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Journal of Chromatography B, 723 (1999) 1–16

JOURNAL OF CHROMATOGRAPHY B

Review

Gas chromatography of bile acids

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Received 21 July 1998; received in revised form 30 October 1998; accepted 5 November 1998

Abstract

Bile acids, the end products of cholesterol metabolism in the liver, are of vital importance in the tissue distribution of cholesterol. Abnormalities in cholesterol biosynthesis or metabolism are often reflected in the proportions, concentrations and conjugation of bile acids in various tissues and determination of bile acids in these tissues is important in the diagnosis of hepatobiliary diseases. Several methods for quantitative determination of bile acids in biological fluids are known and have been reviewed. In this review, we have discussed the gas-chromatographic method for determination of bile acids with special reference to bile acid quantitation in plasma, bile, urine and stool.

Keywords: Reviews; Bile acids

Contents

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0378-4347/99/$ – see front matter
PII: S0378-4347(98)00528-3

1. Introduction

In strict chemical terms, bile acids are a group of steroidal acids with a carboxyl group located in the side chain [1]; however, for general biological purposes, bile acids are the major end products of cholesterol catabolism, formed by the liver, conjugated with amino acids, glycine and taurine and secreted into the bile. The majority of bile acids have the steroidal ring structure, are generally saturated and contain 24 carbons, with the terminal side chain carbon in the form of a carboxyl group. Primary bile acids are formed via the 5β-saturation of the cholesterol double bond by hepatic enzymes, epimerization of the 3β-hydroxyl group to the 3α-configuration and further insertion of a 7α-hydroxyl group with or without a 12α-hydroxyl group, and shortening of the side chain by three carbons and oxidation of the terminal carbon to a carboxyl group.

Bile acids are not only the major catabolic products of cholesterol, they facilitate the excretion of bile lipids, including cholesterol, and the absorption of dietary lipids, including fat-soluble vitamins, via their detergent action. The detergent properties of bile acids result from their unique structure with a non-polar steroid skeleton and polar carboxyl group and α-oriented hydroxyl groups. The polar groups are hydrated with water molecules while the non-polar steroidal moiety emulsifies lipids. The detergency of bile acids is further increased by hepatic conjugation of bile acids with glycine and taurine, before their secretion into the bile. Bile acid conjugates form micelles with phospholipids that solubilize cholesterol in the bile. The primary bile acids, cholic acid and chenodeoxycholic acid, are effectively reabsorbed from the ileum during their enterohepatic circulation, but, approximately 5% seep into the colon, and are subjected to modification by intestinal bacteria to secondary bile acids. These modified bile acids, in particular the 7α-dehydroxylated bile acids, lithocholic acid and deoxycholic acid, also circulate in the enterohepatic circulation, with deoxycholic acid being one of the major plasma and biliary bile acids in humans. Whereas only small amounts of bile acids are excreted into the urine, approximately 500 mg/day are excreted in the stool and form a major catabolic pathway for elimination of body cholesterol.

In hepatobiliary and intestinal diseases, the hepatic synthesis and clearance of bile acids and their intestinal absorption are abnormal, which disturbs both cholesterol synthesis and its metabolism, causing increased plasma, urinary and fecal concentrations of bile acids and, in certain cases, resulting in the accumulation of precursors of cholesterol or bile acids, and serious clinical malformations ensue. Early diagnosis of such conditions is often possible from bile acid analysis in bile, serum, urine and stool. On the other hand, bile acids have therapeutic applications also. Thus, chenodeoxycholic acid and, more recently, ursodeoxycholic acid, have been used as an alternative treatment to surgery in patients with gallstones [2,3]. This latter bile acid is now being increasingly used in a variety of hepatobiliary diseases, like primary biliary cirrhosis [4], primary sclerosing cholangitis [5] and hepatitis C [6], and is suggested to suppress colon polyp formation in experimental animals [7]. Bile acid therapy has been successfully used in conditions with bile acid deficiency and abnormal cholesterol synthesis since both cholic acid and chenodeoxycholic acid are known to suppress the activity of hydroxymethylglutaryl–coenzyme-A reductase, the rate-limiting enzyme for cholesterol synthesis (e.g., [8]). Thus, bile acid analysis may be an important diagnostic tool for diseases of the liver and intestine, and for monitoring bile acid therapy in such diseases.

Several methods have been employed for bile acid analysis in biological fluids, and gas chromatography (GLC), gas chromatography–mass spectrometry

(GC–MS), high-performance liquid chromatography (HPLC), thin-layer chromatography(TLC)/densitometry, colorimetry, fluorimetry and enzymatic methods have all been used [9–15]. However, several of the earlier methods, like colorimetry, fluorimetry, paper and column chromatography and counter-current distribution, are either not sensitive or are non-specific [16] and have been largely displaced by the modern techniques like GLC, HPLC and radioimmunoassay. Of these, radioimmunoassay, although highly sensitive [17,18], is not quite specific and overlaps of different bile acids occur [19]. On the other hand, both GLC and HPLC are highly specific and sensitive to the extent of measuring even a few picomoles of bile acids. In this review, we will describe the practical aspects of bile acid analysis by GLC, with reference to bile acid extraction, derivatization and separation.

In broad terms, GLC works on the same principle as HPLC or adsorption chromatography, however, in GLC, compounds are in gaseous form and an inert gas like nitrogen or helium is used for elution. The various compounds are resolved on the basis of their retention/elution on the stationary phase used in the chromatographic column, which is a liquid phase that is either coated on the inner wall of the column or is absorbed in a solid that is then packed in the column. Thus, there are two basic requirements for GLC analysis, an appropriate column and adsorbent and appropriate derivatization of the compound to cause vaporization under the chromatographic conditions used. Appropriate manipulation of these factors may result in optimum resolution of compounds.

2. Choice of column

2.1. Packed columns

Since the first application of GLC for bile acid analysis by Vanden Heuvel et al. [20], this method is routinely used both for identification and quantitation of bile acids and, until the inception of capillary GLC in the mid 1970s, GLC was performed using metal or glass packed columns. The chromatography is usually carried out on 6 ft long columns packed with the liquid phase, usually on a silica support [21]. The active silicate surfaces in the support and the column are silanized before use, to avoid peak tailing and the partial removal of the protective grouping from the hydroxyl moieties of bile acids and, thereby, improve reproducibility. Various liquid phases have been used depending on polarity, the most common being SE-30 (methyl polysiloxane), OV-17 (methyl phenylsiloxane), QF-1 (trifluoropropyl methylsiloxane) and HI-EFF-8BP (cyclohexanedimethanol succinate), or mixed phases with combinations of two or three liquid phases [21]. Helium is the preferred carrier gas used for chromatography. By appropriate choice of liquid phase, bile acid derivative and temperature programming, most bile acids, and their keto derivatives, have been separated and quantitated [22–24]. However, usually the column has to be packed in the laboratory, and also because of some inherent variabilities in the packing material, the retention times of bile acids are not reproducible from one laboratory to another. Furthermore, the method is not quite sensitive and is usually not applicable for plasma bile acid analysis. Therefore, once capillary columns were introduced, packed columns quickly became obsolete.

