Two Dimensional Thin Layer Chromatographic Separation of Polar Lipids and Determination of Phospholipids by Phosphorus Analysis of Spots

ABSTRACT

Separation of polar lipids by two-dimensional thin layer chromatography providing resolution of all the lipid classes commonly encountered in animal cells and a sensitive, rapid, reproducible procedure for determination of phospholipids by phosphorus analysis of spots are described. Values obtained for brain and mitochondrial inner membrane phospholipids are presented.

Two-dimensional thin layer chromatography (TLC) has been shown to provide good resolution of polar lipids and determination of phosphorus in spots by aspiration and color development without prior elution from TLC adsorbent was found to be a rapid and accurate procedure for determination of molar amounts of phospholipids (1). This report presents a modified TLC procedure providing better resolution of polar lipids, a more accurate and sensitive procedure for determination of phosphorus in TLC spots, and typical analytical data for phospholipids of adult human brain and beef kidney mitochondrial inner membrane.

Silica Gel H (Merck) as a slurry of 20 g in 65 ml of water containing either 0.50 or 1.50 g of magnesium acetate was spread with a 0.25 mm fixed distance spreader and the plates air-dried. Just before use, the plates were placed in a TLC chamber equipped with a cardboard top through which nitrogen of 50% humidity was passed for 20 min. The plates were then transferred for sample application to another humidity controlled chamber constructed of clear plastic supported by a metal rod frame. The desired humidity was obtained by mixing the required amount of dry nitrogen with nitrogen saturated with water by bubbling through a water tower heated electrically at the base.

Immediately after spotting, plates were transferred to TLC chambers (11 3/4 in. long by 2 3/4 in wide by 10 1/2 in. high) lined on all sides with paper saturated with the chromatographic solvent (about 200 ml added per chamber) by tilting the chamber first to one side and then the other about 30 min before use. Plates spread with 1.50 g of magnesium acetate were developed in the first dimension with chloroform-methanol-28% aqueous ammonia 65:25:5, chromatograms were dried for about 10 min in a TLC chamber flushed with nitrogen and then developed in the second dimension with chloroform-acetone-methanol-acetic acid-water 3:4:1:1:0.5. Plates spread with 0.50 g magnesium acetate were developed with the same solvent mixtures in the proportions 65:35:5 and 5:2:1:1:0.5. Plates were air-dried for a few minutes, sprayed with a char reagent composed of 3 vol of 37% formaldehyde and 97 vol of 98% sulfuric acid and heated at 180 C for 30 min. Spots were circled and numbered and each chromatogram photographed.

Spots were aspirated as described previously (1) except that ignition tubes (16 mm o.d. x 125 mm) were substituted for Kjeldahl flasks, the paper filters were discarded in favor of small circles of glass fiber paper (No. 994, H. Reeve Angel Co., Clifton, N.J.) first washed with freshly distilled constant-boiling hydro-

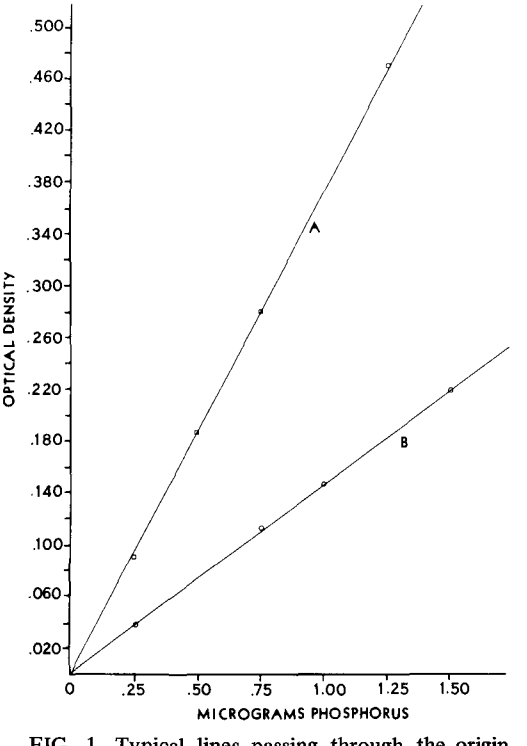

FIG. 1. Typical lines passing through the origin obtained with inorganic phosphate standards by the procedure described in the text for the smaller (A) and larger (B) reagent volumes.

