Novel route to preparation of high purity lysoplasmenylethanolamine

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Abstract A rapid and simple method has been developed for the preparation of highly purified lysoplasmenylethanolamine. The starting material, a phosphatidylethanolamine (PE) sample that contained a mixture of the 1, 2 diacyl- and 1-O-alkenyl-2-acyl forms was subjected to mild alkaline methanolysis for 20 min at room temperature. Addition of chloroform and water with vigorous mixing, but without acidification at this point, led to a preferential retention of the lysoplasmenylethanolamine in the alkaline aqueous phase and complete separation of the methyl esters into the chloroform phase. Neutralization of the alkaline phase with dilute acetic acid, followed by addition of chloroform, allowed recovery of the lysoplasmenylethanolamine in the chloroform phase in very high yields (75–80% based on vinyl ether content of starting material). On the other hand, a preparation of cholineglycerophospholipids enriched in plasmenylcholine, treated in exactly the same manner, gave a lysoplasmenylcholine that was not retained in the alkaline phase, but partitioned primarily into the chloroform-rich phase together with the methyl esters. Characterization of the purified lysoplasmenylethanolamine was achieved by thin-layer chromatography and compositional analysis. In addition, fast atom bombardment mass spectral analysis of the intact lysoplasmenylethanolamine together with gas chromatography–mass spectrometry of the dimethyl acetals derived from the 1-O-alkenyl chains allowed further proof of the structure and an assessment of the purity of this compound.—**Hanahan, D. J., T. Nouchi, S. T. Weintraub, and M. S. Olson.** Novel route to preparation of high purity lysoplasmenylethanolamine. *J. Lipid Res.* 1990. **31:** 2113–2117.

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During an investigation of the biochemical behavior of derivatives of lysoplasmenylethanolamine (1-O-alkenyl-*sn*-glycero-3-phosphoethanolamine) as inhibitors of platelet activating factor-induced platelet aggregation and secretion (Tsuji, H., Olson, M. S. and Hanahan, D. J., unpublished observations), it became necessary to prepare large quantities of this glycerophospholipid in high purity as the starting material for synthetic procedures. The most facile and widely used approach for the preparation of lysoplasmenylethanolamine is base-catalyzed methanolysis of a preparation of ethanolamineglycerophospholipids enriched in plasmenylethanolamine obtained from commercial sources or from freshly collected biological tissues such as heart or brain. Literature reports (1–3) showed considerable variability in recoveries of purified lysoplasmenyl derivatives due in part to the necessity of removing contaminating methyl esters of long chain fatty acids by chromatographic techniques. An additional factor was the high potential for degradation of the vinyl ether bond during both acidification of the alkaline reaction mixture and the chromatographic purification. In an effort to improve this method, it was discovered that if the alkaline methanolysis reaction mixture was not neutralized immediately at the end of the reaction but separated into two phases by the addition of chloroform and water, the lysoplasmenylethanolamine, free of methyl esters, was preferentially retained in the alkaline phase. Subsequent neutralization of the latter extract with dilute acetic acid, followed by extraction with chloroform, led to excellent recovery of highly purified lysoplasmenylethanolamine. On the other hand, as expected, a preparation of cholineglycerophospholipids enriched in plasmenylcholine showed retention of the lysoplasmenylcholine (plus methyl esters) in the first chloroform extract of the alkaline methanolysis reaction mixture. The solubility of the lysoplasmenylethanolamine in an alkaline medium could be attributed to its pKa value (4).

The details of this rather simple and efficient procedure for the preparation of lysoplasmenylethanolamine are described below.

EXPERIMENTAL PROCEDURE

Materials

Plasmenylethanolamine-rich preparations were purchased from Serdary Research Laboratories (London, Ontario) and from Avanti Polar Lipids (Pelham, AL). A plasmenylcholine-

Abbreviations: TLC, thin-layer chromatography; TNS, 6-p-toluidine-2-naphthalene sulfonic acid; GC–MS, gas chromatography–mass spectrometry; FAB, fast atom bombardment.

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rich preparation also was obtained from Serdary Laboratories. Total lipids were extracted from fresh beef heart as described by Pugh, Kates, and Hanahan (5) and were subjected to chromatography on SilicAR CC-7 using 0.5 mg lipid phosphorus per gram adsorbent and a column with a height to diameter ratio of 10–15 to 1. The lipids were eluted with the following solvent systems (v/v): chloroform; chloroform–methanol 20:1; chloroform–methanol 4:1; and chloroform–methanol 1:4. Primary interest centered on the chloroform–methanol 4:1 eluate which was the plasmenylethanolamine-rich fraction.

