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High-performance liquid chromatographic separation of bile acid pyrenacyl esters with cyclodextrin-containing mobile phase

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ABSTRACT

The high-performance liquid chromatographic separation of bile acid pyrenacyl esters with cyclodextrin-containing mobile phase is presented. Compared with conventional methods, inclusion chromatography gives much more satisfactory separation of derivatized bile acids in a short time. The application of this method to the separation of glycine-conjugated bile acids in human bile is also described.

INTRODUCTION

In recent years considerable attention has been directed towards the biodynamics of bile acids in patients with hepatobiliary disease, and high-performance liquid chromatography (HPLC) has been widely used for the analysis of bile acid profiles in biological samples [1,2]. Some skillful derivatization methods have been developed to be able to obtain high sensitivity. Among these, pre-column labelling through a side chain carboxyl or 3α-hydroxyl group is popular. Kamada *et al.* [3] developed an HPLC method with fluorescence detection using 1-bromoacetylpyrene as a pre-column labelling reagent. The method is highly sensitive, but the elution times of the derivatized lithocholic acid or its conjugates are rather long.

In previous papers we reported on inclusion chromatography with cyclodextrin (CD) as a mobile phase additive in HPLC of bile acids [4,5]. As a continuation of this work, the present paper deals with the separation of bile acid pyrenacyl esters by inclusion chromatography. The addition of a suitable CD to the mobile phase in reversed-phase HPLC gave much more satisfactory separation of free or conjugated bile acid pyrenacyl esters in a short time. The application of the method to the separation of glycine-conjugated bile acids in human bile is also described.

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EXPERIMENTAL

Materials

CDs (β and γ) were kindly supplied by Nihon Shokuhin Kako (Tokyo, Japan). Heptakis-(2,6-di-O-methyl)-β-CD (Me-β-CD; 10.5 methyl residues per mol) was prepared and donated by Kao (Tokyo, Japan). Bile acids and 1-bromoacetylpyrene were obtained from Tokyo Kasei Kogyo (Tokyo, Japan) and Wako (Osaka, Japan), respectively. Sep-Pak C_{18} cartridges were obtained from Waters Assoc. (Milford, MA, U.S.A.). Piperidinohydroxypropyl Sephadex LH-20 (PHP-LH-20) was prepared by the method reported by Goto *et al.* [6]. Bile samples were kindly donated by Dr. Matsubara (Department of Laboratory Medicine, Kanazawa University School of Medicine) and Dr. Okumura (Central Clinical Laboratory, Kanazawa University Hospital). Other chemicals used were commercially available.

Instruments

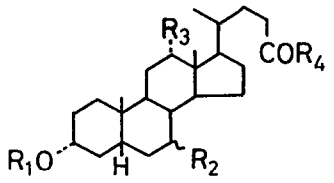
HPLC was carried out on a Shimadzu LC-6A chromatograph (Shimadzu, Kyoto, Japan) equipped with a Hitachi F-1000 fluorescence detector (Hitachi, Tokyo) (λ_{ex} 370 nm, λ_{em} 440 nm) at a flow-rate of 1.0 ml/min unless otherwise stated. A YMC-GEL C_8 (5 μm; 15 cm × 0.4 cm I.D.; Yamamura, Kyoto, Japan) column was used under ambient conditions, and the void volume was measured with methanol (λ_{ex} 280 nm, λ_{em} 320 nm).

Procedure for the separation of glycine-conjugated bile acids in human bile

A bile sample (10 μl) was diluted with 0.5 *M* phosphate buffer (pH 7.0, 20 ml). A 2-ml volume of this solution was added to a solution of the internal standard [I.S.; glycodeoxycholic acid 12-propionate (IVc in Fig. 1); 2 μg in methanol (100 μl)] [7], and applied first to a Sep-Pak C_{18} cartridge and then to a PHP-LH-20 (acetate form) column (1.8 cm × 0.6 cm I.D.) according to the procedure described by Goto *et al.* [8]. The fraction containing glycine-conjugated bile acids was evaporated to dryness, and then one-fifth of the residue was derivatized with 1-bromoacetylpyrene according to the procedure described by Kamada *et al.* [3]. The reaction mixture was applied to a silica gel column (70–230 mesh: 5 cm × 0.6 cm I.D.) to remove the excess or decomposed reagent with benzene–ethyl acetate (2:1; 5 ml). The ethyl acetate–methanol (3:1; 6 ml) eluate was evaporated to dryness and dissolved in methanol, and an aliquot was subjected to HPLC.

