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Note

Determination of bile acids in rat bile by high-performance liquid chromatography

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The determination of individual bile acids in biological fluids is important for studying bile acid metabolism in hepatobiliary and other diseases. The rat has been widely used in studies of bile acid metabolism, in which bile acids have been analysed by gas chromatography (GC) [1—3] and gas chromatography—mass spectrometry (GC—MS) [4]. In recent years, high-performance liquid chromatography (HPLC) has been applied to the analysis of bile acids in human bile [5—7] and serum [8—11]. However, the results for rat samples from this procedure with HPLC have not been satisfactory, because rats, like mice, possess several peculiar bile acids, such as α-, β-, and ω-muricholic acid (MCA), which complicate the bile acid separation.

This report describes a method for separating and quantifying the eight bile acids α-MCA, β-MCA, cholic acid (CA), ursodeoxycholic acid (UDCA), hyodeoxycholic acid (HDCA), chenodeoxycholic acid (CDCA), deoxycholic acid (DCA), and lithocholic acid (LCA) in rat bile by HPLC, with an

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immobilized 3α-hydroxysteroid dehydrogenase (3α-HSD) column reactor for detection.

EXPERIMENTAL

Materials

Most of the standard bile acids and 5β-androstane-3α,11α,17β-triol were purchased from Sigma (St. Louis, MO, U.S.A.) and Steraloids (Wilton, NH, U.S.A.). α-MCA, β-MCA and ω-MCA were synthesized according to the methods of Hsia and co-workers [12, 13]. 3α,7β,12α-Trihydroxy-5β-cholanoic acid was synthesized according to the method of Samuelsson [14]. NAD was obtained from Oriental Yeast Co. (Tokyo, Japan) and 3α-HSD from Sigma. The other chemicals of reagent grade were purchased from Wako (Osaka, Japan).

Apparatus

An HPLC Model LC-3A (Shimadzu, Kyoto, Japan) equipped with a stepwise elution unit SGR-1A (Shimadzu) was used. A Zorbax C_8 column (particle size 5 μm, 250 mm × 4.6 mm I.D.; Dupont Instruments, Wilmington, DE, U.S.A.) was used at 40°C in a column oven CTO-2A (Shimadzu). 3α-HSD-bound amino glass beads (120–200 mesh) prepared by the glutaraldehyde method [15] were packed in a stainless-steel tube (50 mm × 2.1 mm I.D.) and used as a reactor at 20°C in a water bath. The reagent containing NAD was delivered by a peristaltic pump PRR-2A (Shimadzu). A spectrofluorophotometer RF-530 (Shimadzu) was used as a detector to monitor NADH fluorescence. A schematic flow diagram of the system is shown in Fig. 1.

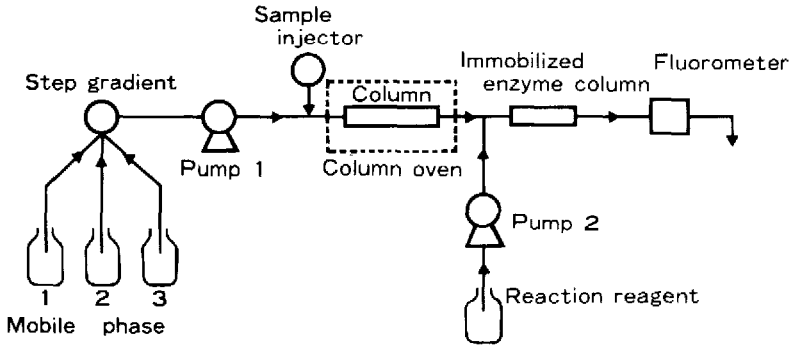

Fig. 1. Schematic flow diagram of the system.

Liquid chromatography

Individual bile acids were separated by reversed-phase chromatography with stepwise elution at a flow-rate of 0.5 ml/min. The initial mobile phase was 100 m*M* potassium phosphate buffer, pH 6.8—acetonitrile (10:4, v/v), the second was 20 m*M* potassium phosphate buffer, pH 6.8—acetonitrile (10:4, v/v), and the last was 1 m*M* potassium phosphate buffer, pH 6.8—acetonitrile (10:5, v/v). The time schedule for the stepwise elution was 8, 12 and 10 min for the first, the second and the third mobile phase, respectively. The eluate from the column was mixed with the NAD solution delivered at a flow-rate of 0.7

ml/min and migrated through the immobilized 3α-HSD column reactor. The NAD solution was prepared by dissolving NAD at the concentration of 0.1% in 100 m*M* Tris—HCl buffer (pH 8.0) containing 0.1% disodium EDTA. NADH formed in the reactor was monitored by the fluorescence detector at excitation 340 nm and emission 460 nm.

