

Quantitative determination of major platelet activating factors from human plasma

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Abstract Platelet activating factor (PAF) is a potent lipid mediator that is involved in many important biological functions, including platelet aggregation and neuronal differentiation. Although an ELISA assay has been used to measure PAF levels, it cannot distinguish between its isoforms. To achieve this, liquid chromatography-tandem mass spectrometry (LC-MS/MS) has been used instead. However, isobaric lysophosphatidylcholine (lyso PC), which is often present in large amounts in complex biological samples and has similar retention times in many LC conditions, can affect the accurate measurement of PAF. The present study examined the fragmentation behavior of major PAF and lyso PC during various MS/MS conditions. Fragment ions at m/z 184 and at m/z 104 were abundantly observed from MS/MS of lyso PCs. PAF provided a dominant fragment ion at m/z 184, but a fragment ion at m/z 104 was almost never produced, regardless of the collision energy. Thus, the two fragment ions at m/z 184 and m/z 104 were used to accurately measure PAF levels. First, the fragment ion at m/z 184 and the retention time of PAF in LC-MS/MS were used to identify and quantitate PAF. However, if there were small retention time shifts, which are common in

multiple sample runs, and lipid composition in a sample is very complicated, the fragment ion at m/z 104 was used to confirm whether the fragment ion at m/z 184 belonged to PAF. This novel method accurately determined the major PAF (C16:0 PAF, C18:0 PAF, and C18:1 PAF) levels in human plasma.

Keywords Platelet activating factor (PAF) · Liquid chromatography (LC) · Tandem mass spectrometry (MS/MS) · Human plasma

Introduction

Lipids play important roles in living cells and their levels can indicate, along with other information, cellular function. Indeed, lipid levels could define the phenotype of a cell or tissue in many diseases, including obesity and atherosclerosis [1–3]. Thus, accurate measurements of the lipids in a biological system are often needed to thoroughly assess their functions and to perform in-depth studies of the disease states that associate with them [4–6, 2, 7–9, 3]. Platelet activating factor (PAF) is a potent lipid mediator that is involved in various physiological and pathological processes, including platelet aggregation and inflammatory responses [10–13]. Since different biological states are associated with disparate PAF molecular species [14–16], it would be useful to be able to accurately measure the individual PAF isoforms; this would greatly improve our understanding of PAF-related biology.

PAF is a subclass of phospholipid mediator and is defined by an alkyl ether group at sn-1, an acetyl residue at sn-2, and a phosphocholine at sn-3. Its level has been measured by various conventional lipid analysis strategies. Initially, lipid extracts that contained PAF were separated by thin-layer chromatography or high-performance liquid chromatography (HPLC), after which the PAF molecules were identified by

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using relatively nonspecific detection techniques, such as UV detection and radiometric measurement [17–19]. However, this method suffers from low resolution and a lack of specificity. Later, an ELISA assay was developed to measure PAF levels; however, the disadvantage of this method is that it cannot quantitate the individual isoforms of PAF [20]. An alternative method that has been used to measure the PAF levels in various biological samples is mass spectrometry (MS) combined with gas chromatography (GC) or HPLC [14, 15, 19]. While GC-MS is the most commonly used MS method for small molecule analysis [21], this technique often involves sample derivatization to improve volatility and is limited in terms of molecular size and type due to volatility and polarity constraints. Another MS method, which was used to measure PAF in tears, is matrix-assisted laser desorption ionization-mass spectrometry (MALDI-MS). However, this technique needs a special matrix to avoid low molecular weight matrix interferences and to improve ionization [22, 23]. It is also not generally suitable for measuring small biomolecules, such as lipids and metabolites. For these reasons, liquid chromatography-mass spectrometry (LC-MS) has become a popular alternative choice for PAF analysis [24–26].

LC-MS can directly analyze various lipids as intact molecules and preserve the information inherent in their chemical structures. Liquid chromatography-tandem mass spectrometry (LC-MS/MS) provides structural information of phospholipids from their fragmentation patterns [27–29]. Collision-induced dissociation (CID) is the most commonly used tandem mass spectrometric technique. Generally, LC-MS/MS of glycerophosphocholine subclasses such as PAF and lysophosphatidylcholine (lyso PC) is performed by using a precursor ion scan of 184 (which represents phosphocholine) in the positive ion mode at various chromatographic conditions [21, 25, 30]. However, distinguishing between PAF and lyso PC in LC-MS/MS can be difficult for several reasons. First, while the concentration of lyso PC in human serum is about several hundred nanomoles per milliliter [31], PAF is generally present at very low concentrations in many biological specimens: the normal range of PAF in human plasma is about 0.03–0.19 pmol/mL [32]. This huge difference in PAF and lyso PC concentrations in biological samples may induce ion suppression of PAF during the electrospray ionization process. The second problem is that major PAFs are isobaric to some lyso PCs, which results in similar retention times. The third problem is that phosphocholine ion (m/z 184) is the most dominant fragment in MS/MS of lyso PCs and PAFs, and these two kinds of phospholipids cannot be distinguished when considering m/z 184 only. Thus, complete separation of PAF from lyso PC is needed for accurately measuring PAF. Alternatively, a different MS/MS strategy is crucial to distinguish between PAF and lyso PC. Another lipid that may hamper PAF measurement is choline-lysoplasmalogen because it has a similar chemical structure to PAF. However, it

is unlikely to have the same molecular weight as PAF: indeed, it would have to have a rare fatty acid composition to be isobaric to PAF. Nevertheless, studies examining the ability of this lipid to hinder PAF measurement by LC-MS/MS are warranted.

