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Group separation of free and conjugated bile acids by pre-packed anion-exchange cartridges

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Abstract: A rapid and simple procedure for the group separation of the major free and conjugated bile acids of man is described. After initial extraction with Bond Elut C_{18} cartridges, the analytes are fractionated into the unconjugated, glycine- and taurine-conjugated forms using disposable Bond Elut SAX columns and methanol–acetate buffer eluents. The method is found to be accurate and reproducible and to afford complete resolution between fractions. Free and conjugated bile acids present in human bile and gastric juice are assayed by high-performance liquid chromatography, after extraction and group separation according to the described procedure.

Keywords: *Anion-exchange cartridges; high-performance liquid chromatography; free and conjugated bile acids; group separation; determination in human bile and gastric juice.*

Introduction

Bile acids are present in human fluids both in the free form and, primarily, as glycine and taurine conjugates [1–4]. The determination of the levels of these compounds and of their state of conjugation is relevant for the diagnosis of hepatobiliary and intestinal diseases [3, 5, 6].

Generally, the chromatographic resolution of unconjugated, glycine- and taurine-conjugated bile acids from biological samples requires a preliminary separation of the three groups; subsequently, the resolution of each fraction into individual bile acids is achieved by thin-layer chromatography [7], gas chromatography after hydrolysis of conjugates and derivatization [8–10] or reversed-phase high-performance liquid chromatography (RP–HPLC) [2, 11–13].

Recently, a HPLC method for the complete simultaneous resolution of the individual free and conjugated bile acids without prior group separation has been reported [14]. This procedure, however, exhibited low sensitivity in the detection of the unconjugated forms. With gas chromatography the fractionation of the free, glyco- and tauro-conjugated bile acid groups prior to analysis is essential owing to the loss of information on the conjugating moiety during hydrolysis.

At present, the group separation of the foregoing compounds is carried out by thin-layer chromatography [11], ion-exchange chromatography [6, 8, 9, 13, 15, 16], or on silica columns [7] or Sep–Pak SIL cartridges [10]. Each of these techniques has distinct disadvantages such as lengthy elution times [6, 7, 13, 15, 16], extensive preparation of the gels [7, 8, 15, 16], overlap between the different fractions [9, 11, 16], inconsistent conjugate recovery [17, 18] and unsatisfactory fractionation reproducibility between batches of Sep–Pak SIL cartridges [10].

In order to overcome these problems, a method for the rapid and complete separation of bile acids into the unconjugated, glycine- and taurine-conjugated groups, has been devised based upon the use of pre-packed silica-based strong anion-exchange cartridges (Bond Elut SAX). The application of this new procedure to the assay of free and conjugated bile acids in human bile and gastric juice also is demonstrated.

Experimental

Reagents

The sodium salts of cholic acid (CA), chenodeoxycholic acid (CDCA), deoxycholic acid (DCA), lithocholic acid (LCA), glycocholic acid (GCA), glycochenodeoxycholic acid (GCDCA), glycodeoxycholic acid (GDCA), glycolithocholic acid (GLCA), taurocholic acid (TCA), taurochenodeoxycholic acid (TCDCA), taurodeoxycholic acid (TDCA) and taurolithocholic acid (TLCA) were purchased from Sigma (St. Louis, MO, USA); the sodium salts of ursodeoxycholic acid (UDCA),

tauroursodeoxycholic acid (TUDCA) and glycoursodeoxycholic acid (GUDCA) were a gift from Gipharmex (Milan, Italy). Their purity was checked by HPLC prior to use. HPLC-grade methanol and water were supplied by Farmitalia Carlo Erba (Milan, Italy). HPLC-grade sodium acetate was from Baker (Phillipsburg, NJ, USA). All other chemicals were of analytical grade (Farmitalia Carlo Erba).

Bond Elut C_{18} (BE–C_{18}) and Bond Elut SAX (BE–SAX) cartridges were obtained from Analytichem International (Harbor City, CA, USA).

Liquid chromatography

The HPLC apparatus consisted of a Jasco chromatographic system (Model Bip-I pump, Model GP-A40 solvent programmer and Model UVIDEC-100-V variable-wavelength UV-detector; Jasco, Tokyo, Japan) linked to an injection valve with a 20-μl sample loop and a chromatographic data processor (Chromatopac C-R3A, Shimadzu, Kyoto, Japan). The detector was set to 210 nm and 0.08 a.u.f.s.

