papers and notes on methodology

Determination of free and amidated bile acids by high-performance liquid chromatography with evaporative light-scattering mass detection

Aldo Roda,[1],* Carolina Cerrè,† Patrizia Simoni,† Carla Polimeni,† Chiara Vaccari,† and Antonio Pistillo†

Dipartimento di Scienze Farmaceutiche,* Istituto di Clinica Medica,† Cattedra di Gastroenterologia, Università di Bologna, Italy

Abstract A simple reverse phase high-performance liquid chromatographic method for a simultaneous analysis of free, glycine- and taurine-amidated bile acids is described. The resolution of ursodeoxycholic, cholic, chenodeocycholic, deoxycholic, and lithocholic acids, either free or amidated with glycine and taurine, is achieved using a C-18 octadecylsilane column (30 cm length, 4 μm particle size) with a gradient elution of aqueous methanol (65→75%) containing 15 mM ammonium acetate, pH 5.40, at 37°C. The separated bile acids are detected with a new evaporative light-scattering mass detector and by absorbance at 200 nm. A complete resolution of the 16 bile acids, including the internal standard nor-deoxycholic acid, is obtained within 55 min. Using the light-scattering mass detector, amidated bile acids and, for the first time, free bile acids can be detected with similar detection limits in the order of 2-7 nmol. The new detector improves the baseline and the signal-to-noise ratio over the UV detection as it is not affected by impurities present in the samples with higher molar absorptivity than bile acids or by the change in the mobile phase composition during the gradient. The method fulfills all the standard requirements of precision and accuracy and the linearity of the mass detector is over 5 decade the detection limit. The new method has been used for the direct analysis of bile acid in stools and bile with only a preliminary clean-up procedure using a C-18 reverse phase extraction.—**Roda, A., C. Cerrè, P. Simoni, C. Polimeni, C. Vaccari, and A. Pistillo.** Determination of free and amidated bile acids by high-performance liquid chromatography with evaporative light-scattering mass detection. *J. Lipid Res.* 1992. **33:** 1393-1402.

Supplementary key words bile acid analysis in bile and stools • light-scattering mass detector

Bile acids (BA) are present in humans as a complex qualitative composition that includes primary BA, i.e., cholic and chenodeoxycholic acids, and secondary BA, i.e., deoxycholic acid and lithocholic acid either free or amidated with glycine and taurine. Other minor BA, such as ursodeoxycholic acid, and BA conjugates, such as glucuronides and sulfate esters, are present in some biological fluids and in some particular disease.

High-performance liquid chromatography (HPLC) has been widely used in BA analysis and its main advantage over other chromatographic procedures, such as, gas-liquid chromatography is its simplicity since some classes of BA (glycine and taurine amidates) can be directly analyzed without preliminary derivatization using conventional UV detectors (1-5).

However, the analytical performance of the direct HPLC analysis of BA has been limited up to now by the detector's low sensitivity and, therefore, a pre-derivatization procedure is required to increase sensitivity (6-11).

The separation of BA can be easily and efficiently achieved using reverse phase C-18 column inasmuch as they differ in lipophilicity and acidity and many methods have been developed so far (12-16).

The main problem is still detection since conventional detectors, including the refractive index and UV spectrophotometer, lack sensitivity.

UV detection of BA at 200-210 nm is limited by the poor absorptivity of BA at that wavelength, and this is particularly true for unconjugated BA whose absorbance is 20-30 times less than amidated BA (17). Impurities and other matrix constituents with high absorptivity at 200 nm greatly affect the identification of the eluted BA, generating unidentified overestimated peaks even when present only in trace amounts.

More recently, other methods have been described and developed that are based on the use of a specific post-

Abbreviations: BA, bile acids; HPLC, high-performance liquid chromatography; ELSD II, evaporative light-scattering mass detector; UV, ultraviolet

[1]To whom reprint requests should be addressed at: Dipartimento di Scienze Farmaceutiche, Università degli Studi di Bologna, Via Belmeloro, 6, 40100 Bologna, Italy.

column detector formed by a BA-specific enzyme (3α-hydroxy steroid dehydrogenase) immobilized on a column and measuring the NADH formed fluorimetrically by bioluminescence (18-20).

The main drawback of the above-reported methods is that they are time-consuming and additional steps are necessary that render the procedure not easily controllable.

In order to simultaneously determine all the 15 naturally occurring BA in humans, we report the use of a new evaporative light-scattering mass detector (ELSD II) (21, 22). With the ELSD II the effluent from the column enters a nebulizer and is converted to a fine mist by a stream of nebulizing carrier gas (nitrogen). The fine droplets are then carried through a temperature-controlled tube in which the mobile phase (volatile ammonium acetate buffer) evaporates. The nonvolatile BA then pass through a laser beam causing light scattering that is detected by a photodiode. The measured light is proportional to the amount of sample in the light-scattering chamber, and the signal is indicative of molecular size and shape but not the chemical identity of the BA passing through the beam.

