Journal of Chromatography, 433 (1988) 243–247
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 4391

Note

Highly sensitive gas chromatographic–mass spectrometric method for the determination of platelet-activating factor in human blood

KOUJI YAMADA*, OSAMU ASANO, TSUTOMU YOSHIMURA and KOUICHI KATAYAMA

Tsukuba Research Laboratories, Eisai Co , Ltd , 1-3, Tokodai-5-chome, Tsukuba, Ibaraki 300-26 (Japan)

(First received May 3rd, 1988; revised manuscript received July 7th, 1988)

Platelet-activating factor (PAF or 1-O-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine), a potent biologically active phospholipid [1], is a lipid mediator derived from numerous cells and able to promote various specific responses, such as platelet secretion and aggregation, neutrophil activation, bronchoconstriction and hypotension [2,3].

Since PAF has multiple potent biological activities, it is important to be able to determine its concentration in blood and tissues. One frequently used method is to measure its ability to aggregate platelets [4]. However, this bioassay is not sufficiently accurate, owing to the variable bioactivity associated with the natural variations in the length of the alkyl ether side-chain of PAF [5]. In recent years, analyses of PAF by fast atom bombardment (FAB) mass spectrometry [6,7] and gas chromatography–mass spectrometry (GC–MS) with selected-ion monitoring [8] have been reported. However, these methods are not sensitive enough to measure accurately the concentration of PAF in biological materials. Ramesha and Pickett [9] have recently described a sensitive GC–negative-ion chemical ionization (NICI) MS method that involves the derivatization of PAF with pentafluorobenzoyl chloride (PFBCl). Although the sensitivity of this method is sufficient to determine the concentration of PAF in biological samples, the authors were not able to measure the distribution of PAF in blood because of the lack of a suitable method for the purification of PAF extracted from blood.

In this paper, we describe a sensitive GC–NICI-MS method for the determination of PAF in blood after a simple and specific purification on Bond Elut SI, with tetradeuterated PAF as an internal standard. This procedure was applied to determine the blood level of PAF in human volunteers.

0378-4347/88/$03.50

EXPERIMENTAL

Chemicals and reagents

1-O-Hexadecyl-2-acetyl-*sn*-glycerophosphocholine (PAF) and 1-O-hexadecyl-2-lysoglycero-3-phosphocholine were synthesized in our laboratories by the method of Fujita et al. [10]. The internal standard, 1-O-(2,3-tetradeuterio)hexadecyl-2-acetyl-*sn*-glycerophosphocholine ([$^2H_4$]PAF), was synthesized as follows. Propargyl alcohol (Wako, Osaka, Japan) was protected with 2,3-dihydropyran (Tokyo Chemical Industry, Tokyo, Japan) by a usual method. This compound was condensed with 1-bromotridecane (Tokyo Chemical Industry) to give hexadec-2-ynyl tetrahydropyranyl ether. The tetrahydropyranyl ether was reduced with deuterium and a platinum catalyst to give 2,3-tetradeuteriohexadecane tetrahydropyranyl ether, and this was transformed into 2,3-tetradeuteriohexadecanol by a usual method. [$^2H_4$]PAF was synthesized by the condensation of 2,3-tetradeuteriohexadecanol with a glycerol derivative by the method of Fujita et al. [10]. Phospholipase C from *Bacillus cereus* was purchased from Boehringer Mannheim (Mannheim, F.R.G.). PFBCl was obtained from Aldrich (Milwaukee, WI, U.S.A.), 1-O-[^{3}H]Hexadecyl-2-acetyl-*sn*-glycerophosphocholine was purchased from New England Nuclear (Boston, MA, U.S.A.). All other reagents were of analytical grade.

Samples

Human blood samples were obtained from normal subjects between 25 and 60 years of age. Each sample was placed immediately in a polypropylene tube containing 3 volumes of ice-cold methanol and centrifuged at 3000 *g* for 20 min. The upper layer was stored at −20°C until analysis.