RESULTS AND DISCUSSION

In a previous paper we reported the much improved separation of bile acids, including glycine and taurine conjugates, using CD as a mobile phase additive in reversed-phase HPLC [4]. The retention behaviour of the examined compounds showed that the functional group at the 12-position of the steroid moiety may be



I : R_1=H, R_2=$\overset{H}{\underset{OH}{}}$, R_3=OH

II : R_1=H, R_2=$\overset{H}{\underset{OH}{}}$, R_3=H

III: R_1=R_2=H, R_3=OH

IV : R_1=R_2=H, R_3=$OCOCH_2CH_3$

V : R_1=H, R_2=$\overset{OH}{\underset{H}{}}$, R_3=H

VI : R_1=R_2=R_3=H

VII: R_1=SO_3H, R_2=$\overset{H}{\underset{OH}{}}$, R_3=OH

VIII: R_1=SO_3H, R_2=$\overset{H}{\underset{OH}{}}$, R_3=H

IX : R_1=SO_3H, R_2=H, R_3=OH

X : R_1=SO_3H, R_2=$\overset{OH}{\underset{H}{}}$, R_3=H

XI : R_1=SO_3H, R_2=R_3=H

a : R_4=OH

b : R_4=OCH_2OC-Pyrene

c : R_4=$NHCH_2COOH$

d : R_4=$NHCH_2COOCH_2OC$-Pyrene

Fig. 1. Structures of bile acids and their derivatives.

an important factor in the formation of inclusion complexes between the solute and CD. In spite of the presence of a bulky fluorophore at the 3-position of the steroid moiety, much improved separation and similar retention behaviour were also observed with 3-(1-anthroyl)bile acids by inclusion chromatography [5]. These data prompted us to examine the chromatographic behaviour of bile acid pyrenacyl esters containing a fluorophore in the side-chain (Fig. 1).

Comparison of retention behaviour of 3-(1-anthroyl)bile acids with that of bile acid pyrenacyl esters

The effect of the CD content in the mobile phase on the capacity factors (k') of 3-(1-anthroyl)bile acids and bile acid pyrenacyl esters were examined using Me-β-CD as a host compound, whose solubility in the organic modifier is much higher than that of other CDs. Typical representative data, in which ursodeoxycholic acid (Va) derivatives were examined, are shown in Table I. Although the values were more or less higher than those for 3-(1-anthroyl)ursodeoxycholic acid, the relative k' (Rk') values of ursodeoxycholic acid pyrenacyl ester (Vb) were de-

TABLE I

COMPARISON OF RETENTION BEHAVIOUR OF 3-(1-ANTHROYL)BILE ACID WITH THAT OF BILE ACID PYRENACYL ESTER

Concentration of Me-β-CD (m*M*)	Derivative of ursodeoxycholic acid (Va)			
	1-Anthroyl ester[a]		Pyrenacyl ester (Vb)[b]	
	k'	R*k'*	*k'*	R*k'*
0	12.66	1.00	12.54	1.00
0.5	9.90	0.78	10.99	0.88
1	8.93	0.71	9.94	0.79
2	7.39	0.58	8.26	0.66
5	4.60	0.36	5.41	0.43

[a] Mobile phase: acetonitrile–0.25% KH_2PO_4 (pH 4.0) (11:5, v/v) containing Me-β-CD; detection: λ_{ex}, 370 nm; λ_{em}, 470 nm.

