Thermospray ionization liquid chromatography-mass spectrometry: a new and highly specific technique for the analysis of bile acids

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Abstract A rapid, highly specific and sensitive combined high performance liquid chromatography-mass spectrometric (LC-MS) method is described for the analysis of bile acids in biological fluids. Ionization of polar bile acid conjugates is achieved in the thermospray interface that is used to directly couple the LC column to the mass spectrometer, thereby allowing continuous monitoring of the LC effluent. Maximum sensitivity (4-10 pmol) is achieved by recording the negative ions generated in the ionization process and mass spectra obtained for the principal bile acid conjugates are characterized by intense $[M-H]^-$ pseudo-molecular ions and fragment ions due to consecutive losses of water corresponding to a number of hydroxyl groups in the molecule. The mass spectrometer thus provides molecular weight and useful structural information for each compound separated by HPLC. Applications of the LC-MS technique to the analysis of bile acids in bile and serum samples after an initial solid-phase extraction step highlight the potential of the thermospray interface for enhancing the specificity and sensitivity of the HPLC technique for bile acid analysis. — **Setchell, K. D. R., and C. H. Vestal.** Thermospray ionization liquid chromatography-mass spectrometry: a new and highly specific technique for the analysis of bile acids. *J. Lipid Res.* 1989. **30:** 1459-1469.

Supplementary key words HPLC-MS • HPLC • bile • serum

Major advances have been made over the last decade in the application of high performance liquid chromatography (HPLC) for bile acid analysis and these have been recently reviewed (1, 2). This technique is potentially the most suitable chromatographic procedure for routine analysis, because unlike gas-liquid chromatography, where there is a need for extraction and purification steps, hydrolysis of conjugates and preparation of volatile derivatives (3, 4), bile acids can be measured directly in simple extracts of biological fluids as intact conjugates. However, despite the early promise indicating that HPLC might supercede other techniques for bile acid analysis, its limited sensitivity and specificity have restricted its widespread use.

As a consequence of the development of the thermospray ionization interface (5, 6), it is now possible to couple the HPLC column directly to the mass spectrometer thereby allowing continuous real-time, in-line monitoring of the effluent by the mass spectrometer, i.e., the mass spectrometer thus serves as a definitive HPLC detector. This communication will describe our experiences with the use of thermospray ionization LC-MS for the analysis of bile acids in biological fluids.

MATERIALS AND METHODS

Extraction of bile acids from biological fluids

Bile acids were rapidly extracted from biological samples by liquid-solid extraction exactly as described by Setchell and Worthington (7). The methanolic extract was evaporated to dryness over nitrogen gas and the sample was redissolved in the HPLC mobile phase (100 μl).

High performance liquid chromatography-mass spectrometry (HPLC-MS)

Bile acids were separated by HPLC using a Waters 600MS system (Waters Inc., Milford, MA) with variable wavelength UV detector (Model 490) housing a 25 × 0.46 cm Hypersil ODS (5 μm particle size) column. Separation was achieved using a mobile phase of methanol-0.4 M ammonium acetate, pH 5.7, 75:25 (by vol) with a flow rate of 1.0 ml/min. The HPLC column was coupled directly to a Vestec 201N dedicated thermospray LC-mass spectrometer (Vestec Corporation, Houston, TX) via a short piece of stainless steel tubing connected to the outlet of the high-pressure UV flow-cell. A schematic of this interface is shown in **Fig. 1**. Ionization was achieved in the thermospray interface (Fig. 1) using the following optimized conditions; control temperature (T_1, measured close to the entrance of the vaporizer) =

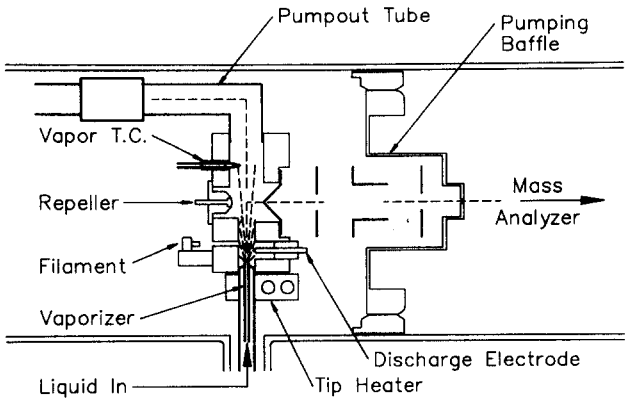

Fig. 1. Schematic illustrating the thermospray ionization interface and ion source of the Vestec 201 LC–MS system.

126°C; vaporizer temperature (T_2, measured at the tip of the vaporizer) = 201°C; source block temperature (T_3) = 318°C; tip heater temperature (T_4, measured at the exit orifice) = 315°C; vapor temperature (T_5, measured downstream of the exit orifice) = 265°C; and lens heater temperature (T_6) = 120°C. The diameter of the exit orifice of the vaporizer was 150 μm. Ionization was assisted by the use of a beam of electrons of 1000 eV energy generated from a filament (filament-on mode). Negative ion spectra generated were continuously recorded over the mass range *m/z* 400–600 daltons using a scan cycle of 2 sec. Where selected ion monitoring was used, specific ions characteristic of each bile acid were monitored using a dwell time of 300 msec/ion.

RESULTS AND DISCUSSION

HPLC has become a popular technique for the analysis of bile acids in biological fluids (1, 2). However, because of the relatively poor UV absorption characteristics of bile acids, most applications of this technique have been restricted to the analysis of biliary bile acids where concentrations are relatively high. Improved sensitivity with a degree of selectivity has been achieved by the use of either pre- or post-column derivatization reactions coupled with more sensitive fluorometric or electrochemical detectors (1, 2) and by this approach it is possible to measure low concentrations of bile acids (1–2 μmol/l) typically found in healthy serum (8). In our experience, such methods however, are less robust, more time-consuming and expensive, and introduce a degree of selectivity to the analysis (2). Irrespective of the HPLC method used, major problems with the technique continue to include interferences from constituents of the biological matrix, restricted specificity, and poor chromatographic resolution, so that presently the method is only suited to the measurement of the principal bile acid conjugates when in relatively high concentrations. Frequently with biological samples, peaks occur in chromatograms that cannot be identified unless fractions are collected and other confirmatory techniques are used. Indeed, the first examples of combining LC and MS for bile acid analysis employed this discontinuous mode (9, 10).

