

Alterations in serum phosphatidylcholine fatty acyl species by eicosapentaenoic and docosahexaenoic ethyl esters in patients with severe hypertriglyceridemia

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Abstract A new and sensitive method has been developed to analyze the molecular species of glycerophospholipids. This method was used to examine the effects of hypolipidemic intervention with n-3 fatty acids on the serum phosphatidylcholine species in severely hypertriglyceridemic patients. The drug treated group (n = 19) received 4 g/day of an 85% concentrate of the ethyl esters of eicosapentaenoic and docosahexaenoic acids for 6 weeks. Control patients (n = 21) received 4 g/day of ethyl esters of corn oil fatty acids. To evaluate the effects of n-3 fatty acids upon serum phosphatidylcholines (PCs), sera from treated and control patients were analyzed before and after 6 weeks of intervention. PCs isolated from sera were digested with phospholipase C to diglycerides, derivatized with 7-methoxycoumarin-3-carbonyl azide, and analyzed by reverse phase high performance liquid chromatography (HPLC) with fluorescence detection. Pre-intervention serum PC species were, in order of decreasing concentration C_{16:0,18:2}, C_{16:0,18:1}, C_{18:0,18:2}, C_{16:0,20:4}, C_{16:0,22:6}, C_{18:0,20:4}, C_{16:0,16:0}, C_{18:0,18:1}, C_{18:1,18:2}, C_{16:0,20:5}, and C_{18:1,20:5}. In the treated patients, mean increases of 300% in C_{16:0,20:5} and of 160% in C_{16:0,22:6} species were observed. There were no significant changes in the molecular species of the serum phosphatidylcholines in the group receiving the corn oil ethyl esters. The cumulative relative percentages for each of the individual fatty acids measured by HPLC were comparable to those determined by gas-liquid chromatography (GLC). In the treated group plasma triglycerides were reduced 26%, while they were increased by 7% in the placebo group. Our data showed that incorporation of eicosapentaenoic and docosahexaenoic acid into the serum PCs occurred within 6 weeks primarily in the C_{16:0,20:5} and C_{16:0,22:6} species and were usually accompanied by a reduction in plasma triglyceride—McKeone, B. J., K. Osmundsen, D. Brauchi, Q. Pao, C. Payton-Ross, C. Kilinç, F. A. Kummerow, and H. J. Pownall. Alterations in serum phosphatidylcholine fatty acyl species by eicosapentaenoic and docosahexaenoic ethyl esters in patients with severe hypertriglyceridemia. *J. Lipid Res.* 1997. **38**: 429–436.

Supplementary key words n-3 fatty acids • hypertriglyceridemia • phospholipid species • high pressure liquid chromatography

Omega-3 (n-3) fatty acids are essential for normal human growth and development. They also have potential use in the treatment of hypertriglyceridemia, coronary artery disease, hypertension, autoimmune diseases, arthritis, and other inflammatory states (for reviews see 1,2). Dietary n-3 fatty acids reduce plasma triglycerides by a mechanism that is thought to involve the inhibition of triglyceride or phosphatidylcholine (PC) synthesis.

Clinical trials of the effects of n-3 fatty acids on lipid metabolism provide information to refine patient management strategies based on this intervention and to guide researchers in the design of mechanistic studies. It is important to measure serum n-3 fatty acid levels because of the associated effects of increased bleeding time and erythrocyte deformability, as well as decreased blood viscosity and fibrinogen levels. Many of these effects are thought to be due to the substitution of n-3 for n-6 fatty acids in the membrane phospholipids of erythrocytes, platelets, neutrophils, monocytes, and liver cells. Increases in membrane n-3 content have also been shown to alter the fluidity and permeability of biomembranes, in addition to altering the activity of membrane-associated proteins (2-4).

Gas-liquid chromatography is one conventional measure of phospholipid acyl composition. However,

Abbreviations: 7-MCCA, 7-methoxycoumarin-3-carbonyl azide; HPLC, high performance liquid chromatography; GLC, gas-liquid chromatography; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid; PC, phosphatidylcholine; VLDL, very low density lipoprotein; HDL, high density lipoprotein.

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this technique gives only the total fatty acid composition of each lipid class, not the identity and concentration of individual molecular species. One successful alternative is to convert isolated PCs to their respective diacylglycerols, followed by derivatization to benzoyl or dinitrobenzoyl esters, which are detected by measuring their absorbance after reverse phase high performance liquid chromatography (HPLC) (5–7). Attempts have been made to improve upon this methodology with fluorescent derivatives of diacylglycerols, however, the necessary derivatization reagents are not readily available or very chemically stable (8, 9). Our method has the advantage of using a single reagent, 7-methoxycoumarin-3-carbonyl azide (7-MCCA), which is commercially available and stable, to convert the diacylglycerols of PCs to highly fluorescent urethane derivatives that are more stable than the ester derivatives used in other studies. We have used this methodology to determine the effects of oral administration of n–3 fatty acids on the serum PC molecular species in 21 control and 19 treated severely hyperlipidemic subjects.

