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Thin-layer chromatography and high-performance liquid chromatography for the assay of fatty acid compositions of individual phospholipids in platelets from non-insulin-dependent diabetes mellitus patients: effect of eicosapentaenoic acid ethyl ester administration

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Abstract

Eight major phospholipids were separated by a TLC method with a one-dimensional developing system without any pretreatment of the plate and the fatty acids incorporated into each phospholipid class were analysed by an improved HPLC method with a simple elution system, which has advantages with respect to resolution and analysis time. The fatty acid compositions of individual phospholipids in platelets were investigated following administration of ethyl *cis*-5,8,11,14,17-eicosapentaenoate for more than 13 weeks to patients with non-insulin-dependent diabetes mellitus. The *cis*-5,8,11,14,17-eicosapentaenoic acid compositions of all phospholipid classes were significantly increased with decreasing platelet aggregation rates after the administration. These results suggested that the present method provides the complete separation of individual phospholipids in sufficient amounts to allow fatty acid analysis on the isolated phospholipid moieties.

Keywords: Fatty acids; Phospholipids; Eicosapentaenoic acid ethyl ester

1. Introduction

An accurate method for the simultaneous separation of different phospholipids is desirable to study the fatty acid compositions, because the phospholipids vary widely between subclasses and different organs [1–3] and variations can also be induced under certain pathological conditions [4,5].

TLC is the most commonly used technique for the separation and identification of phospholipids. Several one-dimensional TLC methods, which are simpler and more rapid than two-dimensional TLC methods, have been developed [6–11]. One drawback of some of these methods is that lengthy and cumbersome pre-

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treatment of the TLC plate is needed for the application of lipids to the plate. Another drawback is that most methods cannot successfully separate phospholipids on a preparative scale owing the the poor resolution of the different phospholipids.

In this paper, we describe a one-dimensional TLC system for the separation of eight major phospholipids classes on a commercially available TLC plate without any pretreatment of the plate, and an improved HPLC method for the analysis of 28 saturated and mono- and polyunsaturated fatty acids ($C_{8:0}$–$C_{22:6}$) including *cis–trans* isomers and positional isomers of double bonds in the fatty acid chain. We also describe the effect of ethyl *cis*-5,8,11,14,17-eicosapentaenoate (EPA-E) on the fatty acid compositions of individual phospholipids in platelets obtained from patients with non-insulin-dependent diabetes mellitus (NIDDM).

2. Experimental

2.1. Chemicals and reagents

Phospholipid standards, L-α-phosphatidylcholine (PC), L-α-phosphatidylethanolamine (PE), L-α-phosphatidyl-L-serine (PS), L-α-phosphatidylinositol (PI), L-α-phosphatidyl-DL-glycerol (PG), L-α-lysophosphatidylcholine (LPC), sphingomyelin (SM) and cardiolipin (CL) were purchased from Sigma (St. Louis, MO, USA). All fatty acid solutions in ethanol were obtained from Yamamura Chemical Labs. (Kyoto, Japan). A 0.02 *M* solution of 2-nitrophenylhydrazine hydrochloride (2-NPH·HCl) (Tokyo Kasei Kogyo, Tokyo, Japan) was prepared by dissolving the reagent in 0.3 *M* hydrochloric acid–ethanol (1:1, v/v). A 0.25 *M* solution of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (1-EDC·HCl) (Sigma) was prepared by dissolving the reagent in a 3% (w/v) solution of pyridine in ethanol. A 10% (w/v) solution of potassium hydroxide in methanol–water (1:1, v/v) and a 0.4 *M* potassium hydroxide–ethanol (1:1, v/v) solution were prepared. All the reagent solutions were stable for at least 3 months when kept below 5°C, and were commercially available from Yamamura Chemical Labs. All other chemicals were of analytical-reagent grade, unless stated otherwise.

