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RAPID AND ACCURATE REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC DETERMINATION OF CONJUGATED BILE ACIDS IN HUMAN BILE FOR ROUTINE CLINICAL APPLICATIONS

THERAPEUTIC CONTROL DURING GALLSTONE DISSOLUTION THERAPY

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SUMMARY

This paper introduces a new method to detect the taurine and glycine conjugates of five different bile acids (cholic acid, deoxycholic acid, chenodeoxycholic acid, ursodeoxycholic acid and lithocholic acid) in human bile. Advantages of this method are sufficient separation of compounds within a short period of time and a high rate of reproducibility. Using a mobile phase gradient of acetonitrile and water, modified with tetrabutylammonium hydrogen sulphate (0.0075 mol/l), we were able to maximize the differentiation between ursodeoxycholic acid and lithocholic acid, which is of primary interest during conservative gallstone dissolution therapy. Use of this gradient reduced analysis time to less than 0.5 h. Recovery rates for this modified method ranged from 94% to 100%, and reproducibility was 98%, sufficient for routine clinical applications.

INTRODUCTION

A rapid and simple high-performance liquid chromatographic (HPLC) method is essential for routine clinical evaluation of alterations in conjugated bile acid pattern during gallstone dissolution therapy.

HPLC is capable of analysing glycine and taurine conjugates and producing accurate data within a short period of time, whereas other methods used to detect bile acids have distinct disadvantages. For example, enzyme kits do not differentiate between conjugated bile acids [1, 2], bioluminescence only

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estimates the total amount of 3-α-hydroxy bile acids [3] and gas chromatography requires a substantial amount of time [4, 5].

During conservative gallstone dissolution therapy it is beneficial to monitor ursodeoxycholic acid (UDCA) and chenodeoxycholic acid (CDCA) via conjugated bile acid levels in biological fluids. For this purpose it was necessary to slightly modify previously published HPLC methods [6—9] in order to increase reproducibility and enhance the separation of conjugates within a shorter period of time. Many authors preferred methanol as the main component of the mobile phase [6—8, 10]. However, in the range of 200 nm, where conjugated bile acids are optimally detected, methanol has a high UV cut-off. Therefore we employed an acetonitrile—water gradient, modified with tetrabutylammonium (TBA) hydrogen sulphate. Using this ion-suppressive and ion-pairing HPLC mobile phase we were able to monitor taurine and glycine conjugates, especially UDCA and lithocholic acid (LCA) components, which are of primary interest during conservative gallstone dissolution therapy.

MATERIALS AND METHODS

Instruments

Two Constametric II HPLC pumps combined with a Gradient Master 1601, were equipped with a variable-wavelength UV spectrophotometer (LDC-Milton Roy, Hasselroth, F.R.G.) and a μBondapak C_{18} steel column (10 μm, 300 × 3.9 mm, Waters Assoc., Milford, MA, U.S.A.). A Hewlett-Packard 3380 A integrator graphed and calculated peak areas. Parameter variables were set as follows: a flow-rate of 1.5 ml/min, wavelength detection at 200 nm, within a range of 0.04 a.u.f.s. and an attenuation of 64.

The mobile phase gradient increased exponentially (mixing gradient, $m = 2$) within 30 min from an initial concentration of 10% solvent B, 90% solvent A, to a final concentration of 60% solvent B. Standards were weighed with a Sartorius Scale 2004, precision, $d = 0.01$ mg, and bile acids from human bile were extracted using Sep-Pak C_{18} cartridges (Waters Assoc.). Extracted samples were filtered through a 0.2-μm disposable filter (Gelman Sciences, Ann Arbor, MI, U.S.A.).

Chemicals

Mobile phase solvents were of HPLC grade (Fluka, Buchs, Switzerland; E. Merck, Darmstadt, F.R.G.), further purified by filtration through a 0.45-μm organic filter (Schleicher and Schüll, Düsseldorf, F.R.G.) and degassed under vacuum in an ultrasonic water bath. Solvent A = acetonitrile—water (30:70) plus 0.0075 mol/l TBA; pH adjusted to 2.5. Solvent B = acetonitrile—water (60:40) plus 0.0075 mol/l TBA; pH adjusted to 2.5.

TBA hydrogen sulphate purissimum was purchased from Fluka. Purified conjugated bile acids were purchased from Calbiochem (La Jolla, CA, U.S.A.) and Sigma (St. Louis, MO, U.S.A.).