EXPERIMENTAL PROCEDURES

Study design

The study protocol was designed for a randomized, placebo-controlled double-blind study of Omacor™, a concentrate of primarily eicosapentaenoic and docosahexanoic acid ethyl esters in patients with severe hypertriglyceridemia. This study was carried out at The Methodist Hospital (Houston, TX) in conjunction with a study to assess the effect of n–3 fatty acids on the serum triglycerides of patients who are at risk for developing pancreatitis. Patient selection was followed by a 6-week run-in period during which patients received instruction in American Heart Association step I diet. At the end of the dietary run-in period, 40 subjects were randomized and treated for 6 weeks with either the drug (4 g/day) or a corn oil placebo (4 g/day) ingested with a meal. The patients were strongly advised to continue the therapeutic diet throughout the treatment period. Inclusion criteria for the study were as follows: 1) male or female subjects between 18 and 70 years of age, and 2) mean serum triglycerides after screening of greater than or equal to 500 mg/dL but less than 2000 mg/dL. Informed written consent was obtained from each subject. The study protocol was approved by the institutional review board and conducted according to the European Guidelines for Good Clinical Practice. An IND was submitted with the FDA.

Patients were excluded from the study if they met any of the following criteria: treatment with fibrates within 3 months prior to the dietary phase; consumption of

lipid-lowering products such as cod liver oil or lipid-lowering fibers in the 4 weeks prior to the dietary phase; eating cold water fish more than once weekly; serum alanine aminotransferase greater than three times upper normal level; a fasting serum glucose of greater than 300 mg/dL; serum creatinine of greater than 2 mg/dL; a platelet count of less than $60 \times 10^9/L$; hemoglobin of less than 10 g/dL; consumption of more than two alcoholic beverages per day; pregnancy or lactation; type I diabetes; experience of myocardial infarction within the past 6 months; or other clinically significant disease as judged by the investigator.

Serum samples were collected by venipuncture in red and grey topped integrated serum separator tubes (Sherwood Medical, St. Louis, MO) both prior to the study and at the end. Aprotinin and phenyl methyl sulfonyl fluoride were added to each sample at a concentration of 10 kallikrein units/mL and 1 mM, respectively. The samples were then stored frozen at -70°C until analyzed. Plasma triglycerides were determined enzymatically using a kit from Boehringer-Mannheim (Indianapolis, IN). Fluorescence excitation and emission spectra were measured using an SLM 8000 spectrofluorometer (Urbana, IL).

Analysis of serum phosphatidylcholine species

In a glass tube with a screw cap, 0.5 mL of serum was diluted to 1 mL with H_2O . The total lipids were then extracted twice with ethyl acetate–acetone 2:1 (v/v) at a ratio of 3 mL of solvent to 1 mL of liquid. The extraction mixture was vortexed and centrifuged at (720 g) to separate the layers. The upper lipid containing layer of the extracts was combined and dried down under nitrogen. Any residual water was removed by lyophilization. The sample was dissolved in 300 μL isooctane–THF 99:1, filtered with 0.2 μm nylon syringe filter and injected onto a 5 μm , 4.5 mm \times 10 cm normal phase HPLC column (Phase Separations Ltd., Norwalk, CT). Individual lipid classes were eluted using a solvent gradient of isooctane–tetrahydrofuran 99:1, isopropanol–methylene chloride 4:1, and isopropanol–water 1:1 (v/v) (10). The elution time of the phosphatidylcholine peak was determined by comparison with the retention time of ^3H -labeled 1-palmitoyl-2-oleoyl-*sn*-glycerol-3-phosphocholine. The peak was collected, the solvent was evaporated, and the PCs were resuspended in 0.5 mL 50 mM potassium phosphate, 30 mM HBO_3 buffer, pH 7.4. Next, 2 mL of diethyl ether was added, followed by 10 μL of phospholipase C (*Bacillus cereus*), approximately 1400 units/mL (Sigma Chemical, St. Louis, MO). The suspension was vortexed vigorously for 1 min and mixed on a rotary mixer for 2 h. At that time 2 mL of hexane was added, the mixture was vortexed, and the upper organic layer was decanted to a screw-cap test tube. The extraction procedure was performed twice