Since the mass spectrometer is a highly specific and sensitive detector, direct coupling of the mass spectrometer to the LC column potentially offers a means of overcoming many of the above limitations. Improvement in chromatographic resolution will only be possible by significant advances in column technology or the use of microbore columns (11–14). In the earliest successful attempts at LC–MS (15, 16), the effluent was spotted onto a continuously circulating belt and, after flash vaporization of the solvent, the solute molecules that deposited on the belt passed into the ion source where spectra were obtained in either electron impact or chemical ionization mode (17). The moving-belt interface works well for many compounds but the major disadvantages of this system include an uneven spreading of the sample on the belt, with consequent loss of chromatographic resolution, the inability to handle thermally labile compounds or mobile phases consisting of nonvolatile solvents, and poor sensitivity because of the low sample transfer. Direct liquid introduction (18) overcomes many of these limitations and the most significant advance to take place in LC–MS was the development of the thermospray interface (5, 6).

Thermospray is defined as the controlled vaporization of a liquid as it passes through a heated capillary tube. In this process a supersonic jet of vapor (aerosol) is created with nonvolatile molecules in the solution being retained within the charged droplets that are formed. Depending upon the flow-rate, temperature, and composition of the mobile phase, ions are generated, facilitated by the ionic composition of the mobile phase. This soft ionization process yields molecular ions of nonvolatile and polar compounds that would otherwise not be amenable to conventional electron impact or chemical ionization mass spectrometric analysis. The technique has been successfully applied to the analysis of steroid sulfates (19) and glucuronides (20, 21), nonsteroidal estrogens (22), glutathione conjugates (23), methylxanthines (24), and many other miscellaneous compounds.

It should be noted that there are certain limitations to the direct thermospray ionization technique. Thermospray ionization is best achieved using mobile phases containing at least a 10% aqueous phase and a high concentration of electrolyte; however, optimal chromatographic separation may not always be possible with such solutions. Furthermore, where organic modifiers are used in relatively high proportions, the sensitivity of the technique is compromised. Some of these problems can be circumvented by the post-column addition of solvents or

electrolyte solutions; alternatively, ionization may be facilitated by utilization of an electron beam (filament) or an electrical discharge placed downstream of the tip orifice (see schematic of ion source, Fig. 1). When external ionization is used (as in filament-on mode used here), positive and negative chemical ionization takes place because the mobile phase is first ionized by the electron beam and behaves as a reagent gas. By this approach the thermospray interface can be usefully extended to cope with mobile phases of pure organic solvents that are typically used in straight-phase chromatography.

Analysis of authentic standards of bile acids

For bile acid conjugates, chromatographic separation is best achieved using reversed-phase chromatography with solvents having a significant proportion of water (1). Such systems are therefore ideally suited to the thermospray interface. Most HPLC methods for bile acids (see 1, 2 and references therein) use mobile phases with phosphate buffers or sodium ions, but these are not desirable for thermospray ionization because the former, due to nonvolatility, cause blockage of the vaporizer, while sodium ions tend to suppress ionization. Because of its volatility, ammonium acetate has proved to be an ideal electrolyte for enhancing the thermospray ionization process (5, 6) and therefore a chromatographic system was utilized incorporating this electrolyte in the mobile phase that afforded an adequate separation of the principal bile acid conjugates. With a mobile phase of methanol–0.4 M ammonium acetate, pH 5.7, 75:25 (by vol) and a flow rate of 1 ml/min, the principal bile acid conjugates eluted within 25 min, although glycochenodeoxycholic and taurodeoxycholic were not completely resolved using this solvent system (**Fig. 2**). Since these two bile acids have different molecular weights they can be readily distinguished and quantified using LC–MS by selected ion monitoring of suitable characteristic ions.

Optimal ionization of bile acid conjugates was achieved in filament-on mode rather than in the direct thermospray ionization mode, and maximum sensitivity for all bile acids studied was obtained by recording the negative ions generated. Positive ions generated in thermospray process were characterized by the production of ammonium adduct ions and mass spectra were characterized by intense ions corresponding in mass to $[M + NH_4]^+$. This was also the case for unconjugated bile acids. On the other hand, negative ion mass spectra obtained by filament-on thermospray ionization for eight of the principal bile acid conjugates (**Fig. 3**) were all characterized by intense pseudo-molecular ions corresponding to the $[M-H]^-$ and,

