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High-performance liquid chromatography with a 3α-hydroxysteroid dehydrogenase postcolumn reactor and isoluminol–microperoxidase chemiluminescence detection

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ABSTRACT

A high-performance liquid chromatographic system with chemiluminescence detection for the determination of bile acids has been developed. Immobilized 3α-hydroxysteroid dehydrogenase was used as a postcolumn reactor and the generated NADH in the eluent was monitored by the chemiluminescence reaction of NADH using a 1-methoxy-5-methylphenazinium methylsulphate–isoluminol–microperoxidase system. A good separation of unconjugated bile acids and their glycine and taurine conjugates could be achieved with a reversed-phase column (Bilepak II) and acetonitrile–methanol–30 m*M* ammonium acetate as the eluent. The detection limit was about 2 pmol for each bileacid.

INTRODUCTION

In recent years, considerable attention has been focused on the development of high-performance liquid chromatography (HPLC) for the analysis of various biological substances, in particular polar and unstable compounds. HPLC is suitable for the separation and determination of unconjugated and conjugated bile acids without prior hydrolysis and/or solvolysis[1]. However, the sensitivity of HPLC using refractometric or ultraviolet spectrometric detection is low for the determination of bile acids in normal human serum. The most significant improvements in the sensitivity of HPLC methods for bile acid determination have been with the use of pre- or postcolumn derivatization and fluorimetric or enzymatic detection. In previous papers we described HPLC methods using 1-bromoacetylpyrene[2] and dansylhydrazine[3] as fluorescent precolumn reagents and an HPLC method using an immobilized 3α-hydroxysteroid dehydrogenase (3α-HSD) column combined with electrochemical detection[4].

Recently, the use of chemi- and bioluminescence analysis has been introduced into biochemistry and clinical chemistry because of their high sensitivity[5]. Several applications of chemiluminescence detection in HPLC have been reported using the peroxyoxalate–hydrogen peroxide[6] and lucigenin[7,8] chemiluminescence reaction

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systems. We have also developed HPLC methods using isoluminol derivative–hydrogen peroxide[9] and phenacyl derivative–lucigenin[10] chemiluminescence detection for the assay of amines and carboxylic acids.

In this study, we have developed an HPLC method for bile acids using a 3α-HSD enzyme reactor and isoluminol–microperoxidase chemiluminescence detection.

EXPERIMENTAL

Materials

Cholic acid, chenodeoxycholic acid, deoxycholic acid, ursodeoxycholic acid, lithocholic acid and their glycine and taurine conjugates were purchased from Sigma (St. Louis, MO, U.S.A.). NAD^+ and NADH were obtained from Boehringer Mannheim–Yamanouchi (Tokyo, Japan), 1-methoxy-5-methylphenazinium methylsulphate (1-MPMS) from Nacalai Tesque (Kyoto, Japan) and isoluminol (IL) from Tokyo Chemical Industry (Tokyo, Japan). 3α-Hydroxysteroid dehydrogenase (3α-HSD) (grade II) and microperoxidase (m-POD) were purchased from Sigma. Amino glass beads used as the solid phase of the immobilized enzyme were Amino Propyl-CPG 180 A from Electro-Nucleonics (Fairfield, NJ, U.S.A.) and Sep-Pak C_{18} cartridges from Millipore (Milford, MA, U.S.A.). All other chemicals were of analytical-reagent grade from commercial sources.

Bile acid stock solution. Each bile acid was dissolved in methanol and made up to 10 μmol/ml with methanol.

NAD^+ solution. A 0.9-m*M* NAD^+ solution was prepared by dissolving NAD^+ in 10 m*M* KH_2PO_4 containing 1 m*M* Na_2EDTA (pH 7.77).

1-MPMS solution. This solution was prepared by dissolving 1-MPMS in redistilled water (4 μg/ml).

