High performance liquid chromatography of platelet-activating factors

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Summary Silica and C18 reverse phase high performance liquid chromatography (HPLC) were used to fractionate synthetic molecular species of 1-O-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine (AGEPC) and semi-synthetic platelet-activating factor (PAF) synthesized from beef heart plasmalogens. A single coincident peak from silica HPLC was observed for either a mixture of synthetic AGEPC's with alkyl chain lengths from C_{12} to C_{18} or for beef heart-derived PAF. This peak was well separated from other classes of phospholipid standards including 2-lysophosphatidylcholine and ^{3}H-labeled lyso-PAF. Subsequently, the synthetic AGEPC mixture or beef heart PAF was separated into individual species on a C18 reverse phase column. Beef heart-derived PAF was fractionated into at least four molecular species of PAF activity which had similar retention times as the radioactivity of ^{3}H-labeled beef heart PAF. Approximately 56% of the radioactivity of ^{3}H-labeled PAF was found in the fraction with a similar retention time as 1-O-hexadecyl-2-acetyl-*sn*-glycero-3-phosphocholine, 10% as 1-O-octadecyl-2-acetyl-*sn*-glycero-3-phosphocholine, 11% as 1-O-pentadecyl-2-acetyl-*sn*-glycero-3-phosphocholine, and 13% in an unidentified fraction which eluted after C_{16}-AGEPC. The unidentified fraction did not correspond to any of the homologous series of synthetic AGEPCs with saturated alkyl chain lengths from C_{12} to C_{18}. Recoveries of radioactive phospholipids from silica or reverse phase columns were greater than 95%.—**Jackson, E. M., G. E. Mott, C. Hoppens, L. M. McManus, S. T. Weintraub, J. C. Ludwig, and R. N. Pinckard.** High performance liquid chromatography of platelet-activating factors. *J. Lipid Res.* 1984. **25:** 753–757.

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The platelet-activating factor (PAF) released by rabbit buffy coat leukocytes has been shown to include AGEPC with a hexadecyl or octadecyl alkyl group in the *sn*-1

Abbreviations: HPLC, high performance liquid chromatography; PI, phosphatidylinositol; PS, phosphatidylserine; PE, phosphatidylethanolamine; LPE, 2-lysophosphatidylethanolamine; PC, phosphatidylcholine; LPC, 2-lysophosphatidylcholine; SPH, sphingomyelin; AGEPC, 1-O-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine; C_{16}-AGEPC, 1-O-hexadecyl-2-acetyl-*sn*-glycero-3-phosphocholine; C_{12}, C_{14}, C_{15}, or C_{18}-AGEPC, 1-O-dodecyl-AGEPC, 1-O-tetradecyl-AGEPC, 1-O-pentadecyl-AGEPC, or 1-O-octadecyl-AGEPC, respectively; PAF, platelet-activating factor; lyso-PAF, 2-lyso-platelet-activating factor (1-O-alkyl-*sn*-glycero-3-phosphocholine); TLC, thin-layer chromatography.

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position (1). Similar semisynthetic PAF has been prepared from bovine heart plasmalogens with the predominant species also reported to contain C_{16} and C_{18} alkyl groupings in the *sn*-1 position of the glycerol backbone (2). PAFs with physicochemical properties similar to AGEPC have been reported to be released from a variety of stimulated cells of humans and experimental animals (3). However, these PAFs have not been characterized chemically, primarily because efficient purification procedures yielding homogeneous populations of PAF molecules have not been available. The recent development of HPLC procedures for the separation and quantitative isolation of phospholipids has permitted detailed characterization of phospholipids in many biological systems (4–6). Several procedures (7, 8) have been used to isolate PAF from other phospholipid classes. We describe in this communication the separation of synthetic AGEPCs and semisynthetic PAFs from other major phospholipid classes by silica HPLC and the subsequent fractionation of PAF activity into several molecular species by reverse phase HPLC.

