Journal of Chromatography, 99 (1974) 553–565

CHROM. 7680

SIMULTANEOUS QUALITATIVE AND QUANTITATIVE ANALYSES OF BILE ACIDS BY MASS CHROMATOGRAPHY

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SUMMARY

For the profile analysis of bile acids by mass chromatography, the extraction and clean-up procedure, hydrolysis and derivatization have been studied. A new method of mass chromatography for qualitative and quantitative determinations, involving the multi-deuterium-labelled conjugated bile acids as internal standards and the reciprocal of the relative intensity of the peaks on the mass chromatogram was developed. This method can be applied to the separation of isomers which have almost identical retention times and which undergo the same fragmentation with different intensities.

INTRODUCTION

In recent years, the relationship between diseases of the gall bladder, such as cholesterol cholelithiasis, and the amount of bile acids and cholesterol in human bile has been investigated by many clinical chemists. Recently, since clinicians have succeeded in dissolving gall stones by oral administration of chenodeoxycholic acid[1,2], the profile analysis of these bile acids in human bile has attracted special interest as an indispensible method for diagnosis and medical treatment of the gall bladder.

For the analysis of bile acids, gas chromatography (GC) gave better separations than other chromatographic methods, as reviewed by Eneroth and Sjövall[3], but it has the disadvantage that no information on chemical structure can be obtained. In order to obviate the disadvantages of GC analysis, a technique for the identification of compounds, based on the relative intensities of mass fragment ions in combination with GC retention times, was developed by Sweeley *et al.*[4], namely the so-called mass fragmentography. The instrument for this technique was developed by Hammer and Hessling[5] and Bonelli[6]. This technique has made rapid progress in the field of biochemical research, as reviewed by Gordon and Frigerio[7].

In addition, in order to compensate for mass range limitations in multiple ion detectors, Hites and Biemann[8] reported an analytical method involving the combination of the mass fragmentographic technique with a computer system, the so-called

mass chromatography. This method has been applied to the analysis of biological substances by Reimendal and Sjövall[9–11].

This paper describes simultaneous qualitative and quantitative analyses of bile acids and cholesterol in bile by mass chromatography. Also, in order to increase the reliability of the assay, we have developed a mass chromatographic technique that can be used to monitor the co-existence of contaminants which might otherwise confuse the analytical results. Moreover, the present technique is able to differentiate easily the peak of a compound of interest from the peaks of its isomers which give the same *m/e* values but which have different relative intensities.

EXPERIMENTAL

Samples and reagents

Cholic, deoxycholic, chenodeoxycholic and lithocholic acids and sodium salts of taurocholic, taurodeoxycholic, taurochenodeoxycholic and glycochenodeoxycholic acids were purchased from Tokyo Kasei Kogyo Co. (Tokyo, Japan), Applied Science Labs. (State College, Pa., U.S.A.) and Sigma (St. Louis, Mo., U.S.A.) Sodium salts of glycocholic and glycodeoxycholic acids were kindly supplied by Dr. J. Sjövall. Deuterium-labelled taurocholic and glycochenodeoxycholic acids, prepared by the methods of Hofmann *et al.*[12] and Lack *et al.*[13], were used as internal standards.

[24-^{14}C]Chenodeoxycholic acid was purchased from Daiichi Pure Chemical Co. (Tokyo, Japan) and its glyco- and tauro-conjugates were prepared by the method of Lack *et al.*[13].

Trimethylsilylimidazole (TSIM), heptafluorobutyrylimidazole (HFBI) and heptafluorobutyric acid were purchased from Tokyo Kasei Kogyo Co. and Pierce (Rockford, Ill., U.S.A.). All reagents were distilled before use.

Instruments

A combined gas chromatograph–mass spectrometer and a data processing system with a mini-computer directly connected to the gas chromatograph–mass spectrometer were used.

The gas chromatograph–mass spectrometer was an LKB 9000S (Shimadzu Seisakusho Ltd., Kyoto, Japan). The data processing system, the GCMSPAC-300 (Shimadzu Seisakusho Ltd.), consisted of the following devices: an OKITAC-4300S mini-computer (Oki Electric Industry Co., Tokyo, Japan) with an 8K core, typewriter, digital plotter, nine-track magnetic tape and interface to the gas chromatograph–mass spectrometer. The software included in the system can be used for the following operations: data acquisition and control for the gas chromatograph–mass spectrometer, mass number calibration, data storage on the magnetic tape, background spectrum subtraction, ion abundance tabulation on the typewriter and its plotting on the digital plotter.

