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Review

Bile acid separation

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Abstract

A review of the methods available for the separation of bile acids is presented, highlighting the most recent developments. The major chromatographic techniques (TLC, GC, HPLC) and combined detection systems for the determination of bile acids are critically evaluated and their advantages and disadvantages discussed. Moreover, future directions in which progress might occur are also indicated. Capillary GC–MS is the more established method since it provides higher efficiency combined with greater sensitivity and specificity and has proven crucial in identifying unusual bile acids. However, it requires deconjugation and derivatization and hence the conjugated species must be inferred from the initial isolation procedure. HPLC is directly amenable to the different forms of bile acids, but it suffers from insufficient resolving power which can be enhanced by exploiting the mobile-phase selectivity. The development of HPLC detection systems with higher sensitivity and specificity than conventional HPLC–UV is reported. In particular, methods for the direct coupling of HPLC to MS are examined with special emphasis on soft ionization processes (thermospray, fast atom bombardment, ion spray). Finally, the analytical potential for bile acid assays of more recent techniques including supercritical fluid chromatography and capillary electrophoresis is evaluated.

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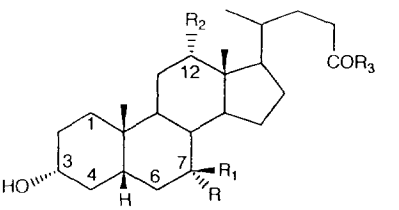
List of abbreviations

CA	Cholic acid
CDCA	Chenodeoxycholic acid
CE	Capillary electrophoresis
DCA	Deoxycholic acid
ED	Electrochemical detection
FAB	Fast atom bombardment
FID	Flame ionization detection
G-	Glycine conjugates
GC	Gas chromatography
HPLC	High-performance liquid chromatography
k'	Capacity factor
LCA	Lithocholic acid
MS	Mass spectrometry
OPTLC	Overpressure TLC
RP	Reversed-phase
SFC	Supercritical fluid chromatography
SIM	Selected-ion monitoring
T-	Taurine conjugates
TLC	Thin-layer chromatography
TSP	Thermospray
UDCA	Ursodeoxycholic acid

1. Introduction

Bile acids are synthesized from cholesterol in the human liver hepatocytes, stored in the gall-bladder, secreted into the small intestine, efficiently reabsorbed from the ileum-proximal colon and returned to the liver via the portal vein [1]. Bile acids play an important physiological role in the elimination of cholesterol from the body and in the intestinal solubilization and absorption of lipids [1]. In mammals the most common bile acids are derivatives of 5β-cholan-24-oic acid [1]. The major compounds present in humans [2] are CA, CDCA, DCA, LCA and UDCA which occur primarily as glycine and taurine amidates at C-24 (Fig. 1). In addition to these bile acids, a wide array of minor components have been identified including C-1, C-4 or C-6 hydroxylated derivatives as well as their epimers, allo bile acids (A/B ring junction in *trans* configuration), C_{23} and C_{27} bile acids, unsaturated compounds, oxo bile acids and



	R	R_1	R_2	R_3
CA	OH	H	OH	
CDCA	OH	H	H	
UDCA	H	OH	H	
DCA	H	H	OH	
LCA	H	H	H	
Free bile acids				OH
Glycine conjugates				$NHCH_2CO_2H$
Taurine conjugates				$NHCH_2CH_2SO_3H$

Fig. 1. Chemical structures of bile acids.

conjugates on any of the hydroxy groups with sulphuric acid, glucuronic acid, glucose or N-acetylglucosamine [2–6]. Since hepatobiliary and intestinal dysfunctions are marked by variation in the concentration and relative proportions of the major bile acids and by increased levels of their minor forms [2–4,7], the individual separation and accurate quantification of these compounds in biological materials are very important prognostic and diagnostic indicators of liver and gastro-intestinal tract diseases in humans and also in other mammals (e.g. cattle, sheep and horses). In addition to their physiological role, CDCA and UDCA are widely used for the treatment of cholesterol gall-stones [8] and, more recently, UDCA has been introduced for the therapy of cholestatic liver diseases [9]. Hence it is important to determine these two bile acids in pharmaceutical raw materials and dosage forms to monitor the manufacturing process, and in human serum to evaluate the bioavailability of drugs containing CDCA or UDCA.

The complex composition of naturally occurring bile acids and the small structural differences between individual components make heavy demands on the analytical technique used. Moreover, since bile acids lack a strong chromo-

phore, conventional spectrophotometric detection systems suffer from limited sensitivity and specificity. The common techniques for the assay of bile acids in biological and pharmaceutical matrices include mainly chromatographic procedures, but also enzymatic, immunological and electrochemical methods. The latter technique has distinct disadvantages such as the complexity of the apparatus and the insufficient specificity and reproducibility [10]. Enzymatic methods using bile acid hydroxysteroid dehydrogenases are particularly suitable for large-scale routine analyses due to their rapidity and simplicity (direct assay of untreated physiological fluids) [11,12]. However, since total bile acids or particular groups of bile acids (i.e., 7α- or 12α-hydroxy bile acids) are measured [12], differentiation between individual components or their state of conjugation is not possible. Moreover, enzymatic techniques generally suffer from poor sensitivity, are subjected to interferences from the biological matrix constituents and are influenced by enzyme contaminants [7,11,12]. Reduced detection limits and enhanced specificity are achieved by radio- and enzyme-immunoassays [7,12], yet cross-reactivity by other structural bile acid analogs is a drawback of the currently available methods [12]. Moreover, only some of the common bile acids (i.e. CA and CDCA) can be accurately determined by the immunological techniques [12], the free forms being usually not detected or partially measured due to the antibody specificity towards the glycine- and taurine-conjugates [12].

Chromatography represents the method of choice for detailed analyses of the bile acid profiles in biological materials. However, no single chromatographic method and detection technique is capable of providing the complete determination of the components of the complex naturally occurring bile acid mixtures and extensive sample pretreatment involving extraction, purification and separation into different groups is needed to increase the sensitivity and specificity of these analyses.

The chromatographic methods for bile acid assays have been examined in several reviews [13,14]. The present report will describe the major techniques for the separation and on-line detection of bile acids with particular attention addressed to the latest developments.

2. Separation methods

2.1. Thin-layer chromatography

TLC has been widely used for the separation of bile acids [15] because it is simple, inexpensive and it can be directly performed on biological fluids without prior sample purification. A number of TLC systems have been developed using plates coated with silica [16–19] or alkyl-bonded silica (RP-TLC) [19–21]. While fractionation of bile acids into the free, glycine- and taurine-conjugated forms has been achieved on silica TLC [16,17], the resolution for the isomeric dihydroxy conjugates (i.e. G-CDCA, G-DCA, T-CDCA, T-DCA) is generally unsatisfactory [16,17,21–23] and consequently quantification of these bile acids in biological samples is not very reliable, particularly when the isomers are present in different proportions [22]. The separation of CDCA and DCA and their conjugates in human bile has been effected on silica TLC with a four-component eluent mixture (chloroform–isopropanol–acetic acid–water) by rather tedious multiple developments [18], or using RP-TLC and acetic acid–methanol–water as the developing solvent after preliminary separation of the conjugates into the glycine and taurine fractions [20]. However, these systems are not of general applicability [23] since the resolution is not complete and the bands tend to overlap when larger amounts of compounds are applied on the plates.

In order to improve the TLC separation of bile acids two-dimensional techniques have been developed. Solvent systems with different selectivities have been employed using the same retention mechanism (adsorption) [17]. Alternatively, other authors have utilized different eluents (non-aqueous and aqueous solvents) and separation principles (i.e. adsorption and partition) for each dimension [20]. The latter ap-

proach achieves adequate resolution of free bile acids and the corresponding glycine and taurine conjugates, but the separation is significantly affected by the number of components in the assayed mixture.