Patients

Bile from three patients with choledochal calculi was obtained by suction via a nasobiliary tube before and after medical dissolution therapy. Three additional patients with gallbladder calculi were treated with 500 mg of urso-

deoxycholic acid (Ursofalk®) per day and bile was obtained by endoscopic intubation of the choledochus.

Isolation

Prior to extraction a Sep-Pak C_{18} cartridge was rinsed with 3 ml of methanol and washed with 10 ml of water. Then 0.1 ml of hepatic bile, diluted with 5 ml of 0.07 mol/l phosphate buffer (pH 7.0, according to Sjörensen) and 120 μg of dexamethasone, added as an internal standard, were loaded onto the cartridge, followed by 10 ml of water, 3 ml of 10% acetone, and an additional 10 ml of water [11, 12]. Bile acids were slowly eluted from the cartridge with 3 ml of methanol. The filtrate was evaporated under a nitrogen stream and the residue dissolved in 0.3 ml of solvent B. After filtration through a 0.2-μm filter assembly, an injection volume of 20 μl was analysed by the HPLC apparatus.

RESULTS AND DISCUSSION

Selectivity

The separation of a standard mixture consisting of dexamethasone and five different bile acids conjugated with glycine and taurine is shown in Fig. 1. Individual injections of purified bile acids are used in order to correlate the retention time with the corresponding bile acid. Column retention behaviour is

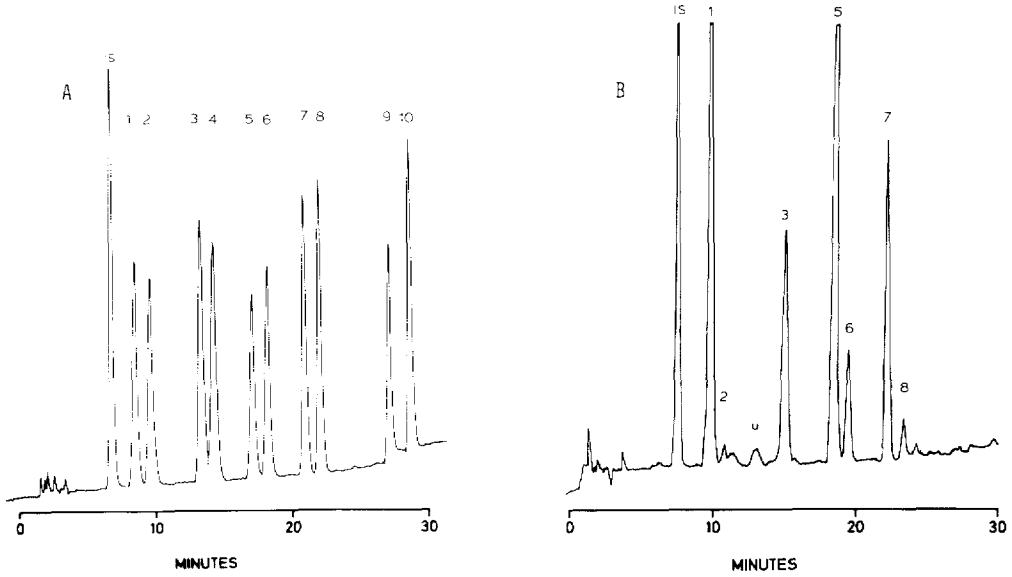

Fig. 1. (A) Chromatogram of a standard mixture of synthetic conjugated bile acids. Peaks: IS = internal standard (dexamethasone); 1 = glycocholic acid (GCA); 2 = glycoursodeoxycholic acid (GUDCA); 3 = taurocholic acid (TCA); 4 = tauroursodeoxycholic acid (TUDCA); 5 = glycochenodeoxycholic acid (GCDCA); 6 = glycodeoxycholic acid (GDCA); 7 = taurochenodeoxycholic acid (TCDCA); 8 = taurodeoxycholic acid (TDCA); 9 = glycolithocholic acid (GLCA); 10 = taurolithocholic acid (TLCA). (B) The distribution of glycine and taurine conjugates in normal human bile obtained by endoscopic choledochal intubation (peaks are labelled as shown in Fig. 1A; u = unknown peak); TUDCA is not detected; GUDCA, GLCA and TLCA are present only in trace amounts.

