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Determination of 1-O-acyl-2-acetyl-*sn*-glyceryl-3-phosphorylcholine, platelet-activating factor and related phospholipids in biological samples by high-performance liquid chromatography–tandem mass spectrometry

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Abstract

Combining normal-phase HPLC separation and tandem mass spectrometric detection, using an ion-spray HPLC–MS interface, a quantitative method for acyl-platelet activating factor (acyl-PAF), platelet-activating factor (PAF) and related phospholipids was developed. Mass spectra, positive ions, showed intense $[M+H]^+$ ions; collision-induced dissociation of protonated molecular ions gave characteristic daughter ions corresponding to the polar head. Detection limits of 0.1–0.3 ng injected were obtained by multiple reaction monitoring. Samples of human endothelial cells treated with compounds modulating the levels of acyl-PAF and PAF have been analyzed by the present technique, proving that this approach is suitable for biochemical studies.

Keywords: 1-O-Acyl-2-acetyl-*sn*-glyceryl-3-phosphorylcholine; Platelet-activating factor; Phospholipids

1. Introduction

1 - O - Alkyl - 2 - acetyl - *sn* - glyceryl - 3 - phosphorylcholine [1] is a platelet-activating factor (PAF) with a broad spectrum of diverse and potent biological activities [2–4]. PAF belongs to a class of phospholipid mediators with complex functions in cellular biochemistry [2].

1 - O - Acyl - 2 - acetyl - *sn* - glyceryl - 3 - phosphorylcholine (acyl-PAF) derivatives, differing from PAF by the presence of an acyl instead of an alkyl in position 1 [5], are particularly interesting since they may compete for PAF receptors [6,7]. The determination of acyl-PAF is difficult; a biochemical assay with radiolabelled compounds is generally used, but it is quite complex [8,9]. A mass spectrometric (MS) method has been also described; however, an enzymatic degradation followed by derivatization is required, in order to permit a gas chromatographic separation of the phospholipids [10].

In a previous study, we described a more practical HPLC–MS method to quantify PAF and lyso-PAF in biological samples [11]; the evaluation of acyl-PAF was not considered. The biological relevance of this class of compounds prompted us to verify if acyl-

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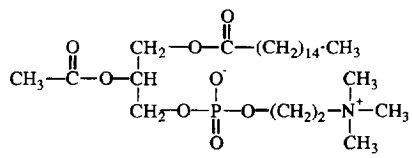
PAF can be measured by a similar analytical approach.

In this paper, we present an HPLC–MS–MS method for quantitation in biological samples of acyl-PAF, PAF, lyso-PAF and lyso-phosphatidylcholine (LPC) species, chemical formulae are reported in Fig. 1; an internal standard, the receptorial PAF antagonist, CV 3988 [12], has been introduced to compensate for the variability deriving from the extraction procedures.

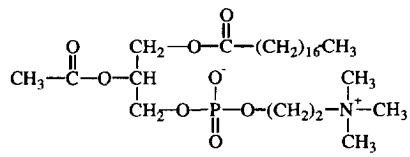
2. Experimental

2.1. Chemicals

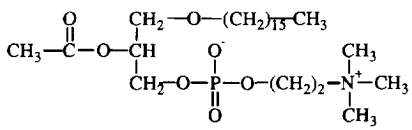
Lysophosphatidylcholine palmitoyl (C16-LPC), lysophosphatidylcholine stearoyl (C18-LPC), 1-O-hexadecyl-*sn*-glyceryl-3-phosphorylcholine (lyso-PAF), 1-O-hexadecyl-2-acetyl-*sn*-glyceryl-3-phosphorylcholine (C16-PAF) and 1-O-octadecyl-2-acetyl-*sn*-glyceryl-3-phosphorylcholine (C18-PAF)



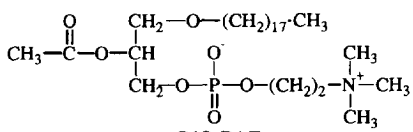
C16-ACYL-PAF



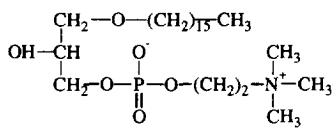
C18-ACYL-PAF



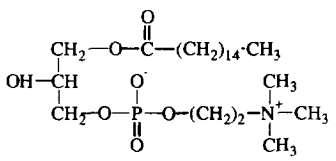
C16-PAF



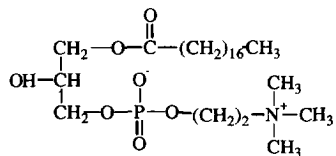
C18-PAF



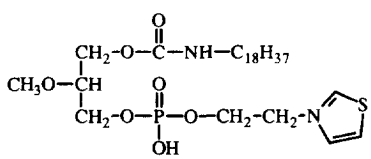
Lyso-PAF



C16-LPC



C18-LPC



CV 3988

Fig. 1. Structures of acyl-PAF, PAF, lyso-PAF, LPC and CV3988.

were purchased from Sigma (Deisenhofen, Germany).