and the combined extracts were dried under nitrogen without heating to prevent rearrangement of the 1,2 diglyceride to the 1,3 diglyceride. The samples were lyophilized again to remove any water. 7-Methoxycoumarin-3-carbonyl azide (Molecular Probes, Eugene OR) dissolved in dry methylene chloride was added to the diglycerides at a 2:1 weight ratio, or an approximately 5:1 molar ratio, and the tube was capped tightly and sealed with parafilm. It was then refluxed in a 60°C heating block for 90 min, cooled in an ice bath, and chromatographed on a K6 silica gel thin-layer chromatography plate (Whatman, Hillsboro, OR) in a solvent of toluene-ethyl acetate 4:1. The bright blue fluorescent spot near the top of the plate (rf 0.85) was visualized by long wave UV illumination and was scraped off of the plate and eluted from the silica gel with 2 mL isopropanol. After measuring the absorbance at 345 nm, the change in volume needed to produce an O.D.₃₄₅ of approximately 0.4 was calculated, the solvent was evaporated, and the sample was redissolved in the previously calculated volume of isopropanol. A 20- μ L sample of this was injected onto a 5 μ m, 4.5 mm \times 25 cm ODS2 C18 column heated to 50°C (Phase Separations Ltd.). The sample was eluted with acetonitrile-isopropanol-water 64:25:11 at flow rate of 1.15 mL/min and detected with a Kratos 980 Spectroflow fluorescence detector equipped with a deuterium lamp. Excitation was at 340 nm and emission was detected with a 370 nm cut on filter. The identity of the PC fatty acyl groups was established by collecting the eluted peaks and performing gas-liquid chromatography, by comparing the retention times of derivatives of commercially available PCs, and by spiking trial serum samples with derivatives made from commercially available PCs. Utilizing this procedure, it is not possible to determine the positional specificity of the fatty acyl chains on the phosphocholine glycerol backbone, the shorter, more saturated, fatty acyl chain is assumed to be in the *sn*-1 position and the longer, more unsaturated, acyl chain is assumed to be in the *sn*-2 position (4).

The fatty acid composition of serum PCs was also analyzed by gas-liquid chromatography. Serum samples (200 μ L) were extracted as above and the PCs were isolated by thin-layer chromatography on silica gel K6 plates (Whatman, Clifton, NJ) using a solvent mixture of iso-octane-ethyl ether-acetic acid 75:25:2. The isolated PCs were converted to their methyl ester derivatives using methanolic base and BF₃ in methanol (Supelco Inc., Bellefonte, PA). Capillary gas-liquid chromatography on the fatty acid methyl esters was then performed using a 30 m \times 0.32 mm Carbowax column (Alltech, Deerfield, IL) and a thermal gradient from 180-245°C. Individual fatty acids were identified by comparison of retention times with standard mixtures of fatty acid methyl esters (Nu-Chek Prep, Elysian MN).

RESULTS

We have developed a new series of fluorescent derivatives of diacylglycerol to enable the detection of small amounts of phospholipids and diglycerides in biological samples. **Figure 1** shows the reaction mechanism, which proceeds by the formation of an isocyanate in the presence of an alcohol (the diacylglycerol) and the subsequent formation of a urethane linkage with the diglyceride. 7-MCCA proved to be better suited than 7-diethylaminocoumarin-3-carbonyl azide (Molecular Probes) for HPLC with fluorescence detection, even though the latter had a higher molar extinction coefficient (44,000 mol⁻¹ cm⁻¹). Both react by the same mechanism, however, the intensity of the detector deuterium source lamp decreases substantially at the excitation maximum for the diethylaminocoumarin-3-carbonyl azide derivative (397 nm) producing a lower output signal at its emission maximum (470 nm) for equimolar amounts of product. The derivatization solution itself was stable for at least 6 months when stored at -20°C in the dark. Additionally, this methodology can be further simplified by using thin-layer chromatography to separate serum lipid groups rather than normal phase HPLC prior to digestion with phospholipase C (5). Normal phase HPLC, however, enabled greater recovery of the PCs (83.5% versus 21.1% for thin-layer chromatography).

Optimal separation of serum PC derivatives by reverse phase HPLC occurred within 2 h using a solvent system of acetonitrile-isopropanol-water 64:25:11 at flow rate of 1.15 mL/min. Elution times of phospholipid derivatives by reverse phase chromatography have been shown to be a predictable function of the carbon number and degree of unsaturation of the derivatized product (5). For PCs containing more saturated fatty acyl species, such as egg yolk lecithin, a more polar solvent is recommended (acetonitrile-isopropanol-water 63:24:13). The fluorescent derivatives of diacylglycerols obtained from serum PCs were detectable with a fluorometric detector at 10 pmol/L. Utilizing the 7-MCCA derivative of dimyristol glycerol, the linear range of the integrated area detectable was determined to be from 5 ng to 1 μ g. Mean overall recovery was 5.74%. The fluorescent PC product had an excitation maximum at 348 nm and an emission maximum of 406 nm. The molar extinction coefficient was 26,000 mol⁻¹ cm⁻¹. Serum samples as small as 25 μ L can be used, however, sample handling and detection of the derivatized diglyceride on the TLC plate is easier when a larger sample is used.

