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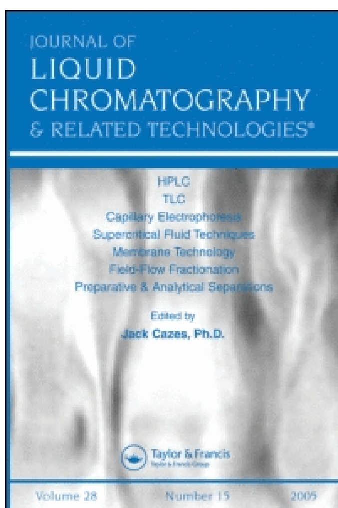

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JOURNAL OF LIQUID CHROMATOGRAPHY, 14(13), 2527-2539 (1991)

HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC BEHAVIOR OF 2-,4- AND 6-HYDROXYLATED BILE ACID STEREOISOMERS

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ABSTRACT

High-performance liquid chromatographic behavior has been investigated for 40 stereoisomeric 2-, 4- and 6-hydroxylated bile acids, most of which possess a *vicinal* glycol structure, as their 4-nitrophthalimidomethyl (NPM) ester derivatives on a reversed-phase column using methanol-water systems as a mobile phase. The bile acid NPM esters were further derivatized to the so-called "mixed" acetonide-NPM esters, whereby the stereochemical relationships (α,α- and β,β-*cis*, diaxial *trans* or diequatorial *trans*) of 2,3-, 3,4- and 6,7-diol groups in the molecules were reflected to changes in the capacity factors of bile acids.

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INTRODUCTION

A number of unusual bile acids have recently been found in significant amounts in biological samples from patients with hepatobiliary diseases and in newborn infants and fetuses. In particular, 1-, 2-, 4- and 6-hydroxylated derivatives of common bile acids (*i.e.*, lithocholic [3α-OH], deoxycholic [3α,12α-$(OH)_2$], chenodeoxycholic [3α,7α-$(OH)_2$], ursodeoxycholic [3α,7β-$(OH)_2$], and cholic [3α,7α,12α–$(OH)_3$] acids, are currently of great interest in biological and metabolic studies (1-3). As a result of our work on a programme of synthesizing potential bile acid metabolites, a series of the 2-, 4- and 6-hydroxylated bile acids, which differ from one another in the number, position, and stereochemical configuration of hydroxyl groups at positions C-2, C-3, C-4, C-6, C-7 and/or C-12, are now available (4).

We have previously reported on the high-performance liquid chromatographic (HPLC) analysis of the mono-, di- and trihydroxy stereoisomers of the common bile acids and their oxo derivatives on a reversed-phase column (5-7). In this paper, in an extension of the HPLC study of unusual bile acids, we describe the HPLC separation of the forty 2-, 4- and 6-hydroxylated bile acids, most of which possess a 2,3-, 3,4-, or 6,7-*vicinal* glycol structure in the molecules. These bile acids were chromatographed as their UV-sensitive 4-nitrophthalimidomethyl (NPM) ester (5) and the so-colled "mixed" acetonide-NPM ester derivatives on a Nova-Pak C_{18} reversed-phase column.

EXPERIMENTAL

Samples and Reagents

Almost all of the 2-, 4- and 6-hydroxylated C_{24} bile acids related to 5α- and 5β-cholanoic acids with two to four

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hydroxyl groups in the nucleus were taken from our laboratory collections, which include new and natural bile acids recently synthesized in these laboratories (4). All solvents were of HPLC grade. *N*-Chloromethyl-4-nitrophthalimide, 18-crown-6 ether, and 2,2-dimethoxypropane were purchased from Tokyo Kasei Kogyo Co. (Tokyo, Japan).

HPLC Instrument and Column

The HPLC apparatus was a Waters M-45 solvent-delivery system (Millipore-Waters, Milford, MA, U.S.A.) equipped with a Waters Model U6K sample loop injector, a Shimadzu SPD-6A UV-spectrophotometric detector (Shimadzu, Kyoto, Japan), and a data-processing system (Shimadzu Chromatopac C-R6A); the wavelength selected for all measurements was 254 nm. A Nova-Pak C_{18} reversed-phase column (15 cm X 3.9 mm I.D.; 5 µm) (Millipore-Waters) was used under ambient conditions. Methanol-water mixtures (9:1-6.5:3.5, v/v) were employed as eluents.

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Derivatization Procedure

NPM ester derivatives

Initially, free bile acid samples were derivatized to their UV-sensitive NPM esters by a procedure described previously (5), using *N*-chloromethyl-4-nitrophthalimide and 18-crown-6 ether.

