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ANALYSIS OF BILE ACIDS BY MICRO HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH POSTCOLUMN ENZYME REACTION AND FLUOROMETRIC DETECTION

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SUMMARY

Free and conjugated bile acids in human sera were analyzed by micro high-performance liquid chromatography with detection by an immobilized hydroxysteroid dehydrogenase postcolumn reaction and a spectrofluorometer. Bile acids could be effectively collected on a precolumn, 10 × 0.2 mm I.D., packed with silica-ODS (octadecylsilane), by passing a dilute phosphate solution of a serum through it. The detection limit was 0.13–0.28 pmole for a signal-to-noise ratio of 2. An amount of 0.1 ml of serum was enough for analysing each bile acid in it.

INTRODUCTION

Analysis of bile acids in serum is required for diagnostic purposes as the abnormal presence of such acids reflects a functional disorder of the liver. High-performance liquid chromatographic (HPLC) analysis of bile acids with 3α-hydroxysteroïd dehydrogenase (3α-HSD) postcolumn derivatization[1–3] seems very promising with respect to resolution, sensitivity and quantitation, compared with other methods, such as gas chromatography[4], gas chromatography–mass spectrometry[5,6], thin-layer chromatography[7] and HPLC with ultraviolet (UV)[8–10], refractive index (RI)[11–15] or other detectors[16,17]. The 3α-hydroxy group in bile acids is oxidized to a keto group by the enzyme reaction, while β-nicotinamide–adenine dinucleotide (NAD) is reduced to NADH, which is subjected to fluorometric detection[1,3]. In previous work[3], we found that the sensitivity of detection was greatly increased by premixing NAD with the mobile phase by using a single pump, mainly thanks to the absence of pulsation of the pump.

Pretreatments for the analysis of bile acids in serum are somewhat time-consuming, *e.g.*, extraction with hot ethanol, followed by evaporation. We found that the micro precolumn concentration method was very useful for the analysis of corticosteroids in serum[18,19]. In that work, serum diluted ten times in distilled water was passed through a micro precolumn for collection prior to the separation. The precolumn concentration method can also be applied to the analysis of bile acids in serum. This paper describes the analysis of bile acids by micro HPLC with an immobilized HSD postcolumn and a fluorometer.

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EXPERIMENTAL

A liquid chromatograph was assembled from a Micro Feeder (Azumadenki-kogyo, Tokyo, Japan), equipped with a gas-tight syringe, GAN-050 (0.5 ml; Terumo, Tokyo, Japan) as a pump, a laboratory-built gradient maker, a micro valve injector (0.011 μl; JASCO, Japan Spectroscopic, Tokyo, Japan), a precolumn, a guard column, a seperating column, an immobilized postcolumn and a spectrophotofluorometer FP-110C (JASCO), equipped with a modified flow cell, as shown in Fig. 1. The gradient maker consisted of a mixing vessel and a magnetic stirrer Model SS-5 (Toyo Kagakusangyo, Tokyo, Japan). The mixing vessel was a modified gas-tight syringe, as described previously[20]. The volume of the mixing vessel was 416 μl. The gradient profile was exponential, and determined by the ratio of the mobile phase flow-rate to the volume of the mixing vessel. The precolumn was composed of a PTFE tubing (10 × 0.20 mm I.D.), packed with Develosil ODS-15/30 (15–30 μm; Nomura Chemical, Seto-shi, Japan). The precolumn was employed for the collection of bile acids. The guard column was made of PTFE tubing (5 × 0.20 mm I.D.), packed with silica ODS SC-01 (5 μm; JASCO). It was changed when it became resistant to flow. The separating column was made from fused-silica tubing (20 cm × 0.26 mm I.D.), packed with silica ODS SC-01. The immobilized postcolumn was made from fused-silica tubing (20 × 0.34 mm I.D.), packed with HSD-immobilized controlled-pore glass beads (200–400 mesh). Procedures for the preparation of immobilized packings were the same as in the previous work[3]. HSD packings were stable over several months, if stored in a refrigerator. The postcolumn was prepared before every chromatographic analysis. The flow cell of the fluorometer was the same as in the previous work[3].

