Analysis of platelet-activating factor by GC–MS after direct derivatization with pentafluorobenzoyl chloride and heptafluorobutyric anhydride

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Abstract Parallel analysis of platelet-activating factor (PAF) using chemical ionization gas chromatography–mass spectrometry after direct derivatization with pentafluorobenzoyl chloride (PFB) and heptafluorobutyric anhydride (HFB) provides a facile and highly sensitive means for detecting and elucidating the structure of the numerous alkyl-chain homologs of this acetylated phospholipid autacoid. In the present study, the PFB derivative was used for initial electron capture negative ion chemical ionization analysis of PAF candidate molecules in human PMN extracts of unknown composition. Subsequent pulsed positive ion/electron capture negative ion chemical ionization evaluation of the HFB derivative furnished a measure of the molecular weight from $[MH]^+$ and yielded the required structural information from characteristic negative ions, in particular $[M-(2HF + \text{ketene})]^-$ and $[M-(HF + \text{acetic acid})]^-$. These procedures easily permitted confirmation of the presence of C16:0-, C17:0-, C18:0-, and C18:1-AGEPC (acetyl glyceryl ether phosphocholine) in extracts of stimulated human PMN and also demonstrated that the C17:0- homolog was comprised of both straight-chain and branch-chain varieties.—**Weintraub, S. T., C. S. Lear, and R. N. Pinckard.** Analysis of platelet-activating factor by GC–MS after direct derivatization with pentafluorobenzoyl chloride and heptafluorobutyric anhydride. *J. Lipid Res.* 1990. **31:** 719–725.

Supplementary key words pulsed positive ion/negative ion chemical ionization mass spectrometry • negative ion chemical ionization mass spectrometry • acetyl glyceryl ether phosphocholine

Platelet-activating factor (PAF) denotes a unique autacoid class of *sn*-2-acetylated phospholipids (1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine, AGEPC). Because of its high potency and remarkably wide spectrum of biological activites, PAF has been implicated in the pathogenesis of numerous human diseases (c.f., 1). Nevertheless, such circumstantial evidence must be complemented by unequivocal documentation that PAF is produced during a given disease process. Currently, platelet bioassay is widely used to detect and quantitate PAF. However, the data obtained from platelet bioassay may not be valid if the samples contain endogenous PAF inhibitors. More importantly, PAF molecular heterogeneity precludes accurate quantitation by bioassay because each of the numerous alkyl-chain homologs of PAF has different platelet stimulating potencies (1). Thus, it is essential to identify each molecular species of PAF in a sample of unknown composition, and mass spectral analysis is the only avenue to accomplish these ends. However, the currently available mass spectrometric procedures have problems in this regard; they either lack the required sensitivity or they are unable to provide structural information. We now describe a novel, highly sensitive GC–MS procedure that permits facile identification of the various molecular species of PAF. The procedure relies on the parallel GC–MS analysis of heptafluorobutyryl and pentafluorobenzoyl derivatives obtained by direct chemical derivatization of PAF.

METHODS

Human polymorphonuclear leukocytes (PMN) totaling 9×10^8 PMN (95–98% purity) from four donors were isolated and suspended in Hank's balanced salt solution (5×10^6 cells/ml) as previously described (2). Each of the PMN suspensions was prewarmed at 37°C for 10 min in the presence of 1.4 mM $CaCl_2$ and 2.5 mg/ml of crystallized human serum albumin (Miles Laboratory, Elkhart, IN). The appropriate amount of $[^3H]$acetic acid (New England Nuclear, Boston, MA, 4.7 Ci/mmol) to give a final concentration of 10 μCi/ml and calcium ionophore A23187 (Sigma Chemical Co., St. Louis, MO) to produce a 2.5 μM solution were then added, and the PMN were

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Abbreviations: PAF, platelet-activating factor; AGEPC, 1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine; GC-MS, gas chromatography-mass spectrometry; PMN, polymorphonuclear leukocytes; HPLC, high performance liquid chromatography; PFB, pentafluorobenzoyl chloride; HFB, heptafluorobutyric anhydride; NICI, negative ion chemical ionization; PPINICI pulsed positive ion NICI; RRT, relative retention time.

