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Note

Novel system for separation of phospholipids by high-performance liquid chromatography

IFTEKHAR ALAM*, J. BRYAN SMITH, MELVIN J. SILVER
Cardeze Foundation and Department of Pharmacology Thomas Jefferson University, Philadelphia, PA (U.S.A.)*
and
DAVID AHERN
New England Nuclear, Boston, MA (U.S.A.)
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Only a few methods for the separation of phospholipids by high-performance liquid chromatography (HPLC) have been reported[1–3] and none of them resolve all the major phospholipids found in cell membranes. We have developed a method which accomplishes this and also separates the newly characterized platelet-activating factor[4,5] from other phospholipids.

Retention times were determined by using radioactive phospholipids and a flow-through radioactivity detector. Use of a stream splitter enabled part of the effluent from the column to be diverted to a fraction collector for confirmation of radioactivity measurements. The system has advantages over thin-layer chromatography (TLC) because resolution of phosphatidylinositol from phosphatidylserine is obtained, recovery of individual phospholipids is excellent and there is no need to elute material from zones on TLC plates for subsequent analyses.

METHODS

A Waters Assoc. liquid chromatograph equipped with a Model 6000A solvent delivery system was used for HPLC. The column employed was a 250 × 4.6 mm I.D. LiChrosorb Si60 (10-μm particle size) purchased from Rainin Instrument, Woburn, MA, U.S.A. The radioactive flow detector was purchased from Radiomatic, Tampa, FA, U.S.A. The system was set up as shown in the flow chart (Fig. 1). It consisted of two solvent delivery pumps, an injector port and a solvent programmer. The effluent from the column was divided by means of a stream splitter. Half went into the radio flow detector and half into the fraction collector for liquid scintillation counting. Miniscint (Radiomatic) was continually pumped through the radio flow detector which was equipped with a built-in integrator. A chart recorder was connected to the above device which recorded the profile of radioactive components as they eluted from the column. HPLC grade solvents were obtained from Fischer Scientific, King of Prussia, PA, U.S.A. Deionized water was filtered through a millipore filter (0.45 μm). All organic solvents were degassed by sonication. Phosphati-

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dylcholine (dipalmitoyl-1-^{14}C, SA 60–100 mCi/mmole), phosphatidylethanolamine (dipalmitoyl-1-^{14}C, SA 95.0 mCi/mmole), sphingomyelin (choline-methyl-^{14}C, SA 40–60 mCi/mmole), prostaglandin-E_2, (5,6,8,11,14,15-^{3}H(N))-, SA 165 Ci/mmol) and [^{3}H]6-keto-prostaglandin $F_{1\alpha}$ (SA 120 Ci/mmol) were purchased from New England Nuclear, Boston, MA, U.S.A. Uniformly labelled [2-^{3}H]arachidonyl phosphatidylinositol (SA 30 Ci/mmole) and ^{3}H-platelet-activating factor (1-O-alkyl[2-^{3}H]acetyl glyceryl-3-phosphorylcholine, SA 50 Ci/mmole), were gifts from New England Nuclear. Phosphatidyl-L-^{14}C-serine (SA 25 mCi/mmole) and [1-^{14}C]arachidonic acid (SA 54 mCi/mmol) were purchased from Amersham, Arlington Heights, IL, U.S.A. [^{14}C]Lysophosphatidylcholine was prepared from [^{14}C]phosphatidylcholine by the methods of Wells and Hanahan[6]. [^{14}C]Lysophosphatidylcholine was purified by TLC on silica gel GH plates (Analtech, Newark, NJ, U.S.A.) using a modification of Skipski's solvent system[7], chloroform–methanol–acetic acid–water (100:44:10:3, v/v).