TABLE I

CAPACITY FACTORS (k', rk') OF TEN CONJUGATED BILE ACIDS AND DEXAMETHASONE IN A STANDARD SAMPLE USING THE PRESENTED HPLC METHOD

Bile acid	k'	rk'
Dexamethasone	4.42	0.35
Glycocholate	5.78	0.46
Glycoursodeoxycholate	6.66	0.53
Taurocholate	9.55	0.76
Tauroursodeoxycholate	10.27	0.82
Glycochenodeoxycholate	12.51	1.00
Glycodeoxycholate	13.37	1.07
Taurochenodeoxycholate	15.43	1.23
Taurodeoxycholate	16.30	1.30
Glycolithocholate	20.32	1.62
Taurolithocholate	21.50	1.72

described by the capacity factor k' and the relative capacity factor rk' (relative to glycochenodeoxycholate) (Table I).

Optimal separation of conjugated bile acids in standard mixtures as well as in biological samples could be obtained using a TBA-modified acetonitrile—water gradient. (TBA is usually applied as a modifier in ion-pair chromatography.)

Da Shi Lu et al. [13] doubt the ion-pair process of TBA for the separation of bile acids at a mobile phase pH lower than 2.85. Our results clearly indicate that an acetonitrile—water gradient modified with TBA at pH 2.5 increases separation as opposed to an acetonitrile—phosphate buffer (0.01 mol/l potassium dihydrogen phosphate) gradient at the same pH, without TBA. Due to the low pK values of taurine conjugates (taurocholic acid: pK = 1.4), TBA may enhance separation at pH 2.5 by ion pairing, while ion suppression may be responsible for separation of conjugates with pK values above 3, such as glycocholic acid (pK = 4.4). Thus, from our experience, we postulate that a combination of ion suppression and TBA ion pairing is responsible for enhancing bile acid conjugate separation. TBA may also interact with free residual silanol groups of the stationary phase, therefore creating an additional factor responsible for optimizing separation [14].

Methanol has an inherently high UV cut-off at 200 nm, where bile acid conjugates are optimally detected. This property causes a high noise-to-signal ratio, which can be significantly reduced by employing acetonitrile.

Dexamethasone proved to be superior to oestrogen [15] and testosterone [16] as internal standard because it does not occur naturally in biological fluids and does not overlap with glycoursodeoxycholic acid (GUDCA) [6] or with glycocholic acid (GCA) (see Fig. 1).

Quantitative analysis

Known amounts of conjugated bile acids (0.5—15.0 μg per injection) were analysed in order to determine the detection response ratio (DRR = response bile acid/response internal standard). Calibration curves were graphed using this

