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SIMULTANEOUS DETERMINATION OF FREE AND CONJUGATED BILE ACIDS IN HUMAN GASTRIC JUICE BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

A high-performance liquid chromatographic procedure has been developed for the simultaneous assay of the major free bile acids and the corresponding glycine and taurine conjugates in man. No preliminary fractionation into the free, glyco- and tauro-conjugated forms was required. An Ultrasphere ODS column with UV detection at 210 nm and methanol–acetate buffer gradient elution were used. The retention volume of the conjugates was dependent on the sodium acetate concentration in the mobile phase. The method is applicable to the quantification of intragastric bile acids with satisfactory sensitivity, selectivity and precision. Unconjugated and conjugated bile acids present in the gastric juice of patients with bile reflux gastritis were determined directly after Sep-Pak C_{18} cartridge purification.

INTRODUCTION

Bile acids occur in human fluids both in the free form and, primarily, as glycine and taurine conjugates [1–4]. The individual separation and quantification of these compounds are very important from a clinical point of view, as the abnormal presence of bile acids in body fluids reflects metabolic disorders and diseases [3,5,6].

Previously, the chromatographic resolution of unconjugated, glyco- and tauro-conjugated bile acids from biological samples has required a preliminary separation of the three classes of steroids, which has been carried out by ion-exchange chromatography [2,7–11] or using Sep-Pak SIL cartridges [12] or silica columns [13]. Subsequently, the resolution of each fraction into individual bile acids was achieved by thin-layer chromatography [13], gas chromatography after hydrolysis of conjugates and derivatization [10–12] or reversed-phase high-performance liquid chromatography (RP-HPLC) [2,7–9].

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Other workers have reported the simultaneous determination of free and conjugated bile acids by RP-HPLC [14–17]; however, none of these procedures affords the complete simultaneous separation of the individual unconjugated and conjugated bile acids.

A method is described here for the simultaneous and baseline resolution of ursodeoxycholic acid, cholic acid, chenodeoxycholic acid, deoxycholic acid, lithocholic acid and of the corresponding glycine and taurine conjugates by RP-HPLC. The application of the method to the determination of the free and conjugated bile acids in human gastric juice is also described.

EXPERIMENTAL

Reagents

The sodium salts of cholic acid (CA), chenodeoxycholic acid (CDCA), deoxycholic acid (DCA), lithocholic acid (LCA), glycocholic acid (GCA), glycodeoxycholic acid (GDCA), glycochenodeoxycholic (GCDCA), glycolithocholic acid (GLCA), taurocholic acid (TCA), taurodeoxycholic acid (TDCA), taurochenodeoxycholic acid (TCDCA) and taurolithocholic acid (TLCA) were purchased from Sigma (St. Louis, MO, U.S.A.); 7α,12α-dihydroxy-5β-cholanic acid was obtained from Calbiochem-Behring (San Diego, CA, U.S.A.). 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 Carlo Erba (Milan, Italy). HPLC-grade sodium acetate was from Baker (Phillipsburg, NJ, U.S.A.). All other chemicals were of analytical-reagent grade (Carlo Erba). Sep-Pak C_{18} cartridges were obtained from Waters Assoc. (Milford, MA, U.S.A.).

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, CA, U.S.A.) and a Chromatopac C-R3A chromatographic data processor (Shimadzu, Kyoto, Japan). The detector was set at 210 nm and 0.08 absorbance units full scale (a.u.f.s.). Sample injections were made with a Hamilton Model 802 RN 10-μl syringe (Hamilton, Bonaduz, Switzerland).