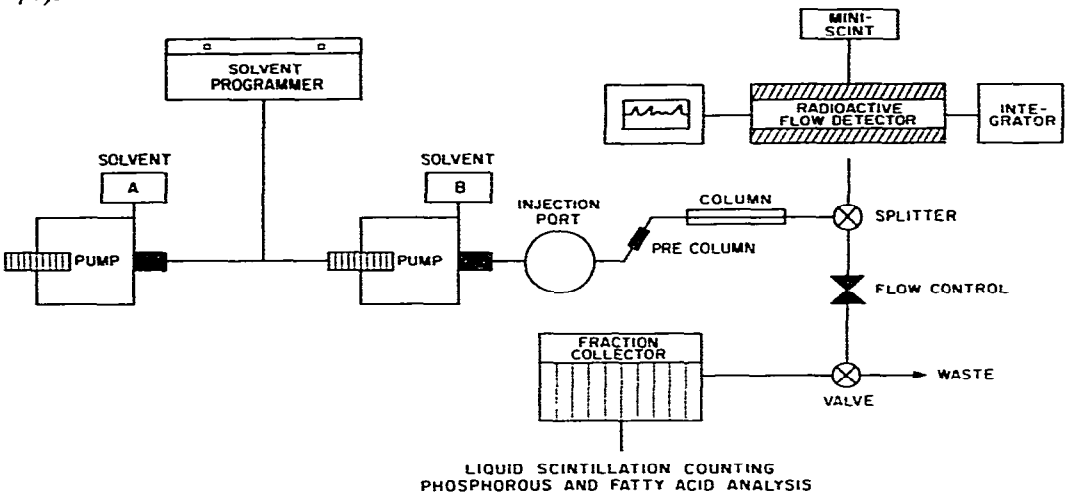

Fig. 1. Flow chart of HPLC system.

RESULTS AND DISCUSSION

The elution profile of the different phospholipids studied is shown in Figs. 2 and 3. Fig. 2 shows the recording of radioactivity (in arbitrary units) obtained directly from the Radio-Flow-One detector. Fig. 3 shows the radioactivity in individual fractions collected at 1-min intervals and measured by liquid scintillation counting. These separations were obtained on a normal-phase LiChrosorb Si60 column under isocratic conditions. The mobile phase consisted of two solvents A and B. Solvent A was a mixture of propanol–ethyl acetate–benzene–water (130:80:30:20, v/v) and solvent B was propanol–toluene–acetic acid–water (93:110:15:15, v/v). Solvent A was pumped at a flow-rate of 1 ml/min for 20 min. Prostaglandins (PGs) and arachidonic acid (AA) were eluted by 7 min (Fig. 3) and a clean phosphatidylethanolamine (PE) fraction came off by 20 min. A change in flow-rate from 1 to 1.5 ml/min enabled the elution of phosphatidylinositol (PI) by 30 min. Elution with solvent B, at 1 ml/min, was started at 36 min to elute phosphatidylserine (PS), followed by phosphatidyl-

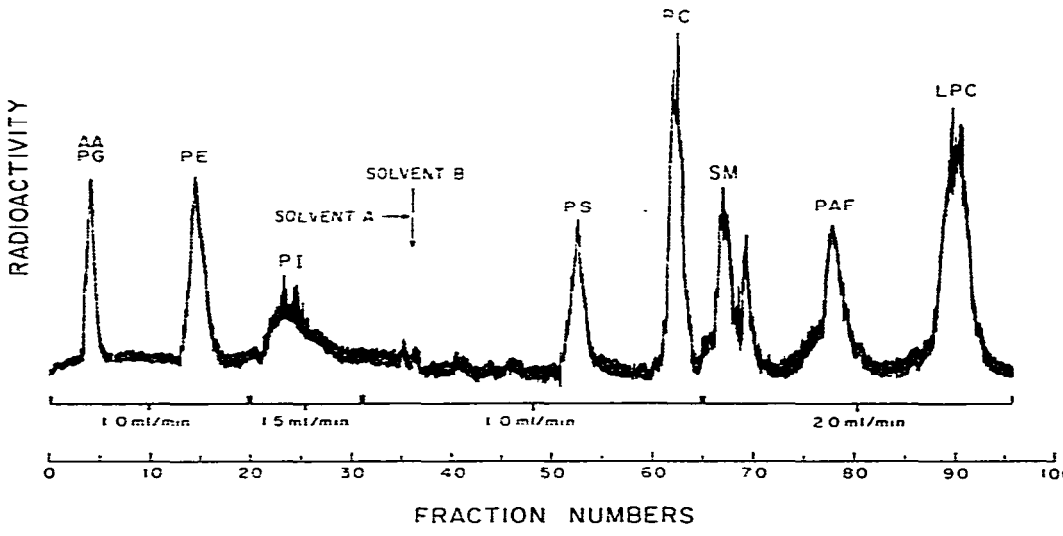

Fig. 2. Radioactivity recording from the Radio-Flow-One detector. For abbreviations, see text.

