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A HIGHLY SENSITIVE DETERMINATION OF INDIVIDUAL SERUM BILE ACIDS USING HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH IMMOBILIZED ENZYME

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SUMMARY

We have developed a highly sensitive method for the simultaneous determination of individual bile acids in serum using high-performance liquid chromatography (HPLC) combined with immobilized 3α-hydroxysteroid dehydrogenase. Both the HPLC column and the immobilized-enzyme column are suitable for use with alkaline solutions necessary in working with this enzyme system. With this method we were able to determine simultaneously fifteen different serum bile acids.

INTRODUCTION

Bile acids are synthesized from cholesterol and conjugated with glycine or taurine in the liver cells. Conjugated bile acids are excreted into the small intestine where about 30% of them are deconjugated and converted into secondary bile acids by intestinal bacteria. Most bile acids are reabsorbed from the terminal ileum by a process of active transport and are returned to the liver via the portal vein. This constitutes the enterohepatic circulation. In vivo, most bile acids are present as conjugates of glycine or taurine with only traces of bile acid present in the unconjugated form. In determining serum bile acids, it

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would be most advantageous not only to be able to determine total concentration but also the concentrations of each individual bile acid since these patterns may yield clinical information in liver disease. Mashige et al. [1] in 1976 reported a simple fluorometric method for determining total serum bile acid concentration and the method became popular among clinicians [2]. In 1978, we reported a method for measuring individual 3α-hydroxy bile acids in serum using high-performance liquid chromatography (HPLC) combined with enzymatic and fluorometric analyses [3]. Because our method was a flow system using enzyme solution, it was expensive. In 1979 Okuyama et al. [4] combined the HPLC method with an immobilized-enzyme system, but it was upon our principle. Although this solves an economic problem, it was difficult to establish optimum conditions for the enzymatic reactions.

In the present paper, we report our experience with a method utilizing a new HPLC column and a new immobilized-enzyme column both of which are suitable for use at the alkaline pH which is optimum for 3α-hydroxysteroid dehydrogenase enzyme activity. In our system the coenzyme NAD fed as the reagent is reduced to NADH by the immobilized 3α-hydroxysteroid dehydrogenase (3α-HSD) in the presence of bile acids as substrate eluting from the HPLC column, and the fluorescence intensity of the NADH thus produced is measured.

EXPERIMENTAL

Materials used for the HPLC column

Tetraethyleneglycol diacrylate (Sin Nakamura Chem. Co., Wakayama, Japan) and tetramethylolmethane triacrylate (Sin Nakamura Chem. Co.) were prepared as monomers. Toluene and benzoyl peroxide were used for the polymerization.

Materials used for immobilized-enzyme column

Beaded cellulose (Cellulofine GC-200 m, Seikagaku Kogyo, Tokyo, Japan) was adopted as solid support because of its chemical stability at alkaline pH and superior mechanical properties. The enzyme was 3α-HSD extracted from *Pseudomonas putida* and purified (Kyowa Hakko, Tokyo, Japan) [5,6].

Eluent

Triammonium phosphate was dissolved in distilled water to make 0.5% and 1.5% (w/v) triammonium phosphate solution. The 0.5% solution was adjusted to pH 9.1 and the 1.5% solution to pH 9.7 with ammonium hydroxide solution. Each solution was mixed with acetonitrile. Three kinds of eluent were made. The volume ratio (v/v) of eluent I was 13:87 (acetonitrile—0.5% triammonium phosphate solution), eluent II was 15:85 (acetonitrile—1.5% solution) and eluent III was 24:76 (acetonitrile—0.5% solution).

Reagents

β-NAD^+, 200 mg/l (Sigma, St. Louis, MO, U.S.A.), 25 mg/l EDTA, 2.3 g/l sodium pyrophosphate, 2.72 g/l sucrose and 500 μl/l mercaptoethanol were dissolved in 0.1 mol/l hydrazine hydrate (pH 9.5).

