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EXTRACTION AND FRACTIONATION OF BILE ACIDS AND THEIR CONJUGATES USING PRE-PACKED MICROPARTICULATE SILICA CARTRIDGES (SEP-PAK SIL® AND BOND-ELUT® C_{18})

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SUMMARY

A new method for the extraction of bile acids from human plasma using acetonitrile precipitation of plasma protein and subsequent use of Bond-Elut C_{18} cartridges is described. After extraction the bile acids can be separated into three fractions: unconjugated, glycine-, and taurine-conjugated, using Sep-Pak SIL cartridges at 4°C, eluting with ethanol—chloroform—water—glacial acetic acid mixtures. These extraction and fractionation procedures were evaluated in terms of recovery, reproducibility and resolution between the fractions. All these parameters were found to be satisfactory. Although the reproducibility of fractionation on Sep-Pak SIL cartridges was found to vary between batches, this did not give rise to significant difficulties. Plasmas from normals and patients with hepatobiliary disease were analysed by capillary gas—liquid chromatography after extraction and fractionation using the procedure described.

INTRODUCTION

Bile acids present in plasma may be unconjugated, or linked with glycine and taurine as N-acyl conjugates on the C_{24} carboxyl group and may in addition form further conjugates on any of the hydroxyls with sulphuric and/or glucuronic acids. The extraction and purification of these bile acids in biological fluids, prior to analysis by capillary gas–liquid chromatography (GLC), is a complex procedure involving a number of chromatographic steps, hydrolysis and derivatisation. Two or even three chromatographic steps are

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necessary to obtain separation of all the bile acid groups. Methods available for the measurement of bile acids have been reviewed elsewhere [1]. At present satisfactory group fractionation procedures prior to GLC require lengthy column chromatography [2, 3]. In recent years, the availability of reversed-phase octadecylsilane bonded-silica cartridges has dramatically improved bile acid extraction efficiency and speed. The use of these cartridges for chromatography of the bile acid groups might similarly improve this step in bile acid analysis. This paper describes the application of a commercially available straight-phase silica cartridge (Sep-Pak SIL) for the fractionation of the unconjugated, glycine- and taurine-conjugated bile acid groups. Previously described methods for the extraction of bile acids from plasma have included the addition of alkali to disrupt protein binding. However, such procedures were not found to be compatible with the subsequent fractionation on Sep-Pak SIL. A new method using acetonitrile protein precipitation and Bond-Elut C_{18} purification for the extraction of bile acids from plasma was therefore developed.

EXPERIMENTAL

Solvents and reagents

All reagents, unless otherwise specified, were analytical reagents from BDH (Poole, U.K.), and were used as supplied except methanol and hexane which were redistilled before use. Ethanol (absolute alcohol 100 from James Burroughs, London, U.K.) was also redistilled.

*Reference bile acids**

Unlabelled bile acids were obtained from a number of sources (Sigma, Poole, U.K.; Koch-Light Labs., Colnbrook, U.K. and Steraloids, Croydon, U.K.) and were used without further purification, except cholanic acid (Steraloids) which was purified by recrystallization from ethanol. Bile acids were dissolved in redistilled ethanol (approx. 1 mg/ml) and stored at −15°C. [2,4-^{3}H(N)]-Cholic acid (16.0 Ci/mmol), [^{3}H(G)]glycocholic acid (2 Ci/mmol) and [^{3}H(G)]taurocholic acid (6.6 Ci/mmol) were obtained from DuPont, NEN Products Division (Steverage, U.K.). [1-^{14}C]Glycocholic acid (52 mCi/mmol) and [carboxyl-^{14}C]lithocholic acid (55 mCi/mmol) were obtained from Amersham International (Amersham, U.K.). Radiolabelled bile acids were purified immediately prior to use by thin-layer chromatography on aluminium sheets precoated with silica gel 60 (Merck, Darmstadt, F.R.G.) using the solvent systems recommended by the manufacturer.