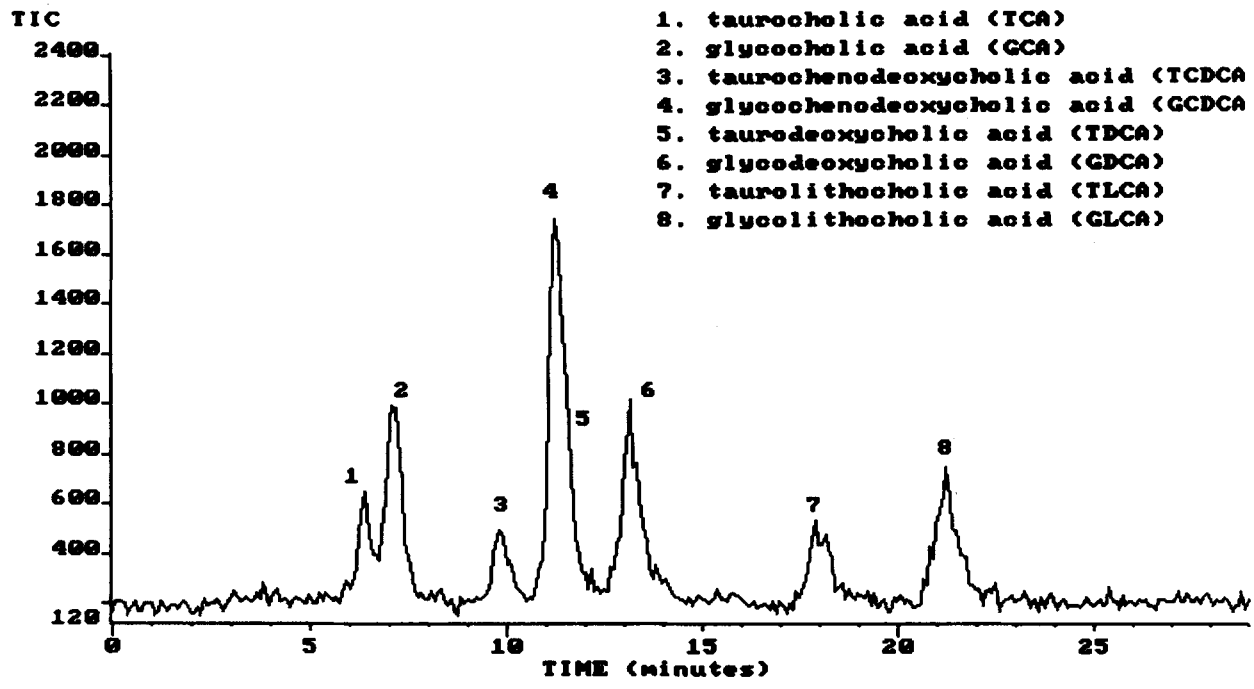

Fig. 2. Total ion current chromatogram obtained by thermospray LC–MS analysis of approximately 5 μg (approx 10 nmol) of eight different bile acid conjugates. Separation was achieved using a Beckman Ultrasphere ODS column (25 cm × 0.46 cm; 5 μ particle size) with an isocratic mobile phase consisting of methanol–0.4 M ammonium acetate, pH 5.7, 75:25 (v/v) using a flow-rate of 1.0 ml/min. Temperatures used to achieve thermospray were as follows: control temp T_1 = 126°C; vaporizer temp T_2 = 201°C; source block temp T_3 = 318°C; tip heater temp T_4 = 315°C; vapor temp T_5 = 265°C; and lens heater temp 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.

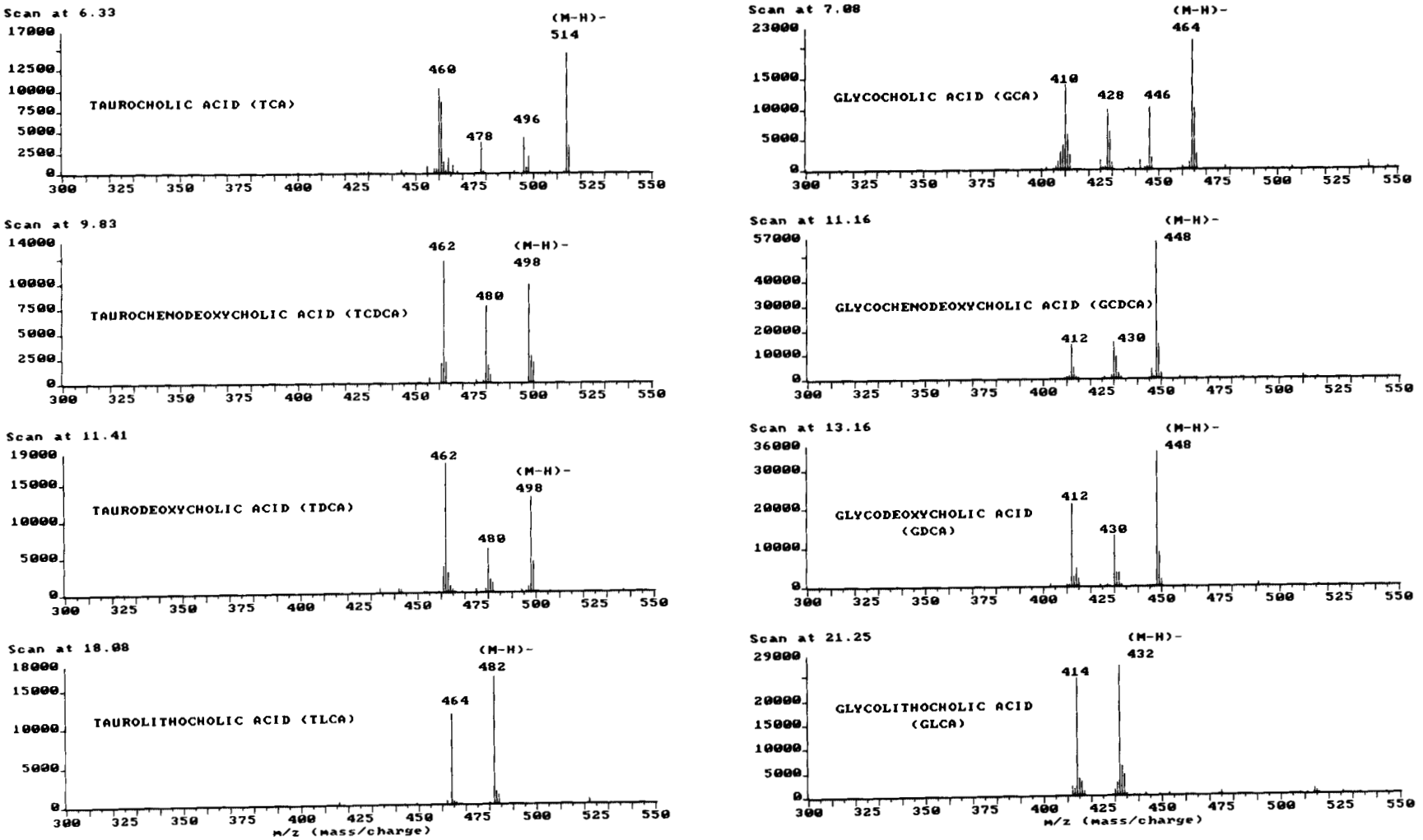

Fig. 3. Negative ion mass spectra obtained by filament-on thermospray LC–MS analysis of eight of the principal bile acid conjugates typically found in biological fluids. Spectra are characterized by intense $[M-H]^-$ ions and fragments arising from consecutive losses of water [– 18 Da] according to the number of hydroxyl groups in the molecule.

