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Review

Separation techniques for bile salts analysis

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Abstract

The analysis of bile salts in biological samples has remained a difficult task, due to the complex nature of the salts and also to their low concentration in common sample fluids such as plasma and urine. Given their importance, the development of accurate and sensitive methods of instrumental analysis has been the subject of intensive research, and recent advances have eliminated or lessened some of the difficulties. Currently available techniques are the following: thin-layer chromatography, gas chromatography, high-performance liquid chromatography, supercritical fluid chromatography, gas chromatography–mass spectrometry and capillary electrophoresis. Liquid chromatography coupled with mass spectrometry (thermospray, fast atom bombardment, electrospray and ionspray), a method undergoing continuous improvement, is also being applied to bile salts analysis. In this paper, these various techniques, which differ greatly in specificity, accuracy and simplicity, are reviewed and discussed, in terms of analytical performance, applicability to a given sample fluid, major limitations, ability to identify uncommon bile salts, including unsaturated oxo derivatives, glucuronides, sulfates, glycosides and bile alcohols.

Keywords: Reviews; Bile salts

Contents

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1. Introduction

Bile salts (BSs) are a complex class of acidic steroids whose qualitative and quantitative composition in different biological fluids has been studied extensively. BSs are found mainly in gallbladder bile, in which they are present at millimolar levels, and in serum and urine, at concentrations at least a thousand-times lower, thanks to high hepatic clearance. The qualitative composition of BSs in bile, serum and urine varies as a result of different physicochemical properties and, consequently, different rate of intestinal absorption and clearance by the liver and kidney. BS hepatic and intestinal metabolism can also be influenced by liver and gastrointestinal diseases [1,2].

Comprehensive analysis requires isolation of the BS from a given matrix, followed by separation by class (unconjugated, glycine or taurine amidated) and, for each class, into the respective sulfate, glucuronide, glucoside and oxo-derivatives. In addition, for each of these types, BS can be mono-, di- or trihydroxylated, in positions 3, 6, 7 or 12, for each of which exist α/β epimeric forms. Among living species, due to differences in hepatic synthesis, intestinal absorption and metabolism, the qualitative and quantitative composition of BSs in bile or serum vary considerably. In addition, in reptiles bile alcohols constitute a large group of bile salt-like steroids present only as metabolites of cholesterol [3].

Because of broad differences in physicochemical properties of the BSs [4] such as lipophilicity and polarity, complete and accurate separation and identification requires the use of sophisticated chromatographic techniques.

Many chromatographic systems have been developed for BS analysis in complex biological matrices, including thin-layer chromatography (TLC), gas chromatography (GC), high-performance liquid chromatography (HPLC), supercritical fluid chromatography (SFC) and capillary electrophoresis (CE), in combination with one of various detectors or with mass spectrometry (MS). The choice of a system is dictated by the analytical task to be performed and to the nature of the target biological fluid to be analyzed. In a recent paper Scalia [5] reviewed major techniques for BS separation and detection and also mentioned previous extensive reviews [6,7]. Other broadly comprehensive reviews have also been published [8,9]. In this paper we would like to review the more important methods developed for BS analysis, discussing their analytical performance, applicability to a given biological fluid, focusing, major limitations, and their ability to identify uncommon BSs, such as unsaturated oxo derivatives, glycosides and bile alcohols.

2. Bile acids in biological fluids

The choice of a given chromatographic method is determined by the expected concentration in a given biological specimen, the complexity of its BS composition as well as the detectability and precision needed.

When BSs are present at millimolar levels and only in conjugated form, as they are in gallbladder or hepatic bile and in the duodenal contents, analysis is relatively easy since it is possible to achieve satisfactory separation and resolution by coupling an HPLC system with a conventional UV–visible detector [10]. To quantify unconjugated BSs which have a very poor absorption in the UV or visible region, an alternative method that has shown good analytical performance, is the evaporative light scattering detection (ELSD) [11]. Moreover, with this detection method a gradient mode can be used, which permits good resolution of more than 16 BSs in a single run (Fig. 1). When a gradient program is used, calibration is required for each BS, due to the fact that the detector response is influenced by the organic component of the mobile phase and by BS retention time. The use of this detector is particularly suitable for those biological fluids in which BSs, whether free or conjugated, are present at millimolar levels.

A complete analytical procedure for BS analysis in bile or duodenal content has been developed, including an appropriate procedure for clean-up from the biological specimen. In highly concentrated gallbladder bile, in which BSs are present in concentrations of up to 150 m*M*, BSs can even be analyzed directly. However, clean-up is recommended by reversed-phase solid-phase extraction (SPE) using a C_{18} cartridge (Bond Elut, Sepack), which efficiently isolates BSs and their conjugates from biological

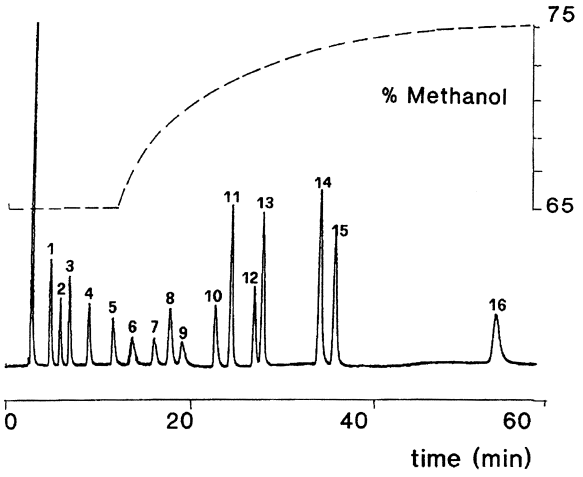

Fig. 1. Separation of glycine, taurine and unconjugated bile acids (6 nmol/injected) using a gradient system and the evaporative light-scattering detector. Column: Nova-Pak C_{18} Waters column (300×3.9 mm I.D., 4 μm particle size) thermostatted at 37±0.2°C. Gradient elution was performed with mixtures of methanol–15 m*M* ammonium acetate, pH 5.4 (65:35, v/v) (solvent A) and increasing methanol percentage to 75% (solvent B). The gradient profile adopted was: 15 min isocratic elution with A, convex gradient from A to B for 35 min, and 20 min isocratic elution with B at a flow-rate of 0.9 ml/min. Set up ELSD: carrier gas flow 40 p.s.i., drift tube temperature 130°C, exhaust gas temperature 85°C. 1=Tauroursodeoxycholic acid; 2=glycoursodeoxycholic acid; 3=taurocholic acid; 4=glycocholic acid; 5=taurochenodeoxycholic acid; 6=taurodeoxycholic acid; 7=glycochenodeoxycholic acid; 8=ursodeoxycholic acid; 9=glycodeoxycholic acid; 10=taurolithocholic acid; 11=cholic acid; 12=glycolithocholic acid; 13=nordeoxycholic acid (internal standard); 14=chenodeoxycholic acid; 15=deoxycholic acid; 16=lithocholic acid. From Ref. [11] with permission.