[b] Mobile phase: acetonitrile–water (5:3, v/v) containing Me-β-CD; detection: λ_{ex}, 370 nm; λ_{em}, 440 nm.

creased significantly by the addition of Me-β-CD. The *k'* values for both derivatives examined obtained without CD were maintained at more than 12 by changing the concentration of the organic modifier. Since the organic modifier competes with the solute for the hydrophobic CD cavity, a change in the proportion of the organic modifier may influence the solute interaction with the CD. However, a change in the proportion was unavoidable in order to obtain the appropriate *k'* value for detection and characterization of the effect of the CD [9].

A similar phenomenon was also observed with the other bile acid pyrenacyl esters (I–III, VIb), and these data showed that inclusion chromatography may be also effective for the separation of these bile acid derivatives.

Retention behaviour and separation of bile acid pyrenacyl esters

Next, effort was directed towards the separation of bile acid pyrenacyl esters, in which unconjugated bile acid (I–III, V, VIa), glycine-conjugated bile acid (I–III, V, VIc), bile acid 3-sulphate (VII–XIa) and glycine-conjugated bile acid 3-sulphate (VII–XIc) derivatives were included (Fig. 1). The effects of Me-β-CD on the retention of these derivatives with 1-bromoacetylpyrene are summarized in Fig. 2. The R*k'* values for all the examined bile acids without a functional group at the 12-position decreased significantly, as has been reported for underivatized bile acids and 3-(1-anthroyl)bile acids [4,5].

The separation of bile acid pyrenacyl esters was much improved by this chromatography, as shown in Fig. 3–5. The elution time is more than 26 min for the derivatized lithocholic acid (VIb) or its conjugates (XIb, d) under the isocratic conditions of conventional chromatography, but the derivatives were eluted in a shorter time in inclusion chromatography.

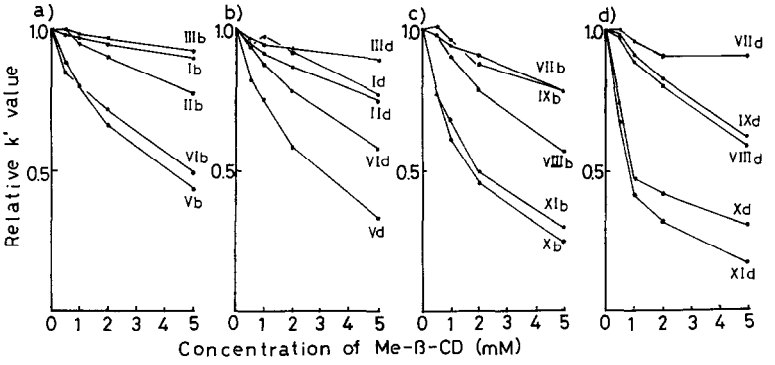


Fig. 2. Effect of Me-β-CD on the retention of the derivatives of bile acids with 1-bromoacetylpyrene: (a) unconjugated bile acids; (b) glycine-conjugated bile acids; (c) bile acid 3-sulphates; (d) glycine-conjugated bile acid 3-sulphates. Mobile phase: (a) acetonitrile–water (Ib: 5:3, v/v; k' 9.15; IIb: 3:1, v/v; k' 7.26; IIIb: 3:1, v/v; k' 7.85; Vb: 5:3, v/v; k' 12.54; VIb: 9:2, v/v; k' 9.84) (b) acetonitrile–water (Id: 1:1, v/v; k' 10.71; IId: 7:5, v/v; k' 10.01; IIId: 7:5, v/v; k' 11.52; Vd: 1:1, v/v; k' 15.61; VId: 2:1, v/v; k' 12.29) (c) acetonitrile–0.5% KH_2PO_4 (pH 4.0) (VIIb: 1:1, v/v; k' 9.65; VIIIb: 5:4, v/v; k' 9.59; IXb: 5:4, v/v; k' 10.05; Xb: 1:1, v/v; k' 10.58; XIb: 5:3, v/v; k' 9.24) (d) acetonitrile–0.5% KH_2PO_4 (pH 4.0) (VIId: 3:4, v/v; k' 7.94; VIIId: 4:5, v/v; k' 11.74; IXd: 4:5, v/v; k' 12.72; Xd: 3:4, v/v; k' 9.04; XId: 1:1, v/v; k' 11.85) containing Me-β-CD as indicated. The k' value obtained without CD, taken as 1.00 for the calculation of the relative k' value, is indicated in parentheses.

