*Journal of Chromatography*, 424 (1988) 367–372
*Biomedical Applications*
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 3960

## Note

# Sensitive high-performance liquid chromatographic method for the determination of phosphatidic acid

KOUJI YAMADA*, SHINYA ABE, KOUICHI KATAYAMA and TADASHI SATO

*Tsukuba Research Laboratories, Eisai Co., Ltd., 1-3, Tokodai-5-chome, Toyosato-machi, Tsukuba-gun, Ibaragi 300-26 (Japan)*

(First received May 5th, 1987; revised manuscript received September 15th, 1987)

Phosphatidic acid (PA) is the precursor of diacylglycerol, which is required in the de novo synthesis of phosphatidylcholine, phosphatidylethanolamine and triacylglycerol [1–3]. In addition, it has been reported that PA has ionophoretic properties in vitro, and it has been suggested that PA derived from the inositol lipids might mediate inward $Ca^{2+}$ movement by acting as an endogenous $Ca^{2+}$ ionophore [4,5]. Recently, Creek at al. [6] have reported mannosylation of PA by liver microsomal membranes. They showed that the endogenous PA acts as a mannosyl acceptor. It is, therefore, important to determine the content of PA in biological materials. In general, the determination of PA has been carried out by using the $^{32}P$ incorporation technique, but this method requires tedious purification procedures. Several high-performance liquid chromatographic (HPLC) methods with UV detection have been reported recently [7–9]. Although these methods are more effective for the separation of the individual molecular species of PA than argentation thin-layer chromatography, which is a commonly used technique [10], they are not sufficiently sensitive for the quantitative analysis of PA in biological materials.

In this paper, we describe a sensitive and direct assay method for PA. The assay is based on the derivatization of PA with 3-(9-anthryl)diazo-2-propene, followed by HPLC on silica with fluorimetric detection. The method is sensitive enough to determine PA in the concentration range of interest in biological research.

0378-4347/88/$03.50 

## EXPERIMENTAL

### *Chemicals*

Dipalmitoyl phosphatidic acid was purchased from Sigma (St. Louis, MO, U.S.A.) and 9-anthryldiazomethane was obtained from Funakoshi (Tokyo, Japan). 3-(9-Anthyl)diazo-2-propene was prepared from 3-(9-anthryl)propenal according to the method of Nakaya et al. [11]. 3-(9-Anthryl)propenal was synthesized as follows. 9-Anthraaldehyde (Aldrich, Milwaukee, WI, U.S.A.) was treated with triethyl phosphonoacetate (Aldrich) to give ethyl 3-(9-anthryl)propeonate. This ester was reduced and oxidized to yield 3-(9-anthryl)propenal. Ethyl acetate, methanol and *n*-hexane were of HPLC grade. All other reagents were of analytical grade.

### *High-performance liquid chromatography*

Chromatographic analyses were performed with a JASCO Twincle pump, a Waters sample processor (Model 710B) and a JASCO FP-110 fluorescence detector. Chromatographic separation was carried out on a Nucleosil column (30 cm × 4.6 mm I.D., 5 μm particle size, Chemco, Tokyo, Japan). The mobile phase was ethyl acetate–*n*-hexane–methanol (70:30:7.5, v/v/v) containing 0.01% diethylamine, and the flow-rate was 1.5 ml/min. Fluorescence was detected with excitation at 254 nm and emission at 430 nm.

### *Reaction of PA with 3-(9-anthryl)diazo-2-propene*

PA was extracted according to the method of Folch et al. [12]. The extracts were evaporated to dryness at 40°C under a stream of nitrogen, and the residue was dissolved in 100 μl of methanol. The solution was mixed with 500 μl of 200 m*M* 3-(9-anthryl)diazo-2-propene in diethyl ether, and the mixture was allowed to stand at 4°C for 8 h until derivatization was completed. The mixture was then evaporated to dryness, and the residue was dissolved in 200 μl of chloroform. The chloroform solution was applied to a Bond Elut SI column (Analytichem International, Harbor City, CA, U.S.A.), the column was washed with 3 ml of chloroform and 3 ml of chloroform–methanol (9:1, v/v), and then eluted with 2 ml of chloroform–methanol (6:4, v/v). The eluate was evaporated to dryness, and the residue was dissolved in 200 μl of ethyl acetate. A 30-μl aliquot of this solution was then analysed by HPLC.

### *Time course of derivatization*

The standard PA (1 μg) and 3-(9-anthryl)diazo-2-propene in diethyl ether (500 μl) were mixed and allowed to stand at 4°C for 12 h. The time course of derivatization of PA was followed by the HPLC method.