in common with fast atom bombardment ionization (FAB-MS) of bile acids (3, 4), relatively little fragmentation is generated by this soft-ionization process. Unlike FAB-MS, however, useful fragments do arise in this thermospray process that result from the consecutive losses of H_2O [– 18 Da] from the pseudo-molecular ion and this provides a useful means of confirming the number of hydroxyl groups in the molecule. The ionization process thus provides limited structural information that may be of value in either resolving bile acids of differing structure that co-elute (see below) or identifying interfering compounds in chromatographic profiles obtained from biological samples.

Repetitive scanning over a large mass range has limited sensitivity. A significant improvement in sensitivity is possible, however, by the continuous monitoring of selected ions that are specific for each bile acid conjugate. For all of the bile acid conjugates thus far studied most of the ionization resides in relatively few ions, usually the $[M-H]^-$ or $[M-H-H_2O]^-$ fragments. Therefore, all of the principal glycine and taurine conjugates of mono-, di-, and trihydroxy bile acids can be measured simultaneously with high sensitivity by monitoring the *m/z* 432, 448, 464, 482, 498, 514. The presence of a double bond or the conversion of a hydroxy group to an oxo group would alter these masses by 2.

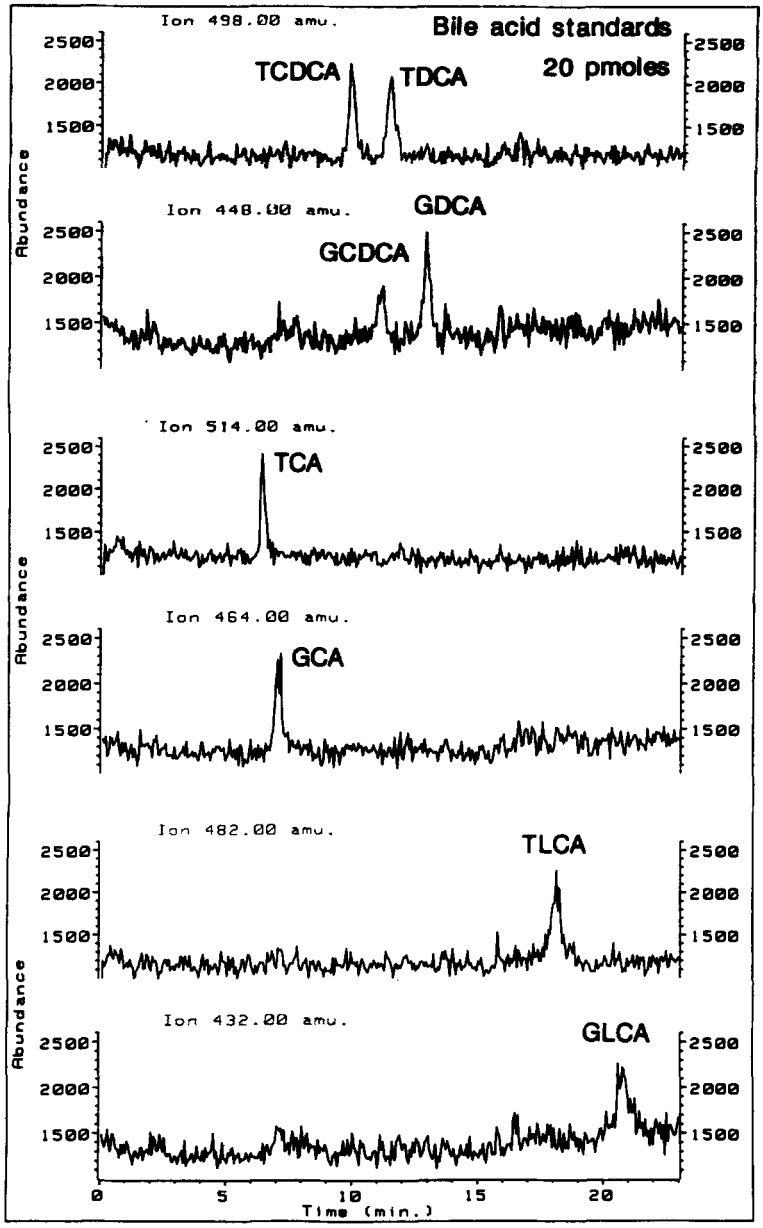

Fig. 4. Ion current chromatograms obtained for the $[M-H]^-$ ions specific for glycine- and taurine-conjugated monohydroxy-, dihydroxy-, and trihydroxy bile acids after analysis by filament-on thermospray LC-MS of a mixture of eight bile acid conjugates (approximately 20 pmol of each bile acid conjugate was injected on-column).

Fig. 4 shows the selected ion current chromatograms obtained from the LC-MS analysis of eight bile conjugates when 10 ng (approx. 20 pmol) of each bile acid is injected on-column. The limit of sensitivity measured at a signal/noise ratio of 3/1 for these bile acid conjugates ranged from 4 to 10 ng (10–20 pmol) on column (**Table 1**). The detection of bile acids at this level would be impossible using conventional UV detection where limits of detection are of the order of 10 nmol.

A linear response between peak area and amount of bile acid injected on-column was obtained in both repetitive scanning operation (by measurement of the peak areas of the total ion current chromatogram) or using selected ion monitoring of the individual masses corresponding to the pseudo-molecular ions. While satisfactory quantification is achieved using this approach, the incorporation of a stable isotope-labeled bile acid conjugate (differing in mass) would be more desirable, but such compounds are not presently commercially available.