Although normal-phase HPLC was able to successfully separate C16:0 and C18:0 PAF from their isobaric lyso PC species (C18:0 and C20:0 lyso PC, respectively) in the positive ion mode [25], palmitoyl-formyl-glycerophosphocholine (PFPC), isobaric with C16:0 PAF, was found to elute at a very close retention time as C16:0 PAF by another research group [26]. PFPC and C16:0 PAF could be distinguished by their different fragmentation patterns in MS/MS in the negative ion mode [24]. However, this strategy is not suitable for routine analysis of PAF because of the lack of sensitivity of MS/MS in the negative ion mode. Thus, it is reasonable to use reverse-phase LC-MS/MS for analysis of PAF in the positive ion mode, due to robustness of reverse-phase LC column and to avoid PFPC interference [26, 15]. But there is a remaining problem that C16:0 and C18:0 PAF elute adjacent to their isobaric lyso PC species (C18:0 and C20:0 lyso PC, respectively) at optimal separation conditions, which causes confusion in the confident identification of PAF from complex biological samples.

A precursor ion scan of 184 was used to detect lipid subspecies containing phosphocholine to quantitate PAF. PAF can be distinguished accurately from lyso PC if the retention times are highly reproducible, regardless of their similar retention times at optimal reverse-phase LC conditions. However, non-linear retention time shifts in chromatograms over runs are often observed. Small retention time shifts may lead to improper matching of peaks, especially when the sample contains many isobaric compounds that yield similar fragmentation patterns in MS/MS and that elute with similar retention times. For this reason, standard addition method was used to measure PAF levels during neutronal differentiation. This allowed to accurately distinguish PAF from other isobaric compounds that eluted closely in various LC conditions [15]. However, this strategy requires additional analysis time and extra sample amount due to several steps of spikes, thus would not be suitable when only a small amount of biological samples are available and the number of sample size is large.

Our goal is to develop a fast and reliable strategy for accurate measurement of PAF. In the present study, the fragmentation behavior of PAF, lyso PC, and choline-lysoplasmalogen during various MS/MS conditions was examined. We found that the CID spectra of lyso PC or choline-lysoplasmalogen were characterized by abundant levels of a fragment ion at m/z 104 that was rarely produced by PAF, regardless of the collision energy. Thus, two MS/MS fragment ions were used to measure PAF levels in biological samples. First, the fragment ion at m/z 184 together with the retention time of PAF in LC-MS/MS was used to quantify PAF from

isobaric choline-containing phospholipids. However, when retention time shifts were experienced over multiple sample runs, the presence of fragment ions at m/z 104 was used to confirm whether the fragment ions at m/z 184 derived from PAF and/or lyso PC. Generally, the amount of PAF in human plasma was low, and the extracted ion chromatogram of m/z 184 in MRM mode generates several isobaric peaks near the expected retention time of the PAF. Thus, further confirmation using m/z 104 would be crucial to avoid misidentification of PAF. This novel strategy successfully measured the PAF levels in human plasma. To the best of our knowledge, this is the first time that the PAF molecules in human plasma have been measured using LC-MS/MS.

Experimental section

Sample preparation

The C16:0, C18:0, and C18:1 PAF were purchased from Avanti Polar lipids (Alabaster, AL, USA). C18:0 PAF- d_4 (Cayman Chemical, MI, USA) served as an internal standard. The C16:0 and C18:0 lyso PC isoforms and choline-lysoplasmalogen were purchased from Avanti Polar lipids (Alabaster, AL, USA). Lyso PC indicates sn-1 acyl-lyso PC unless otherwise indicated. All stock solutions were made in methanol and stored at -20 °C. Standard mixtures containing 0.1–1,000 pmol/mL of each PAF were prepared in methanol with 100 pmol/mL of the internal standard.

The lipids from human plasma were extracted by using the Bligh and Dyer method with minor modifications [33]. In brief, an equal volume of cold methanol was added to 1 mL of human plasma and the supernatant after centrifugation was taken for lipid extraction. Thereafter, 20 μ L of 100 pmol/mL internal standard solution was added to the sample and 3.75 mL of $\text{CH}_2\text{Cl}_2/\text{MeOH}$ (1/2, v/v), 1 mL of CH_2Cl_2 , and 1 mL of H_2O were sequentially added to the vial. Each time, the solution underwent vigorous vortex and centrifugation. The bottom organic layer containing the lipids was taken and dried under vacuum. The dried extract was kept at -20 °C and reconstituted with 20 μ L of MeOH prior to LC-MS/MS analysis.

LC-MS/MS

Tandem mass spectra were obtained by using an ABSciex QTRAP 5500 tandem mass spectrometer (ABSciex, Framingham, MA, USA) equipped with a pneumatically assisted “turbo V” electrospray ionization (ESI)-source. The instrument was operated in positive ion mode with an ion-spray voltage of 4,500 V using 25 psi curtain gas, 50 psi nebulizer gas, and 50 psi drying gas at a temperature of 400 °C. CID mass spectra were collected after direct infusion with a flow rate of 7 μ L/min and summed over 100 scans.

A LC-MS/MS system equipped with 1290 HPLC (Agilent), Qtrap 5500 (ABSciex), and a reverse-phase column (Pursuit 5 C18 150 \times 2.0 mm) was used. Three microliters was injected into the LC-MS/MS system. The LC separation used mobile phase A (5 mM ammonium formate/MeOH/tetrahydrofuran; 500/200/300) and mobile phase B (5 mM ammonium formate/MeOH/tetrahydrofuran; 100/200/700) and proceeded at 150 μ L/min at 35 °C. The separation gradient was as follows: hold at 50 % B for 5 min, 50 to 70 % B in 7 min, hold at 70 % B for 5 min; 70 to 50 % B in 0.1 min, hold at 50 % B for 3 min. This separation gradient and sample run time were slightly modified depending on sample conditions. The MRM mode was used in the positive ion mode and the peak area of the extracted ion chromatogram (EIC) corresponding to the specific transition for each lipid was used for quantitation. Data analysis was performed by using Analyst 1.5.2 software (ABSciex).

