Quantitative determination of lysolecithin and sphingomyelin in phospholipid mixtures by thin-layer chromatography as applied to the thymus of the *fmfm* mouse

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Summary Lysolecithin and sphingomyelin may be readily separated from other phospholipids on thin-layer plates impregnated with silver nitrate using the solvent system chloroform–methanol–concentrated ammonium hydroxide 65:35:8. Quantitation is carried out by determining the phosphorus content of the developed bands. The minimum amount of phospholipid that can be quantitatively determined is that which contains 0.7 μg of phosphorus. This method has been used for the determination of lysolecithin and sphingomyelin in the tissues of mice having foam-cell reticulosis.

Supplementary key words phosphorus · argentation chromatography · foam-cell reticulosis · Niemann-Pick disease

Many solvent systems have been used for the separation by TLC of phospholipids obtained from tissue extracts. However, all present certain difficulties in the quantitative determination of lysolecithin and sphingomyelin. In particular, a rapid quantitative assay that shows no load effects and that can utilize the convenience of commercially available plates is needed.

The acidic solvent system chloroform–methanol–acetic acid–water 50:30:8:4 of Skipski, Peterson, and Barclay (1) has been successfully used for the separation of phospholipids, including sphingomyelin and lysolecithin. However, when this solvent system is used with commercially made silica gel G plates containing calcium sulfate binders, a streaking of serine phospholipids is observed. In neutral solvent systems, e.g., chloroform–methanol–water 70:30:4, serine phospholipids, when heavily loaded, form a diffuse streak from the area above sphingomyelin to the origin (2). In basic solvent systems, e.g., chloroform–methanol–ammonium hydroxide 65:35:8, lysolecithin cochromatographs with sphingomyelin.

We determined that lysolecithin and sphingomyelin may be readily separated from other phospholipids and quantitatively determined on commercially available $AgNO_3$-impregnated silica gel G plates. This technique was then used to analyze the lysolecithin content in the thymus glands of mice with foam-cell reticulosis (*fmfm*), a possible animal model for Niemann-Pick disease.

Chemicals. The following phospholipids were used as standards for TLC: lysolecithin (egg and bovine) from Applied Science Laboratories, Inc., State College, Pa.; sphingomyelin (bovine) from Applied Science or Supelco, Inc., Bellefonte, Pa.; and phosphatidylethanolamine (bovine), phosphatidylserine (bovine), the sodium salt of phosphatidylinositol (plant), and phosphatidylcholine (bovine) from Applied Science.

Procedure. Commerically available 250-μm silica gel G plates (Analtech, Inc., Newark, Del.) are impregnated with $AgNO_3$ by spraying with 10% aqueous $AgNO_3$ until the silica gel just begins to look wet. The plate is then air-dried for 1 hr. Alternatively, silica gel G plates containing 5 to 10% $AgNO_3$ (Analtech) may be used. The plates are activated for 30 min in a vacuum oven at 110°C just before use.

The lipid mixture in a solution of chloroform–methanol 2:1 is spotted onto the plate which is then developed in chloroform–methanol–concentrated NH_4OH 65:35:8. The average developing time is 2 hr. Lysolecithin is clearly separated from sphingomyelin, which migrates ahead of lysolecithin (see Fig. 1). Phosphatidylcholine and phosphatidylethanolamine are also separated, but phosphatidylserine and phosphatidylinositol cochromatograph near the origin. The phospholipid bands may be visualized by spraying the plates lightly with ammonium molybdate reagent (3). Since the blue color of the phospholipid bands is not permanent, the positions of the individual phospholipids should be marked off shortly after spraying.

Quantitation was carried out by scraping off the bands and determining the phosphorus content of each band by a modification of the perchloric acid–ammonium molybdate method of Bartlett (4). The phosphorus determination was

Abbreviations: TLC, thin-layer chromatography.