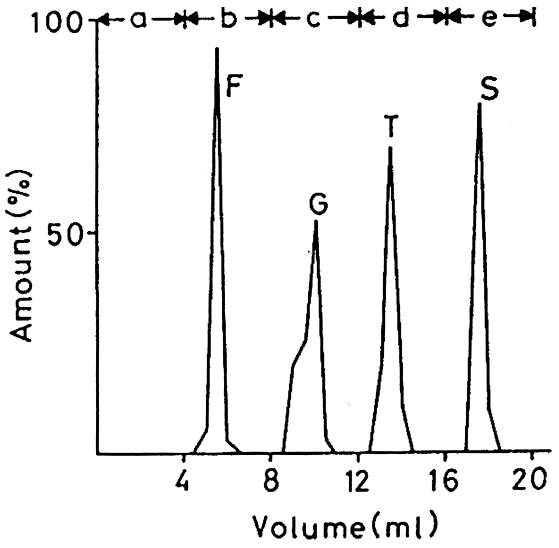

Fig. 2. Group separation of bile acids on PHP-LH-20. Eluent: (a) 90% ethanol; (b) 0.1 *M* acetic acid in 90% ethanol; (c) 0.2 *M* formic acid in 90% ethanol; (d) 0.3 *M* acetic acid–potassium acetate (pH 6.3) in 90% ethanol; (e) 1% ammonium carbonate in 70% ethanol. F=Free, G=glycine conjugate, T=taurine conjugate, S=sulfate. From Ref. [13] with permission.

fluids such as bile, serum, urine, stools, etc. By combining reversed-phase C_{18} with an anionic exchange system (SAX), separation of free, glycine-, taurine- and sulfated-conjugated BSs can be obtained before separative HPLC analysis [12]. Previously, this was accomplished using other resins, such as PHP-LH-20 (piperidinohydroxypropyl) (Fig. 2) [13] or by employing the ion-pair/Lipidex chromatography technique [14,15].

SPE is now the procedure of choice for BS cleaning, even though many endogenous compounds with similar physicochemical properties, such as cholesterol, steroids and bilirubins are co-eluted with the BSs.

BS group separation using SPE ion-exchange cartridges like SAX must be carefully standardized in terms of composition, volume and ion strength of the elution buffers; unfortunately, batch-to-batch variability often occurs among commercially available cartridges [16]. Moreover, various BS fractions containing unconjugated, glycine- or taurine-conjugated sulfated BSs are aqueous solutions enriched with salts that must be removed prior to analysis by this technique.

Wahlén et al. [17] reported that the use of octadecylsilane-bonded silica results in a significant loss of sulfated-taurine and other double conjugates BSs, as well as bile alcohol glucuronides. Thus, C_{18} SPE, previously validated only for common BSs, needs to be further optimized for the determination of complete BS composition, particularly in urine samples [18,19]. It has been shown that the use of triethylamine sulfate in the starting samples improves the recovery of those BSs [17] and, unlike the sodium hydroxide dilution standard method, this procedure permits re-use of the cartridges with no loss in performance [16].

3. Separation method

3.1. Thin-layer chromatography

This technique has been widely used for BS separation and its application to different groups of BSs has been extensively reviewed [5,20,21]. Nowadays, TLC is mainly used during BS synthesis for a rapid check of a given reaction, or for BS identification.

Recently, it has been reported that TLC in combination with densitometry is useful for separating and detecting ^{14}C- or ^{3}H-labeled BSs. Using a radio-chromatographic system it is possible to simultaneously determine, in the same spot, both the concentration and the radioactivity of a given labeled BS, thus providing information about its specific activity. A typical radiochromatogram of a bile sample collected 48 h after intraduodenal administration of 173 kBq of [^{14}C]cholic acid (CA) is shown in Fig. 3. This system has been validated and used for isotope dilution kinetics studies in man after administration of ^{14}C-labeled CA. The validity of the method proposed [22] has been checked by comparison of the results obtained with those of enzymatic spectrophotometric analysis and measurement of the radioactivity by liquid scintillation counting after elution of the separated BSs from a TLC plate. Advantages of this method [22] over the previous one include a reduced number of manipulations, the possibility of automation, better reproducibility, and the possibility of elaborating the radiometric data obtained for the primary BSs, which would serve to better characterize its metabolism in the enterohepatic circulation [22].

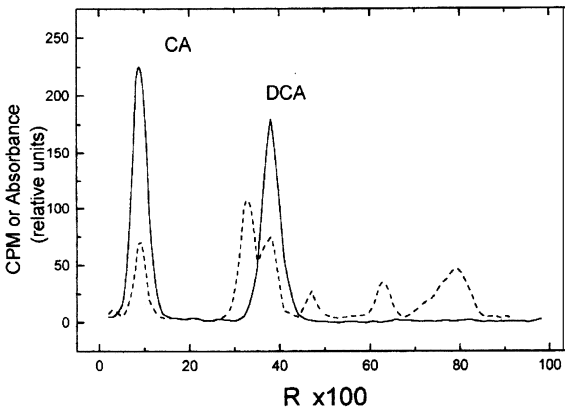

Fig. 3. Radiometric (continuous line) and densitometric (dashed line) profiles obtained for a bile sample collected 48 h after the intraduodenal administration of 173 kBq of [^{14}C] cholic acid. From Ref. [22] with permission.

3.2. Gas chromatography

GC has been extensively used in BS analysis since, before HPLC, it was the only tool available for their identification and quantification in biological fluids. Thanks to this technique, many biological aspects of BSs have been clarified.

The pioneer works of gas–liquid chromatography were based on the use of a packed column with different phases, on which extensive reviews have been published [20,23,24].

More recently, these systems have been replaced by a capillary column in which BS resolution is greatly improved and the analysis time shortened [25,26].

