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Note

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

II*. Separation of free bile acids as a group

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The separation of free (unconjugated) bile acids from their glycine (G) and taurine (T) conjugates is of importance in biological investigation because free bile acids possess different physiological activities[2–4]. For example, free bile acids have been found to play a role in the genesis of acquired monosaccharide intolerance in infants[5], and in several other clinical entities associated with overgrowth of bacteria in the small intestine such as Kwashiorkor[6], and stagnant-loop syndrome[7]. A number of thin-layer chromatographic procedures for bile acids have been suggested[8–21] but only a few of them[8–13] were able to achieve some degree of separation of free bile acids from conjugates based upon the assumption that glycomonohydroxy acids such as glycolithocholic acid (GLC) were present in negligible amounts in biological samples. This assumption appears to be invalid by the observation that GLC may constitute 1–17% of the total bile acids in clinical specimens[22,23]. In this report we propose a new solvent system, H, which gives better separation of free bile acids from their G and T conjugates, including GLC, than previously published procedures using an unidirectional single development system.

MATERIALS AND METHODS

Free and conjugated bile acid standards were obtained from Steraloids (Pawling, N.Y., U.S.A.), Applied Science Labs. (State College, Pa., U.S.A.), Supelco (Bellefonte, Pa., U.S.A.), and Calbiochem (La Jolla, Calif., U.S.A.). [^{3}H]-Cholic acid was purchased from New England Nuclear (Boston, Mass., U.S.A.) with a specific activity of 3.8 Ci/mmole and was found to be 98% pure by thin-layer chromatography. All solvents used were of reagent grade and obtained from Aldrich (Milwaukee, Wisc., U.S.A.) and Mallinckrodt (St. Louis, Mo., U.S.A.). Glass plates (20 × 20 cm), precoated with silica gel G to a thickness of 250 μm were purchased from Brinkmann (Westbury, N.Y., U.S.A.).

The present system (H) was made up of chloroform–methanol–water (70:25:3).

* Part I, see ref. 1.

TABLE I

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

Abbreviations: LC = lithocholic acid; DOC = deoxycholic acid; CDC = chenodeoxycholic acid; C = cholic acid; UrsoDOC = ursodeoxycholic acid; 7-ketoDoc = 7-ketodeoxycholic acid; HyoC = hyocholic acid; 12-keto LC = 12-ketolithocholic acid. For conjugated bile acids: GLC = glycolithocholic acid; GDOC = glycodeoxycholic acid; GCDC = glycochenodeoxycholic acid; GC = glycocholic acid; TLC = taurolithocholic acid; TDOC = taurodeoxycholic acid; TCDC = taurochenodeoxycholic acid; TC = taurocholic acid.

System	*Ref.*	*Composition***	*Ratio (v/v)*
H[§]	Present work	$CHCl_3$–methanol–H_2O	70:25:3
1	8	isooctane–isopropanol–HAC	60:40:1
2	9	*n*-butanol–HAc–H_2O	100:7:5
3	10	isooctane–isopropyl ether–HAc	50:25:40
4	11	toluene–HAc–H_2O	50:50:10
5	12	isooctane–HAc–isopropyl ether–isopropanol	10:6:5:1
6	13	HAc–CCl_4–isopropyl ether–isoamyl acetate–*n*-propanol–benzene	5:20:30:40:10:10
7	14	ethyl acetate–methanol–HAc	70:20:10
8	15	isooctane–isopropyl ether–HAc	100:50:70
9	16	isooctane–ethylene chloride–HAc	60:30:30
10	17	isooctane–isopropyl ether–HAc–isopropanol	2:1:1:1
11	18	isooctane–ethyl acetate–HAc–*n*-butanol	20:10:3:3
12	19	isooctane–isopropyl ether–isopropanol–HAc	1:1:1:1
13	20	isooctane–ethyl acetate–HAc	5:5:1
14	21	$CHCl_3$–ethyl acetate–HAc	45:45:10

* Solvent systems giving inferior separation of GLC, GDOC and C within the same reference are not listed here. References giving negative ΔR_M values for both GLC–C and GDOC–C were omitted also.

** HAc = glacial acetic acid; adsorbent, silica gel G.

*** $\Delta R_M = R_{M,\mathrm{GLC\ or\ GDOC}} - R_{M,\mathrm{C}}$ where $R_M = \log [(1/R_F) - 1]$.

§ $R_F \times 100$ values for other free bile acids are: UrsoDOC, 59; 7-ketoDoc, 52; HyoC, 47; 12-Keto LC, 59.

All thin-layer chromatographic runs were carried out by applying 20–40 μg of the sample in 20–40 μl of ethanol–methanol (95:5) to the plate with a micropipette or by a sample streaker (Applied Science Labs.), 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 detailed procedure of thin-layer chromatography has been described in a previous report by the present workers[1].

In the recovery experiments, the silica gel was scraped off after the run at the zone corresponding to the R_F values of the free and conjugated bile acids. The remaining zones were also removed and examined for radioactivity. Solutions for counting were prepared by dissolving the bile acid in 8 ml of liquid scintillation solution, 12.8 ml of Spectrafluor PPO–POPOP concentrated liquid scintillator (Amersham/Searle, Arlington Heights, Ill., U.S.A.) in 200 ml of toluene. The solution was then transferred to a low-potassium liquid scintillation vial. Each flask was rinsed twice with 5 ml of the solution to effect complete transfer of the bile acid. Counting was done on a Packard Tri-Carb 3003 liquid scintillation spectrometer. Efficiencies were determined by external standardization using acetone-quenched standards (Packard) and results are reported as disintegrations per minute.