Applications of thermospray LC-MS to biological samples

Biliary bile acid analysis. Biliary bile acids can be readily analyzed by HPLC with UV detection (See 1, 2). LC-MS analysis, however, permits useful structural information to be obtained for the eluting components that cannot be obtained by conventional UV detection. **Fig. 5** shows the total ion current (TIC) chromatograms obtained by thermospray LC-MS analysis of a sample of human bile from a patient with gallstones undergoing oral bile acid therapy with ursodeoxycholic acid. Negative ion mass spectra were recorded by repetitive scanning (400–550 daltons) of the eluent. Plots of selected ions corresponding to the pseudo-molecular ions $[M-H]^-$ for the principal glyco- and tauro-conjugates of monohydroxy-, dihydroxy-, and trihydroxy bile acids enable individual bile acids to be distinguished and structures verified from their mass spectra and retention indices. After therapy with UDCA, the major biliary bile acids are the glycine and taurine conjugates of UDCA. In this chromatographic system these two conjugates have short elution times and consequently do not separate. Since these bile acid conjugates differ in molecular weight, computer reconstruction of specific ions, *m/z* 498 (tauroursodeoxycholic acid) and *m/z* 448 (glycoursodeoxycholic acid), permits their resolution and recognition of other bile acids with related structure.

The composition of pig bile is markedly different from that of human bile. Bile acids of this species are predominately conjugated with glycine and extensively 6α-hydroxylated, so that hyodeoxycholic, hyocholic, and 6-oxo-lithocholic acids are the major biliary bile acids of this species. The thermospray LC-MS total ion current chromatogram (**Fig. 6**) indicates a relatively simple mixture of a few components. Computer-reconstructed ion current chromatograms of ions specific for individual bile acid conjugates aided by retention indices permit the resolution and identification of multiple co-eluting components and therefore, despite inadequate chromatographic separation, important information regarding bile acid composition can be obtained. The highly polar compounds eluting rapidly from the column and appearing as a doublet peak between 2 and 3 min can be readily distinguished from the individual chromatograms of the $[M-H]^-$ ions to account for five different co-eluting bile acid conjugates: glycohyocholic (*m/z* 464), taurohyodeoxycholic (*m/z* 498), taurohyocholic (*m/z* 514), glyco-6-oxolithocholic acid (*m/z* 446), and glycohyodeoxycholic (*m/z* 448) acids. **Fig. 7** serves to point out that even a small amount of a co-eluting compound such as taurohyodeoxycholic acid (*m/z* 498) can be readily detected despite the presence of large amounts of other bile acids. In fact taurine-conjugated bile acids, although present, were relatively minor components of pig bile. This type of information is impossible to obtain by conventional HPLC analysis and these examples serve to highlight the dangers of assuming that the appearance of single peaks in HPLC

TABLE 1. Detection limits for various bile acid conjugates analyzed by filament-on thermospray LC-MS

Bile Acid	M_r	$[M-H]^-$ Ion	Retention Time	Detection Limit[a]
			min	
Taurocholic acid (TCA)	515	514	4.35	4.6 ng (8.9 pmol)
Glycocholic acid (GCA)	465	464	7.00	6.0 ng (12.9 pmol)
Taurochenodeoxycholic acid (TCDCA)	499	498	9.81	5.3 ng (10.6 pmol)
Glycochenodeoxycholic acid (GCDCA)	449	448	11.25	10.0 ng (22.0 pmol)
Taurodeoxycholic acid (TDCA)	499	498	11.41	6.0 ng (12.0 pmol)
Glycodeoxycholic acid (GDCA)	449	448	12.06	4.3 ng (9.6 pmol)
Taurolithocholic acid (TLCA)	483	482	18.15	8.2 ng (17.0 pmol)
Glycolithocholic acid (GLCA)	433	432	20.81	10.0 ng (23.0 pmol)

[a]Signal/noise was calculated from manually measured peak heights and detection limits were estimated at a signal/noise ratio of 3:1.

