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Solid-phase extraction on silica cartridges as an aid to platelet-activating factor enrichment and analysis

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SUMMARY

Solid-phase extraction methods using pre-packed silica cartridges and various elution solvents have been developed and evaluated as chromatographic means to enrich biological lipid extracts for platelet-activating factor (PAF). The optimized procedure advanced selectively removed the major tissue/blood neutral lipids and non-choline-containing phospholipids from complex lipid mixtures and yielded thereby a choline phospholipid fraction markedly enriched in bioactive PAF. Some tested solid-phase extraction procedures, while capable of resolving choline phospholipids from other polar and non-polar species, were detrimental to PAF's bioactivity and evidenced considerable loss or degradation of this analyte. It is concluded that, with solvents of appropriate composition, strength and polarity, solid-phase extraction on silica cartridges has several unique advantages over conventional thin-layer and column chromatographic methods presently in use for PAF enrichment from biological sources.

INTRODUCTION

Platelet-activating factor (1-O-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine) (PAF) is a naturally occurring phospholipid, usually containing a 1-hexadecylalkyl or 1-octadecylalkyl ether moiety [1]. Although originally identified as a mediator of anaphylaxis and platelet physiology [2], PAF has a wide spectrum of biological activities which contribute to the progression of many

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important disease states [3]. A range of stimulated cell types synthesize PAF and may subsequently release it into the circulation at sites of tissue injury [3,4]. Even upon appropriate stimulation, though, PAF is produced in trace amounts some five to six orders of magnitude less than the levels of the principal non-PAF tissue and blood lipids: triacylglycerol, cholesterol, cholesterol ester, phosphatidylethanolamine and phosphatidylcholine [5].

PAF cannot be extracted directly from biological tissues and fluids in a selective fashion with respect to non-PAF lipids [5]. The consequent high concentration of non-PAF species in crude biological lipid extracts makes the enrichment of PAF therefrom among the most challenging and laborious facets of analytical lipidology. Such extracts are usually enriched for PAF through silicic acid column chromatography followed by thin-layer chromatography (TLC) to reduce the content of non-PAF neutral and polar glycerides [5,6], but in these methods the great excess of non-PAF lipids often interferes with the chromatographic behavior, unambiguous identification and recovery of tissue-derived PAF [5]. Furthermore, analytical TLC, with a capacity $< 100\ \mu g$ lipid per sample [7], is very sensitive to overloading and, like traditional column chromatography, is cumbersome and time-consuming. Although high-performance liquid chromatography (HPLC) has been used to isolate PAF from lipid mixtures [8,9], the long elution time usually required, the inherently restricted capacity of analytical columns and PAF's limited ultraviolet absorptivity severely undermine the utility of HPLC to routine PAF enrichment.

The need for reliable methods of PAF enrichment stems largely from the unsuitability of biological lipid extracts for functional bioassay as routinely employed to detect and quantify PAF: the large excess of non-PAF lipids in such extracts antagonizes PAF's ability to cause platelet aggregation and to elicit the platelet release reaction [5]. Even more direct PAF assays, such as those developed in this [10] and other [11,12] laboratories, either benefit from or require the removal of at least some non-PAF species from tissue lipid preparations prior to their being analyzed for potential PAF content.

These methodological considerations prompted the present evaluation of solid-phase extraction with disposable, pre-packed silica cartridges to enrich lipid mixtures for PAF with ease, rapidity, high capacity, quantitative PAF recovery and unimpaired PAF bioactivity. In this report, such enrichment techniques are described which readily lend themselves to a variety of potential applications as, for example, adjuncts to PAF bioassay or to molecular PAF analysis by HPLC. The data presented further caution that the PAF molecule can exhibit a sensitivity to chromatographic conditions not shared by other glycerophospholipids and may evidence impaired bioactivity as a result.

EXPERIMENTAL

Reagents and chemicals

Tri[^{14}C]oleoyl glycerol, [^{14}C]palmitic acid, [^{14}C]linoleic acid, [^{14}C]cholesterol, cholesteryl [^{14}C]oleate, [choline-methyl-^{14}-C]-L-α-dipal-