Our main objective was to derive a method that could detect not only amidated BA but also free BA with a similar detector response and to apply this method for the analysis of BA in bile and stools.

Since this mass detector is connected in series with a conventional UV detector, comparative studies in terms of detection limit and overall analytical performance will be reported.

The application of the developed method for BA in the above reported biological fluids and its potential use for other fluids such as serum and urine will be also discussed.

MATERIAL AND METHODS

All chemicals and solvents were of analytical grade unless otherwise noted.

HPLC-grade methanol, acetic acid, and ammonium hydroxide were purchased from Merck, D6100, Darmstadt, Germany.

Glycine- and taurine-amidated and unconjugated BA were purchased from Sigma (St. Louis, MO). Ursodeoxycholic acid was a gift from the Giuliani SpA, Milan and nordeoxycholic acid was purchased from Steraloids Inc. (Wilton, NH).

The commercial available standards have been purified by thin-layer chromatography and dried under vacuum.

Bile and stool specimens were obtained from healthy volunteers in our laboratory and from patients with cholesterol gallstones treated with ursodeoxycholic acid and with BA malabsorption (S. Orsola University Hospital, Bologna, Italy).

A Waters 600E multisolvent delivery system high-performance liquid chromatograph equipped with a U6K Waters sample injector was used. The apparatus was connected with a Waters 484 absorbance detector and in series with an evaporative light-scattering detector ELSD II, Varex Corporation, Burtonsville, MD. The signal was recorded using a Waters 746 data module.

A Nova-Pak C-18 Waters steel column was used (3.9 mm × 300 mm); particle size was 4 μm. The column temperature was kept at 37 ± 0.2°C using a Waters TCM thermostat.

The evaporative light-scattering detector requires the use of a volatile buffer and in the gradient system the ionic strength of the mobile phase must be kept constant.

Simultaneous analysis of amidated and unconjugated BA

For the separation of glycine- and taurine-amidated and unconjugated BA, a gradient system was used. The flow rate was kept constant at 0.9 ml/min. The initial mobile phase composition was 65% (v/v) aqueous methanol containing 15 mM ammonium acetate (solvent A) with an apparent pH of 5.40 ± 0.1. The percentage of methanol was increased to 75% (v/v) keeping constant the pH and the content of ammonium acetate (solvent B). The run was performed with the following gradient program: 15 min isocratic with solvent A; a convex gradient composition from A to B for 35 min; and isocratic for 20 additional min with the solvent B. This configuration allowed efficient separation in 55 min of the standard BA in the following order: **1,** tauroursodeoxycholic acid; **2,** glycoursodeoxycholic acid; **3,** taurocholic acid; **4,** glycocholic acid; **5,** taurochenodeodeoxycholic acid; **6,** taurodeoxycholic acid; **7,** glycochenodeoxycholic acid; **8,** ursodeoxycholic acid; **9,** glycodeoxycholic acid; **10,** taurolithocholic acid; **11,** cholic acid; **12,** glycolithocholic acid; **13,** nor-deoxycholic acid; **14,** chenodeoxycholic acid; **15,** deoxycholic acid; **16,** lithocholic acid (see Fig. 1).

For the analysis of only amidated BA, solvent A can be used under isocratic conditions and in the same way unconjugated BA can be efficiently separated using solvent B (see Fig. 2A, B). Both systems allow a good resolution to be achieved within 40 min.

According to the particular analytical needs, which depend on the expected composition of the particular biological fluids, one of the three described systems can be used.

In both gradient and isocratic conditions the ELSD II detector was set up as follows: nitrogen carrier gas flow, 40 PSI; drift tube temperature, 130°C; exhaust gas temperature, 85°C. Nordeoxycholic acid was used as internal standard.

For comparative studies, isocratic runs with the same analytical conditions using a 15 mM sodium phosphate buffer instead of the ammonium acetate were performed using UV detection at 200 nm. Amidated BA were separated using solvent A (isocratic) and free BA using solvent B (isocratic).

Bile acid analysis in bile

Since the BA in bile are mainly amidated with glycine and taurine, the system can be simplified using only solvent A in an isocratic mode. A complete resolution of all natural occurring BA in human bile, including amidated ursodeoxycholic acid, was obtained within 35 min. The bile samples were submitted to a preliminary clean-up procedure using conventional C-18 reverse phase extraction (C-18 Bond Elut, Analytichem International, Harbor City, CA) (23). In particular, 2 ml of NaOH 0.1 M was added to 10–100 μl of bile and the solution was applied to the column previously activated with methanol (4 ml) and washed with water.

The retained BA were eluted with 4 ml of methanol after a washing with water. The eluted methanolic solution was dried under vacuum and reconstituted with 50–500 μl of methanol according to the expected BA concentration. Varying amounts of this sample (2–10 μl) were then injected in the chromatograph.

Bile samples, and particularly gallbladder bile, can be directly analyzed without any pretreatment.

The complete system can be used if we expect the presence in bile of detectable amounts of unconjugated BA under particular pathological conditions.

