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Short Communication

High-performance liquid chromatographic separation of bile acids and bile alcohols diastereoisomeric at C-25

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ABSTRACT

The high-performance liquid chromatographic separation of the 25*R* and 25*S* diastereoisomers of the bile alcohols 5β-cholestane-3α,7α,26-triol and 5β-cholestane-3α,7α,12α,26-tetrol and the bile acids, 3α,7α-dihydroxy-5β-cholestan-26-oic acid and 3α,7α,12α-trihydroxy-5β-cholestan-26-oic acid is described. A Radial-Pak μBondapak C_{18} reversed-phase cartridge was used for the separations and elutions were carried out with acetonitrile–water–methanol–acetic acid mixtures. All eight diastereoisomeric compounds showed baseline separation when up to 200 μg of the isomeric mixtures were injected into the column and the method can be used for isolation of pure diastereoisomers of these bile acids and bile alcohols.

INTRODUCTION

5β-Cholestane-3α,7α-diol and 5β-cholestane-3α,7α,12α-triol (Fig. 1) are considered obligate intermediates in the biosynthesis of chenodeoxycholic acid and cholic

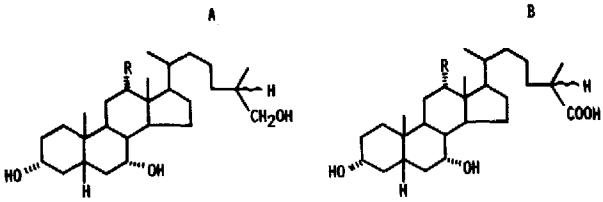

Fig. 1. Structures of bile acids and bile alcohols diastereoisomeric at C-25. (A) 25*R* and 25*S* diastereoisomers of 5β-cholestane-3α,7α,26-triol (R = H) and 5β-cholestane-3α,7α,12α,26-tetrol (R = OH); (B) 25*R* and 25*S* diastereoisomers of DHCA (R = H) and THCA (R = OH).

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acid, respectively, from cholesterol in vertebrates [1]. Shefer *et al.* [2] have recently shown that the biosynthesis of cholic acid involves hydroxylation of 5β-cholestane-3α,7α,12α-triol at C-25 as the initial step, while the classical pathway for the biosynthesis of both primary bile acids is considered to involve intermediates hydroxylated at C-26 [1]. The 26-hydroxylated bile alcohols are then oxidized to 3α,7α-dihydroxy-5β-cholestan-26-oic acid (DHCA) and 3α,7α,12α-trihydroxy-5β-cholestan-26-oic acid (THCA) (Fig. 1), respectively. Further hydroxylation of these C_{27} bile acids at C-24 followed by cleavage of the C-24,25 bond results in the formation of chenodeoxycholic acid and cholic acid, respectively [1]. Traces of the 26-hydroxylated bile alcohols and the cholestanoic acids are present in human bile [3,4]. Large quantities of THCA have been found in the bile of patients with intrahepatic bile duct anomalies [5,6] and Zellweger's syndrome [7]. THCA is also the major biliary bile acid of the alligator [8]. We have recently shown that THCA in both alligator mississippiensis and humans exists as the 25*R* diastereoisomer [9,10] which suggests stereospecificity of the hepatic 26-hydroxylating enzymes. Since, synthetic C_{27} bile acids and bile alcohols are all mixtures of the 25*R* and 25*S* diastereoisomers, it is important to have analytical methods to differentiate between the diastereoisomers of these bile acid intermediates and isolate them in pure form in order to be able to study the stereospecificity of 26-hydroxylation of bile alcohols and metabolism of the pure isomers.

We have developed solvent systems for the thin-layer chromatographic (TLC) separation of the 25*R* and 25*S* diastereoisomers of 5β-cholestane-3α,7α,26-triol,5β-cholestane-3α,7α,12α,26-tetrol and THCA [9,11]. However, the TLC separation requires two or three developments of the TLC plate in the solvent system and bands tend to overlap when larger amounts of compounds are applied on the plate [11]. We report herein separation of these diastereoisomeric bile alcohols and bile acids by high-performance liquid chromatography (HPLC). Diastereoisomers of all compounds are well resolved and the method can be used to isolate quantities of the pure diastereoisomers which may be used as substrates or reference standards to test the substrate specificity of the hepatic 26-hydroxylating enzymes.

