Journal of Chromatography, 273 (1983) 415—420
Biomedical Applications
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROMBIO. 1579

Note

Improved high-performance liquid chromatographic method for isolation of platelet-activating factor from other phospholipids

M.L. BLANK and FRED SNYDER*

Medical and Health Sciences Division, Oak Ridge Associated Universities, Oak Ridge, TN 37830 (U.S.A.)

(Received September 21st, 1982)

High-performance liquid chromatography (HPLC) with silicic acid columns has been utilized for the separation of various classes of intact phospholipids [1—13]. HPLC methods employing ion-exchange columns have also been used for the separation of phospholipid classes [14—16]. Detection of lipids in these systems was based on radioactivity [8, 12], flame ionization [1, 2, 7, 14], collection of fractions followed by phosphorus analysis [6], and UV in the 200—210 nm range [3—5, 9—11, 13, 15, 16]. These methods described a wide range of solvent mixtures; however, the solvents used with the UV detectors were necessarily limited to those that have low absorbance between 200 and 210 nm.

We were primarily interested in selecting a HPLC system that would separate the recently discovered, biologically active 1-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine (alkylacetyl-GPC) (platelet-activating factor) from other normally encountered phospholipids; alkylacetyl-GPC can cause platelet aggregation, hypotension, allergic responses, and anaphylaxis [17—19]. HPLC methods are superior to thin-layer chromatography (TLC) in the separation of alkylacetyl-GPC from other phospholipids by the better resolution from closely related analogues (e.g., lysophosphatidylcholine, LPC) and by having the potential of providing a shorter time for a single analysis of a complex phospholipid mixture.

Earlier, HPLC (using a silica column and a chloroform—methanol—water system) had been used as a final step in the purification of alkylacetyl-GPC [20], however, the alkylacetyl-GPC fraction contained some sphingomyelin (SPG) and LPC as impurities [21]. Alkylacetyl-GPC has also been resolved from total phospholipids by using two separate HPLC runs with different

solvent systems [22]. Recently, tritium-labeled alkylacetyl-GPC was separated from several other radioactive lipids on a silica column with two different solvent mixtures in a single chromatographic run; the time required was about 100 min [12].

Our method uses a gradient elution system, since previous work [4, 5, 9] had suggested that gradients could provide good HPLC separations of phospholipids in the shortest time. Gradient elution systems can be used with UV detectors but since the saturated alkylacetyl-GPC shows little or no absorption at 206 nm, it was also important to find lipid markers that possess UV absorbing properties to serve as reference points for detecting the elution of alkylacetyl-GPC. The advantages of our method over the other HPLC techniques reported earlier for the isolation of PAF are the shorter time (40 min or less), good solvent absorption characteristics, the lack of necessity for any preliminary isolation procedures, and the development of lipid standards as markers with excellent UV-absorbing qualities.

EXPERIMENTAL

Materials

Phosphatidylcholine (PC) from soybean, phosphatidylethanolamine (PE) from egg, phosphatidylglycerol (PG) derived from egg PC, phosphatidic acid (PA) prepared from egg PC, phosphatidylinositol (PI) from soybean, phosphatidylserine (PS) from beef brain, and SPG from egg were all purchased from Sigma (St. Louis, MO, U.S.A.). On the basis of a purity check by TLC on Silica Gel HR plates developed with chloroform—methanol—glacial acetic acid—water (50:25:8:2), only PS had to be further purified (by preparative TLC) before use. LPC was prepared from soybean PC by hydrolysis with phospholipase A_2 [23]. A portion of TLC-purified LPC was acetylated to yield acylacetyl-GPC, which was also purified by TLC [24]. All of these phospholipids contained sufficient unsaturation to be easily detected at levels of 30—50 μg per peak with the UV detector set at 206 nm. 1-Hexadecyl-2-[^{3}H]acetyl-*sn*-glycero-3-phosphocholine (hexadecyl-[^{3}H]acetyl-GPC) was prepared as previously described [25].

Methods

HPLC was performed using a dual-pump Beckman Model 324 M system fitted with a 250 × 4.6 mm Ultrasphere-Si (5 μm) column, which was connected to a Model 155-40 variable UV—visible detector (206 nm) and a Model C-R1A recorder—integrator (all purchased from Beckman Instruments, Norcross, GA, U.S.A.). All samples were dissolved in isopropanol—hexane (1:1) and injected on the column using an Altex Model 210 injector (Beckman Instruments) fitted with a 20-μl sample loop. HPLC grade solvents were from Burdick and Jackson Labs. (Muskegon, MI, U.S.A.). One of the solvent systems used (I) was similar to that of Geurts van Kessel et al. [4] starting with 96% A [isopropanol—hexane (1:1)] and 4% B (water) with a linear gradient to 8% B over a 15-min period after injection. A second solvent system (II) consisted of 96% A [isopropanol—hexane (1:1) containing 0.005% glacial acetic acid] and 4% B (water containing 0.005% glacial acetic acid); the percent B was increased

linearly to 8.5% over a 20-min period. Solvent system III was the same as system II except 0.005% concentrated ammonium hydroxide was substituted for the acetic acid. The flow-rate was 2 ml/min with all solvent systems. At the end of a chromatographic separation, the column was equilibrated with 20 ml of the starting solvent mixture before injecting the next sample. When switching from one solvent system to another, the column was always equilibrated with about 100 column volumes of the new solvent system before use.