Method validation

The calibration range for each PAF was 0.1–1,000 pmol/mL ($r^2 \geq 0.99$), using a $1/x$ weighting factor. The calibration curve for each PAF was generated from one run of a complete set of calibration solutions per batch analysis and is shown in Figure S1 (Electronic Supplementary Material). Two calibration solutions were chosen for the purpose of QC and additional replicates were done as necessary. Human plasma diluted with phosphate-buffered saline (PBS) served as the blank samples for method validation [34]. To ensure that endogenous PAF was not detected, human plasma chosen for method validation had very low PAF concentrations, and 1/5 dilution was made with PBS. The blank samples were spiked with three different concentrations of PAF standard solutions. The spiked samples were extracted as stated above for sample preparation, then reconstituted with 100 μ L of MeOH and analyzed by LC-MS/MS. Three or four spiked samples per concentration group were extracted independently in 1 day and analyzed by LC-MS/MS. The same procedure was repeated with another set of spiked samples on the second day to determine the accuracy and precision of this analytical method.

Results and discussion

MS/MS of PAF, lyso PC, and choline-lysoplasmalogen

In PAF, a fatty acid is attached to the sn-1 position of the phospholipid via an ether bond. By contrast, lyso PC uses an ester bond to link a fatty acid to that position. Thus, certain PAF and lyso PC isoforms can have the same chemical composition. For example, C16:0 and C18:0 PAF have the same molecular weights as C18:0 and C20:0 lyso PC,

respectively. Plasmalogen, another type of phospholipid, has a vinyl ether bond instead of an ether bond at the sn-1 position. Choline-lysoplasmalogen has a similar chemical structure to PAF, although it is unlikely to have the same molecular weight: to be isobaric to PAF, it would have to have a rare fatty acid composition. MS/MS of phosphocholines is known to generate a very dominant fragment ion at m/z 184 (which represents phosphocholine) in the positive ion mode. For this reason, a precursor ion scan of 184 has often been used to detect the choline-containing phospholipids in complex lipid mixtures [21]. However, this approach is hindered in terms of PAF detection if there are also abundant levels of isobaric lyso PC in the biological sample, which is common [35].

Figure 1 showed that, as expected, the most dominant fragmentation pathway in MS/MS of isobaric C16:0 PAF and C18:0 lyso PC was the cleavage that generated the phosphocholine ion of m/z 184. Thus, Fig. 1a shows that at a collision energy of 30 V, CID of C16:0 PAF yielded abundant ions at m/z 184; several other minor fragments at m/z 86 and m/z 124 from this phosphocholine group were also observed. However, the fragment ion at m/z 104 (choline, $(\text{CH}_3)_3\text{NCH}_2\text{CH}_2\text{OH}$) was not observed. By contrast, CID of C18:0 lyso PC (which is isobaric to C16:0 PAF) produced both m/z 184 and m/z 104 as shown in Fig. 1b.

The effect of other collision energies, namely, 20 and 40 V, on PAF fragmentation patterns was then assessed. Phosphocholine was always the most abundant fragment ion, regardless of the collision energy, although other minor fragment ions such as m/z 86 and m/z 124 started to appear at the higher collision energy (Fig. 2). However, the fragment ion at m/z 104 was rarely observed, even in very harsh collision conditions that caused the parent ion to almost disappear as shown in Fig. 2b: even when it was observed, the ion abundance of m/z 104 was always less than 1 % of the ion abundance of m/z 184. By contrast, MS/MS of C18:0 lyso PC always produced quite abundant fragment ions at m/z 104, even at low collision energies such as 20 V shown in Figure S2 (Electronic Supplementary Material). MS/MS of another isobaric PAF:lyso PC pair, namely, C18:0 PAF and C20:0 lyso PC, yielded the same fragmentation patterns at various collision energies (data not shown).

Although choline-lysoplasmalogen is unlikely to have the same molecular weight as PAF, its fragmentation pattern was also explored to assess whether it could interfere with PAF quantitation. Thus, the MS/MS spectra of 18 carbon choline-lysoplasmalogen at various collision energies were obtained. The fragment ion at m/z 104 was the most abundant ion, while the ion abundance of the phosphocholine ion (m/z 184) was much smaller than that at m/z 104 (Fig. S3, Electronic Supplementary Material). Thus, the specific fragmentation pattern of PAF may be useful for discriminating PAF from other isobaric choline-containing phospholipids.

It has been suggested that the fragmentation pathway that generates m/z 104 in CID of lyso PC involves the formation of