Ursodeoxycholic acid (UDC), cholic acid (C), chenodeoxycholic acid (CDC), deoxycholic acid (DC) and lithocholic acid (LC) were purchased from Sigma (St-Louis, MO, U.S.A.). Glycocholic acid (GC), sodium salts of other glycine conjugates [glycoursodeoxycholicacid(GUDC),glycochenodeoxycholicacid(GCDC),glycodeoxycholic acid (GDC), glycolithocholic acid (GLC)] and taurine conjugates [tauroursodeoxycholic acid (TUDC), taurocholic acid (TC), taurochenodeoxycholic acid (TCDC), taurodeoxycholic acid (TDC), taurolithocholic acid (TLC)] were kindly supplied by JASCO. Bile acids were dissolved in ethanol (Wako, Osaka, Japan) and injected with a micro valve injector. NAD and HSD were obtained from Sigma. Other reagents were obtained from Wako, unless stated otherwise.

Human serum was separated from the blood clot after coagulation and centrifugation at a rate of 1600 *g* for 10 min and filtered through a membrane filter (0.45 μm; Toyo Roshi, Tokyo, Japan) before use. The filtered serum was diluted ten times in 10 m*M* phosphate solution (pH = 7–8). One ml of the phosphate solution of serum was drawn into a gas-tight syringe and forced into the precolumn. The

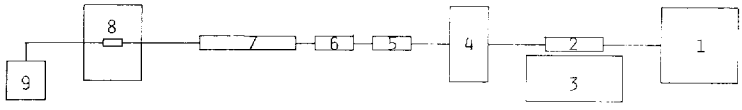

Fig. 1. Block diagram of the apparatus. 1 = pump; 2 = mixing vessel; 3 = magnetic stirrer; 4 = micro valve injector; 5 = precolumn; 6 = guard column; 7 = separating column; 8 = detector; 9 = waste reservoir.

phosphate solution was also filtered through a membrane filter before use, otherwise clogging problems sometimes occurred. Furthermore, PTFE tubing, packed with quartz wool, was connected to the head of the precolumn for filtration. It took 10–20 min to pass 1 ml of a serum sample into the precolumn. Then 10–20 μl of phosphate solution were forced into the precolumn for washing. Prior to analysis, the separating column was conditioned by passing the initial mobile phase through it. The mobile phase was also filtered through a membrane filter (0.45 μm). In precolumn injection, after an adequate volume (equivalent to the dead-volume between the separating column and the mixing vessel) of the mobile phase had been forced through the separating column, the pump was stopped and the flow through the micro valve injector was shut off. Then the precolumn was connected to the head of the guard column and the analysis was started.

In the previous work[3] the mobile phase composition was somewhat complex, *e.g.*, acetonitrile–phosphate solutions containing NAD, 2-mercaptoethanol, disodium ethylenediaminetetraacetate and ammonium carbonate. Simple mobile phase compositions were examined, *viz.*, acetonitrile–phosphate solutions containing only NAD. Potassium dihydrogenorthophosphate and dipotassium monohydrogenorthophosphate were employed as phosphate reagents and the pH was adjusted with potassium hydroxide. In the case of the gradient separation of fifteen bile acids, 60 m*M* phosphate solutions (pH = 9.8 and 9.5) and a 60 m*M* phosphate solution (pH = 8.9) containing 18 m*M* NAD were prepared as stock solutions and stored in a refrigerator. 60 m*M* phosphate solution (pH = 9.8) – 60 m*M* phosphate solution (pH = 8.9) containing 18 m*M* NAD–acetonitrile (70:10:20) was employed as the initial solution (A), and 60 m*M* phosphate solution (pH = 9.5) – 60 m*M* phosphate solution (pH = 8.9) containing 18 m*M* NAD – acetonitrile (30:10:60) was employed as final solution (B). The pH of the mobile phases A and B was 8.8–8.9.

