Bioavailability of marine n-3 fatty acid formulations ✩

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ARTICLE INFO

ABSTRACT

The use of marine n-3 polyunsaturated fatty acids (n-3 PUFA) as supplements has prompted the development of concentrated formulations to overcome compliance problems. The present study compares three concentrated preparations — ethyl esters, free fatty acids and re-esterified triglycerides — with placebo oil in a double-blinded design, and with fish body oil and cod liver oil in single-blinded arms. Seventy-two volunteers were given approximately 3.3 g of eicosapentaenoic acid (EPA) plus docosahexaenoic acid (DHA) daily for 2 weeks. Increases in absolute amounts of EPA and DHA in fasting serum triglycerides, cholesterol esters and phospholipids were examined. Bioavailability of EPA+DHA from re-esterified triglycerides was superior (124%) compared with natural fish oil, whereas the bioavailability from ethyl esters was inferior (73%). Free fatty acid bioavailability (91%) did not differ significantly from natural triglycerides. The stereochemistry of fatty acid in acylglycerols did not influence the bioavailability of EPA and DHA.

1. Introduction

Since our original observations in Greenland Eskimos of an association between dietary intake of marine long-chain poly-unsaturated n-3 fatty acids (n-3 PUFA) and biological and cellular functions [1,2] much interest has been focused on the potential health benefits of marine n-3 PUFA and seafood in the diet [3–7]. Based on this, national heart associations and governmental bodies have recommended an increased intake of oily fish and potentially the use of n-3 PUFA supplements for prevention coronary heart disease [3]. Supplementation with various n-3 PUFA formulations has served as the primary tool for obtaining an exact dose of n-3 PUFA and to perform blinded, controlled studies. Initially, deodorized fish oils, e.g. cod liver oil (CLO) and fish body oils (FBO), were used. In these preparations, the n-3 PUFA are esterified as triglycerides (TG). Problems of patient compliance due to the relatively large amounts of such oils that have to be ingested in order to reach an appropriate dose of n-3 PUFA have prompted the development of more concentrated compounds [8].

Thus, concentrates of marine oils containing up to 30–90% of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) have been developed. The n-3 PUFA are generally present in these formulations as free fatty acids (FFA), ethyl esters (EE) or as re-esterified TG (rTG). The term "re-esterified" is used for products made from FBO, in which the app. 30% TG content is transferred to ethyl esters and then molecularly distilled to remove the short chain and the saturated fatty acids increasing the EPA and DHA contents to around 60%. The ethyl esters are then enzymatically reconverted to glycerides. Some conflicting results have arisen from the rather few studies that have dealt with the bioavailability of EPA and DHA from various concentrated n-3 PUFA formulations [9–14]. The lack of a controlled study comparing the five presently commonly used fish oil supplements (natural TG in fish body oil and CLO, EE, FFA and rTG) led us to undertake a blinded, placebo-controlled study in healthy volunteers, using generally available products. The enrichment of EPA and DHA in plasma TG, cholesterol esters and phospholipids was examined after intake of five different n-3 FA formulations or placebo oil (corn oil, CO) for 2 weeks.

2. Methods

2.1. Subjects

Seventy-two healthy subjects (36 women aged 21–56 years and 36 men aged 23–55 years) volunteered for the study.

Volunteers who had taken fish oil preparations within 2 months prior to the study were excluded. The volunteer subjects were instructed to avoid acetylsalicylic acid 2 weeks prior to and during the study, and to abstain from alcoholic beverages for 1 day before study visits. The study was approved by the local Ethics Committee.

2.2. Experimental design

The subjects were randomized to six groups: four double-blinded groups given concentrated fish oils or placebo, each person taking five capsules twice daily at meal times for 2 weeks and two single-blinded groups given CLO or fish body oil (FBO) capsules twice daily for 2 weeks. The single-blinded design in the two natural fish oil groups was due to different number of capsules taken by these groups. All supplements were from Pronova Biocare, Sandefiord Norway: EPAX 5500 TG consisting of rTG; EPAX 6000 FA consisting of FFA; EPAX 5500 EE consisting of EE; EPAX 3000 TG consisting of a refined fish body oil (FBO); cod liver oil (CLO); and corn oil (CO) as placebo.

The composition and amounts of the supplements are given in Table 1. The daily intake of EPA plus DHA was 3.1–3.6 g. The subjects were examined at baseline and after 2 weeks of supplementation. Each examination was made in the morning after an overnight fast. Blood was drawn from an antecubital vein with minimal stasis. Serum was prepared by clotting whole blood for 1 h at room temperature and centrifuged at 2000g for 15 min. Serum was transferred to plastic tubes and stored at $-70\,^{\circ}$C until analysis. All analytical works were performed before breaking the randomization code.