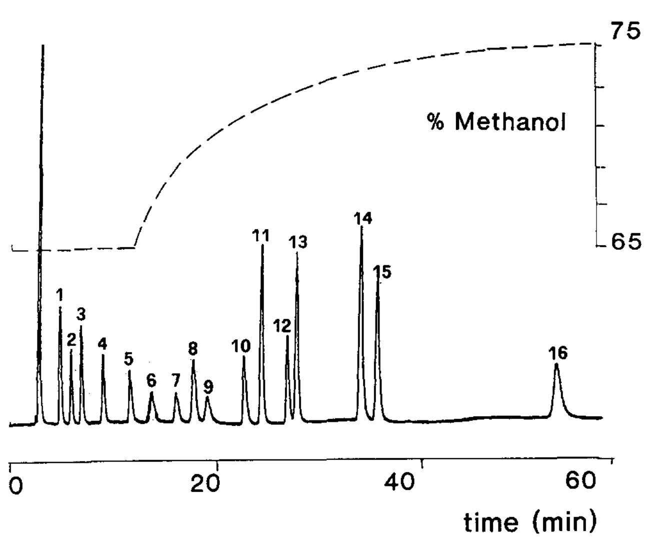

Fig. 1. Separation of glycine, taurine, and unconjugated BA using the gradient system and the ELSD II detector. The internal standard is nordeoxycholic acid. For the analytical conditions see text; 1, tauroursodeoxycholic acid; 2, glycoursodeoxycholic acid; 3, taurocholic acid; 4, glycocholic acid; 5, 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; 14, chenodeoxycholic acid; 15, deoxycholic acid; 16, lithocholic acid.

TABLE 1. Retention time (min) of the glycine- and taurine-conjugated and unconjugated bile acids using the gradient system and the ELSD II detector

Bile Acid	Unconj.	Glycine-Conj.	Taurine-Conj.
LCA	50.4	25.6	21.6
CDCA	32.4	15.3	11.0
DCA	33.9	18.2	12.9
UDCA	17.0	5.6	4.6
CA	23.2	8.6	6.6
norDCA	26.4		

Abbreviations: LCA, lithocholic acid; CDCA, chenodeoxycholic acid; DCA, deoxycholic acid; UDCA, ursodeoxycholic acid; CA, cholic acid; norDCA, nordeoxycholic acid.

Bile acid analysis in stools

BA are present in stools mainly as unconjugated BA and for this reason we used only solvent B in the isocratic mode. The stools were homogenized with 0.02 M phosphate buffer, pH 8, in a v/v ratio to achieve a relatively dense suspension. An aliquot of 2 ml was collected under agitation and 2 ml of NaOH 0.1 M was added. The sample was then centrifuged at 3500 rpm for 5 min and 3 ml of the supernatant was applied to the C-18 Bond Elut cartridge (previously activated) and eluted. The column was washed twice with water and then with acetone and the retained BA were eluted with 4 ml of methanol. The samples were dried and reconstituted with 400 μl of methanol, filtered through a 0.45 μm Millipore membrane, and 2- to 10-μl samples were injected in the chromatograph.

Recovery

Known amounts of glycocholic acid and chenodeoxycholic acid were added to bile and stool homogenates and the samples were submitted to the above-reported clean-up procedures. The analytical recoveries of the exogenous compounds were calculated.

RESULTS

Bile acid separation

The developed method allowed separation within 55 min of all the 15 more common BA present in human biological specimens either amidated or free, including the synthetic BA internal standard nordeoxycholic acid. An efficient separation was obtained within 35 min with the exception of lithocholic acid which had a retention time slightly higher, in the order of 50 min. A typical chromatogram obtained by injecting 5 μl of a mixture of the BA solution is reported in **Fig. 1.** The absolute amount injected for each BA was in the order of 4–6 nmol. The absolute areas were corrected according to the amount of each BA injected. The retention times are listed in **Table 1.**

