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Analysis of platelet-activating factor by gas chromatography–mass spectrometry: low-energy electron impact of the corresponding 3-acetyl-2-*tert.*-butyldimethylsilyl derivative

ANTONIO TRIOLO*, JACOPO BERTINI, CARLO MANNUCCI and ANDREA PERICO

Research Division Analytical Department, A. Menarini Industrie Farmaceutiche Riunite S.R.L., Via Sette Santi 1, 50131 Florence (Italy)

and

VITTORIO PESTELLINI

Chemistry Department, A. Menarini Industrie Farmaceutiche Riunite S.R.L., Via Sette Santi 1, 50131 Florence (Italy)

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ABSTRACT

A method for the analysis of platelet-activating factor in platelets employing gas chromatography and selected-ion monitoring mass spectrometry with low-energy electron impact and stable isotope dilution was developed. The procedure involved Bligh and Dyer extraction of the sample followed by thin-layer chromatographic purification. Platelet-activating factor is successively hydrolysed to the corresponding 2-acetyl-1-O-alkylglycerol by digestion with phospholipase C, and the product is allowed to isomerize to the more thermodynamically stable 3-acetyl-1-O-alkylglycerol before column purification and derivatization of the free OH with *tert.*-butyldimethylchlorosaline-imidazole. This reagent is of common use in platelet-activating factor derivatization, but is made to react with 2-acetyl instead of 3-acetyl isomer. The advantages of using the latter for the final derivatization are discussed and this method is compared with others currently available for gas chromatographic–mass spectrometric analysis of platelet-activating factor.

INTRODUCTION

Platelet-activating factor (PAF) is a biologically active phospholipid acting as a mediator in allergic and inflammatory processes. It may be synthesized by a variety of cell types, in response to several stimuli and produces numerous biological effects [1]. Chemically, it has been demonstrated to be a mixture of ether-linked glycerophospholipids having the common formula of 1-O-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine, with various alkyl chain lengths and degrees of unsaturation. The composition of this mixture varies depending on the animal species and the biological matrix under investigation: however, the most fre-

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quently observed molecular species are 1-O-hexadecyl, 1-O-octadecyl and 1-O-(octadec-9-*cis*-enyl)-2-acetyl-*sn*-glycero-3-phosphocholine, abbreviated respectively as $C_{16:0}$, $C_{18:0}$ and $C_{18:1}$ PAF in this paper.

Because PAF shows biological activity at extremely low concentrations (10^{-10}–10^{-12} *M*), and it has often to be determined in very complex matrices, highly sensitive and specific methods are needed. Several bioassays have been developed for this purpose, measuring platelet aggregation, serotonin release or production of superoxide anion; they all are characterized by good sensitivity, but may be affected by the occurrence of PAF inhibitors and interferences from other substances having the same effects. Several high-performance liquid chromatography (HPLC) methods have been developed for the determination of individual PAF analogues [2]: they show good separation, but their main drawback is their weak UV absorption. In order to enhance the detection limits, benzoate derivatives of PAF have been used by Blank and co-workers [3,4] with a limit of detection of less than 1 μg. Recently, Mita *et al.* [5] described a fluorescent 7-methoxycoumarin derivative, having a limit of detection as low as 100 pg.

Higher selectivity and sensitivity are attainable with mass spectrometric (MS) methods. Analysis of the intact molecule is possible with soft ionization techniques such as fast-atom bombardment (FAB) [6–9] mainly for structural identification. A quantitative approach was reported by Clay *et al.* [10], and more recently by Haroldsen and Gaskell [11], who obtained a detection limit in the low picogram range using FAB ionization coupled with tandem MS.

Gas chromatographic–mass spectrometric (GC–MS) analysis of PAF requires prior derivatization, owing to the thermal instability and low volatility of the molecule; although this necessarily prolongs analysis times, the enhancement in selectivity due to GC separation, and the inherent great sensitivity of this technique make it very suitable for a reliable and sensitive measurement of PAF levels.

