papers and notes on methodology

Capillary gas–liquid chromatography of glycine-conjugated bile acids without prior hydrolysis

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Abstract A method is described for the gas–liquid chromatographic (GLC) analysis of intact glycine conjugates of the major bile acids present in human plasma. It is, therefore, now possible to analyze glycine-conjugated and unconjugated bile acids together on a single GLC column without the necessity for a hydrolytic step. A large number of derivatives of bile acid glycine conjugates were examined, but only acetate- and silyl ether-derivatives of carboxylic acid methyl esters were found initially to be suitable. It was not possible to make acetates consistently, and trimethylsilyl ethers did not allow resolution of the glycine conjugates of cholic and chenodeoxycholic acids. Dimethylethylsilyl ether methyl ester derivatives were subsequently found to give the best results. Chromatographic conditions for successful analysis of these derivatives were examined and it was found to be necessary to use wall-coated capillary columns of thin film thickness (0.12 μm) and very high carrier gas flow rates (ca. 20 ml/min hydrogen). Using acetonitrile and Bond Elut extraction, fractionation on Sep-Pak SIL cartridges, and derivatization as dimethylethylsilyl ether methyl esters, the capillary gas–liquid chromatography of intact glycine-conjugated bile acids from human plasma was demonstrated for the first time. —**Street, J. M., D. J. H. Trafford, and H. L. J. Makin.** Capillary gas–liquid chromatography of glycine-conjugated bile acids without prior hydrolysis. *J. Lipid Res.* 1986. **27:** 208–214.

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There is still considerable controversy over the value of bile acid measurements in clinical medicine. However, such analyses in a variety of body fluids are routinely carried out in laboratories around the world. After reviewing the methodology, Street, Trafford, and Makin (1) concluded that, despite a wealth of differing approaches to this problem, there was no satisfactory simple method applicable to routine clinical analysis of bile acids in human body fluids. A number of new high-performance liquid chromatographic methods have been described which offer many advantages over previous procedures (2, 3). However, most of the methods used in routine laboratories, such as enzymatic assay or radioimmunoassay, provide limited information about the complex mixture of bile acids present in biological fluids.

Gas–liquid chromatography (GLC) with or without mass spectrometry (MS) has been widely used as a method of bile acid analysis (4, 5). In order that information about the type of conjugation should not be lost, the various bile acid classes are usually separated, using DEAP Sephadex LH20 (4), with subsequent hydrolysis of the conjugates prior to derivatization and GLC analysis. Direct GLC analysis of intact conjugates would simplify these analytical procedures considerably. This report describes the development of procedures for the analysis of intact glycine conjugates of the major bile acids present in human plasma by capillary GLC.

MATERIALS AND METHODS

Reference compounds

Unlabeled bile acids and their methyl esters were obtained from a number of sources (Koch Light Laboratories, Colnbrook, Bucks, UK; Steraloids, Croydon, UK; Sigma Chemicals Co. Ltd., Poole, Dorset, UK; and BDH

Abbreviations and trivial names: lithocholic (LCA), 3α-hydroxy-5β-cholanoic; chenodeoxycholic (CDCA) 3α,7α-dihydroxy-5β-cholanoic; ursodeoxycholic (UDCA), 3α,7β-dihydroxy-5β-cholanoic; deoxycholic (DCA), 3α,12α-dihydroxy-5β-cholanoic; cholic (CA), 3α,7α,12α-trihydroxy-5β-cholanoic. The prefix glyco (G) is used for bile acids having glycine in amide linkage at C-24. TMS, trimethylsilyl; TSIM, trimethylsilyl imidazole; DMES, dimethylethylsilyl; DMESI, dimethylethylsilyl imidazole; DMnPS, dimethyl-n-propylsilyl; DMnPSI, dimethyl-n-propylsilyl imidazole; GLC, gas–liquid chromatography; MS, mass spectrometry; FID, flame ionization detector; BA, bile acid.

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Chemicals Ltd., Poole, Dorset, UK), and used without further purification, except cholanic acid which was purified by recrystallization from ethanol. Standard solutions of the bile acids were made up to approximately 1 mg/ml of redistilled ethanol and stored at −15°C.

