Rapid thin-layer chromatographic separation of phospholipids and neutral lipids of serum

WILLIAM C. VOGEL, WILLIAM M. DOIZAKI, and LESLIE ZIEVE

Radioisotope Service and Department of Medicine, Veterans Administration Hospital, University of Minnesota, Minneapolis 17, Minnesota

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» The applications of the original thin-layer chromatography technique of Kirchner, Miller, and Keller (1), in which adsorbents were coated on glass strips, have been reviewed by Demole (2), who used Mallinckrodt silicic acid in his own work. Malins and Mangold (3) have reported studies of the separation of nonphosphatides using the procedure described by Stahl *et al.* (4). The equipment commercially available (Brinkmann Instruments, Inc., Great Neck, Long Island, N.Y.) utilizes a "silica gel G" containing plaster of Paris (3). This gel is prepared in Germany. We were not able to obtain acceptable separations of phospholipids with this silica gel using solvent mixtures effective with filter paper or glass fiber paper impregnated with silicic acid. However, with the substitution of Mallinckrodt silicic acid with plaster of Paris as the binding agent, the thin-layer equipment and technique can be used for the chromatographic separation of phospholipids as well as nonphosphatides in 30 minutes or less.

Mallinckrodt silicic acid, 100 mesh, suitable for chromatography, is shaken on a 200-mesh sieve to yield approximately 60% as 200 mesh. Thirty grams of silicic acid are mixed with 600 mg of dental plaster of Paris (Fisher Scientific Co.). This is placed in a mortar and 59 to 61 ml of water is added with stirring over a 1-minute interval. The slurry is then ground with a pestle and the plates prepared as with silica gel (3). A relatively thick layer of silicic acid is desirable. The thickness obtained is governed by the amount of water added and the manipulation of the slurry applicator. The plates are immediately activated at 95° for 2 hours and then stored in a desiccator

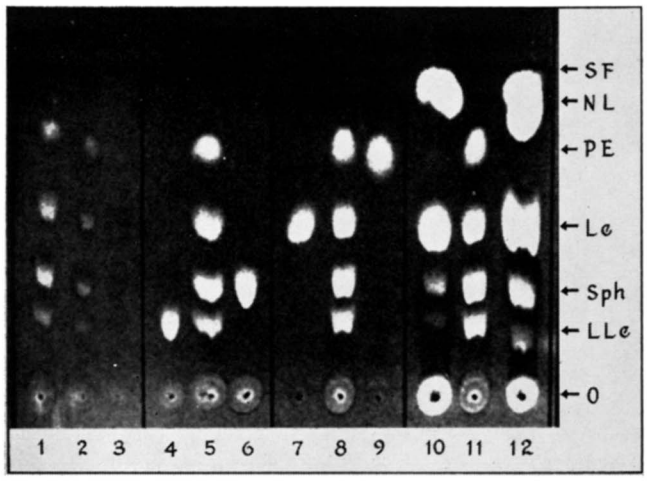

FIG. 1. Separation of phospholipids by thin-layer chromatography. Lipids were applied in chloroform-methanol, 2:1 (v/v). Fifty microliters of the reference solution contained 2 μg phosphorus each of egg phosphatidylethanolamine, lecithin, sphingomyelin, and lysolecithin. This solution in the amount 25, 10, and 5 μl was applied at 1, 2, and 3, respectively. Fifty microliters was applied at 5, 8, and 11, respectively. Suitable aliquots of the egg lysolecithin, sphingomyelin, lecithin, and phosphatidylethanolamine were individually applied at 4, 6, 7, and 9, respectively. Two normal sera were first extracted in ethanol-ether, 3:1 (v/v). An aliquot of each extract was evaporated and taken up in chloroform-methanol, which was applied at 10 and 12. Abbreviations: SF, solvent front; NL, neutral lipids; PE, phosphatidylethanolamine; Le, lecithin; Sph, sphingomyelin; LLe, lysolecithin; and O, origin.

The chromatogram was photographed under the illumination of 2 lamps (Model XX15, Ultraviolet Products Inc., San Gabriel, California) with Kodalith Ortho type 3 film, filter G, at f/11, 20 seconds.