mitoyl phosphatidylcholine, L-α-[dipalmitoyl-1-^{14}C] phosphatidylcholine, L-α-1-palmitoyl 2-[^{14}C]arachidonyl phosphatidylethanolamine, L-α-[myoinositol-2-^{3}H(N)]phosphatidylinositol, [choline-methyl-^{14}C]sphingomyelin, L-1-[palmitoyl-1-^{14}C]lysophosphatidylcholine, [1-O-alkyl-1′,2′-^{3}H]PAF and [1-O-alkyl-1′,2′-^{3}H]lyso-PAF were from New England Nuclear (Boston, MA, U.S.A.). [Serine-^{14}C]phosphatidylserine was from Amersham (Arlington Heights, IL, U.S.A.). L-α-[Dipalmitoyl-1-^{14}C]phosphatidylglycerol was synthesized enzymatically by the transferase action of phospholipase D (Calbiochem, La Jolla, CA, U.S.A.) on L-α-[dipalmitoyl-1-^{14}C]phosphatidylcholine in the presence of 2 *M* glycerol, as described by Dawson [13]. Radiolabeled rat liver [linoleoyl-^{14}C]cardiolipin was prepared by intraperitoneal administration of [^{14}C]linoleic acid (10 mCi) and purification of cardiolipin out of the hepatic lipid extract 24 h later [14]. Radioactive lipids were verified to be >98% pure by HPLC [8,15]. Non-radioactive phospholipid standards were from Avanti Polar Lipids (Burmingham, AL, U.S.A.) and Bachem (Bubendorf, Switzerland) and were >98% pure as purchased. Non-radioactive neutral lipid standards were from NuChek Prep (Elysian, MN, U.S.A.) and were >98% pure as purchased. CV-3988, 3-(4-oxy-7-methoxy-10-oxo-3,5,9-trioxa-11-aza-4-phosphanonacos-1-yl)thiazolium 2-oxide, a specific PAF antagonist [16], was a gift from Takeda Chemical Industries (Tokyo, Japan). Sep-Pak™ cartridges (pre-packed with 600 mg silica; 1.5 ml void volume) were purchased from Waters Chromatography Division (Milford, MA, U.S.A.). Chloroform, methanol, methyl *tert.*-butyl ether (MTBE) and water were HPLC grade (Burdick & Jackson Labs., Muskegon, MI, U.S.A.). Ammonium acetate (pH 8.6) was prepared by mixing 1.0 m*M* ammonium hydroxide with 1.0 m*M* acetic acid, 2:1 (v/v).

Sep-Pak column chromatography

Lipid samples in a volume of $\leq$250 μl chloroform were loaded onto silica Sep-Pak cartridges by using a micropipette whose tip was placed at the upper surface of the adsorbent bed. After adsorption of the sample, syringes were sequentially connected to the cartridge for elution; the syringes contained various solvent systems, as detailed in the text and tables. The columns were eluted manually at a flow-rate of about 30 ml/min at ambient temperature (22°C). The column capacity was at least 5 mg total lipid.

Recovery studies

Tissue lipid extracts were prepared from ventricular muscle of the rat heart as previously detailed [17]. Tissue lipid was quantified by the microchemical method described [17].

For quantitative recovery analysis of the solid-phase extraction procedures, radiolabeled lipid standards (60 000 cpm each) were added to one of a series of identical cardiac muscle lipid extracts containing $\leq$5 mg tissue lipid or to

one of a series of identical mixtures of phospholipid and/or neutral lipid standards containing ≤5 mg total lipid. These samples (in chloroform) were applied to silica Sep-Pak cartridges and eluted with various solvents, as described in the text and tables. The eluates were collected in scintillation vials, evaporated to dryness under nitrogen at ambient temperature and analyzed by liquid scintillation spectrometry in 10 ml of Ecosint scintillant (National Diagnostics, Manville, NJ, U.S.A.) with a Beckman LS 8100 counter. Replicate aliquots of each radiolabeled lipid, equivalent to that chromatographed with the tissue and standard lipids, were likewise counted to enable direct calculation of the recovery of that lipid type in each chromatographic procedure used. The individual radiolabeled lipids were also chromatographed separately on silica Sep-Pak cartridges to establish their behavior in the absence of other lipids. The column separations described were performed at least four times for each individual radioactive lipid, both in the presence and absence of non-labeled tissue/standard lipids.

HPLC of phospholipids

All HPLC analyses were carried out on a ternary gradient system (Beckman Instruments, Palo Alto, CA, U.S.A.) equipped with a Frac-100 fraction collector (Pharmacia, Piscataway, NJ, U.S.A.).