Standard materials

Sodium salts of cholic acid (CA), chenodeoxycholic acid (CDCA), deoxycholic acid (DCA) and lithocholic acid (LCA) were purchased from Calbiochem-Behring (San Diego, CA, U.S.A.). Sodium salts of 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 P.-L. Biochemicals (Milwaukee, WI, U.S.A.). Sodium salts of ursodeoxycholic acid (UDCA), glycoursodeoxycholic acid (GUDCA) and tauroursodeoxycholic acid (TUDCA) were a gift from Tokyo Tanabe Pharmaceutical (Tokyo, Japan). As an internal standard (I.S.), we used N′-(3α,7α,12α-trihydroxy-5β-cholanoyl) glutamate (GLU-CA, Lot No. 9271), a gift from Sekisui Kagaku (Osaka, Japan).

Instruments

A schematic diagram of the method is shown in Fig. 1. HPLC was carried out on an ALC 204 type Waters' system (Waters Assoc., Waltham, MA, U.S.A.). The detector used was a Fluorichrom fluorescence detector (Shimazu Seisakusho, Kyoto, Japan) with an excitation wavelength of 350 nm and an emission wavelength of 460 nm.

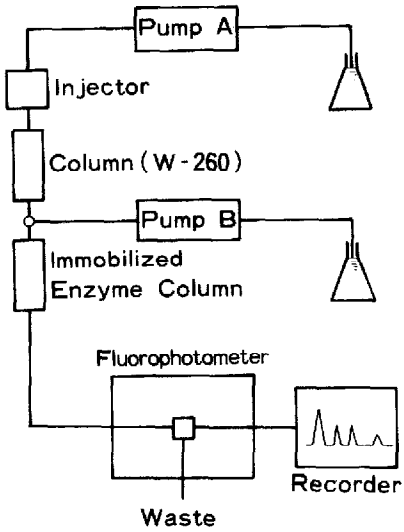

Fig. 1. Flow diagram of the system. Eluent flows from pump A at 1 ml/min, and reagent from pump B at 1 ml/min.

Preparation of W-260 column

Co-polymer beads were synthesized by a standard suspension polymerization method. The aqueous portion of the polymerization mixture was prepared in a reaction flask (5 l) by adding 60 g of polyvinylalcohol as dispersing agent to 2.5 l of distilled water. The monomer phase consisted of 250 g of tetraethyleneglycol diacrylate, 50 g of tetramethylolmethane triacrylate, 300 g of toluene

(diluent), and 3.5 g of benzoyl peroxide. When the aqueous phase reached 50°C, the monomer—diluent—initiator solution was added with stirring. Temperature was maintained at 80°C for 10 h. When the polymerization was complete, the resin beads were filtered and washed with boiling water to remove the dispersing agent. After sieving, about 25 g of the fraction of diameter 10—15 μm were extracted with methanol in order to remove residual diluent and unreacted monomers. The porous beads thus obtained were packed into a stainless-steel column (25 cm × 79 mm I.D.). A slurry containing 10 g of gel in about 100 ml of water was added to a column packing vessel. A total of 120 ml of water was pumped before completing the column packing.

Preparation of immobilized-enzyme column

The activation of beaded cellulose by the addition of CNBr was followed by the coupling reaction with 3α-HSD. The reactions were performed according to the procedure described by March et al. [7]. The enzyme-bound beads were packed into a stainless-steel column (10 × 0.4 cm I.D.) and it was referred to as the immobilized-enzyme column. In this immobilizing method, the yield of immobilized enzyme was over 60%, and the enzyme activity of the gel was stable for two weeks at 15°C in phosphate buffer, pH 11, containing 20% acetonitrile and 0.2% mercaptoethanol.

Preparation of serum material

Serum was collected from a peripheral vein after overnight fasting and frozen (−20°C) until analyzed for bile acids. A 0.5-ml volume of serum was diluted with 4.5 ml of 0.9% NaCl containing 0.1 *M* of NaOH. This solution was applied to a column of Amberlite XAD-7 resin (0.2 g, Lot No. 3933, Rohm and Haas, Philadelphia, PA, U.S.A.). After the serum solution was applied, the column was washed with 5 ml of methanol at the rate of five drops a minute. Then the eluent was evaporated to dryness and the residue was re-dissolved in 0.5 ml of methanol containing 2500 ng/ml N'-(3α,7α,12α-trihydroxy-5β-cholanoyl) glutamate for analyzing.