In most of the experiments described in this report, the Avanti PE preparation, which showed a single spot at R_f 0.73 on TLC in chloroform–methanol–water 65:35:6 (v/v), was the starting material. However, all other samples behaved in a similar manner.

Even though the ethanolamineglycerophospholipid starting material was remarkably stable when stored at −25 °C in chloroform–methanol 20:1 (v/v), it was noted that with time a second lipid phosphorus component, migrating with streaking, developed near the origin on silica gel G plates in chloroform–methanol–water. This material persisted into the alkaline phase and was assumed to be an acidic component, probably a lysophosphatidic acid-like compound. It could be removed easily by thin-layer chromatographic purification of the original PE sample. This component did not form during the alkaline methanolysis procedure as described above, unless incubations at higher temperatures (37 °C) or for longer time periods (1 hr or more) were used.

Methods

Thin-layer chromatography was accomplished using precoated silica gel G plates, 250 μm or 500 μm (Analtech, Newark, NJ) that were prewashed in chloroform–methanol–water 65:35:6 (v/v), air dried, and placed at 110 °C for 15 to 20 min immediately before use. Two solvent systems were used for separation of glycerophospholipids: a) neutral, chloroform–methanol–water 65:35:6 (v/v) and b) basic, chloroform–methanol–ammonia (28%), 80:20:2 (v/v). Subsequent to chromatography on these plates compounds were visualized by spraying with a TNS solution as described by Jones, Keenan, and Horowitz (6), and located under fluorescent light. Qualitative detection of compounds also was effected by spray reagents for phosphorus, free amino groups, vinyl ether bonds, and organic material as described by Kates (7).

A quantitative evaluation of the level of vinyl ether linkages in various phospholipid preparations was achieved by the direct iodine uptake method (8) or after acid hydrolysis by the *p*-nitrophenylhydrazine technique (9). In the latter assay, a palmitaldehyde-bisulfite complex was used as the standard. Total lipid phosphorus was determined, subsequent to perchloric acid digestion of samples, by a modification of the procedure of Allen (10). Qualitative detection of acyl ester and vinyl ether bonds was accomplished through infrared spectroscopic examination of the sample in chloroform (Burdick-Jackson, Muskegon, MI) using a Perkin-Elmer Model 283 B instrument.

Dimethyl acetals were prepared by acid treatment of the vinyl ether-containing phospholipid as outlined by Kates (7). Subsequent to purification on TLC, using a solvent system of petroleum ether (bp 30–60 °C)–diethyl ether–acetic acid 80:20:1 (v/v), the thin-layer plate was sprayed with TNS reagent and the area migrating at an R_f of 0.63 was removed by scraping and then extracted using the Bligh-Dyer technique (11).

Electron impact mass spectra were acquired on a Finnigan-MAT model 4615 quadrupole mass spectrometer in combination with an INCOS data system. The ion source temperature was 180 °C, and the electron energy was 70 eV. Gas chromatographic separation was accomplished by means of a 12 m × 0.32 mm BP-1 fused silica capillary column (SGE, Austin, TX) that was connected directly to the mass spectrometer. Helium was used as the carrier gas at a linear velocity of approximately 70 cm/sec. Both the injector and the interface to the mass spectrometer were maintained at 250 °C. An initial GC oven temperature of 170 °C was held for 2 min and then was increased at 20 °/min to a final temperature of 250 °C. "Split" injections (20 ml/min splitter flow) were utilized.

Fast atom bombardment (FAB) mass spectra were obtained on a Finnigan-MAT model 212 double focusing mass spectrometer in combination with an INCOS data system. An Ion Tech saddle field atom gun was used with xenon at 9 kV. The ion source temperature was approximately 70 °C, and the accelerating voltage was 3 kV. Samples were dissolved in chloroform–methanol 1:1 (v/v) and a few microliters were applied to the copper probe tip. Two μl of thioglycerol was then added and mixed thoroughly with the sample.

