*Journal of Chromatography*, 647 (1993) 261–269
Elsevier Science Publishers B.V., Amsterdam

CHROMSYMP. 2862

# Development of a high-performance liquid chromatographic–mass spectrometric technique, with an ionspray interface, for the determination of platelet-activating factor (PAF) and lyso-PAF in biological samples

L. Silvestro* and R. Da Col

*Res Pharma Pharmacological Research Srl, Via Belfiore 57, 10125 Turin (Italy)*

E. Scappaticci and D. Libertucci

*Servizio di Fisiopatologia Respiratoria e Broncologia, Ospedale San Giovanni, Turin (Italy)*

L. Biancone and G. Camussi

*Laboratorio di Immunopatologia, Cattedra di Nefrologia, Università di Torino e Cattedra di Nefrologia Sperimentale, Dipartimento di Biochimica e Biofisica, I Facolta di Medicina e Chirurgia, Naples (Italy)*

ABSTRACT

An HPLC–mass spectrometric technique with an ionspray interface was developed for the determination of platelet-activating factor (PAF) and PAF-related compounds in biological samples. HPLC separations were performed using a reversed-phase column. The mass spectra showed intense $[M + H]^+$ ions. Collision-induced dissociation of protonated molecular ions gave characteristic daughter ions corresponding to the phosphorylcholine group. By selective-ion monitoring, a detection limit of 0.3 ng was obtained for all molecules; by multiple reaction monitoring, the same sensitivity was achieved for PAF whereas for lyso-PAF the limit was 3 ng. Finally, PAF was comparatively determined by bioassay and HPLC–MS after extraction from the cell pellets and the supernatants of human polymorphonuclear neutrophils unstimulated or stimulated with opsonized zymosan. The good correlation observed between these techniques indicated the reliability of HPLC–MS for biochemical studies on PAF and PAF-related molecules.

## INTRODUCTION

Platelet-activating factor (PAF) is a lipid chemical mediator of inflammation with a broad spectrum of diverse and potent biological activities [1–3]. In addition to its activity on platelets [4], PAF promotes the aggregation, chemotaxis and granule secretion of polymorphonuclear neutrophils (PMN), eosinophils and monocytes [5]. Moreover, PAF enhances vascular permeability and adhesion of PMN to endothelial cells, leading oedema formation and leukocyte accumulation [1–3,5].

On the basis of its multiple biological activities, of its synthesis by a number of cell types involved in the development of inflammatory reaction and of the effect of PAF receptor

* Corresponding author.

0021-9673/93/$06.00 

antagonists, it was postulated that PAF plays a critical role in the physiopathology of inflammation [5] and of endotoxic/septic shock [6,7].

PAF was characterized as an 1-O-alkyl-2-acetyl-*sn*-glyceryl-3-phosphorylcholine (AGEPC) [8], synthesized either via a "remodelling pathway" of membrane phospholipids through acetylation of 2-lyso-PAF generated from 1-O-alkyl-2-acyl-*sn*-glyceryl-3-phosphorylcholine by phospholipase A2 activity or via the "*de novo*" biosynthetic pathway that involves the synthesis of 1-O-alkyl-2-acetyl-*sn*-glycerol which is then converted into PAF by a unique cytidine diphospho (CDP)-choline:l-alkyl-2-acetyl-*sn*-glycerol choline phosphotransferase [9]. The inflammatory cells synthesize PAF mainly via the remodeling pathway [9–11]. The assay of PAF bioactivity performed after TLC and HPLC purification has been extensively used for the quantitative assay of PAF production because of its high sensitivity ($10^{-11}$ *M*) [10–13]. Normal-phase [14] and reversed-phase [15] HPLC have been demonstrated to be valuable techniques for the separation of different molecular species of PAF derived from biological samples. Indeed, it was recently found that PAF belongs to a class of structurally related mediators and that a number of different molecules, some with structures different from AGEPC, share PAF-like bioactivity [16]. Mass spectrometric methods such as GC–MS [17], fast atom bombardment (FAB) MS [18] and FAB-MS–MS [19] were instrumental for the definition of the chemical heterogeneity of PAF molecules. However, the application of these techniques for quantitative studies of PAF extracted from cells or biological fluids was limited by the complexity of these methods. An interesting HPLC–MS technique with thermospray ionization was also developed [20] but the sensitivity was inadequate for biological studies.