This paper describes a modification of an existing method, involving the derivatization of the molecule by cleavage of the phosphocholine moiety and reaction of the free hydroxyl group thus obtained with *tert.*-butyldimethylchlorosilane-imidazole (TBDMSI). This method has been applied to the analysis of PAF and related phospholipids in many biological substrates [12–17], using 70-eV electron-impact ionization. We propose the formation of a slightly different TBDMS derivative, featuring better spectral characteristics for selected-ion monitoring (SIM) analysis, and a simplified method of PAF purification from unstimulated platelets spiked with analytes and deuterated internal standard. We also discuss other GC–MS methods available for PAF analysis.

EXPERIMENTAL

$C_{16:0}$, $C_{18:0}$, $C_{18:1}$ PAF and $C_{16:0}$ lyso-PAF were purchased from Bachem Feinchemikalien (Bubendorf, Switzerland). [$^2H_3$]Acetyl chloride, [$^2H_4$]acetic

acid, lysophosphatidylcholine from egg yolk and phospholipase C (EC 3.1.4.3) from *Bacillus cereus* were obtained from Sigma (St. Louis, MO, USA). Silica gel for chromatography was obtained from Merck (Darmstadt, Germany). Empore 10 cm × 2.5 cm thin-layer chromatography (TLC) plates were from 3M (St. Paul, MI, USA). Tris(hydroxymethyl)aminomethane (Tris) was obtained from BioRad (Richmond, CA, USA). TBDMSI reagent was obtained from Alltech Applied Science (Deerfield, IL, USA). All solvents were purchased from Merck or Carlo Erba (Milan, Italy).

[$^2H_3$] $C_{16:0}$ PAF was prepared as follows [7]: 50 μl of [$^2H_4$]acetic acid and 25 μl of [$^2H_3$]acetyl chloride were added to 1 mg of $C_{16:0}$ lyso-PAF in a reaction vial. After 1 h, the reaction mixture was evaporated under a gentle stream of nitrogen. The product was used without further purification. Its isotopic purity was assayed by GC–MS according to the method described below; the percentages of the deuterium-containing species, measured as the areas of their chromatographic peaks, were: $^2H_0$, 0.11%; $^2H_1$, 0.27%; $^2H_2$, 3.86%; $^2H_3$, 95.76%.

Platelet isolation

Platelets were isolated from fresh human blood according to Korth *et al.* [18]. Briefly, after treatment with ACD (acid citrate dextrose, 7:1, v/v), the blood was centrifuged (200 *g* 15 min, room temperature). The platelet-rich plasma thus obtained was mixed with Tyrode buffer (pH 6.4, "calcium-free"), containing 0.25% bovine serum albumin (BSA) and 1 m*M* EDTA, and centrifuged (850 *g*, 15 min, room temperature). The pellet was washed twice with Tyrode buffer and then suspended in Tyrode buffer (pH 7.4, "calcium-free") containing 0.25% BSA.

Extraction and purification of PAF

Ice-cold methanol (2.5 volumes) and chloroform (1.25 volumes) were added to the platelets suspended in Tyrode buffer; then 50 ng of $C_{16:0}$ [$^2H_3$]PAF (internal standard) were added. PAF was extracted according to the method of Bligh and Dyer [19] by mechanically shaking for 1 h, then 1.25 volumes of chloroform and 1.25 volumes of water were added to obtain phase separation. After centrifugation, the lower phase was evaporated, dissolved in chloroform–methanol (1:1, 3 × 20 μl) and spotted on an Empore silica gel TLC plate (10 cm × 2.5 cm); as a marker, 100 mg of lysophosphatidylcholine from egg yolk were spotted on a adjoining lane of the plate, which was then developed with chloroform–methanol–water (65:35:6) and allowed to dry. A strip corresponding to the lane of the marker was cut and exposed to iodine vapour (under these TLC conditions, lysophosphatidylcholine and PAF have R_F values of 0.15 and 0.19, respectively), then a strip was cut on the lane of the sample starting from the R_F value corresponding to the marker spot and ending 1 cm above; PAF was extracted from this strip by the method of Bligh and Dyer [19] and dried under a stream of nitrogen.

