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HPLC assay of conjugated bile acids in gastric juice during ursodeoxycholic acid (Deursil®) therapy of bile reflux gastritis*

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Abstract: A rapid high-performance liquid chromatographic method for the direct assay of the taurine and glycine conjugated bile acids in human gastric juice is described. After extraction with Sep-Pak C_{18} cartridges, compounds are baseline resolved on a reversed-phase column and detected by UV absorption. The procedure is linear from 10 μmol l^{-1} to 1200 μmol l^{-1}, with recovery rates ranging from 87 to 100%. The present method is applicable to the quantification of bile acid conjugates in human gastric bile with satisfactory sensitivity, selectivity and precision. Intragastric bile acid compositions in 10 patients with bile reflux gastritis during Deursil® or placebo treatment are presented.

Keywords: *High-performance liquid chromatography; bile reflux gastritis; conjugated bile acids; determination in gastric juice; Deursil®.*

Introduction

Gastritis associated with the reflux of bile into the stomach has been attributed to the disruption of a functional gastric mucosal barrier by endogenous bile acids [1–3] which are present primarily as glycine and taurine conjugates [4, 5]. The degree of gastric mucosal damage has been reported to be affected by bile acid hydroxylation and conjugation [5–8]; hence the composition of the refluxing bile plays an important role in the development of the disease.

Published methods for the determination of the intragastric conjugated bile acids in patients with bile reflux gastritis have distinct disadvantages: the enzymatic assay [4, 5, 9, 10] only estimates the total amount of bile acids while gas chromatography after hydrolysis and derivatisation [5, 11] involves time-consuming steps with loss of information on conjugates.

Although several high-performance liquid chromatographic (HPLC) procedures for the separation of bile acids have been described ([12] and references therein), the application of HPLC to the assay of conjugated bile acids in gastric juice has not yet been reported. Through a systematic optimization study of the mobile and stationary phases, a

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novel reversed-phase HPLC (RP–HPLC) method has been developed [13] for the rapid and efficient resolution of the individual glycine and taurine conjugates of bile acids present in man. This paper reports the application of this method, with a new solid phase extraction procedure, to the assay of bile acid conjugates in human gastric juice. Using the method, it has been possible to investigate the composition of gastric conjugated bile acids during ursodeoxycholic acid (Deursil®) therapy of bile reflux gastritis in patients with no previous gastric surgery.

Experimental

Reagents

The sodium salts of glycocholic acid (GC), glycodeoxycholic acid (GDC), glycochenodeoxycholic acid (GCDC), glycolithocholic acid (GLC), taurocholic acid (TC), taurodeoxycholic acid (TDC), taurochenodeoxycholic acid (TCDC) and taurolithocholic acid (TLC) were obtained from Sigma (St. Louis, MO, USA); the sodium salts of tauroursodeoxycholic acid (TUDC), glycoursodeoxycholic acid (GUDC) and glyco-7α,12α-dihydroxy-5β-cholanic acid were purchased from Calbiochem-Behring (San Diego, CA, USA). Their purity was checked by HPLC prior to use. Deursil® was from Gipharmex (Milan, Italy). 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). Sep-Pak C_{18} cartridges were obtained from Water Assoc. (Milford, MA, 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 (Rheodyne, Cotati, USA) and a chromatographic data processor (Chromatopac C-R3A, Shimadzu, Kyoto, Japan). The detector was set to 205 nm and 0.08 a.u.f.s. Sample injections were made with a Hamilton Model 802 RN syringe (10 μl; Hamilton Bonaduz AG, Bonaduz, Switzerland).

Separations were performed according to the method described earlier [13], using a Supelcosil LC-18-DB reversed-phase column (d_p 5-μm, 150 × 4.6 mm i.d.; Supelco, Bellefonte, PA, USA) eluted with methanol–0.02 M aqueous sodium acetate (65:35, v/v) adjusted to pH 4.2 with phosphoric acid, at a flow rate of 1.0 ml min^{-1}. 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 carried out at ambient temperature under isocratic conditions.

The identity of the separated compounds was assigned by co-chromatography with the authentic substances and confirmed by analysis on a different chromatographic column (LiChrospher CH-8, d_p 5-μm, 250 × 4.0 mm i.d.; Merck, Darmstadt, FRG) according to the conditions described in a previous study [13]. Peak areas were quantified using the integrator which was calibrated with standard solutions of pure conjugated bile acids.

