Journal of Chromatography, 363 (1986) 412–417
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 18 747

Note

High-performance liquid chromatographic analysis of platelet activating factor on a cation-exchange column by direct ultraviolet detection

NIKOLAOS K. ANDRIKOPOULOS*
Chemical Department, Social Insurance Foundation (IKA), 8 Ag. Konstantinou Street, GR-102 41 Athens (Greece)
and
CONSTANTINOS A. DEMOPOULOS and ATHANASIA SIAFAKA-KAPADAI
Department of Food Chemistry, National University of Athens, 13a Navarinou Street, GR-106 80 Athens (Greece)
(First received December 9th, 1985; revised manuscript received April 15th, 1986)

Platelet activating factor (PAF) is a common mediator in a variety of acute allergic and inflammatory reactions[1,2] and has been identified as a mixture of 1-O-hexadecyl and 1-O-octadecyl-2-acetyl-*sn*-glycero-3-phosphorylcholine (AGEPC)[3–6]. Current knowledge on AGEPC has recently been reviewed[7,8].

Interest in the improvement of methodologies for PAF separation and purification is a growing. To this end thin-layer chromatography (TLC) and silicic acid column chromatography have been successfully employed, but they are usually, time-consuming. PAF can be effectively separated from other phospholipids by high-performance liquid chromatography (HPLC), the overall analysis time being reduced considerably. The column packings used include adsorption silica[9–16], diol bonded silica[17] and reversed phase[15]. The mobile phases used for isocratic elution include methanol–water in various proportions[9–12,17] and benzene–toluene[13]. Gradient elution was effected with mixtures of isopropanol–hexane and water[14], whilst for reversed-phase HPLC, acetonitrile–methanol–water containing 20 m*M* choline chloride was used as the mobile phase[15]. The latter system was also used for the analysis of 2-acyl-PAFs[18]. Detection was accomplished by measurement of the biological activity[9–12,15,16] or chemokinetic activity[17], by liquid scintillation counting[11–17], by radioactivity flow monitoring[13,17] and by UV spectroscopy[16]. UV detection has also been used for other phospholipids during the analysis of PAF[11,14,17]. A summary of the above mentioned HPLC methods is presented in Table I.

In the present communication we describe the isocratic purification of relatively large amounts of PAF and also its separation from other phospholipids, by HPLC on a silica-based cation-exchange column using acetonitrile–methanol–water as the mobile phase and monitoring directly by means of UV spectroscopy at 205 nm.

EXPERIMENTAL

All reagents used were of analytical grade purchased from Merck (Darmstadt,

0021-9673/86/$03.50 © 1986 Elsevier Science Publishers B.V.

F.R.G.), whilst HPLC-grade solvents were purchased from Rathburn (Walkerburn, U.K.). Standard lipids were purchased from Supelco (Bellefonte, PA, U.S.A.). PAF was synthesized and purified by TLC as described previously[3].

The biological activity towards washed rabbit platelets was measured as described previously[3] employing an aggregometer (Chronolog, CA, U.S.A.) coupled with a Omniscribe recorder (Houston, TX, U.S.A.). The concentration of PAF in methanol was adjusted by a colorimetric phosphorus assay[19].

HPLC was performed on a Series 3B liquid chromatograph (Perkin-Elmer, Norwalk, CT, U.S.A.) equipped with a Model 7105 loop valve injector (Rheodyne, Berkeley, CA, U.S.A.) and a 551 UV–VIS spectrophotometer fitted with 8-μl special flow microcells. The instruments were coupled to a Perkin-Elmer, Model 2, integrator and a Model 550 recorder. A 25 cm × 4.6 mm I.D. PXS 10/25 SCX (Whatman, Clifton, NJ, U.S.A.), packed with 10-μm silica covalently bound to benzenesulphonate residues, was employed. The chromatographic conditions are given in the figure legends.

RESULTS AND DISCUSSION

Two major difficulties are encountered during the separation of semisynthetic PAF (AGEPC, PAF-acether[8,16]), namely its rapid separation from other phospholipids and its direct detection. We have consequently employed a novel HPLC method making use of a cation-exchange column with subsequent UV detection of the eluted semi-synthetic PAF.