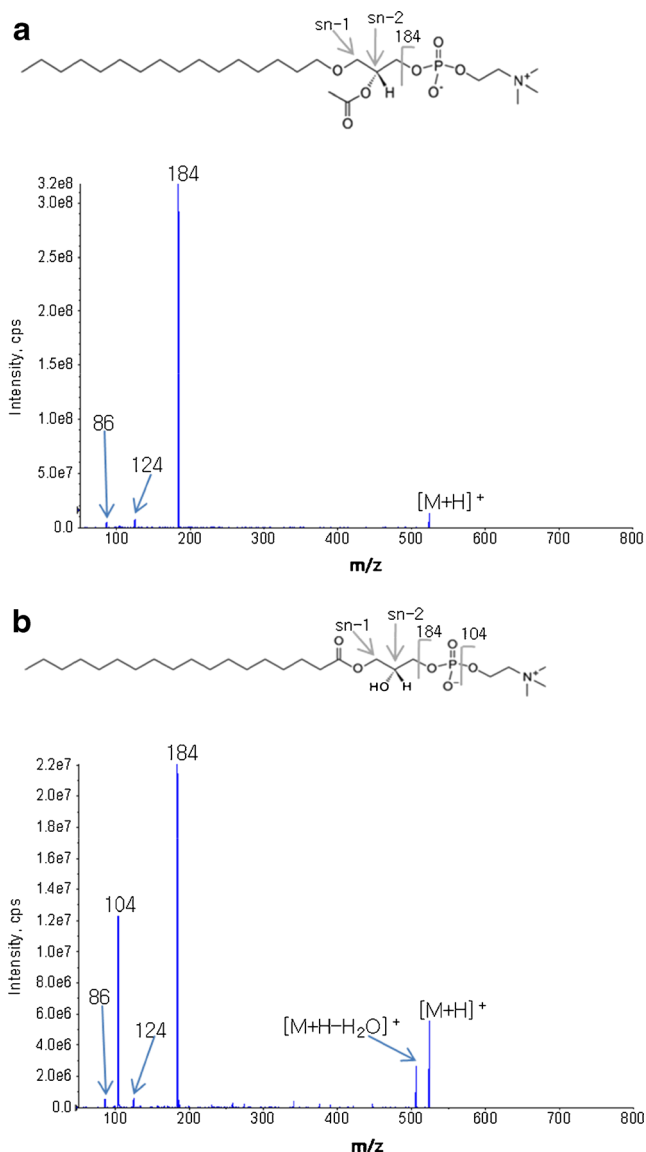


Fig. 1 Collision-induced dissociation of protonated C16:0 platelet activating factor (a) and protonated C18:0 lysophosphatidylcholine (b) at a collision energy of 30 V

five- or six-membered cyclic neutral species and that a hydroxyl group at sn-1 or sn-2 (depending on the lyso PC regioisomer) participates to produce the cyclic neutral species [36, 37]. PAF contains an acetyl group at the sn-2 position and an ether group at the sn-1 position, thus a hydroxyl group is not available to participate in cyclic formation with a phosphate group. This may explain why MS/MS of PAF failed to generate fragment ions at m/z 104.

LC-MS/MS of isobaric PAF and lyso PC

The LC-MS/MS data in Fig. 3 were obtained by using individual standard solutions of C16:0 PAF, C18:0 lyso PC, C18:0 PAF, and C20:0 lyso PC. As expected from the previous MS/

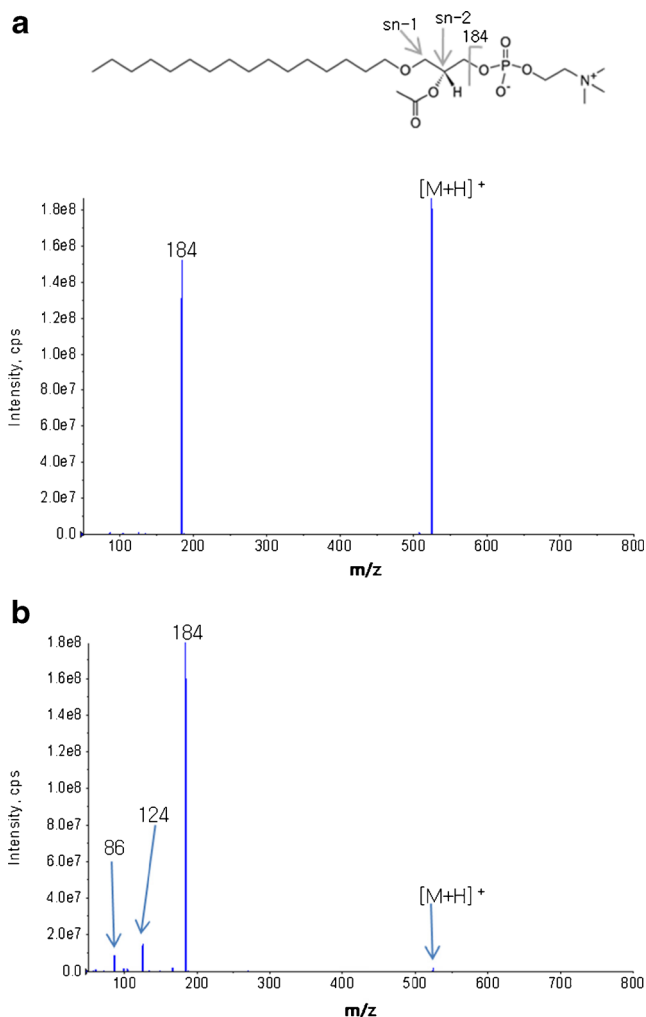


Fig. 2 Fragmentation behavior of C16:0 platelet activating factor at a collision energy of 20 V (a) and 40 V (b)

MS experiments, chromatographic peaks on EIC of m/z 104 were barely shown for C16:0 and C18:0 PAF; however, peaks of m/z 184 were quite abundant: at most, the fragment ion levels at m/z 104 were 0.7 % of the fragment ion levels of m/z 184. By contrast, C18:0 and C20:0 lyso PC had quite abundant fragment ions at m/z 104, although this abundance varied depending on the collision energy and was always less than the abundance at m/z 184. Choline-lysoplasmalogen yielded more abundant fragment ions at m/z 104 than at m/z 184, as expected (data not shown). The efficiency with which fragment ions at m/z 104 are generated may be useful for confirming the identity of PAF in complex lipid mixtures.