Extraction and chromatography materials

Sep-pak SIL cartridges were obtained from Waters Assoc. (Cheshire, U.K.). Bond-Elut C_{18} cartridges and the Vac-Elut apparatus were obtained from Jones Chromatography (Llanbradach, U.K.). Lipidex 1000 and Lipidex 5000 were obtained from Packard Instruments (Reading, U.K.).

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*LCA = lithocholic acid; CA = cholic acid; CDCA = chenodeoxycholic acid; DCA = deoxycholic acid; UDCA = ursodeoxycholic acid; G = glycine conjugated.

Sonication

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

Enzyme hydrolysis

Glycine and taurine conjugates were hydrolysed overnight at 37°C using cholylglycine hydrolase (EC 3.5.1.24, Sigma), a partially purified lyophilized powder from *Clostrium perfringens* (*Welchii*), as described by Nair and Garcia [4]. After deconjugation, the hydrolysis mixtures were diluted with 0.1 *M* sodium hydroxide (5 ml) and extracted using a Bond-Elut C_{18} cartridge (500 mg sorbent) and Vac-Elut apparatus as described by Setchell and Worthington [5].

Gas–liquid chromatography

In this investigation, bile acids were analysed as methyl ester–dimethylethylsilyl (DMES) or trimethylsilyl (TMS) derivatives. Samples were methylated by treating with 200 μl acetyl chloride–methanol [6]. DMES ethers of the hydroxyl groups were then formed by addition of 100 μl dimethylethylsilyl imidazole (DMESI) (Apin Chemicals, Abingdon, U.K.) and incubated at 50°C for 40 min. The unused reagent was removed by an adaptation of the method of Axelson and Sjovall [7] using Lipidex 5000 (3 cm × 0.5 cm) columns prepared in hexane. Samples were applied in two 1-ml volumes of redistilled hexane which were collected. Complete recovery of the derivatised bile acids was ensured by elution with a further 3 ml of this solvent. TMS ethers were formed in a similar fashion using trimethylsilyl imidazole (TSIM). Capillary column GLC was carried out using a Carlo-Erba Fractovap 2900 and auto-solid sampling device (Erba Science (U.K.), Swindon, U.K.) on a 25 m × 0.32 mm CP Sil 5 (CB) column with film thickness of 0.12 μm (Chrompack, London, U.K.). Silanised injection capsules were prepared from glass micro-capillaries (Drummond Scientific, Pennsylvania, PA, U.S.A.) divided into short lengths (approx. 0.7 cm). On injection the capsules are retained in the injection space by a silanized restricted glass lining [8].

Silanisation of glassware

Glassware was silanized by soaking overnight in 1% (v/v) dimethyldichlorosilane (BDH) in toluene, washed with toluene and methanol and dried before use. Silanisation of all glassware was found to be necessary, since adsorption of the bile acids to unsilanised glass surfaces occurred during evaporative steps. Some adsorption to silanised glass still occurred if the bile acids were left dry for longer than 5 min.

Liquid scintillation counting

Liquid scintillation counting was carried out using an Intertechnique Model SL30 (Kontron Intertechnique, St. Albans, U.K.) and 10 ml Cocktail T liquid scintillation fluid (BDH). At least 10 000 counts were collected. Tritium counting efficiency was 40%, with a background count rate of around 40 cpm and ^{14}C-counting efficiency was 95% with a background count rate of 60 cpm. Again, adsorption effects were common. Evaporating the bile acids to dryness

in scintillation vials, particularly when present as bile salts, often gave falsely low count rates. The count rates recovered on standing, sometimes requiring several days to reach stability. It was found that desorption of the bile acid residue with methanol (100 μl) prior to addition of the scintillant overcame these problems.

Analytical procedure

The extraction and fractionation method used is summarised in Fig. 1. Plasma was collected in heparin tubes and stored at −20°C until analysed.