Free and conjugated bile acids were separated on an Ultrasphere ODS (particle diameter, d_p, 5 μm) column (150 $\times$ 4.6 mm I.D.) (Beckman, Berkeley, CA, U.S.A.), eluted with a linear gradient of methanol–aqueous buffer at a flow-rate of 1.0 ml/min. 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 elution programme was as follows: isocratic elution with 15% solvent B, 85% solvent A for 10 min, then a 25-min linear gradient to 90% solvent B; the mobile phase composition was finally maintained at 90% solvent B for 5 min. Samples were injected 0.5 min after the start of the elution programme. The eluents were filtered through type HVLP

filters (0.45 μm) (Millipore, Molsheim, France) and degassed on-line 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 co-chromatography with the authentic substances and confirmed by analysis on a different chromatographic column (Spherisorb ODS-2, d_p 5 μm; 150 $\times$ 4.6 mm I.D.) (Phase Separations, Queensferry, U.K.) using isocratic elution (mobile phase composition: 15% solvent B for conjugated bile acids, 50% solvent B for free bile acids; flow-rate, 1.0 ml/min). Peak areas were quantified using the integrator, which was calibrated with standard solutions of pure bile acids.

Sample processing

Gastric juice samples, obtained from three fasting patients with bile reflux gastritis and no history of previous gastric surgery, were collected before and after treatment for 1 month with ursodeoxycholic acid (300 mg/day). Additional gastric samples were obtained from three fasting patients previously submitted to gastric resection. Gastric juices were aspirated through a sterile catheter inserted down the biopsy channel of the endoscope and stored at $-20°C$ until analysis. Free and conjugated bile acids were extracted from the gastric aspirates by a modification of the method described in a previous study [18]. In brief, a 2-ml aliquot of each aspirate was mixed with 7α,12α-dihydroxy-5β-cholanic acid (8 mg) as an internal standard, homogenized and centrifuged (1000 *g* for 10 min). A 0.5-ml portion of the supernatant was diluted with 2.5 ml of 0.12 *M* phosphate buffer (pH 7.5) and 2 ml of methanol and mixed with a vortex mixer. This solution was passed through a preconditioned (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), 2 ml of water and 2 ml of methanol. The last fraction, which contained the free and conjugated bile acids, was evaporated to dryness under a nitrogen stream, the residue dissolved in 0.5 ml of the initial mobile phase and a portion (10 μl) of this solution injected on to the HPLC column.

Reproducibility

The intra-assay reproducibility was tested by analysing on ten 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.

Recovery

The test samples were prepared by adding known amounts of each free, glyco- and tauro-conjugated bile acid to the gastric juice (0.5 ml) of a patient with previous gastric surgery. The samples were subjected successively to clean-up by Sep-Pak C_{18} and determination by HPLC as described above.

