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Note

Phospholipid analysis and fatty acid content in platelets by the combination of high-performance liquid chromatography and glass capillary gas—liquid chromatography

MICHEL GUICHARDANT and MICHEL LAGARDE*

Institut Pasteur, INSERM U63 and Laboratoire d'Hémobiologie, Faculté Alexis Carrel, 69372 Lyon Cedex 2 (France)

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Complete separation of phospholipids for preparative purposes usually needs a complex and a multistep procedure including single and two-dimensional thin-layer chromatography (TLC) [1—3]. Column chromatography has also been used with a great variety of stationary phases and solvent mixtures [4]. Recently, high-performance liquid chromatography (HPLC) used under various conditions has been introduced to separate phospholipids [5—17]. However, none of these conditions allows a complete separation of these lipids with sufficient resolution.

On the other hand, the great interest in the polyunsaturated fatty acids as precursors of prostaglandins and/or lipoxygenase products requires high-performance gas—liquid chromatography permitting a baseline separation of fatty acid isomers.

We have modified an HPLC technique previously described [9] in order to obtain a complete separation between the different classes of phospholipids and lysoglycerophospholipids from human platelets. The complete profiling of the fatty acid content of resting platelet phospholipids was then determined using a polar-phase column for the capillary gas—liquid chromatography (GLC).

MATERIAL AND METHODS

Reagents

Organic solvents (Analar grade) were provided by Prolabo (Paris, France) and the purest grade of 2,2,4-trimethylpentane, to solubilize fatty acid methyl esters before analysis by GLC, was obtained from Burdick and Jackson Labs. (Muskegon, MI, U.S.A.). N-Methyl-N-nitroso-*p*-toluenesulfonamide, silica

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gel G plates and boron trifluoride—methanol were purchased from Merck (Darmstadt, G.F.R.). Butylated hydroxytoluene (BHT) was provided by Fluka (Buchs, Switzerland).

[5,6,8,9,11,12,14,15-^{3}H]Arachidonic acid (>90 Ci/mol), labelled standard phospholipids L-α-phosphatidyl-[2-^{14}C]ethan-1-ol-2-amine, dioleoyl (PE, 44 Ci/mol), L-α-phosphatidyl-[U-^{14}C]inositol, 1-pentadecaoyl, 2-nonadecaoyl (PI, 270 Ci/mol), L-α-phosphatidyl-L-[U-^{14}C]serine, dioleoyl (PS, 60 Ci/mol), L-α-phosphatidylcholine, di-[1-^{14}C]palmitoyl (PC, 100 Ci/mol), and [N-*methyl*-^{14}C]sphingomyelin (Sph, 50 Ci/mol) were provided by the Radiochemical Centre, Amersham, Great Britain. [^{14}C]lysoPE (LPE) and [^{14}C]-lysoPC (LPC) were obtained by phospholipase A_2 treatment of PE and PC, respectively. [^{14}C]Phosphatidic acid was produced from PC by phospholipase D treatment. Pancreatic phospholipase A_2 and the cabbage phospholipase D were provided by Boehringer (Mannheim, G.F.R.).

Standard fatty acids were furnished by Supelco (Bellefonte, PA, U.S.A.) or Sigma (St. Louis, MO, U.S.A.). Partisil 5 as stationary phase for HPLC was obtained from Whatman (Clifton, NJ, U.S.A.). Silar 7CP as stationary phase for GLC was provided by Applied Science, (Oud- Beijerland, The Netherlands).

High-performance liquid chromatography

A Chromatem 38 apparatus from Touzart et Matignon (Paris, France) was used; this apparatus was equipped with two pumps and a continuous gradient. A 30-cm long, 6.35 mm (O.D.), 4.6 mm (I.D.) column was filled with Partisil 5 (5 μm) according to a published method [18]. A loop of 50 μl, filled with a slight excess of sample was used for injection. The elution was carried out with a mixture of hexane—isopropanol—acetate buffer 1 m*M*, pH 6, in various proportions (gradient on Fig. 1) at a flow-rate of 1 ml/min. Solvents were de-aerated under vacuum before use. The detection was done by counting 0.5 ml from 1-ml aliquots containing 5×10^{-5} *M* BHT to prevent autooxidation.

