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THE MOLECULAR HETEROGENEITY OF SALIVARY PLATELET-ACTIVATING FACTOR (PAF) IN PERIODONTAL HEALTH AND DISEASE

A THESIS

Presented to the Faculty of

The University of Texas Graduate School of Biomedical Sciences

at San Antonio

in Partial Fulfillment of the Requirements for the Degree of

MASTER OF SCIENCE

By

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San Antonio, Texas

May 1995

THE MOLECULAR HETEROGENEITY OF SALIVARY PLATELET-ACTIVATING FACTOR (PAF) IN PERIODONTAL HEALTH AND DISEASE

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DEDICATION

Support of an individual's endeavors in life takes many forms. To my wife Andi - for the past 18 years your love, understanding, listening, and day-to-day motivation has enabled me to pursue and attain many goals. To my children Lindsay and Adam - your artistry, whether on the stage or on the ice provided me with pleasant diversion from the emotional strains of a difficult program. Our best times are yet to come. "Game On!"

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THE MOLECULAR HETEROGENEITY OF SALIVARY PLATELET-ACTIVATING FACTOR (PAF) IN PERIODONTAL HEALTH AND DISEASE

Publication No. _____

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Platelet-activating factor (PAF) is a potent, pro-inflammatory phospholipid which is present in normal human mixed saliva. A role for PAF in periodontal inflammation has been suggested by previous studies. Although PAF has been viewed as a single molecular structure, variations occur in the length of the alkyl chain and the degree of unsaturation. Since the molecular structure of PAF has a profound effect on the biological activities of this autacoid, the structural diversity of PAF in saliva could be of importance in PAF-induced oral inflammation. Thus, the present study was designed to assess the molecular heterogeneity of PAF in saliva from individuals with periodontal disease in comparison to that of normal subjects.

Unstimulated, mixed saliva (5-12 ml) was collected from periodontally normal (N), adult periodontitis (AP), and rapidly progressive periodontitis (RPP) subjects. Following saliva collection, a comprehensive periodontal evaluation was performed by a single examiner. Salivary lipids were extracted and fractionated by sequential high performance liquid chromatography (HPLC); PAF activity was determined in rabbit platelet bioassay or human polymorphonuclear leukocyte (PMN) priming bioassay relative to that of authentic PAF, 1-*O*-hexadecyl-2-acetyl-*sn*-glycero-3-phosphocholine (16:0-alkyl-PAF).

Salivary PAF activity in platelet bioassay for N, AP, and RPP subjects was 4,040 $\pm$ 708, 12,780 $\pm$ 2,621, and 8,445 $\pm$ 1,112 fmole equivalents of 16:0-alkyl-PAF/ml of saliva, respectively (mean $\pm$ SE). The distribution of PAF activity after HPLC fractionation as determined by both platelet and PMN bioassays was not qualitatively different between any of the subject groups. For all subjects, the 16:0-alkyl-PAF region represented the most abundant salivary PAF activity; there were no significant differences in the percentage distributions of 16:0-alkyl-PAF among the subject groups (Table 1). Platelet bioactivity for the 15:0-alkyl-PAF region was significantly increased in the AP and RPP groups as compared to normal subjects; however, only 2 of 8 normal patients exhibited salivary PAF activity in this HPLC region. For the other molecular species of PAF, there were no significant differences between the groups.

When the relationship between clinical indices of periodontal disease and individual PAF molecular species as estimated in platelet bioassay were compared, significant and strong correlations were found between the number of probing depths greater than or equal to 4 mm and 15:0- ($r=0.73$, $p=0.0008$), 16:0- ($r=0.60$, $p=0.01$), 18:1- ($r=0.62$, $p=0.008$), and 18:0-alkyl-PAF ($r=0.64$, $p=0.005$). Additionally, most PAF molecular species significantly correlated with the number of sites with attachment loss 2 mm or greater (15:0-alkyl-PAF: $r=0.54$, $p=0.03$; 16:0-alkyl-PAF: $r=0.53$, $p=0.03$; 18:0-alkyl-PAF: $r=0.57$, $p=0.02$).

Table 1. DISTRIBUTION OF SALIVARY PAF ACTIVITY AFTER HPLC FRACTIONATION

HPLC REGION	BIOASSAY	N (n=7)	AP (n=7)	RPP (n=3)
15:0-alkyl-PAF	PLATELET	0.6 ± 0.4*	2.6 ± 0.5#	1.9 ± 1.0#
	PMN	*1.7 ± 1.3**	*3.4 ± 1.3*	*4.0 ± 3.5*
16:0-alkyl-PAF	PLATELET	68.1 ± 5.7	72.0 ± 2.5	65.4 ± 2.3
	PMN	*75.3 ± 7.0*	*67.2 ± 7.1*	*76.1 ± 4.9*
18:1-alkyl-PAF	PLATELET	23.3 ± 5.0	18.0 ± 1.8	23.2 ± 2.2
	PMN	*22.5 ± 7.4*	*28.5 ± 6.7*	*19.2 ± 5.0*
18:0-alkyl-PAF	PLATELET	8.1 ± 2.1	7.4 ± 1.0	9.6 ± 2.0
	PMN	*0.4 ± 0.4*	*0.8 ± 0.3*	*0.6 ± 0.5*

* Results presented as the mean ± SE

# $p<0.03$ as compared to normal subjects (*t*-test following One-way ANOVA)

Thus, multiple PAF molecules are present in mixed saliva derived from periodontally healthy and diseased subjects. The percentage distribution of individual molecular species of PAF, with the possible exception of 15:0-alkyl-PAF, do not vary in periodontal health and disease. This suggests that periodontal disease is related to the total amount of PAF in saliva rather than differences in PAF molecular species. In addition, clinical features of periodontal disease significantly correlate with the levels of individual PAF molecular species. In combination, these results continue to support the hypothesis that PAF may participate in inflammatory events associated with periodontal tissue injury and disease.

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1. Introduction

Periodontal disease is characterized by inflammation. This inflammation protects the host against local microbial attack. However, it may not be entirely beneficial since surrounding cells, connective tissue and alveolar bone may be damaged. Clinical parameters for the detection of periodontal disease activity have received much attention over the years. Of these, gingival crevicular fluid and its contents have been an area of active research. In 1990, Rasheed and McManus presented evidence for a crevicular source of platelet-activating factor (PAF), a family of structurally-related, potent phospholipid mediators of inflammatory reactions. PAF is produced by a variety of inflammatory cells and has been isolated from inflamed human gingival tissue (Noguchi *et al.*, 1989), and normal, mixed saliva (Cox *et al.*, 1981; Christman and Blair 1989). Cross-sectional studies have demonstrated an increase of PAF in the mixed human saliva of periodontitis patients (Diaz *et al.*, 1990; Braun *et al.*, 1992; McDonnell 1994; Garito *et al.*, 1995; Rasch *et al.*, 1995). Therefore, it seems likely that PAF may participate in inflammatory events associated with periodontal disease.

Past research concerning PAF and periodontal disease has investigated the relationship of PAF in various types of periodontal disease. No studies have attempted to define the molecular species of salivary PAF in relation to periodontal disease. Therefore, the current study was undertaken to evaluate the molecular heterogeneity of salivary PAF in periodontal health and disease.

II. Literature Review

A. Platelet-Activating Factor

1. Historical Background

PAF is a potent phospholipid autacoid, first recognized in studies characterizing the cell-cell interactions following antigen stimulation of rabbit buffy coat leukocytes containing IgE-sensitized basophils (Siraganian and Osler, 1971 and Benveniste *et al.*, 1972). During this immunologically initiated reaction, a fluid-phase intermediate was released as characterized by its ability to induce the aggregation of, and initiate the release reaction from isolated rabbit platelets (*c.f.*, Pinckard *et al.*, 1988). Because its chemical nature was not known, the term platelet-activating factor or PAF was coined to describe its functional activity (Benveniste *et al.*, 1972).

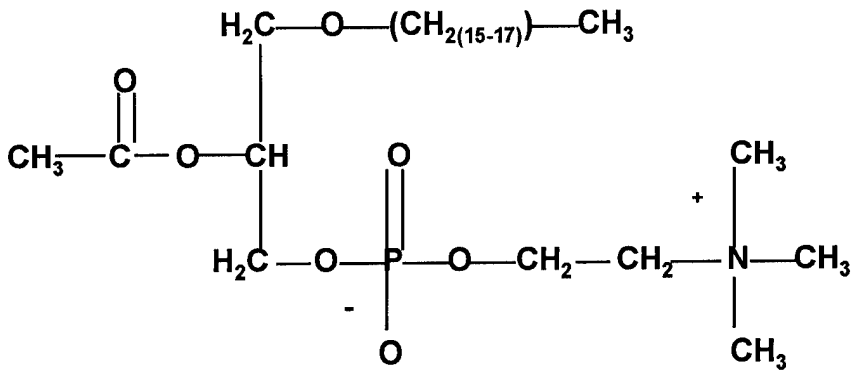
Early investigations demonstrated that hog leukocyte PAF was extractable into chloroform and ethanol, which suggested a lipid-like molecule (Benveniste *et al.*, 1977). The phospholipid-rich fractions containing PAF activity eluted from silicic acid columns and migrated on thin-layer chromatography (TLC) between lysolecithin and sphingomyelin. This migration was unaffected by changes in the solvent system (Pinckard *et al.*, 1979). These characteristics together with the observation that PAF could not be extracted from a chloroform solution with $NaHCO_3$, suggested a neutral lipid molecule. Functional PAF activity and migration on TLC was stable to acid conditions, exposure to air and heating, or treatment with periodic acid or sodium nitrite. These findings eliminated a vinyl ether linkage, a free glycerol, glycol or sugar moiety from the structure. Evidence was provided for a neutral polar lipid containing a fatty acid ester group (Pinckard *et al.*, 1979). Indeed, the sensitivity of PAF to destruction by methanolic sodium hydroxide provided a key into the structural elucidation of PAF as outlined below.

Demopoulos *et al.* (1979), conducted a series of experiments to restore the biologic activity of PAF after treatment with methanolic sodium hydroxide. Thus after extraction into chloroform, the base-catalyzed degradation product(s), acylated with stearic acid anhydride, were not biologically active. In contrast, biologic activity was restored if acetic, proprionic or butyric acid anhydrides were used as the acylating agents; only the acetylated product had TLC behavior identical to native PAF. Since the base-catalyzed PAF degradation product was fully soluble in a mixture of chloroform:methanol:water, these results indicated that PAF contained an alkyl linkage. Together with the TLC migration properties and susceptibility to phospholipase A2 (PLA_2) degradation, the chemical structure of PAF was proposed to be 1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine (alkyl-PAF) (Demopoulos *et al.*, 1979). Subsequent gas chromatography and mass spectrometry analysis of rabbit basophil-derived PAF by Hanahan *et al.*, (1980), identified the chemical structure of PAF as 1-*O*-hexadecyl/octadecyl-2-acetyl-*sn*-glycero-3-phosphocholine (16:0- and 18:0-alkyl-PAF) (Figure 1).

2. Cellular and Tissue Sources of PAF

PAF does not exist within cells as a stored, preformed inflammatory mediator. Upon cell stimulation, PAF is rapidly synthesized by a variety of cells including basophils, eosinophils, lymphocytes, macrophages, mast cells, mesangial cells, monocytes, natural killer cells, polymorphonuclear neutrophilic leukocytes (PMN), platelets, tracheal epithelial cells, and vascular endothelial cells (*c.f.*, McManus *et al.*, 1993). The blood, brain, embryo, kidney, liver, lung, myocardium, retina, saliva, skin, and uterus have been identified as tissue sources of PAF if physiologic conditions such as ischemic injury, allergic reactions, or inflammation are present (*c.f.*, Pinckard *et al.*, 1988).

Figure 1. The Chemical Structure of 1-*O*-hexadecyl/octadecyl-2-acetyl-*sn*-glycero-3-phosphocholine (16:0- and 18:0-alkyl-PAF). This structure consists of a glycerol backbone, with a long chain alkyl group linked to the *sn*-1 position by an ether linkage, an acetate esterified at the *sn*-2 position, and a phosphocholine group at the *sn*-3 position.



1-*O*-hexadecyl/octadecyl-2-acetyl-*sn*-glycero-3-phosphocholine

3. Sources of PAF in Saliva

Although PAF has been demonstrated to be present in human mixed saliva (Cox *et al.*, 1981; Smith *et al.*, 1986; McManus 1988, Christman and Blair 1989, McManus *et al.*, 1993), the exact cellular source of intraoral PAF has not been defined. The contribution of the salivary glands to intraoral PAF is considered to be insignificant. Cox and others in 1981 reported that pure parotid saliva has no PAF activity. In a study comparing edentulous to dentulous subjects (McManus *et al.*, 1990), it was reported that in 60% of the edentulous subjects tested, no salivary PAF was detected; when salivary PAF was present in edentulous subjects, levels were significantly less than in dentate subjects. In addition, the number of PMN in the mixed saliva samples of the edentulous subjects was markedly reduced when compared to dentate subjects. These results suggested that the intraoral source for PAF was the gingival crevice, with the PMN as the likely cell source.

In studies by Garito *et al.*, (1995), histologically identified PMN derived from human mixed saliva were found to progressively increase and significantly correlate with periodontal disease severity. In these same studies, the amount of PAF in saliva sequentially increased as periodontal disease severity increased. Rasheed and McManus (1990) demonstrated a positive correlation between levels of PAF in gingival crevicular fluid and pocket depths greater than 3 mm. In combination, these findings suggest that intraoral PAF likely originates from the crevicular space, possibly produced by inflammatory cells (PMN) from within the gingiva.

4. Synthesis and Degradation

Several pathways for alkyl-PAF biosynthesis have been described: 1) a two-step enzymatic deacylation-reacetylation reaction (remodeling pathway) (Wykle *et al.*, 1980) or 2) a *de novo* enzymatic pathway (Renooij and Snyder, 1981). The former pathway is thought to be the predominant pathway for production of PAF in inflammatory cells and is initiated

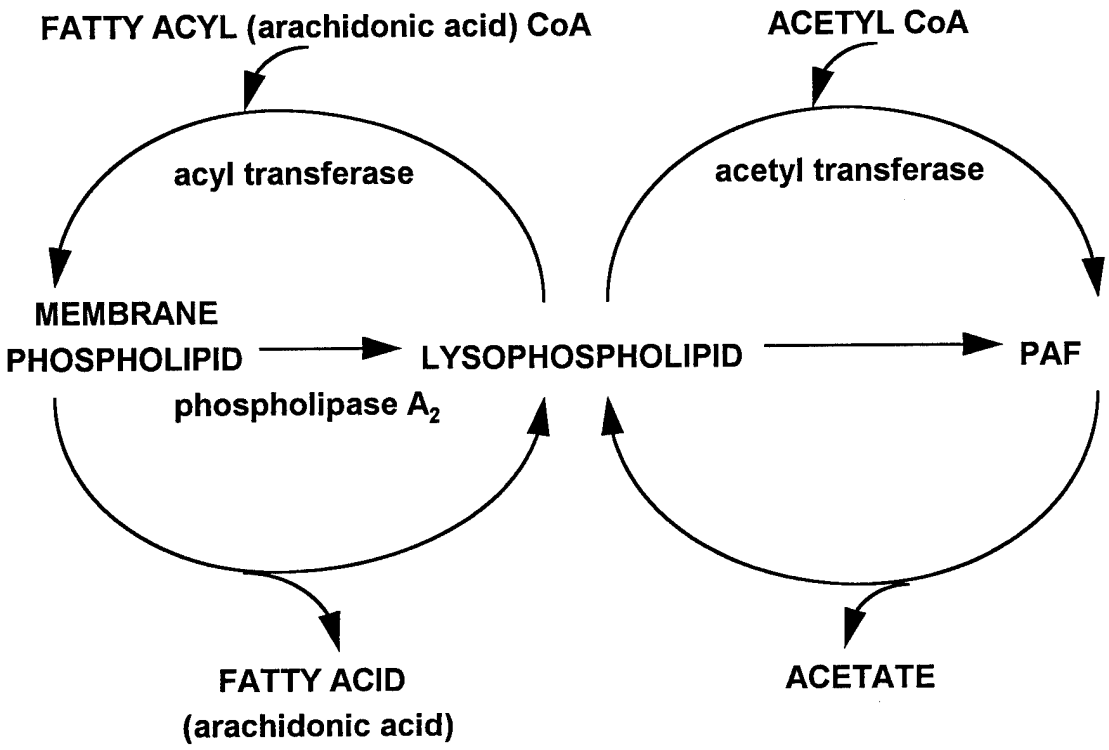
following cell stimulation and subsequent activation of PLA_2. Action of this enzyme on the intracellular precursor 1-*O*-alkyl-2-acyl-*sn*-glycero-3-phosphocholine results in hydrolysis of long-chain acyl residues esterified in the 2 position to form 1-*O*-alkyl-*sn*-glycero-3-phosphocholine (lyso-PAF). Significantly, free arachidonic acid is cleaved at this step, which can be enzymatically converted to various arachidonic acid metabolites including HETES, leukotrienes, thromboxanes, prostacyclin, and prostaglandins (*c.f.*, Venable *et al.*, 1993). Lyso-PAF is then acetylated in the presence of acetyl-CoA by PAF acetyltransferase to form 1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine (alkyl-PAF), the biologically active PAF molecule (Figure 2).

Within this remodeling pathway, recent studies have shown that a coenzyme A-independent transacylase (CoA-IT) may also be involved in the production of PAF (Venable *et al.*, 1993). Arachidonate is transferred from the PAF precursor to Lysophosphatidylethanolamine (Lyso-PE) by CoA-IT; lyso-PAF is formed as a result of this reaction (Venable *et al.*, 1993). Formation of PAF then occurs through the action of PAF acetyltransferase on lyso-PAF.

In the *de-novo* pathway of PAF biosynthesis, 1-*O*-alkyl-*sn*-glycero-3-phosphate is acetylated by 1-*O*-alkyl-*sn*-glycero-3-phosphate:acetyl coenzyme A acetyltransferase (*c.f.* Venable *et al.*, 1993). 1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphate phosphohydrolase then produces 1-*O*-alkyl-2-acetyl-*sn*-glycerol, which is converted to PAF by a CDP-choline phosphotransferase reaction. This pathway appears to be less important for PAF biosynthesis in activated inflammatory cells; enzymes in this pathway are constitutively active and seem to be regulated largely by substrate availability (*c.f.*, Venable *et al.*, 1993).

Although the biosynthesis of PAF can occur by multiple pathways, degradation occurs by a single family of phospholipases. Degradation of PAF is initiated *via* enzymatic removal of the acetyl group at the *sn*-2 position by PAF acetylhydrolase. Both an intracellular and

Figure 2. The Remodeling Pathway of PAF Biosynthesis and Degradation. A two-step enzymatic deacylation-reacetylation reaction is initiated following cell stimulation and subsequent activation of PLA_2. Action of this enzyme on the intracellular PAF precursor, 1-*O*-alkyl-2-acyl-*sn*-glycero-3-phosphocholine, results in the hydrolysis of long-chain acyl residues esterified in the 2 position to form 1-*O*-alkyl-*sn*-glycero-3-phosphocholine (lyso-PAF). Significantly, free arachidonic acid is released at this step. Lyso-PAF is then acetylated in the presence of acetyl-CoA by PAF acetyltransferase to form 1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine (alkyl-PAF), the biologically active PAF molecule. (Taken from, L.M. McManus and S.I. Deavers. Platelet-activating factor in pulmonary pathobiology. Clin. Chest Med. 10:107-118, 1989.)


FATTY ACYL (arachidonic acid) CoA
ACETYL CoA
acyl transferase
acetyl transferase
MEMBRANE
PHOSPHOLIPID
LYSOPHOSPHOLIPID
PAF
phospholipase A_2
FATTY ACID
(arachidonic acid)
ACETATE

plasma form of acetylhydrolase exist (Stafforini *et al.*, 1987; Tselepis and Pinckard 1992), are calcium-independent, specific for short-chain fatty acyl-linked groups at the 2 position, and act to limit the half-life of PAF that is released intravascularly to less than 30 seconds (Pinckard *et al.*, 1979). The result is the formation of the inactive molecule, lyso-PAF, which inside cells, is rapidly reacylated with unsaturated fatty acid *via* acyltransferase (Chilton *et al.*, 1983) (Figure 2). Several enzymes, *i.e.*, acyl-CoA transferase, CoA-dependent transacylase, and CoA-independent transacylase, have each been shown to catalyze this reaction (Robinson *et al.*, 1985). In resting cells, the CoA-independent transacylase pathway predominates, but all three pathways participate after cell stimulation (Venable *et al.*, 1993). Selective reacylation of lyso-PAF by arachidonic acid in the human PMN (Chilton and Murphy, 1986) and platelet (Kramer *et al.*, 1984) is catalyzed by a CoA-independent acyltransferase (Chilton and Murphy, 1986; Wykle *et al.*, 1986). The complete cycle of synthesis by this two-step enzymatic deacylation-reacetylation sequence coupled with degradation is referred to as the remodeling pathway (Wykle *et al.*, 1980; Wykle *et al.*, 1986).

5. PAF Molecular Heterogeneity

In the past, PAF has been viewed as a single molecular structure. However, soon after the structure of PAF was proposed by Demopoulos *et al.* (1979), to be 1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine, Hanahan *et al.*, (1980) described two PAF structures derived from rabbit basophils, 16:0- and 18:0-alkyl-PAF, which differed only in the lengths of the carbon chains at the *sn*-1 position. Since those discoveries, due to advances in techniques for identification, PAF has been found to be a family of structurally related phospholipids that share many pro-inflammatory activities (Ludwig and Pinckard, 1987). However, minor alterations in PAF structure have profound effects on biological activity and metabolic breakdown (Stafforini *et al.*, 1987).

The stimulated human PMN produces multiple molecular species of PAF which include both saturated and unsaturated 1-*O*-alkyl homologs of PAF (alkyl-PAF), 1-*O*-acyl analogs (acyl-PAF), and acetylated phosphoglycerides having polar head groups other than choline (*c.f.*, Pinckard *et al.*, 1988). Mueller *et al.* (1984), examined PAF produced by stimulated human PMN into which tritiated acetate was incorporated. Utilizing reverse-phase high performance liquid chromatography (RP-HPLC) for phospholipid separation, multiple alkyl-chain homologs of PAF were detected. Molecular species and percentages of alkyl-PAF found included 16:0- (40%), two isomers of 17:0- (8% and 5%), 18:0- (16%), and 18:1- (18%). Oda *et al.* (1985), reported that human PMN stimulated with calcium ionophore (A23187) produced only 16:0- and 18:0-alkyl-PAF. Weintraub and others discovered in 1985 that PAF produced by N'-formyl-methionyl-leucyl-phenylalanine (FMLP) or A23187 stimulated human PMN and subjected to fast atom bombardment-mass spectrometry consisted of 16:0-, 17:0-, 18:0-, 18:1-, 15:0-, and 22:2-alkyl-PAF. Thus, multiple alkyl-linked PAF molecular species were demonstrated to be produced by the human PMN.

In addition to alkyl-PAF, stimulated human PMN and other cells produce acyl-PAF. Mueller *et al.* (1984), has shown that PAF produced by human PMN stimulated with A23187 is from 13 to 25% acyl-PAF. Interestingly, 1-*O*-acyl-2-acetyl-*sn*-glycero-3-phosphocholine (acyl-PAF) is the major acetylated lipid produced by stimulated human mast cells, basophils, and vascular endothelial cells (*c.f.*, Venable *et al.*, 1993). Indeed, production of acyl-PAF may be favored if endogenous phospholipase A_1 (PLA_1) is blocked (Sturk *et al.*, 1989) or if there is a relative abundance of acyl-PAF phospholipid precursors present in the cytosol (Venable *et al.*, 1993). Therefore, not only alkyl-linked but also acyl-linked PAF molecular species, all with biological activity, are produced by the human PMN.

Due to the broad substrate specificity in the remodeling pathway, other PAF analogs may also be produced. Tessner and Wykle (1987) have shown that up to 25% of the total phospholipids labeled with tritiated acetate after A23187 stimulation of human PMN were

ethanolamine-containing PAF analogs. Of these, greater than 80% were 1-*O*-alk-1'-enyl-2-acetyl-*sn*-glycero-3-phosphocholine (alkenyl-PAF). Thus, multiple PAF molecular species can be produced by the human PMN upon stimulation.

Quantitatively, the distribution of PAF molecular species produced by activated PMN do not correlate with the predominant intracellular alkyl-chain precursor, 1-*O*-alkyl-2-acyl-*sn*-glycero-3-phosphocholine (Mueller *et al.*, 1984). This is due to the broad substrate specificity that exists for the enzymes involved in PAF biosynthesis. In the remodeling pathway, for example, after activation of PLA_2, numerous lysophospholipids such as 1-*O*-alkyl- and 1-*O*-acyl-lysophospholipid are generated which serve as immediate precursors of PAF. The activities of these numerous PAF biosynthetic enzymes, as well as the availability of their substrates, vary among cells to provide an additional basis for heterogeneity in the molecular species of PAF produced by a given cell or tissue in response to a particular stimulus (*c.f.*, McManus *et al.*, 1993).

The molecular heterogeneity of PAF is not only influenced by biosynthesis pathways, but also by degradation or modification pathways. PAF acetylhydrolase, found intracellularly and in plasma, will selectively catalyze the hydrolysis of the acyl linkage located at the *sn*-2 position of PAF (Tselepis and Pinckard, 1992). Following hydrolysis, these biologically inactive products are reacylated by acyltransferase and available acyl-CoA substrates (*c.f.*, McManus *et al.*, 1993). A distinct degradation pathway for acyl-PAF in PMN is mediated by the action of PLA_1 (*c.f.*, McManus *et al.*, 1993). PLA_1 hydrolyzes long chain fatty acyl residues at the *sn*-1 position to produce 1-lyso-2-acetyl-phospholipids (Triggiani *et al.*, 1991). Catabolism may also occur in inflammatory cells through the action of phospholipase D, which hydrolyzes the base moiety from the phosphodiester in the *sn*-3 position, possibly producing phosphatidic acid PAF analogs (*c.f.*, McManus *et al.*, 1993). The end result of all these anabolic and catabolic pathways is the production of a family of PAF molecules with a wide range of biological activities.

6. PAF Structure-Activity Relationship

The effect of PAF on various cell types is generally one of a pro-inflammatory nature. *In vitro*, PAF has been shown to interact with a variety of cell types. Thus, PAF induces the aggregation and activation of platelets, resulting in secretion of the contents of dense bodies, α-granules, and lysosomal granules (*c.f.*, Pinckard *et al.*, 1988). PAF stimulates human PMN *in vitro* to aggregate, undergo chemotaxis and chemokinesis, and secrete lysozyme and b-glucuronidase (*c.f.*, Pinckard *et al.* 1988). PAF also initiates aggregation of monocytes (Yasaka *et al.*, 1982). Therefore, PAF exhibits agonist-type effects on many different inflammatory cells.

In vivo, PAF causes a multitude of pro-inflammatory effects. Increased vascular permeability, hypotension, decreased cardiac output, stimulation of uterine contraction, leukocyte adhesion to endothelial cells and acute bronchoconstriction resulting from PAF have been demonstrated (*c.f.*, Venable *et al.*, 1993). Thus, PAF exerts a significant pathobiologic effect on numerous organ systems throughout the body.

All of the above *in vitro* and *in vivo* biologic activities of PAF have been shown to be significantly affected by even minor alterations in PAF structure. Hanahan (1986) has rank ordered the biologic potency of various PAF molecular species based on the composition of the molecule at the *sn*-1, 2, and 3 positions. For the *sn*-1 position, 1-*O*-alkyl- > 1-*O*-alk-1'-enyl- > 1-acyl-; at *sn*-2, acetyl = propionyl > butyryl; and at the *sn*-3 position, choline ≥ dimethylethanolamine > monomethylethanolamine ≥ phosphatidic acid > ethanolamine. Changes likely will be made in this rank order scheme because not all molecular species have been studied, few of the many biologic activities of PAF have been evaluated, and the synergistic or antagonistic effect of PAF with other inflammatory mediators has not been fully documented (*c.f.*, McManus *et al.*, 1993).

As indicated above, acyl-PAF molecules have generally been thought to have lower biological activity than corresponding alkyl-PAF species. However, in a recent study by

Pinckard *et al.* (1992), PMN functional responses to 16:0-, 18:0- and 18:1-alkyl-PAF and 16:0-, 18:0- and 18:1-acyl-PAF were investigated. Several molecular species of acyl-PAF were shown to be almost as effective as alkyl-PAF molecular species in priming human PMN to augment the production of superoxide anion after stimulation by either FMLP or human recombinant C5a. The rank order of potency of the alkyl-PAF homologs for inducing lysosomal enzyme secretion and superoxide production was 18:1- $\geq$ 16:0- >> 18:0-alkyl-PAF. The rank order of potency for effecting PMN chemotaxis for alkyl-PAF homologs was 18:0- > 18:1- >> 16:0-alkyl-PAF. Acyl-PAF analogs did not initiate PMN lysosomal enzyme secretion, superoxide production or chemotaxis. However, the acyl-PAF analogs did induce partial PMN desensitization to 16:0-alkyl-PAF. Since these various PAF molecules also stimulate platelets to different degrees and are degraded at different rates by PAF acetylhydrolase (Wardlow et al., 1986), it seems likely that the inflammatory role of PAF *in vivo* will be the result of complex biological interactions of several PAF molecules (Ludwig and Pinckard, 1987).