The molecular identities of the phospholipids eluted from silica Sep-Pak cartridges during solid-phase extraction with the methodology in Tables I–III were checked by HPLC of the eluates which contained radiolabeled phospholipids as internal standards. Each eluted column fraction was evaporated to dryness under nitrogen at ambient temperature and dissolved in 250 μl solvent A (propanol–ethyl acetate–benzene–water, 130:80:30:20, v/v). Each resuspended sample was injected into a stainless-steel sample loop and analyzed by HPLC on a Zorbax™ 5-μm silica column (250 mm×4.6 mm I.D.) (Dupont Chromatography Products, Wilmington, DE, U.S.A.). The HPLC conditions, at ambient temperature, were modified from those detailed [8]: solvent A (above) was pumped at a flow-rate of 1.0 ml/min for 20 min, at which time the flow-rate was increased to 1.5 ml/min and held constant for 15 min. At 35 min into the run, the mobile phase was switched to solvent B (propanol–toluene–acetic acid–water, 93:110:15:15, v/v) at 1 ml/min. At 56 min into the run, the flow-rate was increased to 2.0 ml/min and held constant to the end of the run at 100 min. Alternatively, the fractions from silica Sep-Pak columns which had been eluted in either 10 ml methanol–chloroform–water (2:1:0.8, v/v) (Table II) or 24 ml MTBE–methanol–ammonium acetate (5:8:2, v/v) (Table III) were evaporated to dryness under nitrogen at ambient temperature, resuspended in 250 μl solvent B and subjected to HPLC with solvent B only at a flow-rate of 2 ml/min for 50 min. In some experiments, [^{3}H]PAF alone was analyzed by HPLC with solvent B after having been subjected to solid-phase extraction. For all runs, HPLC column eluates were collected in scintillation

vials at 1-min intervals. The HPLC fractions were evaporated to dryness under nitrogen at ambient temperature, dissolved in 10 ml Ecosint and counted for radioactivity. Recoveries from the HPLC procedure were calculated from the sum of the radioactivity associated with each lipid peak as compared to the known amount of each radioactive lipid injected onto the HPLC column. A mixture of radioactive phospholipid standards (phosphatidylethanolamine, phosphatidylinositol, phosphatidylserine, phosphatidylcholine, sphingomyelin, PAF, lyso-PAF, and lysophosphatidylcholine; 480 cpm each per μl chloroform) was subjected to HPLC as a daily check on the column. The HPLC behavior of these lipids was verified by chromatographing each one separately and determining its elution volume and retention time.

PAF assay

Two independent assays whose specificity for PAF has been established [10,18] were employed. The first was a functional bioassay based upon the pro-aggregatory effect of PAF on washed canine platelets [18]. The second was a radioligand competitive-binding assay developed in this laboratory and based upon the selective inhibition of [^{3}H]PAF specific binding to the canine platelet PAF receptor by non-radioactive PAF [10]. For both assays, the amount of PAF in an experimental sample was calculated from calibration curves of standard PAF constructed daily.

Assessment of PAF-induced platelet aggregation

Platelet aggregation in canine platelet-rich plasma (PRP) was determined by the optical method under conditions, detailed elsewhere [18], specific for PAF. In these assays, the experimental sample (or PAF standard) was solubilized in phosphate-buffered saline, pH 7.4 (PBS) containing 0.1% (w/v) bovine serum albumin (BSA). As required, depending upon the experiment, the order of addition into the aggregation system was: PRP, PAF antagonist (i.e., CV-3988) or PBS, then experimental sample or PAF standard.

RESULTS AND DISCUSSION

Since non-polar glycerides can generally be eluted from stationary silica phases by chloroform, whereas phosphoglycerides are recovered in alcohol [19], the behavior of PAF on silica Sep-Pak cartridges to sequential chloroform and methanol elutions was investigated and compared to that of other tissue neutral lipids and phospholipids (Table I). Neither PAF nor any phospholipid emerged in 20 ml chloroform, which was sufficient to elute the neutral lipid. Phosphatidylcholine, phosphatidylethanolamine, phosphatidylinositol, phosphatidylserine, sphingomyelin and lysophosphatidylcholine could be eluted quantitatively with 30 ml methanol, but only about 40% of the PAF emerged in this fraction. Elution of some 45% more PAF required an additional volume

TABLE I

RECOVERY OF LIPIDS FROM SILICA SEP-PAK CARTRIDGES WITH SEQUENTIAL CHLOROFORM AND METHANOL ELUTIONS

Lipid class	Recovery[a] (%)		
	Fraction 1	Fraction 2	Fraction 3
Neutral lipid[b]	98.8 ± 1.5	0	0
Phosphatidylcholine	0	98.2 ± 1.2	< 1.0
Phosphatidylethanolamine	0	97.9 ± 1.6	< 1.0
Phosphatidylinositol	0	98.3 ± 1.3	< 1.0
Phosphatidylserine	0	97.6 ± 1.4	< 1.0
Sphingomyelin	0	99 0 ± 1.7	< 1.0
Lysophosphatidylcholine	0	96.9 ± 2.0	< 1.0
PAF	< 1.0	39.9 ± 1.4	44.0 ± 2.1

[a]Results are mean + S.D. ($n \geq 4$) of each lipid class as assessed by internal radiolabeled lipid standards added to ≤5 mg heart muscle tissue lipid prior to chromatography. Similar results were obtained in the absence of tissue lipid or in the presence of ≤5 mg non-labeled lipid standard(s). Fraction 1 was eluted in 20 ml chloroform, fraction 2 in 30 ml methanol and fraction 3 in 100 ml methanol.