In order to overcome the problems involved with the insufficient resolution of G-CDCA, G-DCA and T-CDCA, T-DCA by TLC the potential of overpressure TLC on high-performance plates has been investigated [24]. Despite enhanced separation efficiency compared to classical TLC, the complete resolution of unconjugated, glycine- and taurine-conjugated bile acids is not attained by OPTLC (Fig. 2).

In conclusion, because of analysis speed and simplicity but inadequate resolving power, TLC is most suitable for routine bile acid assays which do not require efficient separations [23].

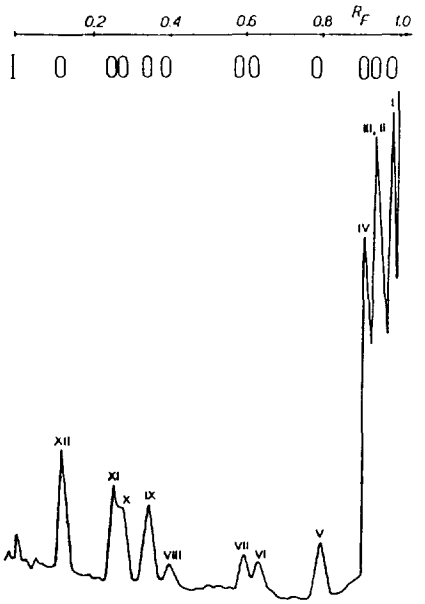


Fig. 2. Separation of bile acids and their conjugates by OPTLC. Labor MIM pressurized ultramicro chamber with Micropump S13; Zeiss PMQ III chromatogram spectrophotometer at 366 nm. Mobile phase, chloroform–butanol–glacial acetic acid–water (2:16:1:1); flow-rate 26 ml/h; pressure 1.2 MPa; 16 cm development in 28 min; plate, HPTLC silica gel 60 F_{254} with impregnated edges. Compounds: I = LCA; II = DCA; III = CDCA; IV = CA; V = G-LCA; VI = G-CDCA; VII = G-DCA; VIII = T-LCA; IX = G-CA; X = T-CDCA; XI = T-DCA; XII = T-CA. From Ref. [24] with permission.

2.2. Gas chromatography

Capillary column gas chromatography has been used more than any other method for bile acid profile analyses [2–4]. The high efficiency of capillary GC and the availability of both universal and selective detections (FID, MS) make it the most powerful separation technique for complex bile acid mixtures in biological extracts [2]. GC coupled with MS is recognised as the reference method [7,12,22,25] because it combines the resolving power of capillary columns with the high sensitivity and conclusive identification provided by MS. Accordingly, correlation studies with the data obtained by GC and GC–MS are established procedures for method validation in bile acid analyses [7,11,12,25–27].

However, since these steroids lack volatility, a derivatization step is required prior to GC analysis [2–4]. In addition, preliminary solvolysis and chemical or enzymatic hydrolysis of conjugated bile acids are generally necessary as the preparation of volatile derivatives can be achieved only for intact glycine, glucoside and glucuronide conjugates [2–6]. Therefore, in order to avoid the loss of information on the conjugating moieties, group separation of the different forms is a prerequisite for GC [2,3,5,6,28]. Moreover, because of the requirements imposed by the high efficiency and reduced sample load of the capillary columns, extensive extraction/purification procedures are performed prior to GC [3,6,28]. These complex and laborious sample pretreatments represent a source of possible errors and artifact formation and are a drawback of the method. Bile acid analysis by capillary GC is a mature technique (for reviews see Refs. [4], [13], [29]) and only refinements are reported in the latest investigations.

Several derivatization procedures for bile acids have been proposed and they are examined in comprehensive reviews (Refs. [4], [30] and references therein). The commonly employed derivatives are the acetates, the trifluoroacetates and the trimethylsilyl ethers of the methyl cholanoates, the latter being the most widely applied [5–7,28,31]. While derivatization is generally accomplished in two stages [5–7,28], the advantage of a one-step reaction has been dem-

onstrated for the hexafluoroisopropyltrifluoroacetate esters [27,32] and for the methyl ester–methyl ether derivatives [33].

Chemically bonded fused-silica capillary columns have replaced conventional packed columns for most applications. Sample introduction by on-column, split or splitless systems have been adopted for the GC analysis of bile acids. The former system [6] provides the most accurate data but requires more stringent sample preparation than split/splitless injection [27,33,34] which is prone to discrimination effects [4]. A solventless glass solid injection device which allows the introduction of a greater proportion of sample and the automation of the injection process has also been employed [4,28,31,35]. Although nonpolar liquid phases (e.g., methyl silicones such as OV-1 and SE-30) have been shown to be more appropriate for the wide range of bile acids present in biological extracts [4,28,31,35] (see Fig. 3), more polar columns (e.g., phenylmethyl- and cyanopropylsilicone) have also been used [4,6,34].

Selection of an appropriate internal standard for quantification is essential in GC and care must be taken to ensure that it is not naturally present in the analysed biological material. Several compounds have been utilized as internal standards for the assay of human samples (e.g. coprostanol [28], nor-deoxycholic acid [34,35], 7α,12α-dihydroxy-5β-cholan-24-oic acid [31]), the most accurate results being obtained with bile acids labelled with stable isotopes [4,28].

In addition to the high separation efficiencies (see Fig. 3), chemically bonded capillary columns exhibit the advantage of direct connection of the column outlet with the MS. This is particularly relevant because of the development of benchtop GC–MS systems which provide the specificity of mass spectra and the sensitivity of SIM at a fraction of the cost of larger MS apparatus and require less operator training and experience.

Complete bile acid profiles in biological samples have been obtained by GC–MS after exhaustive sample pretreatments involving extraction, fractionation in several groups of molecules based on their mode of conjugation, deconjugation and/or solvolysis of each group and derivatization. Hence, sample preparation represents the limiting step in the GC analysis scheme. Development of improved isolation procedures and their automation will increase the analytical potential of the separation power attained by capillary GC.

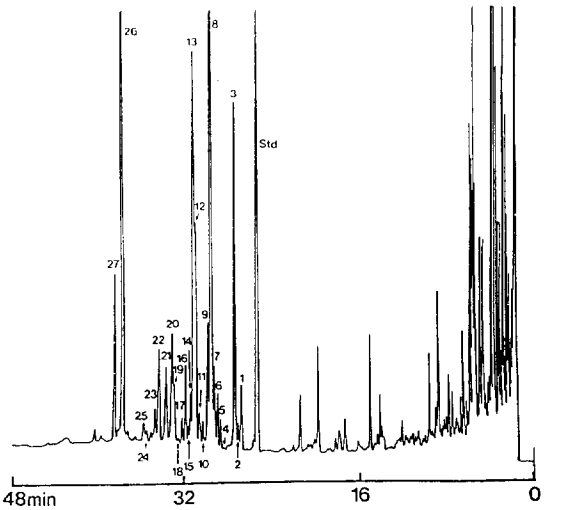


Fig. 3. Profile of methyl esters trimethylsilyl ethers of unconjugated bile acids in rat faeces analyzed on a 25 m × 0.25 mm OV-1 capillary column (220°C isothermal for 5 min, then programmed to 285°C at a rate of 2°C/min. Helium was the carrier gas at a flow-rate of about 2 ml/min and the sample was introduced with a dropping needle system. Compounds: 1 = 3α-hydroxy-5α-cholanoic; 2 = 3β-hydroxy-5β-cholanoic; 3 = LCA; 4 = unknown; 5 = 3α,12α-dihydroxy-5α-cholanoic; 6 = 3α,12β-dihydroxy-5β-cholanoic; 7 = 3α,12β-dihydroxy-5α-cholanoic; 8 = DCA; 9 = 3β-hydroxy-5α-cholanoic; 10 = 3α,6β-dihydroxy-5β-cholanoic and 3-oxo-12α-hydroxy-5β-cholanoic; 11 = unknown; 12 = 3α,6β,7α-trihydroxy-5β-cholanoic; 13 = 3α,6α-dihydroxy-5β-cholanoic; 14 = 3β,6α-dihydroxy-5β-cholanoic; 15 = unknown; 16 = 3β,12α-dihydroxy-5α-cholanoic; 17 = 12-oxo-3α-hydroxy-5α-cholanoic; 18 = unknown; 19 = 3β,12β-dihydroxy-5α-cholanoic; 20 = 12-oxo-3α-hydroxy-5β-cholanoic; 21 = unknown; 22 = 3α,6β,7β-trihydroxy-5β-cholanoic; 23 = unknown; 24 = unknown; 25 = 12-oxo-3β-hydroxy-5α-cholanoic; 26 = 3α,6α,7β-trihydroxy-5β-cholanoic; 27 = 3α,6α,7β-trihydroxy-5α-cholanoic. From Ref. [92] with permission.