Extraction

Plasma (5 ml for normal subjects or 1 ml for patients with hepatobiliary disease) was cooled to 4°C and cold acetonitrile (6 ml or 2 ml, respectively) added and immediately mixed vigorously on a vortex mixer. After

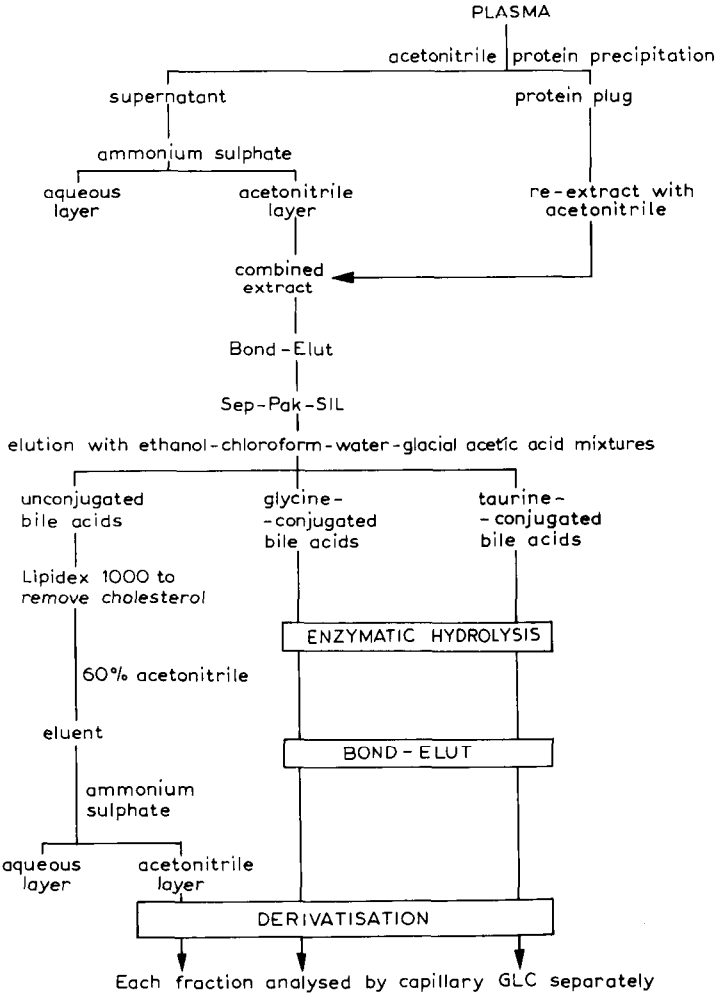

Fig. 1. Schematic representation of the extraction and fractionation procedure.

centrifugation (1500 *g* for 5 min), the supernatant was decanted and ammonium sulphate (0.5 g/ml of plasma) was added. After mixing, two layers were formed which were fully separated by centrifugation (1500 *g* for 5 min). The top acetonitrile layer contained the bile acids. The protein plug obtained after initial acetonitrile extraction and centrifugation was physically dispersed and re-extracted with acetonitrile (4 ml or 1 ml, respectively). The two acetonitrile extracts were combined and evaporated to a small volume (approx. 400 μl) under nitrogen in a small silanized vial and diluted with distilled water (7 ml). A Bond-Elut C_{18} cartridge (500 mg) was primed with redistilled methanol (5 ml) and washed with distilled water (10 ml). After sonication, the diluted extract was applied to the primed Bond-Elut cartridge using the Vac-Elut apparatus at flow-rates of 15—30 ml/min. The small vial was washed with 5 ml distilled water which was also applied to the Bond-Elut cartridge. Bile acids were eluted with 4 ml redistilled methanol at a flow-rate of 15 ml/min. The methanol extract was evaporated to dryness under nitrogen.

Sep-Pak SIL fractionation

All steps in the Sep-Pak SIL fractionation were carried out at 4°C with elution by gravity flow. The Sep-Pak SIL cartridge was primed with 20 ml ethanol—chloroform—water (20:80:1). The plasma extract was redissolved in 1 ml ethanol—chloroform—water (20:80:1) three times by sonication of each aliquot for 3 min, cooled rapidly to 4°C and applied to the cartridge, collecting the eluent as part of the first fraction.

