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Note

New solvent systems for the separation of free and conjugated bile acids

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Free dihydroxycholanic acids in biological samples are generally difficult to separate by thin-layer chromatography. For the quantitative analysis of bile acids in humans, especially for the kinetic determination of bile acid pool and bile acid turnover rate by isotope dilution technique[1], and in the evaluation of C/CDC* ratio for liver function[2,3] it is essential that CDC and DOC, two of three major bile acids in serum[3], duodenal[4] and gallbladder bile[5] be separated from each other as well as from C and LC. Only a few of the thin-layer chromatographic systems[6–11] were capable of separating these two important dihydroxy isomers with various degrees of resolution. We report a new solvent system (A) which gives better resolution between CDC and DOC than previously published procedures using an unidirectional single-development system. The separation of glycine (G) and taurine (T) conjugates is also of importance in systematic analysis of bile acids, especially if the G/T ratio is to be used as a measure of interrupted enterohepatic circulation such as ileal disorder[12,13]. We propose a solvent system (B) which gives better separation of G and T conjugates than the systems previously described[9,14,15].

MATERIALS

Standard, common, free and conjugated bile acids were obtained from Supelco (Bellefonte, Pa., U.S.A.), Steraloids (Pawling, N.J., U.S.A.), Applied Science Labs. (State College, Pa., U.S.A.), and Calbiochem (Los Angeles, Calif., U.S.A.). The purity of the standards was tested by thin-layer chromatography. All solvents used were reagent grade obtained from Aldrich (Milwaukee, Wisc., U.S.A.) and Mallinckrodt (St. Louis, Mo., U.S.A.). Glass plates (20 × 20 cm), pre-coated with silica gel G to a thickness of 250 μm were obtained from Brinkmann (Westbury, N.Y., U.S.A.).

* Abbreviations used in this paper: LC = lithocholic acid; DOC = deoxycholic acid; CDC = chenodeoxycholic acid; C = cholic acid; UrsoDOC = ursodeoxycholic acid; HyoDOC = hyodeoxycholic acid; 7-KetoLC = 7-ketolithocholic acid; 7-KetoDOC = 7-ketodeoxycholic acid. For conjugated bile acids: TCDC = taurochenodeoxycholic acid; GLC = glycolithocholic acid; GCDC = glycochenodeoxycholic acid; GC = glycocholic acid; TLC = taurolithocholic acid; TDOC = taurodeoxycholic acid; TC = taurocholic acid.

METHODS

Pre-coated glass plates were activated in an oven at 135° for 20 min and stored in a desiccated chamber until used. The sample, 20–40 μg in 20–40 μl of ethanol–methanol (95:5), was applied to the plate with a micropipette, allowed to dry, placed in a rectangular glass tank (10 × 30 × 25 cm) and developed by the ascending technique at room temperature (23–25°). The solvent mixture was made up of isooctane–diisopropyl ether–glacial acetic acid–*n*-butanol–water (10:5:5:3:1) for system A and isooctane–diisopropyl ether–glacial acetic acid–*n*-butanol–isopropanol–water (10:5:5:3:6:1) for system B. When the solvent front was 17 cm from the origin (19 cm from the bottom), the plates were taken out of the jars, dried in hot air and sprayed with 80% sulfuric acid saturated with potassium dichromate using Supelco chromatographic glass spray apparatus. The plates were heated for 3 min in an oven at 135°. The time required for a run was about 3 h. The charred plates can be preserved in a desiccated chamber for future reference. Runs carried out in other solvent systems also followed the same procedure.