"Mixed" acetonide-NPM ester derivatives

The above bile acid NPM esters with a 1,2-diol structure were then converted to their "mixed" acetonide-NPM ester derivatives by the following procedure. After NPM esterification, the solvent (acetonitrile) was evaporated under

TABLE I

Rk' values of 2-, 4- and 6-hydroxylated bile acids as their NPM esters on reversed-phase HPLC[a]

Position and configuration of hydroxyls[b]	Conformation of 1,2-*vicinal* hydroxyls[c]	NPM
Dihydroxylated compound		(4:1; 1.0)
2β,3α	e-e	1.76
3α,4β	e-e	1.92
3β,4α	a-a	1.76
3β,4β	a-e	1.76
3α,6α		1.00
3α,6β		0.62
3β,6α		1.13
3β,6α (5α)		1.00
3β,6β		1.13
3α,12α		2.28
Trihydroxylated compound		(7:3; 1.0)
2β,3α,7α	e-e	0.77
2β,3α,12α	e-e	1.42
3α,4β,7α	e-e	1.56
3β,4β,7α	a-e	1.21
3α,4β,12α	e-e	1.05
3β,4α,12α	a-a	0.80
3β,4β,12α	a-e	0.77
3α,6α,7α	e-a	1.00
3α,6α,7β	e-e	0.57
3α,6β,7α	a-a	0.57
3α,6β,7β	a-e	0.63
3α,6β,7β (5α)	a-e	0.81
3β,6α,7α	e-a	0.81
3β,6α,7β	e-e	0.69
3β,6β,7α	a-a	0.57

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Table 1 continued

Position and configuration of hydroxyls[b]	Conformation of 1,2-*vicinal* hydroxyls[c]	NPM
3β,6β,7β	a-e	0.81
3α,6α,12α		0.39
3α,6β,12α		0.16
3β,6α,12α		0.28
3β,6β,12α		0.13
3α,7α,12α		1.32
Tetrahydroxylated compound		(6.5:3.5; 1.0)
2β,3α,7α,12α	e-e	1.39
3α,4β,7α,12α	e-e	1.90
3β,4β,7α,12α	a-e	1.28
3α,6α,7α,12α	e-a	1.00
3α,6α,7β,12α	e-e	0.43
3α,6α,7β,12α (5α)	e-e	0.53
3α,6β,7α,12α	a-a	0.53
3α,6β,7β,12α	a-e	0.43
3α,6β,7β,12α (5α)	a-e	0.53

[a]The capacity factors (*k'*) for di-, tri- and tetrahydroxy bile acids were expressed relative to 3α,6α-di-, 3α,6α,7α-tri- and 3α,6α,7α,12α-tetrahydroxy-5β-cholanoic acid NPM ester derivatives, respectively; values in parentheses refer to the volumetric composition (v/v) of methanol-water mixture as eluent and the flow rate (ml/min).

[b]The designation 5α in parentheses refers to "*allo*" (*trans* 5α-H) bile acids.

[c]e-e (equatorial-equatorial); a-a (axial-axial); a-e (axial-equatorial); e-a (equatorial-axial).

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an N_2 stream, and the residue was treated with 2,2-dimethoxypropane (0.3 ml) and *p*-toluenesulfonic acid (*ca.* 10 μg). The mixture was allowed to stand overnight at room temperature, and then neutralized with 0.1N NH_4OH.

After the reactions, each sample solution was filtered (0.45 μm; Millipore Corp., Bedford, MA, USA), washed with 0.5 ml of methanol, and an aliquot of the sample solution was injected directly into the HPLC.

RESULTS AND DISCUSSION

HPLC Behavior of NPM Ester Derivatives

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Table I shows the relative capacity factors (*rk'* values) for thirty-eight 2-, 4- and 6-hydroxylated bile acids examined plus two common bile acids (deoxycholic and cholic acids) as their NPM esters and the eluent system (methanol-water mixture) on a Nova-Pak C_{18} reversed-phase column. It is evident from the data in Table I that in general the 2,3- and 3,4-hydroxylated compounds and the common bile acids in each group of di-, tri- and tetrols were eluted more slowly than the corresponding 3,6-hydroxylated analogs. With the 3,4-glycols, the diequatorial 3α,4β-glycols were always eluted after the corresponding 3β,4α- and 3β,4β-epimers. In addition, the 3α,4β-glycols were moved more slowly than the corresponding diequatorial 2β,3α-analogs, with an exception of the 3α,4β,12α- *vs.* 2β,3α,12α-triol pair. A comparison between the stereoisomeric 3,6,7- and 3,6,12-triols shows much earlier elution of the latters, regardless of their stereochemical configuration of the hydroxyl groups.