2.2. Capillary columns

Fused-silica capillary columns are very versatile and long-lasting and can stand temperatures up to 400°C. Coupled with the fact that the retention times are highly reproducible, capillary columns have fast become the columns of choice for most chromatographic applications for bile acids [25–30]. The usual capillary column is a 25–50-m-long open tubular column with an internal diameter of 0.22 mm. The inner wall is coated with the liquid phase, which may vary from non-polar (e.g. methyl silicones such as OV-1 and SE-30 [28,29] to more polar ones like phenylmethyl- and cyanopropylsilicone [28,30], depending on the need). Most of the work reported in this manuscript involves the use of capillary columns.

3. Extraction of bile acids

As mentioned above, bile acids are present in unconjugated and/or conjugated form in biological

fluids. They range from 1–2 μg/ml in plasma and urine to significant amounts in the intestinal content and as much as 10 mg/ml in the gallbladder bile. Often, bile acids are present in association with proteins, sterols and their esters, free or esterified fatty acids, bile pigments and water-soluble small molecules, which must be either removed before chromatography or the chromatographic conditions should be such that these compounds do not interfere in bile acid analysis. It is often necessary to isolate and at least partially purify bile acids before preparation for GLC. Since bile acids are present mainly as glycine and taurine conjugate in plasma and bile, unconjugated in feces, and unconjugated as well as conjugated with glycine, taurine, glucuronic acid and sulfuric acid in the urine, the methods for isolation of bile acids from these sources need to be appropriately modified. Described below are a few practical methods that can be used for the isolation of bile acids from plasma, bile, urine and feces.

3.1. Plasma

Bile acids are present in plasma mainly as glycine and taurine conjugates, with unconjugated bile acids being present only in small proportions. However, total amounts of plasma bile acids are of the order of 2 μg/ml. On the other hand, plasma contains approximately 2 mg/ml cholesterol, which, if not completely removed, may interfere in the GLC analysis. Thus, it becomes imperative that in addition to the hydrolysis of bile acid conjugates in the plasma, bile acids are isolated free from neutral sterols. Rigorous alkaline hydrolysis [4 *M* sodium hydroxide, 115°C at 15 p.s.i. (1 p.s.i.=6894.76 Pa) pressure] is usually employed to hydrolyze bile acid conjugates. After hydrolysis, the alkaline solution is extracted exhaustively with *n*-hexane, to remove neutral sterols, and the bile acids are then extracted with ether or ethyl acetate after acidification to pH 1. Under these conditions, the glucuronides and sulfate esters are also hydrolyzed, and a pre-step of solvolysis may not be necessary. In order to correct for losses during extraction, an internal standard, usually nor-deoxycholic acid, or nor-cholic acid, is added before the hydrolysis step.

Another approach for extracting cholesterol in the plasma and concentrating the bile acids is to pass plasma through Sep-Pak, a reversed-phase C_{18} cartridge [31]. In this way, most of the plasma cholesterol is eliminated and bile acids and their conjugates are eluted with methanol. Other methods of concentrating bile acids include lipophilic anion-exchange gel, diethylaminohydroxypropyl (DEAP) Sephadex LH-20 [32–34] and, more recently, size-exclusion chromatography using Sephadex G-75 gel, whereby protein material is removed based on its large size relative to that of bile acids. The bile acid fraction obtained by any of these methods is subjected to rigorous alkaline hydrolysis and the small amounts of cholesterol that are not removed by the above methods are extracted out with *n*-hexane. As an alternative to alkaline hydrolysis, bile acid conjugates can be hydrolyzed with the enzyme, cholylglycine hydrolase, while β-glucuronidase is used to hydrolyze bile acid glucuronides. The commercial β-glucuronidase preparation usually contains approximately 10–15% sulfatase activity also, so that bile acid sulfates that may be present in the plasma are also cleaved in the process.

3.2. Bile

Bile acids in the bile are present predominantly as their glycine and taurine conjugates. However, certain bile acids that are not formed in the particular animal species are handled differently by the hepatocyte, and may be secreted unconjugated or as sulfate and/or glucuronide conjugates [35,36]. Bile acids in the bile are usually present in the same general form as bile acids in the plasma, and may be isolated using an essentially similar methodology. Since bile acids are present in high concentrations in the bile, 0.1 ml or less of bile is usually adequate for quantitation by GLC. Also, since bile contains only 1–2% of cholesterol, it is much easier to obtain pure bile acids free from cholesterol for chromatography.

It is often important to learn the bile acid conjugation pattern in the bile since bile acid conjugation is different in different animals and, in addition, it changes in liver diseases, or during bile acid therapy. Several methods can be adapted for group separation of bile acids, based on their state of conjugation. Thus, after concentration of bile acids using a Sep-Pak column, group separation is achieved using lipophilic anion-exchange chromatography [37]. In a

typical experiment, the bile acid extract from the bile (0.1 ml) is diluted with 5 ml of 70% methanol, passed through the cation-exchange resin Amberlyst A-15, in the [H^+] form, followed by passage through a column of Lipidex–DEAP in 72% ethanol, where the neutral compounds are eluted out while bile acids are retained on the column [38]. Unconjugated, glycine conjugated, taurine conjugated and sulfated bile acids are then isolated by step-wise elution with 0.1 mol/l acetic acid in 72% ethanol, followed by 0.3 mol/l acetic acid in 72% ethanol adjusted to pH 5.0, 0.15 mol/l acetic acid in 72% ethanol adjusted to pH 6.6, and 0.3 mol/l acetic acid in 72% ethanol adjusted to pH 9.6. After evaporation of the buffers, conjugated bile acid fractions are then hydrolyzed either with alkali or enzymatically as described above. In other methods, the bile can be directly applied to thin-layer chromatography plates, and developed in appropriate solvent systems that separate unconjugated bile acids from glycine, taurine and sulfate conjugates [39]. The appropriate bands are scraped from the plate and eluted with methanol and subjected to hydrolysis.

3.3. Urine

Bile acids in the urine are present mainly in conjugated form, however, certain bile acids, when ingested, are excreted in unconjugated form also [40,41]. In healthy humans, urinary excretion of bile acids is only a few milligrams per day, however, in hepatobiliary diseases like primary biliary cirrhosis, urine may become a major pathway for bile acid excretion, and sulfate conjugation of bile acids is increased [39,42–47]. Again, bile acids need to be deconjugated for GLC analysis and methods similar to those for plasma can be used to determine the urinary bile acid composition [39]. On the other hand, to learn the conjugation pattern, methods similar to those described above for the biliary bile acid conjugation pattern can be employed [39,43,45–47].