1 - O - Hexadecyl - 2 - acetyl - *sn* - glyceryl - 3 - phosphorylcholine (C16-acyl-PAF) and 1 - O - octadecyl - 2 - acetyl - *sn* - glyceryl - 3 - phosphorylcholine (C18-acyl-PAF) were prepared by acetylation of C16-LPC and C18-LPC, respectively. Aliquots of each LPC (0.5 mg) were reacted with 0.1 ml of acetic anhydride (Aldrich, Steinheim, Germany) in the presence of 1 ml of pyridine (Fluka, Buchs, Switzerland) for 20 h at room temperature [13]. After lipid extraction by the method of Bligh and Dyer [14], acyl-PAF was purified by the same HPLC analytical method described in this paper.

The internal standard, (*RS*)-2-methoxy-3-(octadecylcarbamoyl-oxy)propyl-2-(3-thiazolo)ethylphosphate (CV 3988), was kindly provided by Takeda (Osaka, Japan).

All other chemicals, of the purest grade available, were purchased from Merck (Darmstadt, Germany); ultrapure water was obtained with a Milli-Q system (Millipore, Molsheim, France).

2.2. Standard solutions

Stock solutions, 1.0 mg/ml, of C16-PAF, C18-PAF, lyso-PAF, C16-LPC, C18-LPC, C16-acyl-PAF, C18-acyl-PAF and the internal standard, were prepared in water–methanol (20:80, v/v). These solutions were prepared freshly each month and stored at −20°C.

2.3. PAF extraction from human umbilical vein endothelial cells (HUVEC)

HUVEC were prepared, as previously described [15] by treating umbilical cord veins with collagenase. Cultures were performed in medium 199 containing 20% fetal calf serum, 50 mg/l porcine heparin (Sigma) and 50 mg/l endothelial cell growth factor (Sigma). HUVEC were biochemically characterized as previously described and experiments were carried out at the second or third passage. HUVEC were grown to confluence in 3.5-cm-diameter wells of cluster plates ($3.6\pm0.6\times10^5$ cells per well) and refed with the same medium every two days and 12 h before PAF synthesis experiments. Just before the start of these experiments, HUVEC monolayers were washed and incubated for 30 min with medium 199 without $NaHCO_3$ and containing 0.25% bovine serum albumin and 20 m*M* HEPES (pH 7.4); in some dishes thrombin (Sigma) was added (at 1 U/ml) 15 min before the end of this incubation. At the end of the incubation, the medium was removed, 33 ng of internal standard (stock solution 1 mg/ml in chloroform) were added per dish, and cells were scraped with a rubber policeman in 1 ml of methanol containing 50 mmol/l of formic acid, wells were washed with other 2 ml of acidified methanol. Lipids were then extracted from cells according to the Bligh and Dyer technique [14]; after extraction, the volumes of chloroform–methanol–water were adjusted to 1:1:0.9 (v/v) to obtain phase separation, and the chloroform rich phase was retained [16]. This fraction, after concentration under a nitrogen stream, was then subjected to thin-layer chromatography (TLC) on pre-coated silica gel plates 60F254 (Merck), using chloroform–methanol–water (65:35:6, v/v) [16] as the solvent system. Subsequently, the area of each plate where acyl-PAF, PAF, lyso-PAF and LPC migrate, as tested with real standards of the compounds run in parallel on the same plate, was scraped and the lipid recovered from silica [16]; the extracts were then evaporated under nitrogen. Finally, samples were redissolved in 0.25 ml of mobile phase before injection.

2.4. Chromatographic separations

HPLC separations were carried out with an Hitachi-Merck L-6200 A pump (Tokyo, Japan) and a Model 231-401 Gilson autosampler (Villiers Le Bel, France). A normal-phase silica column (μPorasil 250×4.6 mm I.D., 5-μm, Millipore, Waters, Milford, MA, USA) was used and eluted, under isocratic conditions at 1.0 ml/min, with a mobile phase [17] composed of chloroform–methanol–water (60:55:5, v/v). To reduce the mobile phase flow-rate to a level acceptable for the MS system (above 50 μl/min), post-column splitting was performed by connecting a piece of silica capillary of adequate length to the splitting port of the MS interface. The samples, both the standards and the lipids recovered after TLC, were injected dissolved in the mobile phase using an injection volume of 250 μl and a 200-μl sample loop.

2.5. Mass spectrometry

Mass spectrometric analyses were performed on a Perkin-Elmer-Sciex (Thornhill, Canada) API III-Plus triple quadrupole mass spectrometer, equipped with an atmospheric pressure articulated ion-spray source. High-purity nitrogen served both as the nebuliser gas, operative pressure was 0.5 MPa, and curtain gas, flow-rate was 0.8 l/min; argon was used as the target gas for the MS–MS experiments, at a collision gas thickness of $3 \cdot 10^{15}$ atoms/cm^2.

To set up the technique, mass spectra, scanning range m/z 100–1000 positive ions, were obtained by direct infusion, at 50 μl/min with a Harvard Apparatus Model 11 syringe pump (Southnatick, MA, USA), of phospholipid standard solutions diluted 1:200 in mobile phase. Then, under MS–MS conditions, positive mode, product ion spectra were obtained by collision-induced dissociation (CID) of precursor ions with m/z values corresponding to $[M+H]^+$ ions of different phospholipids.

