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Direct fluorometric microdetermination of phospholipids on thin-layer chromatograms

The locations of phospholipids separated by thin-layer chromatography (TLC) are often determined by spraying the chromatograms with destructive reagents[1–3]. For quantitative experiments the marked phospholipid spots are scraped from the plate and after complete digestion of the organic material the residual inorganic phosphate is determined colorimetrically. Several authors describe the direct measurement of lipids by densitometry of the TLC plates or their photographs after charring of the organic material[4–11]. The disadvantage of this process is that besides destruction of the sample, variations can occur due to the degree of unsaturation of the fatty acid chain, the presence of hydroxyl groups or evaporation of the sample and conversion to carbon dioxide at high temperature.

This paper presents a sensitive and rapid method for the assay of phospholipids by direct fluorometry after TLC and detection of the spots by the dye 1-anilino-8-naphthalene sulfonate (ANS), which has been found to combine with the apolar regions of the surface of various proteins and with positively charged and neutral lipid micelles[12–15]. The adsorption of the dye on these compounds results in a strong enhancement of its fluorescence.

Experimental

Thin-layer plates. The glass plates (20 × 20 cm), pre-coated with 250-μm layers of silica gel without fluorescence indicator, are commercially available from E. Merck, Darmstadt (G.F.R.). The silica gel microplates (8 × 4 cm) are commercially available from Macherey-Nagel, Düren (G.F.R.).

Separation and detection. All the phospholipids tested were samples from horse erythrocyte plasma membranes, obtained after chloroform–methanol (2:1) extraction. Phospholipid phosphorus was measured by an adaptation of the method of BARTLETT[16].

The monodimensional chromatograms were developed with the solvent system, chloroform–methanol–water (60:25:4). The chromatographic chamber, lined with filter paper, was saturated with the solvent vapor for 1 h before use. Samples were applied to the plates as 2-cm homogeneous lines with a Hamilton microsyringe attached to a Desaga dispenser. Best separations were obtained with TLC plates that were deactivated by equilibrating with air for several days at room temperature. After development, the plates were air-dried and uniformly sprayed with a freshly prepared 0.01% aqueous solution of ANS (Mg salt) (Sigma, St. Louis, Mo., U.S.A.). The lipid components appeared as characteristic green–yellow spots using a short wavelength ultraviolet lamp with Woods glass filter. Quantitative fluorometric determination was carried out directly from thin-layer plates using a Zeiss PMQ-II spectrophotometer with TLC scanner attachment for fluorometry. Measurements were made at an excitation wavelength of 375 nm and emission wavelengths from the sample longer than 440 nm were detected and registered on a recorder. The overall slit of the monochromator was 0.07 × 10 mm. Areas under the peaks were calculated by triangulation.

Results and discussion

As the background fluorescence of the silica gel plates after spraying with ANS was practically zero (Fig. 1), no unsatisfactory irregularities of the baseline were observed by scanning the plates. The recorded tracings of fluorescence emitted from