incubated for an additional 20 min. Lipids were extracted from the cell suspensions as described (3), and the pooled PMN-derived PAF was partially purified by normal-phase high performance liquid chromatography (HPLC) using an 8 × 10 cm 10 μ Radial-PAK silica cartridge in an RCM-100 radial compression module (Waters Assoc., Milford, MA). Isopropanol-hexane-water 1:1:.083 (v/v/v) (i.e., 4% water) was used as the initial mobile phase at a flow rate of 2 ml/min; after sample injection the water content was linearly increased up to 8% during the succeeding 20-min period. The peak region which represented the principal incorporation of [^{3}H]acetate as separated by normal-phase HPLC (eluting between 26 and 30 min) was subsequently fractionated by isocratic C_{18} reversed-phase HPLC, as described (3). Synthetic AGEPC standards and aliquots representing 1-2% of each of the reversed-phase HPLC fractions comprising the principal PAF peak were separately derivatized with pentafluorobenzoyl chloride (PFB, Aldrich Chemical Co., Milwaukee, WS) or heptafluorobutyric anhydride (HFB, Aldrich) as previously described (4), using a 4-h incubation for both PFB and HFB instead of the 18-h period reported earlier. The samples were dissolved in hexane prior to injection into the GC-MS. The sources for AGEPC standards were as follows: C12:0-, Dr. H. Eibl; C14:0- and C15:0-, Calbiochem (La Jolla, CA); C16:0- and C18:0-, Bachem Fine Chemicals (Torrence, CA); C18:1-, Sigma Chemical Co. (St. Louis, MO).

Gas chromatography-mass spectrometry (GC- MS) using methane (0.5 Torr) for chemical ionization was performed on a Finnigan-MAT model 4615 mass spectrometer in combination with an INCOS data system. The ion source temperature was 100°C, the injector temperature was 250°C, and the electron energy was 70 eV. GC separation was accomplished by means of a 12 meter × 0.32 mm i.d. BP-1 column (SGE, Austin, TX), with a linear velocity of helium of 75 cm/sec. Splitless injections using up to 20% of the total volume of each derivatized sample were performed. Specific GC conditions are noted in appropriate figure legends. Mass spectral analysis of PFB derivatives used negative ion detection after electron capture under chemical ionization conditions (abbreviated for convenience as negative ion chemical ionization, NICI). Analysis of HFB derivatives was accomplished by alternately recording the positive ions produced by chemical ionization and the negative ions formed by electron capture (termed pulsed positive ion/negative ion chemical ionization, PPINICI).

RESULTS

HPLC purification

The PAF analyzed in the present study was extracted from stimulated human neutrophils. After initial isolation by normal-phase HPLC, further purification was accomplished by reversed-phase HPLC. The resulting elution profile as determined by incorporation of tritiated acetate was the same as previously described (2). The principal C16:0-AGEPC-containing peak eluted between fractions 18 and 32. (Note: all HPLC fractions mentioned below refer to the reversed-phase separation.)