2.3. High-performance liquid chromatography

2.3.1. Separation

HPLC should represent the method of choice for the assay of bile acids in biological materials since it can perform direct analyses of the natu-

rally occurring different classes of these compounds without prior removal of the conjugating moiety [13,36,37]. However, with typical number of theoretical plates between 10^3 and 10^4, HPLC frequently fails to achieve the component resolution necessary for complex bile acid mixtures. Moreover, since bile acids have low molar absorptivities, the conventional UV detection system for HPLC suffers from limited sensitivity [36] which is an additional disadvantage for this otherwise powerful and versatile analytical method.

Conditions for the HPLC separation of bile acids have been the subject of several reviews [13,14,37,38]. Despite the large number of chromatographic systems described in the literature, determination of the foregoing compounds by HPLC has been carried out almost exclusively on octadecyl-bonded silica stationary phases since they provide a higher degree of resolution than other available packings. Less hydrophobic cyanopropyl-, phenyl- or amino-bonded silica supports fail to produce satisfactory separations for the major glycine- and taurine-conjugates [39,40,41] and for bile acid 3-glucuronides [42]. The use of HPLC silica columns has been hampered by distinct drawbacks including rapid deterioration of their performance [38,43], inadequate resolution of the conjugated dihydroxy isomers [43,44], the need for several columns connected in series [38,45] and the requirement for a preliminary derivatization step [38,45]. Weak or strong anion-exchange silica-based packings have also been tested for the HPLC determination of bile acids using aqueous mobile phases buffered at low pH (3.0–3.6) and containing ethanol or methanol as the organic modifier [46,47]. Fractionation of the foregoing compounds into the free, glycine- and taurine-conjugated groups is achieved on the anion-exchange column (Fig. 4), yet the separation of the individual components of each class is not attainable. The application of a non-siliceous polymeric column with gradient elution at high pH (ca. 13) to the separation of free and conjugated bile acids has been illustrated by Dekker et al. [48], its main advantage being the stability across the entire pH range. Published studies thus

Fig. 4. HPLC separation of bile acid classes. Column, Spherisorb-SAX (250 × 4.5 mm I.D.); flow-rate, 0.9 ml/min; detection, 0.08 AUFS at 210 nm; eluent, 65% methanol in 0.01 *M* phosphate buffer (pH 3.5). Peaks: B = free bile acids, C = glycine conjugates; D = taurine conjugates.

indicate that optimal resolution for bile acids has been obtained by HPLC on octadecyl-silica stationary phases [37,39]. Nevertheless, the use of columns based on different retention mechanisms should be considered as a means of improving selectivity when dealing with complex chromatographic profiles.

A wide range of eluent systems has been proposed for the chromatography of bile acids on the C_{18} sorbent. Mobile phases containing different buffer species ranging in pH from 2.5 to 7.5 [37,38] and methanol [22,39], acetonitrile [37] or isopropanol [38] as the organic modifier have been employed.

The chromatography of bile acids is markedly affected by the pH of the aqueous portion of the eluent [37–39,42]. The retention of unconjugated (pK_a ca. 6) and glycine conjugated (pK_a ca. 4.5) bile acids increases with decreasing pH from 7.5

to 2.5 [37–39,49] owing to ionic suppression, which enhances the lipophilic character of the molecules, hence their interaction with the hydrophobic bonded phase. Due to the lower pK_a (ca. 1.5) of the taurine conjugates their capacity factors are not significantly influenced by the eluent pH within the operating range of chemically bonded silica phases [37–39]. At pH values above 7, free bile acids and the corresponding glycine- and taurine-conjugates exhibit similar k' values [37–39]. This levelling in k' indicates that at a pH which ensures the complete ionization of these acids retention is controlled mainly by the steroidal nucleus of the molecules. Therefore, neutral or weakly alkaline mobile phases are not suitable for the optimal resolution of bile acids [38] requiring gradient elution [41,50,51] or preliminary fractionation into different groups [37], thus reducing the advantages of HPLC over other techniques (e.g., GC). In addition, rapid deterioration of silica-based supports is caused by alkaline eluents [50]. Several investigations have demonstrated that satisfactory RP-HPLC separation of unconjugated [22,27,52], glycine- and taurine-conjugated bile acids [38,39,52–55], bile acid glucosides, glucuronides and sulphates [37,42,55,56] occur in the eluent pH range 3.0–5.0. As an example, the resolution of bile acid 3-glucuronides is illustrated in Fig. 5.

The ionic strength in the mobile phase is an important parameter for the control of peak symmetry [53] and elution order [22,37,39] in RP-HPLC of bile acids. With increasing salt concentration in the eluent the mobility of the undissociated components (e.g. free bile acids at low pH) does not vary appreciably [57], whereas the k' values of the ionized species rise, this effect being more marked the higher the degree of ionization (e.g., taurine conjugates) [39,57,58]. By carefully varying the buffer molarity in the HPLC solvent, conditions have been selected [57] for the simultaneous baseline resolution of UDCA, CA, CDCA, DCA and LCA and their glycine and taurine conjugates in a single chromatographic analysis (Fig. 6).

The chemical nature of the buffering ion also influences the separation efficiency [22,39,41], although the observed effects are limited compared to those produced by the mobile phase pH and ionic strength.

The type of organic modifier affects the chromatographic selectivity, methanol being prefer-

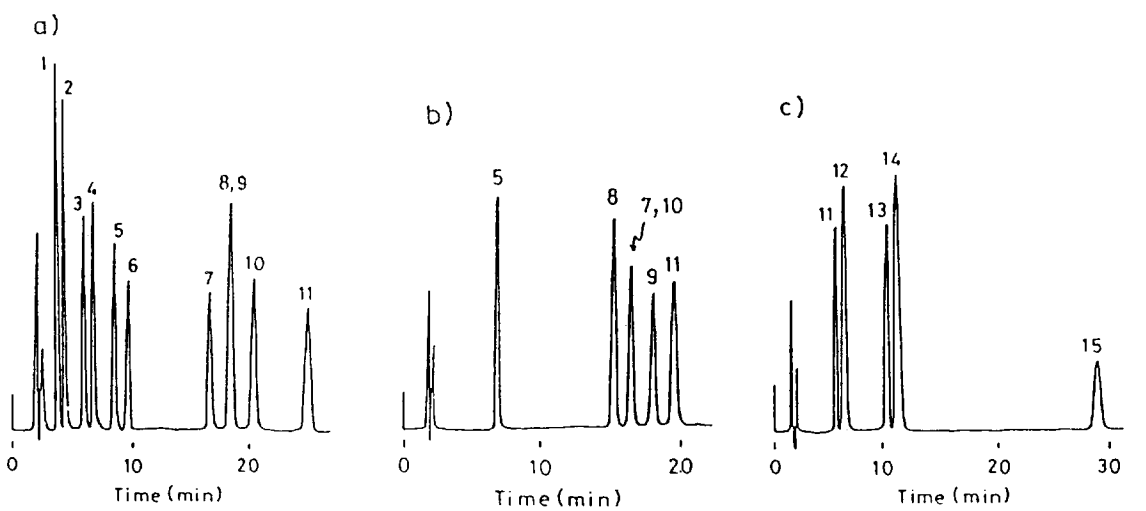


Fig. 5. HPLC separation of a mixture of bile acid 3-glucuronides. Mobile phase: (a) 0.7% ammonium phosphate buffer (pH 3.5)–acetonitrile (150:60); (b) 0.7% ammonium phosphate buffer (pH 3.0)–acetonitrile (135:60); (c) 0.7% ammonium phosphate buffer (pH 5.0)–acetonitrile (125:60). Column, Shodex ODS Pak F-411. Detection, 205 nm. Peaks: 1 = T-UDCA; 2 = T-CA; 3 = G-UDCA; 4 = G-CA; 5 = T-CDCA; 6 = T-DCA; 7 = G-CDCA; 8 = UDCA; 9 = G-DCA; 10 = CA; 11 = T-LCA; 12 = G-LCA; 13 = DCA; 14 = CDCA; 15 = LCA. From Ref. [42] with permission.