Standard bile acid solution

Eight standard bile acids (α-MCA, β-MCA, CA, UDCA, HDCA, CDCA, DCA, and LCA) and 5β-androstane-3α,11α,17β-triol as an internal standard were dissolved in the initial mobile phase.

Rat bile preparation

Wistar strain male rats weighing about 300 g were used; fresh bile was obtained by bile duct cannulation. Rat bile was prepared according to a slight modification of the method described previously [1]. To 10 μl of rat bile were added 100 μl of the internal standard solution (100 μg/ml in ethanol). Ethanol (2 ml) was added to the mixture and the solution was boiled for about 5 min and centrifuged at 650 *g* for 15 min. The supernatant solution was transferred into another tube. The residue was further extracted twice with 2 ml of ethanol twice. The extracts were combined to the supernatant solution and evaporated to dryness under reduced pressure. The residue was dissolved in 2 ml of 1.25 *M* sodium hydroxide solution and hydrolysed at 120°C for 6 h. The reaction mixture was acidified to pH 1.0—2.0 with 2 *M* hydrochloric acid solution and extracted three times with 6 ml of diethyl ether. The combined extracts were evaporated to dryness and the residue was dissolved in 1 ml of the initial mobile phase. A sample of 10—20 μl of the solution was used for HPLC.

RESULTS AND DISCUSSION

In addition to CA, CDCA, DCA, UDCA and LCA, rat bile contains α-MCA, β-MCA which are not found in human bile [1—4]. Since these bile acids could not be separated practically in a short time by HPLC with an isocratic elution system, we adopted a stepwise elution system. Fig. 2 shows a chromatogram of the eight standard bile acids and the internal standard. The components were resolved within 40 min into nine peaks with good separation. The relationship between the amount of each bile acid and the detector response was linear until 1.2 μg and the detection limit ranged from 0.8 to 1.5 ng for a signal-to-noise ratio of 2. Table I shows the reproducibility of the present method for 300 ng of the individual bile acids and the internal standard. The coefficient of variation (C.V.) for the retention time of each bile acid was less than 0.23% and that for the peak area was less than 1.04%.

In the preparation of rat bile, the bile acids were extracted effectively after hydrolysis. Recovery of each bile acid was in the range 93.9—98.3% (C.V. = 0.86—2.31%, $n = 5$). The internal standard was also recovered at 94.3% (C.V. = 0.94, $n = 5$).

Fig. 3 shows a chromatogram of the bile acids in normal rat bile. α-MCA, UDCA, HDCA, CDCA and DCA were detected with CA and β-MCA being the major components, but LCA could hardly be found. Several other unidentified

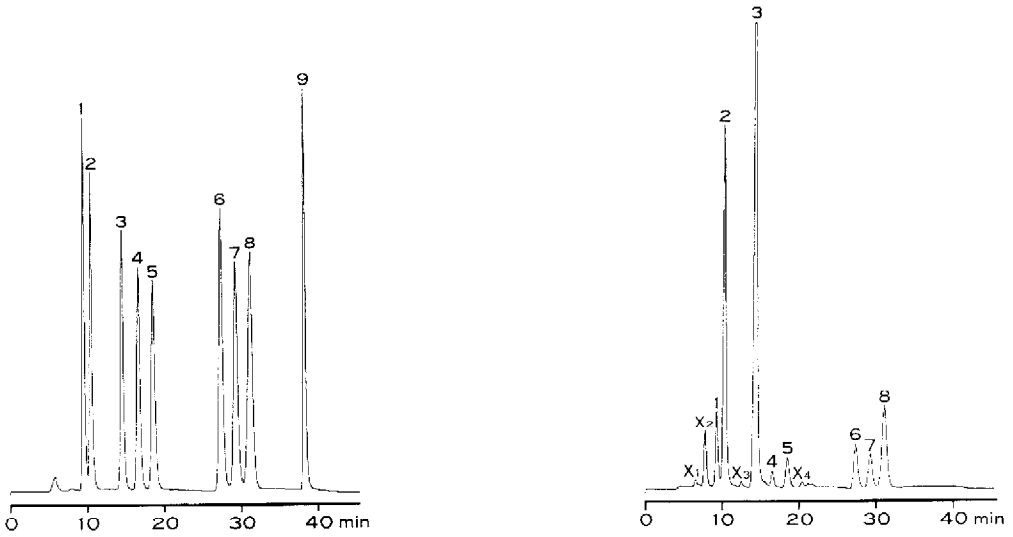

Fig. 2. Separation of standard bile acids and an internal standard. A mixture of 300 ng of each was injected. Peaks: 1 = α-muricholic acid; 2 = β-muricholic acid; 3 = cholic acid; 4 = ursodeoxycholic acid; 5 = hyodeoxycholic acid; 6 = chenodeoxycholic acid; 7 = deoxycholic acid; 8 = 5β-androstane-3α,11α,17β-triol (internal standard); 9 = lithocholic acid.