This technique is particularly suitable for BS analysis in serum or urine where the expected concentration is very low. However the sample must undergo extensive preliminary clean-up, and the BSs under study must be derivatized to make it volatile and thermostable. The BSs are usually isolated by SPE with a C_{18} cartridge and the different groups of BSs (free, glycine, taurine, sulfated, glucuronides), are separated by an ion-exchange system; conjugated BSs undergo enzymatic hydrolysis by cleaving either the C_{24} amide bond (glycine or taurine) with cholylglycine hydrolase and by removing the hydroxy group derivatives (glucuronides or sulfates) using β-glucuronidase, aryl sulfatase. A typical routine procedure for serum BS analysis is described by Setchell and Worthington [27]. The free BSs isolated from the reaction products, usually by liquid–liquid extraction should be converted into methyl esters trimethylsilyl or trifluoroacetyl derivatives [20,24,28–31].

More recently, the formation of hexafluoroisopropyl ester trifluoroacetyl derivatives has proved to be a superior method, thanks to the simplicity of the derivatizing procedure, absence of artifact, higher resolution and increased sensitivity to electron-capture detection [32].

Unfortunately these complex and laborious pro-

cedures are a potential source of error. For example, a deconjugation step results in a loss of information about the type and site of conjugation, and it can produce artifact by inefficient hydrolysis. However direct GC analysis of glycine- [6,33–35] and glucoside-conjugated [36,37] BSs, without the necessity for a hydrolytic step, was achieved. In addition, the combined use of a suitable derivatives (Me-TMS or Me-DMES) and a new type of metal capillary column (Ultra ALLOY) have permitted direct GC determination of free, glycine-, glucoside-, glucuronides-conjugated BSs without prior deconjugation [38].

The most widely used detection method for GC analysis is flame ionization detection; however, despite the linearity of the response with the mass injected, there is some variability among the different BSs.

3.2.1. Mass spectrometry

The combination of GC with MS is currently the reference method for determining of stereochemistry variety of BS structure as well as their analysis in biological fluids.

Mass spectra data for a large variety of BSs was recently reported by Lawson and Setchell [39]. The library is limited to the spectra of methyl ester–trimethylsilyl ether derivatives, but many other derivatives have also been studied and described [40,41].

Electron impact ionization (EI), the most common method, gives a large pattern of fragmentation that is useful for diagnostic purposes and structural information. A conventional mass spectrometer can be used, with most methods using 70 kW for electron impact energy. A reference file of fragmentation profiles useful for comparative purposes could be so obtained.

Chemical ionization (CI) gives a softer impact and, consequently, a reduced fragmentation pattern; its advantage is the increase in sensitivity that results from the ionization being limited to fewer ions. This technique is less used, but could be complementary to EI.

Fast atom bombardment (FAB), a recently introduced technique for BS analysis, has been demonstrated to be very useful and powerful. It can produce both positive and negative ions such as $[M+H]^+$ and $[M-H]^-$, together with cationized species. Unconjugated bile acids (BAs) are readily ionized by FAB, and their mass spectra have been well characterized [42,43]. FAB is not a quantitative method, but is very useful for BS identification. FAB mass spectra of conjugated BAs have been also studied [42–47]. However, it is not possible to differentiate BA isomers from their FAB spectra without a prior chromatographic separation step.

Even more recently, thermospray ionization, ion spray and electrospray ionization (ES) have been developed and applied to BS analysis [48–51]. Our laboratory has also used the latter in combination with HPLC or micro-HPLC; we have found good detectability down to the pmol/injected level of both free and amidated BSs (see Section 3.2.2.).

3.2.2. Gas chromatography–mass spectrometry

Following the introduction of GC–MS in the early 1960s and some early work on BA derivatives [52–54], this technique has contributed more than any other to the elucidation of the structures of BSs in biological fluids by providing a means for separating the complex mixtures of acids encountered in such samples. As a single system, GC has found wide utility for BS identification and quantification, and some conditions for these analyses have been incorporated into those used for GC–MS [41,55–57].

The preparation of volatile derivatives of BAs has been the subject of many studies, and references to these have recently been summarized [55]. The most commonly applied derivatives are the acetates, trifluoroacetates and trimethylsilyl ethers of the methyl cholanoates (Me-TMS); although each has particular advantages, of late the Me-TMS ethers have become the most widely adopted for qualitative purposes. In quantitative studies, where sensitivity and selectivity are the priority, the ammonia CI spectra of methyl acetates [58,59] and the EI spectra of methyl esters alkyldimethylsilyl ethers [60–62] may be a more appropriate choice.

However, the observation of artifacts under the conditions for silyl derivatization in some BS derivatives, such as 3-oxo-Δ^4 and 3-oxo-$\Delta^{4,6}$-BSs, has prompted a search for new derivatizing agents. A newly reported method for derivatization of 3-oxo-Δ^4 and 3-oxo-$\Delta^{4,6}$-BSs employs stable methoxymino derivatization prior to SPE, enzymatic deconjugation

and derivatization to the methyl ester–dimethylethylsilyl ether [63]. With this pretreatment the formation of 3-oxo-$\Delta^{4,6}$-BSs through silyl ether derivatization for the 7α-hydroxylated 3-oxo-Δ^{4}-BSs is completely suppressed, allowing accurate GC–MS analysis of 3-oxo BS in biological fluids [63].

The introduction of capillary columns (15–50 m× 0.25–0.5 mm I.D.) have eliminated many of the problems attendant on the use of packed columns and high gas flow-rates. The need for the carrier gas separator as an interface between GC and MS is now avoided by the column passing without interruption directly into the ion source, thus avoiding the potential absorption and thermal effects of the carrier gas separator, and the ion source pumping system can easily cope with the 0.5–2 ml min^{-1} flow of carrier gas. Chemically bonded liquid phases in capillary columns have much greater thermal stability and can be cleaned by solvents. This has virtually eliminated earlier difficulties with columns whose phase would bleed into the ion source to produce a high background of ions. The sensitivity and scan speed of mass spectrometers have had to be increased to take full advantages of the higher separation efficiencies of capillary columns.

3.2.3. BS kinetics by isotope ratio MS

The use of stable isotope for in vivo BS kinetic studies is a useful technique to determine the BS pool size and fractional turnover rate. By oral administration of [^{13}C]CA labeled in the side chain it is possible to calculate values for the above parameters by measuring the $^{13}C/^{12}C$ isotope ratio in serum samples [64–66].

The BSs must first be isolated from the serum sample, undergo hydrolysis and the trimethylsilyl derivatives analyzed by GC–MS using a capillary column such as DB-1 (J&W Scientific, Rancho Cordova, CA, USA) and a conventional quadrupole mass spectrometer [67].

