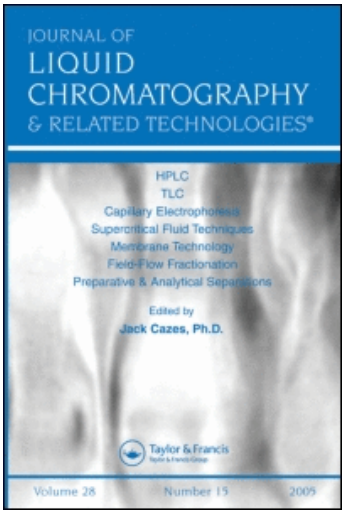

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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF BILE ACIDS. EFFECT OF HYDROXYL GROUPS AT C-3, 6, 7 AND 12 ON BILE ACID MOBILITY

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ABSTRACT

The high-performance liquid chromatographic characteristics of a number of bile acids with hydroxyl groups at C-3, 6, 7 and 12 positions are reported. Using Nova-pak C_{18} reversed-phase columns and mobile phase consisting of acetonitrile/water/methanol/acetic acid mixtures, it was found that compounds with β-hydroxyl groups were eluted much earlier than those with α-hydroxyl groups. Introduction of a hydroxyl group caused a marked increase in polarity of the bile acid, however, the effect was not well defined in bile acids with vicinal glycol system at C-6,7. The retention volumes of the various bile acids were reproducible and can be useful for characterization of bile acids in biological fluids.

INTRODUCTION

Bile acids hydroxylated at C-6 have been isolated in the bile of several animal species. Thus, α-, β- and ω-muricholic acids are present in substan-

tial amounts in rat bile (1) while the major bile acids in the pig are hyodeoxycholic acid and hyocholic acid (2). Although only trace quantities of these compounds are present in humans, substantial amounts are excreted in the urine of patients with hepatobiliary diseases. Several tri- and tetrahydroxylated bile acids with hydroxyl group at C-6 have been reported in the serum and urine of patients with primary biliary cirrhosis and chronic hepatitis (3-8). It is considered that 6-hydroxylation is a mechanism for the liver to get rid of excessive amounts of toxic endogenous bile acids in these diseases (9). These bile acids are further glucuronidated and the glucuronides are preferentially excreted in the urine (10). In order to make a sensible correlation of the presence of these compounds with the disease, it is important to have analytical methods for detection and isolation of the various 6-hydroxy bile acids from biological fluids. A number of bile acids of this series have been recently synthesized (11-15). We report herein high performance liquid chromatographic characteristics of several 6α- and 6β-hydroxy bile acids and compare their retention volumes with those of the corresponding bile acids without a hydroxyl group at C-6. Since most of the bile acids studied are well resolved in the solvent systems employed, we hope that the method will be useful for the isolation of these bile acids from biological fluids.

MATERIALS AND METHODS

Cholic acid, chenodeoxycholic acid, deoxycholic acid, lithocholic acid and 3α,6β-dihydroxy-5β-cholanoic acid were purchased from Steraloids, Inc.

(Wilton, N.H.). Hyodeoxycholic acid and hyocholic acid were from Canada Packers (Toronto, Canada). Ursodeoxycholic acid and ursocholic acid were gifts from Tokyo Tanabe, Japan. The 3β-hydroxy bile acids were synthesized from the corresponding 3α-hydroxy bile acids via azodicarboxylate-epimerization of the 3α-hydroxyl group (16) and the 12β-hydroxy bile acids were prepared via reduction of the corresponding 12-oxo derivatives with potassium/tert. amyl alcohol (17). The α-, β- and ω-muricholic acids were synthesized from chenodeoxycholic acid (12) and 3α,6α,7α,12α-, 3α,6β,7α,12α-, 3α,6β,7β,12α- and 3α,6α,7β,12α-tetrahydroxy-5β-cholanoic acids were synthesized from cholic acid (13,14) following literature methods. The 3α,6β,7α,12β- and 3α,6β,7β,12β-tetrahydroxy-5β-cholanoic acids were synthesized from 3α,12β-dihydroxy-5β-chol-6-enoic acid following methods published by us (15). All compounds were >98% pure as judged by gas-liquid chromatography and all synthesized compounds exhibited mass spectral fragmentation patterns compatible with their structures. Solvents used were HPLC grade and were purchased from Aldrich Chemical Co. (Milwaukee, MA) and deionized water was used to make the solvent systems.