Sample processing

Gastric juice samples were obtained from 10 fasting patients with endoscopic evidence of bile reflux gastritis who had not previously undergone gastric surgery. Samples were

collected during endoscopy performed before and after 1 month's treatment with Deursil® (300 mg day^{-1}) or placebo. Gastric juice was aspirated through a sterile catheter inserted down the biopsy channel of the gastroscope. Gastric aspirates were stored at −20°C until analysis. After thawing, each aspirate was mixed with glyco-7α,12α-dihydroxy-5β-cholanic acid (80 μg) as an internal standard [14], homogenized and centrifuged (3000 r.p.m. for 10 min). A 1-ml portion of the supernatant was diluted with 2 ml of 0.15 M phosphate buffer (pH 6), 2 ml of methanol and mixed by a vortex mixer. This solution was passed through a pre-conditioned (5 ml of methanol and then 5 ml of water) Sep-Pak C_{18} cartridge and eluted successively with 5 ml of 40% (v/v) methanol in phosphate buffer (0.1 M, pH 4.5) and 2 ml of 90% methanol in water. The latter fraction, which contained the conjugated bile acids, was reduced to dryness, redissolved in 0.5 ml of mobile phase and a portion (10 μl) injected onto the HPLC column.

The intra-assay reproducibility was tested by analysing on 10 different days, 10 μl of the same stock sample preparation from gastric juice. The inter-assay variability was evaluated by repeated (N = 10) Sep-Pak extraction and HPLC analyses of the same gastric juice sample.

Statistical analysis of the results was carried out by Student's t-test.

Results and Discussion

Disposable Sep-Pak C_{18} cartridges are currently employed for the extraction of bile acids from biological fluids [11, 12, 15, 16]. This cartridge was therefore examined for the purification of gastric bile acids prior to RP–HPLC analysis. The elution procedures reported in the literature, however, proved not to be applicable to gastric bile owing to overlapping, in the chromatogram, of an unknown peak with TUDC and GUDC (Fig. 1A) which are of primary interest during ursodeoxycholic acid treatment. By examining combinations of phosphate buffer solutions and methanol, a solvent system was selected for the Sep-Pak C_{18} purification (see the Experimental section) which afforded the removal of the interfering peak (Fig. 1B) and the quantitative elution of conjugated bile acids in the 90% methanol fraction.

Representative RP–HPLC chromatograms of the separation of bile acid conjugates from human gastric juice, before and after ursodeoxycholic acid therapy are shown in Figs 2 and 3 respectively. Free ursodeoxycholic acid, which elutes after GCDC and is separated from it, was never detected.

Detection limits at 205 nm ranged from 6.0 μmol l^{-1} to 10 μmol l^{-1} (signal-to-noise ratio 2:1) depending on the degree of retention. There was a linear relation (TUDC: r = 0.999, a = 1.02, b = 0.018; GUDC: r = 0.999, a = 1.56, b = 0.052; TC: r = 0.999, a = 0.91, b = 0.054; GC: r = 0.999, a = 1.52, b = 0.039; TCDC: r = 0.998, a = 0.97, b = 0.043; TDC: r = 0.998, a = 0.85, b = 0.028; GCDC: r = 0.998, a = 1.24, b = 0.027; GDC: r = 0.997, a = 1.19, b = 0.024; TLC: r = 0.995, a = 0.62, b = 0.075) between peak area and concentration up to 1200 μmol l^{-1}. The overall recovery using glyco-7α,12α-dihydroxy-5β-cholanic acid as an internal standard was always >87%. The levels of conjugates were corrected according to the recovery values. The relative standard deviation range was 3.3–6.5% (N = 10) for the intra-assay reproducibility and 5.5–8.6% (N = 10) for the inter-assay reproducibility.

Simultaneous determinations of glycine and taurine conjugated bile acids were carried

