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GAS CHROMATOGRAPHIC DETERMINATION OF THE FATTY ACID PATTERN OF RED CELL MEMBRANE PLASMALOGENS IN HEALTHY CHILDREN

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SUMMARY

A gas chromatographic technique for the determination of the fatty acid pattern of the phosphatidylcholine (PC) and phosphatidyl ethanolamine (PE) plasmalogen and non-plasmalogen fractions in human red cell membranes has been developed. The coefficient of variation lies between 5 and 10%. The technique has been applied to blood samples of healthy Belgian school children (8—10 years old). PE contains 47% plasmalogen and 53% non-plasmalogen form, whereas PC occurs only in its non-plasmalogen form.

The fatty acid pattern of the various fractions and subfractions has been determined.

INTRODUCTION

Appropriate methods for the determination of the fatty acid (FA) pattern of the various phospholipid fractions, using thin-layer chromatography (TLC) and gas—liquid chromatography (GLC) have been developed during the last twenty years [1—3] and have been applied to human red blood cells [4].

Nelson [4] has reviewed reports concerning the FA pattern of the major erythrocyte phospholipids in healthy adults, but for children, only little and mainly incomplete data exist. There has also been little investigation on the occurrence and FA pattern of the individual phospholipid subfractions.

In human red cells, phosphatidyl ethanolamine (PE) and phosphatidylcholine (PC) are possibly present in two subfractions, a plasmalogen and a non-plasmalogen form [4]. A separation method for these two forms, using two-dimensional TLC, has been reported by Owens [5]. A solution of 5 m*M* $HgCl_2$ in water is sprayed on the TLC plate after development of the first dimension. This is not recommended for subsequent determination of the FA pattern of both subfractions, as they are rich in polyunsaturated FA.

In this study, an appropriate TLC—GLC technique for the determination of the FA pattern of red cell membrane plasmalogens is reported.

EXPERIMENTAL

Materials

Organic solvents pro analysis grade were obtained from Merck (Darmstadt, G.F.R.). Sephadex G-25, coarse, was from Pharmacia (Uppsala, Sweden), TLC "Redi Coats", Supelcosil 42A^{R} (silica gel with 10% magnesium silicate), from Supelco (Bellefonte, Pa., U.S.A.), and TLC silica gel H from Merck. The phospholipid standards for TLC were from Sigma (St. Louis, Mo., U.S.A.), the methyl esters of FA from Merck, Fluka (Buchs, Switzerland), Applied Science Labs. (State College, Pa., U.S.A.), Sigma and Supelco. Boron trifluoride—methanol (14% w/v), and the BHT antioxidant (2,6-di-*tert.*-butyl-4-methylphenol) were both from Merck.

Blood collection

After the children had fasted overnight, 10-ml blood samples were taken and placed in ice-cooled heparinised tubes (100 I.U./ml blood), then immediately centrifuged for 30 min at 3000 *g* and 4°, thus separating the red cells.

Preparation of erythrocyte "ghosts"

This was carried out according to the method of Hanahan and Ekholm [6]. An isotonic Tris buffer 310 imOsm (ideal milliosmolar) pH 7.6 and an hypotonic Tris buffer 20 imOsm pH 7.6 were used as washing and hemolysing media, respectively.

Extraction of the total lipids

One volume of the ghost suspension was extracted twice, using 5.5 volumes isopropanol (+ 100 mg/l BHT) and 3.5 vol. chloroform [7].

Purification of the lipid extract

The total lipid extract was purified essentially free of all non-lipid components by partition column chromatography using sephadex G-25, coarse [1]. The lipids were eluted with a chloroform—methanol (19:1) mixture, saturated with water.

