Unidimensional thin-layer chromatography of phospholipids on boric acid-impregnated plates

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Summary A unidimensional thin-layer chromatography system for the separation of phospholipids has been developed. The method employs precoated plates impregnated with boric acid and is capable of resolving eight phospholipid standards, including phosphatidylinositol, phosphatidylserine, and phosphatidylglycerol. Liver, kidney, and platelet phospholipids of the rat were resolved into six major classes using this method. Each was judged to be pure by two-dimensional thin-layer chromatography. The use of boric acid thin-layer chromatography for the separation of phospholipids should find wide application.—**Fine, J. B., and H. Sprecher.** Unidimensional thin-layer chromatography of phospholipids on boric acid-impregnated plates. *J. Lipid Res.* 1982. **23:** 660–663.

Supplementary key words adsorbent modifier • rat kidney phospholipids

One-dimensional thin-layer chromatography is simpler and more rapid than two-dimensional TLC and has the advantage of being adaptable to a preparative scale. Over the last few years, a number of one-dimensional TLC systems have been developed for the separation of phospholipids. Some of these employ novel, five-component solvent systems (1, 2) whereas others utilize multiple solvent development (3) or short-bed continuous development (4). Another TLC method, although less conventional, uses silica-coated quartz rods to separate phospholipids (5).

The first use of boric acid in lipid analysis was in the separation of *threo* and *erythro* isomers of vicinal dihydroxy fatty acids (6). Following this, Horrocks (7) demonstrated an improved separation of certain brain phospholipids, using silica gel G impregnated with sodium borate. Bunn, Keele, and Elkan (8) found a similar improvement in the resolution of phospholipids with both one- and two-dimensional TLC systems, using silica gel G buffered with borate buffer pH 8.0.

We present here a simple unidimensional TLC system for the separation of eight phospholipid classes including PI, PS, and PG. The system utilizes precoated plates impregnated with boric acid. We have, to date, successfully separated liver, kidney, and platelet phospholipids from the rat.

Abbreviations: CL, cardiolipin; LPC, lysophosphatidylcholine; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PI, phosphatidylinositol; PS, phosphatidylserine; SPH, sphingomyelin; TLC, thin-layer chromatography.

MATERIALS AND METHODS

Phospholipid standards were obtained from Avanti Biochemicals, Inc. (Birmingham, AL), except for PG which was obtained from Serdary Research Laboratories, Inc. (London, Ontario). All organic solvents were reagent grade and used as obtained from the supplier. The chloroform contained 0.75% ethanol as a stabilizer. Silicic acid, "Unisil", was purchased from the Clarkson Chemical Co., (Williamsport, PA). Whatman precoated LK5 and LK5D TLC plates with a preadsorbent area (Whatman Inc., Clifton, NJ) were used for one-dimensional TLC, while silica gel H plates with 7.5% magnesium acetate (Analtech Inc., Newark, DE) were used for two-dimensional TLC. All plates were 250 μm thick. One-μl micropipettes, "Microcaps", were obtained from the Drummond Scientific Company (Broomall, PA) and 2′,7′-dichlorofluorescein was from Eastman Organic Chemicals (Rochester, NY).

Lipid extraction and isolation

Liver, kidney or platelet lipids were extracted according to the method of Folch, Lees, and Sloane Stanley (9), and phase partitioned with 0.88% KCl. A phospholipid fraction, free of neutral lipids and glycolipids, was prepared for TLC by fractionation of the total lipid extract on a silicic acid column. Neutral lipids and glycolipids were eluted with chloroform and acetone, respectively. The phospholipids were eluted with methanol. Platelet phospholipids were separated by TLC without prior removal of the neutral lipids and glycolipids.

Boric acid TLC

All Whatman plates were predeveloped with chloroform–methanol 1:1 (v/v) and air-dried overnight, prior to use. The plates were then impregnated with an adsorbent modifier by dipping them upside-down into a solution of 1.2% boric acid in absolute ethanol–water 1:1 (v/v). The preadsorbent area was not immersed in the solution. After air drying for 15 min, the plates were activated at 100°C for 60 min. Samples were either spotted or streaked on the preadsorbent area, then developed in chloroform–methanol–water–ammonium hydroxide 120:75:6:2 (v/v), in paper-lined tanks, previously equilibrated for 60 min. The mobile phase was allowed to ascend to the top of the plate. Lipids were routinely visualized with I_2 vapor.

