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A NEW ANALYTICAL METHOD OF INDIVIDUAL BILE ACIDS USING HIGH PERFORMANCE LIQUID CHROMATOGRAPHY AND IMMOBILIZED 3α-HYDROXYSTEROID DEHYDROGENASE IN COLUMN FORM

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We studied the use of immobilized enzyme, covalently bound to aminopropyl-CPG, to the analysis on free and conjugated individual bile acids. A microcolumn with the immobilized 3α-hydroxysteroid dehydrogenase was prepared and used in a system of high performance liquid chromatography (HPLC). Reduced NAD^+ produced from bile acid by this enzyme was fluorimetrically determined with fluorometer. As the result, the free, glycine- and taurine-conjugated individual bile acids which were separated by the HPLC were determined on high sensitivity and specificity. Clinical trials were done for the serum of patients with hepatobiliary disease.

Since 1976, we have already reported the HPLC analysis of individual bile acids by the derivatization with 1-p-nitrobenzyl-3-p-tolyltriazene, O-(p-nitrobenzyl)-N,N'-diisopropylisourea and 4-bromomethyl-7-methoxycoumarin[1-4]. In order to improve the specificity of these methods, various attempts were done. Total 3α-hydroxy bile acids in serum can be measured quantitatively by the fluorimetric determination of the amount of NADH genarated from NAD^+ and bile acids in the presence of 3α-hydroxysteroid dehydrogenase[5,6]. Therefore, Baba and coworkers[7] described the HPLC analysis of individual bile acids, using 3α-hydroxysteroid dehydrogenase in the form of solution. This method has proven useful for its specificity, but this method consumes cosiderable amount of enzyme in the course of their operation. This makes the assay very expensive.

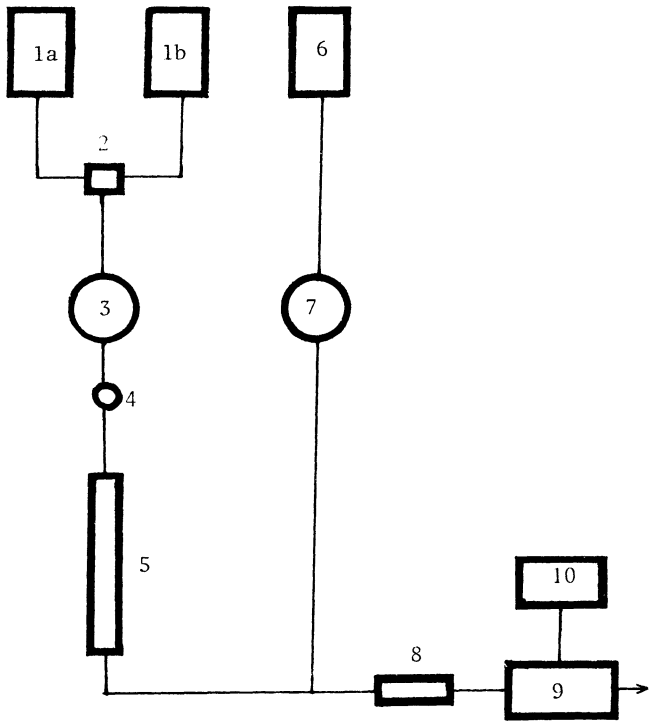
We attempted to use the immobilized enzyme for the HPLC analysis of individual bile acids instead of the enzyme in the form of solution. We prepared immobilized 3α-hydroxysteroid dehydrogenase by binding this enzyme to an aminopropyl-CPG of porous glass, and examined its applicability to HPLC system. A microcolumn of immobilized enzyme with a dimension of 3 mm inner diameter and 25 mm length was prepared, and was placed into a continuous-flow system using HPLC. The system performed well when used to analyze free, glycine- and taurine-conjugated individual bile acids. Speed, precision and accuracy were the same as those obtained by the HPLC methodology using soluble enzyme.

In this report, we describe the system and procedure of continuous-flow analysis combined with HPLC and immobilized enzyme in column form for the determination of

individual free and conjugated bile acids.