In addition to the above operations, several programs for the mass chromatography were provided: a measurement program (F-1) generates the gas chromatograph–mass spectrometer scan start at a pre-determined scan rate, acquires mass spectral data from the gas chromatograph–mass spectrometer output of each scan and stores them successively on the magnetic tape memory. By a plotting program (F-2), mass chromatograms utilizing up to ten fragment ions are plotted simultaneously on

the digital plotter, and the calculation program (F-3) integrates the final concentration by employing a given working curve.

The radiochromatogram scanner employed was a Packard (Downers Grove,

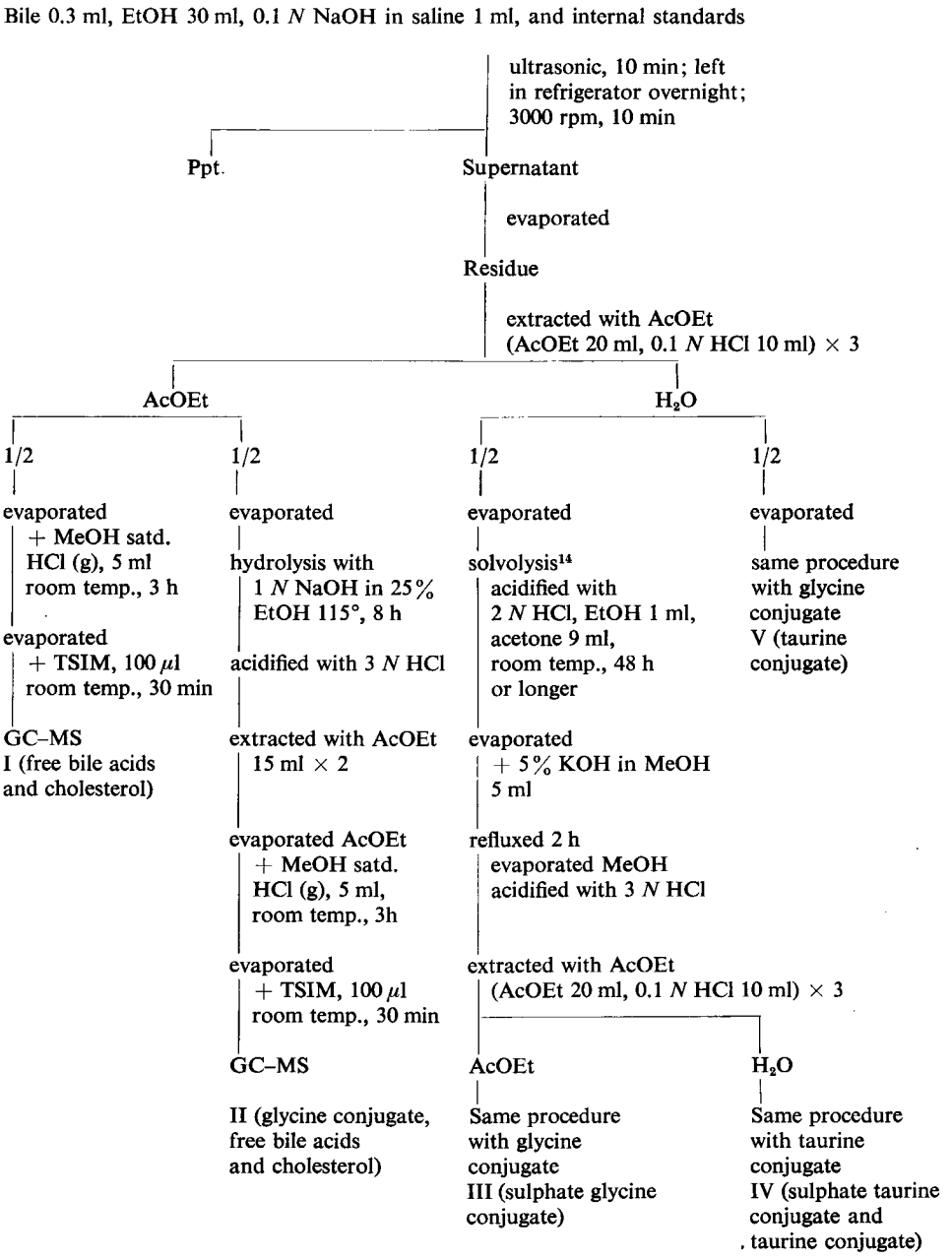

Fig. 1. Flow diagram of extraction and clean-up procedure for bile acids and cholesterol in biological fluids.