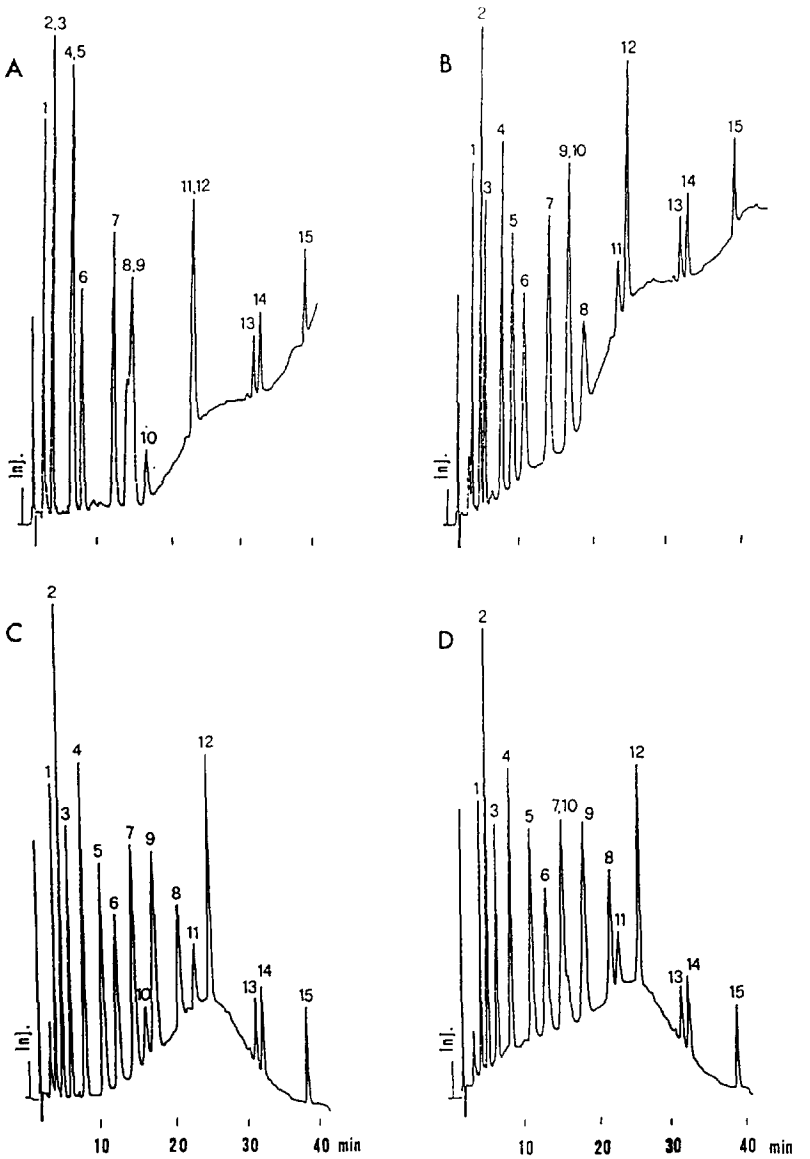


Fig. 6. HPLC elution profiles of free bile acids and their glycine and taurine conjugates on an Ultrasphere ODS column. The gradient was formed between 65% methanol in 0.01 *M* acetate buffer and 90% methanol in 0.024 *M* acetate buffer (A); 65% methanol in 0.02 *M* acetate buffer and 90% methanol in 0.047 *M* acetate buffer (B); 65% methanol in 0.03 *M* acetate buffer and 90% methanol in 0.07 *M* acetate buffer (C); and 65% methanol in 0.04 *M* acetate buffer and 90% methanol in 0.098 *M* acetate buffer (D). Flow-rate, 1.0 ml/min. Detection, 210 nm and 0.08 AUFS. Peaks: 1 = T-UDCA; 2 = G-UDCA; 3 = T-CA; 4 = G-CA; 5 = T-CDCA; 6 = T-DCA; 7 = G-CDCA; 8 = T-LCA; 9 = G-DCA; 10 = UDCA; 11 = CA; 12 = G-LCA; 13 = CDCA; 14 = DCA; 15 = LCA. From Ref. [57] with permission.

red to acetonitrile or isopropanol by several investigators [39,49,53]. However, satisfactory resolutions of bile acids have been obtained with methanol- or acetonitrile-containing mobile phases. The former solvent exhibits higher solvating power for the more hydrophobic bile acids

(e.g. LCA) [39,53], whereas acetonitrile has a lower UV cut-off which is of advantage for spectrophotometric detection [49,59].

The relationship between the structure and the chromatographic mobility on C_{18} stationary phases has been extensively investigated, particularly with relation to the hydroxy- and oxo-substituents in the steroid nucleus. For detailed information the reader is referred to previously published reviews and references therein [37,38,60]. In general, elution is in the order taurine-conjugates < glycine-conjugates < free bile acids [22,37–39,52–55], although reversal of this retention sequence occurs with alkaline mobile phases [41,51]. Within each series, retention increases with decreasing numbers of hydroxyls on the steroid backbone, but modification of this elution order is caused by the presence of hydroxyl groups (e.g. 6α-OH, 7β-OH) which interfere with the interaction of the bile acid hydrophobic β-surface with the C_{18} stationary phase [38,60,61].

Some authors have described the determination of bile acids by RP ion-pair HPLC using acetonitrile–water mobile phases modified with tetrabutylammonium counter ions [49,59,62]. This methodology does not appear to offer any real advantages over conventional RP-HPLC, apart from providing a different separation selectivity with the glycine conjugates eluting before the corresponding taurine forms. Moreover, ion-pair chromatography causes rapid deterioration of column performance [49].

The chromatographic behaviour of bile acids on RP columns eluted with mobile phases containing 1–5 mM β-cyclodextrin has been reported by Shimada et al. [63,64]. The inclusion of β-cyclodextrin in the eluent produces a sharp decrease of the k' values. The retention of the 12α-hydroxylated compounds, however, is not strongly influenced probably due to interference of the hydroxyl at C-12 with the formation of the solute–cyclodextrin inclusion complex. Improved and faster separations have been obtained for free bile acids, glycine- and taurine-conjugates, bile acid 3-glucuronides and 3-sulphates using β-cyclodextrin as a mobile phase additive in RP-HPLC [63,64].

The papers published in the literature indicate that HPLC can be applied to the direct determination of the wide array of the naturally occurring forms of bile acids [13,14,37]. The technique offers many parameters for the control of the separation selectivity including the pH, the chemical nature and concentration of the mobile phase buffer, the type of organic solvent modifier and of packing. The main limitation of HPLC in resolving complex bile acid profiles is the relatively poor chromatographic efficiency which could be improved by advances in column technology (microcolumn HPLC) [37]. The selectivity of the HPLC assay can be enhanced in the sample handling step by efficient clean-up and class separation procedures to remove interferences and fractionate the analytes into smaller groups [28,37,65,66]. The techniques for sample pretreatment have been extensively reviewed by Sjövall and Setchell [66]. Progress in the area of automation, design of extraction and purification procedures based on specific interactions and sample preparation on-line with the analytical column [51,67] will play an important role in the HPLC determination of bile acid profiles in biological samples.

