Development of a mass spectrometric method to quantitate platelet activating factor in mouse urine

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Abstract Platelet activating factor (PAF) is a lipid mediator of inflammation released by a variety of stimulated inflammatory cells. It may be involved in immune glomerulonephritis. Thus, its measurement in urine could give information on the mechanism of this disease. We present here a method to measure PAF in mouse urine, using gas-liquid chromatography-mass spectrometry (GLC-MS) in the selected ion recording (SIR) mode. Before instrumental analysis, the extracted and purified samples were hydrolyzed and derivatized with pentafluorobenzoyl chloride. Different experimental conditions are presented and discussed to corroborate the analytical findings. PAF levels in mouse urine were 2.08 ± 0.46 ng/24 h. This procedure might represent a new experimental tool to establish the possible role of PAF as mediator of tissue damage in renal disease. — **Benfenati, E., D. Macconi, M. Noris, G. Icardi, L. Bettazzoli, G. De Bellis, M. Gavinelli, S. Rotondo, and G. Remuzzi.** Development of a mass spectrometric method to quantitate platelet activating factor in mouse urine. *J. Lipid Res.* 1989. **30:** 1977-1981.

Supplementary key words selected ion recording

Platelet activating factor (PAF; 1-O-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine) is a lipid mediator of inflammatory reactions which has been recently suggested as playing a role in immune glomerulonephritis (1). In acute serum sickness glomerular deposition of circulating immune complexes is, in fact, associated with platelet activation induced by a mechanism related to the degranulation of IgE-sensitized basophils and PAF release (2). Moreover, hyperacute renal allograft rejection is followed by appearance of PAF in the venous effluent of the transplanted kidney (3). Recently PAF has been documented as a mediator of glomerular injury in rabbit nephrotoxic nephritis (4). Finally, PAF is found in plasma of patients with systemic lupus erythematosus (5).

Recent efforts to assess whether PAF is excreted in the urine indicate that PAF is excreted in normal adult (about 50 ng/day) and fetal urine (6, 7). Urinary excretion was above normal in patients with systemic lupus (6). So far, however, all attempts to quantify PAFin urine have involved bioassays. Bioassay based on PAF's ability to induce platelet aggregation and serotonin release is quite sensitive (8, 9), but it is not direct and specific for PAF since several PAF analogs may influence platelet activation as well as other autocoids present as impurities (10).

Gas-liquid chromatography-mass spectrometry (GLC-MS) provides better sensitivity and accuracy (10, 11), making it possible to measure sub-picogram quantities of PAF (10). Its intrinsic accuracy is guaranteed by the chemical basis of the MS measurement: what is measured is the molecular ion of a defined chemical compound and not a biological reaction. Finally, MS is the only method that uses a deuterated internal standard, overcoming problems due to losses during the analytical procedure.

We have developed a GLC-MS quantitative method, in the negative ion chemical ionization (NICI) mode, to detect picogram amounts of PAF excreted in small volumes of biological fluids. Here we report on the application of this method to detect PAF in mouse urine. The results given here represent the first quantitative determination of PAF urinary excretion by GLC-MS.

MATERIAL AND METHODS

Chemicals

Standard PAF and lyso-PAF with the 1-O-hexadecyl chain were obtained from Bachem Feinchemikalien AG (Bubendorf, Switzerland). [^{3}H]PAF (59.5 Ci/mmol) was obtained from New England Nuclear (Boston, MA). Thin-layer chromatography (TLC) plates, precoated with silica gel 60 with concentrating zone, layer thickness 0.25 mm, and 50% hydrofluoric acid were obtained from Merck (Darmstadt, F. R. G.). Pentafluorobenzoyl chloride

Abbreviations: GLC, gas-liquid chromatography; NICI, negative ion chemical ionization; MS, mass spectrometry; SIR, selected ion recording; PAF, platelet activating factor, 1-0-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine; lyso-PAF, 1-0-alkyl-2-lyso-*sn*-glycero-3-phosphocholine; PAF-d_9, 1-0-hexadecyl-2-acetyl-d_9-*sn*-glycero-3-phosphocholine; PFBCl, pentafluorobenzoylchloride; TLC, thin-layer chromatography.

