Journal of Chromatography, 494 (1989) 283–288
Biomedical Applications
Elsevier Science Publishers B V , Amsterdam — Printed in The Netherlands

CHROMBIO 4879

Note

Determination of phosphoethanolamine in animal tissues by gas chromatography with flame photometric detection

HIROYUKI KATAOKA, NORIHISA SAKIYAMA, MITSUYO MAEDA and MASAMI MAKITA*

Faculty of Pharmaceutical Sciences, Okayama University, Tsushima, Okayama 700 (Japan)

(First received April 17th, 1989, revised manuscript received May 30th, 1989)

Phosphoethanolamine (PEA) is known to be present in most animal tissues, but its biological importance is unknown, apart from its role as a possible intermediary in the metabolism of phospholipid [1] However, it is reported that urinary excretion of PEA is significantly increased in patients with hypophosphatasia [1] and metabolic born disease [2], and the PEA content of the brain is significantly decreased in the patients with Alzheimer's disease and Huntington's disease [3] In addition, the influence of PEA on the growth of mammary carcinoma in rats [4,5] deserves attention

The determination of PEA in biological material has been carried out by colorimetric [1,2] and fluorometric [6] assay methods. However, these methods lack sensitivity and usually require time-consuming preliminary clean-up of the sample Chromatographic procedures utilizing an amino acid analyser [7,8] or high-performance liquid chromatography [3,9–11] have also been used for the assay, but when they were applied directly to the biological sample there appears to be difficulty in resolving PEA from interfering components [12–14]

Recently, we developed a convenient and reliable method for the determination of aminoalkyl phosphates by gas chromatography (GC) with flame ionization detection (FID) in which these compounds were analysed as their N-isobutoxycarbonyl (N-isoBOC) methyl ester derivatives [15] This paper describes a sensitive and selective method for the determination of PEA in bio-

0378-4347/89/$03 50

logical samples by GC with flame photometric detection (FPD) The method was used for study the distribution of PEA in several animal tissues.

EXPERIMENTAL

Chemicals

PEA, 2-aminoethylphosphonic acid (AEP), O-phospho-DL-threonine (P-Thr) and O-phospho-L-serine (P-Ser) were obtained from Sigma (St. Louis, MO, U S A.) Standard solutions were prepared containing 10 μg/ml of each compound in distilled water Phosphopropanolamine (PPA), used as an internal standard (I.S), was prepared by a modification of the method of Weisburger and Schneider [16] as follows 0.25 ml of 85% orthophosphoric acid was pipetted into a 10-ml Pyrex glass tube with a PTFE-lined screw-cap, and most of the water was removed in vacuo To the residue were added 0 15 ml of *n*-propanolamine (Nacalai Tesque, Kyoto, Japan) and 0 1 g of phosphorus pentoxide, and the tube was tightly capped and heated in dry block bath (Toyo Kagaku, Tokyo, Japan) at 140°C. After 12 h the reaction mixture was adjusted to pH 7 with 2 *M* sodium hydroxide Subsequently the solution was applied to a 30 cm $\times$0.9 cm I.D Dowex 50W-X8 (H^+) column (100–200 mesh), washed with 100 ml of water, and then eluted with 0 25 *M* hydrochloric acid. The initial 15 ml of the eluate were discarded, and the following 50 ml of the eluate were collected as PPA fraction The PPA fraction was evaporated and the residue was reconstructed in water to prepare a 10 μg/ml solution Isobutylchloroformate (isoBCF) was obtained from Tokyo Kasei Kogyo (Tokyo, Japan) and used without further purification. N-Methyl-N-nitroso-*p*-toluenesulphonamide for use in generation of diazomethane was obtained from Nacalai Tesque. All other chemicals were of analytical grade

Preparation of samples

The following species were used in the experiment; three cuttlefish, three mackerel, five chickens and six mice Immediately after dissection each organ was removed and frozen, and these frozen tissues were stored at -20°C until each assay Each tissue was homogenized with ten volumes of 5% trichloroacetic acid (TCA) using a Model LK-21 ultra-disperser (Yamato Kagaku, Tokyo, Japan) After centrifugation at 2000 *g* for 5 min, the precipitate was re-extracted with five volumes of 5% TCA The supernatants were combined and made up to a constant volume with distilled water, and used for the analysis

Derivatization procedure

Tissue extracts were derivatized as previously described [15] An aliquot of the sample solution containing 0.05–5 μg of PEA was pipetted into a 10-ml Pyrex glass tube with a PTFE-lined screw-cap. After addition of 0.1 ml of 10 μg/ml I.S. solution, the solution was adjusted to pH 10 with 2 *M* sodium hy-

droxide solution and the total reaction volume was made up to 1 0 ml with water if necessary Immediately after the addition of 0.1 ml of isoBCF, the mixture was shaken with a shaker set at 300 rpm (up and down) for 10 min at room temperature The reaction mixture was acidified to pH 1–2 with 2 *M* hydrochloric acid and extracted with 3 ml of diethyl ether in order to remove the excess of reagent, and the ethereal extract was discarded The aqueous layer was saturated with sodium chloride, and then extracted twice with 3 ml of diethyl ether containing 10% 2-propanol. The pooled ethereal extracts were methylated by bubbling diazomethane, generated according to the micro-scale procedure of Schlenk and Gellerman [17], through this solution until a yellow tinge became visible. After standing at room temperature for 5 min, the solvents were evaporated to dryness at 80 °C under a stream of dry air The residue was dissolved in 0.1 ml of ethyl acetate, and 1–2 μl of this resolution were injected into the gas chromatograph