We used HILIC or reverse-phase columns to avoid interference from PFPC [25, 26]. Several HILIC columns from different manufacturers were tested but the results were not successful because of poor separation between PAF and lyso PC and the broad widths of their peaks on chromatograms (data not shown). Finally, a reverse-phase column was chosen and LC-MS/MS was performed with standard lipid solutions

of C16:0 PAF, C18:0 PAF, C18:1 PAF, C18:0 lyso PC, and C20:0 lyso PC. Decent separation between PAF and lyso PC was achieved along with good peak shape. However, PAF and lyso PC still eluted closely, which may be inevitable because of their similar chemical structures; this was observed in most reverse-phase LC-MS/MS conditions, and has been reported by other groups as well [26, 15]. Generally, lyso PC is very abundant in biological samples, unlike PAF [35]. Thus, the close retention times of lyso PC and PAF could lead to the misidentification of PAF in the commonly used MRM mode using the m/z 184 fragment ion. This is likely to be particularly problematic when multiple sample runs are needed because, in such cases, retention time shifts occur quite often for numerous reasons [38]. While there are many software types that align retention times [39–42], not all LC-MS/MS data are compatible with them. In addition, additional data processing is inconvenient and makes interpretation more complex. Thus, the specific fragmentation pattern of PAF should help to accurately identify it in cases of potential retention time shift: the fragment ions at m/z 184 can be used to identify and quantitate PAF, while the fragment ion at m/z 104 can be used to confirm that the fragment ions at m/z 184 truly derive from PAF.

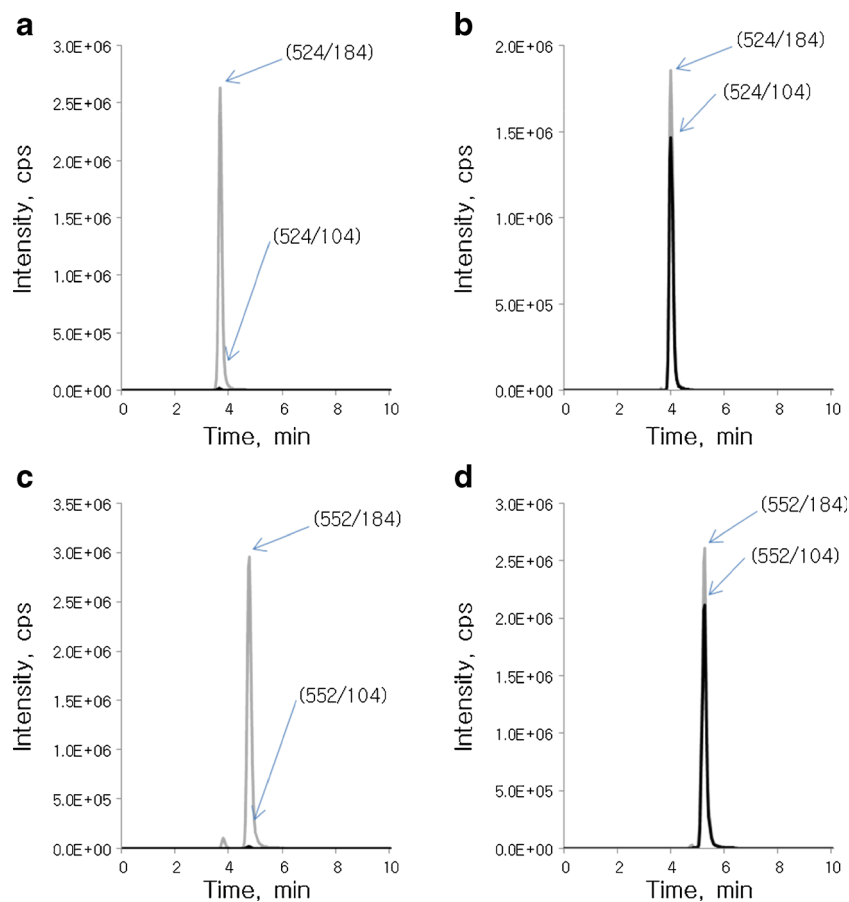
Accuracy and precision of the LC-MS/MS

Table 1 shows the accuracy and precision of the quantitation of each PAF. The accuracies for C16:0, C18:0, and C18:1 PAF were approximately 110, 98, and 109 %, respectively. The precision of this measurement can be evaluated by using the relative standard deviation. LOQ was calculated as ten times the standard deviation of blank samples. The LOQ of C16:0, C18:0, and C18:1 PAF was about 0.06 pmol/mL. Since the normal range of PAF in human plasma is about 0.03–0.19 pmol/mL [32], several hundred micro-liters of human plasma may be needed for accurate quantitation if 20 μ L of reconstitution volume is considered.

Quantitation of PAF in human plasma

Figure 4 shows the LC-MS/MS data to determine major PAF forms from human plasma, where two MRM transitions producing fragment ions of 184 and 104 were used. The expected retention time of C18:0 PAF was 12.3 min, as indicated by the arrow in Fig. 4b. The peak at 12.3 min could not be confidently assigned as being either C18:0 PAF or C20:0 lyso PC when the MRM transition of 552/184 only was considered, due to adjacent peak eluting at 12.7 min. However, the peak abundance eluting at 12.7 min showed quite visible MRM transition of 552/104 and its abundance definitely exceeded 1 % of the peak abundance of the MRM transition of 552/184. Thus, the peak at 12.3 min was confidently assigned as C18:0 PAF, and we also confirmed this by spiking standard solution

