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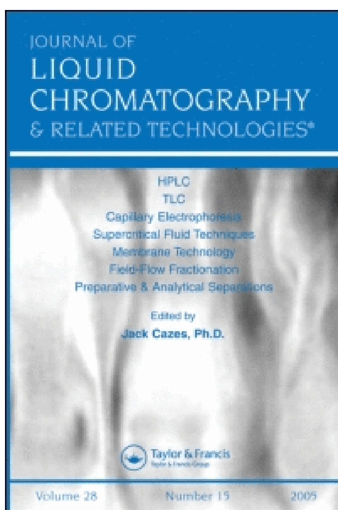

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HIGH PERFORMANCE LIQUID CHROMATOGRAPHIC SEPARATION OF SENSITIVE FLUORESCENT DERIVATIVES OF BILE ACIDS WITH CYCLODEXTRIN-CONTAINING MOBILE PHASE

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ABSTRACT

The high-performance liquid chromatographic behavior of new bile acid fluorescent derivatives, the 7-methoxy-1,4-benzoxazin-2-one-3-methyl esters, with a cyclodextrin-containing mobile phase is compared to those of the bile acid-pyrenacyl and -anthroyl esters. Compared with conventional methods, inclusion chromatography gives a much more satisfactory separation of the bile acid fluorescent derivatives in a short time, but the chromatographic behavior of these derivatives has not been much influenced by the fluorophore used. The detection limits of the new derivatives are in the range of 10-20 fmol at a signal to noise ratio of 5. The application of this method for the separation of glycine-conjugated bile acids in human bile is also described.

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INTRODUCTION

In previous papers we reported on inclusion chromatography with cyclodextrin (CD) as a mobile phase additive for the high-performance liquid chromatography (HPLC) of bile acids [1,2], and its pyrenacyl and anthroyl esters [3]. As a continuation of this work, the present paper deals with the separation of the new bile acid fluorescent derivatives, the 7-methoxy-1,4-benzoxazin-2-one-3-methyl (MB) esters [4], by inclusion chromatography and a comparison of the chromatographic behavior of these derivatives with that of the other fluorescent derivatives. The application of this method for the separation of glycine-conjugated bile acids in human bile is also described.

MATERIALS AND METHODS

Materials

γ-CD was 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). Bile acids were obtained from Tokyo Kasei Kogyo (Tokyo) and Nacalai Tesque (Kyoto, Japan). 3-Bromomethyl-7-methoxy-1,4-benzoxazin-2-one (BrMB) was obtained from Tokyo Kasei Kogyo. Sep-Pak C_{18} cartridges were obtained from Milipore (Milford, MA, USA). Piperidinohydroxypropyl Sephadex LH-20 (PHP-LH-20) was prepared by the method reported by Goto et al. [5]. Bile samples were

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kindly donated by Dr. Okumura (Central Clinical Laboratory, Kanazawa University Hospital).

Instruments

HPLC was carried out using a JASCO TRI ROTAR (JASCO, Tokyo) equipped with a Hitachi F-1000 fluorescence detector (Hitachi, Tokyo)(FL; λex 355 nm, λem 430 nm) at a flow rate of 1.0 ml/min unless otherwise stated. A YMC•GEL C_8 column (5 μm; 15 cm x 0.46 cm I.D.; YMC, Kyoto) was used under ambient conditions unless otherwise stated, and the void volume was measured with methanol (λex 280 nm, λem 320 nm).

Time course for derivatization of bile acids with BrMB

BrMB (0.14 mg) and 18-crown-6 (0.14 mg), each in acetonitrile (0.1 ml), and KF (ca. 1 mg) were added to the screw-capped tube containing glycochenodeoxycholic acid (IIIa) or chenodeoxycholic acid (each 200 ng). The tube was maintained at 40°C with part of its contents being subjected to HPLC at the appropriate time.

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

A bile sample (0.05 ml) was diluted with 0.5 M phosphate buffer (pH 7.0, 5 ml). A 0.1 ml of this solution and the internal standard [I.S.; glycodeoxycholic acid 12-propionate (VIa); 2 μg in methanol (0.1 ml)] were added to the buffer (2 ml), and applied first to a Sep-Pak C_{18} cartridge and then to a

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PHP-LH-20 column (acetate form; 2 cm x 0.6 cm I.D.) according to the procedure described by Goto et al. [6]. About one-fifth of the fraction containing the glycine-conjugated bile acids was evaporated to dryness. BrMB (1.2 mg) in acetonitrile (0.1 ml), 18-crown-6 (1.2 mg) in acetonitrile (0.1 ml) and KF (ca. 1 mg) were added to the residue and the mixture was allowed to stand at 40°C for 1 hr. The reaction mixture was diluted with benzene (3 ml) and applied to a silica gel column (70-230 mesh; 4 cm x 0.6 cm I.D.) to remove the excess or decomposed reagent with benzene-ethyl acetate (5:1, v/v; 2 ml). The chloroform-methanol (7:3, v/v; 10 ml) eluate was evaporated to dryness and dissolved in acetonitrile, and an aliquot was subjected to HPLC [3].

