Note

In situ mineralization and determination of phosphorus in phospholipids on silica gel sintered thin-layer chromatographic plates

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Thin-layer chromatography (TLC) has been used as a simple technique for qualitative and quantitative analyses of lipids. Phospholipids separated by TLC have been determined by colorimetric methods after extraction from the scraped spots[1], by densitometry after visualization using colour reagents or charring reagents[2] or by hydrogen flame ionization detection (FID)[3]. The scraping method is generally accepted but tedious. Most densitometric methods and the FID method are not based on phosphorus determination. Dittmer–Lester reagent[4], a specific reagent for phospholipid detection, is not suitable for densitometry because of the blotchy and grainy sample spots obtained[2].

We therefore designed a method for the determination of lipid phosphorus directly on a TLC plate. Phospholipids were separated on a silica gel sintered TLC plate and mineralized directly on it. The phosphoric acid liberated was determined by densitometry after visualization with Brilliant Green. The determination of amino nitrogen by the ninhydrin method prior to the mineralization procedure did not interfere with the determination of phosphorus by this method. The nitrogen to phosphorus ratio in phosphatidylethanolamine was easily determined on the same silica gel sintered TLC plate after development.

EXPERIMENTAL

Reagents and standard samples

Brilliant Green (BG) was obtained from Wako (Tokyo, Japan). Other reagents were of analytical-reagent grade. Phosphatidylethanolamine (PE), phosphatidylcholine (PC) and sphingomyelin (SM) were prepared from egg yolk[5]. The purity of each lipid was over 98% by high-performance (HP) TLC (silica gel 60, Merck). The determination of lipid phosphorus was performed essentially by the method of Bartlett[6].

TLC plates

Silica gel sintered TLC plates (10 × 10 cm) (Iatron Labs., Tokyo, Japan) were

prepared with uniform-sized particles of silica gel (15 μm) to obtain well defined sample spots with good resolution. The plates were cleaned by immersion in fuming nitric acid overnight at room temperature, rinsed with distilled water and activated at 100°C for 30 min before use.

Solvent systems

The following solvent systems were used: (1) ethyl acetate–n-propanol–chloroform–methanol–water–glacial acetic acid (25:25:25:10:6:0.5, v/v), (2) chloroform–methanol–water–glacial acetic acid (45:15:2:0.2, v/v) and (3) chloroform–methanol–water–28% ammonia (45:15:2:0.1, v/v).

In one-dimensional TLC, the plate was developed 5 cm from the origin with solvent system 2. After drying *in vacuo*, the plate was further developed to the top with solvent system 1. In two-dimensional TLC, the plate was developed in the first direction in the same manner as in one-dimensional TLC, then thoroughly dried *in vacuo* and run in the second direction with solvent system 3.

Spray reagents

Two spray reagents, A and B[7], were used for visualization with Brilliant Green. A was a mixture of 5 ml of 1% aqueous ammonium molybdate solution, 5 ml of 25% hydrochloric acid and 90 ml of acetone. B was a mixture of solutions 1 and 2. Solution 1 was prepared by dissolving 2 g of BG in 350 ml of water and solution 2 by dissolving 4 g of ammonium molybdate in 40 ml of water with heating followed by cooling, addition of 50 ml of 10 M hydrochloric acid and dilution to 100 ml with water. Solutions 1 (350 ml) and 2 (100 ml) were mixed, left for 3 h and filtered into a brown bottle. The solutions were stored at room temperature and were stable for 5 weeks.

Procedures

A 1-μl volume of lipid solution [10–100 ng/μl of lipid in chloroform–methanol (9:1)] was spotted on the TLC plate with a Hamilton 7002N microsyringe and developed with the solvent systems described above. The dried TLC plate was sprayed with 60% perchloric acid, covered with a glass plate and heated at 130°C for 90 min on an aluminium block heater. The TLC plate was further heated at 130°C for 5 min without the cover plate to remove perchloric acid. The cooled plate was sprayed with reagent A and heated at 85°C for 3 min. After cooling, the plate was sprayed with reagent B. As soon as green spots had appeared on an orange background, the TLC plate was covered with a glass plate to keep the concentration of hydrochloric acid constant. After 30 min, the coloured spots were scanned with a Shimadzu CS-910 dual-wavelength TLC scanner (sample, 620 nm; reference, 400 nm; reflection mode). The extinction profiles were recorded with a Hewlett-Packarsd 3390A integrator.

RESULTS AND DISCUSSION

The advantage of the silica gel sintered TLC plate is its durability to treatment with strong acids at high temperatures. The silica gel thin layer did not crumble even after the mineralization procedures. After the analysis, the plate could be used repeatedly by applying the cleaning procedure described above.

Fig. 1 is a two-dimensional thin-layer chromatogram of egg yolk phospholipids. The chromatogram shows well resolved and defined spots. As the adsorptive activity of the sintered TLC plate was less than that of the usual HPTLC plate, the solvent system was modified to reduce the proportions of polar solvents such as methanol and water. The addition of a small amount of glacial acetic acid contributed to a better definition of the sample spots.