Gas—liquid chromatography

An open tubular glass capillary column (70 m × 0.25 mm I.D.) coated with Silar 7CP was used in a Packard Model 427 chromatograph equipped with a solid injector and a flame ionization detector which was connected to a computing integrator. The open tubular glass column was home-made and coated using a static method [19]. Dried helium was used as the carrier gas and the optimal flow-rate was chosen from the Van Deemter curve (arachidonic acid methyl ester at 170°C). Temperature programming was usually 150 to 190°C (1°C/min).

Preparation of biological samples

Human platelets (300,000 per μl) were obtained as previously described [20] and incubated for 1 h at room temperature with 1 μCi/ml [^{3}H]arachidonic acid. Under these conditions, more than 95% of the initial radioactivity was in the phospholipids. Platelets were then extracted twice with 9 volumes of chloroform—ethanol (2:1) containing 5×10^{-5} *M* BHT, and phospho-

lipids were separated from other lipids by TLC on silica gel G. The mixture diethyl ether—methanol—acetic acid (90:1:2) was used for development and the phospholipids remaining at the origin were extracted three times with methanol—water (9:1). They were dried under vacuum and injected onto the HPLC column in solvent A (see Fig. 1).

After their radioactive detection, the glycerophospholipids were dried under vacuum and transmethylated with boron trifluoride—methanol [21]. Fatty acid methyl esters were extracted with 2,2,4-trimethylpentane.

RESULTS AND DISCUSSION

Fig. 1 shows the separation obtained using radioactive standard phospholipids. Amounts of 3—10 nCi of phospholipid (approximately 30 pmol) were injected onto the column. The recovery yield in eluates was higher than 90% for every phospholipid. The use of acetate buffer in the eluent to replace water, as we have done previously [22], gives a better reproducibility of the retention times. Besides, the increase in the column length and the relatively increased proportion of isopropanol in the eluent improved the separation between LPE and PS.

In Fig. 2 one can see a typical radioactivity profile obtained with glycerophospholipids from platelets prelabelled with [^{3}H]arachidonate. Moreover, each phospholipid collected was identified by two-dimensional TLC as we have done previously [23]. The profile was obtained after labelling of only 5×10^8 platelets and the radioactivity detection shows a good sensitivity. The retention times are quite similar to those obtained with tracer amounts of standard phospholipids (Fig. 1) although the quantities obtained from the biological samples were much higher (2—10 nmol, depending on the phospholipid). Using the prelabelling of platelets with [^{3}H]arachidonate we have found a percentage repartition in each glycerophospholipid class of 21.3%,

