J. Lipid Research
October, 1959

Quantitative glass paper chromatography: phosphatidyl choline and sphingomyelin*

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[Received for publication June 18, 1959]

SUMMARY

A rapid chromatographic procedure was developed for the separation of sphingomyelin, phosphatidyl choline, phosphatidyl ethanolamine, phosphatidyl serine, phosphatidyl inositol, and free fatty acids on glass paper coated with sodium silicate. In addition, phosphatidyl choline and sphingomyelin were determined quantitatively by densitometry of the charred chromatogram, which was obtained by spraying the developed chromatogram with sulfuric acid and heating in an oven. The separation of phosphatides on sodium silicate-treated glass paper is more rapid than on silicic acid-impregnated paper, and the former is simpler to prepare. Preliminary application of this quantitative technique to human serum indicates that it may have a wide adaptability for the determination of phospholipids in natural products.

Phospholipids have been separated on cellulose paper impregnated with silicic acid by Lea and Rhodes (1), Witter *et al.* (2), Hack and Ferrans (3), and others using essentially the same technique. Glass fiber paper impregnated with silicic acid has been used by Dieckert and Reiser (4) and Brown *et al.* (5) for separating phospholipids. According to Agranoff *et al.* (6), cellulose and glass paper give similar results for phosphatide separations when impregnated with silicic acid.

Glass paper offers the advantage of increased sensitivity. Its thermal stability permits the use of concentrated sulfuric acid and subsequent charring by heat for detection of most lipids. The detection of as little as 0.2 μg. of lipid material is below the detection limit of most chromogenic reactions. Swartwout *et al.* (7) have shown that the char formed from spraying with sulfuric acid and subsequent heating can be utilized for the quantitative determination of serum cholesterol, and suggested that the method would be useful for the quantitative determination of a wide variety of organic compounds.

It has been found that glass paper coated with a soluble silicate is superior to one coated with silicic acid owing to its simplicity of preparation and also to the ease of reproducing glass papers with the same properties. It has therefore been possible to determine quantitatively phosphatidyl choline and sphingomyelin in pure solutions. Conditions are described that appear to distinguish qualitatively between phosphatidyl choline, sphingomyelin, phosphatidyl ethanolamine, phosphatidyl serine, phosphatidyl inositol, and free fatty acids. Some preliminary results of the determination of phosphatidyl choline and sphingomyelin in a Bloor's extract of human serum are presented.

* This work was supported by grants-in-aid from the National Institutes of Health, Department of Health, Education, and Welfare, Grant 4150; Nutrition Foundation, Inc., New York; and the National Livestock and Meat Board, Chicago.

MATERIALS AND METHODS

Treated Glass Paper. Glass fiber filter paper[1] is cut into rectangles of 10 by 12.5 cm. and heated in a furnace at 600°C for 30 minutes to remove residual organic matter. A 2.0 per cent stock solution of sodium silicate is prepared by dilution of 10.5 ml. of Mallinckrodt sodium silicate solution, 40-42° Be (38 per cent), to 200 ml. with distilled water. The glass paper, suspended by a clip, is wetted by dipping in 0.4 per cent sodium silicate solution, which is made fresh for each use by dilution of the 2 per cent stock solution with water. A clean glass rod is passed across the surface of the paper in such a way as to drain off excess fluid; the papers are suspended over a hot plate until dry, then stored in covered enamel trays until used.

[1] X-934-AH, from H. Reeve Angel and Co., Clifton, N.J.