B. Inflammation and Periodontal Disease

1. Characterization of Inflammatory Changes

Periodontitis is characterized clinically by the presence of gingival inflammation, pocketing, and loss of alveolar bone and probing attachment (Proceedings of the World Workshop in Clinical Periodontics, 1989). The primary etiologic agent in this process is bacterial plaque. Upon the accumulation of bacterial plaque, various host defense mechanisms are stimulated, which contribute to the loss of connective tissue attachment to the teeth. As a result of these cellular and biochemical interactions, histologic changes are seen within the periodontium.

Within four days of bacterial accumulation, an acute inflammatory reaction occurs which is characterized by an increased flow of crevicular fluid and migration of PMN into the

gingival sulcus from the underlying vascular plexus (Page and Schroeder, 1976). This *initial lesion*, demonstrates a collagen loss of 5% to 10% and is not clinically visible. After seven days of plaque accumulation, the *early lesion* appears and may persist for 21 days or longer. Clinically, gingivitis is detectable, while histologically an infiltrate dominated by lymphocytes and macrophages is found. Lymphocytes comprise 75% of the inflammatory cells of this lesion, and occupy 15% of the marginal connective tissue. In this infiltrated region, 60% to 70% of the collagen is destroyed. If treatment is not performed, the early lesion will progress to the *established lesion* after a variable period of time. This lesion is characterized by the presence of plasma cells, further collagen destruction and the proliferation of the junctional epithelium. Established lesions may persist for months or years without progression (Page, 1986). These first three lesions are all characteristic of different stages of gingivitis. However, upon conversion of the established lesion to the *advanced lesion*, periodontitis begins.

Hallmarks of the advanced lesion are apical migration of the junctional epithelium, with resulting pocketing. Loss of connective tissue attachment and alveolar bone are seen, along with a dominant plasma cell infiltrate. Other inflammatory cells such as PMN, lymphocytes and macrophages may be present (Page and Schroeder, 1976).

2. Periodontal Disease Classification

In 1989, The World Workshop in Clinical Periodontics established the following classification of periodontal disease:

I. Adult Periodontitis

II. Early-Onset Periodontitis

III. Periodontitis Associated with Systemic Disease

IV. Necrotizing Ulcerative Periodontitis

V. Refractory Periodontitis

Adult periodontitis may have its onset in adolescence, but is usually not clinically significant until the mid-thirties. Prevalence and severity increase with age while progression of the disease is slow. The presence and severity of adult periodontitis is directly related to the presence of plaque and calculus while PMN and other host defense functions are normal (World Workshop in Clinical Periodontics, 1989).

Early-onset periodontitis is distinguished from adult periodontitis based upon age of onset, rapidity of the disease process, and defects in the host defense system. Included in this group are prepubertal, juvenile, and rapidly progressive periodontitis. Rapidly progressive periodontitis is a subgroup of the early-onset periodontitis classification. This form of periodontitis typically occurs in individuals from their early twenties to mid-thirties. It is characterized by severe gingival inflammation and rapid loss of connective tissue attachment and alveolar bone support. Most teeth are affected and approximately 66% of affected persons have a depressed PMN chemotaxis response (World Workshop in Clinical Periodontics, 1989).

Early-onset forms of periodontal disease may also result from the influence of systemic diseases which appear to predispose an individual to periodontitis. Conditions such as Type 1 diabetes mellitus, Down's Syndrome, Papillon-Lefevre Syndrome and HIV infection are all associated with periodontitis. At the present time, these systemic disorders are insufficiently understood to be placed in a distinctive category for each entity (World Workshop in Clinical Periodontics, 1989). As a result, these conditions are grouped into a single category, *i.e.*, periodontitis associated with systemic disease.

Necrotizing ulcerative periodontitis (NUP) is a distinct form of periodontitis characterized by a distinct erythema of the free gingiva, attached gingiva, and alveolar mucosa. NUP has a severe and rapid course, resulting in loss of periodontal attachment. Because of the extensive soft tissue necrosis, however, deep pocket formation does not occur (Glossary of Periodontal Terms, 1992).

Patients who are unresponsive to any form of therapy, whatever the thoroughness or frequency, are classified in the refractory periodontitis category. This class also contains individuals with recurrent disease at single or multiple sites (World Workshop in Clinical Periodontics, 1989).

3. Periodontal Disease and PAF

Numerous immunologic and inflammatory events have been associated with PAF (see above). Of particular interest relative to a role for PAF in the initiation and/or maintenance of periodontal disease is the effect of PAF on cells such as fibroblasts and osteoclasts found within the periodontium. Interestingly, PAF appears to have no direct effects on human gingival fibroblasts *i.e.*, McAllister *et al.* (1993), in studies which also determined that PMN had approximately 5500 PAF receptors per cell, could not identify specific PAF binding to human gingival fibroblasts. In this same investigation, PAF was shown to significantly stimulate PMN release of kallikrein. The authors concluded that PAF effects in the periodontium are probably initiated by other PAF targets in the periodontal lesion, *e.g.*, inflammatory cells, which then produce secondary mediators that alter periodontal connective tissue.

Conflicting evidence has been published relative to the role of PAF in bone resorption. Wood *et al.*, (1991), have presented evidence that PAF causes release of internal calcium in rat osteoclasts, together with peripheral pseudopod retraction and respreading. Zheng *et al.*, (1993), have shown that PAF stimulates isolated rat osteoclasts to resorb dentin. In combination, these observations suggest that PAF may modulate osteoclastic bone resorption. However, Wuckerpfenny *et al.* (1993), have observed that although PAF caused calcium influx into isolated osteoclasts, no direct effects of PAF on bone resorption occurred. Clearly, further investigations are needed to definitively determine the role of PAF in periodontal bone resorption.

Although few studies have been performed investigating the role of PAF on isolated cells of the periodontium, the pro-inflammatory effects of PAF on numerous other tissues and cells warrants its investigation in the pathogenesis of periodontal disease. Noguchi *et al.* (1989), demonstrated a significant difference in the amount of PAF derived from normal and inflamed gingival tissue. Based on these findings, Noguchi postulated that PAF is likely to play a role in the occurrence and maintenance of periodontal disease. Garito *et al.*, (1990, 1995), demonstrated that salivary PAF levels in periodontitis patients positively correlated with the number of probing depths greater than 4 mm and with the number of sites which bled on probing. In addition, Diaz *et al.*, (1990) has demonstrated that patients with refractory periodontitis exhibit a correlation between probing depths 4 mm or greater and salivary PAF levels. This evidence suggests that PAF may play a role in the pathogenesis of periodontal disease.

Relative to the above, it is important to note that PAF may have a longer half-life in saliva than in plasma. Cooney *et al.*, (1991), have shown that the activity of PAF acetylhydrolase in saliva is 1,000-fold lower than that of plasma. Thus, PAF in the oral cavity may have a longer and/or greater biological potency.

Several studies have examined the effect of periodontal therapy on salivary PAF levels. Braun *et al.* (1992), evaluated salivary PAF levels in 17 patients diagnosed with rapidly progressive periodontitis (RPP), in comparison to 9 age- and sex-matched periodontally healthy control subjects. Initial salivary PAF levels for the RPP and periodontally healthy controls were 18,390 $\pm$ 5,852 and 2,318 $\pm$ 355 fmole equivalents of 16:0-alkyl-PAF/ml of saliva, respectively (mean $\pm$ SE). Prior to treatment, salivary PAF levels within the RPP group were found to positively correlate with bleeding on probing and severity of periodontal disease as estimated by the number of probing depths greater than 3 mm. Following initial and surgical therapy, no difference in salivary PAF levels were found between the groups.

These data provide evidence that a relationship between salivary PAF and periodontal disease exists.

Similar to the above, Rasch (1993, 1995) studied the effect of initial therapy on salivary PAF levels in 15 chronic adult periodontitis patients. Pre-treatment salivary PAF levels decreased from an initial value of 12,084 $\pm$ 2,789 (mean $\pm$ SE) to 9,237 $\pm$ 2,293 fmole equivalents of 16:0-alkyl-PAF/ml of saliva following supragingival plaque control. A further decrease in salivary PAF (5,735 $\pm$ 1,402) was found following scaling and root planing. Thus, initial periodontal therapy reduces salivary PAF levels in parallel with improvements in clinical estimates of marginal and submarginal periodontal inflammation.

McDonnell (1994) performed the first longitudinal study examining the relationship of salivary PAF levels in chronic adult periodontitis patients and clinical parameters of periodontal disease. Fourteen patients with mild to moderate chronic adult periodontitis were treated and followed through maintenance therapy for 12 to 18 months. Pre-treatment salivary PAF levels (10,483 $\pm$ 2,233 fmole equivalents of 16:0-alkyl-PAF/ml of saliva) were significantly reduced following active treatment, and remained stable (3,506 $\pm$ 774) through one year of maintenance therapy. Salivary PAF levels significantly correlated with clinical parameters of periodontal disease and salivary PMN. This confirmed Rasch's conclusions that salivary PAF levels change in parallel with changes in clinical parameters following active treatment. Together, these studies suggest that PAF may play a role in the pathogenesis of periodontal disease.

It is important to note that in all of the above studies, the molecular species of PAF were not identified. That is, the influence of PAF molecular heterogeneity in periodontal inflammation and tissue injury has not been previously examined.

4. Molecular Species of PAF in Human Saliva

Multiple molecular species of salivary PAF have been demonstrated in human saliva. McManus (1988), in a preliminary study utilizing reverse phase high performance liquid chromatography (RP-HPLC) for PAF fractionation, demonstrated a predominance of 16:0-alkyl-PAF in normal human mixed saliva, with a minor amount of 18:0-alkyl-PAF. Christman and Blair (1989) observed only 16:0-alkyl-PAF in normal human mixed saliva when analyzed by gas chromatography and mass spectrometry (GC/MS); however, in one subject with markedly increased salivary PAF levels, 18:0- and 18:1-alkyl-PAF were found in addition to 16:0-alkyl-PAF. In contrast, McManus *et al.* (1993) have demonstrated that patients with radiation-induced oral mucositis have only a single peak of salivary PAF activity as assessed by sequential normal and reverse phase HPLC; the RP-HPLC distribution of this salivary PAF corresponded to that of 16:0-alkyl-PAF. Therefore, subjects with varying types of oral inflammation may produce different molecular species of PAF.

In the first study of salivary PAF molecular heterogeneity documented in periodontally healthy subjects, Woodard *et al.* (1995), examined the PAF molecular species present in unstimulated human mixed saliva following fractionation by HPLC and derivatization by pentafluorobenzoyl (PFB) anhydride. Samples were subsequently analyzed for acyl- and alkyl-PAF by gas chromatography and mass spectrometry (GC/MS). All samples from the five periodontally healthy subjects demonstrated six different molecular species of PAF: 16:0-, 18:0-, and 18:1-alkyl-PAF and 16:0-, 18:0-, and 18:1-acyl-PAF. The predominant molecular species in every sample, representing 27.9% of the total salivary PAF, was 16:0-alkyl-PAF. In addition, independent saliva samples from a given subject showed little variability in PAF molecular heterogeneity. Thus, multiple molecular species of PAF have been demonstrated in human mixed saliva in subjects who are periodontally healthy.

In preliminary studies designed to define differences in the composition of salivary PAF between periodontal health and disease, Lear *et al.*, (1995), examined the molecular

species of salivary PAF present in 14 periodontally healthy subjects compared to 13 adult periodontitis subjects. Salivary PAF was assessed by GC/MS following HPLC fractionation and derivatization with PFB anhydride. Total salivary PAF was significantly increased in adult periodontitis subjects compared to healthy subjects (17.9 $\pm$ 3.9 vs. 4.4 $\pm$ 0.8 pmole/ml of saliva). 16:0-alkyl-PAF represented 35.1 $\pm$ 2.0% and 31.3 $\pm$ 1.8% of the PAF in the adult periodontitis and healthy subjects, respectively, and was the predominant molecular species found for all subjects. However, no significant differences in the proportions of any PAF homolog was found between the groups. These preliminary findings suggest that periodontal disease is related to the total amount of salivary PAF present, rather than differences in individual PAF molecular species.

5. Statement of Purpose

Past research concerning PAF and periodontal disease has investigated the relationship of PAF in various types of periodontal disease. Few studies have been undertaken to identify the molecular species of PAF present in human mixed saliva in various clinical types of periodontal disease. Therefore, the purpose of the present investigation was to determine the molecular species of salivary PAF present in periodontal health and disease in human subjects in comparison to clinical estimates of disease. It was hypothesized that between the subject groups, qualitative and quantitative differences in the molecular species of PAF in saliva would be found.

II. MATERIALS AND METHODS

A. Subject Selection

Subjects that participated in this study were selected from a patient population which presented for periodontal evaluation at MacKown Dental Clinic, Wilford Hall Medical Center (WHMC). Three subject groups were selected for study as follows:

1. Healthy / Mild Gingivitis Group. Subjects in this group presented with less than or equal to 10% of probing depths greater than or equal to 4 millimeters (mm). This group corresponded to the American Academy of Periodontology (AAP) case type I. For this group, eight subjects (seven males, 1 female, mean age 35.88 years, range 21-58 years) were recruited into this group for the study.

2. Adult Periodontitis Group. Subjects in this group presented with greater than 10% of probing depths greater than or equal to 4 mm. This group corresponded to AAP case type III or IV. Seven subjects (six males, one female, mean age 58.43 years, range 43-71 years) were recruited into this group for the study.

3. Rapidly Progressive Periodontitis (RPP) Group. Subjects in this group (two males, two females, mean age 28.50 years, range 19-34 years) were selected on the basis of the following features as described by Page *et al.*, (1983): early age of onset (puberty to mid 30s), generalized periodontal disease without any consistent distribution pattern, severe and rapid bone destruction, and variable amounts of plaque accumulation and inflammation.

General inclusion/exclusion criteria for all groups consisted of the following:

1. Subjects must have been free of systemic diseases which would compromise their immune system or that could be a contributing factor in the severity or progression of periodontal disease (*e.g.*, diabetes mellitus, subjects on immunosuppressive drugs).

2. Subjects must not have taken antibiotics, nonsteroidal antiinflammatory drugs or steroid medications within the previous two weeks prior to the time of clinical evaluation and saliva collection.

3. Patients must have received no periodontal treatment, including oral prophylaxis, within the past six months.

For each subject, the following information was recorded: age, sex, use of medications, tobacco use history, and the presence of oral lesions. The subjects were given a detailed explanation of the research protocol to be completed. In addition, an Institutional Review Board (IRB) approved informed consent form was signed and given to each subject prior to participation in this study.

B. Saliva Collection

Approximately 10 ml of unstimulated mixed saliva was obtained from each subject prior to a comprehensive periodontal examination. The subjects were given written and verbal instructions not to eat, drink, smoke, or perform any oral hygiene procedures for at least one hour prior to saliva collection. The subjects were questioned about their recent use of medications as well as compliance to these instructions prior to and at the time of their appointment.

Saliva samples were collected into 16 x 100 mm disposable glass tubes containing 1.25 ml of chloroform and 2.50 ml of methanol. On each tube, a black line was drawn prior to saliva collection, which represented a total volume of 4.75 ml. The subjects were instructed to expectorate directly into each (maximum of ten) collection tube, with intermittent mixing, until the total volume in the tube reached the black line. This represented the addition of 1 ml of mixed saliva. A final chloroform:methanol:saliva ratio of 1:2:0.8, v/v/v, was thus attained. Immediately following saliva collection, each tube was capped, mixed vigorously, and immediately refrigerated at 4^{o}C. Saliva samples were transported to the Department of Pathology, University of Texas Health Science Center at San Antonio (UTHSCSA) within five days of collection and stored at 4^{o}C until biochemical analysis was initiated.

C. Clinical Assessment

After saliva collection, a comprehensive periodontal examination was completed for all subjects by the same board certified periodontist. This clinical exam included an assessment of: 1) plaque-free surfaces by the modified O'Leary plaque index (Schallhorn and Snider, 1981); 2) full mouth probing depths (PD) and bleeding on probing (BOP) using the Florida Periodontal Probe System (Henry Schein, Inc., Port Washington, NY) interfaced with an IBM PS2 Model 55 SX personal computer for data collection and storage; 3) clinical attachment levels (CAL) (see below); 4) tooth mobility (Miller, 1950); 5) interdental gingival inflammation (see below); and 6) mucosal inflammatory lesions (*i.e.*, apthous lesions, trauma). Probing depths were recorded using a standardized 20 grams of force at six sites per tooth (mesiofacial, midfacial, distofacial, mesiolingual, midlingual, distolingual). Proximal sites were evaluated with the probe adapted as close to the interdental contact as possible, angling it slightly to access the deepest aspect of the site. In evaluating the midfacial and midlingual sites, the probe was placed parallel to the long axis of the tooth. Bleeding on probing was recorded when a site demonstrated bleeding at the gingival margin within 20 to 30 seconds after probing (Ainamo and Bay, 1975). Clinical attachment levels were determined at the same six sites per tooth as probing depths by measuring the position of the gingival margin in relation to the cemento-enamel junction (CEJ) or the margin of a restoration in this region, using a UNC-15 (Hu-Friedy Manufacturing Co., Chicago, IL) periodontal probe and adding this value to the probing depth.

To assess interdental gingival inflammation, the Eastman Interdental Bleeding Index (EIBI) (Caton and Polson, 1985) was performed. The EIBI was performed by inserting a wooden interdental cleaner (Stim-U-Dent) between all teeth and depressing the interdental papilla 1 to 2 mm. The tip of the cleaner was angled slightly coronally in order to prevent trauma to the gingiva, and was inserted and withdrawn four times into each interdental space. Bleeding within 15 seconds was considered a positive response.

D. Laboratory Procedures

Laboratory analysis of all saliva samples was initiated within one week of collection at the Department of Pathology, UTHSCSA.

1. Lipid Extraction

Each sample was warmed to room temperature prior to completion of the extraction of salivary lipids. Any sample that was over or under the desired 4.75 ml volume was marked so that the actual volume of saliva collected could be accurately determined.

PAF was extracted from saliva using the method of Bligh and Dyer (1959). In brief, the collection of tubes containing saliva samples from a given donor was incubated at room temperature for one hour, with vortexing every ten minutes, to extract lipids from the saliva. Tubes were then centrifuged for ten minutes at 1520 x g at room temperature to separate the organic phase containing the lipids from the cells, precipitated protein, cell debris, and bacteria which pelleted at the bottom of the tube. Supernatants were decanted into 16 x 100 mm disposable glass tubes containing 1.25 ml of chloroform and 1.25 ml of distilled, deionized water and vortexed, to obtain a final ratio of 1:1:0.9, v/v/v, of chloroform:methanol:water, which effected phase separation. These phased extracts were capped and stored at -20^{o}C until pooling of all extract tubes from a given saliva sample (see below).

All cell pellets from a given saliva sample were pooled in a 16 x 100 ml disposable glass tube, and two ml of 10% neutral buffered formalin were added with vortexing to ensure a homogeneous sample; this formalin-fixed saliva pellet was subsequently processed for light microscopic examination (see below).

2. Pooling and Concentration of the Salivary Lipids

Prior to pooling of lipid extracts from a given saliva sample, the tubes were removed from the freezer and allowed to warm to room temperature. Tubes were then centrifuged at

room temperature for ten minutes at 1520 x g. The lower chloroform-rich, PAF-containing phase was then removed from a single tube for a given saliva sample, and transferred into a 12 x 75 mm disposable glass tube using a disposable Pasteur pipette. This chloroform layer was then evaporated to near dryness in a 37°C water bath under a stream of nitrogen gas. The chloroform layer of another tube from the same saliva sample was then added and evaporation continued; the procedure was repeated until all of the chloroform-rich layers from a given saliva sample were included in the 12 x 75 mm tube. The final chloroform-rich layer of the sample was then evaporated to dryness. One ml of phased chloroform was added to this tube, and the pooled sample vortexed.

Based on the expected biologic activity of the saliva sample, an aliquot (5 - 100 µl) was removed from the sample and saved for thin-layer chromatography (TLC). To the remaining sample, methanol and distilled deionized water were added. A final ratio of 1:1:0.9, chloroform:methanol:water, v/v/v, was obtained; this sample was vortexed and stored at -20°C.

3. Isolation of PAF by Thin-Layer Chromatography (TLC)

In order to obtain an estimate of PAF bioactivity in each saliva sample, thin-layer chromatographic (TLC) fractionation of salivary phospholipids was performed prior to platelet bioassay. Phospholipids present in the lower, chloroform-rich layer of salivary lipids were fractionated by TLC as described by Pinckard *et al.*, (1979). Migration of the salivary lipids during TLC occurs at different rates depending on the polarity of the salivary lipid molecules (Christie, 1982). Based on this migration, different classes of salivary lipids were separated on the TLC plate.

To the aliquot removed after pooling the lipid extracts from a given saliva sample, 5 µl of [3H]16:0-alkyl-PAF (New England Nuclear, Boston, MA; 50 Ci/mmole in phased

chloroform containing approximately 5000 dpm) was added to allow determination of PAF recovery following TLC fractionation.

A prewashed, heat-activated, 250 μm, 20 cm x 20 cm silica gel G TLC plate (Analtech, Inc., Newark, DE) was divided into 1.5 centimeter (cm) sample lanes, with 0.5 cm blank lanes separating each sample lane. Each sample was spotted onto a sample lane; washing of the sample tube was completed twice with five drops of phased chloroform and subsequent spotting of the wash solution onto the sample lane. A lipid reference standard (Sigma Chemical Co., St. Louis, MO) containing lysophosphatidylcholine (LPC), sphingomyelin (SPH), phosphatidylcholine (PC), and phosphatidylethanolamine (PE) was spotted onto 1.0 cm lanes at both edges of the TLC plate. These reference standards were used to ensure the performance of the lipid separation after TLC and to identify fractions most likely to contain PAF activity. TLC separation was performed in a TLC tank containing an equilibrated solvent system of 65 ml of chloroform, 35 ml of methanol, and 6 ml of water. The solvent front was allowed to migrate approximately 17 cm up the TLC plate, its position was marked, and the TLC plate was removed from the tank and allowed to air dry. A clean glass slab was then used to cover the sample lanes, and the plate was placed into a tank containing iodine crystals to visualize the reference standards. The position of the reference standard was measured, and R_f values were calculated to estimate TLC performance.

Lipids were recovered from the TLC plate by dividing each lane into 1.0 cm fractions. Each fraction was then scraped into individual 12 x 75 mm glass tubes containing 1.9 ml of chloroform, methanol, and water (1:2:0.8, v/v/v), with subsequent Bligh and Dyer lipid extraction. These TLC extracts were then stored at -20^{o}C until platelet bioassay was performed.

4. Normal Phase High-Performance Liquid Chromatographic (NP-HPLC) Isolation of Salivary PAF

Prior to NP-HPLC fractionation of the salivary lipids, a 10% aliquot of the sample was removed and stored in 12 x 75 mm disposable glass tubes at -20°C for future mass spectrometry analysis.

Approximately 20,000 dpm each of [3H]16:0-alkyl-PAF and [3H]18:0-alkyl-PAF (New England Nuclear) was added to each of the extracted salivary lipid samples to serve as a marker for the HPLC elution position of PAF and to quantitate PAF recovery from HPLC.

Major classes of salivary phospholipids were separated by NP-HPLC using a modification of the procedure described by Blank and Snyder (1983) and Weintraub *et al.*, (1990). Separation was performed on a Waters HPLC System (Waters Associates, Milford, MA). This system consisted of an automated gradient controller (Model 680), a temperature control module, two HPLC pumps (Model 510), and a Waters 486 absorbance detector, set at 206 nm (the absorbance scale was set at 2.56). Utilizing a silica column (Rainin Instruments, Woburn, MA; Microsorb-MV, 4.6 mm x 25 cm, five micron steel column in a heater box set at 30°C), gradient elution was performed at a flow rate of 1.5 ml/min. The initial mobile phase was isopropanol:hexane (1:1, v/v) containing 4% water, with a 20 minute linear gradient from the time of injection until reaching 8% water. This mobile phase was then maintained for an additional 20 minutes. The mobile phase was then returned linearly to 4% water content in the next 10 minutes.

Salivary lipids in the chloroform rich, lower phase were dissolved in 80 µl of mobile phase containing 8% water. This sample was then loaded onto the column using an injector with a 200 µl sample loop. Two subsequent rinses of the original tube with 30 µl of solvent were also loaded onto the column for each sample. Fractions were collected at one minute intervals by a LKB Bromma 2211 Superrac for 60 minutes. The collected fractions were subsequently phased by addition of 1.5 ml of chloroform, 1.5 ml of methanol, and 3 ml of

water with vortexing. These phased samples were stored at -20°C until further HPLC fractionation (see below).

NP-HPLC column performance, as measured by PAF retention time and recovery of [3H]16:0-alkyl-PAF, was determined prior to and following NP-HPLC fractionation by the use of a FLO-ONE/beta, on line liquid scintillation counter (Radiomatic, Meriden, CT). The NP-HPLC elution behavior of this authentic [3H]16:0-alkyl-PAF was utilized to determine which NP-HPLC fractions of fractionated salivary phospholipids would be pooled for subsequent reverse phase HPLC (RP-HPLC). Ten NP-HPLC fractions which flanked the expected elution position of [3H]16:0-alkyl-PAF were selected. An aliquot of each sample underwent rabbit platelet and human PMN bioassay to determine PAF biologic activity as described below. Fractions were phased by adding 1.5 ml of chloroform, 1.5 ml of methanol, and 3.0 ml of water with subsequent vortexing and storage at -20°C until RP-HPLC.

5. Reverse Phase High Performance Liquid Chromatographic (RP-HPLC) Separation of Salivary PAF

Reverse phase (RP-HPLC) was performed to effect separation of the choline-containing phospholipids on the basis of the carbon chain length. The technique used was a modification of the procedure by Pinckard *et al.*, (1984). The chloroform layers from the ten NP-HPLC fractions which flanked the expected elution position of [3H]16:0-alkyl-PAF were evaporated and pooled into a 12 x 75 mm disposable glass tube by a stream of nitrogen in a 37°C water bath. For RP-HPLC, methanol:water:acetonitrile (85:10:5) with 20 mM choline chloride was used as the solvent mixture; this solvent was used to dissolve the dried samples. PAF fractionation by RP-HPLC was accomplished using a Waters HPLC System (see above); the sample was loaded onto a C18 column (Rainin Instruments, Microsorb-MV, 4.6 mm x 25 cm, five micron steel column in a heater box at 30°C) using 80 µl of reverse phase solvent with choline chloride. The detector wavelength was set at 204 nm, and the absorbance scale

was set at 2.56. The sample tube was rinsed twice with 30 µl of reverse phase solvent. Using a flow rate of 0.75 ml/minute, fractions were collected at one minute intervals to a relative retention time (RRT) of 4.0 (16:0-alkyl-PAF having a RRT of 1). Fractions were phased by adding 0.75 ml of chloroform and 0.75 ml of water with subsequent vortexing and storage at -20°C until bioassay. Each fraction subsequently underwent evaluation for PAF activity by platelet and PMN priming bioassays (see below).

6. Preparation of Lipid Extracts for Bioassay

All lipid extracts were prepared for bioassay after warming each sample to room temperature. Each TLC or HPLC fraction was centrifuged (1520 x g, 10 minutes, room temperature) to ensure complete phase separation. The overlying methanol-water layer was removed, and the remaining lower chloroform layer was evaporated under a stream of nitrogen gas in a 37°C water bath. At least 60 minutes prior to use in bioassay, each evaporated lipid extract was reconstituted to 100 µl with 0.25% bovine serum albumin (Miles Laboratories, Elkhart, IN) in pyrogen-free saline (0.9% NaCl) (BSA-saline). Any required dilutions of the samples were prepared with this same resuspension medium.

7. PAF Quantification by Rabbit Platelet Bioassay

PAF bioactivity was quantified by the utilization of an assay which measures the amount of radiolabelled [3H]5-hydroxytryptamine ([3H]serotonin) released from rabbit platelets following stimulation with known samples of 16:0-alkyl-PAF or unknown samples containing PAF activity (Henson, 1990).

According to the method of Pinckard *et al.*, (1979), fifty ml of fresh blood was drawn from the central ear artery of California rabbits into sterile conical tubes containing 7 ml of acid citrate dextrose. To effect separation of red blood cells from the platelet-rich plasma (PRP), the tubes were mixed and centrifuged (400 x g, 15 minutes, room temperature). The

upper PRP layer was transferred into 50 ml tubes and incubated for 15 minutes in a 37°C water bath with [3H]5-hydroxytryptamine (1 μCi/ml PRP, 25 Ci/mmole, New England Nuclear, Boston, MA). Layering of the radiolabelled PRP onto Ficoll (2 ml of Ficoll to 10 ml of PRP) was performed, with subsequent centrifugation (650 x g, 20 minutes, room temperature) of the preparation. The platelet layer was then isolated by removing the overlying platelet-depleted plasma and the underlying Ficoll. Tyrode's buffer (NaCl, 8.0 g/l; KCl, 0.195 g/l; $NaHCO_3$, 1.02 g/l; $MgCl_2$ 6H20, 0.213 g/l; and gelatin, 2.5 g/l) containing 0.1 mM EGTA (pH 6.5) was then added to the radiolabelled platelets. These platelets were again layered onto Ficoll and centrifuged for 15 minutes (650 x g, room temperature). The labeled platelets were reisolated and resuspended in Tyrode's buffer without EGTA (pH 6.5). Following centrifugation for 15 minutes (650 x g, room temperature), the washed platelets formed a pellet at the bottom of the centrifuge tubes. The pellet was then resuspended in Tyrode's buffer without EGTA (pH 6.5) to a concentration of 1.2×10^9 cells/ml. Prior to the bioassay, the platelet suspension was diluted with Tyrode's buffer (pH 7.2) containing 1.3 mM Ca^{2+} and warmed in a 37°C water bath for 10 minutes.

