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Note

Thin-layer chromatography of human platelet phospholipids with fatty acid analysis

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A major problem in membrane biochemical studies is the gathering of information about the phospholipid fatty acyl chains that contribute significantly to membrane structure and function [1–3]. The role of lipids in platelet function is also highly relevant to the analysis of the structure of phospholipids in connection with their metabolism [4,5].

Several solvent systems for two-dimensional thin-layer chromatography (TLC) of polar lipids have been published [6–10]. All these methods are time-consuming, not very reproducible and subject to error because of excessive handling and critical steps. The oxidation of polyunsaturated fatty acids cannot be prevented owing to several drying periods and to alternate migrations in basic and acidic solvents. On the other hand, the amount of polar lipids that can be resolved by two-dimensional TLC is relatively low and, generally, individual components are near the lower limit of their determination by gas chromatography (GC).

One-dimensional TLC is simpler and more rapid than two-dimensional TLC, but there remains a high risk of contamination between two or several compounds leading to significant errors in the interpretation of fatty acid patterns. Thus, normal TLC plates yield poor separation of critical pairs such as phosphatidylserine (PS) and phosphatidylinositol (PI), phosphatidic acid (PA) and cardiolipin (CL), phosphatidylglycerol (PG) and phosphatidylethanolamine (PE)

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[11–15]. If high-performance TLC allows good separations [11,16,17] then the amounts of membrane lipids that can be analysed are too small. Recently one-dimensional systems have been improved by the use of boric acid-impregnated plates [18] or inclusion of boric acid [19] or triethylamine [15] in conventional solvent systems.

In this study we describe a procedure combining the use of boric acid and triethylamine, which permits complete resolution of membrane phospholipids in pure form with special attention to platelets.

EXPERIMENTAL

Phospholipid, glycolipid standards and primulin were obtained from Sigma (St. Louis, MO, U.S.A.), N-acylphosphatidylethanolamine was isolated from soybean lipids as previously described [20]. All organic solvents were reagent grade and used as obtained from the suppliers. Silica gel 60 for column chromatography, triethylamine and ethyl gallate were from Merck (Darmstadt, F.R.G.). Silica gel LK5 plates (250 μm thick) with a preabsorbent area were from Whatman (Whatman, Clifton, NJ, U.S.A.). A solution of 14% BF3 in methanol was from Pierce (Rockford, IL, U.S.A.).

Lipid extraction and isolation

Human platelets were isolated and washed by differential centrifugation from acid–citrate–dextrose anticoagulated blood from human volunteers who had taken no drugs for at least 10 days. During the isolation procedure, which has been described previously [21], the platelets are not activated, remain functional and maintain a discoid shape. Platelet lipids (10^{10} platelets in 1 ml of physiological saline) were extracted according to Folch's method [22] and partitioned with 0.9% potassium chloride. A phospholipid fraction, free of neutral lipids and glycolipids, was prepared by fractionation on a 300 mg silicic acid column. Neutral lipids and glycolipids were eluted with 10 ml of chloroform and 15 ml of acetone–methanol (19:1), respectively. Phospholipids were eluted with 10 ml of methanol. All solvents contained 10 mg ethyl gallate per litre.

Thin-layer chromatography

Plates were predeveloped with chloroform–methanol (1:1), air-dried and kept in a closed tank before use. The silica gel-coated part was impregnated by wetting with a solution of 2.3% boric acid in absolute ethanol. After air-drying for 5 min, the plates were activated at 110°C for 15 min. Samples were streaked on the preadsorbent area, then developed up to the top in chloroform–ethanol–water–triethylamine (30:35:6:35, v/v), in paper-lined tanks at room temperature. After air-drying for 5 min, the lipid fractions were detected under UV light after spraying with a solution of 5 mg of primulin in 100 ml of acetone–water (4:1).