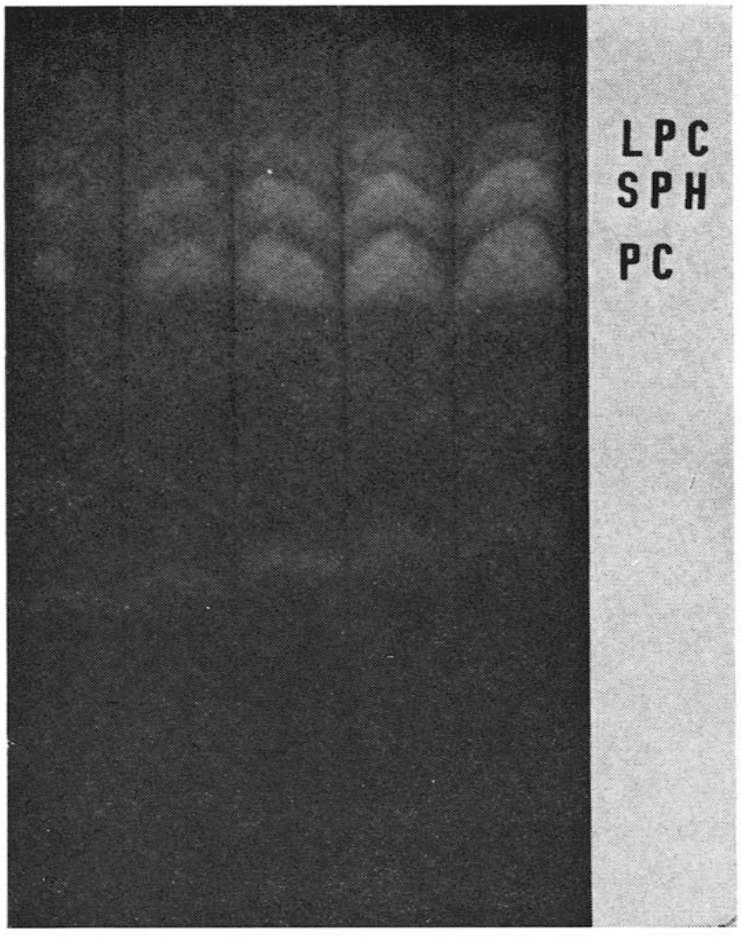

Fig. 1. TLC chromatogram on silica gel of phospholipids after detection with 1-anilino-8-naphthalenesulfonate. Solvent system: chloroform–methanol–water (60:25:5)

TABLE I

THE ANS FLUORESCENCE OF PHOSPHOLIPIDS AND VARIOUS OTHER LIPIDS ON SILICA GEL PLATES

Lipid	*Concentration (nmoles)*	*Relative fluorescence*[a]
PC	50	100
PE	50	172
PI	50	88
SPH	50	105
LPC	50	33
Tristearate	50	17
Cholesterol	50	16.5
Cholesterol acetate	50	27
Palmitic acid	50	12

[a] Values are percentages of the fluorescence of 50 nmoles PC.

ANS-stained phospholipids were integrated and the resulting peak area was plotted in a diagram against the determined concentration of each component. Fig. 2 shows the calibration curves of phosphatidylethanolamine (PE), phosphatidylcholine (PC), lysophosphatidylcholine (LPC) and sphingomyeline (SPH), in the concentration range of 1–100 μg of phospholipid. By increasing the sensitivity of the photomultiplier and using a slit of 0.2 mm, 0.1 μg of phospholipid can easily be detected. While the curves of PE and PC are rectilinear, the curves of SPH and LPC become curvilinear at high concentrations. The fluorescence of standards may vary somewhat from plate to plate. However, the relative positions of the curves obtained for the different phospholipid classes remained unchanged. Therefore, for accurate quantitation of an unknown mixture it is necessary to include a standard on each plate Then the proposed fluorometric TLC method for phospholipids gives a good reproducibility when the assay is made on different plates relative to added standards.