Ill., U.S.A.) Model 7200 and the nuclear magnetic resonance (NMR) spectrometer was a Varian (Palo Alto, Calif., U.S.A.) HA-100.

Procedure

Bile was treated by the extration and clean-up procedure illustrated in Fig. 1. Methyl ester O-trimethylsilyl ether derivatives of I, II, III, IV and V were subjected to gas chromatography–mass spectrometry (GC–MS).

Mass chromatography

A sample was injected into the GC inlet of the mass spectrometer, and the components were separated with a 2-m coiled glass column packed with 3% Poly I-110 on Gas-Chrom Q, 80–100 mesh (Applied Science Labs.). The temperature of the column oven was maintained at 260° and the carrier gas (helium) flow-rate was 30 ml/min. The eluted components from the GC column were enriched through a Ryhage-type carrier gas separator at 280°, then passed into the ionization source, maintained at 290°, in the mass spectrometer. The ionization energy was 60 eV and the trap current was 60 μA.

The mass spectrometer was scanned under computer control at 6-sec intervals for 20 min, starting 8 min after injection of the sample. All peaks between *m/e* 200 and 500 were collected and stored in the data processing system. Mass chromatograms were then obtained on the digital plotter upon assignment of the mass numbers by the operator. Subsequently, the peak areas of the fragments of interest were integrated and the concentrations of the components were calculated by means of working curves.

The concentrations of free acids, cholesterol and conjugates were obtained as follows. Free acid and cholesterol = I; glycine conjugate = II − I; taurine conjugate = V; sulphate glycine conjugate = III; sulphate taurine conjugate = IV − V.

RESULTS AND DISCUSSION

Derivatization

Many papers on the GC analysis of bile acids have been published. An important problem is the method of derivatization because the reliability of qualitative and quantitative analyses is influenced by the GC characteristics of the derivatives.

In the present work, we selected only the alkyl ester O-trimethylsilyl ether derivatives of bile acids, which were obtained easily and quantitatively, because it was impossible to eliminate minor by-products in the case of the alternative O-acylation procedure. When the carboxyl group of bile acids was treated with diazomethane in order to effect esterification, a minor by-product appeared in the gas chromatogram. In order to confirm the structure of this by-product, the reaction product of methyl chenodeoxycholate treated with diazomethane was subjected to silica gel column chromatography[15]. The mass spectrum of this isolated by-product is suggested as methyl 3- or 7-O-methylchenodeoxycholate (Fig. 2). In order to clarify the position and conformation of the hydroxyl group in this isolated compound, it was subjected to a trimethylsilyl (TMS) via heptafluorobutyryl (HFB) exchange reaction, which can identify the conformation of hydroxyl groups on a steroidal skelton[16,17]. The structure was elucidated to be methyl 3α-methoxy-7α-hydroxycholanate from the

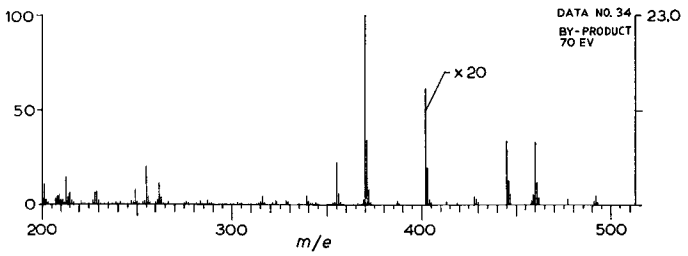

Fig. 2. Mass spectrum of methyl ester O-trimethylsilyl ether derivative of 3α-methoxy-7α-hydroxy-5β-cholanic acid obtained as by-product by esterification of chenodeoxycholic acid with diazomethane.

fact that the compound did not undergo the TMS-via-HFB exchange reaction by GC–MS. The structural assignment received additional support from NMR spectrometry.

We attempted to avoid the formation of this by-product obtained from the treatment with diazomethane by treating the hydroxyl groups with TSIM prior to esterification with diazomethane. However, this approach was unsuccessful because the reaction products of bile acids were confirmed by MS to be the persilyl derivatives in which even the carboxyl groups were trimethylsilylated. Hence it was found to be very difficult to trimethylsilylate the hydroxyl group selectively with TSIM.