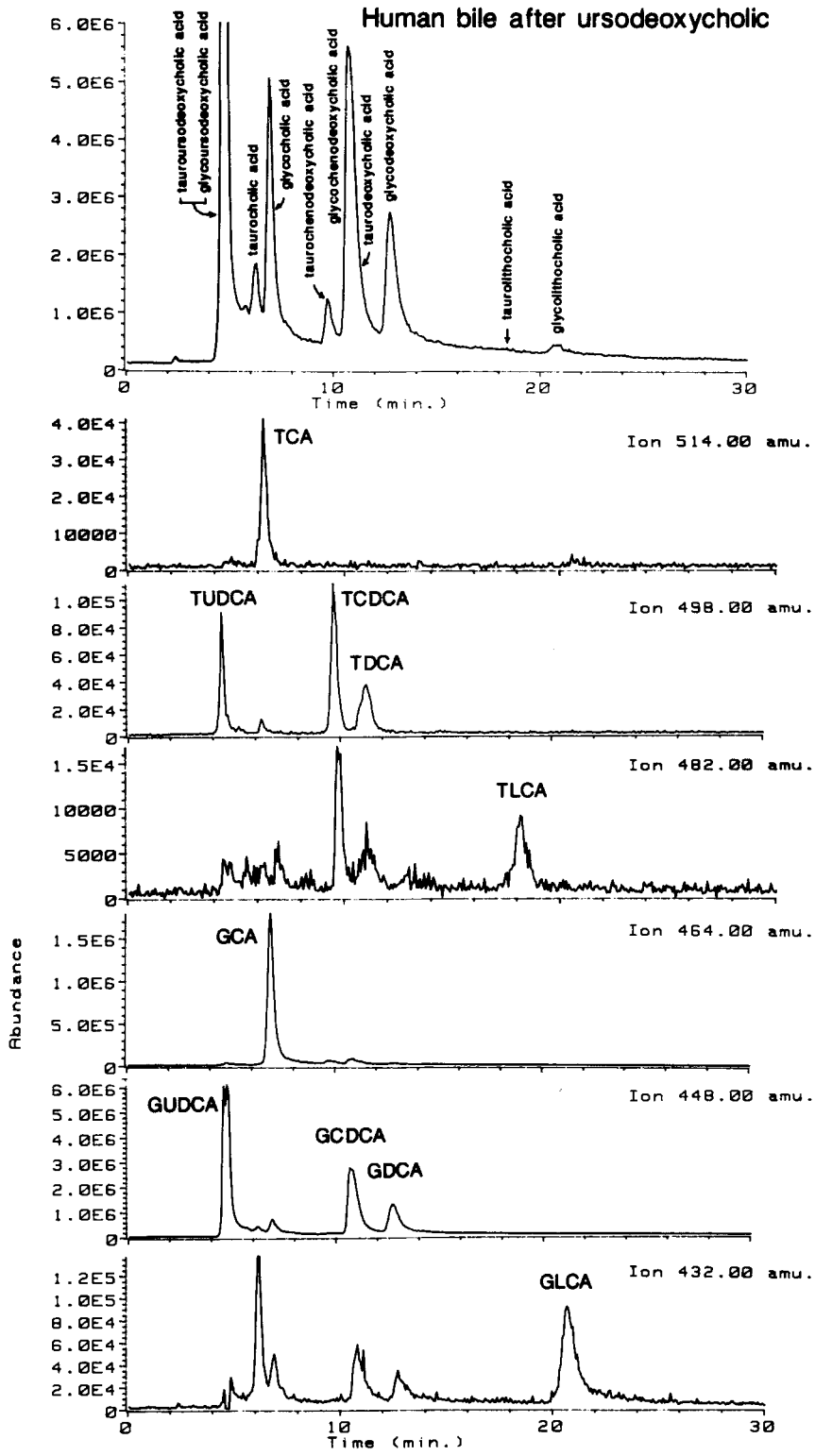

Fig. 5. Thermospray LC-MS analysis of the biliary bile acids from a patient with gallstones after oral bile acid therapy with ursodeoxycholic acid. Thermospray LC-MS was carried out in filament-on mode and negative ions were recorded by repetitive scanning over the mass range *m/z* 400–550 during continual elution of components from the HPLC column. LC-MS conditions were as described in Fig. 2. Total ion current chromatograms are shown in the top panel and computer reconstructed ion current chromatograms (normalized) corresponding to specific $[M-H]^-$ ions for glyco- and tauro-conjugates permit the identification of individual bile acid conjugates as indicated by abbreviations. For comparison of the relative amounts of each bile acid present, allowance must be made for the differences in ion abundances of the various channels. These profiles were obtained from the analysis of 1 μl of bile injected on-column.