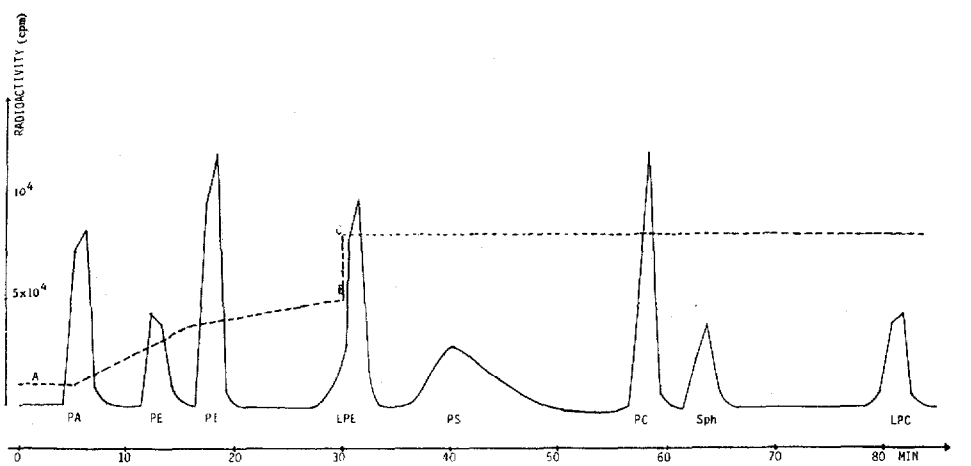

Fig. 1. Profile of radioactive standard phospholipids (3—10 nCi of each). Gradient elution at 1 ml/min on Partisil 5. (———) gradient. (A) Hexane—propanol-2—acetate buffer 1 m*M*, pH 6 (60:120:10). (B) Hexane—propanol-2—acetate buffer 1 m*M*, pH 6 (60:120:16). (C) Hexane—propanol-2—acetate buffer 1 m*M*, pH 6 (60:110:21).

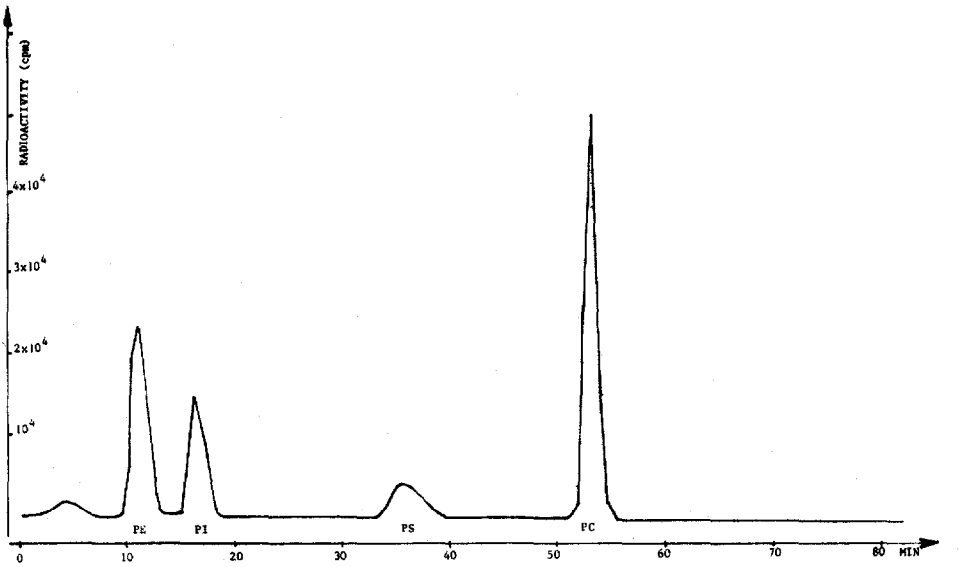

Fig. 2. Typical profile of phospholipids from platelets which were prelabelled with tracer doses of ^{3}H-labelled 20:4ω6 (see Methods).