Fig. 3 Comparison of the liquid chromatography-tandem mass spectrometric spectra of isobaric platelet activating factor (PAF) and lysophosphatidylcholine (lyso PC) pairs, namely, C16:0 PAF (a) vs. C18:0 lyso PC (b), and C18:0 PAF (c) vs. C20:0 lyso PC (d). A collision energy of 30 V was used. Since standard solutions were used, the liquid chromatography gradient was modified to save analysis time. The concentration of each PAF was 1 nmol/mL. The gray and black lines show the extracted ion chromatograms of multiple reaction monitoring transitions of 524/184 (552/184) and 524/104 (552/104), respectively



of C18:0 PAF to this sample (data not shown). The peak that appeared adjacent to the expected retention time for C18:0 PAF could be from a regioisomer of C20:0 lyso PC: while sn-1 acyl-lyso PC is the most common form of lyso PC, sn-2 acyl- lyso PC has also been observed in biological samples. It has been reported that CID of the sn-1 and sn-2 regioisomers of lyso PC generates fragment ions at both m/z 184 and m/z 104, although their relative abundances may differ depending on the collision energy and the isomers involved [36, 43].

Moreover, sn-2 acyl-lyso PC has been reported to elute a little earlier than its regioisomer [36]. Thus, based on studies from other groups, the small peak at 12.7 min, which eluted earlier than C20:0 lyso PC (whose retention time was 13.2 min), could be its sn-2 C20:0 lyso PC regioisomer. Likewise, C16:0 PAF and C18:1 PAF were measured from the LC-MS/MS data shown in Fig. 4a, c. The C16:0, C18:0, and C18:1 PAF levels in this sample were 0.39, 0.08, and 0.05 pmol/mL, respectively.

Table 1 Accuracy and precision of the measurement of C16:0, C18:0, and C18:1 platelet activating factor (PAF) ($n=4$ per group for the first day and $n=3$ per group for the second day). The percentages of accuracy are shown in parentheses

Concentration, spiked (pmol/ml)		Observed concentration (pmol/ml)					
		C16:0 PAF		C18:0 PAF		C18:1 PAF	
		Mean (%)	RSD	Mean (%)	RSD	Mean (%)	RSD
1st day	2	2.1 (105)	10.8	1.8 (90)	9.7	2.1 (106)	9.4
	20	22.6 (113)	10.2	18.6 (93)	5.4	23.1 (116)	10.8
	200	194.7 (97)	13.2	239.0 (120)	6.0	236.5 (118)	7.2
2nd day	2	2.4 (121)	12.8	1.7 (86)	8.8	2.0 (101)	7.8
	20	24.6 (123)	5.1	19.4 (97)	10.6	23.5 (117)	12.6
	200	201.9 (101)	10.3	198.7 (99)	7.3	186.5 (93)	7.9

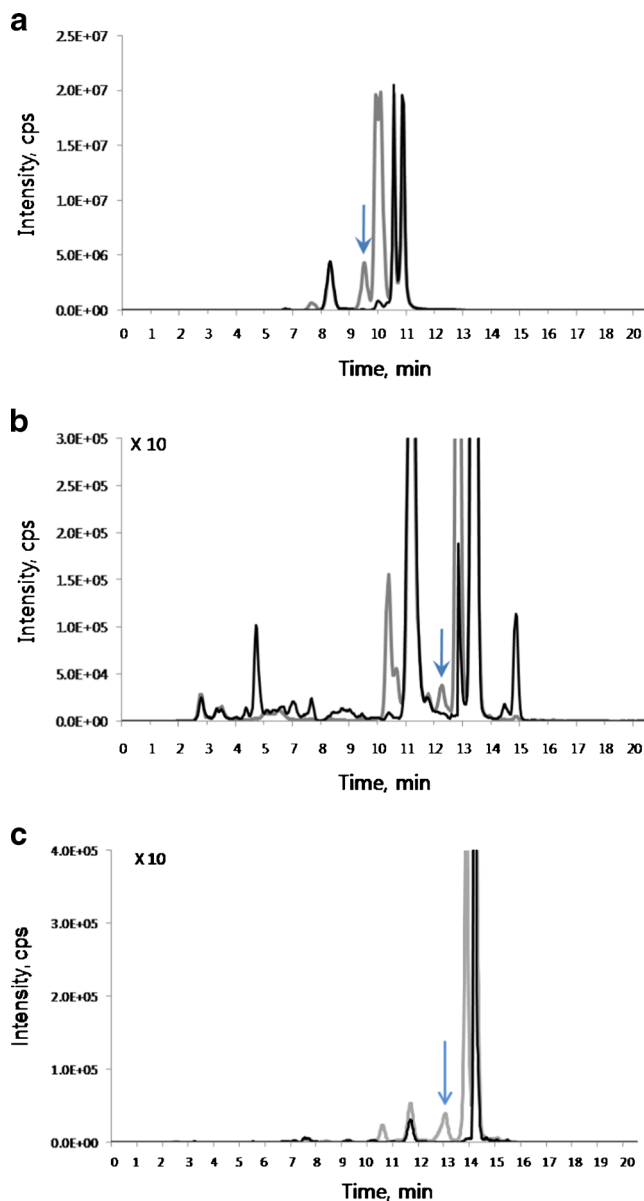


Fig. 4 Liquid chromatography-tandem mass spectrometry to measure **a** C16:0, **b** C18:0, and **c** C18:1 platelet activating factor (PAF) in a human plasma sample. $\times 10$ the y-axis was zoomed ten times. The *gray and black lines* show the extracted ion chromatograms of multiple reaction monitoring transitions of 524/184 and 524/104, respectively, for C16:0 PAF (552/184 and 552/104 for C18:0 PAF, 550/184 and 550/104 for C18:1 PAF). The *arrow* indicates the expected retention time of each PAF

Conclusion

PAF exists in most biological samples at low levels, while other phosphocholines such as lyso PC is often present at high levels in biological samples. This can cause problems in the accurate measurement of PAF, because the abundant lyso PC molecules are isobaric to the major forms of PAF and elute closely at optimum reverse-phase LC conditions. The novel strategy introduced in this paper would be valuable for confident measurement of PAFs from complex biological samples.