Gas chromatography–mass spectrometry

GC–MS analyses were carried out on a JEOL DX-300 with a post-acceleration-type high-sensitivity detector (conversion dinode +8.6 kV), a JMA-3500 data system (Tokyo, Japan), and a solvent-less injection system. GC was performed on an HP-1 (Yokogawa, Tokyo, Japan) cross-linked methyl silicone capillary column (25 m × 0.31 mm I.D.). The column temperature was 255°C and the GC–MS spectrometric interface lines were at 290°C. The carrier gas was helium and the flow-rate was 0.2 kg/cm^2. The MS conditions were: emission current, 0.3 mA; ionizer temperature, 290°C. The mass spectrometer was operated in the NICI mode using isobutane ($5 \cdot 10^{-5}$ Torr) as the reagent gas. The spectra were obtained either by scanning *m/z* 50 to 800 or by monitoring selected ions. When the selected-ion monitoring (SIM) mode was used, the molecular anions at *m/z* 552 for the PAF derivative and *m/z* 556 for [$^2H_4$]PAF were monitored.

Extraction

A sample (3 ml) was mixed with the internal standard solution, 1 ml of distilled water and 4 ml of chloroform. The lower phase was collected and evaporated to dryness at 35°C under a stream of nitrogen. The residue was dissolved in 200 μl of chloroform and then applied to a Bond Elut SI column. The column was washed

with 3 ml of chloroform, 2 ml of chloroform–methanol (6:4, v/v) and 3 ml of chloroform–methanol–28% aqueous ammonia (70:35:7, v/v/v), then eluted with 2 ml of chloroform–methanol–28% aqueous ammonia (50:50:7, v/v/v). The eluate was evaporated to dryness, and the residue was dissolved in 200 μl of saline containing 0.5% Triton X-100.

Preparation of the pentafluorobenzoyl derivative

The reaction mixture, consisting of 0.8 U of phospholipase C, 72 m*M* calcium chloride and 50 m*M* Tris–HCl buffer (pH 7.5), was added to the extract and incubation was performed at 37°C for 60 min. The hydrolysed mixture was extracted by the method of Folch, and the lower phase was evaporated at 35°C under a stream of nitrogen. The residue was treated with 3 m*M* PFBCl in 150 μl of distilled acetonitrile. The reaction mixture was allowed to stand at 35°C for 60 min, by which time derivatization was complete, then evaporated to dryness. The residue was dissolved in 3 ml of *n*-hexane, and the solution was washed twice with 1 ml of 5% aqueous citric acid and again evaporated to dryness. The residue was dissolved in 100 μl of ethyl acetate, and 5–20 μl of this solution were injected into the GC–MS apparatus.

Calibration curve

Samples containing 10–200 pg of PAF and 50 pg of internal standard in 1 ml of distilled water were prepared. The samples were extracted, and the extract was hydrolysed, derivatized and analysed by GC–MS. Calibration curves of the peak-area ratio of PAF to internal standard against PAF concentration were constructed.

RESULTS AND DISCUSSION

GC–NICI-MS method

Recently, Ramesha and Pickett [9] have reported the measurement of PAF by the GC–NICI-MS method. The sensitivity of this method appeared to be sufficient to determine the concentration of PAF in blood. Therefore, we synthesized the pentafluorobenzoyl (PFB) derivative of PAF as a standard. Fig. 1 shows the NICI mass spectrum of the PFB derivative of PAF. The peak at *m/z* 552 corre-

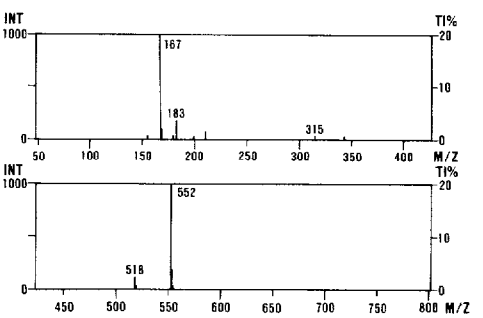

Fig. 1. NICI mass spectrum of the PFB derivative of PAF.

