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Note

Two-dimensional thin-layer chromatography of bile acids

SHIRO IKAWA and MIDORI GOTO
Department of Biochemistry, Tottori University, School of Medicine, Yonago (Japan)
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Many methods for the separation of bile acids and salts by one-dimensional[1–13] and two-dimensional thin-layer chromatography (TLC)[14] have been reported. However, the presence of phospholipids (chiefly lecithin), cholesterol and fatty acids in bile has impeded the use of solvent systems for the direct separation of conjugated bile acids and salts.

Direct group separation of the components of small amounts of ethanol or methanol extracts of bile (especially from small experimental animals such as bile-fistula rats) is desirable, as complicated procedures for purification and hydrolysis of conjugated bile acids may cause some loss of the bile acids and may also produce artifacts. Other advantages are the possibilities of ascertaining, simultaneously, the composition of the bile (particularly as regards conjugated bile acids, lecithin and cholesterol) and of displaying the profile of biliary metabolites of labelled compounds, especially when the separation is used in combination with autoradiography.

In applying two-dimensional TLC to the direct group separation of conjugated bile salts from bile, it is desirable that (1) separation of the bile salts, especially taurine conjugates, should be good; (2) the use of relatively involatile organic acids and solvents should be avoided in the first development, as they require long periods for drying before the second development and are also undesirable when sensitive and non-destructive staining and extraction of the separated substances are required; and (3) the development times should be as short as possible. To meet these requirements, the present study was undertaken. The application of the method to human gall-bladder bile was demonstrated.

EXPERIMENTAL

Materials

Reference bile acids and salts obtained as described below were checked by TLC, by their melting points and by IR spectroscopy.

Lithocholic acid and chenodeoxycholic acid were prepared from cholic acid (Eiken-Kagaku, Tokyo, Japan) according to Fieser and Rajagopalan[15]; hyodeoxycholic acid was prepared from hog bile[16]. Ursodeoxycholic acid was obtained from Tokyo Tanabe (Tokyo, Japan) and deoxycholic acid from Eiken-Kagaku.

Conjugated bile acids and salts were synthesized by the methods of Bergström and Norman[17] and of Hofmann[18]. Sodium lithocholate sulphate and its taurine con

jugate were kindly donated by Professor G. A. D. Haslewood (Guy's Hospital Medical School, London, Great Britain).

Cholesterol was purchased from Wako (Osaka, Japan), and palmitic acid, stearic acid, lecithin and lysolecithin were obtained from Nakarai (Kyoto, Japan). 7α-Hydroxycholesterol was synthesized according to Bide *et al.*[19] and Henbest and Jones[20]. Cholesteryl palmitate and stearate were prepared according to the method of Page and Rudy[21].

Pyrene was purchased from Hopkin & Williams, (Chadwell Heath, Great Britain) and kieselgel G and H (type 60) from E. Merck (Darmstadt, G.F.R.).

All solvents were of analytical grade and were redistilled before use.

Procedure

Layers, 0.25 mm thick on glass plates (20 × 20 cm), were prepared by using a uniform slurry of 30 g of kieselgel H (type 60) in 75 ml of water, or of 30 g of kieselgel G in 60 ml of water, and were activated at 120° for 1 h and stored in a desiccator over silica gel.

All runs were performed in a chromatography tank lined with filter paper at room temperature (19–20°); when the solvent front had run 15 cm from the origin (the time required for this being designated as the development time), the plates were removed from the tank, dried in a stream of air for 30 min and then subjected to a second development perpendicular to the first.

Sample application

A 5-mg portion of sample in 1.0 ml of chloroform - methanol (1:3, v/v) was applied with a micro-pipette to the starting point for two-dimensional TLC, located 15 cm in from two adjacent edges of the plate; the amount of material actually applied is given in the legend to each Figure.

