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Note

Densitometric analysis of ethanolamine- and serine-containing phosphoglycerides

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The simplicity, good separating ability and rapidity of thin-layer chromatography (TLC) make this technique excellent for the qualitative examination of phospholipids, but quantitative evaluation of members of this class of polar lipids separated by TLC is more difficult. The problem is generally approached in one of the following three ways.

(1) The lipid spots (visualized by exposure to iodine) are scraped from the plate and ashed, and the ash is analysed for inorganic phosphate[1,2].

(2) The phospholipid spots are located with a special spray reagent and darkened with subsequent elution of the zones for analysis[3–5].

(3) Photodensitometry of the charred chromatogram[6–8].

All these methods have their disadvantages. Elution is time-consuming (several extractions and centrifugations, and evaporation of eluents are needed). The photodensitometric method also has limitations, basically attributable to the unequal photo-electric response to the same molar concentration of different phospholipids; the reason for this is the unequal numbers of carbon atoms in the individual phospholipids being determined. This limitation can be to some extent mitigated by using a calibration graph for each phospholipid, but problems still remain due to the different fatty acids esterified in naturally occurring phospholipid molecules. Privett and Blank[8] partially overcame some of these disadvantages by hydrogenating the phospholipids before separation, but the subsequent charring needed a temperature of about 200° and often led to loss of substance.

In this paper we describe a simple densitometric determination of ethanolamine- and serine-containing phospholipids in form of their dinitrophenyl (DNP) derivatives. The DNP derivative of phosphatidylethanolamine (DNP-PE) was first described by Wheeldon and Collins[9], but their method for its estimation had two great disadvantages, namely, the derivative was formed in a medium of light petroleum, which was several times distilled off and renewed, and the excess of 1-fluoro-2,4-dinitrobenzene (FDNB) reagent and by-products were distilled on to a "cold finger" at a pressure of less than 0.1 Torr and at about 80°.

These facts made quick and simple analysis impossible. In our method, the DNP derivatives of the complex phospholipid mixture are formed in about 30 min at 40°; the compounds are then separated by TLC, and, after drying the plate, the yellow spots are immediately measured. All these manipulations can be accomplished within 1 h.

MATERIALS AND METHODS

Standards

Phosphatidylethanolamine (PE) was isolated from soya-bean oil and purified as described by Rhodes and Lea[10], the purity being established from the ratio of ester to phosphorus and by TLC. Phosphatidylserine (PS) was isolated from pig brain and purified, and its purity was established in a similiar way to that used for PE. Lyso-phosphatidylethanolamine (Lyso-PE) was prepared by treatment of PE with phospholipase A_2 and purified by TLC. The FDNB and triethylamine were obtained from Merck (Darmstadt, G.F.R.) and purified by distillation.

Thin-layer chromatography

The TLC plates (Merck 60F-254) were activated before use by heating at 115° for 30 min. The developing system was chloroform–methanol–water (65:25:4, v/v).

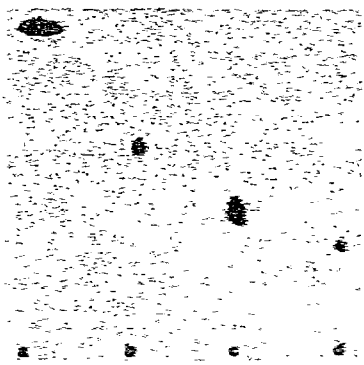
Preparation of derivatives

To establish the calibration graphs, the individual standard phospholipids (10–100 nmoles of each) were dried over phosphorus pentoxide in a vacuum desiccator. To each were added 0.1–0.25 ml of 0.1 % (w/v) FDNB solution in diethyl ether and then 2–5 μl of triethylamine, and the mixture was shaken vigorously at 37° for 30 min. The reaction products were purified as follows: 5 volumes of chloroform–methanol (2:1, v/v) were equilibrated with 1 volume of water, the phases were allowed to separate, the upper phase was discarded, and 10 ml of the lower phase were added to the reaction mixture. The excess of reagent was removed by thrice washing the solution with saturated sodium bicarbonate solution. The lower phase was evaporated to dryness under a stream of nitrogen, and the residue was finally purified by TLC. With unknown phospholipids, the reaction mixture was applied directly to the TLC plate.

RESULTS

The DNP derivatives of pure phospholipids were characterized by TLC on a silica gel plate developed with chloroform–methanol–water (65:25:4, v/v), the R_F values being 0.60, 0.29, 0.33 and 0.95 for the derivatives of PE, PS and Lyso-PE and for FDNB, respectively. Fig. 1 shows a chromatogram of the derivatives of Lyso-PE and PS and FDNB. The derivatives appeared as yellow spots on a white background and did not give a positive colour reaction with ninhydrin. The recovery of the phospholipids (based on determination of phosphorus) after derivative formation varied between 98 and 102 %. The molar absorptivity of DNP-PE was 17,300 and that of DNP-PS was 15,200 at 345 nm (the wavelength of max. absorption). The photoelectric response was linear in the range 0.02–0.4 μmole of pure DNP derivative (see Fig. 2).