Free and conjugated bile acids were separated according to the method described earlier [14], using an Ultrasphere ODS column (d_p = 5 μm, 150 × 4.6 mm i.d.; Beckman, Berkeley, USA) eluted with a linear gradient of methanol–aqueous buffer at a flow-rate of 1.0 ml min^{-1}. Solvent A was 65% methanol in 0.03 M sodium acetate adjusted to pH 4.3 with phosphoric acid and solvent B was 90% methanol in 0.07 M sodium acetate adjusted to pH 4.3 with phosphoric acid. The eluents were filtered through type-HVLP filters (0.45 μm; Millipore S.A., Molsheim, France) and on-line degassed by a model ERC-3311 automatic solvent degasser (Erma, Tokyo, Japan). Chromatography was performed at ambient temperature.

The identity of the separated compounds was assigned by retention coincidence using authentic substances and confirmed by analysis on a different chromatographic column (Supelcosil LC-18-DB, d_p = 5 μm, 150 × 4.6 mm i.d.; Supelco, Bellefonte, PA, USA) using isocratic elution (mobile phase composition: 80% solvent A and 20% solvent B for conjugated bile acids, 40% solvent A and 60% solvent B for free bile acids; flow-rate, 1.0 ml min^{-1}). Peak areas were quantified using the integrator which was calibrated with standard solutions of pure bile acids.

Sample extraction

Gastric juice samples were obtained from two fasting patients with endoscopic evidence of bile reflux gastritis and collected, as previously reported [19], before and after 1 month's treatment with UDCA (300 mg/day). Human bile was obtained by gall bladder puncture at laparotomy from subjects without biliary disease. The specimens were stored at −20°C until required for analysis and the bile acids extracted by a modification of the method described in a previous study [19]. In brief, after thawing each sample was homogenized and centrifuged (3000 r.p.m. for 10 min). An aliquot of the supernatant (0.5 ml for gastric aspirates and 0.05 ml for gall bladder bile, <3.0 μ mol of total bile acids) was diluted with 5.0 ml of 0.05 M phosphate buffer (pH 7.5) and mixed in a vortex mixer. This solution was passed through a pre-conditioned (5 ml of methanol and then 5 ml of water) BE–C_{18} cartridge (sorbent weight, 200 mg) and eluted successively with 5 ml of 40% (v/v) methanol in acetate buffer (0.04 M, pH 4.3) and 10 ml of water. The free bile acids and their conjugates were recovered from the cartridge by elution with methanol (2.0 ml).

Bond Elut SAX fractionation

The BE–SAX column (sorbent weight, 500 mg) was primed successively with 5 ml of methanol, 5 ml of water and 5 ml of methanol. The eluate from the BE–C_{18} extraction column (2.0 ml of methanol) was applied directly to the BE–SAX cartridge, collecting the eluent as part of the first fraction. Further elution for the separation of the bile acid groups was carried

Table 1
Solvent mixtures for the group separation of bile acids on BE–SAX cartridges

Fraction	Bile acid	Eluting solvent
1	Unconjugated	2 ml of MeOH (application of sample) 3.5 ml of MeOH
2	Glyco-conjugates	5.5 ml of 65% (v/v) MeOH in acetate buffer (20 mM, pH 3.7)
3	Tauro-conjugates	5.5 ml of 65% (v/v) MeOH in acetate buffer (80 mM, pH 3.7)

out using sequential application of the solvent mixtures shown in Table 1, at a flow-rate of *ca* 1.5 ml min^{-1}.

The unconjugate fraction was evaporated under nitrogen, redissolved in 0.2 ml of the initial mobile phase and a portion (20 μl) injected onto the HPLC column. The glycine and taurine fractions were reduced to dryness *in vacuo*, the residue dissolved in 0.5 ml of the initial mobile phase and subjected to HPLC analysis.

Recovery and reproducibility

The test samples were prepared by adding known amounts of each free, glyco- and tauro-conjugated bile acid to human gall bladder bile (0.05 ml). The samples were subjected successively to extraction by BE–C_{18}, group separation on BE–SAX and determination by HPLC as described above.

