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Platelet-Activating Factor Quantification Using Reversed Phase Liquid Chromatography and Selected Reaction Monitoring in Negative Ion Mode

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Abstract

Platelet-activating factor (PAF) is a potent biologically active phospholipid that mediates human physiological and pathophysiologic responses. PAF levels increase transiently and are typically assessed by techniques with limitations related to expense, sensitivity, pre-analysis derivatization and interference with isobaric molecules. This study elucidates a facile, accurate liquid chromatography-mass spectrometry analytical method for PAF. In negative ion mode using electrospray ionization, collisionally-activated dissociation analysis showed a unique product ion for acetate adducts of PAF molecular species representing the loss of methyl acetate from the polar head group and loss of a part of the acetate group from the *sn*-2 position. This product ion was exploited for selected reaction monitoring of PAF molecular species following separation by reversed-phase liquid chromatography. Standard calibration responses were determined, and this method was able to detect as low as 100 fmol of PAF. Finally, PAF molecular species were quantified in human neutrophils and monocytes.

Keywords

Platelet-activating factor; mass spectrometry; neutrophils; monocytes

Introduction

Platelet activating factor (PAF) is a choline glycerophospholipid in the alkyl ether subclass containing acetate at the *sn*-2 position. The predominant PAF molecular species have either a hexadecyl (16:0), octadecyl (18:0) or octadec-*cis*-9-enyl (18:1) aliphatic alkyl ether at the *sn*-1 position. PAF is an important biological mediator in physiological and pathophysiological responses involving inflammation [1–4]. In response to a stimulus PAF is rapidly synthesized via remodeling of 1-O-alkyl-2-acyl-*sn*-glycero-3-phosphocholine precursors, and is rapidly degraded by PAF acetylhydrolases [5–7]. Several techniques for

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Conflict of Interest

No conflict exists with the authors.

PAF quantitation have been applied in the past, which have their merits and limitations. PAF was originally quantitated by biological responsiveness by platelets, which, while sensitive and ground-breaking, lacked structural detail regarding molecules responsible for platelet activation [8]. An enzyme-linked immunosorbent assay (ELISA) is commercially available and is sensitive to ~200 fmol, but is expensive, lacks structural detail of PAF molecular species, and is susceptible to preparative loss of PAF by PAF acetylhydrolase. A sensitive gas chromatography-mass spectrometry method for PAF quantification includes phospholipase C conversion of PAF to diglycerides and subsequent conversion of the diglyceride to a pentafluorobenzoyl derivative [9]. This method is time consuming with the multiple derivatization steps. Finally, several liquid chromatography-mass spectrometry (LC-MS) methods have been reported. Electrospray ionization (ESI) in the positive ion mode has been used to quantitate PAF species with selected reaction monitoring (SRM) detection using the common choline glycerophospholipid product ion m/z 184 [10]. While this method reportedly detects PAF as low as 2 fmol, this method is highly dependent on adequate resolution of 16:0 PAF from isobaric 18:0 lysophosphatidylcholine isomers, which have the same precursor and product ion as 16:0 PAF. ESI in the negative ion mode has been used to quantitate PAF species following straight phase liquid chromatography with SRM detection using the acetate product ion m/z 59 and precursor ion of the deprotonated m/z -15 ion with detection levels as low as 1 pmol [11]. The present study used reversed-phase liquid chromatography (RPLC) and detection of acetate adducts in negative ion mode employing a PAF-specific product ion with SRM detection at levels as low as 100 fmol.