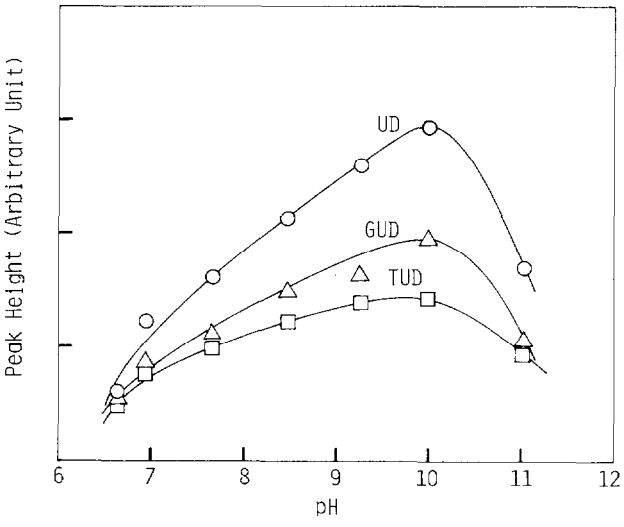

Fig. 2. Effect of the mobile phase pH on peak height. Postcolumn: 20 × 0.34 mm I.D. Mobile phase: acetonitrile–10 m*M* phosphate solution (20:80) containing 1 m*M* NAD. Flow-rate: 1.4 μl/min.

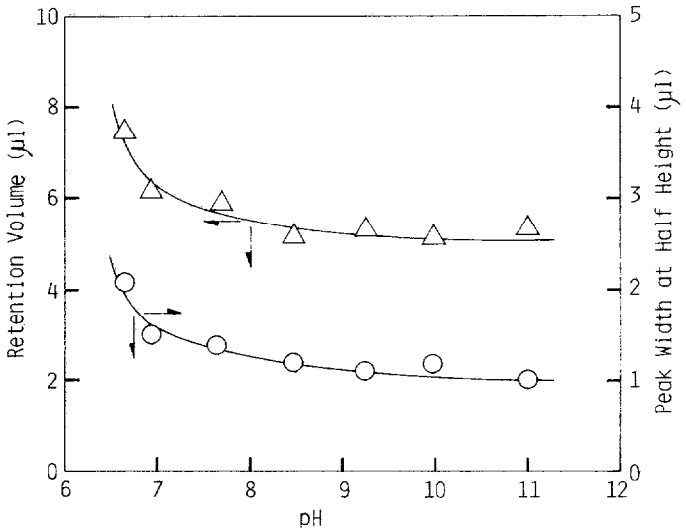

Fig. 3. Effects of the mobile phase pH on retention volume and peak width. Operating conditions as in Fig. 2.

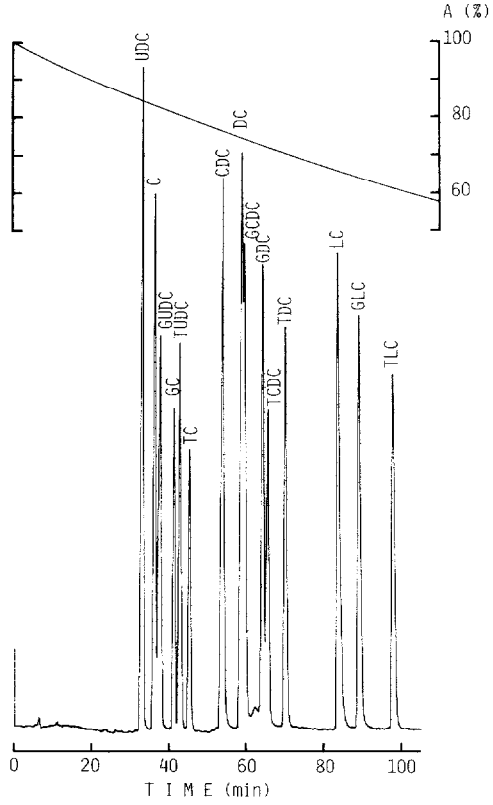

Fig. 4. Separation of standard bile acids with valve injection. Column: silica ODS SC-01, 20 cm × 0.26 mm I.D. Guard column: silica ODS SC-01, 5 × 0.20 mm I.D. Mobile phase: see text; gradient profile as indicated. Flow-rate: 2.1 μl/min. Postcolumn: 20 × 0.34 mm I.D. Wavelength: excitation 365 nm; remission, 470 nm. Sample: 0.011 μl of ethanol solution containing 21.7 ng of UDC, 24.0 ng of C, 22.0 ng of GUDC, 20.4 ng of GC, 21.1 ng of TUDC, 21.7 ng of TC, 22.6 ng of CDC, 21.7 ng of DC, 20.9 ng of GCDC, 19.9 ng of GDC, 20.2 ng of TCDC, 18.7 ng of TDC, 20.6 ng of LC, 20.1 ng of GLC and 18.8 ng of TLC.

