDCA and ΔUDCA.

The ion at *m/z* 255 corresponds to the dihydroxy bile acid from which two acetic acid groups and side-chains have been removed by cleavage. This ion is seen in HDCA, UDCA, ΔHDCA and ΔUDCA, which indicates that the double bond is present in the side-chains. If the double bond were to be present in the steroid skeleton, ΔHDCA and ΔUDCA would give an MC peak at *m/z* 253. ΔCDCA is also present in dihydroxy bile acid.

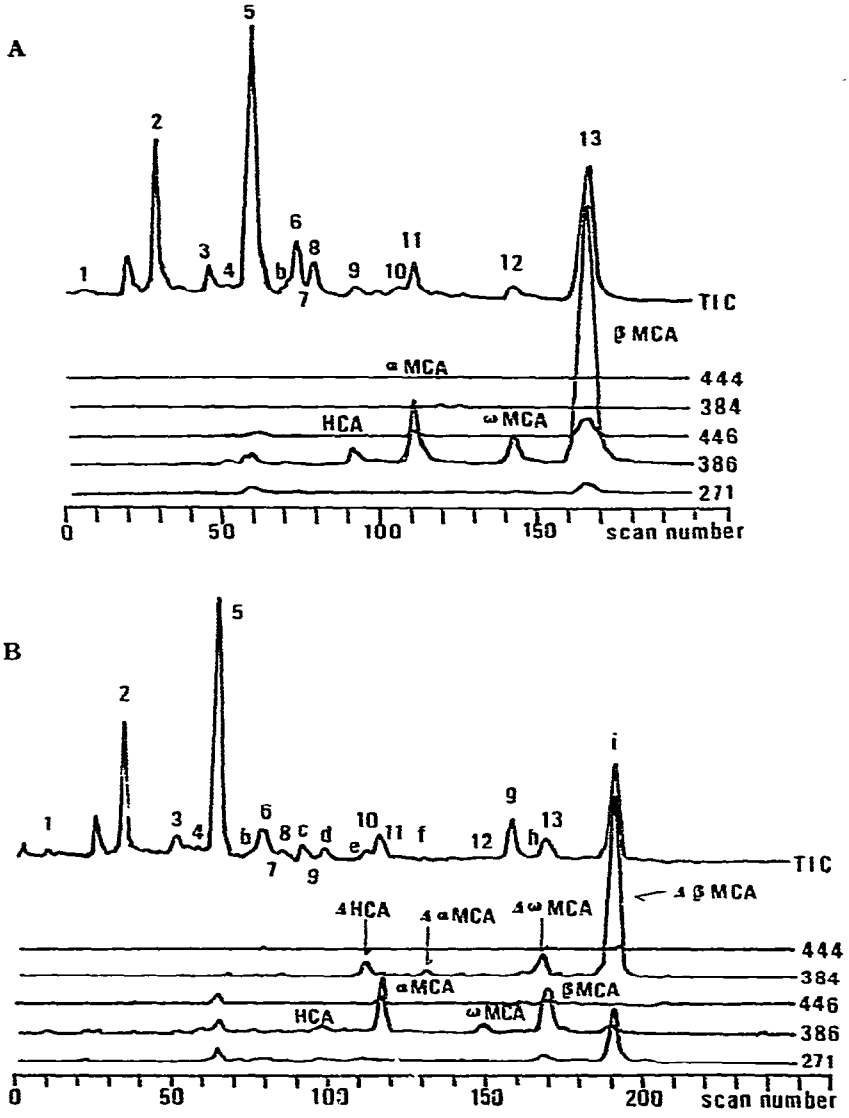

Fig. 7. MC of 3,6,7-trihydroxy bile component. In (A), HCA, α-MCA, ω-MCA and β-MCA are seen. In (B), ΔHCA, Δα-MCA, Δω-MCA and Δβ-MCA are seen in addition to these components.

The ion at *m/z* 329 corresponds to 7-keto-DCA from which one acetic acid group and side-chain have been cleaved off. It is the MC of the mass spectra of HDCA and 7-keto-DCA shown in Fig. 3.

Only component b gives the $(M+NH_4)^+$ ion at *m/z* 566 (molecular weight 548), is not influenced by catalytic hydrogenation and gives an EI mass spectrum similar to that of CA and allo-CA: the base peak is recorded at *m/z* 253 and the main fragment peaks at *m/z* 313, 368 and 428. This indicates that the component b is 3,7,12-trihydroxy bile acid. We presumed it to be $C_{24}5\beta3\beta OAc7\alpha OAc12\alpha OAc$.

TABLE I

IDENTIFICATION OF BILE ACIDS BY GLASS CAPILLARY COLUMN GAS CHROMATOGRAPHY–ELECTRON IMPACT, CHEMICAL IONIZATION MASS SPECTROMETRY