[b]Represented by a mixture of triacylglycerol, non-esterified fatty acid, cholesterol, and cholesterol ester radiolabeled internal standards.

of methanol over 60-fold greater than the void volume of the column. Neither the elution pattern of PAF nor its recovery was influenced by the absence of tissue lipid or by the presence of excess PAF or other standard lipid(s) in the chromatographed sample. Although this procedure afforded excellent separation of neutral lipid from phospholipid (including PAF), the <90% recovery of PAF and, especially, the large volume of methanol required to elute it made this system unattractive for routine analytical use. However, if quantitative PAF recovery were not critical, the method could be used preparatively to obtain a fraction highly enriched in PAF, but representing only about 45% of that in the chromatographed sample.

Mixtures of methanol and chloroform which have been shown [20–23] to elute phosphoglycerides from silica with high efficiency were next studied for their ability to elute PAF from the Sep-Pak column. Of the solvent systems investigated, one [23] was modified to afford quantitative PAF recovery, unimpaired PAF bioactivity and marked PAF enrichment with minimal solvent volumes and a limited number of fractionation steps (Table II). PAF could be quantitatively eluted along with other choline phospholipids and free of neutral lipid in 10 ml methanol–chloroform–water (2:1:0.8, v/v). With the exception of some phosphatidylserine, a minor membrane phosphoglyceride usually representing <5% of total tissue phospholipid [24], negligible non-choline-containing phosphoglyceride emerged in fraction 3. The elution patterns and

TABLE II

ELUTION AND RECOVERY OF LIPIDS FROM SILICA SEP-PAK CARTRIDGES USING CHLOROFORM–METHANOL

Lipid class	Recovery[a] (%)			
	Fraction 1	Fraction 2	Fraction 3	Fraction 4
Neutral lipid[b]	99.8 ± 0.5	0	0	0
Phosphatidylcholine	0	< 0.1	99.2 ± 0.7	0
Phosphatidylethanolamine	0	97.2 ± 1.0	< 0.1	< 0.1
Phosphatidylinositol	0	98.3 ± 0.9	< 0.1	< 0.1
Phosphatidylserine	0	62.3 ± 0.9	37.1 ± 0.8[c]	< 0.1
Phosphatidylglycerol	0	99.3 ± 0.5	0	0
Cardiolipin	0	99.7 ± 0.6	0	0
Sphingomyelin	0	< 0.1	98.6 ± 0.6	0
Lysophosphatidylcholine	0	< 0.1	99.1 ± 0.8	< 0.1
Lyso-PAF	0	< 0.1	98.3 ± 0.5	< 0.1
PAF	0	< 0.1	100.2 ± 2.4	0

[a]Results are mean ± S.D. ($n \geq 4$) of each lipid class as assessed by internal radiolabeled lipid standards added to ≤ 5 mg heart muscle tissue lipid prior to chromatography. Similar results were obtained in the absence of tissue lipid or in the presence of ≤ 5 mg non-labeled lipid standard(s). Fraction 1 was eluted in 16 ml chloroform–acetic acid (100.1, v/v); fraction 2 was eluted in 5 ml methanol–chloroform (2.1, v/v); fractions 3 and 4 were each eluted in 10 ml of methanol–chloroform–water (2 1 0.8, v/v).

[b]Represented by a mixture of triacylglycerol, non-esterified fatty acid, cholesterol and cholesterol ester radiolabeled internal standards.

[c]This phosphatidylserine (along with ~ 1.5% of the total PAF) could be removed from fraction 3 by passing 5 ml methanol–water (3:1, v/v) through the column after eluting fraction 2. Fraction 3 would subsequently emerge as a PAF-enriched, choline phospholipid fraction.

recoveries in Table II were not affected by the absence of tissue lipid or the presence of excess PAF or other standard lipid(s) in the chromatographed sample. If desired, the phosphatidylserine remaining on the column after elution of fraction 2 could be removed by passing 5 ml methanol–water (3:1, v/v), through the Sep-Pak prior to eluting the choline lipids in fraction 3. This intermediary step was not routinely employed, however, since some 2% of the total PAF was also eluted with the phosphatidylserine. The lipids in the PAF-enriched fraction could immediately be reextracted into chloroform by adding 4 ml water and 3 ml chloroform to fraction 3.