For the separation of hexadecyl-[^{3}H]acetyl-GPC, a fraction collector (Model 1220, Instrumentation Specialties Company, Lincoln, NE, U.S.A.) was attached to the UV flow cell and fractions collected at 0.2-min intervals. The amount of tritium in each fraction was determined with a liquid scintillation spectrometer and the radioactivity per fraction was plotted manually at the appropriate position on the UV recorder tracing.

RESULTS AND DISCUSSION

Hexadecyl-[^{3}H]acetyl-GPC was completely separated from other commonly encountered phospholipids with all three solvent systems (Figs. 1—3). It was eluted between SPG and LPC with a retention time of about 30 min in solvent systems I and III and at 36 min in system II. The resolution of hexadecyl-[^{3}H]acetyl-GPC, acylacetyl-GPC, and LPC was better using either the neutral system (I) or the basic system (III) than using the acetic system (II). Regardless of whether small amounts of acetic acid or ammonia were included in the solvents, PE was eluted at the same time (ca. 10 min). However, elution times of PG, PI, and PA were increased when acetic acid was included in the solvents (Fig. 2), i.e., relative to their retention times in the neutral system (Fig. 1).

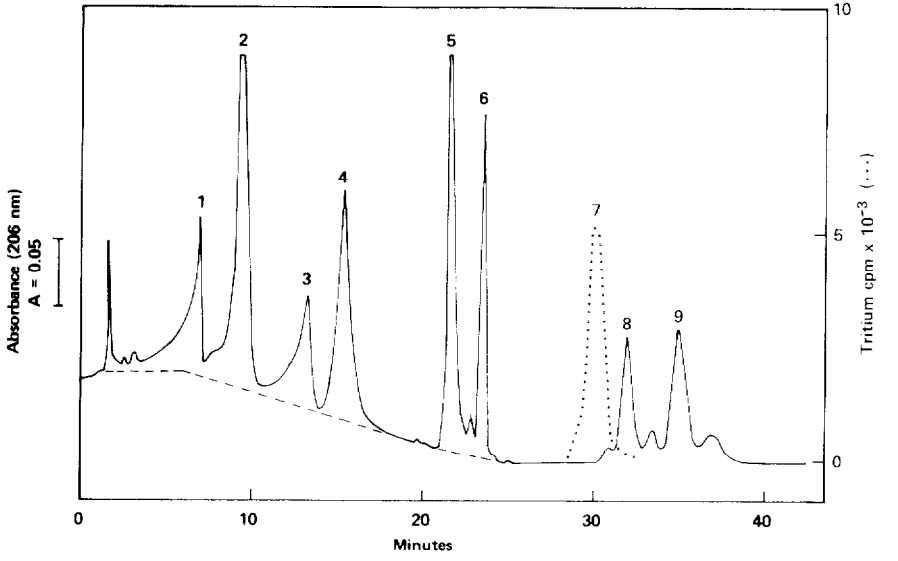

Fig. 1. HPLC separation of (1) PG, (2) PE + PI, (3) PA, (4) PS, (5) PC (and some SPG), (6) SPG, (7) hexadecyl-[^{3}H]acetyl-GPC, (8) acylacetyl-GPC, and (9) LPC using solvent system I described in Methods.