Effect of CD on the fluorescence response

The effect of the CD in the mobile phase on the fluorescence response was examined by using unconjugated bile acid pyrenacyl esters (Table II). The fluorescence intensity of derivatized ursodeoxycholic acid (Vb), whose Rk' value was most influenced by the addition of host compound, was 1.4 times higher upon addition of 5 mM Me-β-CD to the mobile phase. On the contrary, that of derivatized cholic acid (Ib) was only 1.1 times higher when the same amount of the host compound was added to the mobile phase, in which the Rk' value of Ib was

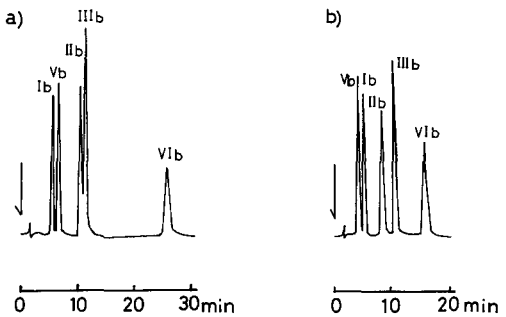


Fig. 3. Separation of the derivatives of unconjugated bile acids with 1-bromoacetylpyrene. Mobile phase: (a) acetonitrile–water (3:1, v/v); (b) acetonitrile–water (3:1, v/v) containing 5 mM Me-β-CD.

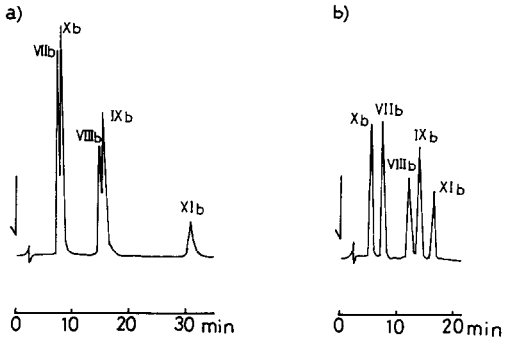


Fig. 4. Separation of the derivatives of bile acid 3-sulphates with 1-bromoacetylpyrene. Mobile phase: (a) acetonitrile–0.5% KH_2PO_4 (pH 4.0) (6:5, v/v); (b) acetonitrile–0.5% KH_2PO_4 (pH 4.0) (6:5, v/v) containing 1.5 m*M* Me-β-CD.

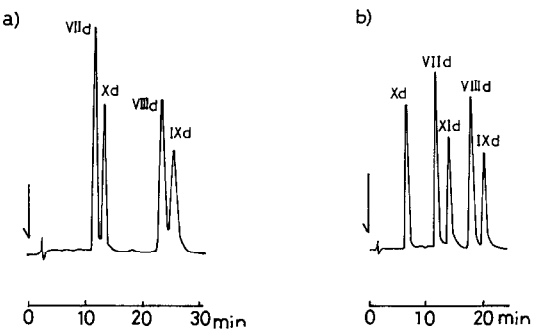


Fig. 5. Separation of the derivatives of glycine-conjugated bile acid 3-sulphates with 1-bromoacetylpyrene. Mobile phase: (a) acetonitrile–0.5% KH_2PO_4 (pH 4.0) (3:4, v/v); XId was not eluted within 50 min under these conditions; (b) acetonitrile–0.5% KH_2PO_4 (pH 4.0) (3:4, v/v) containing 2 m*M* Me-β-CD.