TABLE I

Values Obtained in Quadruplicate Determinations of Different Amounts of Phosphorus

	O.D.[a]	Blank[b]	Corrected O.D.	Ave. μg P[c]	Per cent total P	Std. Dev.[d]
Phosphatidyl ethanolamine	0.588	0.006	0.582	3.751	31.26	±0.47
	0.581	0.006	0.575			
	0.568	0.006	0.562			
	0.572	0.006	0.568			
Phosphatidyl inositol	0.151	0.013	0.138	0.325	2.08	±0.13
	0.141	0.013	0.128			
	0.143	0.013	0.130			
	0.136	0.013	0.123			
Lysophosphatidyl choline	0.035	0.013	0.022	0.044	0.34	±0.05
	0.033	0.013	0.020			
	0.030	0.013	0.017			
	0.031	0.013	0.018			

[a]Optical density values for three brain lipids.

[b]The total blank including TLC adsorbent. Reagent blanks (excluding adsorbent) are very low or zero with carefully acid-washed glassware and the purest reagents. Glass fiber paper washed with hydrochloric acid and the formaldehyde-sulfuric acid spray do not contribute to blank values.

[c]The lower limit for positive detection of phosphorus is about 0.003 μg.

[d]Maximum reproducibility is obtained when: (a) the amount of TLC adsorbent in each tube is kept constant, the desired total amount being obtained by aspiration of additional adsorbent from a blank plate for spots moving close together; (b) variable loss of perchloric acid is prevented by neutralization with hydrochloric acid of silicate in the adsorbent and avoidance of loss of fumes during digestion; (c) before aspiration of small spots the aspirator is carefully cleaned by aspiration from a blank plate of adsorbent which is then discarded; (d) spurious color production after addition of ascorbic acid is prevented by immediate and thorough mixing; (e) accidental inclusion of sedimented TLC adsorbent is prevented by centrifugation in relatively small bore tubes; and (f) OD readings are reproduced to ± 0.001 units.

chloric acid and then water, dried and cut to uniform size with a paper punch. Water (0.5 ml) was added to each tube to serve as a liquid trap for the adsorbent. The glass fiber filter was held in place by suction during aspiration and allowed to fall into the test tube by release of vacuum after aspiration of a spot was complete. Large spots (total area 4.5 cm^2) were aspirated first and the aspirator carefully cleaned by aspirating adsorbent from a blank plate before aspiration of small spots (total area 2.25 cm^2). After aspiration, 0.5 ml of concentrated hydrochloric acid was added to each tube for neutralization of silicate, and water and excess acid removed by placing the tubes for 10 min in an electrically heated metal block (heating base No. 2127-A, Model 100-300 C; tube blocks No. 2127-B-2; Hallikainen Instruments, Richmond, Calif.) maintained at 180 C.

The procedure for large spots (4.5 cm^2) was then as follows. Perchloric acid (0.65 ml, 70% triple distilled into Vycor, G. Frederick Smith Chemical Co., Columbus, Ohio) was added and the lipid digested by heating for 20 min at 180 C in the heated metal block with the upper one half of each tube extending outside of the block to prevent loss of perchloric acid fumes. After cooling, reagents added in order were: water (3.30 ml), 2.5% ammonium molybdate (0.50 ml), and 10% ascorbic acid solution (0.50 ml). The additions were made rapidly and accurately with RePipettes (Lab Industries, Berkeley, Calif.) and the tube contents were mixed after each addition with a vibrator mixer (De Luxe Mixer, S8220, Scientific Products, Evanston, Ill.). Small spots (2.25 cm^2) were treated similarly except that 0.26 ml of perchloric acid was used for digestion and 0.92 ml of water, 0.40 ml of 1.25% ammonium molybdate, and 0.40 ml of 5% ascorbic acid solution were added for color development.

Color was developed by heating for 5 min in a boiling water bath (Renwall electric water bath, No. 3025, Scientific Products, Evanston, Ill.). Adsorbent was sedimented by brief centrifugation, the solutions from the smaller reagent volumes first being transferred to 3 ml centrifuge tubes. After centrifugation, the solutions were transferred to cuvettes with Pasteur pipettes with care not to disturb the sedimented adsorbent, and the color intensity was determined at 797 mμ in a Gilford Model 240 spectrophotometer equipped with a digital readout. Both reagent blanks and reagent-TLC adsorbent blanks were carried through the procedure. The optical density values were read against a water blank and the reagent-TLC adsorbent blank was subtracted to give the cor-