When additional analysis was required, lipids were visualized with 0.1% 2′,7′-dichlorofluorescein and the

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appropriate silica gel bands were scraped from the plate. Phospholipids were eluted from the adsorbent with 5.0 ml of chloroform–methanol–water 5:5:1 (v/v). Following centrifugation, the silica gel was re-extracted in an identical manner. The 10 ml of extract was washed by the addition of 4.5 ml of chloroform and 2.0 ml of 0.05 M Tris buffer, pH 9.0 (10), and the upper dichlorofluorescein-containing phase was removed. After evaporation of the lower phase, the lipids were dissolved in 5.0 ml of chloroform–methanol 2:1 (v/v) and partitioned with 1.0 ml of water to remove any remaining Tris buffer. The lower phase was evaporated under reduced pressure and the lipids were redissolved in chloroform–methanol.

Two-dimensional TLC

To verify the purity of the phospholipids separated on a boric acid-impregnated plate, the phospholipids were rechromatographed in the two-dimensional system of Rouser, Fleischer, and Yamamoto (11) with slight modifications. Plates were "activated" for 3 hr in a chamber with nitrogen of 55% relative humidity. Lipid samples were spotted directly in this controlled humidity chamber. In between the first and second dimensions, plates were dried in a nitrogen atmosphere for 30 min. Phospholipids were visualized with I_2 vapor.

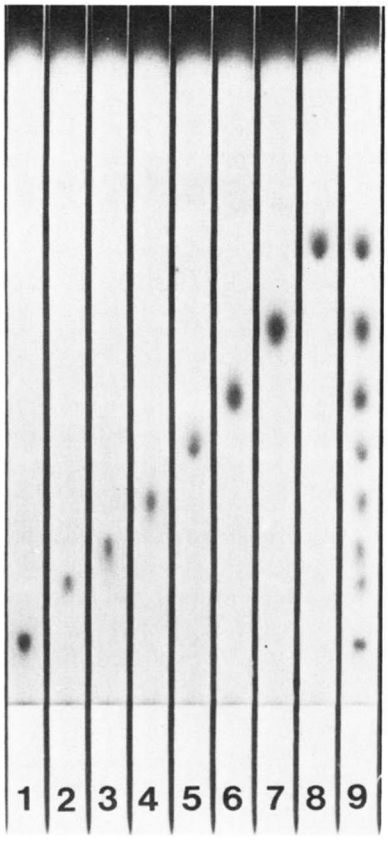

Fig. 1. One-dimensional TLC of phospholipid standards on boric acid-impregnated plates. One μl of a 0.3% solution of each standard in chloroform–methanol 2:1 (v/v) was applied to the preadsorbent area of the plate with a Drummond Microcap. 1, PI; 2, LPC; 3, PS; 4, SPH; 5, PG; 6, PC; 7, PE; 8, CL; 9, a mixture of 1–8.

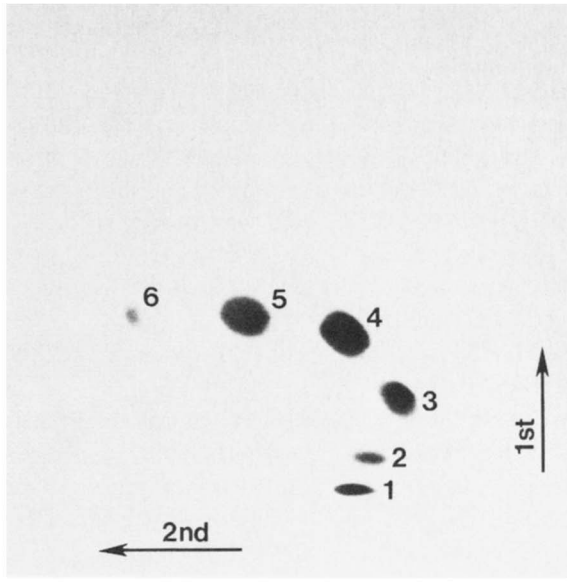

Fig. 2. Two-dimensional TLC of total kidney phospholipids. 350 μg of phospholipid was applied at the lower right corner of the plate. The solvent systems used were chloroform–methanol–28% aqueous ammonia 65:25:5 (v/v) for the first dimension, and chloroform–acetone–methanol–acetic acid–water 3:4:1:1:0.5 (v/v) for the second dimension. 1, PS; 2, PI; 3, SPH; 4, PC; 5, PE; 6, CL.