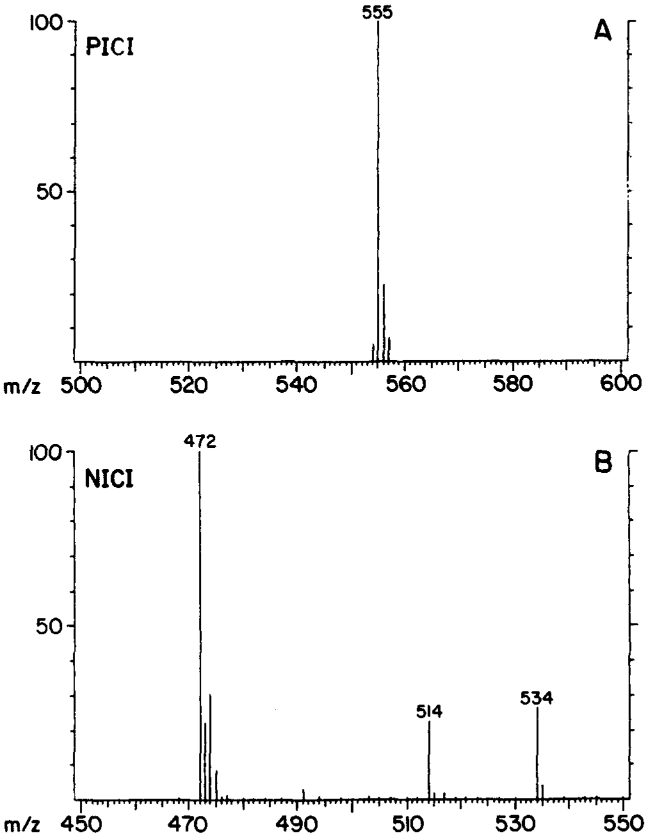

Fig. 1. Chemical ionization mass spectra (pulsed detection of positive and negative ions) of C16:0-AGEPC/HFB derived from stimulated human PMN (reversed-phase HPLC fraction 21): A, positive ion detection; B, negative ion detection.

Identification of C16:0-AGEPC

Direct PFB derivatization of HPLC fraction 21, which had a relative retention time (RRT) of 0.95 as compared to a synthetic C16:0-AGEPC standard, yielded a single product as viewed by GC-MS, scanning negative ions from 490 to 640 amu. The GC retention time was 4.48 min, and the mass spectrum was comprised of predominantly one ion, *m/z* 552, the molecular anion of C16:0-AGEPC/PFB[1]. The HFB derivative of this fraction was then prepared and analyzed by pulsed positive ion/negative ion chemical ionization (PPINICI) GC-MS,

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[1] Terms such as C16:0-AGEPC/PFB are used to signify the product formed by reacting C16:0-AGEPC with PFB, i.e., 1-*O*-hexadecyl-2-acetyl-3-pentafluorobenzoyl-*sn*-glycerol. Similar nomenclature, such as C16:0-AGEPC/HFB, is used in an analogous manner for HFB derivatives.

scanning a limited mass range which encompassed the ions of interest. This mass range selection was based on the prior GC-MS data from the corresponding PFB derivative. An intense GC peak was observed with a retention time of 2.70 min. The positive ion spectrum is shown in **Fig. 1A**, where *m/z* 555 represents $[MH]^+$ for C16:0-AGEPC/HFB. More structural information was obtained using negative ion detection (Fig. 1B). In this mode, characteristic ions at *m/z* 534 ($[M–HF]^-$), *m/z* 514 ($[M–2HF]^-$), *m/z* 474 ($[M–(HF + \text{acetic acid})]^-$, and *m/z* 472 ($[M–(2HF + CH_2CO)]^-$) were observed. The latter two ions are of particular importance since they document the presence of the acetate moiety in the compound under investigation. The selected ion retrieval profile for the preceding analysis is shown in **Fig. 2**. The upper trace (A) represents *m/z* 555 from the positive ion analysis, and the lower trace (B) shows *m/z* 472 from the negative ion scan.

Identification of C18:1-AGEPC

Several other HPLC fractions from the descending portion of the principal C16:0-AGEPC-containing PAF peak were then chosen for similar analysis. These fractions were selected because our previous studies using fast atom bombardment mass spectrometry indicated that C18:1-AGEPC also eluted in this region (2). To illustrate a representative example from this region, the PFB derivative of HPLC fraction 24 exhibited two major species. The resulting selected ion retrieval traces, as seen in **Fig. 3**, indicated the presence of C16:0-AGEPC/PFB (*m/z* 552, Fig. 3A) and C18:1-AGEPC/PFB (*m/z* 578, Fig. 3B). Subsequent evaluation of this fraction after HFB treatment confirmed the identity of both components; the resulting positive and negative ion spectra of the 18:1 homolog are shown in **Fig. 4**.