(PFBCl) was purchased from Janssen Chimica (Beerse, Belgium). Acetic anhydride-d_6 was obtained from Sigma Chemical Co. (St. Louis, MO). Sep-Pak silica cartridges were purchased from Waters Associates (Milford, MA). Silicic acid was obtained from Bio-Rad Laboratories (Richmond, CA). All solvents were analytical grade.

Preparation of deuterated AGEPC

Lyso-PAF, 1.5 mg, was added with 200 μl of acetic anhydride-d_6 and heated at 80°C for 3 h. After the reaction, the mixture was extracted by the method of Bligh and Dyer (12). The chloroform phase was dried under a nitrogen stream and purified by HPLC. Aliquots of the sample were injected onto a Perkin Elmer Silica A Column (2.6 × 250 mm) using a Beckman model 342 Gradient Liquid Chromatograph. Analysis was performed by isocratic elution with acetonitrile–methanol–85% phosphoric acid 130:5:1.5 (v/v/v), as described by Chen and Kou (13). Flow rate was 1.5 ml/min. PAF-d_3 fractions were collected on the basis of the retention time of the corresponding tritiated standard. The purity of the PAF-d_3 obtained was checked by TLC, GLC–MS, and FAB.

Animals

CD-1 (ICR) BR mice (30–40 g) (Charles River Italia S. p. A., Calco, Italy) had free access to standard diet and water.

Collection of urine

The animals were housed in metabolic cages for collection of 24-h urine samples at room temperature. In some experiments sodium azide 1/10000 (wt/vol, final concentration) or hydrochloric acid (300 μl) was added to the urine reservior.

Extraction and purification of urine

Five (or 20) ng of PAF-d_3 as internal standard was added to the 24-h urine sample which was extracted according to Pinckard, Farr, and Hanahan (14). Briefly, methanol was added to each sample (1.5 times the urine volume) and, after gentle mixing for 30 min, the samples were centrifuged. Chloroform and water were added to obtain a solvent mixture containing chloroform–methanol–water 1:1:0.9 (v/v/v). The organic layer was removed and evaporated to dryness under a nitrogen stream.

Samples were purified by the method of Pinckard et al. (14), with the following modification: the extract was purified on a silicic acid column prepared with 0.5 g of silicic acid (100–200 mesh) activated at 100°C overnight. PAF was eluted using a sequential solvent system of chloroform (15 ml), acetone (15 ml), acetone–methanol 1:1 (v/v) (15 ml), and chloroform–methanol 1:4 (v/v) (30 ml). The fourth fraction containing PAF was evaporated to dryness under a nitrogen stream.

Recovery was assessed by addition of tritiated PAF to the sample before processing. It was about 94% for the extraction and 89% for the silicic acid column. These values are in good agreement with similar yields for another biological matrix (15).

Derivatization of PAF

After purification, samples were hydrolyzed with hydrofluoric acid to 1-O-alkyl-2-acetyl-glycerol. Samples were treated with 0.2 ml of 50% hydrofluoric acid at 25°C; after 4 h 0.5 ml of hexane was added and the organic phase was removed and evaporated under N_2 (16). For derivatization (10) 50 μl of distilled pentafluorobenzoyl chloride (PFBCl) was added; the system was heated at 100°C for 40 min. After the reaction, the excess reagent was evaporated under a nitrogen stream. The residue was dissolved in hexane for MS analysis.