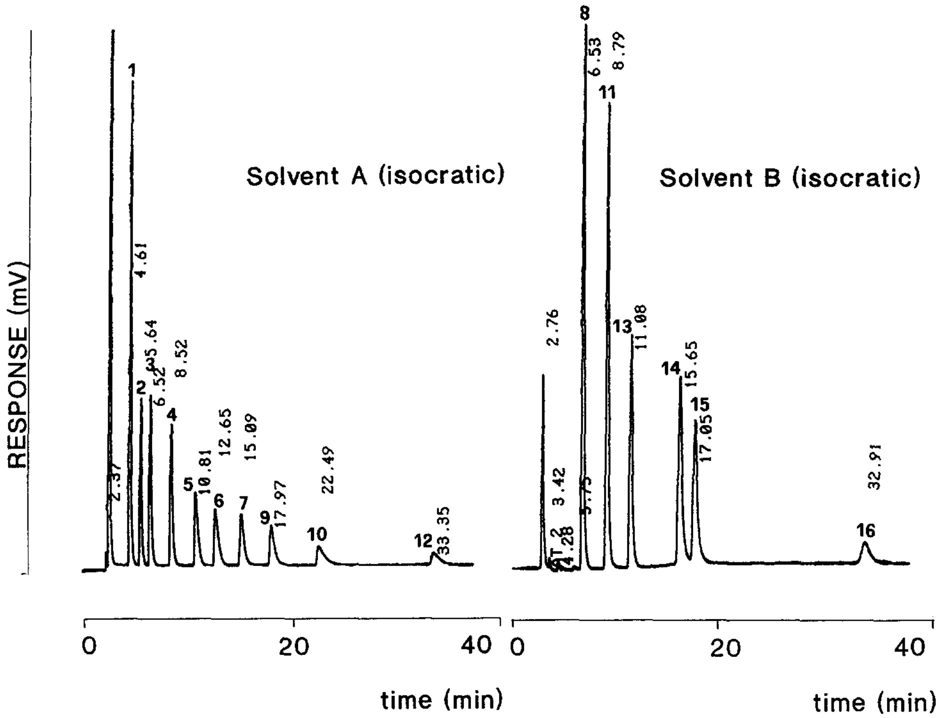

Fig. 2. Typical chromatograms of BA standards obtained using ELSD II detector. Left: separation of amidated BA using solvent A in isocratic mode. Right: separation of unconjugated BA using solvent B in isocratic mode. For the analytical conditions see text.

The efficient resolution of all the different classes of BA was achieved by an optimization of the pH of the mobile phase, percentage of methanol, ionic strength, flow rate, and column temperature.

The optimal analytical conditions, which account for differences in the pKa, lipophilicity, and polarity of the eluted BA, are as reported in the Material and Methods section. An apparent pH of 5.40 for the mobile phase ensured a resolution of glycine and taurine BA since the former are partially protonated at this pH.

The convex gradient program used allows a reduction of the retention time and an increased sharpness of free BA, which at the pH of the mobile phase are partially protonated and consequently more lipophilic than amidated BA. Unconjugated BA are eluted after the taurine and glycine BA with the exception of ursodeoxycholic acid which is eluted between glycochenodeoxycholic acid and glycodeoxycholic acid.

The ionic strength of the mobile phase plays an important role in determining the retention time and the resolution of the studied BA. We added ammonium acetate to the mobile phase since the mass detector requires a volatile buffer and its concentration in both solvent A and B must be kept constant at 15 mM levels.

TABLE 2. Detection limit (nmol/5 μl injected) of the evaporative light-scattering mass detector (ELSD II) and the UV detector at 200 mm for glycine- and taurine-conjugated and unconjugated bile acid analysis

Bile	Unconjugated			Glycine-Conjugated			Taurine-Conjugated		
Acid	ELSD	UV_1^a	UV_2^b	ELSD	UV_1^a	UV_2^b	ELSD	UV_1^a	UV_2^b
LCA	7	280	250	4	6	5	3	6	4
CDCA	5	250	220	2	5	4	4	5	4
DCA	5	250	220	2	5	4	3	5	3
UDCA	5	250	230	2	4	4	3	5	4
CA	2	220	200	2	5	3	3	5	4
UCA	2	220	210	2	5	2	3	5	3
norDCA	5	250	220	2	5	4	3	5	4

Abbreviations: LCA, lithocholic acid; CDCA, chenodeoxycholic acid; DCA, deoxycholic acid; UDCA, ursodeoxycholic acid; CA, cholic acid; UCA, ursocholic acid; norDCA, nordeoxycholic acid.

[a] UV_1, values obtained with the UV detector in series with the ELSD II using 15 mM ammonium acetate buffer.

[b] UV_2, values obtained with the UV detector using 15 mM sodium phosphate buffer.

By lowering the concentration of buffer, the retention times of amidated BA were shortened and a poor resolution was obtained, as a result of a reduced polarity of the mobile phase.

On the other hand the presence of ammonium acetate in the mobile phase increased the absorptivity of this solution at 200 nm, thus rendering the detection of BA using the UV detector slightly less sensitive. The best performance of the UV detector was obtained using a mobile phase containing conventional buffers such as sodium phosphate, which in turn cannot be used with the ELSD II detector.

The temperature is also important since the solubility of some ionized BA, such as lithocholate or glycolithocholate, in the mobile phase, is poor and increases with temperature. Moreover, a precise control of the temperature (37 ± 0.1 °C) improves the precision of the analysis, and a 10–15% reduction of the working pressure can be obtained (3800 PSI).

The intraassay and day-to-day variation in the retention time over a 1-month period was less than 2% for all the BA that were analyzed.

According to the expected composition of the biological fluids under study, the system can be used in isocratic condition. Using only solvent A, a good separation of amidated BA is achieved within 35 min while unconjugated BA can be efficiently separated with solvent B within 35 min (**Fig. 2**).