Four μl of known amounts of 16:0-alkyl-PAF (2-50 fmole) (Bachem Fine Chemicals, Torrance CA) or 4 μl aliquots of each unknown lipid extract fraction were placed into 12 x 75 polystyrene reaction tubes. Positive and negative controls were established by adding 20 μl of thrombin or four ul of BSA-saline, respectively, to separate reaction tubes. The maximum amount (100%) of [3H]serotonin in each platelet preparation was determined by adding 0.02% Triton X-100 (final concentration) to separate reaction tubes. Two hundred μl of prewarmed (37°C), radiolabelled platelets were added to each reaction tube. The platelet secretion reaction was terminated sixty seconds later, by the addition of 20 μl of cold 1.5M formalin and immediately cooling each tube to 0°C. The reaction tubes were centrifuged (1000 x g, 10 minutes, 4°C), and by liquid scintillation spectrometry, the amount of

[3H]serotonin released in the cell-free supernatant was determined. The percent secretion of [3H]serotonin was then assessed.

PAF bioactivity in each TLC, NP-HPLC, or RP-HPLC fraction of salivary lipid was determined directly from the standard dose-response curve established from the [3H]serotonin release from the known amounts of synthetic 16:0-alkyl-PAF (2-50 fmole). These standard curves were always performed in parallel with unknown samples. For each fractionated saliva sample, PAF bioactivity was expressed as fmole equivalents of 16:0-alkyl-PAF/ml saliva. Losses of PAF during lipid extraction and fractionation procedures were estimated by determining the amount of [3H]-alkyl-PAF tracer recovered in each sample.

8. PAF Quantification by Human PMN Bioassay

a) Isolation of Polymorphonuclear Leukocytes (PMN)

PMN were obtained by collecting 200 ml of blood from the antecubital vein of healthy adult human subjects of both genders. All subjects had fasted for 12 hours and had not taken any medication during the previous two weeks. Donors were excluded if within the previous two months they had donated one unit of blood, or donated 200 ml of blood in the past month. An Institutional Review Board approved consent form was signed and given to each subject prior to participation in this study.

Human PMN were prepared as described by Ludwig *et al.*, (1984). Venous blood was immediately anticoagulated with 1/6 volume of 0.65 M citric acid, 0.85 M trisodium citrate, 0.110 M dextrose, and 2 U heparin (grade I, Sigma Chemical Company) per ml of anticoagulated blood. All isolation procedures were conducted in polypropylene test tubes. After initial centrifugation (10 minutes, 1780 x g, room temperature), the supernatant and the buffy coat were removed. The remaining cell pellet was divided equally and placed into two sterile 50 ml tubes, to which 10 ml of 3% dextran and as much saline as sufficient to bring the volume to 50 ml was added. Upon resuspension, the tubes were incubated in a 37^{o}C water

bath for 30 minutes to permit erythrocyte sedimentation, after which, supernatants were transferred to a new sterile 50 ml tube and saline was added to bring the volume to 50 ml. Centrifugation (20 minutes, 1730 x g, 24°C) was performed, the supernatant was poured off and discarded, and 5 ml of cold erythrocyte lysing solution (1.667 grams of NH_4Cl, 0.0074 grams of Na_2 EDTA, 0.2 grams $KHCO_3$ in 200 ml of deionized water, pH 7.4) was added to each tube to resuspend the pellet. Four tubes were combined into one tube, placed in ice for 10 minutes, and then brought to 50 ml of volume with Hank's buffered saline solution (HBSS). After centrifugation (10 minutes, 1130 x g, 4°C), the supernatant was discarded and the cell pellet was washed with 5 ml of HBSS. The resuspended pellet was centrifuged (10 minutes, 1130 x g, 4°C), the supernatant was discarded, and the cell pellet was resuspended in 5 ml of HBSS. PMN were isolated by overlaying the 5 ml of HBSS on Ficoll-Hypaque cushions in 16 x 95 mm sterile polypropylene tubes and centrifuging for 35 minutes (1360 x g, 4°C). After discarding the supernatant, the pellet was resuspended in 5 ml of HBSS, brought to 10 ml of HBSS, and centrifuged (10 minutes, 1130 x g, 4°C). The supernatant was discarded, and the pellet was resuspended to a cell density of 2.5 x 10^6 PMN/ml HBSS. PMN were maintained at 4°C until 10 minutes before stimulation at which time they were preincubated with 1.4 mM Ca^{2+} at 37°C.

b) <u>PMN Priming for Enhanced Superoxide Anion (O2-) Production</u>

PMN priming was conducted as described by Pinckard *et al.*, (1992). In brief, 10 µl of each NP-HPLC or RP-HPLC fractionated sample was added to 1.0 ml of PMN (2.5 x 10^6 PMN/ml HBSS containing 1.4 mM Ca^{2+} and 100 uM cytochrome *c* at 37°C) in polypropylene tubes for 2.5 minutes. The PMN in each reaction tube were then stimulated with N'-formyl-methionyl-leucyl-phenylalanine (FMLP) (Penisula Laboratories, Belmont, CA) dissolved in dimethyl sulfoxide (DMSO) (Sigma Chemical Company), 100 nM final concentration, with incubation for 10 minutes in a 37°C water bath. Unstimulated PMN

(negative controls) were run in parallel. The net increase in superoxide anion production (nanomoles of superoxide anion produced from FMLP-stimulated PMN minus nanomoles of superoxide anion produced by unstimulated, control PMN) was determined spectrophotometrically (at 550 nm) and expressed as nanomoles of superoxide anion/2.5 x 10^6 PMN.

9. Histologic Analysis of Saliva Pellets

Saliva pellets were prepared for histologic analysis following the protocol of McManus *et al.*, (1990). Saliva pellets preserved in formalin following Bligh and Dyer lipid extraction and stored at room temperature (see above) were transferred to polypropylene microcentrifuge tubes (Beckman Instruments, Inc., Palo Alto, CA), centrifuged (10,000 x g, 4 minutes, room temperature) to remove excess formalin, and resuspended in 3.5% gelatin (Sigma Chemical Co., St. Louis, MO) at 60°C. Samples were immediately centrifuged (10,000 x g, 4 minutes, room temperature) and then the centrifuge tubes were immersed in an ice bath for 10 to 15 minutes. The gelatin embedded pellets were placed in histologic cassettes and stored in 10% NBF prior to embedding in paraffin. Sections of 6 μm thickness were stained with hematoxylin and eosin for light microscope examination.

Polymorphonuclear leukocytes (PMN) were quantified using a square reticule in a 10X eyepiece with a 40X objective. Three representative fields were examined for each specimen and the mean number of PMN/mm^2 was estimated. The magnification factor for a high power field was calculated to be 0.0625; the mean value of identified PMN was divided by the magnification factor to arrive at the corrected number of PMN/mm^2 for each sample. Histologic specimen were coded to allow for blind PMN quantification.

10. Statistical Analysis

All estimates of PAF activity from the platelet and PMN bioassays of RP-HPLC fractionated saliva samples were plotted using Sigma Plot software (Jandel Scientific, San Rafael, CA). These graphs were then scanned utilizing a Hewlett-Packard scanner, with data storage on a 486 personal computer (486 Datacom). In order to estimate the biological activity of individual molecular species of PAF after RP-HPLC, graphed and scanned data were further processed utilizing Un-Scan-It software (Silk Scientific Corporation, Orem, Utah) to calculate the areas under the curves.

Data were analyzed to establish a measure of central tendency and variability and are presented as means $\pm$ standard error (mean $\pm$ SE). Statistical analyses were performed on a personal computer using Statistical Analysis System software (SAS Institute, Inc., Cary, NC).

Pearson correlation coefficients were computed to determine the relationship between any two variables for each subject group. Partial correlation coefficients were used when comparing any two variables after adjustment for group, gender, smoking, age and/or the number of teeth. One-way analysis of variance (ANOVA) was calculated to compare group means using the least squares means. Log transformation was performed when necessary to allow for equal variances between groups. Residuals were evaluated to check for validity of assumptions for analysis of variance. *P* values less than 0.05 were considered significant.

IV. RESULTS

A. Subject Population

Nineteen subjects were enrolled in this study. Eight subjects comprised the healthy / mild gingivitis (Normal) group, seven subjects comprised the adult periodontitis (AP) group, and four subjects comprised the rapidly progressive periodontitis (RPP) group.

The age of the normal subjects was 35.9 $\pm$ 4.5 years (Table 1). One female and seven males comprised this group with one of these eight subjects reporting a smoking history. The age of the AP subjects was 58.4 $\pm$ 3.1 years with one female and six males in this group. A smoking history was reported by two of the seven subjects in this group. For the RPP group, the age of the subjects was 28.5 $\pm$ 3.4 years. Two females and two males comprised this group with two of the four subjects reporting a smoking history.

B. Clinical Features of the Subject Population

The eight normal subjects had an average of 27.8 $\pm$ 0.6 teeth and demonstrated clinical features consistent with periodontal health (Table 2). Thus, the modified O'Leary plaque index indicated that 91.4 $\pm$ 3.5% of tooth surfaces were plaque-free. The percentage of sites positive for supragingival bleeding as assessed by the Eastman Interdental Bleeding Index (EIBI) was 8.8 $\pm$ 2.5, and the percentage of sites positive for bleeding on probing (BOP) was 14.0 $\pm$ 2.8. The number of probing depths $\geq$ 4 mm was 0.9 $\pm$ 0.4, or 0.5 $\pm$ 0.3% of all sites. Thus, the average probing depth for all sites was 2.03 $\pm$ 0.07 mm and the average attachment level was 1.27 $\pm$ 0.22 mm. The number of sites with attachment loss $\geq$ 2 mm was 36.5 $\pm$ 12.9.

The seven AP subjects presented with 26.0 $\pm$ 0.6 teeth, which was not significantly different from that of normal subjects (Table 2). In contrast, the modified O'Leary plaque index for this group revealed considerable plaque inasmuch as only 58.9 $\pm$ 10.7% of tooth

Table 2

CHARACTERISTICS OF SUBJECT POPULATION

	Normal Subjects (n=8)	**Adult Periodontitis (AP) Subjects (n=7)**	**Rapidly Progressive Periodontitis (RPP) Subjects (n=4)**
Age (years)	35.9 ± 4.5*	58.4 ± 3.1	28.5 ± 3.4
Sex	1 Female 7 Males	1 Female 6 Males	2 Females 2 Males
Smoking Status	1 Smoker 7 Non-Smokers	2 Smokers 5 Non-Smokers	2 Smokers 2 Non-Smokers

* Results are presented as the mean ± SE

Table 3

COMPARISON OF CLINICAL FEATURES BETWEEN GROUPS

	Normal Subjects (n=8)	Adult Periodontitis (AP) Subjects (n=7)	Rapidly Progressive Periodontitis (RPP) Subjects (n=4)
Teeth (#)	27.8 ± 0.6*	26.0 ± 0.6	27.3 ± 0.8
Plaque Index (%)	91.4 ± 3.5	58.9 ± 10.7#	51.0 ± 7.0#
EIBI (%)	8.8 ± 2.5	29.4 ± 11.0	42.5 ± 11.7#
BOP (%)	14.0 ± 2.8	57.7 ± 8.2#	73.0 ± 5.9#
PD (mm)	2.04 ± 0.07	3.05 ± 0.18#	3.35 ± 0.19#
PD ≥ 4 mm (%)	0.5 ± 0.3	26.1 ± 4.7#	31.0 ± 7.4#
AL (mm)	1.27 ± 0.22	3.04 ± 0.35#	3.01 ± 0.34#
AL ≥ 2 mm (#)	36.5 ± 12.9	102.0 ± 9.7#	103.3 ± 11.7#

* Results are presented as the mean ± SE

# $p<0.05$ as compared to results derived from normal subjects (*t*-test following One-way ANOVA)

Plaque Index = Modified O'Leary Plaque Index (% plaque-free surfaces)

EIBI = Eastman Interdental Bleeding Index (% of interdental sites with inflammation)

BOP = Bleeding on Probing (bleeding within 20 to 30 seconds after probing by Florida Probe @ 20 grams of force)

PD = Probing Depth (Florida Probe @ 20 grams of force)

AL = Attachment Level

surfaces were plaque-free. The percentage of sites with supragingival bleeding as assessed by EIBI was 29.4 $\pm$ 11.0 with BOP present at 57.7 $\pm$ 8.2% of sites. The number of probing depths $\geq$ 4 mm was 40.4 $\pm$ 7.3 which represented 26.1 $\pm$ 4.7% of all sites. Thus, the average probing depth for all sites (3.05 $\pm$ 0.18 mm) and the average attachment level (3.04 $\pm$ 0.35 mm) were significantly increased as compared to the normal subjects. The number of sites with attachment loss $\geq$ 2 mm was 102.0 $\pm$ 9.7.

Four RPP subjects presented with 27.3 $\pm$ 0.8 teeth (Table 3). The modified O'Leary plaque index score for the RPP subjects was 51.0 $\pm$ 7.0% while 42.5 $\pm$ 11.7% of sites were positive for bleeding by the EIBI. The percentage of sites with BOP was 73.0 $\pm$ 5.9, and the number of probing depths $\geq$ 4 mm was 49.8 $\pm$ 10.5, the latter reflecting 31.0 $\pm$ 7.4% of all sites. Thus, the average probing depth for all sites was 3.35 $\pm$ 0.19 mm, and the average attachment level was 3.01 $\pm$ 0.34 mm. The number of sites with attachment loss $\geq$ 2 mm was 103.3 $\pm$ 11.7.

There were no differences in the average number of teeth between the three groups (Table 3). However, when comparing the groups on the basis of clinical features, significant differences were observed. Thus, subjects with AP or RPP had significantly lower plaque-free surfaces and a significantly greater percentage of sites with BOP and number of probing depths greater than or equal to 4 mm. For interproximal inflammation as assessed by the EIBI, only the RPP group was significantly different than the group of normal subjects; although the EIBI was increased in the AP group as compared to that of the normal group, this increase was only marginally significant (p=0.07).

C. Salivary Polymorphonuclear Leukocyte (PMN) Levels

Of interest was a comparison of the three groups with respect to the number of histologically defined polymorphonuclear leukocytes (PMN) present in the saliva pellet (Table 4). For the normal group, 206.0 $\pm$ 77.9 PMN/mm^2 were present. The AP group and RPP

Table 4

COMPARISON OF SALIVARY PAF ACTIVITY AFTER THIN-LAYER CHROMATOGRAPHY (TLC) AND SALIVARY PMN LEVELS IN PERIODONTAL HEALTH AND DISEASE

	Normal Subjects (n=8)	Adult Periodontitis (AP) Subjects (n=7)	Rapidly Progressive Periodontitis (RPP) Subjects (n=4)
PMN/mm^2	206 ± 78*	433 ± 186	287 ± 35
PAF	4040 ± 708	12,780 ± 2621#	8445 ± 1112^

* Results are presented as the mean ± SE

# $p<0.05$ as compared to results derived from normal subjects (*t*-test following One-way ANOVA)

^ $p<0.05$ as compared to results derived from normal subjects after log transformation (*t*-test following One-way ANOVA)

PMN - PMN in saliva pellet following lipid extraction were histologically quantified.

PAF - After TLC fractionation of salivary lipids, PAF activity was determined in rabbit platelet bioassay and expressed as fmole equivalents of 16:0-alkyl-PAF/ml of saliva

group had 432.8 ± 185.8 and 287.3 ± 34.7 PMN/mm^2, respectively. After log transformation of the data, the AP group was only marginally different (p=0.07) than the normal group while the RPP group was not significantly different (p=0.16).

D. Salivary PAF Following Thin Layer Chromatography (TLC)

An aliquot of each mixed saliva sample was subjected to TLC fractionation for subsequent estimation of PAF activity in rabbit platelet bioassay. Total salivary PAF activity ranged from 1612 to 7678 fmole equivalents of 16:0-alkyl-PAF/ml of saliva in the normal group, with a mean of 4040 ± 708. In the AP group, total PAF ranged from 5806 to 27,304 fmole equivalents of 16:0-alkyl-PAF/ml of saliva, with a mean of 12,780 ± 2621. In the RPP group, total PAF ranged from 5630 to 11,068 fmole equivalents of 16:0-alkyl-PAF/ml of saliva, with a mean of 8445 ± 1112. Thus, salivary PAF levels were significantly increased for both the AP and RPP groups (p<0.05) in comparison to normal subjects (Table 4).

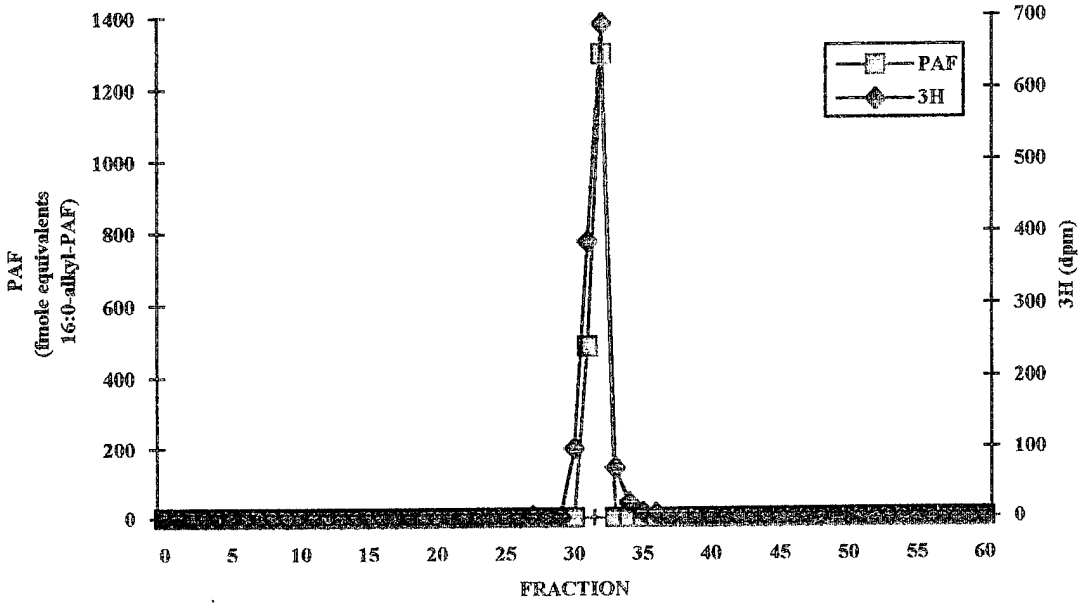
Tritiated 16:0-alkyl-PAF recovery in saliva samples after TLC fractionation ranged from 42.8 to 63.8% with a mean of 54.1 ± 1.5%.

E. Normal Phase (NP) HPLC Fractionation of Salivary PAF

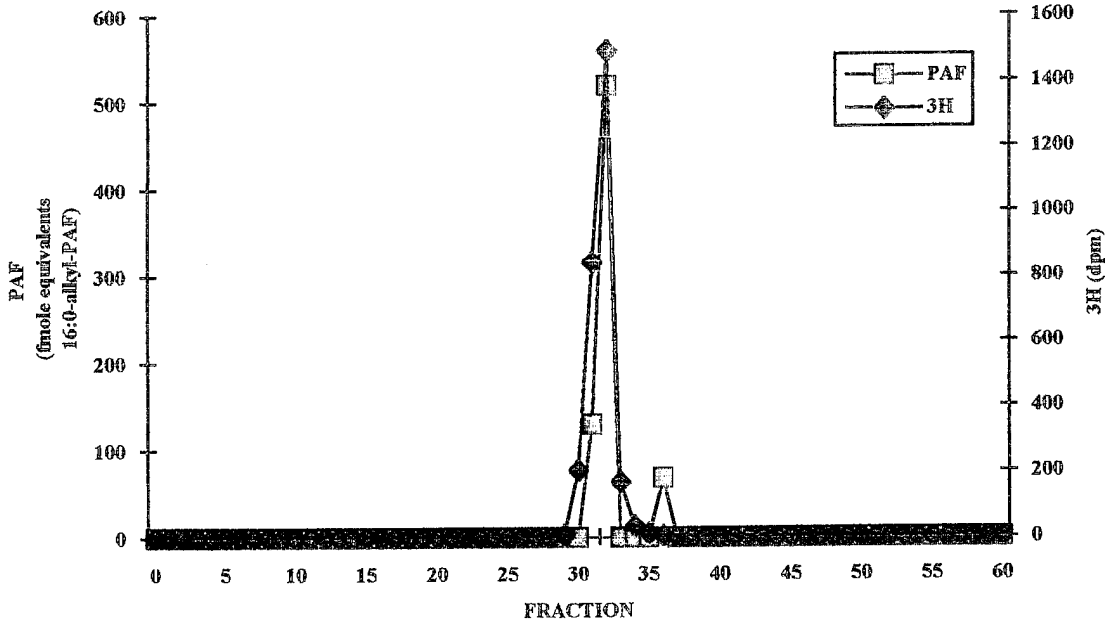
Normal Phase HPLC (NP-HPLC) was used to fractionate the major classes of salivary phospholipids. The elution positions of known phospholipid standards were determined prior to fractionation of salivary phospholipids; these standards included phosphatidylcholine, sphingomyelin, and lysophosphatidylcholine. A single peak of salivary PAF activity (as estimated in platelet bioassay) was observed in all saliva samples. The elution position of salivary PAF corresponded to the elution position of the tritiated 16:0- and 18:0-alkyl-PAF tracer which was included in each sample (Figure 3). Thus, salivary PAF was separated from most other major classes of phospholipids during NP-HPLC.

Figure 3. The Distribution of Salivary PAF Activity (by Platelet Bioassay) after Fractionation by NP-HPLC. After NP-HPLC fractionation of salivary lipids derived from a normal subject, an aliquot of each NP-HPLC fraction was utilized to determine PAF activity in rabbit platelet bioassay (in fmole equivalents of 16:0-alkyl-PAF) as well as ^{3}H recovery (in dpm). This profile (which is representative of the results obtained with all 19 saliva samples) reveals a single peak of PAF activity which corresponds to the elution position of tritiated 16:0- and 18:0-alkyl-PAF tracer. The peak elution position of phosphatidylcholine, sphingomyelin and lysophosphatidylcholine was fraction number 24, 26, and 34, respectively. For comparison, the corresponding PMN bioassay is provided in the lower panel. Two peaks of PAF activity were present, with the greatest peak corresponding to the elution position of the tritiated 16:0- and 18:0-alkyl-PAF tracer.

PLATELET BIOASSAY



PMN BIOASSAY



In contrast to the results derived from platelet bioassay after NP-HPLC fractionation, the PMN bioassay demonstrated more than one peak of salivary PAF activity in samples derived from five normal subjects, four AP, and one RPP subjects. In each sample demonstrating more than one peak, there was a single peak of greatest PAF activity which corresponded to the elution position of tritiated PAF (Figure 4).

The total salivary PAF activity determined in platelet bioassay following NP-HPLC was 4164 $\pm$ 1218, 14332 $\pm$ 2772, and 7868 $\pm$ 1880 fmole equivalents of 16:0-alkyl-PAF/ml of saliva for normal, AP and RPP subjects, respectively (Table 5). Salivary PAF was significantly (p<0.0019) increased in the AP group whereas the RPP group was not significantly increased. PAF activity determined in PMN bioassay following NP-HPLC indicated 4967 $\pm$ 1264, 16,897 $\pm$ 7307, and 19,229 $\pm$ 11,994 fmole equivalentsof 16:0-alkyl-PAF/ml of saliva for the normal, AP and RPP subjects, respectively. Although these estimates of PAF activity determined in PMN bioassay were somewhat greater than that determined in platelet bioassay for all groups, these differences did not achieve statistical significance.

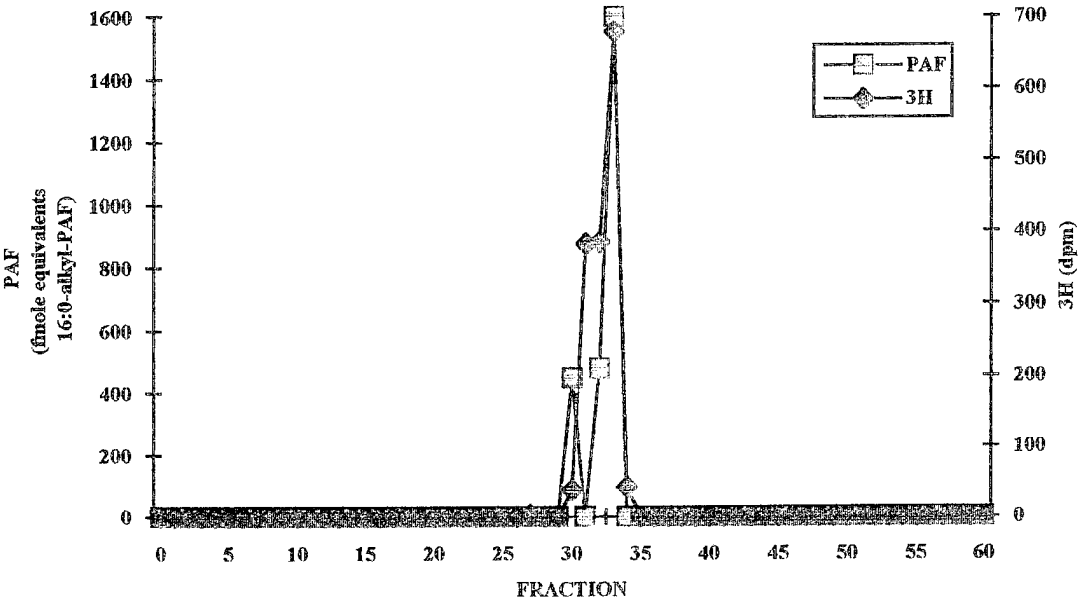
Following NP-HPLC, recovery of tritiated 16:0- and 18:0-alkyl-PAF for all groups ranged from 71.5% to 90.4%, *i.e.*, 78.9 $\pm$ 1.8%, 82.8 $\pm$ 2.0%, and 83.7 $\pm$ 2.6% for the normal, AP and RPP subjects, respectively (Table 5). Thus, recovery of radiolabeled PAF (tracer) was not significantly different between the groups.

F. Reverse Phase (RP) HPLC Fractionation of Salivary PAF

After RP-HPLC fractionation of salivary PAF, platelet bioassay revealed multiple peaks of salivary PAF activity for the normal subjects (Figure 5 and Appendices A-G). For all normal subjects, a predominant peak of PAF activity corresponding to 16:0-alkyl-PAF was found. In addition a shoulder region eluted after the peak of 16:0-alkyl-PAF which corresponded to the elution position of 18:1-alkyl-PAF. Five of the eight normal subjects with greater total salivary PAF demonstrated a peak of activity corresponding to the elution

Figure 4. The Distribution of Salivary PAF Activity (by PMN Bioassay) after Fractionation by NP-HPLC. After NP-HPLC fractionation of salivary lipids, an aliquot of each NP-HPLC fraction was utilized to determine PAF activity in human PMN bioassay (in fmole equivalents of 16:0-alkyl-PAF) as well as ^{3}H recovery (in dpm). This profile (which is representative of results obtained with five normal, four AP, and 1 RPP saliva samples reveals two peaks of PAF activity. Nevertheless, a single peak of greatest PAF activity was found in all saliva samples which corresponded to the elution position of tritiated 16:0- and 18:0-alkyl-PAF tracer. For comparison, the platelet bioassay is presented in the lower panel. In contrast to the PMN bioassay (upper panel), a single peak of PAF activity was present in the platelet bioassay which corresponded to the elution position of tritiated 16:0- and 18:0-alkyl-PAF.

PMN BIOASSAY



PLATELET BIOASSAY

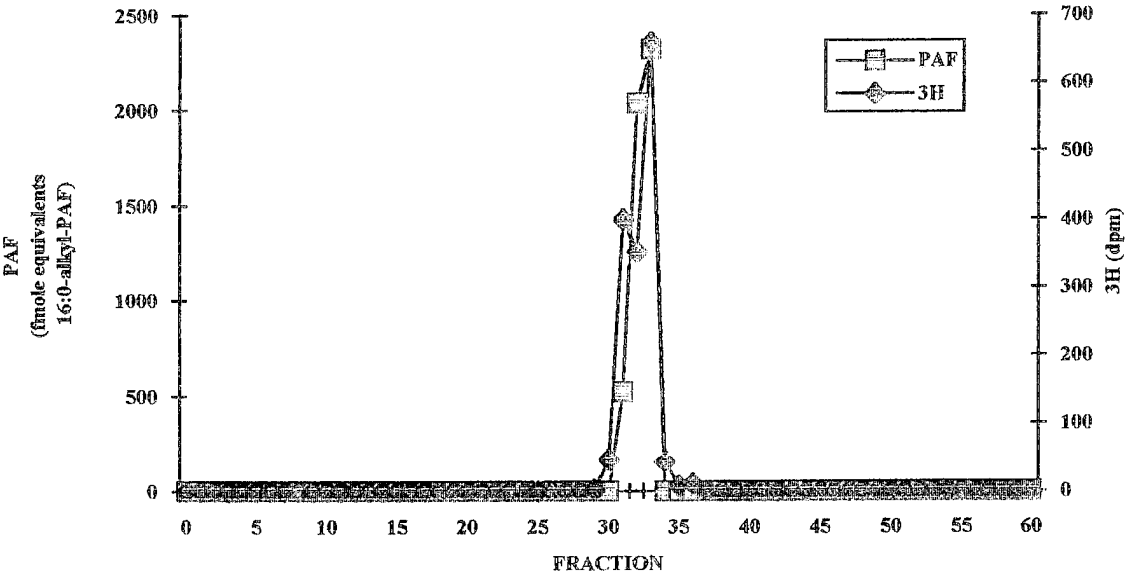


Table 5

COMPARISON OF SALIVARY PAF ACTIVITY AND [^{3}H]PAF RECOVERY AFTER NP-HPLC FRACTIONATION IN PERIODONTAL HEALTH AND DISEASE

	Normal Subjects (n=8)	Adult Periodontitis (AP) Subjects (n=7)	Rapidly Progressive Periodontitis (RPP) Subjects (n=4)
Platelet Bioassay	4164 ± 1218*	14,332 ± 2772#	7868 ± 1880
PMN Bioassay	4967 ± 1264	16,897 ± 7307	19,229 ± 11,994
[^{3}H] 16:0-, 18:0-alkyl-PAF (%)	78.9 ± 1.8	82.8 ± 1.9	83.7 ± 2.6

* Results are presented as the mean ± SE

# $p<0.0019$ as compared to results derived from normal subjects (*t*-test following One-way ANOVA)

Salivary PAF activity after NP-HPLC fractionation was defined in both platelet and PMN bioassay relative to the activity of authentic 16:0-alkyl-PAF evaluated in parallel. All PAF activity in NP-HPLC fractions from a given saliva sample were totaled and expressed in fmole equivalents of 16:0-alkyl-PAF/ml of saliva; for each saliva sample, values were corrected for recovery of [^{3}H]PAF tracer. The recovery of [^{3}H]PAF tracer is expressed as a percentage of the radiolabel included in the sample prior to HPLC.

position of 18:0-alkyl-PAF. Further, the two samples with the greatest amount of total salivary PAF activity demonstrated peaks corresponding to the elution position of 15:0-alkyl-PAF.