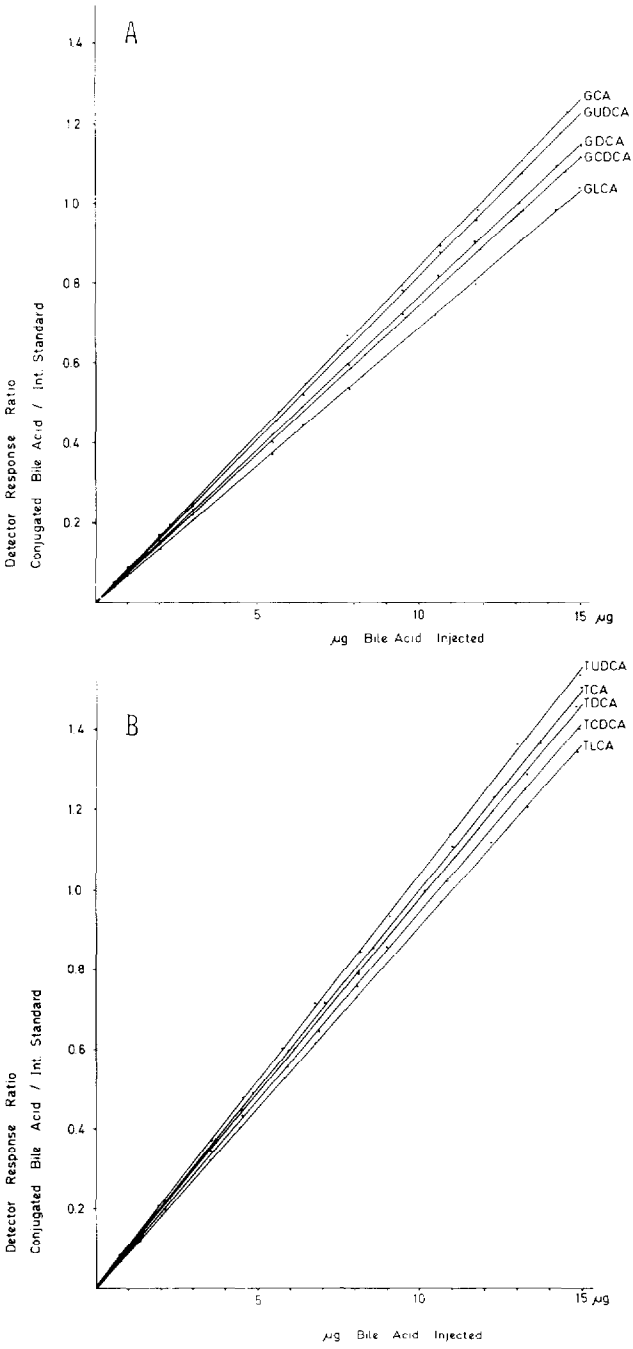

Fig. 2. Calibration curves for glycine (A) and taurine (B) conjugates (see Fig. 1 for abbreviations). The ratio of conjugated bile acid to internal standard (int. standard) was plotted against bile acid concentrations ranging from 0.5 to 15 μg per injection. The linearity of the graph indicates an excellent correlation between peak height ratio and amount injected. Correlation factors are: $r_{GCA} = 0.998$; $r_{TCA} = 0.999$; $r_{GCDCA} = 0.999$; $r_{TCDCA} = 0.999$; $r_{GDCA} = 0.998$; $r_{TDCA} = 0.999$; $r_{GUDCA} = 0.996$; $r_{TUDCA} = 0.999$; $r_{GLCA} = 0.996$; $r_{TLCA} = 0.998$.

information. The accuracy of the presented HPLC method is demonstrated in the graphs by the small deviations of the plotted points from linearity. Correlation factors (r) range from 0.996 to 0.999 (see Fig. 2).

A correlation of quantitative analysis between our HPLC method and gas chromatography was not performed due to the inconsistent conjugate recovery when Sephadex columns are employed for gas chromatography [17].

Recovery

The test samples were prepared by adding known amounts of different synthetic bile acids to human hepatic bile of a UDCA-treated patient. The results are shown in Table II. Our recovery range of 94.6—100% is sufficient for clinical application.

Reproducibility

Reproducibility was tested by analysing 20 μl on ten different days taken from the same stock sample which was isolated from hepatic bile. Results are

TABLE II

RECOVERY OF CONJUGATED BILE ACIDS ADDED TO HUMAN BILE OF A UDCA-TREATED PATIENT

Bile acid	Bile (μg per 100 μl)	Added (μg per 100 μl)	Expected (μg per 100 μl)	Found (μg per 100 μl, mean ± S.D., n = 4)	Recovery (%, mean ± S.D., n = 4)	Recovery (%, mean ± S.D., n = 12)
Glycocholate	12.275	81.90	94.175	92.87 ± 1.56	98.40 ± 1.90	99.00 ± 1.54
		40.95	53.225	52.75 ± 0.77	99.10 ± 1.45	
		13.65	25.925	25.80 ± 0.38	99.51 ± 1.47	
Glycoursodeoxycholate	71.05	96.00	167.05	165.15 ± 1.30	98.86 ± 0.78	99.29 ± 1.05
		48.00	119.05	118.85 ± 1.74	99.83 ± 1.46	
		16.00	87.05	86.77 ± 0.52	99.19 ± 0.83	
Taurocholate	2.59	84.60	87.19	88.26 ± 0.70	101.22 ± 0.79	100.65 ± 1.43
		42.30	44.89	45.06 ± 0.69	100.38 ± 1.54	
		14.30	16.89	16.95 ± 0.33	100.36 ± 1.96	
Tauroursodeoxycholate	4.15	81.90	86.05	87.43 ± 1.01	101.59 ± 1.16	100.39 ± 2.29
		40.95	45.10	45.60 ± 0.64	101.12 ± 1.41	
		13.65	17.80	17.53 ± 0.52	98.47 ± 2.90	
Glycochenodeoxycholate	39.13	75.30	114.43	112.44 ± 2.70	98.25 ± 2.36	98.30 ± 1.52
		37.65	76.78	75.80 ± 0.74	98.72 ± 0.97	
		12.55	51.68	50.61 ± 0.66	97.93 ± 1.27	
Glycodeoxycholate	4.29	75.00	79.29	77.45 ± 0.64	97.68 ± 0.81	97.20 ± 1.22
		37.50	41.79	40.64 ± 0.58	97.24 ± 1.40	
		12.50	16.79	16.26 ± 0.26	96.70 ± 1.50	
Taurochenodeoxycholate	7.4	76.35	83.75	80.92 ± 0.34	96.62 ± 0.41	96.65 ± 1.49
		38.18	45.58	44.34 ± 0.54	97.94 ± 1.51	
		12.73	20.13	19.31 ± 0.44	95.93 ± 2.21	
Taurodeoxycholate	0	76.65	76.65	77.73 ± 0.84	101.40 ± 1.09	100.20 ± 2.13
		38.33	38.33	37.54 ± 0.90	98.61 ± 2.11	
		12.78	12.78	12.88 ± 0.33	100.59 ± 2.35	
Glycolithocholate	0.93	79.95	80.88	76.95 ± 1.04	95.15 ± 1.28	94.61 ± 1.98
		39.98	40.91	38.82 ± 0.98	94.89 ± 2.38	
		13.33	14.26	13.37 ± 0.35	93.79 ± 2.40	
Taurolithocholate	0	78.15	78.15	76.28 ± 1.61	97.60 ± 2.05	95.86 ± 2.77
		39.08	39.08	37.60 ± 0.61	96.22 ± 1.54	
		12.03	12.03	11.28 ± 0.47	93.76 ± 2.39	