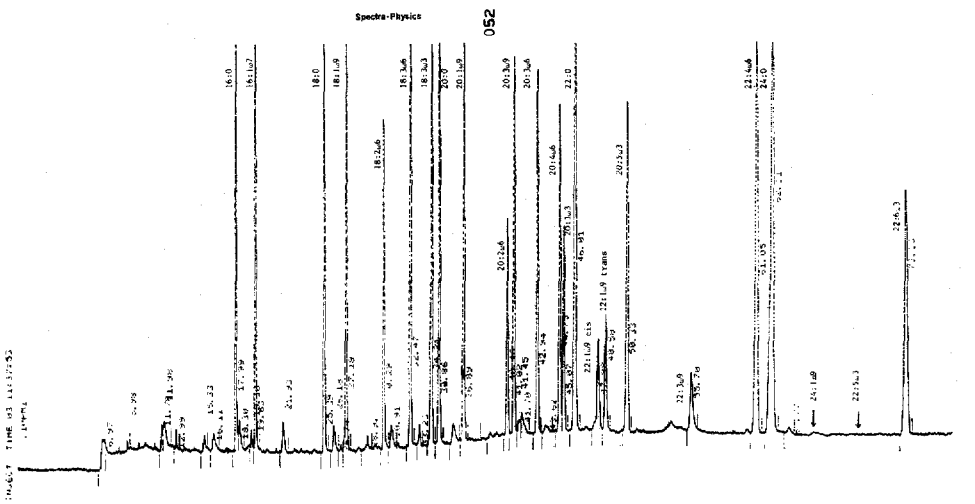

Fig. 3. Profile of standard fatty acid methyl esters (5—15 ng of each). Carrier gas flow: helium 1 ml/min. Temperature gradient: 150—190°C (1°C/min). Approximately 130,000 theoretical plates (calculated with 20:4ω6).

14.8%, 6.9% and 54.9% (mean of five determinations), for PE, PI, PS and PC, respectively. These values are in a good agreement with those reported elsewhere [23, 24]. The method we have used did not allow us to detect lysophospholipids which are not labelled by tracer doses of arachidonate and in any case are present in negligible quantities in resting platelets [25]. A way to detect them, especially after platelet stimulation, would be to prelabel platelets with [^{3}H]glycerol.

The HPLC technique presents two major advantages. Firstly, it takes around

1.5 h for complete separation of phospholipids while about 4 h are usually required for two-dimensional TLC. Secondly, phospholipids and their polyunsaturated fatty acids are protected against oxygen during the separation whereas they are exposed during TLC development and mainly during their localization on the plate, usually by autoradiography. This second point is very important for the further characterisation of the phospholipids and especially for analysis of their fatty acid content. A fatty acid methyl ester separation with capillary GLC (Fig. 3) was obtained with fatty acid standards methylated by ethereal saturated diazomethane treatment [26]. The column we used was very efficient with around 130,000 theoretical plates when calculated using the arachidonate methyl ester (20:4ω6) peak, obtained at 170°C; 5—15 ng of each component were injected. Quantitation of each fatty acid was corrected for its own response factor by the detector. This high sensititivy, which may be extrapolated to a limit of 1 ng, was obtained because of a high column efficiency. The separation was highly resolutive especially for C_{20} polyunsaturated fatty acids which are of great interest for the cell prostaglandin synthetase and lipooxygenase pathways. Particularly, a baseline separation can be observed between 20:2ω6 and 20:3ω9, two fatty acids usually not totally separated using free fatty acid phase of Carbowax columns. This point is very important since 20:3ω9 recently appeared to be of special interest in the regulation of platelet aggregation [27]. Fig. 4 represents a run of fatty acids obtained from platelet PE, showing the retention times of the dimethyl acetals of 16:0, 18:0 and 18:1 contained in that phospholipid.

Finally, using a combination of the two analytical techniques described above, we have determined the fatty acid profile of each glycerophospholipid from unstimulated human platelets. The results are shown in Table I and appear quite similar to those published elsewhere [28, 29] except that ours give more details about the minor fatty acids which are relevant to fatty acid dietary manipulations. The weak standard deviation seen in Table I sug-

