An HPLC procedure for separating polyphosphoinositides on hydroxylapatite

Larry E. Boyle, Larry A. Sklar, and Alexis E. Traynor-Kaplan

Department of Immunology, IMM12, Research Institute of Scripps Clinic, 10666 North Torrey Pines Road, La Jolla, CA 92037

Abstract We describe a method for separation of several phosphoinositides by high performance liquid chromatography (HPLC) for the purposes of identification, analysis, and possible purification of cell phospholipid extracts. The phosphoinositides were separated on an hydroxylapatite column using a solvent system consisting of tetrahydrofuran–ethanol–water with a gradient of triethylamine phosphate ranging from 1 to 100 mM. Increasing triethylamine phosphate concentrations over a series of isocratic steps resulted in the resolution of [^{3}H]phosphoinositide standards (phosphatidylinositol, phosphatidylinositol-4-phosphate, phosphatidylinositol-4,5-bisphosphate) and their lyso-derivatives. The eluted peaks were collected and quantitated using scintillation counting, and the samples of the eluates were subjected to further analysis by thin-layer chromatography to verify their integrity and identity. —**Boyle, L. E., L. A. Sklar, and A. E. Traynor-Kaplan.** An HPLC procedure for separating polyphosphoinositides on hydroxylapatite. *J. Lipid Res.* 1990. **31:** 157–159.

Supplementary key words phosphoinositide • HPLC • hydroxylapatite • lyso-phosphoinositide

Several techniques for separating phosphoinositides have been described and have been reviewed by Hajra, Fisher, and Agranoff (1). For example, column chromatography of phospholipids on hydroxylapatite has been used to separate phosphatidylserine, phosphatidylethanolamine, phosphatidylinositol, phosphatidylcholine, cardiolipin, and sphingomyelin (2), and DEAE cellulose has been used to resolve individual phosphoinositides after they have been converted to their alkali metal salts (3). Schacht (4) developed a column chromatography technique on neomycin-adducted glass beads for phospholipids, but the procedure is not adaptable to HPLC at this time. Hydroxylapatite HPLC columns have been widely used for nucleic acid and peptide chromatography, with isolated reports for use in lipid purification. HPLC techniques have also successfully resolved several phospholipids (5) and some success has been made resolving derivatized polyphosphoinositides (6). However, none of the procedures have shown resolution of the more phosphorylated phosphoinositides, phosphatidylinositol-4-phosphate (PIP) and phosphatidylinositol-4,5-bisphosphate (PIP_2) in their native form nor have they analyzed their lysophosphoinositide derivatives. Primes and coworkers (7) described a technique for separating phospholipids on hydroxylapatite using a gradient of triethylamine phosphate (TEAP) in ether–ethanol–water.

Here we have modified the procedure of Primes et al. (7) to resolve PI, PIP, PIP_2, and their lyso-derivatives on an hydroxylapatite column. The method used tetrahydrofuran instead of ether and higher concentrations of triethylamine phosphate. There are several advantages to using THF instead of ether. The volatility of ether makes it difficult to maintain a constant concentration in a mixture. THF is much less volatile but has a similar dielectric constant. Furthermore, triethylamine phosphate is more soluble in THF than in ether. The presence of peroxides in either THF or ether, however, can cause lipid oxidation which must be kept to a minimum.

MATERIALS AND METHODS

Reagents, phospholipase A_2 from Russell's Viper venom (PLA_2), mixed phosphoinositides (bovine brain), PIP, PI-4,5-P_2, were all obtained from Sigma Chemical Company, St. Louis, MO. HPLC grade tetrahydrofuran, chloroform, methanol, ethanol, and triethylamine were from Fisher. [2-^{3}H(N)]Myo-inositol-labeled PI, [2-^{3}H(N)[inositol-labeled PIP and -PI-4,5-P_2 were obtained from New England Nuclear Corporation, Boston, MA.

The phosphoinositides were chromatographed over a period ranging up to 75 min at room temperature at a constant flow of 1 ml/min, using a Perkin-Elmer Chromatograph Series 4 and a 500 μL injection loop. Samples were filtered through Xydex Teflon filters before injection. All of the solvents were degassed before use. The hydroxylapatite column was a 100 × 78 mm Bio-Gel HPHT model from Bio-Rad labs of Richmond, CA. No guard column was used. We used a multistep gradient starting with a 1 mM solution of TEAP in THF–ethanol–water 54:31:15 (v/v/v), solvent (A). The elution solvent (B) contained 100 mM TEAP in the same solvent mixture as (A).