The most popular method for determining organic phosphorus is colorimetric measurement of the blue colour developed by reducing phosphomolybdic acid. However, on spraying with reducing reagents such as 1-amino-2-naphthol-4-sulphonic acid reagent, L-ascorbic acid solution and amidol reagent, the background of the chromatogram developed the same blue colour as the sample spots, which then could not easily be distinguished from the background. The examination of other dyes showed BG to be optimal. BG is both an acid–base and a redox indicator, and an analogue of Malachite Green, which has been used for qualitative and quantitative analyses of lipid phosphorus[8,9]. The mechanism of the coloration of BG was elucidated by Trujillo *et al.*[10]. After the visualization procedure, green spots appeared on an orange background. The absorption maxima were 620 nm for the sample spot and 400 nm for the background. The developed colour is stable for about 40 min from 20 min after the colour development. After 60 min, the background intensity increases with increase in both the colour intensity of sample spots and its coefficient of var-
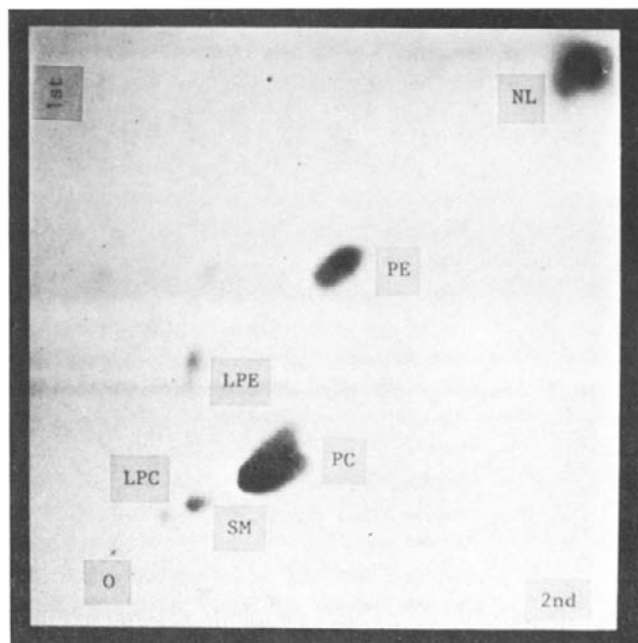

Fig. 1. Two-dimensional thin-layer chromatogram of phospholipids using the silica gel sintered TLC plate. Egg yolk phospholipid fraction (200 ng of total lipid phosphorus) was developed. PE = phosphatidylethanolamine; LPE = lysophosphatidylethanolamine; PC = phosphatidylcholine; LPC = lysophosphatidylcholine; SM = sphingomyelin. Phospholipids were visualized with 50% sulphuric acid. Chromatographic conditions as in text.
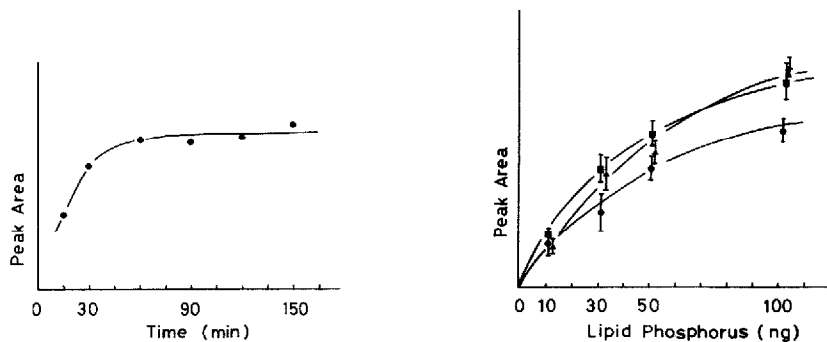
Fig. 2. Effect of time of heating on the mineralization. PE (100 ng of lipid phosphorus) was mineralized at 130°C and visualized with BG.

Fig. 3. Calibration graphs in the range 10–100 ng of lipid phosphorus for (▲) PE, (■) PC and (●) SM. Phospholipids were separated by one-dimensional TLC, followed by mineralization and visualization with BG. Abbreviations as in text.

iation (C.V.) value. Therefore, densitometric scanning between 20 and 60 min after colour development is to be preferred.

The effect of the time of heating on the mineralization performance of the organic phosphorus compound was investigated (Fig. 2). Although the colour density reached a plateau at around 60 min, a time of 90 min was adopted to ensure complete mineralization. In the TLC scraping method, phospholipids were mineralized at 180°C[1]. On the sintered TLC plate, phospholipids were mineralized sufficiently at 130°C. Fig. 3 shows the calibration graphs for PE, PC and SM, determined after one-dimensional TLC. The colour development was saturated at around 100 ng of phosphorus. In the range 10–100 ng of phosphorus, the coefficient of variation of the peak area for each phospholipid was 5–15%. Unfortunately, the individual calibration graphs were not the same for all phospholipids on a phosphorus basis. Macala et al.[11] reported that the densitometric responses were affected by diffusion of the sample spots during development. In this method, the spot grew broarder not only
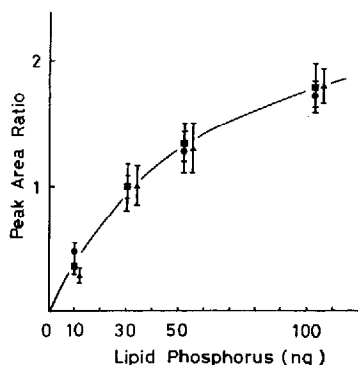