Following these preliminary experiments, the ion-spray needle voltage was set at 5 kV, the orifice voltage at 50 V and the MS–MS collision energy at 25 V, which were optimal parameters for these analytes. Finally, quantitative analysis was performed in multiple reaction monitoring (MRM) using the following characteristic reactions: 538→184 (C16-acyl-PAF), 566→184 (C18-acyl-PAF), 524→184 (C16-PAF), 552→184 (C18-PAF), 482→184 (Lyso-PAF), 496→184 (C16-LPC), 524→184 (C18-LPC) and 594→210 (CV 3988, internal standard).

2.6. Calibration and quantitation procedures

To determine the limits of sensitivity and for quantitative analyses, calibration curves were prepared following the extraction procedure described in Section 2.3. The spiked samples were prepared from aliquots (250 μl) of a 1% albumin solution in water added with C16-acyl-PAF, C18-acyl-PAF, C16-PAF,

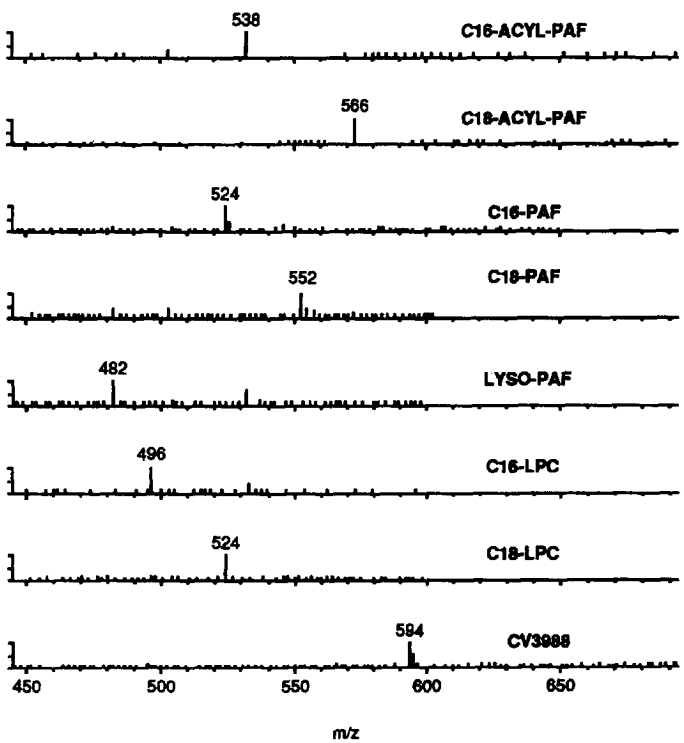


Fig. 2. MS spectra, positive ions, obtained by direct injection of standards dissolved in mobile phase at 5 μg/ml, C16-acyl-PAF C18-acyl-PAF, C16-PAF, C18-PAF, lyso-PAF, C16-LPC, C18-LPC and CV 3988.

C18-PAF, lyso-PAF, C16-LPC and C18-LPC at concentrations of 0.1, 0.33, 1, 3.3, 10 and 33 ng each and 33 ng of internal standard in all samples. The albumin solution was preferred to HUVEC to avoid interferences in the calibration graphs from C16-acyl-PAF, C18-acyl-PAF, C16-PAF, C18-PAF, lyso-PAF, C16-LPC and C18-LPC physiologically present in the cells. The amount of proteins contained in these albumin solutions is similar to that of cell samples.

3. Results

Fig. 2 shows the mass spectra obtained by direct infusion of 5 μg/ml solutions of C16-acyl-PAF, C18-acyl-PAF, C16-PAF, C18-PAF, Lyso-PAF, LPC palmitoyl, LPC stearoyl and the internal standard, CV-3988; with all molecules the most intense ion had the m/z corresponding to the protonated molecular ion $[M+H]^+$. Fig. 3 shows the mass spectra of daughter ions obtained by CID of protonated ions from the same analytes as shown in Fig. 2, using

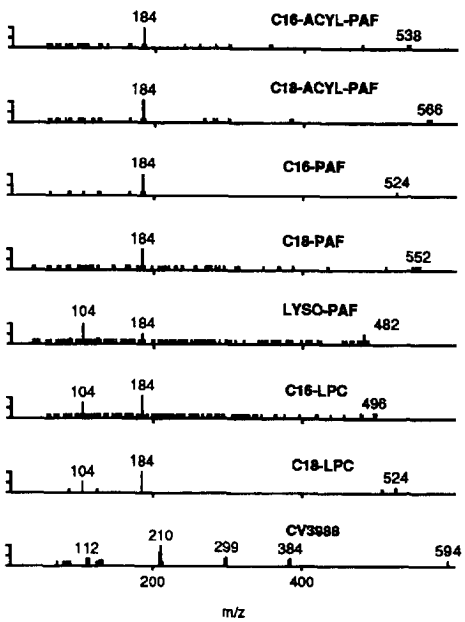


Fig. 3. MS–MS spectra, daughter ions, from CID of the protonated molecular ions of C16-acyl-PAF, C18-acyl-PAF, C16-PAF, C18-PAF, lyso-PAF, C16-LPC, C18-LPC and CV 3988. Samples were infused as indicated in Fig. 2.