Mass spectrometry

GLC–MS analyses were carried out on a VG 70-250 mass spectrometer (VG Analytical, Manchester, U. K.). A Dani 6500 gas chromatograph (Dani, Monza, Italy) was coupled to the mass spectrometer. A fused silica capillary column CPSil 5 CB, 25 m, 0.32 mm I.D. (Chrompack, Middelburg, The Netherlands) was used, with a programmed temperature vaporizer (PTV) injector. The chromatographic program was: oven temperature: 140°C, initial, for 1.3 min; then from 140 to 280°C programmed temperature, 18°C/min. For chemical ionization MS, ammonia was used as the reagent gas. SIR analyses were done monitoring the ions at *m/z* 552 and 555 for PAF and PAF-d_3, respectively. Fast atom bombardment (FAB) mass spectra were acquired with the VG 70-250 instrument, with its standard FAB gun operating at 8 kV. Glycerol was used as liquid matrix and xenon as bombarding gas.

RESULTS AND DISCUSSION

Standards

The NICI-MS method of quantifying PAF gives good sensitivity and selectivity. The calibration curve using PAF-d_3 (400 pg) as internal standard was linear from 2 to 800 pg (**Fig. 1**); the correlation coefficient was 0.999. Similar calibration curves were obtained using smaller amounts of PAF-d_3 on narrower ranges. The selectivity was achieved considering three specific characteristics of this PAF quantification method: *1*) the chromatographic retention time; *2*) the detected ion, corresponding to the molecular weight of PAF (SIR technique); and *3*) the typical doublet, due to the two chromatographic peaks obtained after derivatization of PAF (see **Fig. 2**). These two compounds correspond to two positional isomers bearing

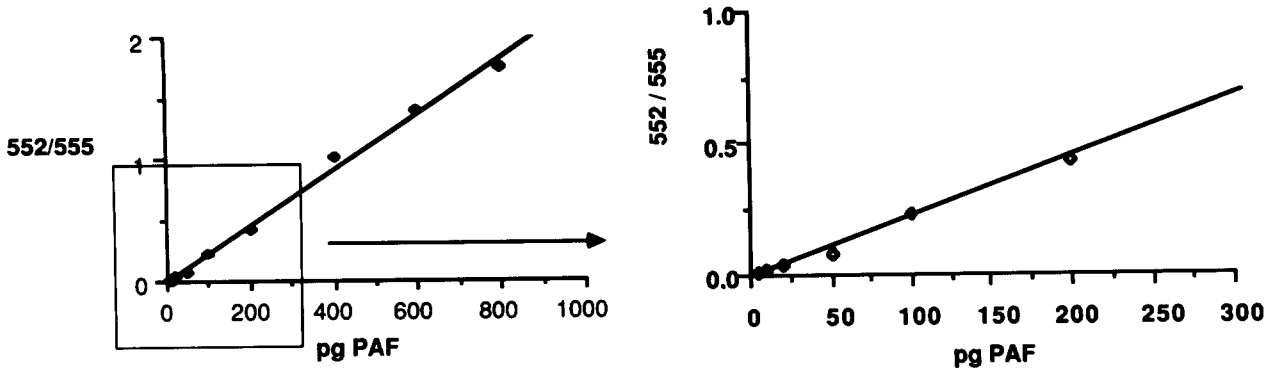

Fig. 1. Calibration curve for quantification of PAF by GLC-MS using 400 pg (injected) of PAF-d_3 as internal standard (*m/z* 555) and different amounts (2–800 pg injected) of PAF (*m/z* 552); on the right, an expanded region of the curve.

the acetyl moiety in the *sn*-2 or *sn*-3 position resulting from intramolecular shift of the acetyl group, as demonstrated by Clay and Lobmeyer (17). The chromatographic retention time of PAF was checked and confirmed by the presence of the added internal standard, PAF-d_3, whose retention time is almost identical to that of natural PAF (it is about 1 s shorter). This behavior is in agreement with what has been found for deuterated analogs of other compounds (18).