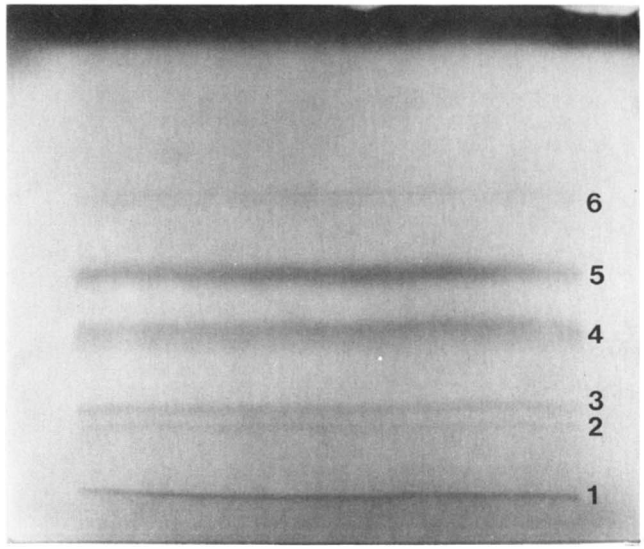

Fig. 3. Boric acid TLC of total kidney phospholipids. 350 μg of phospholipid in chloroform–methanol 2:1 (v/v) was streaked on the preadsorbent area of the plate. 1, PI; 2, PS; 3, SPH; 4, PC; 5, PE; 6, CL.

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RESULTS AND DISCUSSION

Eight phospholipid standards were resolved using boric acid-impregnated plates (**Fig. 1**). It was found that plates impregnated with sodium borate were not as effective in resolving these compounds. The chromatographic behavior of PI and PG appeared to be due to the glycol-complexing effect of boric acid, since increasing concentrations of boric acid produced lower R_f values for both phospholipids.

Fig. 2 and **Fig. 3** compare the resolution of total kidney phospholipids in a two-dimensional system with that obtained by boric acid TLC. When 350 μg of kidney phospholipid was chromatographed on a boric acid-impregnated plate, there was good resolution of six major phospholipids. Minor phospholipids were not detected even when plates were charred with a sensitive copper acetate reagent (12). Each of the six kidney phospholipids purified by boric acid TLC migrated as a single compound when analyzed by two-dimensional TLC (**Fig. 4**). Comparable results were obtained with liver and platelet phospholipids. In the latter case, neutral and glycolipids, which were not removed prior to boric acid TLC, did not interfere with the phospholipid separation. More importantly, neutral lipids, including free fatty acids, have an R_f substantially greater than the farthest migrating class of phospholipid, i.e., CL. Glycolipids, however, comigrate with PI as was determined by a cerebroside standard.

The resolution of kidney phospholipids posed a unique problem due to the high concentration of sphingomyelin found in this tissue (13). This, coupled with the relatively close migration of SPH and PS in our system, caused these two compounds to comigrate when more than 350 μg of kidney phospholipid was applied to the plate. This could be overcome by increasing the concentration of boric acid, although this caused the LPC and PS standards, as well as the PG and SPH standards, to comigrate. With liver and platelet phospholipids, the resolution of SPH and PS was not as sensitive to sample size.