Fig. 3. Typical chromatogram of bile acids extracted from normal rat bile. Peaks: 1 = α-muricholic acid; 2 = β-muricholic acid; 3 = cholic acid; 4 = ursodeoxycholic acid; 5 = hyodeoxycholic acid; 6 = chenodeoxycholic acid; 7 = deoxycholic acid; 8 = 5β-androstane-3α,11α,17β-triol (internal standard); X_{1-4} = unknown.

TABLE I

REPRODUCIBILITY OF THE PRESENT METHOD FOR THE DETERMINATION OF INDIVIDUAL BILE ACIDS AND AN INTERNAL STANDARD

Column: Zorbax C_8, 250 × 4.6 mm I.D. Mobile phase: stepwise gradient programmed 8, 12 and 10 min for the first, second and third mobile phase, respectively. First mobile phase: 100 m*M* potassium phosphate, pH 6.8—acetonitrile (10:4, v/v); second mobile phase: 20 m*M* potassium phosphate, pH 6.8—acetonitrile (10:4, v/v); third mobile phase: 1 m*M* potassium phosphate, pH 6.8—acetonitrile (10:5, v/v). Flow-rate: 0.5 ml/min.

	Retention time		Relative peak area*	
	Mean (min)	C.V.** (%)	Mean (min)	C.V.** (%)
Internal standard***	31.08	0.12	—	—
α-Muricholic acid	9.35	0.10	0.74	1.03
β-Muricholic acid	10.32	0.15	0.69	0.71
Cholic acid	14.38	0.07	0.64	0.44
Ursodeoxycholic acid	16.53	0.13	0.62	0.54
Hyodeoxycholic acid	18.43	0.11	0.65	0.41
Chenodeoxycholic acid	27.22	0.07	0.93	0.49
Deoxycholic acid	29.11	0.10	0.82	0.32
Lithocholic acid	37.94	0.22	0.85	0.35

*Relative peak area was defined as the ratio of the peak area of individual bile acids to the area of the internal standard.

**$n = 5$.

***5β-Androstane-3α,11α,17β-triol.

peaks (X_1—X_4) were also present. These peaks were due to 3α-hydroxysteroids, since none of them appeared when the rat bile was analysed without NAD in the reagent.

Table II shows the retention times of other bile acids which should be present in rat bile. Their values differ from those of the eight standard bile acids and the internal standard we studied. The unidentified peak X_2 seemed to correspond to 3α-hydroxy-7,12-dioxo-5β-cholanoic acid. Peak X_3 appeared very close to that of hyocholic acid, but differed in its retention time. The small peak X_4 corresponded to 3α-hydroxy-7-oxo-5β-cholanoic acid or 3α-hydroxy-12-oxo-5β-cholanoic acid, which could not be separated by the present method. These unknown peaks, however, require further investigation to be identified. Peaks X_1 and X_3 and the small peaks behind X_4 had different retention times from any bile acid we tested.

TABLE II

RETENTION TIMES OF OTHER BILE ACIDS

Conditions as in Table I.

Bile acids	Retention time (min)	C.V.* (%)
3α,7β,12α-Trihydroxy-5β-cholanoic acid	7.16	0.21
3α-Hydroxy-7,12-dioxo-5β-cholanoic acid	7.78	0.47
ω-Muricholic acid	8.52	0.05
Hyocholic acid	12.29	0.06
3α-Hydroxy-12-oxo-5β-cholanoic acid	20.24	0.02
3α-Hydroxy-7-oxo-5β-cholanoic acid	20.25	0.04

*$n = 3$.

The present method using HPLC in combination with the immobilized 3α-HSD enzymatic detection offers selectivity for 3α-hydroxysteroids and does not require any derivatization of bile acids, which is essential in GC analysis. Deterioration of immobilized enzyme is generally of concern. The immobilized 3α-HSD column reactor in the present method reduced its sensitivity of detection to about 80% but it maintained almost the same reproducibility of intra-assay even after 200 times repetition of analysis. This indicates that our method is a reliable and simple one for analysing individual bile acids in rat bile.

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