Detection reagents

After air-drying, fatty acids, phospholipids and sterols were revealed by spraying the chromatogram with 0.02% Rhodamine 6GO (Chroma-Gesellschaft, Stuttgart, G.F.R.) in ethanol–water (95:5, v/v) or a 0.05% solution of pyrene in light petroleum[22] and examining it under UV radiation. After UV examination, the plate was sprayed with molybdophosphoric acid–acetic acid–sulphuric acid reagent (1:20:1, w/v/v)[3] and heated briefly to reveal bile acids, phospholipids and sterols. Dragendorff's reagent[23] was also used in some experiments to detect lecithin and lysolecithin.

RESULTS AND DISCUSSION

Adsorbents

Glycine- and taurine-conjugated mono-, di- and tri-hydroxy bile acids and salts were separated on kieselgel G or H (type 60) with Hofmann's solvent system No. 2 (see ref. 2).

Good separations of conjugated bile acids and salts were achieved on kieselgel G, but 120 min development time was needed; on the other hand, development for 90 min on kieselgel H (type 60) gave almost the same R_F values as on kieselgel G, and, as the former gel contained no binder, it was more suitable for use when the bile

TABLE I

SOLVENT SYSTEMS FOR TLC OF CONJUGATED BILE ACIDS AND SALTS ON KIESELGEL H (TYPE 60)

System No.	*Solvents*	*Ratio (v/v)*	*Development time (min)**	*Application***
1	Ethyl acetate–ethanol–acetic acid	7:3:1	43	G, T
2	Chloroform–ethanol–ethyl acetate–acetic acid–water	8:6:5:4:1	70	G, T, S-2
3	Chloroform–ethanol–acetic acid–water	17:4:3:1	82	G
4	Chloroform–ethanol–acetic acid–water	12:8:4:1	90	T, G
5	Chloroform–methanol–ethanol	5:4:1	60	S-1
6	Chloroform–ethanol–28% aqueous ammonia***	25:35:1	62	S-1

* Each value is the mean of five experiments.

** Abbreviations: G, glycine conjugates; T, taurine conjugates; S-1, S-2, first and second developing solvents, respectively, for two-dimensional TLC.

*** Analytical grade, sp. gr. 0.88.

TABLE II

R_F VALUES FOR UNCONJUGATED AND CONJUGATED BILE ACIDS IN DIFFERENT SOLVENT SYSTEMS ON KIESELGEL H (TYPE 60)

The following abbreviations are used: L, lithocholic acid; CD, chenodeoxycholic acid; D, deoxycholic acid; U, ursodeoxycholic acid; HD, hyodeoxycholic acid; C, cholic acid; G, glycine conjugates; T, taurine conjugates; S, sulphate. Each value is the mean of five experiments.

Compound	*Solvent system*					
	1	*2*	*3*	*4*	*5*	*6*
Unconjugated						
L	0.86	0.87	1.00	0.97	0.56	0.30
CD	0.81	0.82	0.90	0.92	0.49	0.20
D	0.82	0.82	0.90	0.92	0.47	0.20
U	0.81	0.82	0.91	0.92	0.49	0.20
HD	0.77	0.80	0.84	0.88	0.49	0.20
C	0.74	0.72	0.67	0.84	0.34	0.09
Conjugated						
GL	0.78	0.85	0.85	0.94	0.29	0.25
NaGCD	0.70	0.73	0.63	0.84	0.21	0.16
NaGD	0.72	0.73	0.63	0.77	0.20	0.16
GHD	0.65	0.72	0.60	0.77	0.23	0.16
NaGC	0.50	0.57	0.33	0.69	0.13	0.08
NaTL	0.32	0.37	0.07	0.47	0.53	0.58
NaTCD	0.21	0.24	0.03	0.33	0.43	0.50
NaTD	0.21	0.24	0.03	0.33	0.43	0.50
NaTU	0.21	0.25	0.03	0.34	0.46	0.50
NaTC	0.10	0.12	0.01	0.17	0.35	0.36
Sulphate						
NaLS	—	0.40	—	0.65	—	0.32
NaTLS	—	0.03	—	0.12	—	0.12

acids were to be eluted from the adsorbent. In subsequent work, therefore, kieselgel H (type 60) was mainly used.