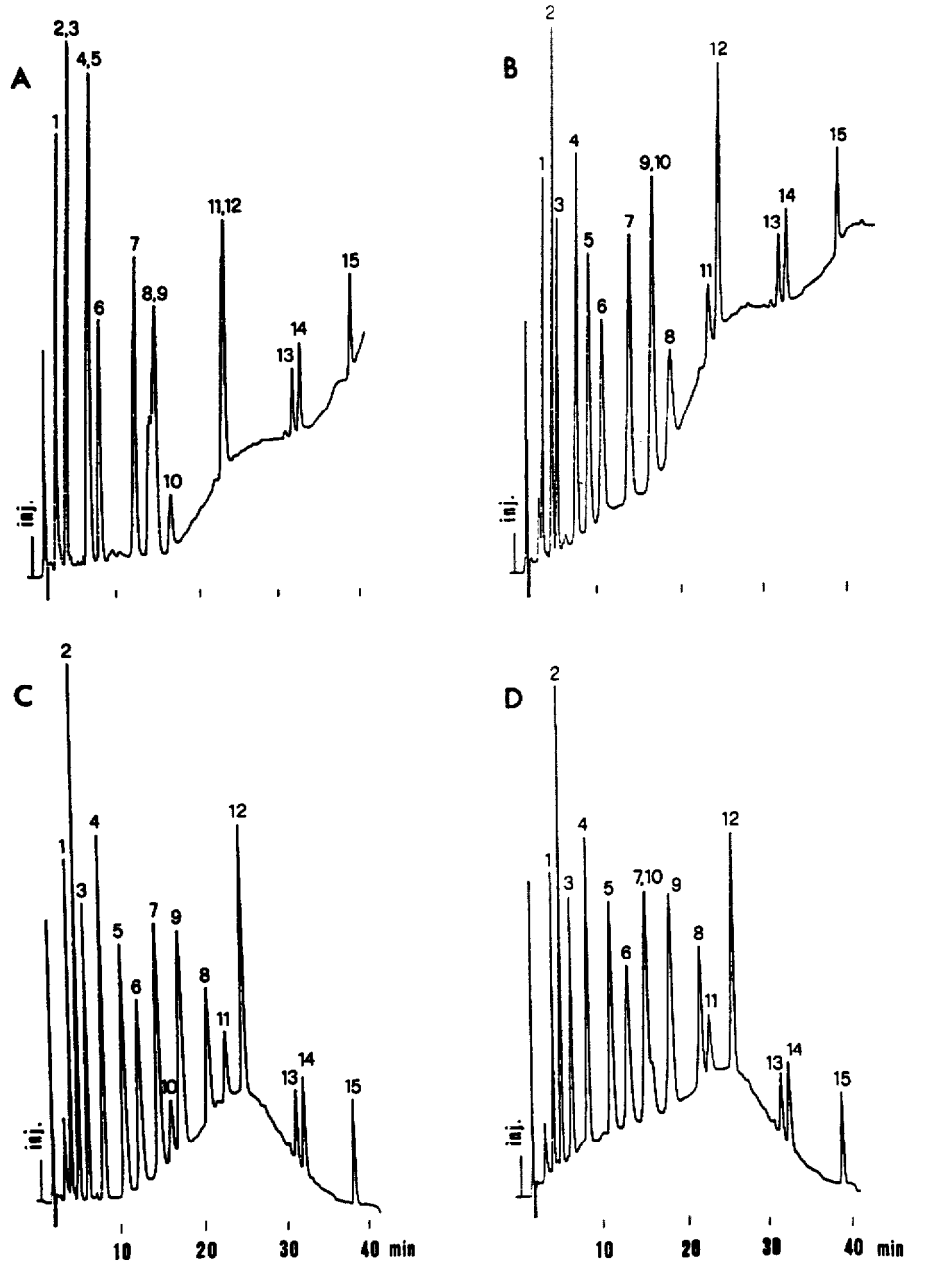

Fig. 1. Elution profiles of free bile acids and their glycine and taurine conjugates on an Ultrasphere ODS column. The gradient (see Experimental) was formed between 65% methanol in 0.01 *M* acetate buffer and 90% methanol in 0.024 *M* acetate buffer (A); 65% methanol in 0.02 *M* acetate buffer and 90% methanol in 0.047 *M* acetate buffer (B); 65% methanol in 0.03 *M* acetate buffer and 90% methanol in 0.07 *M* acetate buffer (C); and 65% methanol in 0.04 *M* acetate buffer and 90% methanol in 0.098 *M* acetate buffer (D). Other chromatographic conditions are described under Experimental. Peaks: 1 = TUDCA; 2 = GUDCA; 3 = TCA; 4 = GCA; 5 = TCDCA; 6 = TDCA; 7 = GCDCA; 8 = TLCA; 9 = GDCA; 10 = UDCA; 11 = CA; 12 = GLCA; 13 = CDCA; 14 = DCA; 15 = LCA.

RESULTS AND DISCUSSION

Chromatographic separation

In a previous paper [19] on the optimization of the mobile and stationary phases for the isocratic RP-HPLC of bile acid conjugates, we showed that rapid and efficient resolution of the foregoing compounds is obtained on C_{18} columns with an eluent consisting of methanol–0.02 *M* sodium acetate (60:30) adjusted to pH 4.3 with phosphoric acid. Under these conditions, however, the complete separation of unconjugated and conjugated bile acids is not obtained. Moreover, CDCA, DCA and LCA exhibit excessively long retention times (chromatograms not shown).