Although the persilyl derivatives were formed advantageously by a one-step reaction with TSIM, they could not be completely separated from each other by the use of any stationary phase which is suitable for high-temperature operation. As described above, the derivatization methods that produce by-products create difficulties for MS analysis. For good reproducibility, it has been found necessary to develop a derivatization method that is able to convert bile acids into certain derivatives without these problems. We found that bile acids treated with dry hydrogen chloride–methanol solution were converted quantitatively into their esters without the formation of any by-products, and they were trimethylsilylated smoothly with TSIM to give single sharp peak. The fact that esterification is performed quantitatively at room temperature was confirmed radiochemically by utilizing [24-^{14}C]chenodeoxycholic acid, which produced no by-products following esterification.

Various stationary liquid phases were examined for separating the above derivatives, and Hi-Eff 8B and Poly I-110 were selected for the analysis of bile acids. Poly I-110 was adopted for mass chromatography because it was found to be suitable for use at higher temperatures (*e.g.* 270°).

As shown in Fig. 3, authentic bile acids were separated completely but chenodeoxycholic acid and cholesterol were eluted simultaneously. In response to this problem, it was tried to utilize mass chromatography for the analysis of bile acid methyl ester O-trimethylsilyl ether derivatives and trimethylsilylcholesterol.

Calibration curves

The reliability of qualitative and quantitative results has been confirmed in previous work on polychlorinated biphenyls[18] by using the ratio of the characteristic natural abundances of chlorine isotopes. In that work, we noticed that even if multi-isotopic elements were not present in the compounds of interest, characteristic fragment ions from their mass spectra could be utilized instead of the natural abundance

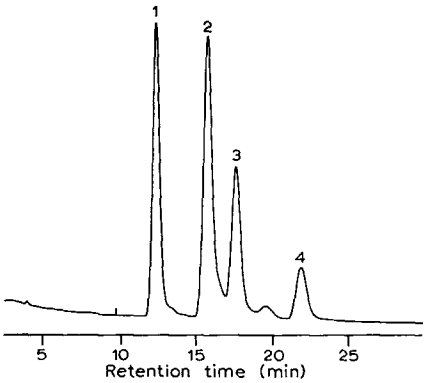

Fig. 3. Gas chromatogram of total ion current record of methyl ester O-trimethylsilyl ether derivatives of authentic bile acids. Glass coil column (2 m × 2 mm) packed with 3 % Poly I-110 coated on 80–100-mesh Gas-Chrom Q. Column temperature, 260°; flash heater temperature, 280°; separator temperature, 290°; ion source temperature, 290°; carrier gas (helium) flow-rate, 30 ml/min; and exit and collector slit widths, 0.1 mm. A mixture of 1 μg each of authentic bile acids was injected. 1 = Cholic acid; 2 = deoxycholic acid; 3 = chenodeoxycholic acid; 4 = lithocholic acid.

of isotope ions. Fortunately, if the operating conditions of the mass spectrometer are kept constant, it is well known that the relative intensities must also remain constant.

For the preparation of calibration curves for bile acids and cholesterol, mass chromatography was used. The mass spectrometer was automatically scanned at an ionization voltage of 20 eV. After mass chromatograms had been plotted by the use of these characteristic fragment ions[19–21], each peak area was calculated by program F-3, job = 2.

In order to illustrate the calibration curves, the areas of the peak in question and of that of the internal standard recalled from the computer were plotted on paper.

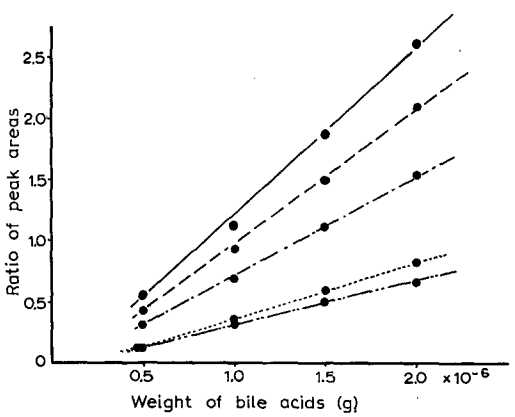

Fig. 4. Calibration curves for bile acids and cholesterol. The weight of a component relative to the internal standard is plotted on the x-axis and the ratio of peak areas on the mass chromatograms of characteristic fragment ions of the component to the internal standard is plotted on the y-axis. ●—·—·—●, Cholic acid (m/e = 368); ●————●, deoxycholic acid (m/e = 255); ●— — —●, chenodeoxycholic acid (m/e = 370); ●— · · —●, lithocholic acid (m/e = 372); ●· · · · · ·●, cholesterol (m/e = 329).