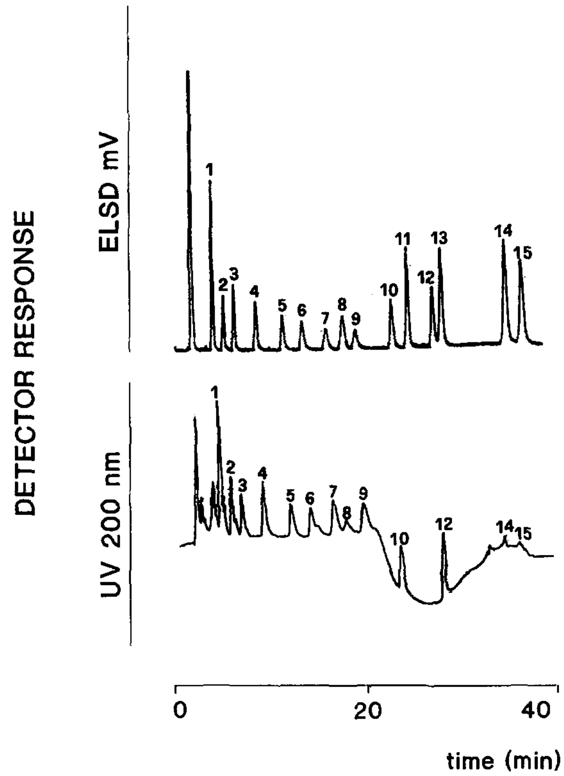

Fig. 4. Effect of the gradient system on the baseline using the ELSD II (upper panel) and UV detector (lower panel). A lower detector response for unconjugated BA using the UV detector is shown. The analysis was carried out by injecting 5 nmol of each BA.

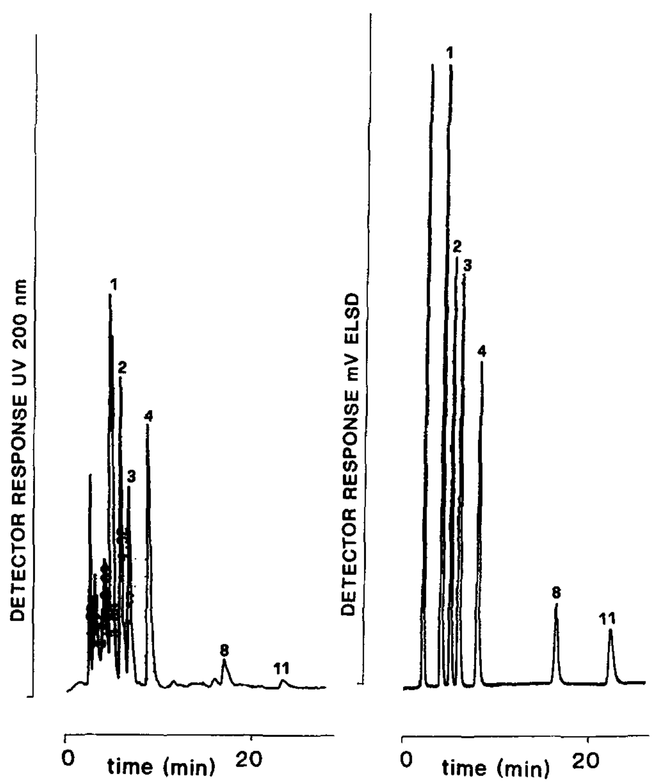

Fig. 3. Example of the different sensitivity of the UV detector (left) and ELSD II detector (right) for unconjugated BA in respect to amidated BA. The chromatogram was recorded by using 5 nmol of each amidated and unconjugated BA and using the ELSD II detector in series with the UV detector.

Bile acid detection

The new evaporative light-scattering mass detector ELSD II used in the present work represents an important improvement in BA analysis over conventional UV or refractive index detectors.

Unconjugated and glycine- and taurine-amidated BA can be detected with similar detection limits as reported in **Table 2**, which shows the detection limits using the ELSD II detector in comparison with a UV detector at 200 nm.

The ELSD II was connected in series with the UV detector so the results were derived from a single run. The detection limit of the ELSD II detector was similar for all BA studied and was in the order of 2–7 nmol for either free or amidated BA (Table 2). With the UV detector the detection limit of amidated BA was only slightly higher: 4–6 nmol. Free BA had a detection limit 30–50 times higher in the order of 220–280 nmol (Table 2) which was greatly improved using the ELSD II detector (2–7 nmol). A typical separation of some BA recorded simultaneously with the ELSD II and UV detectors is reported in **Fig. 3.** The chromatograms were recorded with the same signal output and under the same analytical conditions; only cholic and ursodeoxycholic acids, free, glycine- and taurine-amidated are shown. When a mobile phase containing sodium phosphate instead of ammonium acetate was used, the detection limit was slightly improved (2–5 nmol for amidated BA and 200–250 nmol for free BA) (Table 2).

The chromatogram obtained using the ELSD II detector shows an improvement not only in the detection limit for unconjugated BA, but also in signal-to-noise ratio and in the baseline output.

The gradient system that was used, which increased the percentage of methanol in the mobile phase from 65 to 75% (v/v), generated a distortion in the baseline due to the different absorbance of solvent B (more enriched in methanol). This was evident with detection at 200 nm, but did not occur using the ELSD II detector (**Fig. 4**).