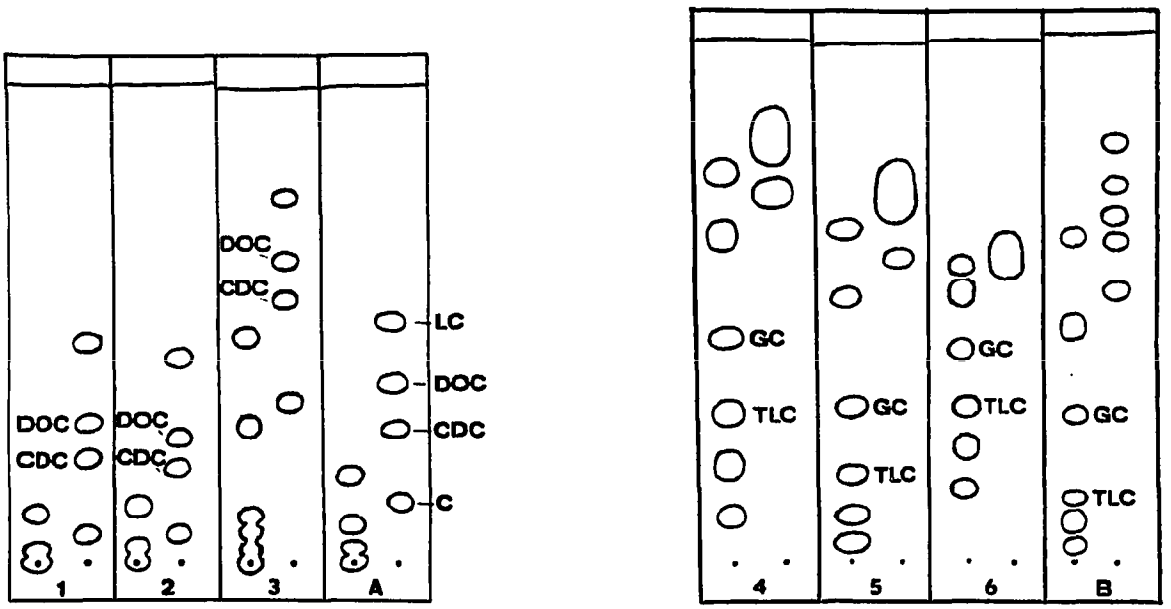

Fig. 1. Thin-layer chromatograms developed in various solvent systems under identical conditions. On the left, from the bottom up, are TC, TCDC, TDOC, TLC, and GC (overlapping), GCDC and GDOC (overlapping), and GLC for present system A and system 3 (ref. 8); TC, TCDC, TDOC, TLC, GC, GCDC and GDOC (overlapping), and GLC for systems 1 (ref. 6) and 2 (ref. 7). On the right, from the bottom up, are C, CDC, DOC and LC for all four systems.

Fig. 2. Thin-layer chromatograms developed in various solvent systems under identical conditions. On the left, from the bottom up, are TC, TCDC and TDOC (overlapping), TLC, GC, GCDC, and GDOC (overlapping), GLC for all four systems. On the right, from the bottom up, are C, UrsoDOC, CDC, DOC, and LC for present system B; free bile acids are not completely resolved in systems 4 (ref. 14), 5 (ref. 15), and 6 (ref. 9).

TABLE I

COMPARISON OF VARIOUS SOLVENT SYSTEMS FOR SEPARATION OF FREE DIHYDROXY BILE ACIDS BY THIN-LAYER CHROMATOGRAPHY

The numbers in parentheses are R_F values for the corresponding methyl esters. Solvent systems giving inferior separation of CDC and DOC within the same reference are not listed here.

System	*Reference*	$R_F \times 100$ values				$\Delta R_M \times 100$
		C	*CDC*	*DOC*	*LC*	*CDC – DOC**
A**,***	This work	12 (18)	27 (34)	37 (39)	50 (57)	20 (9)
1	6	6 (7)	20 (21)	26 (26)	43 (46)	15 (12)
2	7	6 (10)	22 (30)	29 (34)	46 (52)	16 (8)
3	8	33 (33)	55 (55)	63 (63)	77 (80)	14 (14)
7	10	16 (16)	33 (34)	38 (38)	52 (53)	10 (8)
8	9	30 (30)	48 (49)	54 (54)	64 (66)	11 (9)
9	11	13 (15)	36 (39)	41 (41)	56 (58)	9 (4)
10	16	10 (12)	30 (32)	36 (37)	52 (55)	12 (10)
11	17	9 (13)	31 (35)	39 (39)	53 (57)	15 (8)
12	18	9 (9)	27 (28)	33 (34)	51 (52)	12 (12)
13	19	2 (3)	12 (14)	16 (17)	31 (35)	15 (10)
14	15	8 (9)	27 (28)	30 (32)	58 (59)	6 (8)
15	20	13 (13)	31 (32)	37 (37)	54 (55)	12 (10)
16	21	39 (39)	49 (50)	54 (54)	59 (60)	9 (7)

* $\Delta R_M = R_{M,\,CDC} - R_{M,\,DOC}$, where $R_M = \log [(1/R_F) - 1]$.