Figure 2 shows the distribution of PC species in the serum of a typical subject prior to treatment with the drug (panel A) and after 6 weeks of treatment with the drug (panel B). In both placebo and drug groups,

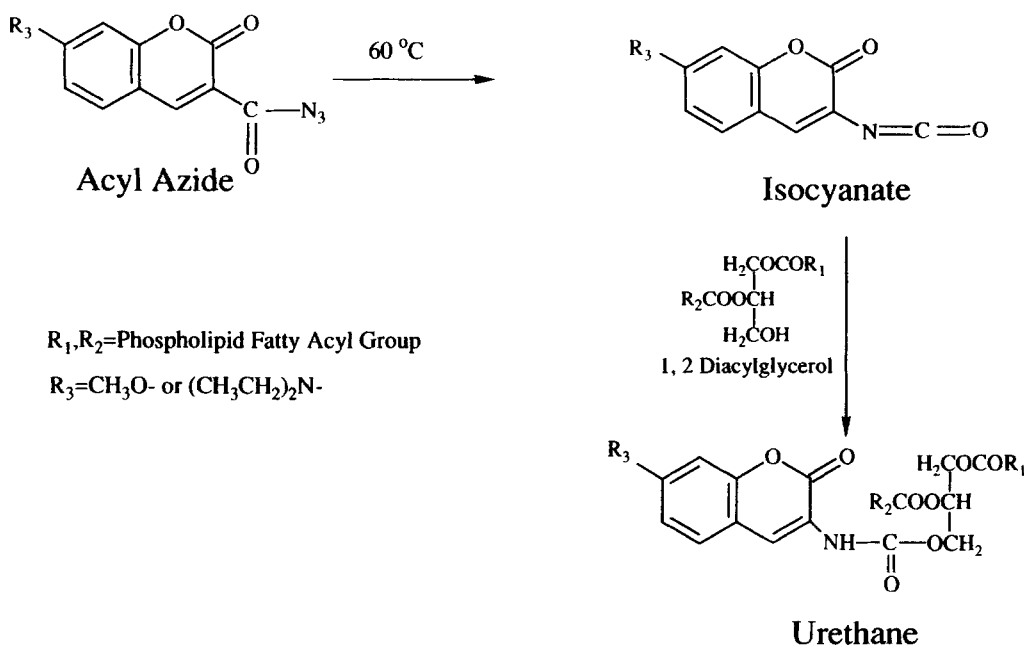


Fig. 1. Reaction mechanism of the fluorescent coumarin carbonyl azides with diacylglycerol. The carbonyl azides rearrange in the presence of alcohol (the diacylglyceride) to form an isothiocyanate that reacts with the hydroxyl group to form an urethane linkage.

the most prominent species was $\text{C}_{16:0,18:2}$, followed by $\text{C}_{16:0,18:1}$, $\text{C}_{18:0,18:2}$, and $\text{C}_{16:0,20:4}$. Lesser amounts of $\text{C}_{16:0,22:6}$, $\text{C}_{18:0,20:4}$, $\text{C}_{16:0,16:0}$, $\text{C}_{18:0,18:1}$, $\text{C}_{18:1,18:2}$, $\text{C}_{16:0,20:5}$, and $\text{C}_{18:1,20:5}$ were present in most of the samples. Occasionally traces of $\text{C}_{18:1,18:1}$, $\text{C}_{18:1,22:6}$, and $\text{C}_{18:0,22:6}$ were detected. The most significant increases in n-3 content after 6 weeks of treatment with the drug were in the $\text{C}_{16:0,20:5}$ and the $\text{C}_{16:0,22:6}$ species.

Table 1 shows the relative percentage distribution of serum PCs in both the placebo control group and the drug-treated group prior to treatment and after 6 weeks on placebo or drug. In the treated group, there was an approximately 3-fold increase in $\text{C}_{16:0,20:5}$ and a 1.6-fold increase in $\text{C}_{16:0,22:6}$. Overall, there were statistically significant increases in the percentages of both the $\text{C}_{16:0,20:5}$ and the $\text{C}_{16:0,22:6}$ species with concomitant statis-