*Fast atom bombardment mass spectrometry*

Di[3-(9-anthryl)-2-propenyl]PA (ca. 10 $\mu$g), which was obtained as described above, was analysed by fast atom bombardment (FAB) mass spectrometry (MS) using a JEOL HX-100 instrument (Tokyo, Japan).

*Calibration curve*

Samples containing 0.1–25 ng of PA in 1 ml of distilled water were prepared. These samples were extracted, derivatized, and analysed by HPLC. Calibration curves of the peak height of PA against PA concentration were constructed.

*Preparation of washed rabbit platelets*

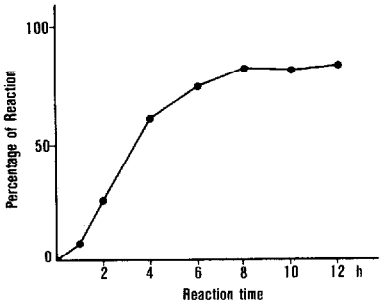
Washed rabbit platelets were prepared by the method of Ardlie et al. [13]. Platelets sedimented from platelet-rich plasma were resuspended in 10 ml of calcium- and phosphate-free Tyrode's albumin solution (pH 6.35) containing 5 m*M* 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid (HEPES) and 0.27 m*M* ethylene glycol tetraacetate (EGTA). After centrifugation at 1500 *g* for 10 min, the platelets were resuspended in calcium- and phosphate-free Tyrode's albumin at ca. $10^7$ platelets per ml of suspension.

## RESULTS AND DISCUSSION

Fluorimetric detection is generally expected to provide better sensitivity than UV detection and, therefore, the derivatization of PA with a fluorescent chromophore, 3-(9-anthryl)diazo-2-propene, was investigated. First, PA was treated with 9-anthryldiazomethane, which is generally used to measure fatty acids with fluorescence detection [14]. However, the fluorescence intensity of the product was low (the detection limit was 2 ng/ml at a signal-to-noise ratio of 4). Thus, PA was next derivatized with 3-(9-anthryl)diazo-2-propene. The fluorescence intensity of the resulting di[3-(9-anthryl)-2-propenyl]PA was ca. ten-fold higher than that of di(9-anthryl)PA. Between the derivatization of fatty acids with 9-anthryldiazomethane and 3-(9-anthryl)diazo-2-propene there was no difference in the fluorescence intensity (the detection limit was 0.05 ng/ml at a signal-to-noise ratio of 4).

Fig. 1 shows the time course of formation of di[3-(9-anthryl)-2-propenyl]PA. The percentage of the products increased with time and reached an almost constant value at 8 h. The percentage formation was $82.1 \pm 2.32\%$ (mean $\pm$ S.D., $n=3$).

Fig. 2 shows an HPLC profile of the di[3-(9-anthryl)-2-propenyl] derivative of the standard PA. The derivative of PA was eluted within 25 min. The peak was collected, pooled and analysed by FAB-MS (Fig. 3). The mass peak at 1081 corresponds to the molecular mass of di[3-(9-anthryl)-2-propenyl]PA. Fig. 4 shows the excitation spectrum of the di[3-(9-anthryl)-2-propenyl] derivative of PA; there are two excitation peaks at 254 and 360 nm, and excitation at 254 nm pro-



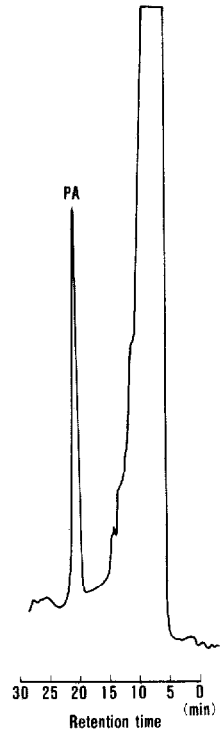



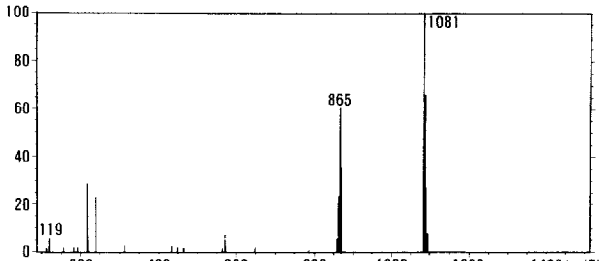

Fig. 1. Time course of formation of di[3-(9-anthryl)-2-propenyl]PA, measured by HPLC with fluorimetric detection. Each point is the mean of three determinations.