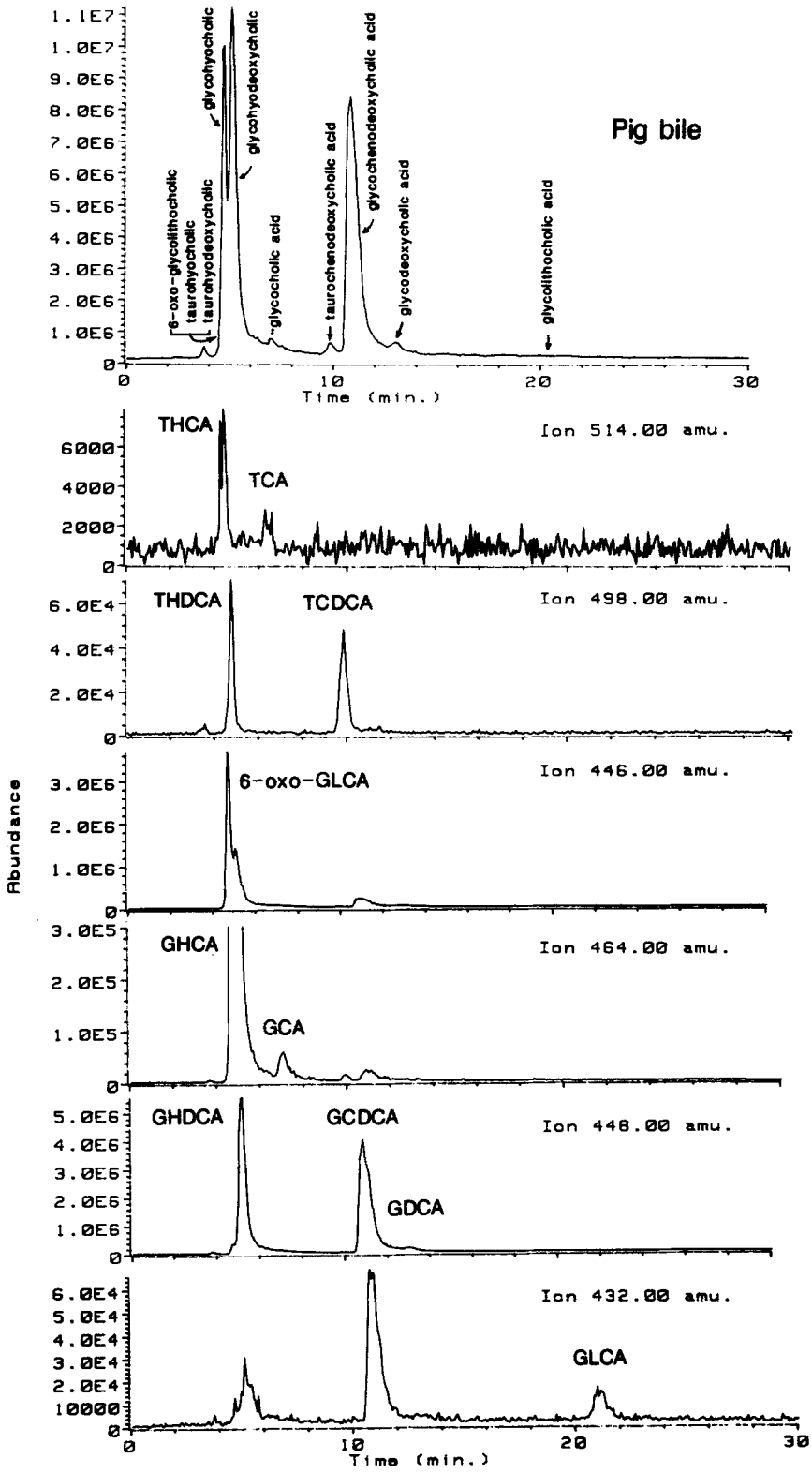

Fig. 6. Thermospray LC-MS analysis of the biliary bile acids from 1 μl of pig bile. Thermospray LC-MS was carried out in filament-on mode and negative ions were recorded by repetitive scanning over the mass range *m/z* 400-550 during continual elution of components from the HPLC column. LC-MS conditions were as described in Fig. 2. Total ion current chromatograms are shown in the top panel and computer reconstructed ion current chromatograms (normalized) corresponding to specific $[M-H]^-$ ions for glyco- and tauro-conjugates permit the identification of individual bile acid conjugates as indicated by abbreviations. For comparison of the relative amounts of each bile acid present, allowance must be made for the differences in ion abundances of the various channels.

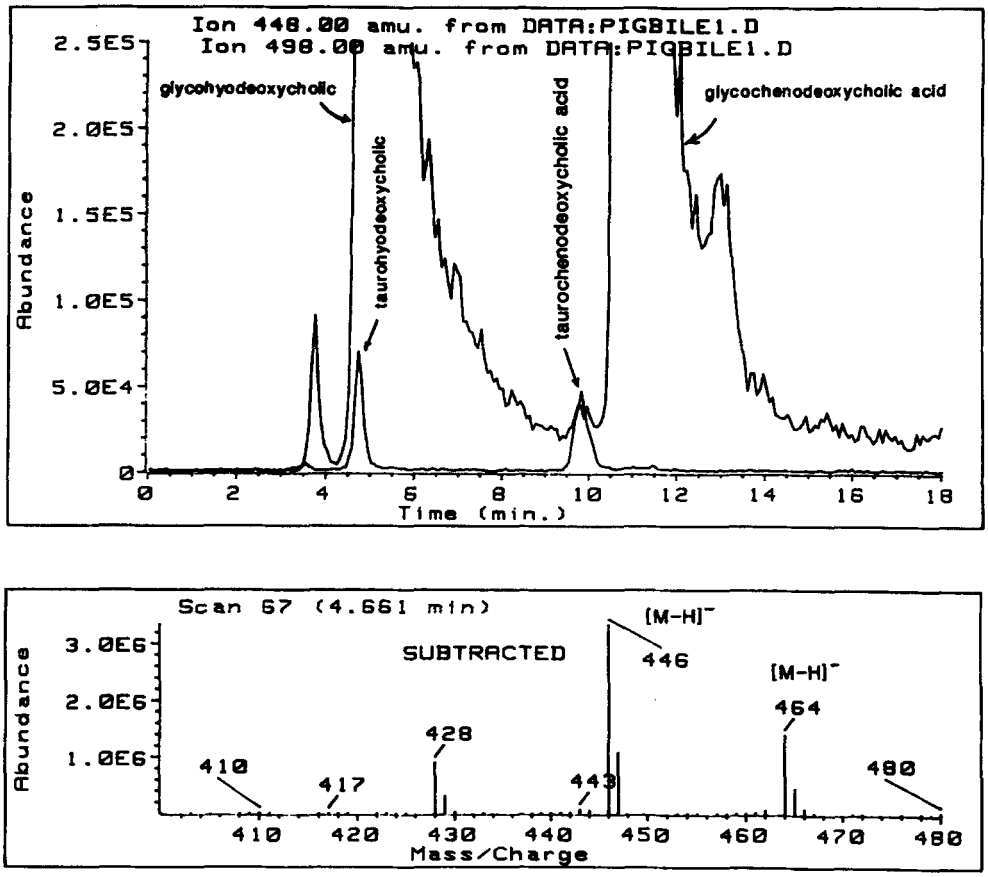

Fig. 7. Reconstructed ion current chromatograms of the ions *m/z* 448 (specific for glyco-dihydroxy bile acids) and *m/z* 498 (specific for tauro-dihydroxy bile acids) after repetitive scanning thermospray LC-MS analysis of a sample of pig bile (1 μl injected on-column). The major peaks (*m/z* 448) arise from the presence of glycochenodeoxycholic acid (retention time 12 min) and glycohyodeoxycholic acid (retention time approx. 4.6 min). However, this latter peak also contains 6-oxo-glycolithocholic acid (*m/z* 446), that co-elutes with the same retention time and this is confirmed by the mass spectrum (scan 67, below) which is a mixture of glycohyodeoxycholic acid ($[M-H]^-$ = 448) and 6-oxo-glycolithocholic acid ($[M-H]^-$ = 446). Computer reconstruction of *m/z* 498 permits the detection of small amounts of taurohyodeoxycholic acid even in the presence of large amounts of other bile acids, while taurodeoxycholic acid is evident from the peak eluting at approx. 9.8 min.

chromatograms reflects homogeneity in composition. With the relatively poor resolving power of conventional HPLC columns, it is not surprising to obtain significant overlap of bile acid conjugates, but despite this problem the combined HPLC-MS technique can serve to provide increased confidence in compound identity.