IL–m-POD solution. This solution contained $2.4 \cdot 10^{-4}$ *M* IL and $1 \cdot 10^{-6}$ *M* m-POD in 0.4 *M* carbonate buffer (pH 9.5).

Immobilized 3α-HSD column. 3α-HSD was coupled to amino glass beads by the glutaraldehyde method as described previously[4] and packed into a stainless-steel column (25 mm × 4.6 mm I.D.).

Apparatus and chromatographic conditions

A schematic diagram of the apparatus is shown in Fig. 1. It consisted of a Model CCPM 8000 chromatograph (Tosoh, Tokyo, Japan), a JASCO (Tokyo, Japan) Bilepak-II (5 μm) column (100 mm × 5 mm I.D.) and a JASCO Model 825 CL chemiluminescence detector.

The mobile phases were acetonitrile–methanol–30 m*M* ammonium acetate [(A) 30:30:40 (pH 7.10) and (B) 20:20:60 (pH 6.80)], with isocratic elution with B for 5 min and then linear gradient elution from 100% B to 100% A in 32 min at a flow-rate of 1.0 ml/min. The eluent from the column was mixed with NAD^+ solution (1 ml/min). 1-MPMS solution and IL–m-POD solution were mixed into the eluent from the immobilized 3α-HSD column by pumps at a flow-rate of 1.0 ml/min. The generated chemiluminescence was monitored with the chemiluminescence detector.

Sample preparation

Extraction of bile acids from bile. Bile (20 μl) was diluted using 0.05 *M* phosphate

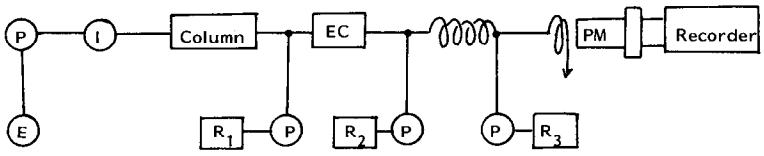

Fig. 1. HPLC system with chemiluminescence detection for the assay of bile acids. P = Pump; E = eluent; I = injection valve; column = Bilepak-II; EC = Enzymepak-3α-HSD; R_1 = 0.9 m*M* NAD^+–10 m*M* KH_2PO_4–1 m*M* Na_2EDTA (pH 7.77), flow-rate 1.5 ml/min; R_2 = 1-MPMS (4 μg/ml), flow-rate 1.0 ml/min; R_3 = isoluminol ($2.4 \cdot 10^{-4}$ *M*)–m-POD ($1.0 \cdot 10^{-6}$ *M*), flow-rate 1.0 ml/min. Mobile phase: acetonitrile–methanol–30 m*M* ammonium acetate [(A) 30:30:40 (pH 7.10) and (B) 20:20:60 (pH 6.80)] with isocratic elution with B for 5 min followed by linear gradient elution from 100% B to 100% A in 32 min at a flow-rate of 1.0 ml/min.

buffer (pH 7.0) containing internal standard (I.S.). The diluted bile (1.0 ml) was applied to a Sep-Pak C_{18} column. After washing with 2% methanol (2 ml), the bile acids were eluted with 90% ethanol (5 ml). The solvent was evaporated under a stream of nitrogen at 40°C. The residue was dissolved in methanol (1 ml) and the solution evaporated to dryness under a stream of nitrogen. The residue was dissolved in methanol 100 μl and a 10-μl portion was injected into the HPLC system.

Extraction of bile acids from serum. Serum (500 μl) was mixed with methanol (3 ml) and ultrasonicated for 15 min. The mixture was centrifuged at 1200 *g* for 10 min at 4°C, the supernatant was transferred into a test-tube and the precipitated residue was extracted with methanol (2 ml) in the same manner. The combined supernatant was evaporated to dryness under a stream of nitrogen. The residue was dissolved by adding 0.05 *M* phosphate buffer (pH 7.0) and applied to a Sep-Pak C_{18} column, after which bile acids were extracted in the same manner as above.