RESULTS AND DISCUSSION

In previous papers, we reported the much improved separation of bile acids [1,2], its pyrenacyl and anthroyl esters [3], using CD as a mobile phase additive in reversed-phase HPLC. The retention behavior of the examined compounds showed that the functional group at the 12-position of the steroid moiety may be an important factor of the inclusion complexes between the solute and CD. The chromatographic behavior of bile acids and its derivatives has not been much influenced by the fluorophore used or conjugated forms (unconjugates, glycine- or taurine-conjugates). Recently, Nakanishi et al. reported BrMB as a

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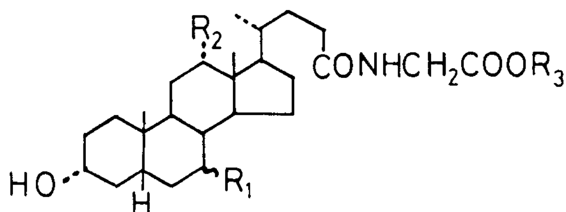

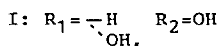

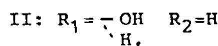

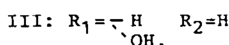

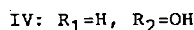

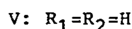

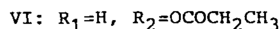

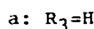

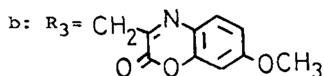

Fig. 1. Structures of glycine-conjugated bile acids and their MB esters.

Ia: Glycocholic acid, IIa: glycoursodeoxycholic acid, IIIa: glycochenodeoxycholic acid, IVa: glycodeoxycholic acid, Va: glycolithocholic acid, VIa: glycodeoxycholic acid 12-propionate (I.S.).

highly sensitive fluorescence derivatization reagent for fatty acids in HPLC [4]. These data prompted us to examine the chromatographic behavior of bile acid MB esters containing a fluorophore in the side-chain (Fig. 1).

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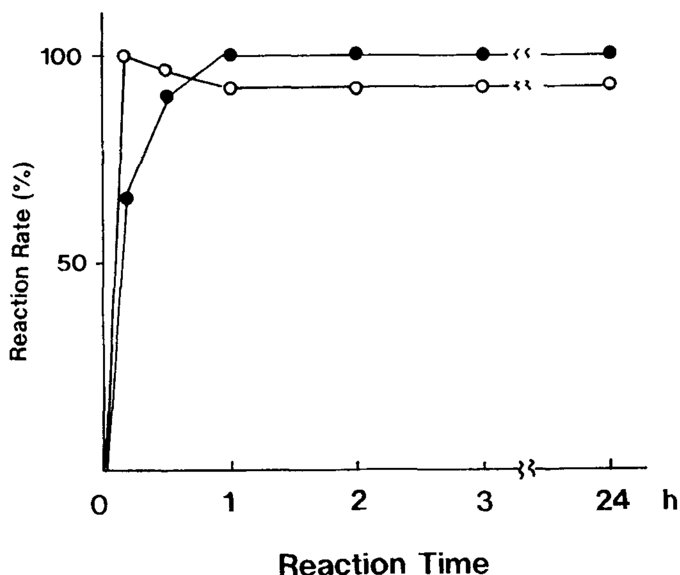

Fig. 2. Time course for derivatization of chenodeoxycholic acid (o) and its glycine-conjugate (●).

HPLC conditions: YMC•GEL C_8 (5 μm: 15 cm x 0.4 cm I.D.), solvent system (o) acetonitrile-water (3:2, v/v), t_R= 13.5 min, (●) methanol-water (4:1, v/v), t_R=10.0 min.

Derivatization of bile acids with BrMB

The derivatization reaction rate of bile acids with BrMB (about 1000 molar ratio excess) was examined according to the previously described procedure except for using potassium fluoride instead of potassium carbonate [4]. The results using unconjugated and glycine-conjugated bile acids are shown in Fig. 2. The rate of formation of the fluorescent esters with

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unconjugated bile acids was rapid, the reaction being completed within 10 min, and a slow decrease in the fluorescence intensity was observed, but the fluorescent derivatives were stable for at least 1 day as previously described [4]. On the contrary, it took 1 hr to derivatize glycine-conjugated bile acids and the stable derivatives were obtained.

Detection limit of derivatized bile acids

The detection limits of the unconjugated and glycine-conjugated bile acid MB esters were 10 fmol and 20 fmol (signal to noise ratio=5), respectively. These data are lower than those of the pyrenacyl esters, 60 fmol (unconjugates) and 190 fmol (glycine-conjugates), respectively [3]. On the other hand, that of the bile acid anthroyl esters was reported to be 20 fmol [6], which is almost the same as those for the new derivatives.

