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Separation of phosphatidyl ethanolamine, phosphatidyl serine, and other phospholipids by thin-layer chromatography*

VLADIMIR P. SKIPSKI, ROBERT F. PETERSON, and MARION BARCLAY

Division of Experimental Chemotherapy, Sloan-Kettering Institute for Cancer Research, Rye, New York

and

Sloan-Kettering Division, Graduate School of Medical Sciences, Cornell University Medical College, New York, N. Y.

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SUMMARY

Thin-layer chromatography of phospholipids and cerebrosides was performed on Silica Gel G plates using a mixture of chloroform–methanol–acetic acid–water as development solvent. Two types of chromatoplates were used: "neutral" plates, prepared from Silica Gel G slurry made in water, and "basic" plates, prepared from Silica Gel G slurry made in 0.01 M sodium acetate or sodium carbonate solutions. Only chromatograms run on "basic" plates showed good and reproducible separations of phosphatidyl serine from other phospholipids, independent of the amount of phosphatidyl serine present in the sample. However, "neutral" plates gave better separation of cerebrosides from phospholipids. A practical method of applying these systems for separation of phospholipids extracted from rat liver and human serum is presented.

Thin-layer chromatography, introduced recently into lipid research, has proven to be the quickest and simplest procedure for identification of neutral lipids (1–6). Several investigators have applied this technique for separation of various phospholipids and glycolipids (6–15). However, none has reported successful separation of phosphatidyl serine by thin-layer chromatography. This paper describes a procedure for the separation of phosphatidyl serine along with other phospholipids by applying a sample on a "basic" Silica Gel G plate using as developing solvent an acidified mixture of chloroform, methanol, and water. A procedure for separation of phospholipids from rat liver and human serum by application of this system is presented.

EXPERIMENTAL METHODS

Materials. The following were used as standard compounds: phosphatidyl serine isolated from beef brains (generous gift from Dr. Donald G. Therriault, U. S. Army Medical Research Laboratory, Fort Knox, Kentucky); synthetic dipalmitoyl-L-α-glycerylphosphorylethanolamine (Sigma Chemical Company, St. Louis, Mo.), purified by silicic acid column chromatography; egg phosphatidyl ethanolamine and vegetable lysolecithin (generous gifts from Dr. Maurice M. Rapport, Albert Einstein College of Medicine, Yeshiva University, New York, N.Y.); synthetic dimyristoyl-L-α-glycerophosphorylethanolamine and dimyristoyl-L-α-lecithin (generous gifts from Dr. Erich Baer, University of Toronto, Canada); synthetic β-palmitoyl-

* This manuscript was completed before the abstract of work by W. D. Skidmore and C. Entenman (*Federation Proc.* **22**: 292, 1962) was published. The authors described separation of phosphatidyl serine and other phospholipids by two-dimensional thin-layer chromatography.

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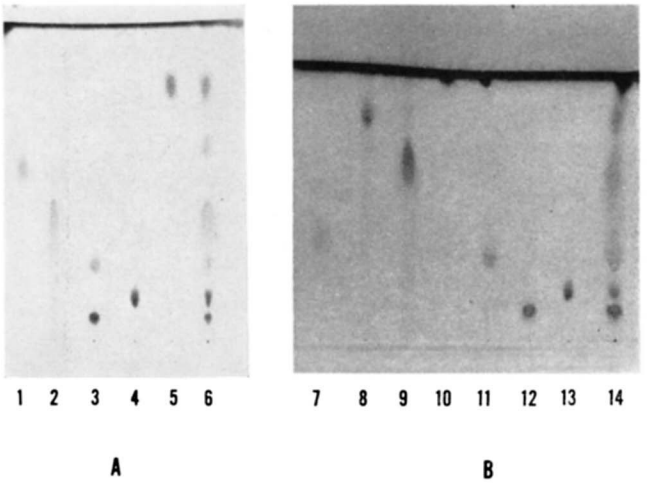