High-Performance Liquid Chromatography (HPLC)

HPLC of the bile acids was performed on a Waters Associates (Millford, MA) Model M-6000 reciprocating pump and a Model UK6 septumless loop injector. A Waters Associates Model 401 differential refractometer was used and the detector response was recorded with a Spectra-Physics (San Jose, CA) Model SP 4290 integrator. A Waters Associates Radial-Pak μBondapak

C_{18} reversed-phase column (100 x 8 mm I.D., 5 μm particle size) was employed for the chromatography. A guard column (Waters Associates) with C_{18} reversed-phase material was placed before the separation column.

A 3-10 μg amount of the bile acid dissolved in 5 μl methanol was injected into the HPLC column. Solvent systems containing the following proportions of acetonitrile/water/methanol/acetic acid (v/v) were used for the analysis depending on the polarity of the bile acid under study: 70:70:20:1 (solvent system A); 60:70:20:1 (solvent system B); 50:70:20:1 (system C); 40:70:20:1 (system D); 30:70:20:1 (system E). The flow rate was maintained at 2 ml/min (operating pressure, ca 10.3 x 10^3 KPa).

RESULTS AND DISCUSSION

Table 1 shows the HPLC retention volumes of various bile acids epimeric at C-3, C-7 and C-12. It is clear from the table that β - orientation of the hydroxyl group renders the bile acid more hydrophilic and it is eluted earlier from the column than the corresponding α-hydroxy derivative (18,19). Thus, isobile acids have greatly reduced retention volumes than the corresponding 3α -hydroxy bile acids and the effect is even more pronounced in case of the 7β - and 12β-hydroxy derivatives of the bile acids (Table 1). This is in accordance with the finding of Shaw et al (18). The retention volumes of the various compounds were greatly altered with change in the polarity of the solvent system. Thus, reducing the volume of acetonitrile in the solvent system (solvent systems B, C and D) resulted in greatly increased retention volumes for all compounds (Table 1), the effect was more pronounced on the more hydrophobic bile acids with all axially oriented hydroxyl groups.

TABLE 1

HPLC Retention Volumes of Bile Acids Epimeric at C-3, C-7 and C-12 [a].

5β-Cholanoic acid	HPLC retention volume (ml)			
	A [b]	B	C	D
3α-Hydroxy-	110.6	- [c]	-	-
3β-Hydroxy-	96.6	-	-	-
3α,7α-Dihydroxy-	29.8	43.0	89.4	-
3β,7α-Dihydroxy-	17.0	23.4	43.5	74.6
3α,7β-Dihydroxy-	11.7	16.0	26.0	48.0
3β,7β-Dihydroxy-	10.8	14.0	24.0	38.8
3α,12α-Dihydroxy-	34.1	49.4	103.2	-
3α,12β-Dihydroxy-	15.4	21.0	34.0	70.0
3α,7α,12α-Trihydroxy-	12.1	16.6	30.6	56.2
3β,7α,12α-Trihydroxy-	6.8	8.0	13.4	25.8
3α,7β,12α-Trihydroxy-	5.2	6.1	7.9	12.9
3α,7α,12β-Trihydroxy-	5.2	6.0	7.8	12.8
3α,7β,12β-Trihydroxy-	3.6	3.8	4.4	5.3

[a] HPLC of the bile acids was performed on a μBondapak 5 μm reversed-phase C_{18} column. For HPLC operating conditions, see 'Experimental' section.

[b] Solvent systems: A, acetonitrile-water-methanol-acetic acid (70:70:20:1); B, acetonitrile-water-methanol-acetic acid (60:70:20:1); C, acetonitrile-water-methanol-acetic acid (50:70:20:1); D, acetonitrile-water-methanol-acetic acid (40:70:20:1). Flow rate, 2 ml/min. Operating pressure, ca 10.3 x 10^3 KPa.