Ions at m/z 459/458 for CA and at 371/370 for deoxycholic acid containing the side chain are measured by selected ion monitoring (SIM) from the samples after administration of ^{13}C isotope and then compared with those of unenriched samples. The linear correlation between enrichment in isotope ratio and molar ratio of labeled to unlabeled CA permits determination of the steady-state kinetics of the BA. This procedure does not require quantitative preinstrumental steps, but precise evaluation of the isotope ratio is necessary. Indeed, when significant error is introduced into the measurement of isotope ratios, such as occurs at low serum concentrations of bile acids or at low isotopic enrichment, the determinations of the kinetic will be inaccurate. Usually the coefficient of variation of isotope ratio ranges from 0.1 to 8% as a function of the mass spectrometer used [67].

Fig. 4 shows a capillary gas chromatogram of a sample of normal human serum obtained in our laboratory following the proposed method [67].

3.3. High-performance liquid chromatography

This technique has been widely applied in BS analysis only in the last 20 years. The main advantage over GC is that some BS classes (glycine or taurine conjugates) can be analyzed directly without preliminary derivatization procedures. The most popular stationary phase utilizes a C_{18} reversed-phase silica gel and, thanks to continuous increases in the column technology (controlled uniformity of the particles), many BSs can be separated with high-resolution [68–74]. Fig. 5 shows a typical HPLC resolution of more than 12 conjugated BSs obtained in less than 40 min under isocratic conditions [10].

The main drawback of HPLC is limited ability to detect the separated BSs; UV detection can be used at 200–210 nm with moderate sensitivity for amidated BSs, but it useless for unconjugated BSs due to their markedly low absorbance. To increase sensitivity the BSs can be pre- or post-column derivatized to form compounds that are more sensitive to UV or fluorimetric detection.

The pre-column derivatization reaction can be carried out on the 3α-OH group of free, glycine- and taurine-conjugated BSs, or on the carboxylic group on the side chain of free and glyco-BS. Table 1 lists the most common pre-column derivatizing reagents. Methods that use fluorescence detection are the most promising because extremely high sensitivity is possible with reasonable selectivity. Esterification of the carboxyl group with *N*-(9-acridinyl)-bromoacetamide gives detection limits of 10 to 100 fmol injected [86], while 3-(4-bromomethylphenyl)-7-

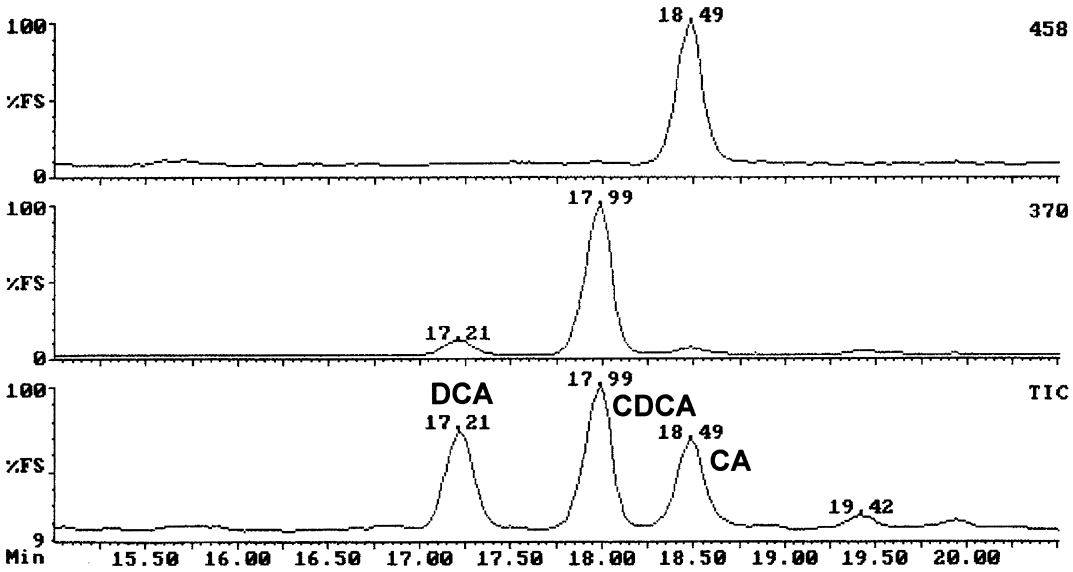

Fig. 4. Total ion current chromatogram and selected ion monitoring of ions at 370 and 458 of a serum sample analyzed by GC–MS.

diethylaminocoumarin, a newly synthesized labeling reagent for analysis of stereoisomeric C_{27}-BAs, permits detection limits of about 15 fmol [87]. Recently proposed as such a reagent, 2-bromoacetyl-6-methoxynaphthalene [88] has proven to be suitable for the determination of ursodeoxycholic acid (UDCA) and chenodeoxycholic acid (CDCA) in their pharmaceutical forms as well as for the analysis of conjugated BSs and the major unconjugated BSs in human serum. Fig. 6 shows the chromatogram of free and glycoconjugated BSs derivatized with 2-bromoacetyl-6-methoxynaphthalene.

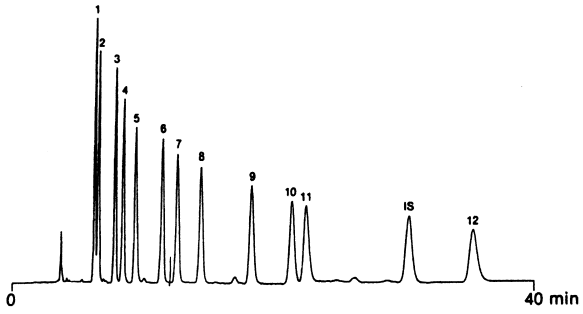

Fig. 5. HPLC chromatogram of standard mixture of conjugated bile acid: Altex Ultrex C_{18} column (250×4.6 mm I.D., 5 μm particle size); isocratic elution with methanol–0.01 *M* phosphate buffer, pH 5.35 (75:25, v/v), at a flow-rate of 0.7 ml/min, and spectrophotometric detection at 200 nm. 1=Ursodeoxycholyl taurine; 2=sulfolithocholyl taurine; 3=ursodeoxycholyl glycine; 4=cholyl taurine; 5=sulfolithocholyl glycine; 6=cholyl glycine; 7=chenodeoxycholyl taurine; 8=deoxycholyl taurine; 9=chenodeoxycholyl glycine; 10=deoxycholyl glycine; 11=lithocholyl taurine; 12=lithocholyl glycine; I.S.=3α,12α,5β-cholanoil glycine (internal standard). From Ref. [10] with permission.