TABLE I

REPRODUCIBILITY OF RETENTION TIME (VALVE INJECTION)

S.D. standard deviation; R.S.D. = relative standard deviation.

Solute	*Retention time (min)*	*S.D. (min)*	*R.S.D. (%)*
UDC	33.7	0.76	2.3
C	36.7	0.72	2.0
GUDC	38.0	0.65	1.7
GC	41.5	0.71	1.7
TUDC	43.3	0.69	1.6
TC	45.8	0.76	1.7
CDC	54.2	0.87	1.6
DC	59.3	0.91	1.5
GCDC	59.9	0.86	1.4
GDC	64.6	0.89	1.4
TCDC	65.9	0.91	1.4
TDC	70.4	0.95	1.3
LC	84.6	1.05	1.2
GLC	89.6	1.14	1.3
TLC	98.4	1.26	1.3

RESULTS AND DISCUSSION

The pH of the mobile phase was the most significant parameter. It affected both selectivity and sensitivity. Fig. 2 shows the effect of the mobile phase pH on peak height without using a precolumn, a guard column and a separation column. A pH of around 10 gave the highest sensitivity. However, NAD is unstable above pH 10. Thus, the pH of the mobile phase should be lower than 10, which is also required in view of the stability of the enzyme and silica-ODS packings.

Fig. 3 shows the effect of the mobile phase pH on the retention volume and

TABLE II

REPRODUCIBILITY OF PEAK HEIGHT (VALVE INJECTION)

Operating conditions as in Fig. 4.

Solute	*Amount (ng)*	*Mean peak height (Arbitrary units)*	*R.S.D. (%)*
UDC	21.7	173.2	0.95
C	24.0	140.0	1.4
GUDC	22.0	102.5	1.7
GC	20.4	83.5	2.3
TUDC	21.1	100.9	1.8
TC	21.7	73.2	2.3
CDC	22.6	143.2	1.5
GDC	19.9	117.9	2.5
TCDC	20.2	80.4	2.2
TDC	18.7	105.1	2.7
LC	20.6	124.5	2.3
GLC	20.1	107.0	2.8
TLC	18.8	91.5	3.4

peak width. The operating conditions are the same as in Fig. 2. These results indicate that bile acids are retarded on the postcolumn and that both retention volume and peak width decrease with increasing pH of the mobile phase. Therefore, a higher pH value would be preferred.

The capacity factor (k') of free bile acids decreased with increasing pH of the mobile phase, while the dependence of k' of conjugate bile acids on pH was slight. The resolution of fifteen bile acids was improved by increasing the pH of the mobile phase.

The concentration of NAD also affected the sensitivity. The optimum concentration was around 1.8 mM. Thus, an 18 mM NAD solution was prepared as the stock solution and diluted ten times in the mobile phase prior to use.

A typical separation of bile acids is demonstrated in Fig. 4. The resolution of bile acids was satisfactory, except for DC and GCDC. The sample was loaded by the micro valve injector with an injection volume of 0.011 μl. Around 20 ng of each bile acid can be detected. In addition, a peak appeared between GCDC and GDC which originated from the ethanol employed as a solvent.