Measurement procedure

Aliquots (100 μl) of the samples were applied to the W-260 column from the injector with a microsyringe (Hamilton, Reno, NV, U.S.A.). Eluent was passed through at a rate of 1 ml/min. In order to shorten the retention time, the mobile phase was changed from eluent I to eluent II at 30 min after injection and from eluent II to eluent III at 68 min. Reagent was passed through at a constant rate of 1 ml/min. Eluent and reagent together flow into the immobilized-enzyme column, where 3α-hydroxysteroids are converted to 3-ketosteroids and NAD is converted into NADH. This reaction is carried out at 27°C in a water bath. NADH produced by this reaction is measured by the fluorophotometer connected to the immobilized-enzyme column. The chart speed is 2.5 mm/min. Fig. 2 shows a standard chromatogram.

Standard curves

Four concentrations of a mixture of each of the standard bile acids were made: 10, 5, 2.5 and 1.25 μmol/l. These samples were dissolved in distilled

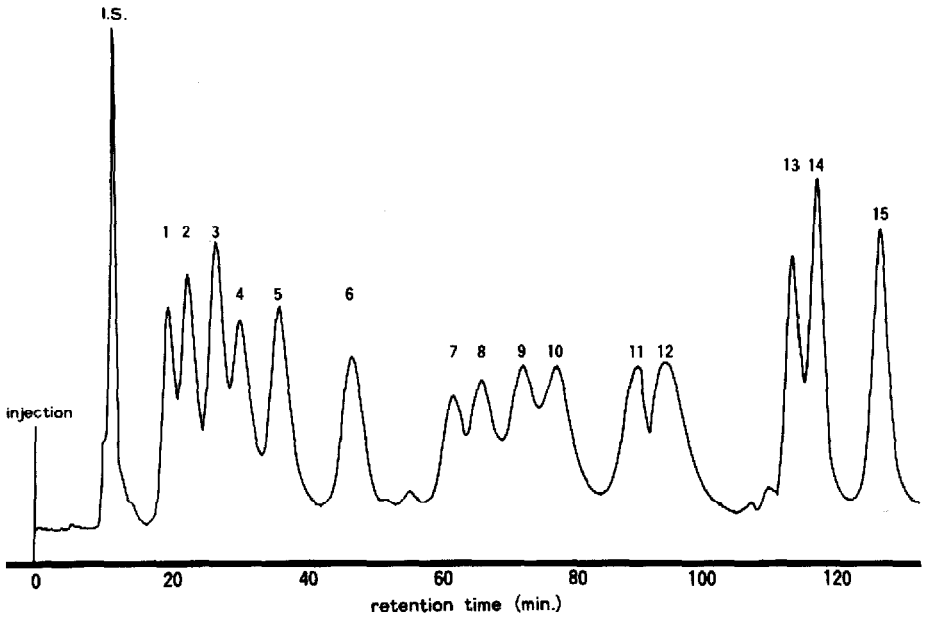

Fig. 2. Standard chromatogram of individual bile acids (125 ng each). I.S. = N'-(3α,7α,12α-trihydroxy-5β-cholanoyl) glutamate, 1 = cholic acid, 2 = glycocholic acid, 3 = taurocholic acid, 4 = ursodeoxycholic acid, 5 = glycoursodeoxycholic acid, 6 = tauroursodeoxycholic acid, 7 = chenodeoxycholic acid, 8 = deoxycholic acid, 9 = glycochenodeoxycholic acid, 10 = glycodeoxycholic acid, 11 = taurochenodeoxycholic acid, 12 = taurodeoxycholic acid, 13 = lithocholic acid, 14 = glycolithocholic acid, 15 = taurolithocholic acid.

water and treated with Amberlite XAD-7. The standard curves were established by plotting the peak height against the amount of each bile acid.

Recovery experiments

Recovery experiments were performed by adding a mixture of standard samples to the sera of healthy subjects. This mixture contained 5 μmol/l of each bile acid. The sera with added standards were then treated with Amberlite XAD-7.