After oxidation of the 3α-hydroxy group of free and conjugated BSs to a 3-oxo group by a 3α-hydroxysteroid dehydrogenase enzyme, selective derivatization using dansylhydrazine is possible. The detection limit of dansylhydrazone derivative is 0.5 pmol [89,90]. The same technique has been also successfully utilized for the separation of stereoisomers of bile alcohols determined by the configuration of hydroxy groups on the side chain using 2,4-dinitrophenylhydrazine as the labeling agent [91]. The 3α-hydroxy group can also be directly transformed into a 3-(1-anthroyl) ester with a detection limit of 20 fmol [86,92–95].

The reversed-phase principle allows the use of aqueous methanol or acetonitrile as a mobile phase, making it possible to use a BS specific and sensitive enzymatic post-column detector formed by immobilizing a BS specific enzyme (3α-hydroxysteroid dehydrogenase) on a second column [96–98]. 3α-Hydroxysteroid dehydrogenase transforms the 3α-

Table 1
Most common pre-column derivatizing reagents for HPLC detection of bile salts

Reagent	Detection			Ref.
	UV (nm)	Fluorimetric		
		ex (nm)	em (nm)	
Carboxyl group				
2-Phenacyl bromide	254			[75]
O-(*p*-Nitrobenzyl)-*N*,*N*′-diisopropylurea	254			[76,77]
p-Bromo-phenacyl bromide	254			[78–81]
N-Chloromethyl-4-nitrophthalimide	254			[78]
4-Bromomethyl-7-methoxycoumarin		360	410	[81–83]
1-Bromoacetylpyrene		370	440	[84,85]
N-(9-Acridinyl)-bromoacetamide		357	570	[86]
3-(4-Bromomethylphenyl)-7-diethylaminocoumarin		400	475	[87]
2-Bromoacetyl-6-methoxynaphthalene		300	460	[88]
3α-Hydroxy group				
Dansylhydrazine		436	520	[89,90]
2,4-Dinitrophenylhydrazine	364			[91]
1-Anthroylnitrile				[86,92–95]

hydroxy group of BSs in the 3-oxo group in the presence of NAD^+. The NADH produced is monitored by UV (340 nm) [99,100], fluorescence [101], or chemiluminescence [102] detection. The enzymatic reaction can be further coupled with electrochemical detection if a solution of phenazine methasulfate is added; however no significant difference in the detection limit has been found with respect to the fluorescence detection [103,104].

The main drawbacks of the above reported methods of derivatization are that they are time consuming, sometimes laborious, and, due to matrix effect, they do not assure complete sample conversion.

The fact that MS is an invaluable method for providing definitive qualitative and quantitative information in BS analysis has inspired efforts to produce a satisfactory combination of HPLC–MS instrumentation. In the past, HPLC–MS combination was most commonly employed in the discontinuous mode, in which chromatographic fractions were subjected to MS individually [105,106]. An important development in MS technology has been the introduction of FAB; utilizing a high-energy ionizing atom beam, quasimolecular ions are almost always produced, although sufficient energy is generally transferred to the ionized molecules to produce fragmentation. The formation of adducts and matrix ions and the inability of direct FAB to differentiate isomeric structures, made the combination with on-line separation desirable. Ito and co-workers [107,108] were the first to apply FAB (frit-FAB) in ionization of BA effluent from a capillary column.

In 1993, micro HPLC continuous-flow FAB was developed for the analysis of urinary BS (free, conjugated and oxo derivatives, this last after reduction with sodium borodeuteride) [109]. With this technique negative pseudomolecular ions were produced. When combined with chromatographic separation, it could identify and quantify positional and stereoisomers of urinary BSs not distinguishable by their FAB spectra. The method is particularly suitable for high sample throughput; it allows sample analysis with a picogram range of detectability, at 4-min intervals [109].

The development in the early 1980s of the thermospray ionization interface permitted coupling of the HPLC column directly to the MS thereby allowing continuous real-time in-line monitoring of the effluent [110,111]. Setchell and Vestal [48] developed a method for qualitative and quantitative analysis of free, glycoconjugated and tauroconjugated BSs in bile and serum utilizing negative thermospray ionization.

By SIM of the pseudomolecular ion it is possible to achieve a sensitivity of 10 to 20 pmol per injection. Fig. 7 shows the total ion current chro-

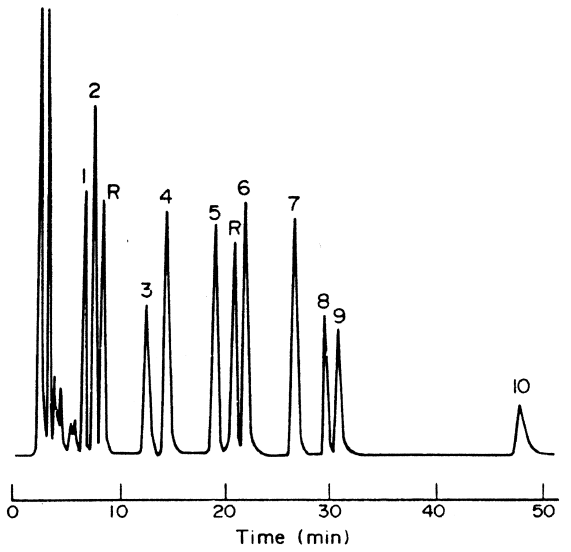

Fig. 6. Representative HPLC separation at ambient temperature of a standard mixture of free and glycoconjugated bile acids (30 μ*M*) derivatized with 2-bromoacetyl-6-methoxynaphthalene. 1=Glycocholic acid; 2=glycoursodeoxycholic acid; 3=glycochenodeoxycholic acid; 4=glycodeoxyxholic acid; 5=cholic acid; 6=ursodeoxycholic acid; 7=glycolithocholic acid; 8=chenodeoxycholic acid; 9=deoxycholic acid; 10=lithocholic acid; R=reagent peak. Column: Hypersil RP-18 (250×4.6 mm I.D., 5 μm particle size). Gradient elution with mixtures of water (A), acetonitrile (B) of varying composition (v/v). The gradient profile adopted was: t=0 min, 60% B; t=10 min, 60% B; t=20 min, 80% B; t=45 min, 80% B; t=50 min, 60% B at a flow-rate of 1.0 ml/min. Fluorescence detection λ_{ex}=300 nm; λ_{em}=460 nm. From Ref. [88] with permission.

matogram obtained by thermospray ionization HPLC–MS of eight conjugated BSs; in the same figure the mass spectrum of one eluted BS indicates the presence of pseudomolecular ions with relatively little fragmentation (consecutive losses of water, due to each hydroxy group in the molecule) [48].