Recently, an HPLC system interfaced to a tandem triple-quadrupole mass spectrometer by an electrospray pneumatically assisted ionization source was successfully used for structural analysis and chemical characterization of PAF extracted from clinical samples [21,22]. The aim of this study was to optimize this HPLC–MS technique for the quantitative evaluation of PAF and related compounds in biological samples. For this purpose, cell-associated PAF and PAF released in the supernatant were extracted from human PMN unstimulated or stimulated with opsonized zymosan.

## EXPERIMENTAL

### *Chemicals*

1-O-Hexadecyl-*sn*-glyceryl-3-phosphorylcholine (lyso-PAF), 1-O-hexadecyl-2-acetyl-*sn*-glyceryl-3-phosphorylcholine ($C_{16}$-PAF) and 1-O-octadecyl-2-acetyl-*sn*-glyceryl-3-phosphorylcholine ($C_{18}$-PAF) were purchased from Bachem, Feinchemikalien (Bubendorff, Switzerland).

Deuterium-labelled ($^2H_3$) PAF ($d_3$-$C_{16}$-PAF) was prepared by acetylation of lyso-PAF in the presence of 1 ml of deuterated acetic-anhydride (Aldrich Chemie, Steinheim, Germany) and 1 ml of pyridine for 20 h at room temperature [23]. After lipid extraction by the method of Bligh and Dyer [14], $d_3$-$C_{16}$-PAF was purified by TLC and HPLC as described previously [21]. Acetonitrile and all other chemicals, of the purest grade available, were purchased from Fluka (Buchs, Switzerland); ultrapure water was obtained with a Milli-Q system (Millipore, Bedford, MA, USA).

### *Standard solutions*

Stock solutions of 2.5 mg/ml of $C_{16}$-PAF, $C_{18}$-PAF and lyso-PAF were prepared in water–methanol (20:80, v/v). The internal standard, $d_3$-$C_{16}$-PAF, was dissolved at 2.5 mg/ml in deuterated chloroform. These solutions were prepared freshly each month and stored at −20°C.

### *PAF extraction from human PMN*

Human PMN were prepared as described previously [10,11] and resuspended at $2.5 \cdot 10^6$/ml in Tris-buffered Tyrode's solution containing 0.25% of delipidated bovine serum albumin (BSA). Cells were stimulated at 37°C for 20 min with 0.2 mg/ml complement-activated zymosan. Lipids were extracted from the supernatant and cells according to Bligh and Dyer's technique [14]. After extraction, chloroform–methanol–water (1:1:0.9, v/v/v) was used for phase separation and the chloroform-rich phase was retained

[13]. The extracted lipids were divided into two samples to compare the PAF determined, after TLC and HPLC purification, by the bioassay and that determined by HPLC–MS. The first sample was submitted to TLC on silica gel plates 60 $F_{254}$ (Merck, Darmstadt, Germany) developed with chloroform–methanol–water (65:35:6, v/v/v) [13] and to HPLC on a μPorasil column (Millipore Chromatographic Division, Waters, Milford, MA, USA) eluted with chloroform–methanol–water (60:55:5, v/v/v) at a flow-rate of 1 ml/min as described previously [21]. The overall recovery of PAF, evaluated in parallel experiments by addition of 10 nCi of [$^3$H]$C_{16}$-PAF (New England Nuclear, NET 910; 30 Ci/m*M*) to the cells or supernatants followed by extraction as described above, was 90–95% [10,21]. After extraction and purification, the PAF bioactivity was determined by aggregometry using washed rabbit platelets in the presence of 10 μ*M* indomethacin, which inhibits cyclooxygenase, and of a creatinine phosphate (312.5 μg/ml)–creatinine phosphokinase (152.5 μg/ml) enzymatic system which converts ADP to ATP [10,11,21]. The specificity of PAF-induced aggregation was evaluated by inhibition with the PAF-receptor antagonists WEB 2170 and CV 3128 [21]. The amount of PAF was expressed in ng/ml and calculated from a calibration graph for synthetic PAF constructed for each test. PAF extracted from biological samples shared with synthetic $C_{16}$-PAF the same TLC and HPLC chromatographic patterns and physico-chemical characteristics such as inactivation by strong bases, resistance to acidic and weakly basic conditions [13,24] and inactivation by phospholipase A2 but not phospholipase A1 [25].

The second sample (0.5 ml of the chloroformic phase) was applied to a disposable silica column (Alltech, Milan, Italy; extra-clean silica column, 200 mg) as described [13], eluted sequentially with chloroform, acetone–methanol (1:1, v/v) and finally with chloroform–methanol (1:4, v/v) which retained PAF activity [13], and then submitted to HPLC–MS analysis.