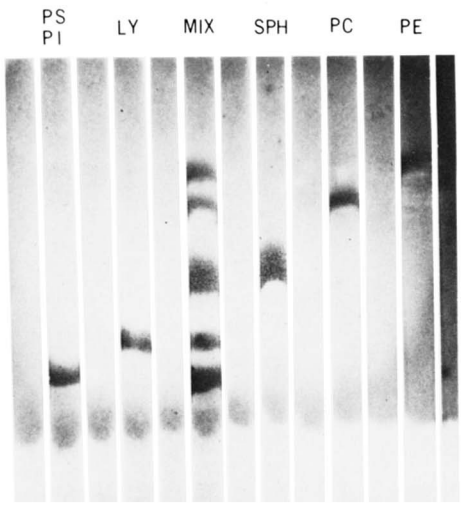

Fig. 1. Separation of lysolecithin and sphingomyelin from other phospholipids on a silica gel G plate containing 5% $AgNO_3$. The TLC plate was developed in $CHCl_3$–MeOH–concd NH_4OH 65:35:8. PS, phosphatidylserine; PI, phosphatidylinositol; LY, lysolecithin; MIX, mixture of phospholipids; SPH, sphingomyelin; PC, phosphatidylcholine; PE, phosphatidylethanolamine.

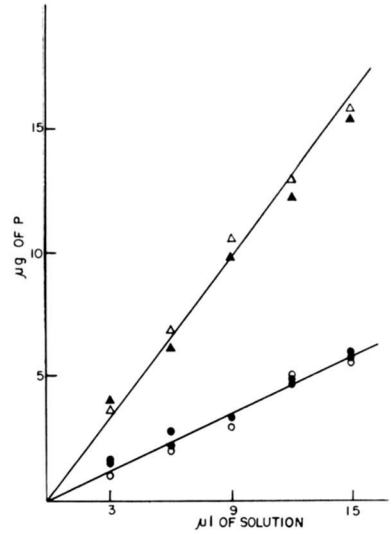

Fig. 2. Standard curves for lysolecithin and sphingomyelin. The concentrations of lysolecithin and sphingomyelin were 7.0 and 26.0 mg/ml, respectively. ●, Lysolecithin aliquot assayed from TLC plate; ○, lysolecithin aliquot put into test tube and assayed; ▲, sphingomyelin aliquot assayed from TLC plate; △, sphingomyelin aliquot put into test tube and assayed.

carried out without eluting the phospholipids from the silica gel, as described by Parker and Peterson (5). It was necessary to subtract the value of a plate blank from the values obtained by scraping a blank band of silica gel at the same distance from the origin as the phospholipid being determined, then assaying the phosphorus content of that band. The phosphorus values of the plate blanks ranged from 0.5 to 1.5 μg.

The determination of phospholipid in this manner on $AgNO_3$-containing plates is quantitative. The standard curves determined by spotting various amounts of lysolecithin and sphingomyelin, eluting, scraping off the bands, and determining phosphorus content were linear. The phosphorus content of identical amounts of phospholipids put into test tubes was ascertained for comparison, and the values obtained closely corresponded to those values for sphingomyelin or lysolecithin put on the thin-layer plate (see Fig. 2).

The determination of phosphorus in samples of sphingomyelin and lysolecithin scraped from a TLC plate is reproducible (Table 1) on samples containing as little as 0.7 μg of phosphorus (12 μg of lysolecithin or 17 μg of sphingomyelin). The spray used for development is sensitive to as little as 0.3 μg of phosphorus, but such low values cannot be quantified.

Up to 250 μg each of phosphatidylserine, lysolecithin, and sphingomyelin may be spotted on a plate before any tailing is seen. At these high concentrations, the three phospholipids are still clearly separated and no load effect is observed.

Although the use of argentation chromatography for the separation of lipids according to the degree of unsaturation is well known (6–9), the method presented in this paper separates the phospholipids only by functional group. Thus, the standards bovine lysolecithin, bovine sphingomyelin, bovine phosphatidylethanolamine, bovine phosphatidylserine, and bovine phosphatidylcholine each migrate as a single spot. In contrast, $AgNO_3$-containing thin-layer systems that separate phospholipids according to the degree of unsaturation are known to separate bovine lecithin into from three to six components (8). The presence of NH_4OH in the elution solvent must result in the complexing of Ag^+ ions with ammonia, thereby preventing the complexing of Ag^+ with double bonds in the lipids to provide separation according to the degree of unsaturation.