Derivatization of PAF

The residue was resuspended in 2 ml of peroxide-free diethyl ether, then 1 ml of phospholipase C dissolved in 0.5 *M* pH 7.6 Tris buffer (1 U/ml) was added; the mixture was mechanically shaken at room temperature for 3 h. After centrifugation for 5 min at 650 *g*, the upper phase was evaporated and the residue dissolved in 1 ml of chloroform–methanol–water (1:2:0.8) and mixed with 0.25 ml of chloroform and 0.2 ml of water. The lower phase was evaporated, and the residue was dissolved in 100 μl of chloroform; 1 ml of *n*-hexane was added, and the solution was applied to a small glass column filled with 1 g of silica gel, previously washed with 10 ml of chloroform and 2 ml of *n*-hexane. The sample was left at room temperature overnight in order to allow acetyl migration from position 2 to 3 of 1-O-alkyl-2-acetylglycerol generated by the action of phospholipase C. Then it is washed with 5 ml of *n*-hexane, eluted with 5 ml of chloroform, collected in a reaction vial and evaporated. Next, 100 μl of TBDMSI were added, and the vial was capped and kept for 15 min at 110°C. After cooling, 0.5 ml of chloroform were added, and the mixture washed with two 1-ml volumes of water; the lower phase was evaporated and the residue redissolved in 20 μl of *n*-heptane for GC–MS analysis.

A scheme of the whole procedure is shown in Fig. 1.

Gas chromatography–mass spectrometry

GC–MS was performed on an HP-5890 gas chromatograph coupled to an HP-5988A mass spectrometer (Hewlett-Packard, Palo Alto, CA, USA). For the chromatographic separation, a HP-1 (100% methylsilicone) fused-silica capillary column (12 m × 0.2 mm I.D., 0.33 μm film thickness) was used, equipped with a 1 m × 0.32 mm I.D. inactivated fused-silica pre-column. The analytical column

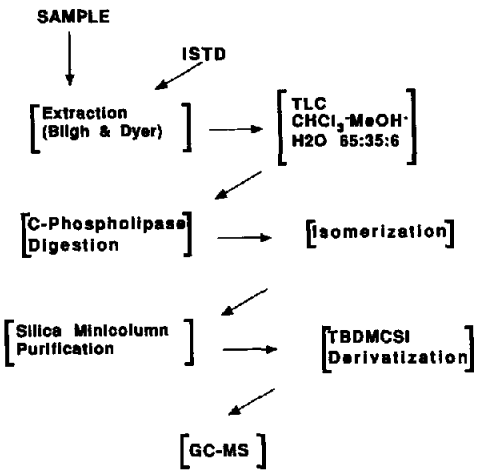

Fig. 1. Scheme of the whole analytical procedure adopted for analysis of PAF species in human platelets.

was directly connected to the ion source. The oven temperature was programmed from 100°C (0.5 min hold) to 200°C at 45°C/min and at 30°C/min up to 270°C. A 2-μl volume of sample was injected on to the column. Helium was used as the carrier gas at 35 kPa inlet pressure. The ion source and transfer line temperatures were set at 200 and 290°C, respectively. Both full-scan mass spectra and SIM experiments for PAF quantitation were performed in the electron-impact mode at an ionizing energy of 20 eV. Selected ions monitored were m/z 415 ($C_{16:0}$ PAF), m/z 441 ($C_{18:1}$ PAF), m/z 443 ($C_{18:0}$ PAF) and m/z 418 ($C_{16:0}$ [$^2H_3$]PAF). Dwell times were 100 ms for all ions. Standard curves were prepared by combining various amounts of each molecular species of PAF in the range 2–100 ng, with 50 ng of deuterated internal standard, and subjecting them to the same derivatization steps as the sample.

RESULTS

Typical parameters for the regression line equations of $C_{16:0}$, $C_{18:1}$ and $C_{18:0}$ PAF are listed in Table I: they show a linear response in the range 200–1000 pg as absolute amounts. The values of the intercept were not significant different from zero. The limit of detection, calculated as the amount of $C_{16:0}$ PAF giving a peak with a signal-to-noise ratio of 3:1, is 20 pg. This is in agreement with the limit of detection determined by analysing the $C_{16:0}$ PAF regression line: in this case too it has been estimated to be 20 pg, calculated as the upper 95% confidence limit of the intercept. The detection limits for $C_{18:1}$ and $C_{18:0}$ PAF, estimated with the latter method, are 13 and 32 pg, respectively.