In selected experiments, $d_3$-$C_{16}$-PAF was added to cells and supernatants (10 ng per sample) as an internal standard and then extracted as above.

*Chromatographic separations*

All the HPLC separations were performed with Applied Biosystems Model 140A HPLC syringe pumps and a Perkin-Elmer ISS-101 autosampler. In the first experiments we used reversed-phase chromatography [21,22], with a water–acetonitrile gradient containing trifluoroacetic acid (TFA), but we subsequently found that better results could be achieved with isocratic conditions, following the methods described by Kim and Salem [20]. Separations were obtained with a reversed-phase column (Phase Separations, Spherisorb $C_{18}$, 5 μm, 100 × 1 mm I.D.). The mobile phase was methanol–2-propanol–hexane–0.1 *M* aqueous ammonium acetate (100:10:2:5, v/v); the flow-rate was 50 ml/min.

*Mass spectrometry*

The effluent from the HPLC micropore column was connected to a PE-Sciex API III triple quadrupole mass spectrometer (Sciex, Thornhill, Canada) equipped with an atmospheric pressure articulated ionspray source. To set up the technique, analyses were previously performed scanning in the range *m/z* 100–600 in the positive-ion mode. Then, under MS–MS conditions, daughter ions, obtained by collision-induced dissociation (CID) of parent ions with *m/z* values corresponding to $[M+H]^+$ of PAF and PAF-related molecules, were acquired in the positive-ion mode; CID was optimized by collision with argon at a collision gas thickness of $2.7 \cdot 10^{12}$ atoms/cm$^2$ and at an impact energy of 70 eV. Finally, quantitative analyses were performed both by selective-ion monitoring (SIM), with *m/z* corresponding to protonated molecular ions, and by multiple reaction monitoring (MRM), following the reactions *m/z* 524→184, 552→184, 482→184 and 527→185, characteristic of $C_{16}$-PAF, $C_{18}$-PAF, lyso-PAF and $d_3$-$C_{16}$-PAF, respectively; the conditions of collision were the same as given previously.

*Calibration and quantification procedures*

Samples of unstimulated PMN, cellular or supernatant fractions, were spiked with $C_{16}$-PAF, $C_{18}$-PAF and lyso-PAF, using stock solutions containing 0.3, 1, 3, 30 and 100 ng of each

per sample; appropriate volumes of solvent [methanol–water (80:20, v/v)] were also added to ensure an equivalent total volume (0.5 ml) in each instance. In another series of spiked samples, to be analysed only by HPLC–MS, an internal standard was also added. Both series of standard additions were treated in the same way as the unknown samples. Calibration graphs, separately for $C_{16}$-PAF, $C_{18}$-PAF and lyso-PAF, were constructed as concentration *versus* peak area for these molecules. In the samples spiked with the internal standard the results were corrected by an extraction factor calculated from the internal standard areas. Calibration graphs for bioassay could measure only the PAF-like bioactivity without separating $C_{16}$-PAF from $C_{18}$-PAF and without evaluating lyso-PAF.

## RESULTS

Fig. 1A shows the mass spectra obtained from 100 ng each of $C_{16}$-PAF, lyso-PAF, $d_3$-$C_{16}$-PAF and $C_{18}$-PAF analysed, as described above, during an HPLC separation; with all molecules the most intense ion had the $m/z$ corresponding to the protonated molecular ion $[M+H]^+$. Fig.1B shows the mass spectra of daughter ions obtained by CID of protonated ions from the same molecules as in Fig. 1A using identical HPLC conditions. In these spectra the most intense daughter ion, with the exception of $d_3$-$C_{16}$-PAF, had $m/z$ 184 corresponding to the mass of phosphorylcholine; for $d_3$-$C_{16}$-PAF the most intense ion was shifted by 1 u, corresponding to the phosphorylcholine fragment with an increase in mass due to the redistribution of a deuterium atom within the molecule during the fragmentation process, as described previously [19].