Platelet bioassay of RP-HPLC fractionated salivary PAF for AP as well as RPP subjects also revealed multiple peaks of salivary PAF activity (Appendices H-P). Thus, when the profile of PAF activity in the saliva from a normal subject (Figure 5) was compared to that from an AP subject (Figure 6) or RPP subject (Figure 7), a similar pattern was revealed. Peaks of PAF activity corresponding to the elution position of authentic 15:0-, 16:0-, 18:1-, and 18:0-alkyl-PAF, respectively, were observed.

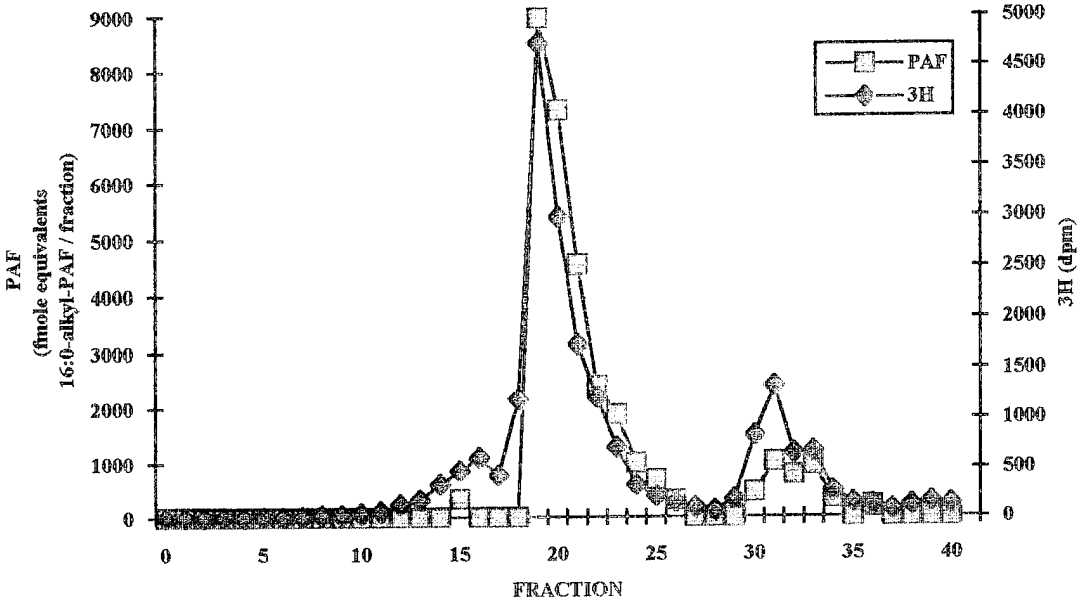
The human PMN bioassay for salivary PAF activity yielded a fractionation pattern after RP-HPLC similar to that found in the rabbit platelet bioassay (Figure 5, 6, and 7). Thus, for the normal, AP and RPP subjects, multiple peaks corresponding to the elution position of authentic 15:0-, 16:0-, 18:1-, and 18:0-alkyl-PAF, respectively, were present.

The total amount of PAF activity determined in platelet bioassay following RP-HPLC was 3330 $\pm$ 795, 13,520 $\pm$ 1466, and 8783 $\pm$ 2785 fmole equivalents of 16:0-alkyl-PAF/ml of saliva for normal, AP and RPP subjects, respectively. Thus, the total amount of salivary PAF after RP-HPLC was significantly increased ($p<0.04$) in AP and RPP subjects when compared to normal subjects. PAF activity in PMN bioassay following RP-HPLC 19,391 $\pm$ 4269, 116,900 $\pm$ 31,931, and 62,156 $\pm$ 38,646 fmole equivalents of 16:0-alkyl-PAF/ml of saliva for normal, AP and RPP subjects, respectively. Significant increases ($p<0.01$) in total PAF was found in the AP subject group when compared to the normal subject group. For all groups, PAF activity after RP-HPLC as determined in PMN bioassay was significantly elevated as compared to that in platelet bioassay (Table 6).

Following RP-HPLC, recovery of radiolabeled tracer PAF (tritiated 16:0- and 18:0-alkyl-PAF) for all groups ranged from 61.8% to 104.8%, *i.e.*, 81.9 $\pm$ 5.3%, 81.9 $\pm$ 4.2%, and 93.0 $\pm$ 3.8% for the saliva samples derived from normal, AP and RPP subjects, respectively

Figure 5 . Representative RP-HPLC Fractionation of Salivary PAF From a Normal Subject. After RP-HPLC, an aliquot of each RP-HPLC fraction was utilized to determine PAF activity in rabbit platelet and human PMN bioassay (in fmole equivalents of 16:0-alkyl-PAF) as well as ^{3}H recovery (in dpm). As determined in platelet bioassay (upper panel), a peak of PAF activity was present at fractions 15, 19, and 31; these HPLC regions corresponded to the elution position of 15:0-alkyl-PAF, 16:0-alkyl-PAF, and 18:0-alkyl-PAF. The shoulder region of the main peak of PAF activity at fraction 23 corresponded to the elution position of 18:1-alkyl-PAF. As determined in PMN bioassay (lower panel), peaks of PAF activity were present in fractions 15, 20 and 31. The shoulder region of the main peak of PAF activity at fraction 22 corresponded to the elution position of 18:1-alkyl-PAF.

PLATELET BIOASSAY



PMN BIOASSAY

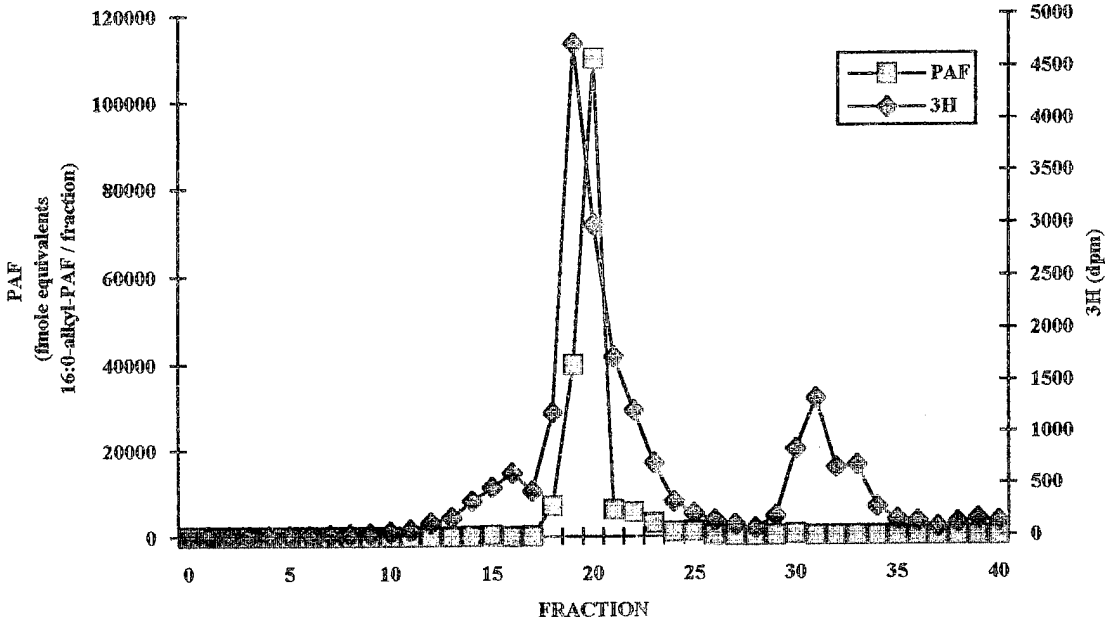
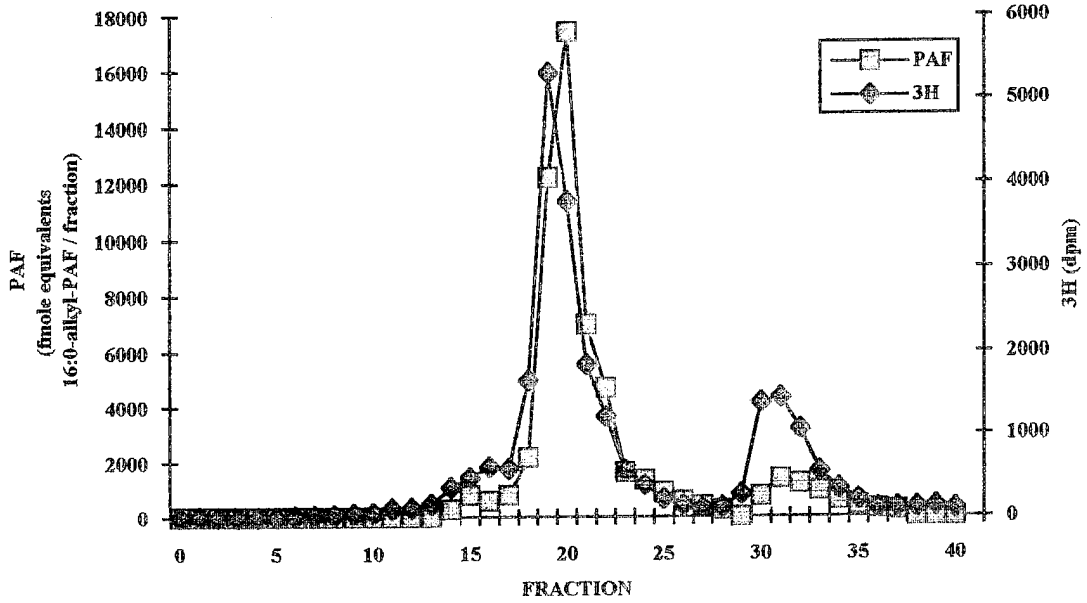


Figure 6 . Representative RP-HPLC Fractionation of Salivary PAF From an AP Subject. After RP-HPLC, an aliquot of each RP-HPLC fraction was utilized to determine PAF activity in rabbit platelet and human PMN bioassay (in fmole equivalents of 16:0-alkyl-PAF) as well as ^{3}H recovery (in dpm). As determined in platelet bioassay (upper panel), a peak of PAF activity was present at fractions 15, 20, and 31; these HPLC regions corresponded to the elution position of 15:0-alkyl-PAF, 16:0-alkyl-PAF, and 18:0-alkyl-PAF. The shoulder region of the main peak of PAF activity at fraction 22 corresponded to the elution position of 18:1-alkyl-PAF. As determined in PMN bioassay (lower panel), peaks of PAF activity were present in fractions 15, 20 and 31. The shoulder region of the main peak of PAF activity at fraction 23 corresponded to the elution position of 18:1-alkyl-PAF.

PLATELET BIOASSAY



PMN BIOASSAY

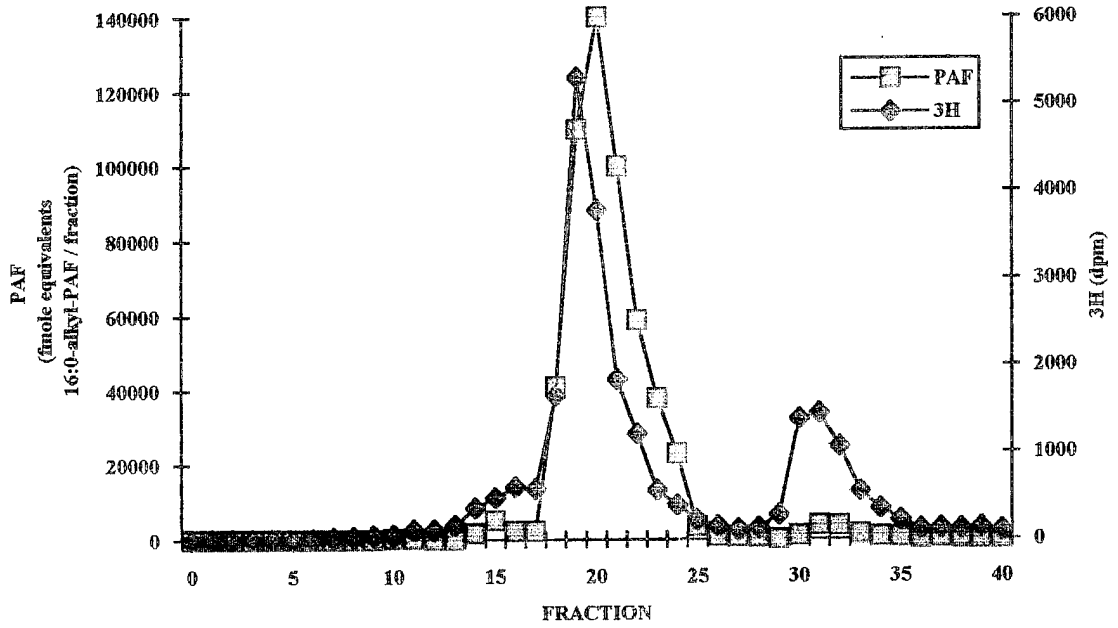
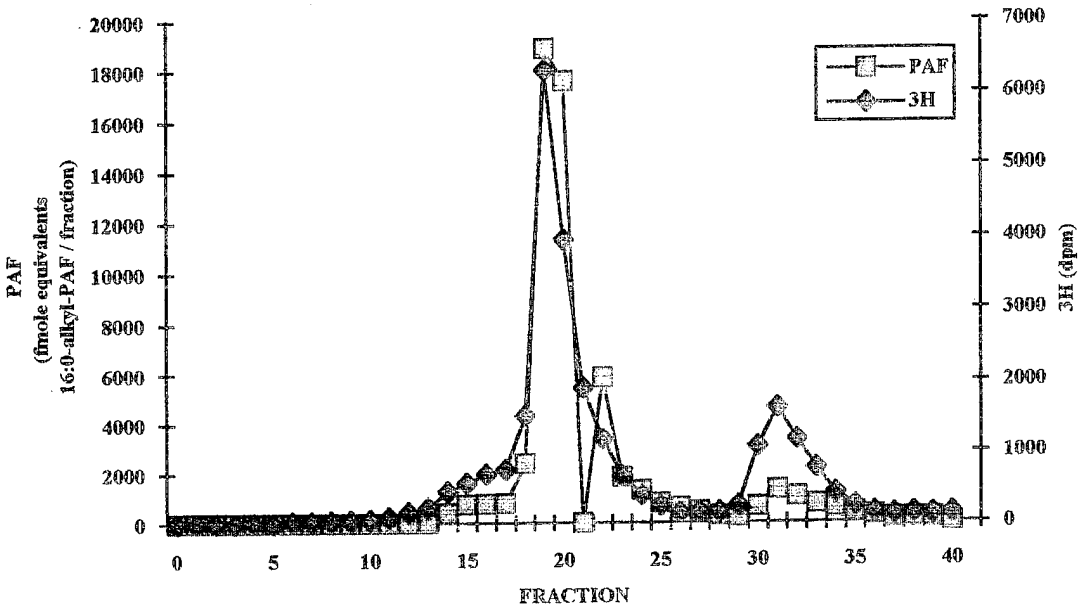


Figure 7 . Representative RP-HPLC Fractionation of Salivary PAF From a RPP Subject. After RP-HPLC, an aliquot of each RP-HPLC fraction was utilized to determine PAF activity in rabbit platelet and human PMN bioassay (in fmole equivalents of 16:0-alkyl-PAF) as well as ^{3}H recovery (in dpm). As determined in platelet bioassay (upper panel), a peak of PAF activity was present at fractions 17, 19, and 31; these HPLC regions corresponded to the elution position of 15:0-alkyl-PAF, 16:0-alkyl-PAF, and 18:0-alkyl-PAF. The shoulder region of the main peak of PAF activity at fraction 22 corresponded to the elution position of 18:1-alkyl-PAF. As determined in PMN bioassay (lower panel), peaks of PAF activity were present in fractions 15, 20 and 32. The shoulder region of the main peak of PAF activity at fraction 23 corresponded to the elution position of 18:1-alkyl-PAF.

PLATELET BIOASSAY



PMN BIOASSAY

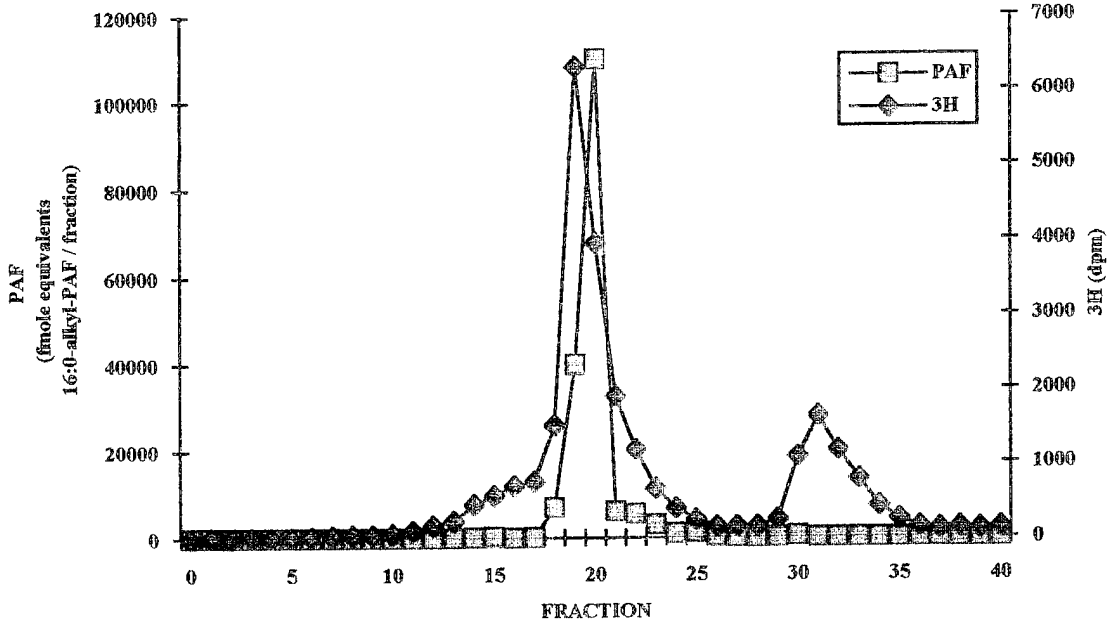


Table 6

COMPARISON OF SALIVARY PAF ACTIVITY AND [^{3}H]PAF RECOVERY AFTER RP-HPLC FRACTIONATION IN PERIODONTAL HEALTH AND DISEASE

	Normal Subjects (n=7)	Adult Periodontitis (AP) Subjects (n=7)	Rapidly Progressive Periodontitis (RPP) Subjects (n=3)
Platelet Bioassay	3330 ± 795*	13,520 ± 1466#	8783 ± 2785#
PMN Bioassay	19,391 ± 4269	116,900 ± 31,931^	62,156 ± 38,646
[^{3}H] 16:0-, 18:0-alkyl-PAF (%)	81.9 ± 5.3	81.9 ± 4.2	93.0 ± 3.8

* Results are presented as the mean ± SE

# $p<0.04$ as compared to results derived from normal subjects (*t*-test following One-way ANOVA)

^ $p<0.01$ as compared to results derived from normal subjects (*t*-test following One-way ANOVA)

Salivary PAF activity after RP-HPLC fractionation was defined in both platelet and PMN bioassay relative to the activity of authentic 16:0-alkyl-PAF evaluated in parallel. Results derived from two subjects (one each from the normal and RPP group) were deleted from these analyses due to excessive [^{3}H] recovery thought to be due to lab error. All PAF activity in RP-HPLC fractions from a given saliva sample were totaled and expressed in fmole equivalents of 16:0-alkyl-PAF/ml of saliva; for each saliva sample, values were corrected for recovery of [^{3}H]PAF tracer. The recovery of [^{3}H]PAF tracer is expressed as a percentage of the radiolabel included in the sample prior to HPLC.

(Table 6). Thus, the recovery of radiolabeled tracer PAF after RP-HPLC was not significantly different between the groups.

G. Distribution of Individual Molecular Species of PAF in Periodontal Health and Disease

Efforts were undertaken to compare the level and distribution of individual molecular species of salivary PAF in periodontal health as compared to periodontal disease. Overall, whether estimated by platelet or PMN bioassay, every PAF molecular species was increased in the saliva of subjects with disease as compared to normal subjects (Table 7). Significant increases were found when comparing normal to AP subjects in the platelet ($p \leq 0.05$) and PMN ($p \leq 0.02$) bioassays. For RPP subjects, only 16:0-alkyl-PAF activity in platelet bioassay was significantly increased in comparison to normal subjects; however, AP subjects had significantly greater ($p=0.04$) amounts of 16:0-alkyl-PAF activity as compared to RPP subjects. In contrast to the above, when each PAF molecular species was expressed as a percentage of total salivary PAF, there were few differences between the groups for most PAF molecules (Table 8 and Figures 8 and 9). For all groups, in both the platelet and PMN bioassay, 16:0-alkyl-PAF represented the majority of the biologic activity. Thus, for the platelet bioassay, 16:0-alkyl-PAF represented 68.1 $\pm$ 5.7%, 72.0 $\pm$ 2.5%, and 65.4 $\pm$ 2.3% of the total PAF activity in the normal, AP and RPP groups, respectively. Similarly in the PMN bioassay, 16:0-alkyl-PAF represented 75.3 $\pm$ 7.0%, 67.2 $\pm$ 7.1%, and 76.1 $\pm$ 4.9% of the total PAF activity in the normal, AP and RPP groups, respectively. In the platelet bioassay, the percentage of 15:0-alkyl-PAF was significantly increased in the diseased subjects when compared to normal subjects; however, 15:0-alkyl-PAF activity was only detected in two of eight normal subjects (this may reflect limitations in the bioassay detection system since there was a relatively smaller amount of PAF in the saliva of normal subjects in comparison to that observed in subjects with periodontal disease. For all other PAF molecular species, there

Table 7

COMPARISON OF THE ACTIVITY OF INDIVIDUAL PAF MOLECULAR SPECIES IN PERIODONTAL HEALTH AND DISEASE

	Normal Subjects (n=7)	Adult Periodontitis (AP) Subjects (n=7)	Rapidly Progressive Periodontitis (RPP) Subjects (n=3)
PLATELET BIOASSAY			
15:0-alkyl-PAF	31.9 ± 21.7*	353.7 ± 71.9#	214.9 ± 107.7
16:0-alkyl-PAF	2278.1 ± 581.2	9664.9 ± 1038.0#	5797.0 ± 1923.5#^
18:1-alkyl-PAF	730.3 ± 261.2	2503.9 ± 375.3#	2037.1 ± 669.7
18:0-alkyl-PAF	289.3 ± 107.7	997.2 ± 136.0#	734.3 ± 153.8
PMN BIOASSAY			
15:0-alkyl-PAF	290.1 ± 186.3*	2994.8 ± 964.6**	5208.2 ± 5016.0
16:0-alkyl-PAF	14,549.5 ± 3954.2	87,430.8 ± 27,838.8**	46,188.5 ± 29,161.3
18:1-alkyl-PAF	4455.1 ± 1557.7	25,467.0 ± 7099.7**	10,446.2 ± 4930.5
18:0-alkyl-PAF	96.1 ± 83.5	1007.9 ± 381.9**	313.5 ± 184.4

* Results are presented as the mean ± SE

$p \leq 0.05$ as compared to results derived from normal subjects (*t*-test following One-way ANOVA)

^ $p = 0.04$ as compared to results derived from AP subjects (*t*-test following One-way ANOVA)

** $p \leq 0.02$ as compared to results derived from normal subjects after log transformation of data (*t*-test following One-way ANOVA)

See legend for Table 6 for an additional discussion of platelet and PMN bioassay.

Table 8

COMPARISON OF THE PERCENTAGE DISTRIBUTION OF TOTAL PAF

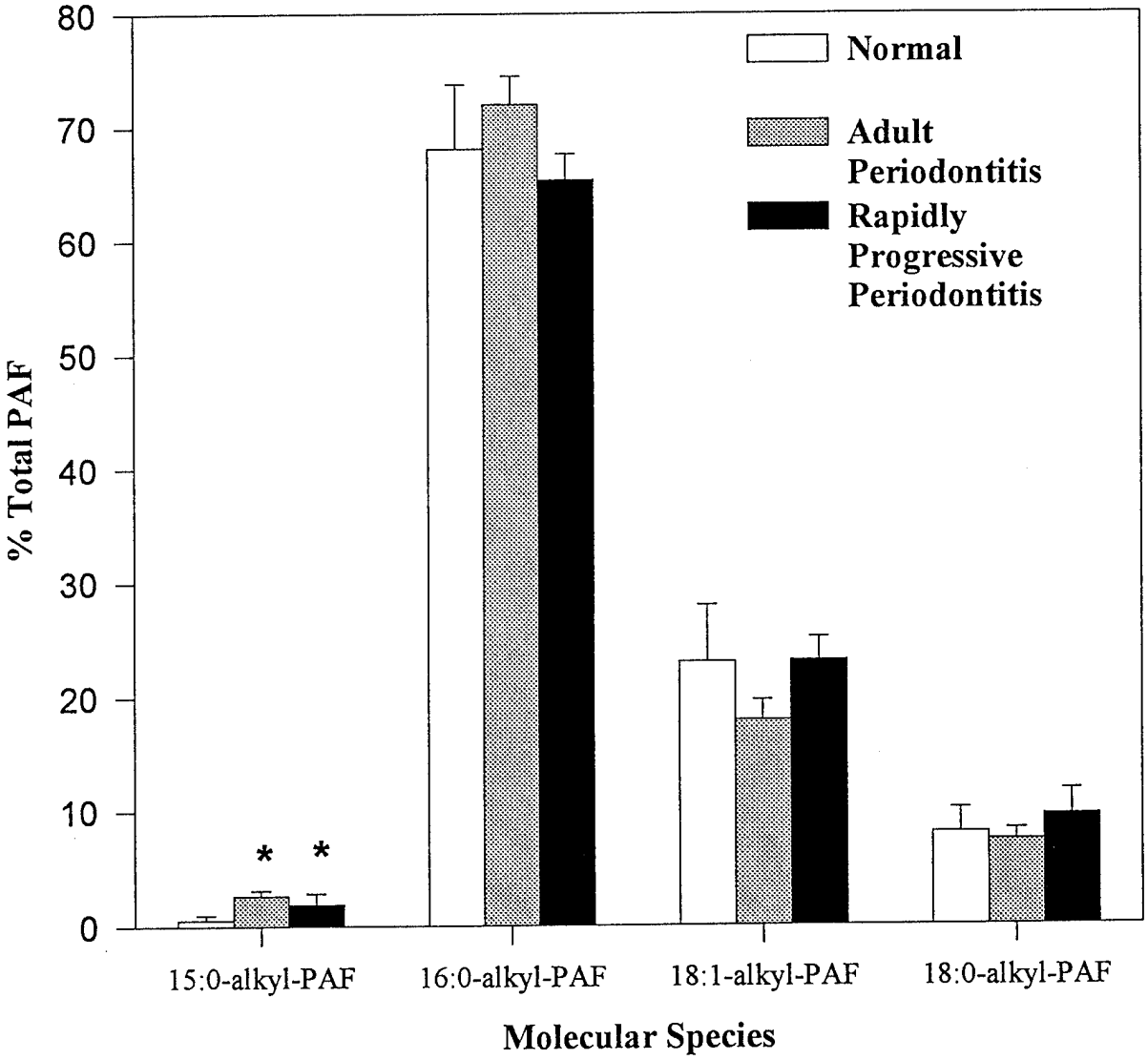
	Normal Subjects (n=7)	Adult Periodontitis (AP) Subjects (n=7)	Rapidly Progressive Periodontitis (RPP) Subjects (n=3)
PLATELET BIOASSAY			
15:0-alkyl-PAF	0.6 ± 0.4*	2.6 ± 0.5#	1.9 ± 1.0#
16:0-alkyl-PAF	68.1 ± 5.7	72.0 ± 2.5	65.4 ± 2.3
18:1-alkyl-PAF	23.3 ± 5.0	18.0 ± 1.8	23.2 ± 2.2
18:0-alkyl-PAF	8.1 ± 2.1	7.4 ± 1.0	9.6 ± 2.0
PMN BIOASSAY			
15:0-alkyl-PAF	1.7 ± 1.3	3.4 ± 1.3	4.0 ± 3.5
16:0-alkyl-PAF	75.3 ± 7.0	67.2 ± 7.1	76.1 ± 4.9
18:1-alkyl-PAF	22.5 ± 7.4	28.5 ± 6.7	19.2 ± 5.0
18:0-alkyl-PAF	0.4 ± 0.4	0.8 ± 0.3	0.6 ± 0.5

* Results are presented as the mean ± SE

$p \leq 0.03$ as compared to results derived from normal subjects (*t*-test following One-way ANOVA)

See legend for Table 6 for an additional discussion of platelet and PMN bioassay. The total amount of PAF activity in all RP-HPLC fractions was determined and utilized to calculate the relative (percent) distribution of PAF activity in RP-HPLC fractions which corresponded to the elution position of authentic molecular species of alkyl-PAF.