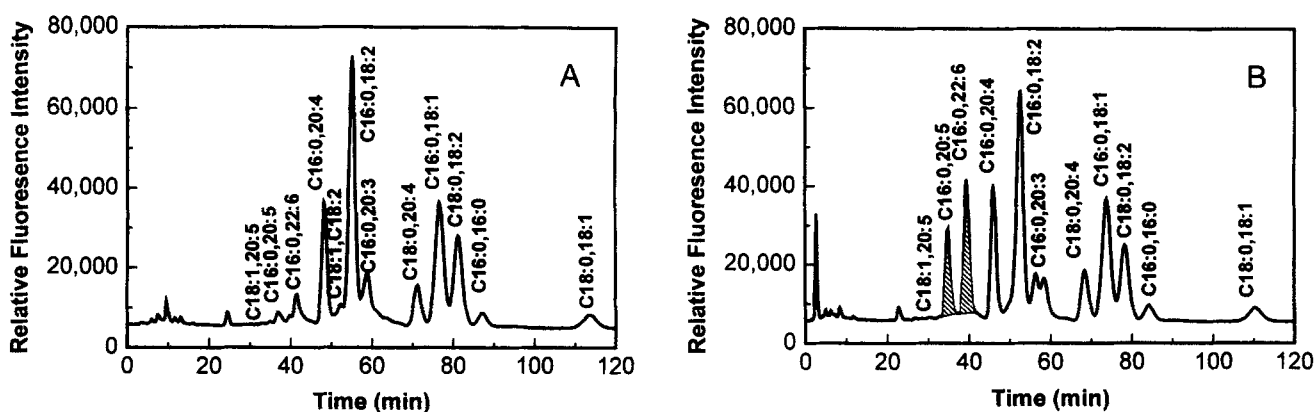


Fig. 2. Changes in serum phosphatidylcholine species after 6 weeks of treatment with drug (OmacorTM). Panel A shows a chromatograph of the distribution of PCs from the serum of a typical subject at the end of a 6-week run-in period during which the patient received dietary counseling according to guidelines established by the AHA for a step 1 diet. Panel B displays the distribution for the same subject after 6 weeks of treatment with drug, four 1-g, capsules per day. The most substantial increases were seen in the relative percentages of $\text{C}_{16:0,20:5}$ and $\text{C}_{16:0,22:6}$ species, ($1.51\% \pm 0.94\%$, vs. $5.04\% \pm 1.92\%$) and ($5.09\% \pm 1.55\%$ vs. $8.12\% \pm 1.88\%$). Samples were prepared as in Methods.

TABLE 1. Relative percentage distribution of serum phosphatidylcholine species in patients receiving placebo or drug (Omacor™) prior to treatment and after 6 weeks of treatment

Phosphatidylcholine Species	Placebo Group prior to Treatment (n=21)	Placebo Group after 6 Weeks of Treatment (n=21)	Drug Group prior to Treatment (n=19)	Drug Group after 6 Weeks of Treatment (n=19)
	<i>relative %</i>			
C18:1,20:5	0.59 ± 0.25	0.67 ± 0.28	0.79 ± 0.78	0.61 ± 0.41
C16:0,20:5	1.47 ± 0.52	1.36 ± 0.35	1.51 ± 0.94	5.14 ± 1.80 ^a
C16:0,22:6	5.25 ± 1.35	4.83 ± 1.59	5.09 ± 1.55	8.35 ± 1.74 ^a
C16:0,20:4	11.03 ± 2.80	11.01 ± 2.43	10.39 ± 3.48	10.36 ± 2.31
C18:1,18:2	2.07 ± 0.99	1.65 ± 1.20	1.96 ± 0.87	1.42 ± 1.06
C16:0,18:2	30.27 ± 4.38	29.54 ± 4.53	31.59 ± 3.14	27.84 ± 2.71 ^a
C16:0,20:3	9.68 ± 1.18	9.26 ± 0.96	9.44 ± 1.80	9.27 ± 0.96
C18:0,20:4	4.51 ± 1.46	4.73 ± 1.40	4.95 ± 1.53	4.85 ± 1.19
C16:0,18:1	13.82 ± 4.53	14.49 ± 2.81	15.21 ± 2.90	14.22 ± 2.34
C18:0,18:2	14.41 ± 2.73	14.32 ± 2.59	14.99 ± 2.79	12.64 ± 2.17 ^a
C16:0,16:0	2.81 ± 1.55	2.22 ± 0.52	2.57 ± 0.59	2.27 ± 0.47
C18:0,18:1	2.74 ± 0.95	2.98 ± 0.66	2.83 ± 0.66	3.01 ± 0.65

Values are given as mean ± SD.

^a*P* < 0.01 relative to drug group prior to treatment as determined by Mann-Whitney U test.

tically significant reductions in the C_{16:0,18:2} and the C_{18:0,18:2} species. The increases in n-3-containing species were confined to PCs in which the other acyl chain is derived from palmitic acid. There was no increase in the species where the other acyl chain was derived from either oleic or stearic acid. The C_{18:1} and C_{18:0} adducts of either EPA or DHA never amounted to more than 1% of the total integrated area of the chromatograph and frequently were not detectable at all. In the placebo controls, there were no significant differences in any of the PC species after 6 weeks of ingestion of 4 g of corn oil per day.