The intra-assay reproducibility was tested by analysing, on 10 different days, 20 μl of the same stock sample preparation from gall bladder bile. The inter-assay variability was evaluated by repeated (n = 10) BE–C_{18} extractions, BE–SAX fractionation and HPLC analyses of the same gall bladder sample.

Results and Discussion

Group separation of bile acids by Bond Elut SAX

A synthetic mixture of UDCA, CA, CDCA, DCA and LCA and of the corresponding glycine and taurine conjugates was dissolved in methanol, applied to a pre-packed BE–SAX cartridge and the eluted fractions assayed directly by gradient elution RP–HPLC. The unconjugated bile acids were not retained on the anion exchanger, whereas their glycine and taurine forms were completely adsorbed. The stepwise desorption of the conjugates from the BE–SAX cartridge was performed by increasing the ionic strength of the eluent. Initially, combinations of phosphate buffer solutions, ranging in pH from 2.5 to 3.0, and methanol were examined. Under these conditions, however, satisfactory resolution of the glyco- and tauro-conjugated groups was not achieved; in particular considerable overlap of GLCA with the taurine conjugate fraction occurred. When sodium phosphate was replaced by sodium acetate as the buffering ion in the eluent (see Table 1), the complete separation of free, glycine- and taurine-conjugated bile acids into the three groups was achieved (Fig. 1). The elution profile obtained with the BE–SAX disposable column is illustrated in Fig. 2. The recovery of all bile acids examined, after BE–SAX fractionation, was quantitative (see Table 2) and their elution achieved with only small volumes of the appropriate solvent mixtures. Moreover the recovery and separation of the unconjugated, glyco- and tauro-conjugated fractions were found to be reproducible between different lots of BE–SAX. By contrast, other investigators, using Sep–Pak SIL cartridges, have observed that resolution of the three bile acid groups varied between batches [10]; re-evaluation of the elution profile was thus necessary when a new batch of cartridges was used. Furthermore, the latter authors did not investigate the elution pattern of UDCA and of its glycine and taurine conjugates, which are of primary interest during UDCA treatment [18, 19]. The procedure developed in this study is rapid and less laborious than others reported in the literature. The group separation of bile acids on BE–SAX is performed at ambient temperature, whereas chromatography at 4°C is required when Sep–Pak SIL cartridges are used [10] to prevent considerable overlap between the unconjugated and glycine-conjugated bile acid fractions. In addition no preparation of the pre-packed BE–SAX column is necessary, apart from conditioning it with methanol and water, thus circumventing time-consuming preparation and washing steps [7, 8, 15, 16].

Other researchers [20] have reported the fractionation of steroid glucuronides and sulphates to be strongly influenced by varying residual water concentrations in the methanol sample when BE–SAX in the original chloride-ion form was used. This is not found to be the case; the BE–SAX fractionation of bile acids was not affected by the water content (up to 30%, v/v) of the applied sample.

Application

The group fractionation procedure described above was applied to the assay of free and conjugated bile acids in human gall bladder bile and gastric juice. The bile acids were extracted from the samples by BE–C_{18} cartridges, directly separated into the unconjugated, glyco- and tauro-conjugated forms by BE–SAX columns and analysed by RP–HPLC (see the Experimental section). The initial BE–C_{18} extraction step also afforded the re-