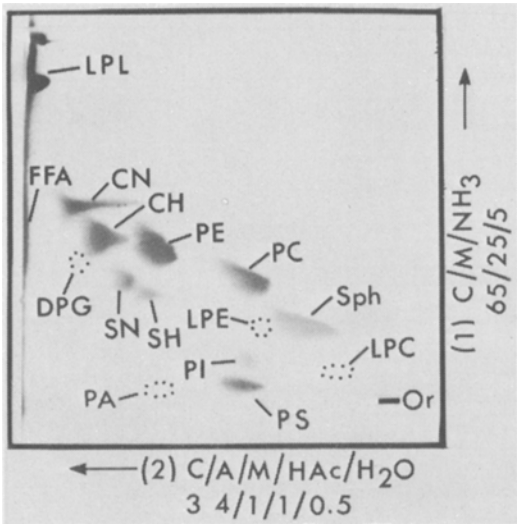

FIG. 2. Two-dimensional TLC of normal human (23 year old male) whole brain lipids. Lipid (800 μg) was applied at the lower right (see text for further details). Abbreviations and percentage of the total phosphorus ± standard deviation: LPL, less polar lipid (cholesterol, triglyceride, etc.); CN and CH, cerebroside with normal and hydroxy fatty acids; SN and SH, sulfatide with normal and hydroxy fatty acids; PE, phosphatidyl ethanolamine (31.3 ± 0.5); PC, phosphatidyl choline (29.2 ± 0.5); PS, phosphatidyl serine (15.4 ± 0.5); PI, phosphatidyl inositol (2.1 ± 0.1); LPE, lysophosphatidyl ethanolamine (1.14 ± 0.06); PA, phosphatidic acid (0.50 ± 0.02); Sph, sphingomyelin (13.2 ± 0.1); DPG, diphosphatidyl glycerol (0.9 ± 0.07). Total phosphorus recovery 97.5% (including areas devoid of spots but containing phosphorus).

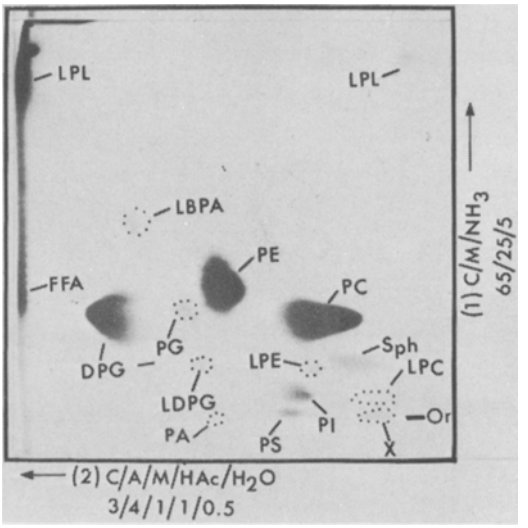

FIG. 3. Beef kidney mitochondrial inner membrane lipid class separation as described in the text. Abbreviations (as for Fig. 2 except as noted) and percentage of the total phosphorus ± standard deviation: PE (35.7 ± 0.3); PC (35.0 ± 0.4); PS (1.36 ± 0.3); PI (3.40 ± 0.07); LPE (0.98 ± 0.12); PA (0.22 ± 0.04); Sph (2.43 ± 0.02); DPG (15.8 ± 0.06); LPC, lysophosphatidyl choline (0.8 ± 0.07); PG, phosphatidyl glycerol (1.17 ± 0.04); LDPG, lysodiphosphatidyl glycerol (0.71 ± 0.05); LBPA, lysobisphosphatidic acid (0.58 ± 0.02). Total phosphorus recovery 99.4%. Note that some of the less polar lipid (LPL) is insoluble in the solvent mixture used in the second dimension and does not migrate.

rected optical densities. The latter were converted to micrograms of phosphorus by multiplication by a factor which was determined from known amounts of disodium hydrogen phosphate spotted onto TLC plates and, except for chromatography, treated in the same manner as the sample spots. Phosphorus recovery was determined by analysis of eight separate 100-200 μg applications of the lipid mixture to a TLC plate that was not developed with solvent but which was otherwise treated in the same manner as the phosphate standards and TLC spots.

Typical results with inorganic phosphate standards are shown in Figure 1 and typical values obtained for various amounts of phosphorus are shown in Table I. The improved TLC separation of polar lipids and the reproducibility of quadruplicate determinations of phospholipids are shown for brain (Fig. 2) and a beef kidney mitochondrial inner membrane preparation (Fig. 3). In both cases, complete resolution of all detectable components was obtained. This was judged by TLC of diethylaminoethyl cellulose column fractions which also disclosed the presence of trace components below the level of detectability by two-dimensional TLC of the total lipid mixture.

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