Fig. 2A shows chromatograms obtained in the SIM mode for $m/z$ values corresponding to the $[M+H]^+$ of lyso-PAF, $C_{16}$-PAF, $d_3$-$C_{16}$-PAF and $C_{18}$-PAF; a mixture containing 10 ng of each molecule was injected. Under these mass spectrometric conditions the same sensitivity was

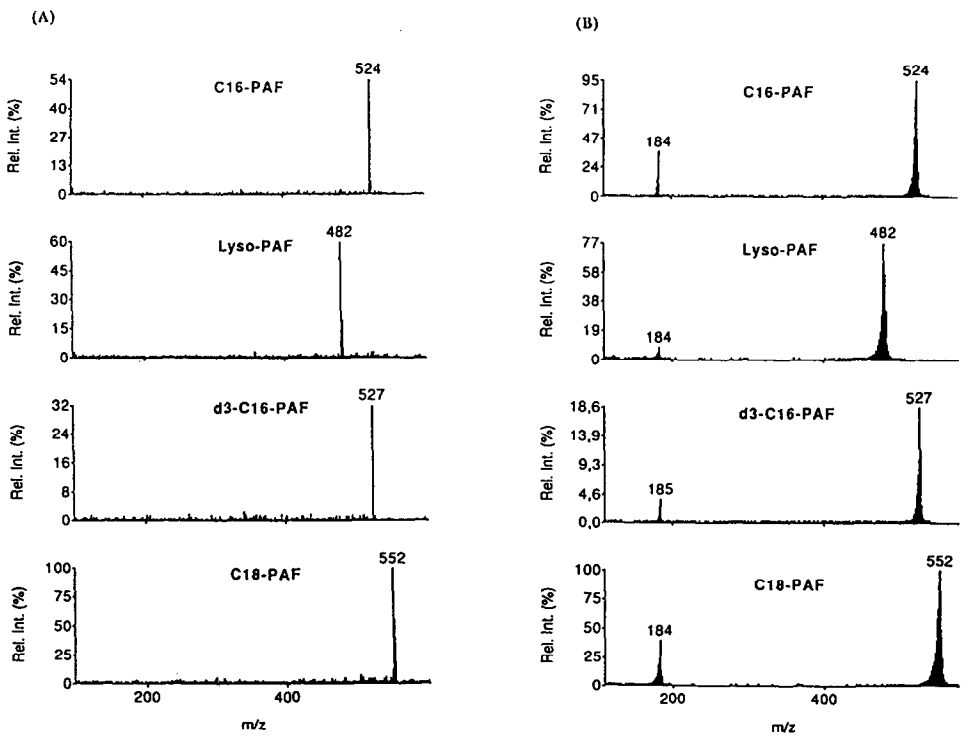


Fig. 1. (A) Mass spectra, positive-ion mode, obtained during an HPLC separation of a standard mixture containing $C_{16}$-PAF, lyso-PAF, $d_3$-$C_{16}$-PAF and $C_{18}$-PAF, 100 ng each. (B) Tandem mass spectra, daughter-ion mode, from CID of the protonated molecular ions of $C_{16}$-PAF, lyso-PAF, $d_3$-$C_{16}$-PAF and $C_{18}$-PAF.