From Fig. 1 it can be seen that semisynthetic PAF and lyso-PAF, purified by TLC, exhibit absorbance at $\lambda_{max.}$ 204 and 206 nm respectively. Solutions of the phospholipid standards, semisynthetic PAF and lyso-PAF were injected into a cation-exchange HPLC column monitored by a UV detector at 205 nm. A cation-exchange column has previously been employed for the separation of various phospholipids[20], but our improved method provides for satisfactory separation of PAF from the usually coeluting sphingomyelin (SM), lysophosphatidylcholine (LPC) and lyso-PAF using the solvent system acetonitrile–methanol–water.

As shown in Fig. 2, PAF was eluted in about 25 min. The detection of PAF was accomplished with the UV detector at 205 nm. As little as 0.25 mg of PAF can easily be detected by these means and up to 2.5 mg of PAF can also be separated and detected in one step. Flow programming can also be used for the reduction of the analysis time, as shown in Fig. 3. Subsequent studies along the same lines have provided evidence for the reproducibility of the chromatograms obtained; the recovery of the lipid standards and semi-synthetic PAF and lyso-PAF was 98%.

Wardlow[16] employed the method of Chen and Kou[21] for the separation of micro quantities of PAF (10 μg) from other phospholipids, by UV detection at 203 nm, but SM coeluted with LPC, the other phospholipids —as usually obtained in biological materials— were partially overlapped and lyso-PAF was not separated from SM.

The cation-exchange HPLC analysis of PAF meets the criteria of a short analysis time with isocratic elution, clear separation of the major phospholipid classes among themselves, including PAF and lyso-PAF, column stability and reproducible retention times. The UV detection has the advantage of non-specific instrumentation and of one-step purification of relatively large amounts of PAF.

TABLE I

HPLC METHODS USED FOR THE ANALYSIS OF PAF

Abbreviations: BA = biological activity; LSC = liquid scintillation counting; RA = radioactivity; CA = chemokinetic activity; UV = ultraviolet.

Column	*Mobile phase composition*	*Elution (flow-rate, ml/min)*	*Retention time of PAF (min)*	*Detection*	*Ref.*
MicroPak Si 5 (5 μm)	Chloroform–methanol–water	Isocratic	–	BA	9
MicroPak Si 5 (5 μm), 25 cm × 8.0 mm I.D.	Chloroform–methanol–water (60:50:5)	Isocratic (3.33)	17	BA	10
LiChrosorb Si 60 (5-μm), 25 cm × 9.0 mm I.D.	1st: Acetonitrile–methanol–water (60:30:10)	Isocratic (5.0)	23	BA, LSC	11
	2nd: *n*-Hexane–isopropanol–water (39:52:9)	Isocratic (5.0)	30		
Microporasil	Dichloromethane–methanol–water (60:50:5)	Isocratic (1.7)	14	BA, LSC	12
LiChrosorb Si 60 (10 μm) 25 cm × 4.5 mm I.D.	A = Propanol–ethyl acetate–benzene–water (130:80:30:20) B = Propanol–toluene–acetic acid–water (93:110:15:15)	Isocratic Flow programming A = 1 to 1.5 in 35 min, B = 1 to 2 in 65 min)	77	LSC, RA	13
Ultrasphere Si (5 μm) 25 cm × 4.6 mm I.D.	A = Isopropanol–hexane (1:1) B = Water or A and B + 0.005% acetic acid or conc. ammonia	Gradient B = 4 to 8% in 15 min or 4 to 8.5% in 20 min (2.0)	20	LSC	14
LiChrosorb-DIOL (10 μm) 25 cm × 4.6 mm I.D.	*tert.*-Butyl methyl ether–methanol–water–ammonia (200:100:10:0.02)	Isocratic (1.0 or 2.0)	8	LSC, RA, CA	17
Radial-Pak silica (10 μm) 10 cm × 8 mm I.D.	Acetonitrile–methanol–85% phosphoric acid (130:5:1.5)	Isocratic (2.0)	20	LSC	15
Radial-Pak C_{18} (10 μm) 10 cm × 8 mm I.D.	Choline chloride (20 m*M*) in methanol–water–acetonitrile (85:10:5)	Isocratic (1.0)	20	BA, LSC	15
MicroPak Si 10, LiChrosorb Si 60, μPorasil (10 μm); 25 or 30 cm × 4 mm I.D.	Acetonitrile–methanol–85% phosphoric acid (130:5:1.5)	Isocratic (1.0)	13	BA, LSC, UV	16