Reproducibility

As an intra-assay reproducibility, the same samples were determined six times within a day, and for inter-assay reproducibility the same samples were determined during another six days. The coefficients of variation (C.V., %) were calculated from the mean values and S.D. values.

RESULTS

Fig. 2 shows a chromatogram of a mixture of free, glycine- and taurine-conjugates of each of five bile acids. The separation of these bile acids was satisfactorily obtained in the order CA, GCA, TCA, UDCA, GUDCA, TUDCA, CDCA, DCA, GCDCA, GDCA, TCDCA, TDCA, LCA, GLCA and TLCA. The

resolution value of TCDCA and TDCA, in which separation was most difficult, was 0.7. Fig. 3 shows the standard curve of each bile acid. Linear correlations were obtained between the peak height and the amount of each bile acid.

Table I indicates the recovery ratios and reproducibility. Satisfactory recovery ratios were obtained ranging between 88.7% and 103.8%. The intra-assay C.V. value of each bile acid ranged from 0.3 to 4.5% in six replicate determinations and from 0.6 to 7.8% in six replicate determinations for inter-assay C.V. The sensitivity limits of each bile acid by this method ranged from 0.01 to 0.04 μmol/l.

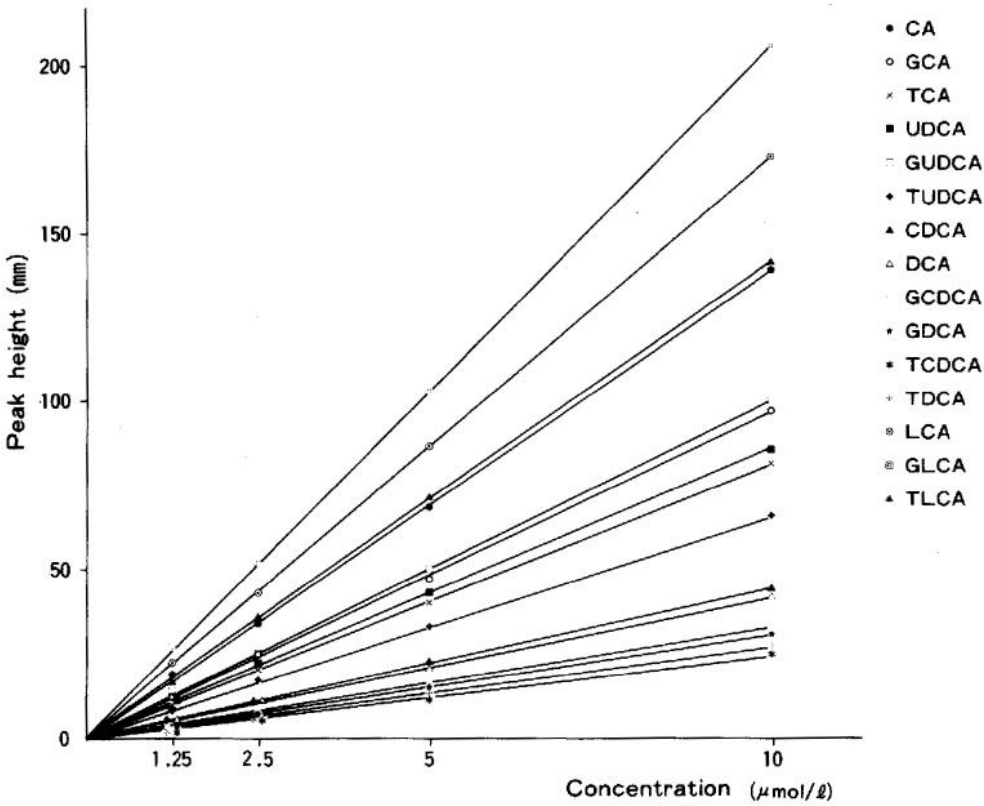

Fig. 3. Standard curves of individual bile acids.

A normal-serum analysis by this method is presented in Fig. 4. Also an example of serum analysis in a patient with gallstones is presented in Fig. 5. A typical bile acid pattern of extrahepatic cholestasis was obtained. Normal values analyzed in this system are compared in Table II with values obtained from other methods including our previous HPLC method using 3α-HSD reagent solution [3,8—10].