Fig. 2. Chromatogram of di[3-(9-anthryl)-2-propenyl]PA, obtained under the conditions described in Experimental.

duced a fluorescence emission signal ca. six-fold greater than that obtained with excitation at 360 nm. A calibration curve obtained by this method showed excellent linearity with amounts of PA in the range 0.5–25 ng. The minimum detectable amount of PA by this method was 0.1 ng/ml. The sensitivity of this HPLC–fluorimetric method is ca. 50-fold that of the HPLC–UV technique.

By using this HPLC–fluorimetric method, we have measured the amount of PA in unstimulated washed rabbit platelets. Fig. 5 shows an HPLC profile of PA in unstimulated washed rabbit platelets. The concentration of PA was $21 \pm 1.8$ ng per $10^7$ platelets ($n=4$, mean $\pm$ S.D.), and was in good agreement with the data of Vickers et al. [15]. Thus this method is suitable for the measurement of the amount of PA in stimulated platelets.

In conclusion, we have developed a highly sensitive assay for determination of PA by HPLC with fluorimetric detection. This method should be useful for investigations of the biological functions of phosphatidic acid.



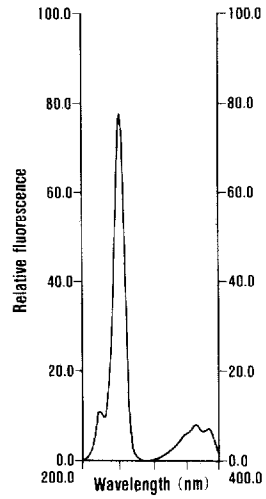




Fig. 3. Fast atom bombardment mass spectrum of di[3-(9-anthryl)-2-propenyl]PA isolated by HPLC. The mass peak at 1081 corresponds to the molecular mass of di[3-(9-anthryl)-2-propenyl]PA.

Fig. 4. Excitation spectrum of di[3-(9-anthryl)-2-propenyl]PA, determined at the emission wavelength of 430 nm using a Shimadzu RF-540 spectrofluorophotometer (Kyoto, Japan).

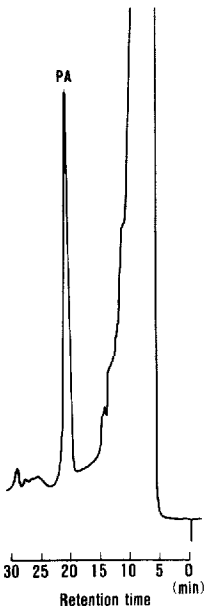



Fig. 5. Chromatogram of PA in unstimulated washed rabbit platelets, obtained under the conditions described in Experimental.

ACKNOWLEDGEMENT

The authors thank Prof. Toshio Nambara, Pharmaceutical Institute, Tohoku University, for his help in the preparation of the manuscript.

REFERENCES

1 P.A. Weinhold, J. Lipid Res., 9 (1968) 262.
2 P.M. Farrell, M.F. Epstein, A.R. Fleischmann, G.F. Oakes and R.A. Che, Biol. Neonat., 29 (1976) 238.
3 B. Rustow, D. Kunze, H. Rabe and G. Reichmann, Biochim. Biophys. Acta, 835 (1985) 465.
4 C.N. Serhan, J. Fridovich, E.J. Goetzl, P.B. Dunham and G. Weismann, J. Biol. Chem., 257 (1982) 4746.
5 A. Imai, Y. Ishizuka, K. Kawai and Y. Nozawa, Biochem. Biophys. Res. Commun., 108 (1982) 752.
6 K.E. Creek, D. Rimoldi, A.J. Clifford, C.S. Silverman-Jones and L.M. De Luca, J. Biol. Chem., 261 (1986) 3490.
7 H. Kato, K. Ishidate and Y. Nakazawa, Biochim. Biophys. Acta, 796 (1984) 262.
8 M.L. Blank, M. Robinson, V. Fitzgerald and F. Synder, J. Chromatogr., 298 (1984) 473.
9 Y. Nakagawa and K. Waku, J. Chromatogr., 381 (1986) 225.
10 O. Renkonen, Biochim. Biophys. Acta, 125 (1966) 288.
11 T. Nakaya, T. Tomomoto and M. Imoto, Bull. Chem. Soc. Jpn., 40 (1967) 691.
12 J. Folch, M. Lees and G.H. Sloane-Stanley. J. Biol. Chem., 226 (1957) 497.
13 N.G. Ardlie, D.W. Perry, M.A. Packham and J.F. Mustard, Proc. Soc. Exp. Biol. Med., 136 (1971) 1021.
14 N. Nimura and T. Kinoshita, Anal. Lett., 13 (1980) 191.
15 J.D. Vickers, R.L. Kinlough-Rathbone and J.F. Mustard, Biochem. J., 219 (1984) 25.