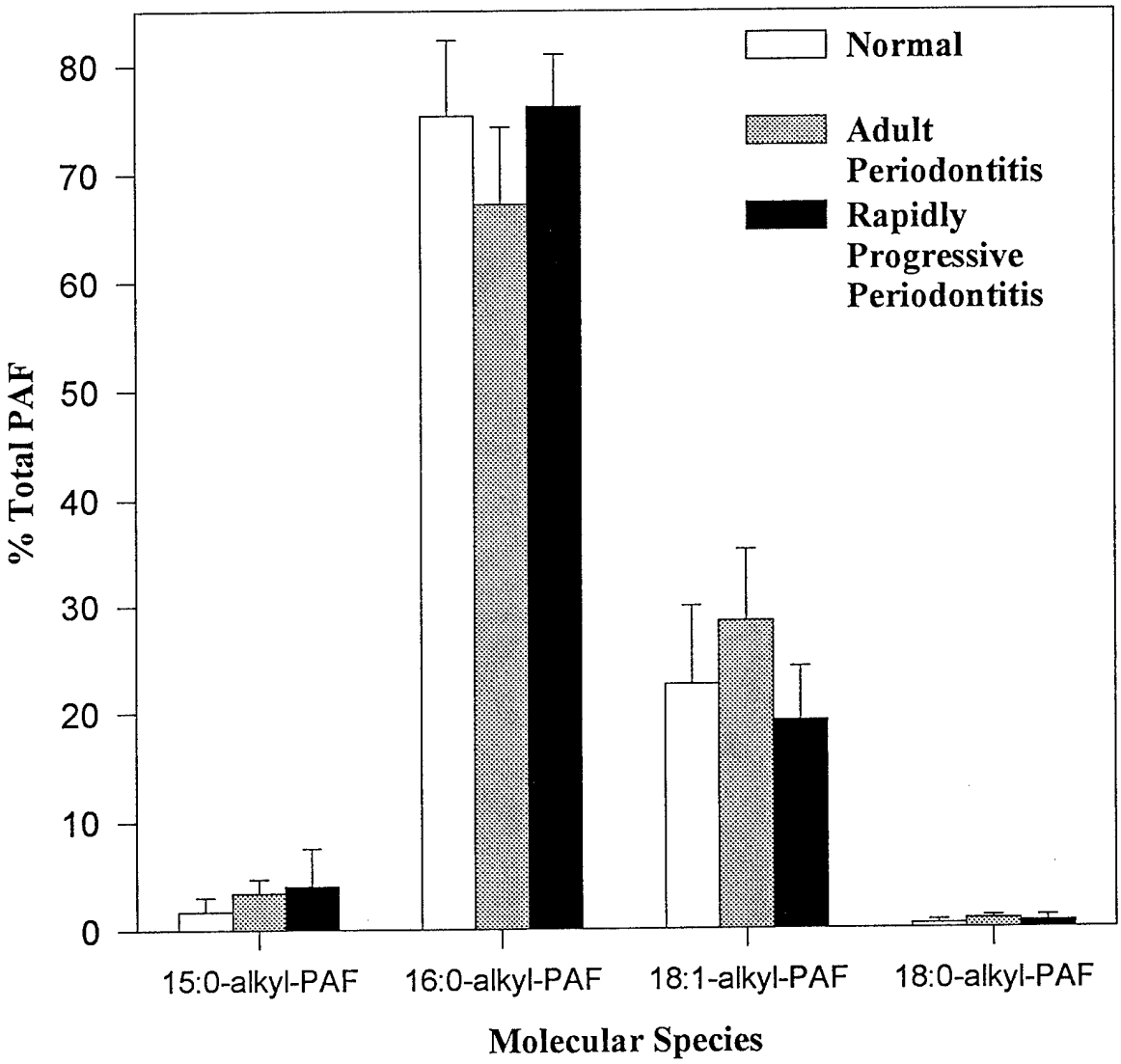
Figure 8. Comparison of the Percentage Distribution of Individual PAF Molecular Species in Periodontal Health and Disease. Platelet bioassay was used to determine the amount of PAF activity after RP-HPLC fractionation of salivary lipids previously purified by NP-HPLC. The total amount of PAF activity in all RP-HPLC fractions was determined and utilized to calculate the relative (percent) distribution of PAF activity in RP-HPLC fractions which corresponded to the elution position of authentic molecular species of alkyl-PAF. 16:0-alkyl-PAF represented the majority of biologic activity for all subject groups. Significant increases ($p<0.03$) in 15:0-alkyl-PAF were found in the AP and RPP subject groups when compared to normal subjects (*). No significant differences existed between the groups relative to the other molecular species of PAF.



Mean ± S.E.

***p*<0.03 as compared to normal (least squares means)**

Figure 9. Comparison of the Percentage Distribution of Individual PAF Molecular Species in Periodontal Health and Disease. PMN bioassay was used to determine the amount of PAF activity after RP-HPLC fractionation of salivary lipids previously purified by NP-HPLC. The total amount of PAF activity in all RP-HPLC fractions was determined and utilized to calculate the relative (percent) distribution of PAF activity in RP-HPLC fractions which corresponded to the elution position of authentic molecular species of alkyl-PAF. 16:0-alkyl-PAF represented the majority of biologic activity for all subject groups. No significant differences existed between the groups relative to the molecular species of PAF.


Normal
Adult Periodontitis
Rapidly Progressive Periodontitis
% Total PAF
0
10
20
30
40
50
60
70
80
15:0-alkyl-PAF
16:0-alkyl-PAF
18:1-alkyl-PAF
18:0-alkyl-PAF
Molecular Species

Mean ± S.E.

were no significant differences between periodontally healthy subjects as compared to those with periodontal disease relative to the percentage distribution of PAF molecules as determined in both platelet and PMN bioassay.

Interestingly, when comparing the percentage distribution of individual PAF molecules as determined in platelet and PMN bioassay, differences were found in the activity of 18:0-alkyl-PAF. For the platelet bioassay, 18:0-alkyl-PAF represented 8.1 $\pm$ 2.1%, 7.4 $\pm$ 1.0%, and 9.6 $\pm$ 2.0% of the total PAF activity for normal, AP, and RPP subjects, respectively; in the PMN bioassay 18:0-alkyl-PAF represented a lower percent of total PAF activity with 0.4 $\pm$ 0.4%, 0.8 $\pm$ 0.3%, and 0.6 $\pm$ 0.5%, respectively, in the normal, AP, and RPP subjects.

H. Relationship Between Clinical Indices of Periodontal Disease and the Salivary Levels of Individual PAF Molecular Species

The relationship between the amount of individual PAF molecular species and the extent of clinically measured indices of periodontal inflammation was examined. The levels of 15:0-, 16:0-, 18:1-, and 18:0-alkyl-PAF as determined in platelet bioassay were found to significantly correlate with the number of probing depths (PD) greater than or equal to 4 mm (Table 9). Additionally, all of the molecular species with the exception of 18:1-alkyl-PAF were found to significantly correlate with the number of sites with attachment loss (AL) 2 mm or greater. When the relationship between subject age and individual molecular species was examined, all molecular species significantly correlated. Only 15:0- and 18:0-alkyl-PAF were found to significantly correlate with the number of histologically identified PMN present in the saliva pellet. No significant correlations were found for any of the molecular species and the Eastman Interdental Bleeding index (EIBI), number of sites with bleeding on probing (BOP), and the modified O'Leary plaque index (PlI).

The relationship between the levels of individual PAF molecules determined in the PMN bioassay and the clinical features for 17 subjects was also examined (Table 10). In

Table 9

RELATIONSHIP BETWEEN CLINICAL INDICES OF PERIODONTAL DISEASE AND THE SALIVARY LEVELS OF INDIVIDUAL PAF MOLECULAR SPECIES - PLATELET BIOASSAY (n=17)

	PD ≥ 4 mm (#)	AL ≥ 2 mm (#)	AGE	SALIVARY PMN	EIBI (%)	BOP (#)	PII (%)
15:0-alkyl-PAF	r=0.73 *p*=0.0008	r=0.54 *p*=0.03	r=0.53 *p*=0.03	r=0.51 *p*=0.04	r=0.30 *p*=0.24	r=0.45 *p*=0.07	r=-0.44 *p*=0.07
16:0-alkyl-PAF	r=0.60 *p*=0.01	r=0.53 *p*=0.03	r=0.64 *p*=0.005	r=0.40 *p*=0.11	r=0.19 *p*=0.46	r=0.44 *p*=0.08	r=-0.37 *p*=0.15
18:1-alkyl-PAF	r=0.62 *p*=0.008	r=0.47 *p*=0.06	r=0.52 *p*=0.03	r=0.52 *p*=0.03	r=0.25 *p*=0.33	r=0.41 *p*=0.10	r=-0.27 *p*=0.30
18:0-alkyl-PAF	r=0.64 *p*=0.005	r=0.57 *p*=0.02	r=0.63 *p*=0.007	r=0.31 *p*=0.22	r=0.22 *p*=0.39	r=0.46 *p*=0.06	r=-0.33 *p*=0.20

PD = Probing Depth (Florida Probe at 20 grams of force)

AL = Attachment Loss

EIBI = Eastman Interdental Bleeding Index

BOP = Bleeding on Probing (Florida Probe at 20 grams of force)

PII = Modified O'Leary Plaque Index

Table 10

RELATIONSHIP BETWEEN CLINICAL INDICES OF PERIODONTAL DISEASE AND THE SALIVARY LEVELS OF INDIVIDUAL PAF MOLECULAR SPECIES - PMN BIOASSAY (n=17)

	PD ≥ 4 mm (#)	AL ≥ 2 mm (#)	AGE	SALIVARY PMN	EIBI (%)	BOP (#)	PII (%)
15:0-alkyl-PAF	r=0.40 *p*=0.13	r=0.24 *p*=0.36	r=0.05 *p*=0.84	r=0.24 *p*=0.36	r=0.07 *p*=0.79	r=0.33 *p*=0.19	r=-0.28 *p*=0.27
16:0-alkyl-PAF	r=0.52 *p*=0.03	r=0.52 *p*=0.03	r=0.58 *p*=0.01	r=0.23 *p*=0.38	r=0.29 *p*=0.27	r=0.45 *p*=0.07	r=-0.50 *p*=0.04
18:1-alkyl-PAF	r=0.60 *p*=0.01	r=0.46 *p*=0.07	r=0.58 *p*=0.02	r=0.05 *p*=0.85	r=0.53 *p*=0.03	r=0.50 *p*=0.04	r=-0.55 *p*=0.02
18:0-alkyl-PAF	r=0.57 *p*=0.02	r=0.56 *p*=0.02	r=0.57 *p*=0.02	r=0.35 *p*=0.17	r=0.19 *p*=0.46	r=0.23 *p*=0.38	r=-0.31 *p*=0.22

PD = Probing Depth (Florida Probe at 20 grams of force)

AL = Attachment Loss

EIBI = Eastman Interdental Bleeding Index

BOP = Bleeding on Probing (Florida Probe at 20 grams of force)

PII = Modified O'Leary Plaque Index

contrast to the results obtained by platelet bioassay, significant correlations were found between, the EIBI and 18:1-alkyl-PAF, the PlI and 16:0- and 18:1-alkyl-PAF, and the number of sites with BOP and 18:1-alkyl-PAF. Also, all molecular species except 15:0-alkyl-PAF significantly correlated with the number of sites with PD greater than or equal to 4 mm. When the relationship between the individual molecular species and the number of sites with AL 2 mm or greater was examined, it was found that only 16:0- and 18:0-alkyl-PAF significantly correlated with this parameter. All PAF molecular species with the exception of 15:0-alkyl-PAF were found to significantly correlate with age, while none of the molecular species were found to correlate with the number of histologically identified PMN in the saliva pellet.

Partial correlations were compared between clinical indices of periodontal disease and individual PAF molecular species. Adjustments were made for group, gender, smoking, age, and number of teeth to verify the correlations were not due to any of these factors. These results are summarized as follows:

1. Eastman Interdental Bleeding Index (EIBI)

Significant partial correlations were found for the EIBI and all PAF molecular species ($r \geq 0.53$, $p \leq 0.03$) when adjustments were made for group, gender, smoking, age, and number of teeth.

2. Number of Sites with Bleeding on Probing (BOP)

No significant partial correlations were found when adjustments for group were made for any molecular species as assessed in either platelet or PMN bioassay (Table 11). However, when adjusted for gender, molecular species except 16:0-alkyl-PAF in the platelet bioassay and except 15:0- and 18:0-alkyl-PAF in the PMN bioassay had significant partial correlations with the number of sites with bleeding on probing. Upon adjustment for smoking, only 18:1-alkyl-PAF in the PMN bioassay ($r=0.51$, $p=0.05$) was significant. Adjustment for age revealed significant partial correlations for 16:0- and 18:1-alkyl-PAF in

Table 11

PARTIAL CORRELATIONS FOR THE NUMBER OF SITES WITH BLEEDING ON PROBING AND THE MOLECULAR SPECIES OF SALIVARY PAF

Variable Adjusted For:	Group	Gender	Smoking	Number of Teeth
PLATELET BIOASSAY				
15:0-alkyl-PAF	r=-0.18 *p*=0.52	r=0.52 *p*=0.04	r=0.44 *p*=0.09	r=0.51 *p*=0.04
16:0-alkyl-PAF	r=-0.40 *p*=0.14	r=0.48 *p*=0.06	r=0.42 *p*=0.10	r=0.44 *p*=0.09
18:0-alkyl-PAF	r=-0.25 *p*=0.37	r=0.51 *p*=0.04	r=0.44 *p*=0.09	r=0.50 *p*=0.05
18:1-alkyl-PAF	r=-0.40 *p*=0.19	r=0.55 *p*=0.03	r=0.40 *p*=0.13	r=0.43 *p*=0.09
PMN BIOASSAY				
15:0-alkyl-PAF	r=-0.18 *p*=0.52	r=0.27 *p*=0.31	r=0.36 *p*=0.18	r=0.34 *p*=0.20
16:0-alkyl-PAF	r=0.23 *p*=0.42	r=0.52 *p*=0.04	r=0.45 *p*=0.08	r=0.45 *p*=0.08
18:0-alkyl-PAF	r=-0.15 *p*=0.59	r=0.40 *p*=0.13	r=0.20 *p*=0.45	r=0.20 *p*=0.45
18:1-alkyl-PAF	r=0.40 *p*=0.14	r=0.61 *p*=0.01	r=0.51 *p*=0.05	r=0.50 *p*=0.051

the PMN bioassay ($r \geq 0.50$, $p \leq 0.05$), and 18:0-alkyl-PAF in the platelet bioassay ($r=0.51$, $p=0.04$). When adjusted for the number of teeth, only 15:0- and 18:0-alkyl-PAF in the platelet bioassay ($r \geq 0.50$, $p \leq 0.05$) had significant partial correlations.

3. Number of Sites with Probing Depths > 4 mm

When compared by group, significant correlations were found ($r \geq 0.54$, $p=0.04$) for 15:0-alkyl-PAF in the platelet bioassay and 18:0-alkyl-PAF in the PMN bioassay (Table 12). When comparing on a gender basis in both platelet and PMN bioassay, all molecular species except 15:0-alkyl-PAF in the PMN bioassay significantly correlated ($r \geq 0.54$, $p \leq 0.03$). When adjusted for smoking, all molecular species in both platelet and PMN bioassay except 15:0- and 16:0-alkyl-PAF in the PMN bioassay significantly correlated ($r \geq 0.53$, $p \leq 0.03$). Significant correlations were found ($r \geq 0.52$, $p \leq 0.03$) for most PAF molecular species after adjustment for age in both platelet and PMN bioassay except for 15:0- and 16:0-alkyl-PAF in the PMN bioassay. When adjusted for the number of teeth, significant correlations ($r \geq 0.52$, $p \leq 0.04$) were found for all molecular species in both platelet and PMN bioassay except for 15:0- and 18:0-alkyl-PAF in the PMN bioassay, and 16:0- and 18:1-alkyl-PAF in the platelet bioassay.

4. Number of Sites with Attachment Loss (AL) > 2 mm

Comparison by group found no significant correlations between any of the molecular species in both platelet and PMN bioassay and this clinical index (Table 12). However, when adjusted for gender, most PAF molecular species except 15:0-alkyl-PAF in the PMN bioassay were significantly correlated with the number of sites with AL $\geq$ 2 mm. When adjusted for smoking, only 15:0-alkyl-PAF ($r=0.53$, $p=0.04$) in the platelet bioassay, and 18:0- and 18:1-alkyl-PAF ($r \geq 0.49$, $p=0.05$) in the PMN bioassay were significantly correlated with the number of sites with AL $\geq$ 2 mm. Adjusted for age, none of the molecular species in both platelet and PMN bioassay significantly correlated with the number of sites with AL $\geq$ 2 mm, and only 16:0-alkyl-PAF in the PMN bioassay ($r=0.54$, $p=0.03$) significantly correlated with the number of sites with AL $\geq$ 2 mm when adjusted for the number of teeth.

Table 12

PARTIAL CORRELATIONS FOR THE NUMBER OF SITES WITH PROBING DEPTH ≥ 4 MILLIMETERS AND THE MOLECULAR SPECIES OF SALIVARY PAF

Variable Adjusted For:	Group	Gender	Smoking	Number of Teeth
PLATELET BIOASSAY				
15:0-alkyl-PAF	r=0.53 *p*=0.04	r=0.74 *p*=0.001	r=0.72 *p*=0.002	r=0.67 *p*=0.005
16:0-alkyl-PAF	r=0.07 p=0.79	r=0.60 *p*=0.01	r=0.57 *p*=0.02	r=0.48 *p*=0.06
18:0-alkyl-PAF	r=0.24 *p*=0.40	r=0.64 *p*=0.007	r=0.60 *p*=0.02	r=0.53 *p*=0.04
18:1-alkyl-PAF	r=0.19 *p*=0.50	r=0.65 *p*=0.007	r=0.57 *p*=0.02	r=0.50 *p*=0.05
PMN BIOASSAY				
15:0-alkyl-PAF	r=-0.005 *p*=0.97	r=0.39 *p*=0.14	r=0.43 *p*=0.10	r=0.39 *p*=0.13
16:0-alkyl-PAF	r=0.30 *p*=0.27	r=0.54 *p*=0.03	r=0.47 *p*=0.07	r=0.52 *p*=0.04
18:0-alkyl-PAF	r=0.54 *p*=0.04	r=0.63 *p*=0.009	r=0.53 *p*=0.03	r=0.46 *p*=0.08
18:1-alkyl-PAF	r=0.50 *p*=0.06	r=0.62 *p*=0.01	r=0.62 *p*=0.01	r=0.59 *p*=0.02

Table 13

PARTIAL CORRELATIONS FOR THE NUMBER OF SITES WITH ATTACHMENT LOSS ≥ 2 MILLIMETERS AND THE MOLECULAR SPECIES OF SALIVARY PAF

Variable Adjusted For:	Group	Gender	Smoking	Number of Teeth
PLATELET BIOASSAY				
15:0-alkyl-PAF	r=0.08 *p*=0.78	r=0.56 *p*=0.02	r=0.53 *p*=0.04	r=0.20 *p*=0.47
16:0-alkyl-PAF	r=-0.08 *p*=0.76	r=0.53 *p*=0.04	r=0.49 *p*=0.06	r=0.29 *p*=0.27
18:0-alkyl-PAF	r=0.10 *p*=0.73	r=0.58 *p*=0.02	r=0.47 *p*=0.07	r=0.27 *p*=0.31
18:1-alkyl-PAF	r=-0.09 *p*=0.75	r=0.51 *p*=0.04	r=0.34 *p*=0.19	r=0.13 *p*=0.64
PMN BIOASSAY				
15:0-alkyl-PAF	r=-0.17 *p*=0.54	r=0.20 *p*=0.50	r=0.28 *p*=0.29	r=0.22 *p*=0.41
16:0-alkyl-PAF	r=0.25 *p*=0.36	r=0.54 *p*=0.03	r=0.42 *p*=0.11	r=0.54 *p*=0.03
18:0-alkyl-PAF	r=0.42 *p*=0.12	r=0.65 *p*=0.007	r=0.49 *p*=0.05	r=0.35 *p*=0.19
18:1-alkyl-PAF	r=0.15 *p*=0.60	r=0.49 *p*=0.05	r=0.50 *p*=0.05	r=0.44 *p*=0.09

V. DISCUSSION

Platelet-Activating Factor (PAF) has been viewed in the past as a single molecular species. It is now known that PAF is comprised of a large family of structurally related phospholipid compounds that each have unique biologic activities which also differ in potency. The combined action of these different molecular species of PAF must be considered when evaluating their effects on target cells and tissues.

No studies to date have attempted to define the molecular species of salivary PAF present in periodontal health and disease. With this in mind, the hypothesis of this study was that qualitative and quantitative differences in PAF molecular species would be found in human mixed saliva during periodontal health as compared to periodontal disease. Results of this study demonstrate that qualitative differences do exist in the total amount of salivary PAF present in periodontal health and disease, with greater amounts of salivary PAF present in disease. However on a percentage basis, with the possible exception of 15:0-alkyl-PAF, no significant differences exist in the molecular species of salivary PAF present in periodontal health, adult periodontitis and rapidly progressive periodontitis.

In the present study, no significant differences were found between periodontally healthy and diseased subjects when comparing the number of PMN present in the saliva pellet. In contrast, Garito (1991) with a larger sample size found that salivary PMN progressively increased and significantly correlated with periodontal disease severity. In addition, both Rasch (1993) and McDonnell (1994) have demonstrated strong correlations between salivary PAF and salivary PMN. Reasons for the lack of significant differences in this study population may be related to the small number of subjects, the variability in the number of PMN found in the adult periodontitis group, or the suppressive effect of smoking on PMN emigration from vascular structures into the oral environment.

Total salivary PAF activity following TLC for the normal and adult periodontitis group in the current study was consistent with that found from other investigations from this laboratory. Braun *et al.* (1992), reported 2318 $\pm$ 355 fmole equivalents of 16:0-alkyl-PAF/ml of saliva in normal patients. In the 15 AP subjects studied by Rasch (1993), salivary PAF was reported as 12,084 $\pm$ 2789 fmole equivalents of 16:0-alkyl-PAF/ml of saliva. However, the RPP group in the current study demonstrated significantly less salivary PAF than that found in the subjects of Braun *et al.* (1992), *i.e.*, 8445.0 $\pm$ 1111.5 vs. 18,390 $\pm$ 5852 $\pm$ 2789 fmole equivalents of 16:0-alkyl-PAF/ml of saliva respectively. These investigators do not report the number of PMN present within the saliva pellets of their subject group, and since this cell has been implicated by McManus *et al.* (1990), as the likely source for intraoral PAF in periodontal disease, no comparisons can be made.

Normal phase (NP) HPLC was performed to fractionate PAF from other salivary lipids within each sample. The PAF behavior in all saliva samples in platelet bioassay following normal phase HPLC was similar for all subjects from each subject group. This is consistent with the past history of this technique for the lab. For instance, Pinckard *et al.* (1984), demonstrated that PAF obtained from cell-free supernatants or cell pellets of FMLP-stimulated human PMN obtained from peripheral blood eluted from the normal phase HPLC column as a "single, homogenous peak of PAF activity." In a later study by McManus *et al.* (1993), in which saliva was collected from edentulous patients with oral mucositis secondary to radiation therapy, "a single peak of PAF activity was observed and closely corresponded to the elution profile of the [3H]16:0-alkyl-PAF internal standard" following normal phase HPLC. In the current study, comparable results were observed for all saliva samples after NP-HPLC when assessed in platelet bioassay. In contrast, however, PMN bioassay of 10 NP-HPLC fractionated saliva samples demonstrated multiple peaks of salivary PAF activity. This likely reflects the presence of different classes of salivary lipids along with PAF species present in the samples, which may be underestimated in platelet bioassay.

Of particular interest in this study was the consistent RP-HPLC fractionation pattern of salivary PAF molecular species for every subject in each subject group as assessed in the platelet bioassay. This finding is of even greater significance since this study is one of the first to provide definitive assessment of the periodontal health status of the subjects. Pinckard *et al.* (1984), demonstrated that FMLP-stimulated human PMN from peripheral blood produced multiple molecular species of PAF. The greatest amount of PAF activity was found to elute in a similar position as the [3H]16:0-alkyl-PAF internal standard and confirmed by mass spectral analysis to be 16:0-alkyl-PAF. McManus *et al.* (1993), reported that multiple peaks of biologic activity which correspond to various choline-containing PAF molecular species were always identified following reverse phase HPLC of salivary lipids derived from normal dentate subjects. However, clinical data to support the periodontal health of these subjects were not provided, and in contrast to the findings of this study, four peaks and one shoulder region of biologic activity were found, which suggests large total amounts of PAF.

In contrast to this study, McManus *et al.* (1993), reported that subjects with oral mucositis secondary to head and neck irradiation for oral cancer had only a single peak of PAF activity following reverse phase HPLC; the peak of this salivary PAF activity corresponded to the retention time of [3H]16:0-alkyl-PAF. Therefore, oral inflammation due to different etiologies demonstrate qualitative differences in molecular species. This may be explained on the basis of PAF biosynthesis and degradation. Either through selective degradation of other PAF molecular species, or differences in the precursors available for biosynthesis, PAF molecular species may be modulated. Alternatively, different cell populations may be present in oral mucositis as compared to oral health and periodontal disease, reflecting differences in PAF molecular species produced (McManus *et al.* 1993).

In the current study, the PMN bioassay yielded reverse phase fractionation chromatograms which were not as consistent as the platelet bioassay. Nevertheless, multiple molecular species of salivary PAF were present in every saliva sample, with the greatest

biologic activity seen in a peak which corresponded to the elution position for the [^{3}H]16:0-alkyl-PAF. Greater variability in these chromatograms was seen as the total amount of PAF increased which suggests that other PAF molecular species, most notably acyl-PAFs, that would be underestimated in the platelet bioassay, contributed to this finding. In addition, biologic activity was 8 to 10 times greater in the PMN bioassay as compared to platelet bioassay. This suggests a synergistic effect among molecular species, resulting in an amplification of PAF bioactivity, as assessed by this bioassay.

These findings of multiple molecular species of PAF in both bioassays in the present study are particularly interesting when compared to the results of Christman and Blair (1989). These investigators studied the molecular species of salivary PAF present in seven normal subjects. Analysis of PAF molecular species present in salivary lipids was undertaken following phospholipase C cleavage of PAF, TLC separation of the diglyceride product, and derivatization with pentafluorobenzoyl (PFB) chloride prior to gas chromatography and mass spectrometry. Only 16:0-alkyl-PAF was detected in six of the subjects. In the seventh subject, 16:0-, 18:1- and 18:0-alkyl-PAF were found in the saliva sample. However, this subject reported having a recent upper respiratory infection, and salivary PAF levels were increased 10-fold over the other subjects. Several differences in methodology between the current study and the Christman and Blair (1989) investigation may explain differences in results. First, the subjects in the Christman and Blair study provided saliva samples after they had rinsed their mouths multiple times with water which may have reduced the total salivary PAF as well as individual molecular species present. Second, their multi-step procedure prior to quantification of PAF molecular species may have resulted in selective loss of individual molecular species. Additionally, no clinical data were provided for these subjects to document the intraoral and periodontal health status of the "normal subjects" in that study.

When the activity of individual PAF molecular species is compared on a femptomole basis, significant increases were found for all molecular species in both bioassays when AP

subjects are compared to normal subjects in the current study. However, when comparing RPP subjects to normal subjects, the only molecular species significantly increased was 16:0-alkyl-PAF in the platelet bioassay. This is appears to represent the great variability seen in a limited number of subjects in the RPP group. Additional studies are necessary to evaluate this possibility.

In contrast to the above, examination of the percent distribution of salivary PAF molecular species revealed that for all subject groups, in both the platelet and PMN bioassay, 16:0-alkyl-PAF represented the majority of the biologic activity. This is significant in that 16:0-alkyl-PAF appears to be one of the more biologically potent PAF species. For instance, Pinckard and co-workers (1992) demonstrated that 18:0-alkyl-PAF was 30-fold less potent in inducing PMN lysozyme secretion than 16:0-alkyl-PAF. 16:0-alkyl-PAF was also found to produce a 10-fold increase in superoxide production when compared to 18:0-alkyl-PAF.