3.4. Stool

Excessive amounts of intestinal bile acids, in particular, deoxycholic acid, have long been implicated in colon carcinogenesis, even though there is only circumstantial evidence for this involvement of bile acids. Thus, increased amounts of deoxycholic acid have been reported in patients with colon cancer compared with controls, by some authors, but not all [48–50]. However, the fecal bile acid pattern is highly complex, due to extensive bacterial metabolism of bile acids during intestinal transit. Thus, the intestinal bacteria deconjugate bile acid conjugates, cleaving the glycine and taurine conjugates as well as glucuronides and sulfate esters. The majority of the unconjugated bile acids formed are 7α-dehydroxylated, and lithocholic and deoxycholic acids are the predominant fecal bile acids. However, other manipulations are also observed, thus, oxidation at carbons 3, 7 and/or 12 to form mono-, di- and/or trioxo compounds have all been isolated from stool [51,52]. Furthermore, epimerization of hydroxyl groups at C-3, -7 and -12 is possible and iso-(3β-hydroxy), urso- (7β-hydroxy) and lago-(12β-hydroxy) bile acids have been reported [51–53]. The secondary bile acids, deoxycholic acid and lithocholic acid are reabsorbed from the colon and further modified by hepatic enzymes and circulate in the enterohepatic circulation. Whereas scores of metabolites of bile acids are formed in the colon, bile acids in the jejunum remain predominantly conjugated, due to the absence of bacteria in the small intestine, and mirror biliary bile acids.

Thus, although increased amounts of deoxycholic acid may be associated with colon polyp formation, it is possible that some of the other secondary bile acids may also be co-carcinogenic. Current studies have focused on the fecal water-soluble bile acids, as the important fraction of the fecal bile acids, since this fraction is the one that is actually in contact with the intestinal epithelium, and increased concentrations of certain bile acids in this fraction may be related to carcinogenesis. Recently, ursodeoxycholic acid has been shown to suppress colon polyp formation in experimental rats [7,54] and it has been shown that the proportion of deoxycholic acid in the fecal water-soluble fraction is significantly reduced in these rats after ursodeoxycholic acid feeding.

Evidently, it is important to study fecal bile acid composition in order to better understand the role of bile acids in colon carcinogenesis. Since fecal bile acid output parallels daily hepatic synthesis, and is thus the main excretory pathway for body cholesterol

elimination, bile acid outputs and their transit times may affect cholesterol absorption and its fecal excretion. Deconjugation of bile acids is nearly complete in the colon, so that fecal bile acids are present mainly in unconjugated form. However, a major difficulty in quantitative analysis of fecal bile acids is their strong binding with the bacterial debris in the stool, and quantitative extraction is difficult. Furthermore, stool contains, in addition to bile acids, neutral sterols, including cholesterol and its bacterial metabolites and plant sterols and their bacterial metabolites, and also fatty acids, which need to be removed before GLC. Several methods have been reported for bile acid extraction from feces, and most are quite complex. Thus, Eneroth et al. [55] used continuous soxhlet extraction of aliquots of homogenized stool using chloroform–methanol, hydrolyzed the extract with strong alkali in aqueous dioxane, acidified the hydrolysate and finally isolated bile acids by continuous extraction with ethyl ether for 16 h. The ether extract was washed free of mineral acid and evaporated to dryness. The residue was subjected to methyl ester formation followed by preparative TLC using freshly activated silicic acid and respective bands were eluted and prepared for GLC. In another method developed by Grundy et al. [56], stool is homogenized with an equal volume of water, an internal standard of ^{14}C-labeled deoxycholic acid is added, followed by 1 *M* sodium hydroxide in 90% ethanol and the mixture is refluxed for 1 h. After dilution with an equal volume of water, the neutral sterols are extracted out with petroleum ether and 10 *M* sodium hydroxide is added to the aqueous layer to give a final alkali concentration of around 3 *M*. The aqueous phase is then heated at 115°C for 3 h, cooled to room temperature, acidified to pH 2, and extracted with chloroform–methanol (2:1, v/v). After evaporation of solvents, the residue is subjected to careful chromatography over Florisil and the purified bile acid fraction is subjected to methyl ester formation, which is then subjected to preparative TLC. A reference standard of methyl cholate is applied to the side of the plate and the TLC plate is first developed in benzene, to separate the fatty acid methyl esters, and then in a solvent system of isooctane–isopropanol–acetic acid (120:40:1, v/v/v). The reference spot of methyl cholate is revealed by exposure to iodine vapor, and a broad band below the band for the fatty acid methyl esters and including the area up to methyl cholate is scraped from the plate and eluted with methanol. After the addition of an internal standard of 5α-cholestane, an aliquot is used for GLC while another aliquot is subjected to radioactivity measurement, to correct for recovery during the procedure. In alternate methods, stool has been extracted with ammoniacal alcohol, methanol–hydrochloric acid, acetic acid–toluene, and bile acids have been extracted after removal of neutral sterols [38,56–59]. Since fatty acids are less strongly retained on the capillary columns, bile acids can usually be quantitated in the presence of fatty acids. In an attempt to simplify the method for routine screening of fecal samples, we digested 50 mg of stool to which 50 μg of the internal standard, nordeoxycholic acid, were added, with 1 ml of 1 *M* sodium hydroxide for 1 h at 80°C in a test tube, extracted out neutral sterols by repeated extraction with *n*-hexane (4×3 ml), acidified the aqueous layer to pH 1 and extracted the bile acids with ethyl acetate (4×3 ml). Ethyl acetate was washed with water to remove mineral acid, evaporated to dryness and the residue was subjected to methyl ester formation. One-tenth of the aliquot was then used for GLC after trimethylsilylation. The method, however, is not applicable to samples where bile acid conjugates are suspected.

4. Derivatization

4.1. Derivatization of the carboxyl group

Since the basic requirement of GLC is that the compounds are in gaseous form at the column temperature, it is imperative that the polar functional groups are appropriately derivatized. The carboxyl group in bile acids is most often converted into the methyl ester. Treatment with diazomethane in ether converts bile acids instantly into their methyl esters. However, due to the hazards of using diazomethane and the formation of small amounts of methyl ethers as artefacts, alternative methods have been used. Methyl esters are quantitatively formed with methanol–5% sulfuric acid or with methanol in the presence of *p*-toluene sulfonic acid, however, the reaction product needs to be extracted with a solvent

and the extract washed with water to remove the acid. On the other hand, treatment of an extract containing bile acids at room temperature with 3% anhydrous methanolic hydrochloric acid for 1–2 h and evaporation of the solvent at 55°C converts them into the methyl esters quantitatively. We have found that, with the use of anhydrous reagents, methyl esters are almost exclusively formed, whereas the use of an older reagent of methanolic hydrochloric acid, which may have absorbed some atmospheric moisture, up to 5% of less polar, mainly dehydration products, are formed. Alternatively, 2,2-dimethoxypropane–hydrochloric acid may be used for derivatization [60].

In addition to methyl esters, other bile acid esters have also been used for GLC. Thus, ethyl, *n*-propyl, isobutyl and *n*-butyl esters have all been employed for the GLC of bile acids [61–63]. The advantage of esterification with higher homologs of alcohols is that the retention times of bile acids are increased and, sometimes, resolutions are improved.

