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Note

Separation of allo bile acid stereoisomers by thin-layer and high-performance liquid chromatography

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Numerous studies have been reported the separation, identification and quantification of natural and synthetic bile acids as well as their conjugates by thin-layer chromatography (TLC)[1–7] and high-performance liquid chromatography (HPLC)[7–10] on both normal-phase (NP) and/or reversed-phase (RP) adsorbents. However, they were concerned primarily with "normal" (5β, A/B-*cis*) cholanoic acids. Similar studies on the epimeric "allo" (5α, A/B-*trans*) acids, which, although also of considerable biochemical interest, have been limited by their restricted availability or non-existence. However, as a result of the recently reported preparations[11–14] of the rare and new acids needed to complete the set, we have available for further studies the 26 theoretically possible allocholanoic acids substituted by one to three hydroxyl groups at positions 3, 7 and 12.

In this paper we describe the separation of the 26 allo acids as their methyl esters by NP- and RP-TLC, and their 4-nitrophthalimidemethyl (NPM) esters by RP-HPLC. A comparison of the resulting data with those for the corresponding 5β epimers[5,10] is included.

EXPERIMENTAL

Samples and reagents

Almost all of the 5α and 5β bile acids as well as their methyl ester derivatives were from the collection in our laboratory, and the new 5α-cholanoic acids were synthesized and characterized recently by us[11–14]. All solvents were of HPLC grade and used without further purification.

TLC

NP-TLC plates precoated with silica gel 60F_{254} (20 cm × 20 cm, layer thickness 0.2 mm) and RP-TLC plates precoated with octadecyl (C_{18})-bonded silica gel RP-18 F_{254s} (10 cm × 10 cm for nano-TLC, layer thickness 0.2 mm, particle size 7

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μm) were obtained from E. Merck (Darmstadt, F.R.G.). TLC was carried out by essentially the same procedures as described previously[5]. For the developing solvent, hexane–ethyl acetate or hexane–ethyl acetate–acetic acid mixtures were employed for NP-TLC and methanol–acetonitrile–water–formic acid mixtures for RP-TLC.

HPLC

The HPLC apparatus used was a Waters M-45 solvent-delivery system (Waters Assoc., Milford, MA, U.S.A.) equipped with a Model U6K sample loop injector and a Shimadzu SPD-2A UV detector (Shimadzu Corp., Kyoto, Japan); the wavelength

TABLE I

R_F VALUES ON NP- AND RP-TLC OF 5α AND 5β BILE ACID METHYL ESTERS

Compound	*NP-TLC**		*RP-TLC***	
	5α	*5β*	*5α*	*5β*
Monohydroxy compound				
3α	0.30	0.21	0.41	0.50
3β	0.22	0.29	0.30	0.40
7α	0.41	0.54	0.45	0.46
7β	0.37	0.37	0.43	0.49
12α	0.49	0.58	0.43	0.46
12β	0.48	0.53	0.38	0.43
Dihydroxy compound				
3α, 7α	0.06	0.07	0.48	0.54
3α, 7β	0.09	0.08	0.63	0.66
3β, 7α	0.06	0.12	0.59	0.58
3β, 7β	0.06	0.09	0.58	0.63
7α, 12α	0.25	0.46	0.52	0.52
7α, 12β	0.63	0.74	0.67	0.67
7β, 12α	0.53	0.59	0.67	0.69
7β, 12β	0.62	0.61	0.67	0.70
3α, 12α	0.11	0.08	0.52	0.53
3α, 12β	0.20	0.15	0.61	0.64
3β, 12α	0.12	0.12	0.61	0.61
3β, 12β	0.14	0.22	0.59	0.64
Trihydroxy compound				
3α, 7α, 12α	0.28	0.38	0.21	0.26
3β, 7α, 12α	0.33	0.51	0.40	0.38
3α, 7α, 12β	0.35	0.63	0.46	0.52
3α, 7β, 12α	0.53	0.54	0.51	0.54
3β, 7α, 12β	0.56	0.69	0.62	0.61
3β, 7β, 12α	0.49	0.56	0.64	0.65
3β, 7β, 12β	0.54	0.62	0.68	0.73
3α, 7β, 12β	0.66	0.63	0.68	0.74

* In TLC on silica gel $60F_{254}$; mono-, di- and trihydroxy compounds were developed in hexane–ethyl acetate (83:17, v/v), hexane–ethyl acetate (60:40, v/v) and hexane–ethyl acetate–acetic acid (10:40:2, v/v/v), respectively.

