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Short communication

High-performance liquid chromatographic separation of ultraviolet-absorbing bile alcohol derivatives

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Abstract

This paper describes the high-performance liquid chromatographic separation of UV-absorbing bile alcohol derivatives. Bile alcohols were treated with 3α-hydroxysteroid dehydrogenase to form the corresponding 3-keto bile alcohols. The 3-keto bile alcohols produced were converted to the 2,4-dinitrophenylhydrazone derivatives, separated using a Nova-Pak Phenyl column, and monitored at 364 nm. The separation of stereoisomers related to the configuration of hydroxyl groups on the side chain of the bile alcohols, which was not achieved by gas chromatography, could also be accomplished.

Keywords: Alcohols; Bile alcohols; Cholic acid; Cholestane

1. Introduction

Bile alcohols are known to be the major biliary constituents of evolutionary primitive vertebrates such as amphibians and fishes [1], and are considered to be intermediates in the pathway for bile acid biosynthesis from cholesterol in mammals. Until recently, bile alcohols have not been found in considerable amounts in mammals, especially humans. However, increased amounts of bile alcohols have been observed in urine, serum and bile of patients with a rare inherited lipid storage disease, cerebrotendinous xanthomatosis [2–4], and of patients with liver dysfunction, such as acute hepatitis or liver cirrhosis [5–8]. Studies of bile alcohols present in primitive vertebrates and patients with bile alcohol-accumulating diseases require a convenient micro-technique for their separation and identification. Thus, gas chromatography [3,4], gas chromatography–mass spectrometry [9] and radioimmunoassay [10] were developed for the analysis of bile alcohols. However, the stereoisomers related to the configuration of hydroxyl groups in the side chain could not be resolved by these methods. The present study was carried out in order to develop a method for the analysis of bile alcohols using high-performance liquid chromatography after derivatization with UV-absorbing 2,4-dinitrophenylhydrazone following the enzymatic oxidation of the 3α-hydroxyl group of bile alcohols.

2. Experimental

2.1. Bile acids and bile alcohols

Cholic acid was purchased from Sigma (St. Louis, MO, USA). Bile alcohols, 5β-cholestane-

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$3\alpha,7\alpha,12\alpha$-triol (THC) [11], 22*R*- and 22*S*-5β-cholestane-$3\alpha,7\alpha,12\alpha$,22-tetrol (22-Tetrol) [11], 23*R*- and 23*S*-5β-cholestane-$3\alpha,7\alpha,12\alpha$,23-tetrol (23-Tetrol) [12], 24*R*- and 24*S*-5β-cholestane-$3\alpha,7\alpha,12\alpha$,24-tetrol (24-Tetrol) [13], 5β-cholestane-$3\alpha,7\alpha,12\alpha$,25-tetrol (25-Tetrol) [14], 25*R*- and 25*S*-5β-cholestane-$3\alpha,7\alpha,12\alpha$,26-tetrol (26-Tetrol) [15], 22*R*- and 22*S*-5β-cholestane-$3\alpha,7\alpha,12\alpha$,22,25-pentol (22,25-Pentol) [16], 23*R*- and 23*S*-5β-cholestane-$3\alpha,7\alpha,12\alpha$,23,25-pentol (23,25-Pentol) [17,18], 24*R*- and 24*S*-5β-cholestane-$3\alpha,7\alpha,12\alpha$,24,25-pentol (24,25-Pentol) [14], 5β-cholestane-$3\alpha,7\alpha,12\alpha$,26,27-pentol (5β-cyprinol) [19] and 5β-cholestane-$3\beta,7\alpha,12\alpha$,26,27-pentol (latimerol) [20] were prepared as reported previously. 5α-Cholestane-$3\alpha,7\alpha,12\alpha$,26,27-pentol (5α-cyprinol), 5α- and 5β-cholestane-$3\alpha,7\alpha,12\alpha$,25,26-pentols (5α- and 5β-bufols) and 5α-cholestane-$3\beta,7\alpha,16\alpha$,26-tetrol (myxinol) were isolated from the solvolyzed bile of carp [21], newt [22], toad [23,24] and hagfish [25], respectively.

