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Note

Formation and separation of fluorescent derivatives of diacylglycerols

P. J. RYAN
Department of Psychiatry and Pharmacology, Medical College of Pennsylvania, 3200 Henry Ave., Philadelphia, PA 19129 (U.S.A.)
and
T. W. HONEYMAN*
Department of Physiology, University of Massachusetts Medical School, 55 Lake Avenue North, Worcester, MA 01605 (U.S.A.)
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The routine separation and quantitation of glycerophospholipids and related substances is difficult owing to the lack of a suitable reporting group in such molecules. Thus separation of these lipids into component molecular species has, for the most part, relied on thin-layer chromatography (TLC)[1–7]. Resolution of these compounds by high-performance liquid chromatography (HPLC) has become increasingly popular although available detection methods (UV absorption, refractive index, mass spectrometry) are either insensitive or expensive[8–13]. Recently several studies have appeared whereby UV absorbing or fluorescent derivatives of diacylglycerol were prepared and separated by HPLC[14–16]. This strategy has the advantages of greater sensitivity and application to a variety of glycerophospholipids by first converting them to diacylglycerol.

We have previously reported the separation of fluorescent derivatives of phosphatidic acid by HPLC[17]. This method esterified phosphatidate with the 5-dimethylaminonapthalene-1-sulfonyl chloride derivative of ethanolamine (Dns-ethanolamine) to form the fluorescent phospholipid, Dns-phosphatidylethanolamine. In the present report, we describe the formation of the same compound, Dns-phosphatidylethanolamine, by an esterification of diacylglycerol with Dns-ethanolamine phosphate. Using this scheme diacylglycerols or phosphatidic acids may be converted to the corresponding fluorescent Dns-phosphatidylethanolamines and their molecular species resolved by HPLC.

MATERIALS AND METHODS

Ethanolamine phosphate, 1,2-dipalmitoyl-*sn*-glycerol, 1,2-distearoyl-*sn*-glycerol, egg phosphatidylcholine, egg phosphatidylethanolamine, and phospholipase C (*Bacillus cereus*) were obtained from Sigma (St. Louis, MO, U.S.A.). 5-Dimethylaminonapthalene-1-sulfonyl chloride (Dns-Cl), 2,4,6-triisopropylbenzenesulfonyl chloride (TPS), triethylamine and pyridine were obtained from Aldridge (Milwaukee, WI, U.S.A.). Dry pyridine was prepared by distillation over potassium hydroxide

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and stored in the presence of molecular sieves 4A (Sigma). HPLC-grade solvents were supplied by J. T. Baker (Philipsburg, PA, U.S.A.). TLC was performed on silica plates (Silica 60, 0.25 mm; E. Merck, Darmstadt, F.R.G.) or reversed-phase plates (Silica C_{18}; J. T. Baker). Reagent-grade sodium carbonate, potassium dihydrogen phosphate and dipotassium hydrogen phosphate were obtained from Fisher Scientific (Somerville, NJ, U.S.A.).

Formation of N-Dns-ethanolamine phosphate

Ethanolamine phosphate was reacted with Dns-Cl at room temperature by mixing equal volumes of acetone containing Dns-Cl (50 mg/ml) with 20 mg/ml ethanolamine phosphate dissolved in 0.02 *M* sodium carbonate (pH > 8). The product, Dns-ethanolamine phosphate, was separated from ethanolamine phosphate by extraction into chloroform and further purified by TLC on Silica 60 plates with chloroform–methanol–ammonium hydroxide (spec. grav. 0.88), (65:25:4, v/v) as solvent. The Dns-ethanolamine phosphate appeared as a green-yellow spot below the blue spot of Dns-OH (R_F *ca.* 0.2).

Formation of diacylglycerols

Up to 10 mg of egg phosphatidylcholine or phosphatidylethanolamine were hydrolysed with phospholipase C according to the procedure of Renkonen[2]. The reaction mixture was extracted twice with diethyl ether and the diacylglycerols were purified by TLC on Silica 60 plates developed with light petroleum–diethyl ether–glacial acetic acid (60:20:1.5, v/v; R_F *ca.* 0.6).

Formation of N-Dns-phosphatidylethanolamines

Dns-phosphatidylethanolamine was synthesized from phosphatidylethanolamine essentially in the manner described by Chen *et al.*[18]. Briefly 0.1 to 10 mg of phosphatidylethanolamine was dissolved in 50 μl of chloroform containing Dns-Cl (10 mg/ml). Triethylamine (10 μl) was added and the reaction allowed to proceed at room temperature protected from light.

Dns-phosphatidylethanolamine was synthesized from diacylglycerol by the esterification of the hydroxyl group with the phosphoric acid of Dns-ethanolamine phosphate in the presence of TPS. This reaction was adapted from the synthesis of nucleotide phosphoesters described by Lohrmann and Khorana[19], the synthesis of phosphatidylethanolamine from phosphatidic acid described by Aneja *et al.*[20] and is similar to the esterification of phosphatidic acid with Dns-ethanolamine we have previously reported[17].