PAF chromatographed in this solid-phase extraction system retained full biologic activity which could be inhibited by a specific PAF antagonist (Fig. 1) and was detectable as PAF by a specific receptor-binding assay [10] (data not shown). PAF eluted in fraction 3 emerged as a single peak on HPLC with a retention time and an elution volume identical to those of PAF standard not subjected to solid-phase extraction (Fig. 2). Consequently, the interaction

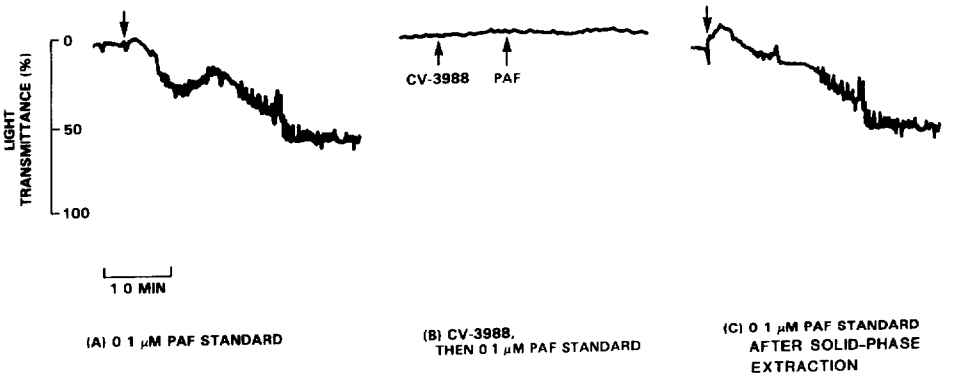

Fig. 1. Bioactivity of PAF after solid-phase extraction on silica Sep-Pak cartridges. (A) Canine platelet aggregation in response to 0.1 μM standard PAF. (B) Inhibition of PAF-induced canine platelet aggregation by the specific PAF antagonist CV-3988 (100 μM final concentration). (C) Canine platelet aggregation in response to 0.1 μM standard PAF which had been subjected to the solid-phase extraction procedure described in Table II.

among PAF, the silica adsorbent of the Sep-Pak cartridge and the elution solvents in Table II did not lead to any apparent alteration of this analyte during chromatography. The resolution and recovery of PAF observed were clearly a function of lipid polarity as well as solvent strength and polarity, for methanol alone (Table I) was not as efficient as methanol–chloroform–water (Table II) in eluting PAF and separating it from less polar, non-choline-containing phospholipids.

It has recently been reported that elution of silica adsorbent with basic solvents containing MTBE also affords excellent resolution of less polar phospholipids from phosphatidylcholine while obviating the use of chloroform [25]. Consequently, the elution and recovery of PAF from silica Sep-Pak cartridges by the MTBE-based solvent mixtures described [25] were evaluated (Table III). In accord with the results reported [25], acidic MTBE selectivity eluted neutral lipid from phospholipid, which remained on the column. MTBE–methanol–ammonium acetate (10:4:1, v/v) eluted the less polar phosphoglycerides, such as phosphatidylethanolamine, with high recovery; no choline phospholipids emerged in this fraction. The relatively more polar, choline-containing phospholipids, including PAF, appeared to be eluted quantitatively by 24 ml of MTBE–methanol–ammonium acetate (5:8:2, v/v). The elution pattern and recoveries in Table III were not influenced by the absence of tissue lipid or the presence of excess standard lipids in the chromatographed sample. Fraction 3 thus appeared to represent a choline phospholipid fraction enriched for PAF.

When standard PAF subjected to the solid-phase extraction procedure in Table III was assayed for bioactivity, a significant (about 60%) reduction was noted in its ability to elicit platelet aggregation (cf. Fig. 1), despite the appar-

TABLE III

ELUTION AND RECOVERY OF LIPIDS FROM SILICA SEP-PAK CARTRIDGES USING MTBE, METHANOL AND AMMONIUM ACETATE

Lipid class	Recovery[a] (%)		
	Fraction 1	Fraction 2	Fraction 3
Neutral lipid[b]	98.3 ± 1.9	0	0
Phosphatidylcholine	0	< 0.1	99.6 ± 0.5
Phosphatidylethanolamine	0	97 7 ± 1.5	0
Phosphatidylinositol	0	98.1 ± 1.4	0
Phosphatidylserine	0	97.9 ± 1.6	0
Sphingomyelin	0	< 0.1	98.2 ± 0.6
Lysophosphatidylcholine	0	< 0.1	94.3 ± 0.8
Lyso-PAF	0	< 0.1	98.3 ± 0.8
PAF	0	0	99.5 ± 0.7[c]

[a]Results are mean ± S.D. ($n \geq 4$) of each lipid class as assessed by internal radiolabeled lipid standards added to ≤ 5 mg heart muscle tissue lipid prior to chromatography. Similar results were obtained in the absence of tissue lipid or in the presence of ≤ 5 mg non-labeled lipid standard(s). Fraction 1 was eluted in 14 ml MTBE–acetic acid (100 0.2, v/v); fraction 2 was eluted in 12 ml MTBE–methanol–ammonium acetate (10 4 1, v/v); fraction 3 was eluted in 24 ml MTBE–methanol–ammonium acetate (5:8 2, v/v).