identical conditions of direct infusion. In all these spectra, with the exception of the internal standard, the most intense daughter ion had m/z 184 corresponding to the mass of phosphorylcholine; in the case of the internal standard, an intense ion corresponding to the polar head of this compound was observed at m/z 210. In the spectra of lyso derivatives (lyso-PAF, C16-LPC, C18-LPC) another intense product ion had m/z 104. This ion corresponds to the choline fragment that is preferentially released in phospholipids with a hydroxyl group in position 2, favoring the formation of a cyclic derivative with the phosphate of phosphocholine.

Fig. 4 reproduces the chromatograms obtained in MRM by injection of a mixture of phospholipid standards and internal standard dissolved together in mobile phase at a concentration of 120 ng/ml (amount injected was 24 ng of each). It can be observed that C16- and C18-acyl-PAF elute first, close to C18- and C16-PAF. C16- and C18-LPC are more retained and an optimal separation between the isobaric compounds C16-PAF and C18-LPC has been obtained; the presence of the product ion m/z 104 in the last eluting peak permitted an unambiguous identification as C18-LPC.

Fig. 5 displays chromatograms, obtained in MRM, from an extracted spiked albumin solution containing 333 pg of each analyte and 33 ng of the internal standard. All analytes can be observed and the retention times are the same as previously observed by injecting the unextracted standard.

Calibration graphs obtained by analyzing extracted samples spiked with different amounts of analytes, normalized by the internal standard, showed a very good linearity in the whole range of tested concentrations with regression coefficients always $\geq$0.995; the results are summarized in Table 1.

Considering a signal-to-noise ratio of 3 as the limit of sensitivity, all analytes, except lyso-PAF, can be adequately analyzed in the sample spiked with 100 pg, while with lyso-PAF the lower limit is 333 pg, as can be observed in Fig. 5.

The recovery of these analytes by the current extraction was already evaluated in previous reports, by means of radiolabelled compounds [10,11,17], therefore no other assays have been performed here to verify this procedure again. However, comparisons made between unextracted and extracted stan-