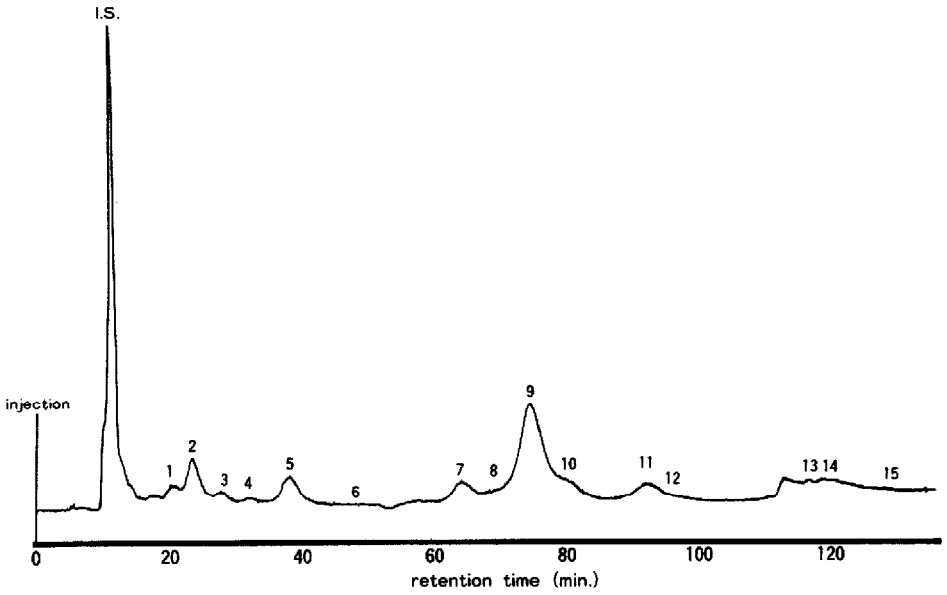

Fig. 4. A bile acid pattern of normal serum. Peak numbers as in Fig. 2.

TABLE I

REPRODUCIBILITY AND RECOVERY

Bile acids	Reproducibility of each bile acid assay		Recovery rate (%)
	Intra-assay C.V. (%) (n = 6)	Inter-assay C.V. (%) (n = 6)	
Cholic acid	0.6	2.9	89.2
Glycocholic acid	2.1	2.5	96.9
Taurocholic acid	1.7	5.0	92.4
Ursodeoxycholic acid	2.7	0.6	95.9
Glycoursodeoxycholic acid	2.9	3.8	90.9
Touroursodeoxycholic acid	0.2	5.5	90.4
Chenodeoxycholic acid	2.3	4.6	88.7
Glycochenodeoxycholic acid	2.7	3.4	96.2
Taurochenodeoxycholic acid	4.5	3.8	94.7
Deoxycholic acid	2.3	1.3	92.5
Glycodeoxycholic acid	4.2	4.5	89.0
Taurodeoxycholic acid	0.3	7.0	103.8
Lithocholic acid	2.7	6.8	98.9
Glycolithocholic acid	2.3	2.5	102.9
Taurolithocholic acid	2.1	7.8	94.7

DISCUSSION

The data indicate that our HPLC method and immobilized-enzyme column operate well under the severely alkaline conditions which are optimum for 3α-hydroxysteroid dehydrogenase activity. Columns could be used more than

TABLE II

NORMAL VALUES OF SERUM BILE ACIDS*

Values are given in μmol/l (mean ± S.E.M.).