Base-catalyzed methanolysis

In a typical reaction, a phosphatidylethanolamine sample (200–400 μg P), containing both diacyl and 1-O-alkenyl-2-acyl forms, was evaporated to dryness under nitrogen. The sample was resuspended in 0.2 ml $CHCl_3$ and 1.0 ml 0.25 M KOH in CH_3OH was added. The mixture was allowed to stand at room temperature for 25 min, at which point 0.8 ml $CHCl_3$, 1.0 ml CH_3OH, and 0.8 ml H_2O were added. The mixture was vortexed well, and 1.0 ml each of $CHCl_3$ and H_2O was added. To facilitate phase separation, CH_3OH was added dropwise to help clear any emulsion. The reaction mixture was vortexed well and centrifuged at 800 *g* for 10 min. The chloroform-rich (CI) (bottom) layer was removed and saved for phosphorus determination and/or further extraction. The pH of the water-rich (upper) layer was adjusted to 6.0 by dropwise addition of 1 M acetic acid. (The water-rich fraction had an initial pH of near 12.0.) The water-rich fraction became very turbid upon

addition of the acetic acid. After this treatment, 1.5 ml $CHCl_3$ was added and the mixture was mixed well and centrifuged at 800 *g* for 10 min. The water-rich (AII) fraction was saved for phosphorus analysis. The chloroform-rich (CII) (lower) layer was removed and evaporated to dryness under nitrogen. The residue was redissolved in $CHCl_3$–CH_3OH 1:1 (v/v). Approximately 75–80% of the lysoplasmenylethanolamine was recovered at this point. The remainder was recovered from CI, as described below.

The entire chloroform-rich (CI) layer (1.5 ml total volume) was washed with 2 ml CH_3OH–H_2O 10:9 (v/v). The mixture was vortexed well and centrifuged at 800 *g* for 10 min. The washed chloroform-rich (CI) fraction was saved for phosphorus determination. The water-rich (upper) layer was removed and 2 ml hexane (saturated with CH_3OH) was added to extract any remaining methyl esters. The sample was mixed well and centrifuged as above. If an emulsion formed, CH_3OH was added dropwise to facilitate phase separation. The hexane (AIHX) layer (upper) was removed and the remaining water-rich fraction (lower) was neutralized with 1 M acetic acid, then 1.5 ml $CHCl_3$ was added. The sample was mixed and centrifuged as above. The chloroform-rich (AIC) layer (lower) was removed, evaporated under nitrogen, and dissolved in $CHCl_3$–CH_3OH 1:1 (v/v). The water-rich upper layer (AI) was saved for phosphorus analysis only.

All fractions were assayed for phosphorus, and except for A-I and A-II fractions, were examined by thin-layer chromatography and mass spectrometry.

RESULTS

General comments

This procedure represents a particularly effective and facile approach to preparation of lysoplasmenylethanolamine. As shown in **Table 1**, the distribution of phosphorus-containing material in the various fractions (described above) was very consistent, and the overall yield was excellent. As is evident, the C-II fraction which contained the pure lysoplasmenylethanolamine, showed a recovery of approximately 75–80% of the calculated amount. Essentially the remainder of the lysoplasmenylethanolamine was obtained from the washes of C-I as described in the experimental section above. Thus, one can isolate nearly all of the vinyl ether derivative by following the entire procedure. Several other preparations using different sources of ethanolamineglycerophospholipids gave very similar results.

Examination of the A-II fraction demonstrated the presence of organic phosphorus and ninhydrin-reactive material, but not any vinyl ether (Schiff base-reactive) component. While there was no further preparative or analytical treatment of this fraction, it was assumed that the major component was glycerophosphoethanolamine.

TABLE 1. Partitioning of phosphate after mild alkaline methanolysis of an ethanolamineglycerophospholipid preparation enriched in plasmenylethanolamine

Starting Sample	Fraction					
	C-I	C-II	AIHX	AIC	AI	AII
μg P						
315	7.0	40	1.0	13	10	28
316	9.5	41	0.9	9.7	11	28
320	8.0	38	0.8	8.0	18	28

Results are based on percent of starting phosphatidylethanolamine-phosphorus and represent three separate experiments using an Avanti Polar Lipid bovine heart preparation. Briefly the protocol was as follows. The starting material was subject to mild alkaline methanolysis and, without acidification, was extracted with chloroform. The latter lower phase was termed C-I and contained primarily the methyl esters and a small amount of lysoplasmenylethanolamine. The upper, water-rich alkaline phase was neutralized and extracted with chloroform. The lower, chloroform-rich phase, termed C-II, contained only the lysoplasmenylethanolamine component. The other fractions, AI, AII, etc. represent fractions resulting from manipulations designed to remove methyl ester contamination and to allow full recovery of lysoplasmenylethanolamine. Further details are given in the text.

