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Note

High-performance liquid chromatographic separation of higher bile acids*

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Biles from amphibians and reptiles contain in place of the usual bile acids (C_{24}) higher bile acids which nevertheless have the usual type of nuclear structure and the extended side chain[1–4]. The occurrence in nature of higher bile acids is especially interesting since they have been shown to be intermediates or simple modifications of intermediates in the biogenetic sequence between cholesterol and the usual bile acids in mammals[1].

Studies on higher bile acids present in primitive vertebrates and on bile acid biosynthesis in mammals require a convenient micro-technique for the separation and identification of higher bile acids. Gas–liquid chromatography (GLC), which has been widely used for the analysis of the usual bile acids, permits the separation of higher bile acids differing in the length of their side chains and in the presence and absence of the Δ^{22}- or Δ^{23}-double bond[2]. However, the resolution of steroisomers, such as two isomers at C-25 of 3α,7α,12α-trihydroxy-5β-cholestan-26-oic acid (THCA) and four isomers at C-24 and C-25 of 3α,7α,12α,24-tetrahydroxy-5β-cholestan-26-oic acid (TeHCA), is, in practice, not satisfactory[2], besides, recovery of the injected samples is quite difficult, since a destructive flame ionization detector is used.

In recent years, high-performance liquid chromatography (HPLC) of the usual bile acids has been developed[5–11]. This method offers the possibility of separating and qualifying individual higher bile acids. Detection by a non-destructive ultraviolet (UV) detector also permits recovery of the injected higher bile acids for further analysis. In the present paper we report a reversed-phase HPLC separation of higher bile acids and the application of the method to biological samples.

EXPERIMENTAL

HPLC

The instrument used was a Shimazu 830 liquid chromatograph equipped with a single-wavelength (254 nm) UV detector. A reversed-phase column, TSK GEL LS-

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410 (Toyo Soda, Tokyo, Japan), 30 cm × 4 mm I.D., particle size 5 μm, was used for analysing all higher bile acids. It was operated at ambient temperature. The eluent consisted of methanol–water (85:15 or 80:20, v/v), flow-rate 0.7 ml/min.

Preparation of p-bromophenacyl (PBP) esters of higher bile acids

The bile acid (about 1 mg) to be chromatographed was dissolved in 5 ml of anhydrous acetonitrile–methanol (9:1) containing 2.5 mg of *p*-bromophenacyl bromide and 5 μl of N,N-diisopropylethylamine. The reaction mixture was heated at 50–60°C for 1 h. After cooling, the solvent was evaporated to dryness under reduced pressure. The residue was dissolved in 0.5 ml of methanol and filtered through a membrane filter (TM-2P, 0.45 μm; Toyo Roshi). An aliquot of the filtrate was injected into the chromatograph.

Higher bile acids

Cholic acid used as the internal standard was from Sigma. The higher bile acids listed in Table I were isolated from natural sources or were synthesized in this laboratory according to methods described previously[12–19]

Analysis of higher bile acids of Rana catesbeiana

Ten gall-bladders from the bullfrog, *Rana catesbeiana*, were cut under ethanol, and the ethanol extract was filtered. Evaporation of the solvent from the filtrate left a residue (512 mg) which was dissolved in 100 ml of water and extracted with three 50-ml portions of diethyl ether acidified to pH 1 with hydrochloric acid. The ethereal extracts were combined, washed with water until neutral, dried over anhydrous sodium sulphate and concentrated to dryness, leaving a residue. A part of the residue was treated with *p*-bromophenacyl bromide as described above and the resulting PBP derivative was analyzed by HPLC. To elucidate whether isomerization at C-25 of THCA occurs, similar experiments were carried out with 1.0 mg of each of (25*R*)- and (25*S*)-THCAs. These standards were estimated by HPLC to be more than 99% pure. The higher bile acids were dissolved in 0.1 *M* sodium hydroxide solution at room temperature, and were extracted with diethyl ether acidified to pH 1. The samples were then converted into the PBP esters which were analyzed by HPLC.