Reference Compounds and Reagents. Phosphatidyl choline, sphingomyelin, phosphatidyl ethanolamine, and a concentrate of phosphatidyl serine were generous gifts of Dr. Donald J. Hanahan. The properties of these compounds as provided by him are as follows: Phosphatidyl choline from bovine plasma: phosphorus, 3.74 per cent; fatty acid to phosphorus ratio, 1.97; nitrogen to phosphorus ratio, 0.97; choline to phosphorus ratio, 0.95. Phosphatidyl ethanolamine from bovine red blood cells: nitrogen bases, 98 per cent ethanolamine; phosphorus, 4.07 per cent; fatty acid to phosphorus ratio, 2.00; nitrogen to phosphorus ratio, 0.98. Sphingomyelin from bovine red blood cells: nitrogen to phosphorus ratio, 1.97; choline to phosphorus ratio, 0.96. Phosphatidyl serine from bovine red blood cells: contains phosphatidyl serine, phosphatidyl inositol, and some phosphatidyl ethanolamine.

Other reference compounds used were a sample of purified phosphatidyl choline, which was a generous gift of the Upjohn Company, phosphatidyl choline and phosphatidyl ethanolamine obtained from egg yolk by silicic acid chromatography (1) in this laboratory, and a mixture of fatty acids obtained by saponification of corn oil.

The following reagents were Baker Analyzed Reagents and all except pyridine were redistilled before use: Pyridine, benzene, chloroform, diethyl ether, and methanol. U.S.P. reagent absolute ethanol was obtained from U.S. Industrial Chemicals Company and was redistilled before use.

Chromatographic Procedure. The general technique has been previously described (7). Standard reference compounds were dissolved in chloroform-methanol, 2:1,[2] in concentrations ranging from 0.005 to 0.015 per cent. Ten μl. of each standard was applied to the chromatogram giving amounts of each lipid ranging from 0.5 to 1.5 μg. per spot. Other lipid extracts were applied in 10 μl. volumes to contain 0.5 to 1.5 μg. of the desired phosphatide. After spotting, the glass papers were allowed to air-dry for a minimum of 8 minutes.

The chromatogram was first developed in benzene, which moved all the neutral lipids with the solvent front and left the phosphatides at the origin. The chromatogram was air-dried for 8 minutes and developed in benzene-pyridine, 1:1, with varying amounts of water. The chromatogram was air-dried approximately 15 minutes until free of pyridine. The benzene sweep and the solvent development with benzene-pyridine-water each required 7 minutes.

[2] All solvent ratios are given as volume ratios.

The dried chromatogram was sprayed with fresh reagent grade concentrated sulfuric acid so that both sides were evenly but not heavily coated. The sprayed chromatogram was hung in an oven at 230°C for 4 minutes. The density of each charred spot was read in the specially constructed densitometer (7).

Spot Tests. Since the sulfuric acid char has a much lower limit of sensitivity than the various color tests, it was often convenient to separate the phosphatides by chromatography and then to concentrate the separated compounds by the following device. Five, ten, or more 10 μl. aliquots of the lipid extract or standard were applied along a line 2 cm. from the bottom of a coated paper, which was then developed in the desired solvents as described above. After drying in air, this paper was developed in the second dimension, this time in a solvent which moves the phosphatides with or near the front. In this manner the amount of material in a given region on the chromatogram could be increased by a factor of five, if five spots were applied, by ten, if ten spots were applied, and so on. The procedures described by Hack and Ferrans (3) were employed for identifying choline with phosphomolybdate, amino compounds with ninhydrin, and plasmalogens with fuchsin-sulfurous acid-mercuric chloride. Choline containing lipids were also identified by the Dragendorff periodobismuthate reagent as described by Brown *et al.* (5).

RESULTS

Qualitative Separation of Phosphatides. The R_f values in Table 1 were obtained by chromatography on glass paper coated with sodium silicate. The R_f value of any given phosphatide can be varied over a wide range by varying the water content of the developing solvent. A chromatographic separation of phosphatidyl choline, sphingomyelin, and phosphatidyl ethanolamine is shown in Figure 1. These standard samples are chromatographically homogeneous except for phosphatidyl ethanolamine, which leaves a small amount of material at the origin. This probably represents decomposition products formed subsequent to the isolation of the compound.