[^{3}H(G)]Glycocholic acid (2 Ci/mmol) (NEN Chemicals GmbH, West Germany) was purified as necessary by thin-layer chromatography on aluminium sheets precoated with silica gel 60 (Merck, Darmstadt, Germany) using the solvent system recommended by the manufacturer.

Solvents and reagents

Solvents and reagents were, unless otherwise specified, of Analytical Reagent grade from BDH Chemicals Ltd. and were used as supplied, except methanol, hexane and ethanol (absolute alcohol 1000, James Burroughs Ltd., London, UK) which were redistilled before use. All solvents and reagents used were checked to ensure that they did not contain interfering peaks with GLC retention times similar to those of the bile acid derivatives under investigation. Dimethylethylsilyl imidazole (DMESI) and dimethyl-n-propylsilyl imidazole, were from Apin Chemicals Ltd., Abingdon, UK; hexamethyldisilazane and trimethylchlorosilane were from Koch-Light Laboratories; trimethylsilyl imidazole (TSIM) was a preparation from Pierce & Warriner (UK) Ltd., Chester, Cheshire, UK.

Bond Elut (C18) cartridges (500 mg) and Vac Elut apparatus were obtained from Jones Chromatography, Llanbradach, UK. Lipidex 5000 was obtained from Packard Instrument Co. Inc., Reading, UK, and Sep-Pak SIL cartridges were from Waters Associates, Cheshire, UK.

All glassware was silanized by soaking overnight in 1% (v/v) dimethyldichlorosilane in toluene, washed with toluene and methanol, and dried before use. Bile acid glycine conjugates were hydrolyzed as described by Nair and Garcia (6) using cholyl glycine hydrolase (EC 3.5.1.24) as partially purified lyophilized powder from *Clostridium perfringens* (Welchii) (Sigma Chemical Co. Ltd.).

Sonication was carried out for 5 min using an ultrasonic bath (Model T-14; L & R Manufacturing Co., Kearny, NJ).

Capillary GLC analysis of bile acids was carried out with a Carlo Erba Fractovap (Erba Science UK Ltd., Swindon, UK), using a solid injection system essentially the same as that described by Shackleton and Honour (7). The oven temperature was raised from 150–280°C at 2°C/min with injector and detector temperatures at 290°C. CP Sil 5(CB) WCOT (25 m × 0.32 mm/df −0.12 μm), CP Sil 8 WSCOT (25 m × 0.32 mm/df −1.25 μm), and CP Sil 19 (CB) WCOT (25 m × 0.24 mm/df 0.13 μm) capillary columns were from Chrompack UK Ltd, London, UK. Optimum conditions for the chromatography of silyl derivatives of the glycine-conjugated bile acid methyl esters were as follows: carrier gas (H_2), 22 ml/min; auxiliary H_2 gas, 25 ml/min; air flow to jet, 250 ml/min. Carrier gas flow was measured at room temperature at the jet using a bubble flowmeter. To achieve hydrogen carrier gas flow rates of 20 ml/min, the pressure at the column inlet was about 3.5 kg/cm^2.

Derivatization

Carboxyl group. Lillington, Trafford, and Makin (8) showed that, using acetyl chloride–methanol mixtures for the esterification of steroid carboxylic acid groups, the rapidity with which the ester was formed was directly related to the length of the side chain. Thus, methyl esters of the glycine-conjugated bile acids were found to be formed very rapidly at 50°C. However, to ensure complete derivatization, incubation was carried out for 15 min at 50°C. No destruction of the glycine conjugates was observed with this reagent, even using prolonged reaction times of up to 2 hr. Various other esters including ethyl, propyl, isopropyl, butyl, amyl, and 2-ethoxyethyl were also formed by substituting the appropriate alcohol for methanol in the reagent mixture (7). In general, the use of these derivatives caused prolonged retention times and broad peaks without any improvement in resolution. Methyl esters were therefore used throughout.