Moreover, potentially present impurities in the matrix that strongly absorb at 200 nm (even 100–1000 times more than BA) can generate false peaks thus affecting the correct interpretation of the BA composition. This is particularly evident for hydrophilic compounds with low retention time (1–5 min) that are poorly retained by the column.

The method was designed for the ELSD II detector in terms of composition of the mobile phase that requires the use of a volatile buffer (ammonium acetate). In turn, this compound reduces the analytical performance of the UV detector due to its absorption at 200 nm.

The best analytical performances of the ELSD II were obtained by a correct optimization of the evaporization temperature and the carrier gas flow rate.

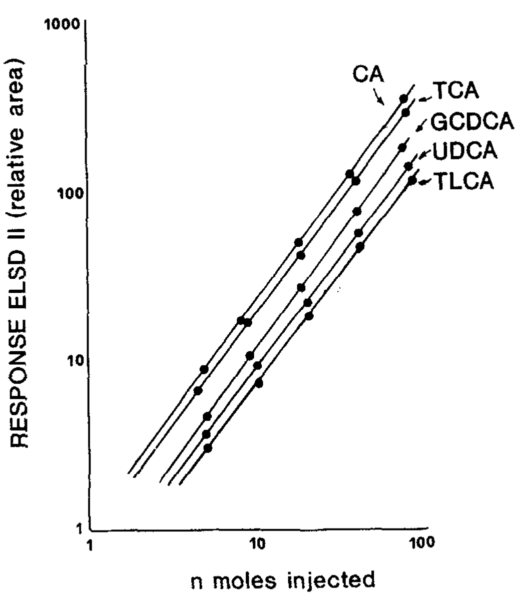

Fig. 5. Linearity of the ELSD II in terms of detector response for some amidated and unconjugated BA.

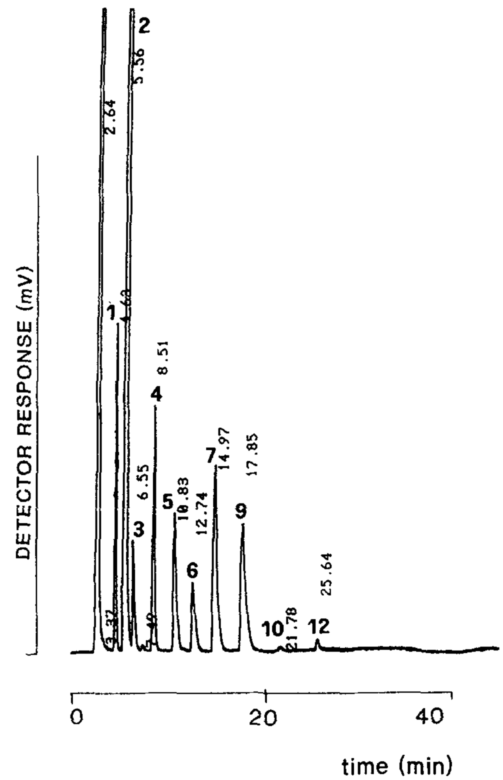

Fig. 6. Example of separation of BA in a bile sample of a cholesterol gallstone subject treated with UDCA.

The temperature must be sufficiently high to ensure complete vaporization of the volatile mobile phase and we have found that 130°C is the best compromise. The main parameter affecting the sensitivity is the gas flow rate which was set up at 40 PSI to obtain an acceptable signal-to-noise ratio achieving the highest sensitivity.

The linearity of the ELSD II detector was tested by analyzing known amounts of standard BA. The results obtained for five representative BA are reported in **Fig. 5.**

Application

Biliary bile acids. The present method can be easily used for BA analysis in bile. For this biological fluid it is appropriate to use the simplified method with solvent A in isocratic conditions. A typical chromatogram for BA analysis in a bile sample of a patient with cholesterol gallstones treated with ursodeoxycholic acid is reported in **Fig. 6.** Unconjugated BA were not present in bile.

Fecal bile acids. BA in stools are mainly unconjugated and in the healthy subject are composed mostly of secondary BA, i.e., lithocholic acid, deoxycholic acid, and with trace amounts of primary unconjugated BA (**Table 3**). In patients with BA malabsorption, high concentrations of cholic and chenodeoxycholic acid are present together with trace amounts of the same BA amidated with glycine or taurine.

