Journal of Chromatography, 342 (1985) 359—362
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 2638

Note

Fatty acid analysis in phosphatidylethanolamine subclasses of human erythrocyte membranes by high-performance liquid chromatography

YASUNOBU ANTOKU*, TETSUO SAKAI and HIROSHI IWASHITA

Department of Neurology, National Chikugo Hospital, 515 Kurakazu, Chikugo City 833 (Japan)

(First received January 2nd, 1985; revised manuscript received March 12th, 1985)

Erythrocyte membrane lipid are composed of cholesterol, phospholipids and a small amount of glycolipids [1], and the phospholipids contain mainly sphingomyelin and three glycerophospholiplid classes: phosphatidylcholine, phosphatidylethanolamine (PE) and phosphatidylserine. Erythrocyte membranes are known to include plasmalogen form in glycerophospholipids, especially in PE [2—4].

Human erythrocyte membrane fatty acids in phospholipid classes have been described in detail [3, 5—9], but there have been only a few reports of fatty acid analysis on phospholipid subclasses; plasmalogen form and diacyl form [4- 6]. Therefore, we studied fatty acids of PE subclasses of human erythrocyte membranes by high-performance liquid chromatography (HPLC).

EXPERIMENTAL

Heparinized blood samples were obtained from seven healthy adults fasted overnight, and were immediately cooled in ice-water for ca. 1 h. The erythrocyte membranes were prepared as described previously [10] and suspended in 10 m*M* Tris–HCl buffer solution (pH 7.4) to bring the protein concentration to ca. 4 mg/ml. They were stored at −80°C until use.

Lipids were extracted from 0.5 ml of the membrane suspension by the method of Folch et al. [11], and the lower chloroform phase was evaporated to dryness in a stream of dry nitrogen. The lipid extracts were separated into phospholipid classes on thin-layer plates coated with silica gel G (Uniplate, Analtech, Newark, NJ, U.S.A.; chloroform—methanol—28% ammonia—water,

0378-4347/85/$03.30

65:25:0.5:2). The silica gel plates were exposed to the fumes from 36% hydrochloric acid and dried in a stream of ambient air for 10 min [12]. Phospholipid spots were located with iodine vapour. Scraped PE was further separated into diacyl-PE and lyso-PE (cleavage product from ethanolamine plasmalogen) by thin-layer chromatography (Uniplate, Analtech; chloroform- methanol- 28% ammonia, 100:50:12). The PE subclass spots were located with iodine vapour and scraped. Scraped PE subclasses were saponified by heating at 98°C for 30 min in 2 ml of potassium hydroxide- methanol (50 g of potassium hydroxide, 500 ml of methanol, 450 ml of water). After acidification with hydrochloric acid, the fatty acids were extracted in chloroform, dried in a stream of nitrogen, and were esterified with a fluorescent marker, 9-anthryldiazomethane (Funakoshi, Tokyo, Japan) [13–15]. An aliquot of the esterified fatty acids was subjected to HPLC.

HPLC was performed with a Shimadzu LC-4A system equipped with a fluorescence spectrophotometer monitoring at 412 nm with excitation at 365 nm (Shimadzu, Kyoto, Japan). The column was a 25 cm × 4.6 mm I.D. stainless-steel tube prepacked with Zorbax C_8 (particle size, 5–6 μm; Shimadzu). The column temperature was maintained at 60°C. Fatty acids were eluted by a programmed linear gradient elution with acetonitrile–water (0 min, 80:20; 36 min, 80:20; 80 min, 100:0; 90 min, 100:0) within 90 min, and the flow-rate was 1.6 ml/min. Each fatty acid was identified by the retention time of standards, purchased from Serdary Research Lab. (London, Canada) or Funakoshi.

All organic solvents and water (Wako, Osaka, Japan) were commercial HPLC-grade materials, and butylated hydroxytoluene (an antioxidant) was added to methanol and chloroform (5 mg per 100 ml).

RESULTS

Nine fatty acid peaks were identified (Fig. 1). Major fatty acid components were $C_{16:0}$, $C_{18:1}$ and $C_{20:4}$ in diacyl-PE, and were $C_{20:4}$, $C_{22:6}$ and $C_{18:1}$ in

TABLE I

FATTY ACID COMPOSITIONS OF PHOSPHATIDYLETHANOLAMINE SUBCLASSES IN ERYTHROCYTE MEMBRANES

Values are area percentages (means ± S.D.)