** In TLC on C_{18}-bonded silica gel RP-18 F_{254S}; mono-, di- and trihydroxy compounds were developed in methanol–acetonitrile–water–formic acid, 47.5:47.5:5.0:0.5, 45:45:10:0.5 and 40:40:20:0.5 (v/v/v/v), respectively.

selected for all measurements was 254 nm. A Nova-Pak C_{18} RP column (15 cm × 3.9 mm I.D., 5 μm; Waters Assoc.) was used under ambient conditions. Methanol–water mixtures (ratios from 90:10 to 70:30, v/v) were used as the eluent at flow-rates of 0.5–1.0 ml/min unless otherwise noted.

Free bile acids were derivatized as their UV-sensitive NPM esters[10], with N-chloromethyl-4-nitrophthalimide as reagent and 18-crown-6 ether as the catalyst.

RESULTS AND DISCUSSION

Table I shows the R_F values on NP- and RP-TLC of the two sets of 26 bile acid methyl esters of the 5α and 5β series. Separation of the twelve monohydroxy esters of the two series by NP-TLC, while still not complete, was more satisfactory than by RP-TLC. The following order of increasing mobility was observed on the NP plates: 3α (5β) ≈ 3β (5α) < 3β (5β) ≈ 3α (5α) < 7β (5α) ≈ 7β (5β) < 7α (5α) < 12β (5α) ≈ 12α (5α) < 12β (5β) ≈ 7α (5β) < 12α (5β). In both series, compounds having an axial hydroxyl group in positions 3, 7 or 12 move faster than the corresponding equatorially substituted stereoisomers. In addition, 7- and 12-hydroxy compounds in the 5β series have larger R_F values than those of the corresponding 5α series, but at the 3-position the mobility depends on the conformation of the hydroxyl substituent rather than on the configuration at C-5; the axial pair of isomers 3α-OH (5α) and 3β-OH (5β), and the equatorial pair, 3β-OH (5α) and 3α-OH (5β), each have nearly identical R_F values. This observation is at variance with data published previously in which a different developing solvent was used (acetone–benzene; ref. 15, p. 59).

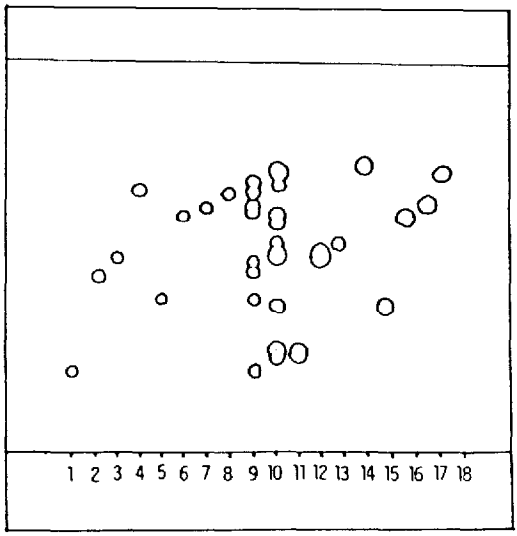

Fig. 1. RP-TLC of sixteen trihydroxylated bile acid stereoisomers as their methyl esters. Eluent: methanol–acetonitrile–water–formic acid (40:40:20:0.5, v/v/v/v). Spot identification, position and configuration of hydroxyls: 1 = ααα (5α); 2 = ααβ (5α); 3 = αβα (5α); 4 = αββ (5α); 5 = βαα (5α); 6 = βαβ (5α); 7 = ββα (5α); 8 = βββ (5α); 9 = mixture of 1–8; 10 = mixture of 11–18; 11 = ααα (5β); 12 = ααβ (5β); 13 = αβα (5β); 14 = αββ (5β); 15 = βαα (5β); 16 = βαβ (5β); 17 = ββα (5β); 18 = βββ (5β).