Separation of the phospholipids by TLC

Two-dimensional TLC on Supelcosil-42A was carried out to separate the phospholipids. For the first dimension a mixture of chloroform—methanol—ammonium hydroxide (65:25:5, v/v) was used and for the second dimension chloroform—methanol—acetone—acetic acid—water (30:40:10:10:5, v/v) [8]. The phospholipid spots were detected by spraying the plate with a non-destructive Rhodamine 6G solution (0.001% in water), and by inspection of the plate in day light.

Separation of PE_{total} and PC_{total} in plasmalogen and non-plasmalogen form

The scrapings of the PE_{total} and PC_{total} spots were extracted with chloroform—methanol (2:1) + 100 mg/l BHT and hydrolysed with 90% acetic acid for 18 h at 37° under an atmosphere of nitrogen [9]. After elimination of the excess acetic acid by evaporation (Rotavapor, Büchi) of the binary azeotrope

with carbon tetrachloride (30—35°, under vacuum), the hydrolysis products were separated by TLC on silica gel H with chloroform—methanol—water (70:30:4, v/v) [10]. The phospholipid spots were detected with the Rhodamine 6G solution in day light.

Determination of the FA pattern of both subfractions

The PE, LPE (lyso-PE), PC and LPC (lyso-PC) spots were scraped off, extracted with chloroform—methanol (2:1, v/v) + 100 mg/l BHT, transmethylated with boron trifluoride—methanol and then extracted with pentane [11]. The FA methyl esters were separated by GLC using a column filled with 5% EGSS-X on Chromosorb W HP (100—200 mesh). Gas chromatographic conditions: the temperature was raised from 130° to 210° at 3°/min. Dual columns (2 m × 1/8 in. I.D.) were used to compensate for column bleeding. The flow-rates were: nitrogen, 15 ml/min; hydrogen, 28 ml/min and air, 400 ml/min. A Perkin-Elmer F17 gas chromatograph with a dual flame ionization detection system was used. Peaks were identified by their relative retention times versus that of the internal standard ($C_{17:0}$) and quantitated with a Hewlett-Packard Model 3380A digital integrator.

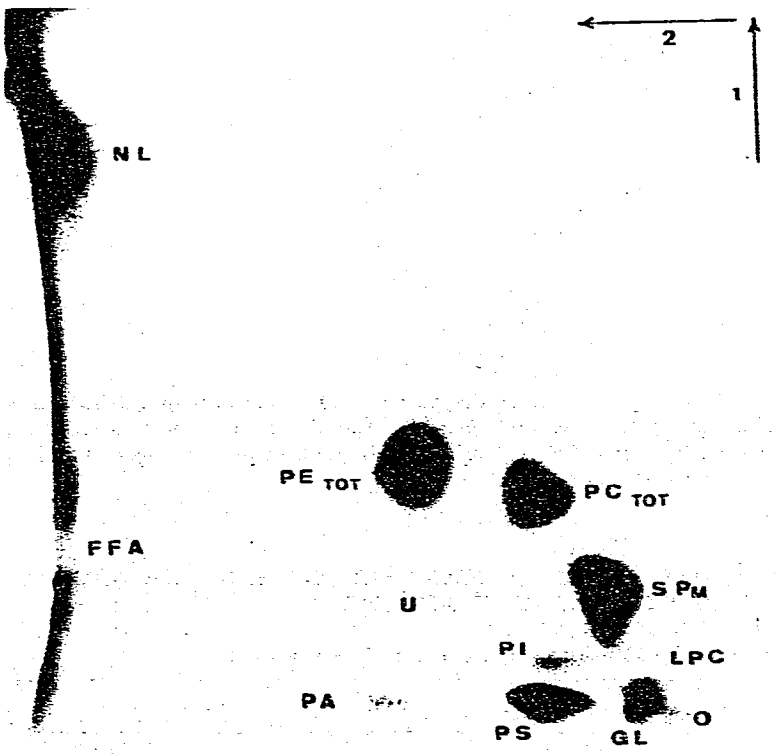

Fig. 1. Two-dimensional TLC of red cell membrane phospholipids. NL, neutral lipids; FFA, free fatty acids; U, unknown; PE_{tot}, phosphatidyl ethanolamine (total); PC_{tot}, phosphatidylcholine (total); SP_M, sphingomyelin; LPC, lyso-PC; PI, phosphatidylinositol; PS, phosphatidylserine; PA, phosphatidic acid; GL, globoside and O, origin. Conditions are as described in Methods.