Materials and Methods

Materials, RPLC and Mass Spectrometry

16:0, 18:0, 18:1 PAF and [d_4] 16:0 PAF were purchased from Biomol. All solvents and chemicals were purchased from either Fisher Scientific or Sigma Aldrich. RPLC was performed with a Phenomenex Onyx monolithic C-18 column. Mobile phase A was comprised of methanol/water/acetonitrile (57/23/20) supplemented with 10 mM ammonium acetate. Mobile phase B was methanol supplemented with 10 mM ammonium acetate. Flow rates were 300 l/min with initial conditions with 100% A for 10 min followed by a linear gradient to 20% B over the next 5 min, and then a 1 min linear gradient to 100% B, which was then held for 10 min. Samples were resuspended in 70 l of mobile phase A and 35 l were injected. SRM detection of PAF molecular species was performed using a Thermo Fisher TSQ Ultra mass spectrometer (Thermo Fisher, Waltham, MA). SRM transitions were $582.5 \rightarrow 466.4$, $586.5 \rightarrow 470.4$, $608.5 \rightarrow 492.4$, and $610.5 \rightarrow 494.4$ for 16:0, [d_4] 16:0, 18:1, and 18:0 PAF, respectively. Q2 was set at 31 eV at 1.0 atm argon. ESI was in the negative ion mode with spray voltage at 3200 eV, vaporizer temperature at 290°C, sheath gas at 33 psi, auxiliary gas at 10 psi, and capillary temperature at 270°C. Initial mass spectrometry studies elucidated the fragmentation of PAF molecular species using direct infusion and collisionally-activated dissociation (CAD).

Human Neutrophil and Monocyte Isolation and Stimulation

Human neutrophils were isolated from peripheral blood in the presence of 5.4 mM EDTA and subjected to Polymorph Prep™ density gradient centrifugation (density 1.113 g/mL; 500

× *g* for 30 min). Neutrophils were prepared according to Polymorph manufacturer's directions and as previously described [12, 13]. Cells were counted on a hemocytometer and diluted to 1×10⁶ cells/mL in Hanks' buffered saline solution (HBSS) containing Mg²⁺ and Ca²⁺. Human monocytes were isolated from peripheral blood (3.8 units heparin/mL blood) and subjected to Ficoll-Paque density gradient centrifugation (density, 1.077g/mL; 400 X *g* for 20 min). Cells were diluted to 1×10⁶ cells/mL in Iscove's modified Dulbecco's medium (IMDM) (10% FBS, penicillin-streptomycin) and then were subjected to a 46% Percoll density gradient centrifugation in IMDM media with 10% fetal bovine serum and penicillin-streptomycin (550 X *g* for 30 min). Monocytes were washed twice with ice-cold HBSS and then diluted to 1×10⁶ cells/mL. For both monocytes and neutrophils, reactions were assembled with 2 mL of cells in HBSS and treated with vehicle, 2.5 μM A23187, or 10 μg/mL LPS. Incubations were for 30 min at 37°C and stopped with the addition of methanol. Lipids were subsequently subjected to a modified Bligh-Dyer extraction with 4.74 pmol of [*d*₄] 16:0 PAF added [14]. Lipids in the chloroform extract were dried under nitrogen and suspended in 70 l of mobile phase A prior to LC-MS analyses of PAF molecular species.

Results and Discussion

One of the major problems with mass spectrometry of PAF is that the predominant PAF molecular species, 16:0 PAF is isobaric with 18:0 LPC and their positive charged ions and the primary ion fragment is associated with the phosphocholine headgroup [15]. Thus, the presence of 18:0 LPC at abundances 2–3 fold greater than that of 16:0 PAF in biological matrices confounds the quantification of PAF in positive ion mode. Accordingly, initial ESI-MS analysis was performed in the negative ion mode with 16:0 PAF in mobile phase A (containing ammonium acetate). The predominant negative ion *m/z* 582.5 (acetate adduct) was observed in negative ion mode, and CAD analysis of *m/z* 582.5 (Figure 1A) revealed a predominant fragment ion at *m/z* 466.4 resulting from the loss of methyl acetate from the head group, and part of the acetate group at the *sn*-2 position. The other assignments for fragment ions observed in Figure 1A are shown in Figure 1B. Since the fragment ion *m/z* 466.4 is novel to acetate adducts of 16:0 PAF and similar fragmentation patterns are observed for [*d*₄] 16:0, 18:1, and 18:0 PAF, SRM detection was used for these molecular species that were resolved by reversed phase HPLC. Figure 1C shows analysis of 100 fmol of 16:0 PAF which was detected at a signal to noise ratio greater than 8.