2.2. Preparation of platelet phospholipids

After overnight fasting, to measure platelet aggregation rates and fatty acid compositions in platelet phospholipids, blood was drawn from NIDDM patients by use of a vacuumed venous aspirator containing Na_2EDTA anticoagulant (Terumo, Kyoto, Japan). Platelet aggregation rates were measured by nephelometry according to Born [12] by using ADP and collagen at final concentrations of 1 m*M* and 0.25 mg/ml, respectively. The quantity of light penetrating the sample was used for maximum aggregation rate. The blood (ca. 20–30 ml) was centrifuged at 225 *g* for 6 min to obtain platelet-rich plasma (PRP). The PRP was washed twice with 0.9% sodium chloride and centrifuged at 1000 *g* for 20 min after each washing, to obtain a platelet pellet. The lipid fraction was extracted by the method of Folch et al. [13], using a chloroform–methanol solution (2:1, v/v).

2.3. TLC separation

Individual phospholipids were separated from the other lipids by TLC in one dimension on a 20 cm × 10 cm × 0.25 mm silica plate (LK-5 with a preadsorbent area; Whatman, Clifton, NJ, USA), which was developed in methyl acetate–1-propanol–chloroform–methanol–0.25% (w/v) potassium chloride solution (25:35:20:10:10, v/v). After detection on the TLC plates with iodine vapour, each phospholipid class was scraped off and extracted with 5 ml of methanol. The solvent was removed under a stream of nitrogen at room temperature, and the fatty acids of each phospholipid class were analysed as their hydrazides.

2.4. Assay procedures

The residue was dissolved in 100 μl of ethanol containing 50 nmol of heptadecanoic acid as the internal standard and was saponified with 50 μl

of 0.4 *M* potassium hydroxide–ethanol (1:1, v/v) at 80°C for 20 min. To the saponified sample, 100 μl of 2-NPH·HCl solution and 100 μl of 1-EDC·HCl solution were added and the mixture was heated at 80°C for 5 min. After the addition of 100 μl of 10% (w/v) potassium hydroxide solution, the mixture was further heated at 80°C for 5 min and then cooled. To the resultant hydrazide mixture, 4 ml of 1/30 *M* phosphate buffer (pH 6.4)–0.5 *M* hydrochloric acid (7:1, v/v) were added. The fatty acid hydrazides were extracted with 5 ml of *n*-hexane, and the solvent was evaporated under a stream of nitrogen at room temperature. The residue was dissolved in 50 μl of methanol and an aliquot (5–20 μl) was injected into the chromatograph.

2.5. HPLC analysis

Chromatographic analyses were carried out using a Shimadzu LC-6A liquid chromatograph (Shimadzu Seisakusho, Kyoto, Japan) equipped with an ERC-3310 on-line degasser (Erma, Tokyo, Japan) and a Shimadzu SPD-6AV variable-wavelength UV–visible detector ($\lambda = 400$ nm). The detector signals were recorded on a Rikadenki multi-pen recorder (Tokyo, Japan). The column temperature was kept constant at 50°C using a Shimadzu GTO-6A column oven. The column consisted of a J'sphere ODS-M 80 main column (particle size 4 μm, 250 × 4.6 mm I.D.) with a guard cartridge (J'sphere ODS-M 80), packed at Yamamura Chemical Labs.

The separation of the fatty acid hydrizides was achieved isocratically using acetonitrile–water (86:14, v/v) as the eluent at a flow-rate of 2.0 ml/min. The pH of the eluent was maintained at 4–5 by adding 0.1 *M* hydrochloric acid. The eluent was filtered through a Fluoropore filter (pore size 0.45 μm) (Sumitomo Electric, Osaka, Japan).

2.6. Calculation

Previous works [14,15] had demonstrated that the calibration graphs for individual fatty acids were linear over a wide concentration range with good correlation coefficients. Thus, the amounts of individual fatty acids (FA) in platelet phospholipids were calculated by the internal standard method.

3. Results and discussion

The aim of this study was to establish a standaridized procedure for the analysis of the fatty acid compositions of different phospholipid subclasses in biological materials. We have already described an HPLC method for the analysis of 25 saturated and mono- and polyunsaturated fatty acid hydrazides ($C_{8:0}$–$C_{22:6}$) including *cis–trans* isomers [14,15] and its application to the determination of the fatty acid composition of total phospholipid in platelets [14].