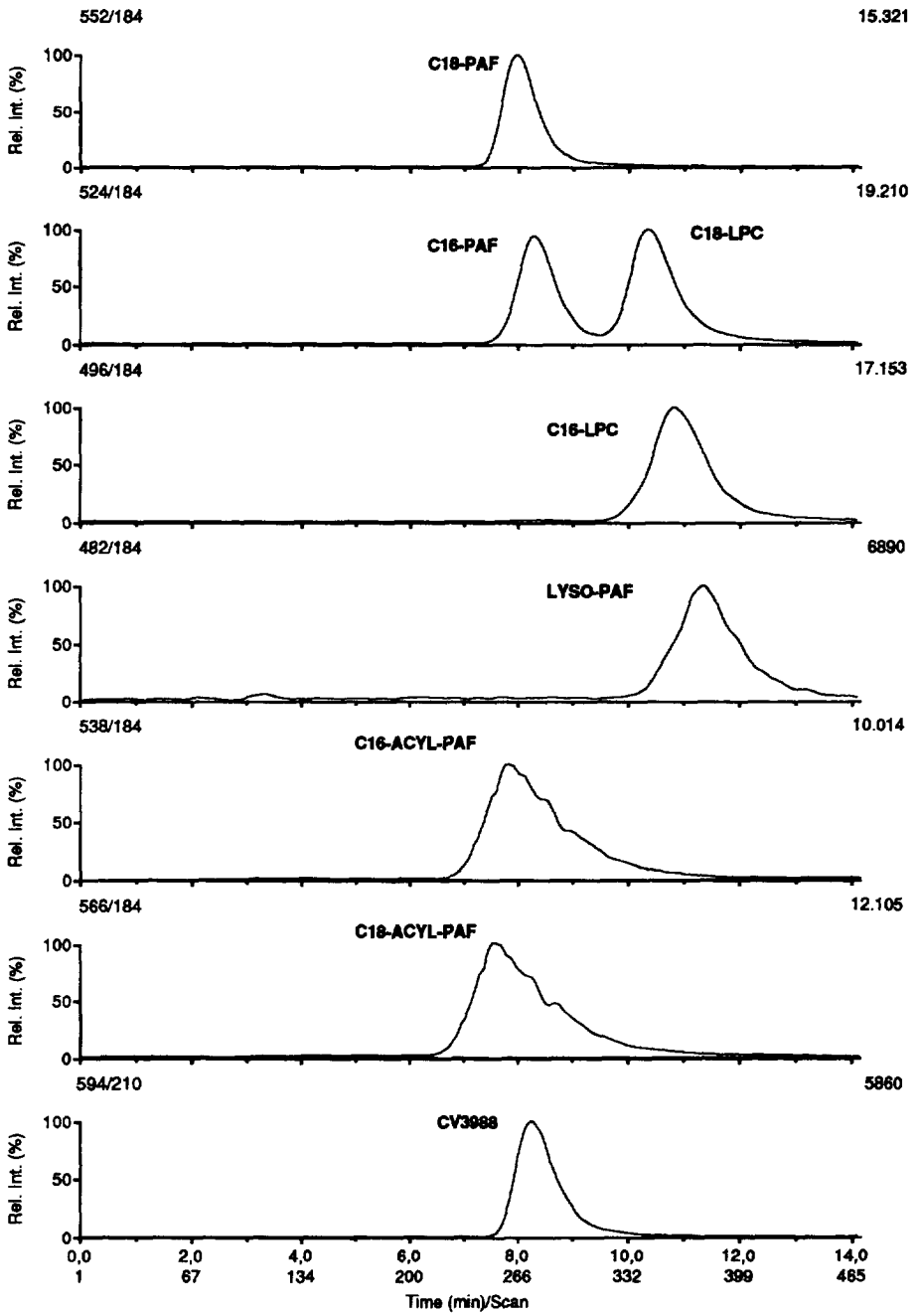


Fig. 4. Chromatographic traces by MRM detection at m/z (parent→daughter) 552→184, 524→184, 496→184, 482→184, 538→184, 566→184, 594→210 (corresponding respectively to C18-PAF, C16-PAF and C18-LPC, C16-LPC, lyso-PAF, C16-acyl-PAF, C18-acyl-PAF, CV 3988), obtained by injection of a standard containing C16-acyl-PAF, C18-acyl-PAF, C16-PAF, C18-PAF, lyso-PAF, C16-LPC, C18-LPC and CV 3988 (24 ng each).

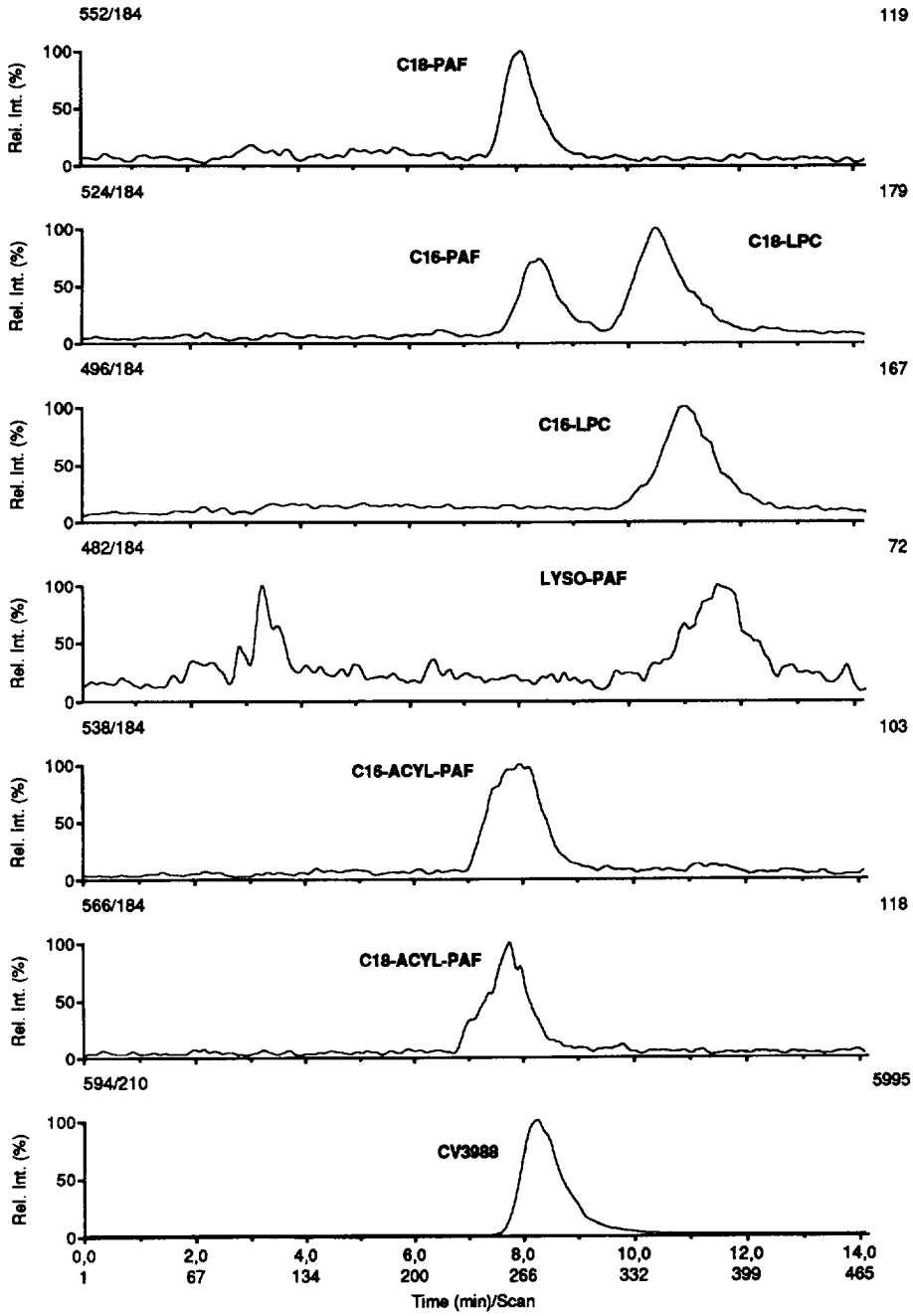


Fig. 5. Chromatographic traces by MRM detection, with the MS parameters reported in Fig. 4, obtained by injection of an extracted sample spiked with 333 pg each of C16-acyl-PAF, C18-acyl-PAF, C16-PAF, C18-PAF, lyso-PAF, C16-LPC and C18-LPC and with 33 ng of CV 3988.