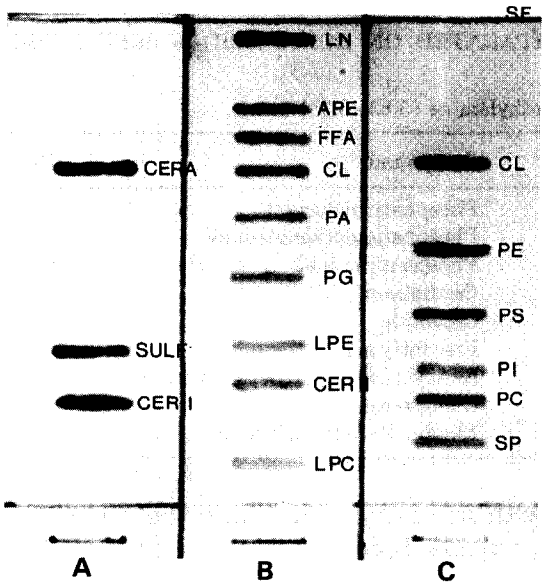

Fig. 1. Chromatogram of lipid compounds separated on boric acid-impregnated plates (LK5, Whatman) with the solvent system chloroform–ethanol–water–triethylamine (30:35:6:35). (A,B) Purified standards; (C) platelet phospholipids. SF = Solvent front; APE = N-acyl phosphatidylethanolamine; CERA = ceramide; CER I = cerebroside Type I; CER II = cerebroside Type II; CL = cardiolipin; FFA = free fatty acids; LN = neutral lipids (mono-, di-, triacylglycerol, cholesterol). LPC = lysophosphatidylcholine; LPE = lysophosphatidylethanolamine; PC = phosphatidylcholine; PE = phosphatidylethanolamine; PI = phosphatidylinositol PS = phosphatidylserine; SP = sphingomyelin; Amounts applied were 70 μg of platelet phospholipids and 10 μg of each purified standard. Lipids were visualized by exposure to iodine vapour, and photographed with a high contrast film.

Fatty acid analysis

The appropriate silica gel bands were scraped off and transferred to a screw-capped glass tube. The fatty acid compositions of the individual phospholipids were determined by GC after direct transmethylation with 1 ml of 14% BF3 in methanol [23] in the presence of known amounts of heptadecanoate as internal standard. GC was conducted with a Perkin-Elmer Sigma 1 gas chromatograph equipped with a bonded fused-silica open tubular column (50 m × 0.32 mm I.D.; Superox; Alltech Assoc.). Peak areas were determined by the in-line Perkin-Elmer Sigma 10 chart integrator, and results are given as molar percentages of the total fatty acids.

RESULTS AND DISCUSSION

Eleven phospholipid standards were resolved with this technique (Fig. 1). The mobilities of the various compounds tested, expressed as R_F values, are given in Table I. The celite–silica gel boundary is taken as the origin. It can be seen that the important pairs PS–PI and PA–CL were well resolved. Otherwise, N-acyl phosphatidylethanolamine can easily be separated from CL and neutral lipids

TABLE I

R_F VALUES OF LIPID COMPOUNDS SEPARATED ON BORIC ACID-IMPREGNATED PLATES (LK5, WHATMAN)

Solvent system, chloroform–ethanol–water–triethylamine (30:35:6:35).

Compound	R_F	Compound	R_F
Lysophosphatidylcholine	0.08	Phosphatidylglycerol	0.47
Sphingomyelin	0.11	Phosphatidylethanolamine	0.51
Phosphatidylcholine	0.21	Phosphatidic acid	0.58
Cerebroside Type I	0.22	Cardiolipin	0.68
Cerebroside Type II	0.25	Ceramide	0.70
Phosphatidylinositol	0.26	Free fatty acid	0.74
Sulphatides	0.32	N-Acyl phosphatidylethanolamine	0.81
Lysophosphatidylethanolamine	0.32	Cholesterol	0.96
Phosphatidylserine	0.38	Mono-, di-, triacylglycerol	0.98

without any possible contamination with free fatty acids. Thus, this technique can be of value for the analysis of this particular lipid appearing in injured tissues [20]. It must be emphasized that glycolipids co-migrated with some phospholipids (i.e. cerebrosides with PE and phosphatidylcholine). A previous purification step on a silica gel column is thus necessary to prevent erroneous results in fatty acid analysis of phospholipids.

As an example of an application of the method, Fig. 1C shows the separation of total platelet phospholipids. There was a good resolution of six compounds, which could be detected and analysed when up to 70 μg of lipids were spotted per centimetre of initial streak. As little as 100 μg of platelet phospholipids (spotted along an 8-cm streak) was required for the detection and analysis of these six compounds. Table II gives the phospholipid composition of the human platelet, which is not statistically different from data recently reported [24–26] but slightly different from data obtained from platelet homogenates [27]. The absence of lysophosphatidylcholine in our samples is probably related to the integrity of the platelets obtained through an efficient and non-activating isolation and washing procedure [21]. Table III gives the fatty acid profile of each phospholipid from unstimulated human platelets. Our results, including several minor fatty acids,

TABLE II

PHOSPHOLIPID COMPOSITION OF HUMAN PLATELETS

Values are given as mean ± S.E. for seven subjects (S.E. = standard error). Total platelet phospholipid was 3.28 ± 0.27 μmol per 10^{10} platelets.

Phospholipid	Mol%
Cardiolipin	1.1 ± 0.07
Phosphatidylethanolamine	21.1 ± 2.0
Phosphatidylserine	9.3 ± 0.7
Phosphatidylinositol	6.8 ± 0.6
Phosphatidylcholine	37.8 ± 2.0
Sphingomyelin	16.8 ± 1.1

TABLE III

FATTY ACID COMPOSITION OF HUMAN PLATELET PHOSPHOLIPIDS

Values are given as mol% (mean ± S.E., $n = 7$). Fatty acids contributing less than 0.5 mol% have been omitted. CL = Cardiolipin; PE = phosphatidylethanolamine; PS = phosphatidylserine; PI = phosphatidylinositol; PC = phosphatidylcholine; SP = sphingomyelin; DMA = dimethyl acetals.