The intra- and inter-assay precision and accuracy were investigated by assaying unstimulated human platelets spiked with known amounts of the analytes and 50 ng of internal standard: data are shown in Tables II and III, respectively.

Fig. 2 (upper traces) illustrates the selected-ion chromatograms obtained by analysing a sample of $5 \cdot 10^8$ unstimulated platelets spiked with 50 ng of each molecular species of PAF and the internal standard. In the chromatograms of the spiked sample, the small peaks preceding all the peaks of interest correspond to the 2-acetyl-3-TBDMS derivatives: they are formed during the last step of deriv-

TABLE I

TYPICAL PARAMETERS FOR THE REGRESSION LINE EQUATIONS OF $C_{16:0}$, $C_{18:1}$ AND $C_{18:0}$ PAF

Compound	Slope	Intercept	r^2	Number of points
$C_{16:0}$ PAF	1.09	0.00718	1.000	5
$C_{18:1}$ PAF	1.15	0.00342	1.000	5
$C_{18:0}$ PAF	1.32	0.000419	0.999	5

TABLE II

INTRA-ASSAY PRECSION AND ACCURACY FOR $C_{16:0}$, $C_{18:1}$ AND $C_{18:0}$ PAF

Amount given (ng)	Accuracy (%)	R.S.D. (%)	*n*
$C_{16:0}$ PAF			
10	101.0	7.6	4
50	96.9	5.2	4
$C_{18:1}$ PAF			
10	90.4	7.4	4
50	95.0	6.5	4
$C_{18:0}$ PAF			
10	110.3	6.1	4
50	101.6	3.8	4

atization from the small amount of 1-O-alkyl-2-acetylglycerol remaining in the equilibrium mixture after migration of the acetyl group from the 2- to the 3-position of the glycerol backbone. The peaks of the two isomers are well separated, allowing accurate integration of the main peak. The relative amounts of isomers at equilibrium are 95 and 5, as measured from the peak areas of the TBDMS derivatives; once these are formed, they are stable, and there is no more variation of their relative amounts.

The selected-ion chromatograms of an unspiked sample show the absence of interfering peaks at the retention times of the PAF derivatives (Fig. 2, lower traces).

DISCUSSION

We chose to derivatize PAF as its 3-acetyl-2-TBDMS isomer rather than its 2-acetyl-3-TBDMS isomer for several reasons. The spontaneous migration of the acetyl group from the initial product of phospholipase C cleavage to the more stable 3-acetyl isomer is a well known phenomenon [20,21]: it occurs during the