[b]Represented by a mixture of triacylglycerol, non-esterified fatty acid, cholesterol and cholesterol ester radiolabeled internal standards.

[c]Subsequent molecular analysis by HPLC (cf Fig. 3) revealed that most (~60%) of this radioactivity applied as [^{3}H]PAF was associated with lyso-PAF after elution.

ently excellent recovery of PAF as assessed by a radiolabeled internal standard (Table III). This discrepancy was resolved by HPLC analysis to determine the molecular nature of the material eluted in the MTBE–methanol–ammonium acetate of fraction 3 (Fig. 3). Some 60% of the PAF subjected to chromatography on a silica Sep-Pak, as outlined in Table III, displayed a conspicuous shift in HPLC behavior from the PAF region to co-migrate with [^{3}H]lyso-PAF, which is not bioactive [5,17,18]. Therefore, it is evident that significant PAF deacetylation occurred during this solid-phase extraction procedure which would preclude its use in quantitative PAF analysis despite the integrity of non-PAF lipids. Since the PAF internal standard was ^{3}H-labeled at positions other than the acetyl moiety and since PAF and lyso-PAF co-eluted from the Sep-Pak cartridge (Table III), the deacetylation was not detected as a reduction of PAF-associated ^{3}H label. It has been reported [5] that aluminum oxide adsorbent degrades PAF. The results in Fig. 3 demonstrate that considerable PAF degradation (i.e., deacetylation) may also occur during solid-phase extraction on silica adsorbent with basic solvents, although no degradation of non-PAF phosphoglycerides was observed.