2.3.2. Detection

Since bile acids possess weak chromophores with absorption around 200 nm, conventional HPLC–UV suffers from limited sensitivity and requires the selection of short UV wavelengths [27,36,39,55] which result in increased interference from the biological matrix constituents. Even trace amounts of endogenous components with much stronger absorptivities than bile acids would generate large peaks thus limiting the accuracy of UV detection. Moreover, identification of a peak from a biological extract based solely on its retention time in HPLC–UV analysis is not sufficient and additional confirmatory methods are required. Another convenient detection system for HPLC is the differential refractometer [22,38,61] but the sensitivity obtainable is generally insufficient for assays of biological fluids [37].

Precolumn labelling of bile acids enables sensitive UV and also specific fluorescence and elec-

trochemical detections [13,37]. Various derivatization procedures have been devised for UV detection including the formation of strongly absorbing *p*-bromophenacyl or *p*-nitrobenzyl esters of free and glycine-conjugated bile acids (Ref. [37] and references therein). A dramatic gain in sensitivity has been achieved by the labelling of bile acids with a fluorophore via the 3α-OH group (free, glycine- and taurine-conjugated bile acids) using 1-anthroyl nitrile or via the carboxyl group (free, glycine conjugates and deconjugated taurine forms) with fluorescent reagents such as 1-bromoacetylpyrene or the commercially available 4-bromomethyl-7-methoxycoumarin [13,37]. Derivatization to an electrochemically active form (e.g. *p*-hydroxyanilide of deconjugated bile acids) has also been reported (Ref. [68] and references therein). However, because of laborious sample pretreatments and possible incomplete conversion due to matrix effects, such methods are time-consuming and not robust [36,48]. Moreover, the loss of functional groups that might participate in selectivity-based separations is a disadvantage [37]. However, the review of the derivatization methods for bile acids is outside the scope of this article which concentrates on direct determination procedures.

High sensitivity and selectivity is achieved by on-line coupling of HPLC with a detector formed by a bile acid-specific enzyme (e.g. 3α-hydroxysteroid dehydrogenase) immobilized on a column and measuring the NADH produced, following the conversion of bile acids to the 3-oxo derivatives, with fluorimetric [37,51] or electrochemical detection [68,69]. The requirement for additional pumps and weakly alkaline mobile phases [51,69] and problems with the reproducibility of the enzymic reaction [37,54] are drawbacks of the method and render it impractical for routine analysis.

HPLC combined with electrochemical detection represents a very sensitive technique providing also enhanced specificity as a result of the limited number of solutes which can undergo redox reactions under certain conditions. To date, few examples of the use of ED for the direct HPLC analysis of bile acids have appeared in the literature. Alternating voltage polarographic detection for the HPLC determination of free bile acids has been reported by Kemula and Kutner [70]. The complexity of the apparatus, the inadequate resolution between the isomeric dihydroxy bile acids and the modest gain in sensitivity are major defects of this system. Dekker et al. [48] developed a HPLC method for the simultaneous separation of free and conjugated bile acids on a resin-based RP column with a gradient of acetonitrile in dilute sodium hydroxide and amperometric detection at a gold electrode using a sequence of three pulses. Enhanced sensitivity (almost two orders of magnitude higher than UV detection) was obtained in conjunction with satisfactory specificity as indicated by the absence of interfering matrix peaks in the chromatograms of biological samples (bile, ileal fluid). Disadvantages of this technique include the requirement for a high pH (approx. 13) eluent, the complexity of the procedure and the laborious electrode refurbishments [48]. A more convenient ED system has been developed by Scalia et al. [71] for the HPLC determination of free bile acids in pharmaceutical dosage forms. The compounds were separated on a RP silica-based column and monitored at a porous graphite electrode set at an oxidation potential of +1.4 V. Detection limits were at least four times lower than those reported for UV [37,54] and the ED response was not affected by pharmaceutical excipients which generated interfering peaks in the UV traces. The application of this technique to the assay of biological samples was not investigated.

RP-HPLC coupled with an evaporative light-scattering mass detector has been applied to the analysis of free and conjugated bile acids in biological materials [54]. Higher sensitivity for free bile acids and improved baseline output are achieved compared to UV. However, the lack of specificity (the signal is related to the bulk molecular size), the lower response for the late eluting peaks and the limited choice of usable mobile-phase buffers hamper this detection system.

Direct combination of a mass spectrometer with the HPLC column offers a means of en-

hancing the sensitivity and specificity of the HPLC determination of bile acids. One of the main problems to be addressed in utilizing the MS as an on-line detector for HPLC is the incompatibility between the large gas volume generated from the evaporated column eluent and the high vacuum of the MS source. Another obstacle is the low volatility and thermal lability of bile acids. In fact, the first examples [45,72] of combining HPLC with MS were based on collection of fractions eluted from the column followed by introduction into the MS using a direct insertion probe. Several interfaces have been designed for the on-line coupling of HPLC with MS.

One of the earliest approaches involves the spraying of the column effluent on a continuously circulating polyimide belt, removal of the solvent in vacuum lock chambers during the mechanical transport and then transfer of the sample into the ion source region of the MS where it is flash-vaporized [73]. The main limitations of this technique are recycle peaks due to incomplete desorption, solvent interference, poor sensitivity, loss of chromatographic integrity from uneven spreading of the sample on the belt and inability to handle mobile phases with high percentages of water. HPLC–MS using the moving belt interface and chemical ionization has been applied by Games et al. [73] to the analysis of a standard mixture of CA, LCA, CDCA, T-CA and G-CA, but the poor resolution and sensitivity are drawbacks.

Determination of bile acids by micro-HPLC interfaced with MS via continuous-flow fast atom bombardment has been described by different researchers [74,75]. The total effluent from a 0.26 mm I.D. column (flow-rate <2 μl/min) [74] or only a fraction (5%) of the eluent from a 1 mm I.D. column (flow-rate, 60 μl/min) [75] has been introduced to the ion source through the interface made of a capillary tubing with a porous frit at the end. The solvent is immediately vaporized on the surface of the frit while the solute and the matrix (commonly glycerol premixed with the mobile phase) are bombarded by an argon or xenon beam. Intense positive [74] or negative [75] pseudo-molecular ions are produced with little fragmentation, thus yielding mainly molecular mass information. The chromatographic separation provides retention values for the differentiation of positional- and stereo-isomers of bile acids which are not distinguishable from their FAB spectra [75]. On the other hand, the specificity of MS allows the resolution and identification of coeluting components with different molecular mass from the reconstructed current plots of specific ions. Microbore RP-HPLC–FAB-MS provides high sensitive (picogram range) determination of bile acids in human fluids [75] allowing direct analysis of intact polar conjugates (e.g. taurine amidated bile acids, bile acid glucuronides) [74,75]. However, the rather complex and specialised instrumentation required limit the analytical ruggedness of the technique.