[c] Retention volume was not determined.

The retention volumes of some 6α- and 6β-hydroxylated bile acids are shown in Table 2. Addition of a hydroxyl group at C-6 resulted in reduced retention volume of the bile acid. Like bile acids with α-oriented hydroxyl groups at C-3, C-7 and C-12 (Table 1), hyodeoxycholic acid with a 6α-hydroxyl group had a substantially higher retention volume as compared with 3α,6β-dihydroxy-5β-cholanoic acid which has a 6β-hydroxyl group. In

TABLE 2

HPLC Retention Volumes of 6-Hydroxylated Bile Acids.

5β-Cholanoic acid	HPLC retention volume (ml)			
	B [a]	C	D	E
3α,6α-Dihydroxy-	17.5	33.2	59.4	- [b]
3α,6β-Dihydroxy-	12.5	21.2	42.0	-
3α,6α,7α-Trihydroxy-	12.7	18.8	38.8	100.6
3α,6β,7α-Trihydroxy-	8.2	11.6	21.8	50.6
3α,6α,7β-Trihydroxy-	7.6	10.8	20.2	46.3
3α,6β,7β-Trihydroxy-	9.4	13.7	26.0	59.3
3α,6α,7α,12α-Tetrahydroxy-	-	-	11.9	26.8
3α,6β,7α,12α-Tetrahydroxy-	-	-	7.6	14.5
3α,6α,7β,12α-Tetrahydroxy-	-	-	5.6	11.0
3α,6β,7β,12α-Tetrahydroxy-	-	-	7.4	14.1
3α,6β,7α,12β-Tetrahydroxy-	-	-	4.0	4.8
3α,6β,7β,12β-Tetrahydroxy-	-	-	4.3	5.4

[a] Solvent system: B, C and D, see Table 1. Solvent System E, acetonitrile-water-methanol-acetic acid (30:70:20:1). Flow rate, 2 ml/min. Operating pressure, ca 10.3 x 10^3 KPa.

[b] Retention volume was not determined.

their attempt to define a structure-mobility relationship of bile acids, Shaw et al used the terms 'α- and β-surface' of the bile acid molecule (18). 'α-Surface' is the term used to describe the side below the plane of the ring system while 'β-surface' denotes the side above the plane of the ring system and contributes most to the hydrophobicity of the compound. They observed that for bile acids with hydroxyl groups present only on the α-surface of the molecule, there was a good agreement between the calculated and observed mobilities of the various bile acids, provided the hydroxyl groups were spaced sufficiently apart. The 6α-hydroxy group in hyodeoxycholic

acid is, however, equatorial and coplanar with the β-surface of the compound and therefore the hydrophobicity due to the β-surface is reduced. This renders the compound more hydrophilic than the dihydroxy bile acids like chenodeoxycholic acid and deoxycholic acid that have the 7α- and 12α-hydroxyl groups on the α-side of the molecule (19). The retention volume of hyodeoxycholic acid is expected to be more in line with those of 3α,7β-dihydroxy- and 3α,12β-dihydroxy bile acids with equatorial hydroxyl group in ring B of the nucleus. This is indeed what was observed on HPLC. As is seen from Tables 1 and 2, the retention volume of hyodeoxycholic acid is quite close to that of ursodeoxycholic acid or lagodeoxycholic acid (3α,12β-dihydroxy-5β-cholanoic acid) in all solvent systems employed (systems B , C and D) and is much lower than that of chenodeoxycholic acid or deoxycholic acid. Thus, hyodeoxycholic acid was eluted at 17.5 ml in solvent system B as compared with a retention volume of 16.0 ml for ursodeoxycholic acid and 21.0 ml for lagodeoxychoic acid. In contrast, chenodeoxycholic acid eluted at 29.8 ml and deoxycholic acid at 34.1 ml in this system.