For the sake of comparison, we have summed the contribution of each individual fatty acid and compared it with data obtained by the more traditional method of capillary gas-liquid chromatography of fatty acid

methyl esters. **Table 2** shows that both methods produce comparable results and that in both instances administration of the drug produced a statistically significant increase in the content of the n-3 fatty acids. Some minor differences were observed; the GLC data showed statistically significant reductions in oleic, eicosatrienoic, and arachidonic acids after drug treatment. On the other hand, the HPLC data showed statistically significant reductions in stearic and linoleic acids. Overall, the GLC analysis produced higher values for stearic and arachidonic acids, while the HPLC method gave higher values for palmitic and linoleic acids. Occasionally it was possible to detect myristic, palmitoleic, and linolenic acids by GLC, but they were always less than 1% of the total fatty acid composition.

In the drug-treated group, there was a statistically sig-

TABLE 2. Comparison of the relative percentage phospholipid fatty acid composition obtained by GLC versus that obtained by HPLC in patients receiving the drug (Omacor™) prior to treatment and after 6 weeks of treatment

Fatty Acid	GLC prior to Treatment with Drug (n=19)	GLC after Treatment with Drug (n=19)	HPLC prior to Treatment with Drug (n=19)	HPLC after Treatment with Drug (n=19)
	<i>relative %</i>			
C16:0	32.06 ± 0.10	32.32 ± 3.22	37.58 ± 0.98	37.78 ± 1.13
C18:0	16.23 ± 3.04	17.53 ± 2.37	11.12 ± 1.37	10.25 ± 1.39 ^a
C18:1 (n-6), <i>cis</i> -9	10.52 ± 2.33	9.07 ± 1.41 ^a	9.71 ± 2.76	9.63 ± 1.63
C18:2 (n-6), <i>cis</i> -9,12	21.74 ± 4.53	19.50 ± 2.55	24.33 ± 2.39	20.95 ± 1.99 ^b
C20:3 (n-6), <i>cis</i> -8,11,14	4.10 ± 0.90	3.14 ± 0.63 ^b	4.84 ± 0.59	4.63 ± 0.48
C20:4 (n-6), <i>cis</i> -5,8,11,14	11.39 ± 1.95	8.88 ± 1.56 ^b	7.45 ± 2.34	7.61 ± 1.55
C20:5 (n-3), <i>cis</i> -5,8,11,14,17	0.58 ± 0.28	3.66 ± 1.26 ^b	1.15 ± 0.71	2.88 ± 0.99 ^b
C22:6 (n-3), <i>cis</i> -4,7,10,13,16,19	2.06 ± 0.63	4.87 ± 1.11 ^b	2.54 ± 0.78	4.18 ± 0.87 ^b

Values are given as mean ± SD.

^a*P* < 0.05 as determined by the Mann-Whitney U test.

^b*P* < 0.01 as determined by the Mann-Whitney U test.

nificant 26% mean reduction in plasma triglycerides. After 6 weeks of drug treatment, all 19 of the patients had an increase in $C_{16:0,20:5}$ and $C_{16:0,22:6}$ PCs. Of those 19 patients, 14 had a reduction in plasma triglyceride, while 5 had an increase in TG. After 1 month of a 1 year extension of the trial, 4 out of the 5 nonresponsive patients did have a reduction in plasma triglyceride while the remaining patient's triglyceride level increased. In the placebo control group, 13 out of 21 subjects had a reduction in TG, while 8 showed no reduction or an increase, to give a 7% increase in mean TG. There were also statistically significant reductions in VLDL (28%) and total cholesterol (11%), along with a 14% increase in HDL cholesterol in patients that received the drug (data not shown). In the placebo control group, none of these parameters changed significantly.

DISCUSSION

The 7-MCCA derivatives of phospholipids and diacylglycerols represent an improvement over previous methodology for three reasons. 1) A single chemically stable reagent is used that produced a product with a more chemically stable urethane linkage to the diacylglycerol; 2) the fluorescent adducts of serum glycerides are detectable in the 1–10 pmol/L concentration range; and 3) the derivatization product is easily detected on thin-layer chromatography plates without the use of visualization reagents. When compared to the traditional method of derivatization with benzoic anhydride and 4-dimethylaminopyridine in benzene, the percentage distribution of the 7-MCCA derivatives in the control serum was remarkably similar (6). The results obtained by this method also compared favorably with those obtained by gas-liquid chromatography (Table 2). The differences observed between the GLC and HPLC results may be due to minor differences in the reactivity of either the methylating or fluorescent derivatizing agents with their respective fatty acid or diglyceride species. Data from a previous study confirm that the increase in phospholipid n-3 fatty acid content after n-3 fatty acid ingestion does occur primarily at the expense of oleic and linoleic acid (3).