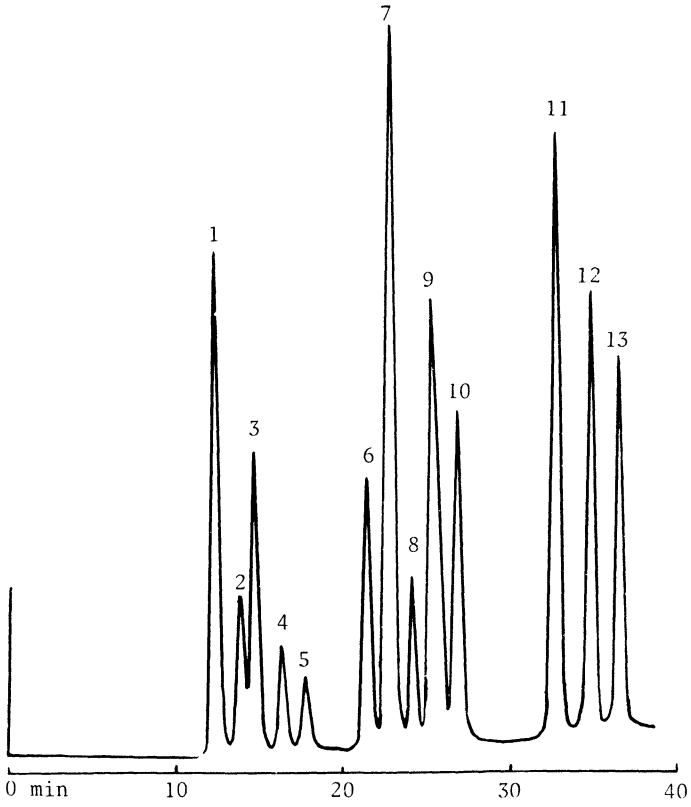
Materials and Methods

The bile acids, hydroxysteroid dehydrogenase (Grade II), and NAD were obtained from Sigma, St. Louis. Mo. and Calbiochem, San Diego, Calif. A Jasco Trirotar high pressure liquid chromatograph was used throughout this work. The instrument was fitted with a Jasco FP-110 spectrometer, and a gradient elution accessory GP-A30. μBondapak/phenyl column (3.9 mm i.d. x 300 mm) which was commercially available from Waters Associates, Inc., was used for the separation of individual bile acids. On the separation of individual free and conjugated bile acids, the operating conditions were as follows: mobile phase, A-0.3% $(NH_4)_2CO_3$, B-CH_3CN; gradient B/A ratio, 20/80 at 0 time, and 44/56 at 64 min; slope, linear; flow rate, 1ml/min; detection, fluorescence, λex=365 nm, λem= 465 nm; pressure, 70 kg/cm^2; temperature, ambient. Immobilized enzyme was prepared by coupling 3α-hydroxysteroid dehydrogenase to aminopropyl-CPG glass (Electro-Nucleonics, Inc.) with glutaraldehyde through Schiff base formation[8,9]. This immobilized enzyme was packed in a small glass tubing, and then this column was put into a system of HPLC as shown in its diagram (Fig. 1). Furthermore, the NAD^+ solution introduced in this system was prepared as follows: 0.3μM/l NAD^+ was soluted in 10mM/l potassium phosphate buffer (pH7.0) containing ethylenediaminetetraacetic acid solution (1mM/l) and 2-mercaptoethanol (0.05%,V/V). The mixture of the individual free and conjugated bile acids containing each 10 mg in 1000 ml methanol was made up. This standard solution of the mixture of bile acids was injected into the system of HPLC (Fig.1). The extraction of bile acids in serum was done using Amberlite XAD-2 column.



1a, 1b: Solvent Reservoir
2: Gradient Elution Accessory
3, 7: Pump
4: Sample Injector
5: Column
6: Reservoir of NAD^+ Solution
8: Column Packed with Immobilized 3α-Hydroxysteroid Dehydrogenase
9: Fluorometer
10: Recorder

Fig.1. Diagram of the system of HPLC.



*1: Ursodeoxycholic acid
2: Cholic acid
3: Glycoursodeoxycholic acid
4: Glycocholic acid
5: Taurocholic acid
6: Chenodeoxycholic acid
7: Deoxycholic acid
8: Glycochenodeoxycholic acid
9: Glycodeoxycholic acid
Taurochenodeoxycholic acid
10: Taurodeoxycholic acid
11: Lithocholic acid
12: Glycolithocholic acid
13: Taurolithocholic acid

* These numbers were also used in subsequent figures.

Fig.2. HPLC analysis of individual bile acids.