TABLE III

INTER-ASSAY PRECISION AND ACCURACY FOR $C_{16:0}$ PAF

Amount given (ng)	Accuracy (%)	R.S.D. (%)	*n*
10	94.6	9.0	12
50	95.1	7.1	12

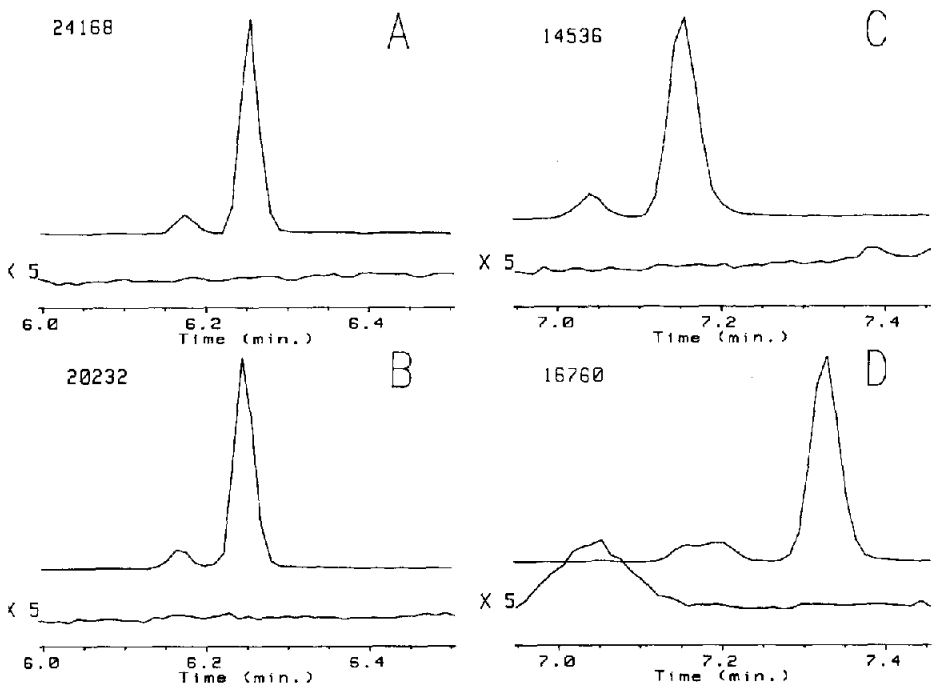

Fig. 2. Selected-ion chromatograms obtained by analysing a sample of human platelets spiked with 50 ng each of $C_{16:0}$, $C_{18:0}$ and $C_{18:1}$ PAF, and 50 ng of deuterated $C_{16:0}$ PAF as internal standard; the lower traces in each window are the corresponding blanks: (A) *m/z* 415, $C_{16:0}$ PAF; (B) *m/z* 418, internal standard; (C) *m/z* 441, $C_{18:1}$ PAF; (D) *m/z* 443, $C_{18:0}$ PAF.

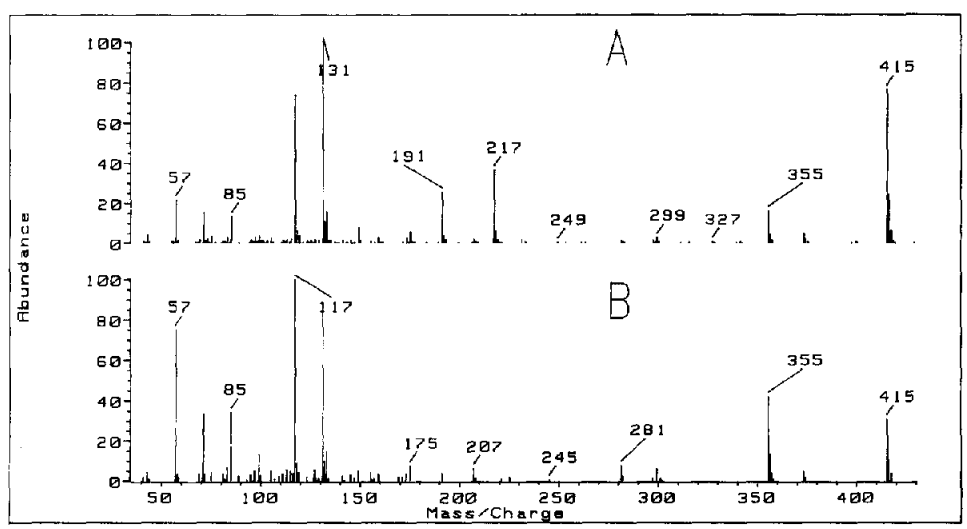

Fig. 3. Full-scan mass spectra, registered at 20 eV, ø(A) 3-acetyl-2-TBDMS derivative of $C_{16:0}$ PAF and (B) 2-acetyl-3-TBDMS derivative of $C_{16:0}$ PAF.

purification steps, leading to the formation of two peaks in the final chromatogram, as described before. Although they are well separated, it is preferable, for sensitivity, precision and accuracy purposes, to control acetyl migration, either by preventing it or by allowing it to reach completeness. We chose the latter approach owing to the more favourable characteristics of the mass spectrum of 3-acetyl isomer for SIM GC–MS analysis. This is depicted in Fig. 3, which shows the full-scan low-energy EI spectra of 3-acetyl and 2-acetyl derivatives of $C_{16:0}$ TBDMS-PAF: the *m/z* 415 ion being monitored in SIM analysis, arising from a *tert.*-butyl radical loss from the molecular ion, is more abundant in the 3-acetyl isomer (76% instead of 31%), and it is 13.7% of total ion abundance instead of 4.8%. Assuming equal ionization efficiencies for both derivatives, this yields an almost three-fold gain in sensitivity.