However, the development of a simple and accurate TLC method for the preparative-scale isolation of platelet lipid complexes is extremely important for the analysis of fatty acid compositions of the different phospholipids in platelets. Eight phospholipid standards and platelet phospholipids were separated with a one-dimensional developing system on a commercially available TLC plate without any pretreatment (Fig. 1). Vapour saturation and/or the temperature of the chromatographic chamber did not have any significant effect on the separation, which was always excellent provided that an appropriate amount of lipids was applied to the plate.

The detection of a very small amount of fatty acid hydrazides by the HPLC method sometimes suffered interference from a few components in PE. Therefore, an improved HPLC method is needed to overcome this problem.

The commonly used HPLC separations of fatty acid derivatives were carried out on reversed-phase columns with an isocratic or gradient elution system using a mixture of acetonitrile, methanol and water. In our previous work [14,15], a mixture of 25 fatty acid hydrazides was separated isocratically using as the mobile phase acetonitrile–methanol–water (75:11:14, v/v/v) at a flow-rate of 1.2 ml/min at 35°C on a YMC-FA (C_8) column (250 × 6 mm I.D.). Acetonitrile and methanol have different effects on two

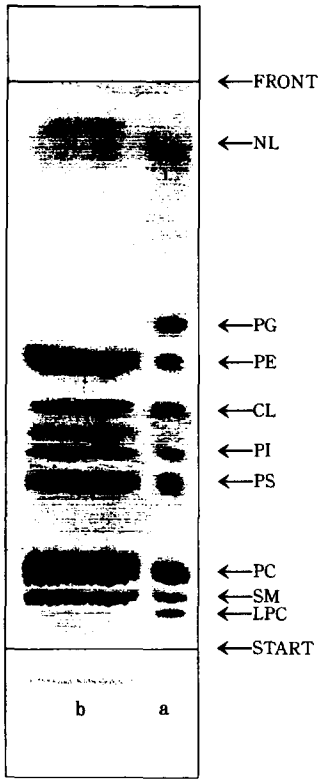

Fig. 1. Thin-layer chromatogram of (a) eight phospholipid standards and (b) whole lipids of platelet from a NIDDM patients on an LK-5 plate (20 cm × 5 cm × 0.25 mm). Abbreviations: NL = neutral lipids; PG = L-α-phosphatidyl-DL-glycerol; PE = L-α-phosphatidylethanolamine; CL = cardiolipin; PI = L-α-phosphatidylinositol; PS = L-α-phosphatidyl-L-serine; PC = L-α-phosphatidylcholine; SM = sphingomyelin; LPC = L-α-lysophosphatidylcholine. Staining: iodine vapour.

parameters for the elution volumes of fatty acid hydrazides, namely the number of carbon atoms and the number of unsaturated bonds in the fatty acid chain. Therefore, the resolution of fatty acid hydrazides on the column was significantly affected by the proportion of acetonitrile and methanol in the mobile phase.

By introducing the newly developed J'sphere ODS-M80 column (250 × 4.6 mm I.D.), these problems are overcome and the separation of the 25 fatty acids and positional isomers of double bonds in the fatty acid chain, such as γ-linolenic acid, which is of wide interest with regard to its physiological effects and the possibility of health benefits, has progressed remarkably. The separation is achieved within only 22 min using a simple isocratic elution system consisting of acetonitrile–water (86:14, v/v) (Fig. 2) at a flow-rate of 2.0 ml/min at 50°C, whereas the same separation has hitherto been achieved only by capillary GLC with a longer analysis time.

In order to evaluate the separation procedure, a standard solution containing known amounts of PC, PE, PS and PI (1 mg/ml of each in chloroform solution) was prepared as a model of individual phospholipids in platelets. From this standard solution, six aliquots of 200 μl were developed on the TLC plates and analysed as described in the assay procedures. Another six aliquots of 200 μl were analysed directly. The differences in the fatty acid compositions incorporated into the four phospholipids with and without the TLC isolation procedure were less than 2.1%. The relative recoveries of the individual phospholipids were in the range 97.2–103.8% and the coefficients of variation were in the range 0.4–3.2%. The present method has a satisfactory precision in analysing the fatty acid compositions of individual platelet phospholipids.