TABLE 1. Reproducibility of phosphorus determination on samples of lysolecithin and sphingomyelin

Phospholipid Standard	Phosphorus[a]
	μg
Lysolecithin	0.70 ± 0.04 (3)
	1.23 ± 0.06 (4)
	1.80 ± 0.06 (4)
	2.15 ± 0.07 (3)
Sphingomyelin	0.74 ± 0.03 (3)
	1.24 ± 0.03 (3)
	2.17 ± 0.07 (3)
	2.66 ± 0.10 (3)

Phosphorus determinations were performed on various aliquots of solutions of lysolecithin and sphingomyelin standards that had been spotted onto silica gel G plates containing 5% $AgNO_3$, developed, and scraped into tubes.

[a] Mean values are shown for the number of determinations in parentheses.

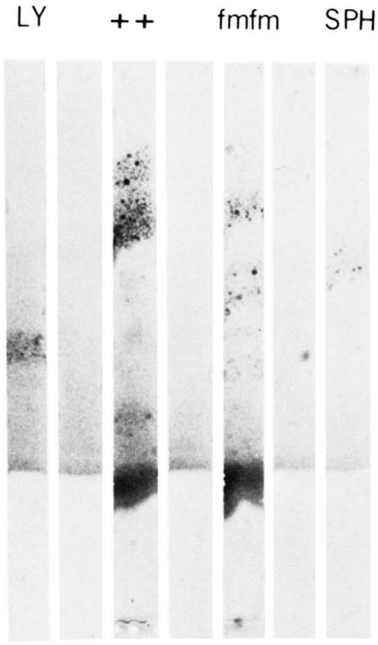

Fig. 3. Phospholipids in the thymus of *fmfm* and normal (++) mice. Silica gel G plate containing 10% $AgNO_3$ eluted with $CHCl_3$–MeOH–concd NH_4OH 65:35:8. LY, lysolecithin; SPH, sphingomyelin.

This method is currently being used for the determination of lysolecithin and sphingomyelin in the tissues of mice having foam-cell reticulosis, a genetically transmitted lipid storage disease first reported by Lyon, Hulse, and Rowe (10). A controversy exists as to the nature of the lipid that accumulates in the thymus of the *fmfm* mouse, the lipid having been tentatively identified as lysolecithin by Lyon et al. (10) but later reported to be sphingomyelin by Fredrickson, Sloan, and Hansen (11).

The thymus glands of 4-month-old *fmfm* and control mice were extracted with chloroform–methanol 2:1. An aliquot containing 10 μg of phosphorus was spotted onto a silica gel G plate containing 10% $AgNO_3$, and the plate was developed in chloroform–methanol–concentrated NH_4OH 65:35:8 (see Fig. 3). The phospholipids were visualized with ammonium molybdate spray and scraped off the plate, and the quantity of phosphorus in each band was determined.

Table 2 shows that there was a 30-fold increase in sphingomyelin and an 11-fold increase in lysolecithin in the *fmfm* thymus. The cholesterol and the phosphatidylserine and inositol were also increased, but to a much lesser extent.

Unless it can be established that there is a significant increase of lysolecithin in the viscera in Niemann-Pick disease, one is not justified in considering the *fmfm* mouse an animal model.

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TABLE 2. Phospholipid and cholesterol distribution in thymus of *fmfm* and normal mice

Lipid	*fmfm*	Normal
	μmoles/g dry wt	
Total phospholipid	126	59.3
Total cholesterol	154	42.5
Lysolecithin	23.6	2.0
Sphingomyelin	43.6	1.4
Phosphatidylserine and phosphatidylinositol	14.4	4.8
Lecithin	39.0	36.4
Phosphatidylethanolamine	15.9	13.2

Each of these determinations (*fmfm* and normal) was made on the pooled thymuses from four mice.

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