Hydroxyl groups. Many methods for the derivatization of the hydroxyl groups, summarized by Street et al. (1), were studied. In our hands, only acetates and silyl ethers could be successfully formed with the glycine-conjugated bile acids. Difficulty was experienced in obtaining consistent results in the formation of acetate derivatives of these compounds and therefore attention was focused on silyl ethers. Silyl ether derivatives of the methyl ester glycine-conjugated bile acids were formed using a variety of procedures (1). The use of the appropriate silylimidazole was found to be the most suitable method. A reaction time of 40 min at 50°C gave complete derivatization using 100 μl of the silylimidazole reagent. For the reasons described by Shackleton and Honour (7), Lipidex 5000 columns were used to remove excess reagents (9). However, it was found to be necessary to increase the volume of hexane eluent to 15 ml to obtain complete elution because of the prolonged retention of the least polar glycine conjugates and glycocholanic acid on the column.

Using trimethylsilyl ether derivatives, single symmetrical peaks were obtained with standard curves linear over the range 2–12 ng. However, GCDCA and GCA derivatives did not separate on the CP Sil 5(CB) column used. More polar stationary phases, e.g., CP Sil 8(CB) and CP Sil 19(CB), were examined in an attempt to resolve these compounds, but no peaks corresponding to the glycine-conjugated bile acids could be seen on these columns. DMES derivatives (10) of methyl ester glycine conjugates gave greatly improved resolution but somewhat broader peaks. DMnPS derivatives gave broaker peaks and decreased sensitivity, while offering no improvement in resolution over that obtained for the DMES derivatives.

Improvement in peak shape for all the silyl ether derivatives studied was achieved using increased gas flows as described below. **Fig. 1** shows the separation of DMES ether methyl esters of the unconjugated and glycine-conjugated bile acids. Reaction times with imidazole reagents in excess of the recommended 40 min should not be used since reduced recovery of the glycine-conjugated bile acids has been observed in these circumstances.

Using DMES derivatives and GLC conditions described in the Materials and Methods section, linear standard curves (over the range 0–30 ng injected) were obtained for methyl ester DMES ethers of the glycine conjugates of LCA, CDCA, DCA, and CA as shown in **Fig. 2**.

Chromatographic conditions

In all the GLC studies described, hydrogen was used as the carrier gas since it is considerably cheaper than helium. Response and peak shape for all the silyl derivatives of unconjugated and glycine-conjugated bile acids examined (DMS, TMS, DMES, DMnPS) were found to be dependent on hydrogen carrier gas flow. This is particularly apparent with the glycine-conjugated acids. Carrier gas flow rates for capillary GLC are normally in the range of 2–5 ml/min. At these flow rates the glycine-conjugated bile acid methyl ester DMES derivatives gave very poor peaks with reduced response and long retention times (Fig. 1). Increasing the carrier gas flow improved resolution and decreased retention times of the glycine-conjugated bile acids without apparent loss of resolution of the unconjugated bile acid derivatives. The width at half-height of all the glycine-conjugated bile acid peaks decreased with increasing carrier gas flow. Furthermore, the glycine conjugates eluted within 60 min at carrier gas flows of 20 ml/min as compared with retention times of 85 min with low carrier gas flow (2.3 ml/min). The resolution of unconjugated bile acids did not appear to deteriorate at high flow rates since the relative retention time of CDCA:DCA, the most difficult of the bile acids to resolve, was shown to be unchanged or even slightly increased with faster carrier gas flow.

FID response of these derivatives was also considerably improved by increasing carrier gas flow. The peak height ratio of each glycine-conjugated bile acid relative to their corresponding unconjugated bile acid (GBA/BA) and of each unconjugated bile acid relative to LCA (BA/LCA) was measured over a range of carrier gas flow from 2.3–25.7 ml/min keeping the auxillary H_2 flow at 24.5 ml/min. FID response of unconjugated bile acids increased with increased H_2 carrier gas flow reaching a plateau at 10 ml/min, while FID response of glycine conjugates also increased with faster carrier gas flow but did not reach a plateau until 20 ml/min (**Fig. 3**).