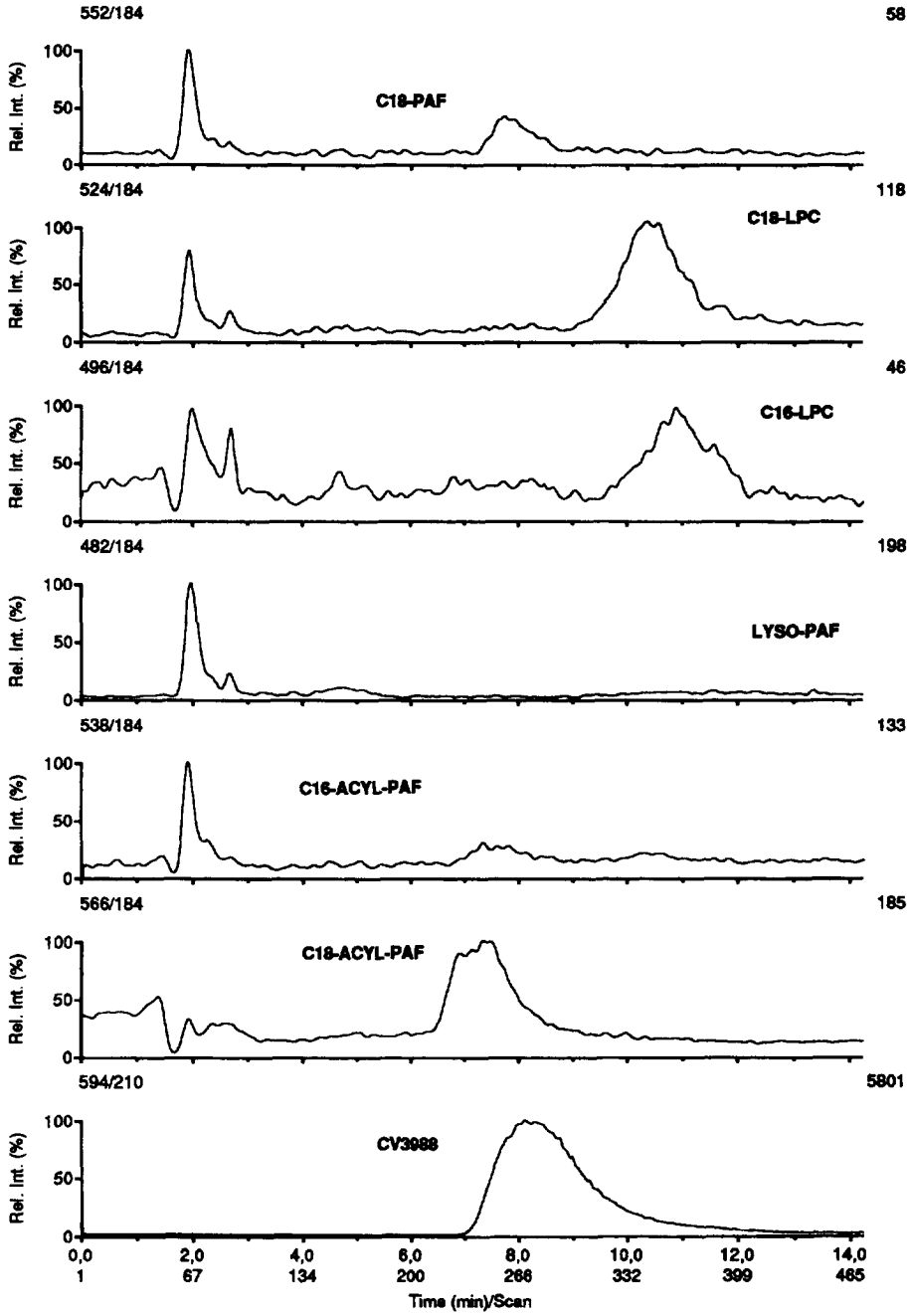


Fig. 6. Chromatographic traces by MRM at *m/z* values corresponding to C16-acyl-PAF, C18-acyl-PAF, C16-PAF, C18-PAF, lyso-PAF, C16-LPC, C18-LPC and CV 3988 on lipid extracts from cellular pellets of unstimulated HUVEC.