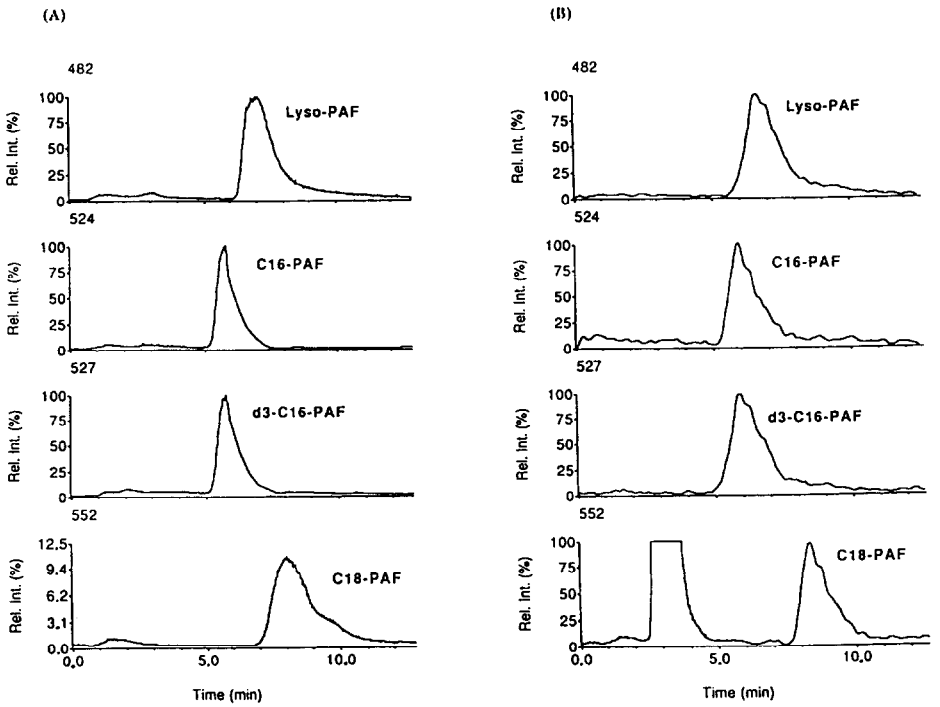


Fig. 2. Chromatographic traces, SIM at $m/z$ 482, 524, 527 and 552 (corresponding to lyso-PAF, $C_{16}$-PAF, $d_3$-$C_{16}$-PAF and $C_{18}$-PAF, respectively), obtained by injection of (A) a pure standard containing $C_{16}$-PAF, lyso-PAF, $d_3$-$C_{16}$-PAF and $C_{18}$-PAF (10 ng each) in comparison with (B) an analysis performed, with the same conditions, on a cellular PMN pellet spiked with 3 ng of each analyte and then extracted.

observed for each molecule. The separation obtained was sufficient to identify our analytes; if necessary, modifications of the mobile phase, as described by Kim and Salem [20], permitted (data not shown) the chromatographic resolution to be improved. By analysing samples of decreasing concentrations (data not shown), a detection limit of 0.3 ng, with a signal-to-noise ratio of 3:1, was obtained for all molecules.

Fig. 2B shows the chromatograms obtained with a sample of cellular pellet spiked with 3 ng each of lyso-PAF, $C_{16}$-PAF, $d_3$-$C_{16}$-PAF or $C_{18}$-PAF and extracted as described above. The retention times of PAF and PAF-related compounds were reproducible in both sample in Fig. 2A and B, showing that in spiked samples no relevant effects arose from compounds present in the biological samples. Only at $m/z$ 552, corresponding to $C_{18}$-PAF, was a peak from an interfering compound observed, but it was eluted early in the chromatogram. This peak was also detected in the blank cellular pellets, indicating that it is not derived from $C_{18}$-PAF added but rather from a component of the cellular extract.

Fig. 3 shows the regression lines of the peak areas, obtained with standard mixtures of different concentrations, to evaluate the analytical linearity for quantitative analysis by SIM (A) on pure standards or (B) on PMN pellets spiked with the same concentrations of $C_{16}$-PAF, $d_3$-$C_{16}$-PAF, $C_{18}$-PAF and lyso-PAF and then analysed after extraction and, finally, (C) by MRM on pure standards. Good linearity was observed with the different analytical conditions tested in a range of concentrations far below the amounts detectable in biological samples. A comparison of the areas in Fig. 3A and B confirmed the high efficiency of the extraction procedure; in fact, considering that only part of the extracted samples was injected, a mean recovery of 85–90% was calculated. The results obtained in MRM showed that the detection limits were different depending on the molecule; for $C_{16}$-PAF and the internal standard the detection limit was the

