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Note

Rapid isocratic procedure for the separation of platelet-activating factor from phospholipids in human saliva by high-performance liquid chromatography

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Platelet-activating factor (PAF) is an extremely potent ether-linked phospholipid which can initiate a wide range of biological activities including platelet activation, the cardiovascular and pulmonary changes of anaphylaxis and systemic hypotension [1—3]. The structure of PAF as derived from rabbit basophils [4], dog leukocyte [5] and rat medullary hypotensive lipid [3] has the structure 1-O-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine (AGEPC, alkyl acetyl-GPC, PAF-acether).

The separation of PAF from other phospholipids has been done using thin-layer chromatography (TLC) [4]. Recently, high-performance liquid chromatographic (HPLC) methods, using silica gel columns, for the separation of PAF from other phospholipids have been reported [6, 7]. The advantage of HPLC over TLC includes higher resolution of phospholipids, excellent recovery of individual phospholipids, and there is no need to elute material from TLC plate zones for subsequent analysis. These HPLC methods involve gradient elution with two different solvent systems [6, 7], but UV detection of phospholipid peaks can be used with only one [7].

The method we employ is a technique based on the procedure described by Chen and Kou [8]. This system has the advantage of an isocratic gradient, good separation of PAF from other phospholipids, a short run time and UV detection of phospholipid markers since PAF has low UV absorbance.

EXPERIMENTAL

Materials

Soybean phosphatidylinositol (PI), bovine brain phosphatidylserin (PS),

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egg yolk phosphatidylethanolamine (PE), phosphatidylcholine (PC) and lysophosphatidylcholine (LPC), synthetic PAF (1-O-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine, AGEPC) and lyso-PAF (1-O-alkyl-2-hydroxy-*sn*-glycero-3-phosphocholine, LAGEPC) were purchased from Avanti Polar Lipids (Birmingham, AL, U.S.A.). [^{3}H-Cholinemethyl]phosphatidylcholine (^{3}HPC), [^{14}C-1-dipalmitoyl]phosphatidylethanolamine (^{14}CPE), [^{14}C-cholinemethyl]sphingomyelin (^{14}CSPH) and [^{3}H-1′,2′-alkyl]1-O-alkyl-2-acetyl-*sn*-glyceryl-3-phosphocholine (^{3}HAGEPC) from New England Nuclear (Boston, MA, U.S.A.). [^{14}C-1]-L-α-Phosphatidylserine (^{14}CPS), [^{3}H-2]L-α-phosphatidylinositol (^{3}HPI), [^{14}C-1]L-α-lysophosphatidylcholine (^{14}CLPC) and [^{3}H-1′,2′-octadecyl]lyso-PAF (^{3}HLAGEPC) were purchased from Amersham (Arlington Heights, IL, U.S.A.). HPLC-grade acetonitrile, chloroform, methanol and 85% phosphoric acid were purchased from Fisher (St. Louis, MO, U.S.A.); alternatively HPLC-grade acetonitrile and methanol were purchased from Burdick & Jackson Labs. (Muskegon, MI, U.S.A.).

Preparation of salivary lipids

Collection and extraction into chloroform of unstimulated mixed human saliva was performed as described elsewhere [9]. The chloroform phases of the saliva extracts were pooled and dried to a 1-ml volume in a Buchler vortex-evaporator (Bucki-Brinkman Instruments, Westbury, NY, U.S.A.). The sample was then filtered through a MSD cameo II, 0.45-μm filter (Fisher Scientific, St. Louis, MO, U.S.A.) into a clean 75 × 12 mm glass tube and then taken to dryness in the vortex-evaporator. The dried saliva extract was then resuspended in 250 μl of HPLC-grade chloroform and chromatographed as described below.

Chromatographic conditions

The equipment used was a Waters Assoc. (Milford, MA, U.S.A.) liquid chromatography system consisting of a Model 6000 solvent delivery system, a Model U6K injector, a Model 450 variable-wavelength detector and a Model M730 data module. The chromatographic columns used were a 30 cm × 4 mm I.D. prepacked stainless-steel Micro-Pak SI-10 column (Varian Assoc., Palo Alto, CA, U.S.A.), a 25 cm × 4 mm I.D. prepacked stainless-steel Hibar LiChrosorb Si 60 column (Rainin, Woburn, MA, U.S.A.), or a 30 cm × 4 mm I.D. prepacked stainless-steel μPorasil column (Waters Assoc.). All three contained 10 μm particle size silica gel. The mobile phase was acetonitrile—methanol—85% phosphoric acid (130:5:1.5) using initially Fisher solvents followed by re-equilibration of the column and testing using Burdick & Jackson Labs. solvents (acetonitrile and methanol with Fisher phosphoric acid). Flow-rate through the column was 1 ml/min. All phospholipid standards and salivary lipids were dissolved in HPLC-grade chloroform and 1.5 and 10 μl, respectively, were injected into the column. Sample mixtures and recorder response are indictated in figure legends.