4.2. Derivatization of hydroxyl groups

The derivative of choice for the hydroxyl group is the trimethylsilyl ether derivative and kits are available for quantitative derivatization of even hindered and tertiary hydroxyl groups. Thus, the bile acids from 1–2 ml of plasma are converted into the methyl esters and the residual product is treated with 100 μl of trimethylsilylating reagent at 55°C for 30 min. After evaporation of excess reagent, the residue is taken up in *n*-hexane and an aliquot is injected into the gas chromatograph. A variety of trimethylsilylating reagents are commercially available (Supelco, Alltech, etc.) for derivatization of unhindered as well as hindered hydroxyl groups. Thus, *N*,*O*-bis(trimethylsilyl)acetamide, in combination with trimethylchlorosilane, can convert relatively unhindered hydroxyl groups into their trimethylsilyl ether derivatives, while, when mixed also with trimethyliodosilane, the reagent is highly potent and can silylate hydroxyl groups in all positions in the bile acid. Bis(trimethylsilyl)trifluoroacetamide is equally as reactive as *N*,*O*-bis-(trimethylsilyl)acetamide, but the reagent and the by-products are more volatile. The older silylating reagent, a mixture of 1,1,1,3,3,3-hexamethyldisilazane, trimethylchlorosilane and pyridine (3:1:9, v/v/v) is still commonly used for trimethylsilyl ether derivatization. Although trimethylsilyl ethers have gained general applicability in bile acid analysis, other silylating groups may have some distinct advantages. Thus, dimethylethylsilyl and dimethylpropylsilyl ether derivatives have longer retention times than the corresponding trimethylsilyl ether derivatives [61,64–66], while *tert*.-butyldimethylsilyl ether derivatives are very stable and can be stored, and also show significantly longer retention times so that better resolutions can often be obtained.

In addition to silylation, hydroxyl groups are also protected as acyl derivatives, and formate, acetate as well as trifluoroacetate derivatives are all employed for GLC [67–70]. Although the trifluoroacetyl derivatives are more commonly used due to the ease of formation and volatility, the simple acetates are more stable, with lower molecular masses, and have been successfully used in bile acid analysis (Fig. 1). A one-step derivatization of the hydroxyl and carboxyl groups with heptafluorobutyric anhydride, which also results in the simultaneous hydrolysis of the glycine and taurine conjugates of the bile acids, has been reported [71,72].

4.3. Derivatization of oxo groups

Oxo bile acids are formed by bacterial modification of bile acids during their intestinal transit and are therefore found in the intestinal content. Small amounts of oxo bile acids are absorbed and thus enter into the enterohepatic circulation, however, the hepatic enzymes convert them into the hydroxy bile acids. Oxo bile acids, in particular, 3-oxo bile acids, are vulnerable to the rigorous alkaline hydrolysis conditions [73] and, therefore, enzymatic hydrolysis of bile acid conjugates is preferred when oxo bile acids are suspected in the sample. The methyl esters of oxo bile acids can be chromatographed without further derivatization (the hydroxyl groups in the partially oxidized compounds must be derivatized) [74], however, sometimes artifacts are produced and it may be better to protect the oxo groups as the *O*-methyloxime [67] or the dimethylhydrazone [75] derivatives.

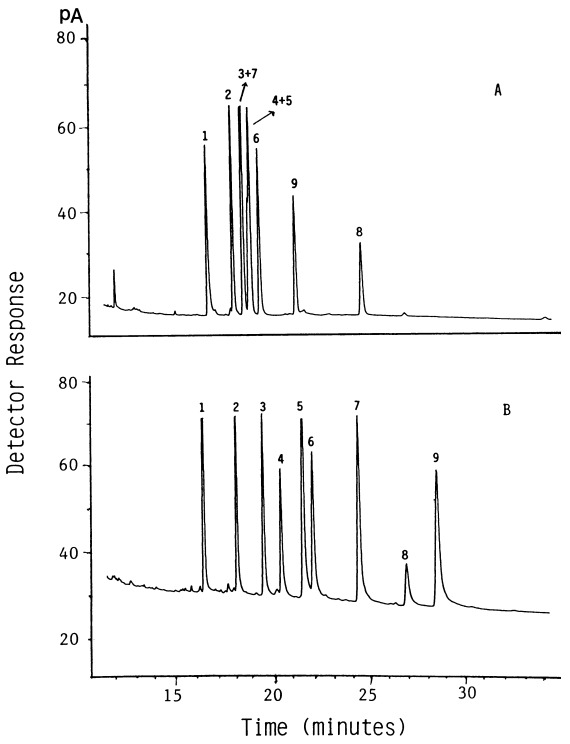

Fig. 1. Capillary GLC profile of the methyl ester–trimethylsilyl ether and acetate–methyl esters of bile acids. The derivatized bile acids were injected on a fused-silica capillary CP-Sil-5 CB column, using the conditions described in Table 1. Fig. 1A, methyl ester–trimethylsilyl ether derivatives and Fig. 1B, acetate–methyl ester derivatives. Peak identification is as follows: peak 1, lithocholic acid; 2, deoxycholic acid; 3, chenodeoxycholic acid; 4, hyodeoxycholic acid; 5, cholic acid; 6, ursodeoxycholic acid; 7, α-muricholic acid; 8, β-muricholic acid and 9, ω-muricholic acid.

5. Bile acid analysis in biological fluids

5.1. Plasma

As mentioned above, there are two main considerations in plasma bile acid analysis: low bile acid levels and very high cholesterol levels. Although cholesterol is well resolved from bile acids on packed glass columns, low sensitivity practically precludes their use [21]. Newer gas chromatographs that use capillary columns have greatly enhanced sensitivity so that plasma bile acids are now routinely quantitated by GLC on capillary columns. The predominant bile acids in human plasma are cholic and chenodeoxycholic acids and their 7-dehydroxylated products, deoxycholic and lithocholic acids. There are numerous reports on the quantitation of plasma bile acids in healthy humans and in patients with hepatobiliary diseases and other pathological conditions. Usually, bile acids in plasma are determined as the methyl ester–trimethylsilyl ether derivatives, and fused-silica capillary columns, such as OV-10, CP-Sil-5 CB and the polar CP-Sil-19 CB columns, are used. Base-line resolutions are obtained for the predominant plasma bile acids using these columns, however, cholesterol may sometimes interfere and, since the GLC retention time of the trimethylsilyl ether of cholesterol is not substantially different from that of methyl lithocholate, plasma lithocholic acid may be overestimated. We encountered this paradox during our study of plasma bile acids in patients with primary biliary cirrhosis and found that approximately 5% of the samples showed abnormally high amounts of lithocholic acid, a situation corrected later by repeating the analyses following the more thorough extraction of neutral sterols. We have now developed a method in which bile acids are chromatographed as their *n*-butyl ester–trimethylsilyl ether derivatives [76]. Since the butyl esters show substantially longer retention times than the corresponding methyl esters [63], the trimethylsilyl ether of lithocholic acid *n*-butyl ester was very well resolved from the trimethylsilyl ether of cholesterol and, so, lithocholic acid could be quantitated in the presence of cholesterol. The method was applicable to plasma bile acid analysis and the work-up was greatly simplified. In a typical analysis, internal standard (nor-cholic acid or nor-deoxycholic acid, 10 μg in 100 μl of methanol) is added to plasma (1 ml) followed by the addition of acetate buffer, pH 5.6 (2 ml), 1.86% EDTA (1 ml), 0.87% mercaptoethanol (1 ml) and the enzymes cholylglycine hydrolase (1–2 U) and β-glucuronidase (40 U) suspended in 1 ml of acetate buffer. The incubation mixture is kept at 37°C for 18 h and then passed through a pre-washed C_{18} reversed-phase Sep-Pak cartridge and bile acids are eluted with acetone (methanol may not be used for elution to avoid the formation of partial methyl esters of the bile acids during further work-up [76]). After evaporation of acetone, the residue is treated with 100 μl of *n*-butanol and 20 μl of a 40% solution of hydrogen chloride in dioxane, and heated at 60°C for