The two fragment ions at m/z 184 and m/z 104 were used to quantitate PAF: the fragment ion at m/z 184 and the PAF retention time in LC-MS/MS were used to identify and quantitate PAF. However, if lipid composition in a sample was very complicated and multiple sample runs caused small retention time shifts, the fragment ion at m/z 104 would be crucial to confirm whether the fragment ions at m/z 184 truly did derive from PAF. Three major PAF isoforms (C16:0 PAF, C18:0 PAF, and C18:1 PAF) were successfully measured in human plasma. However, this strategy should be applied to measure other PAFs, as long as standard lipids of the corresponding PAF isoforms are available.

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References

- Wymann MP, Schneider R (2008) Lipid signalling in disease. *Nat Rev* 9(2):162–176
- Farooqui AA, Horrocks LA (1998) Lipid peroxides in the free radical pathophysiology of brain diseases. *Cell Mol Neurobiol* 18(6):599–608
- Kim HY, Wang TC, Ma YC (1994) Liquid chromatography/mass spectrometry of phospholipids using electrospray ionization. *Anal Chem* 66(22):3977–3982
- Ikeda H, Ohkawa R, Watanabe N, Nakamura K, Kume Y, Nakagawa H, Yoshida H, Okubo S, Yokota H, Tomiya T, Inoue Y, Nishikawa T, Ohtomo N, Tanoue Y, Koike K, Yatomi Y (2010) Plasma concentration of bioactive lipid mediator sphingosine 1-phosphate is reduced in patients with chronic hepatitis C. *Clin Chim Acta Int J Clin Chem* 411(9–10):765–770
- Dugo P, Favoino O, Tranchida PQ, Dugo G, Mondello L (2004) Off-line coupling of non-aqueous reversed-phase and silver ion high-performance liquid chromatography-mass spectrometry for the characterization of rice oil triacylglycerol positional isomers. *J Chromatogr A* 1041(1–2):135–142
- Prestwich GD (2005) Visualization and perturbation of phosphoinositide and phospholipid signaling. *Prostaglandins Other Lipid Mediat* 77(1–4):168–178
- Anderson HA, Maylock CA, Williams JA, Paweletz CP, Shu H, Shacter E (2003) Serum-derived protein S binds to phosphatidylserine and stimulates the phagocytosis of apoptotic cells. *Nat Immunol* 4(1):87–91
- Wenk MR (2005) The emerging field of lipidomics. *Nat Rev Drug Discov* 4:594–610
- Wenk MR (2010) Lipidomics: new tools and applications. *Cell* 143(6):888–895
- Prescott SM, Zimmerman GA, Stafforini DM, McIntyre TM (2000) Platelet-activating factor and related lipid mediators. *Annu Rev Biochem* 69:419–445
- Yost CC, Weyrich AS, Zimmerman GA (2010) The platelet activating factor (PAF) signaling cascade in systemic inflammatory responses. *Biochimie* 92(6):692–697
- Farbiszewski R, Dudek H, Skrzydlewska E, Lewko J (2002) The role of platelet activating factor (PAF) in physiology and pathology of the central nervous system. *Neurol Neurochir Pol* 36(4):801–808
- Demisch L, Gerbaldo H, Gebhart P, Georgi K, Bochnik HJ (1987) Incorporation of ^{14}C -arachidonic acid into platelet phospholipids of