The possibility that the observed improvements in FID response could be due to increased H_2 flow through the

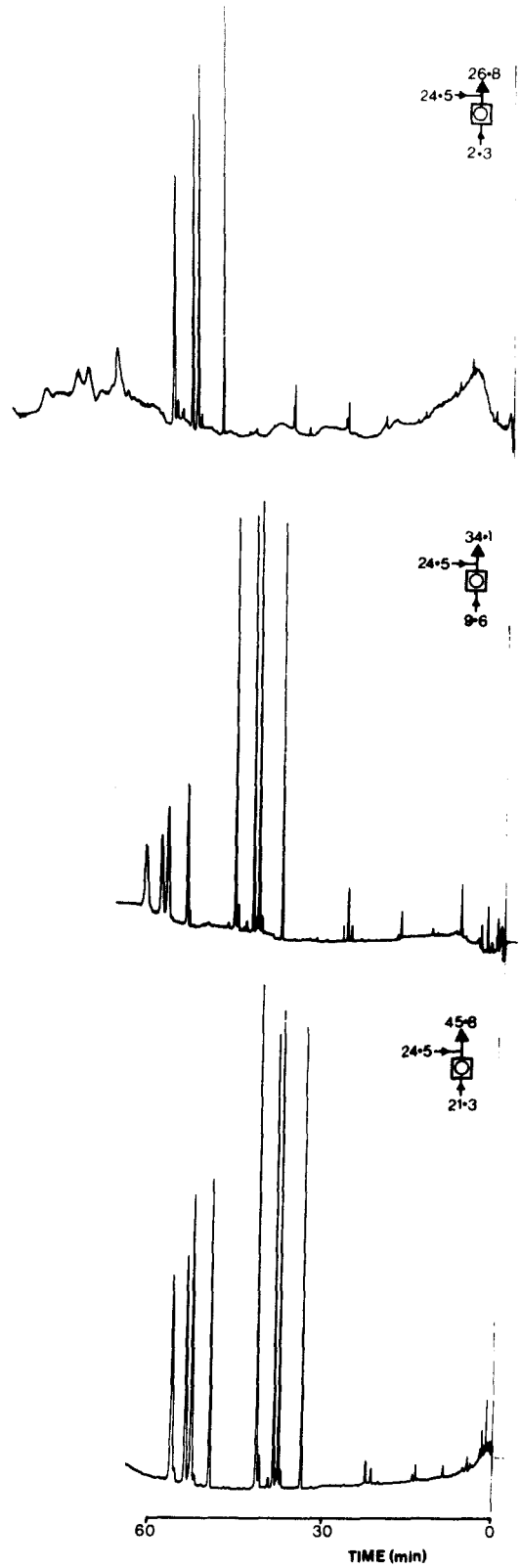

Fig. 1. Gas-liquid chromatography of DMES ethers of carboxylic acid methyl esters of unconjugated and glycine-conjugated bile acids on CP SIL 5. Conditions for GLC are described in the text. Hydrogen carrier gas was varied from 2.3 ml/min (top trace) to 21.3 ml/min (bottom