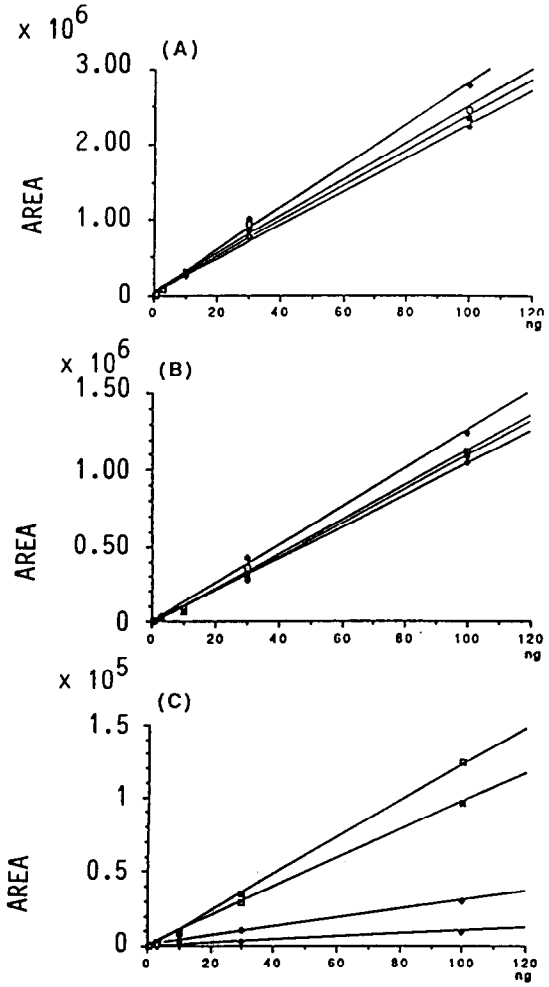


Fig. 3. Linear regressions, peak area *versus* amount of PAF and PAF-related compounds, estimated from SIM analysis of (A) pure standards □ = $C_{16}$-PAF ($R^2 = 0.994$); ◆ = lyso-PAF ($R^2 = 0.996$); ■ = $d_3$-$C_{16}$-PAF ($R^2 = 0.995$); ◇ = $C_{18}$-PAF ($R^2 = 0.998$); or (B) spiked samples: □ = $C_{16}$-PAF ($R^2 = 0.997$); ◆ = lyso-PAF ($R^2 = 0.996$); ■ = $d_3$-$C_{16}$-PAF ($R^2 = 0.997$); ◇ = $C_{18}$-PAF ($R^2 = 0.998$); and (C) MRM analysis of pure standards.

same as observed with SIM whereas with $C_{18}$-PAF and lyso-PAF the sensitivities were substantially lower (1.5 and 3 ng, respectively). Modifications of the CID conditions did not improve the fragmentation of these compounds, in accordance with MS–MS data reported elsewhere for lyso-PAF [26].

Fig. 4 shows the chromatograms obtained with SIM at $m/z$ corresponding to $C_{16}$-PAF and lyso-PAF from a sample of (A) stimulated or (B) unstimulated PMN pellet extracts. A peak corresponding to $C_{16}$-PAF was observed only in the sample of stimulated PMN whereas a small amount of lyso-PAF was present in both samples; $C_{18}$-PAF (data not reported) was undetectable in both samples. The results obtained by the analysis of the supernatants of the stimulated PMN are presented in Fig. 4C in comparison with the unstimulated sample Fig. 4D. Again, a significant peak of $C_{16}$-PAF but not of $C_{18}$-PAF was observed in the stimulated PMN sample.

Table I shows the net amounts of $C_{16}$-PAF and lyso-PAF detected by SIM, as cell-associated or as released in the supernatants, in unstimulated and stimulated PMN samples. These amounts are comparable to those observed by bioassay [11] or other MS techniques [19].

Fig. 5 presents the results obtained from the stimulated PMN samples, (A) cellular pellets and (B) supernatants, by MRM. Using this technique the presence of $C_{16}$-PAF was confirmed in both samples without any interfering peak from the background. In contrast, lyso-PAF, despite its presence as shown by SIM (Fig. 4), could not be quantified by MRM because of the lower sensitivity of this technique compared with SIM.

The linear regression analysis of the same samples determined by bioassay or by HPLC–MS using SIM, reported in Fig. 5C, gave a good correlation ($r = 0.979$) between the techniques, confirming the reliability of HPLC–MS for quantitative analysis of biological samples.

The mean recovery, evaluated by standard addition to the biological samples ($n = 15$) of $d_3$-$C_{16}$-PAF as internal standard, was $84 \pm 5.1\%$. These results confirmed the recovery evaluated by bioassay or [$^3$H]$C_{16}$-PAF as described under Experimental.

## DISCUSSION

The mass spectra in Fig. 1 show that good ionization can be obtained for PAF-related phospholipids; these findings are not unexpected considering the high polarity of the phosphorylcholine group. A molecular ion is always observed without relevant fragmentations in the source.