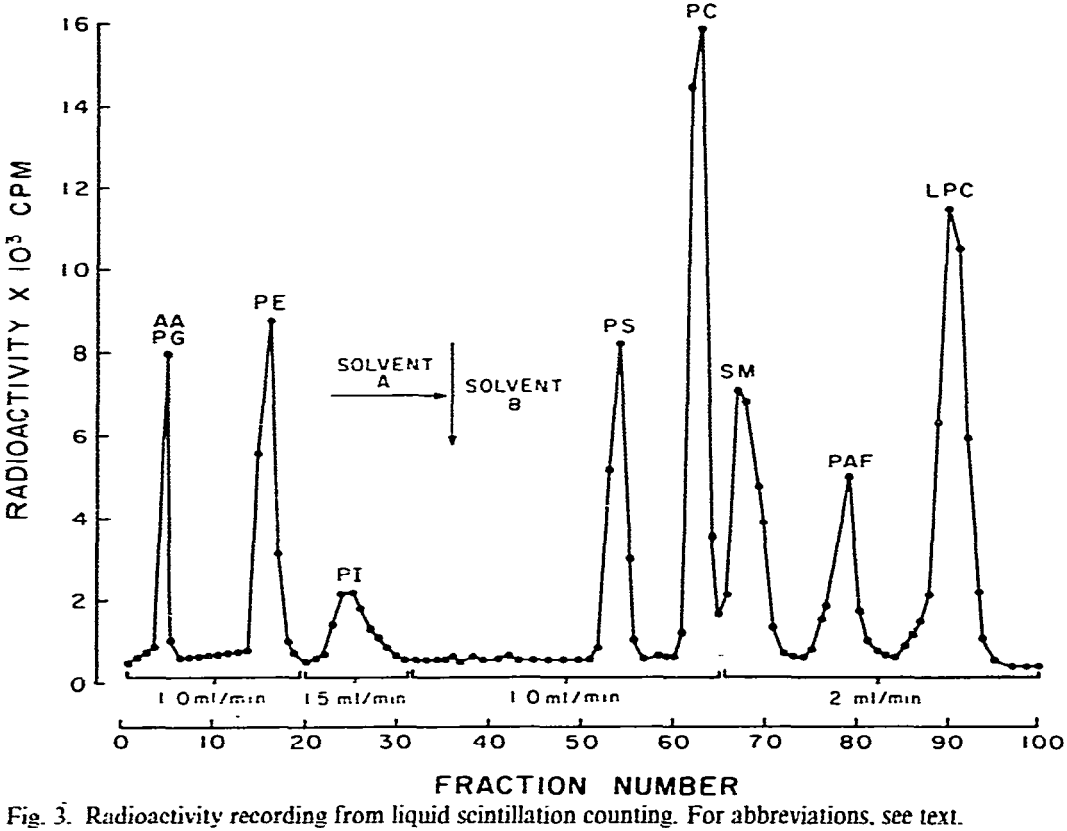

Fig. 3. Radioactivity recording from liquid scintillation counting. For abbreviations, see text.

choline (PC). At 65 min, after the elution of phosphatidylcholine, the flow-rate was increased to 2 ml/min to obtain complete resolution of sphingomyelin (SM), platelet activating factor (PAF) and lysophosphatidylcholine (LPC). The total analysis time was 100 min. The retention times of individual phospholipids analysed alone or in mixtures were highly reproducible and rarely varied by more than 1 min. Recovery of radioactivity was always greater than 90%.

Our approach for the complete separation of PE from PS and PI was based on the use of the non-amphoteric solvent ethylacetate in combination with polar solvents on a column of deactivated silica. Although detection of phospholipids by UV absorption has been used in combination with HPLC it is inappropriate when solvents such as benzene and toluene, which are excellent for separation of phospholipids but which strongly absorb UV light, are used. We obviated this difficulty by using a flow-through detector to monitor continuously the radioactivity in the effluent from the HPLC column.

The use of a stream splitter allowed us to collect fractions and count the radioactivity to validate the results obtained with the flow-through detector. This analysis revealed that the flow-through detector was superior because it monitors radioactivity every 4 sec, whereas samples were collected only at 1-min intervals by the fraction collector. For example, the radio-labelled sphingomyelin standard, which was 99% homogeneous by TLC and appeared as a single peak using the fraction collector (Fig. 3) was resolved into two components by the flow-through detector (Fig. 2). The stream splitter also will allow for collection of samples, free of silicic acid or TLC binders, which can be used for phosphorus, fatty acid or other analyses. Finally, this system should be of value in experiments involving radioactive labelling of cell membrane phospholipids as well as for the purification of individual phospholipids.

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