RESULTS

The reproducibility of the complete technique was investigated. The coefficient of variation of each FA lies between 5 and 10% ($n = 6$). No oxidation of the polyunsaturated FA could be detected during the whole procedure.

A two-dimensional thin-layer chromatogram of human red cell membrane phospholipids is shown in Fig. 1. A complete separation of the various phospholipid fractions was obtained. The PE_{total} and PC_{total} spots contain plas-

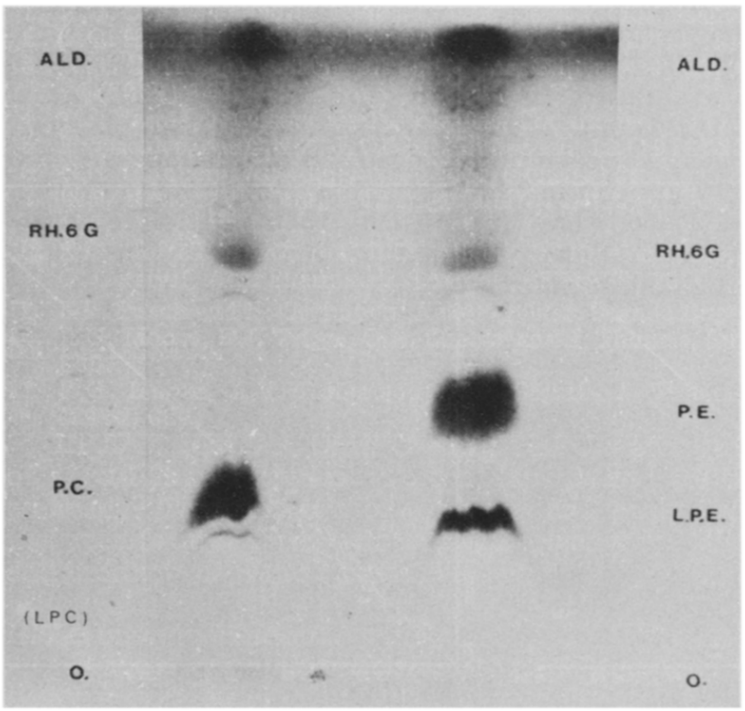

Fig. 2. Separation of PE_{total} and PC_{total} into plasmalogen and non-plasmalogen subfractions by TLC on silica gel H. ALD, aldehydes; RH6G, Rhodamine 6G and antioxidant BHT; PE, phosphatidyl ethanolamine; LPE, lyso-PE; PC, phosphatidylcholine; LPC, lyso-PC and O, origin. Conditions are as described in Methods.

TABLE I

PLASMALOGEN AND NON-PLASMALOGEN CONTENT OF TOTAL PE AND PC FRACTIONS IN RED CELL MEMBRANES OF HEALTHY CHILDREN

Conditions are as described in Methods. Results are mean ± S.D. with $n = 6$.

	Plasmalogen (%)		Non-plasmalogen (%)	
	girls	boys	girls	boys
PE_{total}	47.6 (±1.4)	47.3 (±1.2)	52.4 (±1.3)	52.7 (±1.4)
PC_{total}	0.0 (±0.0)	0.0 (±0.0)	100.0 (±0.0)	100.0 (±0.0)

malogen and non-plasmalogen forms. A further separation, after acid hydrolysis, was obtained by one-dimensional TLC as shown in Fig. 2. A spot equivalent to LPC (i.e. the plasmalogen form of PC) was never found.