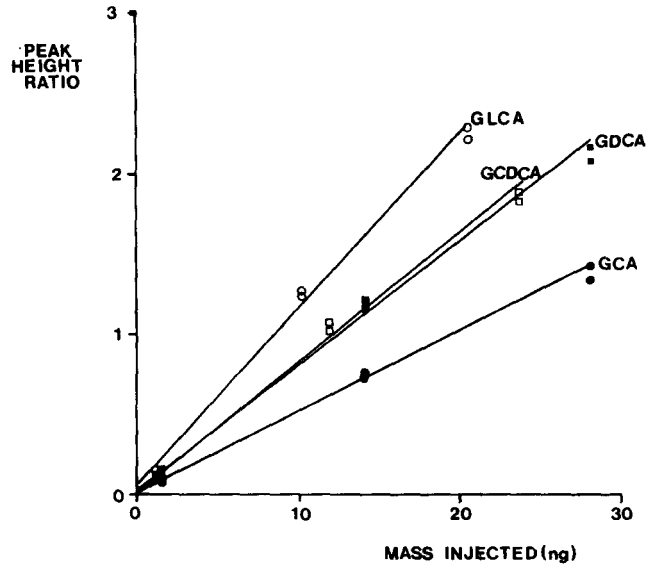

Fig. 2. Graph relating peak height ratio* to mass for DMES ethers of carboxylic acid methyl esters of glycine-conjugated bile acids. Linear correlation coefficients of these lines were all >0.997 ($P < 0.05$). *Peak height of bile acid derivative/peak height of glycocholanic acid derivative.

jet, resulting in improved combustion of the sample, was investigated. Reduction of hydrogen carrier gas flow to 2 ml/min, with a concomitant increase in auxiliary hydrogen flow to the flame, still gave unsatisfactory peaks. Various other parameters were investigated such as increasing the injection temperature from 290°C to 365°C, but this did not alter the peak characteristics of the glycine-conjugated bile acids where low carrier gas flow was used. Similarly, a more rapid rise in oven temperature during chromatography, although reducing retention times greatly, did not improve the peak shape or response for the glycine-conjugated bile acids, and resolution of the unconjugated bile acids was adversely affected. This suggests that the length of time the glycine-conjugated bile acids are retained on the column is not an important factor in determining their resolution and peak shape.

The theoretical basis of these empirical observations eludes us but it has been suggested (D. W. Grant, personal communication) that these effects may only be seen with columns with very thin stationary phase coating (in the CP SIL 5 column used, it was 0.12 μm) enabling unhindered mass transfer to occur between the phases at these high flow rates.

trace), keeping auxiliary hydrogen gas at 24.5 ml/min. Bile acid derivatives elute in the following order LCA, DCA, CDCA, CA, GLCA, GDCA, GCDCA, GCA, with retention times depending upon carrier gas flow rate. UDCA runs midway between CA and CDCA from which it is completely resolved. At low carrier gas flow rates (upper trace), glycine-conjugated bile acid derivatives give a very poor response.

Application to plasma analysis

To demonstrate that this procedure could be applied to the analysis of plasma, some samples from apparently normal laboratory workers and patients with hepatobiliary disease were examined using the following procedure.

Plasma (5 ml for a normal subject or 1 ml for patients with hepatobiliary diseases) was cooled to 4°C and cold acetonitrile (6 ml or 2 ml, respectively) was added and immediately mixed vigorously on a vortex mixer. After centrifugation (1500 *g* for 5 min using an MSE Major centrifuge), the supernatant was decanted and the protein plug was physically dispersed and washed by resuspension in a further volume of acetonitrile (4 ml or 1 ml, respectively). After vigorously shaking on a vortex mixer, the mixture was again centrifuged (2000 *g* for 10 min) and the supernatant was removed. Ammonium sulfate (0.5 g/ml of plasma) was added to the first extraction supernatant. After mixing, two layers were formed which were fully separated by centrifugation (1500 *g* for 5 min). The top layer of acetonitrile was removed and combined with the second acetonitrile extraction supernatant. The combined extract was evaporated to a small volume (ca. 400 μl) under N_2 in a small silanized vial, diluted with distilled water (7 ml), and extracted using Bond Elut cartridges as described by Setchell and Worthington (11).

