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ANALYSIS OF CONJUGATED BILE ACIDS IN HUMAN SERUM BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY USING POST-COLUMN ENZYME REACTION AND OFF-LINE FLUORIMETRIC DETERMINATION

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SUMMARY

An assay for glycine and taurine conjugates of cholic (GC, TC), chenodeoxycholic (GCDC, TCDC), deoxycholic (GDC, TDC) and ursodeoxycholic (GUDC, TUDC) acids in human serum by high-performance liquid chromatography using an immobilized 3-α-hydroxysteroid dehydrogenase post-column reaction and an off-line spectrofluorimetric detection is described. The detection limit is 0.2 μM. Recoveries of extraction and chromatographic analysis range from 89.50 to 91.68% and from 96.75 to 98.83%, respectively. The analysis in serum reveals a coefficient of variation for reproducibility between 16.2% for GC and 4.7% for TDC; inter-assay variability ranges between 4.06% for TCDC and 8.24% for TUDC.

INTRODUCTION

Bile acids in serum are usually conjugated to glycine and taurine, except in some liver diseases. The analysis of the serum bile acid profile is required for diagnostic purposes in these liver diseases, cholestasis and primary biliary cirrhosis [1, 2]. It is also of major interest in the therapeutic control of gallstone

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dissolution therapy with urso- and chenodeoxycholic acid. The correlation between the biliary and the serum bile acid patterns in these patients is the subject of our work.

Recently, several methods have been used to analyse bile acids in serum, such as thin-layer chromatography [3], gas–liquid chromatography [4], radioimmunoassay [5], reversed-phase high-performance liquid chromatography (RP-HPLC) with UV detection [6–9] and HPLC with fluorimetric detection [10, 11].

This paper describes a newly developed HPLC analysis, using an immobilized 3α-hydroxysteroid dehydrogenase (3α-HSD) post-column reaction, fraction collection and off-line fluorimetric determination, which allows the quantitative analysis of eight major bile acid conjugates in human serum.

EXPERIMENTAL

Reagents and chemicals

The bile acids [glycine- and taurine-conjugated cholate (GC, TC), chenodeoxycholate (GCDC, TCDC), deoxycholate (GDC, TDC) and ursodeoxycholate (GUDC, TUDC)], 3α-hydroxysteroid dehydrogenase and NAD were purchased from Sigma (St. Louis, MO, U.S.A.) and Calbiochem (San Diego, CA, U.S.A.). Tetrabutylammonium hydrogensulphate (TBA) was purchased from Fluka (Buchs, Switzerland). Mobile phase solvents and NAD solution reagent were of HPLC grade (Merck, Darmstadt, F.R.G.), further purified by filtration through a 0.45-μm organic filter (Schleicher and Schuell, Düsseldorf, F.R.G.) and degassed under vacuum in an ultrasonic water-bath.

The HPLC solvents were: solvent A, acetonitrile–water (30:70, v.v) containing 0.0075 *M* TBA (pH adjusted to 2.5); solvent B, acetonitrile–water (60:40, v/v) containing 0.0075 *M* TBA (pH adjusted to 2.5).

The NAD stock solution contained 1 m*M* NAD, 50 m*M* Tris–HCl, 1 m*M* EDTA, 3.15 m*M* dithiothreitol and 0.1 *M* hydrazine hydrate (pH adjusted to 8.5).

Apparatus

A schematic flow diagram of the applied system is shown in Fig. 1. Two HPLC pumps (Constametric I and Constametric II, LDC Milton Roy, Hasselroth, F.R.G.) were combined with a gradient elution controller (Gradient Master 1601, LDC Milton Roy) and connected to a guard column, a separating column (μBondapak C_{18}, 10 μm, 30×3.9 cm I.D., Waters, Milford, MA, U.S.A.), a T-connector and an immobilized 3α-HSD enzyme column (post-column reaction) (100×1.8 mm I.D. steel column, Bischoff, Leonberg, F.R.G.). The system was additionally equipped with a fluorescence HPLC monitor (RF-530, Shimadzu, Kyoto, Japan), an integrator (C-R3A Chromatopac, Shimadzu) and a fraction collector (FRAC-100, Pharmacia, Freiburg, F.R.G.). NAD reagent was delivered by a third pump (Constametric III, LDC Milton Roy).