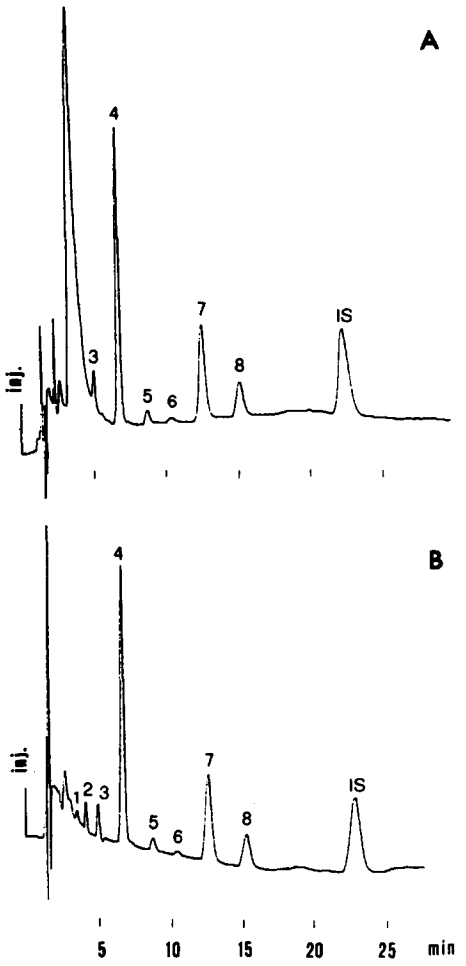

Figure 1
Chromatograms showing the separation of conjugated bile acids from a sample of human gastric juice purified according to: (A) the method reported in the literature [11, 12, 15, 16] and (B) the procedure here developed. Compounds: 1 = TUDC; 2 = GUDC; 3 = TC; 4 = GC; 5 = TCDC; 6 = TDC; 7 = GCDC; 8 = GDC; IS = glyco-7α,12α-dihydroxy-5β-cholanic acid (internal standard). Operating conditions: column, Supelcosil LC-18-DB; mobile phase, methanol–0.02 M sodium acetate (65:35) adjusted to pH 4.2 with phosphoric acid; flow-rate, 1.0 ml min^{-1}; UV detection, 205 nm.

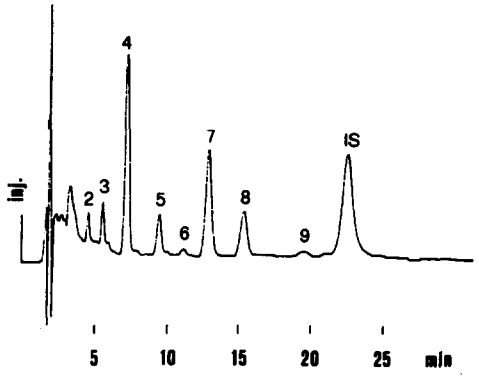

Figure 2
RP–HPLC separation of intragastric conjugated bile acids from a patient with bile reflux gastritis before Deursil® therapy. Conditions and peak identification as in Fig. 1B; 9 = TLC.

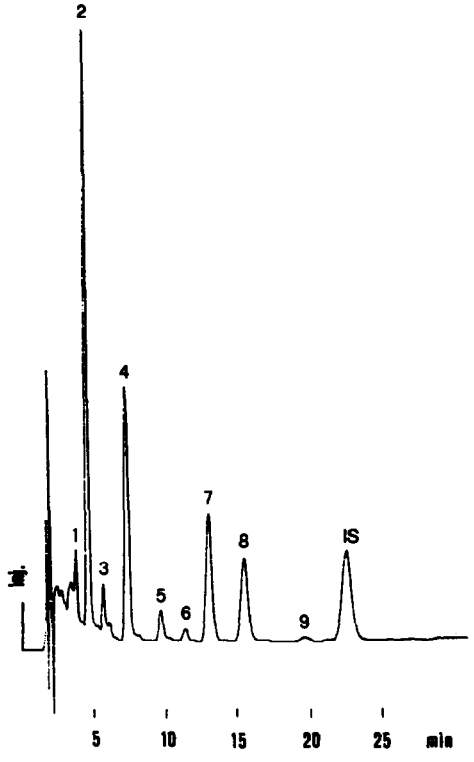

Figure 3
RP–HPLC separation of intragastric conjugated bile acids from the same patient as in Fig. 2 after Deursil® (300 mg day^{-1}) therapy. Conditions and peak identification as in Figs 1B and 2.

out on gastric juice samples from 10 patients with bile reflux gastritis during Deursil® or placebo treatment. The results are presented in Table 1.

Virtually identical values were obtained for the same sample when the foregoing Supelcosil LC-18-DB column and a packing (i.e. LiChrospher CH-8) which exhibits different selectivity [13] were used. This indicates that the method is not subjected to interferences by endogenous substances.

Total conjugated bile acid concentrations measured by summing the quantities of individual conjugates determined on HPLC (Table 1) were within the concentration range reported in previous studies [4, 9, 10]. The percentage composition of the individual glycine and taurine conjugates showed considerable inter-individual variability as also found by others [5].

Before Deursil® treatment, the major bile acid conjugates refluxed into the stomach were GC (mean 35.8%, S.D. 14.2%), GCDC (mean 27.3%, S.D. 11.7%) and GDC (mean 16.2%, S.D. 12.8%). GLC (retention time, 27.2 min) was not detected in the gastric bile and TLC was identified only in trace amounts. The percentage of TUDC and GUDC was below 1.3 and 4.6% respectively. The values obtained by summing the percentage of both conjugates of each bile acid (Table 1) are in good agreement with those measured by gas chromatography in earlier studies [5, 11] with the exception of deoxycholic acid conjugates present in lower proportions.