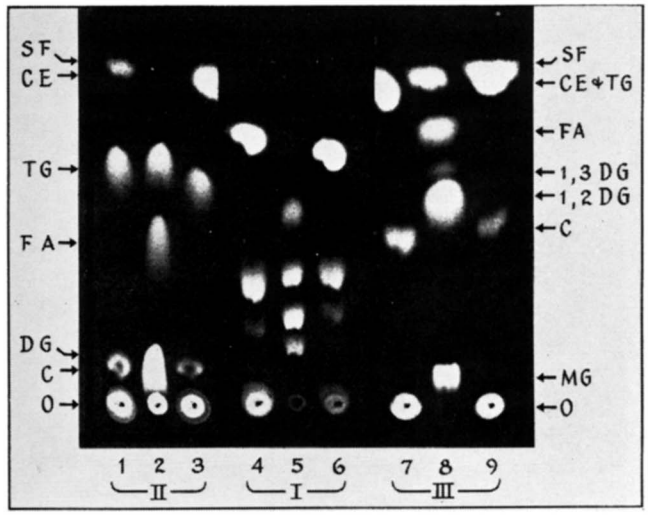

FIG. 2. Separation of phospholipids and neutral lipids on the same thin-layer silicic acid plate. The plate was divided into three "lanes" (I, II, and III) and the chromatograms were developed as described in the text. The "lanes" were numbered in the order in which the three parts of the plate were prepared and chromatographed. Thus the center section was developed first, then the left-hand section, and finally the right-hand section. Reference lipid extracts and total serum lipid extracts were applied in chloroform-methanol to the numbered positions, as in Figure 1. These were arbitrarily numbered from left to right for purposes of this photograph and do not indicate the order in which the extracts were applied. The order of application and the material applied at each position were as follows: Position 4, 150 μl of a serum extract from a case of pancreatitis. Position 5, 50 μl of the phospholipid standard used in Figure 1. Position 6, 150 μl of a normal serum extract. Position 1, 100 μl of the serum extract from the case of pancreatitis. Position 2, 25 μl of a reference solution containing known neutral lipids as described in the text. Position 3, 100 μl of the normal serum extract. Position 7, 100 μl of the serum extract from the case of pancreatitis. Position 8, 25 μl of the reference solution containing known neutral lipids. Position 9, 100 μl of the normal serum extract.

Abbreviations on the left apply to "lane" II, on the right to "lane" III. The separated components in "lane" I are the same as those in Figure 1 and can be identified by the abbreviations of Figure 1. Fatty acids were observed in both "lanes" II and III with both sera, but faded before photography. Abbreviations: SF, solvent front; CE, cholesterol esters; TG, triglyceride; FA, fatty acids; DG, diglyceride; C, cholesterol; MG, monoglyceride; and O, origin.

over anhydrous calcium chloride. Separation of applied phospholipids is accomplished in 30 minutes or less at room temperature using chloroform-methanol-water, 80:25:3 (v/v). Two glass plates covered with filter paper are placed at each side of the chamber and wetted with the solvent just before the chromatogram is to be developed. After chromatography, the plate is air dried for 1 minute and sprayed with 0.2% 2′, 7′-dichlorofluorescein in ethanol (3). The fluorescent phospholipid spots are observed under ultraviolet light.

Separations of the phospholipids from egg yolk, as prepared by the method of Rhodes and Lea (5) and from extracts of normal serum, are shown in Figure 1. Since it is difficult to prepare a completely uniform thin layer of silicic acid so as to have uniform movement of the solvent front across the plate and since R_f values may vary considerably from plate to plate, it is necessary to apply known reference phospholipids on each chromatogram. Construction of "lanes" on the plates as shown in the figures minimizes lateral movement of compounds. While the separation in Figure 1 was accomplished on an unactivated plate with the solvent mentioned in the ratio 80:20:1 (v/v), the best conditions we have found for separation of phospholipids are as described above. The amount of water required in the solvent is dependent on the dryness and thickness of the silicic acid layer.

The separations of nonphosphatides on the silicic acid plates do not give the sharply delineated areas obtained with the silica gel. Adequate separations may be obtained, however, as shown in Figure 2.

In order to permit direct comparison of the mobilities of various phosphatide and nonphosphatide components in different solvent systems and yet avoid the variability introduced by using different silicic acid plates, the following device was employed. The plate was divided into three "lanes." Lipids extracted from human serum and a reference mixture of phospholipids were applied in the center "lane" ("lane" I) and a chromatogram was developed with chloroform-methanol-water as described above. The plate was dried at 95° for 1 hour. Then the silicic acid was scraped away from the plate below the origins of "lane" I so that in succeeding runs the spots already chromatographed in that "lane" would not be shifted. Then aliquots of total lipid from human serum, together with a reference mixture of the neutral lipids, were applied in the left-hand "lane" ("lane" II) and the chromatogram was developed in petroleum ether-ethyl ether-acetic acid, 90:10:1 (v/v) for 30 minutes (1). Again the plate was dried at 95° for 1 hour and the silicic acid scraped from the area below the origins in "lane" II. Finally aliquots of human serum lipid and the reference mixture of neutral lipids were applied in "lane" III and the chromatogram was developed for 30 minutes using petroleum ether-ethyl ether-acetic acid, 60:40:1 (v/v). The plate was dried and all three "lanes" were then sprayed with dichlorofluorescein. It should be noted that although the silicic acid in "lanes" II and III had been exposed to the solvent used in the preceeding runs, the separations observed are typical of those obtained when each run is made on a separate plate.

Cholesterol and esterified cholesterol are identified through their mobility as reported (3). The reference solution in II and III contained oleic acid, triglycerides of olive oil, and di- and monoglyceride obtained by the action of lipase on olive oil with subsequent elution from silicic acid as described by Papariello *et al.* (6). Marinetti (7) and Privett and Blank (8) have demonstrated the separation of isomeric diglycerides. We have found that the component identified as 1,3-diglyceride appeared in a preparation of 1,2-diglyceride obtained by the action of phospholipase D on lecithin, as described by Hanahan *et al.* (9).

Separation of esterified cholesterol and triglycerides, and movement of monoglyceride from the origin, may be accomplished on the same plate by using successive solvent systems as reported by Weicker (10). This is effectively done on unactivated silica gel plates by a first separation for about 35 minutes followed by a second separation for about 20 minutes, using petroleum ether-ethyl ether-acetic acid, 90:10:1 and 40:60:1 (v/v), respectively. This procedure, however, may not effectively separate the isomeric diglycerides.

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