Interestingly, this procedure also was applied effectively to a preparation of choline glycerophospholipids enriched in plasmenylcholine except that the resulting lysoplasmenylcholine was extracted into the C-I fraction along with the methyl esters derived from the fatty acyl groups present in the starting material. Consequently, this reaction mixture was subjected to further purification, usually by thin-layer chromatography, to obtain pure lysoplasmenylcholine.

Characteristics of lysoplasmenylethanolamine

Upon thin-layer chromatography, the C-II fraction in a solvent system of chloroform-methanol-water 65:35:6 (v/v) showed a single band at R_f 0.33. The other lysoplasmenylethanolamine-containing fractions, C-I, and AIC, all had similar R_f values. In addition, these samples gave only one spot for phosphorus, ninhydrin, and Schiff base (vinyl ether)-reactive material. When the pure lysoplasmenylethanolamine was examined by infrared spectroscopy, it showed a strong C–H stretching doublet at 2925 cm^{-1} and 2858 cm^{-1}, a deep sharp band at 1655 cm^{-1}, indicative of a *cis*-vinyl (alkenyl) group and a strong, rather broad band, in the region from 1078 to 1030 cm^{-1} composed most probably of a phosphate and an ether moiety. Even in very concentrated solutions (1–2% in chloroform in a 1-mm cell) the lysoplasmenylethanolamine showed no absorption at 1735 cm^{-1} characteristic of a fatty acyl ester. Finally, using the techniques of Pietruszko and Gray (1) and Wells and Dittmer (12), no evidence for the occurrence of any cyclic acetal was obtained.

The determination of the presence of vinyl ether bonds using the iodine method or the *p*-nitrophenylhydrazine technique indicated a vinyl ether/phosphorus molar ratio of

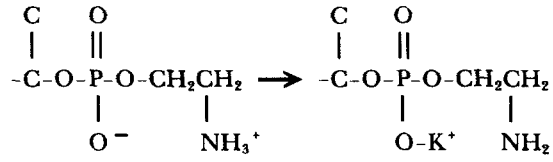
0.95 to 0.97 in the purified lyso derivative (data not shown). In further support of the high level of vinyl ether bonds, acid treatment of the purified lysoplasmenylethanolamine (0.1 N HCl in 95% methanol for 30 min at 37 °C) showed that less than 2% was stable under these conditions.

GC-MS analysis of the dimethyl acetals derived from the alkenyl (vinyl ether) residues by acid methanolysis revealed that the vinyl ether chains contained 16:0, 18:0, and 18:1 (two isomers) moieties. A minor amount of a heptadecenyl homolog also was observed. In addition, there was a GC peak eluting at a time appropriate for a 22-carbon homolog that exhibited the characteristic *m/z* 75 base peak in its mass spectrum. However, the intensities of the other fragments were too low to determine whether the corresponding $[M-31]^+$, seen in dimethal acetals, was also present.

Examination of the intact lysoplasmenylethanolamine by FAB mass spectrometry revealed that protonated molecular ions were observed at *m/z* 438, *m/z* 464, and *m/z* 466, representative of the 16:0, 18:1, and 18:0 homologs (**Fig. 1**). Also, there were equally intense thioglycerol adduct ions at $[MH + 108]^+$: *m/z* 546, *m/z* 572, and *m/z* 574. A smaller amount of the 17-carbon homolog was indicated by a weak ion at *m/z* 452 along with the corresponding thioglycerol adduct at *m/z* 560. The base peak was seen at *m/z* 142, representative of protonated phosphoethanolamine. An additional intense ion was found at *m/z* 216, formed by the loss of the alk-1-enyl side chain and concomitant proton transfer. Ions were also recorded at *m/z* 331, *m/z* 357, and *m/z* 359 ($[MH-107]^+$) which had intensities similar to the molecular ions (approximately 30% of the base peak). These fragments apparently contain the hydrocarbon side chain as evidenced by their relative *m/z* values, but their structure has not yet been determined.