Author and method	No.	Total	UDCA group			CA group			CDCA group			DCA group			LCA group		
			UDCA	GUDCA	TUDCA	CA	GCA	TCA	CDCA	GCDCA	TCDCA	DCA	GDCA	TDCA	LCA	GLCA	TLCA
Present method HPLC (using immobilized enzyme)	12	3.90 ±0.85	0.08 ±0.03	0.14 ±0.05	0.07 ±0.02	0.26 ±0.05	0.30 ±0.08	0.21 ±0.03	0.75 ±0.20	0.97 ±0.38	0.45 ±0.13	0.20 ±0.06	0.23 ±0.04	0.23 ±0.05	0.02 ±0.01	0.01 ±0.00	0.01 ±0.00
Baba et al. [3] HPLC (using enzyme solution)	8	2.88 ±0.74	0.07 ±0.04			0.14 ±0.05	0.16 ±0.07	0.16 ±0.05	0.42 ±0.16	0.47 ±0.05	0.36 ±0.08	0.30 ±0.05	0.46 ±0.26	0.36 ±0.08	N.D.	N.D.	N.D.
Demers and Hepner [8] RIA**	25						0.27 ±0.03			0.20 ±0.03			0.06 ±0.01				
Sandberg et al. [9] GLC** (ranges)	18	0.9—6.9		N.D.***			0.09—1.95			0.15—3.9			0.18—1.35			N.D.	
Sino et al. [10] GC—MS** (mean)	4			1.02			0.55			1.81			1.12			0.11	

*Abbreviations, see text.
**RIA = radioimmunoassay, GLC = gas—liquid chromatography, GC—MS = gas chromatography—mass spectrometry.
***N.D. = not determined.

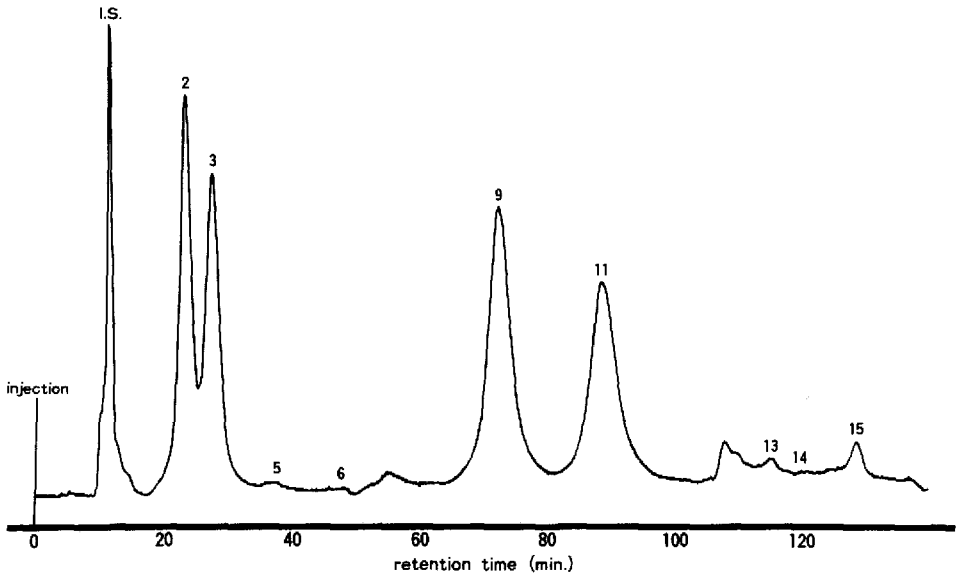

Fig. 5. A bile acid pattern of serum in the patient with gallstones. Peak numbers as in Fig. 2.

300 times without a significant change in their operating characteristics. The enzyme which we used was purified from *Pseudomonas putida* instead of *Pseudomonas testeroni*. The former enzyme is specific for the 3α-hydroxy group whereas the latter enzyme has some activity towards the 3β-hydroxy group as well as the 3α-hydroxy group [11]. The acetonitrile used in our eluent probably decreases the life span of the immobilized enzyme to some extent. Okuda and Hashimoto [12] reported a method using methanol instead of acetonitrile for the eluent. Although their method separated many bile acids, it did not separate TCDCA and TDCA completely. It may be possible in the future to devise methods which combine excellent separation with long enzyme life span. Until then, the present method appears to provide the best separation specificity and sensitivity available for the analysis of serum bile acids. We hope that the ability to analyze quickly and specifically individual circulating bile acid species will become clinically useful in differentiating various kinds of liver disease. We have shown the reproducibility of this method and have defined its practicality in clinical use. We have also established normal values against which we can compare values in patients with various kinds of liver disease.

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