Two-dimensional chromatograms of the phosphatidyl serine concentrate were obtained by developing first in benzene-pyridine-water, 100:100:10, then in benzene-pyridine-water, 100:100:14. From these chromatograms the phosphatidyl ethanolamine content of the phosphatidyl serine concentrate was estimated to be 10 to 20 per cent. Only two other spots were detected; the faster moving component, the major constituent of the mixture, has been designated in

TABLE 1. R_f VALUES OF PHOSPHATIDES AND FATTY ACID ON GLASS PAPER TREATED WITH 0.4 PER CENT SODIUM SILICATE

Lipid	Volume of Water Added to Benzene-Pyridine Solvent 100:100								
	0	2	4	6	8	9	10	11	14
Free fatty acids	0.65	0.60	0.60	0.60	0.70	0.82	0.85	0.85	—
Phosphatidyl choline	0	0	0	0.25	0.50	0.60	0.75	0.80	0.85
Sphingomyelin	0	0	0	0.04	0.25	0.40	0.50	0.65	0.85
Phosphatidyl ethanolamine	0	0	0	0.02	0.10	0.20	0.25	0.50	0.85
Phosphatidyl serine	0	0	0	0	0	0	0	0	0.60

Table 1 as phosphatidyl serine, and the other component is presumably phosphatidyl inositol.

Quantitative Chromatography of Phosphatidyl Choline and Sphingomyelin. The absorbance of the charred spots obtained after spraying the chromatogram with sulfuric acid and heating was read in the densitometer and was found to be directly proportional to the amount of phosphatide applied to the glass paper. The established useful range of concentrations of sphingomyelin and phosphatidyl choline solutions, applied in 10 μl. volumes, in these studies extends from 0.005 to 0.015 per cent for sphingomyelin, and from 0.005 to 0.020 per cent for phosphatidyl choline solutions. Standard curves for phosphatidyl choline and sphingomyelin are shown in Figure 2. It should be pointed out that the background in the photographs of the chromatograms is not dirt or char but only an artifact of the photographic process. The extreme contrast of the photographic process records the nonuniformity of the glass paper as apparent char spots on the paper. This nonuniformity is easily compensated for in the quantitative densitometry (7).

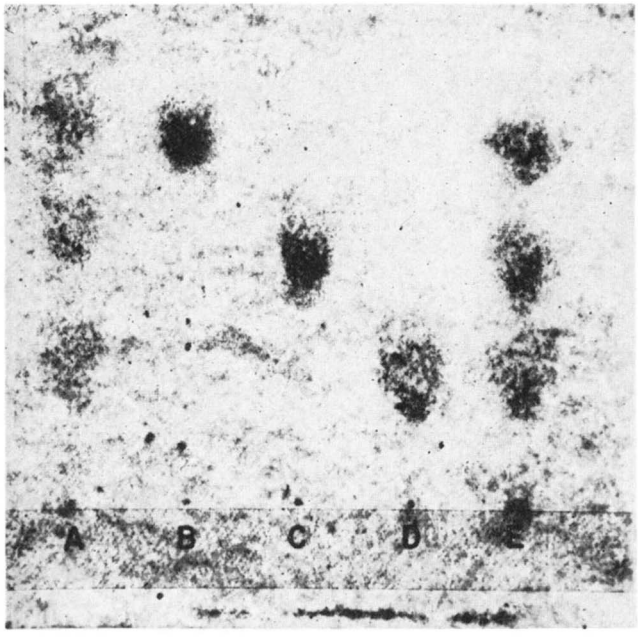

FIG. 1. Photograph of a chromatogram showing the separation of phosphatidyl choline, sphingomyelin, and phosphatidyl ethanolamine. Developing solutions: Benzene followed by benzene-pyridine-water, 100-100-10. Developing time was 7 minutes. A. 0.5 μg. each of phosphatidyl choline, sphingomyelin, and phosphatidyl ethanolamine. B. 1.0 μg. of phosphatidyl choline. C. 1.0 μg. of sphingomyelin. D. 1.0 μg. of phosphatidyl ethanolamine. E. 1.0 μg. each of phosphatidyl choline, sphingomyelin, and phosphatidyl ethanolamine. A contact negative was made on Kodalith film and the print was made on No. 4 contrast projection paper.