In case of 3α,6β-dihydroxy-5β-cholanoic acid, the 6β-hydroxyl group is axially oriented. This hydroxyl group lies just above the plane of the ring system and therefore strongly interferes with the hydrophobicity of the β-surface of the compound. The result is that this compound is even more polar than hyodeoxycholic acid (Table 2). Thus, it can be concluded that a hydroxyl group on the α-side of the ring system will increase the polarity of the bile acid less than a hydroxyl group that is coplanar with the β-side while the effect will be strongest for a β-hydroxyl group that is axially oriented. On this basis, it can be predicted that the axial 11β-hydroxyl group will increase polarity of the bile acid more than the equatorial 11α-hydroxyl group.

In the bile acids with C-6,7 glycol system the retention volumes followed a different pattern due to hydrogen bonding (18). Thus, the bile acids with the cis-equatorial-axial 6α,7α-diol system are expected to be the least hydrophilic and with the largest retention volumes whereas those with the axial-equatorial 6β,7β-diol system should be the most hydrophilic and with the smallest retention volumes. As is seen from Table 2, both 3α,6α,7α-trihydroxy- and 3α,6α,7α,12α-tetrahydroxy-5β-cholanoic acids have the largest retention volumes in the series. However, instead of the compounds with the 6β,7β-dihydroxy system, the two compounds with a diequatorial 6α,7β-dihydroxy system show the smallest retention volumes. This may be due to the fact that the polarity of the bile acids with cis-equatorial-axial (6α,7α-) and axial-equatorial (6β,7β-) diol systems is reduced due to strong hydrogen bonding between the neighbouring cis-diol system while hydrogen bonding is weak in the trans-diequatorial 6α,7β-dihydroxy compounds. Hydrogen bonding is very weak in the trans-diaxial 6β,7α-diol system since the two hydroxyl groups are farthest apart, however, the 7α-hydroxyl group has a relatively small effect on the polarity of the compound. Despite stronger hydrogen bonding, the relatively strong effects due to 6α- and 7β-hydroxyl groups on the retention volume renders 3α,6α,7β-trihydroxy- and 3α,6α,7β,12α-tetrahydroxy-5β-cholanoic acids more hydrophilic (Table 2).

Due to recent interest in 6-hydroxylated bile acids, it seems desirable to have a method for the separation of various bile acids containing 6α- and 6β-hydroxyl groups. As can be seen from Fig. 1, all four isomeric 3α,6,7-trihydroxy-5β-cholanoic acids show baseline separation in solvent system D and are resolved in less than 20 min (flow rate, 2 ml/min). Attempts to

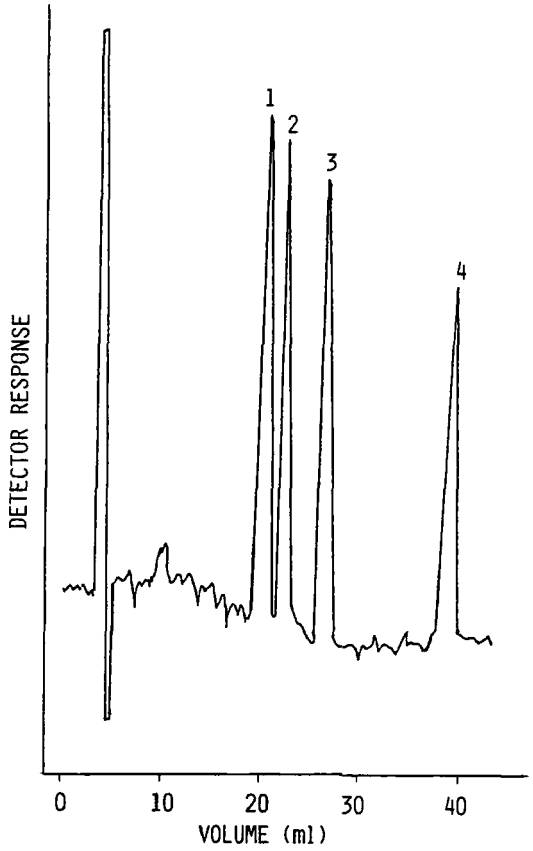

FIGURE 1. HPLC chromatogram of epimeric 3α,6,7-trihydroxy-5β-cholanoic acids. Column:100 x 8 mm I.D. Radial-Pak μBondapak C_{18} reversed-phase cartridge (5μm). Eluent: acetonitrile-water-methanol-acetic acid, 40:70:20:1 (v/v). Flow rate, 2 ml/min. The bile acids were dissolved in methanol and 5 μl of the solution containing 5 μg of each compound was injected into the column. Peaks: 1, 3α,6α,7β-trihydroxy-5β-cholanoic acid; 2, 3α,6β,7α-trihydroxy-5β-cholanoic acid; 3, 3α,6β,7β-trihydroxy-5β-cholanoic acid; 4, 3α,6α,7α-trihydroxy-5β-cholanoic acid.