TABLE III

REPRODUCIBILITY OF THE CHROMATOGRAPHIC PROCEDURE FOR DETERMINATION OF BILE ACIDS IN HUMAN BILE

The sample was injected ten times on different days.

Bile acid conjugate	Mean ± S.D. (mmol/l) (n = 10)	Percentage deviation of the mean
Glycocholic acid	3.576 ± 0.035	0.98
Glycoursodeoxycholic acid	7.655 ± 0.055	0.72
Taurocholic acid	0.861 ± 0.026	3.02
Tauroursodeoxycholic acid	0.659 ± 0.032	4.86
Glycochenodeoxycholic acid	5.611 ± 0.017	0.30
Glycodeoxycholic acid	5.643 ± 0.014	0.25
Taurochenodeoxycholic acid	1.021 ± 0.020	1.96
Taurodeoxycholic acid	0.841 ± 0.012	1.43
Glycolithocholic acid	0.390 ± 0.028	7.17
Taurolithocholic acid	0.253 ± 0.021	8.30

shown in Table III. The reproducibility ranged from 0.3% for the primary bile acid glycochenodeoxycholic acid (GCDCA) up to 8.3% for the secondary bile acid taurolithocholic acid (TLCA). The larger deviation of secondary bile acids is due to their generally lower concentrations.

Interassay variability

Interassay variability includes repeated Sep-Pak extractions and HPLC analyses of the same bile sample in order to determine the consistency of the entire method. Results are shown in Table IV. In particular, GUDCA and GCDCA showed excellent reproducibility and low inter-assay variability.

TABLE IV

INTERASSAY VARIABILITY OF THE CHROMATOGRAPHIC PROCEDURE INCLUDING THE ISOLATION STEPS FOR DETERMINATION OF BILE ACIDS IN HUMAN BILE

The conjugated bile acids were isolated ten times from an identical bile sample and determined by HPLC

Bile acid conjugate	Mean ± S.D. (mmol/l) (n = 10)	Percentage deviation of the mean
Glycocholic acid	3.551 ± 0.073	2.05
Glycoursodeoxycholic acid	7.368 ± 0.176	2.38
Taurocholic acid	1.198 ± 0.048	4.00
Tauroursodeoxycholic acid	0.598 ± 0.046	7.69
Glycochenodeoxycholic acid	5.460 ± 0.075	1.35
Glycodeoxycholic acid	5.425 ± 0.063	1.16
Taurochenodeoxycholic acid	1.023 ± 0.023	2.25
Taurodeoxycholic acid	0.844 ± 0.021	2.49
Glycolithocholic acid	0.331 ± 0.028	8.45
Taurolithocholic acid	0.220 ± 0.020	9.09

Patient data

The varying amounts of conjugates in human bile are shown in Table V. Patients 1—3 had choledochal calculi; in patient 3 partial obstruction of the bile duct was present. Due to the obstruction, elevated taurine conjugate levels could be observed (see Fig. 3).