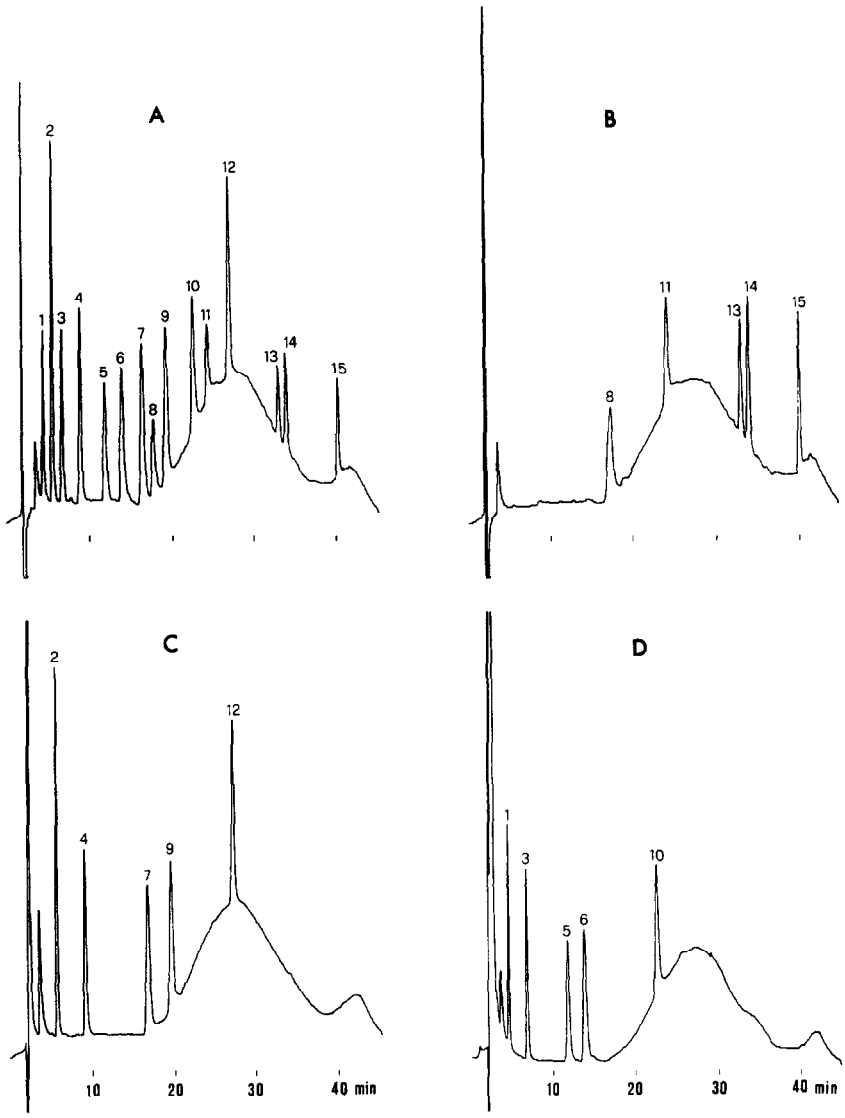

Figure 1
RP-HPLC profiles of free and conjugated bile acid standards (A) and of the fractions obtained by their group separation on BE-SAX cartridge. (B) Unconjugated fraction; (C) glycine conjugated fraction; (D) taurine conjugated fraction. Chromatographic conditions as described under Experimental. Peaks: 1 = TUDCA, 2 = GUDCA, 3 = TCA, 4 = GCA, 5 = TCDCA, 6 = TDCA, 7 = GCDCA, 8 = UDCA, 9 = GDCA, 10 = TLCA, 11 = CA, 12 = GLCA, 13 = CDCA, 14 = DCA, 15 = LCA.

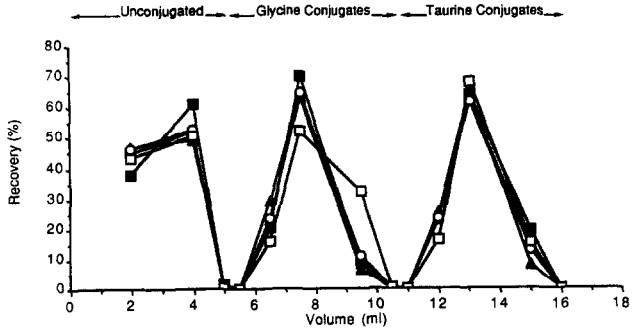

Figure 2
Elution profiles of UDCA (▲), CA (■), CDCA (●), DCA (○) and LCA (□) and of their glycine and taurine conjugates from BE-SAX cartridges. Values are means of duplicate experiments. Elution conditions as in Table 1.