Coupling of HPLC with MS for bile acid assays has been effected with commercially available thermospray interfaces by Setchell and Vestal [36] and Eckers et al. [76,77]. The eluent from the column is passed through a heated capillary tube (vaporizer) resulting in the formation of a vaporized jet composed of a mist of fine charged droplets. It is generally accepted that ion evaporation from the micro-droplets is induced by the electric field generated at their surface. Ionization of bile acids in thermospray HPLC–MS has been facilitated by utilization of an electron beam (filament-on mode) [36] or an electrical discharge [76]. Maximum sensitivity for the bile acids has been obtained in the negative-ion mode using ammonium acetate buffer. Little fragmentation is generated by this soft ionization process, with spectra consisting of prominent $[M-H]^-$ pseudo-molecular ions as well as ions due to losses of water. The latter ions provide some structural information (i.e. number of hydroxyl groups) unlike FAB-MS. The specificity of mass spectral detection permits the resolution and identification of multiple co-eluting bile acids and the detection of minor components in the presence of major, overlapping peaks in human bile and serum and in pig bile [36]. HPLC–MS–MS using the thermospray interface has also been tested [77] for a group of bile acids and their glycine conjugates as a means of obtaining

diagnostic fragment ions to enhance the specificity of HPLC–MS alone. The TSP interface maintains the chromatographic integrity, is compatible with conventional column flow-rates (1–2 ml/min) and yields molecular ions for the non-volatile bile acids. Although improved sensitivity (10 ng) is achieved [36] compared to UV, the detection limits are one order of magnitude higher than those obtained by HPLC–FAB-MS [75]. Moreover, the limitation in the selection of buffer, the requirement for careful control of vaporizer and ion source temperatures and the ion current instability reduce the reliability and reproducibility of the technique.

The most recent example of bile acid analysis by HPLC combined with MS is based on the ion spray interface [78]. The total effluent from a microbore column or a fraction (5%) of the mobile phase from a conventional column [78] is fed through a small capillary held at several kilovolts and dispersed into fine charged droplets in dry nitrogen at atmospheric pressure. The ions emitted from the charged droplets due to surface electric field gradients are then sampled through an orifice from the atmospheric pressure into the vacuum region of the mass spectrometer. At variance with thermospray methods, the ion spray approach does not require the input of heat during the spray-ionization process and thus is particularly suitable for labile and polar samples. Intense $[M + H]^+$ pseudo-molecular ions and $[M + CH_3CN + H]^+$ solvent adduct ions are observed in the mass spectra of bile acids with little fragmentation [78]. The evaluation of the relative abundance of these adduct ions in some cases permits differentiation of the isomeric components. Detection limits for bile acids (ca. 200 pg) by ion spray microbore HPLC–MS [78] are two orders of magnitude lower than those reported for thermospray [36]. In addition, the ion spray interface affords improved peak shape and baseline output compared to the other HPLC–MS techniques (Fig. 7). As illustrated in Fig. 7, overlapping peaks can be resolved by analysis of specific ion current chromatograms. Separation and identification of bile acids in monkey bile has been performed with a commercial ion spray HPLC–MS instrument [78]. Although more applications are necessary to fully evaluate the potential of this technique, existing data suggest that it may develop into an easily maintained, reliable and sensitive HPLC–MS method for bile acids.

In summary, the use of MS as a detector for HPLC overcomes HPLC's inherent disadvantages in specificity and sensitivity providing molecular mass identification, SIM detection limits and in some cases, structural information. Although commercial HPLC–MS systems are available, there is still a need for reduced cost and improvements in analytical ruggedness and reproducibility.

2.4. Supercritical fluid chromatography

SFC employs fluids above their critical temperature and pressure as mobile phases. Supercritical fluids have some potentially useful properties for chromatography. They exhibit solvation strengths approaching those of liquids, yet they offer lower viscosities and higher analyte diffusivities and hence higher efficiency per unit time [79]. Moreover, the density of a supercritical fluid and hence its elution power can be modified by simply changing the applied pressure and/or temperature [79]. Carbon dioxide is the most commonly used supercritical mobile phase because it has a comparatively low critical temperature (31.1°C) and pressure (73.8 bar), is non-flammable, is chemically inert and cost-effective and presents no disposal problems. The solvating power of CO_2 is restricted to relatively non-polar molecules. However, the addition of a few percent of a polar solvent (e.g. methanol, acetonitrile) extends the application of supercritical CO_2 to more polar solutes [79,80]. SFC is useful for the analysis of thermally labile and non-volatile compounds that are not directly amenable to GC but require higher chromatographic efficiencies and faster analyses than those available in HPLC [80]. Given these advantages it is perhaps surprising that only few investigations on the SFC separation of bile acids have appeared in the literature.

The separation of free, glycine- and taurine-conjugated bile acids by SFC has been reported

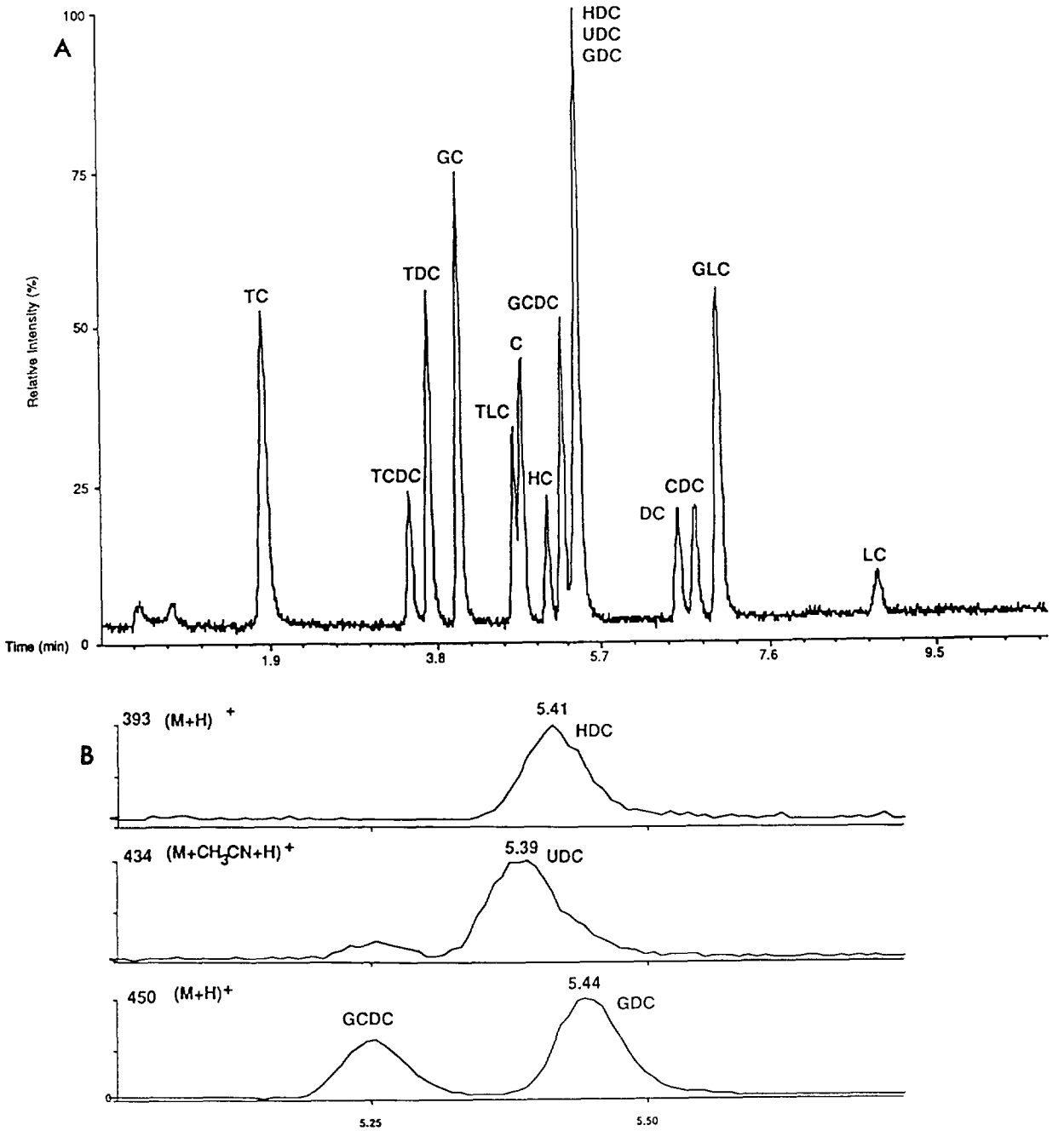


Fig. 7. (A) Ion spray HPLC–MS using SIM analysis of an injection of 40 ng each of 15 bile acid standards. Column, C_{18} cartridge (3 cm × 4.6 mm) packed with 3-μm particles. Gradient elution from 30 to 70% acetonitrile in 0.1% trifluoracetic acid in 15 min at 1 ml/min. The HPLC eluent was split approximately 19:1 prior to MS. Ion spray voltage, +4800 V; interface plate, +650 V; orifice, +60 V. SIM data were acquired with a duel time of 100 ms. Peaks: TC = T-CA; TCDC = T-CDCA; TDC = T-DCA; GC = G-CA; TLC = T-LCA; C = CA; HC = hyocholic acid (3α,6α,7α-trihydroxy-5β-cholan-24-oic acid); GCDC = G-CDCA; HDC = hyodeoxycholic acid (3α,6α-dihydroxy-5β-cholan-24-oic acid); UDC = UDCA; GDC = G-DCA; DC = DCA; CDC = CDCA; GLC = G-LCA; LC = LCA. (B) Expansion of the region around 5.5 min showing single ion traces for m/z 393, hyodeoxycholic acid; 434, UDCA; and 450, G-DCA. From Ref. [78] with permission.