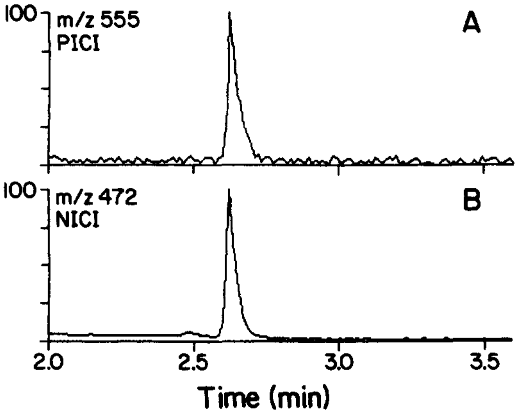

Fig. 2. Selected ion retrieval profiles for the HFB derivative prepared from PMN reversed-phase HPLC fraction 21. GC conditions were: initial column temperature of 180°C held for 1 min followed by heating at 30°C/min to reach a final temperature of 225°C.

Identification of C17:0-AGEPC

Examination of the PFB derivative of HPLC fraction 28 proved to be quite interesting and informative (**Fig. 5**). In addition to trailing amounts of the 16:0- and 18:1-AGEPC homologs, at least two species of C17:0-AGEPC were suggested by peaks with GC relative retention times of 1.055 and 1.076 (as compared to the retention time of C16:0-AGEPC/PFB) which exhibited essentially identical mass spectra (intense ions at *m/z* 566). The spectrum of the later-eluting substance is included in Fig. 5. Analysis of PFB and HFB derivatives of individual HPLC fractions from number 25 through number 31 supported these assignments. To further understand the nature of these compounds, portions representing 2.5% of HPLC fractions 25, 26, and 27 and fractions 29, 30, and 31 were

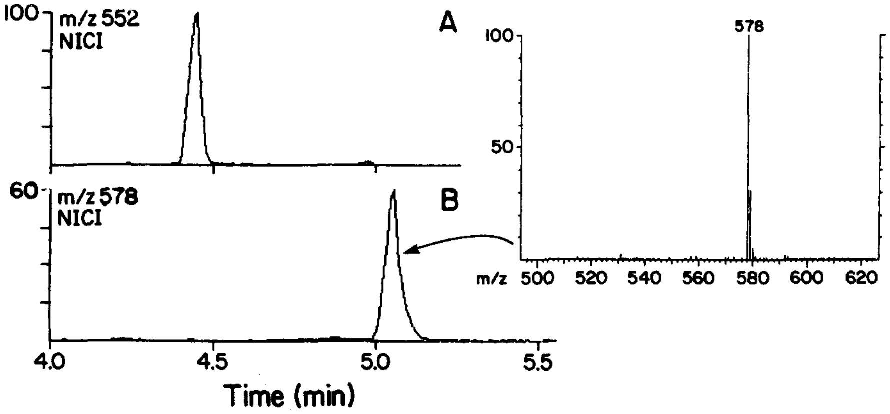

Fig. 3. Selected ion retrieval profiles (negative ion detection) for the PFB derivatives prepared from PMN reversed-phase HPLC fraction 24. GC conditions were: initial column temperature of 180°C held for 1 min followed by heating at 30°C/min to 250°C and then additional heating at 10°C/min to reach a final temperature of 270°C.