Technological advances in interfacing HPLC–MS have led to the development of ES systems [112–114], which have turned out to be extremely useful for the analysis of proteins and other high-molecular-mass substances. We have developed an HPLC–MS method with an ES interface for the qualitative and quantitative analysis of BSs in biological fluids. In this study [115] good separation of free as well as glycine- and taurine-conjugated BSs was achieved with a conventional HPLC system equipped with a post-column splitter that diverted part of eluate into the ES source at a flow-rate of 18 μl/min. When BSs were ionized in the ES interface, operating in the negative-ion mode, only $[M-H]^-$ molecular ions were generated; the detection limit was 15 pg injected for all BS studied. Alternatively, a microbore HPLC was utilized; with this system, a head-column enrichment technique was used that permitted improvement in the detection limit to 5 pg injected. Figs. 8 and 9 give total ion chromatograms from the injection of a mixture of BS standards using, respectively, the conventional HPLC and the microbore HPLC–ES-MS systems [115].

ES mode has proved to be a powerful tool for simultaneous determination and detection of many BS derivatives. Ikegawa and co-workers developed a method for separatory determination of BS 3-sulfated [116] or 3-glucuronides [117] in human urine by HPLC–ES-MS. These derivatives are characterized by pseudomolecular ions $[M-H]^-$ and $[M-2H]^{2-}$; the ratio of these ions is influenced by the acidic component of salt added to the mobile phase according to the pK_a value of the side chain. Therefore, with a suitable mobile phase and operating in selected ion monitoring, a good separation can be achieved with a detection limit of 200 fmol, 1000-times lower than that obtained with UV detection [116,117].

Another example of BS analysis by HPLC combined with MS is based on ion spray ionization. Warrack and DiDonato [51] reported a method for qualitative and quantitative analysis of free and conjugated BSs in monkey bile using an ion-spray interface coupled with HPLC or micro HPLC system. Intense and reproducible $[M+H]^+$ ions and $[H+CH_3CN+H]^+$ adducts were observed, providing, on the basis of their relative abundance, a means for identification of several BSs and their isomeric forms. Adduct ions were most abundant for free acids, but were reduced for glycine and taurine conjugates; detection limits by ion-spray microbore HPLC–MS and SIM from 40 to 100 fmol were calculated.

LC–MS is improving on a seemingly daily basis; one example is the combined use of soft ionization techniques (FAB and ES) for the successful qualitative analysis of BSs and bile alcohols conjugates in urine from infant with cholestatic liver disease in which more than 150 of these substances were