In contrast with the monohydroxy esters, the trihydroxy compounds were found to be much better separated by RP-TLC than by NP-TLC, although the resolution was not complete (Fig. 1). The relative mobilities of the individual analogues in the two series lay in similar orders.

As for the dihydroxy compounds, the eight 5-stereoisomers in each group of 3,7-, 3,12- and 7,12-dihydroxy esters were only fairly well resolved on RP-plates and poorly on NP-plates, but the latter plates are useful for group separation, as the 7,12-diols move much faster than the other two groups of diols.

These TLC experiments suggest that a combined use of NP- and RP-TLC

TABLE II

rk' VALUES ON RP-HPLC OF 5α and 5β BILE ACID NPM ESTERS

Nova-Pak C_{18} column; mobile phase, methanol–water (75:25, v/v). *rk'* is the ratio of the capacity factor of deoxycholic acid ester (3α, 12α-dihydroxy-5β-cholanoate) to those of the bile acid esters; *Δrk'* shows the difference in the *rk'* values between the 5α and 5β series. The tree recalcitrant pairs, 3α, 7β–7α, 12β (5α), 3β, 7β–3β, 12α (5α) and 3β, 7β–7α, 12β (5β), were nicely separated by a change of eluent to acetonitrile–methanol–water (35:35:30, v/v/v); in each pair, the former before the latter. The use of a Zorbax ODS column (methanol–water, 80:20, v/v) resulted in the separation of an epimeric pair, 3α, 12β–3β, 12β (5α); the former before the latter.

Compound	*5α*	*5β*	*Δrk'*
Monohydroxy compound			
3α	2.18	1.98	0.20
3β	1.86	1.72	0.14
7α	3.29	3.51	0.22
7β	3.64	2.95	0.69
12α	3.64	3.51	0.13
12β	5.09	4.42	0.67
Dihydroxy compound			
3α, 7α	1.02	0.90	0.12
3α, 7β	0.32	0.28	0.04
3β, 7α	0.38	0.45	0.07
3β, 7β	0.35	0.30	0.05
7α, 12α	1.29	1.21	0.08
7α, 12β	0.32	0.31	0.01
7β, 12α	0.28	0.26	0.02
7β, 12β	0.25	0.21	0.04
3α, 12α	0.98	1.00	0.02
3α, 12β	0.49	0.45	0.04
3β, 12α	0.34	0.45	0.11
3β, 12β	0.49	0.48	0.01
Trihydroxy compound			
3α, 7α, 12α	0.60	0.51	0.09
3β, 7α, 12α	0.17	0.21	0.04
3α, 7α, 12β	0.14	0.12	0.02
3α, 7β, 12α	0.10	0.09	0.01
3β, 7α, 12β	0.06	0.07	0.01
3β, 7β, 12α	0.045	0.05	0.005
3β, 7β, 12β	0.04	0.035	0.005
3α, 7β, 12β	0.03	0.025	0.005