TABLE 3. Fecal bile acid composition evaluated by HPLC using the gradient system in six healthy subjects and six patients with bile acid malabsorption

Code	LCA	DCA	CDCA	CA	GCDCA	TDCA	GCA	TCA	Unknown
					%				
Healthy subjects									
1	38	60							2
2	42	56		1					1
3	40	55	1	2					2
4	42	58							
5	45	50	1	1					3
6	46	45	1	1					7
Mean	42.17	54	0.5	0.83					2.5
± SD	3.0	5.5	0.5	0.75					2.4
Patients with bile acid malabsorption									
7	39	40	5	10			1	1	4
8	42	40	6	9	1		2		
9	37	32	10	14	1		3	1	2
10	35	41	7	12	1	2	1	1	
11	30	38	10	18			3		1
12	30	32	12	15	2		3		1
Mean	35.5	37.2	8.3	13.0	0.83	0.33	2.16	0.50	1.33
± SD	4.8	4.1	2.7	3.3	0.75	0.81	0.98	0.54	1.50

Abbreviations: LCA, lithocholic acid; DCA, deoxycholic acid; CDCA, chenodeoxycholic acid; CA, cholic acid; GCDCA, glycochenodeoxycholic acid; TDCA, taurodeoxycholic acid; GCA, glycocholic acid; TCA, taurocholic acid.

The analysis can be carried out on less than 1 g of stools and in **Fig. 7** a typical chromatogram recorded in a healthy subject is shown.

Recovery. The analytical recovery of glycocholic acid and chenodeoxycholic acid added to bile and stool samples is shown in **Table 4.** After addition of 100-900 nmol of glycocholic acid and chenodeoxycholic acid to aliquots of human bile, followed by the entire clean-up procedure, the average total recovery was found to be 97.9 ± 1.7 and 97.3 ± 3.8, respectively. Similar results were obtained when the stools enriched with the exogenous compound were analyzed with a recovery range of 96.9 ± 4.8 and 99.4 ± 4.2 for glycocholic and chenodeoxycholic acids, respectively.

Detector comparison

Fig. 8 shows the analysis of amidated BA in bile using the ELSD II and UV detectors. The peak areas have been corrected according to the detector response of the individual BA for each of the two detectors and for the recovery using the internal standard.

As discussed above, the analytical conditions chosen for the ELSD II detector are not the best for the UV detector. The detection limits obtained with the ELSD II detector using ammonium acetate buffer and with the UV detector using sodium phosphate buffer are reported in Table 2.

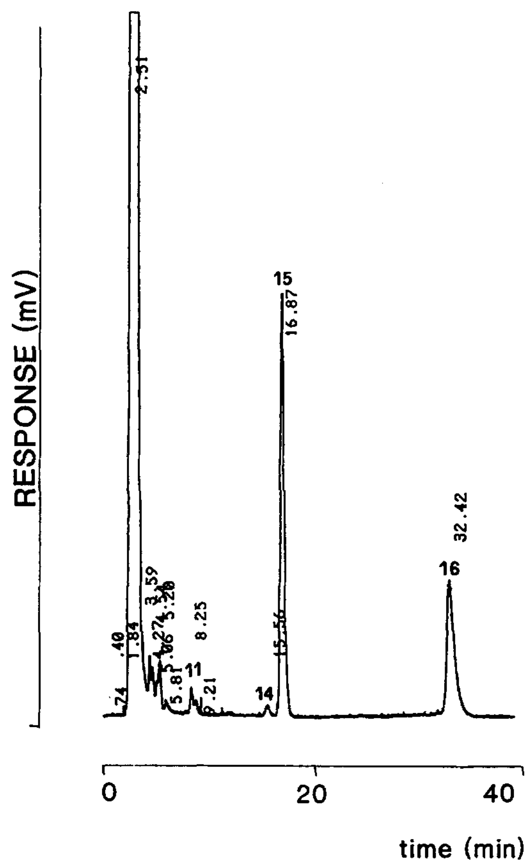

Fig. 7. Example of separation of unconjugated BA in the stool of a healthy subject. Solvent B in isocratic mode and the ELSD II detector have been used.

DISCUSSION

The method described in the present study appears to be appropriate for the simultaneous analysis in human

TABLE 4. Analytical recovery of the developed method for bile acid analysis in stools and bile

Sample	Glycocholic Acid			Chenodeoxycholic Acid		
	Added[a]	Found	Recovery	Added[a]	Found	Recovery
	nmol		%	*nmol*		%
Bile	11.3	11.0	97.3	10.2	9.4	92.2
	22.6	22.1	97.8	20.4	20.7	101.4
	45.2	45.3	100.2	40.8	40.1	98.3
	90.4	87.0	96.2	81.6	79.4	97.5
Mean ± SD			97.9 ± 1.7			97.3 ± 3.8
Stools	11.3	10.2	90.5	10.2	9.6	94.2
	22.6	21.8	96.6	20.4	20.0	98.2
	45.2	44.5	98.4	40.8	41.4	101.4
	90.4	92.3	102.1	81.6	84.9	104.0
Mean ± SD			96.9 ± 4.8			99.4 ± 4.2

[a] Injected.

specimens of the 15 major amidated and free BA with comparable detection limits.

For the first time an HPLC direct method can be applied for an adequate analysis of free BA with a detection limit 50 times lower than previously described methods using a conventional UV detector (17).

The efficient resolution of all BA is achieved with a convex gradient system allowing a reduction in analysis time to less than 60 min. The retention time of the internal standard nordeoxycholic acid is intermediate between amidated and free BA.