2.3. Fatty acid analysis

Total lipids were extracted from serum according to Bligh and Dyer [15]. Serum (400 µL) was mixed briefly with 500 µL chloroform ($CHCl_3$) containing internal standards (diheptadecanoyl phosphatidylcholine, cholesteryl heptadecanoate, and triheptadecanoin) and 1000 µl methanol containing butylated hydroxytoluene as antioxidant. After the addition of 500 µl $CHCl_3$ and 500 µL H_2O and brief mixing, the tubes were centrifuged at 1000g for 2 mins for phase separation; 550 µl of the $CHCl_3$ phase was transferred to a SepPak NH_2 column (Waters Corporation, Milford, MA, USA), which had been prewashed with hexane. Lipid classes were separated into phospholipids (PL), cholesterol esters (CE) and monoglycerides, diglycerides and TGs as described by Kaluzny et al. [16], except that the glycerides were collected as a single class. The extracted lipids were dried under nitrogen (N_2) and redissolved in 100 µL toluene. Transmethylation was carried out overnight at 45 $^{\circ}$C under N_2 after addition of 200 µl

methanolic sulphuric acid 1% [17]. The methylated FA were extracted after addition of 500 µL NaCI 5% and 1500 µL hexane. The hexane phase was washed with 2% $NaHCO_3$, dried under N_2 and redissolved in 60 µL dichloromethane. Gas chromatography was performed isothermally at 220 $^{\circ}$C on an HP 5700 gas chromatograph (Hewlett Packard, Avondale, PA, USA) supplied with a 25 m × 0.53 mm FFAP-CB capillary column (Chrompack, Middelburg, The Netherlands) with N_2 as the carrier gas (2 ml/minute).

When comparing the bioavailability of different n-3 FA preparations, it should be noticed that despite the fact that the total amounts of EPA plus DHA given to the volunteers were almost equal (Table 1), the relative amounts of EPA and DHA varied to some extent, for example, between FFA and CLO. The sum of the increase in EPA and DHA is consequently the best marker in comparisons to the bioavailability of the products.

2.4. Statistics

The SPSS for Windows statistical software package, version 6.1, was applied. Statistical comparisons between groups at baseline and after 2 weeks of supplementation were made using the Mann–Whitney U-test. A two-sided p value < 0.05 was considered to be statistically significant.

3. Results

The increases in the amounts of EPA and DHA in plasma CE, PL and TG, and in the sum of EPA and DHA in each lipid class are given in Table 2. The increases in plasma total lipids (CE+PL+TG) of EPA, DHA and in EPA plus DHA are illustrated in Fig. 1. Each n-3 PUFA preparation produced a significant increase in both EPA and DHA in all lipid classes compared to placebo oil. The volunteers in the CLO group received approximately 0.5 g less EPA and approximately 0.5 g more DHA per day compared to the other groups. Consequently, the sum of EPA and DHA gives a more accurate picture of the differences in bioavailability (Fig. 1). By not considering the increase in EPA and DHA individually in CE, PL and TG, but calculating the sums of EPA plus DHA in each class of plasma lipids, and using the grand total as a measure of bioavailability, EE was the formulation giving the lowest assimilation. The differences in the grand total of EPA plus DHA (Fig. 1) were significant when comparing EE with rTG ($p=0.000I$) and EE with fish body oil ($p=0.024$), but not when comparing EE with CLO ($p=0.13$) and EE with FFA ($p=0.29$). Comparing the assimilation of the other preparations (rTG, fish body oil, CLO and FFA), again using the grand total sums of EPA plus DHA as an index, the bioavailability of rTG was significantly better than that of FFA ($p=0.006$) and of CLO ($p=0.002$), whereas it did not differ

Table 1
Dose and composition of capsules and relative and absolute amounts of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) administered in the six study groups.

	rTG	FFA	EE	FBO	CLO	CO
Capsule weight (mg)	650	650	650	1000	500	650
Capsules/day (morning+evening)	5+5	5+5	5+5	6+7	17+7	5+5
EPA (%)	28.5	33.5	28.8	15.7	8.1	0
DHA (%)	19.8	21.5	21.4	11.4	11.0	0
n-6 FA (%)	4.0	2.2	3.9	2.5	2.2	56.7
Monounsaturated FA (%)	13.3	12.1	15.7	25.9	51.8	29.0
Saturated FA (%)	1.0	2.8	6.0	27.5	16.0	13.4
Tocopherols (mg/g)	3.7	3.5	3.9	1.1	1.0	2.8
EPA (g/day)	1.85	2.18	1.87	2.04	1.38	0
DHA (g/day)	1.29	1.40	1.39	1.48	1.87	0
EPA+DHA (g/day)	3.1	3.6	3.3	3.5	3.2	0

Abbreviations: rTG: re-esterified triglycerides. FFA: free fatty acids. EE: ethyl esters. FBO: fish body oil. CLO: cod liver oil. CO: corn oil. FA: fatty acids.

Table 2
Mean difference between baseline and end of study regarding the amount of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) in serum lipids. Values are mg/L, with standard error of the mean (SEM) in brackets.