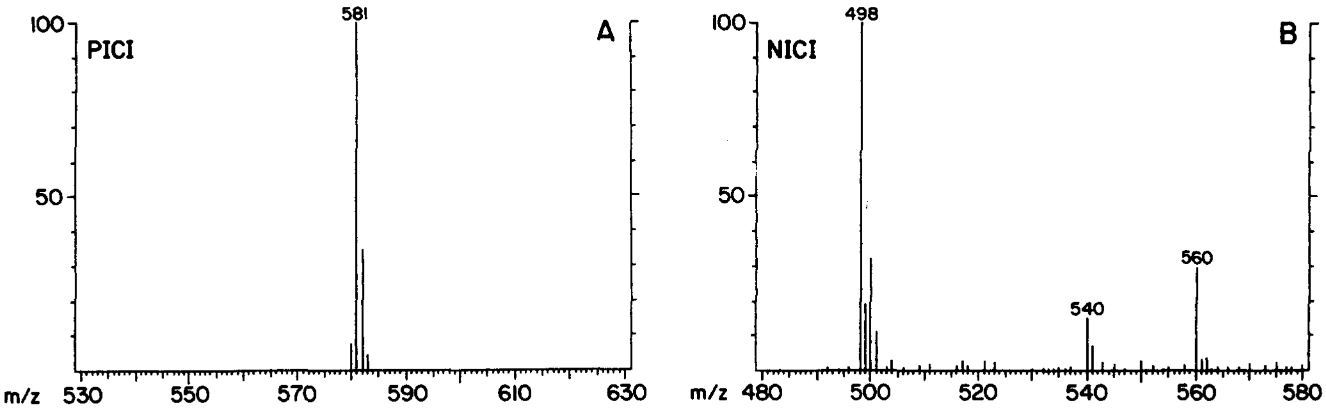

Fig. 4. Chemical ionization mass spectra (pulsed detection of positive and negative ions) of C18:1-AGEPC/HFB derived from stimulated human PMN (reversed-phase HPLC fraction 24): A, positive ion detection; B, negative ion detection.

pooled separately, derivatized with HFB, and analyzed by GC-MS. The resulting selected ion retrieval traces for *m/z* 486 ($[M-(2HF + CH_2CO)]^-$ for C17:0-AGEPC/HFB) are shown in **Fig. 6**, along with the corresponding positive and negative ion mass spectra. The identity of each of these two components was thus confirmed as C17:0-AGEPC by the following observations: an intense ion appropriate for $[M]^-$ was present at *m/z* 566 in the negative ion spectrum of the PFB derivative; the PPINICI spectra exhibited positive ions at *m/z* 569, consistent with $[MH]^+$, and negative ions with the expected fragmentation pattern for HFB derivatives of AGEPC. Based on the GC retention times, we postulated that the later-eluting compound was the straight chain C17:0-AGEPC homolog and the material with the shorter retention time was a branched chain variety. Further substantiation for this assignment was obtained from a plot of GC relative retention time versus carbon number for PFB and HFB derivatives of synthetic alkyl-chain homologs of AGEPC; with the GC conditions used in this study, a straight line was obtained from the analysis of standard unbranched C12:0-, C14:0-, C15:0-, C16:0-, and C18:0-AGEPC, using either PFB or HFB. Evaluation of the retention time data for the PFB derivatives of biological origin revealed that a GC peak with an RRT of 1.076 fit the plot of the saturated straight chain AGEPC/PFB homologs at the precise location for a 17-carbon chain component; however, an RRT of 1.055 fell significantly below the line at that position and, therefore, represented a member of a different structural series, most likely a branched-chain isomer. The corresponding HFB derivatives yielded comparable results.

Additional observations

The results from comprehensive GC-MS analyses of the separate reversed-phase HPLC fractions were in agreement with the HPLC peak assignments of the alkyl-chain PAF homologs previously detected by platelet bio-

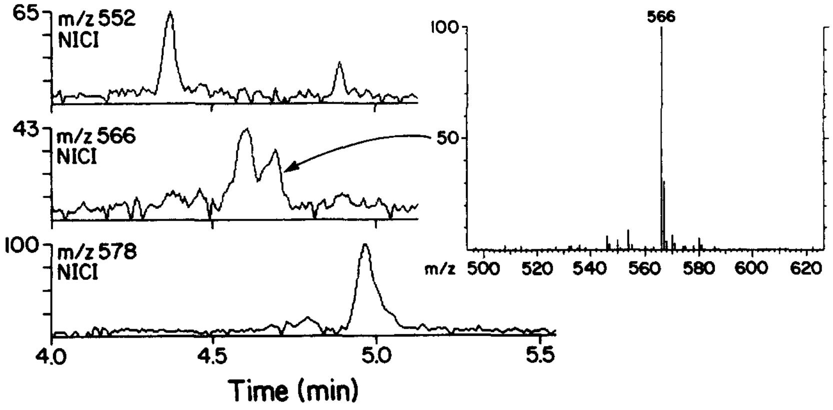

Fig. 5. Selected ion retrieval profiles (negative ion detection) for the PFB derivatives from PMN reversed-phase HPLC fraction 28. GC conditions were the same as described in the legend to Fig. 3.