The conditions for acetyl migration were investigated. It was found that isomerization is catalysed by silica and reaches equilibrium after *ca.* 10 h at room temperature; it was also found that neither increased temperature (40°C) nor traces of acid had a significant effect on that isomerization time.

Although low-energy EI is rarely used in SIM GC–MS, since it gives lower ionization efficiencies and consequently higher detection limits with respect to the electron energies usually employed, in this case it has a positive effect: in fact decreasing the electron energy from 70 to 15 eV yields fewer ions in total, but also a lower degree of fragmentation. Performing several injections of a standard solution of PAF at various ionization energies, it was found that maximum peak areas were obtained at 20 eV. As shown in Fig. 4, working at this electron energy the PAF $C_{16:0}$ peak area is *ca.* 2.5 times greater than when working at 70 eV; similar results were obtained with the other molecular species of PAF.

Other methods are currently used for GC–MS analysis of PAF. The most sensitive involves the formation of the pentafluorobenzoyl (PFB) derivative and its analysis in the negative-ion mode under chemical-ionization conditions (NI-CI). Using this method Ramesha and Pickett [22] detected amounts of PAF of less than 100 fg, owing to the "softness" of this ionization technique, which gives

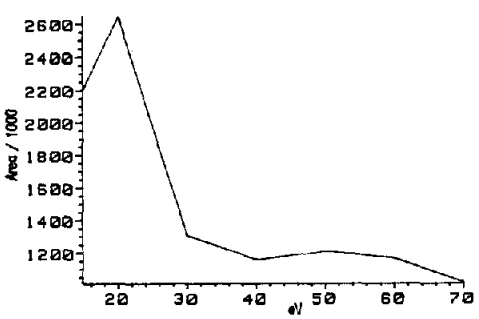

Fig. 4. Plot of chromatographic peak area of 3-acetyl-2-TBDMS derivative of $C_{16:0}$ PAF *versus* electron energy.

spectra with very little fragmentation, and to its intrinsically high ionization efficiency. With respect to this method, the method described in this paper is less sensitive, and cannot measure very small amounts of PAF. On the other hand, while the NICI spectra of the PFB derivatives show practically only the molecular anion, whose abundance is more than 92% of the total ion current, the EI spectra of TBDMS derivatives yield both the molecular mass and structural information. As shown by Satouchi and co-workers [12,17], it is possible to identify every structural characteristic of PAF and related phospholipids as TBDMS derivatives by monitoring only a few ions, and this feature can be very useful when structural confirmation is needed.

Moreover, EI methods require generally simpler operation and instrumentation than CI methods, allowing use by less experienced operators and with less sophisticated instruments.

Another EI GC–MS method involves PAF analysis via its trimethylsilyl derivative [7]: the full-scan spectra for all the molecular species of PAF give an abundant ion at m/z 175 (m/z 178 for deuterated internal standard), a value too low for SIM analysis since it may suffer from interferences coming from the matrix. Furthermore, the molecular mass-indicating fragments, which are 15 units below the molecular mass, have too little abundance to be useful for a sensitive assay. Another drawback for TMS derivatives is their very high instability to moisture, whereas TBDMS derivatives allow work-up of the reaction mixture in the presence of water.

In spite of their higher molecular mass, TBDMS derivatives show also good GC properties, *i.e.* symmetrical peak shapes and reasonably short retention times.

CONCLUSIONS

Low-energy EI GC–MS analysis of PAF via its TBDMS derivative cannot be useful if very low levels of PAF have to be determined, in which case the NICI GC–MS approach has a largely better performance. It can be a valid analytical tool when greater concentrations of PAF have to be measured, such as in stimulated substrates, and when structural information is needed. For this purpose, it can be considered a good complementary technique to NICI GC–MS, which can only give molecular mass information.

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