After TLC, phosphorus determination of the PE, LPE, PC and LPC spots was carried out, using the Bartlett method [12]. To determine the LPC spot, a known area of the TLC plate with a R_F value corresponding to a reference LPC spot, was scraped off and its phosphorus content quantitated. The results for healthy Belgian school children (8—10 years old) are shown in Table I. The FA patterns of the PE_{total}, PE, LPE, PC_{total} and PC spots were each determined for the same healthy boys and girls. No sex-dependent differences were detected. The GLC separation of the FA methyl esters of the PE plasmalogen and non-plasmalogen forms are shown in Fig. 3a and b. The antioxidant BHT used in the extraction has an identical retention time to the palmitoleic acid methyl ester, hence the latter can not be quantitated. The FA compositions of PC_{total}, PE_{total} and subfractions in the red cell membranes of healthy girls are shown in Table II (n = 8). Identical

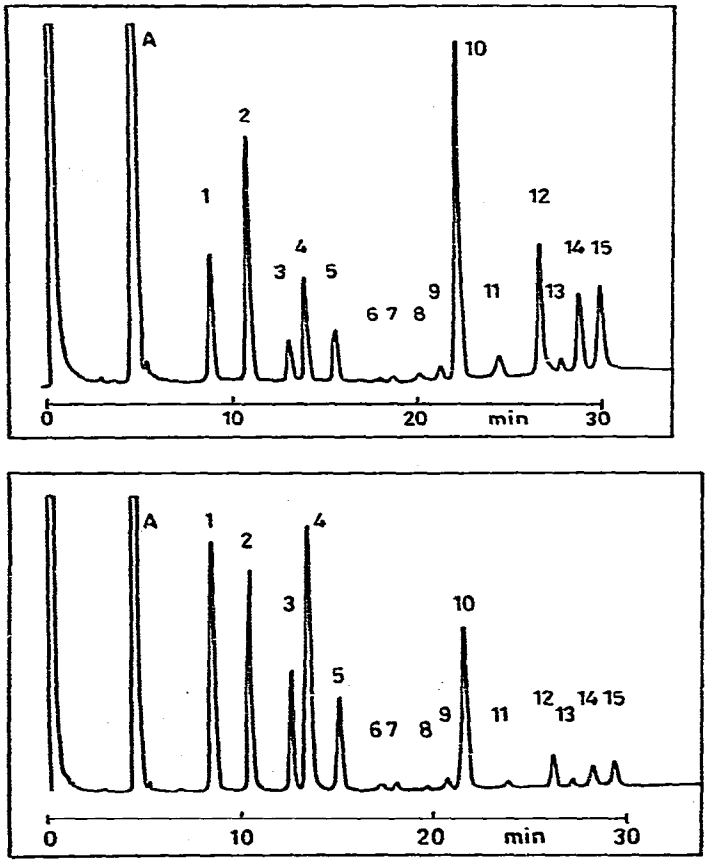

Fig. 3. Gas chromatograms of (a) the PE plasmalogen and (b) non-plasmalogen form of red cell membranes of healthy children. The column packing used was 5% EGSS-X and conditions are as described in Methods. Peaks: A, BHT derivative; 1, $C_{16:0}$; 2, $C_{17:0}$; 3, $C_{18:0}$; 4, $C_{18:1\omega9}$; 5, $C_{18:2\omega6}$; 6, $C_{18:3\omega3}$ + $C_{20:0}$; 7, $C_{20:1\omega9}$; 8, $C_{20:2\omega6}$; 9, $C_{20:3\omega6}$; 10, $C_{20:4\omega6}$; 11, $C_{20:5\omega3}$; 12, $C_{22:4\omega6}$; 13, $C_{22:5\omega6}$; 14, $C_{22:5\omega3}$; 15, $C_{22:6\omega3}$.

TABLE II

FATTY ACID COMPOSITION OF TOTAL PC, PE AND SUBFRACTIONS IN RED CELL MEMBRANES OF HEALTHY GIRLS

Conditions are as described in Methods. Results are mean ± S.D. with $n = 8$.