Fatty acid	CL	PE	PS	PI	PC	SP
14:0	8.3 ± 1.1	0.9 ± 0.1	0.9 ± 0.1	1.6 ± 0.3	0.7 ± 0.03	3.2 ± 0.6
16:0 DMA	–	3.1 ± 1.1	–	–	–	–
16:0	14.7 ± 1.2	5.0 ± 0.2	2.2 ± 0.2	6.6 ± 1.5	31.3 ± 0.8	29.6 ± 2.8
18:0 DMA	–	3.4 ± 1.5	–	–	–	–
18:0	9.0 ± 1.0	14.8 ± 1.0	40.4 ± 1.2	18.3 ± 1.1	13.9 ± 0.7	7.8 ± 0.8
20:0	1.3 ± 0.1	0.7 ± 0.1	2.2 ± 0.3	7.3 ± 0.1	1.1 ± 0.1	7.2 ± 0.6
22:0	–	–	0.6 ± 0.1	–	–	25.5 ± 1.6
24:0	–	–	–	–	–	9.0 ± 0.8
Total sat.	35.4 ± 2.2	28.0 ± 2.2	46.3 ± 1.3	47.3 ± 1.2	46.9 ± 0.2	82.4 ± 0.5
16:1 DMA	–	5.3 ± 0.9	–	–	–	–
16:1 ($n-9$)	3.5 ± 1.0	0.5 ± 0.2	–	0.6 ± 0.2	0.7 ± 0.06	–
18:1 DMA	–	9.7 ± 1.3	–	–	–	–
18:1 ($n-9$)	12.3 ± 0.6	5.0 ± 0.4	21.0 ± 0.8	3.5 ± 0.4	21.9 ± 0.3	1.5 ± 0.1
20:1 ($n-9$)	–	0.6 ± 0.1	1.1 ± 0.1	0.6 ± 0.1	1.5 ± 0.1	–
20:3 ($n-9$)	2.2 ± 0.4	–	0.6 ± 0.1	1.3 ± 0.3	–	–
22:1 ($n-9$)	–	–	–	–	–	0.8 ± 0.2
24:1 ($n-9$)	–	–	–	–	–	11.5 ± 0.4
Total ($n-9$)	19.1 ± 1.5	19.6 ± 2.6	23.2 ± 0.9	5.5 ± 0.8	24.1 ± 0.4	14.8 ± 0.5
18:2 ($n-6$)	34.2 ± 1.2	2.2 ± 0.2	1.1 ± 0.1	1.1 ± 0.04	8.3 ± 0.3	0.7 ± 0.1
18:3 ($n-6$)	0.7 ± 0.1	–	–	0.5 ± 0.1	–	–
20:2 ($n-6$)	–	–	–	–	0.6 ± 0.08	–
20:3 ($n-6$)	1.6 ± 0.3	0.5 ± 0.04	1.4 ± 0.1	0.5 ± 0.04	1.6 ± 0.6	–
20:4 ($n-6$)	8.7 ± 0.9	35.6 ± 1.2	22.9 ± 1.5	43.5 ± 1.5	15.2 ± 0.5	1.9 ± 0.1
22:4 ($n-6$)	1.1 ± 0.2	5.2 ± 0.2	1.4 ± 0.1	0.9 ± 0.2	1.1 ± 0.06	–
22:5 ($n-6$)	–	0.6 ± 0.1	0.6 ± 0.05	–	–	–
Total ($n-6$)	45.4 ± 2.1	44.4 ± 1.1	28.0 ± 1.5	46.2 ± 1.5	27.3 ± 0.3	2.8 ± 0.1
18:3 ($n-3$)	–	0.6 ± 0.1	–	1.2 ± 0.1	–	–
18:4 ($n-3$)	–	1.1 ± 0.1	–	–	–	–
22:5 ($n-3$)	–	3.1 ± 0.2	0.8 ± 0.08	–	0.7 ± 0.05	–
22:6 ($n-3$)	–	2.7 ± 0.1	1.5 ± 0.1	–	0.8 ± 0.07	–
Total ($n-3$)	–	7.6 ± 0.3	2.4 ± 0.05	1.0 ± 0.1	1.7 ± 0.1	–

are in general agreement with those published elsewhere [24,26–29] for the main components. The differences observed are probably caused either by the influence of the dietary lipids on the membrane composition or by the platelet processing. New data are given for cardiolipin, which displayed a marked enrichment in linoleic acid, as had been reported previously for many mammalian tissues [30]. Despite its low amount in human platelets this phospholipid might be a suitable target molecule with regard to its ability to be modified by dietary manipulations [31].

It is apparent that the technique described here has several advantages, including complete separation of phospholipid classes on a microscale and the determination of their fatty acid patterns. It compares well with new high-performance

liquid chromatographic techniques in presenting the possibility of analysing several samples in a short time and with substantial cost saving.

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