4 h and then kept overnight at room temperature. After evaporation of solvents at 60°C, the product is subjected to trimethylsilyl ether formation and one-twentieth of the volume is used for GLC. Fig. 2 shows a typical gas chromatogram on a 25-m fused-silica CP-Sil-5 CB capillary column when a mixture of bile acids, as their *n*-butyl ester–trimethylsilyl ether (Fig. 2A) or as the methyl ester–trimethylsilyl ether (Fig. 2B) derivatives, was employed, while a typical gas chromatogram of plasma bile acids as the *n*-butyl ester–trimethylsilyl ether derivatives in a patient with primary biliary cirrhosis is shown in Fig. 3A. Cholesterol is obviously very well resolved from lithocholic acid. Under these conditions, the commonly encountered sterols, including sitosterol, campesterol and cholesterol are all eluted from the column before the major plasma and biliary bile acids and also the internal standard, nor-cholic acid. Thus, bile acids can be determined in the presence of

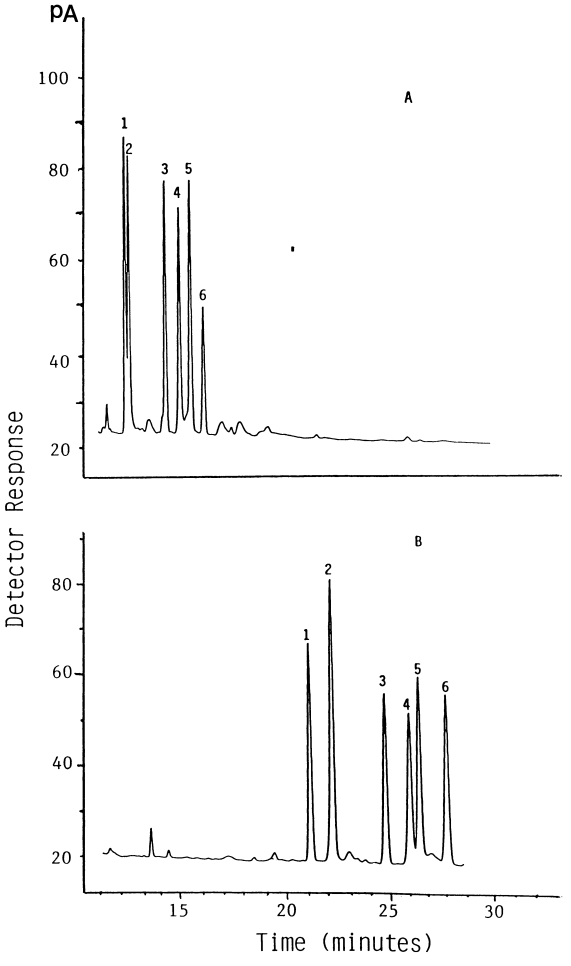

Fig. 2. Capillary GLC profile of the methyl ester–trimethylsilyl ethers and *n*-butyl ester–trimethylsilyl ethers of bile acids. The derivatized bile acids were injected onto a fused-silica capillary (CP-Sil-5 CB) column, using the conditions described in Table 1. Fig. 2A, Methyl ester–trimethylsilyl ethers and Fig. 2B, *n*-butyl ester–trimethylsilyl ethers. Peak identification is as follows: peak 1, nor-cholic acid; 2, lithocholic acid; 3, deoxycholic acid; 4, chenodeoxycholic acid; 5, cholic acid and 6, ursodeoxycholic acid.

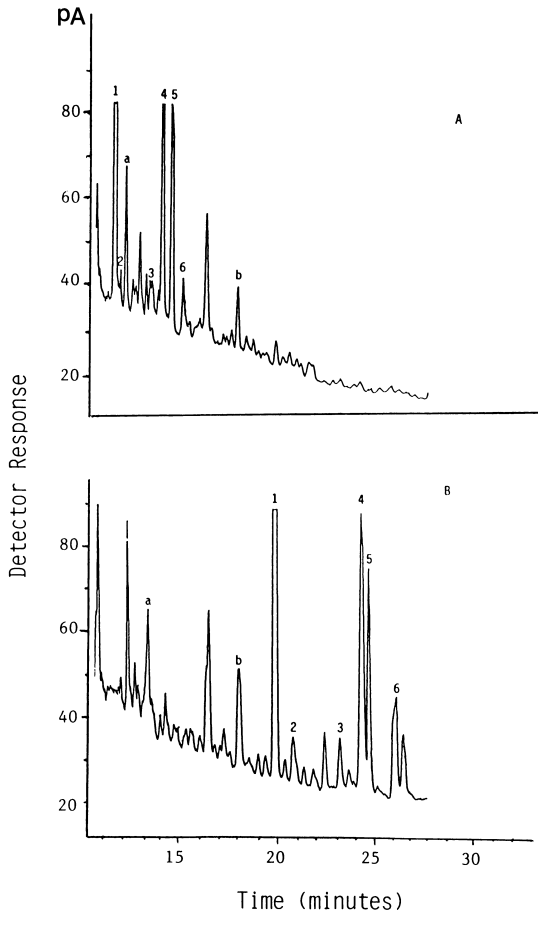

Fig. 3. Capillary GLC profile of plasma bile acids. Fig. 3A, patient with primary biliary cirrhosis; Fig. 3B, patient with sitosterolemia. The *n*-butyl ester–trimethylsilyl ether derivatives of bile acids were injected onto a fused-silica capillary (CP-Sil-5 CB) column, using the conditions described in Table 1 above. Peak identification is as follows: peak 1, nor-cholic acid; 2, lithocholic acid; 3, deoxycholic acid; 4, chenodeoxycholic acid; 5, cholic acid; 6, ursodeoxycholic acid; a, cholesterol and b, sitosterol.

sitosterol, a situation observed in patients with the rare lipid storage disease, sitosterolemia, where large amounts of plant sterols are absorbed and circulate in the blood (Fig. 3B). The method represents a modification of the method of Child et al. [63], who prepared the *n*-butyl ester-acetates of bile acids and used them for capillary GLC on a non-polar DB-5 or a more polar DB-1701 capillary column. Although the acetylated derivatives of sterols are well resolved from the *n*-butyl ester-acetates of bile acids, the retention times of acetates are higher than those of the corresponding trimethylsilyl ethers, so that the *n*-butyl ester-acetates will have significantly higher elution times compared with the *n*-butyl ester–timethylsilyl ethers. Furthermore, the lower limit of detection of acetate derivatives is generally higher than that of the trimethylsilyl ethers, in particular, for the trihydroxy bile acids (e.g., cholic acid) and, therefore, the method may not be sensitive enough to be applied to plasma bile acid analysis.