In our gas chromatographic conditions, the two chemical isomers obtained by hydrolysis and derivatization are separated. In other conditions, these two compounds did not separate (10). When the experimental conditions of hydrolysis and derivatization were kept constant (including the time between these two steps), the relative abundance of the two positional isomers was constant and equal for natural and deuterated PAF. This characteristic shape of derivatized PAF, producing two separated peaks, with constant relative intensities and retention times, thus contributes to correct identification of the peaks. Any sample presenting two peaks with different intensity ratios for the natural and deuterated compounds should be rejected, as this indicates the probable presence of interfering substances. For quantitation we used both peaks.

Biological samples

The GLC–MS method of analysis of PAF in mouse urine described here appears accurate and sensitive and can be used to detect amounts as low as 20 pg.

Since in biological fluids and tissues the detectable amount of PAF is principally regulated by acetylhydrolase, a highly active enzyme that converts PAF into its deacetylated metabolite, lyso-PAF (19, 20), we first investigated whether urine has acetylhydrolase activity. The plasma acetylhydrolase is acid-labile (21) so acidification of serum facilitates detection of PAF in blood. Thus, to investigate the presence of acid-labile acetylhydrolase in urine, we added 0.3 ml of 1 N HCl to the urine collection tube. The final pH was about 3. Failure to find any significant difference between PAF levels in samples collected with or without acid (2.51 ± 0.55 ng vs 2.08 ± 0.46 ng) led us to conclude that there is no significant acetylhydrolase activity in urine.

The second step was to exclude the contribution of bacteria (22) to the amounts of PAF found in urine. We performed some experiments on urine collected with sodium azide 1:10000 (wt/vol). No significant difference was found in samples collected with or without the antibacterial substance (**Table 1**).

Finally, we studied the effect of freezing on PAF measured in the samples. Urine samples were frozen about 1 month before the analysis. No significant differences appeared between PAF measured in fresh and frozen urine (Table 1).

As regards hydrolysis, we tested several solvents to extract 1-O-hexadecyl-2-O-acetyl-glycerol from the hydrofluoric acid: hexane, methylene chloride, and benzene. Hexane gave good recovery in extraction of 1-O-hexadecyl-2-O-acetyl-glycerol from the hydrolysis of PAF

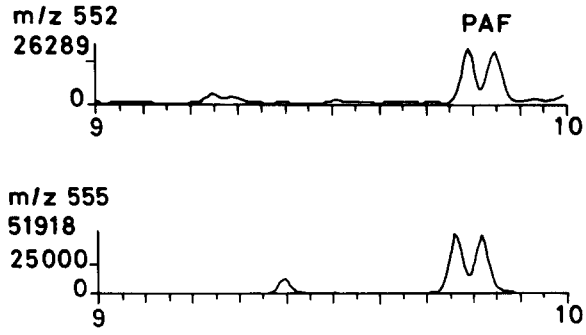

Fig. 2. Selected ion chromatograms monitoring the M^- ion of the pentafluorobenzoyl derivatives of PAF excreted in a 24-h urine sample (*m/z* 552) and at 555 for PAF-d_3 internal standard.

TABLE 1. PAF excretion in 24-h urine[a]

Urine	PAF[b]	n
	ng/day	
Fresh	2.08 ± 0.46	4
Frozen	2.41 ± 0.85	7
Fresh + HCl	2.51 ± 0.55	5
Fresh + NaN_3	2.08 ± 0.48	4

[a]Comparison of fresh and frozen urine and effect of addition of HCl or NaN_3 before urine collection.
[b]Mean ± SD. Each determination is from individual animals.

(76% calculated on [^{3}H]PAF recovery). Methylene chloride and benzene gave similar recoveries, but were discarded: methylene chloride for practical reasons (it represents the lower phase) and benzene for its toxicity.

