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Note

Formation and separation of fluorescent derivatives of phosphatidic acid

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Current interest in the metabolism of phospholipids has highlighted the difficulties in separating and quantitating glycerophospholipids and related membrane lipids. Separation of phospho- and acylglycerolipids into component classes (*e.g.* phosphatidylcholine, phosphatidylethanolamine) and molecular species is generally accomplished by thin-layer chromatography (TLC) although several recent reports have documented the use of normal and reversed-phase high-performance liquid chromatography (HPLC)[1–9]. As most lipids lack a suitable reporting group, available detection methods are expensive (mass spectrometry) or generally require large amounts of lipid and limit the use of certain solvents (*e.g.* UV absorption and refractive index). Several previous studies have analysed phospholipids containing free amino groups subsequent to the formation of UV absorbing or fluorescent derivatives[10,11]. However, as the chemical synthesis of phospholipids by esterification of phosphatidic acid with various alcohols (*e.g.* choline, ethanolamine, serine) can be readily achieved[12], it seemed possible to couple phosphatidate to a fluorescent alcohol. Such a procedure would greatly expand the range of phospholipids which could be analysed by fluorescence. To this end we have reacted Dns-ethanolamine with phosphatidic acid and separated the product, N-Dns-phosphatidylethanolamine (Dns-PE) by reversed-phase chromatography.

MATERIALS AND METHODS

Phosphatidic acids, phosphatidylethanolamines, phosphatidylcholines and phospholipase D (peanut) were obtained from Sigma (St. Louis, MO, U.S.A.). Ethanolamine, 5-dimethylaminonaphthalene-1-sulfonyl chloride (Dns-Cl), 2,4,6-triisopropylbenzene sulfonyl chloride (TPS) and triethylamine were obtained from Aldrich (Milwaukee, WI, U.S.A.). HPLC-grade solvents were supplied by J. T. Baker (Philipsburg, PA, U.S.A.). Glass distilled water was further purified by passage over ion-exchange resin and a C_{18} Sep Pak (Waters Assoc., Milford, MA, U.S.A.) prior to

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use. KH_2PO_4, K_2HPO_4 and pyridine were obtained from Fisher Scientific (Somerville, NJ, U.S.A.).

Formation of N-Dns-ethanolamine

Dns-ethanolamine was prepared by reacting Dns-Cl with a large excess of stirred ethanolamine. Dns-ethanolamine precipitated and was collected by filtration. Recrystallization from methanol yielded an amorphous solid. A stock solution (40 mg/ml) in chloroform was stored refrigerated.

Formation of Dns-PE

Dns-PE was prepared by reacting phosphatidylethanolamine with Dns-Cl essentially in the manner described by Chen *et al.*[11]. Dry phosphatidylethanolamine (100 μg) was combined with 50 μl of Dns-Cl (1 mg/ml in chloroform) and 15 μl triethylamine in a small reaction vial. The mixture was tightly capped, vortexed briskly and allowed to react for 2–3 h at 50°C protected from light.

Formation of Dns-PE from phosphatidic acid and Dns-ethanolamine

The esterification between the hydroxyl group of Dns-ethanolamine and the phosphoric acid of phosphatidate was accomplished using TPS. This reaction was adapted from the synthesis of phosphatidylethanolamine described by Aneja *et al.*[12]. Typically 100 μg of dry phosphatidic acid was allowed to react with 2 molar equivalents of Dns-ethanolamine and 5 molar equivalents of TPS in pyridine solution at room temperature protected from light. The reaction was monitored by TLC (see below).

Chromatographic analysis

TLC analyses were performed on silica plates (Silica 60, Merck, Darmstadt, F.R.G.) and reversed-phase plates (Silica C_{18}, J. T. Baker), R_F values and solvents are as indicated in Tables I and II.

HPLC analyses were performed at room temperature on C_{18} reversed-phase columns (Econosphere, 25 × 4.5 cm, Alltech, Deerfield, IL, U.S.A.) with a Varian

TABLE I

SEPARATION OF Dns-PE ON SILICA 60 TLC

Dns-PE I was prepared by reaction of egg phosphatidylethanolamine with Dns-Cl. Dns-PE II was prepared by reaction of Dns-ethanolamine with egg phosphatidate. Dns-PE III was prepared from phosphatidate formed by enzymatic hydrolysis of egg phosphatidylcholine. Dns-lysophosphatidylethanolamine (Dns-lysoPE) was formed by reaction of lysophosphatidylethanolamine with Dns-Cl. Solvent composition was methylene chloride–methanol–17 *M* NH_4OH (80:15:2).

	R_F
Dns-PE I	0.71
Dns-PE II	0.72
Dns-PE III	0.73
Dns-lysoPE	0.62
Dns-Cl	>0.9
Dns-ethanolamine	>0.9
Dns-OH	0.4

TABLE II

SEPARATION OF Dns-PE ON REVERSED-PHASE TLC (C_{18})

Dns-PE I was prepared by reaction of egg phosphatidylethanolamine with Dns-Cl. Dns-PE II was prepared by reaction of Dns-ethanolamine with egg phosphatidate. Dns-PE III was prepared from phosphatidate formed by enzymatic hydrolysis of egg phosphatidylcholine. Dns-lysoPE was formed by reaction of lysophosphatidylethanolamine with Dns-Cl.