In summary, the chromatographic system described in Table II can be used

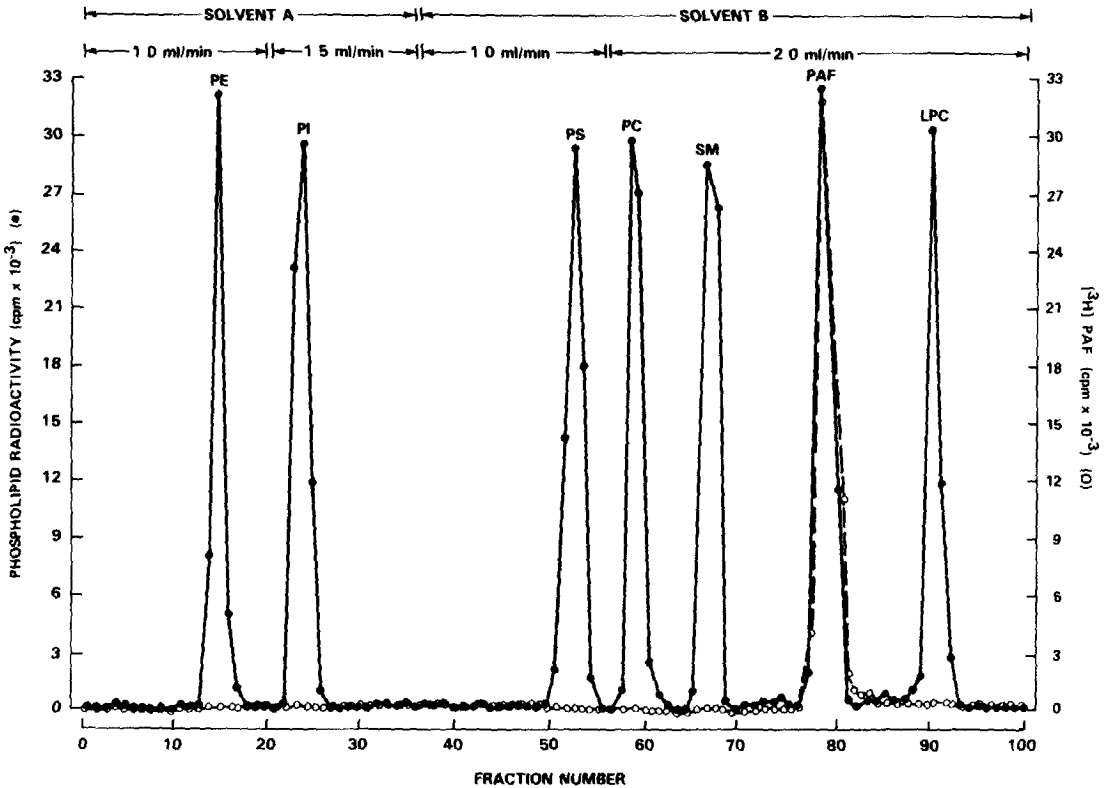

Fig. 2. Molecular identification of PAF after solid-phase extraction. A mixture of seven radiolabeled phospholipids (60 000 cpm each) was subjected to HPLC as detailed under Experimental. [^{3}H]PAF standard alone (60 000 cpm) was subjected to the solid-phase extraction procedure outlined in Table II, and fraction 3 from this procedure was recovered and likewise subjected to HPLC. HPLC eluates were collected at 1-min intervals over a 100-min run and counted by liquid scintillation spectrometry. The elution profiles of the phospholipid mixture (●) and of PAF after solid-phase extraction (○) are plotted here and are representative of three independent runs. Identities given were confirmed by the HPLC elution volume and retention time of each radiolabeled lipid standard chromatographed separately. The recovery of each lipid through the HPLC procedure was >94% in all cases; the recovery of PAF was ~99%. Abbreviations: solvent A = propanol-ethyl acetate-benzene-water (130·80 30.20, v/v); solvent B = propanol-toluene-acetic acid-water (93:110 15 15, v/v); PE = phosphatidylethanolamine; PI = phosphatidylinositol; PS = phosphatidylserine; PC = phosphatidylcholine; SM = sphingomyelin; PAF = platelet-activating factor; LPC = lysophosphatidylcholine.

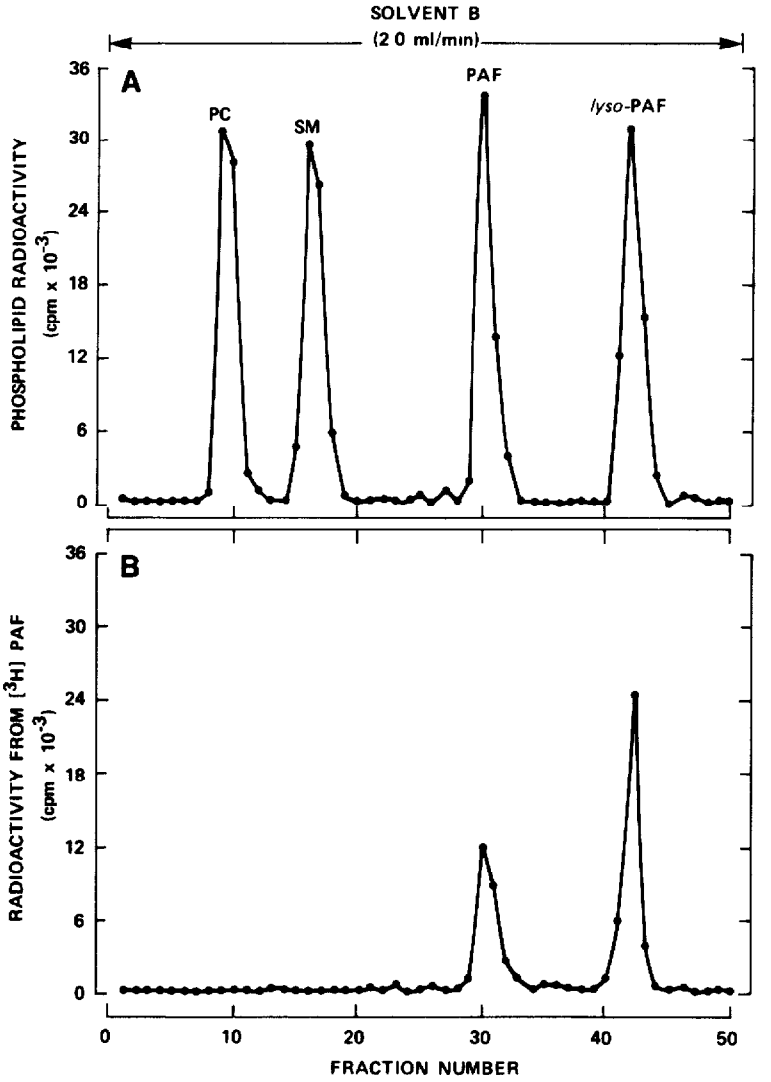

Fig. 3. Deacetylation of PAF during solid-phase extraction with MTBE–methanol–ammonium acetate. A mixture of four radiolabeled phospholipids (phosphatidylcholine, spingomyelin, PAF and lyso-PAF; 60 000 cpm each) was subjected to the HPLC procedure detailed under Experimental with solvent B only at a flow-rate of 2 ml/min. [^{3}H]PAF standard alone (60 000 cpm) was subjected to the solid-phase extraction procedure outlined in Table III, and fraction 3 eluted from the Sep-Pak™ cartridge was recovered and likewise subjected to HPLC. HPLC eluates were collected at 1-min intervals over a 50-min run and counted by liquid scintillation spectrometry. The elution profiles of the mixture (A) and of the PAF which had been subjected to solid-phase extraction (B) are given here and are representative of three independent runs. Identities were confirmed by the HPLC elution volume and retention time of each radiolabeled lipid standard chromatographed separately. Abbreviations are as defined in the legend to Fig. 2.