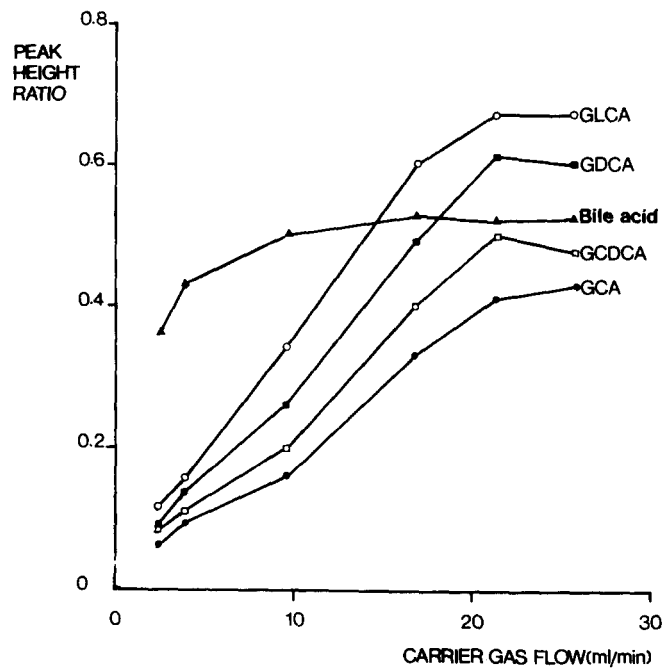

Fig. 3. Graph relating peak height ratio (see Fig. 2 legend) to hydrogen carrier gas flow rate of the DMES ethers of carboxylic acid methyl esters of unconjugated and glycine-conjugated bile acids. The mean peak height ratio (peak height BA/peak height LCA) for the unconjugated bile acids (DCA, CDCA, and CA) is indicated as "Bile acid" in the figure. Peak height ratios for the glycine-conjugated bile acids (peak height GBA/peak height of equivalent BA) are recorded as a mean of duplicate values.

The glycine-conjugated bile acids fraction was separated using a Sep-Pak SIL cartridge at 4°C with elution by gravity flow. The Sep-Pak SIL cartridge was primed with 20 ml of ethanol–chloroform–water 20:80:1 (v/v/v). The acetonitrile extract was redissolved in 3 × 1 ml of ethanol–chloroform–water 20:80:1 (v/v/v) by sonication of each aliquot for 3 min, cooled to 4°C for 5 min, and applied to the cartridge; the eluent contained some unconjugated bile acids. These were further eluted with an appropriate volume of ethanol–chloroform–water–acetic acid 20:80:1:0.02 (v/v/v/v). Glycine-conjugated bile acids were eluted with an appropriate volume of ethanol–chloroform–water–acetic acid 20:80:3:5 (v/v/v/v). Selection of appropriate volumes for quantitative fractionation of the bile acid groups was carried out using radiolabeled CA and GCA. The glycine bile acid fractions were evaporated under N_2 in small silanized vials and analyzed as DMES ether methyl esters using capillary GLC as described above.

In each case the glycine-conjugated fraction was also hydrolyzed using cholyl glycine hydrolase prior to GLC analysis. **Fig. 4** shows an example of a comparison between the capillary GLC profile of the glycine conjugate fraction from a normal plasma sample, before and after hydrolysis. **Fig. 5** shows the capillary GLC of glycine-conjugated bile acids from patients with jaundice and liver failure.

In order to assess the recovery of glycine-conjugated bile acids through this procedure, [^{3}H]GCA was added to plasma samples (5 ml) in duplicate. The samples were analyzed and recoveries were assessed by liquid scintillation counting of aliquots removed at various stages of the procedure. Recoveries are shown in **Table 1**.

DISCUSSION

Steroid conjugates are usually hydrolyzed prior to GLC analysis. Early workers were restricted by the limitations of packed column GLC, which was unable to reach the high temperatures required for the analysis of these high molecular weight intact conjugates. Problems were also experienced with early capillary columns because of the instability of stationary phase at high temperature and adsorption problems due to inadequate coating techniques. The development of fused silica, chemically bonded columns has overcome many of these problems. These more robust columns permit the use of high temperature programs and are stable for long periods.