To confirm the practical utility of the present method in the assay of biological materials, we applied it to the determination of fatty acids incorporated into each major phospholipid class (PC, PE, PS and PI) of platelets obtained from ten fasting NIDDM patients (age range 32–66 years). In the TLC, glycolipids and neutral lipids including free fatty acids, which have an R_F substantially greater than the furthest migrating class of phospholipid, such as PG, did not interfere with the phospholipid separation (Fig. 1).

The fatty acid profile of PE in platelets obtained from an NIDDM patient after EPA-E administration is shown in Fig. 3. In the chromatogram monitored by visible absorbance, a few unknown peaks appeared, but the fatty acids in the samples were easily identified by comparison of the retention times of their hydrazides with those of standards. These results suggested that the TLC–HPLC method described here reduced the overall analysis time and decreased the sample volume required.

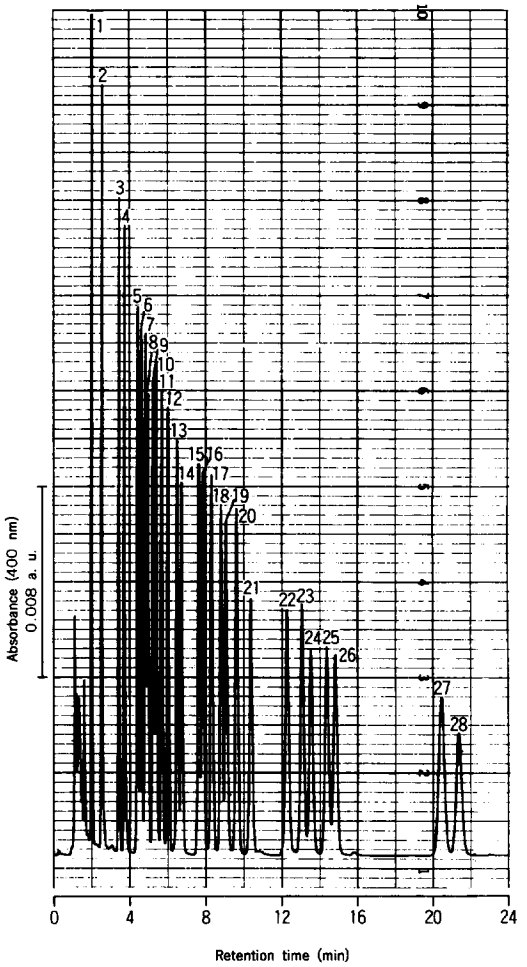

Fig. 2. Chromatogram of the 2-nitrophenylhydrazides of a standard mixture of 25 fatty acids obtained with visible detection. Peaks: 1 = octanoic ($C_{8:0}$); 2 = decanoic ($C_{10:0}$); 3 = dodecanoic ($C_{12:0}$); 4 = *cis*-9-tetradecanoic ($C_{14:1}$); 5 = *cis*-5,8,11,14,17-eicosapentaenoic ($C_{20:5}$); 6 = *cis*-9,12,15-octadecatrienoic ($C_{18:3}$); 7 = *cis*-6,9,12-octadecatrienoic ($C_{18:3}$); 8 = tetradecanoic ($C_{14:0}$); 9 = *cis*-4,7,10,13,16,19-docosahexaenoic ($C_{22:6}$); 10 = *cis*-9-hexadecenoic ($C_{16:1}$); 11 = *cis*-5,8,11,14-eicosatetraenoic ($C_{20:4}$); 12 = *cis*-9,12-octadecadienoic ($C_{18:2,c}$); 13 = *trans*-9,12-octadecadienoic ($C_{18:2,t}$); 14 = *cis*-8,11,14-eicosatrienoic ($C_{20:3}$); 15 = hexadecanoic ($C_{16:0}$); 16 = *cis*-7,10,13,16-docosatetraenoic ($C_{22:4}$); 17 = *cis*-9-octadecenoic ($C_{18:1,c}$); 18 = *trans*-9-octadecenoic ($C_{18:1,t}$); 19 = *cis*-11,14-eicosadienoic ($C_{20:2}$); 20 = heptadecanoic ($C_{17:0}$) (I.S.); 21 = *cis*-13,16,19-docosatrienoic ($C_{22:3}$); 22 = octadecanoic ($C_{18:0}$); 23 = *cis*-11-eicosenoic ($C_{20:1}$); 24 = *cis*-8-eicosenoic ($C_{20:1}$); 25 = *cis*-13,16-docosadienoic ($C_{22:2}$); 26 = *cis*-5-eicosenoic ($C_{20:1}$); 27 = eicosanoic ($C_{20:0}$); 28 = *cis*-13-docosaenoic ($C_{22:1}$) acid hydrazide. Each peak corresponds to 150 pmol.