Recovery test

A synthetic mixture of 500 pmol of each bile acid containing I.S. was added to 20 μl of normal bile or 500 μl of normal serum and then assayed by the proposed method. Recoveries were calculated against a pure standard bile acid mixture carried through the procedure.

RESULTS AND DISCUSSION

Recently, we reported a highly sensitive chemiluminescent assay of NADH and NADPH based on the chemiluminescence reaction using an electron mediator and isoluminol–microperoxidase[11], and also developed a flow system for the assay of total bile acids in serum by using an enzyme column containing immobilized 3α-HSD on amino glass beads. In this study, we have developed an HPLC method with chemiluminescence detection for the assay of individual bile acids based on the flow detection system.

The assay principle is illustrated schematically in Fig. 2. This method utilizes an immobilized 3α-HSD enzyme column reactor in a flow mode that converts the 3α-hydroxyl group of bile acid to the corresponding 3-oxo bile acids in the presence of

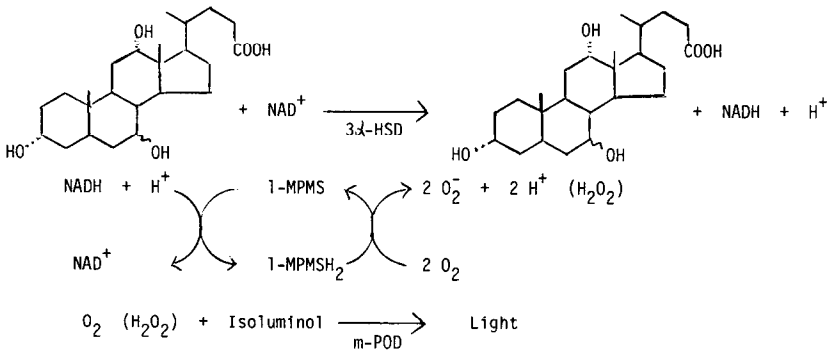

Fig. 2. Principle for the assay of bile acids using 3α-HSD and NADH chemiluminescence reaction.

NAD^+ after chromatographic separation by HPLC on an ODS column. In the enzyme column reactor NADH is generated and monitored by the chemiluminescence reaction with 1-MPMS–IL–m-POD.

Several parameters were examined in order to determine the optimum conditions for the enzymatic reaction of bile acids by using the flow system without the separation column shown in Fig. 1.

The first step of this method is the enzymatic oxidation of bile acids to 3-oxo bile acids in the immobilized 3α-HSD enzyme column reactor. Fig. 3 shows the effects of the concentration and flow-rate of NAD^+ solution on the chemiluminescence intensity (peak height in the chromatogram). The peak height increased with increasing concentration and flow-rate and reached a maximum at 0.9 m*M* NAD^+ concentration and a flow-rate of 1.5 ml/min; therefore, these conditions were adopted for the method.

The second step is the reaction of NADH with 1-MPMS in the reaction coil. The concentration of 1-MPMS solution and the length of the reaction coil were examined. As shown in Fig. 4, the maximum peak occurred at a 1-MPMS concentration

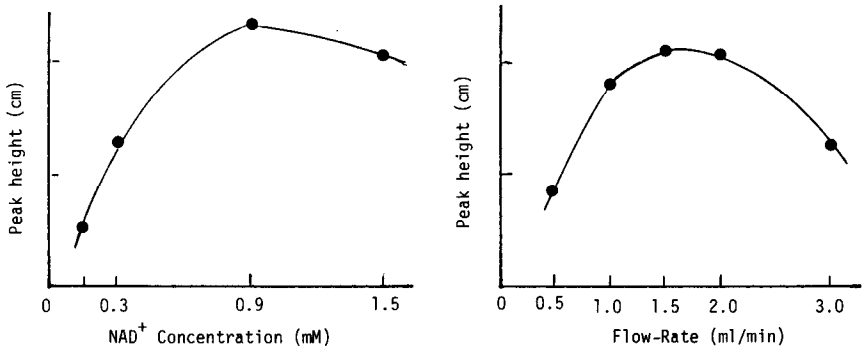

Fig. 3. Effects of the concentration and the flow-rate of NAD^+ solution on chemiluminescence intensity. Sample used: glycoursodeoxycholic acid (300 pmol).