Gas chromatography

GC analysis was carried out with a Shimadzu 7A gas chromatograph equipped with a flame ionization detector and flame photometric detector (P-filter) The column packing, 3% DCQF-1 on Uniport HP (100–120 mesh), was prepared using toluene as a coating solvent according to the solution-coating technique [18], and was poured into a silanized glass column (1.5 m×3 mm I D) conditioned at 260 °C for 24 h with hydrogen at a flow-rate of 30 ml/min The operating conditions were as follows: column temperature, programmed at 2 °C/min from 210 °C to 235 °C, injection and detector temperature, 250 °C, nitrogen flow-rate, 50 ml/min. The peak heights of PEA and the I.S were measured, and the peak-height ratios against the I.S. were calculated for the construction of calibration curve.

Gas chromatography–mass spectrometry (GC–MS)

A Hewlett-Packard 5890A gas chromatograph was operated in connection with a VG Analytical 70-SE mass spectrometer and VG 11-250J mass data system. The GC column was of the same type as used for GC analysis, with an ionizing voltage of 20 eV, an ion-source temperature of 250 °C, and a helium flow-rate of 40 ml/min

RESULTS AND DISCUSSION

PEA could be conveniently converted into its N-isoBOC methyl ester derivative as previously described [15] As shown in Fig. 1A, PEA was completely separated from other biological phosphorus compounds, such as AEP, P-Thr and P-Ser on 3% DCQF-1 The PEA derivative provided an excellent FPD response, the minimum detectable amount of PEA to give a signal three times as high as the noise under our instrumental conditions was ca 30 pg The GC–

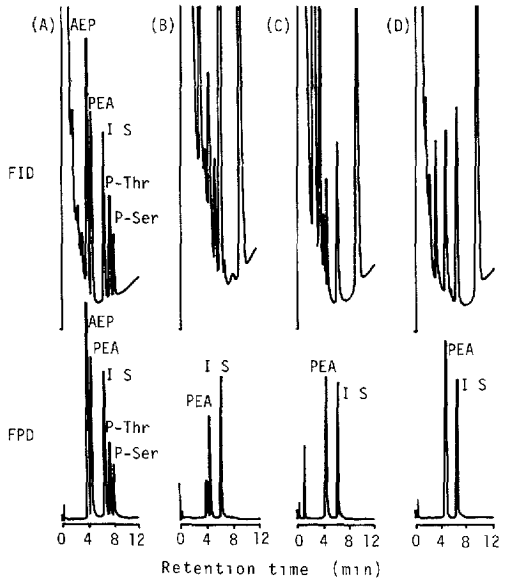

Fig 1 Gas chromatograms obtained from standard solution and animal tissues (A) standard (containing 1 μg of each compound), (B) mackerel kidney, (C) chicken heart, (D) mouse brain The derivatized samples were analysed by both GC-FID (top, 4 μl) and GC-FPD (bottom, 0 5 μl) GC conditions are given in Experimental Attenuation GC-FID, $4 \cdot 10^2$, GC-FPD, 128 10

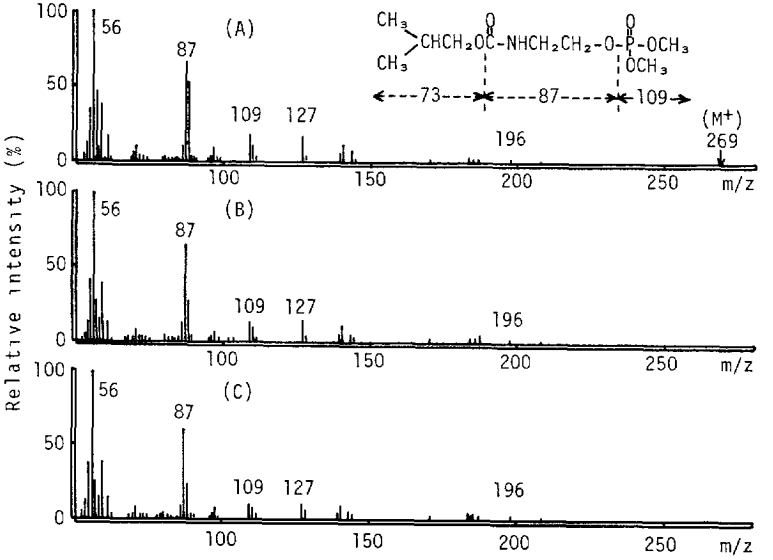

Fig 2 GC-MS spectra obtained from the N-isoBOC methyl ester derivative of authentic PEA and from the peak identified with PEA in animal tissues (A) authentic PEA, (B) chicken spleen, (C) mouse brain