(Ref. [80] and references therein) using an SE-30 capillary column at 77°C and performing a CO_2 pressure gradient from 7.1 to 22.3 MPa or from 5.6 to 18.2 MPa for taurine conjugates and for free and glycine conjugates, respectively. Since preliminary derivatization of bile acids is required, no real advantage over capillary GC is afforded. Moreover, only qualitative analysis of human serum bile acids has been performed.

The potential application of packed-column SFC with UV detection for the separation of bile acids has been investigated by Scalia and Games [81,82]. A HPLC apparatus modified for SFC operations (the pump heads were cooled to −5°C and a back-pressure regulator was connected at the outlet of a high-pressure flow-cell to maintain supercritical conditions in the column) and conventional chemically bonded silica HPLC columns were used. The addition of methanol to the supercritical CO_2 eluent was required in order to elute the bile acids from non-polar and polar packings. The influence of the stationary phases, methanol concentration, column pressure and temperature on retention was investigated. The effect of the last two parameters on the k' values of free bile acids on a cyanopropyl column is reported in Fig. 8. A similar pattern was observed for the conjugated forms. Optimal separation for free bile acids was obtained on a phenyl bonded phase with 15% methanol in CO_2 at 20.3 MPa and 40°C (Fig. 9A) [81]. The more polar conjugates were analysed on a cyanopropyl column at 25.3 MPa and 40°C using a methanol gradient from 20 to 28% (Fig. 9B) [82]. Under the conditions used the mobile phase is in a subcritical rather than in a supercritical state. However, there is no difference in the solvent properties of a just supercritical dense gas or a just subcritical dense fluid, provided the densities of the two fluids are similar. Applicability of the SFC technique to the assay of biological samples was demonstrated for conjugated bile acids [82]. The separations of bile acids by packed-column SFC (Fig. 9) are much faster than those typically obtained by the HPLC procedures reported in the literature [22,38,52,55,57]. Moreover, SFC offers the advantage of speed in method development as the

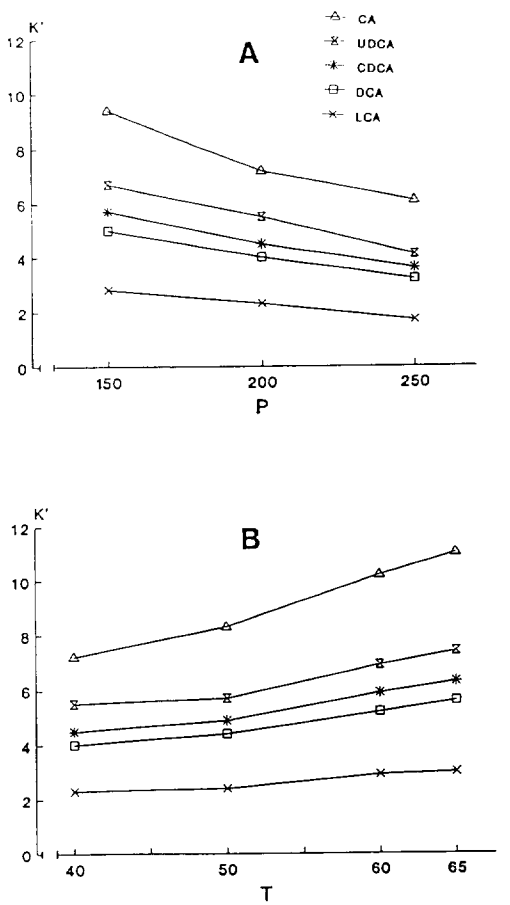


Fig. 8. Effect of column (A) pressure (bar) at 40°C and (B) temperature at 200 bar on the k' of free bile acids in SFC. Mobile phase, 15% methanol in carbon dioxide. Column, cyanopropyl Zorbax; flow-rate, 4 ml/min; UV detection, 210 nm. From Ref. [81] with permission.

column re-equilibration following change in mobile-phase composition is rapid [79,82]. In addition, SFC provides an alternative separation selectivity to the currently used RP-HPLC techniques [37–39] since the bile acids are eluted in order of increasing polarity (Fig. 9), hence following a normal-phase mechanism. The use of a sensitive and universal FID detector is one of the advantages of SFC over HPLC which cannot be exploited for bile acids because CO_2 modified with methanol has to be applied. Baseline separation is achieved for free bile acids (Fig. 9A) by SFC, yet the conjugated dihydroxy isomers are not completely resolved (Fig. 9B). Due to prob-

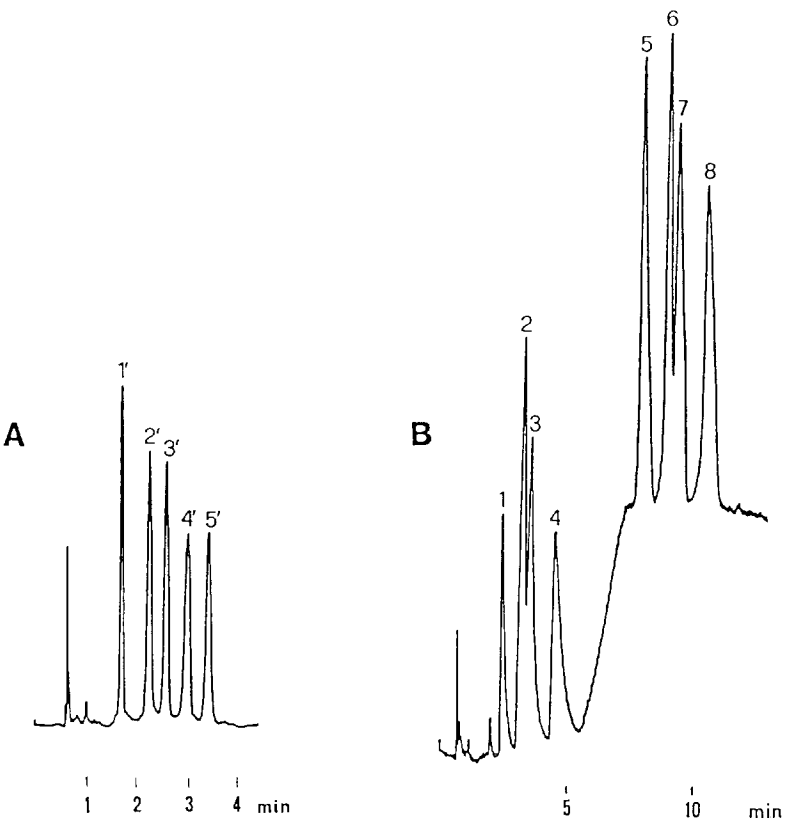


Fig. 9. (A) SFC separation of free bile acids on a phenyl Zorbax column. Mobile phase, 15% methanol in carbon dioxide; flow-rate, 4 ml/min; inlet pressure, 20.3 MPa; temperature, 40°C; UV detection, 210 nm. Peaks: 1′ = LCA; 2′ = DCA; 3′ = CDCA; 4′ = UDCA; 5′ = CA. (B) SFC separation of conjugated bile acids. Column, cyanopropyl Zorbax; mobile phase, gradient from 20 to 28% methanol in carbon dioxide; flow-rate, 4 ml/min; inlet pressure, 25.3 MPa; temperature 40°C; UV detection, 210 nm. Peaks: 1 = G-LCA; 2 = G-DCA; 3 = G-CDCA; 4 = G-CA; 5 = T-LCA; 6 = T-DCA; 7 = T-CDCA; 8 = T-CA. From Refs. [81] and [82] with permission.