There are two limitations to this method. This method does not indicate which fatty acyl substituent is located in the *sn*-1 or *sn*-2 position of the diglyceride. In most situations, however, it has been shown that the *sn*-1 position is predominantly occupied by the more saturated substituent (11). The second limitation is that it does not distinguish between diacyl, alkylacyl, and alk-1-enylacyl linkages with the glycerol backbone. Ether-linked glycerophospholipids are present in plasma in

low concentrations, but are more prevalent in the membranes of lymphocytes, macrophages, and polymorphonuclear leukocytes (4, 12). This second disadvantage can be overcome by the use of argentation thin-layer chromatography (5). This methodology was straightforward enough to enable the analysis of the large number of serum samples produced by a clinical trial. Approximately 20 samples per week were analyzed using this method. With the addition of ditridecanoyl phosphatidylcholine to the initial extraction step as an internal standard, quantitative values can be obtained. All 19 of the patients who were given the drug exhibited increases in their serum $C_{16:0,20:5}$ and $C_{16:0,22:6}$ levels. None of the patients who were given the corn oil placebo had any significant changes in PC species composition.

It is interesting to note that only n-3 phospholipids containing palmitic acid (presumably in the *sn*-1 position) increased in the patients given the drug. The non-random distribution of fatty acids in phospholipids suggests that distinctive mechanisms must exist within cells to produce such a distribution. The main mechanism of de novo PC synthesis, the CDP-choline pathway, appears to have only a minor amount of selectivity. Cellular positional selectivity in PCs occurs mainly through deacylation and reacylation with a minor amount occurring by transacylation (11). The main source of phospholipid in the intestinal lumen has been shown to be bile, 11–12 g/day, while dietary sources supply 1–2 g/day (13). Pancreatic phospholipase A_2 digests PCs to lysophosphatidylcholine and free fatty acids. The mechanism of intestinal fatty acid uptake and phospholipid synthesis has not yet been elucidated in a definitive manner. However, in addition to passive diffusion across a concentration gradient, lysophosphatidylcholine and long chain fatty acid uptake may be facilitated by a microvillus membrane fatty acid binding protein (14). Intracellularly, lysophosphatidylcholine is either resynthesized into PC or converted to glycerol-3-phosphate and free fatty acid. Short and medium chain fatty acids (chain lengths less than C14) are not often incorporated into complex lipids (i.e., triglycerides, cholesterol esters, and phospholipids) either because they do not bind well to cytosolic fatty acid binding proteins or because they are not activated to acyl CoA derivatives (15). Yang et al. (16) have shown that in rats fed fatty acid esters from menhaden oil, chylomicron phospholipids isolated from the lymph ducts are synthesized via the phosphatidic acid pathway rather than the monoacyl glycerol pathway.

In the phosphatidic acid pathway, the final step is the reaction of CDP-choline with diacylglycerol by 1,2 diacylglycerol:CDP-choline phosphotransferase. Van Greevenbroek et al. (17) have shown that in particles secreted from CaCo-2 cells, palmitic acid is incorporated preferentially into cellular phospholipid and its precursor.

sors diacylglycerol and phosphatidic acid rather than triglyceride. This may explain the greater abundance of species containing palmitic acid (Table 1). The lack of substrate specificity of CDP-choline phosphotransferase for diglycerides containing eicosapentaenoic and docsa-hexaenoic esters and the greater availability of $C_{20:5}$ and $C_{22:6}$ provided by the 4 g/day of the drug may explain why only $C_{16:0,20:5}$ and $C_{16:0,22:6}$ were increased in patients given the drug. Postsynthetic deacylation–reacylation reactions may also have a role in the selection of the $C_{16:0,20:5}$ and $C_{16:0,22:6}$ species. The transfer of acyl groups to 1-acyl-glycerophosphocholine is catalyzed by acyl CoA:1 acyl-glycerophosphocholine transferase. This enzyme has been shown to have a high specificity for unsaturated fatty acyl-CoA in liver microsomal preparations, whereas in lung microsomes it prefers palmitoyl-CoA over oleoyl-CoA (18). Enzyme activity also appears to be dependent upon the phospholipid composition and the fluidity of the microsomal membrane.