To date, the use of boric acid TLC in lipid analysis has been restricted primarily to the resolution of isomeric mono- and diglycerides (14), isomeric polyhydroxy fatty acids (15), and ceramides (16). The chromatographic system described here extends the use of boric acid TLC to phospholipids, where it should find wide application. It is rapid and reproducible, and resolves all the major phospholipids. The ability to resolve PI and PS simply

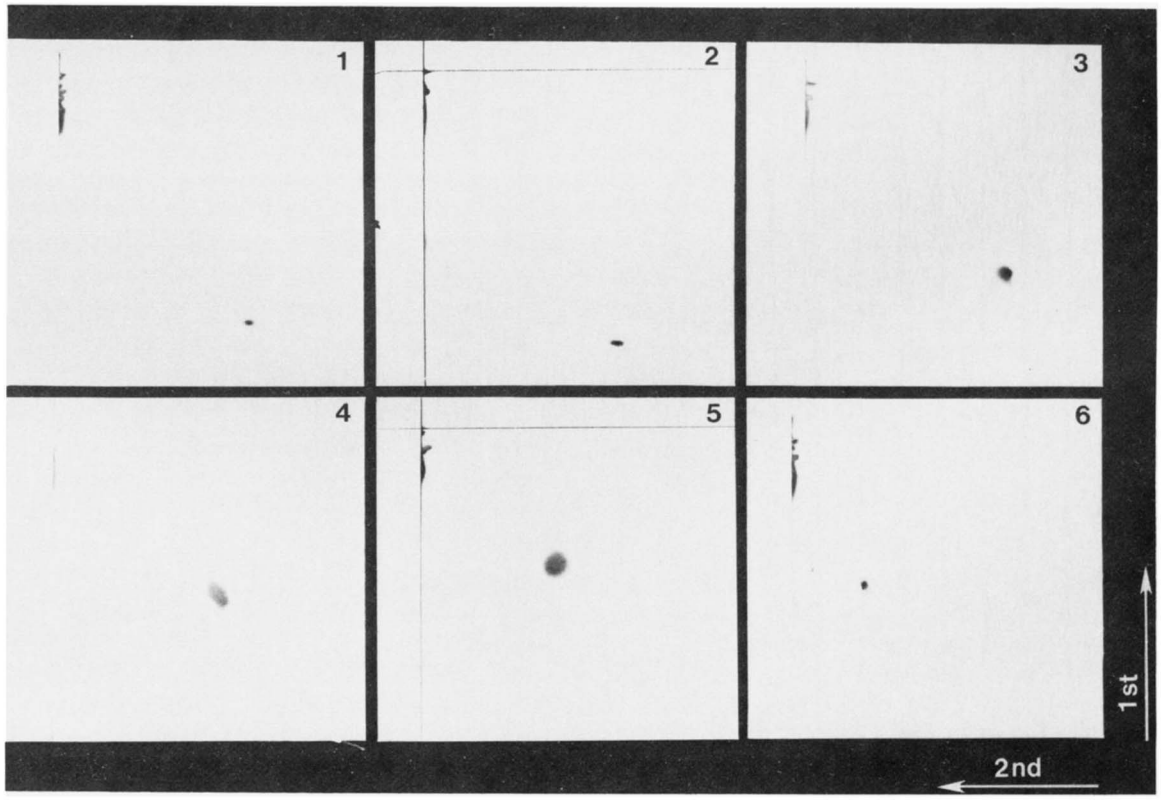

Fig. 4. Two-dimensional TLC of phospholipids purified by boric acid TLC. Kidney phospholipids resolved by boric acid TLC (Fig. 3), were eluted from the adsorbent, and rechromatographed two-dimensionally according to Rouser et al. (11). 1, PI; 2, PS; 3, SPH; 4, PC; 5, PE; 6, CL.

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and quickly should be particularly useful in studies of PI metabolism. We have not, however, ascertained where phosphatidic acid and polyphosphoinositides migrate relative to PI. Phosphatidic acid is normally present only in very small quantities in animal tissues and could not be detected due to the low level of phospholipid required for TLC. Polyphosphoinositides, on the other hand, are not believed to be present in our samples since they cannot be extracted from tissues unless acidic conditions are used.

While we have not worked with any tissues containing significant amounts of PG, the discrete migration of this compound in our system (Fig. 1) suggests that the technique may be useful to those requiring the resolution of PG in amniotic fluid for the assessment of fetal lung maturity (17, 18).

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