Direct qualitative GLC analysis of steroid glucuronides (12, 13) and bile acid glucuronides (14) has been reported. Direct derivatization of steroid sulfates as perfluoroesters (15) or replacement of the sulfate group with a TMS group has also been demonstrated (13). So far no success-

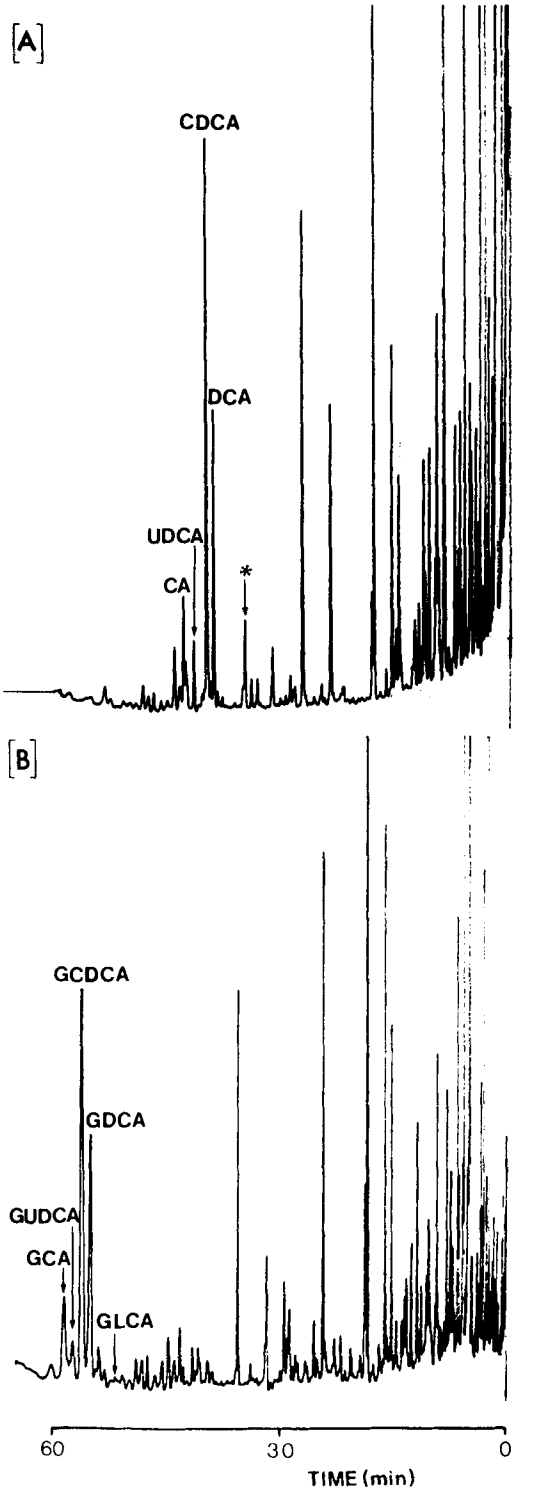

Fig. 4. Gas-liquid chromatography of DMES ethers of carboxylic acid methyl esters of glycine-conjugated bile acids from normal human plasma. [A], After hydrolysis with cholylglycine hydrolase; [B], without prior hydrolysis. Conditions for GLC are described in the text. (*) Indicates position of LCA derivative which may be contaminated with cholesterol. Peaks are tentatively identified from their retention times.