Analysis of higher bile acids in Bombina orientalis

The bile obtained from six gall-bladders of *Bombina orientalis* was diluted in 30 ml of water, acidified with hydrochloric acid to pH 1 and extracted with diethyl ether. Evaporation of the solvent from the ethereal extract left a residue (10.9 mg). A part of the residue was converted into the PBP ester which was analyzed by HPLC.

RESULTS AND DISCUSSION

Since free higher bile acids do not have a strong UV absorbance, their PBP esters were prepared according to the procedures employed for the derivatization of the usual bile acids[7]. The PBP esters have an intense UV absorbance and are easily detected at 254 nm. Simultaneous quantitation of less than 20 ng of each higher bile acid is possible. Reversed-phase HPLC has made possible the efficient separation of the PBP esters of higher bile acids. Table I summarizes the HPLC retention times for

TABLE I

RELATIVE RETENTION TIMES OF HIGHER BILE ACIDS BY HPLC

Column: TSK GEL LS-410, 30 cm × 4 mm I.D. Flow-rate: 0.7 ml/min. Mobile phase: methanol–water (80:20). Each sample was analysed as the *p*-bromophenacyl ester.

Higher bile acid (reference in parentheses)	*Relative retention time**
3α,7α,12α-Trihydroxy-26,27-dinor-5β-cholestan-25-oic acid (12)	1.29
3α,7α,12α-Trihydroxy-27-nor-5β-cholestan-26-oic acid (13)	1.65
(25*R*)-3α,7α,12α-Trihydroxy-5β-cholestan-26-oic acid (14)	1.92
(25*S*)-3α,7α,12α-Trihydroxy-5β-cholestan-26-oic acid (14)	1.89
3α,7α,12α-Trihydroxy-5β-cholestane-24-carboxylic acid (15)	2.16
3α,7α,12α-Trihydroxy-5β-cholest-23-en-26-oic acid (16)	1.68
(24*E*)-3α,7α,12α-Trihydroxy-5β-cholest-24-en-26-oic acid (17)	1.55
(24*Z*)-3α,7α,12α-Trihydroxy-5β-cholest-24-en-26-oic acid (17)	1.66
3α,7α,12α-Trihydroxy-5β-cholest-22-ene-24-carboxylic acid (18)	1.96
(24*S*)-3α,7α,12α,24-Tetrahydroxy-27-nor-5β-cholestan-26-oic acid (19)	0.80
(24*R*)-3α,7α,12α,24-Tetrahydroxy-27-nor-5β-cholestan-26-oic acid (19)	0.83
(24*R*,25*R*)-3α,7α,12α,24-Tetrahydroxy-5β-cholestan-26-oic acid (17)	1.00
(24*R*,25*S*)-3α,7α,12α,24-Tetrahydroxy-5β-Tetrahydroxy-5β-cholestan-26-oic acid (17)	1.08
(24*S*,25*R*)-3α,7α,12α,24-Tetrahydroxy-5β-cholestan-26-oic acid (17)	0.90
(24*S*,25*S*)-3α,7α,12α,24-Tetrahydroxy-5β-cholestan-26-oic acid (17)	0.98

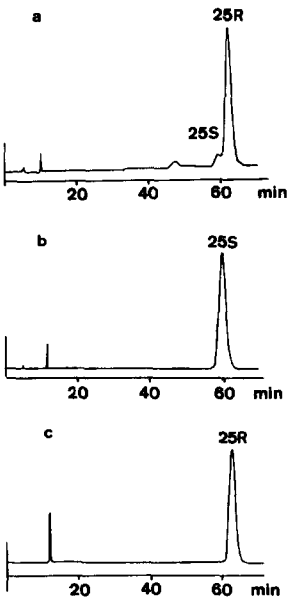
* Relative to the *p*-bromophenacyl ester of cholic acid.

fifteen different higher bile acids using 85% aqueous methanol as the eluent. The order of elution reflects the increasing polarity of the higher bile acids. The retention times of a homologous series of higher bile acids having the cholic acid type nucleus increased with the number of carbon atoms in their side chains. The presence of a double bond in the side chain tends to increase the mobility of a compound and the effect of the double bond is dependent on its position and geometry. The order of elution was $\Delta^{24}E$, $\Delta^{24}Z$ and Δ^{23}.