Once in circulation, phosphatidylcholines are redistributed from chylomicron remnants to the lipoprotein subfractions via the phospholipid transfer activity of phospholipid and cholesterol ester transfer proteins as well as by passive diffusion. In plasma, phosphatidylcholine remodeling is carried out by lecithin:cholesterol acyltransferase (LCAT). We have shown that the most prevalent species of PC in human sera is $C_{16:0,18:2}$. In circulating human lipoproteins, cholesterol linoleate is the most abundant species of cholesteryl ester. Cholesteryl ester synthesized intracellularly by acyl CoA:cholesterol O-acyltransferase is primarily cholesteryl oleate (3, 19). Oleic and linolenic acid have both been shown to be better substrates than linoleic acid for LCAT (20). However, the relative abundance of linoleic acid which is usually found in the PC *sn*-2 position may account for the higher concentrations of cholesterol linoleate in plasma. The process of reverse cholesterol transport may be driven in part by the constant replenishment of HDL surface pools of PC transferred from chylomicrons synthesized in the intestine.

Hepatic uptake of *n*-3 fatty acid-containing lipoprotein particles reduce VLDL triglyceride synthesis in the liver by competing with *n*-6 fatty acids for the final step in triacylglycerol synthesis performed by the enzyme triacylglycerol acyl-CoA:diacylglycerol acyltransferase. De novo synthesis of PC also appears to be necessary for VLDL secretion. In hepatocytes from rats treated with a choline–methionine-deficient diet, less VLDL was secreted while HDL secretion remained unaffected (21).


The effects of *n*-3 fatty acids upon cell physiology are just beginning to be understood. Within the cell, there appears to be stricter control of the positional specificity of fatty acids within phospholipid species. In leukocytes isolated from the plasma buffy coat the most prominent PC species is $C_{16:0,18:1}$ (data not shown). Membrane flu-

idity and the asymmetric distribution of phospholipids within cell membranes also appear to be closely regulated. Yet most of the proteins of intestinal phospholipid biosynthesis seem to be constitutively expressed (22). Alteration in phospholipid synthesis occurs mainly by changes in the supplied substrate or by changes in enzyme activation and inactivation. This contrasts with the regulation of HMG-CoA reductase, the rate-limiting step in cholesterol synthesis which is under stringent genetic control via the sterol regulatory elements.

Another issue of importance is the regulation of the balance between phospholipid biosynthesis and catabolism. It has been calculated that fluid phase endocytosis results in the turnover of the entire macrophage plasma membrane within 33 min under steady state conditions (23). Evidence suggests that phospholipid synthesis and degradation are coordinated with the cell cycle. In cells undergoing mitosis, phospholipid synthesis increases early in the G1 phase and then decreases as the cell progresses through the S and G2/M phase. Membrane phospholipid degradation is also periodic; it decreases as the cell progresses from the G1 to S phase and increases again as the cell reenters the G1 phase. The result is net accumulation of PC in the S phase (24).

In programmed cell death, control of cell phospholipid homeostasis is lost. Increased membrane permeability occurs as a result of enhanced degradation of membrane PCs by cellular phospholipases and decreased synthesis (25). Loss of membrane asymmetry is also apparent in the exposure of phosphatidylserine on the cell surface of apoptotic lymphocytes (26). The effect of *n*-3 fatty acids upon cellular proliferation appears to depend upon the type of cell. Finstad et al. (27) have shown that while HL-60 promyelocyte proliferation is inhibited by EPA and arachidonate, it is not inhibited in U-937, THP-1, or CaCo-2 cells. EPA was shown to increase the number of HL-60 cells undergoing apoptosis and necrosis (27). In the future, we plan to use the strategy outlined in the Methods section to analyze the cellular distribution of the molecular species of diglycerides and phospholipids. We believe this will prove to be a valuable tool in the investigation of cell-phosphoglycerolipid turnover.

In summary, we have developed a new and sensitive method for the determination of the molecular species of phospholipids and diacylglycerols. This method was applied to examine changes in serum phosphatidylcholines in hypertriglyceridemic subjects given a mixture of *n*-3 fatty acids versus patients given a corn oil placebo. The changes observed in $C_{16:0,20:5}$ and $C_{16:0,22:6}$ species in the treated group demonstrate that the drug was taken up and incorporated into plasma PCs. The magnitude of incorporation was similar to that seen by the more traditional method of gas–liquid chromatogra-

phy. The principal advantage of this method is that it enables the determination of the distribution of fatty acids within individual molecular species of diglycerides and phospholipids. 

The authors wish to thank Kendall Campbell for technical assistance and Brook Watts for editorial assistance. This work was supported by Pronova Biocare a.s., and The Methodist Hospital Foundation (FDN 38776-0010). Some of the data in this manuscript was presented at the XII International Symposium on Drugs Affecting Lipid Metabolism (Houston, TX), in November 1995.

Manuscript received 21 August 1996 and in revised form 4 December 1996.

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