FIG. 1. Thin-layer chromatogram of different reference phospholipids and cerebrosides. Detection method: ammonium molybdate–perchloric acid spray (for details see text). (A) "Neutral" plate. Developing solvent: chloroform–methanol–glacial acetic acid–water 65:25:8:4 (v/v). Compounds: (1) synthetic dipalmitoyl-L-α-glycerylphosphorylethanolamine, 40 μg; (2) phosphatidyl serine, 25 μg (faint lower spot is apparently lysophosphatidyl serine); (3) synthetic dipalmitoyl-L-α-lecithin, 25 μg (upper spot), and lysolecithin, 25 μg (lower spot); (4) sphingomyelin, 43 μg; (5) phrenosine, 25 μg; (6) mixture of 1–5 in same amounts. (B) "Basic" plate. Developing solvent: chloroform–methanol–glacial acetic acid–water 50:25:8:4 (v/v). Compounds: (7) synthetic β-palmitoyllysophosphatidylethanolamine, 20 μg; (8) phosphatidyl ethanolamine, 25 μg; (9) phosphatidyl serine, 20 μg (lower faint spot is apparently lysophosphatidyl serine); (10) phrenosine, 25 μg; (11) lecithin, 35 μg (middle spot), cardiolipin, 30 μg (spot at front), spot at bottom represents lysolecithin impurities; (12) lysolecithin, 25 μg; (13) sphingomyelin, 43 μg; (14) mixture of 7–13 in same amounts.

lysophosphatidylethanolamine and α-myristoyllysophosphatidylethanolamine (generous gifts from Dr. T. H. Bevan, University of Bristol, Great Britain); synthetic dipalmitoyl-L-α-lecithin, "chromatographically pure," (Mann Research Laboratories, New York City); beef heart lecithin, sphingomyelin, and cardiolipin, (Sylvana Chemical Company, Orange, N.J.); synthetic stearoyl sphingomyelin and palmitoyl sphingomyelin (generous gifts from Dr. H. Flowers, Weizmann Institute of Sciences, Israel); phrenosine, isolated from bovine spinal cord (16); natural kerasin (Delta Chemical Works, New York City), purified by solvent fractionation. The following isolated lipid mixtures were studied: total lipids extracted from rat liver with methanol–chloroform 1:2, followed by Folch washing; phospholipids from the same total lipid extract further separated on silicic acid column into neutral lipid and phospholipid fractions; and total lipids extracted from human serum by the same procedure.

Methanol, absolute, Merck reagent, was redistilled under anhydrous conditions; chloroform, Merck reagent, was washed with water, dried with anhydrous calcium chloride, and redistilled, adding 1% absolute methanol as a preservative; acetic acid, glacial, Merck reagent, was used without redistillation.

Silica Gel G for thin-layer chromatography (prepared according to Stahl, E. Merck, AG, Darmstadt, Germany) was used for preparation of chromatoplates, on 200 x 200 mm carrier slides. The nonadjustable Desaga applicator was used to apply the layer of Silica Gel G to about 250 μ thickness. All thin-layer chromatograms were run in rectangular chambers of the following dimensions: length 29 cm, height 27.5 cm, and width 10 cm.

Preparation of Chromatoplates. Two types of Silica Gel G plates were used: "neutral" and "basic." The "neutral" plates were prepared by making a slurry of 30 g of Silica Gel G with 65 ml distilled water and applying it as described by Stahl (17). Plates were allowed to dry on the template at room temperature for 20–25 min and stored in a cabinet (without drying agent). Just prior to application of samples, chromatoplates were activated at 80° for 45-60 min. When separation of phosphatidyl serine from other phos-