After fraction collection, every bile acid conjugate was quantified with a spectral fluorimeter (Hitachi 650-10S, Colora, Lorch, F.R.G.) after renewed fluorescent labelling with a commercially available enzyme kit (Sterognost-3α Flu®, Nyegaard, Oslo, Norway).

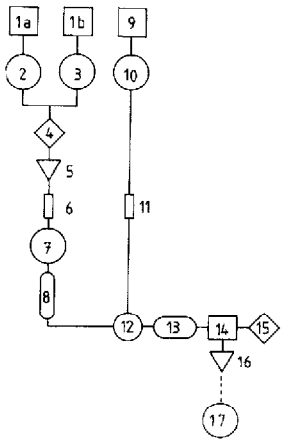

Fig. 1. Diagram of the HPLC system. 1a, 1b = Mobile phase solvents; 2, 3, 10 = pumps; 4 = gradient elution controller; 5 = mixer; 6, 11 = precolumn; 7 = sample injector; 8 = column; 9 = NAD reservoir; 12 = T-connector; 13 = immobilized enzyme (post-column qualitative analysis); 14 = HPLC fluorimeter; 15 = integrator; 16 = fraction collector; 17 = spectral fluorimeter for off-line reaction (post-column quantitative analysis).

Chromatographic conditions

The flow-rates of the mobile phase solvents and the NAD reagent were set to 1 and 0.5 ml/min, respectively. The mobile phase gradient was linear (mixing gradient, $m = 1$) during a 40-min period from an initial ratio of 95% solvent A to 5% solvent B to a final ratio of 35% solvent A to 65% solvent B. The excitation wavelength was set at 350 nm and the emission wavelength at 465 nm, with the sensitivity "high" and an attenuation of 4.

Serum samples

Serum samples were obtained from six subjects, a healthy man, three patients with cirrhosis of the liver, a patient with cholestasis due to carcinoma of the Sphincter of Oddi and a patient with choledochal calculi.

Extraction

A 5-ml volume of serum and 20 ml of ethanol–diethyl ether (3:1, v/v) were mixed, dispersed by sonication, heated for several minutes and decanted to remove protein, mucus and bile pigment precipitates. The decanted fraction was evaporated to dryness at 40°C under vacuum. The residue was then dissolved in 5 ml of 0.1 *M* sodium hydroxide in 0.9% sodium chloride. A reversed-phase C_{18} cartridge was activated with 3 ml of methanol and 10 ml of water. The cartridge was filled with the solution and then neutralized with 10 ml of water, followed by 3 ml of 10% acetone and 3 ml of water [9, 12]. Next, the bile acids were slowly eluted with 3 ml of methanol. The eluate was evaporated to dryness under a stream of nitrogen at 60°C, and the residue was dissolved in 200 μl of solvent B. After filtration through a 0.2-μm filter, 20-μl samples were injected for analysis.