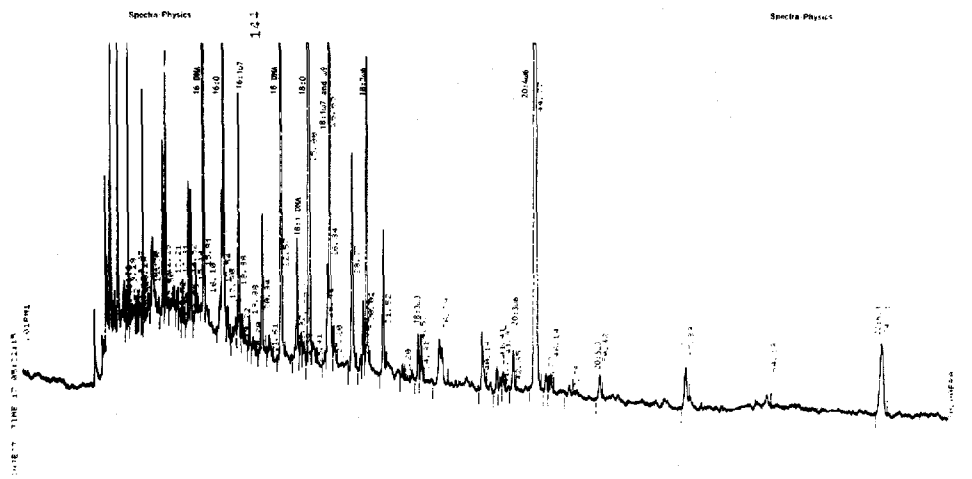

Fig. 4. Typical profile of fatty acid methyl esters obtained from platelet PE. The analytical conditions were those of Fig. 3.

TABLE I

FATTY ACID PROFILES OF THE DIFFERENT PHOSPHOLIPID CLASSES IN HUMAN PLATELETS

Results are given as percentages (mean ± S.D.) of the total fatty acids (n = 5).

	PE	PI	PS	PC
16 DMA	5.70 ± 0.4	—	—	—
18 DMA	9.9 ± 0.6	—	—	—
18:1 DMA	1.1 ± 0.4	—	—	—
16:0	1.9 ± 2.5	11.5 ± 2.3	19.9 ± 6.9	26.0 ± 2.5
16:1ω7	3.4 ± 1.2	4.4 ± 2.2	5.3 ± 2.9	1.9 ± 0.2
18:0	13.8 ± 0.3	29.6 ± 4.1	27.1 ± 5.0	14.7 ± 0.6
18:1ω7 + 18:1ω9 *	6.6 ± 1.2	11.0 ± 3.2	18.5 ± 3.1	18.9 ± 3.0
18:2ω6	3.4 ± 1.0	4.7 ± 2.1	4.6 ± 1.7	12.4 ± 2.6
18:3ω6	0.6 ± 0.8	<0.1	0.1 ± 0.3	<0.1
18:3ω3	0.2 ± 0.3	0.6 ± 0.5	1.2 ± 1.0	0.8 ± 0.5
20:0	0.7 ± 0.2	2.8 ± 1.5	1.7 ± 1.0	1.2 ± 0.7
20:1ω9	0.2 ± 0.2	<0.1	<0.1	0.8 ± 0.7
20:2ω6	0.2 ± 0.2	<0.1	<0.1	0.7 ± 0.1
20:3ω9	0.3 ± 0.3	N.D.	<0.1	0.2 ± 0.2
20:3ω6	0.6 ± 0.1	0.6 ± 0.3	1.0 ± 0.4	2.2 ± 0.2
20:4ω6	42.5 ± 4.6	32.4 ± 3.5	16.7 ± 3.3	15.7 ± 0.4
20:3ω3	N.D.**	N.D.	N.D.	N.D.
22:0	0.1 ± 0.1	0.8 ± 1.0	1.1 ± 0.7	1.7 ± 1.3
22:1ω9(*cis*)	N.D.	N.D.	N.D.	N.D.
22:1ω9(*trans*)	N.D.	N.D.	N.D.	N.D.
20:5ω3	0.5 ± 0.3	<0.1	N.D.	0.2 ± 0.1
22:4ω6	0.7 ± 0.6	<0.1	<0.1	0.9 ± 0.9
24:0	0.1 ± 0.1	N.D.	<0.1	0.3 ± 0.4
24:1	N.D.	N.D.	N.D.	N.D.
22:5ω3	<0.1	N.D.	N.D.	N.D.
22:6ω3	1.8 ± 2.3	N.D.	<0.1	0.7 ± 0.7

*We have summed the 18:1 isomers because they are not completely separated.
**N.D. = not detected.

gests that the combined analysis gives a good reproducibility of the measurements.

We can conclude that the combination of HPLC and capillary GLC used as described above represents a quick, reproducible and very sensitive approach to the study of phospholipids of animal cells and their fatty acid composition, especially with regard to the oxygenated metabolism of polyunsaturated fatty acids.

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