At the end of the placebo period (patients 1–4, Table 1) no significant variation of the gastric bile acid pattern was observed; on the other hand after Deursil® therapy (patients 5–10, Table 1) GUDC increased substantially ($P < 0.001$) while TUDC did not show

Table 1
Conjugated bile acid composition of gastric juice of patients with bile reflux gastritis during placebo (1–4) and Deursil® (5–10) treatment

Patient		Percentage of total bile acids TUDC	GUDC	TC	GC	TCDC	TDC	GCDC	GDC	TLC	TBA§ (mmol l^{-1})
1	B*	—	1.0	6.0	43.5	3.6	0.5	39.7	5.5	—	16.01
	A*	—	1.1	8.9	32.7	10.7	0.7	43.5	2.3	—	6.75
2	B*	0.3	3.2	6.8	26.1	7.9	1.8	38.8	14.6	0.5	17.09
	A*	0.2	3.9	4.7	28.6	6.1	1.7	41.9	12.5	0.4	7.38
3	B*	0.7	4.0	3.9	28.9	3.5	1.8	38.3	18.9	—	7.20
	A*	0.5	3.3	4.4	32.1	2.3	1.5	40.9	15.0	—	12.30
4	B*	—	0.8	11.7	62.3	3.2	1.0	17.9	3.1	—	0.14
	A*	—	1.0	10.1	55.7	2.5	1.2	21.8	7.6	—	0.12
5	B*	0.7	2.8	6.7	27.4	12.6	2.5	31.7	14.9	0.7	1.18
	A*	4.1	34.9	4.1	18.1	5.1	1.5	17.8	13.3	1.0	4.01
6	B*	1.3	4.6	5.2	42.9	2.1	8.7	16.5	18.6	—	0.42
	A*	2.4	50.1	1.6	14.9	1.5	2.2	19.2	7.9	—	2.37
7	B*	—	1.0	7.2	45.2	3.5	1.0	28.3	11.2	2.4	2.13
	A*	—	24.5	5.2	25.3	3.9	0.6	29.5	9.9	1.2	1.83
8	B*	—	1.7	20.7	41.4	4.3	7.1	7.7	16.9	—	0.76
	A*	—	18.2	9.1	51.6	0.9	1.2	8.5	10.3	—	0.55
9	B*	0.8	3.7	3.5	10.2	4.6	9.2	16.8	49.5	1.5	1.21
	A*	6.0	34.8	5.9	17.8	5.4	2.7	14.9	11.0	1.2	0.84
10	B*	—	1.1	9.4	29.7	10.9	1.6	37.6	9.0	1.0	3.48
	A*	2.2	20.6	6.9	24.0	7.8	1.2	29.1	7.8	0.7	2.37

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

any relevant change. A statistically significant decrease in the mean percentage composition of both glyco- and tauro- conjugates of deoxycholic acid (from 25.0 ± 17.7% to 11.6 ± 2.2%; $P < 0.05$) and of the taurine-conjugated bile acids (from 21.5 ± 6.2% to 14.2 ± 5.2%; $P < 0.05$) was also detected.

In parallel, the endoscopic appearance of the gastric mucosa showed a marked improvement in patients treated with Deursil®, whereas no significant change in the endoscopic picture was observed in placebo-treated patients.

The foregoing results indicate that Deursil®-therapy of gastritis, caused by bile reflux, effects an improvement of the gastric mucosal aspect by reducing the proportion of both deoxycholic acid conjugates and taurine-conjugated bile acids which have been found to be, by far, the most damaging to the gastric mucosa [6–8].

An earlier investigation [11] was carried out by gas chromatography on the pattern of intragastric bile acids during ursodeoxycholic acid therapy of reflux gastritis in a different group of patients (i.e. subjects with a history of previous gastric surgery). The gas chromatographic assay, however, suffers from several drawbacks including incomplete recovery and production of artifacts during the vigorous hydrolysis step, lengthy derivatization procedures and the inability to differentiate between unmetabolized ursodeoxycholic acid and free ursodeoxycholic acid released by the alkaline hydrolysis of TUDC and GUDC. Moreover, the loss of information on the conjugated bile acid moiety during hydrolysis, is a disadvantage since the nature of the conjugated group, as well as the number and type of hydroxy substitutions, influences the mucosa-destroying ability of bile acids [5–8].

In conclusion, the method here developed allows the rapid (analysis time, less than 30 min) and simultaneous determination of the individual glycine and taurine conjugated bile acids present in gastric bile. The accuracy and reproducibility of the assay make it suitable for routine clinical applications.

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