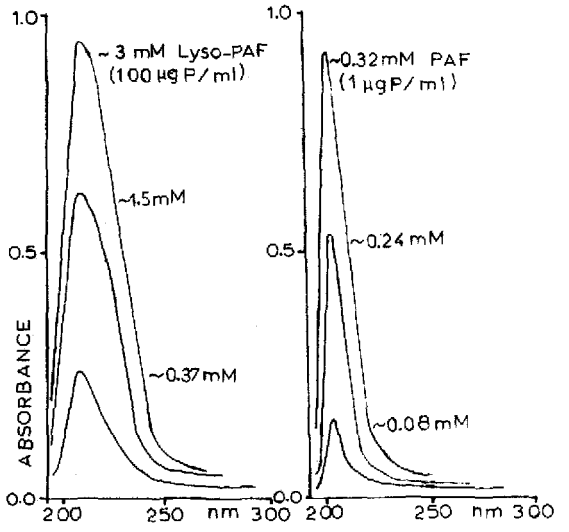

Fig. 1. UV spectra of semisynthetic lyso-PAF and PAF in methanol. The concentrations were based on phosphorus determinations.

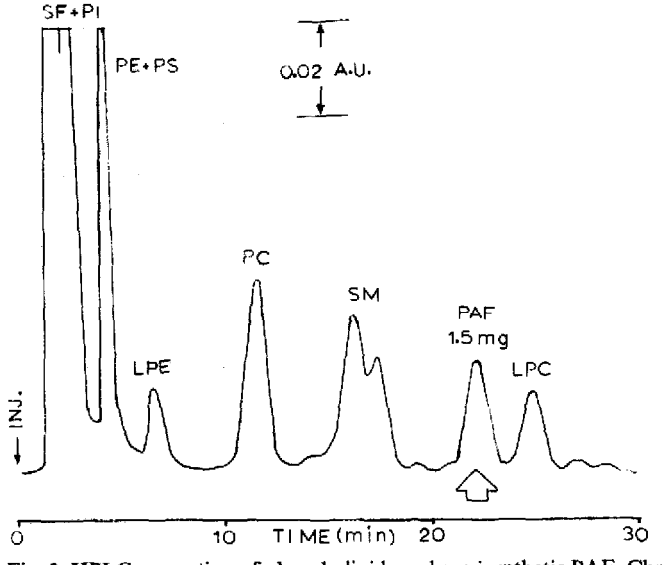

Fig. 2. HPLC separation of phospholipids and semisynthetic PAF. Chromatographic conditions: column, 10-µm Partisil PSX/SCX (Whatman), 25 cm × 4.6 mm I.D.; elution, isocratic (1.5 ml/min), acetonitrile–methanol–water (300:150:35); detection, UV at 205 nm, 0.2 a.u.f.s.; injections, 50 µl of 0.1% phospholipid mixture in chloroform and 100 µl of 1.5% PAF in methanol. The arrow indicates the collected fraction with biological activity. SF = Solvent front; PI = phosphatidylinositol; PE = phosphatidylethanolamine; PS = phosphatidylserine; LPE = lysophosphatidylethanolamine; PC = phosphatidylcholine; SM = sphingomyelin; PAF = platelet activating factor; LPC = lysophosphatidylcholine.