Although the resolution of two diastereoisomers at C-25 of THCA has been accomplished by thin-layer chromatography[20], the present method gives better separation, easier quantitation and simpler sample recovery. Mabuti[21,22] isolated both isomers of THCA from gall-bladder bile of bullfrog, *Rana catesbeiana*. There remains some uncertainty as to the occurrence of both isomers within a species, since the procedures involved in the isolation of THCA may cause isomerization at C-25, thus giving rise to both diastereoisomers. Thus, we have re-examined the bile acids in bullfrog with diethyl ether of the diluted and acidified bile. Analysis of the bile acid mixture by HPLC (Fig. 1a) confirmed the previous finding[21,22] that bullfrog bile contains both isomers of THCA. When the present procedure was carried out with each of the isomers of THCA, the isomerization of THCA did not occur (Fig. 1b and c).

An outstanding example of the power of the present method is the resolution of four stereoisomers at C-24 and C-25 of TeHCA. Fig. 2a shows the separation of these isomers. The elution sequence was (24*S*,25*R*), (24*S*,25*S*), (24*R*,25*R*), and (24*R*,25*S*).

Kuramoto *et al.*[2] analysed bile acids in gall-bladder bile of the frog, *Bombina*



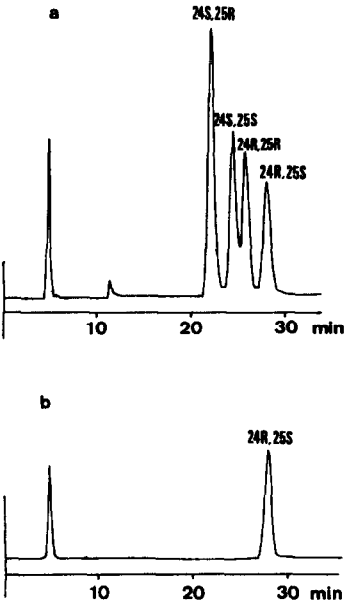


Fig. 1. HPLC chromatograms of higher bile acids of *Rana catesbeiana* (a) and (25*S*)- (b) and (25*R*)-3α,7α,12α-trihydroxy-5β-cholestan-26-oic acid (c) after the extraction procedure (see text). Column: TSK GEL LS-410, 30 cm × 4 mm I.D. Flow-rate: 0.7 ml/min. Mobile phase: methanol–water (80:20). Each sample was analysed as the *p*-bromophenacyl ester.

Fig. 2. HPLC chromatograms of a standard mixture of four diastereoisomers of 3α,7α,12α,24-tetrahydroxy-5β-cholestan-26-oic acid (a) and higher bile acids of *Bombina orientalis* (b). Conditions as in Fig. 1.

orientalis, by combined GLC and mass spectrometry, and found that its major bile acid had the same retention time as and identical mass spectrum to TeHCA. However, it has not been determined whether the frog contains one or two and more of the four stereoisomers at C-24 and C-25 of TeHCA, since in GLC the four isomers were eluted together with the same retention time and the mass spectra of these isomers were completely identical to one another.

Fig. 2b shows a HPLC separation of the bile acids in *Bombina orientalis* gallbladder bile. The major peak had the same retention time as (24*R*,25*S*)-TeHCA. No peak was observed at the retention times corresponding to the other three isomers. It can, therefore, be concluded that *Bombina orientalis* contains only one isomer of the four TeHCAs, *i.e.*, (24*R*,25*S*).

THCA, 3α,7α,12α-trihydroxy-5β-cholest-24-enoic acid and TeHCA have been recognized as intermediates in the biosynthesis of cholic acid from cholesterol in mammals[23]. The resolution of the stereoisomers of these higher bile acids permits us to study their stereospecific formation from cholesterol and their transformation into cholic acid. Such a study is now in progress.

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