Further elution of the bile acids was carried out using sequential application of appropriate volumes of the solvent mixtures shown in Table I. Selection of appropriate volumes for fractionation of the bile acid groups was carried out for each Sep-Pak SIL batch as described below. Each fraction was collected separately and evaporated under nitrogen in small silanized vials.

Unconjugated bile acid fraction

Cholesterol present in plasma is eluted from the Sep-Pak SIL in the unconjugated fraction. In the GLC system used in this study, cholesterol co-elutes

TABLE I

SOLVENT MIXTURES FOR THE SEPARATION OF BILE ACID GROUPS ON SEP-PAK SIL CARTRIDGES

Fraction	Bile acids	Eluting solvent*
1	Unconjugated	3 ml E—C—W (20:80:1) (application of sample) 5.5 ml E—C—W—AA (20:80:1:0.02)
2	Glycine conjugates	10 ml E—C—W—AA (20:80:3:5)
3	Taurine conjugates	6 ml E—C—W—AA (60:40:3:5)

*All mixtures are v/v. Volumes are subject to change depending on Sep-Pak SIL batch although composition of solvent mixtures remains constant. E = Ethanol; C = chloroform; W = water; AA = glacial acetic acid.

with LCA and thus it is necessary to remove it prior to GLC analysis. To remove cholesterol, Lipidex 1000 was used as described by Setchell and Matsui [9] but using a modified solvent system. The unconjugated bile acid extracts were redissolved in 100 μl methanol followed by 5-ml washes of 0.01 *M* hydrochloric acid, four times. Each aliquot was sonicated for 5 min and applied to a Lipidex 1000 column. The column was washed with 10 ml distilled water and the conjugated bile acids eluted with 20 ml acetonitrile—water (60:40). The eluent was shaken with ammonium sulphate (1 g) and centrifuged (1500 *g*, 5 min). The upper acetonitrile layer was removed and evaporated to dryness in a small silanized vial under nitrogen.

Excess ammonium sulphate interfered with the subsequent derivatisation procedure and was removed by passing the extract resuspended in three 0.5-ml volumes of acetonitrile through the sinter in a Bond-Elut cartridge from which the silica had been removed. The bile acids were derivatised as methyl ester DMES derivatives and analysed by capillary GLC.

Glycine- and taurine-conjugated fractions

Glycine and taurine conjugates were hydrolysed with cholylglycine hydrolase and deconjugated acid extracted using Bond-Elut C_{18} as described above, converted to their methyl ester—DMES ethers and analysed by capillary GLC.

RESULTS

Extraction from plasma

Bile acids are bound to plasma protein and this binding must be disrupted before Bond-Elut C_{18} extraction is carried out in order to achieve adequate recovery, particularly of LCA. This disruption of protein binding has been achieved by incubation of plasma diluted with alkali at 65°C [5]. However, this procedure was found to be incompatible with the Sep-Pak SIL fractionation procedure described here, since it caused spreading of peaks and considerable overlap between the fractions.

To overcome this difficulty, an alternative extraction procedure, using acetonitrile precipitation of plasma protein followed by purification of the extract

TABLE II

RECOVERY OF RADIOLABELLED BILE ACIDS ADDED TO PLASMA, AFTER ACETONITRILE EXTRACTION, AMMONIUM SULPHATE PARTITION AND BOND-ELUT C_{18} PURIFICATION

Bile acid	Percentage recovery*	
	After acetonitrile extraction	After acetonitrile extraction, partition and Bond-Elut C_{18}
LCA	86.3	84.0
GCA	95.6	92.5
TCA	93.8	89.1

*Mean values are recorded (n = 2). Values did not differ from the mean by more than 1%.

on Bond-Elut C_{18} cartridge, was developed and is described above. Using this technique, quantitative recovery of a number of bile acids was obtained and these are summarised in Table II. The use of methanol, instead of acetonitrile, for washing the protein plug, resulted in much higher recoveries of the less polar bile acids but also increased the extraction of cholesterol. Chromatograms obtained were also cleaner when acetonitrile, rather than methanol, was used. Recoveries of added bile acids were found to be most reproducible when extraction was carried out at 4°C.