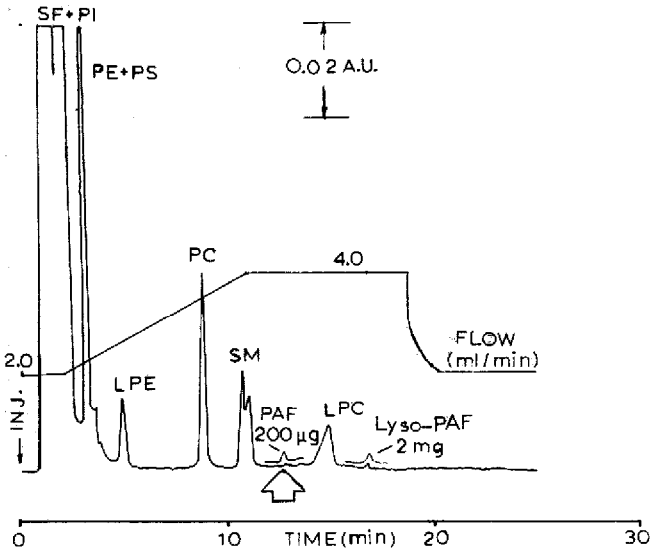

Fig. 3. HPLC separation of phospholipids, semisynthetic PAF and lyso-PAF. Flow programming is indicated. Injections: 50 µl of 0.1% phospholipid mixture in chloroform and 100 µl of a mixture of 0.2% PAF and 2% lyso-PAF in methanol. Other conditions and abbreviations as in Fig. 2.

It is interesting that, in contrast to previous HPLC analytical methods[9–17], by the present method relatively large amounts of PAF and lyso-PAF can easily be separated and therefore the method is especially suitable for the purification of PAF during its synthesis in the laboratory.

ACKNOWLEDGEMENT

The comments of Dr. M. C. Moschidis are appreciated.

REFERENCES

1 P. M. Henson and C. G. Cochrane, *J. Exp. Med.*, 129 (1969) 167.
2 P. M. Henson, *J. Exp. Med.*, 131 (1970) 287.
3 C. A. Demopoulos, R. N. Pinckard and D. J. Hanahan, *J. Biol. Chem.*, 254 (1979) 9355.
4 J. Benveniste, M. Tence, P. Varenne, J. Bidault, C. Boullet and J. Polonsky, *C.R. Acad. Sci., Ser. D*, 289 (1979) 1037.
5 M. L. Blank, F. Snyder, W. Byers, B. Brooks and E. E. Muirhead, *Biochem. Biophys. Res. Commun.*, 90 (1979) 1194.
6 D. J. Hanahan, C. A. Demopoulos, J. Liehr and R. N. Pinckard, *J. Biol. Chem.*, 255 (1980) 5514.
7 R. N. Pinckard, L. M. McManus and D. J. Hanahan, in G. Weissmann (Editor), *Advances in Inflammation*, Vol. 4, Raven Press, New York, 1982, pp. 147–180.
8 F. Spener, J. Benveniste and B. B. Vargaftig, in H. K. Mangold and F. Paltauf (Editors), *Ether Lipids, Biochemical and Biomedical Aspects*, Academic Press, New York, 1983, pp. 252–257, 355–376.
9 J. Benveniste, J. P. Le Couedic, J. Polonsky and M. Tence, *Nature (London)*, 269 (1977) 170.
10 M. Tence, J. Polonsky, J. P. Le Couedic and J. Benveniste, *Biochemie*, 62 (1980) 251.
11 H. Chap, G. Mango, M. F. Simon, J. Benveniste and L. Douste-Blazy, *Nature (London)*, 289 (1981) 312.
12 E. Ninio, J. M. Mencia-Huerta, F. Heymans and J. Benveniste, *Biochim. Biophys. Acta*, 710 (1982) 23.

13 I. Alam, J. B. Smith, M. J. Silver and D. Ahern, *J. Chromatogr.*, 234 (1982) 218.
14 M. L. Blank and F. Snyder, *J. Chromatogr.*, 273 (1983) 415.
15 E. M. Jackson, G. E. Mott, E. Hoppens, L. M. McManus, S. T. Weintraub, J. G. Ludwig and R. N. Pinckard, *J. Lipid Res.*, 25 (1984) 753.
16 M. L. Wardlow, *J. Chromatogr.*, 342 (1985) 380.
17 A. I. Mallet, F. M. Cunningham and R. Daniel, *J. Chromatogr.*, 309 (1984) 160.
18 F. H. Chilton, J. T. O'Flaherty, J. M. Ellis, C. L. Swendsen and R. L. Wykle, *J. Biol. Chem.*, 258 (1983) 6357.
19 G. R. Bartlett, *J. Biol. Chem.*, 234 (1959) 466.
20 R. W. Cross and B. E. Sobel, *J. Chromatogr.*, 197 (1980) 79.
21 S. S.-H. Chen and A. Y. Kou, *J. Chromatogr.*, 227 (1982) 25.