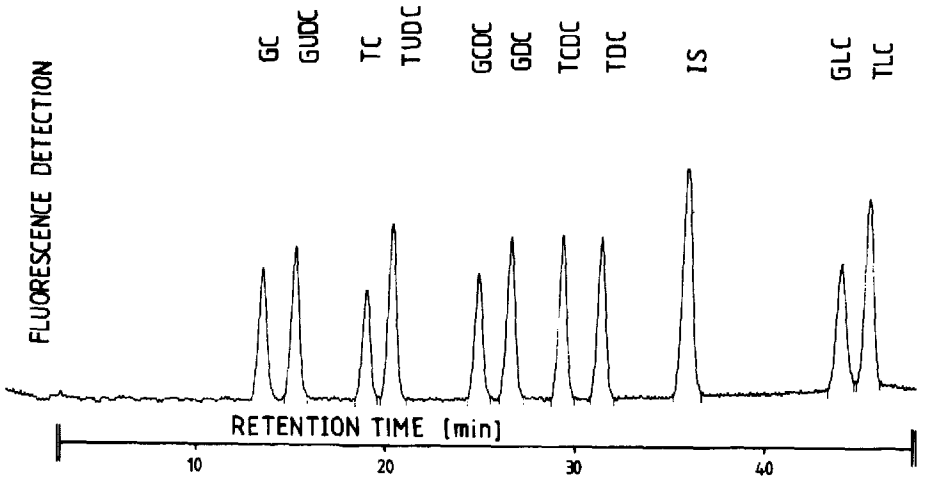

Fig. 2. Chromatogram of a standard mixture of conjugated bile acids. Peaks: GC = glycocholic acid; GUDC = glycoursodeoxycholic acid; TC = taurocholic acid; TUDC = tauroursodeoxycholic acid; GCDC = glycochenodeoxycholic acid; GDC = glycodeoxycholic acid; TCDC = taurochenodeoxycholic acid; TDC = taurodeoxycholic acid; IS = internal standard (5β-pregnane-3α,17α-diol-20-one); GLC = glycolithocholic acid; TLC = taurolithocholic acid.

Enzyme immobilization

The 3α-HSD was immobilized with glutaraldehyde on aminated porous glass beads (AMP-CPG 170 Å, 200–400 mesh, Fluka) by Schiff base formation and was packed in a steel column (100 × 1.8 mm I.D.). Except when in use, the enzyme column was disconnected from the apparatus, sealed at both ends and stored in a refrigerator.

RESULTS

Selectivity

The separation of a reference mixture of conjugated bile acids (GC, TC, GUDC, TUDC, GCDC, TCDC, GDC, TDC, internal standard, GLC, TLC) at a concentration of 8 μM is shown in Fig. 2 and Table I.

Detection limit

A 5-ml volume of serum was extracted and dissolved in 200 μl of solution B; 20 μl of this solution were injected. The injection volume corresponded to 0.5 ml of serum. The detection limit was 0.2 μM. The signal-to-noise ratio at the detection limit was 2, allowing for the detection of 5 μg of ursodeoxycholic acid and 10 μg of taurocholic acid.

Off-line reaction

The quantification of serum bile acids by HPLC post-column reaction is not reproducible, because the enzyme 3α-HSD loses its activity very quickly. The decrease in peak height within days is remarkable, especially for bile acids possessing a 7α-hydroxy group (cholic acid and chenodeoxycholic acid conjugates). Obviously, the speed of the enzymatic reaction is rapidly decreased owing to this

TABLE I

ABSOLUTE CAPACITY FACTORS AND SELECTIVITY FACTORS OF TEN CONJUGATED BILE ACIDS AND INTERNAL STANDARD IN A STANDARD SAMPLE

Bile acid	k'	α
GC	6.90	–
GUDC	8.65	1.25
TC	12.43	1.44
TUDC	13.78	1.11
GCDC	18.36	1.33
GDC	20.10	1.09
TCDC	22.85	1.14
TDC	24.95	1.09
Internal standard	29.48	1.18
GLC	37.77	1.28
TLC	39.20	1.04

7α-hydroxy group, because of its position with respect to the 3α-OH group in the bile acid molecule. Therefore, bile acid concentrations are quantified by an additional off-line reaction with 3α-HSD in excess (Sterognost 3α Flu) after peak-triggered fractional collection. Each bile acid fraction was evaporated to dryness at 40°C under vacuum and dissolved in 1.2 ml of Tris–HCl buffer (0.1 *M*, pH 9.0). Then 250 μl of Sterognost-3α Flu sample reagent were added. The tubes were incubated at room temperature for 15 min. The following reactions took place:

3α-hydroxy bile acid

$$+\mathrm{NAD^+} \underset{\text{pH 5.5, 3}\alpha\text{-HSD}}{\overset{\text{pH 9, 25°C}}{\rightleftharpoons}} \text{3-keto bile acids} + \mathrm{NADH} + \mathrm{H^+} \quad (1)$$

$$\mathrm{NADH} + \mathrm{H^+} + \text{resazurin} \xrightarrow{\text{diaphorase}} \mathrm{NAD^+} + \mathrm{H_2O} + \text{resorufin} \quad (2)$$

The concentration of resorufin was measured within 20 min after the termination of the incubation period, using a Hitachi spectrofluorimeter set at an excitation wavelength of 569 nm and an emission wavelength of 580 nm. A standard solution of taurocholic acid was used for calibration purposes. Exact triggered fractioning was essential for reproducible results.

Reproducibility

In order to examine the reproducibility of the whole procedure (HPLC analysis, enzyme post-column reaction, fraction collection and off-line fluorescence detection), 20-μl samples taken from the same standard mixture of eight bile acids (GC, TC, GCDC, TCDC, GDC, TDC, GUDC, TUDC) at a concentration of 8 μM each were tested on ten different days. Results are shown in Table II. The coefficient of variation (C.V.) ranged from 16.2% for GC to 4.7% for TDC.

Internal standard and conjugated lithocholic acids do not dissolve in the Tris buffer and therefore could not be quantified.

TABLE II

REPRODUCIBILITY

Bile acid	Concentration (mean ± S.D., $n = 10$) (μM)	Coefficient of variation (%)
GC	6.88 ± 1.11	16.2
GUDC	7.53 ± 0.46	6.1
TC	6.95 ± 1.07	15.4
TUDC	7.58 ± 0.45	6.0
GCDC	7.01 ± 0.99	14.2
GDC	7.61 ± 0.40	5.2
TCDC	7.05 ± 1.02	14.5
TDC	7.72 ± 0.36	4.7

TABLE III

INTER-ASSAY VARIABILITY

Bile acid	Concentration (mean ± S.D., $n = 10$) (μM)	Coefficient of variation (%)
GC	2.36 ± 0.067	4.98
GUDC	0.75 ± 0.045	6.02
TC	0.99 ± 0.056	5.62
TUDC	0.34 ± 0.028	8.24
GCDC	3.73 ± 0.144	3.87
GDC	1.22 ± 0.059	4.83
TCDC	3.18 ± 0.130	4.06
TDC	0.59 ± 0.040	6.83

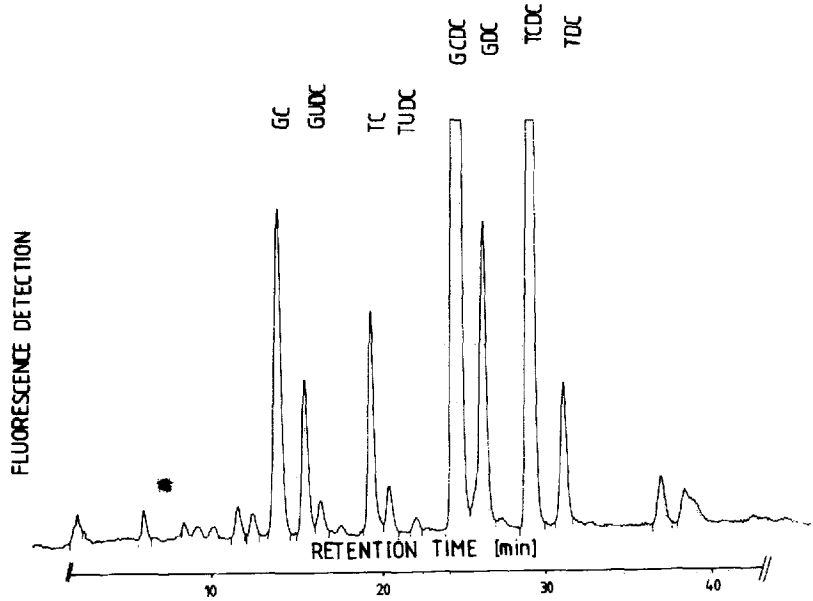

Fig. 3. Chromatogram of a serum sample from a patient with cirrhosis of the liver. Peak labels as in Fig. 2.