For the nine 3α,6,7- and 3β,6,7-triols and six 3α,6,7,12α-tetrols differing in the stereochemical configuration of the hydroxyl groups at C-6 and C-7, the isomers having both α-oriented hydroxyls (equatorial-axial 6α,7α-glycols) showed decidedly the least mobility of each group. Further, the triols

and tetrols in the 5β series were eluted earlier than the corresponding 5α-analogs. However, the separation of these compounds was unsatisfactory, where several stereoisomeric pairs (*e.g.*, 3α,6α,7β-, 3α,6β,7α- and 3β,6β,7α-triols) overlapped completely under the HPLC conditions used. Fig. 1 shows a typical chromatogram of a mixture of the stereoisomeric 3,6,12- and 3,6,7-triols as their NPM esters.

HPLC Behavior of "Mixed" Acetonide-NPM Ester Derivatives

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Cyclic derivatives for the gas chromatographic analysis of *vicinal* glycols of bile acids (8) and for HPLC determination of *vicinal* hydroxylated steroids such as brassinosteroids (9-11) suggested their applications to the bile acids under study. Formation of arylboronate cyclic derivatives of the *cis*-glycols (3β,4β–, 6α,7α- and 6β,7β-) with 1-naphthaleneboronic or 9-phenanthreneboronic acid (9-11) under various experimental conditions was incomplete.

Difficulties encountered with the cyclization reaction of the 1,2-glycols in the 5β-steroid nucleus would be ascribable to the steric hindrance due to the bulky aromatic groups. In analogy with the previous preparation of the "mixed" alkylboronate ester derivatives (8), the *cis*-glycols, regardless of their α,α- or β,β-configuration, as their NPM esters, reacted easily and completely to form cyclic acetonides, which in HPLC have much larger *rk'* values than the corresponding NPM esters, improving the separation of overlapping pairs. The preparation procedure consisted of treatment of the bile acid NPM esters with 2,2-dimethoxypropane catalyzed by *p*-toluenesulfonic acid (12).

As for the *vicinal* glycols of other configurations the same procedure gave somewhat different products, providing useful diagnostic informations. The diaxial *trans*-glycols (3β,4α- and 6β,7α-) were incapable of forming cyclic derivatives,

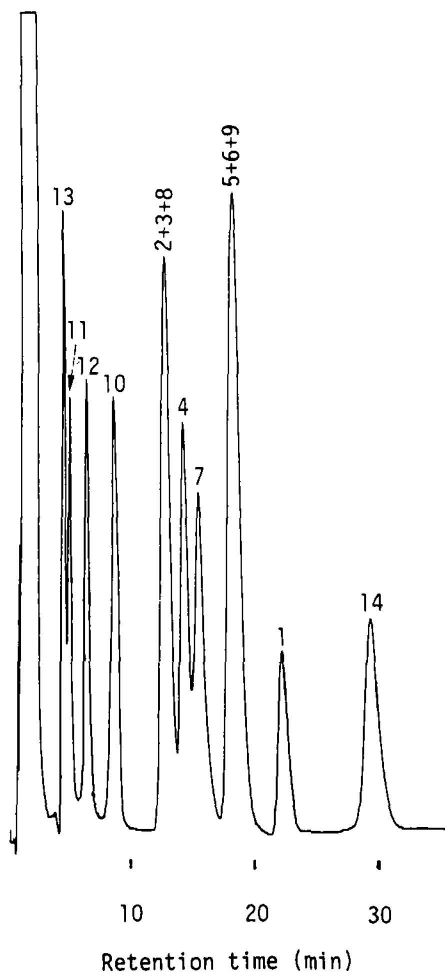

FIGURE 1. HPLC of a mixture of stereoisomeric 3,6,7-triols as their NPM ester derivatives (mobile phase, methanol-water [7:3, v/v]; flow rate, 1.0 ml/min). Peak identification, position and configuration of hydroxyls: 1 = 3α,6α,7α; 2 = 3α,6α,7β; 3 = 3α,6β,7α; 4 = 3α,6β,7β; 5 = 3α,6β,7β(5α); 6 = 3β,6α,7α; 7 = 3β,6α,7β; 8 = 3β,6β,7α; 9 = 3β,6β,7β; 10 = 3α,6α,12α; 11 = 3α,6β,12α; 12 = 3β,6α,12α; 13 = 3β,6β,12α; 14 = 3α,7α,12α.