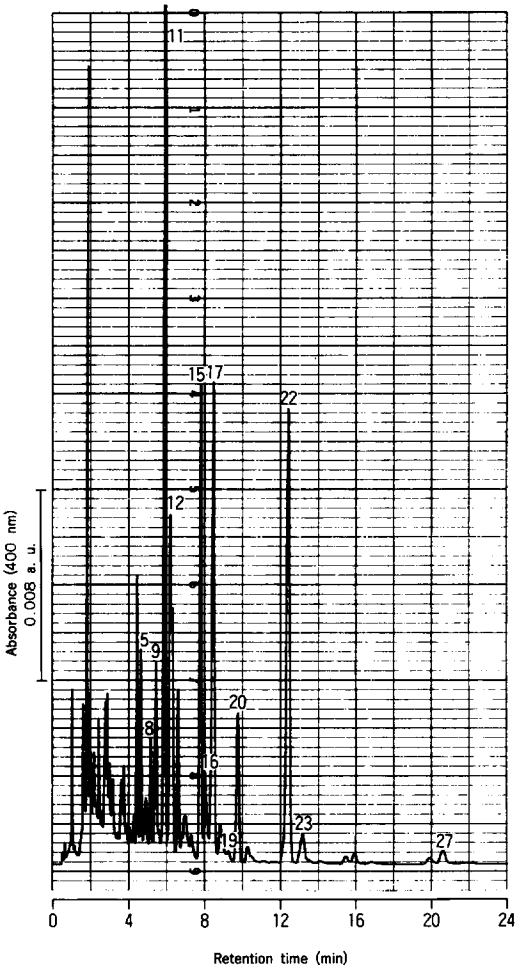

Fig. 3. Fatty acid profile of L-α-phosphatidylethanolamine of platelet obtained from an NIDDM patient after EPA-E adminstration. Peak numbers correspond to those in Fig. 2.

Table 1 listed the mean values for the relative fatty acid compositions of the four major phospholipids in platelets obtained from NIDDM patients before and after EPA-E adminstration (Epadel, Mochida Pharmaceuticals, Tokyo, Japan; administration 1.8 g/day) for 13–22 weeks (mean 17 weeks). The fatty acid compositions showed different patterns, with a significant increase of EPA in all phospholipid classes. The EPA composition of platelet PC in the patients increased significantly with administration of EPA-E, while the *cis*-5,8,11,14-eicosatetraenoic acid (arachidonic acid, AA) composition de-

Table 1
Effect of EPA-E administration on the fatty acid compositions of platelet phospholipids obtained from NIDDM patients