Solvent systems

Solvent systems for the first and second developments in two-dimensional TLC for group separation of the mono-, di- and tri-hydroxy bile acid conjugates of glycine and taurine are listed in Table I. An advantage of these solvents is that the plates are easy to dry in air. The R_F values are summarized in Table II.

Glycine conjugates migrate faster than taurine conjugates, except in solvent systems 5 and 6, in which glycine conjugates remain in ionic form[7]. Within each group, bile acids and salts move more slowly as the number of hydroxyl groups on the molecule increases.

System 1 has the advantage of a particularly short development time, but sometimes gives poor separation of taurine conjugates.

System 2 separates conjugated bile acids and salts into glycine and taurine groups, and, in this system, glycine-conjugated lithocholic acid has an R_F value of 0.85. This means, in practice, that system 2 is particularly suitable for the second solvent in two-dimensional TLC, as it leaves room for sterols, fatty acids and unconjugated bile acids and salts in the R_F region 0.85–1.0.

System 3 gives incomplete separation of taurine conjugates, but is suitable for the separation of glycine conjugates, showing differences of about 0.2 to 0.3 in R_F value (see Table III).

System 4 is suitable for the separation of taurine conjugates and more polar compounds, and, by using this solvent, sodium lithocholate sulphate and its taurine conjugate can be completely separated from other conjugated bile acids and salts.

Combination of solvent systems for two-dimensional TLC

The R_F values of some fatty acids, phospholipids and sterols were investigated in the solvent systems used; the results are listed in Table IV, which shows that solvent system 5 is not suitable for use as the first solvent in two-dimensional TLC, as it does not separate lecithin completely from the taurine conjugates of dihydroxy bile acids.

A chromatogram obtained by the proposed procedure is shown in Fig. 1 and demonstrates that unconjugated, conjugated and conjugated sulphated bile acids and

TABLE III

R_F VALUES OF CONJUGATED BILE ACIDS AND SALTS IN DIFFERENT SOLVENT SYSTEMS ON KIESELGEL H (TYPE 60)

For abbreviations, see Table II; each value is the mean of five experiments.

Compounds	*Solvent system*				
	No. 2 of Hofmann[2]	*System B of Huang and Nichols*[13]	*2*	*3*	*4*
GL-NaGCD	0.11	0.17	0.12	0.22	0.10
NaGCD-NaGC	0.17	0.23	0.16	0.30	0.15
NaGC-NaTL	0.19	0.20	0.20	0.26	0.22
NaTL-NaTCD	0.08	0.06	0.13		0.13
NaTCD-NaTC	0.09	0.05	0.12		0.17

TABLE IV

R_F VALUES OF SOME FATTY ACIDS, PHOSPHOLIPIDS AND STEROLS ON KIESELGEL H (TYPE 60)

Each value is the mean of five experiments.

Compound	*Solvent system*		
	2	*5*	*6*
Palmitic acid	0.93	0.60	0.28
Stearic acid	0.93	0.60	0.28
Cholesteryl palmitate	1.00	0.80	0.90
Cholesteryl stearate	1.00	0.85	0.90
7α-Hydroxycholesterol	0.88	0.74	0.79
Cholesterol	0.91	0.79	0.83
Lecithin	0.09	0.43	0.02
Lysolecithin	0.03	0.03	0.01