Fatty acid	PE_{total}	$PE_{non\text{-}plasmalogen}$	$PE_{plasmalogen}$	PC_{total}
$C_{16:0}$	16.8 ± 1.0	21.7 ± 1.4	7.7 ± 2.1	34.9 ± 2.5
$C_{18:0}$	8.9 ± 1.2	10.3 ± 0.7	2.0 ± 1.1	12.1 ± 0.7
$C_{18:1\omega9}$	19.1 ± 0.6	24.4 ± 1.1	9.2 ± 1.0	19.4 ± 0.5
$C_{18:2\omega6}$	7.0 ± 1.0	9.5 ± 1.1	3.8 ± 0.7	22.0 ± 1.5
$C_{18:3\omega3}$	0.4 ± 0.1	0.5 ± 0.1	0.3 ± 0.1	0.4 ± 0.1
$C_{20:1\omega9}$	0.6 ± 0.1	0.7 ± 0.1	0.4 ± 0.1	0.3 ± 0.1
$C_{20:2\omega9}$ $\omega6$	0.4 ± 0.1	0.5 ± 0.2	0.3 ± 0.2	0.4 ± 0.1
$C_{20:3\omega6}$	1.4 ± 0.2	1.6 ± 0.3	1.4 ± 0.3	1.9 ± 0.4
$C_{20:4\omega6}$	24.0 ± 0.5	17.7 ± 0.6	36.3 ± 1.5	4.9 ± 0.9
$C_{20:5\omega6}$	1.2 ± 0.3	0.7 ± 0.1	2.0 ± 1.0	0.4 ± 0.1
$C_{22:4\omega6}$	7.7 ± 0.7	4.5 ± 0.4	14.6 ± 1.4	0.5 ± 0.2
$C_{22:5\omega6}$	1.2 ± 0.2	1.0 ± 0.2	1.7 ± 0.3	0.5 ± 0.3
$C_{22:5\omega3}$	4.8 ± 0.4	2.6 ± 0.3	9.4 ± 0.7	0.6 ± 0.3
$C_{22:6\omega3}$	6.6 ± 1.0	4.6 ± 0.8	11.0 ± 1.8	1.8 ± 0.8

results were found for healthy boys of the same age group ($n = 6$). Since the FA pattern of the PC_{total} is not significantly different from that of the $PC_{non\text{-}plasmalogen}$ and a spot for $PC_{plasmalogen}$ was never detected, we conclude that there is no plasmalogen subfraction in the red cell membranes of 8—10 year old healthy children.

DISCUSSION

After determination of the phosphorus content of the various PL fractions in red cell membranes, it appears that for healthy children the PE_{total} spot contains 47% plasmalogen and 53% non-plasmalogen form. PC occurs only in its diacyl form. For healthy adults, Farquhar [13] found 67% plasmalogen form in PE and 10% in PC, Williams et al. [14] reported 52% and 4%, Cohen and Derksen [10] 46% and 3%, and Hill et al. [15] 36% and 4%, respectively. These variations are probably due to the different analytical methods used and to difficulties in achieving a clean separation of the PL components present, especially those techniques using column chromatography instead of TLC. The FA pattern of the total PC and PE fractions of erythrocyte membranes in healthy adults has been described in detail [10, 13, 14, 16—18], whereas the FA composition of their subfractions has only been analysed by a few investigators [10, 14].

As far as we know, no data are available for the FA pattern of the erythrocyte PL subfractions of children. Kobayashi et al. [19] have determined the FA composition of the major red cell PL fractions in healthy children of 10 years old, but no further separation into the subfractions has been carried out. Our results showing the FA pattern of the total PE and PC fractions are in good agreement with theirs. The FA compositions of the PE subfractions

of healthy children are comparable with those described for healthy adults [10, 14].

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