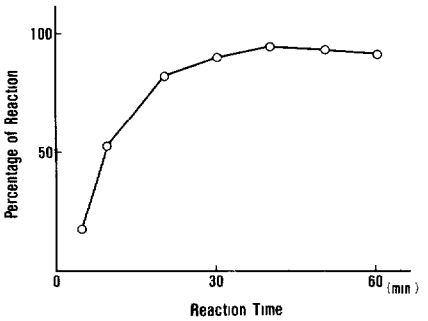

Fig. 2. Time course of hydrolysis of PAF with phospholipase C.

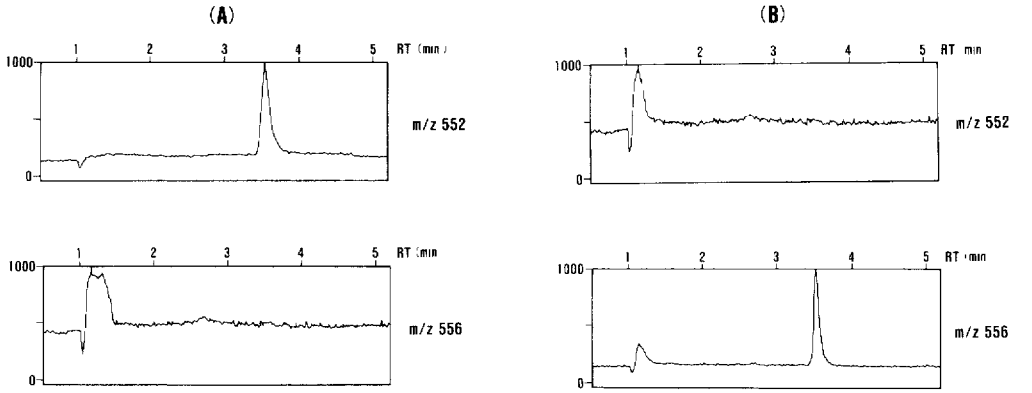

Fig. 3. NICI selected-ion chromatograms of (A) PAF (m/z 552) and (B) the internal standard (m/z 556).

sponds to the molecular anion of the PFB derivative of PAF. The limit of detection of this derivative was 50 fg at a signal-to-noise ratio of 5, when the mass spectrometer was operated in the SIM mode. These results are in good agreement with the data of Ramesha and Pickett [9]. It seems that the sensitivity of this method is indeed sufficient to determine the concentration of PAF in human blood. Thus we investigated the extraction and purification of PAF from blood by using a Bond Elut SI column. The purification procedure on the Bond Elut SI column following extraction was indispensable for good GC–MS spectra, and the recovery of PAF was ca. $82 \pm 5.40\%$ (mean $\pm$ S.D., $n=4$) when [^{3}H]PAF (0.005 μCi) was tested.

Fig. 2 shows the time course of hydrolysis of PAF with phospholipase C. The resulting 1-O-hexadecyl-2-acetylglycerol was separated by thin-layer chromatography (developed with benzene–ethyl acetate, 85:15), and then the concentration of 1-O-hexadecyl-2-acetylglycerol was measured with a liquid scintillation counter (Aloka, Tokyo, Japan). The percentage of 1-O-hexadecyl-2-acetylglycerol increased with time and attained an almost constant level at 40 min. The conversion from PAF at 40 min was $93.4 \pm 1.96\%$ (mean $\pm$ S.E., $n=3$) when [^{3}H]PAF (0.005 μCi) was tested.