The appropriate choice of the percentage of methanol, pH, and total ionic strength is fundamental for the efficient separation of the BA, together with the accurate

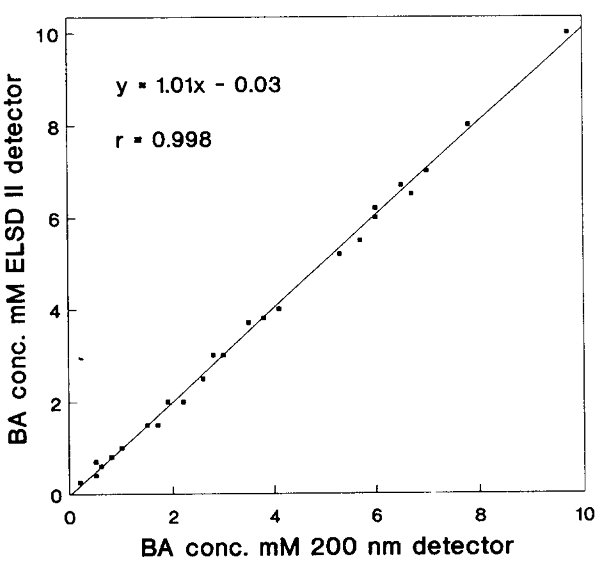

Fig. 8. Relationship between amidated BA concentration in bile samples measured using the ELSD II detector (y) and the UV at 200 nm detection (x).

control of the column temperature. Under these conditions the reproducibility of the analysis in terms of retention time is satisfactory, allowing a precise identification of the eluted peaks.

The new evaporative light-scattering mass detector, used for the first time for BA analysis, offers the main advantage of detecting all the BA with similar sensitivity.

All the BA scatter the light according to their size, which is a function of the number of molecular substituents and to a less extent of the side chain length. Only slight differences were noted among the studied BA and are the function of the real size of the molecule that interacts with the laser light.

The new detector requires the use of a complete volatile mobile phase and thus the use of ammonium acetate at 15 mM concentration was appropriate.

The ELSD II detector offers the unique possibility of using a gradient system with mobile phases at different methanol compositions without affecting the baseline, as observed with the UV detector, as the effluent from the column is converted to a very fine mist by passage through a nebulizer into a stream of nitrogen carrier gas. The volatile solvent constituents (methanol, water, ammonium acetate) are vaporized leaving the particles of BA in the carrier gas, and a light beam is scattered by those particles, detected, and measured by a sensitive photodetector.

Consequently, the response of the ELSD II detector depends on the momentary concentration of the BA in the light beam and not, as in the UV detector, on the presence of a particular functional group (amide bond). For this reason, the detector response is similar for all the BA studied and slightly higher for trihydroxy BA with higher molecular weight and size and for the corresponding amidated BA.

Another advantage of the ELSD II detector over the UV detector is that impurities present in the sample could greatly affect UV detection results. This is particularly true for molecules with physicochemical properties similar to those of BA and with functional groups that strongly absorb at 200 nm. Overestimation of these compounds or false peaks that can be attributed to BA can be avoided or properly estimated with the ELSD II.

For this reason the ELSD II can be connected in series with the UV detector, facilitating the interpretation of unknown peaks observed in the UV detector or vice versa. This is particularly important in BA quality control studies and pharmaceutical analysis.

When results obtained with the ELSD II detector and the UV detector were compared, good agreement was obtained only if the areas of bile peaks for both UV and ELSD II detectors were corrected according the their corresponding detector response, which is a function of different theoretical principles. As reported in Table 2, the detection limit of the ELSD II detector for amidated BA is similar to that of the UV detector (even using sodium phosphate buffer), while it is 50 times lower for unconjugated BA.

For unconjugated BA with poor UV molar absorptivity, the ELSD II has been demonstrated to be a more universal, accurate, and precise method of detection and its application to BA analysis in stools offers a simple and convenient assay.

Moreover, the detection limit, even if lower when compared with UV detector, is in the order of nmoles and not yet competitive with prederivatization procedures, which in turn are not direct and more time consuming.

The system can be successfully applied with adequate sensitivity for BA analysis in stools and bile requiring minute amounts of sample. A direct analysis, without any preanalytical step, is also possible for relatively concentrated bile samples (gallbladder bile). The present method could be applied for BA analysis in serum and urine. The main problem is the detection limit: 1-2 ml of specimen is required to achieve a reasonable accuracy and precision. Moreover, the main advantage will be the possibility to detect free BA that are present mainly in human serum. Detailed application for serum and urine BA analysis will be the object of further studies.

With the ELSD II detector other BA can be detected, such as those in bile of other species or sulfated BA, with the use of an appropriate mobile phase.

A single analysis of free BA can also be performed in bile or other biological samples in which conjugated BA are hydrolyzed (enzymatically or chemically) to form free BA.

In conclusion, the ELSD II detector can be used for BA analysis and particularly for those analytical determinations that require an easy, simple, and rapid method allowing for many analyses per day.

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