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Note

Improved two-dimensional solvent system for thin-layer chromatographic analysis of polar lipids on silica gel 60 precoated plates

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A major problem in lipid analysis concerns the reliability and reproducibility of the analytical methods. Several solvent systems for two-dimensional thin-layer chromatography (TLC) of polar lipids have been published (for a review, see ref. 1), but they are generally useful only when the lipid distribution is homogeneous. Whenever one of the lipid components (*e.g.* phosphatidylcholine) reaches a high concentration, there is a loss of resolution and some overlapping occurs with the minor components.

We recently developed a solvent system for two-dimensional TLC separation and quantitation of phospholipids extracted from fish tissues[2], using a first-dimensional solvent derived from that described by Gray[3], and a second-dimensional solvent modified from Rouser's[1]. This solvent system was then applied to the determination of phospholipids and glycolipids of rabies viruses[4]. However, two or three analyses have to be made to give an accurate average value and, if availability of lipid material is limited, additional analyses are difficult to perform.

EXPERIMENTAL

The lipids were extracted and purified from NIL 2 cells in culture as already described[4]. All solvents used were of analytical grade (Merck, Darmstadt, G.F.R.). Silica gel N-HR was purchased from Macherey-Nagel (Duren, G.F.R.) and applied as a slurry in 0.1 % Na_2CO_3 (w/v) on 10 × 20 cm glass plates with a Desaga applicator, to a thickness of 0.5 mm. The plates were dried overnight at 25°, then activated and stored at 110° until use. Silica gel 60, 10 × 20 cm precoated chromatoplates (Merck) were cut into 10 × 10 cm plates and stored in a vacuum desiccator at ambient temperature. For lipid analysis with the silica gel H plates, 3 mg of total lipids (*i.e.* 2 mg of polar lipids) were applied near the corner of the plate, and the development was made in a Desaga chromatotank with the following solvent systems: I, tetrahydrofuran–acetone–methanol–water–ammonia (28 %), 55:25:43:7:1 (by vol.), in the first (10 cm) dimension; II, chloroform–acetone–methanol–glacial acetic acid–water, 50:20:10:

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10:4.5 (by vol) in the second (20 cm) dimension The plate was thoroughly dried for 5 min in a vacuum desiccator after the first development. The thin-layer plate was then dried and spots on the chromatogram were visualized successively with two specific spray reagents: orcinol–H_2SO_4 for detection of glycolipids, 1 min at 180°, then the phosphorus-specific spray reagent of Dittmer and Lester[5]. When the glycolipids were to be detected by a non-destructive method, a primuline solution was first sprayed and the spots marked under UV light[6], before spraying with the phosphorus-specific reagent. The phosphorus-positive spots were scraped off into Pyrex glass test tubes and mineralized with 0.4 ml of H_2SO_4–$HClO_4$ (4:1, v/v) containing 0.1% (w/v) of V_2O_4 as a catalyst. Quantitation was carried out using the procedure of Bartlett[7], simplified as follows: a mixture of aminonaphtholsulphonic acid, sodium metabisulphite, sodium sulphite, and ammonium molybdate, 1:60:2:20 (by weight), was homogenized in a mortar (the mixture is stable for months when kept in a dry and dark place), and an aqueous solution was made with the mixture at a concentration of 0.83 g per 100 ml of twice-distilled water. 10 ml of this solution were added to each mineralized sample and the test tubes were heated at 100° in boiling water for 8 min. After cooling, the blue colouration was read at 830 nm on an SP 800 Unicam spectrophotometer (6 μg phosphorus gave an O.D. of 0.510). The glycolipid-containing spots detected as primuline-reacting and phosphorus-negative, were scraped off and the sphingosine content was assayed with fluorescamine (Hoffman-La Roche, Basel, Switzerland), as described by Naoi *et al.*[8].

The same lipid analysis was performed on silica gel 60 precoated 10×10 cm chromatoplates. Only 0.5–1 mg of total lipids was applied to a plate and development was made in cylindrical one litre glass tanks with the two-dimensional solvent system modified as follows: I, tetrahydrofuran–acetone–methanol–water, 50:20:40:8 (by vol); II, chloroform–acetone–methanol–glacial acetic acid–water, 50:20:10:15:5 (by vol.). The plate was thoroughly dried between the two developments. The visualization procedure was as described above. For mineralization of the phosphorus-positive spots, the acid–vanadium tetroxide mixture was diluted with twice-distilled water (22 ml of the mixture with 18 ml of water) and 0.4 ml of the diluted mixture was used for each sample. Only 5 ml of the phosphorus-reactive solution were added. After heating and cooling, 3 μg of phosphorus gave an O.D. of 0.510. The glycolipidic sphingosine was assayed as described above.