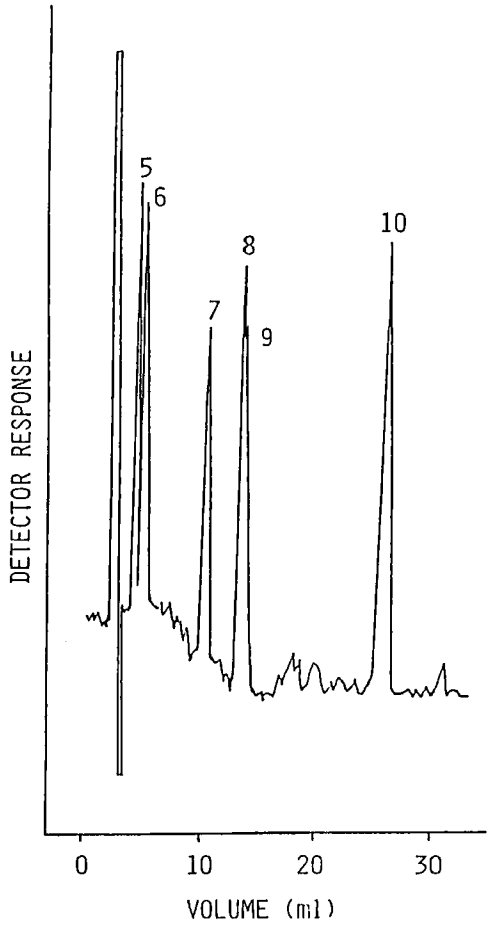

FIGURE 2. HPLC chromatogram of epimeric 3α,6,7,12-tetrahydroxy-5β-cholanoic acid. For HPLC conditions, see Fig. 1. Eluent: acetonitrile-water-methanol-acetic acid, 30:70:20:1 (v/v). The bile acids were dissolved in methanol and 5 μl of the solution containing 3-10 μg of each compound was injected into the column. Peaks: 5, 3α,6β,7α,12β-tetrahydroxy-5β-cholanoic acid; 6, 3α,6β,7β,12β-tetrahydroxy-5β-cholanoic acid; 7, 3α,6α,7β,12α-tetrahydroxy-5β-cholanoic acid; 8, 3α,6β,7β,12α-tetrahydroxy-5β-cholanoic acid; 9, 3α,6β,7α,12α-tetrahydroxy-5β-cholanoic acid; 10, 3α,6α,7α,12α-tetrahydroxy-5β-cholanoic acid.

resolve the four isomers of 3α,6,7,12α-tetrahydroxy-5β-cholanoic acid failed. Although 3α,6α,7α,12α- and 3α,6α,7β,12α-tetrahydroxy-5β-cholanoic acids showed baseline separation from other isomers in solvent system E (Fig. 2), 3α,6β,7α,12α- and 3α,6β,7β,12α-tetrahydroxy-5β-cholanoic acids could not be resolved in the solvent systems used. As expected, the two compounds with 12β-hydroxyl group, 3α,6β,7α,12β-tetrahydroxy- and 3α,6β,7β,12β-tetrahydroxy-5β-cholanoic acids were highly polar and were eluted with less than 6 ml of solvent system E. These compounds did not show baseline resolution in this system although they were almost completely resolved (Fig. 2). Based on the pattern of mobilities of the various bile acids in Tables 1 and 2, it is likely that these two compounds will show better resolution if their retention volumes are increased, e.g., by increasing the proportion of water in the solvent system. It is hoped that the availability of these solvent systems will facilitate isolation of various isomers of 6-hydroxylated bile acids in hepatobiliary diseases and help study their role in these diseases.

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