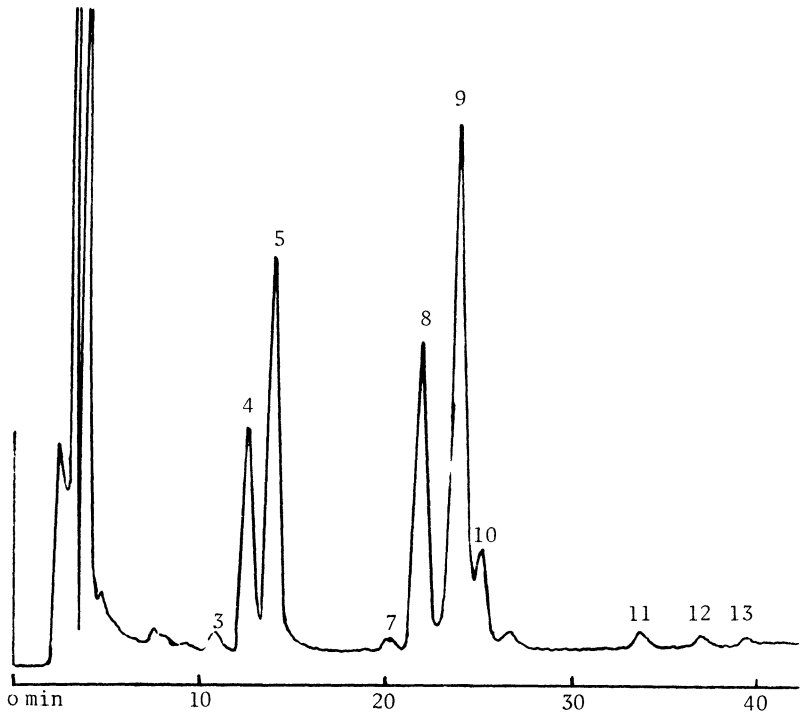


Fig.3. HPLC analysis of bile acids in serum of patient with cholestasis.

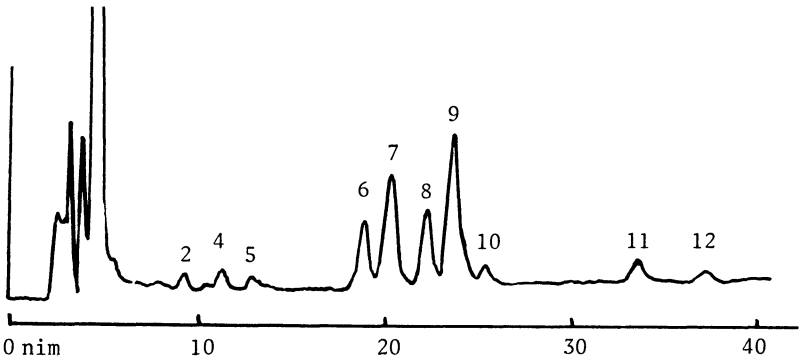


Fig.4. HPLC analysis of bile acids in serum of a patient with liver cirrhosis.

Results and Discussion

The separation of the individual bile acids including unconjugated (free), glycine- and taurine-conjugated bile acids was investigated by using HPLC and immobilized enzyme in column form. The separation is very clear except glycodeoxycholic acid and taurochenodeoxycholic acid as shown in Fig. 2. Each peak is equivalent with 100 ng of bile acids. The estimated detection limit is 10 ng approximately. From these results, we could achieve the improvement of the detectability of bile acids by means of the specificity of enzymatic system. Simplification of the procedure, high reliability and high reproducibility at low cost were performed by the use of the immobilized enzyme packed in column, compared with existing method. Furthermore, the valuable analysis of bile acids in serum of patients with cholestasis due to primary biliary cirrhosis and liver cirrhosis are shown in Fig. 3 and 4, respectively. In cholestasis, the increase of glycocholic acid and taurocholic acid was observed. On the other hand, in liver cirrhosis the amount of glycocholic acid and taurocholic acid decreased and then the increase of free chenodeoxycholic acid, glycochenodeoxycholic acid, and taurochenodeoxycholic acid plud glycodeoxyxholic acid was indicated. The existence of lithocholic acid and its conjugates in serum of the patients was recognized.

We are able to expect that this method may be used in clinical medicine, in order to elucidate disorder of the conjugation and hydroxylation of bile acids in hepatobiliary disease.

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