As shown in Fig. 4, these calibration curves showed good linearity in the range from 0.5 to $2.0 \cdot 10^{-6}$ g. In practice, all calibration curves were stored in the computer and the analysis was run with the aid of program F-3, job = 1 and 2. Also, several calibration curves utilizing characteristic fragment ions were prepared for each compound in order to ensure the reliability of the qualitative and quantitative analyses.

Qualitative analysis

The amounts of bile acids and cholesterol in human bile were determined by the above procedure. As shown in Table I, satisfactory agreement for cholic and deoxycholic acid contents was obtained with the calculated results obtained by use of different fragment ions. With chenodeoxycholic acid, the contents estimated from its ions disagreed with each other; these different values might be due to the abnormal fragmentation caused by simultaneous ionization of chenodeoxycholic acid and cholesterol.

TABLE I

COMPARISON OF RATIO OF PEAK AREA OBTAINED FROM STANDARD BILE ACIDS AND THAT OF HUMAN BILE, AND CONTENTS MEASURED BY USING A FEW FRAGMENT IONS FOR EACH BILE ACID

Conditions as in Fig. 3.

Compound	*m/e*	*Ratio of peak area*		*Weight of bile acids and cholesterol (mg/ml)*
		Standard	*Sample*	
Cholic acid	253	0.78	0.80	36.86
	343	0.31	0.31	35.89
	368	1	1	35.84
Deoxycholic acid	255	1	1	18.36
	345	0.17	0.17	18.23
	370	0.27	0.27	17.93
Chenodeoxycholic acid	255	0.13	0.18	30.65
	370	1	1	39.16
Cholesterol	329	1	1	18.92
	458	0.88	0.79	16.99

Moreover, the same phenomenon was observed with trimethylsilylcholesterol and the *n*-propyl ester O-trimethylsilyl ether derivative of cholic acid, which were eluted simultaneously under the above-mentioned mass chromatographic conditions. Therefore, it may be possible that this phenomenon is a chemical ionization-like process that differs from the chemical self-ionization reported by Dougherty and Dalton[22], which also involves simultaneous ionization of more than two components in the ionization chamber.

In order to examine this phenomenon in detail, a mixture of trimethylsilyl derivatives of methyl chenodeoxycholate and cholesterol were measured by mass chromatography at various ionization voltages. With an increase in ionization voltage, as shown in Fig. 5, the ratio of the contents of chenodeoxycholic acid which were calculated from different fragment ions gradually approached 1.0, and this phenomenon finally disappeared at voltages above 50 eV. Therefore, the quantitative analysis of bile acids in human bile must be carried out by means of an ionizing voltage of more

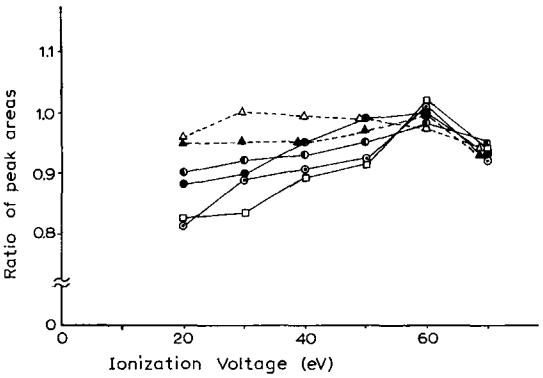

Fig. 5. Relationship between ionization voltage and fluctuation of peak areas caused by ionizing voltage on chenodeoxycholic acid and cholesterol individually or simultaneously. Mass chromatography was carried out for cholesterol, chenodeoxycholic acid, a mixture of them and cholic acid as internal standard at different ionization voltages. Each peak area on the mass chromatogram for characteristic fragment ions was computed, and then the ratios (same time ionization/individual ionization) were calculated. Cholesterol: ●, $m/e = 329$; ⦿, $m/e = 353$; ◐, $m/e = 368$; □, $m/e = 458$. Chenodeoxycholic acid: △, $m/e = 355$; ▲, $m/e = 370$.

than 50 eV. As the chemical ionization-like process may be caused by the co-existence of another biological component even if a single peak is plotted on the mass chromatogram, it is necessary to recognize this situation, because it decreases the reliability of the mass chromatography. Therefore, in order to carry out quantitative analyses by mass chromatography, it may be necessary to establish the purity of each chromatographic peak.