TABLE I

RECOVERIES OF PHOSPHOETHANOLAMINE ADDED TO SEVERAL ANIMAL TISSUES

Sample	Added (μg/g)	Amount found (mean ± S D , $n=3$) (μg/g)		Recovery (%)
		Non-addition	Addition	
Cuttlefish liver	84 4	61 3±0 1	142 4±2 8	96 1
Mackerel kidney	75 2	34 2±0 4	107 1±3 4	97 0
Chicken heart	79 2	49 0±1 2	126 8±3 8	98 2
Mouse brain	217 4	230 3±3 8	447 9±8 3	100 1

TABLE II

PHOSPHOETHANOLAMINE CONTENT IN SEVERAL ANIMAL TISSUES

Tissue	Phosphoethanolamine content (mean ± S D , $n=3$) (μg/g wet weight)			
	Cuttlefish	Mackerel	Chicken	Mouse
Brain	-	43 3±0 5	383 0±6 3	230 2±3 8
Heart	21 9±1 1	13 8±0 3	49 0±1 2	28 6±1 8
Gill	5 5±0 3	23 7±0 4	-	-
Lung	-	-	74 2±1 3	193 9±4 2
Liver	61 3±0 1	16 2±0 7	52 7±2 0	98 2±2 6
Spleen	-	361 2±10 8	453 4±13 6	767 3±2 9
Pancreas	-	-	420 5±5 9	662 1±20 1
Kidney	-	34 2±0 4	-	169 8±0 5
Stomach	6 0±0 3	45 3±3 0	128 7±6 1	166 2±1 7
Muscle	1 5±0 1	14 6±0 1	16 2±0 1	8 0±0 4

FPD method described here was over 200 times more sensitive than our GC–FID method previously reported [15] In order to test the linearity of the calibration curve, various amounts of PEA ranging from 0 05 to 5 μg were derivatized and aliquots representing 0 5–50 ng of PEA were injected A linear relationship was obtained, and the regression line was $y=1\ 140x-0\ 070$ ($r=0\ 9998$, $n=14$), where y is the peak-height ratio and x is the amount of PEA

The method developed was successfully applied to biological materials Fig 1B–D show the chromatograms obtained from animal tissues by GC–FID (top) and GC–FPD (bottom) It was difficult to determine the PEA in tissue samples by GC–FID, because of the interfering peaks However, the PEA in tissue samples could be analysed by GC–FPD without any such interference The PEA peak obtained from each tissue sample was confirmed by GC–MS (Fig 2) As shown in Table I, the recoveries of PEA added to animal tissues were

96 1–100.1%, and the relative standard deviations were 0.2–3.2% ($n=3$), indicating that this method is accurate and precise. The PEA contents of several animal tissues (Table II) shows that PEA is widely distributed. spleen, pancreas and brain contain considerable amounts whereas muscle, heart and liver do not. Similar results were reported by Hayase et al. [10].

In conclusion, these experiments have conclusively demonstrated that PEA can be accurately and precisely determined by GC–FPD as its N-isoBOC methyl ester derivative This method is selective and sensitive, and complex biological materials can be analysed without any interference from other substances We believe that this method provides a useful tool in biochemical and biomedical research where PEA assay is required.

REFERENCES

1 K Rasmussen, Dan Med Bull , 15 (1968) 1
2 A A Licata, N Radfar, F C Bartter and E Bou, Am J Med , 64 (1978) 133
3 D W Ellison, M F Beal and J B Martin, Brain Res , 417 (1987) 389
4 T Kano-Sueoka, D M Cohen, Z Yamaizumi, S Nishimura, M Mori and H Fujiki, Proc Natl Acad Sci U S A , 76 (1979) 5741
5 T Kano-Sueoka and J E Errick, Exp Cell Res , 136 (1981) 137
6 G Gundlach, A Kabisch, R. Voegeli, H Pralle and S Hartenfels, Anal Biochem , 142 (1984) 511
7 M V Sande and K V Camp, Invest Urol , 16 (1979) 445
8 J R Eastman and D Bixler, Clin Chem , 26 (1980) 1757
9 Y Hayase, M. Murakami, I Kashiwakura and Y Takagi, Yakugaku Zasshi, 106 (1986) 615
10 Y Hayase, M Murakami and Y Takagi, Yakugaku Zasshi, 106 (1986) 694
11 D W Ellison, M F Beal and J B Martin, J Neurosci Methods, 19 (1987) 305
12 K H Tachiki, H C Hendrie, J Kellams and M H Aprison, Clin Chim Acta, 75 (1977) 455
13 B R Larsen, D S Grosso and S Y Chang, J Chromatogr Sci , 18 (1980) 223
14 H F Erbersdobler, H G Greulich and E Trautwein, J Chromatogr , 254 (1983) 332
15 H Kataoka, N Sakiyama and M Makita, J Chromatogr , 436 (1988) 67
16 J H Weisburger and W C Schneider, J Org Chem , 26 (1961) 1658
17 H Schlenk and J L Gellerman, Anal Chem , 32 (1960) 1412
18 E C Horning, W J A VandenHeuvel and B G Creech, Methods Biochem Anal , 11 (1963) 69