Typically, 0.1 to 2.0 mg of diacylglycerol was dissolved in 50 μl of anhydrous pyridine. Dns-ethanolamine phosphate (2 mequiv.) and TPS (5 mequiv.) were added and the reaction was allowed to proceed at 60–80°C for 24 h protected from light. The formation of Dns-phosphatidylethanolamine was monitored by TLC (see below). The excess TPS was hydrolysed by the addition of water, the mixture dried and the Dns-phosphatidylethanolamines extracted with chloroform.

Fatty acid analyses

The fatty acid composition of the major species of phospholipids was determined by collection of HPLC peaks fractions, liberation of fatty acids by treatment

with alkali, and the formation of fluorescent derivatives of fatty acids. We have previously reported the separation and identification of fluorescent fatty acid derivatives by HPLC[21].

Chromatographic analyses

Dns-phosphatidylethanolamine was isolated by TLC on Silica 60 plates developed with chloroform–methanol–ammonium hydroxide (spec. grav. 0.88) (65:25:4, v/v; R_F *ca.* 0.80) or on reversed-phase developed with methanol–10 m*M* aqueous potassium phosphate, pH 7 (9.5:0.5, v/v; R_F *ca.* 0.2).

HPLC analyses were performed at room temperature on C_8 reversed-phase columns 250 × 4.5 mm I.D. (Econosphere, Alltech Assoc., Deerfield, IL, U.S.A.) with a Varian Model 5000 instrument (Varian, Palo Alto, CA, U.S.A.) equipped with a fluorescent detector (Fluorochrome, Varian; excitation 360 nm, emission above 420 nm). The derivatives were separated isocratically with a mobile phase of methanol aqueous potassium phosphate, pH 7 (83:17) with a flow-rate of 1 ml/min.

RESULTS AND DISCUSSION

Initial attempts at reacting diacylglycerol and Dns-ethanolamine phosphate at room temperature were unsuccessful and warming the reaction to 60–80°C was found

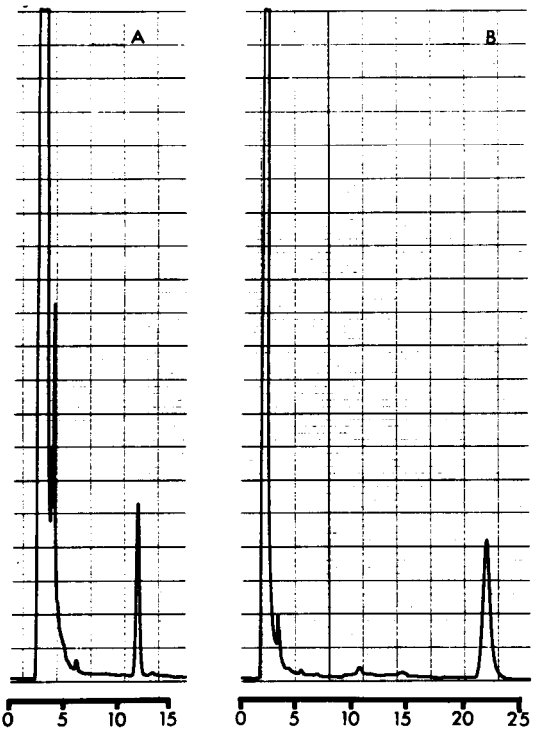

Fig. 1. HPLC separation of Dns-dipalmitoyl phosphatidylethanolamine (A) and Dns-distearoyl phosphatidylethanolamine (B) formed by esterification of the corresponding diacylglycerols with Dns-ethanolamine phosphate.

to be necessary. The reaction proceeded slowly and maximal yields were obtained only after 24 h or more. Fig. 1 depicts the chromatograms of Dns-dipalmitoyl phosphatidylethanolamine (Fig. 1A) and Dns-distearoyl phosphatidylethanolamine (Fig. 1B) prepared from the corresponding diacylglycerols. In each case a single major product was formed indicating that acyl chain migration was minimal during the course of the reaction.

Fig. 2 compares the chromatograms of Dns-phosphatidylethanolamines formed directly by derivatization of phosphatidylethanolamine with Dns-Cl (Fig. 2A) to that formed indirectly by hydrolysis of phosphatidylethanolamine to diacylglycerol and esterification with Dns-ethanolamine phosphate (Fig. 2B). Four major molecular species were observed. Fatty acids contained in peak 1 were 16:0 and 18:2; peak 2, 16:0 and 18:1; peak 3, 18:0 and 18:2; peak 4, 18:0 and 18:1. Assuming the saturated fatty acid was present in the 1′ position, the molecular species were 16:0–18:2, 16:0–18:1, 18:0–18:2 and 18:0–18:1. The relative amount of each major species was nearly identical regardless of whether the Dns-phosphatidylethanolamines were formed directly by reaction of phosphatidylethanolamines with Dns-Cl or indirectly by esterifying the diacylglycerols with Dns-ethanolamine. Thus the derivatization scheme appears to produce products in proportion to the relative amount of each molecular species.