In order to ascertain the absence of the above effect for the biological sample used, we compared the relative intensities from purified and non-purified samples with those of the authentic samples by setting the ionization voltage at 60 eV. Fortunately, the ratio of the relative intensities for the three samples agreed very well, and the determination of bile acids and cholesterol in human bile can therefore be carried out without any clean-up operation when using mass chromatography.

Quantitative analysis

Although there are many analytical methods for the determination of conjugated bile acids, their isolation has been investigated in the present work by use of a liquid–liquid extraction technique. As shown in Fig. 1, free bile acids and their glycine conjugates can be isolated from other conjugated compounds in the first extraction step. [24-^{14}C]Glyco- and taurochenodeoxycholic acids were used for measuring the recovery in this extraction and the radioactive recoveries in the fractions of glyco- and taurochenodeoxycholic acids were 96.2 and 93.8%, respectively.

For the purpose of compensating for analytical errors that arise in this procedure, multi-deuterium-labelled glycochenodeoxycholic and taurocholic acids were utilized as internal standards.

The mass spectra of methyl ester O-trimethylsilyl ether derivatives of deuterium-labelled cholic and chenodeoxycholic acids are shown in Fig. 6. These derivatives were obtained as a mixture in which each molecule was labelled with more than four deuterium atoms. As the fragment ions of the internal standard have no

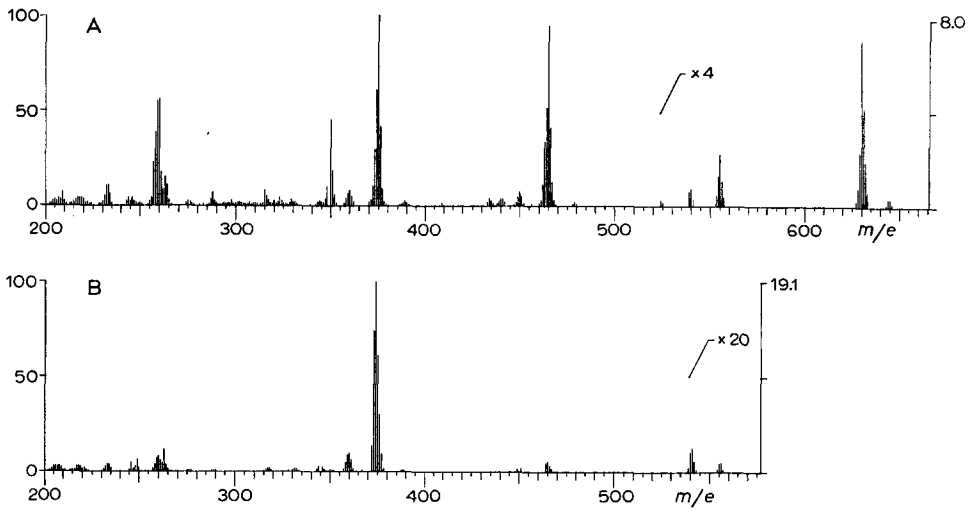

Fig. 6. Mass spectra of methyl ester O-trimethylsilyl ether derivatives of multi-deuterium-labelled cholic (A) and chenodeoxycholic (B) acids. Ionization voltage, 20 eV; ion source temperature, 290°; and trap current, 60 μA.

d_0-fragment ions which were produced from native bile acids, they can be available as the internal standard in this method.