Sample testing

Volumes of 1 ml, representing 1-min fractions, were collected in glass 75 × 12 mm test tubes from the HPLC column. To each tube 2 ml of chloroform - methanol—water (1:2:0.8) were added. The test tubes were vortexed and 0.5

ml each of chloroform and water were added to effect phasing. The lower phase was removed and dried completely in a glass 75 × 12 mm test tube using a vortex-evaporator. The dried sample was resuspended in 100 μl of pH 7.2 0.15 *M* phosphate-buffered saline (PBS) containing 5 mg/ml human serum albumin fraction V (Sigma, St. Louis, MO, U.S.A.). The samples were tested for PAF activity using platelet aggregometry as described elsewhere [9]. Fractions containing PAF are indicated on the figures.

RESULTS

Synthetic PAF (AGEPC) was completely separated from other phospholipids by this system. As indicated by 203-nm absorbance in Fig. 1, AGEPC eluted between PC and LPC/SPH with a retention time of 13—14 min. The same elution pattern and retention times were found when using ^{3}HAGEPC and radioactive phospholipid standards (Fig. 2). Recovery of radioactive phospholipid standards and ^{3}HAGEPC from the HPLC column typically were in excess of 97% of the amounts injected. No difference in retention times were noted with any of the three columns used or with solvents from either Fisher or Burdick & Jackson Labs.

HPLC fractions of human saliva extracts containing PAF activity corresponded to two small peaks having retention times of 13 and 14 min (Fig. 3 A and B).

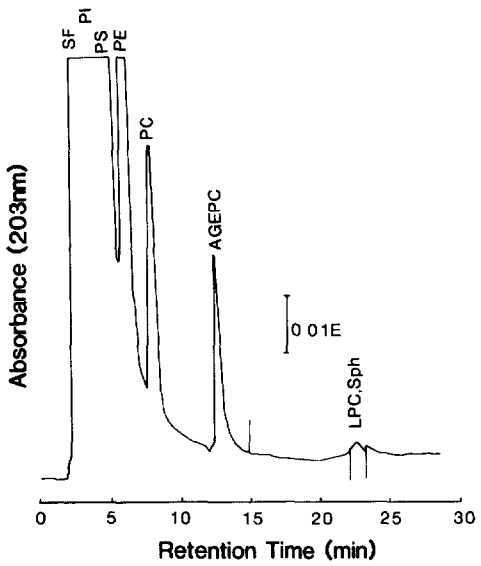

Fig. 1. Chromatogram of phospholipid standards. The amount injected was 1.5 μl of chloroform containing 0.5 μg each of PS, PE and PL, 2.5 μg each of PI and SPH, 5 μg each of LPC and LAGEPC and 10 μg of AGEPC. Chromatographic conditions: flow-rate 1 ml/min, mobile phase, acetonitrile—methanol—85% phosphoric acid (130.5:1.5); UV detection at 203 nm; recorder response 0.1 a.u.f.s.; and ambient temperature. Peaks: SF = solvent front; PI = phosphatidylinositol; PS = phosphatidylserine; PE = phosphatidylethanolamine; PC = phosphatidylcholine; AGEPC = alkyl glyceryl phosphorylcholine or PAF, LPC = lysophosphatidylcholine; LAGPEC = lyso-AGEPC or lyso-PAF, Sph = sphingomyelin.