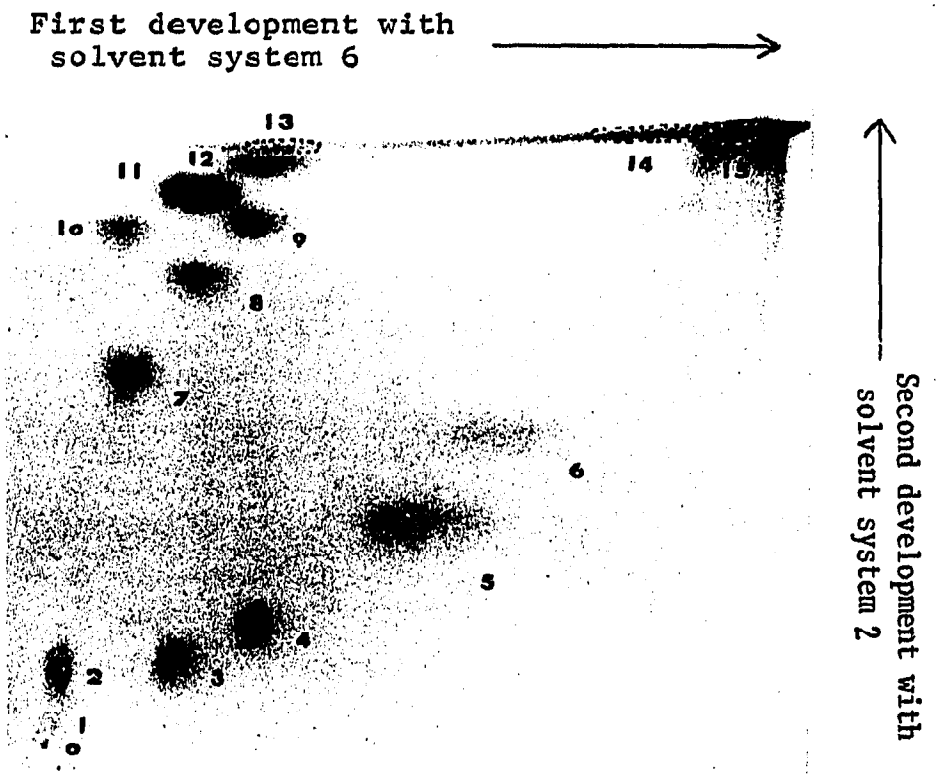

Fig. 1. Two-dimensional thin-layer chromatogram of bile acids and salts (10 μg of each), fatty acids (10 μg of each), phospholipids (10 μg of each) and sterols (10 μg of each) on kieselgel H (type 60). The first development was with solvent system 6 and the second with solvent 2. The following reference compounds were applied to the origin (O): 1, lysolecithin; 2, lecithin; 3, sodium taurolithocholate sulphate; 4, sodium taurocholate; 5, sodium taurochenodeoxycholate and sodium taurodeoxycholate; 6, sodium taurolithocholate; 7, sodium glycocholate; 8, sodium glycochenodeoxycholate and sodium glycodeoxycholate; 9, glycolithocholic acid; 10, cholic acid; 11, chenodeoxycholic acid and deoxycholic acid; 12, lithocholic acid; 13, palmitic and stearic acids; 14, cholesterol; 15, cholesteryl palmitate and cholesteryl stearate. The areas revealed with Rhodamine 6GO spray are outlined by dashed circles.