As shown in Fig. 7, there is good agreement between the mass spectra of multi-

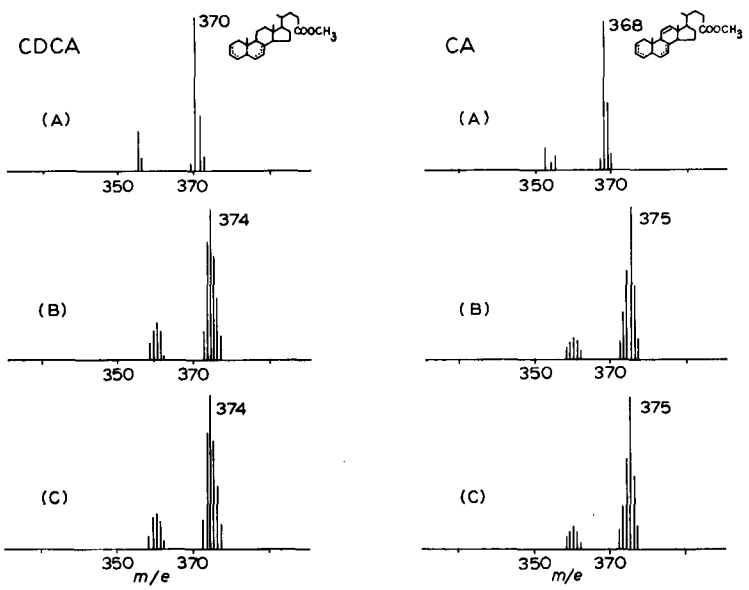

Fig. 7. Partial mass spectra of methyl ester O-trimethylsilyl ether derivatives of cholic acid (CA) and chenodeoxycholic acid (CDCA) of (A) authentic compounds, (B) multi-deuterium-labelled CA and CDCA, and (C) materials obtained by hydroxylation of their conjugates. These mass spectra suggest no elimination of deuterium by hydroxylating the conjugates as internal standards in alkaline solution.

deuterium-labelled cholic and chenodeoxycholic acids after derivatization and those of the conjugated bile acids after hydrolysis and derivatization. It is obvious that no elimination of deuterium atoms from these compounds occurred as a result of this procedure.

Experiments to measure the recovery were carried out by adding definite amounts of native conjugated bile acids and cholesterol to human bile. Satisfactory recoveries of more than 96% were obtained, and the standard deviation calculated from five measurements of each component was less than 3%. In particular, excellent standard deviations were obtained for taurocholic and glycochenodeoxycholic acids by utilizing the corresponding internal standards. The results show the utility of stable isotope-labelled compound as internal standards in mass chromatography, as reported by Samuelson *et al.*[23].

Although no interfering substances were found in the above experiments, it may be unsuitable to apply the technique to other biological samples, because samples prepared from biological fluid for mass chromatography may be contaminated with unexpected impurities which cannot be eliminated completely by the usual clean-up procedures, and because of the existence of different kinds of impurities due to the nature of the particular diseases involved and individual differences between patients.

Monitoring system using mass chromatography

We attempted to establish a monitoring system which could detect easily the existence of interfering substances by mass chromatography. This was achieved by utilizing the relative intensities of the objective compound for identifying the peaks with high reliability. A coefficient, defined as the reciprocal of the relative intensity, was determined for each characteristic fragment ion of the sample.

If these coefficients were used for the identification of deoxycholic acid in a mixture of dihydrocholanoic acids, only its characteristic fragment ions should be obtained with almost equal intensities, as shown in Table II. The coefficients can be calculated automatically by inputting the relative intensity into this system and storing it in the computer memory core. As the peaks for these fragment ions are observed as only a single peak, deoxycholic acid was identified easily from the relative intensity in combination with the GC retention time. Moreover, the presence and distribution of contaminants was checked simultaneously. Thus, when the intensities

TABLE II

"COEFFICIENTS" TO BE FED INTO COMPUTER FOR CONSTRUCTING A PROPOSED MASS CHROMATOGRAM

DCA = deoxycholic acid; CDCA = chenodeoxycholic acid; UDCA = ursodeoxycholic acid.

m/e	*Relative intensity*			*Coefficient*	*Converted intensity*		
	DCA	*CDCA*	*UDCA*		*DCA*	*CDCA*	*UDCA*
255	100	25	50	100/100	1.0	0.25	0.5
355	5	27	40	100/5	1.0	5.4	8.0
370	16	100	100	100/16	1.0	6.3	6.3
535	13	0.4	16	100/13	1.0	0.03	1.2

of the compound to be identified were fed into the computer with this program, the mass chromatogram can be plotted as if it consists of only one peak, as shown in Fig. 8, (marked by a "fish hook") while the peaks of other compounds ought to be plotted as several peaks, including both small and large peaks. This means that the qualitative analysis was performed with the inherent relative intensities of characteristic fragment ions in addition to conventional mass chromatography. Therefore, it will be expected that, even if only one fragment ion is used, the compound can be quantified with almost the same reliability as that which can be obtained by measuring every characteristic fragment ion.