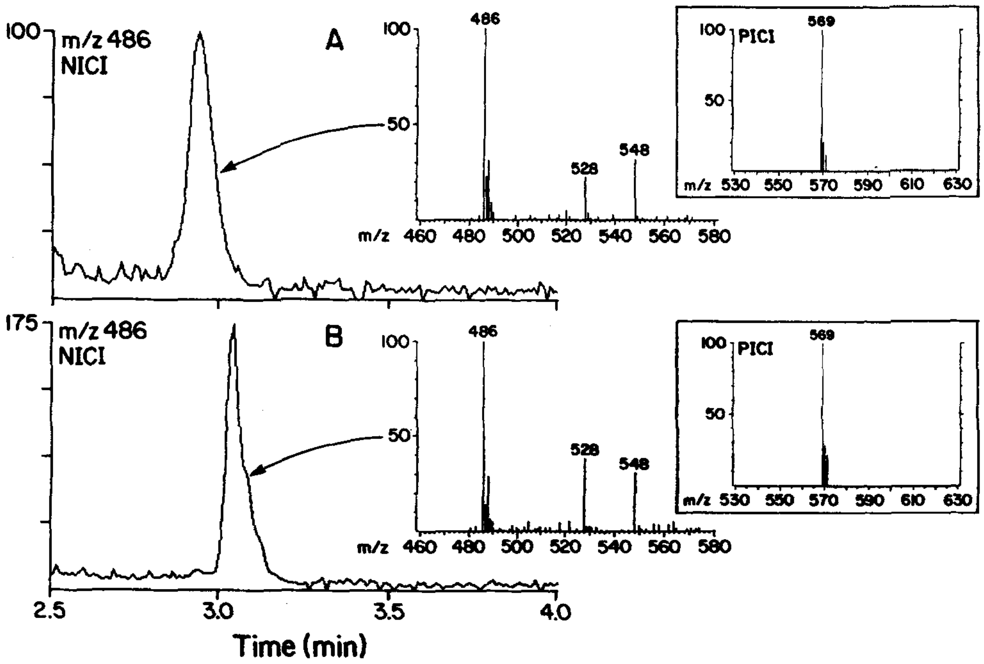

Fig. 6. Selected ion retrieval profiles and mass spectra for the HFB derivatives prepared from: A, pooled PMN reversed-phase HPLC fractions 25, 26, and 27; B, pooled PMN reversed-phase HPLC fractions 29, 30, and 31. GC conditions were the same as described in the legend to Fig. 2.

assay (2). The GC–MS data revealed that the individual homologs of AGEPC displayed their peak HPLC concentrations as follows: 16:0, HPLC fraction 22 (RRT 1.00); 18:1, fraction 26 (RRT 1.18); 17:0 (peak 1), fraction 28 (RRT 1.27); 17:0 (peak 2), fraction 30 (RRT 1.36); 18:0, fraction 38 (RRT 1.72). In addition, the PFB and HFB derivatives of the compounds identified as C16:0-, C18:0-, and C18:1-AGEPC isolated from PMN exhibited the same GC retention times and mass spectral behavior as corresponding authentic standards.

The importance of using the PPINICI mode for alternate positive and negative ion detection of molecular species of PAF in samples of unknown composition was particularly emphasized in the analysis of the HFB derivative of pooled HPLC fractions 29, 30, and 31, as shown in **Fig. 7**. Here it can be seen that monitoring *m/z* 472 alone would have erroneously indicated the presence of two C16:0 species. However, by including $[MH]^+$ as well in the measurement, it was evident that only the later-eluting peak was authentic C16:0-AGEPC/HFB because the earlier peak did not exhibit a positive ion at *m/z* 555.