Evaporation of the acetonitrile extract to dryness is a lengthy procedure because of the large amount of water present. Although rotary evaporators may be used, this spreads the bile acids over large areas of glass which may cause adsorption problems. Ammonium sulphate was therefore added to acetonitrile extracts from plasma, causing separation into two immiscible layers. The upper layer, being acetonitrile free of water, could be evaporated to a small volume more rapidly. It was not necessary to evaporate the sample completely before application to Bond-Elut C_{18}. Using [H^3]TCA and [^{14}C]LCA added to acetonitrile—water mixtures, it was found that dilution to less than 5% (v/v) acetonitrile in water permitted quantitative adsorption of bile acids of wide polarity range to the Bond-Elut C_{18} (see Fig. 2), all of which could then be eluted with methanol.

Sep-Pak SIL fractionation

In order to investigate the fractionation of the bile acid groups on Sep-Pak cartridges, two representative bile acids from each group, i.e. LCA, CA and their glycine and taurine conjugates, representing the extremes of polarity, were studied.

Initially a number of solvent mixtures were examined in conjunction with Sep-Pak C_{18} cartridges but no separation of the bile acid groups could be

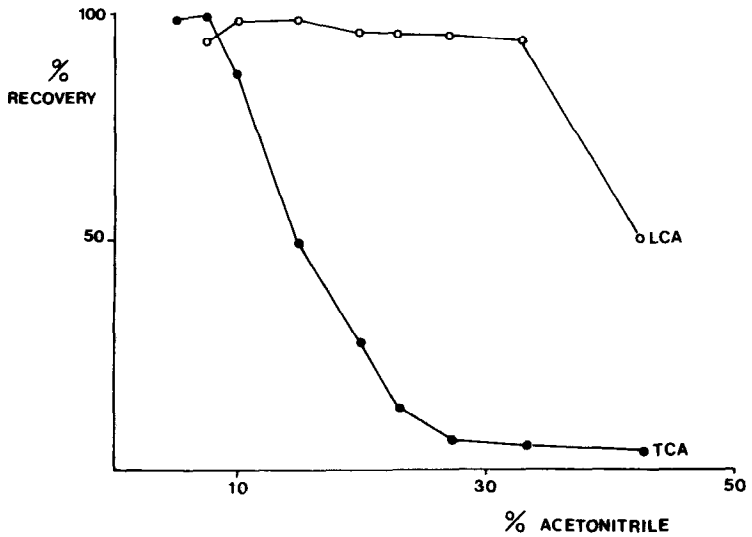

Fig. 2. Percentage recovery of [^{14}C]LCA and [^{3}H]TCA in acetonitrile—water mixtures used to elute Bond-Elut cartridges. Each point represents the mean of two determinations.

obtained. Sep-Pak SIL, used in conjunction with methanol—isopropanol solvent systems, resolved taurine-conjugated bile acids but the glycine-conjugated and unconjugated bile acids did not separate. A solvent system of ethanol—chloroform—distilled water—glacial acetic acid adapted from a method described by Ikawa [10] was found to be successful in separating the unconjugated, glycine-conjugated and taurine-conjugated bile acid groups.

Increasing the proportion of acetic acid in the solvent mixture eluted the glycine-conjugated bile acids and in the final fraction, taurine-conjugated bile acids eluted with increased proportion of ethanol. A certain amount of water in the elution mixture was necessary in order to obtain quantitative recoveries. Initially water-saturated chloroform [10] was used in the elution solvent mixture, but was found to be unsatisfactory since results were not reproducible. Therefore standard mixtures of chloroform and water were adopted.