The finding of no significant differences between the three subject groups in the percent distribution of total PAF molecular species in both bioassays, with the possible exception of 15:0-alkyl-PAF in the platelet bioassay, is also of interest when considering the differential responses of host cells and presumably tissues to different PAF molecular species. Pinckard *et al.* (1992), have shown that the rank order of potencies for alkyl-PAF homologs in effecting PMN chemotaxis was 18:0- > 18:1- >> 16:0-alkyl-PAF. In addition, the rank order of potency of the alkyl-PAF homologs for inducing lysosomal enzyme secretion and superoxide production was 18:1- $\geq$ 16:0 >> 18:0-alkyl-PAF. This variance in biologic activity and potency suggests that the molecular species produced by a stimulated cell interact with either more than one PAF-receptor subtype, or a PAF receptor having more than one affinity or conformational state (Pinckard *et al.* 1994). Thus, the individual PAF molecular species differ from one another in their biologic activity and potency, thereby effecting the response at the cellular as well as tissue level.

Of interest was the finding of a lesser amount of 18:0-alkyl-PAF on a percentage basis in the PMN bioassay when compared to the platelet bioassay. This is reflected in percentage increases when the PMN bioassay is compared to the platelet bioassay for other molecular species, except for 18:1-, 16:0- and 18:1-alkyl-PAF in the normal, AP, and RPP subjects, respectively. This suggests a synergistic effect of the alkyl- and acyl-PAF species in the PMN bioassay.

Recovery of total salivary PAF activity for the platelet bioassay demonstrated no selective losses between TLC, NP-HPLC, and RP-HPLC. However, the PMN bioassay showed large increases in total salivary PAF activity between NP-HPLC and RP-HPLC. There are three possibilities for this phenomenon. One, RP-HPLC separation of individual PAF molecular species permits a greater biological response as a result of lack of competition for PMN receptors. Secondly, there may be synergistic effects between selective combinations of PAF molecular species, which provide for a greater biological response. Finally, RP-HPLC separation may allow for greater expression of the acyl-PAF species. It is of note that the differences in salivary PAF biological activity after RP-HPLC occurred in samples where the recovery of tritiated 16:0- and 18:0-alkyl-PAF demonstrated no differences between NP-HPLC and RP-HPLC.

When the relationship of subject age was compared to the individual PAF molecular species for all subjects, significant correlations were found for all of the salivary PAF molecular species in both platelet and PMN bioassay (except 15:0-alkyl-PAF in the PMN bioassay). It has been demonstrated by Grossi *et al.,* (1995), in a cross-sectional study of 1361 subjects, that as age increases so does the percentage of subjects with severe bone loss. As demonstrated by Garito (1995), as periodontal disease severity increases, so does salivary PAF. Therefore, as age increases, periodontal disease severity would be expected to increase, reflecting the presence in saliva of multiple PAF molecular species.

Perhaps most important was the finding that significant correlations existed for all molecular species (except 15:0-alkyl-PAF in the PMN bioassay) with the number of probing depths greater than or equal to 4 mm. In addition, most PAF molecular species were significantly correlated with the number of sites with attachment loss greater than or equal to 2 mm, except for 15:0-alkyl-PAF in the PMN bioassay, and 18:1-alkyl PAF in the platelet and PMN bioassay. These findings suggest that individual molecular species of salivary PAF may act as markers for periodontal disease. However, to identify a specific inflammatory mediator as a key effector mechanism in the pathogenesis of periodontal disease requires identification of concurrent disease activity. Only through site specific, longitudinal studies can these relationships be established.

When clinical indices were compared to individual salivary PAF molecular species adjusting for group, no significant partial correlations were found. However, when results were adjusted for gender, significant partial correlations were found for bleeding on probing, number of sites with probing depth greater than or equal to 4 mm, and number of sites with attachment loss greater than or equal to 2 mm. In addition, adjustment for smoking yielded significant partial correlations for probing depth greater than or equal to 4 mm. These findings must be considered in light of the limited number of females and smokers within this study.

Samples used in this study (as well as additional samples collected for all three subject groups) have undergone derivatization with pentafluorobenzoyl anhydride prior to gas chromatography/mass spectrometric analysis (Lear *et al.*, 1995). Results from fourteen periodontally healthy and thirteen adult periodontitis subjects have confirmed the findings of this study. Total salivary PAF was significantly increased in adult periodontitis subjects compared to healthy subjects (17.9 ± 3.9 vs. 4.4 ± 0.8 pmole/ml of saliva). 16:0-alkyl-PAF represented $35.1 \pm 2.0\%$ and $31.3 \pm 1.8\%$ of the PAF in the adult periodontitis and healthy subjects, respectively, and was the predominant molecular species found for all subjects.

However, no significant differences in the proportions of any PAF homolog were found between the groups. These data obtained from a more sensitive method of analysis when combined with the findings of this study suggest that periodontal disease is related to the total amount of salivary PAF present, rather than differences in individual PAF molecular species.

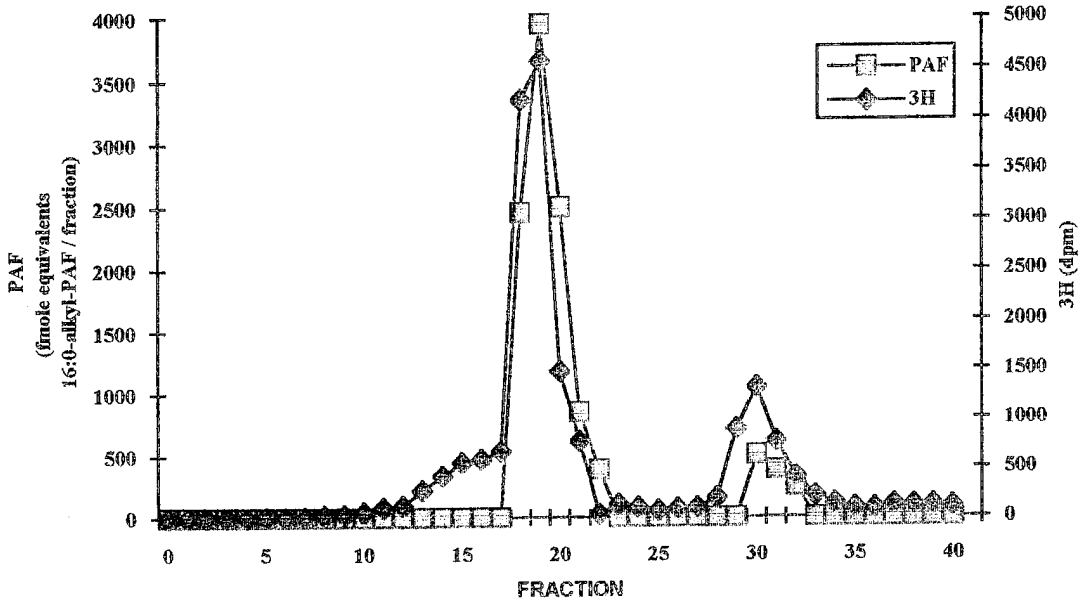
PAF is produced in parallel with other mediators of inflammation such as arachidonic acid, and also has been shown to work in a synergistic manner with prostaglandins and leukotrienes. Prior to this study, it was hypothesized that similar to the specific plaque hypothesis, which states that a single pathogenic species causes periodontal disease, that a particular molecular species of PAF may play a role in the pathogenesis of periodontal disease. Clinically, this would mean that detection of this particular molecular species of PAF might be used to assess disease activity, with end points of treatment based upon elimination of this species. However based on the results of this study and others, salivary PAF molecular species are analogous to the nonspecific plaque hypothesis, in that periodontal disease is due to an increase in all molecular species.

In conclusion, this study has demonstrated that individual molecular species of salivary PAF correlate with clinical parameters of periodontal disease. In addition, this study in conjunction with the study of Lear suggests that periodontal disease is related to the total amount of salivary PAF present, rather than differences in individual PAF molecular species.

VI. APPENDICES

Appendix A. Representative RP-HPLC Fractionation of Salivary PAF From a Normal Subject (3690). After RP-HPLC, an aliquot of each RP-HPLC fraction was utilized to determine PAF activity in rabbit platelet and human PMN bioassay (in fmole equivalents of 16:0-alkyl-PAF) as well as ^{3}H recovery (in dpm). As determined in platelet bioassay (upper panel), a peak of PAF activity was present at fractions 19, and 30; these HPLC regions corresponded to the elution position of 16:0-alkyl-PAF, and 18:0-alkyl-PAF. The shoulder region of the main peak of PAF activity at fraction 21 corresponded to the elution position of 18:1-alkyl-PAF. As determined in PMN bioassay (lower panel), peaks of PAF activity were present in fractions 14, 19 and 30. The shoulder region of the main peak of PAF activity at fraction 22 corresponded to the elution position of 18:1-alkyl-PAF.

PLATELET BIOASSAY



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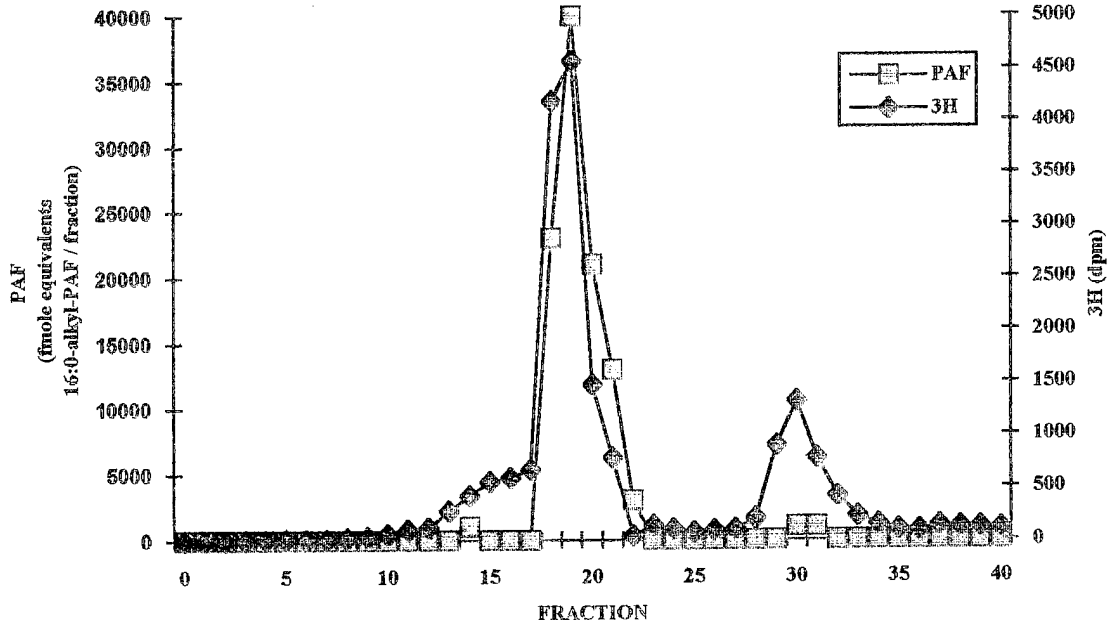
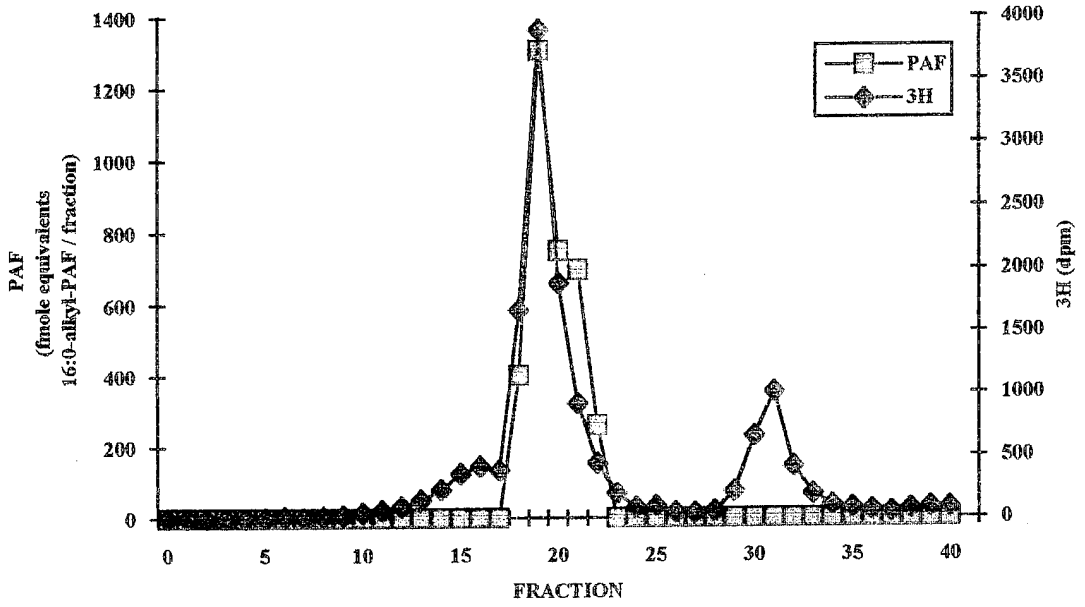


Appendix B. Representative RP-HPLC Fractionation of Salivary PAF From a Normal Subject (3691). After RP-HPLC, an aliquot of each RP-HPLC fraction was utilized to determine PAF activity in rabbit platelet and human PMN bioassay (in fmole equivalents of 16:0-alkyl-PAF) as well as ^{3}H recovery (in dpm). As determined in platelet bioassay (upper panel), a peak of PAF activity was present at fraction 19; this HPLC region corresponded to the elution position of 16:0-alkyl-PAF. The shoulder region of the main peak of PAF activity at fraction 20 corresponded to the elution position of 18:1-alkyl-PAF. As determined in PMN bioassay (lower panel), multiple peaks of PAF activity were present.

PLATELET BIOASSAY



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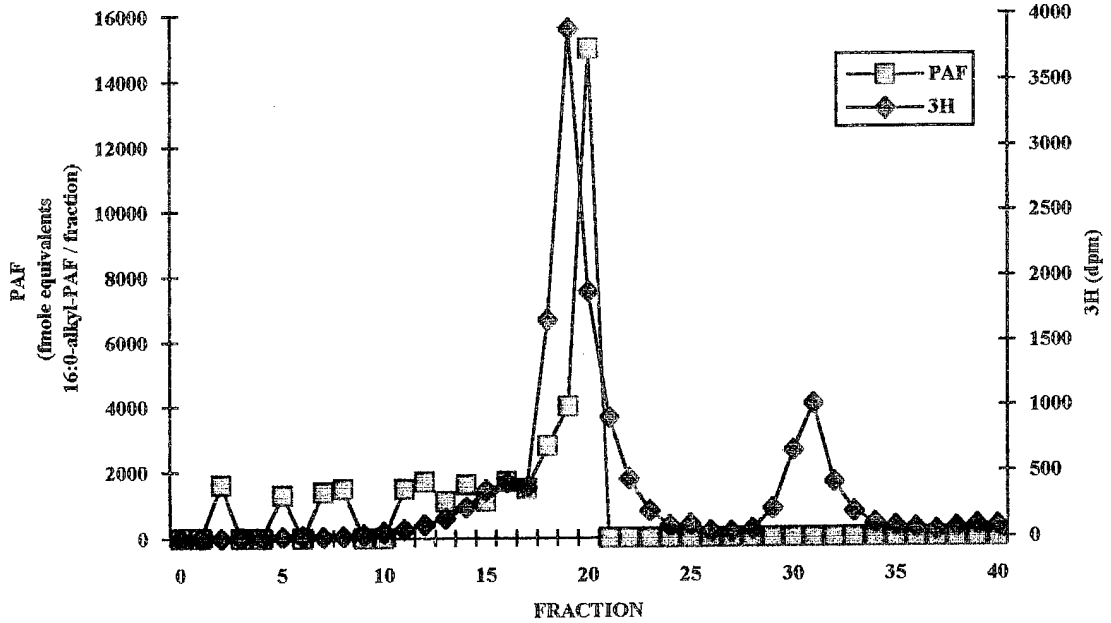
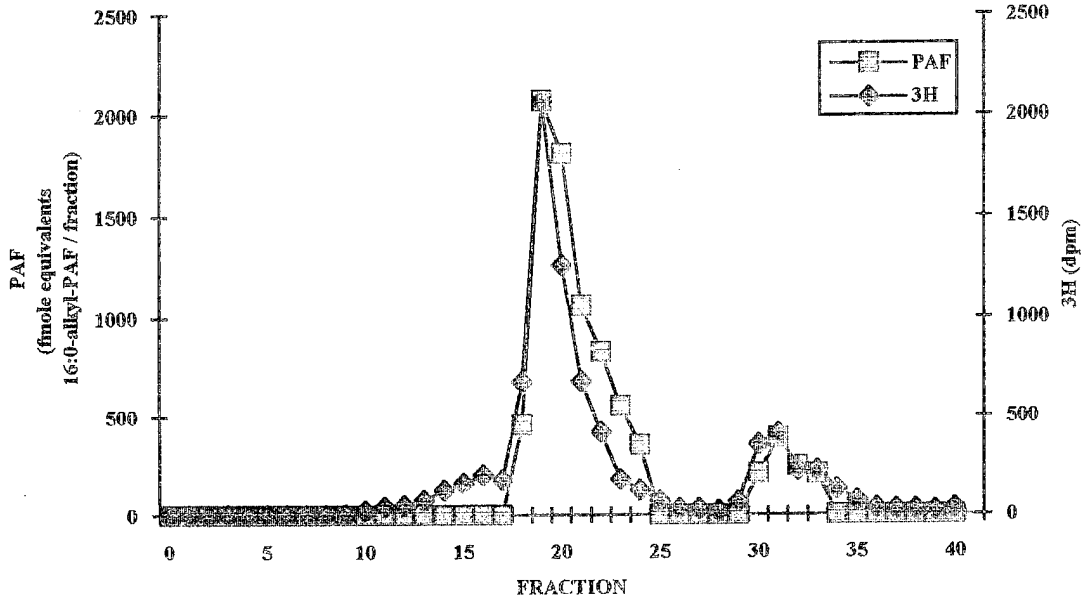


Appendix C. Representative RP-HPLC Fractionation of Salivary PAF From a Normal Subject (3695). After RP-HPLC, an aliquot of each RP-HPLC fraction was utilized to determine PAF activity in rabbit platelet and human PMN bioassay (in fmole equivalents of 16:0-alkyl-PAF) as well as ^{3}H recovery (in dpm). As determined in platelet bioassay (upper panel), a peak of PAF activity was present at fractions 19, and 31; these HPLC regions corresponded to the elution position of 16:0-alkyl-PAF, and 18:0-alkyl-PAF. The shoulder region of the main peak of PAF activity at fraction 20 corresponded to the elution position of 18:1-alkyl-PAF. As determined in PMN bioassay (lower panel), peaks of PAF activity were present in fractions 16, and 19.

PLATELET BIOASSAY



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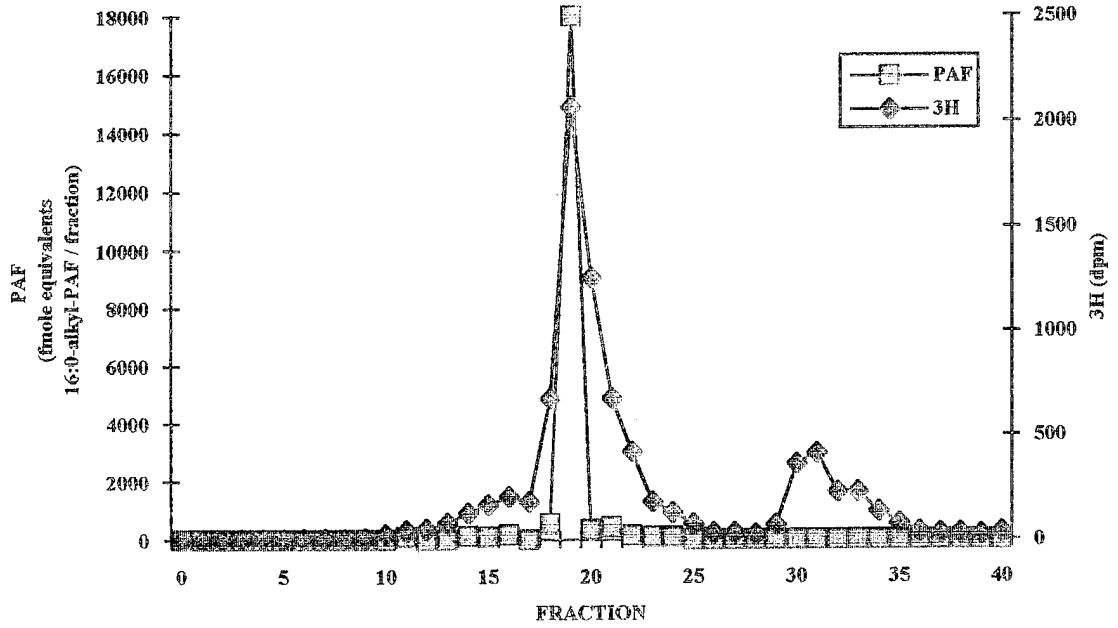
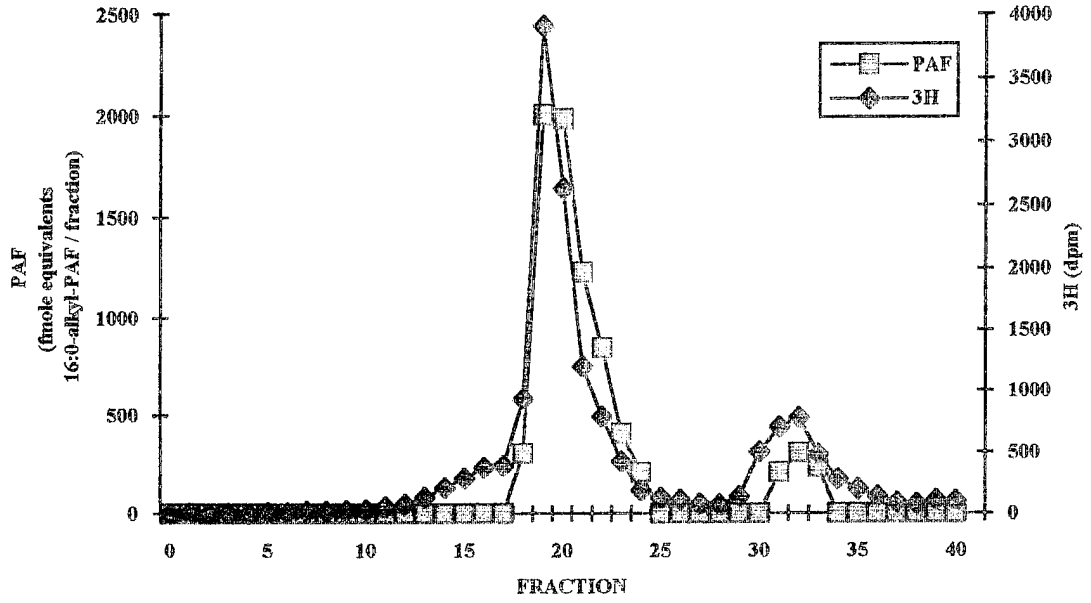


Appendix D. Representative RP-HPLC Fractionation of Salivary PAF From a Normal Subject (3696). After RP-HPLC, an aliquot of each RP-HPLC fraction was utilized to determine PAF activity in rabbit platelet and human PMN bioassay (in fmole equivalents of 16:0-alkyl-PAF) as well as ^{3}H recovery (in dpm). As determined in platelet bioassay (upper panel), a peak of PAF activity was present at fractions 19, and 32; these HPLC regions corresponded to the elution position of 16:0-alkyl-PAF, and 18:0-alkyl-PAF. The shoulder region of the main peak of PAF activity at fraction 21 corresponded to the elution position of 18:1-alkyl-PAF. As determined in PMN bioassay (lower panel), peaks of PAF activity were present in fractions 5, 10, and 19. The shoulder region of the main peak of PAF activity at fraction 22 corresponded to the elution position of 18:1-alkyl-PAF.

PLATELET BIOASSAY



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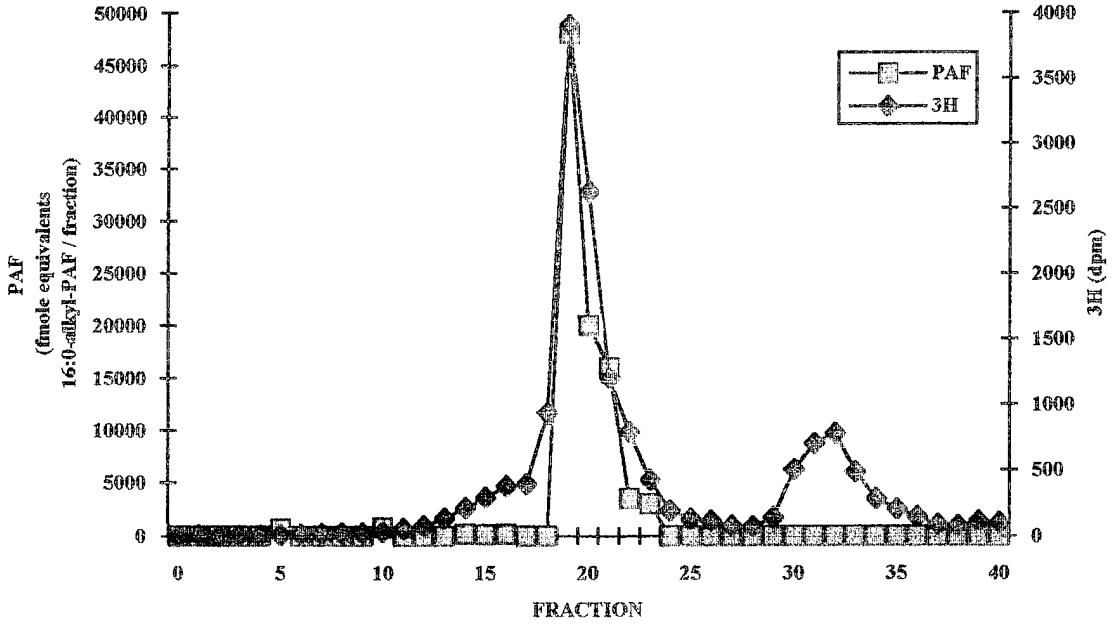
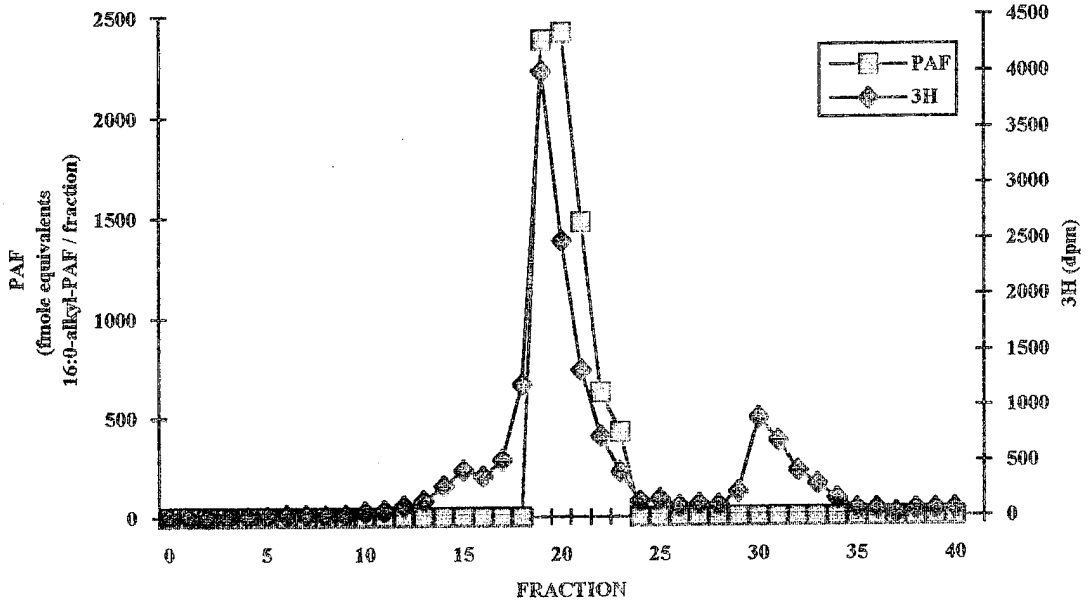


Appendix E. Representative RP-HPLC Fractionation of Salivary PAF From a Normal Subject (3704). After RP-HPLC, an aliquot of each RP-HPLC fraction was utilized to determine PAF activity in rabbit platelet and human PMN bioassay (in fmole equivalents of 16:0-alkyl-PAF) as well as ^{3}H recovery (in dpm). As determined in platelet bioassay (upper panel), a peak of PAF activity was present at fraction 20; this HPLC region corresponded to the elution position of 16:0-alkyl-PAF. The shoulder region of the main peak of PAF activity at fraction 22 corresponded to the elution position of 18:1-alkyl-PAF. As determined in PMN bioassay (lower panel), a peak of PAF activity is present in fraction 19. The shoulder region of the main peak of PAF activity at fraction 21 corresponded to the elution position of 18:1-alkyl-PAF.