METHODS

Materials

All solvents and phosphoric acid were HPLC grade and were purchased from Fisher Scientific Co. (Pittsburgh, PA). PS and SPH were obtained from Supelco Inc. (Bellefonte, PA). Bovine brain PE, ovine LPE, soybean LPC and PI, synthetic dioleoyl PC, semisynthetic beef heart-derived PAF, and choline chloride (3× crystallized) were purchased from Sigma Chemical Co. (St. Louis, MO). C_{16}-AGEPC was purchased from Bachem Fine Chemicals (Torrance, CA), C_{18}-AGEPC was a gift from Dr. Donald Ayer of the Upjohn Co. (Kalamazoo, MI), C_{12}-AGEPC was prepared as described (9), and C_{14} and C_{15}-AGEPC were purchased from Calbiochem-Behring (San Diego, CA). Tritium-labeled beef heart-derived PAF (30–60 Ci/mmol) and lyso-PAF, 1-O-alkyl-*sn*-glycero-3-phosphocholine, (30–60 Ci/mmol) were obtained from New England Nuclear (Boston, MA). Both of these radiolabeled compounds were purified immediately prior to use by thin-layer chromatography (TLC) on 250 μ Silica Gel G plates (Analtech Inc., Newark, DE) using a solvent system of chloroform–methanol–water 65:35:6 (v/v/v), as described (10). Such purification was necessary since 10–20% of the ^3H was associated with degradation products that migrated with the solvent front. In this communication, we refer to the pure, synthetic alkyl acetyl phospholipids as AGEPC because their chemical structures are well defined. We refer to the semisynthetic compounds prepared from bovine heart as PAF inasmuch as they have not been fully characterized.

Mass spectrometry

Fast atom bombardment (FAB) mass spectra of the AGEPC standards were obtained using a Finnigan MAT 212/INCOS 2200 system equipped with an Ion Tech saddle field atom gun operating at 8 kV with argon used for the atom beam. The accelerating voltage in the mass spectrometer was 3 kV and the ion source temperature was 70°C. The sample was applied in 1–2 μl of methanol to a copper probe tip coated with glycerol. The structures of the individual standard AGEPC homologues were verified by the presence of an $[MH]^+$ ion, an $[MH-42]^+$ ion and a base peak at m/z 184 from the phosphocholine moiety. For the AGEPC standards in the present study the $[MH]^+$ ions were: C_{12}, m/z 468; C_{14}, m/z 496; C_{15}, m/z 510; C_{16}, m/z 524; and C_{18}, m/z 552.

HPLC procedures

Phased solvents were prepared by mixing chloroform–methanol–water in a volume ratio of 1:1:0.9 (11) and recovering the lower, chloroform-rich phase and the upper, methanol–water phase. For normal phase HPLC separation, phospholipid standards, AGEPC species, and beef heart PAF were dissolved separately in the lower phase solvent and loaded into a 100-μl sample loop of a Rheodyne 7125 injector. A 10-μ Radial-Pak silica cartridge, 8 mm × 10 cm, protected with a silica Guard-Pak was used in a Z-Module or RCM-100, radial compression module (Waters Associates, Milford, MA). The elution solvent was acetonitrile–methanol–85% phosphoric acid 130:5:1.5 (v/v/v), as described by Chen and Kou (5). The column was equilibrated either overnight by pumping the elution solvent with a M6000A HPLC pump (Waters Associates) at a flow rate of 0.3 ml/min or for 4 hr at 2 ml/min before the samples were injected. Samples were eluted at a flow rate of 2.0 ml/min.

Phospholipid standards with unsaturated fatty acids were detected at 203 nm. ^3H-labeled PAF and ^3H-labeled lyso-PAF were detected by liquid scintillation spectrometry of fractions using Scintisol (Isolabs Inc., Akron, OH) as cocktail. The various AGEPC species were isolated from the normal phase HPLC fractions by adding one volume of chloroform, one volume of methanol, and two volumes of water. After mixing, the upper phase was discarded and the lower phase was washed three times with two volumes of upper phase solvent (see above). After concentration of the lower phase the samples were assayed for PAF activity as described below. The silica column was regenerated by washing as follows: methanol–water 1:1, 40 ml; methanol, 40 ml; dichloromethane, 40 ml; Reactivation Agent for silica columns (Alltech Associates, Deerfield, IL), 40 ml; dichloromethane, 80 ml; and finally hexane, 40 ml. The column was stored in hexane.

Reverse phase HPLC was performed on a 10-μ Radial-Pak C18 cartridge, 8 mm × 10 cm, with a C18 Guard-Pak (Waters Associates) in a Z-module or RCM-100, radial compression module. The solvent system was a modification of that described by Patton, Fasulo, and Robins (6). Choline chloride (20 mM) in methanol–water–acetonitrile 85:10:5 (v/v/v) was pumped at a flow rate of 1.0 ml/min for 4 hr to equilibrate the column. Several injections of 20–50 μg of LPC during the equilibration time improved the performance of the column. AGEPC or beef heart-derived PAF which had been purified by silica HPLC was dried under N_2 and was dissolved in the reverse phase elution solvent without choline chloride prior to injection on the reverse phase column. Fractions were eluted at 1.0 ml/min and were extracted by adding one volume of chloroform and one volume of water and recovering the lower phase.