Resolution of bile acids on Sep-Pak SIL was found to be dependent on temperature and the particular batch used. At room temperature, considerable overlap between the unconjugated bile acid and glycine-conjugated bile acid fraction occurred, in particular with CA and GLCA. Resolution of these bile acids was greatly improved by chromatography at reduced temperature (4°C, see Fig. 3). A similar effect of temperature has been observed in the chromatography of vitamin D metabolites on Sep-Pak SIL [11]. Recovery of all bile

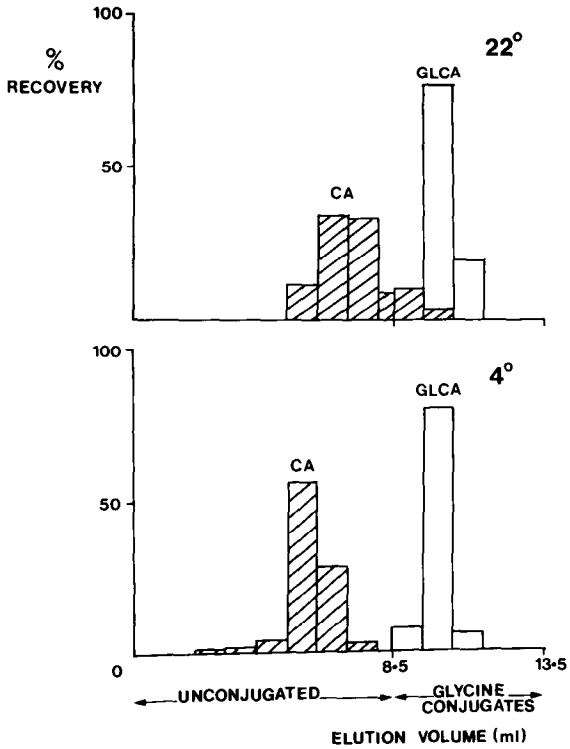

Fig. 3. Effect of temperature on the resolution of CA and GLCA using Sep-Pak SIL cartridges. Means of duplicate values are given.

acids examined (LCA, GLCA, TLCA, CA, GCA, TCA) after Sep-Pak SIL fractionation was quantitative, although a small overlap of GLCA into the unconjugated bile acid fraction occurred (see Table III). This overlap was not seen when 1-ml aliquots were collected (Fig. 4). Fig. 4 illustrates the separation achieved on Sep-Pak SIL cartridges using the method described. Apart from priming the cartridges with ethanol—chloroform—water (20:80:1) (20 ml), no preparation or washing was necessary. Cartridges were discarded after use, although it may be possible to re-use them. It may also be possible to obtain similar fractionation using other pre-packed microparticulate silica cartrdiges (e.g. Bond-Elut silica) but these have not been evaluated.

Selection of elution volumes

Although elution and recovery of bile acids were highly reproducible within a single batch of Sep-Pak SIL, some variation was found to occur between batches. The elution profile obtained with a new batch of Sep-Pak SIL was therefore re-evaluated before use, particularly to ensure complete resolution

TABLE III

PERCENTAGE RECOVERY OF BILE ACID STANDARDS FROM SEP-PAK SIL CHROMATOGRAPHY

Recoveries shown are the mean ± 1 S.D. of five determinations.

Bile acid	Fraction 1	Fraction 2	Fraction 3
LCA	96.2 ± 5.0	—	—
CA	102 ± 3.5	—	—
GLCA	2.7 ± 0.7	98.3 ± 2.8	—
GCA	—	91.6 ± 0.8	—
TLCA	—	—	91.6 ± 1.7
TCA	—	—	93.0 ± 2.6