Serum bile acids. Analysis of bile acids in serum using HPLC is particularly challenging, because of the relatively low concentrations. Selected ion monitoring thermospray LC-MS of the pseudo-molecular ions of bile acid conjugates yields sufficient sensitivity (Table 1) to permit the determination of the principal bile acid conjugates in relatively small volumes of serum. **Fig. 8** shown the thermospray LC-MS analyses obtained for serum samples from two patients with cholestatic liver disease when the equivalent of only 50 μl of serum was injected on-column. These analyses were carried out after a simple and rapid liquid-solid extraction on a Bond-Elut C_{18} cartridge (6) and reconstitution of the extract in the mobile phase. Selected ion monitoring of the ions *m/z* 432 (glyco-monohydroxy), *m/z* 448 (glyco-dihydroxy), *m/z* 464 (glyco-trihydroxy), *m/z* 482 (tauro-monohydroxy), *m/z* 498 (tauro-dihydroxy), and *m/z* 514 (tauro-trihydroxy) enables the detection of the principal bile acid conjugates in these samples of serum. Concentrations were determined from the peak area responses for each bile acid and values obtained for glycocholic acid compared favorably with the values given by a specific radioimmunoassay (Fig. 8).

To conclude, while there has been an explosive increase in recent years in the use of the thermospray ionization in-

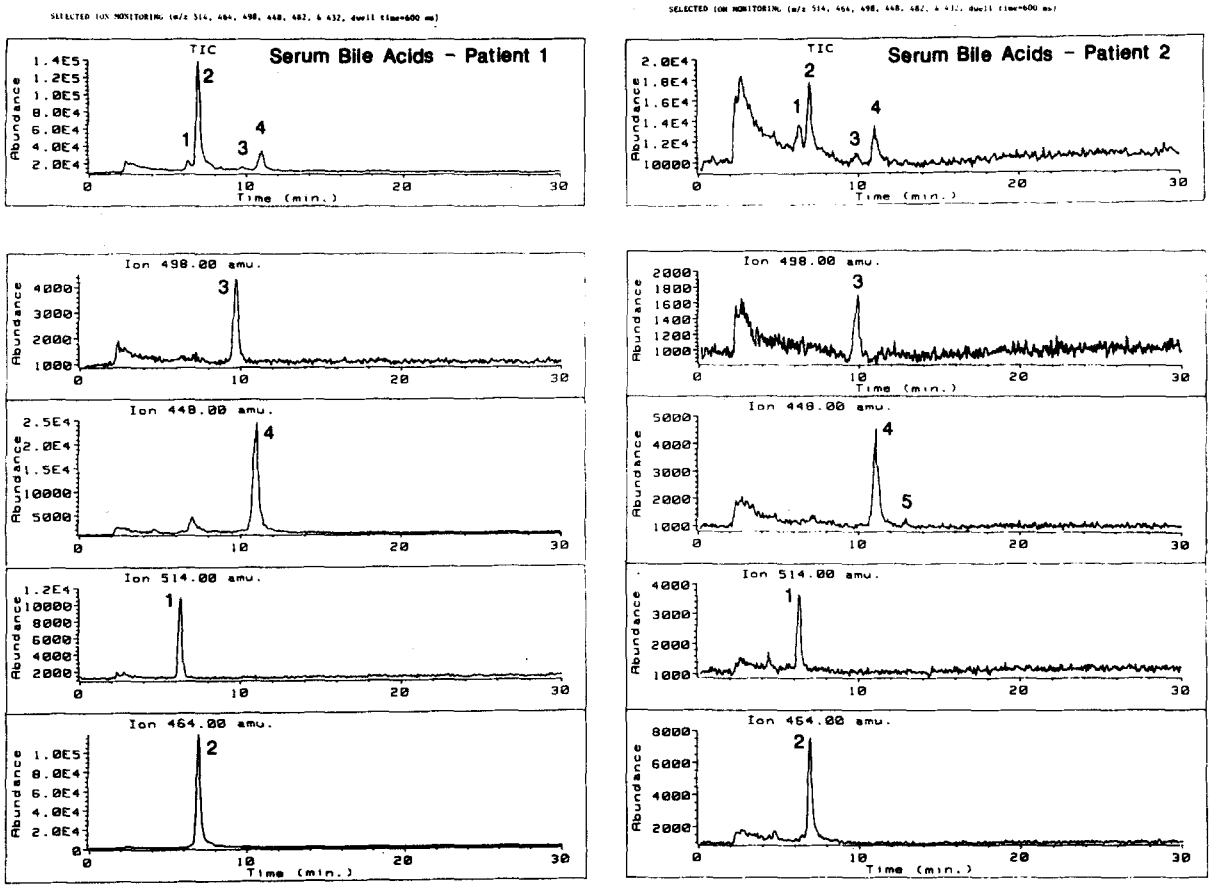

Fig. 8. Thermospray LC-MS analyses of the serum bile acids of two patients with cholestatic liver disease. Selected ion monitoring of the $[M-H]^-$ ions *m/z* 432, 448, 464, 482, 498, and 514 (dwell time 600 msec) was carried out and the summed ion current chromatogram (upper panel) and individual ion current chromatograms are plotted. LC-MS conditions were exactly as indicated in Fig. 2. Bile acids were first extracted from serum using a Bond-Elut cartrdige and the extract was dissolved in mobile phase. These chromatograms were obtained from the analysis of 50 μl of serum injected on-column. Bile acid concentrations of the principal conjugates were determined for the peak area responses of the respective $[M-H]^-$ ions relative to a series of calibration standards. The following concentrations were obtained:

Bile Acid	Patient S1	Patient S2
	μmol/l	
Glycocholic acid (GCA)	8.2	36.70
Taurocholic acid (TCA)	2.76	2.44
Glycochenodeoxycholic (GCDCA)	6.23	9.86
Taurochenodeoxycholic (TCDCA)	1.03	1.06
Glycodeoxycholic (GDCA)	0.37	
(Cholyglycine concentration by RIA)	18.2	50.0

terface for the mass spectrometric analysis of polar molecules, data presented here represent the first demonstration of the applicability of the thermospray LC-MS technique to the qualitative and quantitative analysis of bile acids in biological fluids. Thermospray LC-MS is clearly a useful and rapid technique for the determination of unconjugated bile acids and their glycine and taurine conjugates, but conditions for the analysis of the doubly charged bile acid sulfates remain to be established. The examples shown here serve to highlight the potential of this technique for extending the scope of HPLC for the analysis of bile acids.

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