Fatty acid	Fatty acid composition (mol-%)							
	Phosphatidylcholine		Phosphatidylethanolamine		Phosphatidylserine		Phosphatidylinositol	
	Before EPA-E	After EPA-E	Before EPA-E	After EPA-E	Before EPA-E	After EPA-E	Before EPA-E	After EPA-E
$C_{14:0}$	0.90 ± 0.30	1.95 ± 3.14	3.25 ± 0.85	3.17 ± 1.02	1.88 ± 1.19	2.33 ± 1.67	2.90 ± 0.96	3.48 ± 1.58
$C_{16:0}$	43.54 ± 3.57	44.70 ± 4.63	14.36 ± 1.93	13.10 ± 1.64[b]	6.98 ± 3.36	8.06 ± 3.11	10.27 ± 2.27	12.53 ± 3.89
$C_{18:0}$	16.11 ± 2.04	16.16 ± 0.99	32.51 ± 3.73	29.12 ± 4.09[a]	53.83 ± 6.42	53.03 ± 7.06	54.27 ± 8.16	53.20 ± 8.01
$C_{18:1,c}$	22.70 ± 3.32	21.45 ± 4.46	11.29 ± 1.13	9.94 ± 1.25[a]	20.95 ± 5.08	19.11 ± 5.40	4.35 ± 0.70	4.59 ± 1.26
$C_{18:2,c}$	7.64 ± 1.50	7.89 ± 1.25	8.05 ± 0.48	10.60 ± 2.28[b]	2.19 ± 0.70	2.39 ± 0.70	4.71 ± 1.14	4.46 ± 1.86
$C_{20:0}$	1.11 ± 0.26	1.19 ± 0.23	1.17 ± 0.24	0.80 ± 0.14[b]	2.11 ± 0.20	1.90 ± 0.23	0.63 ± 0.17	0.58 ± 0.14
$C_{20:1}$	1.05 ± 0.30	1.08 ± 0.20	0.87 ± 0.18	0.72 ± 0.22	0.48 ± 0.25	0.54 ± 0.21	0.30 ± 0.11	0.36 ± 0.08
$C_{20:2}$	0.35 ± 0.08	0.32 ± 0.12	0.48 ± 0.09	0.62 ± 0.11				
$C_{20:4}$	5.30 ± 0.73	3.71 ± 0.60[c]	23.47 ± 2.51	24.83 ± 4.72	10.70 ± 2.39	11.79 ± 2.07	22.04 ± 7.83	20.12 ± 4.97
$C_{20:5}$	0.66 ± 0.22	0.94 ± 0.19[a]	1.16 ± 0.52	3.10 ± 1.33[c]	0.13 ± 0.07	0.22 ± 0.07[a]	0.26 ± 0.14	0.47 ± 0.20[a]
$C_{22:4}$			0.96 ± 0.20	1.24 ± 0.35				
$C_{22:6}$	0.64 ± 0.23	0.61 ± 0.14	2.43 ± 0.72	2.76 ± 0.70	0.75 ± 0.32	0.63 ± 0.38	0.27 ± 0.18	0.21 ± 0.07
EPA–AA	0.125 ± 0.054	0.253 ± 0.063[b]	0.049 ± 0.021	0.125 ± 0.041[c]	0.012 ± 0.012	0.019 ± 0.007[a]	0.012 ± 0.005	0.023 ± 0.008[b]

Data are expressed as the means ±S.D. ($n = 10$). The significant difference between the two groups are assessed.
[a] $P < 0.05$.
[b] $P < 0.01$.
[c] $P < 0.001$.

creased markedly. In platelet PE the drastically increased incorporation of EPA was associated with a significant decrease in the proportions of hexadecanoic, octadecanoic and eicosanoic acids. The proportion of *cis*-9,12-octadecadinoic acid was significantly increased only in PE. These results showed that the differences in the fatty acid compositions of platelet phospholipids obtained from the patients before and after EPA-E administration were limited mainly to PE. The EPA-to-AA ratios were significantly increased in all phospholipid classes following oral EPA-E administration.

Needleman et al. [16] described the mechanism of the inhibitory effect of oral EPA administration on platelet aggregation. They indicated that oral EPA-E administration was effective at the level of cyclooxygenase to reduce TXA_2 production from AA and, therefore, to decrease the platelet aggregation. Concerning our observations, platelet aggregation rates induced by both ADP and collagen decreased significantly ($P < 0.05$) from 67.6 ± 2.5 and 67.3 ± 3.2 (mean $\pm$ S.D.) to 55.7 ± 3.2 and 51.7 ± 4.3 with EPA-E administration, respectively. These results were identical with those in their study and indicated that the fatty acid compositions incorporated into individual phospholipids of platelet are directly related to the platelet function.

4. Conclusion

A TLC–HPLC method has been developed for the analysis of fatty acid compositions incorporated into individual phospholipids of human platelets. The method has two important advantages over previously published methods. First, individual phospholipids on a preparative scale are directly isolated by the one-dimensional developing system on a commercially available TLC plate without any pretreatment of the plate. Second, the introduction of a new HPLC column markedly improved the separation of 28 fatty acid hydrazides by the simple isocratic elution system. Using the present method, it was observed that EPA compositions of all phospholipids in platelets obtained from NIDDM patients increased with EPA-E administration, whereas platelet aggregation rates decreased.

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