Fig. 4. Modified calibration graphs for (▲) PE, (■) PC and (●) SM. In place of the colour response itself, ratios of the response at each amount of lipid phosphorus amount to that of a fixed amount (30 ng) were plotted. Abbreviations as in text.

TABLE I

MAJOR PHOSPHOLIPID COMPOSITION OF EGG YOLK

Phospholipid	Lipid phosphorus* (ng)	wt. %	
		Present method**	Reference***
PC	53.3 ± 2.50	75.9 ± 2.1	80.7
PE	18.0 ± 0.82	20.2 ± 2.0	16.5
SM	3.1 ± 0.14	3.8 ± 0.15	2.8

* Values are means ± standard deviations of three determinations. Amounts of phosphorus in PC and PE were determined after the development of egg yolk phospholipid fraction (80.8 ng of total lipid phosphorus). For SM, the amount of sample was increased 3-fold and the value of phosphorus in SM was divided by three.

** Lipid composition represented as weight percentage for comparison with reference values. The molecular weight of each lipid was calculated by assuming that each lipid contained only stearic acid.

*** Amount of PE, PC and SM were taken from ref. 12 and calculated as weight percentage of major components.

during development but also in the mineralization. To correct for these influences, the ratios of the responses of varying amounts of phosphorus to the response at 30 ng were plotted (Fig. 4). For all the phospholipids investigated the relationship between this ratio and the amount of phosphorus agreed well. Therefore, an external standard mixture consisting of phospholipids, all of which contained 30 ng of phosphorus, was developed alongside unknown samples.

The phospholipid composition of egg yolk was measured very easily by this method. The results obtained are in close agreement with literature values[12] (Table I). A further advantage of this method is that the determination of other constituents such as the amino group prior to the mineralization procedure did not interfere with the subsequent determination of phosphorus. The amount of nitrogen in PE was
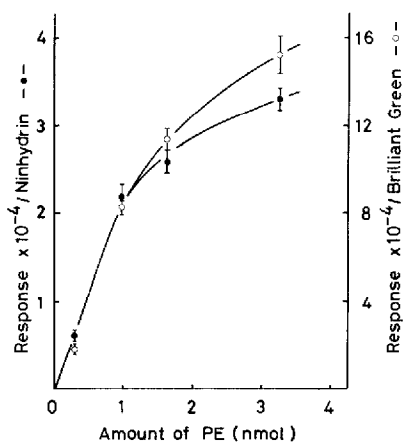

Fig. 5. Calibration graphs for PE in the range 0.3–3 nmol. After development, PE was determined by the ninhydrin method, followed by mineralization and determination by this method on the same plate. Chromatographic conditions and abbreviations as in text.

determined after development on the sintered TLC plate by the ninhydrin method. After the densitometry (570 nm), the same plate was submitted to mineralization and applied to the phosphorus determination (Fig. 5). The nitrogen:phosphorus ratio for PE was 1.06 ± 0.19 at 1 nmol, which is very close to the theoretical value of 1.00.

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REFERENCES

1 Y. H. Itoh, T. Itoh and H. Kaneko, *Anal. Biochem.*, 154 (1986) 200–204.
2 J. Sherman and S. Bennett, *J. Liq. Chromatogr.*, 6 (1983) 1193–1211.
3 T. Terabayashi, T. Ogawa, Y. Kawanishi, M. Tanaka, K. Takase and J. Ishii, *J. Chromatogr.*, 367 (1986) 280–285.
4 J. C. Dittmer and R. L. Lester, *J. Lipid Res.*, 5 (1964) 126–127.
5 A. R. Johnson and J. B. Davenport, *Biochemistry and Methodology of Lipids*, Wiley-Interscience, Toronto, 1971, p. 155.
6 G. R. Bartlett, *J. Biol. Chem.*, 234 (1959) 466–468.
7 F. Jungnickel, *J. Chromatogr.*, 31 (1967) 617–618.
8 H. H. Hess and J. E. Derr, *Anal. Biochem.*, 63 (1975) 607–613.
9 M. Goppelt and K. Resch, *Anal. Biochem.*, 140 (1984) 152–156.
10 A. Trujillo, S. W. Kang and H. Freiser, *Anal. Chim. Acta*, 182 (1986) 71–81.
11 L. J. Macala, R. K. Yu and S. Ando, *J. Lipid Res.*, 24 (1983) 1243–1250.
12 F. D. Gunstone, J. L. Harwood and F. B. Padley, *The Lipid Handbook*, Chapman & Hall, London, 1986, p. 166.