EXPERIMENTAL

Materials

25*R* and 25*S* diastereoisomers of 5β-cholestane-3α,7α,26-triol and 5β-cholestane-3α,7α,12α,26-tetrol were synthesized from chenodeoxycholic acid and cholic acid, respectively, as described before [12,13]. Chromic acid oxidation of (25*R* or 25*S*)3α,7α-diformyloxy-5β-cholestane-26-ol [prepared during the synthesis of (25*R* or 25*S*)5β-cholestane-3α,7α,26-triol] followed by alkaline hydrolysis yielded the 25*R* and 25*S* diastereoisomers of DHCA. The 25*R* and 25*S* diastereoisomers of THCA were isolated from the bile of alligator mississippiensis via rigorous hydrolysis of the bile with 25% sodium hydroxide (which resulted in partial isomerization of the 25*R* into the 25*S* diastereoisomer) followed by fractional crystallization [9]. All compounds were >98% pure as judged by TLC and HPLC.

High-performance liquid chromatography

HPLC of the bile acids and bile alcohols was performed on a Waters Assoc. (Millford, MA, U.S.A.) Model M-6000 reciprocating pump and a Model UK6

septumless loop injector. A Waters Assoc. Model 401 differential refractometer was used and the detector response was recorded with a Spectra-Physics (San Jose, CA, U.S.A.) Model SP 4290 integrator. A Waters Assoc. Radial-Pak μBondapak C_{18} reversed-phase column (100 × 8 mm I.D., 5 μm particle size) was employed for all separations. A guard column (Waters Assoc.) prepacked with C_{18} reversed-phase material was placed before the separation column.

A 10–200-μg amount of the bile acid or bile alcohol dissolved in 5–20 μl of methanol was injected into the HPLC column. Solvent systems containing the following proportions of acetonitrile–water–methanol–acetic acid (v/v) were used for analysis: 60:70:20:1 (solvent system A); 70:70:20:1 (system B); 75:70:20:1 (system C) and 80:70:20:1 (system D). The solvents used were HPLC grade and were purchased from Waters Assoc. The flow-rate was maintained at 2.5–3.0 ml/min (operating pressure, *ca.* $13.8 \cdot 10^3$ KPa).

RESULTS AND DISCUSSION

Fig. 2 shows the HPLC analysis of the 25*R* and 25*S* diastereoisomers of the bile acids and bile alcohols with solvent system C as the mobile phase. As is seen from the figure, the diastereoisomers of all four compounds were well resolved in approximately 40 min at a solvent flow-rate of 3 ml/min. 5β-Cholestane-3α,7α,12α,26-tetrol and THCA with three hydroxyl groups in the ring system were eluted significantly earlier than 5β-cholestane-3α,7α,26-triol and DHCA which have two hydroxyl groups in the