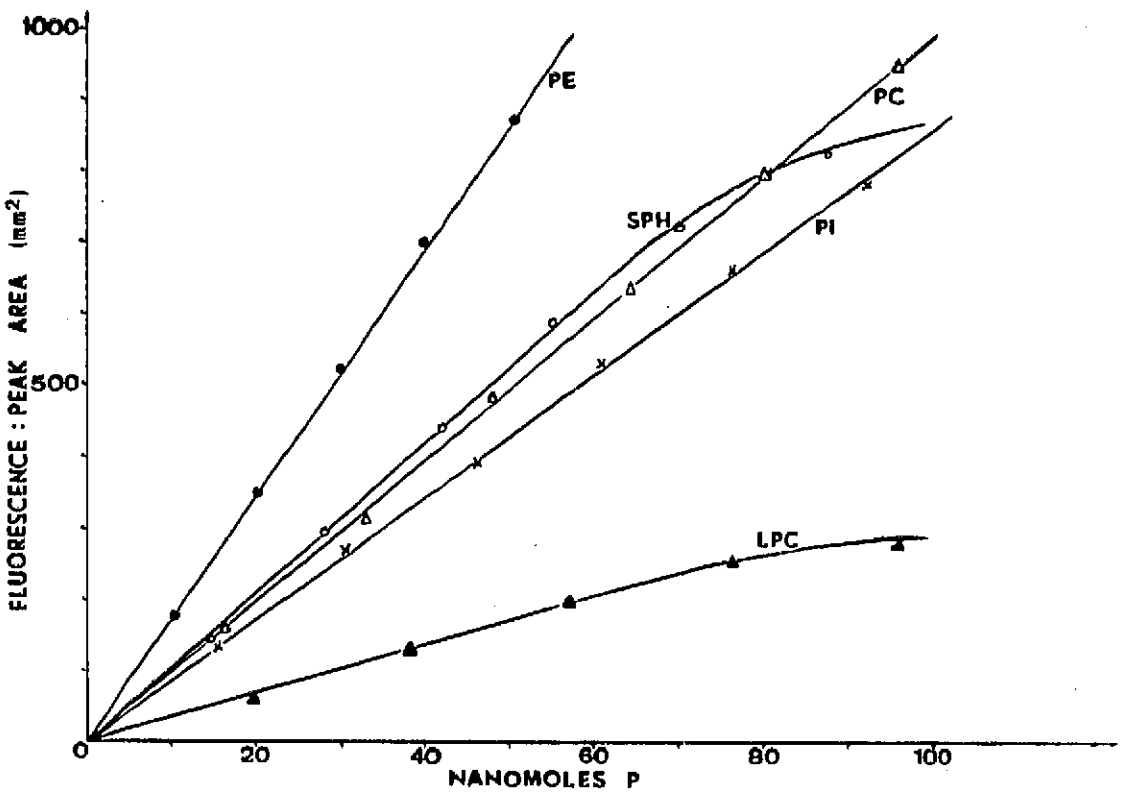

Fig. 2. Calibration curve for phosphatidylethanolamine (PE), sphingomyeline (SPH), phosphatidylcholine (PC), phosphatidylinositol (PI) and lysophosphatidylcholine (LPC).

The fluorescence is stable for several hours when the plates are kept in the dark. It disappears when the chromatograms are exposed to daylight or artificial light.

In Table I a comparison is made of results obtained by fluorescence measurements of phospholipids with those of various other lipid classes. The results show that the fluorescence obtained with phospholipids is generally about ten times higher than the average recording of equimolar quantities of other lipid components.

It has been demonstrated that minute amounts of phospholipids can be analysed by a simple, rapid and non-destructive technique.

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1 K. RANDERATH (Editor), *Thin-Layer Chromatography*, Academic Press, New York, 1966.
2 E. STAHL (Editor), *Dünnschicht-Chromatographie*, 2. Aufl., Springer-Verlag, Berlin, 1967.
3 B. L. WALKER, *J. Chromatogr.*, 56 (1971) 320.
4 R. KLAUS, *J. Chromatogr.*, 16 (1964) 311.
5 D. T. DOWNING, *J. Chromatogr.*, 38 (1968) 91.
6 N. NESKOVIC, *J. Chromatogr.*, 27 (1967) 488.
7 M. L. BLANK, J. A. SCHMIT AND O. S. PRIVETT, *J. Amer. Oil Chem. Soc.*, 41 (1964) 371.
8 O. S. PRIVETT, M. L. BLANK, D. W. CODDING AND E. C. NICKELL, *J. Amer. Oil Chem. Soc.*, 42 (1965) 381.
9 H. RASMUSSEN, *J. Chromatogr.*, 27 (1967) 142.
10 J. C. TOUCHSTONE, A. K. BALIN, T. MURAWEC AND M. KASPAROW, *J. Chromatogr. Sci.*, 8 (1970) 443.
11 J. J. BIEZENSKI, W. POMERANCE AND J. GOODMAN, *J. Chromatogr.*, 38 (1968) 149.
12 T. GULIK-KRZYWICKI, E. SHECHTER, M. IWATSUBO, J. L. RANCK AND VITTORIO LUZATTI, *Biochim. Biophys. Acta*, 219 (1970) 1.
13 W. HASSELBACH AND K. W. HEIMBERG, *J. Membrane Biol.*, 2 (1970) 341.
14 E. DANIEL AND G. WEBER, *Biochem. J.*, 5 (1966) 1893.
15 D. F. H. WALLACH, E. FERBER, D. SELIN, E. WEIDEKAMM AND H. FISCHER, *Biochim. Biophys. Acta*, 203 (1970) 67.
16 G. R. BARTLETT, *J. Biol. Chem.*, 234 (1959) 466.

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