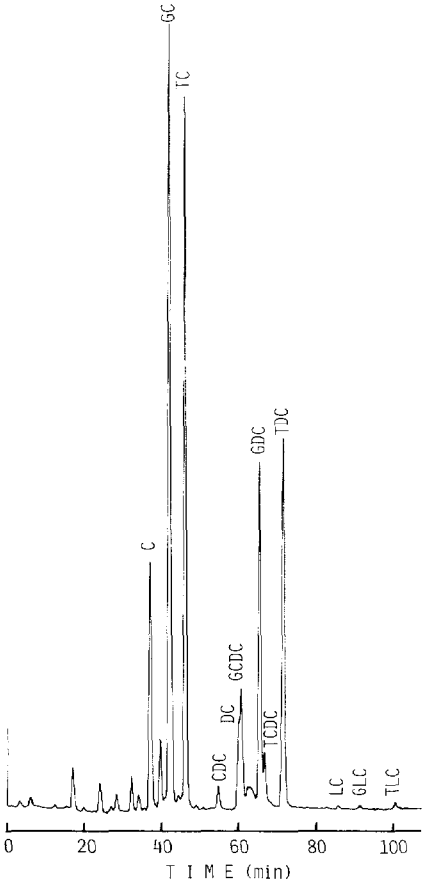

Fig. 5. Separation of bile acids in dehydrated gall bladder. Operating conditions as in Fig. 4 except the sample. Sample: see text.

The gradient profile was exponential but almost linear. The accuracy of the gradient profile has previously been confirmed[20]. The reproducibility of retention time with valve injection for eleven measurements is demonstrated in Table I. Relative standard deviations were 1.2–2.3%, which are acceptable values.

The reproducibility of peak height for five measurements is shown in Table II. Operating conditions were the same as in Fig. 4. Deviation of peak height arising from the fluctuation of the postcolumn reaction efficiency may be observed, although the postcolumn was freshly prepared before each chromatographic analysis. When the postcolumn was used repeatedly, the peak heights of bile acids gradually decreased, owing to the deactivation of the enzyme. The decrease in peak heights was remarkable, especially for bile acids possessing a 7α-hydroxy group. The reproducibility of peak heights was improved by preparing the postcolumn freshly before each chromatographic analysis. Linear relationships between sample amounts and peak heights were observed.

The sensitivity is much higher in this micro HPLC system than with other methods. The detection limit for a signal-to-noise ratio of 2 was 0.13–0.28 pmole (10^{-12} mole). This result is mainly due to premixing NAD with the mobile phase and optimization of the mobile phase.

This system was applied to the separation of bile acids in 0.5 g of dehydrated gall bladder, as shown in Fig. 5. The dehydrated gall bladder was extracted with 10 ml of ethanol under ultrasonic vibration and filtered through a membrane filter (0.45 μm). The filtrate was diluted to twice the volume in ethanol, and a portion was injected with a micro valve injector. Cholic acids, chenodeoxycholic acids and deoxycholic acids were the main constituents, but peaks due to material other than bile acids appeared in the chromatogram.

Bile acids are present in serum at low concentration. Thus, pretreatments are indispensable to the analysis. The micro precolumn concentration method is convenient for micro HPLC in the analysis of dilute components[18,19]. The total amount of

TABLE III

REPRODUCIBILITY OF RETENTION TIME (PRECOLUMN INJECTION)

Solute	*Retention time (min)*	*S.D. (min)*	*R.S.D. (%)*
UDC	33.0	0.53	1.6
C	36.1	0.55	1.5
GUDC	37.4	0.51	1.4
GC	41.0	0.57	1.4
TUDC	42.8	0.61	1.4
TC	45.2	0.60	1.3
CDC	53.6	0.74	1.4
DC	58.7	0.64	1.1
GCDC	59.3	0.81	1.4
GDC	64.0	0.84	1.3
TCDC	65.3	0.84	1.3
TDC	69.7	0.91	1.3
LC	83.7	0.99	1.2
GLC	89.0	1.14	1.3
TLC	97.5	1.19	1.2