DISCUSSION

The method described in this report represents a very simple, rapid, and gentle procedure for the preparation of pure lysoplasmenylethanolamine in high yields. A distinct advantage of this approach is that it avoids an additional thin-layer (or column) chromatography procedure to remove methyl esters derived from the fatty acid esters present in the starting material. An additional advantage of this technique derives from the preferential solubility of the lysoplasmenylethanolamine in an alkaline (aqueous) medium. This result is consistent with the observations of Garvin and Karnovsky (4) who reported a "pKa" near 9.1 for dimyristoyl-glycerophosphoethanolamine. Thus, under the experimental conditions described here, the lysoplasmenylethanolamine must be converted to a water-soluble potassium salt as shown in the following partial equation:



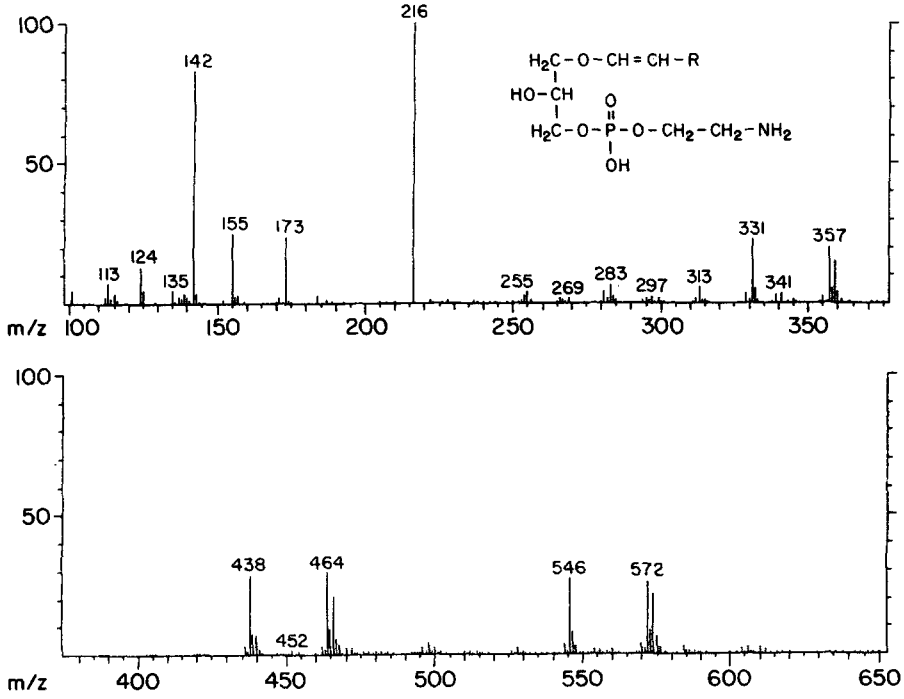


Fig. 1. Fast atom bombardment mass spectrum of purified lysoplasmenylethanolamine (C-II). The contributions from the thioglycerol matrix have been subtracted from the spectrum. Alkyl chains (R) were: $C_{14}H_{29}$ (*m/z* 438), $C_{15}H_{31}$ (*m/z* 452), $C_{16}H_{31}$ (*m/z* 464), and $C_{16}H_{33}$ (*m/z* 466).

In support of the above reasoning on the structure of the ethanolamine polar head group under alkaline conditions, it was shown that a preparation of cholineglycerophoshoplipids enriched in plasmenylcholine, which behaves as a zwitterion essentially over the entire pH range (4) and which was subjected to base-catalyzed methanolysis as above, yielded lysoplasmenylcholine that remained in the C-I fraction along with the methyl esters. Hence, as noted above, a chromatographic step was needed to obtain a pure lysoplasmenylcholine.

A further significant advantage of the experimental approach described here is that methyl esters are very effectively separated from the desired product, lysoplasmenylethanolamine, essentially in a single operation. It is interesting that Wells and Dittmer (12) noted a significant loss of "chloroform"-soluble plasmalogen from an alkaline hydrolysate of brain phospholipids when the reaction was not neutralized. The most probable explanation of this observation is that extraction of the ethanolamine derivatives into the alkaline phase occurred.

In summary, starting with an ethanolamineglycerophospholipid preparation enriched in plasmenylethanolamine free of other acidic components, a pure lysoplasmenylethanolamine can be obtained in a facile manner.

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