5.2. Bile

Bile acids in the bile are almost exclusively present conjugated with glycine and/or taurine. The hepatic conversion of cholesterol into bile acids is species-specific and, therefore, bile acid patterns and their conjugation are different in different animals. Thus, whereas pig, rat and mouse are able to further 6-hydroxylate chenodeoxycholic acid, most other animals can not. Humans produce both cholic acid and chenodeoxycholic acid in almost equal amounts, guinea pigs lack 12α-hydroxylation and make predominantly chenodeoxycholic acid, whereas rabbits have very efficient 12α-hydroxylation and cholic acid is the major bile acid prepared by hepatic enzymes. However, secondary bile acids formed by intestinal bacteria may also circulate in the enterohepatic circulation and significantly modify the biliary bile acid pattern. This is especially evident in the rabbit where the gallbladder bile contains deoxycholic acid as the major bile acid due to very efficient 7-dehydroxylation of cholic acid in the intestine and the efficient intestinal absorption of deoxycholic acid. However, after prolonged bile fistula, cholic acid becomes the predominant biliary bile acid in the rabbit and represents hepatic synthesis. Several species, such as rat, mouse, dog, cat and ox, have predominantly taurine-conjugated bile acids, while rabbits and guinea pigs have mainly glycine conjugates. On the other hand, several animals, including humans, make both glycine and taurine conjugates. Under certain disease or therapeutic conditions, the conjugation pattern can be altered and, therefore, it is important to know the bile acid conjugation pattern in the bile. Thus, under certain conditions of reduced bile acid synthesis, such as cholestatic liver disease or cerebrotendinous xanthomatosis (a rare lipid storage disease with defective bile acid synthesis characterized by accumulation of bile alcohol glucuronides), taurine conjugation of bile acids increases. On the other hand, feeding of bile acids results in greatly increased glycine conjugation. Both alkaline and enzymatic deconjugation methods are generally applicable to the hydrolysis of bile acid conjugates in the bile, however, higher homologs of bile acids (C_{25}- or C_{27}-bile acids) are resistant to enzymatic deconjugation [77]. After deconjugation, neutral sterols are extracted with *n*-hexane and bile acids are extracted with ethyl acetate, derivatized and injected into the GLC column. Gallbladder bile is usually several-fold more concentrated than duodenal bile aspirate, so that smaller aliquots of gallbladder bile need to be used. In order to study the bile acid conjugation pattern, bile components have to be first separated into the glycine and taurine conjugates before hydrolysis.

5.3. Urine

Urinary bile acids are usually present as sulfate esters or as glucuronides, and are first converted into the unconjugated form before GLC analysis. The sulfate esters can be solvolyzed and both glucuronides and sulfates can be hydrolyzed with a crude preparation of the enzyme β-glucuronidase, which also has sulfatase activity. In a typical analysis, 1 ml of urine with 10 μg of internal recovery standard is incubated for 18 h with cholylglycine hydrolase and β-glucuronidase in acetate buffer, pH 5.6, and the contents are then passed through a pre-washed Sep-Pak cartridge [39]. The liberated unconjugated bile acids are eluted with methanol. After appropriate derivatization, one-twentieth of the volume is injected into the capillary gas chromatography column

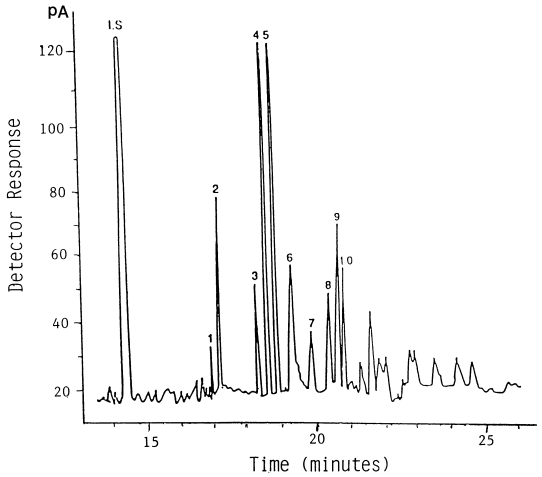

Fig. 4. Capillary GLC profile of urinary bile acids in a patient with primary biliary cirrhosis. The methyl ester–trimethylsilyl ether derivatives of the bile acids were injected on a fused-silica capillary (CP-Sil-5 CB) column, using the conditions described in Table 1. Peak identification is as follows: peak 1, nor-cholic acid; 2, lithocholic acid; 3, deoxycholic acid; 4, chenodeoxycholic acid; 5, cholic acid; 6, ursodeoxycholic acid; 7, ursocholic acid; 8, 1β,3α,12α-trihydroxy-5β-cholan-24-oic acid; 9, hyocholic acid; 10, 1β,3α,7α,12α-tetrahydroxy-5β-cholan-24-oic acid; I.S., 5α-cholestane.

(Fig. 4). In order to quantitate the fraction of bile acids present as the sulfate, glucuronide and/or glycine/taurine conjugate, urine may be subjected to ion-exchange chromatography and the various fractions are isolated before hydrolysis/derivatization. Thus, a urine sample (10 ml) is passed through a Sep-Pak C_{18} cartridge and bile acids are eluted with methanol. The methanol extract is diluted to 70% with water and tracer amounts of ^{14}C-labeled cholic acid and taurocholate are added as recovery standards. The solution is passed through a Lipidex–DEAP column in acetate form in 70% methanol and group separation of bile acids is achieved by stepwise elution with acetic acid and ammonium acetate/ammonium hydroxide, with an apparent pH of 5.0, 6.6 and 10, in 70% methanol [45]. In this way, unconjugated bile acids, glycine-conjugated bile acids, taurine-conjugated bile acids and sulfated unconjugated plus conjugated, and glucuronidated conjugated bile acids are obtained. Each fraction is then directly derivatized, or after appropriate hydrolysis of the conjugate and/or glucuronide/sulfate moieties, and used for GLC.