lems with the mixing of the injection solvent (e.g. methanol, acetonitrile) with the supercritical fluid mobile phase, the volume of sample that can be introduced in SFC without causing peak broadening is limited (<10 μl), which is a disadvantage for sensitivity. Moreover, the separation of bile acids by SFC was found to be less reproducible than those attainable by RP-HPLC, requiring extensive column purging [82] and exhibiting deterioration of resolution and marked dependence on stationary phase parameters such as surface area, carbon content and residual silanols [83]. The disadvantages outlined above and the fact that the majority of separations of bile acid mixtures can be performed by existing HPLC or GC methods are probably the reasons for SFC not being widely adopted and developed more intensively.

Interfacing of SFC with MS should be easier than with HPLC since the gas volumes generated from the mobile phase are lower [84]. The direct coupling of packed column SFC with MS using a modified TSP interface in the filament-on mode has been studied for the determination of conjugated bile acids [84]. Maximum response was obtained in the negative ion mode with spectra consisting only of the $[M-H]^-$ pseudo-molecular ions. Thermospray SFC–MS provided enhanced specificity for the analysis of bile acids in biological samples [84] and achieved higher sen-

sitivity than UV for the taurine derivatives but produced weak and broad bands for the glycine forms. Moreover, the temperature of the vaporizer and its crimping, necessary to maintain supercritical fluid conditions in the interface [85], were not easily controllable, thus affecting the reproducibility of the technique.

2.5. Capillary electrophoresis

Capillary electrophoresis is a rapidly developing separation technique based on the differential migration of solutes in an electric field. In CE, electrophoresis is performed in narrow-bore capillaries which results in high separation efficiency and speed of analysis [86]. Since the difference in electrophoretic mobilities is the mechanism responsible for separation in CE, only charged compounds can be analyzed. With the introduction of micellar electrokinetic capillary chromatography [87] in which a micellar solution of surfactant is employed instead of the buffer solution in CE, the separation of neutral compounds as well as ionic ones is possible. The high resolving power of CE and its alternative selectivity principle to the chromatographic techniques should be of advantage for the discrimination of the small structural differences between individual bile acids. Although these compounds have been widely used as surfactants in micellar electrokinetic capillary chromatography [87], few reports have appeared on their direct resolution by capillary electrophoretic techniques [88–90]. Earlier investigations have been based on capillary isotachophoresis in which solutes migrate in discrete zones between a leading and a terminating electrolyte [88,89]. These techniques have been hampered by the low solubility of bile acids under aqueous isotachophoresis conditions. In order to overcome this problem, a non-aqueous medium (i.e. 95% methanol) [88] or aqueous electrolyte systems containing cyclodextrins [89] have been proposed. The latter approach produced satisfactory resolution for some free, oxo and C_{23} bile acids (Fig. 10). However, these procedures are technically demanding and of scarce reliability and hence not suitable for routine analyses of biological samples. Recently,

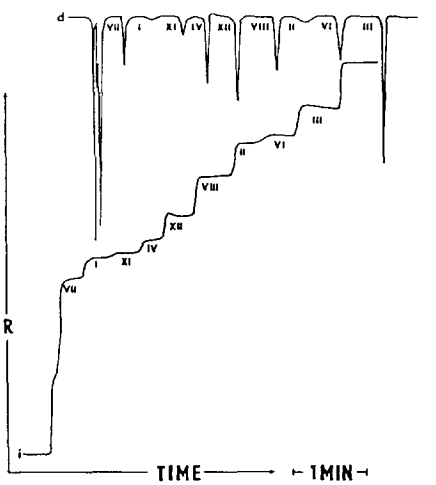


Fig. 10. Separation of bile acid mixture by isotachophoresis. Leading electrolyte, 5 m*M* HCl containing 0.4% hydroxyethylcellulose, histidine and 4 m*M* trimethyl-β-cyclodextrin (pH 6.4). Terminating electrolyte, 10 m*M* 4-(2-hydroxyethyl)-1-piperazinethanesulphonic acid with tris(hydroxymethyl)aminomethane to pH 8.3. Capillary, 230 mm × 0.55 mm I.D.; current, 100 μA for 5 min; temperature, 18°C; detection, conductivity; R = response of detector; i = conductivity; d = differential conductivity. Compounds: I = CA; II = CDCA; III = UDCA; IV = DCA; VI = 3α-hydroxy-7-oxo-5β-cholanoic; VII = 3,7,12-trioxo-5β-cholanoic; VIII = 3,7-dioxo-5β-cholanoic; XI = 3,7-dioxo-24-nor-5β-cholan-23-oic; XII = 3α,7α-dihydroxy-24-nor-5β-cholan-23-oic. From Ref. [89] with permission.

a simpler and more versatile form of CE, free solution CE, has been reported for the determination of free and conjugated bile acids [90]. Through the systematic evaluation of several operational parameters including the applied voltage, the temperature, the pH and concentration of the running buffer and the type of organic modifier, optimal resolution was obtained at 25°C and 25 kV using 15% methanol in 75 m*M* phosphate buffer (pH 6.5) containing 5 m*M* 1,4-diaminobutane [90]. The electropherograms obtained, however, show incomplete resolution of several components and co-migration of TCDCA and TDCA. CE of bile acids is still at an early developmental stage and more studies are required to assess the potential of this technique. Moreover, improvements may be produced by emerging technologies such as electro-

chromatography [91] which involves both electrophoretic mobility and partition.

3. Conclusions

The modern techniques for bile acid separation and determination including TLC, GC, HPLC, SFC and CE have been reviewed. Prior to assay of bile acids in biological matrices, a sample preparation step is often required to remove interfering endogenous components and to pre-concentrate the analytes and fractionate them into groups. Significant progress has been made in the pretreatment methodology (for a comprehensive review see Ref. [66]) based on the use of solid-phase extraction with classical adsorbents (e.g. Amberlite XAD resins, Lipidex gels, lipophilic ion exchange gels), silica or chemically modified silicas (e.g. octadecyl-, quaternary amino-bonded silicas). Integration of these procedures on-line with the instrumental analysis and automation of the full process will be of importance for the assay of the large number of samples from routine clinical studies.

Among the separation techniques available for bile acids, GC and HPLC represent the methods of choice. In fact, despite its rapidity and simplicity, TLC exhibits insufficient resolving power for the complex composition of biological extracts. SFC, on the other hand, provides faster but less robust and effective separations than HPLC. Because of the high peak efficiency and resolution speed, CE represents one of the most important analytical developments and the success achieved in interfacing it with MS has further expanded its potential. However, because of the limited number of reports, the impact of CE on bile acid analysis remains to be evaluated.

HPLC should be the preferred method for clinical evaluation of bile acid profiles since it has less requirements than GC (i.e. preliminary hydrolysis and derivatization are circumvented), but the unsatisfactory sensitivity, specificity and resolution have hampered its use. These inherent disadvantages are likely to be overcome by the rapid advances in HPLC–MS and HPLC–MS–MS techniques, especially if their commercial cost and analytical ruggedness will become comparable to those of GC–MS. Nevertheless, for the analysis of very complex bile acid mixtures, multi-mode separation and detection systems would provide more accurate data than any single method.

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