Bioassay for PAF activity

PAF activity was assessed by the capacity of a sample to stimulate the release of [^{3}H]serotonin from washed rabbit platelets (250,000/μl) as described (10). One unit of PAF activity was defined as the amount of a sample required to effect 50% [^{3}H]serotonin secretion in 60 sec from 200 μl of platelets. The following are femtomole (fm) amounts of each AGEPC species that are equivalent to 1 unit of PAF activity: C_{12}-AGEPC, 380; C_{14}-AGEPC, 270; C_{15}-AGEPC, 30; C_{16}-AGEPC, 30; and C_{18}-AGEPC, 110.

RESULTS

TLC-purified, beef heart-derived ^{3}H-labeled PAF was well separated from the major classes of phospholipids and also from ^{3}H-labeled lyso-PAF by silica HPLC (**Fig. 1**). ^{3}H-labeled PAF had a retention time between those for PC and LPC. ^{3}H-labeled lyso-PAF overlapped SPH on silica HPLC. Recovery of both radioactive standards after silica HPLC exceeded 95% of the amounts injected. The peak of ^{3}H-labeled PAF was further fractionated by reverse phase HPLC as shown in **Fig. 2.** Four distinct peaks of radioactivity were recovered which accounted for 90% of the total radioactivity applied on the column, with the remainder distributed throughout the chromatogram. The fractions corresponding to the leading edge of the largest ^{3}H peak with retention times from 19–20 min were combined and used as a radioactive marker (highly purified ^{3}H-labeled PAF) in subsequent experiments. Reverse phase HPLC also was used to fractionate unlabeled beef heart-derived PAF with highly purified ^{3}H-labeled PAF used as a marker. Four peaks of PAF biological activity were observed for the unlabeled beef heart-derived PAF (**Fig. 3**). These peaks corresponded to those observed by radioactive counting of HPLC fractions of beef heart-derived ^{3}H-labeled PAF (Fig. 2).

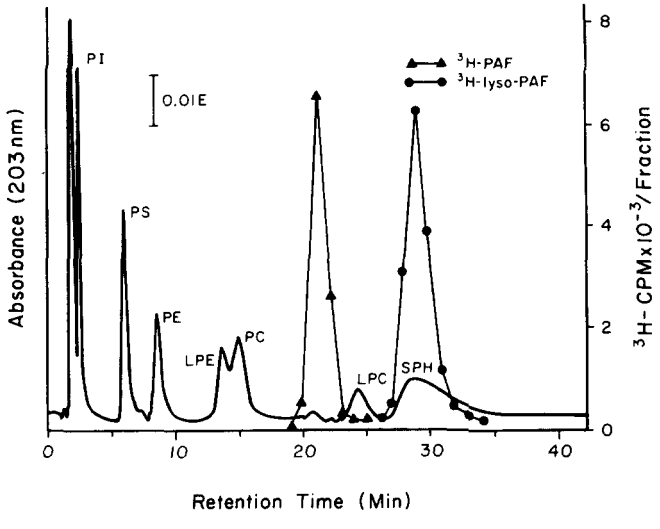

Fig. 1. Separation of phospholipid standards, beef heart-derived ^{3}H-labeled PAF, and beef heart-derived ^{3}H-labeled lyso-PAF by silica HPLC.

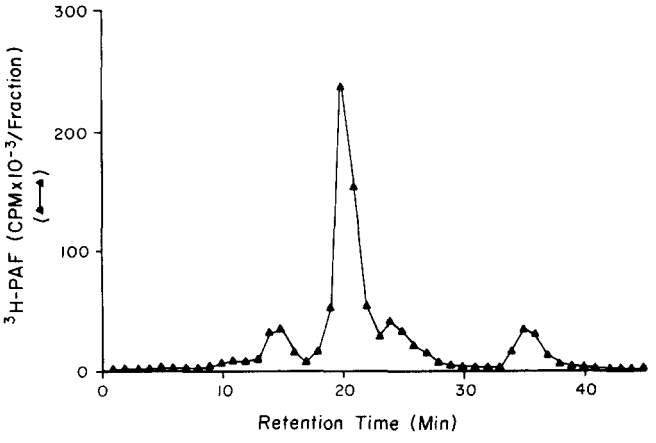

Fig. 2. Fractionation of beef heart-derived ^{3}H-labeled PAF by C18 reverse phase HPLC.