2.2. Treatment of cholanoids with 3α-hydroxysteroid dehydrogenase (3α-HSD)

Cholanoids (100 μg) were incubated at 25°C for 20 h in pyrophosphate buffer (pH 8.9, 2 ml) containing NAD (39.6 mg) and 3α-HSD (EC 1.1.1.50, Sigma, 0.006–0.12 units). The incubation mixture was passed through a Sep-Pak C_{18} cartridge (Waters) primed with water. The cartridge was then washed with 10 ml of water, and the cholanoids were eluted with 5 ml of methanol.

2.3. Preparation of 2,4-dinitrophenylhydrazone (DNPH) derivatives of cholanoids

To a solution of the 3-ketocholanoid (100 μg) dissolved in methanol (1 ml), 2,4-dinitrophenylhydrazine (200 μg) was added, and the reaction mixture was allowed to stand at 60°C for 1 h. The reaction mixture was evaporated to dryness. The residue was dissolved in 2 ml of a mixture of *n*-hexane–ethyl acetate (2:1, v/v) and the solution was loaded onto a Sep-Pak silica cartridge (Waters, Milford, MA, USA) and the column was washed with 30 ml of the mixture of *n*-hexane–ethyl acetate (2:1, v/v) in order to remove the excess reagents. Then, the DNPH derivative of 3-ketocholanoids was eluted with 20 ml of ethyl acetate.

2.4. High-performance liquid chromatography (HPLC)

The apparatus used was a Waters M-45 solvent-delivery system equipped with a Shimadzu SPD-1 UV detector, and the wavelength used was 364 mn. A Nova-Pak Phenyl (150×3.9 mm I.D., Waters) column and a TSK GEL ODS-80-TM (150×4.6 mm I.D., Tosoh, Tokyo, Japan) column were used. The mobile phase was as follows: 85% methanol for the analysis of tetrahydroxy bile alcohols; 82% methanol for the analysis of pentahydroxy bile alcohols; 83% methanol for the simultaneous analysis of both tetra- and pentahydroxy bile alcohols.

3. Results and discussion

The analysis of bile alcohols by HPLC has been neglected due to the limited detection, since these compounds do not possess an apparent UV absorbance and it is hard to introduce an UV-absorbing or fluorescent agent specifically to a hydroxyl group. The present HPLC method for bile alcohols involves initial oxidation of the 3α-hydroxy group of bile alcohols with 3α-HSD and subsequent derivatization of the keto groups.

The reaction activity of bile alcohols toward 3α-HSD was studied and the results are summarized in Table 1. Under the conditions used, in which cholic acid is almost completely oxidized to the corresponding 3-keto derivative, more than 95% of the 3α-hydroxyl group of all of the pentahydroxy- and tetrahydroxy bile alcohols was converted to the 3-keto group, except for THC, which was oxidized as a somewhat lower rate. Two bile alcohols possessing the 3β-hydroxyl group, myxinol and 5β-latimerol, remained unreacted as expected.

3-Keto bile alcohols obtained from the enzymic reaction were converted to the corresponding DNPH derivatives and subjected to HPLC. HPLC separation using two different columns of the 25-tetrol DNPH derivative is shown in Fig. 1. When HPLC was performed using a C_{18} column, the chromatogram showed two peaks corresponding to the anti and syn