DISCUSSION

The results in the present report demonstrate the ease and wide utility of GC–MS analysis of biologically relevant levels of PAF after direct derivatization with PFB and HFB. Using the PFB derivative of a small portion of a partially purified PAF sample of biological origin, initial high sensitivity electron capture negative ion chemical ionization GC–MS screening is easily accomplished by scanning a mass range which encompasses the ions of interest. In this way, it is not necessary to use selected ion monitoring which requires a priori selection of target compounds; rather, selected ion retrieval techniques can be used to identify PAF candidates in a mixture of unknown composition. Subsequent analysis of the HFB derivative is then used to obtain structural elucidation of the compounds of interest. If sufficient quantities of PAF are present, limited mass scanning can be used simul-

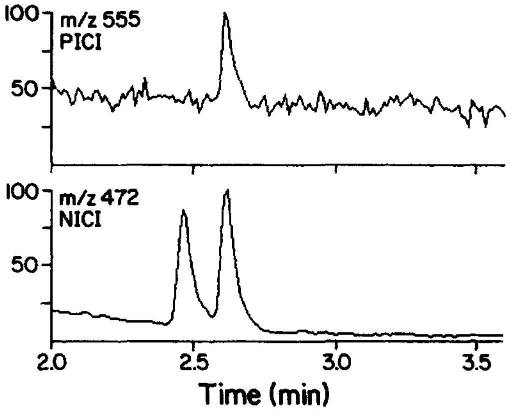

Fig. 7. Selected ion retrieval profiles for PPINICI analysis after HFB derivatization of pooled PMN reversed-phase HPLC fractions 29, 30, and 31: A, positive ion detection; B, negative ion detection. GC conditions were the same as described in the legend to Fig. 2.

taneously in the positive and negative ion detection modes. Otherwise, selected monitoring of predicted values for $[MH]^+$ and $[M-(2HF + ketene)]^-$ can be utilized to gain significantly enhanced sensitivity while maintaining the necessary specificity.

Direct derivatization of PAF with HFB or PFB affords numerous distinct advantages over other currently available methods in regards to ease of operation and degree of recovery. First, from a practical point of view, direct derivatization obviates the need for either hydrogen fluoride or phospholipase C hydrolysis to remove the polar head group and phosphate prior to derivatization. Hydrogen fluoride is a highly reactive and hazardous reagent which can cause partial hydrolysis of the *sn*-2 acetyl group and could also damage unsaturated homologs of AGEPC. Phospholipase C treatment, on the other hand, exhibits differential substrate reactivity (5) and requires extensive, time-consuming sample purification which is often associated with low yields. Furthermore, isolation of an intermediate diglyceride or alkyl-acyl-glycerol is accompanied by isomer formation (6, 7), resulting in splitting of GC peaks, thereby presenting potential difficulties in data interpretation. Use of direct derivatization, on the other hand, retains the original isomeric configuration and also permits facile recovery and analysis of the polar head group (4), a task that is exceedingly difficult after enzymatic cleavage of the phosphodiester at the *sn*-3 position. Finally, with this method there are no interferences caused by contamination with 2-lysophospholipids because direct derivatization does not remove the polar head group in the absence of an acyl moiety esterified at carbon-2 of glycerol (Satsangi, R. K., unpublished observations).

Several investigators have used the PFB derivative for GC-MS identification and quantification of PAF homologs because of the strong electron-capturing capability of PFB coupled with the high proportion of molecular anion in the NICI mass spectrum of the derivative. Ramesha and Pickett (8) first described PFB derivatization of PAF after phospholipase C treatment. Yamada and co-workers (9) subsequently used this procedure to measure PAF in blood, and Christman and Blair (7) similarly analyzed PAF in saliva. However, it should be pointed out that acquisition of data reflecting the putative molecular weight alone is neither sufficient for identification nor structurally informative; indeed, the tentative identification of PAF structure from $[M]^-$ of a PFB derivative must be made by comparing GC retention times of unknowns with those of appropriate authentic standards. Thus, the PFB derivative provides no unequivocal information about the nature of the acyl group esterified at the *sn*-2 position, and it is this group which is of critical importance in the extraordinarily potent biological activities of AGEPC.

As a means of capitalizing on the enhanced responsiveness of electron capture, Bossant and associates (10) prepared the HFB derivative after phospholipase C treatment of PAF. They showed that excellent sensitivity could be obtained with GC analysis using electron-capture detection (ECD). However, identification of PAF homologs by GC-ECD relies solely on GC retention time. Without the added dimension of correlation of a GC peak with mass spectral data, GC-ECD lacks the specificity to be satisfactory for analysis of PAF isolated from biological samples, as evidenced by numerous unidentified and partially resolved GC peaks (11).