Fig. 3 depicts a chromatogram of Dns-phosphatidylethanolamine synthesized from egg phosphatidylcholine. Phosphatidylcholine had the same four major species

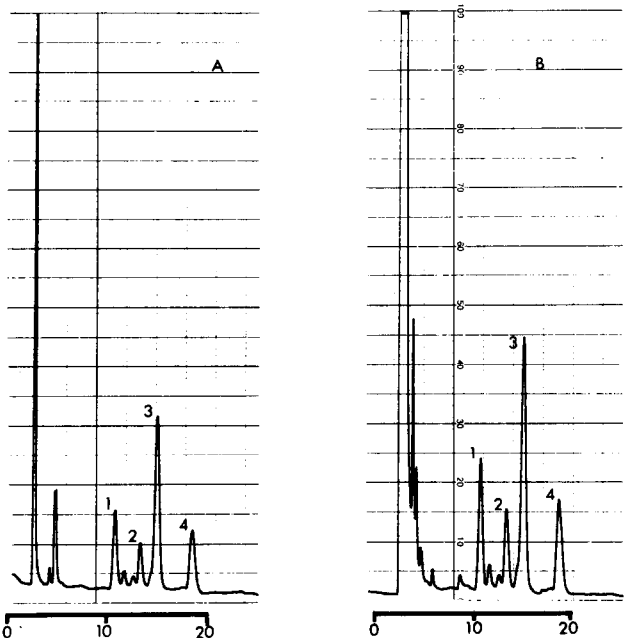

Fig. 2. HPLC separation of Dns-phosphatidylethanolamine prepared by reacting egg phosphatidylethanolamine with Dns-Cl (A) and by esterification of diacylglycerols derived from phosphatidylethanolamine with Dns-ethanolamine phosphate (B). Fatty acid composition of peaks 1–4 were 16:0–18:2, 16:0–18.1, 18:0–18:2 and 18:0–18:1, respectively.

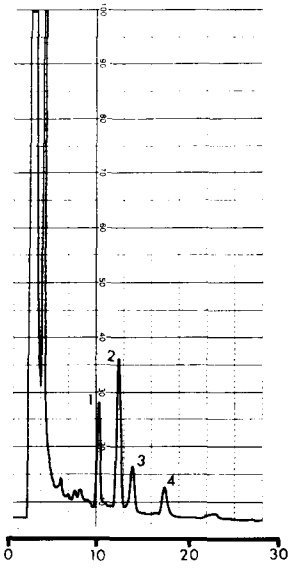

Fig. 3. HPLC separation of Dns-phosphatidylethanolamine prepared by reacting diacylglycerols derived from egg phosphatidylcholine with Dns-ethanolamine phosphate. Fatty acid composition of peaks 1–4 were 16:0–18:2, 16:0–18:1, 18:0–18:2 and 18:0–18:1, respectively.

as observed in phosphatidylethanolamine, although the relative amounts were quite different. The observation that the majority of egg phosphatidylcholine is composed of 16 and 18 carbon mono- and dienes is in agreement with other studies[1,5–7].

The present report complements our previous study[17] documenting the formation of Dns-phosphatidylethanolamine from phosphatidic acid. In the previous study, which utilized C_{18} reversed-phase columns, incomplete resolution of molecular species was noted. Much superior resolution, with an equivalent overall retention time of some 20 min, was achieved with the C_8 column. Several recent reports have described the formation and separation of UV and fluorescent derivatives of diacylglycerols. For example Batley *et al.*[14] prepared and separated *p*-nitrobenzoate derivatives of diacylglycerols by HPLC while Kruger *et al.*[16] separated fluorescent naphthylurethane derivatives. Blank *et al.*[15] separated UV absorbing benzoate derivatives of diadrylglycerols prepared from diacyl, alkylacyl, and alk-1-enylacyl glycerophosphatides. The present approach offers the advantage of forming a derivative which may be readily compared to phosphatidylethanolamine, excellent resolution on C_8 columns, and, as Dns derivatives emit light at visible wavelengths, the reaction can be conveniently monitored by TLC.

Esterification of diacylglycerol to Dns-ethanolamine phosphate was chosen in the present studies to allow comparison of equivalent derivatives of phosphatidate or phosphatidylethanolamine. As TPS has been used for the formation of a variety of esters, this approach should be suitable for the formation of other esters of diacylglycerols.

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