Using the optimized mobile phase reported above, a linear gradient elution and an Ultrasphere ODS column, the influence of the buffer concentration on the chromatography of free bile acids and of their glycine and taurine conjugates was examined. The range of sodium acetate concentration studied was 0.01–0.04 *M*. With increasing ionic strength in the mobile phase the retention volumes for each conjugate increase, whereas the retention of unconjugated bile acids is unaffected over the entire range of mobile phase salt concentration (Fig. 1A–D). This is further illustrated in Table I where, for each sodium acetate concentration, the retention volumes measured from the chromatograms in Fig. 1 are listed. The

TABLE I

EFFECT OF MOBILE PHASE SALT CONCENTRATION ON RP-HPLC OF FREE AND CONJUGATED BILE ACIDS

Chromatographic conditions as in Fig. 1.

Bile acid	Retention volume (ml)			
	Sodium acetate concentration (*M*)			
	0.01 (Fig. 1A)	0.02 (Fig. 1B)	0.03 (Fig. 1C)	0.04 (Fig. 1D)
TUDCA	3.1	3.7	4.1	4.3
GUDCA	4.4	4.8	5.1	5.2
TCA	4.4	5.4	6.1	6.5
GCA	6.9	7.7	8.1	8.5
TCDCA	6.9	9.2	10.5	11.4
TDCA	8.2	10.8	12.4	13.4
GCDCA	12.4	14.1	14.7	15.5
TLCA	14.1	18.8	20.9	21.9
GDCA	14.7	16.6	17.5	18.3
UDCA	16.6	16.6	16.3	16.2
CA	23.1	23.5	23.1	23.0
GLCA	23.1	24.8	25.3	26.0
CDCA	31.9	32.0	31.7	31.7
DCA	32.9	32.9	32.6	32.7
LCA	39.0	39.0	38.8	38.9

increase in retention of the conjugates with sodium acetate in the mobile phase can be traced to ionic suppression, which enhances the lipophilic character of the molecules, hence their interaction with the bonded phase. Owing to the low pK values of the taurine conjugates (p$K \approx 2$) [20], their retention is influenced more distinctly by the ionic strength of the eluent, at pH 4.3, than that of the glycine conjugates (p$K \approx 4$) [20], as also found by other workers [2]. Conversely, the chromatography of free bile acids is not affected by the molarity of the acetate buffer, as their carboxyl groups (p$K \approx 6$) [20] are non-ionized under the above pH elution conditions. The reversal in elution order for the triplet TLCA (8), GCDCA (9) and UDCA (10) as the buffer concentration increases (Fig. 1) reflects the increase in retention of the conjugated bile acids.

The complete baseline resolution of UDCA, CA, CDCA, DCA, LCA and the corresponding glycine and taurine conjugates, using the system described here, is