Solvent	R_F	
	Methanol–10 mM KH_2PO_4 (95:5)	*Acetone–10 mM KH_2PO_4 (95:5)*
Dns-PE I	0.33	0.37
Dns-PE II	0.33	0.36
Dns-PE III	0.34	0.37
Dns-lysoPE	0.65	0.66
Dns-Cl	0.69	0.67
Dns-ethanolamine	0.78	0.77
Dns-OH	0.88	0.87

(Palo Alto, CA, U.S.A.) Model 5000 HPLC equipped with a fluorescent detector (Fluorchrome, Varian; Excitation 360 nm, emission above 420 μm).

RESULTS AND DISCUSSION

The esterification between phosphatidate and Dns-ethanolamine proceeded smoothly and yielded a product chromatographically indistinguishable from that produced by reacting Dns-Cl with phosphatidylethanolamine. Tables I and II present the R_F values of the Dns-PEs prepared in these ways chromatographed on normal and reversed-phase TLC. Dns-PE formed from phosphatidate prepared by phospholipase D hydrolysis of phosphatidylcholine also exhibited identical R_F values. Although both chromatography systems resolve N-Dns-lysophosphatidylethanolamine, none was discernible in the reaction mixtures.

Comparison of phosphidate and phosphatidylethanolamine derived Dns-PE was also made with HPLC on octadecyl silane (Fig. 1) reversed-phase columns. Fig. 1A depicts the chromatograms of Dns-PE prepared by reacting Dns-Cl with egg phosphatidylethanolamine. Fig. 1B depicts the chromatograms of Dns-PE derived from egg phosphatidate. Fig. 1C depicts the chromatogram of Dns-PE formed from egg phosphatidylcholine. While conditions of complete separation of the Dns-PE into molecular species was not accomplished in the present study, limited resolution was observed. All three sources of Dns-PE exhibited similar chromatographic profiles with four major peaks. Indeed the pattern observed for egg phosphatidylethanolamine was nearly identical to that observed for egg phosphatidylcholine. On the other hand while egg phosphatidate appeared to have the same major species, their relative amounts were quite different.

The procedure reported here provides a facile method for preparing fluorescent derivatives of phosphatidic acid by reaction with Dns-ethanolamine to form Dns-PE. The formation of phosphate esters with TPS has been employed in the synthesis of many substances of biological interest including nucleotide phosphates[13] and

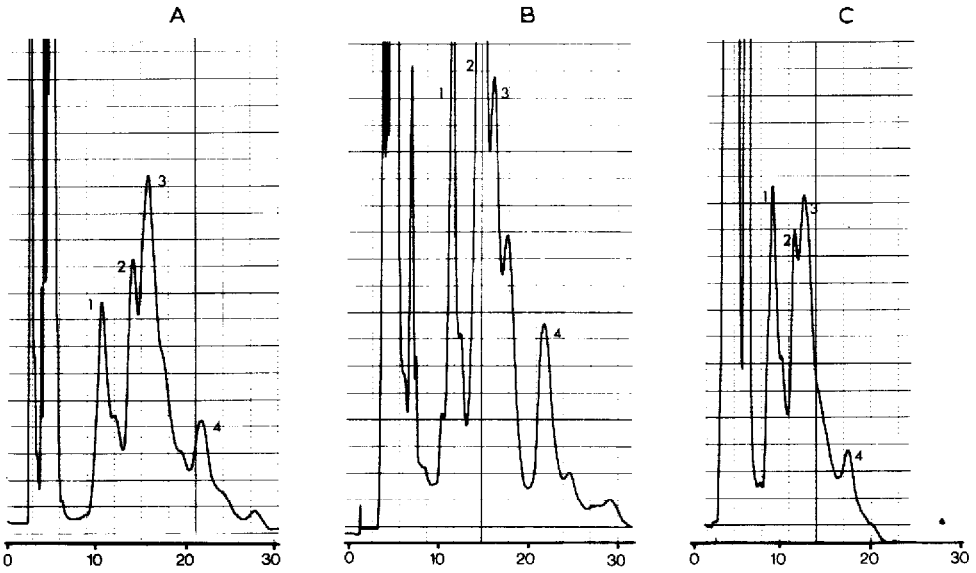

Fig. 1. Analysis of Dns-PE by HPLC with C_{18} reversed-phase column. Mobile phase was methanol–10 m*M* KH_2PO_4 (93:7). Flow-rate: 1 ml/min. (A) Dns-PE prepared by reaction of egg phosphatidylethanolamine with Dns-Cl. Major peaks are marked 1 through 4. (B) Dns-PE prepared by reaction of egg phosphatidate with Dns-ethanolamine. Major peaks correspond with those indicated in (A). (C) Dns-PE prepared by reaction of phosphatidate with Dns-ethanolamine. Phosphatidate was prepared by hydrolysis of phosphatidylcholine with phospholipase D. Major peaks correspond with those indicated in (A).

phospholipids[12]. The derivatization procedure is by no means limited to the use of Dns-ethanolamine. Rather the present scheme was chosen to yield a product, Dns-PE, which can be really compared to Dns-PE prepared from phosphatidylethanolamine. In the original studies Aneja *et al.*[12], it was found that blocking the amino group of ethanolamine was necessary to prevent reaction with TPS. As might be expected the presence of the Dns-group appears to serve this same purpose in the present synthetic scheme.Although the present study focused on formation of Dns-PE from phosphatidate, it should be noted that the method is readily adaptable for the conversion of virtually any other phospholipid to Dns-PE by first converting the lipid to phosphatidate by enzymatic or other means. In this regard Krüger *et al.*[14] recently published a scheme whereby fluorescent derivatives of diacylglycerols (or diacylglycerols prepared by enzymatic hydrolysis of phospholipids) were prepared and separated by HPLC. Thus quantitation of trace amounts of individual molecular species of phospholipids by fluorescence is possible.

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