For optimal separation of the phosphoinositides, the steps of the gradient were as follows. After loading the lipid material onto the column for 3 min with 100% buffer A, the concentration of TEAP was increased linearly over 18 min to 16 mM. This concentration was maintained for an additional 15 min. The TEAP concentration was then increased linearly to 100 mM over a period of 30 min and maintained at 100 mM for an additional 10 min. Keeping the buffers at pH 6.5 provided the best combination of HPHT column life and phosphoinositide stability. TEAP was prepared by adding the triethylamine to the desired final proportion in buffer A or B, adjusting the pH with HPLC grade phosphoric acid, and then adding the THF.

Abbreviations: HPLC, high performance liquid chromatography; TLC, thin-layer chromatography; TEAP, triethylamine phosphate; THF, tetrahydrofuran; PI, phosphatidylinositol; PIP, phosphatidylinositol-4-phosphate; PIP_2, phosphatidylinositol-4,5-bisphosphate; PLA_2, phospholipase A_2; BHT, butylated hydroxytoluene.

Stock solutions of the standards for injection were prepared by suspending 7.5 μl of ^3H standard (0.01 mCi/ml) in 2 ml of $CHCl_3$–MeOH 1:1 with 0.02 mg/ml of unlabeled phosphoinositides (bovine braine) added as a carrier. The lysophosphoinositides were prepared from the ^3H standards using PLA_2 by the following method. 7.5 μl of the ^3H standard was dissolved in H_2O-saturated $CHCl_3$ and dried under nitrogen. Four hundred and fifty μl of buffer (30 mM HEPES, 110 mM NaCl, 10 mM KCl, 1 mM $MgCl_2$, 10 mM glucose, 1.53 mM Ca^{2+}, pH 6.5) and 1.38 units of PLA_2 were then added. After incubating the solution overnight at 36°C, cold ethanol was added and the mixture was filtered through a 0.45 μm Xydex Teflon filter syringe to remove the protein.

The HPLC eluates were prepared for TLC analysis by the use of a phospholipid extraction procedure. The eluates (0.5 ml) were added to 5 ml of a mixture of chloroform–methanol–BHT 1:2:5 containing 0.04 mg phosphoinositides per ml. Chloroform (3 ml) was then added. Subsequent lipid extraction was carried out according to Schacht (4). Then the combined lower phases of each fraction were dried under nitrogen and resuspended in chloroform for spotting on EM Science silica gel 60 TLC plates. The plates were impregnated with a 1.2% solution of potassium oxalate in methanol–water 2:3 and activated at 110°C for 20 min. The plates were developed in chloroform–acetone–methanol–acetic acid–water 80:36:26:24:14. After the plates were dried the phosphoinositides were visualized by iodine staining. The regions corresponding the authentic PI, PIP, and PIP_2 were scraped from the plates and counted by scintillation counting.

RESULTS AND DISCUSSION

The goal of these studies was twofold: *1*) to define a rapid HPLC separation condition for polyphosphoinositides; and *2*) to develop a procedure for purification of lysoPIP after its enzymatic production. We found that the degree of phosphorylation of the phosphoinositides was a factor in their interaction with the hydroxylapatite such that elution times were reliably manipulated according to the TEAP concentration. Separation of PI, PIP, and PIP_2 is shown in (**Fig. 1**). We were able to achieve essentially baseline separation of PI, PIP, and PIP_2 peaks. The PI standard eluted at approximately 5 mM TEAP, with PIP following at 16 mM TEAP and PIP_2 at 35–45 mM TEAP. All of the standards were also run separately to verify elution times and sample purity. Percent recovery evaluated from these runs consistently ranged from 90 to 99%. As suggested from further experiments (shown below), the small peak near 40 min and the shoulder on the PIP_2 were probably associated with lysoPIP and lysoPIP$_2$, small amounts of which might have been present as impurities.