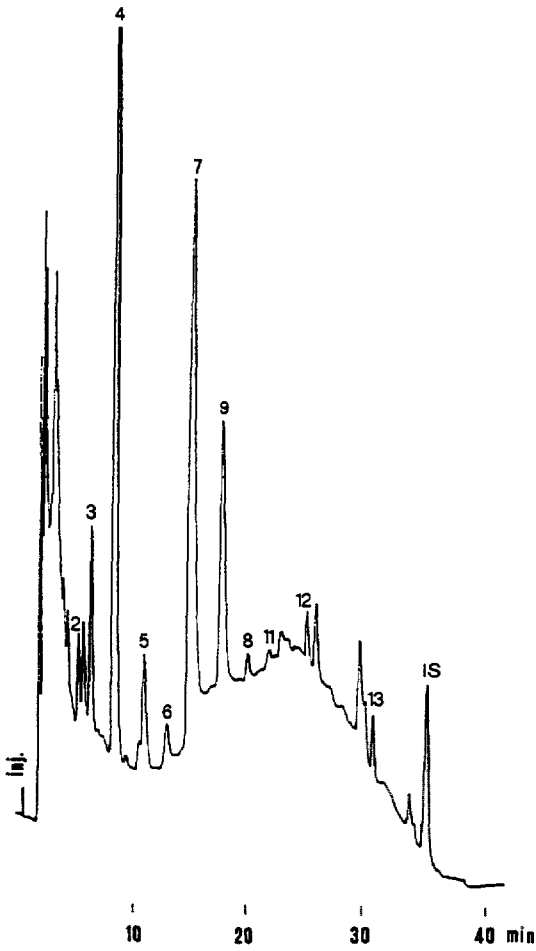

Fig. 2. Chromatogram of the separation of intragastric bile acids from a patient submitted to gastric surgery. Conditions and peak identification as in Fig. 1C; IS = 7α,12α-dihydroxy-5β-cholanic acid (internal standard).

shown in Fig. 1C. The foregoing bile acids were resolved into fifteen well defined peaks in 40 min using a gradient of methanol in 0.03 *M* sodium acetate. Methanol has an inherently high UV cut-off at 200 nm where conjugated bile acids are optimally detected [4]. This property causes baseline instability during gradient elution [4], which is significantly reduced by operating at 210 nm. Moreover, the molarity of the buffer in the stronger solvent (legend to Fig. 1) has to be accurately adjusted in order to avoid severe drift of the baseline reflecting the gradient profile of methanol in the mobile phase.

The baseline separation of unconjugated, glyco- and tauro-conjugated bile acids in a single chromatographic run has not been reported before; in fact, previously published HPLC methods [14–17] for the simultaneous analysis of the foregoing compounds produced unsatisfactory resolution of several component peaks and required longer elution times (50 and 120 min).

Application

The 0.03 *M* sodium acetate–methanol gradient RP-HPLC system was applied to the assay of free and conjugated bile acids in gastric juice samples from patients with endoscopic evidence [18] of bile reflux gastritis. The bile acids were extracted from the gastric aspirates by Sep-Pak C_{18} cartridges (see Experimental) and their unconjugated, glyco- and tauro-conjugated forms were separated by RP-HPLC in a single analysis. A chromatogram of a representative gastric juice sample from a subject with a history of previous gastric surgery is shown in Fig.

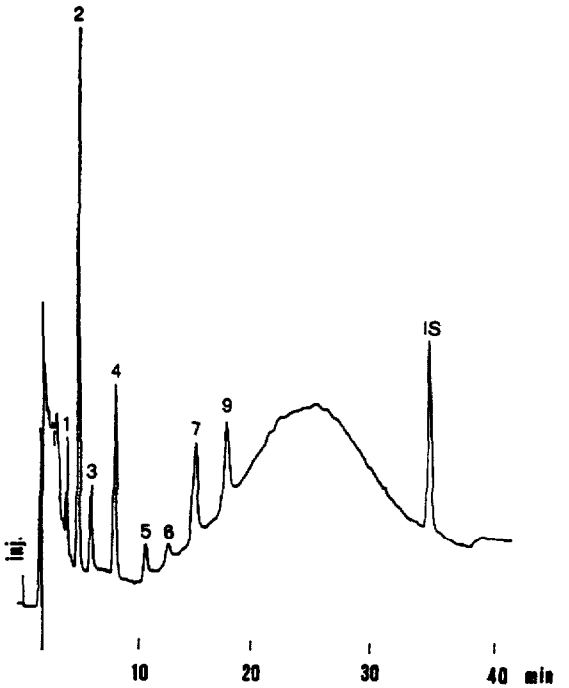

Fig. 3. RP-HPLC separation of intragastric bile acids from a patient with no previous gastric surgery after UDCA therapy (300 mg/day). Conditions and peak identification as in Figs. 1C and 2.