Table 2
Recovery of bile acid standards after BE–SAX fractionation

Bile acid	Percentage recovery (mean ± SD, n = 6) Fraction 1	Fraction 2	Fraction 3
UDCA	98.2 ± 2.5	—	—
CA	101.1 ± 2.0	—	—
CDCA	96.9 ± 2.1	—	—
DCA	98.5 ± 3.4	—	—
LCA	94.6 ± 1.2	—	—
GUDCA	—	99.5 ± 2.2	—
GCA	—	98.5 ± 2.6	—
GCDCA	—	100.7 ± 2.5	—
GDCA	—	100.2 ± 1.5	—
GLCA	—	98.0 ± 2.2	—
TUDCA	—	—	98.6 ± 3.0
TCA	—	—	100.1 ± 1.9
TCDCA	—	—	97.1 ± 3.2
TDCA	—	—	99.1 ± 2.6
TLCA	—	—	98.1 ± 2.5

moval of proteins and inorganic ions which interfere with the anion-exchange chromatography [6, 20]. Representative chromatograms of the bile acid fractions from human gall bladder bile are shown in Fig. 3. In accordance with previous studies [2, 3, 12, 21] no bile acids were detected in the unconjugated group. For the linearity and sensitivity data the reader is referred to the previously published method for the simultaneous determination of free and conjugated bile acids by gradient elution RP–HPLC [14]. Efficient resolution of the group-separated bile acids also is obtained by isocratic RP–HPLC [22], circumventing the problems of baseline instability during gradient elution [4, 14]. This allows detection within a range of 0.02 absorbance units full scale, improving the sensitivity achieved with the gradient system [14] by a factor of 4 (detection limits, 0.12 mmol l^{-1} for free bile acids and 0.006 mmol l^{-1} for conjugated bile acids).

Known quantities of standard bile acids were added to human gall bladder bile and their recovery values calculated. As shown in Table 3, recoveries for each bile acid were at least 87%. Applying the reported procedure to human gall bladder bile, bile acids were determined with a relative standard deviation range of 1.1–4.2% (n = 10) for the intra-assay reproducibility and 3.7–7.9% (n = 10) for the inter-assay reproducibility.

Determination of bile acids was carried out on a sample of gall bladder bile and on gastric juice specimens from two patients during UDCA-therapy of bile reflux gastritis and the results are presented in Table 4.

The values for the biliary bile acid composition determined in this study (Table 4, subject 1) are in agreement with those measured in previous investigations [3, 21], with the exception of UDCA conjugates present in higher proportions.

The levels of intragastric bile acids recorded here are within the concentration range reported in earlier studies [14, 19]. After UDCA-

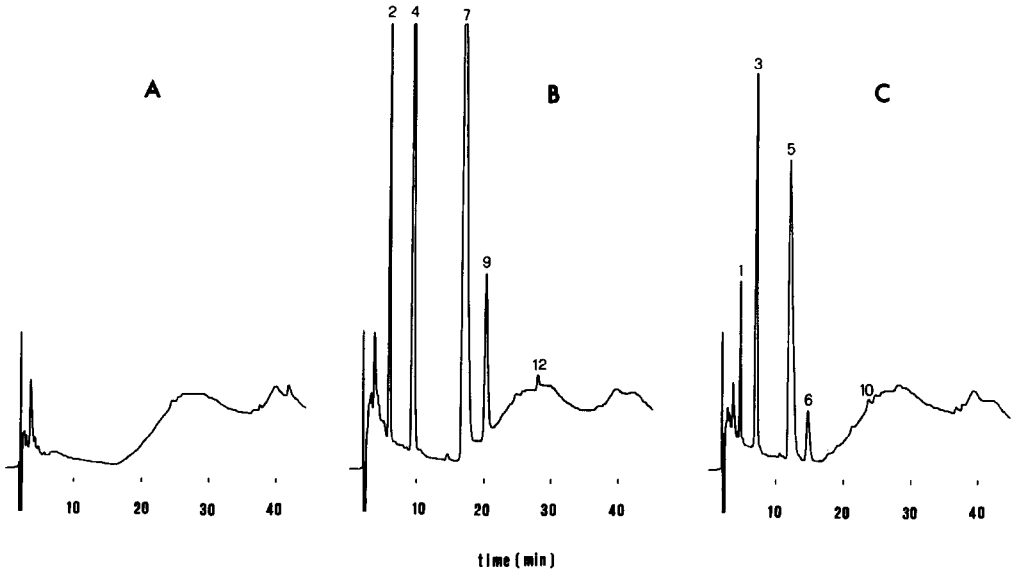

Figure 3
Chromatograms of the separation of biliary bile acids. (A) Free bile acids; (B) glycine conjugates; (C) taurine conjugates. Conditions and peak identification as in Fig. 1.