Preliminary Results with Human Serum. A Bloor's extract of serum is made by pipetting 1 ml. of serum into a 25 ml. volumetric flask. Six ml. of ethanol-diethyl ether, 3:1, are added in a fine stream and brought just to boiling on a water bath. The solution is cooled, made to volume with ethanol-diethyl ether, and filtered. Suitable aliquots of this extract (1, 2, and 3 ml.) are dried under nitrogen and dissolved in 1 ml. of chloroform-methanol, 3:1. Two 10 μl. aliquots of this solution and similar volumes of three standards are applied to the same chromatogram. This provides a standard curve for each determination. The aliquot whose concentration falls within the desired range is selected for quantitative densitometry.

Two different serum samples having a phospholipid concentration of 280 mg. per 100 ml. (mg. of lipid phosphorus per 100 ml. $\times$ 25) were analyzed by this procedure. The 2 ml. aliquot can be used for the determination of both phosphatidyl choline and sphingomyelin. One sample gave a phosphatidyl choline value of 151 mg. per 100 ml. and a sphingomyelin value of 105 mg. per 100 ml. The other gave a phosphatidyl choline value of 157 mg. per 100 ml. and a sphingomyelin value of 71 mg. per 100 ml. Although the ratios of phosphatidyl choline to sphingomyelin of these two sera are 1.5

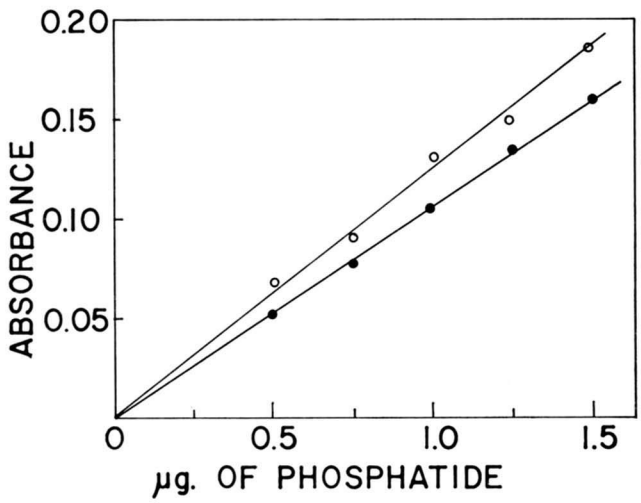

FIG. 2. Standard curves of phosphatidyl choline (●) and sphingomyelin (○). The absorbance of the charred spot is plotted against the amount of phosphatide spotted on the chromatogram.

and 2, ratios as high as 5 have been reported. The chromatogram in Figure 3 shows that both phosphatidyl choline and sphingomyelin are separable and could be determined at ratios as high as 5. The serum sample with a ratio of 2 is included in the figure for comparison. In a single determination on one serum 84 per cent of added phosphatidyl choline and 100 per cent of added sphingomyelin was recovered.

DISCUSSION

Conventional separations of phosphatides on acidic adsorbents, i.e., papers impregnated with silicic acid, are run in acidic or neutral solvents. It is not surprising, therefore, that chromatography of phosphatides on a basic paper, coated with silicate, developed in a basic solvent containing pyridine reverses the order of adsorption. In neutral solvents phosphatides are adsorbed in the same order on silicate paper as in solvents containing pyridine. Although the separation of phosphatidyl ethanolamine and phosphatidyl serine on silicic acid paper is accomplished with time-consuming chromatograms, it was anticipated that they would be easily and rapidly separated on a basic glass fiber paper. The speed of the present method constitutes an important advantage in the handling of the amino phosphatides because of the ease with which these compounds undergo oxidative and hydrolytic decomposition. Chromatographic separation and spot tests of the phosphatidyl serine concentrate indicate that phosphatidyl ethanolamine and phosphatidyl serine are separable by this technique, although further evidence is necessary for absolute proof.