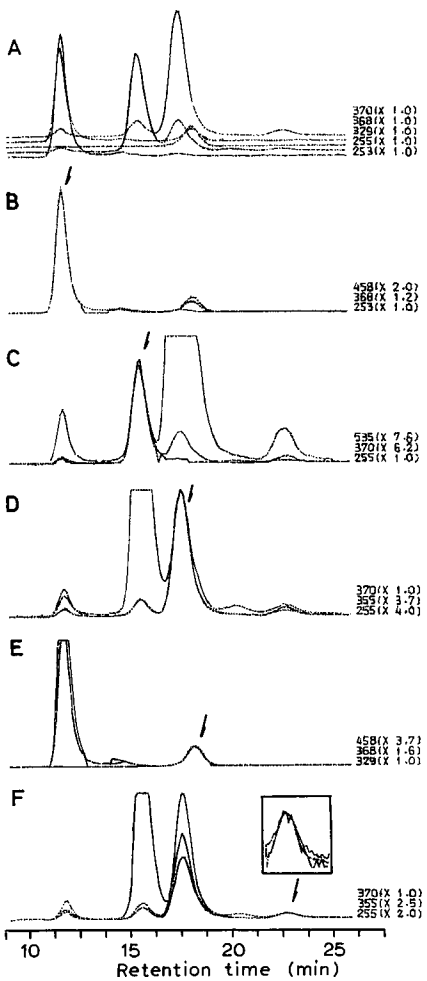

Fig. 8. Conventional and new mass chromatograms of bile acids and cholesterol in human bile. Conventional mass chromatogram is illustrated in (A). New mass chromatograms are drawn out to identify the peaks corresponding to (B) cholic, (C) deoxycholic, (D) chenodeoxycholic and (F) ursodeoxycholic acids and (E) cholesterol.

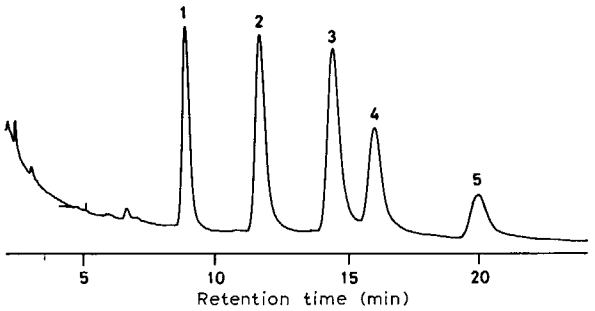

Fig. 9. Gas chromatogram of total ion current record of *n*-butyl ester O-trimethylsilyl ether derivatives of authentic bile acids and cholesterol. Conditions as in Fig. 3, except column temperature, 260° and 1% Poly I-110. 1 = Cholesterol; 2 = cholic acid; 3 = deoxycholic acid; 4 = chenodeoxycholic acid; 5 = lithocholic acid.

In spite of carrying out further investigations, the separation between the methyl ester O-trimethylsilyl ether derivative of chenodeoxycholic acid and trimethylsilylcholesterol has not yet been achieved. On the other hand, complete separation could be achieved by converting the bile acids into *n*-butyl ester O-trimethylsilyl ether derivatives, as shown in Fig. 9. However, these derivatives must be analyzed at high temperatures and therefore, as the stationary phase cannot be used over a long period of time without deterioration of its efficiency, it may be unsuitable for routine use in this analysis.

CONCLUSION

Simultaneous qualitative and quantitative analyses can be carried out by comparing the ratio of the peak areas with that of the relative intensities of a reference compound. The technique described, which involves the use of the reciprocal of relative intensity, can be used not only to identify simply compounds of interest but also to monitor easily the purity of the peak in question without the need to calculate the ratio of the peak areas. By using deuterium-labelled taurocholic and glycochenodeoxycholic acids as internal standards, bile acids in biological fluids can be determined with high reliability.

We believe that this technique will be very useful in the field of clinical pharmacology and drug metabolism.

ACKNOWLEDGEMENTS

The authors are grateful to W. Tanaka, Research Laboratories of Nippon Kayaka Co., for his encouragement, to J. Sjövall, Department of Chemistry, Karolinska Institutet, for profitable discussion of this work and for samples of glycocholic and glycodeoxycholic acids, and to S. Asai, M. Kiri and K. Kobayashi, Systems Department, Shimadzu Seisakusho Ltd., for production of the computer program for mass chromatography.

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