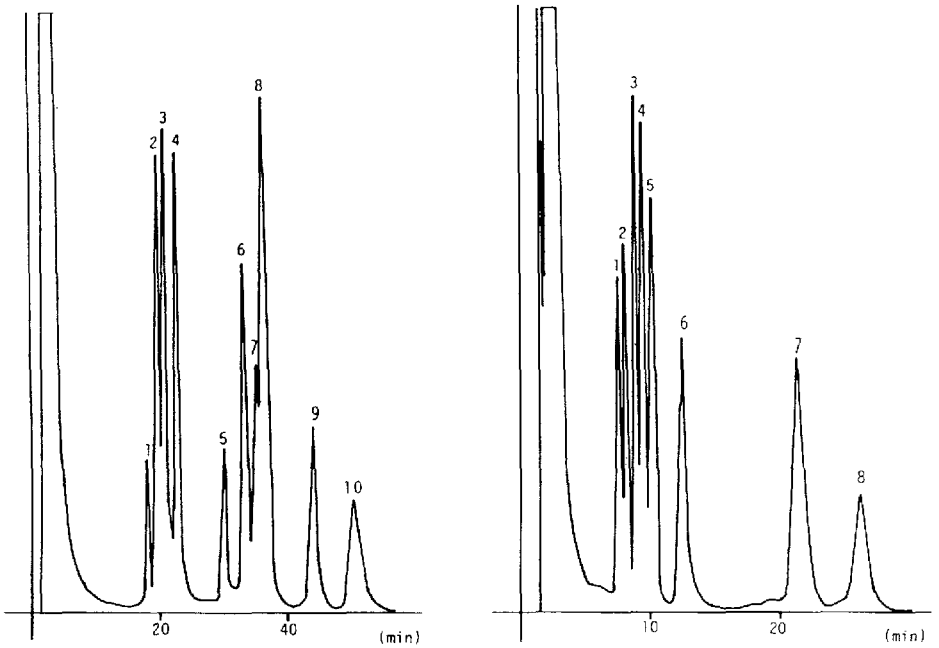

Fig. 2. RP-HPLC of a mixture of twelve monohydroxylated isomers as their NPM esters. Conditions: column, Nova-Pak C_{18}; mobile phase, methanol–water, 90:10 (v/v); flow-rate, 0.5 ml/min; detection, UV at 254 nm. Peak identification, position and configuration of hydroxyls: 1 = 3β (5β); 2 = 3β (5α); 3 = 3α (5β); 4 = 3α (5α); 5 = 7β (5β); 6 = 7α (5α); 7 = 7α and 12α (each 5β); 8 = 7β and 12α (each 5α); 9 = 12β (5β); 10 = 12β (5α).

Fig. 3. RP-HPLC of a mixture of twelve dihydroxylated 5α bile acid isomers as their NPM esters. Conditions: column, Nova-Pak C_{18}; mobile phase, methanol–water, 80:20 (v/v); flow-rate, 0.7 ml/min; detection, UV at 254 nm. Peak identification, position and configuration of hydroxyls: 1 = 7β, 12β; 2 = 7β, 12α; 3 = 3α, 7β and 7α, 12β; 4 = 3β, 7β and 3β, 12α; 5 = 3β, 7α; 6 = 3α, 12β and 3β, 12β; 7 = 3α, 7α and 3α, 12α; 8 = 7α, 12α.

would be helpful in a supplementary analysis of hydroxylated bile acids, as a preliminary to a subsequent more definitive analysis by RP-HPLC.

As shown by the data summarized in Table II and Figs. 2–4, analysis by RP-HPLC of the same group of acids, as their C-24 NPM ester derivatives, in appropriate solvent systems, offers a high degree of resolution, and can provide additional confirmatory evidence for the structure of the individual compounds.

A mixture of the twelve monohydroxy NPM esters afforded ten resolved peaks when eluted from the Nova-Pak C_{18} column with methanol–water (90:10, v/v) (Fig. 2), emerging in the following order: 3β (5β) < 3β (5α) < 3α (5β) < 3α (5α) < 7β (5β) < 7α (5α) < 7α (5β) = 12α (5β) < 7β (5α) = 12α (5α) < 12β (5β) < 12β (5α). This order of mobility is not a direct reversal of that found on NP-TLC, as might be expected. It is generally accepted that on RP columns an C-3 equatorially substituted monohydroxy steroid is less strongly adsorbed than its axial counterpart[8]. This generalization was found to hold for the C-3 epimers of the 5α series*, as well as for the

* However, from a Zorbax ODS RP column (25 cm × 4.6 mm I.D.; Du Pont Co., Wilmington, DE, U.S.A.), the axial 3α-hydroxy ester of the 5α series was eluted before its equatorial 3β-epimer.

C-7 epimers of the 5β series, but not for the other epimeric pairs (C-3 and C-12, 5β), (C-7 and C-12, 5α), in which the equatorially hydroxylated stereoisomers were eluted more slowly than their axial epimers.

Unlike their poorer resolution by TLC, the eight 5-stereoisomeric NPM esters in each group of 3,7- and 7,12-dihydroxy compounds were well resolved on Nova-Pak C_{18} when eluted by methanol–water (75:25, v/v). However, with the 3, 12 group of eight, the separation was incomplete; two of the pairs, 3α, 12β (5α)–3β, 12α (5α) and 3α, 12β (5β)–3β, 12α (5β), overlapped under the conditions used. The order of mobility of the 5α and 5β series of each group of diols is essentially identical (Fig. 3)[10] and corresponded well with the order observed on RP-TLC: 3,7-diols, $\alpha\beta < \beta\beta < \beta\alpha \ll \alpha\alpha$; 7,12-diols, $\beta\beta < \beta\alpha < \alpha\beta \ll \alpha\alpha$; 3,12-diols, $\beta\alpha \leqslant \alpha\beta \leqslant \beta\beta \ll \alpha\alpha$.