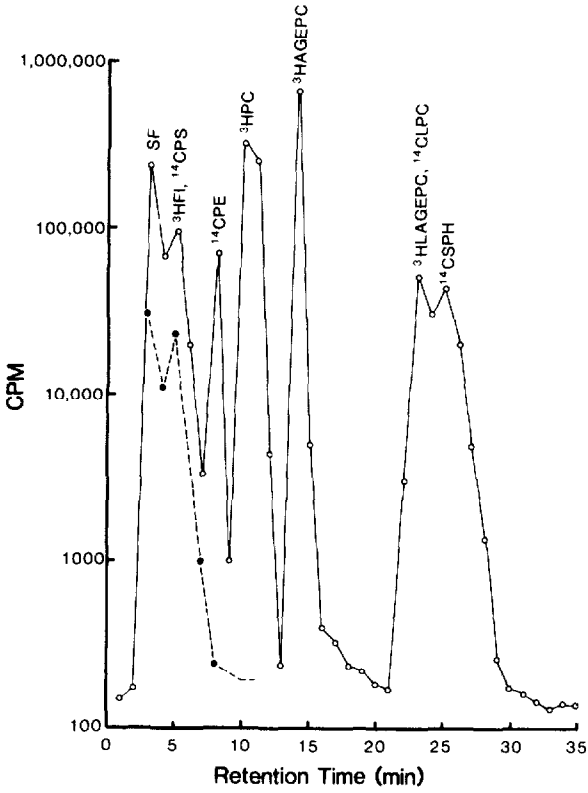

Fig. 2. Elution profile of different ^{3}H- or ^{14}C-labeled phospholipids separated by HPLC. *Radioactivity in individual fractions collected at 1-min intervals and measured by liquid* scintillation counting. For the first run (—), the amount injected was 1.5 μl of chloroform containing 18 ng of ^{14}CPS; 9 ng each of ^{14}CLPC, ^{14}CSPH and ^{14}CPE, 0.30 ng ^{3}HPC, 0.03 ng each ^{3}HPI and ^{3}HAGEPC, 0 01 ng ^{3}HLAGEPC. Other conditions are the same as in the legend to Fig. 1. Peaks· ^{3}HPI = [^{3}H-2]phosphatidylinositol; ^{14}CPS = [^{14}C-1]phosphatidylserine; ^{14}CPE = [^{14}C-1]phosphatidylethanolamine; ^{3}HPC = [^{3}H-cholinemethyl]phosphatidylcholine, ^{3}HAGPEC = [^{3}H-1′,2′-alkyl]AGEPC or PAF; ^{14}CLPC = [^{14}C-1]lysophosphatidylcholine; ^{3}HLAGEPC = [^{3}H]-1′,2′-alkyl]lyso-AGEPC or lyso-PAF, ^{14}CSPH = [^{14}C-cholinemethyl]sphingomyelin. For the second run (- - -), the amounts of ^{3}HPI and ^{14}CPS were reduced to 0.025 and 15 ng, respectively.

DISCUSSION

The presence of a lipid with characteristics of PAF in human saliva has been previously reported [9]. Fractionation of phospholipid species from human saliva indicates at least two species having platelet aggregating ability. These phospholipids had retention times very similar to synthetic PAF (AGEPC), confirming previous studies [9] that human salivary PAF (HS-PAF) and AGEPC have structural similarities.

The concentrations of HS-PAF in normal human mixed saliva (< 4 fmol/ml of saliva) is many times lower than other phospholipid species present [10—12]. These low concentrations of HS-PAF make normal detection and isolation extremely difficult. The use of HPLC silica columns with the solvent

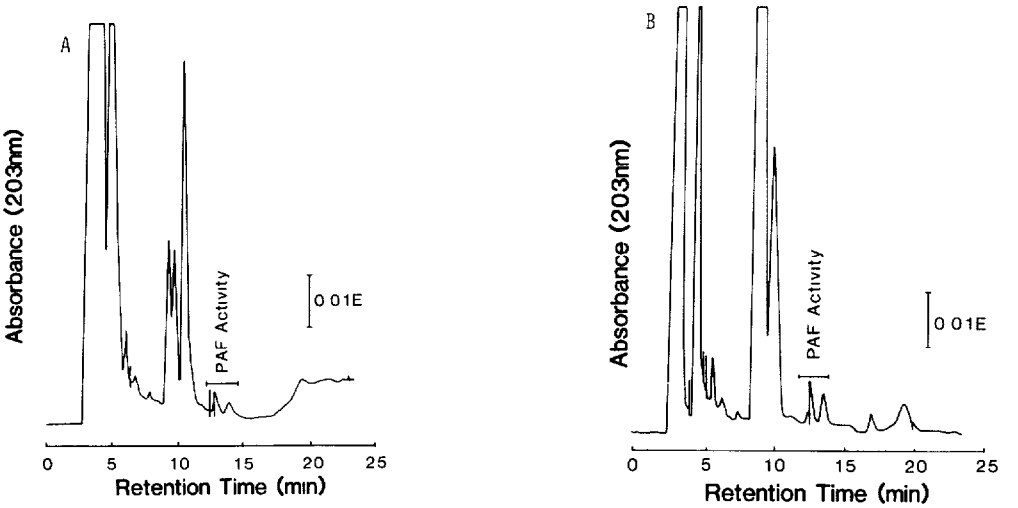

Fig. 3. Examples of chromatograms of human saliva extracted from two normal subjects (A and B) separated by HPLC. The amount injected was 10 μl of extract in chloroform. Chromatographic conditions were as in Fig. 1. PAF activity was determined by collecting fractions at 1-min intervals and testing as described in text.

system of Chen and Kou [8] results in the separation of synthetic PAF and HS-PAF from other common phospholipids with excellent recovery.

The use of the Chen and Kou [8] solvent system and silica HPLC columns should also be applicable to the separation and purification of PAF from contaminating phospholipids in other biological samples.

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