5.4. Stool

Due to efficient bacterial deconjugation, bile acids in the stool are present almost completely in the unconjugated form. There are two important considerations in the quantitation of fecal bile acids, the large number of oxo- and epimeric bile acids in the feces and the tight binding of bile acids to bacterial debris. Several epimers of common bile acids have been resolved by GLC using standard capillary columns (Tables 1 and 2), and Iida et al. [74] have reported on the resolution of a number of oxo bile acids by capillary GLC. The major problem in fecal bile acid analysis lies in their quantitative extraction from the feces and, as described above, highly

Table 1
GLC retention indices of methyl ester–trimethylsilyl ethers of bile acids[a]

5β-Cholanoic acid	Retention index[b]	
	CP-Sil-19 CB	CP-Sil-5 CB
3α-Hydroxy-	3187	3179
3α-Hydroxy-Δ^6-	3157	3136
3α-Hydroxy-Δ^7-	3186	3177
3β-Hydroxy-	3187	3162
3α,7α-Dihydroxy-	3270	3316
3α,7β-Dihydroxy-	3306	3397
3β,7α-Dihydroxy	3232	3319
3β,7β-Dihydroxy	3316	3405
3α,12α-Dihydroxy-	3247	3260
3α,12β-Dihydroxy-	3221	3331
3α,7α,12α-Trihydroxy-	3288	3344
3α,7β,12α-Trihydroxy-	3332	3464
3α,7α,12β-Trihydroxy-	3268	3453
3α,7β,12β-Trihydroxy-	3313	3531

[a] A Hewlett-Packard model 5880A gas chromatograph equipped with a flame ionization detector and an injector with a split/splitless device for capillary columns was used for all separations. The chromatographic column consisted of a chemically bonded fused-silica CP-Sil-19 CB (stationary phase, 85% dimethyl,7% cyanopropyl, 7% phenyl and 1% vinylsiloxane) or CP-Sil-5 CB (stationary phase, 100% dimethylsiloxane) capillary column (25 m×0.22 mm I.D.; Chrompack, Raritan, NJ, USA) and helium was used as the carrier gas. The GLC operating conditions were as follows. Injector and detector temperatures were 260 and 290°C, respectively. After injection, the oven temperature was kept at 100°C for 2 min, then programmed at a rate of 35°C/min to a final temperature of 265°C when using a CP-Sil-19 CB column and 278°C when using a CP-Sil-5 CB column.

[b] Kovats values, determined by the prior injection of a hydrocarbon mixture C_{31}–C_{37} under identical GC conditions, and comparison of retention times.

Table 2
GLC retention indices of methyl ester–trimethylsilyl ethers of 6-hydroxylated bile acids[a]

5β-Cholanoic acid	Retention index	
	CP-Sil-19-CB	CP-Sil-5-CB
3α,6α-Dihydroxy-	3422	3256
3α,6β-Dihydroxy-	3389	3242
3α,6α,7α-Trihydroxy-	3471	3336
3α,6β,7α-Trihydroxy-	3359	3246
3α,6α,7β-Trihydroxy-	3608	3453
3α,6β,7β-Trihydroxy-	3486	3348
3α,6α,7α,12α-Tetrahydroxy-	3445	3340
3α,6β,7α,12α-Tetrahydroxy-	3338	3254
3α,6α,7β,12α-Tetrahydroxy-	3570	3447
3α,6β,7β,12α-Tetrahydroxy-	3468	3310
3α,6β,7α,12β-Tetrahydroxy-	3342	3243
3α,6β,7β,12β-Tetrahydroxy-	3467	3356

[a] The GLC operating conditions, chromatographic columns and calculation of retention indices are described in Table 1.

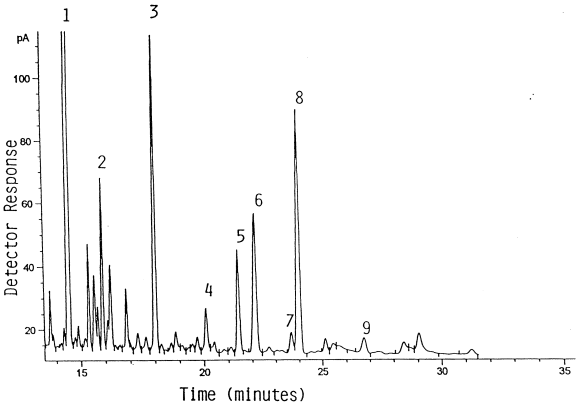

Fig. 5. Capillary GLC profile of human fecal bile acids as *n*-butyl ester–trimethylsilyl ether derivatives. The derivatized bile acids were injected on a fused-silica capillary (CP-Sil-5 CB) column, using the conditions described in Table 1. Peak identification is as follows: peak 1, coprostanol; 2, cholesterol; 3, coprositostanol; 4, sitosterol 5, nor-cholic acid (internal standard); 6, lithocholic acid; 7, iso-deoxycholic acid; 8, deoxycholic acid and 9, ursodeoxycholic acid.

cumbersome methods have been used for this purpose. Although such methods may be used to analyze a few samples, they are inconvenient for the analysis of large numbers of samples or for routine sample screening. In an attempt to avoid elaborate extractions, Child et al. [63] prepared the *n*-butyl ester–acetate derivatives of bile acids present in human stool without prior purification. They used the method for routine fecal bile acid analysis in patients with colon polyps. Considering several drawbacks of acetate–*n*-butyl ester derivatives, including reduced sensitivity and greatly increased retention times, we recently prepared the *n*-butyl ester–trimethylsilyl ether derivatives of bile acids and showed that the method could be conveniently employed to quantitate fecal bile acids without any prior treatment. Fig. 5 shows a typical GLC chromatogram of human fecal bile acids. As can be seen, sterols are all eluted before bile acids and there is no overlap. The method was compared with a solvent extraction method where bile acids were continuously extracted with basic ethanol for 18 h, neutral sterols were extracted with *n*-hexane before isolation of bile acids and the bile acids were then quantitated as the trimethylsilyl ether–*n*-butyl ester derivatives, and as shown in Table 3, the two methods were compatible. As can be seen from the table, methanol extraction results in incomplete recovery of lithocholic acid. The weakness of the method is that it cannot be used for fecal bile acid analysis under certain pathological conditions, where bile acids in the stool may remain conjugated with glycine and/or taurine.

6. Analysis of bile acid precursors

In most animal species, the end products of cholesterol metabolism are the C_{24}-bile acids. However, certain amphibian species are not capable of metabolizing the intermediate C_{27}-bile acids that accumulate as the end products of cholesterol biosynthesis [78–83]. In certain disease conditions of peroxisomal deficiency, like Zellweger's syndrome, the C_{27}-bile acid, 3α,7α,12α-trihydroxy-5β-cholestanoic acid, is found as the predominant biliary and plasma bile acid [84]. Furthermore, in hepatobiliary diseases and in neonates, several unnatural, extrahydroxylated bile acids are excreted in the urine [40,85]. Table 4 shows the GLC retention times of several unusual bile acids. In certain species of fish, the C_{27}-bile alcohols, which are the 27-hydroxylated bile alcohols, are not converted into bile acids, and bile alcohols like schymol are present in the bile as sulfate esters [86,87]. In humans, the presence of increased amounts of bile alcohols would represent

Table 3
Bile acid analysis in human stool. Comparison of different methods

Bile acid	Direct derivatization[a]	Soxhlet extraction[b] (μg/mg dry stool)	Methanol extraction[c]
LC+isoLC[d]	3.12±1.22	2.52±0.88	1.80±0.82
IsoDC	1.04±0.56	0.91±0.53	0.95±0.48
DC	3.10±1.35	3.22±1.52	3.15±1.28
Other	0.49±0.49	0.25±0.25	0.40±0.36
Total	7.75±2.80	6.90±2.74	6.30±2.42

[a] Stool (10–15 mg) was directly subjected to *n*-butyl ester formation followed by trimethylsilyl ether derivatization. After the addition of hexane (100 μl), 1 μl was injected into the gas chromatograph.