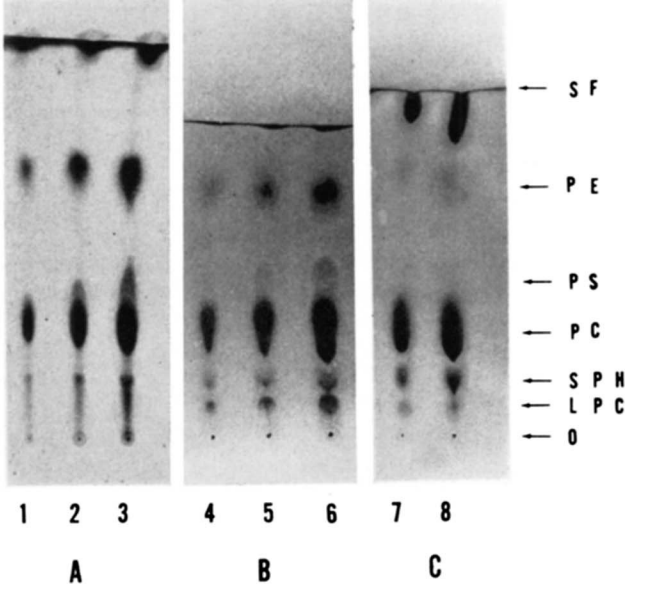

FIG. 2. Thin-layer chromatogram of lipids extracted from tissues. Developing solvent and spray same as those used for "basic" and "neutral" plates in Fig. 1. (A) "Neutral" plate, Total lipid extracted from rat liver: (1) 100 μg, (2) 250 μg, (3) 500 μg. (B) "Basic" plate. Phospholipids extracted from rat liver: (4) 120 μg, (5) 240 μg, (6) 360 μg. (C) "Basic" plate. Total lipid extracted from human serum: (7) 160 μg, (8) 320 μg. Spots at solvent front in A and C include neutral lipids, polyglycerophosphatide, and phosphatidic acid; in B these spots include polyglycerophosphatide and phosphatidic acid. Abbreviations: SF = solvent front, PE = phosphatidyl ethanolamine, PS = phosphatidyl serine, PC = phosphatidyl choline, SPH = sphingomyelin, LPC = lysophosphatidyl choline, and O = origin.

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pholipids was desired, "basic" plates were prepared. Silica Gel G was slurried with 0.01 M aqueous solutions of sodium carbonate or sodium acetate instead of distilled water.

Application of Samples. The samples in methanol–chloroform or methanol solution were applied with micro pipettes 3.5–3.6 cm from the bottom of the plate. Amounts of standard compounds varied according to the nature of the material and in most cases were in the range 20–40 μg. The unknowns were usually applied in greater quantities, depending on the amounts of expected minor constituents to be detected. Application of 100 μg of phospholipid mixture permitted the detection of all components accounting for more than 3–5% of the total. The amount of the applied sample can be increased up to 1000 μg without appreciably affecting the separation of components. The chromatographic chambers were prepared 45–60 min prior to insertion of the plate. In order to prevent drying of the solvent at the front, the chamber was lined on three sides with Whatman 3 MM paper wetted with developing solvent. In our experience, equilibration of the chromatoplate before development was not necessary. The chromatogram was allowed to develop at room temperature until the solvent front reached approximately 11–13 cm from the origin of spots, requiring about 70 min. On both types of plates—"neutral" and "basic"—the best separation was achieved with solvent mixtures of chloroform–methanol–glacial acetic acid–water 65:25:8:4 and 50:25:8:4, respectively.

Detection of Spots. Five different detection methods were employed.

(1) Phospholipids with free amino groups were revealed by ninhydrin spray (0.2% ninhydrin in butanol saturated with water). After spraying, the chromatoplates were heated for about 5 min at 100–105° in atmosphere saturated with water.

(2) Choline-containing phospholipids were detected by the Dragendorff procedure modified by Wagner *et al.* (10).

(3) Plasmalogens were detected by spraying plates with 2,4-dinitrophenylhydrazine according to Reitsema's procedure (18).

(4) Rhodamine 6 G or rhodamine B, 0.03% in 95% ethanol, was used as a universal spray reagent. Spots were detected under ultraviolet light.