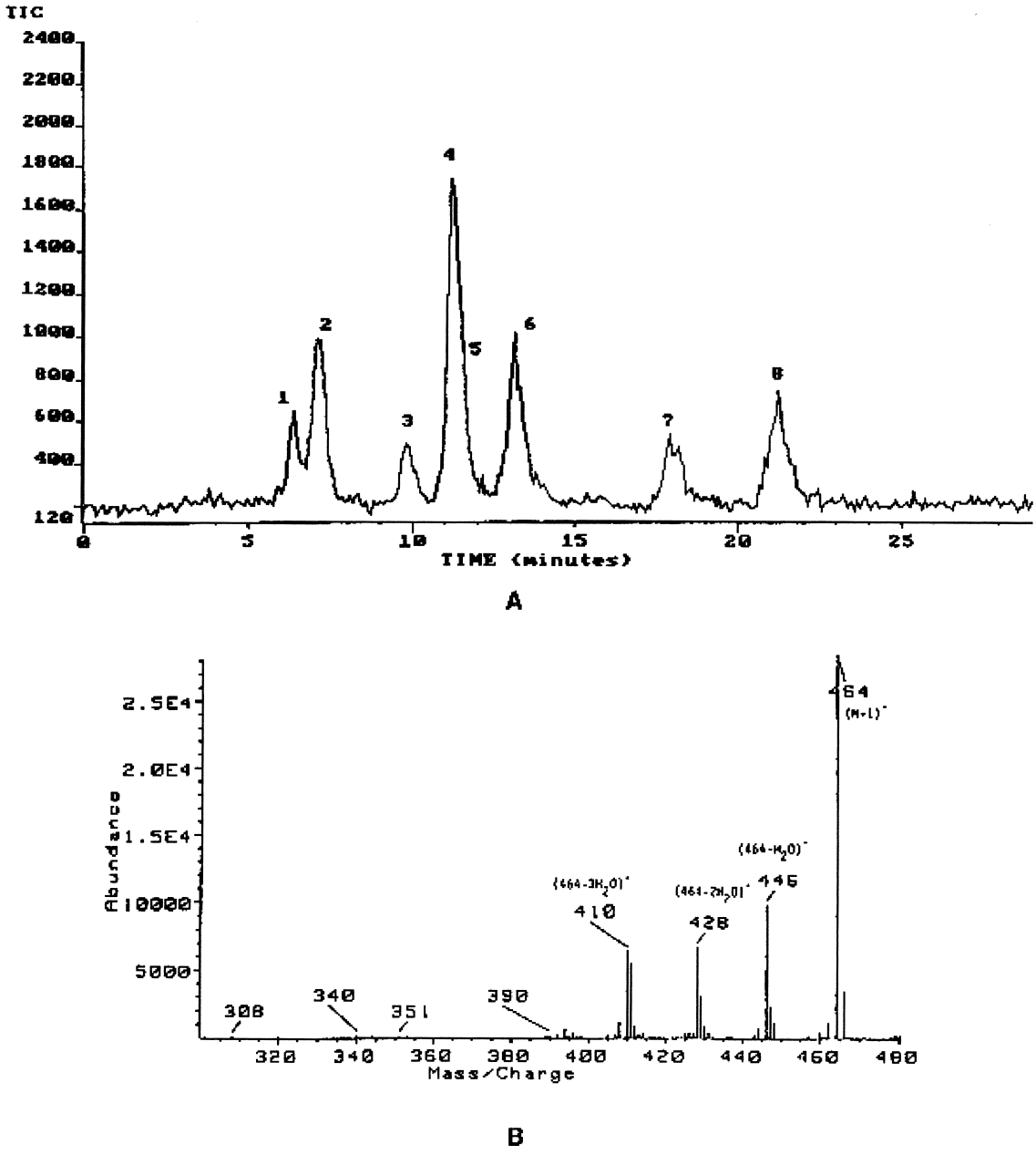

Fig. 7. (A) Total ion current chromatogram obtained by thermospray ionization HPLC–MS of a mixture of eight conjugated BAs (5 μg). Ultrasphere ODS column (250×4.6 mm I.D., 5 μm particle size). Isocratic elution with methanol–ammonium acetate, pH 5.7, (75:25, v/v) at a flow-rate 1 ml/min. Temperatures used to achieve thermospray were as follows: control temperature T_1=126°C, vaporizer temperature T_2=201°C, source block temperature T_3=318°C, tip heater temperature T_4=315°C, vapor temperature T_5=265°C, lens heater temperature T_6=120°C. Ionization was facilitated by filament-on mode and negative ion spectra were recorded by continuous repetitive scanning over the mass range m/z=400–550 Da. 1=Taurocholic acid; 2=glycocholic acid; 3=taurochenodeoxycholic acid; 4=glycochenodeoxycholic acid; 5=taurodeoxycholic acid; 6=glycodeoxycholic acid; 7=taurolithocholic acid; 8=glycolithocholic acid. (B) Negative ion thermospray ionization mass spectrum of peak 2 (glycocholic acid). From Ref. [48] with permission.

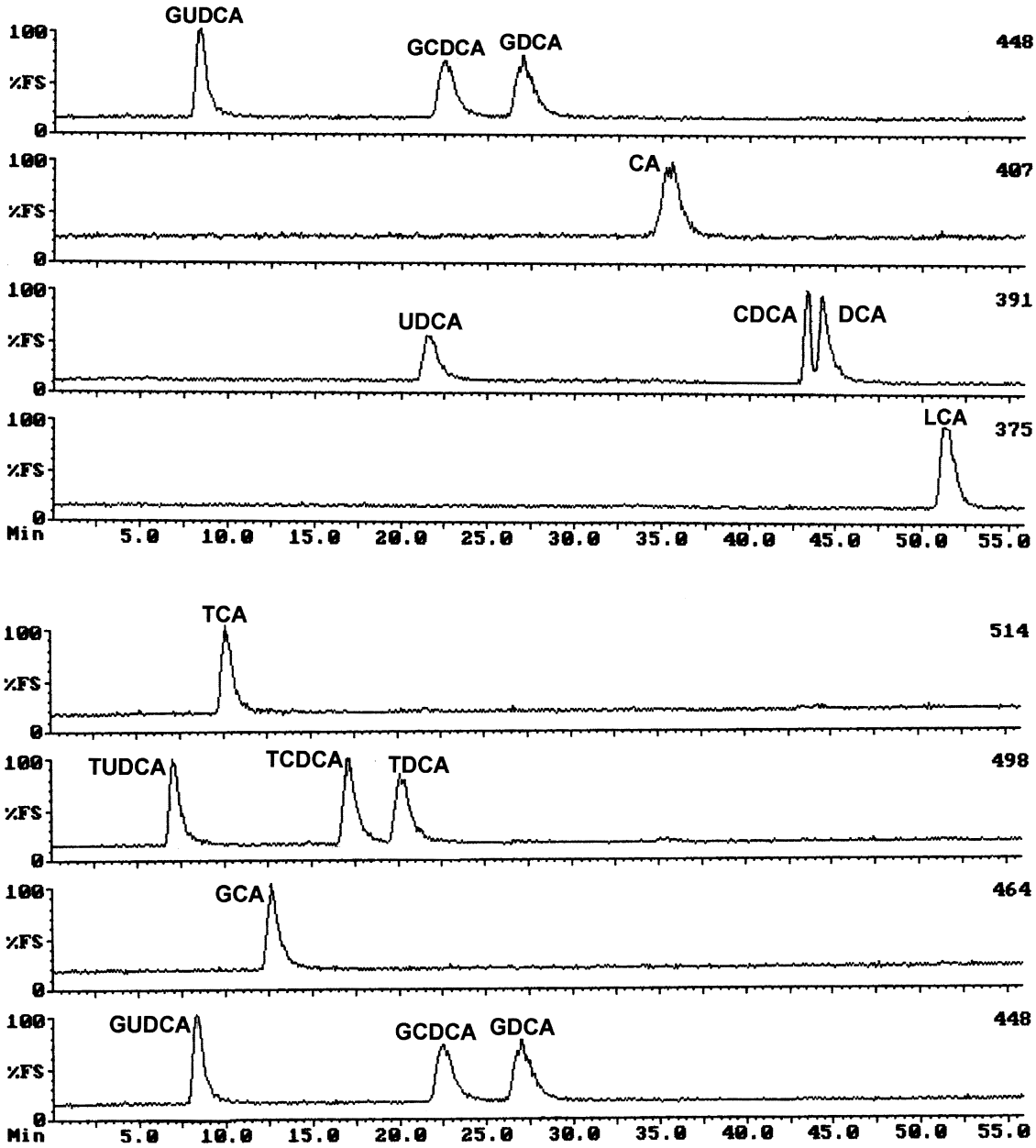

Fig. 8. Total ion chromatograms from the injection of a mixture of bile acids standards (free, glycine- and taurine-conjugated) at the 40 pg level. Column: Ultrasphere XL C_{18}, 3 μm particle silica size (70×4.6 mm I.D.). Gradient elution with mixtures of different solvent systems: (A) methanol–15 m*M* ammonium acetate solution (66:34, v/v, pH 5.4); (B) methanol–15 m*M* ammonium acetate solution (75:25, v/v, pH 6.0); (C) methanol 100%. The gradient profile adopted was: $t=0$ min, 90% A, 0% B, 10% C; $t=15$ min, 90% A, 0% B, 10% C; $t=23$ min, 100% A; $t=40$ min, 0% A, 80% B, 20% C; $t=50$ min, 100% B; $t=60$ min, 0% A, 65% B, 35% C; $t=70$ min, 90% A, 0% B, 10% C at a flow-rate of 0.3 ml/min. Probe voltage, 3.19 kV; counter electrode, 0.54 V; cone voltage, 56 V; source temperature, 68°C; flow-rate at source, 18 μl/min. From Ref. [115] with permission.