[b] Stool (10–15 mg) was continuously extracted for 18 h with ammoniacal ethanol, in a soxhlet extractor, sterols were removed by extraction with *n*-hexane, and bile acids were extracted with ethyl acetate and subjected to *n*-butyl ester formation followed by trimethylsilyl ether derivatization. After the addition of hexane (100 μl), 1 μl was injected into the gas chromatograph.

[c] Stool (10–15 mg) was extracted with methanol at 65°C for 2 h (4×5 ml) and sterols were removed by extraction with *n*-hexane, and bile acids were extracted with ethyl acetate and subjected to *n*-butyl ester formation followed by trimethylsilyl ether derivatization. After the addition of hexane (100 μl), 1 μl was injected into the gas chromatograph.

[d] LC, 3α-hydroxy-5β-cholanoic acid; Iso-LC, 3β-hydroxy-5β-cholanoic acid; DC, 3α,12α-dihydroxy-5β-cholanoic acid; Iso-DC, 3β,12α-dihydroxy-5β-cholanoic acid.

abnormal bile acid synthesis, and several bile alcohols hydroxylated in the side-chain at C-23,24 and 25 accumulate in the bile, plasma, urine and stool of patients with cerebrotendinous xanthomatosis [88,89]. Bile alcohols in the plasma, bile and urine of these patients are usually present as glucuronides, while those in the stool are present predominantly in unconjugated form. Bile alcohols can be extracted from biological fluids (urine, bile or plasma) by passing them through a pre-washed Sep-Pak cartridge, and are collected together with bile acids by elution with methanol. The glucuronides can then be hydrolyzed and bile alcohols isolated and derivatized as the trimethylsilyl ethers for GLC [90]. Table 5 shows the retention indices of several bile alcohols on two capillary columns. Most compounds are resolved on the individual columns, but it was found that the more polar CP-Sil-19 CB columns generally showed better resolutions between the various compounds. It should also be noted that, whereas the bile alcohols with 7α,12α-dihydroxy groups are eluted later than the bile alcohols without the 12α-hydroxyl group on the CP-Sil-5 CB column, the trend was reversed when a CP-Sil-19 CB column was used [89]. This was also found to be the case when bile acids with a 7α-hydroxyl group and with 7α,12α-dihydroxyl groups were chromatographed on the two columns. Thus, as shown in Table 1, chenodeoxy-

Table 4
Retention indices of the methyl ester trimethylsilyl ether derivatives of bile acids with longer or shorter side chains[a]

Bile acid	Retention index (Kovats)	
	CP-Sil-19-CB	CP-Sil-5-CB
24-Nor-3α,7α-dihydroxy-5β-cholanoic acid	3271	3119
24-Nor-3α,7β-dihydroxy-5β-cholanoic acid	3312	3151
24-Nor-3α,7α,12α-trihydroxy-5β-cholanoic acid	3269	3151
3α,7α-Dihydroxy-5β-homocholanoic acid	3491	3339
3α,7β-Dihydroxy-5β-homocholanoic acid	3534	3376
3α,7α,12α-Trihydroxy-5β-homocholanoic acid	3470	3350
3α,7α-Dihydroxy-5β-cholestanoic acid	3606	3473
3α,7α,12α-Trihydroxy-5β-cholestanoic acid	3582	3480

[a] The GLC operating conditions, chromatographic columns and calculation of retention indices are described in Table 1.

Table 5
GLC retention indices of the trimethylsilyl ethers of bile alcohols[a]

Bile alcohol	Retention indices (Kovats)	
	CP-Sil-19	CP-Sil-5
24-Nor-5β-cholane-3α,7α,23-triol	3199	3146
24-Nor-5β-cholane-3α,7α,12α,23-tetrol	3186	3166
5β-Cholane-3α,7α,24-triol	3298	3251
5β-Cholane-3α,7α,12α,24-tetrol	3278	3260
5β-Homocholane-3α,7α,25-triol	3405	3360
5β-Homocholane-3α,7α,12α,25-tetrol	3385	3370
24-Nor-5β-cholestane-3α,7α,25-triol	3339	3311
24-Nor-5β-cholestane-3α,7α,12α,25-tetrol	3314	3311
5β-Cholestane-3α,7α-diol	3212	3174
5β-Cholestane-3α,7α,12α-triol	3195	3187
5β-Cholestane-3α,7α,25-triol	3462	3436
5β-Cholestane-3α,7α,12α,25-tetrol	3447	3451
5β-Cholestane-3α,7α,26-triol	3526	3491
5β-Cholestane-3α,7α,12α,26-tetrol	3502	3496
5β-Cholestane-3α,7α,24R,25-tetrol	3624	3615
5β-Cholestane-3α,7α,12α,24R,25-pentol	3597	3619
5β-Cholestane-3α,7α,24S,25-tetrol	3635	3628
5β-Cholestane-3α,7α,12α,24S,25-pentol	3607	3627

[a] The GLC operating conditions, chromatographic columns and calculation of retention indices are described in Table 1.

cholic acid was eluted earlier than cholic acid on a CP-Sil-5 CB column but later on the CP-Sil-19 CB column [91].

7. Conclusion

In this review, we have described some practical aspects of bile acid analysis by gas–liquid chromatography. We have described the need for bile acid quantitation and the importance of GLC, in particular, capillary GLC, for bile acid analysis. Emphasis is placed on the steps involved in bile acid analysis, like isolation of bile acids from biological fluids and their derivatization, and some practical applications, including literature and recent methodologies for bile acid analysis in various biological fluids, are described. We hope that this review will help readers understand the various steps involved in the quantitation of bile acids by GLC and for making modifications to suit particular needs.

7.1. Abbreviations

The following abbreviations and trivial names have been used:

GLC	gas–liquid chromatography
HPLC	high-performance liquid chromatography
TLC	thin-layer chromatography
Lithocholic acid	3α-hydroxy-5β-cholanoic acid
Deoxycholic acid	3α,12α-dihydroxy-5β-cholanoic acid
Chenodeoxycholic acid	3α,7α-dihydroxy-5β-cholanoic acid
Cholic acid	3α,7α,12α-trihydroxy-5β-cholanoic acid
Ursodeoxycholic acid	3α,7β-dihydroxy-5β-cholanoic acid
Ursocholic acid	3α,7β,12α-trihydroxy-5β-cholanoic acid
Hyodeoxycholic acid	3α,6α-dihydroxy-5β-cholanoic acid
α-Muricholic acid	3α,6β,7α-trihydroxy-5β-cholanoic acid
β-Muricholic acid	3α,6β,7β-trihydroxy-5β-cholanoic acid
ω-Muricholic acid	3α,6α,7β-trihydroxy-5β-cholanoic acid

Acknowledgements

This work was supported by a grant from the Veterans Affairs Research Service, Washington, DC, USA, and National Cancer Institute grant PO1-CA-41108. The contents of this publication are solely the responsibility of the authors and do not necessarily

represent the official views of the National Cancer Institute.

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