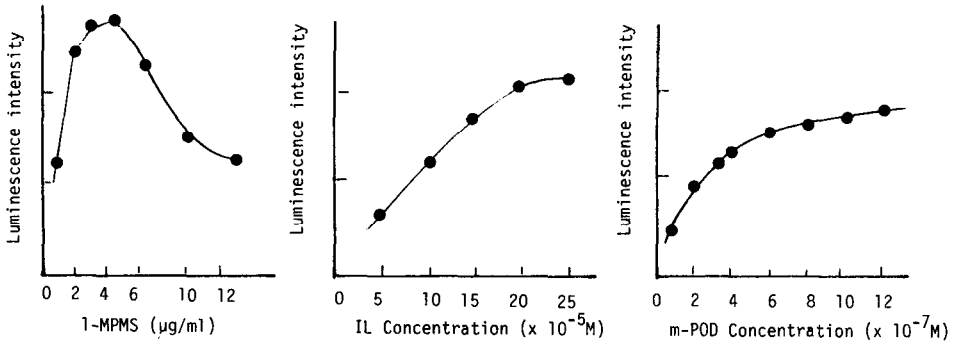

Fig. 4. Effects of the concentrations of 1-MPMS, isoluminol and microperoxidase solutions on chemiluminescence intensity.

3–4 μg/ml and the sensitivity decreased sharply on either side of this concentration; therefore 4 μg/ml 1-MPMS solution was used. The reaction rate of NADH with oxygen depends on the electron mediator used. In this system, 1-MPMS was used because the reaction rate was faster than with other electron mediators and 1-MPMS was stable towards light. The chemiluminescence intensity increase with increasing length of reaction coil, but the peak band became wider. Therefore, a 12-m reaction coil was used as a compromise between sensitivity and separation efficiency.

The last step is the chemiluminescence reaction of hydrogen peroxide with IL and m-POD. As shown in Fig. 4, the chemiluminescence intensity increased with increasing concentration of IL and m-POD and reached a nearly constant value at concentrations of $2 \cdot 10^{-4}$ and $1 \cdot 10^{-6}$ M, respectively. Therefore, these conditions were selected for the method.

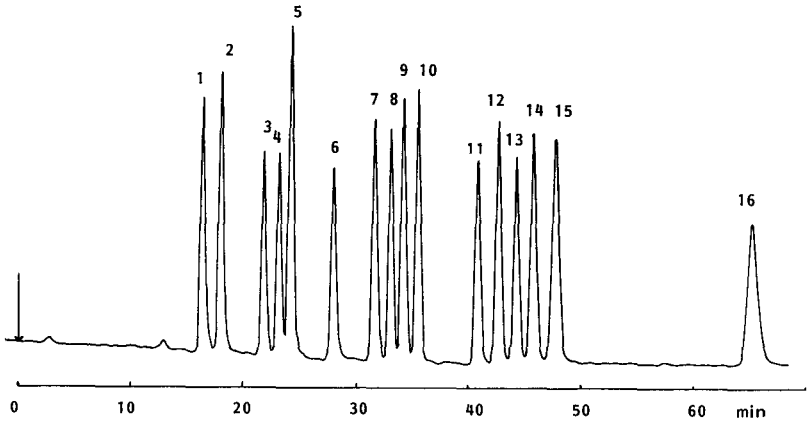

Fig. 5. Typical chromatogram of bile acids obtained with the proposed method. Peaks: 1 = GUDC; 2 = TUDCA; 3 = GCA; 4 = TCA; 5 = UDCA; 6 = CA; 7 = GCDCA; 8 = TCDCA; 9 = GDCA; 10 = TDCA; 11 = CDCA; 12 = DCA; 13 = GLCA; 14 = TLCA; 15 = I.S.; 16 = LCA [CA = cholic acid, DCA = deoxycholic acid, CDCA = chenodeoxycholic acid, UDCA = ursodeoxycholic acid, LCA = lithocholic acid, G = glyco-, T = tauro-, I.S. = internal standard (5α-pregnane-3α,17α,20α-triol)].