Samples containing known amounts of phosphatidylcholine (PC) and PE, or PC and PS, were submitted to derivative formation and analyzed by densitometry; for this purpose, six samples and three standards were applied to the TLC plate. Table I shows the calculated amounts of PE, Lyso-PE and PS, together with those



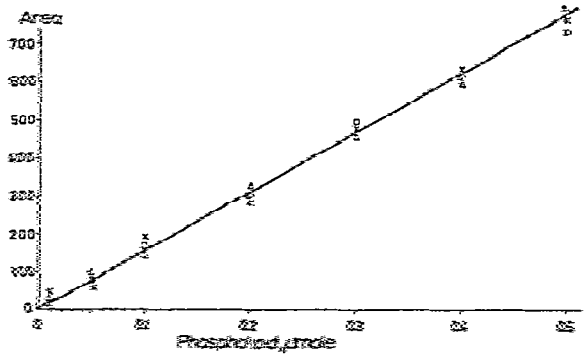


Fig. 1. Chromatogram showing positions of (a) FDNB, (b) DNP-PE, (c) DNP-Lyso-PE and (d) DNP-PS after development with chloroform–methanol–water (65:25:4, v/v).

Fig. 2. Graph showing photo-electric response (as area) to increasing amounts of pure DNP derivatives: △, DNP-PE; ○, DNP-PS; ×, DNP-Lyso-PE.

TABLE I

CALCULATED AND MEASURED RESULTS FOR PS, PE AND Lyso-PE

Each value is expressed as the percentage (by weight) in the mixture.

PE			*Lyso-PE*			*PS*		
Calculated	*Found*	*Difference*	*Calculated*	*Found*	*Difference*	*Calculated*	*Found*	*Difference*
9.61	9.94	+3.43	10.0	9.8	−2.0	10.00	10.23	+2.30
19.13	19.01	−0.63	20.0	19.2	−4.0	19.35	19.67	+1.65
28.79	27.88	−3.16	29.9	29.2	−2.4	29.80	28.94	−2.92
38.68	39.47	+2.04	40.0	40.0	0	40.14	40.78	+1.59
48.61	48.82	+0.43	50.0	48.9	−2.2	49.26	48.87	−0.79
58.90	60.64	+2.95	60.2	62.4	+3.6	58.98	57.63	−2.29
68.85	66.48	−3.44	70.0	72.3	+3.3	69.85	70.68	+1.19
79.10	77.42	−2.13	80.0	81.2	+1.5	80.04	79.95	−0.12
89.41	89.79	+0.43	89.9	90.0	−0.1	90.10	92.07	+2.19
100.00	98.94	−1.06	100.0	101.4	+1.4	100.00	98.54	−1.46
$\bar{X} \pm s$	−0.114 ± 2.42		−0.1 ± 2.71			+0.13 ± 1.92		

found by densitometry, and the differences between these values expressed as relative percentages. In all instances, the mean difference was ± 0.1%, with a max. standard deviation of ± 2.7%.

Finally, the PE contents of several natural phospholipid mixtures were determined by the method of Rouser *et al.*[2] (estimation of phosphorus) and by densitometry of the DNP derivatives; Table II shows the good agreement between these methods.

CONCLUSIONS

The procedure described here is suitable for the rapid preparation of derivatives of phospholipids containing amino-groups. The amount of FDNB used for deriv-

TABLE II

THE CONTENTS FOUND IN SOME NATURAL PHOSPHOLIPID MIXTURES

Each value is expressed as the percentage (by weight) in the mixture.

Sample	*PE content found by*		*Difference*
	Densitometry	*Method of Rouser et al.*[2]	
Soybean raw lecithin	15.7	15.3	+2.6
Oil-free soybean raw lecithin	20.8	21.4	−2.9
Ethanol-soluble part of soybean raw lecithin	17.7	17.3	−2.26
Technical PE	90.2	87.4	+3.1

ative formation is dependent on the amounts of PE and PS in a mixture. A 10-molar excess of the reagent over the aminophospholipids was more than sufficient for derivative formation and did not interfere with the analysis. For unknown amounts of such phospholipids present in *e.g.*, tissue extracts, it was assumed that 40% of the total phospholipids were those containing amino-groups. For many technical and pharmaceutical purposes, phospholipid products with low contents of PE and PS are used. When the amount of PE and PS is below 1%, it is very difficult to determine these phospholipids by any known method. The proposed procedure gives very accurate results for such samples, and the minimum amount of phospholipids required is about 0.02 μmole. A rapid and exact analysis for PS and PE can be achieved with 200 μg of natural phospholipids.

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