Table 1
Oxidation rates of cholanoids by 3α-hydroxysteroid dehydrogenase

Bile alcohol	Formation of 3-oxo-cholanoids			
	0.006 U[a]	0.03 U	0.06 U	0.12 U
Cholic acid	25.0	63.1	99.8	99.8
5β-Cholestane-$3\alpha,7\alpha,12\alpha$-triol	6.3	2.7	33.6	85.4
$(22R)$-5β-Cholestane-$3\alpha,7\alpha,12\alpha$,22-tetrol	16.6	61.6	55.1	95.3
$(23R)$-5β-Cholestane-$3\alpha,7\alpha,12\alpha$,23-tetrol	nt	nt	nt	96.8
$(24R)$-5β-Cholestane-$3\alpha,7\alpha,12\alpha$,24-tetrol	27.3	89.7	91.4	95.8
5β-Cholestane-$3\alpha,7\alpha,12\alpha$,25-tetrol	7.6	50.4	95.9	93.9
5β-Cholestane-$3\alpha,7\alpha,12\alpha$,26-tetrol	nt	nt	nt	98.5
5α-Cholestane-$3\beta,7\alpha,16\alpha$,26-tetrol	nd	nd	nd	nd
$(22R)$-5β-Cholestane-$3\alpha,7\alpha,12\alpha$,22,25-pentol	nt	nt	nt	98.9
$(23R)$-5β-Cholestane-$3\alpha,7\alpha,12\alpha$,23,25-pentol	nt	nt	nt	98.8
$(24R)$-5β-Cholestane-$3\alpha,7\alpha,12\alpha$,24,25-pentol	55.9	95.6	96.3	97.4
5α-Cholestane-$3\alpha,7\alpha,12\alpha$,25,26-pentol	nt	nt	nt	98.5
5α-Cholestane-$3\alpha,7\alpha,12\alpha$,25,26-pentol	nt	nt	nt	96.5
5α-Cholestane-$3\alpha,7\alpha,12\alpha$,26,27-pentol	nt	nt	nt	98.9
5β-Cholestane-$3\beta,7\alpha,12\alpha$,26,27-pentol	nd	nd	nd	nd

[a] Enzyme amounts.
Details were described in Section 2. nt: not tested. nd: not detected.

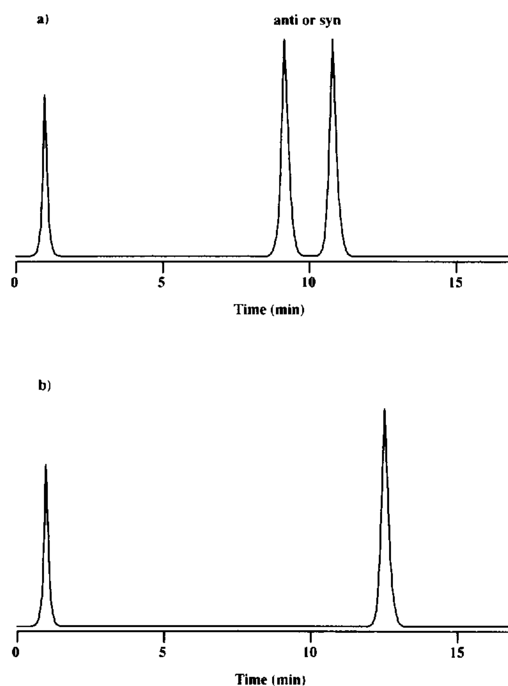

Fig. 1. Chromatograms of $7\alpha,12\alpha,25$-trihydroxy-5β-cholestane-3-(2,4-dinitrophenylhydrazone). Column, (a) A TSK-GEL ODS-80TM, 150×4.6 mm I.D., (b) Nova-Pak Phenyl, 150×3.9 mm I.D.; mobile phase, 85% methanol; flow-rate, 1.0 ml/min.

isomers (Fig. 1a). However, since these isomers of the hydrazone were not separated on the Nova-Pak Phenyl column (Fig. 1b), it was found that this column was suitable for the analysis of the DNPH derivatives.

It is known that GC is useful for the quantitative and qualitative analysis of bile alcohols as well as of bile acids. However, the separation of the stereoisomers related to the configuration of hydroxyl groups on the side chain of bile alcohols is not necessarily achieved using GC. As shown in Fig. 2, two isomers at C-23 of 23-tetrols and two isomers at C-24 of 24-tetrols, which could not be distinguished satisfactorily by GC, were separated completely from each other. The elution volumes of the bile alcohols studied are summarized in Table 2. This method provided satisfactory separation of 3α-hydroxylated bile alcohols differing not only in number and position but also in the configuration of their hydroxyl groups. Furthermore, 5α- and 5β-isomeric bile alcohols, 5α- and 5β-cyprinols, and 5α- and 5β-bufols were also eluted with different elution volumes. The limit of detection of this analytical method was 20 ng.

