Isolation and Identification of Palmitoylphosphocholinepropanediol from γ-Irradiated Dipalmitoylphosphatidylcholine

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1-Palmitoyl-3-phosphocholinepropanediol was isolated from γ-irradiated aqueous suspensions of dipalmitoylphosphatidylcholine. The product was positively identified by comparison of its high-performance liquid chromatography elution time and its mass spectra with the synthetic compound.

KEY WORDS: **Dipalmitoylphosphatidylcholine, high-performance liquid chromatography, liquid secondary-ion mass spectrometry, palmitoylphosphocholinepropanediol, γ-radiation.**

Our previous research into the effects of γ-radiation on phospholipids in aqueous suspension (1) led to the isolation from γ-irradiated dipalmitoylphosphatidylcholine (PPPC) of a previously unreported radiolytic product 1-O-hexadecanoyl-3-O{[(2-trimethylamino) ethyl]phosphoryl}-propanediol, more commonly referred to as 1-palmitoyl-3-phosphocholinepropanediol (PPNPD). Chemical synthesis of PPNPD was reported in 1967 (2) and later improved by direct amination (3). Synthetic PPNPD has been shown to be a phospholipase inhibitor (4,5), a uridine-5′-diphosphate (UDP) glucuronosyltransferase activator (6) and a good detergent for the purification of UDP-glucuronosyltransferase (7); yet no mention of endogenous PPNPD appears in the literature.

MATERIALS AND METHODS

Chemicals. Synthetic PPNPD, the structure of which was confirmed by nuclear magnetic resonance, infrared and mass spectra (4), was graciously contributed by Dr. M.K. Jain (Department of Chemistry, University of Delaware). Synthetic PPPC and monopalmitoylphosphatidylcholine [lysophosphatidylcholine (LPC)] were obtained from Avanti Polar Lipids, Inc. (Alabaster, AL). Synthetic dipalmitoylphosphatidic acid (PPPA) in the acid form was obtained from Sigma Chemical Co. (St. Louis, MO). Chromatography-grade solvents were obtained from Burdick and Jackson (Muskegon, MI). Ultrapure reagent-grade ammonium hydroxide (22.9%) was obtained from J.T. Baker Chemical Co. (Phillipsburg, NJ).

Phospholipid suspensions. Aliquots (250 μL) of a stock solution of PPPC in chloroform (20 μmol/mL) were evaporated to dryness with nitrogen, and 1.0 mL water was added for a suspension of 5.0 μmol/mL. PPPC was suspended by vortexing (1 min) and sonicating (5 min) twice in sequence. Samples were kept at 0–4°C and vortexed prior to irradiation.

γ-Radiation. PPPC suspensions were irradiated at 0–4°C with a ^{137}Cs source (0.114 kGy/min) to a final dose of 9.66 kGy. Irradiated and control samples (nonirradiated suspensions kept at 0–4°C) were frozen (−80°C), lyophilized and stored (−80°C) until analyzed.

High-performance liquid chromatography (HPLC) instrumentation. HPLC was performed on a Chrompack (Raritan, NY) ChromSep 7-micron LiChrosorb Si 60 silica glass column (10 cm × 3.0 mm) with a silica guard column (10 × 2.1 mm) in a metal cartridge system, with a Waters (Milford, MA) Millipore 510 pump operated with an Autochrom (Milford, MA) Model 300 static gradient controller, followed by a Spectra-Physics (San Jose, CA) SP8500 dynamic mixer. Samples were reconstituted in 2:1 chloroform/methanol (250 μL) prior to injection of 40 μL into a 50-μL loop on a Rheodyne (Cotati, CA) Model 7125 injector. A Varex (Rockville, MD) Modell IIA evaporative light-scattering detector (ELSD) was operated at 80°C (exhaust at 54.7°C) with nitrogen as the nebulizing gas (43 psi).

Chromatographic methods. The HPLC method by Becart *et al.* (8) was modified. PPNPD was isolated from irradiated PPPC by two sequential binary-gradient HPLC methods (Table 1). Method 1 isolated PPPA and PPNPD from PPPC, and Method 2 separated the PPPA and PPNPD for identification. Elution times were determined with the ELSD attached, and timed collections were done at the column outlet prior to the ELSD. For Method 1 the elution times of PPPA, PPNPD and LPC were 20.5, 20.4 and 33.6 min, respectively; sample collection was from 17.8 to 29.0 min. For Method 2 the elution times of PPPA, 480-amu peak #1, 480-amu peak #2 (PPNPD) and LPC were 6.8, 9.6, 10.8 and 18.9 min, respectively, as seen in Figure 1. The following regions were collected for Method 2: for PPPA, 5.4–8.0 min; for 480-amu #1, 9.4–10.6 min, for 480-amu #2, 10.7–13.0 min; and for LPC, 13.3–21.1 min. For both methods, several timed collections were pooled to increase the final sample concentration so that adequate chromatograms and mass spectra could be obtained. By using Method 1, the PPPA region, from injections of irradiated PPPC, was collected and evaporated. By means of Method 2, the PPPA, 480-amu peak #1, 480-amu peak #2 and LPC regions, from injections of the PPPA region collected by Method 1, were collected and evaporated for mass spectrometry (MS). An aliquot of each collected sample was individually re-injected with the

TABLE 1

Mobile Phase Gradients for the Isolation of PPNPD from Irradiated PPPC[a]

Time (min)	Mobile phase (percent, vol/vol) Eluant A[b]	Eluant B[c]	Flow rate (mL/min)
Method 1			
0	75	25	0.4
28	30	70	
31	75	25	
Method 2			
0	55	45	0.5
4	45	55	
6	55	45	

[a]Injections were made every 35 min. PPNPD, 1-palmitoyl-3-phosphocholinepropanediol; PPPC, dipalmitoylphosphatidylcholine.
[b]Eluant A: chloroform/methanol/ammonium hydroxide (80:19.3:0.7).
[c]Eluant B: chloroform/methanol/water/ammonium hydroxide (60:34:5.3:0.7).