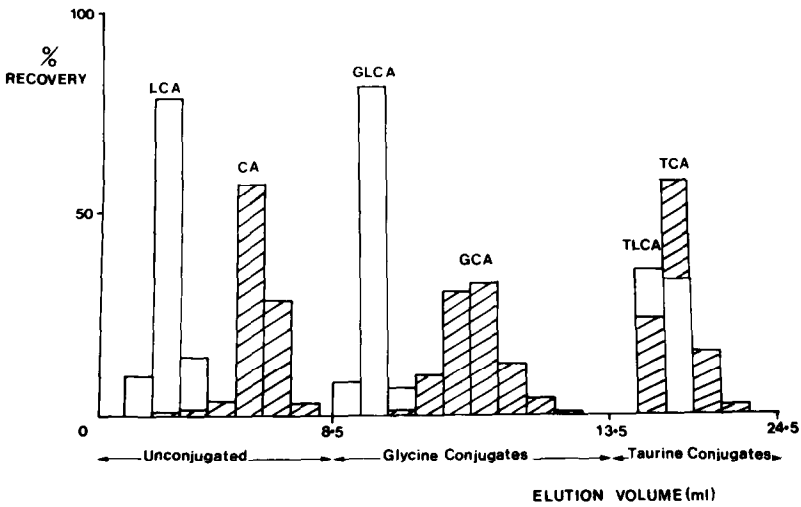

Fig. 4. Elution of LCA, CA, and their glycine and taurine conjugates from Sep-Pak SIL cartridges. Means of duplicate values are given.

of CA and GLCA which is the most crucial separation. It was not found to be necessary to change solvent composition but only to alter the volumes of eluent used.

[^{3}H]CA (20 000 cpm) was applied at 4°C to a primed Sep-Pak SIL cartridge as described above. The SIL cartridge was eluted with ethanol–chloroform–water–glacial acetic acid (20:80:1:0.02) solvent, and 1-ml fractions were collected. Each fraction was evaporated to dryness and counted and the total volume of eluent required to elute CA, the most polar unconjugated bile acid, determined. The final fraction of the elution profile of CA was defined as the first fraction in the tail of the elution peak which contains less than 2% of the total radioactivity. Only 0.5 ml of this fraction is included in the total elution volume used for the fraction 1 elution.

The separation of CA and GLCA was evaluated using unlabelled standards (approx. 100 μg of each), applied to Sep-Pak SIL as above and eluted with