A major problem in the solvent separations of lipids arises from their mutual solubility. Marinetti *et al.* (8) pointed out the desirability of adjusting the composition of the solvent system for phosphatide extracts of different tissues, which is interpreted by us as evidence of lipid mutual solubility effects. The mutual solubility effect is quite pronounced on sodium silicate paper in the benzene-pyridine-water solvent system. However, this difficulty is minimized in the present method by the benzene sweep, a preliminary development of the chromatogram in benzene, which removes all the neutral lipids by sweeping them to the solvent front. This procedure was found to be essential in the analysis of complex lipid mixtures, e.g., serum extract and egg yolk extract, to avoid excessive streaking. It was not found to be necessary for the separation of mixtures of pure phosphatides.

Although this method has the advantage of rapidity, simplicity, reproducibility, and sensitivity, there are limitations which should be pointed out. Satisfac-

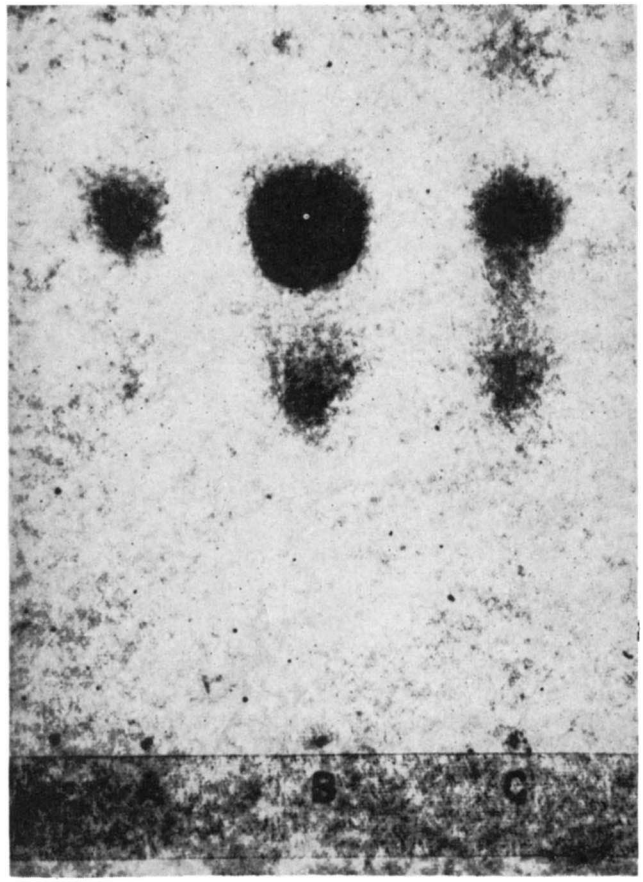

FIG. 3. Photograph of a chromatogram showing the separation of phosphatidyl choline and sphingomyelin at a 5 to 1 ratio and an extract of serum. A. 1.0 μg. of phosphatidyl choline and 0.2 μg. of sphingomyelin. B. 5.0 μg. of phosphatidyl choline and 1.0 μg. of sphingomyelin. C. 10.0 μl. of a 1:12.5 serum extract containing 157 mg./100 ml. of phosphatidyl choline and 71 mg./100 ml. of sphingomyelin.

tory chromatographic separations of phosphatides can be achieved at concentrations as high as 60 mg. of phosphatide per 100 ml. of solution, applied in 10 μl. volumes (cf. *B* in Fig. 3). When the useful range of phosphatide concentrations is maintained between 5 and 15 mg. of individual phosphatide per 100 ml. for quantitative densitometry, it is a simple matter to prepare the indicated dilutions of the lipid mixture and perform the quantitative chromatography separately for each phosphatide on the appropriately diluted extracts. Conversely, when phosphatides are more dilute than 5 mg. per 100 ml., they are concentrated before chromatography. Although this approach increases the number of chromatograms over the requirements of the more conventional cellulose paper chromatography, the time expended is much less.