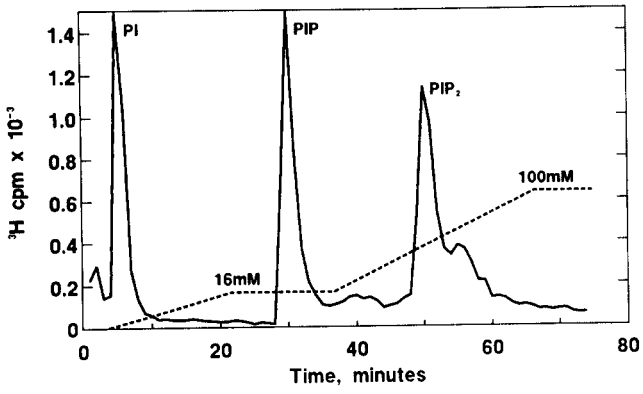

Fig. 1. HPLC resolution of phosphoinositide standards. ^3H-labeled PI, PI-4-P, and PI-4,5-P_2 were injected together, and the eluates were collected over 75 min in 1-min fractions. The PI standard can be seen at 5 min (at approximately 5 mM TEAP), followed by PIP at 30 min (16 mM), and PIP_2 at 50 min (35–45 mM). The standards were contaminated with small amounts of lysoPIP (40 min) and lysoPIP$_2$ (55 min) respectively. This figure is representative of four elution profiles.

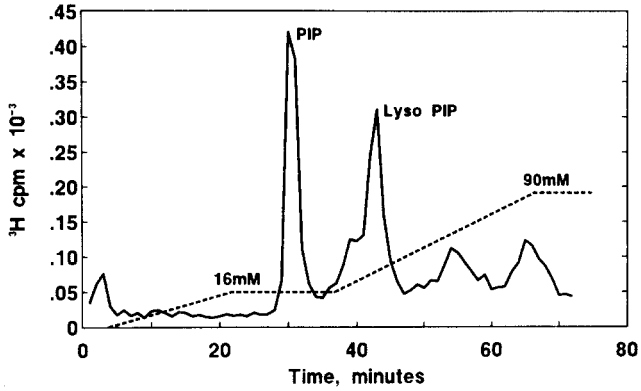

Fig. 2. Resolution of PIP from LysoPIP. The PIP standard was combined and injected with newly prepared lysoPIP. The PIP peak can be seen at 30 min with the lysoPIP at 43 min. Smaller unknown peaks are at 55 and 65 min. The gradient used was a modification of that seen in Fig. 1; the amount of water as part of the total solvent mixture was increased isocratically by 10% after 36 min to favor the complete elution of the lysoPIP.

To check the integrity of the resolved phosphoinositides, predetermined amounts of the HPLC peak eluates were subjected to a lipid extraction and spotted on TLC after adding nonradioactive phosphoinositides. The nonradioactive lipids served both as carrier during the extraction and for visualization of mass by iodine staining on the plates. In all cases the bulk (typically greater than 80%) of the eluted radioactivity from HPLC comigrated on TLC with the authentic radioactive standard spotted directly onto the plate. These results verified the identity of the eluted materials and confirmed that their integrity was maintained through the HPLC, extraction, and recovery protocols.

Because we and other investigators (8, 9) have been interested in possible roles of lysolipids as intracellular signals derived from the activation of PLA_2, we defined HPLC conditions to resolve lysoPIP prepared from PIP and lysoPIP$_2$ from PIP_2 as illustrated in **Fig. 2** and **Fig. 3**, respectively. The gradient used for PIP and lysoPIP was slightly modi-

fied from the conditions shown in Fig. 1. While the PIP standard again eluted at 30 min, the lysoPIP yielded a consistent peak eluting at 43 min. By increasing the water concentration after 36 min, we were able to improve resolution of the lyso-derivatives, probably a result of their greater hydrophilicity compared to their parent compounds. Smaller unknown peaks, probably contaminants, migrated in Fig. 2 at 55 and 65 min.

In order to resolve lysoPIP$_2$ (Fig. 3), the gradient used a higher TEAP concentration while following the same time profile as Figs. 1 and 2 to speed the elution of the more slowly migrating species, PIP$_2$ and lysoPIP$_2$. The PIP$_2$ peak was centered at 30 min while the lysoPIP$_2$ eluted at 43 min. Under these new conditions, the elution pattern closely followed that of PIP and lysoPIP. Since no authentic standards of the lysophospholipids were available, we did not attempt to verify further the integrity of the eluates.

It is worthwhile to comment about the anticipated utility of these HPLC procedures at their current stage of development. Because of the hope of using these methods on phosphoinositides extracted from cells or membranes labeled with ^{32}P, we characterized the behavior of several ^{32}P-labeled compounds using the gradient described for Fig. 1. These included ^{32}P-labeled ATP, pyrophosphate, and orthophosphate. These phospholipid precursors were all resolved from the [^{3}H]phosphoinositide standards. All three of the phosphate compounds eluted in broad peaks at approximately 50 to 65 min, the center of the peak appearing at or near 60 min.