Comparison of retention behavior of glycine-conjugated bile acid MB esters with those of other fluorescent derivatives

The effect of the CD content in the mobile phase on the relative capacity factors (Rk') of glycine-conjugated bile acid MB esters was examined using Me-β- or γ-CD as a host compound. A significant difference has not been observed on the used host compounds and the results using γ-CD are summarized in Table 1. The Rk' values of the derivatives having a hydroxyl group at 12-position (Ib, IVb) were bigger than those of the other steroids (IIb, IIIb, Vb). All these data are compatible with those

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TABLE 1. Retention Behavior of Glycine-conjugated Bile Acid MB Esters During Inclusion Chromatography

Bile acid MB ester	γ-CD 0	γ-CD 5 mM
Ib[a]	9.12[c]	8.02 (0.88)[d]
IIb[a]	12.11	5.11 (0.42)
IIIb[b]	6.89[e]	4.99 (0.72)
IVb[b]	7.96	6.65 (0.84)
Vb[b]	19.19	7.89 (0.41)

a) Mobile phase: acetonitrile-water (2:3, v/v) containing γ-CD as indicated.
b) Mobile phase: acetonitrile-water (1:1, v/v) containing γ-CD as indicated.
c) k' (t_o=1.77 min).
d) Rk'; k' value obtained without CD was taken as 1.00.
e) k' (t_o=1.64 min)

previously obtained, in which other fluorophores were used during the derivatization [3]. These phenomena were also observed using unconjugated bile acid fluorescent derivatives including the MB esters. That is, the functional group at the 12-position of the bile acids may be an important factor for the chromatographic behavior during this inclusion chromatography.

<u>Separation of glycine-conjugated bile acid MB esters in human bile</u>

In order to investigate the applicability of the present method, the separation of the glycine-conjugated bile acid MB

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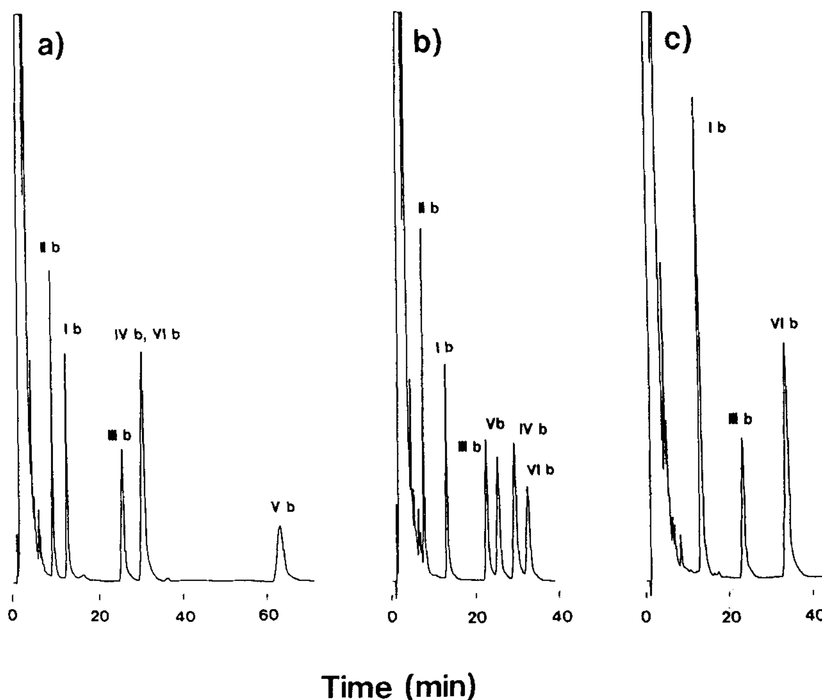

Fig. 3. Separation of glycine-conjugated bile acid MB esters.

a, b) Authentic sample c) bile sample.

HPLC conditions: flow rate, 1.5 ml/min, solvent system

a) acetonitrile-methanol-water (6:7:8, v/v/v),

b), c) acetonitrile-methanol-water (6:7:8, v/v/v) containing 2.5 mM γ-CD.

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esters (Ib-Vb) was carried out on a human bile sample from a patient with liver disease. Initially, the authentic glycine-conjugated bile acids (Ia-Va) including I.S. (VIa) were derivatized, eluted through a small silica gel column [3] and then injected into a conventional HPLC system. The separation of derivatized glycodeoxycholic acid (IVb) and the I.S. (VIb) was unsatisfactory, and the glycolithocholic acid derivative (Vb) was eluted at 63 min (Fig. 3a). In contrast, inclusion chromatography overcame these problems and gave a satisfactory separation as shown in Fig. 3b. In addition to acetonitrile, methanol was used as an organic modifier, it was effective for the separation of decomposed or excess reagents from the derivatives. All these data are compatible with those obtained with bile acid pyrenacyl esters [3]. The bile sample was treated as described before [3], derivatized with BrMB and then subjected to HPLC. A typical chromatogram is shown in Fig. 3c. Glycocholic acid (Ia) and glycochenodeoxycholic acid (IIIa) were detected as the main glycine-conjugated bile acids in this bile specimen.

CONCLUSION

The retention behavior of bile acid MB esters was examined using reversed-phase HPLC with a CD-containing mobile phase. The behavior was compatible with those of the previously reported 3-(1-anthroyl)bile acids and bile acid pyrenacyl esters [3]. The

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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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