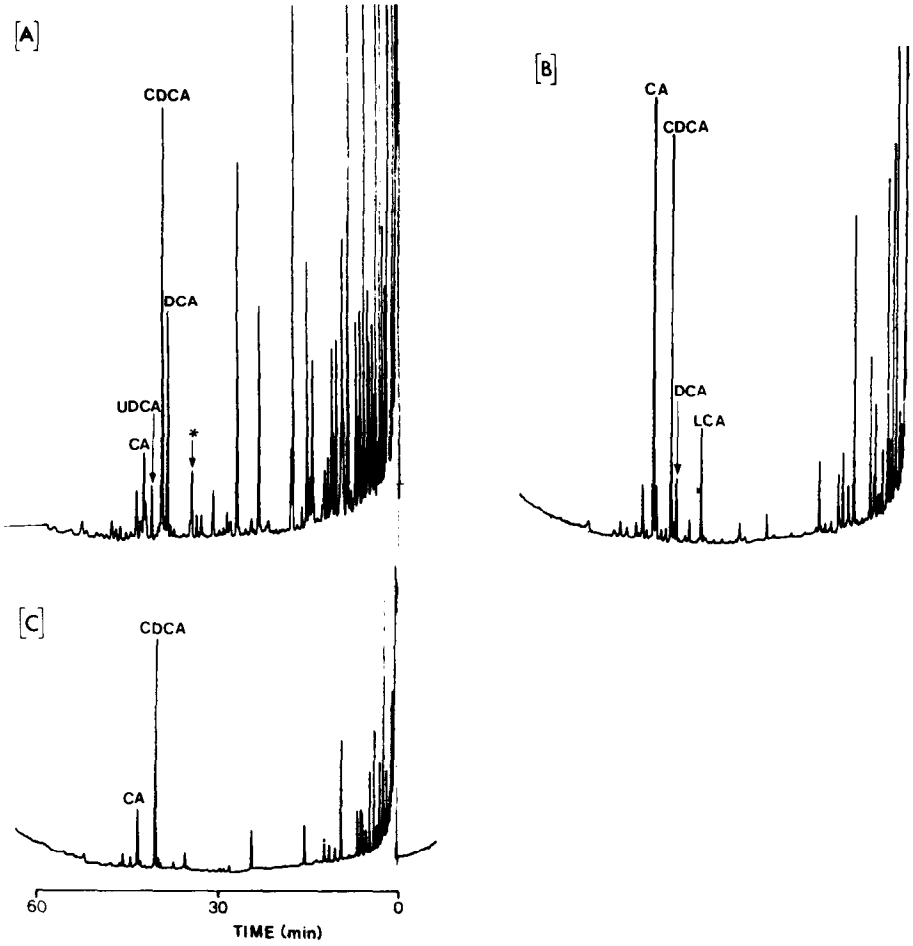

Fig. 5. GLC profiles of plasma bile acids in the glycine conjugated fraction of normal plasma (A) and in the taurine-conjugated fractions in the plasma of a patient with jaundice (B) and with liver failure (C). Bile acids are tentatively identified by their retention times. Star in A represents the position of LCA which may be contaminated with cholesterol.

the selected volume of fraction 1 solvent mixture followed by 5 ml fraction 2 (glycine-conjugated fraction) solvent mixture. After addition of an aliquot of glycocholanic acid as an internal standard the two fractions were dried under nitrogen in small silanized vials. Methyl ester–TMS derivatives were formed as described above and the extracts were analysed using capillary GLC. Determination of the volume of fraction 2 (glycine-conjugated fraction) solvent mixture required to obtain complete elution of GCA can be assessed using [^{3}H]GCA in a similar fashion to that described for [^{3}H]CA in fraction 1.

As shown in Table I, the polarity of the eluting solvents is increased greatly in order to achieve the elution of the taurine conjugates. Overlap of TLCA into fraction 2 has not been observed. Large quantities of SIL cartridges from the same batch can be purchased from the manufacturers if required, and under these circumstances evaluation of the solvent volumes should be necessary only infrequently.

Application to plasma samples

In order to evaluate the use of the bile acid fractionation procedure described above, some plasma samples were analysed as described above. Fig. 5 shows examples of plasma bile acid profiles obtained by capillary GLC from a normal person and two patients with hepatobiliary disease. No bile acids were detected in the unconjugated fraction in any of the three samples analysed.

DISCUSSION

The many techniques of chromatography used for the purification and separation of bile acids have been discussed previously [1]. Although quantitative recoveries, high capacity and large separation factors have been reported using ion-exchange columns, these methods have not been generally adopted, probably because of the large volumes of strongly acidic solvent required for quantitative elution of some of the bile acids [12]. The development of the lipophilic strong anion-exchange gel, DEAP Sephadex LH20, allowed the use of relatively small columns and reduced elution volumes. Stepwise elution of unconjugated, gycine-conjugated, taurine-conjugated and sulphated bile acid groups was achieved by increasing the pH of the eluting buffer [2]. Although quantitative recoveries of several radiolabelled bile acids were found, with minimal overlap between the fractions, non-sulphated or sulphated bile acids other than CA and its conjugates were not studied. With small adaptations, glycine and taurine conjugates were eluted together and analysis time further reduced, although information about the conjugation pattern was lost [9]. Goto et al. [3] experienced difficulty with the use of DEAP Sephadex LH 20 due to insufficient resolution, and separated the bile acids using an alternative lipophilic ion-exchange gel piperidino-hydroxypropyl (PHP) Sephadex LH 20. However, this method required large volumes of eluting buffers and a lengthy elution time was necessary [3, 13]. Both methods require prior removal of cations, which interfere with separation [2, 10]. Extensive preparation of the gels is also necessary although the columns can be regenerated.

Silica gel columns have been used for the separation of the bile acids into mono- and di-, and trihydroxy fractions, and group separation of the glycine and taurine conjugates [10]. However, these methods use large volumes of eluting solvent and have encountered difficulties with standardising flow-rates because of inconsistent mesh sizes of the silicic acid used [12, 14]. The use of Sep-Pak SIL cartridges containing microparticulate carefully graded silica overcomes many of the problems encountered with silica columns. Relatively small volumes of eluting solvent are required and results are reproducible. In our hands, the relatively inexpensive silica cartridges have proved to be an extremely useful method of separating bile acid conjugates prior to subsequent hydrolysis and GLC analysis.

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