The CID spectra show a characteristic frag-

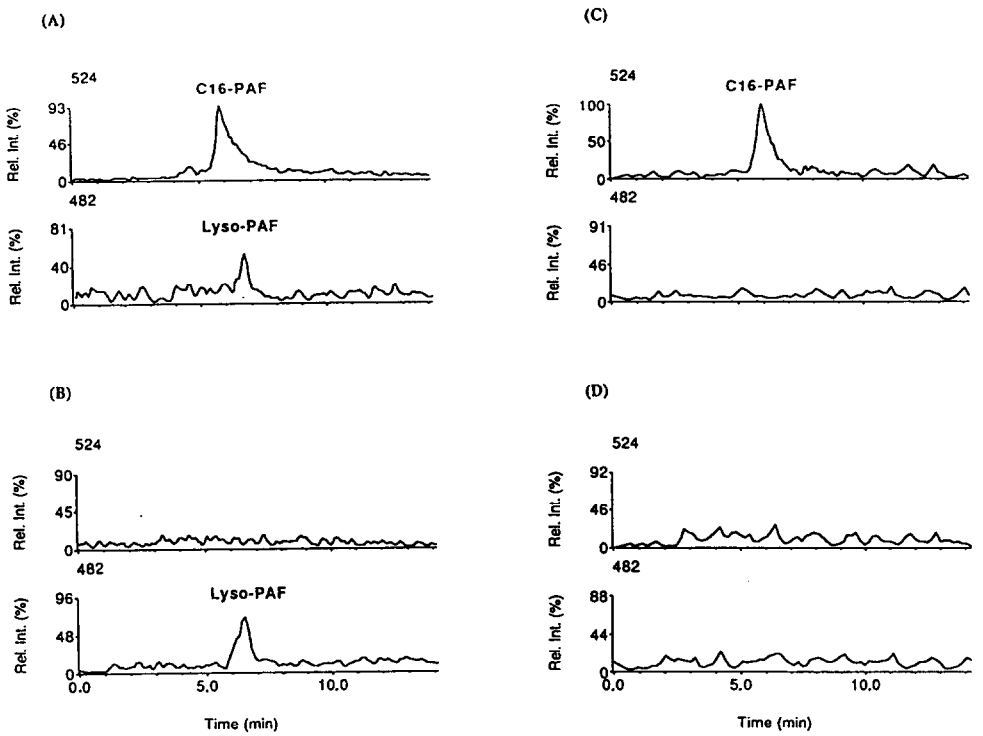


Fig. 4. Chromatographic traces obtained by SIM at $m/z$ values corresponding to $C_{16}$-PAF (524) or lyso-PAF (482) on cellular pellets of (A) stimulated or (B) unstimulated PMN and supernatants of (C) stimulated or (D) unstimulated PMN.

mentation giving an intense daughter ion corresponding to the phosphorylcholine group; the presence of such a typical product ion can be useful in detecting other uncommon PAF-related phospholipids, such as unsaturated or 1-acyl derivatives, performing acquisition under MS–MS conditions and monitoring the parent ions undergoing fragmentation with daughter ions of $m/z$ 184.

In previous experiments [21,27] we used a reversed-phase column eluted with a mobile phase gradient (water–acetonitrile containing

TABLE I

NET AMOUNTS OF $C_{16}$-PAF AND LYSO-PAF IN UNSTIMULATED ($n = 5$) AND STIMULATED ($n = 5$) PMN SAMPLES AS DETERMINED BY HPLC–MS AND SELECTIVE-ION MONITORING

| Sample | $C_{16}$-PAF | | Lyso-PAF | |
|---|---|---|---|---|
| | Cell-associated (ng)[a] | Released in the supernatant (ng)[b] | Cell-associated (ng)[a] | Released in the supernatant (ng)[b] |
| Unstimulated PMN | NE [c] | NE [c] | 1.1 ± 0.2 | NE [c] |
| Stimulated PMN | 6.1 ± 0.9 | 4.3 ± 0.7 | 0.6 ± 0.2 | NE [c] |

[a] $C_{16}$-PAF and lyso-PAF in PMN pellets ($10^6$ cells); mean ± standard deviation.
[b] $C_{16}$-PAF and lyso-PAF in PNM supernatants ($10^6$ cells); mean ± standard deviation.
[c] NE = Non-evaluable concentrations.