- untreated patients with schizophreniform or schizophrenic disorders. *Psychiatry Res* 22(4):275–282
14. Sugatani J, Fujimura K, Miwa M, Satouchi K, Saito K (1991) Molecular heterogeneity of platelet-activating factor (PAF) in rat glandular stomach determined by gas chromatography/mass spectrometry. PAF molecular species changes upon water-immersion stress. *Lipids* 26(12):1347–1353
 15. Whitehead SN, Hou W, Ethier M, Smith JC, Bourgeois A, Denis R, Bennett SA, Figeys D (2007) Identification and quantitation of changes in the platelet activating factor family of glycerophospholipids over the course of neuronal differentiation by high-performance liquid chromatography electrospray ionization tandem mass spectrometry. *Anal Chem* 79(22):8539–8548
 16. Surles JR, Wykle RL, O'Flaherty JT, Salzer WL, Thomas MJ, Snyder F, Piantadosi C (1985) Facile synthesis of platelet-activating factor and racemic analogues containing unsaturation in the sn-1-alkyl chain. *J Med Chem* 28(1):73–78
 17. Wientzek M, Arthur G, Man RY, Choy PC (1985) A sensitive method for the quantitation of lysophosphatidylcholine in canine heart. *J Lipid Res* 26(9):1166–1169
 18. Mitoma J, Kasama T, Furuya S, Hirabayashi Y (1998) Occurrence of an unusual phospholipid, phosphatidyl-L-threonine, in cultured hippocampal neurons. Exogenous L-serine is required for the synthesis of neuronal phosphatidyl-L-serine and sphingolipids. *J Biol Chem* 273(31):19363–19366
 19. Cao HC, Chen XM, Xu W (2005) Determination of platelet-activating factor by reverse phase high-performance liquid chromatography and its application in viral hepatitis. *World J Gastroenterol* 11(46):7364–7367
 20. Daniil G, Phedonos AA, Holleboom AG, Motazacker MM, Argyri L, Kuivenhoven JA, Chroni A (2011) Characterization of antioxidant/anti-inflammatory properties and apoA-I-containing subpopulations of HDL from family subjects with monogenic low HDL disorders. *Clin Chim Acta Int J Clin Chem* 412(13–14):1213–1220
 21. Pulfer M, Murphy RC (2003) Electrospray mass spectrometry of phospholipids. *Mass Spectrom Rev* 22(5):332–364
 22. Ham BM, Cole RB, Jacob JT (2006) Identification and comparison of the polar phospholipids in normal and dry eye rabbit tears by MALDI-TOF mass spectrometry. *Invest Ophthalmol Vis Sci* 47(8):3330–3338
 23. Mulvenna I, Stapleton F, Hains PG, Cengiz A, Tan M, Walsh B, Holden B (2000) Low molecular weight analysis of tears using matrix assisted laser desorption ionization-time of flight mass spectrometry. *Clin Exp Ophthalmol* 28(3):205–207
 24. Harrison KA, Clay KL, Murphy RC (1999) Negative ion electrospray and tandem mass spectrometric analysis of platelet activating factor (PAF) (1-hexadecyl-2-acetyl-glycerophosphocholine). *J Mass Spectrom* 34(4):330–335
 25. Savu SR, Silvestro L, Sorgel F, Montrucchio G, Lupia E, Camussi G (1996) Determination of 1-O-acyl-2-acetyl-sn-glycerol-3-phosphorylcholine, platelet-activating factor and related phospholipids in biological samples by high-performance liquid chromatography–tandem mass spectrometry. *J Chromatogr B Biomedicinal Appl* 682(1):35–45
 26. Owen JS, Wykle RL, Samuel MP, Thomas MJ (2005) An improved assay for platelet-activating factor using HPLC-tandem mass spectrometry. *J Lipid Res* 46(2):373–382
 27. Ho YP, Huang PC, Deng KH (2003) Metal ion complexes in the structural analysis of phospholipids by electrospray ionization tandem mass spectrometry. *Rapid Commun Mass Spectrom* 17(2):114–121
 28. Hsu FF, Turk J (2005) Studies on phosphatidylserine by tandem quadrupole and multiple stage quadrupole ion-trap mass spectrometry with electrospray ionization: structural characterization and the fragmentation processes. *J Am Soc Mass Spectrom* 16(9):1510–1522
 29. Yoo HJ, Hakansson K (2011) Determination of phospholipid regiochemistry by Ag(I) adduction and tandem mass spectrometry. *Anal Chem* 83(4):1275–1283
 30. Xu H, Valenzuela N, Fai S, Figeys D, Bennett SA (2013) Targeted lipidomics—advances in profiling lysophosphocholine and platelet-activating factor second messengers. *FEBS J* 280(22):5652–5667
 31. Kishimoto T, Soda Y, Matsuyama Y, Mizuno K (2002) An enzymatic assay for lysophosphatidylcholine concentration in human serum and plasma. *Clin Biochem* 35(5):411–416
 32. Sugatani J, Miwa M, Komiyama Y, Murakami T (1993) Quantitative analysis of platelet-activating factor in human plasma. Application to patients with liver cirrhosis and disseminated intravascular coagulation. *J Immunol Methods* 166(2):251–261
 33. Bligh EG, Dyer WJ (1959) A rapid method of total lipid extraction and purification. *Can J Biochem Physiol* 37(8):911–917
 34. Yang J, Schmelzer K, Georgi K, Hammock BD (2009) Quantitative profiling method for oxylipin metabolome by liquid chromatography electrospray ionization tandem mass spectrometry. *Anal Chem* 81(19):8085–8093
 35. Psychogios N, Hau DD, Peng J, Guo AC, Mandal R, Bouatra S, Sinelnikov I, Krishnamurthy R, Eisner R, Gautam B, Young N, Xia J, Knox C, Dong E, Huang P, Hollander Z, Pedersen TL, Smith SR, Bamforth F, Greiner R, McManus B, Newman JW, Goodfriend T, Wishart DS (2011) The human serum metabolome. *PLoS One* 6(2):e16957
 36. Dong J, Cai X, Zhao L, Xue X, Zou L, Zhang X, Liang X (2010) Lysophosphatidylcholine profiling of plasma: discrimination of isomers and discovery of lung cancer biomarkers. *Metabolomics* 6:478–488
 37. Hong J, Kim YH, Gil JH, Cho K, Jung JH, Han SY (2002) Structural determination of hexadecanoic lysophosphatidylcholine regioisomers by fast atom bombardment tandem mass spectrometry. *Rapid Commun Mass Spectrom* 16(22):2089–2093
 38. Barwick VJ (1999) Sources of uncertainty in gas chromatography and high-performance liquid chromatography. *J Chromatogr A* 849:13–33
 39. Ahmad I, Suits F, Hoekman B, Swertz MA, Byelas H, Dijkstra M, Hoofit R, Katsubo D, van Breukelen B, Bischoff R, Horvatovich P (2011) A high-throughput processing service for retention time alignment of complex proteomics and metabolomics LC-MS data. *Bioinformatics* 27(8):1176–1178
 40. Katajamaa M, Miettinen J, Oresic M (2006) MZmine: toolbox for processing and visualization of mass spectrometry based molecular profile data. *Bioinformatics* 22(5):634–636
 41. Katajamaa M, Oresic M (2005) Processing methods for differential analysis of LC/MS profile data. *BMC Bioinforma* 6:179
 42. Tautenhahn R, Patti GJ, Rinehart D, Siuzdak G (2012) XCMS Online: a web-based platform to process untargeted metabolomic data. *Anal Chem* 84(11):5035–5039
 43. Han XL, Gross RW (1996) Structural determination of lysophospholipid regioisomers by electrospray ionization tandem mass spectrometry. *J Am Chem Soc* 118(2):451–457