Next this method was applied to detect PAF molecular species in human neutrophils. Figures 2A and B show chromatograms with SRM detection of PAF molecular species from neutrophils that were either untreated or treated with the calcium ionophore A23187, respectively. Figure 2C shows the chromatogram of synthetic PAF molecular species, which had similar retention times to PAF molecular species detected in activated neutrophils. In order to accurately quantify PAF molecular species, response factors were determined by comparing ratios of authentic PAF molecular species to that of the internal standard, [*d*₄] 16:0. Response factors are shown in the inset (line parameters) of Figure 3A-C. Data shown in Figure 3A-C are for the SRM response of authentic PAF molecular species compared to that of [*d*₄] 16:0 PAF at various molar ratios. Additional response factors were assessed in the presence of neutrophil extract, and analyses of PAF molecular species in the absence of activators and A23187 were 90 and 21,921 fmol/ million neutrophils for 16:0 PAF; 126 and

10,320 fmol/million neutrophils for 18:0 PAF; and 125 and 9,948 fmol/million neutrophils for 18:1 PAF. These amounts are comparable to previous studies using negative ion mode mass spectrometry, but with straight phase chromatography and a SRM of the M-15 precursor and the acetate anion product ion fragment [16]. Although this previous study separated the molecular species produced in the presence of A23187, they reported the total PAF (sum of the amounts of the molecular species measured). Further studies used this RPLC method to determine the accumulation of PAF molecular species in monocytes treated with LPS and A23187 (Table I). It should be noted that plasmalogen PAF has previously been reported in the plasmenylethanolamine pool [17], and has been suggested to be present in the plasmenylcholine pool [18]. Thus, if plasmenylcholine analog of PAF with 18:0 at the *sn*-1 position is produced in neutrophils and monocytes it likely would have a similar fragmentation pattern and SRM as 18:1 PAF. This potential artifact could be assessed using the described analytical approach in samples pretreated with acid to remove plasmalogens. Collectively, these studies provide an accurate technique to measure PAF molecular species in biological specimens at levels as low as 100 fmol.

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Abbreviations

CAD	Collisionally-activated dissociation
ESI	Electrospray ionization
ELISA	Enzyme-linked immunosorbent assay
HBSS	Hanks' buffered saline solution
IMDM	Iscoe's modified Dulbecco's medium
LC-MS	Liquid chromatography-mass spectrometry
PBS	Phosphate buffered saline
PAF	Platelet-activating factor
RPLC	Reversed-phase liquid chromatography

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Fig. 1. ESI-MS analysis of acetate adduct of PAF in negative ion mode. CAD analysis of 16:0 PAF (*m/z* 582) (A) showing the predominant product ion at *m/z* 466. Fragment ion assignments from CAD analysis and the structure of 16:0 PAF are shown in B. RPLC with SRM (582.5 → 466.4) detection of 100 fmol 16:0 PAF (arrow) as described in “Materials and Methods”.

Fig. 2. ESI-MS detection of PAF molecular species present in human neutrophils and comparisons to synthetic standards. Two million human neutrophils were treated with either vehicle (A) or 2.5 μ M A23187 (B), and prepared for RPLC and SRM detection of PAF molecular species as described in “Materials and Methods”. Neutrophils were extracted in the presence of 4.74 pmol of [d_4] 16:0 PAF. Panel C shows the injection of 2.5 ng each of authentic synthetic 16:0, [d_4] 16:0, 18:1, and 18:0 PAF. Each molecular species was monitored in each

panel as indicated by the SRM. Each panel was normalized such that 100% relative intensity is equal in each panel as well as each SRM within a panel.

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Fig. 3. Calibration response of PAF molecular species compared to the deuterated internal standard, [d_4] 16:0 PAF. Selected amounts (0.2 pmol-96 pmol) of either 16:0 (A), 18:1 (B) or 18:0 (C) PAF were mixed with 4.74 pmol of [d_4] 16:0 PAF and subjected to RPLC with SRM detection of each PAF molecular species as indicated in the y-axis of each of the panels. Line parameters are shown in the inset of each panel.

Table 1

Quantitation of Human Monocyte PAF Levels

Condition	Monocytes		
	16:0	18:0	18:1
Control	565±11	503±28	452±14
LPS	989±144	507±20	521±27
A23187	33,911±3,639	4,889±323	6,079±458

Values are means ± standard error (n=3) and are expressed as fmol/2×10⁶ cells. Monocytes were treated with either vehicle, LPS (10 g/mL) or A23187 (2.5 M) for 30 min. Individual molecular species were quantified by LC/MS in negative ion mode using SRM as described in “Materials and Methods”.