In order to ascertain the purity of each phospholipid and glycolipid assayed, all spots visualized with primuline spray under UV light were scraped off, eluted with chloroform–methanol–water, 10:10:1 (by vol.), and rechromatographed on silica gel 60 precoated 10×20 cm plates in two different monodimensional solvents: chloroform–methanol–glacial acetic acid–water, 60:30:7:3 (by vol.) for phospholipids, and chloroform–methanol–water, 65:30:8 (by vol.), lower phase[9], for glycolipids. The spots were visualized with orcinol–sulphuric acid, then with Dittmer and Lester's spray reagent.

RESULTS

Table I compares the phospholipid and glycolipid distributions of NIL 2 cells determined with the two methods described. Figs. 1 and 2 show the chromatograms obtained on silica gel H and silica gel 60 plates, respectively. Fig. 3 shows the glyco-

TABLE I

PHOSPHOLIPID AND GLYCOLIPID DISTRIBUTION DETERMINED AFTER TLC ON SILICA GELS H AND 60

Lipid fraction	*Silica gel H*	*Silica gel 60*
Phospholipids*		
Phosphatidic acid	0.3	0.3
Diphosphatidylglycerol	1.2	1.0
Phosphatidylglycerol	0.7	0.9
Phosphatidylethanolamine	22.4	22.1
Phosphatidylserine	6.0	5.9
Phosphatidylinositol	6.7	6.9
Lysophosphatidylethanolamine	3.1	2.9
Lysophosphatidylcholine	1.3	1.1
Sphingomyelin	9.2	9.2
Phosphatidylcholine	49.0	49.7
Glycolipids**		
Ceramide monohexoside	4.6	4.2
Ceramide dihexoside	31.2	30.6
Ceramide trihexoside	48.0	48.9
Gangliosides	16.2	16.3

* Expressed as μmoles phosphorus per 100 μmoles of total phosphorus recovered.
** Expressed as nmoles sphingosine per 100 nmoles of total sphingosine assayed in glycolipids.

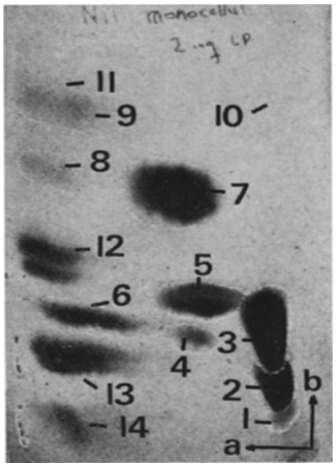

Fig. 1. Two-dimensional TLC on basic silica gel H of polar lipids extracted from NIL 2 cells. 1 = Lysophosphatidylcholine; 2 = sphingomyelin; 3 = phosphatidylcholine; 4 = lysophosphatidylethanolamine; 5 = phosphatidylserine; 6 = phosphatidylinositol; 7 = phosphatidylethanolamine; 8 = phosphatidylglycerol; 9 = diphosphatidylglycerol; 10 = phosphatidic acid; 11 = ceramide monohexoside; 12 = ceramide dihexoside; 13 = ceramide trihexoside; 14 = gangliosides. Visualization: orcinol–H_2SO_4, then reagent of Dittmer and Lester. Solvent a: tetrahydrofuran–acetone–methanol–water–ammonia (28%), 55:25:43:7:1 (by vol.). Solvent b: choroform–acetone–methanol–glacial acetic acid–water, 50:20:10:10:4.5 (by vol.).

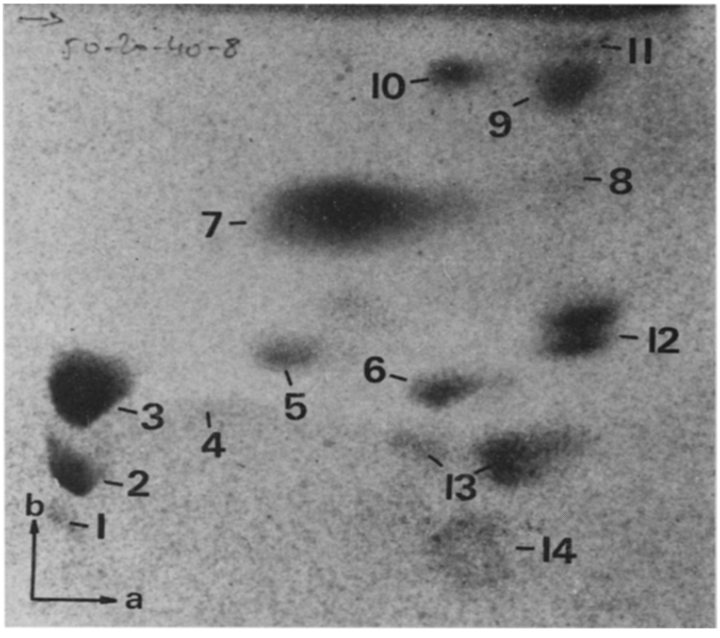

Fig. 2. Two-dimensional TLC on silica gel 60 precoated plate of total lipids extracted from NIL 2 cells. Nomenclature and visualization as in Fig. 1. Solvent a: tetrahydrofuran–acetone–methanol–water, 50:20:40:8 (by vol.), Solvent b: chloroform–acetone–methanol–glacial acetic acid–water, 50:20:10:15:5 (by vol.).