2. The chromatogram of a sample from a patient after UDCA treatment is illustrated in Fig. 3.

Linear correlations were obtained between peak areas and concentrations of bile acids in the range 0.55–50 mmol/l for free bile acids (UDCA, $r = 0.998$, $a = 0.13$, $b = 0.11$; CA, $r = 0.999$, $a = 0.097$, $b = 0.041$; CDCA, $r = 0.999$, $a = 0.087$, $b = 0.03$; DCA, $r = 0.998$, $a = 0.091$, $b = 0.034$; LCA, $r = 0.999$, $a = 0.098$, $b = 0.059$) and 0.025–3.5 mmol/l for bile acid conjugates (TUDCA, $r = 0.999$, $1 = 2.6$, $b = 0.002$; GUDCA, $r = 0.999$, $a = 4.03$, $b = 0.034$; TCA, $r = 0.999$, $a = 2.15$, $b = 0.006$; GCA, $r = 0.999$, $a = 4.3$, $b = 0.002$; TCDCA, $r = 0.999$, $a = 2.5$, $b = 0.04$; TDCA, $r = 0.999$, $a = 3.0$, $b = 0.067$; GCDCA, $r = 0.999$, $a = 3.5$, $b = 0.026$; GDCA, $r = 0.999$, $a = 3.8$, $b = 0.028$; TLCA, $r = 0.998$, $a = 2.6$, $b = 0.065$;

TABLE II

RECOVERY OF FREE AND CONJUGATED BILE ACIDS ADDED TO HUMAN GASTRIC JUICE

Bile acid	Gastric juice (μmol/ml)	Added (μmol/ml)	Expected (μmol/ml)	Recovery (mean ± S.D., $n = 5$) (%)
TUDCA	0.0	0.038	0.038	94.7 ± 1.4
		0.196	0.196	100.5 ± 0.8
GUDCA	0.11	0.050	0.16	103.7 ± 0.8
		0.245	0.355	98.6 ± 1.5
TCA	0.611	0.058	0.669	96.1 ± 2.4
		0.296	0.906	97.8 ± 1.1
GCA	2.81	0.047	2.857	98.4 ± 1.7
		0.239	3.049	89.4 ± 1.3
TCDCA	0.452	0.081	0.533	95.9 ± 2.9
		0.419	0.869	95.5 ± 2.2
TDCA	0.127	0.056	0.183	101.6 ± 1.9
		0.294	0.421	92.2 ± 1.3
GCDCA	2.949	0.068	3.017	99.5 ± 2.5
		0.339	3.288	102.2 ± 1.4
UDCA	0.0	1.171	1.171	90.2 ± 3.8
		2.897	2.897	98.8 ± 2.2
GDCA	1.149	0.067	1.216	98.8 ± 0.8
		0.345	1.494	93.4 ± 1.5
TLCA	0.07	0.052	0.122	86.9 ± 2.1
		0.254	0.324	90.7 ± 2.7
CA	1.341	1.248	2.589	87.2 ± 3.4
		4.313	5.654	89.2 ± 3.1
GLCA	0.127	0.051	0.178	91.0 ± 1.8
		0.259	0.386	88.6 ± 1.2
CDCA	3.79	1.009	4.799	94.9 ± 2.9
		2.531	6.321	102.7 ± 2.5
DCA	0.0	1.110	1.110	88.5 ± 3.0
		2.802	2.802	94.1 ± 2.8
LCA	0.0	1.264	1.264	86.2 ± 2.2
		3.054	3.054	88.7 ± 2.4

GLCA, $r = 0.998$, $a = 4.2$, $b = 0.015$). The detection limits at 210 nm ranged from 0.48 to 0.55 mmol/l for unconjugated bile acids and from 0.015 to 0.025 mmol/l for conjugated bile acids.