Table 1
Linear regressions, internal standard peak-areas ratios versus amounts of analytes added to samples, estimated on MRM analysis of a series ($n=3$) of extracted spiked samples. Parameters a and b of the equation $y=a+bx$, reported as mean±R.S.D., and correlation coefficients (r)

Compound	a	b	r
C16-acyl-PAF	0.0628±0.0015	0.1564±0.0068	0.99471
C18-acyl-PAF	0.0054±0.0003	0.1745±0.0053	0.99915
C16-PAF	0.1052±0.0073	0.2201±0.0048	0.99578
C18-PAF	0.0609±0.0002	0.1909±0.0063	0.99724
Lyso-PAF	0.0073±0.0001	0.0722±0.0011	0.99891
C16-LPC	0.1166±0.0033	0.2029±0.0037	0.99527
C18-LPC	0.1489±0.0101	0.2282±0.0039	0.99478

dards confirmed an extraction efficiency >90%; the mean recovery of the internal standard, calculated in ten spiked samples and ten real samples, was 90.7±3.3% and 91.5±3.8%, respectively.

Assays performed on non-stimulated endothelial cells and stimulated endothelial cells are reported in Fig. 6 and Fig. 7, respectively.

In the results of the unstimulated HUVEC, a relevant peak, corresponding to C18-acyl-PAF, can be observed, while only a minimal amount of C18-PAF is present. After stimulation, the situation changes completely: acyl-PAFs are undetectable and both C18- and C16-PAF increased significantly. LPCs are present in both samples in similar amounts while lyso-PAF is always undetectable.

The concentrations of different phospholipids observed in replicate ($n=6$) experiments, performed as in Fig. 6 and Fig. 7, from HUVEC obtained from the same source and cultured in parallel are summarized in Table 2. The mean concentrations of PAF are similar to those reported in the literature and measured by the bioassay [18].

Table 2
Net amounts of cell-associated C16-acyl-PAF, C18-acyl-PAF, C16-PAF, C18-PAF, lyso-PAF, C16-LPC and C18-LPC in unstimulated ($n=6$) and stimulated ($n=6$) HUVEC samples as determined by HPLC–MS–MS

Compound	Amount (mean±S.D.) (ng/10^6 cells)	
	Unstimulated	Stimulated
C16-acyl-PAF	0.18±0.016	N.E.[a]
C18-acyl-PAF	1.41±0.083	N.E.
C16-PAF	N.E.	0.38±0.021
C18-PAF	0.28±0.019	1.48±0.032
Lyso-PAF	N.E.	N.E.
C16-LPC	0.25±0.016	0.27±0.013
C18-LPC	0.65±0.031	0.68±0.026

[a] N.E. = Not evaluable concentrations

These results provide evidence of another relevant aspect of this method. The HPLC–MS–MS analyses have been performed on three different working days using different calibration graphs; the C.V. is below 10%, showing that this technique has good reproducibility even with real samples presenting a biological variability.

4. Discussion

The MS spectra of Fig. 2 prove that an effective ionization of these phospholipids can be obtained also in normal phase; the more relevant ions are always protonated pseudomolecular ions and fragmentations cannot be observed.

The MS–MS spectra in Fig. 3 show that an effective fragmentation can be induced in these compounds, as proved by the low intensity of the parent ions. In our previous study, carried out with an API III PE-Sciex mass spectrometer instead of an API III-Plus instrument, the parent ions were more intense as a result of the less effective fragmentation typical of the collision cell used in the API III instrument. The fragmentation of lyso-PAF was particularly critical in the past and the fragment at m/z 104, characteristic of lyso-phospholipids, was not observed. As a consequence of the improved fragmentation in the new instrument, the analytical sensitivity, using MRM, has been clearly increased with all compounds, including lyso-PAF.

The decision to apply a normal-phase separation instead of a reversed-phase method was largely suggested by the need to separate the isobaric compounds C18-LPC and PAF-C16. In the past, we used polymorphonuclear neutrophils (PMNs) as a model of study and the low levels of LPC present in those samples enabled us to avoid the TLC purification step, and the separation of C18-LPC from C16-PAF attained in reversed-phase was enough to obtain correct quantitation of C16-PAF. However, in HUVEC samples, high amounts of LPC were present and an optimal separation, like that achieved by