TABLE IV

RECOVERY OF CONJUGATED BILE ACIDS

Bile acid	Added (ng per 20 μl)	Found (mean ± S.D., $n=4$) (ng per 20 μl)	Recovery (mean ± S.D., $n=4$) (%)	Recovery (mean ± S.D., $n=12$) (%)
GC	20	19.27 ± 0.656	96.35 ± 3.28	97.69 ± 2.59
	80	78.06 ± 2.25	97.58 ± 2.81	
	200	198.26 ± 3.34	99.13 ± 1.67	
GUDC	20	19.55 ± 0.75	97.74 ± 3.76	98.69 ± 2.57
	80	78.70 ± 1.62	98.38 ± 2.02	
	200	199.90 ± 3.84	99.95 ± 1.92	
TC	20	19.08 ± 0.87	95.38 ± 4.35	96.75 ± 3.14
	80	77.02 ± 2.60	96.27 ± 3.25	
	200	198.36 ± 2.10	99.18 ± 1.05	
TUDC	20	19.36 ± 0.61	96.82 ± 3.04	97.74 ± 2.33
	80	77.78 ± 2.33	96.27 ± 3.25	
	200	198.36 ± 2.10	99.18 ± 1.05	
GCDC	20	19.39 ± 0.70	96.95 ± 3.49	97.95 ± 2.52
	80	78.30 ± 1.87	97.87 ± 2.34	
	200	198.06 ± 3.44	99.03 ± 1.72	
GDC	20	19.40 ± 0.78	97.02 ± 3.88	98.56 ± 2.86
	80	78.71 ± 2.62	98.39 ± 3.28	
	200	200.54 ± 2.86	100.27 ± 1.43	
TCDC	20	19.22 ± 0.79	96.12 ± 3.93	97.60 ± 2.83
	80	77.95 ± 2.43	97.44 ± 3.04	
	200	198.50 ± 3.04	99.25 ± 1.52	
TDC	20	19.50 ± 0.77	97.49 ± 3.86	98.83 ± 2.93
	80	78.42 ± 2.51	98.03 ± 3.14	
	200	201.96 ± 3.58	100.98 ± 1.79	

TABLE V

SERUM BILE ACID CONCENTRATIONS

Values are μM.

Patient	GC	GUDC	TC	TUDC	GCDC	GDC	TCDC	TDC
1★	0.33	0.44	–	–	1.03	0.75	< 0.2	< 0.2
2	2.37	0.76	1.35	0.32	10.75	1.24	6.69	0.61
3	0.44	< 0.2	0.27	–	0.93	0.55	0.54	0.32
4	1.04	–	0.88	–	2.50	–	2.38	–
5	2.07	0.92	1.81	0.70	2.64	0.99	1.47	1.06
6	> 16	0.35	> 16	–	13.49	< 0.2	> 16	< 0.2

★Healthy volunteer.