The calibration curve was constructed by using the calculated peak-area ratio

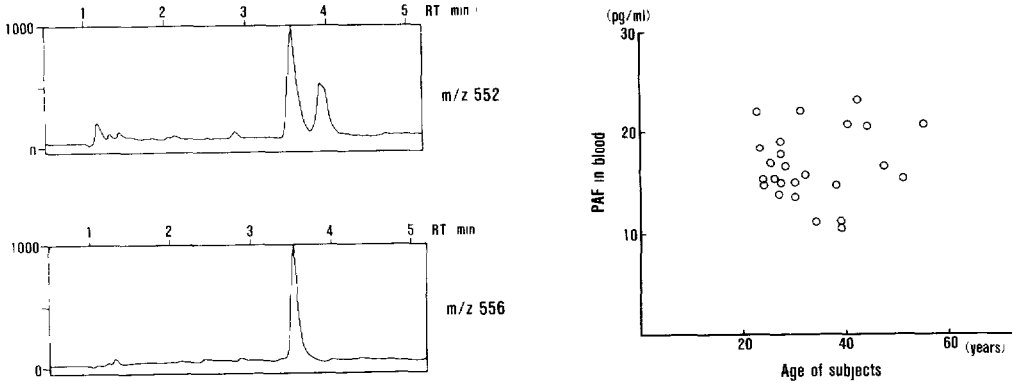

Fig. 4. NICI selected-ion chromatograms of PAF in human blood. The blood sample and internal standard (50 pg) were extracted as described in text.

Fig. 5. Concentration of PAF in human blood. Each point is the mean of three determinations.

of PAF to the internal standard. The peak-area ratio was linearly correlated with the PAF concentration in the range 10–200 pg. The minimum detectable amount of PAF was 5 pg by this method. The intra-experimental coefficient of variation was 3.8% (at 50 pg/ml, $n=6$). Fig. 3 shows NICI selected-ion chromatograms of PFB derivatives of standard PAF (50 pg) and the internal standard (50 pg). The derivatives of PAF (*m/z* 552) and the internal standard (*m/z* 556) appeared at 3.5 min, and there was no interfering peak.

PAF in human blood

PAF in human blood was analysed by the GC–NICI-MS method, and a representive NICI chromatogram is shown in Fig. 4. The derivatives of PAF and the internal standard were well resolved, and no endogenous interfering peaks were seen. Fig. 5 shows the concentration of PAF in blood from human subjects. Each point is the mean of three determinations. The blood level of PAF was in the range 10–30 pg/ml. The value varies widely from subject to subject, but there is no apparent change with age and no significant sex difference.

The present investigation has demonstrated that the blood concentration of PAF can be determined by the GC–NICI-MS method. The method should be useful for clinical investigations into the physiological role of PAF.

REFERENCES

1 R.M. Pickard, L.M. McManus and D.J. Hanahan, Adv. Inflam. Res., 4 (1982) 147–180.
2 F. Snyder, Med Res. Rev., 5 (1985) 107–140.
3 P.O. Clark, D.J. Hanahan and R.N. Pickard, Biochim. Biophys. Acta, 628 (1980) 69–75.
4 C.A. Demopoulos, R.N. Pickard and D.J. Hanahan, J. Biol. Chem., 254 (1979) 9355–9358.
5 K. Satouchi, R.N. Pickard and D.J. Hanahan, Arch. Biochem. Biophys., 211 (1981) 683–688.
6 K.L. Clay, D.O. Stene and R.C. Murphy, Biomed. Mass Spectrom., 11 (1984) 47–49.
7 P. Verenne, B.C. Das and M. Tence, Biomed. Mass Spectrom., 12 (1985) 6–10.
8 M. Oda, K. Satouchi, K. Yasunaga and K. Saito, J. Immunol., 134 (1985) 1090–1093.
9 C.S. Ramesha and W.C. Pickett, Biomed. Mass Spectrom., 13 (1986) 107–111.
10 K. Fujita, H. Nakai, S. Kobayashi, K. Inoue, S. Nojima and M. Ohno, Tetrahedron Lett., 23 (1982) 3507–3510.