Table 3
Recovery of free and conjugated bile acids added to human bile

Bile acid	Bile	Added (μmol per 0.05 ml)	Expected	Recovery (%) (mean ± SD, $n = 5$)
UDCA	0	0.198	0.198	90.3 ± 2.3
		0.989	0.989	97.4 ± 4.1
CA	0	0.213	0.213	92.2 ± 2.5
		1.064	1.064	94.1 ± 1.9
CDCA	0	0.135	0.135	88.2 ± 0.2
		0.675	0.675	95.8 ± 4.8
DCA	0	0.162	0.162	89.6 ± 3.0
		0.810	0.810	90.7 ± 5.0
LCA	0	0.177	0.177	87.6 ± 2.1
		0.887	0.887	91.3 ± 1.3
GUDCA	0.092	0.017	0.109	96.0 ± 5.2
		0.086	0.178	99.4 ± 0.9
GCA	0.249	0.015	0.264	93.6 ± 1.4
		0.075	0.324	102.0 ± 1.3
GCDCA	0.483	0.022	0.505	93.8 ± 1.5
		0.108	0.591	100.9 ± 1.4
GDCA	0.081	0.018	0.099	93.7 ± 2.6
		0.090	0.171	97.1 ± 3.4
GLCA	0.003	0.014	0.017	92.0 ± 6.0
		0.073	0.076	88.3 ± 4.3
TUDCA	0.038	0.011	0.049	99.8 ± 5.8
		0.057	0.095	102.4 ± 1.9
TCA	0.15	0.015	0.165	94.7 ± 3.9
		0.075	0.225	98.3 ± 2.8
TCDCA	0.226	0.010	0.236	91.7 ± 4.2
		0.052	0.278	97.8 ± 1.6
TDCA	0.028	0.018	0.046	93.0 ± 3.2
		0.088	0.116	96.4 ± 2.3
TLCA	0.001	0.016	0.017	89.5 ± 7.1
		0.083	0.084	87.3 ± 6.7

Table 4
Bile acid composition of human gall bladder bile (1) and of gastric juice of patients under UDCA-therapy (2,3)

	Percentage of total bile acid				
	Patient				
Bile acid	1	2		3	
		B*	A*	B*	A*
GUDCA	6.4	0.9	23.7	4.1	33.8
GCA	18.6	32.6	19.8	10.9	17.1
GCDCA	36.9	23.1	27.4	17.8	14.9
GDCA	6.4	26.2	20.7	49.4	11.0
GLCA	0.2	—	—	—	—
TUDCA	2.5	—	1.9	—	6.8
TCA	10.4	6.4	2.4	3.7	5.8
TCDCA	15.7	9.9	2.7	4.7	5.4
TDCA	2.6	0.8	1.0	9.2	2.8
TLCA	0.1	—	0.2	—	2.2
TBA (mmol/l)	28.73	4.1	6.1	1.2	0.9

B*, Before treatment.
A*, After treatment.
TBA, Total bile acid concentration.

therapy (Table 4, patients 2 and 3), GUDCA increased significantly while GDCA and TDCA decreased. This is in accordance with previous reports [19, 23]. Moreover virtually identical values were obtained for bile acid conjugates when the present method and the previously adopted procedure, without group fractionation [14], were used on the same sample of gastric juice.

Conclusions

A method has been developed for the rapid (taking less than 20 min to perform) and efficient group separation of bile acids by means of disposable BE–SAX cartridges. This new procedure is simpler than those previously reported since time-consuming preparations of the gels [7, 8, 15, 16], lengthy elutions [7, 15, 16] or several sample handling steps [6, 8, 15] are not required. Moreover multiple samples can be processed simultaneously with specially designed vacuum manifolds. The accuracy and high reproducibility of the BE–SAX fractionation method make it particularly suitable for the routine assay of bile acids.

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