Peak	*Compound*	M^+	$M + NH_4^+$
1	Lithocholate (C_{24} 5β3αOAc)	432	450
2	Deoxycholate (C_{24}5β3αOAc12αOAc)	490	508
3	Chenodeoxycholate (C_{24}5β3αOAc7αOAc)	490	508
4	7-Ketolithocholate (C_{24}5β3αOAc7C=O)	446	464
5	Cholate (C_{24}5β3αOAc7αOAc12αOAc)	548	566
6	Hyodeoxycholate (C_{24}5β3αOAc6αOAc)	490	508
7	7-Ketodeoxycholate (C_{24}5β3αOAc7C=O12αOAc)	504	522
8	Ursodeoxycholate (C_{24}5β3αOAc7βOAc)	490	508
9	Hyocholate (C_{24}5β3αOAc6αOAc7αOAc)	548	566
10	Allo-cholate (C_{24}5α3αOAc7αOAc12αOAc)	548	566
11	α-Muricholate (C_{24}5β3αOAc6βOAc7αOAc)	548	566
12	ω-Muricholate (C_{24}5β3αOAc6αOAc7βOAc)	548	566
13	β-Muricholate (C_{24}5β3αOAc6βOAc7βOAc)	548	566
a	Δ-Chenodeoxycholate (C_{24}5β3αOAc7αOAc)	488	506
b	– (C_{24}5β3βOAc7αOAc12αOAc)	548	566
c	Δ-Hyodeoxycholate (ΔC_{24}5β3αOAc6αOAc)	488	506
d	Δ-Ursodioxycholate (ΔC_{24}5β3αOAc7βOAc)	488	506
e	Δ-Hyocholate (C_{24}5β3αOAc6αOAc7αOAc)	546	564
f	Δα-Murichole (C_{24}5β3αOAc6βOAc7αOAc)	546	564
g	Unidentified (not bile acid)	368	369
h	Δω-Muricholate (ΔC_{24}5β3αOAc6αOAc7βOAc)	546	564
i	Δβ-Muricholate (ΔC_{24}5β3αOAc6βOAc7βOAc)	546	564

3,6,7-Trihydroxy bile acid gives the base peak at *m/z* 386 and main fragment peaks at *m/z* 368, 428 and 446. Fig. 7 shows the mass chromatograms of the base peak (*m/z* 386) and of the peak at *m/z* 384. Sample A gives the MC peaks of HCA, α-MCA, ω-MCA and β-MCA attributable to the *m/z* 386 ion. Sample B gives, in addition to all of these peaks, the MC peaks of ΔHCA, Δα-MCA, Δω-MCA, and Δβ-MCA.

The MC of the ion at *m/z* 253 shows that 3,6,7-trihydroxy bile acid has a double bond in its side-chains. 3,7,12-Trihydroxy bile acid has no components that have a double bond.

Table I shows the identification results.

REFERENCES

1 S. Bergström, R. Ryhage and E. Stenhagen, *Sv. Kem. Tidskr.*, 73 (1961) 566.
2 P. Eneroth, B. Gorden, R. Ryhage and J. Sjövall, *J. Lipid Res.*, 7 (1966) 511.
3 P. Eneroth, B. Gordon and J. Sjövall, *J. Lipid Res.*, 7 (1966) 524.
4 J. Sjövall, *Application of Gas Chromatography–Mass Spectrometry to the Analysis of Bile Acids in Biological Materials*, Cambridge University Press, London, 1967, p. 243.
5 J. Sjövall, P. Eneroth and R. Ryhage, *Mass Spectra of Bile Acids, Vol. I, Chemistry*, Plenum Press, New York, 1971, p. 209.
6 H. Miyazaki, M. Ishibashi, M. Inoue, M. Itoh and T. Kubodera, *J. Chromatogr.*, 99 (1974) 553.
7 H. Miyazaki, M. Ishibashi and K. Yamashita, *Biomed. Mass Spectrom.*, 5 (1978) 469.
8 B. Munson and F. H. Field, *J. Amer. Chem. Soc.*, 88 (1966) 2621.

9 B. Munson, *Anal. Chem.*, 43 (1971) 28A.
10 P. A. Szczepanik, D. L. Hacheg and P. D. Klein, *J. Lipid Res.*, 17 (1976) 314.
11 G. M. Muschik, L. H. Wright and J. A. Schroer, *Biomed. Mass Spectrom.*, 6 (1979) 266.
12 K. Kuriyama, Y. Ban, T. Nakashima and T. Murata, *Steroids*, 34 (1980).
13 T. Murata, T. Takahashi, T. Takeda, S. Onishi, T. Nakashima, Y. Ban and K. Kuriyama, *Proceedings of the Fifth Meeting of the Japanese Society for Medical Mass Spectrometry*, Japanese Society for Medical Mass Spectrometry, Kurume, 1980, p. 205.
14 A. Maquestion, R. Flammang and L. Nielson, *Org. Mass Spectrom.*, 15 (1980) 379.
15 D. I. Carroll, J. G. Nowlin, R. N. Stillwell and E. C. Horning, *Anal. Chem.*, 53 (1981) 2007.
16 J. Yanagisawa, M. Itoh, M. Ishibashi, H. Miyazaki and F. Nakayama, *Anal. Biochem.*, 104 (1980) 75.
17 S. Barnes, D. G. Pritchard, R. L. Settine and M. Geckle, *J. Chromatogr.*, 183 (1980) 269.
18 O. J. Rosseurand and C. M. van Gent, *Clin. Chim. Acta*, 66 (1976) 269.
19 K. Tsuda, Y. Sato, N. Ikekawa, S. Tanaka, H. Higasshikuze and R. Ohsawa, *Chem. Pharm. Bull.*, 13 (1965) 720.
20 P. N. J. van den Berg and Th. P. H. Cox, *Chromatographia*, 5 (1972) 301.
21 T. Murata and S. Takahashi, *Carbohydr. Res.*, 62 (1978) 1.
22 T. Murata and S. Takahashi, *Anal. Chem.*, 49 (1977) 728.