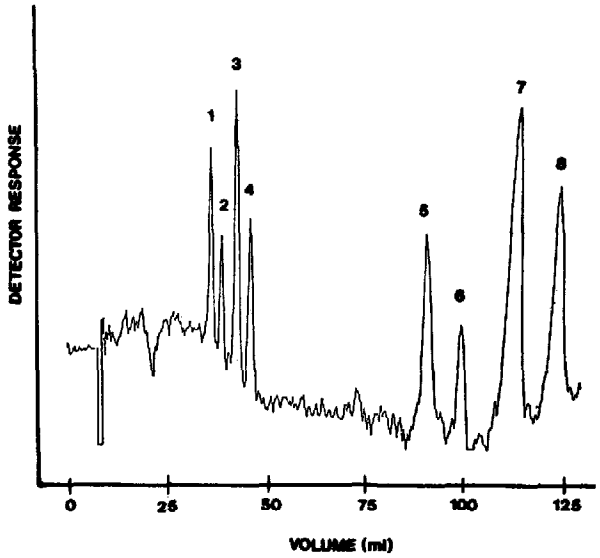

Fig. 2. HPLC of diastereoisomeric bile acids and bile alcohols. Column: 100 × 8 mm I.D. Radial-Pak μBondapak C_{18} reversed-phase cartridge (5 μm). Eluent: acetonitrile–water–methanol–acetic acid (75:70:20:1, v/v). Flow-rate: 3 ml/min. The bile acids and bile alcohols were dissolved in methanol and 20 μl of the solution containing 10–100 μg of each compound was injected into the column. Peaks: 1 = (25*S*)THCA; 2 = (25*R*)THCA; 3 = (25*S*)5β-cholestane-3α,7α,12α,26-tetrol; 4 = (25*R*)5β-cholestane-3α,7α,12α,26-tetrol; 5 = (25*S*)DHCA; 6 = (25*R*)DHCA; 7 = (25*S*)5β-cholestane-3α,7α,26-triol; 8 = (25*R*)5β-cholestane-3α,7α,26-triol.

ring system. Thus, introduction of an hydroxyl group in the ring system greatly increased the polarity of the compound. On the other hand, the retention volume changed only slightly when a bile alcohol and the corresponding bile acid were injected. The retention volumes were highly reproducible and remained within a 2% range when fresh solvent system was used for analysis. Less than 2 μg of the bile acid or bile alcohol could be detected and the detection limit for THCA and 5β-cholestane-3α,7α,12α,26-tetrol was as low as 0.5 μg. In order to isolate bile acids or bile alcohols for biological experiments, 200 μg of each compound could be injected onto the column without appreciable loss of resolution.

It was found that the polarity of the solvent system had profound effect on the retention volumes of the various compounds and the effect was more pronounced for the less polar compounds which were eluted later. Thus, as shown in Table I, reducing the volume of acetonitrile from 75 ml in the solvent system C to 70 ml in system B substantially increased the retention volumes for all compounds and 25*S* and 25*R* diastereoisomers of 5β-cholestane-3α,7α,26-triol were eluted at approximately 143 and 157 ml, respectively. Although the two diastereoisomers of 5β-cholestane-3α,7α,26-triol were very well resolved in this system, the large retention volumes resulted in broad peaks. Further reduction in the amount of acetonitrile in the solvent system (system A) resulted in very high retention volumes for both 5β-cholestane-3α,7α,26-triol and DHCA but the system was quite suitable for the resolution of the diastereoisomers of 5β-cholestane-3α,7α,12α,26-tetrol and THCA. Obviously, larger retention volumes resulted in better resolution between the two diastereoisomers of all compounds. We found that although systems C and D could be employed for the

TABLE I

HPLC RETENTION VOLUMES OF THE DIASTEREOISOMERIC BILE ACIDS AND BILE ALCOHOLS

Bile acids and bile alcohols were subjected to HPLC on a μBondapak 5 μm reversed-phase C_{18} column. For HPLC operating conditions, see Experimental. Solvent systems: (A) acetonitrile–water–methanol–acetic acid (60:70:20:1, v/v), flow-rate, 2.5 ml/min; (B) acetonitrile–water–methanol–acetic acid (70:70:20:1, v/v), flow-rate, 2.5 ml/min; (C) acetonitrile–water–methanol–acetic acid (75:70:20:1, v/v), flow-rate, 3 ml/min; (D) acetonitrile–water–methanol–acetic acid (80:70:20:1, v/v), flow-rate, 3 ml/min.

Compound	HPLC retention volume (ml)			
	A	B	C	D
5β-Cholestane-3α,7α,26-triol (25*R*)	–	157.1	123.1	108.1
5β-Cholestane-3α,7α,26-triol (25*S*)	–	143.0	112.3	98.8
5β-Cholestane-3α,7α,12α,26-tetrol (25*R*)	79.5	52.0	42.8	37.6
5β-Cholestane-3α,7α,12α,26-tetrol (25*S*)	72.4	47.6	39.4	34.7
DHCA (25*R*)	–	124.2	96.8	82.5
DHCA (25*S*)	–	112.2	87.8	74.6
THCA (25*R*)	68.6	43.8	35.5	31.1
THCA (25*S*)	63.1	40.4	32.9	28.7

resolution and characterization of all compounds, systems A and B were more suited for the isolation of pure diastereoisomers of 5β-cholestane-3α,7α,12α,26-tetrol and THCA and baseline resolutions were obtained when 10–200 μg of the diastereoisomeric mixtures were injected into the column.

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