Fatty acid	Diacyl form (n = 7)	Plasmalogen form (n = 7)
$C_{16:0}$	30.27 ± 1.39	11 78 ± 2.68
$C_{18:0}$	10.81 ± 0.89	4.04 ± 0.89
$C_{18:1}$	24.07 ± 2.75	16.36 ± 3.29
$C_{18:2}$	10.19 ± 1.06	11.15 ± 1.42
$C_{20:3}$	1.01 ± 0.14	0 97 ± 0 17
$C_{20:4}$	14.54 ± 1.14	29.99 ± 1.47
$C_{20:5}$	1.40 ± 0.58	4.47 ± 2 05
$C_{22:4}$	2 25 ± 0.73	4.58 ± 1 32
$C_{22:6}$	7 81 ± 1.24	17.63 ± 3.70

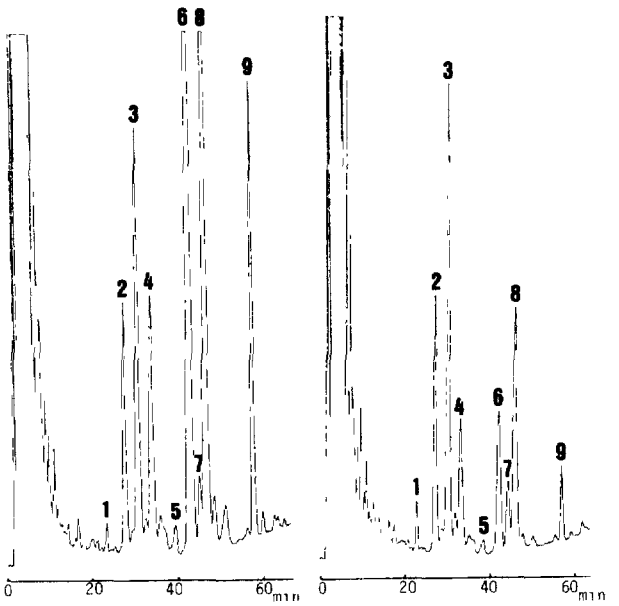

Fig. 1. Separations of fatty acids from diacyl (left) and plasmalogen (right) forms in PE of human erythrocyte membranes by HPLC. Peaks: 1 = $C_{20:5\omega9}$; 2 = $C_{22:6\omega3}$; 3 = $C_{20:4\omega6}$; 4 = $C_{18:2\omega6}$; 5 = $C_{20:3\omega6}$, 6 = $C_{16:0}$; 7 = $C_{22:4\omega6}$; 8 = $C_{18:1\omega9}$; 9 = $C_{18:0}$.

plasmalogen form of PE (Table I). Saturated long-chain fatty acids ($C_{20:0}$, $C_{22:0}$, $C_{24:0}$ and $C_{26.0}$) were trace components in both subfractions.

DISCUSSION

Human erythrocyte membrane fatty acids have been analysed in phospholipid fractions by gas chromatography (GC) [3, 5—9], and there have been also several reports on fatty acids in phospholipid subfractions [4—6]. Recently, HPLC has been introduced into fatty acid analyses [13- 15]. There has been no study on fatty acid compositions of phospholipid subclasses in human erythrocyte membranes by HPLC, as far as we know.

Our study showed a dramatic difference in the fatty acid composition between diacyl and plasmalogen forms in PE. The fatty acids in plasmalogen form have more unsaturation than those in diacyl form, which agrees with the GC data obtained by previous authors [4—6]. There are some differences in the fatty acid compositions of both PE subclasses between our data and those in the previous reports [4—6]. In particular, the content of polyunsaturated fatty acids in both PE subclasses of the present study is less than that reported previously [4—6]. This discrepancy may be attributable to a difference in the preparation procedures rather than to a difference in the analytical modes (HPLC and GC), because the treatment with 36% hydrochloric acid used in our study may have destroyed not only vinyl ethers but also polyunsaturated fatty acids in part.

Erythrocyte membranes may be used as purified plasma membrane samples for studying membrane components and their metabolism. Therefore, it seems

important to bear in mind the facts that phosphlipid classes contain their subclasses and that there are marked differences between their fatty acid compositions. HPLC may be useful for studying fatty acids of biological samples.

REFERENCES

1 P Ways and D.J. Hanahan, J. Lipid Res., 5 (1964) 318.
2 R.M.C. Dawson, N Hemington and D.B. Lindsay, Biochem. J., 77 (1960) 226.
3 J.W. Farquhar, Biochim. Biophys. Acta, 60 (1962) 80.
4 V. Rogiers, J. Chromatogr., 182 (1980) 27.
5 J.H. Williams, M. Kuchmak and R.F. Witter, Lipids, 1 (1966) 391.
6 P. Cohen and A. Derksen, Brit. J. Haematol., 17 (1969) 359.
7 J.T. Dodge and G.B. Phillips, J. Lipid Res., 8 (1967) 667.
8 L. Marai and M. Kuksis, J. Lipid Res., 10 (1969) 141.
9 W. Ruitenbeek, Clin. Chim. Acta, 89 (1978) 99.
10 S. Mawatari, Y. Antoku and Y. Kuroiwa, J. Neurol. Sci., 53 (1982) 23.
11 J. Folch, M. Lees and G.H. Sloane Stanley, J. Biol. Chem., 226 (1957) 497.
12 L.A Horrocks, J. Lipid Res., 9 (1968) 469.
13 N. Nimura and T. Kinoshita, Anal. Lett., 13 (1980) 191.
14 Y. Antoku, T. Sakai, I. Goto, H. Iwashita and Y Kuroiwa, Neurology (Cleveland), 34 (1984) 1499.
15 Y. Antoku, T. Sakai, I. Goto, H. Iwashita and Y. Kuroiwa, Exp. Neurol., 87 (1985) 206.