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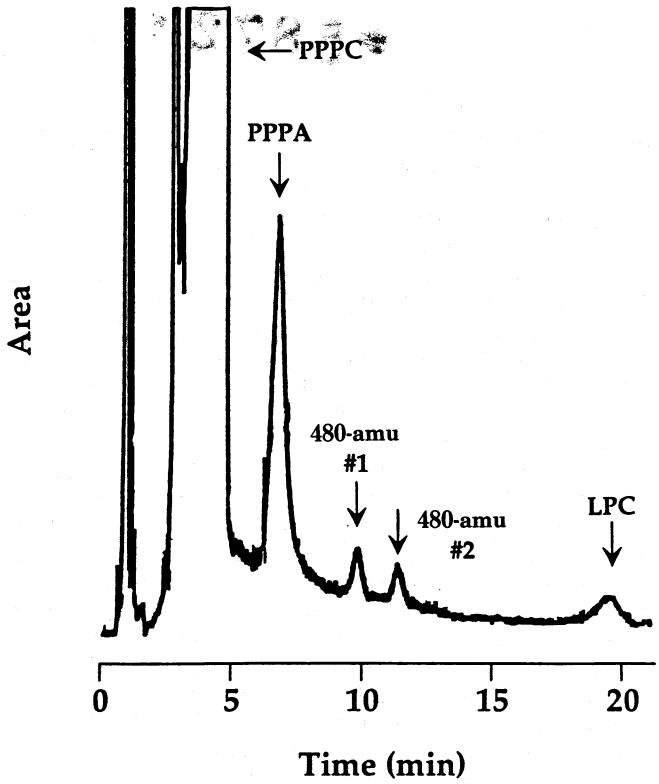

FIG. 1. High-performance liquid chromatography Method 2 chromatogram of the dipalmitoylphosphatidic acid (PPPA) region collected from irradiated dipalmitoylphosphatidylcholine (PPPC). LPC, lysophosphatidylcholine.

appropriate HPLC method to confirm that the desired region or peak had been collected.

MS. Positive (+) mode liquid secondary-ion MS (LSIMS) was performed in a matrix of triethanolamine on a VG Analytical (Manchester, United Kingdom) ZAB-T mass spectrometer. The LSIMS was performed by the Center for Advanced Food Technology, Cook College, Rutgers University (New Brunswick, NJ).

RESULTS AND DISCUSSION

The first indication of PPNPD as a radiolytic product of PPC was seen when two unexplainable ions of 480.4 amu and 959.9 amu were detected in the +LSIMS spectra of radiolytically-generated PPPA, collected by HPLC Method 1. In LSIMS, the presence of a dimeric ion is indicative of a molecular ion, thus the two ions were interpreted as being due to a compound that co-elutes with PPPA and generates a molecular ion of 480.4 amu and a dimer of 959.9 amu. The actual molecular structure of the 480-amu compound was suggested when the MS-MS of the 480-amu ion revealed a spectrum similar to that of LPC, but with many of the ions shifted by the loss of 16 amu, suggesting that the 480-amu ion had a structure similar to LPC minus one oxygen.

Method 1 was not only instrumental in initially detecting the presence of the 480-amu compound in irradiated PPPC, but also in separating PPNPD from the majority of the excess PPPC present in the original irradiated sample. Because PPNPD co-elutes with PPPA in Method 1, Method 2 was then used to separate PPNPD from PPPA. Method 2, however, separated two small peaks out from under the PPPA peak (Fig. 1) and LSIMS of each HPLC peak yielded identical spectra with a molecular ion of 480.4 amu and a dimer of 959.9 amu. Because LSIMS is an ion bombardment technique, the LSIMS spectrum of synthetic PPNPD yielded no significant structural ions besides the 480.4-amu molecular ion and the dimer of 595.9 amu. These ions were also seen in the LSIMS spectra of both isolated HPLC peaks. Also, due to the presence of column matrix which were collected with the two HPLC peaks, additional ions were seen in the spectra of these samples. The additional ions matched those seen in the spectra of the column matrix. Because palmitoylphosphocholinepropanediol can exist as the two positional isomers PPNPD or 1-phosphocholine-2-palmitoylpropanediol, two 480-amu HPLC peaks were not totally unexpected. Because peak #2 has the same retention time as synthetic PPNPD, and only peak #2 increased in area when a Method 1 sample was spiked with synthetic PPNPD, HPLC peak #2 has been assigned the structure of authentic PPNPD, and peak #1 is probably 1-phosphocholine-2-palmitoylpropanediol. The structural assignment is further supported by considering the results from two other structurally similar isomeric compounds. Like the two 480-amu peaks, both positional isomers of LPC can be separated by HPLC on silica columns (9,10), and MS of the positional isomers of palmitoylpropanediol diesters are identical (11). The structures of PPPA and LPC were previously confirmed by LSIMS (1). The PPPC control samples taken through the entire procedure show no extra peaks in the Method 2 chromatogram. Therefore, PPNPD is not an artifact of the methodology.

Nawar (12) has reported on the formation of propanediol diesters from irradiated triglycerides and meats and has postulated a plausible mechanism for this reaction. It seems likely that a similar mechanism may be involved in the radiolytic formation of PPNPD, yet it is not clear what effect the polar functional group, phosphocholine, may have on the relative concentrations of the two radiolytic products.

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[Received February 15, 1993; accepted June 18, 1993]