Because of the small quantities available we have not yet attempted to resolve even more highly phosphorylated compounds, such as PIP$_3$ recently observed by us (10). It is likely, however, that the elution conditions of Fig. 1 could be modified such that PIP$_2$ is eluted by a step to 35 mM TEAP and that PIP$_3$ could be resolved and subsequently eluted by a step to 100 mM TEAP.

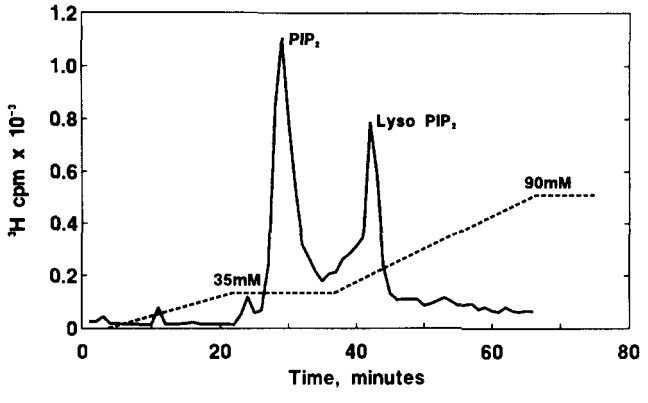

Fig. 3. Resolution of PIP$_2$ from LysoPIP$_2$. PIP$_2$ standard was combined with lysoPIP$_2$. The PIP$_2$ peak is centered at 30 min, while the lysoPIP$_2$ is at 43 min. The gradient corresponded to that of Fig. 2 but with higher starting TEAP concentrations to expedite elution.

Our initial success at resolving phosphoinositide standards by HPLC, maintaining their integrity through extraction and subsequent TLC analysis, and finally the resolution of the standards from synthetically derived lyso compounds as well as from ^{32}P-labeled phospholipid precursors suggests that these methods should find application in analysis and preparation of phosphoinositides from whole cell and tissue extracts.

We would like to express our appreciation to Dr. Robert Sanchez for suggesting hydroxylapatite as a means for separating polyphosphoinositides. This work was supported by NIH grants AI 17354, AI 19032, and RR00833. This is publication #6005-IMM from the Department of Immunology, Research Institute of Scripps Clinic.

Manuscript received 20 March 1989 and in revised form 7 August 1989.

REFERENCES

1. Hajra, A. K., S. K. Fisher, and B. W. Agranoff. 1988. Isolation, separation, and analysis of phosphoinositides from biological sources. In Neuromethods 7: Lipids and Related Compounds. A. A. Bolton, G. B. Baker, and L. A. Horrocks, editors. Humana Press, Clifton. N. J. 211–225.
2. Slomiany, B. I., and M. I. Horowitz. 1970. Separation of polar lipids by column chromatography of hydroxylapatite. *J. Chromatogr.* **49:** 455–461.
3. Hendrickson, H. S., and C. M. Ballou. 1964. Ion exchange chromatography of intact brain phosphoinositides on DEAE-cellulose by gradient salt elution in a mixed solvent system. *J. Biol. Chem.* **239:** 1369–1373.
4. Schacht, J. 1978. Purification of polyphosphoinositides by chromatography on immobilized neomycin. *J. Lipid Res.* **19:** 1063–1067.
5. Kaitaranta, J. K., and S. P. Bessman. 1981. Determination of phospholipids by a combined liquid chromatography-automated phosphorus analyzer. *Anal. Chem.* **53:** 1232–1235.
6. Nakamura, T., Y. Hatori, K. Yamada, M. Ikeda, and T. Yuzuriha. 1989. A high-performance liquid chromatographic method for the determination of polyphosphoinositides in brain. *Anal. Biochem.* **179:** 127–130.
7. Primes, K. J., R. A. Sanchez, E. K. Metzner, and K. M. Patel. 1982. Large-scale purification of phosphatidylcholine from egg yolk phospholipids by column chromatography on hydroxylapatite. *J. Chromatogr.* **236:** 519–522.
8. Wheeler, J. J., and W. F. Boss. 1987. Polyphosphoinositides are present in plasma membranes isolated from fusogenic carrot cells. *Plant Physiol.* **85:** 389–392.
9. Thomas, L. M., and B. J. Holub. 1982. The formation of lysophosphatidylinositol phosphate in human platelet microsomes. *Lipids.* **22:** 144–147.
10. Traynor-Kaplan, A. E., A. L. Harris, B. L. Thompson, P. Taylor and L. A. Sklar. 1988. An inositol tetrakisphosphate-containing phospholipid in activated neutrophils. *Nature.* **334:** 353–356.