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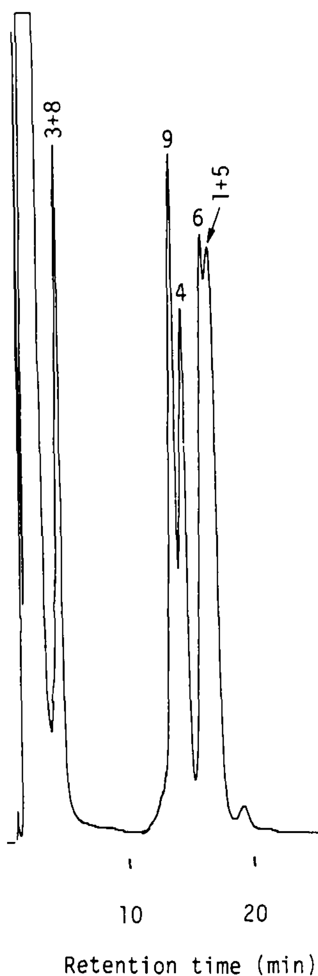

FIGURE 2. HPLC of a mixture of the acetonide-NPM ester derivatization products formed from the *cis*- and diaxial *trans*-glycol types of stereoisomeric 3,6,7-triols (mobile phase, methanol-water [4:1, v/v]; flow rate, 0.7 ml/min).
Peak identification, position and configuration of hydroxyls: see legend in FIGURE 1; compounds 3 and 8 and comounds 1, 4, 5, 6 and 9 were identified as the NPM and acetonide-NPM ester derivatives, respectively.

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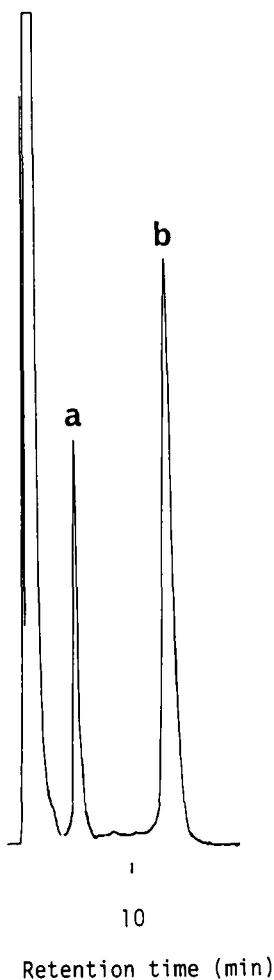

FIGURE 3. HPLC of the acetonide-NPM ester derivatization products formed from the diequatorial *trans*-glycol type of 3α,4β,7α-trihydroxy-5β-cholanoic acid (mobile phase, methanol-water [83:17, v/v]; flow rate, 0.7 ml/min). Peak identification: (a) NPM ester; (b) acetonide-NPM ester.

TABLE II

Rk' values of 2-, 4- and 6-hydroxylated bile acids as their "mixed" acetonide-NPM esters on reversed-phase HPLC[a]

Position and configuration of hydroxyls	Acetonide-NPM
Dihydroxylated compound	(9:1; 0.7)
2β,3α	9.88
3α,4β	10.1
3β,4β	11.8
Trihydroxylated compound	(4:1; 0.7)
2β,3α,7α	3.36
2β,3α,12α	4.07
3α,4β,7α	4.64
3β,4β,7α	5.76
3α,4β,12α	4.07
3β,4β,12α	4.07
3α,6α,7α	3.32
3α,6α,7β	2.62
3α,6β,7β	2.75
3α,6β,7β (5α)	3.32
3β,6α,7α	3.17
3β,6α,7β	2.62
3β,6β,7β	2.62
Tetrahydroxylated compound	(7:3; 1.2)
2β,3α,7α,12α	4.79
3α,4β,7α,12α	5.10
3β,4β,7α,12α	6.58
3α,6α,7α,12α	4.67
3α,6α,7β,12α	3.45
3α,6α,7β,12α (5α)	4.26
3α,6β,7β,12α	3.76
3α,6β,7β,12α (5α)	4.26

[a]See footnotes to TABLE I.

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exhibiting only the NPM ester peaks on HPLC. Fig. 2 illustrates a typical chromatogram of a mixture of the acetonide-NPM ester derivatization products formed from stereoisomeric 3,6,7-triols having the *cis*- and diaxial *trans*-glycols.

However, each of the diequatorial *trans*-glycols (2β,3α-, 3α,4β- and 6α,7β-) showed two well-separated peaks (Fig. 3). The peak with a larger *rk'* value corresponded to the mixed acetonide-NPM ester and that with the smaller to the NPM ester, indicating the occurrence of partial cyclization under the derivatization conditions employed. The NPM/acetonide-NPM ratios of the two peaks (*ca*. 9:1 to 2:8) were dependent upon the structures of bile acids. The present result is in sharp contrast with the previous finding (13) that under an anhydrous cyclization condition on a large scale preparation the *trans*-glycol type of bile acids forms the acetonide derivatives almost quantitatively (13). The *rk'* values of the bile acids which form the "mixed" acetonide-NPM esters are listed in Table II.

This procedure may be helpful for characterizing the structures including stereochemistry of the 2,3- 3,4- and 6,7-hydroxylated bile acids in biological fluids.

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