The chromatographic system herein reported also gives a good separation of free fatty acids. R_f values of fatty acids from corn oil are listed in Table 1. Fatty acids from serum are visible as a spot above phosphatidyl choline in *C* of Figure 3. The feasibility of the determination of free fatty acids in serum by this approach is currently under investigation in this laboratory.

No evidence was obtained to indicate that the chromatographic procedure described hydrolyzes or decomposes the phosphatides. Two-dimensional chromatograms of the pure phosphatides, following the exact procedure as above, were compared with duplicate chromatograms which were developed in identical solvents but from which the solvents were rapidly removed by evaporation in a high vacuum in order to minimize chance of oxidative and hydrolytic decomposition. The chromatograms run under these two sets of conditions were identical by visual inspection.

The identity of the substances from serum which chromatograph the same as pure phosphatides was established by conventional spot tests reagents as follows: phosphatidyl choline and sphingomyelin gave positive choline tests with phosphomolybdate and Dragendorff's periodobismuthate. Phosphatidyl ethanolamine and phosphatidyl serine gave positive ninhydrin reactions. Feulgen's reaction indicated that the major portion of serum plasmalogen ran with phosphatidyl choline. Chromatographic identity of the phosphatides in serum extracts was further established by recovery experiments in which phosphatidyl choline and sphingomyelin appeared in the predicted locations on the chromatogram.

Although the lysophosphatides have not been determined in these solvent systems, from results obtained by others on silicic acid paper, the R_f of lysophosphatidyl choline may be similar to that of sphingomyelin. Because of the basic nature of the paper and the solvent systems, phosphatidyl ethanolamine and phosphatidyl serine have smaller R_f values than phosphatidyl choline and sphingomyelin. Lysophosphatides of phosphatidyl ethanolamine and phosphatidyl serine would be expected to have even smaller R_f values, and therefore not interfere with the determination of phosphatidyl choline and sphingomyelin. The feasibility of applying this method to the determination of all phosphatides in serum and other biological samples is under investigation in this laboratory.

The authors are indebted to Dr. Donald J. Hanahan for generous gifts of reference phosphatides and to Dr. M. H. Hack for his interest and advice during the progress of this work The technical assistance of Mrs. M. S. Galiotto and Mr. M. L Barberot is gratefully acknowledged.

REFERENCES

1. Lea, C. H., and D. N. Rhodes. *Biochem. J.* **57:** xxiii, 1954.
2. Witter, R. F., G. V. Marinetti and E. Stotz. *Federation Proc.* **15:** 386, 1956.
3. Hack, M. H., and V. J. Ferrans. *Z. physiol. Chem. Hoppe-Seyler's* **315:** 57, 1959.
4. Dieckert, J. W., and R. Reiser. *Federation Proc.* **14:** 202, 1955.
5. Brown, M., D. A. Yeadon, L. A. Goldblatt, and J. W Dieckert. *Anal. Chem.* **29:** 30, 1957.
6. Agranoff, B. W., R. M. Bradley and R. O. Brady, *J. Biol. Chem.* **233:** 1077, 1958.
7. Swartwout, J. R., J. W. Dieckert, O. N. Miller, and J. G. Hamilton. *J. Lab. Clin. Med.* Submitted for publication.
8. Marinetti, G. V., J. Erbland and J. Kochen. *Federation Proc.* **16:** 837, 1957.