After elution from silica HPLC in a single peak, the mixture of C_{12}, C_{14}, C_{15}, C_{16}, and C_{18} synthetic AGEPCs or the highly purified ^{3}H-labeled PAF from beef heart was injected on the reverse phase column (**Fig. 4**). The retention times of the synthetic AGEPCs as determined by platelet activation were: C_{12}, 8 min; C_{14}, 12 min; C_{15}, 16 min; C_{16}, 20 min; and C_{18}, 36 min. The times were unchanged when each analogue was chromatographed individually. These retention times then were compared to the peaks of both ^{3}H and PAF activities observed after reverse phase HPLC of the beef heart-derived PAF (Figs. 2 and 3). Approximately 56% of the radioactivity of beef heart-derived ^{3}H-labeled PAF was found in the fraction with a retention time of 19–21 min and was similar to that for C_{16}-AGEPC. Ten percent corresponded to C_{18}-

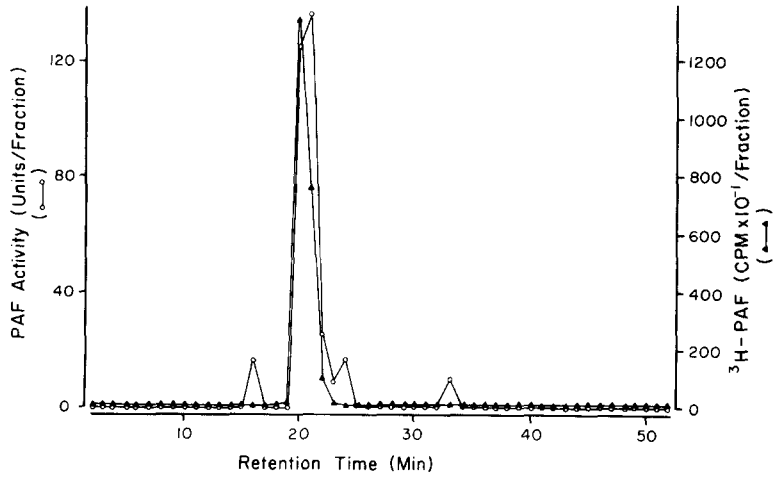

Fig. 3. Fractionation of unlabeled beef heart-derived PAF (isolated from silica HPLC) by C18 reverse phase HPLC. Highly purified beef heart-derived ^{3}H-labeled PAF was included as an internal marker (see Results).

AGEPC, 11% to C_{15}-AGEPC, and 13% to an unidentified fraction with a retention time of 22–24 min.

DISCUSSION

The isolation and identification of PAF in biological samples is extremely difficult because these phospholipids are many times lower in concentration than other endogenous phospholipid species. The two-step HPLC procedure described in this report first separates most of the known classes of phospholipids from synthetic AGEPC or from beef heart-derived PAF. Subsequent fractionation by C18 reverse phase HPLC provides good resolution of several synthetic, saturated AGEPCs or beef heart-derived PAFs. The structures of several minor components of PAF activity have not been determined, but the retention times of the major peaks corresponded to known synthetic standards. In several separate runs the highly purified beef heart-derived ^{3}H-labeled PAF (which co-eluted with C_{16}-AGEPC) did not have exactly the same retention time as the major peak of PAF activity from unlabeled beef heart-derived PAF (see Fig. 3). This suggests the probability of heterogeneity in the 1-O-hexadecyl-AGEPC region of the chromatogram of beef heart-derived PAF.

The results of the present investigation confirm and extend previous studies indicating that beef heart-derived PAF is a heterogeneous preparation (2, 12). Thus, use of beef heart-derived PAF as a biological probe, e.g., in binding or metabolic studies (12–16), should be interpreted with some reservation inasmuch as the different components of this PAF mixture may be processed differently in various biological systems. In addition, the results of the current study suggest that heterogeneity in PAF produced by a variety of cells could be detected using reverse phase HPLC. Demonstration of such molecular heterogeneity may be important in understanding the diverse biological activities of these inflammatory mediators.

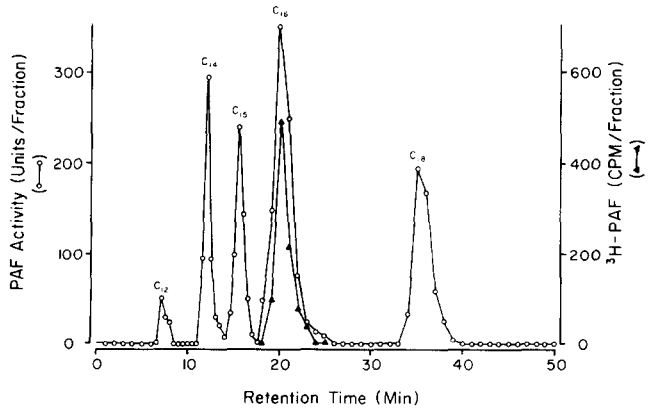

Fig. 4. Fractionation of homologues of synthetic AGEPC by C18 reverse phase HPLC. Highly purified beef heart-derived ^{3}H-labeled PAF was included as an internal marker (see Results).

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