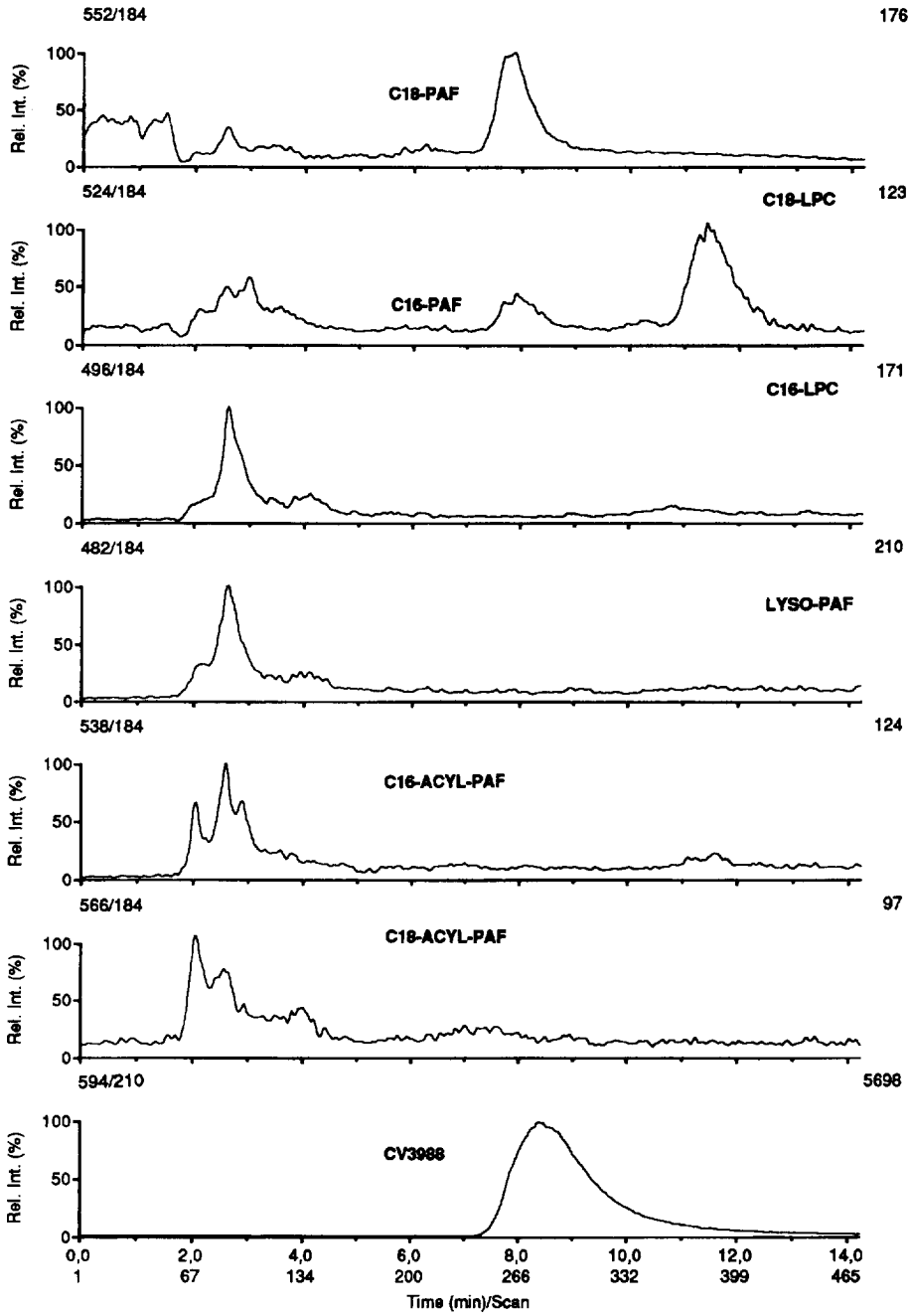


Fig. 7. Chromatographic traces by MRM, at *m/z* values corresponding to C16-acyl-PAF, C18-acyl-PAF, C16-PAF, C18-PAF, lyso-PAF, C16-LPC, C18-LPC and CV 3988, on lipid extracts from cellular pellets of thrombin-stimulated HUVEC, 1 U/ml for 15 min.

normal-phase chromatography, was particularly important to reach reliable quantitative results.

In the present study, we preferred to maintain the TLC purification step to avoid column overloading from other lipids present in our biological samples and to improve the sample purification; extraction on a disposable silica column was previously described [11], however, the sample clean-up was less efficient. Concerning interferences from other lipids, sphingomyelins are a group of phospholipids that are not adequately separated by the different purification steps, solvent extraction or by TLC purification, and, having a phosphocholine as the polar group, may interfere in the analysis. In reversed-phase chromatography, these compounds eluted last and, if present at high concentrations, an adequate interval between injections was required. In normal-phase chromatography, with the present analytical conditions, sphingomyelins elute early, between 4 and 6 min, without interference with the other compounds.

The results obtained on real samples prove that this HPLC–MS–MS method has adequate sensitivity to measure acyl-PAF, PAF and related phospholipids at the concentrations present in biological samples. The increase of PAF levels, measured after stimulation with thrombin, was similar to the results reported by other authors [8,10]. In apparent contrast with these authors [8,10], acyl-PAF decreased following thrombin treatment. However, in the present study, a different incubation time with thrombin was used. The reproducibility observed in these experiments shows that this technique is suitable for biochemical studies requiring quantitation of acyl-PAF and PAF.

With the exception of MS methods, no other techniques have been proposed at present to detect acyl-PAF and PAF in biological samples at the same time. Regarding other MS techniques, the approach by GC–MS [10] has been used more often. However, it requires a complex sample preparation including an enzymatic hydrolysis to remove the polar head. This last step may introduce analytical interferences from other phospholipids having a different cationic group.

In conclusion, this new HPLC–MS–MS method permits a direct analysis of acyl-PAF and related phosphocholine derivatives in a single HPLC run with high specificity. Thus, it is a useful tool for investigating the biochemistry of acyl-PAF and PAF in biological systems.

Acknowledgments

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