(5) Ammonium molybdate–perchloric acid spray (3 g ammonium molybdate dissolved in 50 ml water, 5 ml 6 N HCl and 13 ml 70% perchloric acid) was another universal reagent for detection of all phospholipids and cerebrosides used (10). This spray turned all phospholipids and cerebrosides to blue-gray or dark blue with white background after heating at 80° for 10 min. When ammonium molybdate–perchloric acid spray was used after treatment of the chromatoplate with ninhydrin, the background became grayish but the spots remained dark blue. This spray for detection of phospholipids and cerebrosides was superior to the rhodamine sprays.

RESULTS AND DISCUSSION

Fig. 1A shows a typical thin-layer chromatogram of individual reference compounds and a mixture of them on a "neutral" plate. Phosphatidyl ethanolamine and phosphatidyl serine are separated from each other as well as from other lipids tested on this chromatogram. The position of phosphatidyl serine, however, is dependent upon the amount of this compound spotted. Such dependence is shown on Fig. 2A, where varying amounts of total lipid extracted from rat liver are chromatographed. This load-dependence has been observed only for phosphatidyl serine. The effect is observed on "neutral" plates whether the compound is applied as a pure standard or in a mixture.

On the other hand, chromatograms performed on "basic" plates with the solvent system chloroform–methanol–glacial acetic acid–water 50:25:8:4 (v/v) have shown constant R_f values for phosphatidyl serine. Figure 2B shows different amounts of phospholipids extracted from rat liver chromatographed on "basic" plates. The positions of phosphatidyl serine spots are practically unaffected by the amount of material spotted. This was also verified for pure phosphatidyl serine applied in quantities from 5 to 80 μg.

The separation of different standard phospholipids and their mixture chromatographed on a "basic" plate is shown on Fig. 1B. Synthetic β-palmitoyl-lysophosphatidylethanolamine was chromatographed along with other phospholipids. Experiments with other chromatograms on "basic" plates demonstrated that β-palmitoyllysophosphatidylethanolamine has the same R_f value as another synthetic lysophosphatidyl-ethanolamine: α-myristoyllysophosphatidylethanolamine.

Figs. 2B and 2C illustrate the application of thin-layer chromatography on "basic" plates for the qualitative analyses of phospholipids extracted from rat liver and total lipids extracted from human serum, respectively. The chromatogram of phospholipids from rat liver reveals the presence of phosphatidyl ethanolamine, phosphatidyl serine, lecithin, sphingomyelin, and lysolecithin. The main bulk of phospholipids is represented by lecithins. Chromatography of serum lipids (Fig. 2C) reveals the presence of phosphatidyl

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ethanolamine, lecithin, sphingomyelin, and lysolecithin. Polyglycerophosphatide and phosphatidic acid move with the solvent front together with neutral lipids (cholesterol, cholesterol esters, triglycerides, diglycerides, monoglycerides, free fatty acids) and therefore their presence or absence could not be established.

Several tests were performed to determine the sensitivity of methods for the detection of spots. In general, 2–3.5 μg of material per spot could be detected with most sprays, except Dragendorff reagent, which required more material (up to 10 μg). Application of a specific test for plasmalogens in the lecithin fraction from beef heart and other natural phospholipids indicated that these compounds moved together with their phosphatidyl analogs. No extensive hydrolytic decomposition of plasmalogens was found during the development in our system on "basic" plates.

The method described facilitates the separation and identification of a mixture containing phosphatidyl ethanolamine, phosphatidyl serine, lysophosphatidyl ethanolamine, lecithin, lysolecithin, sphingomyelin, and cerebrosides from a mixture. Kerasine and phrenosine moved near the solvent front in "basic" plates, but gave good spots on "neutral" plates. Neither system described in this paper permitted the separation of the mixture of these two cerebrosides. Two forms of lysophosphatidyl ethanolamine: α-, and β-, on both systems moved together and gave reliable separation from other phospholipids. Although the described thin-layer chromatographic procedure does not permit the separation of all phospholipids and glycolipids present in animal tissue extracts on a single one-dimensional chromatogram, it should be useful in that it permits the separation of phosphatidyl serine and lysophosphatidyl ethanolamine along with other phospholipids.

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