PLATELET BIOASSAY



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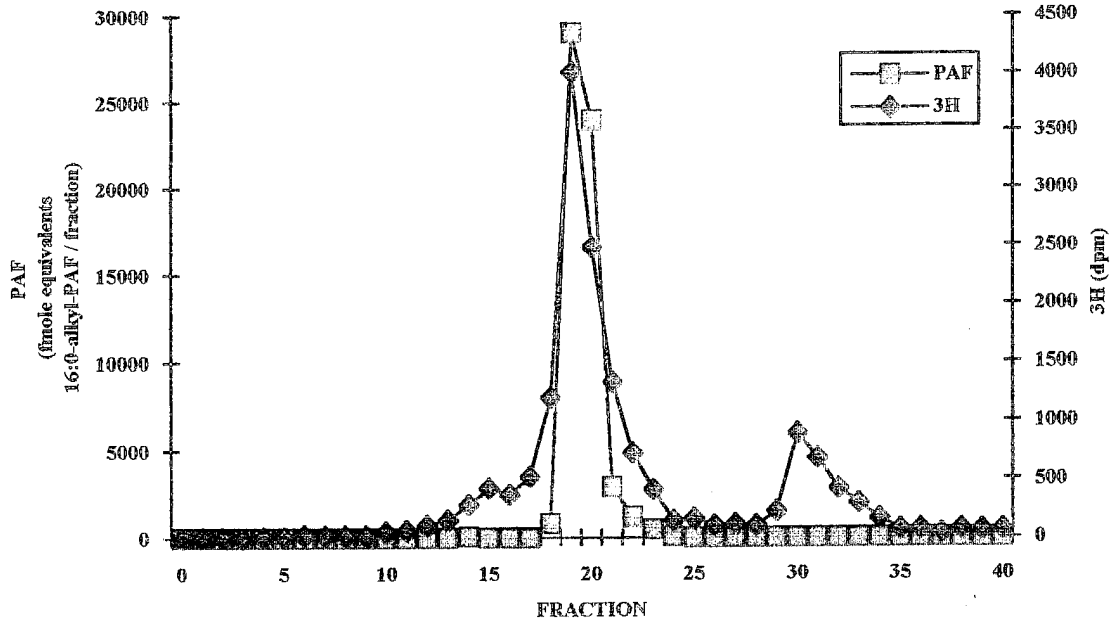
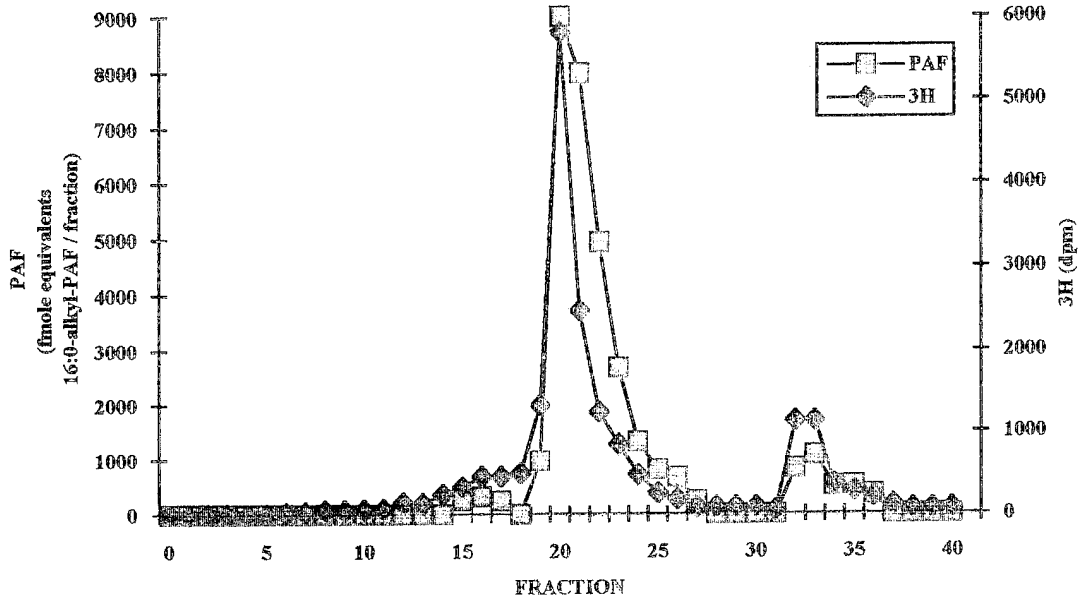


Appendix F. Representative RP-HPLC Fractionation of Salivary PAF From a Normal Subject (3705). After RP-HPLC, an aliquot of each RP-HPLC fraction was utilized to determine PAF activity in rabbit platelet and human PMN bioassay (in fmole equivalents of 16:0-alkyl-PAF) as well as ^{3}H recovery (in dpm). As determined in platelet bioassay (upper panel), a peak of PAF activity was present at fractions 16, 20, and 33; these HPLC regions corresponded to the elution position of 15:0-alkyl-PAF, 16:0-alkyl-PAF, and 18:0-alkyl-PAF. The shoulder region of the main peak of PAF activity at fraction 22 corresponded to the elution position of 18:1-alkyl-PAF. As determined in PMN bioassay (lower panel), peaks of PAF activity were present in fractions 16, and 20. The shoulder region of the main peak of PAF activity at fraction 22 corresponded to the elution position of 18:1-alkyl-PAF.

PLATELET BIOASSAY



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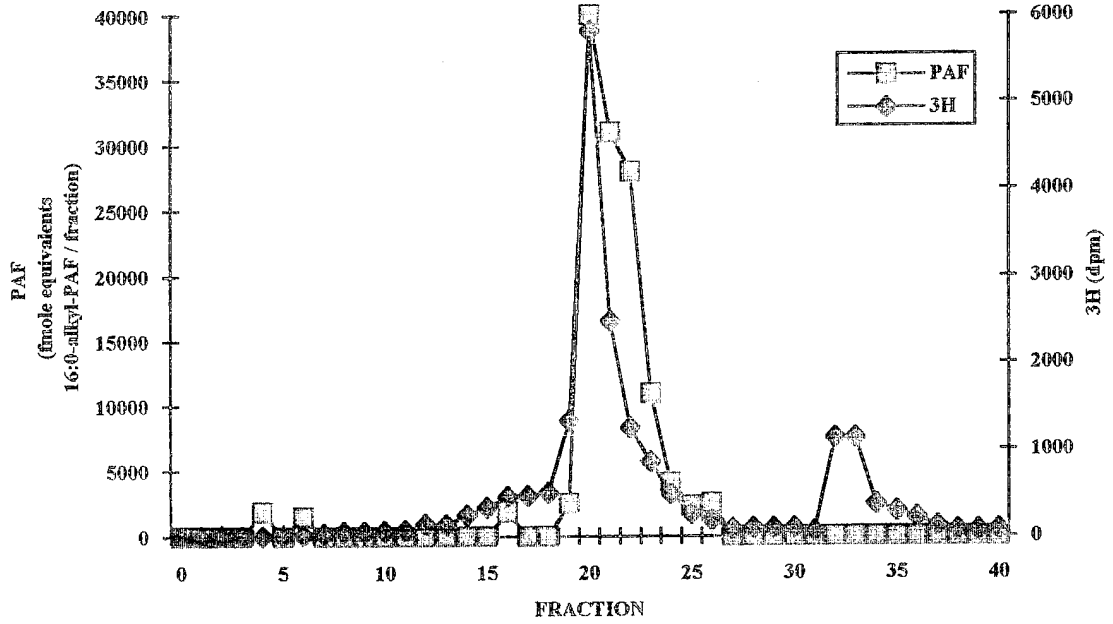
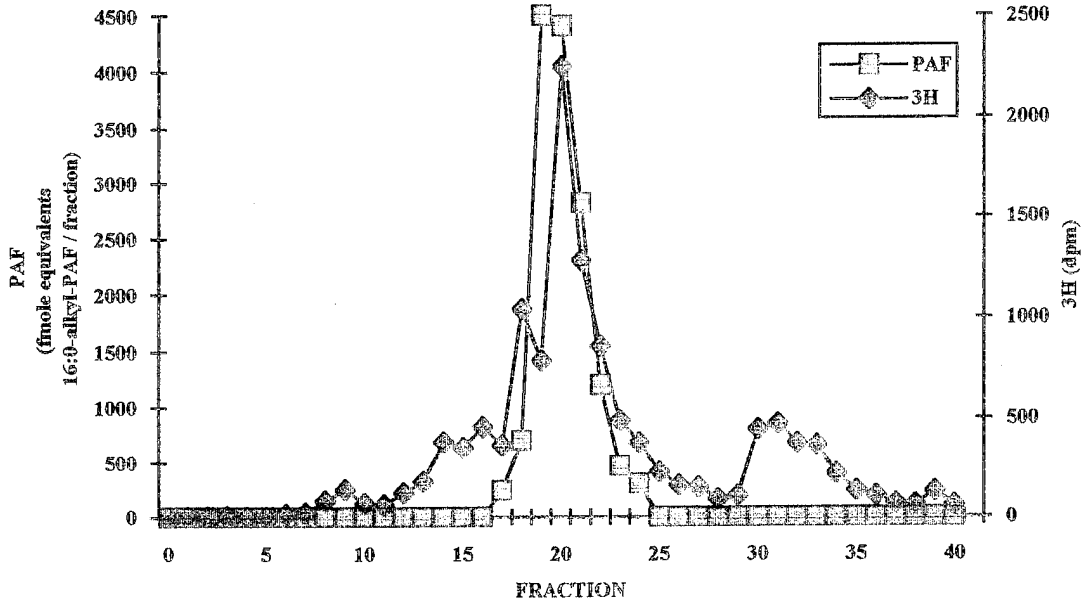


Appendix G. Representative RP-HPLC Fractionation of Salivary PAF From a Normal Subject (3708). After RP-HPLC, an aliquot of each RP-HPLC fraction was utilized to determine PAF activity in rabbit platelet and human PMN bioassay (in fmole equivalents of 16:0-alkyl-PAF) as well as ^{3}H recovery (in dpm). As determined in platelet bioassay (upper panel), a peak of PAF activity was present at fraction 19; this HPLC region corresponded to the elution position of 16:0-alkyl-PAF. The shoulder region of the main peak of PAF activity at fraction 22 corresponded to the elution position of 18:1-alkyl-PAF. As determined in PMN bioassay (lower panel), a peak of PAF activity was present in fraction 19. The shoulder region of the main peak of PAF activity at fraction 22 corresponded to the elution position of 18:1-alkyl-PAF.

PLATELET BIOASSAY



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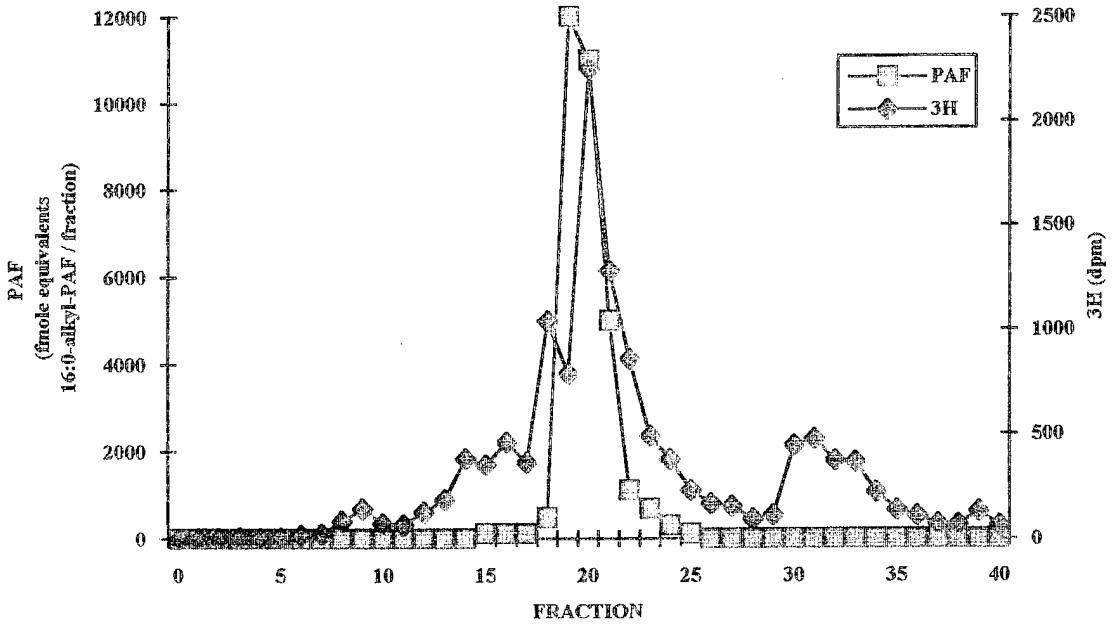
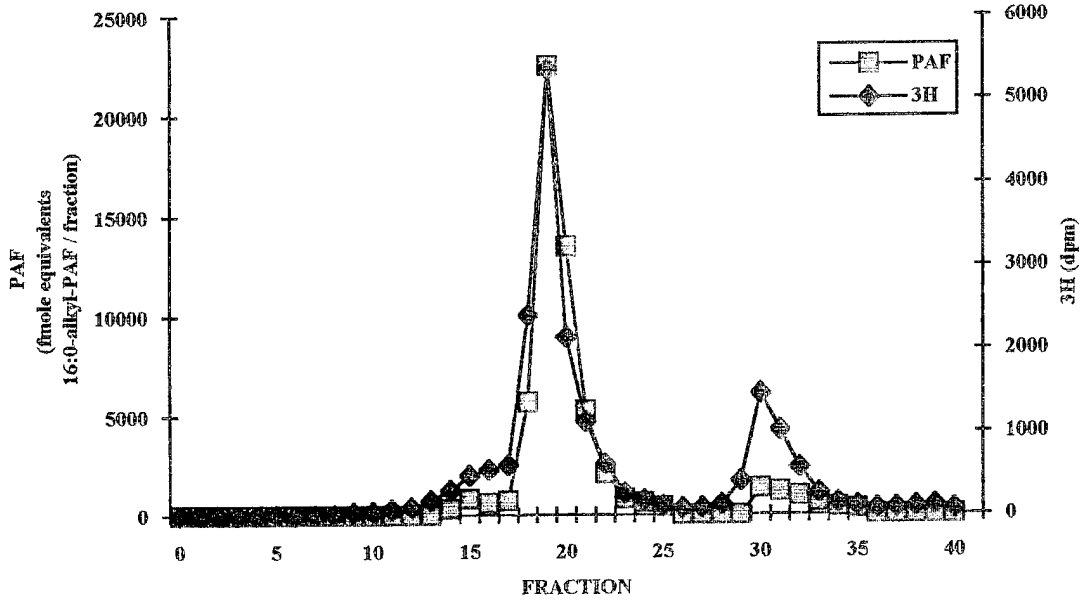


Appendix H. Representative RP-HPLC Fractionation of Salivary PAF From an AP Subject (3689). After RP-HPLC, an aliquot of each RP-HPLC fraction was utilized to determine PAF activity in rabbit platelet and human PMN bioassay (in fmole equivalents of 16:0-alkyl-PAF) as well as ^{3}H recovery (in dpm). As determined in platelet bioassay (upper panel), a peak of PAF activity was present at fractions 15, 19, and 30; these HPLC regions corresponded to the elution position of 15:0-alkyl-PAF, 16:0-alkyl-PAF, and 18:0-alkyl-PAF. The shoulder region of the main peak of PAF activity at fraction 21 corresponded to the elution position of 18:1-alkyl-PAF. As determined in PMN bioassay (lower panel), peaks of PAF activity were present in fractions 15, 19 and 31. The shoulder region of the main peak of PAF activity at fraction 21 corresponded to the elution position of 18:1-alkyl-PAF.

PLATELET BIOASSAY



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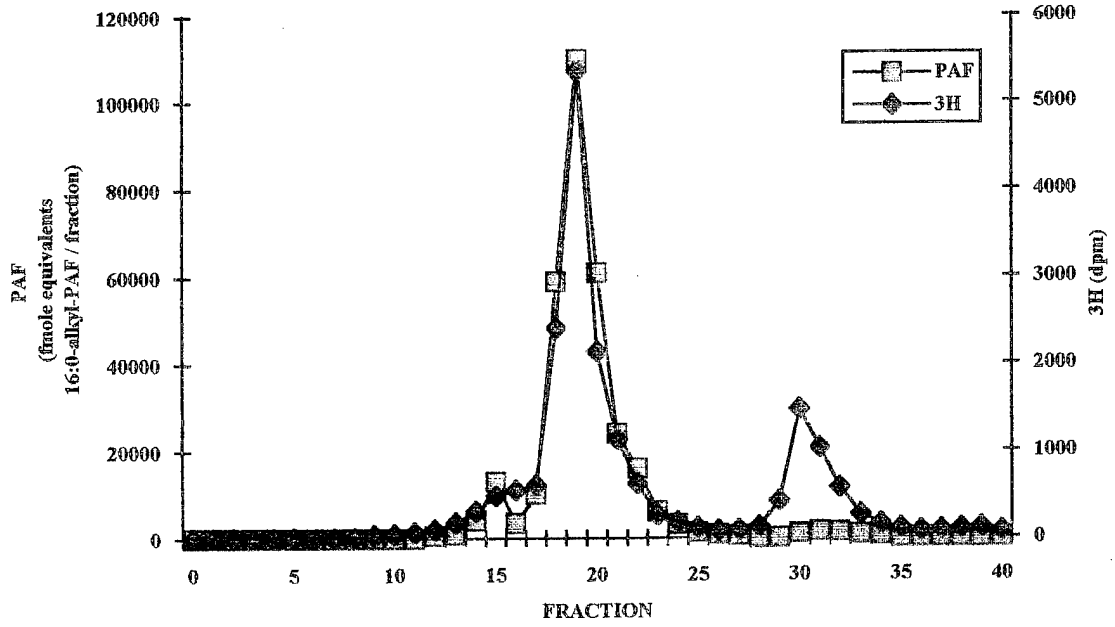
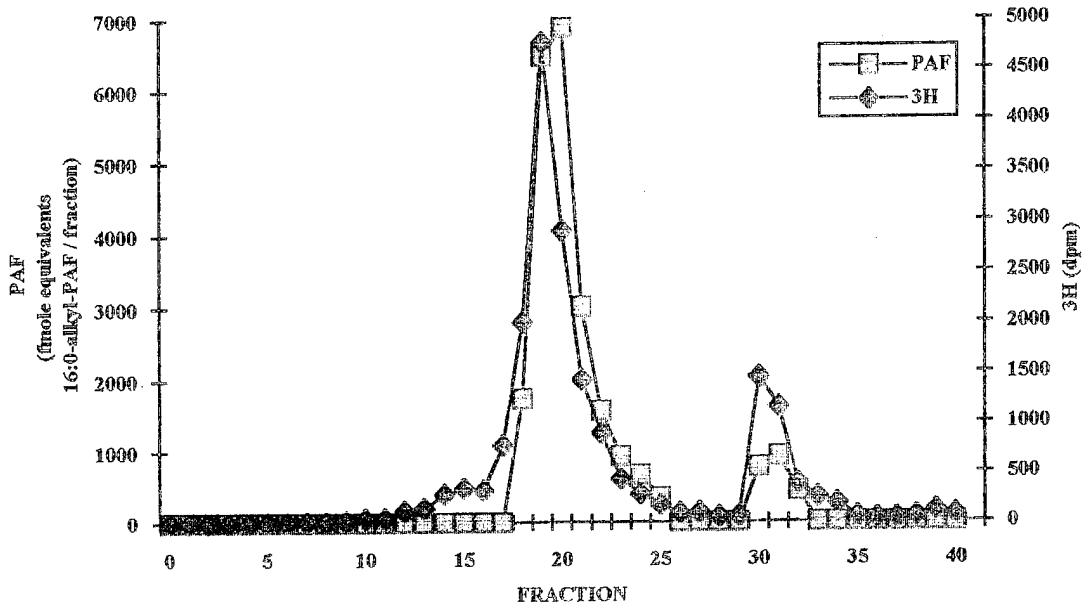


Appendix I. Representative RP-HPLC Fractionation of Salivary PAF From an AP Subject (3706). After RP-HPLC, an aliquot of each RP-HPLC fraction was utilized to determine PAF activity in rabbit platelet and human PMN bioassay (in fmole equivalents of 16:0-alkyl-PAF) as well as ^{3}H recovery (in dpm). As determined in platelet bioassay (upper panel), a peak of PAF activity was present at fractions 20 and 31; these HPLC regions corresponded to the elution position of 16:0-alkyl-PAF and 18:0-alkyl-PAF. The shoulder region of the main peak of PAF activity at fraction 22 corresponded to the elution position of 18:1-alkyl-PAF. As determined in PMN bioassay (lower panel), peaks of PAF activity were present in fractions 15, 19 and 31. The shoulder region of the main peak of PAF activity at fraction 21 corresponded to the elution position of 18:1-alkyl-PAF.

PLATELET BIOASSAY



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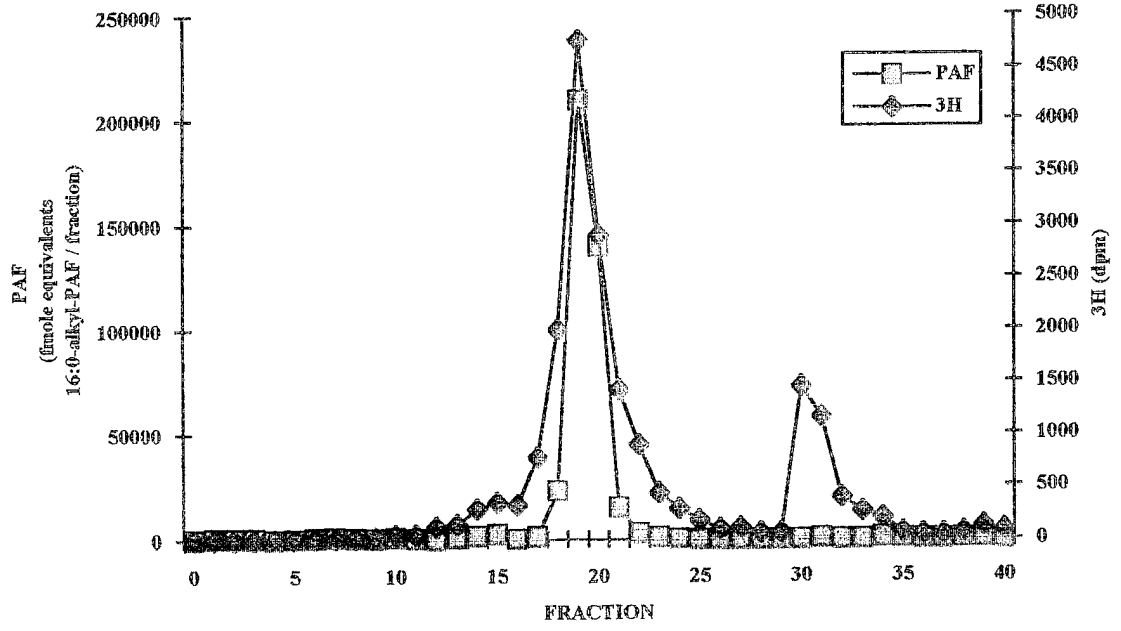
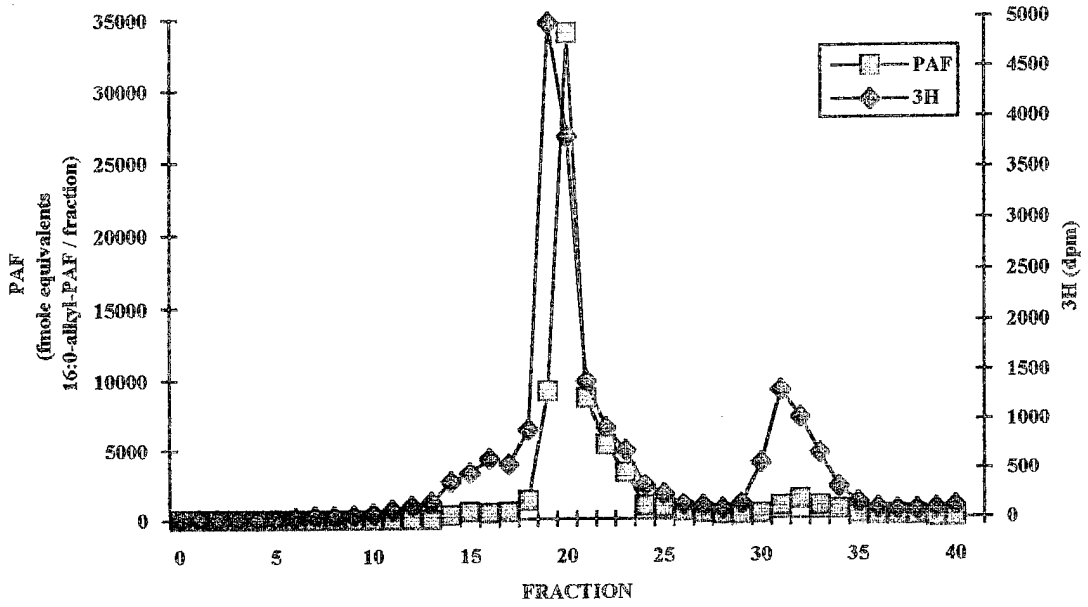


Appendix J. Representative RP-HPLC Fractionation of Salivary PAF From an AP Subject (3718). After RP-HPLC, an aliquot of each RP-HPLC fraction was utilized to determine PAF activity in rabbit platelet and human PMN bioassay (in fmole equivalents of 16:0-alkyl-PAF) as well as ^{3}H recovery (in dpm). As determined in platelet bioassay (upper panel), a peak of PAF activity was present at fractions 15, 20, and 32; these HPLC regions corresponded to the elution position of 15:0-alkyl-PAF, 16:0-alkyl-PAF, and 18:0-alkyl-PAF. The shoulder region of the main peak of PAF activity at fraction 21 corresponded to the elution position of 18:1-alkyl-PAF. As determined in PMN bioassay (lower panel), peaks of PAF activity were present in fractions 15, 19 and 33. The shoulder region of the main peak of PAF activity at fraction 21 corresponded to the elution position of 18:1-alkyl-PAF.

PLATELET BIOASSAY



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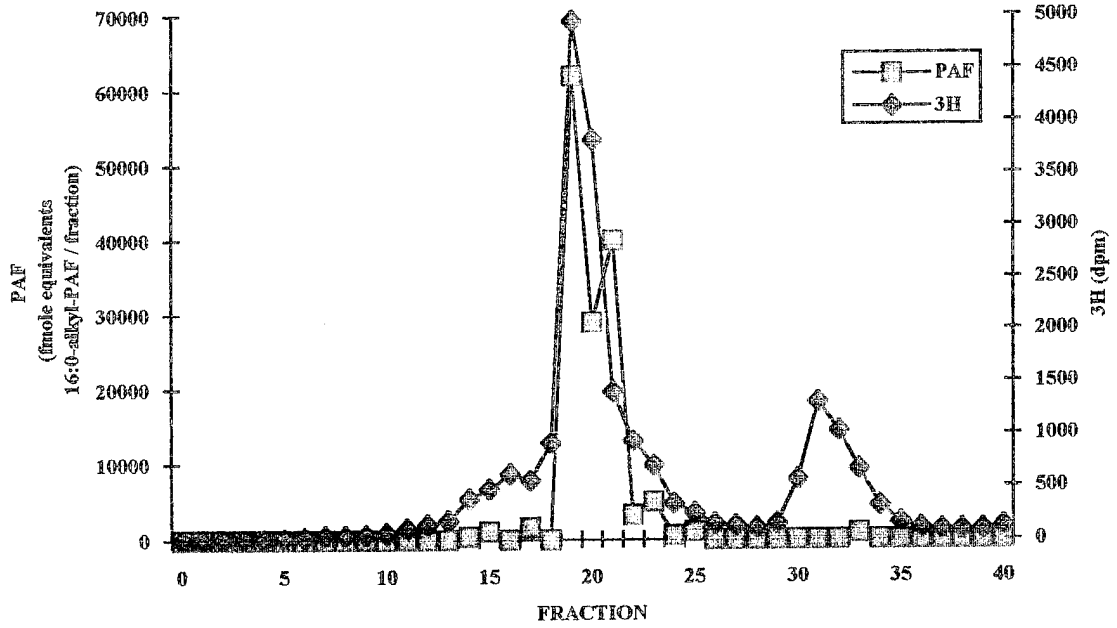
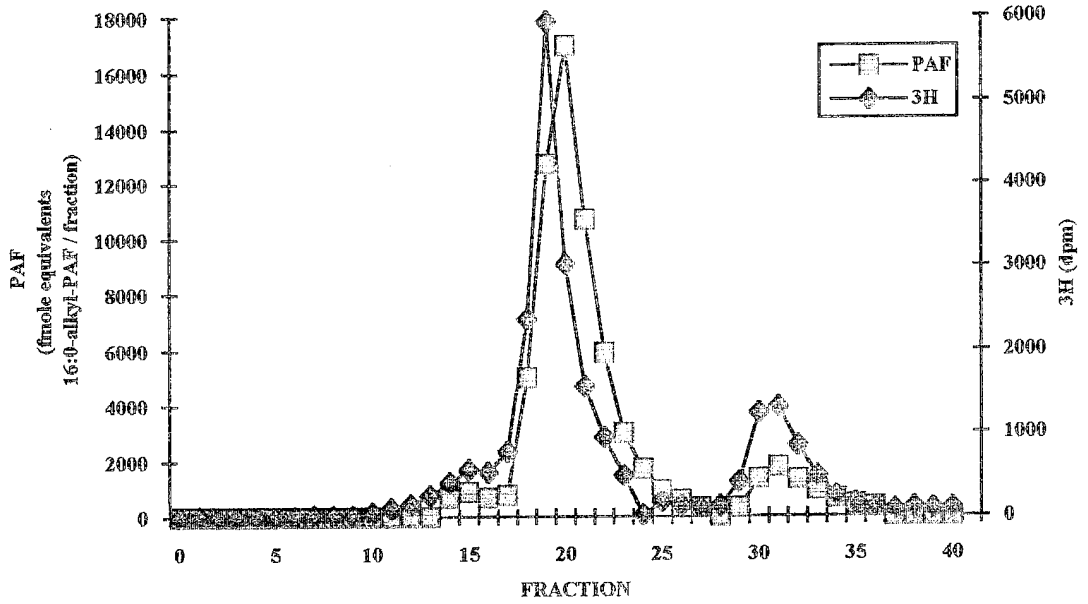


Appendix K. Representative RP-HPLC Fractionation of Salivary PAF From an AP Subject (3719). After RP-HPLC, an aliquot of each RP-HPLC fraction was utilized to determine PAF activity in rabbit platelet and human PMN bioassay (in fmole equivalents of 16:0-alkyl-PAF) as well as ^{3}H recovery (in dpm). As determined in platelet bioassay (upper panel), a peak of PAF activity was present at fractions 15, 20, and 31; these HPLC regions corresponded to the elution position of 15:0-alkyl-PAF, 16:0-alkyl-PAF, and 18:0-alkyl-PAF. The shoulder region of the main peak of PAF activity at fraction 22 corresponded to the elution position of 18:1-alkyl-PAF. As determined in PMN bioassay (lower panel), peaks of PAF activity were present in fractions 15, 19 and 31. The shoulder region of the main peak of PAF activity at fraction 21 and possibly the peak at fraction 24 corresponded to the elution position of 18:1-alkyl-PAF.