TABLE II

EFFECT OF Me-β-CD ON THE FLUORESCENCE DETECTOR RESPONSE

Values are peak-area ratios relative to the value obtained without Me-β-CD. Mobile phase, acetonitrile–water (5:2, v/v) containing Me-β-CD.

Concentration of Me-β-CD (m*M*)	1-Bromoacetylpyrene derivative	
	Ib	Vb
0	1.0	1.0
2	1.0	1.2
5	1.1	1.4

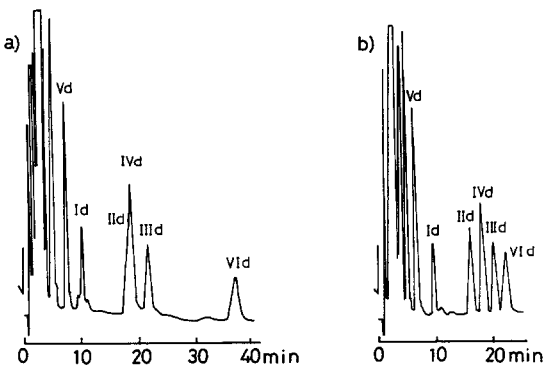


Fig. 6. Separation of the derivatization reaction mixture of glycine-conjugated bile acids with 1-bromoacetylpyrene. Mobile phase: (a) methanol–acetonitrile–water (30:10:13, v/v); (b) methanol–acetonitrile–water (30:10:13, v/v) containing 2 m*M* γ-CD. Flow-rate, 1.5 ml/min.

not so influenced. These data suggest that the change in the Rk' value is compatible with the perturbation of the spectrum.

Separation of glycine-conjugated bile acids in human bile

In order to investigate the applicability of the present method, the separation of glycine-conjugated bile acids was carried out on a human bile sample from a patient with liver disease. Initially the authentic glycine-conjugated bile acids were derivatized, eluted through a small silica gel column and then injected into a conventional HPLC column. The separation of derivatized glycochenodeoxycholic acid (IId) and the I.S. (IVd) was unsatisfactory, and the glycolithocholic acid derivative (VId) was eluted at 36.5 min (Fig. 6a). In contrast, inclusion chromatography overcame all the problems and gave a satisfactory separation as

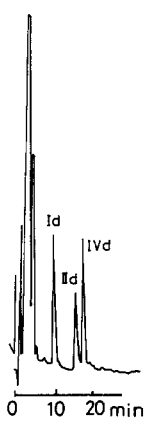


Fig. 7. Typical chromatogram of bile from a patient. Conditions as in Fig. 6b.

shown in Fig. 6b. In addition to acetonitrile, methanol was also used as an organic modifier: it was effective for the separation of decomposed or excess reagents from the derivatives. γ-CD was used as a host compound because it is more effective than Me-β-CD for the elution of the glycolithocholic acid derivative (VId) in a short time. The bile sample was treated with a Sep-Pak C_{18} cartridge and a PHP-LH-20 column, and then derivatized with 1-bromoacetylpyrene according to the procedures reported by Goto *et al.* [8] and Kamada *et al.* [3], respectively. A typical chromatogram is shown in Fig. 7. Peaks were assigned by comparison of the chromatographic behaviour with that of the authentic sample using several solvent systems. Glycocholic acid (Ic: 2.83 mg/ml) and glycochenodeoxycholic acid (IIc: 1.33 mg/ml) were detected as main glycine-conjugated bile acids in this bile specimen.

CONCLUSION

The retention behaviour of bile acid pyrenacyl esters was examined in reversed-phase HPLC with a CD-containing mobile phase. The behaviour was compatible with that of 3-(1-anthroyl)bile acids as reported previously [5]. The separation of these derivatives by inclusion chromatography was much improved, and the elution times of the derivatives, especially derivatized lithocholic acid and its conjugates, were shortened. The method was applied to the separation of glycine-conjugated bile acids in a human bile sample and gave satisfactory results in a short time.

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