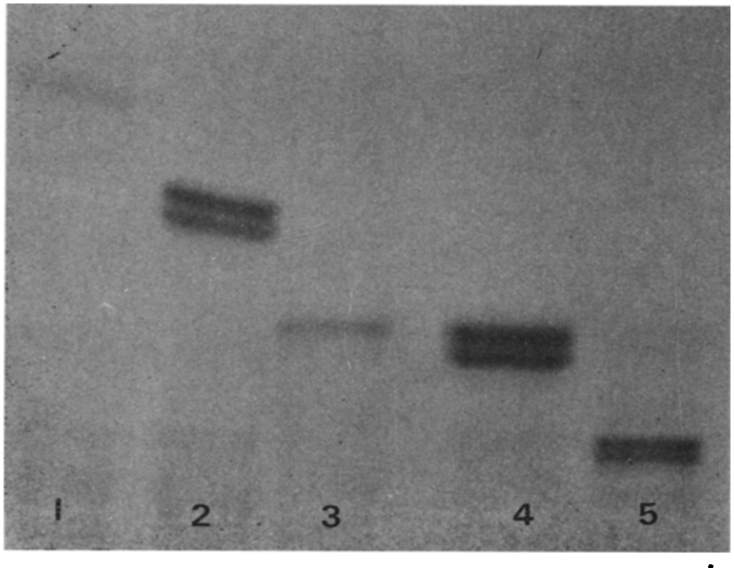

Fig. 3. TLC on silica gel 60 precoated plate of glycolipids eluted from chromatogram 2 (see Experimental procedures). 1 = Ceramide monohexoside; 2 = ceramide dihexoside; 3 and 4 = ceramide trihexoside; 5 = ganglioside. Visualization: orcinol–H_2SO_4. Solvent: chloroform–methanol–water, 65:30:8 (by vol.), lower phase.

lipids eluted from silica gel 60 precoated plates after two-dimensional development and rechromatography.

Table I reveals no significant difference in the lipid repartition between the two methods. A major problem concerned the visualization on 0.5 mm thick silica gel H plates. Minor spots containing less than 0.5 μg phosphorus or less than 1 μg carbohydrates were difficult to detect, because the spray reagents reacted only with the lipid compounds at the surface of the silica gel layer. Whenever the bluish background colouration hampered the phospholipid detection with the Dittmer and Lester's reagent, we were able to remove this background colouration and obtain a sharp contrast by spraying some distilled water on the plate. On 10×10 cm silica gel 60 precoated thin-layer plates, the spray reagents penetrated the whole silica gel layer and the detection limit was lowered about four-fold. Furthermore, the silica gel blank value for phosphorus was negligible, whereas the blank value for fluorescence assay on sphingosine was markedly increased. Development time was reduced to 10 min for each direction with these small plates.

Ammonia was omitted from the first developing solvent used with silica gel 60 precoated plates in order to increase the migration of phosphatidylserine and phosphatidylinositol[1] and to prevent the tailing of glycolipids. The relative humidity of precoated plates was found to be an important factor: the amount of water had to be reduced from 8 to 6 volumes in the tetrahydrofuran-containing solvent when the plates were not kept in a vacuum desiccator until use.

No modification of the migration rates of the polar lipids was observed when the neutral lipid content represented up to 85% of the total lipids. However, the neutral lipids were found to migrate in diethyl ether prior to the two-dimensional development of the polar lipids.

Fig. 3 shows that rechromatography of the glycolipids eluted from the chromatogram shown in Fig. 2 confirmed the purity of each component. Ceramide trihexoside was separated into two spots on Fig. 2 and recovered at the same R_F on Fig. 3. A similar phenomenon was observed with phosphatidylinositol contained in the lipids extracted from fish tissues (Zwingelstein and Meister, unpublished observation). Moreover, when a ganglioside fraction with higher molecular weight than hematoside (sialyldihexosylceramide) was present, a gross separation was obtained.

DISCUSSION

The main difference between the two developing solvent systems concerned the loading of the thin-layer plates. With 0.5 mm thick silica gel H on 10×20 cm plates, a preparative separation of polar lipids might be performed on 4 mg of lipid material. The absence of organic binder in silica gel H makes it more suitable for preparative purposes, and the removal of contaminants from the gel might be achieved by the procedure described by Carreau *et al.*[9], thus allowing the addition of formic acid to the elution mixture in order to increase the lipid recovery[10].

Silica gel 60 precoated 10×10 cm plates are preferable for analytical purposes. Because of the standardization of these plates, this procedure was found to be more reliable and reproducible. In fact, the solvent system was even applicable to 5×5 cm precoated plates, with a developing time reduced to 5 min in each direction. The tetrahydrofuran-containing solvent was more stable when acetone was added instead

of methylal, which was previously used by Gray[3] and replaced by butan-2-one, as described by Kijimoto and Hakomori[11].

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