There are several GC-MS methods for analysis of PAF which can furnish both structural and quantitative information. Satouchi and co-workers (12) and Tokumura and associates (13) have employed phospholipase C treatment of PAF followed by *t*-butyldimethylsilyl (TBDMS) derivatization, while Clay and co-workers (14) used the trimethylsilyl (TMS) derivative after removal of the polar head group of AGEPC by hydrogen fluoride. Electron impact GC-MS evaluation of the TBDMS product (which is similar to but more stable than the comparable TMS derivative) yields much more structural information than does NICI GC-MS of AGEPC/PFB. The relatively intense $[M-57]^+$ (loss of the *t*-butyl group) provides a measure of the molecular weight of the compound; furthermore, there are ions which not only reflect the size of the alkyl chain in the *sn*-1 position ($[M-(t\text{-butyl} + \text{acetic acid})]^+$ for AGEPC/TBDMS) but also identify the nature of any acyl moieties present in the molecule (e.g., *m/z* 117, $[CH_3COOSi(CH_3)_2]^+$). However, electron impact GC-MS measurements of the TBDMS or TMS derivatives obtained from AGEPC are orders of magnitude less sensitive than electron capture negative ion detection of AGEPC/PFB. Thus, even though excellent structural information can be obtained from these derivatives, their utility for qualitative or quantitative measurements of PAF homologs other than C16:0-AGEPC isolated from biological samples is hampered by practical considerations of analytical sensitivity.

Fast atom bombardment mass spectrometry (FAB-MS) has been used successfully both for qualitative (2, 14) and quantitative (15, 16) evaluation of PAF. While these methods are extremely valuable for analysis of intact phospholipids, static FAB-MS suffers from potentially significant background interferences and requires relatively high sample levels ($>$10 ng). In addition, prior reversed-phase HPLC fractionation to separate individual PAF homologs is essential in order to obtain interpretable results. The tandem mass spectrometric measurements reported by Haroldsen and Gaskell (16) obviate some of these difficulties; and if MS-MS is combined with continuous-flow FAB, as suggested by these investigators, the needed chromagraphy for separation of isomers and homologs of AGEPC would be attainable in an on-line mode. At present, however, continuous-flow FAB MS-MS instruments are not readily available. Furthermore, it is likely that the sensitivity required for evaluation of biological samples will only be realized using selected ion monitoring techniques. This latter factor is a serious limitation for the analysis of samples of unknown composition.

In the present report, we show results that confirm the identity of a straight-chain C17:0-AGEPC homolog isolated from stimulated human PMN. In addition, we show evidence for the existence of at least one branched-chain C17:0-AGEPC isomer. Mueller, O'Flaherty, and Wykle (17) have previously found a reversed-phase HPLC peak of [^{3}H]acetate-labeled PAF with a retention time that corresponded to a branched-chain 17-carbon AGEPC isomer derived from beef heart. Inspection of the reconstructed selected ion monitoring trace for *m/z* 566 published by Ramesha and Pickett (18) indicated that additional species of C17:0-AGEPC which eluted earlier than the straight-chain homolog might be present in their sample as well; however, only a value for the putative molecular weight can be obtained from NICI mass spectrum of the PFB derivative. Unless the assignment can be deduced based on the GC retention time of the unknown as compared to the GC behavior of authentic standards, unambiguous identification cannot be made because analysis of the PFB derivative does not provide sufficient structural information. Parallel utilization of the HFB derivative with PPINICI GC-MS, on the other hand, complements and extends the PFB data and greatly facilitates structural elucidation of unknown compounds. This is illustrated in the present report where these techniques were used to identify both straight and branched alkyl chain C17:0-AGEPC in PAF isolated from stimulated human PMN (Fig. 6). Indeed, these procedures now provide a facile and rigorous means to fully characterize the myriad of molecular species of PAF synthesized in biological systems and will be invaluable for elucidation of the role of these phospholipid autacoids in modulating normal and pathological processes.

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