In each of the six groups of three diols, the isomer having both hydroxyls substituted α, regardless of the conformation or position of the substituents, or whether the A/B ring junction is *cis* or *trans*, had decidedly the lowest mobility of the group. This finding is consistent with that of Shaw *et al.*[9] who reported on the RP-HPLC of many bile acids, including several pairs of the compounds studied by us. They rationalized that the lowest mobility of the all α-hydroxylated bile acids in each group is not caused by a special property of the α-hydroxy substituent, but rather because a β-substituent reduces the hydrophobic area of the reversed-phase surface. Accordingly, the α-trihydroxylated stereoisomers in both 5α and 5β series also had markedly the lowest mobility of each group.

The sixteen trihydroxy stereoisomers of the two series were completely resolved

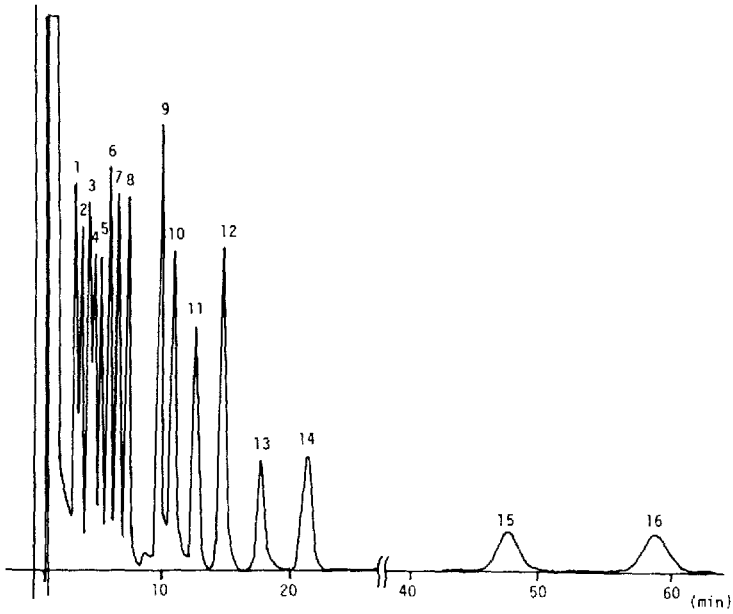

Fig. 4. RP-HPLC of a mixture of sixteen trihydroxylated bile acid stereoisomers as their NPM esters. Conditions: column, Nova-Pak C_{18}; mobile phase, methanol–water, 70:30 (v/v); flow-rate, 1.0 ml/min; detection, UV at 254 nm. Peak identification, position and configuration of hydroxyls: 1 = $\alpha\beta\beta$ (5β); 2 = $\alpha\beta\beta$(5α); 3 = $\beta\beta\beta$ (5β); 4 = $\beta\beta\beta$ (5α); 5 = $\beta\beta\alpha$ (5α); 6 = $\beta\beta\alpha$ (5β); 7 = $\beta\alpha\beta$ (5α); 8 = $\beta\alpha\beta$ (5β); 9 = $\alpha\beta\alpha$ (5β); 10 = $\alpha\beta\alpha$ (5α); 11 = $\alpha\alpha\beta$ (5β); 12 = $\alpha\alpha\beta$ (5α); 13 = $\beta\alpha\alpha$ (5α); 14 = $\beta\alpha\alpha$ (5β); 15 = $\alpha\alpha\alpha$ (5β); 16 = $\alpha\alpha\alpha$ (5α).

with methanol–water (70:30) as solvent (Fig. 4). The order of elution of the individual compounds in both series was identical, *i.e.*, $\alpha\beta\beta < \beta\beta\beta < \beta\beta\alpha < \beta\alpha\beta < \alpha\beta\alpha < \alpha\alpha\beta < \beta\alpha\alpha \ll \alpha\alpha\alpha$, and also corresponds to the order found by RP-TLC.

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