Finally, as an application to a biological sample, the results of the analysis of biliary bile alcohols

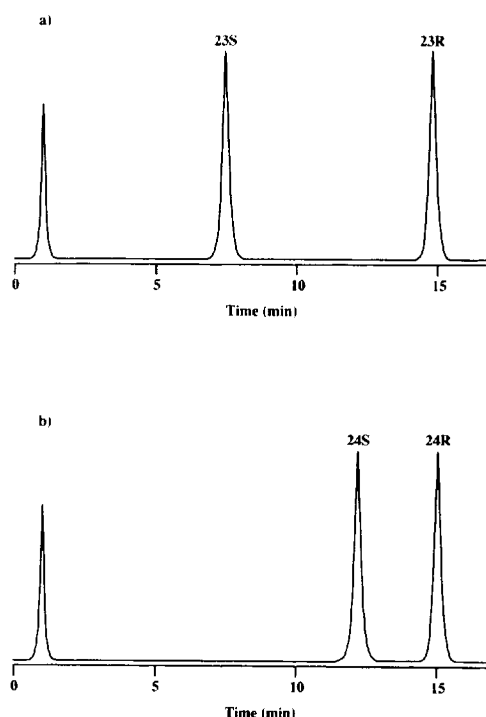

Fig. 2. Separation of stereoisomers of (23*R*)- and (23*S*)-5β-cholestane-3α,7α,12α,23-tetrols (a) or (24*R*)- and (24*S*)-5β-cholestane-3α,7α,12α,24-tetrols (b) as DNPH derivatives by HPLC. For conditions, see Section 2.

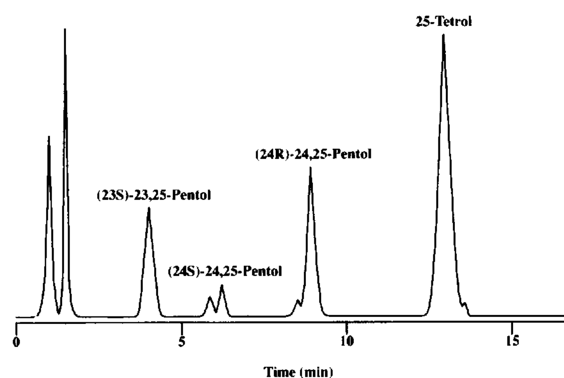

Fig. 3. Analysis of bile alcohols from bile of a CTX patient. For conditions, see Section 2.

from a patient with cerebrotendinous xanthomatosis is shown in Fig. 3.

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Table 2
Elution volumes of bile alcohols

Bile alcohol	Elution volume (ml)
(22*R*)-5β-Cholestane-3α,7α,12α,22-tetrol	8.08
(22*S*)-5β-Cholestane-3α,7α,12α,22-tetrol	23.57
(23*R*)-5β-Cholestane-3α,7α,12α,23-tetrol	14.94
(23*S*)-5β-Cholestane-3α,7α,12α,23-tetrol	7.76
(24*R*)-5β-Cholestane-3α,7α,12α,24-tetrol	15.43
(24*S*)-5β-Cholestane-3α,7α,12α,24-tetrol	12.47
5β-Cholestane-3α,7α,12α,25-tetrol	12.80
(25*R*)-5β-Cholestane-3α,7α,12α,26-tetrol	13.47
(25*S*)-5β-Cholestane-3α,7α,12α,26-tetrol	13.90
(22*R*)-5β-Cholestane-3α,7α,12α,22,25-pentol	6.17
(22*S*)-5β-Cholestane-3α,7α,12α,22,25-pentol	9.89
(23*R*)-5β-Cholestane-3α,7α,12α,23,25-pentol	6.30
(23*S*)-5β-Cholestane-3α,7α,12α,23,25-pentol	3.99
(24*R*)-5β-Cholestane-3α,7α,12α,24,25-pentol	9.06
(24*S*)-5β-Cholestane-3α,7α,12α,24,25-pentol	6.30
5α-Cholestane-3α,7α,12α,25,26-pentol (5α-bufol)	7.75
5β-Cholestane-3α,7α,12α,25,26-pentol (5β-bufol)	9.12
5α-Cholestane-3α,7α,12α,26,27-pentol (5α-cyprinol)	7.66
5β-Cholestane-3α,7α,12α,26,27-pentol (5β-cyprinol)	8.77

Column, Nova-Pak Phenyl; mobile phase, 83% methanol; flow-rate, 1.0 ml/min.
Each sample was analysed as the dinitrophenylhydrazone derivative.

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