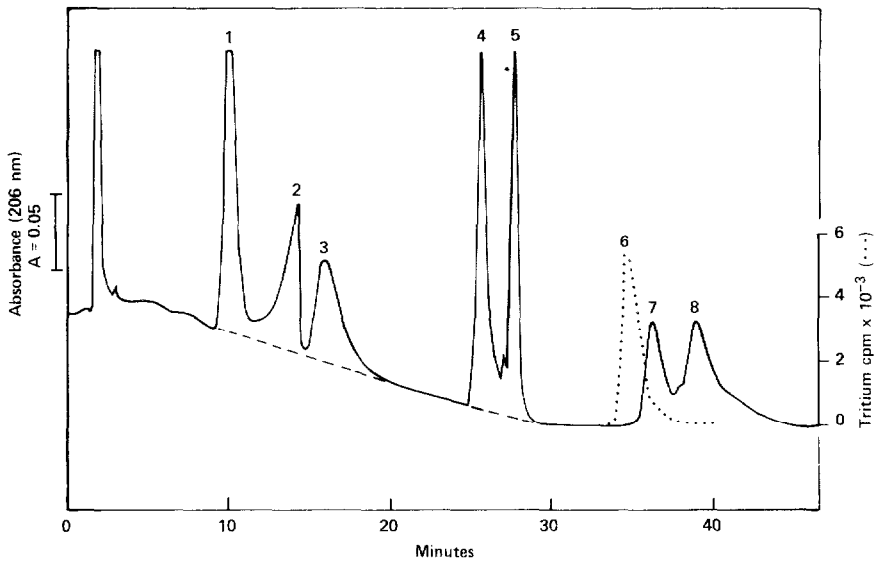

Fig. 2. HPLC separation of (1) PE + PG, (2) PI, (3) PS (also PA if present), (4) PC (and some SPG), (5) SPG, (6) hexadecyl-[^{3}H]acetyl-GPC, (7) acylacetyl-GPC, and (8) LPC using solvent system II described in Methods.

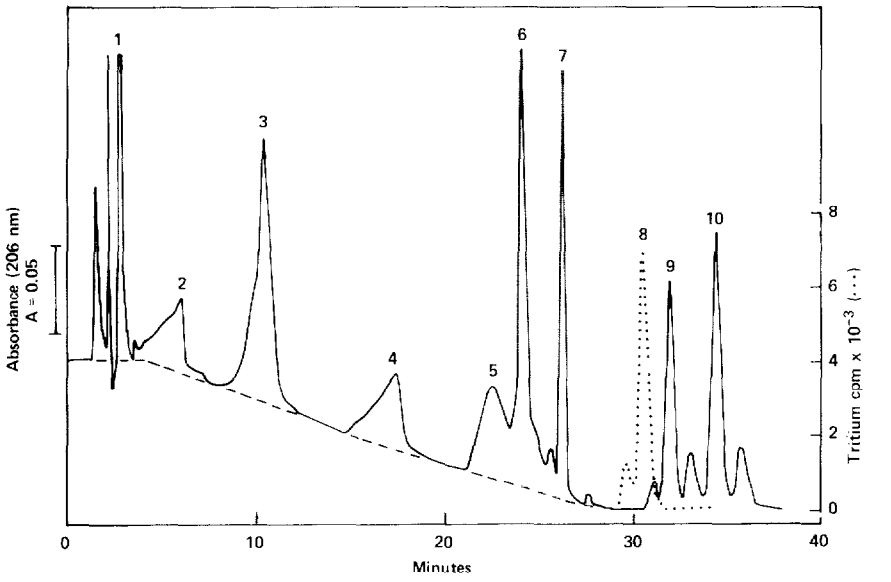

Fig. 3. HPLC separation of (1) fatty acids, (2) PG, (3) PE + PI, (4) PA, (5) PS, (6) PC (and some SPG), (7) SPG, (8) hexadecyl-[^{3}H]acetyl-GPC, (9) acylacetyl-GPC, and (10) LPC using solvent system III described in Methods.

When ammonium hydroxide was in the solvents, the retention of PS was greatly increased (Fig. 3) compared to its retention volume in either of the other solvent systems. However, the acetic system (II) produced better separations of PE, PI, and PS than systems I and III. SPG from egg and the acylacetyl-GPC or LPC prepared from soybean PC serve as good, UV-absorbing

reference peaks for the isolation of alkylacetyl-GPC by HPLC in the solvent systems described. Although the UV recorder tracing shows good separation of PC and SPG in all solvent systems, there was a significant amount (ca. 16%) of SPG eluted with PC as shown by TLC of the collected HPLC peak; this may be related to the extent of unsaturation and acyl chain lengths in the SPG species.

The use of the Ultrasphere-Si column and solvent systems I—III provide a rapid and apparently complete separation of alkylacetyl-GPC (platelet-activating factor) from other commonly encountered phospholipids. The time required to separate alkylacetyl-GPC from other phospholipids, using solvent systems I—III, is shorter than other published HPLC systems [12, 20—22]. SPG from egg and acylacetyl-GPC or LPC prepared from soybean PC are sufficiently unsaturated to provide useful reference compounds that can be used with UV detection to "bracket" the elution time of the non-UV-absorbing alkylacetyl-GPC; this could not be done with some of the systems previously described for the separation of alkylacetyl-GPC because the solvents were not transparent at 200—210 nm [12, 20, 21].

ACKNOWLEDGEMENTS

This work was supported by the Office of Energy Research, US Department of Energy (Contract No. DE-AC05-76OR00033), the National Heart, Blood, and Lung Institute (Grant HL-27109-02), and the American Cancer Society (Grant No. BC-70M).

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