** $R_F \times 100$ values for UrsoDOC, 24 (29); HyoDOC, 21 (26); 7-KetoDOC, 12 (16); 7-KetoLC, 25 (31); 3β-hydroxy-5-cholenoic acid, 45 (55); GC, 3; GCDC, 8; GLC, 19; TC, TCDC, TDOC and TLC, 0.

*** Adsorbent: silica gel G. Solvent mixture: isooctane–diisopropyl ether–glacial acetic acid–*n*-butanol–water (10:5:5:3:1).

TABLE II

COMPARISON OF VARIOUS SOLVENT SYSTEMS FOR SEPARATION OF CONJUGATED BILE ACIDS BY THIN-LAYER CHROMATOGRAPHY

Solvent systems giving inferior separation of TLC and GC within the same reference are not listed here.

System	*Reference*	$R_F \times 100$ values								$\Delta R_M \times 100$
		TC	*TCDC*	*TDOC*	*TLC*	*GC*	*GCDC*	*GDOC*	*GLC*	*TLC – GC**
B**,***	This work	3	7	8	12	28	44	46	62	46
4	14	9	19	19	29	44	63	63	74	28
5	15	5	11	13	20	33	55	57	70	29
6	9	14	22	23	30	41	51	53	58	21
17	22	21	31	31	40	51	63	64	70	19
18	23	16	26	27	34	44	56	57	61	18
19	24	1	6	6	12	18	41	41	60	21
20	25	19	29	29	38	49	58	59	64	20
21	26	16	26	26	35	48	61	61	68	23

* $\Delta R_M = R_{M,\,TLC} - R_{M,\,GC}$, where $R_M = \log [(1/R_F) - 1]$.

** Adsorbent: silica gel G. Solvent mixture: isooctane–diisopropyl ether–glacial acetic acid–*n*-butanol–isopropanol–water (10:5:5:3:6:1).

*** $R_F \times 100$ values for free bile acids are: C, 52; UrsoDOC, 62; CDC, 66; DOC, 72; LC, 80; 3β-hydroxy-5-cholenoic, 79; 7-KetoLC, 64; 7-KetoDOC, 52.

RESULTS AND DISCUSSION

Figs. 1 and 2 show the positions of different free and conjugated bile acids on thin-layer plates after development in some solvent systems tested under identical conditions. Tables I and II give the R_F values of various bile acids and their methyl esters. It is apparent from Table I that the resolution between CDC and DOC, in unmethylated, unconjugated forms, appears to be better in system A (relative mobility: $\Delta R_M = 20$) than in any other system, although the solvent system proposed by Gregg[8] does give superior separation of methyl esters of DOC and CDC ($\Delta R_M = 14$). Thus the present system A provides the optimum resolution between these two biologically important isomers without undergoing the time-consuming procedure of methylation. The separation of G and T conjugated bile acids as a group appears to be best in present system B as judged by the migration of glycocholic and taurolithocholic acids (relative mobility $\Delta R_M = 46$). This is a definite advantage over Hofmann's system[15], probably the most quoted system in the literature. Furthermore, system B offers adequate resolution between various free (unconjugated) bile acids while Hofmann's system failed to achieve this. However, the latter system does provide somewhat better resolution between various T conjugated bile acids than the present one. Although the present systems A and B seem to offer significant advantage over other published systems and should be useful in a number of biological and clinical applications where maximum separations of CDC and DOC, G and T conjugates, respectively, are required, they do not separate conjugated dihydroxy isomers from each other. To the authors' knowledge, no reported solvent system is able to do so on thin-layer chromatography.

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