Known amounts of standard bile acids were added to human gastric juice and their recoveries calculated. As shown in Table II, the recoveries of individual bile acids were at least 86%. The lowest values were recorded for some of the free bile acids which were incompletely extracted by the Sep-Pak procedure. The relative standard deviation of each bile acid concentration ranged from 3.5 to 5.1% ($n = 10$) for the intra-assay reproducibility and from 5.5 to 8.6% ($n = 10$) for the inter-assay reproducibility.

The simultaneous determination of free, glyco- and tauro-conjugated bile acids was carried out on gastric juice samples from six patients with bile reflux gastritis. The levels of bile acids were corrected according to the recoveries of the internal standard and the results are presented in Table III.

In accordance with earlier studies [21–23], free bile acids were detected only in the group of subjects submitted to gastric surgery (Table III, patients 1 and 2); the levels determined by the RP-HPLC procedure described here are in good agreement with those measured enzymatically by the 3α-hydroxysteroid dehydrogenase method [22,23]. Moreover, total bile acid titres recorded in this study (Table III, patients 1–3) are within the concentration range reported in previous investigations [23,24].

TABLE III

BILE ACID COMPOSITION OF GASTRIC JUICE OF PATIENTS SUBMITTED TO GASTRIC SURGERY (1-3) AND PATIENTS UNDER UDCA THERAPY (4-6)

Bile acid	Percentage of total bile acids								
	Patient								
	1	2	3	4		5		6	
				B*	A*	B*	A*	B*	A*
TUDCA	-	-	-	1.4	7.2	-	3.0	-	-
GUDCA	0.8	2.5	-	4.2	36.2	0.8	24.2	1.4	16.8
TCA	4.5	3.6	5.4	3.1	5.3	7.0	2.2	17.8	10.8
GCA	20.8	14.3	55.1	8.9	16.2	28.7	16.9	41.3	51.1
TCDCA	3.3	2.9	13.0	5.3	6.0	14.3	2.8	4.1	2.1
TDCA	1.0	2.1	5.9	8.0	2.9	1.0	2.0	8.1	1.0
GCDCA	21.7	11.1	13.6	18.5	15.3	22.6	26.5	8.0	7.6
GDCA	8.5	22.6	6.9	50.6	10.9	25.5	21.0	17.9	10.4
TLCA	0.5	-	-	-	-	-	-	1.4	-
CA	9.9	38.7	-	-	-	-	-	-	-
GLCA	1.0	2.1	-	-	-	-	1.4	-	-
CDCA	28.0	-	-	-	-	-	-	-	-
TBA** (mmol/l)	13.53	2.79	0.16	0.98	0.63	1.57	8.55	0.86	1.18

*B, before treatment; A, after treatment.

**TBA, total bile acid concentration.

During UDCA therapy of bile reflux gastritis in subjects with no history of previous gastric surgery (Table III, patients 4–6), GUDCA increased substantially whereas the percentage composition of both glycine and taurine conjugates of deoxycholic acid decreased. This is in accordance with other reports [18,25]. Furthermore, virtually identical values were obtained for bile acid conjugates when the present method and the previously adopted procedure [18] were used on the same sample of gastric juice.

CONCLUSION

An HPLC method without prior group separation has been developed for the simultaneous determination of the free bile acids and of the corresponding glycine and taurine conjugates in human gastric juice. The only limitation of this technique is the low sensitivity in the detection of unconjugated bile acids; however, samples containing a higher proportion of the latter compounds [22,23] can be satisfactorily assayed (see Table III). The advantages offered by this RP-HPLC procedure are minimal sample preparation, circumventing preliminary fractionation into the free and conjugated forms of these steroids. Further, the well resolved peaks permit unequivocal identification and accurate and rapid quantification of the bile acids suitable for routine clinical applications.

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