PLATELET BIOASSAY



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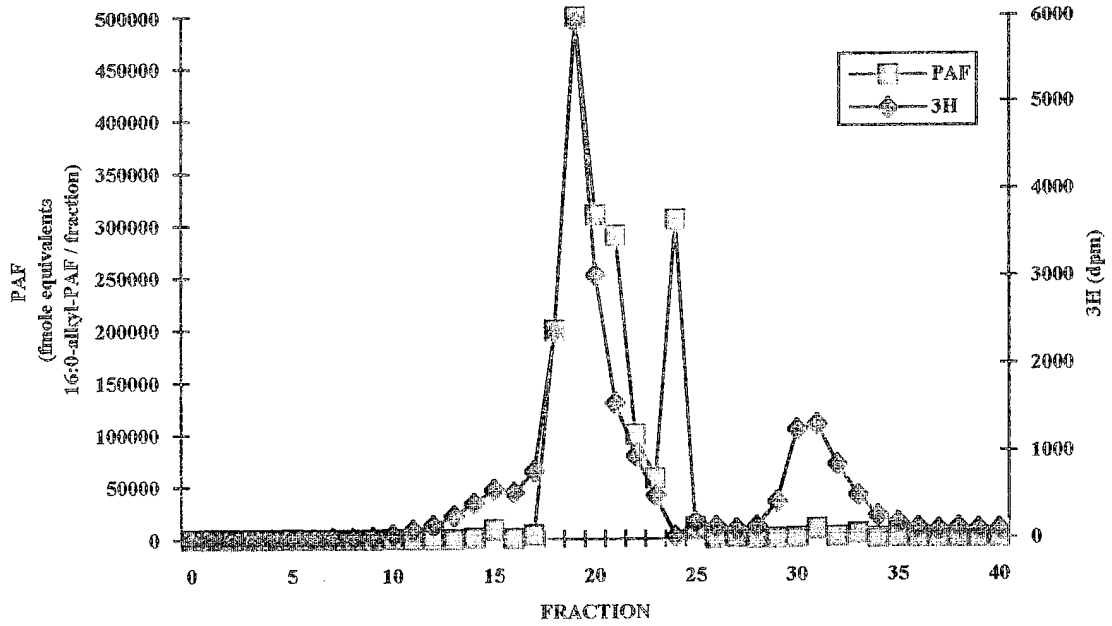
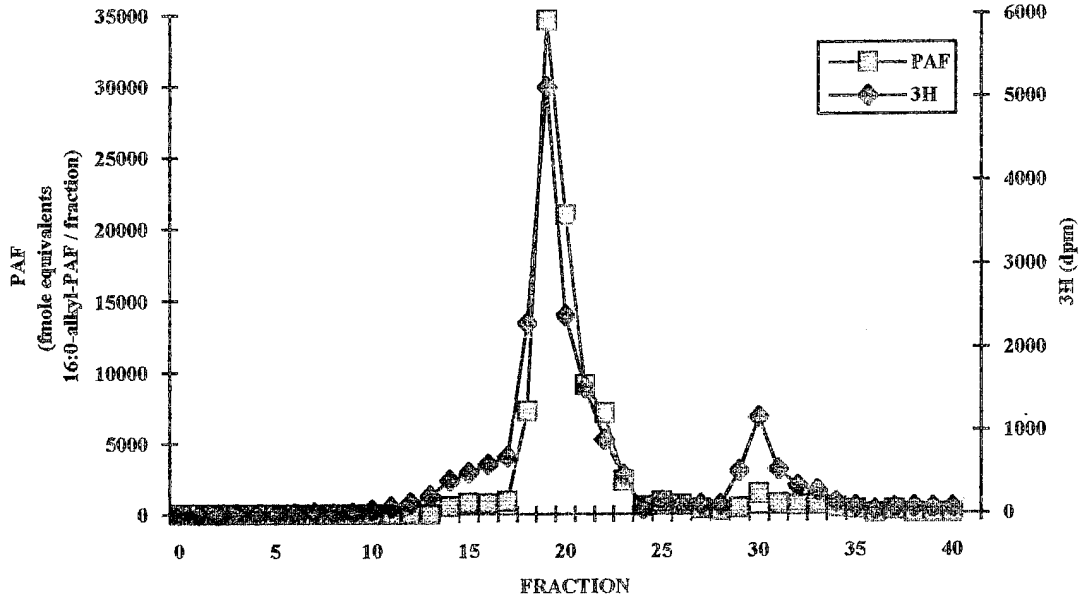


Appendix L. Representative RP-HPLC Fractionation of Salivary PAF From an AP Subject (3720). After RP-HPLC, an aliquot of each RP-HPLC fraction was utilized to determine PAF activity in rabbit platelet and human PMN bioassay (in fmole equivalents of 16:0-alkyl-PAF) as well as ^{3}H recovery (in dpm). As determined in platelet bioassay (upper panel), a peak of PAF activity was present at fractions 15, 19, and 30; these HPLC regions corresponded to the elution position of 15:0-alkyl-PAF, 16:0-alkyl-PAF, and 18:0-alkyl-PAF. The shoulder region of the main peak of PAF activity at fraction 21 corresponded to the elution position of 18:1-alkyl-PAF. As determined in PMN bioassay (lower panel), peaks of PAF activity were present in fractions 15, 19 and 31. The additional peak of PAF activity at fraction 21 corresponded to the elution position of 18:1-alkyl-PAF. Multiple additional peaks were present.

PLATELET BIOASSAY



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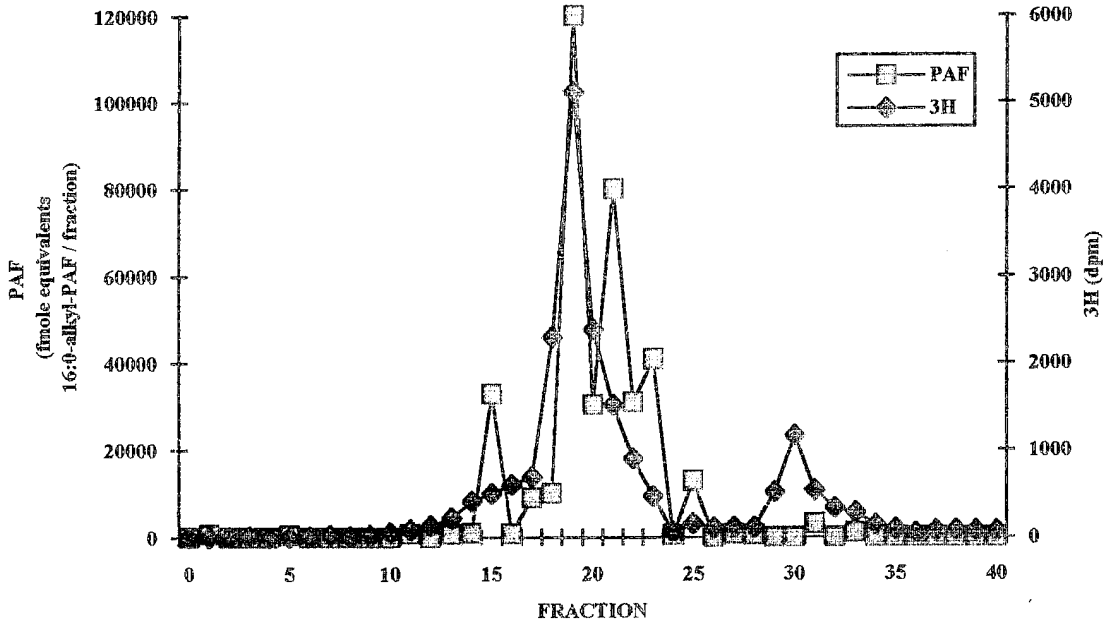
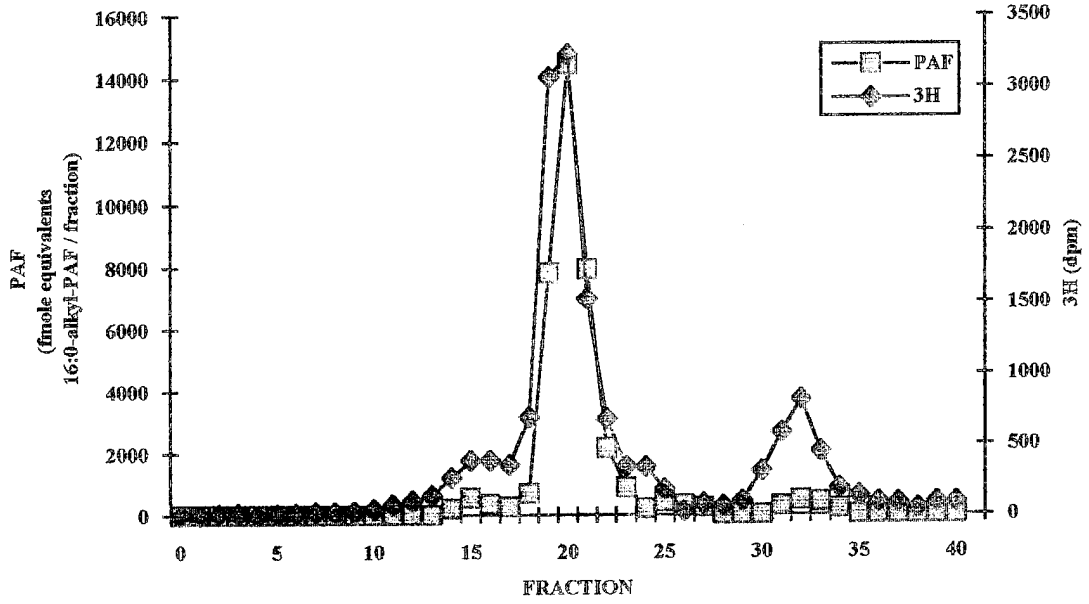


Appendix M. Representative RP-HPLC Fractionation of Salivary PAF From an AP Subject (3735). After RP-HPLC, an aliquot of each RP-HPLC fraction was utilized to determine PAF activity in rabbit platelet and human PMN bioassay (in fmole equivalents of 16:0-alkyl-PAF) as well as ^{3}H recovery (in dpm). As determined in platelet bioassay (upper panel), a peak of PAF activity was present at fractions 15, 20, and 32. These HPLC regions corresponded to the elution position of 15:0-alkyl-PAF, 16:0-alkyl-PAF, and 18:0-alkyl-PAF. The shoulder region of the main peak of PAF activity at fraction 22 corresponded to the elution position of 18:1-alkyl-PAF. As determined in PMN bioassay (lower panel), peaks of PAF activity were present in fractions 15 and 19. The shoulder region of the main peak of PAF activity and possibly the additional peak at fraction 21corresponded to the elution position of 18:1-alkyl-PAF.

PLATELET BIOASSAY



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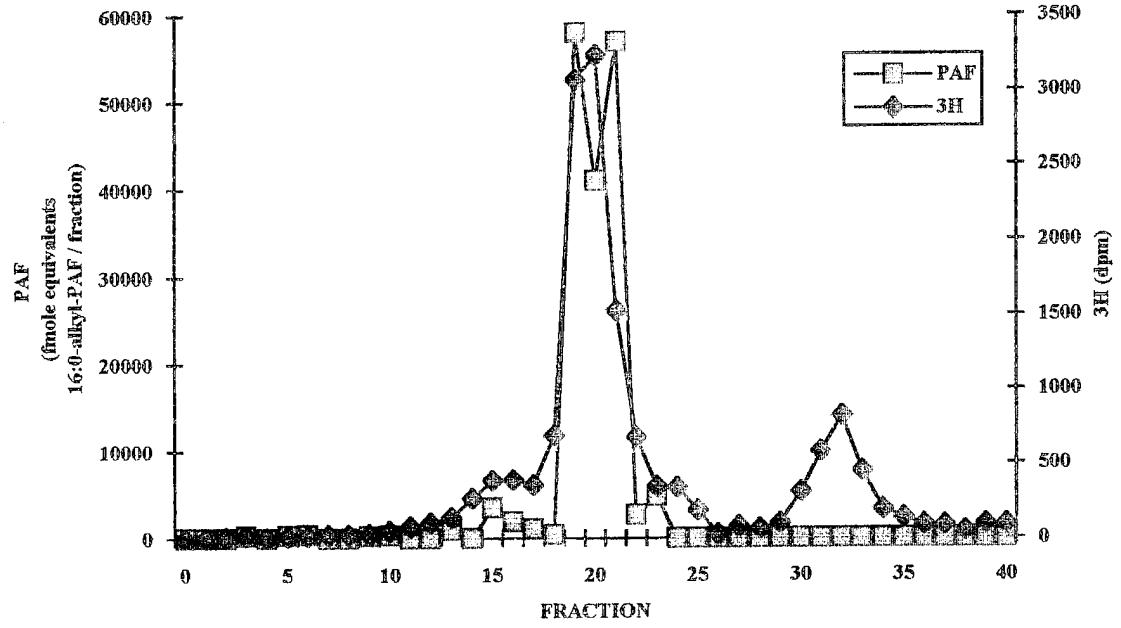
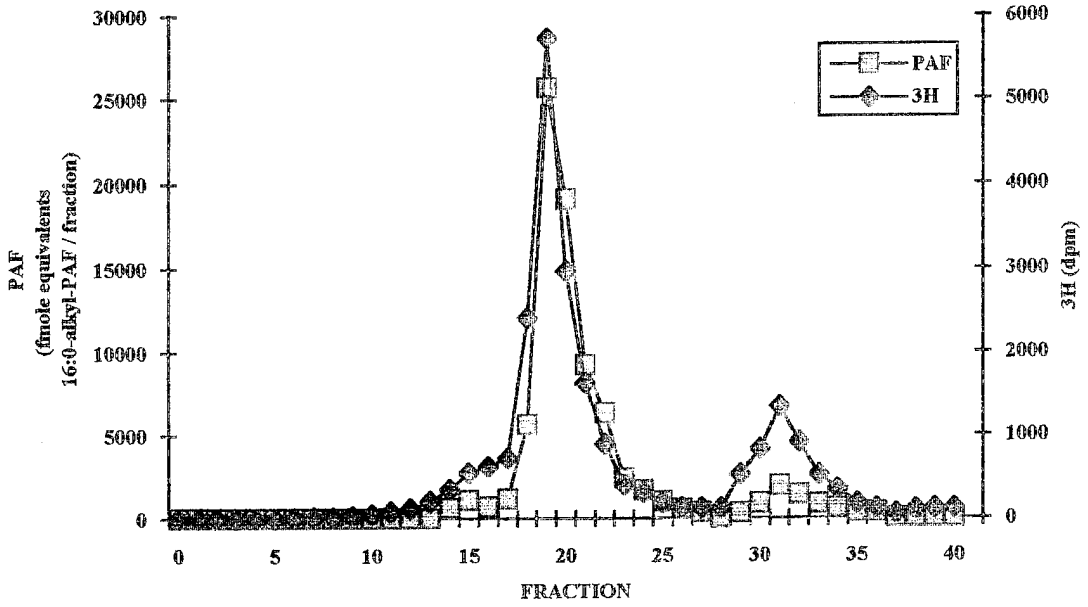


Appendix N. Representative RP-HPLC Fractionation of Salivary PAF From a RPP Subject (3742). After RP-HPLC, an aliquot of each RP-HPLC fraction was utilized to determine PAF activity in rabbit platelet and human PMN bioassay (in fmole equivalents of 16:0-alkyl-PAF) as well as ^{3}H recovery (in dpm). As determined in platelet bioassay (upper panel), a peak of PAF activity was present at fractions 15, 19, and 31; these HPLC regions corresponded to the elution position of 15:0-alkyl-PAF, 16:0-alkyl-PAF, and 18:0-alkyl-PAF. The shoulder region of the main peak of PAF activity at fraction 21 corresponded to the elution position of 18:1-alkyl-PAF. As determined in PMN bioassay (lower panel), peaks of PAF activity were present in fractions 15, 19 and 31. The shoulder region of the main peak of PAF activity at fraction 21 corresponded to the elution position of 18:1-alkyl-PAF.

PLATELET BIOASSAY



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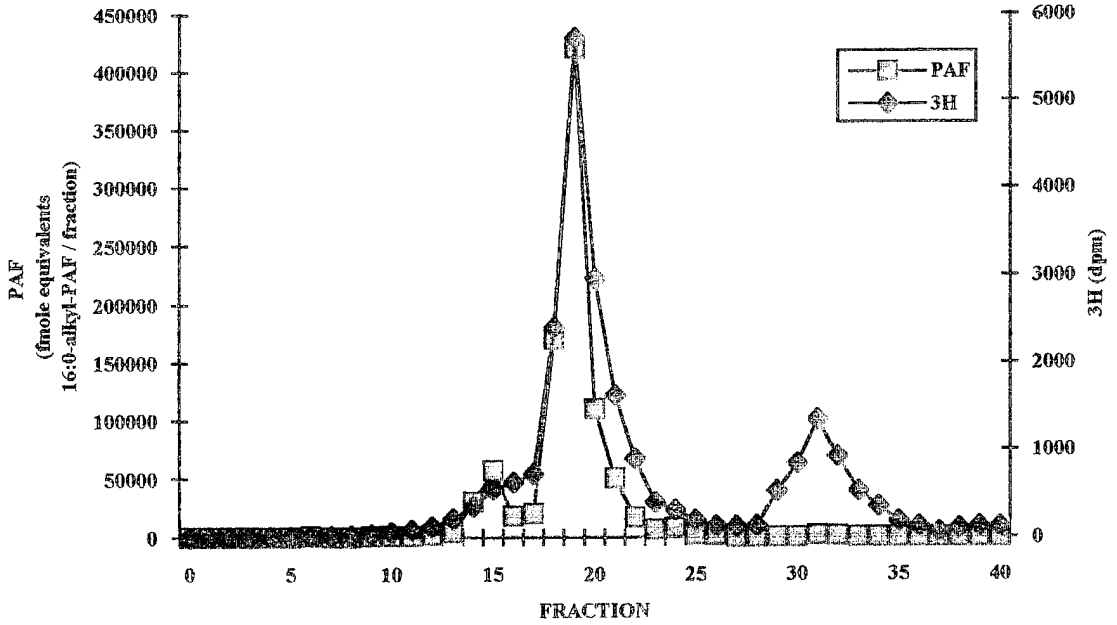
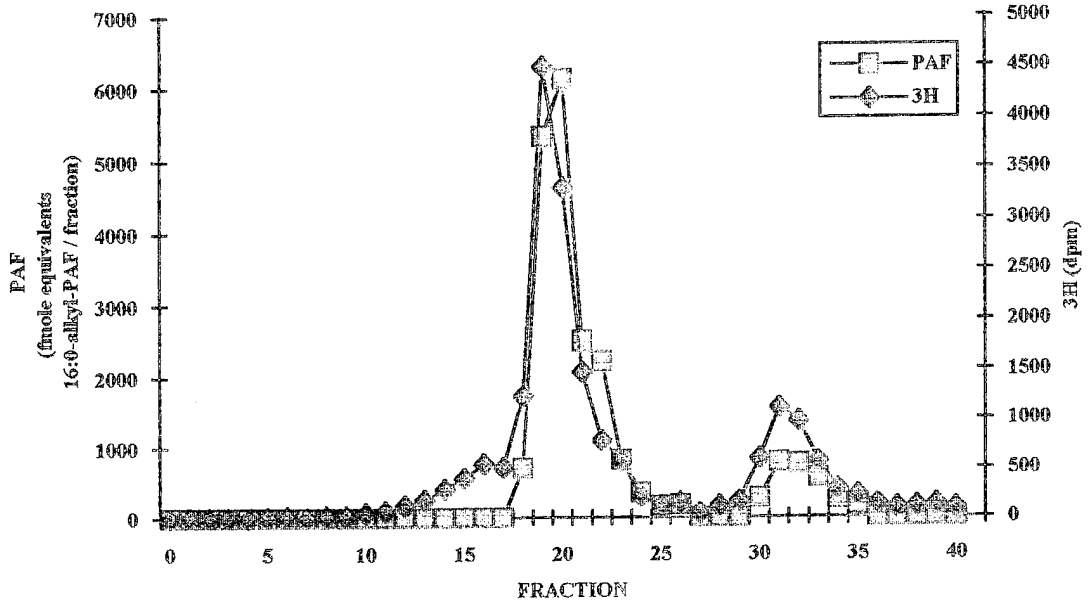


Appendix O. Representative RP-HPLC Fractionation of Salivary PAF From an RPP Subject (3745). After RP-HPLC, an aliquot of each RP-HPLC fraction was utilized to determine PAF activity in rabbit platelet and human PMN bioassay (in fmole equivalents of 16:0-alkyl-PAF) as well as ^{3}H recovery (in dpm). As determined in platelet bioassay (upper panel), a peak of PAF activity was present at fractions 20 and 31; these HPLC regions corresponded to the elution position of 16:0-alkyl-PAF, and 18:0-alkyl-PAF. The shoulder region of the main peak of PAF activity at fraction 22 corresponded to the elution position of 18:1-alkyl-PAF. As determined in PMN bioassay (lower panel), a single peak of PAF activity was present in fraction 20.

PLATELET BIOASSAY



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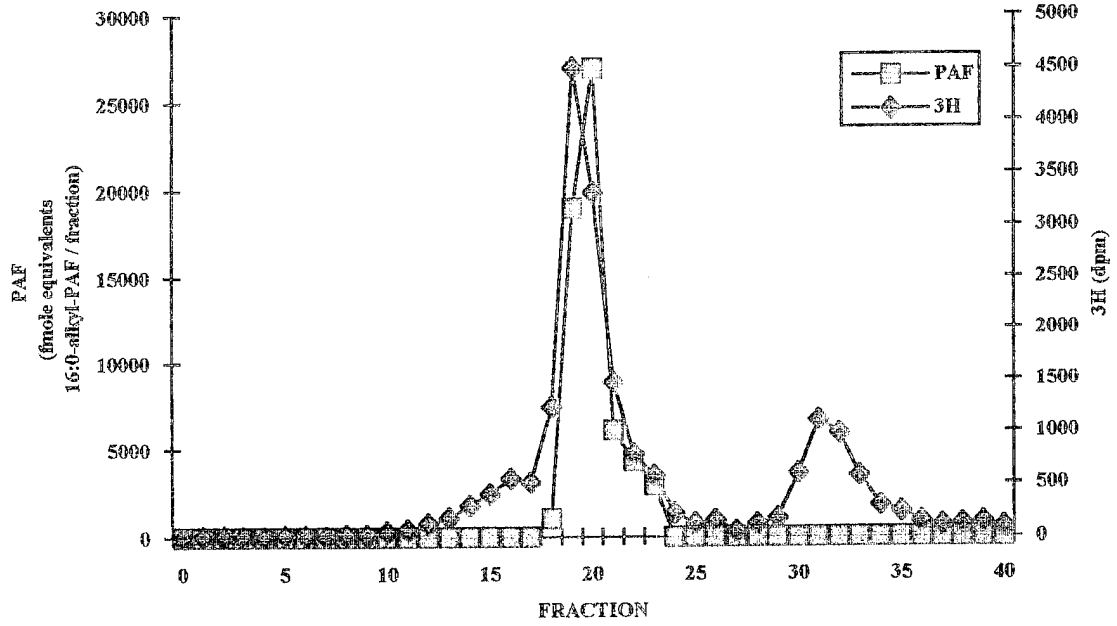
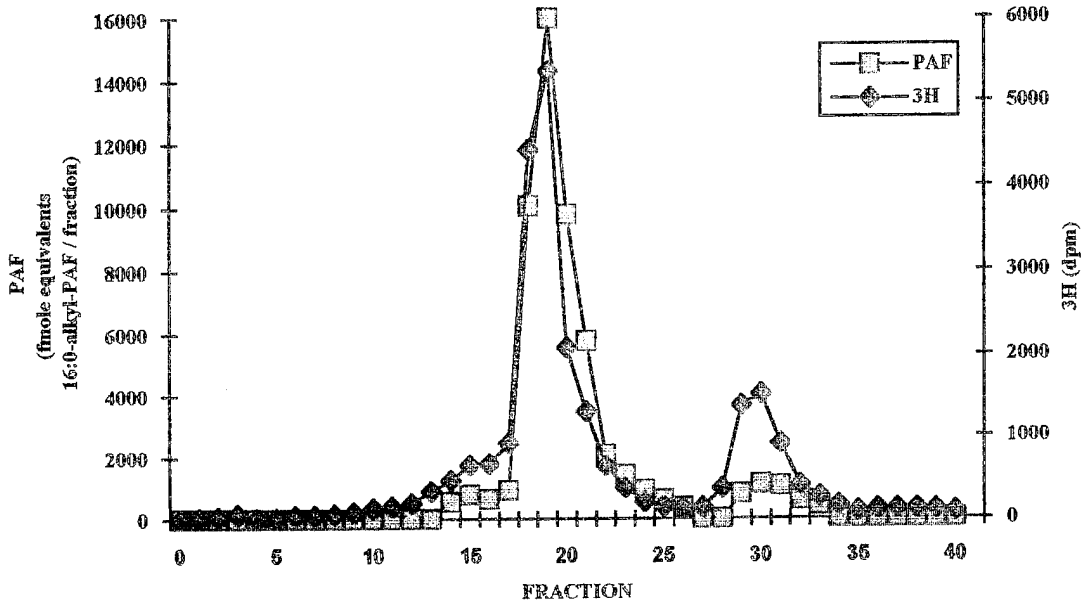


Appendix P. Representative RP-HPLC Fractionation of Salivary PAF From an RPP Subject (3750). After RP-HPLC, an aliquot of each RP-HPLC fraction was utilized to determine PAF activity in rabbit platelet and human PMN bioassay (in fmole equivalents of 16:0-alkyl-PAF) as well as ^{3}H recovery (in dpm). As determined in platelet bioassay (upper panel), a peak of PAF activity was present at fractions 15, 19, and 30; these HPLC regions corresponded to the elution position of 15:0-alkyl-PAF, 16:0-alkyl-PAF, and 18:0-alkyl-PAF. The shoulder region of the main peak of PAF activity at fraction 22 corresponded to the elution position of 18:1-alkyl-PAF. As determined in PMN bioassay (lower panel), peaks of PAF activity were present in fractions 14, 19 and 31. The shoulder region of the main peak of PAF activity at fraction 20 corresponded to the elution position of 18:1-alkyl-PAF.

PLATELET BIOASSAY



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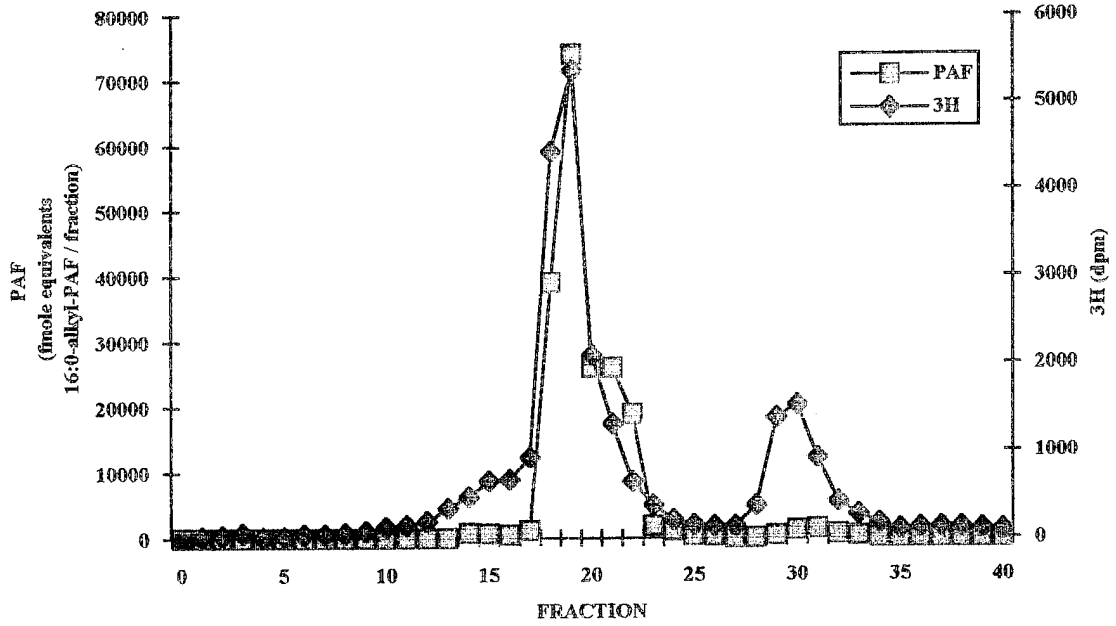

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VITA

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