For the derivatization, we observed that PFBCl was, in some cases, a source of undesirable impurities with peaks on the 552 trace, mainly at short retention times. These impurities did not directly interfere with PAF peaks, but they did dirty the GLC-MS system. The impurities were already present in the derivatizer (before heating) and did not evaporate under nitrogen. Thus, we preferred to distill the derivatizer. Alternatively, to eliminate these interfering substances, the sample could be purified on Sep-Pak silica cartridges. The sample, previously dissolved in 1 ml of hexane, was poured into the cartridge. Then, after a first fraction of 10 ml of hexane, the derivatized PAF was eluted with 7.5 ml of chloroform.

Fig. 1 presents SIR traces of PAF and PAF-d_3. No interfering peaks appeared at the retention time of PAF. This is because of the purification steps and the gas-liquid chromatographic conditions that were used. Distillation of the derivatizer avoided the presence of peaks eluting at about 5 min.

As previously discussed, the presence of the two peaks, with a fixed intensity ratio between them, produced by PAF, helped to confirm the absence of interfering peaks. We also constantly monitored the ions at *m/z* 551 and 554, to check the absence of peaks on these traces; these peaks could give interference from the isotopic contribution at *m/z* 552 and 555. Again, we did not find any peaks at the PAF retention time.

TABLE 2. PAF excretion in 24-h urine[a]

Urine	PAF-d_3	PAF[b]	n
	ng	*ng/day*	
Fresh	5	2.08 ± 0.46	4
Fresh	20	2.22 ± 0.77	4

[a]Comparison of the values after adding two different amounts of deuterated internal standard.
[b]Mean ± SD. Each determination is from individual animals.

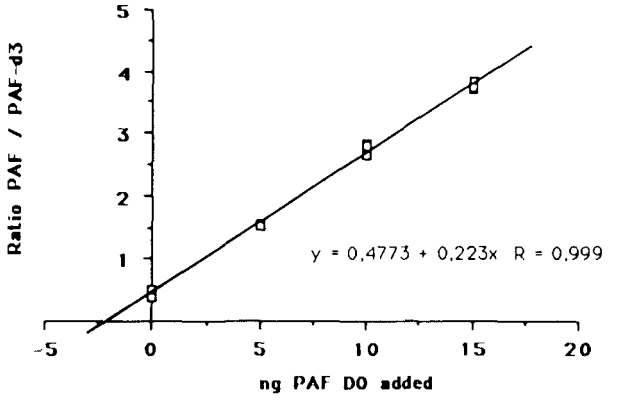

Fig. 3. Comparison of various amounts of PAF added (x) to urine and the ratio (y) of PAF/PAF-d_3 measured. Straight line intersects abscissa at a value equivalent to 2.14 ng of PAF/day. Each sample was injected three times.

To assess whether our deuterated internal standard when added to the urine could somehow originate protonated PAF, in some experiments we added two different amounts of PAF-d_3 (5 or 20 ng) to the urine samples. No differences were observed (**Table 2**).

To assess the accuracy of the assay, urine from four animals was pooled and then separated into four equal fractions. All were spiked with 5 ng of PAF-d_3 and three with 5, 10, and 15 ng of PAF. The samples were purified as usual, and the results, illustrated in **Fig. 3**, showed a good correlation ($r > 0.99$) between the amount of PAF added and the ratio PAF/PAF-d_3. The straight line intersects the abscissa at a value equivalent to 2.14 ng/day, which is in very close agreement with that found using the method described above.

The data appeared reproducible under all the conditions used, so the values measured can be taken as an exact assessment of PAF excretion in urine.

No studies have yet addressed the significance of PAF urinary excretion. Whether PAF in the urine reflects renal synthesis or whether structures in the urinary tract more distal than the kidney are responsible for the production of the PAF detected in the urine is not known. Given its short plasma half-life (20) and its rapid distribution into the various organs and tissues (23), PAF in the urine may reflect events in the kidney. However, the possibility that PAF is filtered in the glomerulus and remains in an aqueous milieu during the transit through the kidney tubules to the bladder is open to speculation. Future research is needed to clarify the pathophysiological significance of PAF excretion in order to establish whether it may represent an interesting tool for investigating renal disease.

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