Bile was extracted from patients 4—6 by endoscopic choledochal intubation during medical dissolution therapy (500 mg UDCA per day) of gallbladder calculi. During therapy, GUDCA increased substantially, while primary bile acid levels, in particular GCA, decreased (see Fig. 4). This is in accordance with other reports in the literature [18]. A less substantial increase in taurine conjugates, especially UDCA and LCA, was also observed.

In conclusion, the presented method allows the conjugated bile acid pattern in human bile to be monitored in a short period of time. The separation module employed is less laborious than others presented in the literature and guarantees good reproducibility. Using this method concentrations as low as 0.5 μg per injection can be detected, possibly allowing analysis of serum samples.

TABLE V

CONJUGATED BILE ACIDS IN HUMAN BILE IN PATIENTS WITH CHOLEDOCHAL CALCULI (1—3) AND PATIENTS UNDER DISSOLUTION THERAPY OF GALLBLADDER CALCULI (4—6)

Values are expressed in mmol/l.

Patient	GCA	GUDCA	TCA	TUDCA	GCDCA	GDCA	TCDCA	TDCA	GLCA	TLCA
1	10.14	1.69	3.67	0.58	9.25	6.32	3.98	2.735	—	—
2	9.89	0.98	4.63	0.29	8.35	6.59	4.07	1.36	0.27	—
3	7.03	—	9.35	—	3.37	0.83	3.46	0.95	—	—
4	3.55	7.37	1.20	0.60	5.46	5.42	1.02	0.84	0.33	0.22
5	3.82	13.53	1.61	2.14	6.35	3.60	2.29	0.68	—	—
6	2.92	9.86	1.34	1.65	6.13	4.83	1.85	0.73	0.42	—

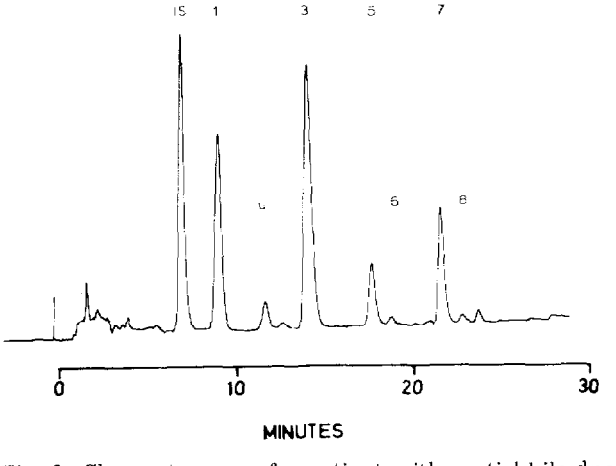

Fig. 3. Chromatogram of a patient with partial bile duct obstruction. In particular, TCA (3) and TCDCA (7) conjugate levels are elevated. LCA is not detected. Complete pattern reversal was observed after endoscopic removal of the obstruction. Peaks are labelled as in Fig. 1; u = unknown peak.

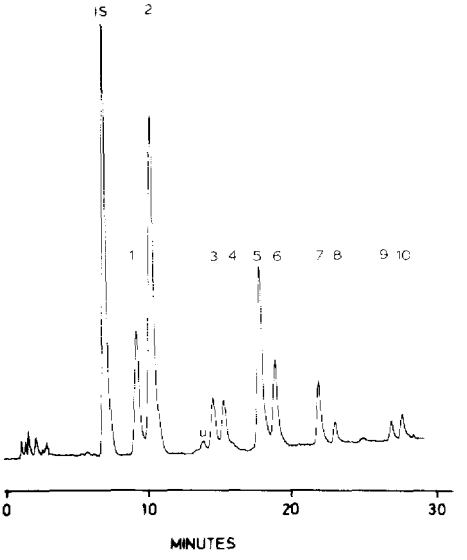

Fig. 4. Bile acid conjugate pattern from a UDCA-treated patient (500 mg per day). An increase in GUDCA (2) and TUDCA (4) peaks is due to therapy. In comparison to Fig. 1B, cholic acid conjugate levels are decreased. Peaks are labelled as in Fig. 1; u = unknown peak.

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