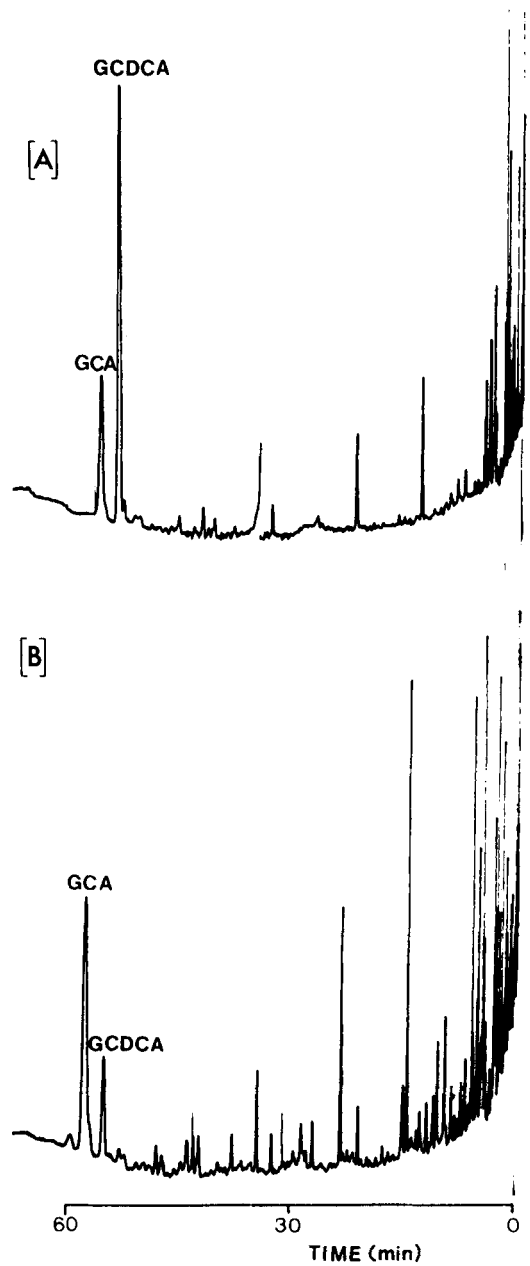

Fig. 5. Gas-liquid chromatography of DMES ethers of carboxylic acid methyl esters of glycine-conjugated bile acids from plasma from patients with [A] jaundice and [B] liver failure. Conditions for GLC are described in the text. Bile acids are tentatively identified from their retention times.

ful analysis of glycine-conjugated bile acids by GLC has been described, although preliminary work on analyzing derivatized bile acid glycine conjugates on packed columns was published by Hanaineh and Brooks (16) and Hanaineh (17). Good separation of glycine conjugates derivatized as methyl ester acetates was demonstrated, but the methyl ester TMS derivatives did not resolve on any of the packed columns studied. Furthermore, the chromatograms obtained were poor and no application of the method to biological samples was attempted.

The work described here is the first report of direct GLC analysis of glycine-conjugated bile acids from plasma without prior hydrolysis, although preliminary studies on the use of methyl ester TMS derivatives analyzed by capillary GLC in conjunction with MS have been described by Street et al. (18). Although Hedenborg and Norman (19) have described the use of GLC of trimethylsilyl ether derivatives of glycine-conjugated bile acids, little detail was given. To achieve the very high carrier gas flow rates described here, high inlet pressures are required, which may not be possible to achieve with alternative injection systems.

At this stage in our investigations, it has not proved possible to analyze taurine-conjugated bile acids by GLC without prior hydrolysis. However unconjugated, glycine-, and taurine-conjugated bile acids can be easily separated by the use of Sep-Pak SIL cartridges (20). The unconjugated and glycine-conjugated fractions can be analyzed by GLC in a single run without the necessity of a hydrolytic step, and the taurine conjugates can be analyzed after enzyme hydrolysis. This procedure requires fewer chromatographic and hydrolytic steps than are necessary if the three fractions are isolated and analyzed individually.

The very high flow rates apparently needed for successful analysis of glycine-conjugated bile acids as described here may cause difficulties if combined GLC-MS is required. With capillary column GLC-MS, the end of the column is usually inserted directly into the ion source of the mass spectrometer and the vacuum systems are not always designed to deal with such high flow rates. It may, however, be possible to overcome this problem. Many mass spectrometers are still used in conjunction with conventional packed columns, connected via a jet separator, which use carrier gas flow rates similar to those described

TABLE 1. Recovery of [^{3}H]GCA added to plasma at various stages in the extraction prior to GLC

Stage in Extraction	Cumulative Recovery (%) after Each Stage[a]
Acetonitrile preparation	89.5
Plug wash	6.9
Total	95.6
Bond Elut	92.5
Sep-Pak SIL fractionation	
Fraction 1	0
Fraction 2	88.3
Fraction 3	2.1
Prior to GLC analysis	79.6

[a] Mean of two determinations is recorded.

here. Our GLC system could, therefore, be connected to a mass spectrometer via such a jet separator, although possible loss through adsorption might occur. A profile of unconjugated and glycine-conjugated bile acids, by the method described here, may also prove useful if used in conjunction with fast atom bombardment-MS which gives good spectra for taurine and sulfate conjugates (21).

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