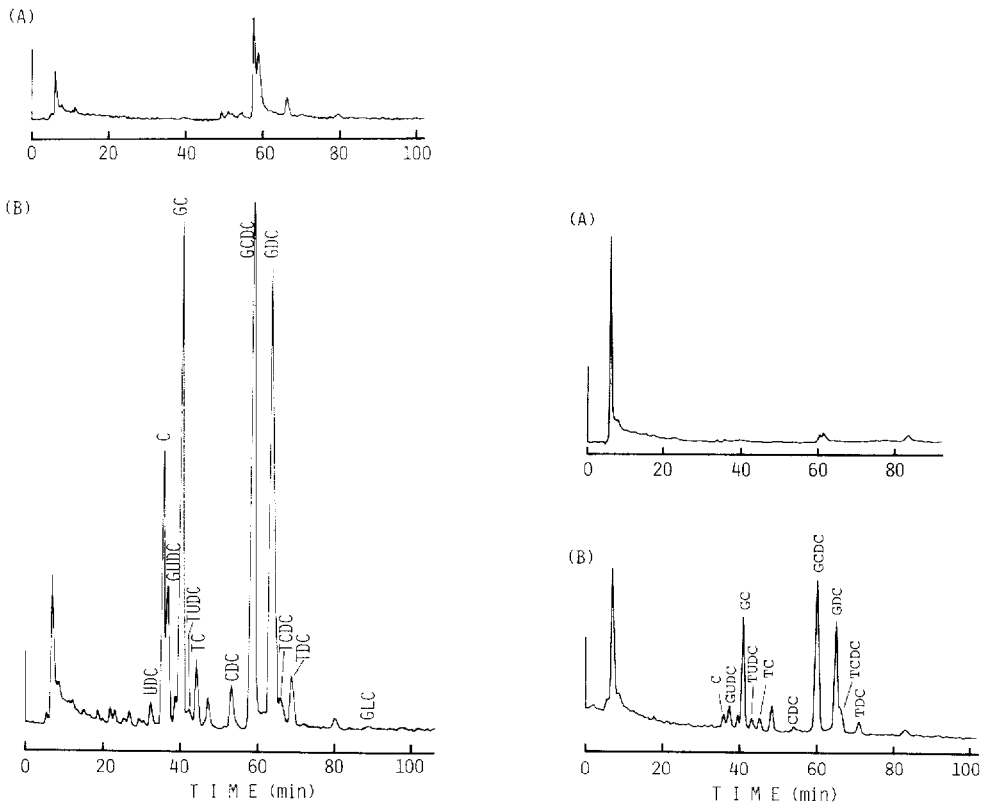

Fig. 6. Separation of bile acids in the serum of a patient with alcoholic cirrhosis. Operating conditions as in Fig. 4 except for sample and precolumn. Sample: 0.1 ml of serum from a patient with alcoholic cirrhosis. A, Operation without a postcolumn; B, operation with a postcolumn. Precolumn: Develosil ODS-15/30, 10 × 0.2 mm I.D.

Fig. 7. Separation of bile acids in the serum of a healthy volunteer. Operating conditions as in Fig. 6 except the sample. Sample: 0.1 ml of serum from a healthy volunteer.

bile acids in 1 ml of a healthy human serum is around 1 μg. Thus, 0.1 ml of a serum is a large enough sample for this system. The concentration of bile acids by a micro precolumn was examined. An ethanol stock solution of bile acids (each *ca.* 2 μg/ml) was diluted 100 times in 10 m*M* phosphate solution (pH = 7.8), the concentration of each bile acid being around 20 ng/ml. The prepared sample was placed in a gas-tight syringe (1 ml) and then passed through the precolumn. This took 10–20 min.

Peak heights were proportional to concentrations in the range from 10 ng/ml to 40 ng/ml when a 10 × 0.20 mm I.D. precolumn was used. Recovery was defined as the ratio of the slopes of calibration curves for valve and precolumn injection methods. The recovery was 88–103% depending on the solutes. The recoveries of solutes with long retention times were slightly low, probably due to adsorption on the surface of the gas-tight syringe during concentration. Deterioration of resolution arising from precolumn injection was not observed, compared with valve injection. In the separation of bile acids in serum, there was no interference, since ethanol was not contained in the sample solution.

The reproducibility of retention times for precolumn injection is shown in Table III. Relative standard deviations for eleven measurements were 1.1–1.6%, *i.e.*, lower than those for valve injection.

Separations of bile acids in the serum of a patient with alcoholic cirrhosis and in a healthy volunteer are shown in Figs. 6 and 7, respectively. The upper tracings were obtained without a postcolumn, in which fluorescent compounds other than NADH were detected. The difference in amounts of bile acids between the two chromatograms is distinct.

Mixtures of 3α- and 3β-HSD were immobilized in these experiments, therefore steroids possessing a 3β-hydroxyl group can also be detected.

CONCLUSION

Bile acids in serum could be analyzed by micro HPLC with the use of a simple pretreatment. The sensitivity was highly increased, so that the amount of sample required was decreased to 0.1 ml of a serum. The resolution of bile acids was satisfactory, except for DC and GCDC.

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