$R_F \times 100$ values												$\Delta R_M \times 100$ values***	
CC	DOC	CDC	C	GLC	GDOC	GCDC	GC	TLC	TDOC	TCDC	TC	GLC–C	GDOC–C
75	86	53	38	18	12	12	5	18	12	12	8	45	65
69	54	49	39	32	20	20	15	8	5	5	1	13	41
67	64	64	62	61	57	56	44	34	27	26	16	2	9
52	38	33	16	22	9	9	2	0	0	0	0	−17	29
88	29	23	11	16	6	6	1	0	0	0	0	−19	29
54	37	31	13	21	8	7	2	1	0	0	0	−25	24
64	43	35	10	30	6	5	1	0	0	0	0	−59	24
88	85	81	73	74	63	63	44	29	19	19	9	− 2	20
51	33	27	9	17	6	6	1	0	0	0	0	−32	19
43	26	20	6	12	4	4	1	0	0	0	0	−33	19
77	63	55	33	47	25	24	9	6	2	2	0	−26	17
52	36	30	10	26	7	7	0	0	0	0	0	−50	17
74	66	61	46	60	41	41	18	12	6	6	1	−25	9
46	29	22	6	17	5	5	0	0	0	0	0	−51	8
53	39	31	9	30	8	8	1	0	0	0	0	−64	6

RESULTS AND DISCUSSION

Table I gives a comparison of various solvent systems for the separation of free bile acids from G and T conjugates tested under identical conditions, and Fig. 1 shows the positions of different free and conjugated bile acids on thin-layer plates after development in system H and in systems 1–6 of Table I. It is clear from Table I that the resolution between free and conjugated bile acids is better in system H (relative mobility, $\Delta R_M = 45$ and 65 for GLC–C* and GDOC–C, respectively) than in any other system tested regardless of the presence of GLC. This is an advantage over other published systems because quantitative determination of total free bile acids as a group are possible even in the presence of glycomonohydroxy isomers. The results of the run using 2,4-[^{3}H]-cholic acid as a radioactive marker are shown in Table II. The recovery of radioactive cholic acid is nearly complete in the zone corresponding to the R_F values of the free bile acids (*ca.* 96%). This indicates the validity of system H as a tool for satisfactory isolation of free bile acids.

* For abbreviations, see Table I.

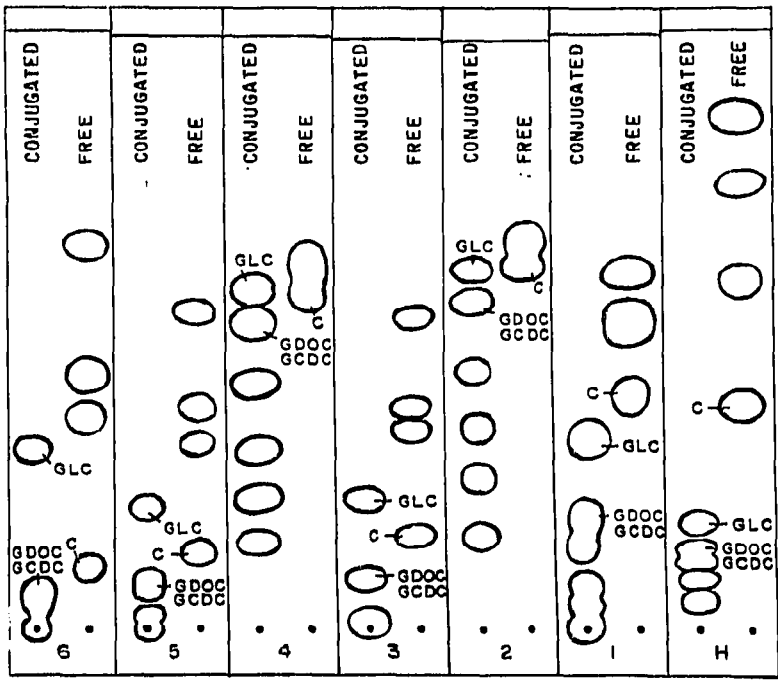

Fig. 1. Thin-layer chromatograms developed in various solvent systems under identical conditions. For abbreviations of compounds see Table I. On the left, from the bottom up, are TC, TCDC and TDOC (overlapping), TLC, GC, GCDC and GDOC (overlapping) and GLC for systems 2 and 4. Taurine-conjugated bile acids and GC are not resolved in systems 1, 3, 5, 6 and system H. On the right, from the bottom up, are C, CDC, LC and DOC for system H; C, CDC, DOC and LC for systems 3, 5 and 6. Free bile acids are not completely resolved in systems 1, 2 and 4.

TABLE II

CHROMATOGRAPHIC DISTRIBUTION OF RADIOACTIVITY OF 2,4-[^{3}H]-CHOLIC ACID AS RUN IN SYSTEM H

Region of plate	*Total Activity (%)*
Above cholic acid	2.9
Cholic acid zone	93.2
GLC zone*	2.3
Below GLC	1.6
Total	100.0

* Based on 10 determinations; average activity found in conjugated bile acid region (GLC and below) was 3.8 ± 1.2%.

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