The chromatographic conditions were examined in order to obtain a complete separation of all unconjugated bile acids and their glycine and taurine conjugates. From the results, the gradient elution mode and the acetonitrile–methanol–30 m*M* ammonium acetate as the mobile phase were selected. A typical chromatogram is shown in Fig. 5. All the bile acids were completely separated from each other. Calibration graphs were costructed from the chromatograms obtained by injecting a standard mixture of bile acids and I.S. and were linear for all the bile acids over the range 10–500 pmol. The detection limit was about 2 pmol per injection (signal-to-noise ratio = 2). This value is comparable to those obtained with previous methods[2–4], with fluorescence prederivatization and electrochemical detection. The relative standard deviations at 250 and 500 pmol ranged from 0.9 to 13.2% and from 1.5 to 9.4%, respectively.

In order to determine the recovery for each bile acid, standard solutions of bile acids were added to normal bile and serum and assayed using the HPLC system after extraction as described under Experimental. The mean recoveries of each bile acid (n = 5) ranged from 85.1 to 112.1% for bile and from 93.7 to 103.7% for serum.

The applicability of the method to clinical samples was examined by the assay of bile acids in bile and serum samples from patients. Typical chromatograms for patients with liver cancer and chirrhosis are shown in Fig. 6. Characteristic patterns of individual serum bile acids were observed in patients with these diseases. Unconjugated bile acids and glycine and taurine conjugates were significally elevated in patients with liver chirrhosis and carcinoma. The results suggested that the method may

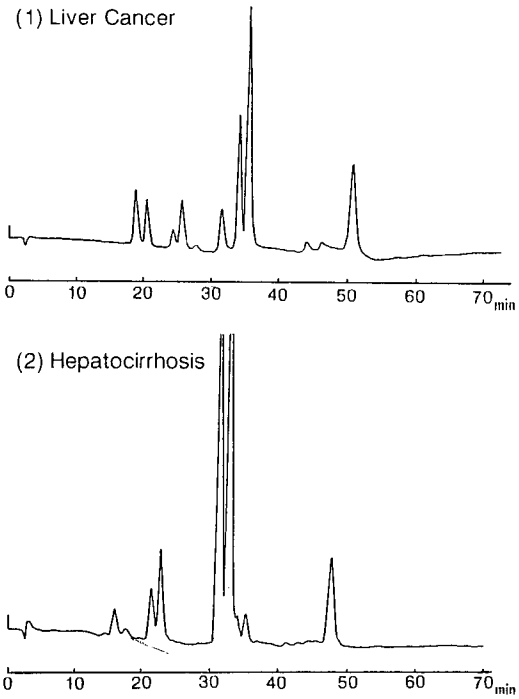

Fig. 6. Typical chromatograms of bile acids in serum from patients.

provide more precise information on the metabolic profile of bile acids in patients with various diseases, including hepatobiliary diseases.

CONCLUSION

The HPLC of bile acids using the immobilized 3α-HSD enzyme reactor and the NADH chemiluminescence reaction for detection is sensitive enough for the determination of bile acids in human serum. This metod has advantages over previous HPLC methods as since unconjugated bile acids and their glycine and taurine conjugates can be determined simultaneously in a single step using a simple linear methanol gradient without prior fractionation of the sample and prior hydrolysis of the conjugatad bile acids. Although the complicated detection system using three pumps is a disadvantage, this problem may be overcome by improving the system for the delivery of the postcolumn reagent solutions.

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