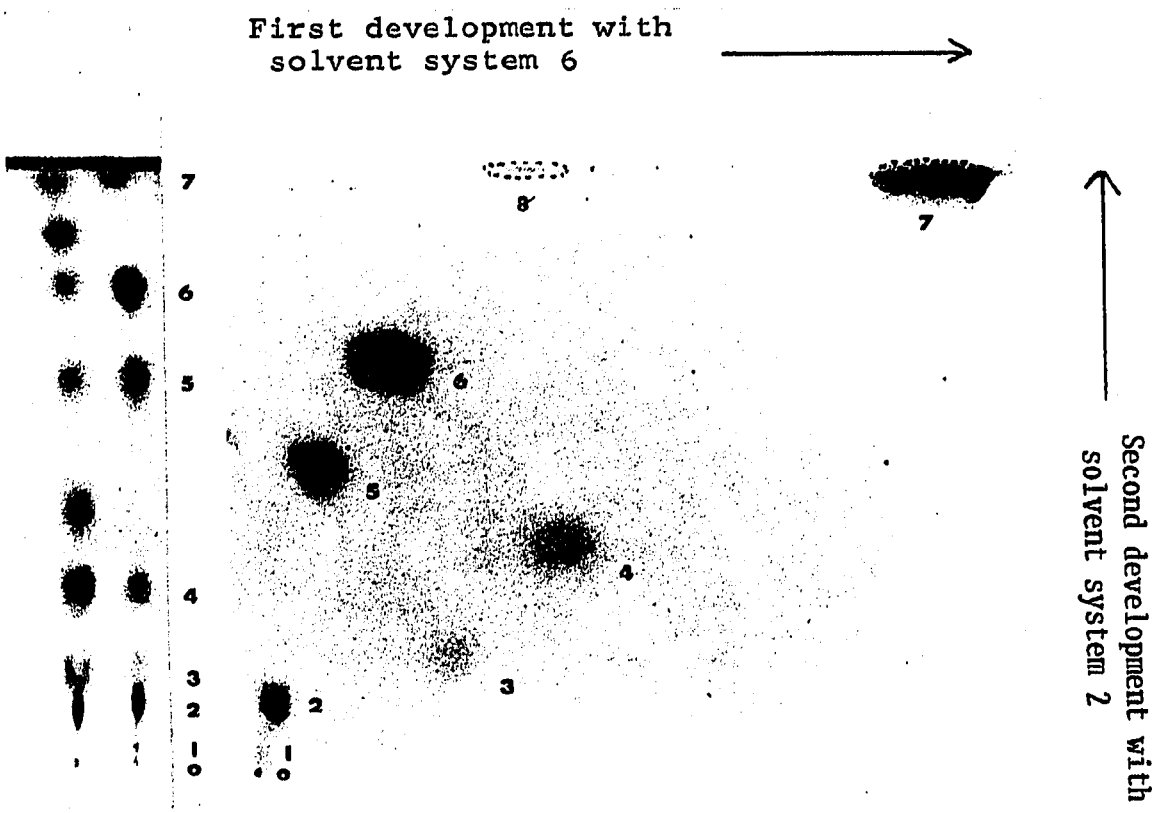

Fig. 2. Two-dimensional thin-layer chromatogram of an extract of human gall-bladder bile (150 μg) made according to the method of Haslewood[24] (on the right), and a one-dimensional thin-layer chromatogram of the same sample (50 μg) in solvent system 2 (on the left). In the left-hand lane of the one-dimensional chromatogram, reference samples (20 μg of each) represent in ascending order, lecithin, taurocholate, taurochenodeoxycholate and taurodeoxycholate, taurolithocholate, glycocholate, glycochenodeoxycholate and glycodeoxycholate, and glycolithocholic acid. Spots on the plates are: O, origin; 1, unidentified compounds; 2, lecithin; 3, taurine-conjugated trihydroxycholanate; 4, taurine-conjugated dihydroxycholanate; 5, glycine-conjugated trihydroxycholanate; 6, glycine-conjugated dihydroxycholanate; 7, cholesterol; 8, fatty acids. The areas revealed with pyrene spray[22] are outlined by dashed circles.

salts, and some fatty acids, phospholipids and sterols, can be simultaneously separated from each other as groups.

An application to extracts of human gall-bladder bile prepared by the method of Haslewood[24] is shown in Fig. 2, together with a one-dimensional chromatogram of the same sample developed with solvent system 2. One-dimensional TLC gives incomplete separation of taurocholate and lecithin in acidic solvent systems (solvent systems 1–4 and No. 2 of Hofmann[2]) and incomplete separation of taurolithocholate sulphate and glycine-conjugated bile acids and salts in neutral and alkaline solvent systems (solvent systems 5 and 6). Two-dimensional TLC overcomes these disadvantages and also permits free bile acids to be separated from a mixture of conjugated mono-, di- and tri-hydroxy bile acids and salts.

If pyrene is used as spray reagent, the positions of the bile acids and phos-

pholipids can be marked, then the pyrene can be removed according to the method of Eastwood and Hamilton[22]; subsequent quantitative analysis is then possible.

For the assay of radioactivity found in the marked areas, it is possible to transfer gel from the areas into counting vials. Similar two-dimensional chromatograms were obtained on kieselgel G plates, which were suitable for autoradiography, since they have abrasion resistance.

The proposed method should be particularly useful in studies on unstable metabolites in bile acid biosynthesis.

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