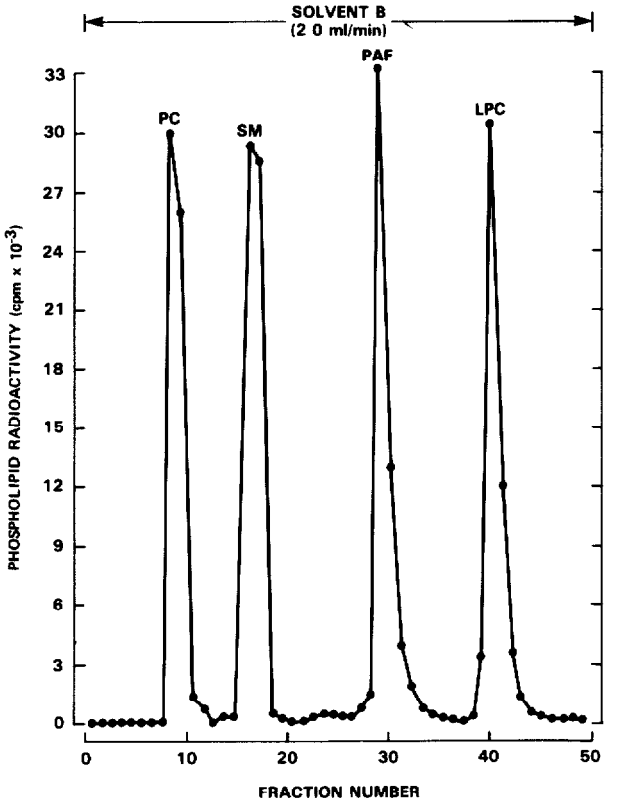

Fig. 4. Solid-phase extraction on silica Sep-Pak cartridges as an aid to PAF isolation by HPLC. A mixture of seven radiolabeled phospholipids (60 000 cpm each) identical to that used in Fig 2 was subjected to the solid-phase extraction procedure outlined in Table II. PAF-enriched fraction 3 was subjected to the HPLC procedure described under Experimental with solvent B only at a flow-rate of 2 ml/min. The HPLC eluate was collected at 1-min intervals over a 50-min run and counted by liquid scintillation spectrometry. The elution profile given is representative of three independent runs. Peak identities were established by the HPLC elution volume and retention time of each radiolabeled lipid standard chromatographed separately. Abbreviations are as defined in the legend to Fig. 2.

to enrich crude biological lipid extracts or lipid mixtures for PAF with ease, economy of materials, quantitative PAF recovery and low solvent volumes. During the procedure, the major tissue and blood neutral lipids along with virtually all of the non-choline-containing phospholipids are selectively removed from the starting lipid mixture, thereby yielding a fraction markedly enriched for PAF (Fig. 4). The high (at least 5 mg lipid) capacity of the method is noteworthy in comparison to the far (> 50-fold) lower capacity of analytical TLC. The quantitative recovery of PAF in this solid-phase extraction system

is also compelling, since PAF recovery from multi-step column chromatography–TLC procedures may be as low as 30% [5].

The attractiveness of this solid-phase extraction technique as an alternative to routine PAF enrichment on conventional columns followed by TLC [4–6] can perhaps best be appreciated from the considerable enhancement of sample throughput afforded: a PAF-enriched fraction suitable for PAF bioassay, PAF radioligand receptor assay or subsequent molecular analysis can be prepared from a tissue extract or complex lipid mixture by the methodology outlined in Table II within minutes in one chromatographic step, whereas conventional column chromatography–TLC systems require several hours of time and multiple cumbersome manipulations [4–6]. As exemplified by comparison of Fig. 2 with Fig. 3, solid-phase extraction can be coupled with subsequent HPLC to facilitate PAF isolation/purification and thereby greatly reduce the length of the HPLC run and the total analysis time. Clearly, with solvents of appropriate composition, strength and polarity, solid-phase extraction on pre-packed silica cartridges has several unique advantages over conventional chromatographic methods for PAF enrichment from lipid mixtures and thus constitutes and improvement over such methods.

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