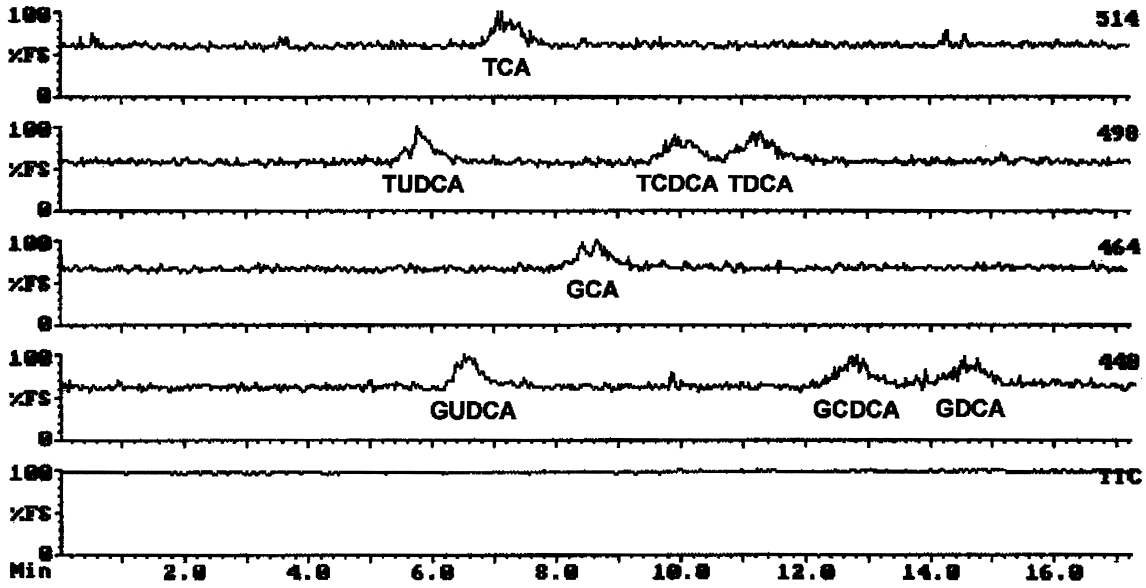

Fig. 9. Total ion chromatograms obtained by microbore-HPLC–ES-MS analysis of a mixture of eight bile acids standards, at a level of 20 pg component. Column: C_{18} microbore column Fusica with 0.3 mm I.D. Mobile phase and gradient profile adopted are as described in Fig. 8. Mobile phase flow-rate 1.4 μl/min. From Ref. [115] with permission.

detected, after anion-exchange chromatography [50]. Other interface are under study for BS analysis, including ion-trap.

3.4. Supercritical fluid chromatography

SFC is a technique suitable for the analysis of non-volatile and thermally labile compounds that cannot be analyzed by GC, or that require analysis time faster than that obtainable with HPLC [118]. Employing substances such as carbon dioxide above their critical temperature and pressure as mobile phase, relatively non-polar molecules can be efficiently separated; by adding a few percent of polar solvent to the mobile phase, SFC can be extended to more polar solutes [118,119].

In the past few years, SFC has been applied to the separation of free, glyco- and tauro-BSs [118,120,121]; in terms of the separation rate of BS, it is faster than reversed-phase HPLC, and it provides alternative separation selectivity with respect to conventional HPLC systems. For example, by adding methanol to supercritical CO_2 and using a phenyl-bonded phase or cyanopropyl columns for free and conjugated BSs, respectively [120,121], these compounds are eluted following a normal-phase mechanism.

Unfortunately, some drawbacks have also been reported; conjugated dihydroxy isomers are not completely resolved and, in general, BS separation is less reproducible than that obtainable by reversed-phase HPLC [121,122]. These disadvantages, plus the fact that most separations involving BS mixtures can be performed by existing HPLC or GC methods, have relegated SFC to a less important role in BS analysis.

3.5. Capillary electrophoresis

CE is a relatively new technique under continuous improvement.

In this separative technique, BSs are often used in the CE buffer to set up micellar electrokinetic capillary chromatography, thanks to the ability of BSs to form aqueous micellar solution in which critical micelle concentration (CMC) size and aggregation number is related to the BS structure [4]. Using these systems, it has been possible to achieve an enhanced selective and resolutive power.

Until now, few papers report the use of CE for BS

separation and limited only to pure BS solutions [123].

The main limitation for BS analysis is the poor molar absorption of conjugated BSs (and even less for free BSs). Snopek et al. [124] reported a relatively good separation of a series of free BSs and oxo derivatives, using isotachophoresis with an aqueous electrolyte buffer containing β-cyclodextrins and conductivity as a detection system [124].

The analysis of free and conjugated BSs has been reported but some BS showed incomplete resolution.

More recently Quaglia et al. [125] describe an indirect UV detection of ursodeoxycholic acid in pharmaceutical forms using high-performance capillary zone electrophoresis (HPCE). The background electrolyte contains UV absorbing ions, such as benzoic acid or 5,5-diethylbarbituric acid and β-cyclodextrins to improve the BS resolution. The non-absorbing BSs will displace the absorbing species at the UV detector resulting in a decrease of absorbance related to their concentration in the buffer. The authors reported that, with this system, they increase the BS detectability about 100-times.

Unfortunately, until now CE has been applied only to pure BS solutions and no data are available about its applicability to complex biological fluids where HPLC or GC techniques are still superior.

The recent introduction of combined techniques which take advantage of different principles, such as electrochromatography [126], could improve the analytical performance of this technique. Moreover the interface between CE and MS, using electrospray interface, could resolve problem of BS detection in terms of sensitivity and detectability.

4. Conclusions

Despite great improvements in both separative and detection techniques, there is still much room for improvement in BS quali–quantitative analysis in complex biological matrices.

Clean-up of sample such as urine or tissue remains problematic due to the broad differences in BS physicochemical properties and to the fact that commercially available materials need to be further standardized.

The choice of separation technique must take into account the concentration and composition in BS of the sample to be analyzed and, at the same time, combine easy use and eventually clinical practicability.

Among the analytical techniques for BS analysis here reviewed, HPLC and GC are more suitable than TLC, SFC or CE: TLC does not have sufficient resolutive power, while SFC is limited by lower resolution and reproducibility than HPLC. CE, until now, has been applied only to pure BS solutions and no data are available about its applicability to complex biological fluids.

GC–MS is widely employed for identifying and quantifying many BSs in various metabolic stages, but use of this technique entails a series of laborious pre-analytical steps, including preliminary separation of BSs by class, hydrolysis and derivatization, which limit its analytical performance considerably. HPLC, offers an excellent method of separation and does not require preliminary derivatization procedures, but it is hampered by unsatisfactory sensitivity and the need for specific detector systems. Because of persistent need for a rapid and accurate means of screening BSs and their minor metabolites in biological samples, many efforts have been made in recent years to improve HPLC technology, especially in terms of sensitivity. HPLC–MS techniques have been developed, and like GC–MS, now represent a powerful tool for analysis of biological fluids. Nevertheless, combined separation and detection systems are the current better choice for obtaining complete and accurate data about BS composition in a complex biological matrix.

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