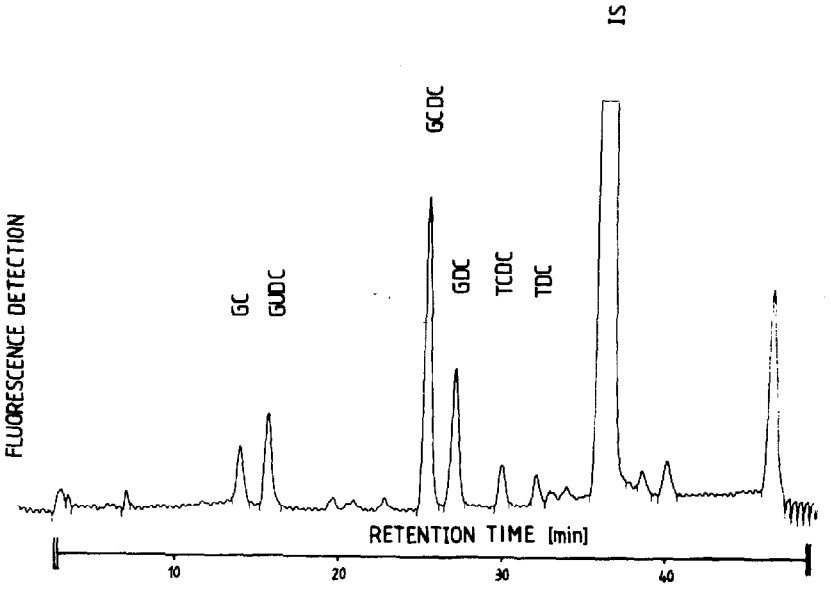

Fig. 4. Chromatogram of a serum sample of a healthy volunteer. Peak labels as in Fig. 2.

Inter-assay variability

To examine the inter-assay variability, a serum sample from a patient with cirrhosis of the liver was extracted ten times, the bile acids were separated on a C_{18} column, fractionated according to peak areas and determined by off-line reaction. The inter-assay variability is shown in Table III, and the representative chromatogram is shown in Fig. 3.

Recovery

For the determination of the recovery of the method, (serum extraction, HPLC analysis, enzyme post-column reaction, fraction collection and off-line reaction) standard mixtures of three different concentrations (2, 8 and 20 μM) of eight bile acids were analysed. The results are shown in Table IV. Recovery rates well above 95% are sufficient for routine clinical application.

Serum concentration of bile acids

The concentrations of individual bile acids (GC, GUDC, TC, TUDC, GCDC, GDC, TCDC, TDC) were determined in five patients and a healthy volunteer. The corresponding data are given in Table V and Fig. 4.

DISCUSSION

The separation of conjugated bile acids by RP-HPLC with UV detection has been described by several authors [6–9]. However, the detection sensitivity is insufficient for the quantitative analysis of bile acids in serum, because most bile acids do not have strong UV light-absorbing properties. Therefore, interference

from serum components is likely to occur. Total 3α-hydroxy bile acids in serum can be measured quantitatively by the fluorimetric determination of the amount of NADH generated from NAD and bile acids in the presence of 3α-HSD. Baba et al. [10] described an HPLC analysis of individual bile acids, using 3α-HSD in solution. This method is suitable with respect to specificity but requires a large amount of enzyme, thus making the assay very expensive. Okuyama et al. [11] attempted to use immobilized enzyme and their system has advantages when compared with Baba's method using soluble enzyme. Despite recent improvements, reliability and reproducibility became a serious problem with this technique, because the enzyme lost its activity very quickly [13–15]. Linnet [16] published a method for bile acid determination in serum too. The disadvantage of this approach is that it is necessary to fractionate the bile acids "blindly", according to prefixed retention times, because it is not possible to monitor the peaks of the bile acids with on-line UV detection. This difficulty is overcome in the present method by on-line fluorescence detection. Goto et al. [17] also labelled serum bile acids through the 3α-hydroxy group, but used 1-anthroylnitrile as fluorescent agent. The preparation before HPLC analysis is more laborious compared with the present method. Furthermore, the bile acid peaks of patient's serum samples are located on tip of the elution front.

Improvements in enzyme immobilization and the selection of adequate mobile phase components have led to enhanced separation and sensitivity in our method, in comparison with Goto's [17] and Okuyama's [11] work.

The present HPLC analysis with off-line fluorimetric determination for serum bile acid conjugates is probably too laborious for routine clinical use. However, the separate determination of the glycine and taurine conjugates of the major bile acids in serum offers the possibility of investigating serum bile acid profiles in selected clinical applications, e.g. during gallstone dissolution therapy.

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