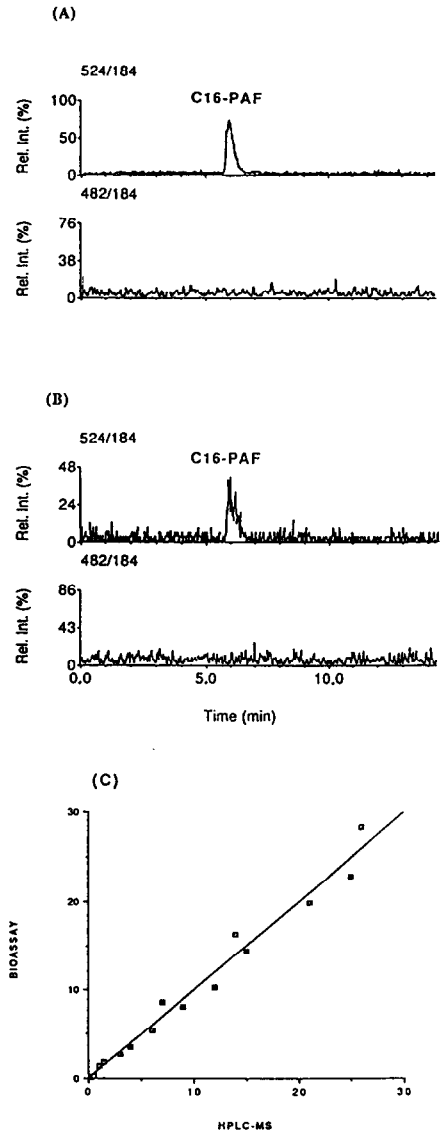


Fig. 5. Chromatographic traces obtained by MRM, following the fragmentations characteristic of $C_{16}$-PAF $m/z$ (524→184) and lyso-PAF ($m/z$ 482→184) on samples of (A) cellular pellets or (B) supernatants of stimulated PMN. (C) Regression line analysis comparing the results obtained by HPLC–MS or bioassay on the same samples: $y = -0.10277 + 0.99891x$; $R^2 = 0.979$.

TFA). Chromatographic runs of 30 min were necessary to obtain a reasonable separation; moreover, the acidic environment could degrade some PAF-related molecules. Normal-phase chromatography, tested in preliminary studies (unpublished data), was shown to enhance the separation but only provided that biological samples were submitted to an additional purification step by TLC to avoid rapid deterioration of the column. The reversed-phase method, described in this paper, gave good separations without the need for a gradient or pH conditions detrimental to the samples.

The use of an effective separative technique, according to Haroldsen and Gaskell [19], is particularly important for obtaining a correct determination of different PAF-related compounds having the same molecular mass.

Both the SIM and MRM modes showed good linearity of plots of peak area *versus* concentration within the range tested. The amount of PAF detected by bioassay in the biological samples was within 1–10 ng per sample. Despite the fentomole sensitivity of the bioassay, a widely accepted technique for PAF determination, the range of linearity was significantly lower than that obtained by mass spectrometry. Moreover, compounds without a PAF-like activity such as lyso-PAF could not be directly determined by bioassay but an additional step to generate active PAF was required [21]. Comparison of the results obtained from extraction of biological samples and those from pure standards clearly showed the absence of matrix interference in the ionization. Evaluations of the recovery of the extraction technique used confirmed the reliability of this method for both PAF-related molecules and the internal standard as previously reported [10,21]. The sensitivity observed with MRM on $C_{16}$-PAF suggests a possible application of this technique also for determinations in biological samples. In contrast, the low sensitivity observed for other PAF-related molecules, notably lyso-PAF, is a restriction for studies of low concentrations.

The peak of $C_{16}$-PAF produced by stimulated PMN was clearly detected using the SIM and MRM modes, both in the cellular fractions and in the supernatants; interferences from other molecules were never found. The HPLC–MS technique described here was applied in preliminary experiments to the characterization of PAF in broncoalveolar lavage [21] and cultured mesangial cells [27] with good results. To attain the optimization of HPLC–MS for a quantitative evaluation of PAF in biological samples, this technique was compared with the bioassay. The

results illustrated in Fig. 5C show a good correlation between $C_{16}$-PAF values determined by HPLC–MS and those obtained by a bioassay on the same samples.

Several studies were previously performed using MS techniques. GC/MS gave reliable results and the technique was very sensitive; however, an enzymatic degradation step and a subsequent derivatization were required in order to obtain volatile products amenable to GC separation. These steps, which require a skilled operator, are time consuming and complicate the method. The already proposed HPLC–MS, with a thermospray interface, and FAB-MS techniques do not require enzymatic or derivatization steps and therefore minimize the problems related to these procedures. However, thermospray ionization exhibits a low sensitivity [20]; concerning FAB-MS the detection limits are good but HPLC interfacing is complex and TLC separation is generally used to discriminate isobaric compounds.

In conclusion, the results of this study indicate that the proposed HPLC–MS technique, based on a pneumatically assisted electrospray interface, may be useful for further research on PAF and PAF-related molecules in biological samples. The sensitivity and the reliability when compared with the bioassay and the relative simple methodology are the relevant features of this method. The wide possibilities offered by this ionization technique for the selection of mobile phases is a further advantage on the assumption that with certain samples different reversed-phase or normal phase separations may be required to overcome problems of interference by other molecules.

## ACKNOWLEDGEMENT

This work was supported by the National Research Council (CNR), Targeted Project "Prevention and Control of Disease Factors", Subproject "Causes of Infective Disease" (CT 920037.PF4 1).

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