	CE			PL			TG		
	EPA	DHA	EPA+DHA	EPA	DHA	EPA+DHA	EPA	DHA	EPA+DHA
rTG	71.0 (9.0)	4.8 (1.3)	75.8 (9.6)	60.5 (6.7)	27.1 (7.1)	87.6 (12.2)	15.9 (3.4)	12.8 (3.1)	28.7 (6.2)
EE	46.3 (7.4)	3.2 (1.5)	49.6 (8.7)	37.3 (5.4)	6.9 (5.6)	44.2 (9.6)	10.4 (2.2)	8.4 (2.1)	18.8 (4.2)
FFA	53.1 (6.4)	3.5 (1.6)	56.6 (7.6)	52.8 (6.0)	5.6 (5.8)	58.4 (10.9)	15.4 (2.7)	11.0 (1.5)	26.4 (3.9)
FBO	55.0 (11.1)	4.2 (1.6)	59.2 (11.8)	53.7 (7.1)	25.9 (6.4)	79.6 (11.7)	11.7 (2.9)	12.0 (3.2)	23.7 (5.8)
CLO	48.4 (3.6)	5.6 (1.8)	54.0 (4.8)	38.8 (4.2)	21.2 (5.2)	60.0 (8.8)	11.8 (2.0)	20.1 (3.1)	31.9 (15.6)
CO	2.7 (3.7)	1.5 (0.9)	4.2 (4.1)	0.4 (3.9)	0.3 (4.0)	0.7 (7.0)	−1.2 (0.9)	−0.9 (1.4)	−2.1 (1.9)

Abbreviations: CE: cholesterol esters. PL: phospholipids. TG: triglycerides. rTG: re-esterified triglycerides. EE: ethyl esters. FFA: free fatty acids. FBO: fish body oil. CLO: cod liver oil. CO: corn oil.
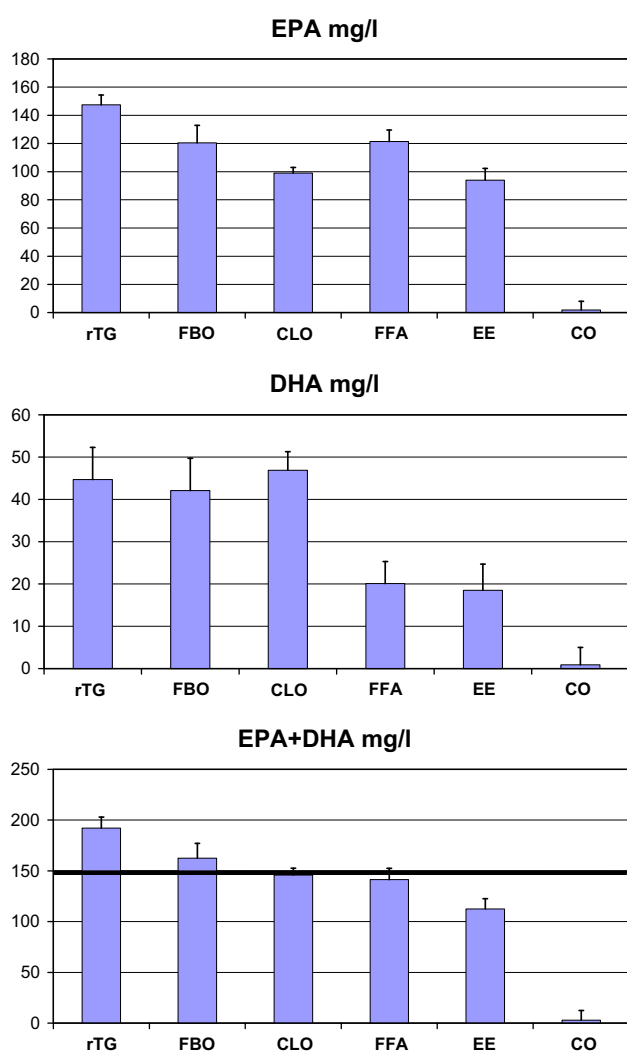

Fig. 1. Differences between end of study and baseline values of eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA), and EPA plus DHA content in mg/L serum, in the sum of serum lipid fractions (cholesterol esters, triglycerides, and phospholipids). A bioavailability index of 100 is indicated in the EPA+DHA plot, corresponding to 150 EPA+DHA mg/l (solid line) indicating the mean value of increases (mg/L) after intake of natural fish oils: Fish body oil (FBO) and cod liver oil (CLO). The differences in the grand total of EPA plus DHA were significant when comparing EE with rTG ($p=0.0001$) and EE with fish body oil ($p=0.024$), but not when comparing EE with CLO ($p=0.13$) and EE with FFA ($p=0.29$). Values are means +SEM (bars). rTG=re-esterified triglycerides, FFA=free fatty acids, EE=ethyl esters, and CO=corn oil.

from fish body oil ($p=0.24$). There was only a modest difference in bioavailability between fish body oil and CLO (Fig. 1). There were no significant differences between fish body oil, CLO and the FFA preparations.

4. Discussion

The present study aimed at examining the somewhat conflicting data on the bioavailability of EPA and DHA from various forms of fish oil [9–14]. El Boustani et al. [9] reported a delayed and reduced incorporation of EPA into plasma TG when administered as EE, compared to the incorporation of EPA administered as natural TGs or as FFA. Lawson and Hughes [10] compared the time course for the increase in concentration of EPA in plasma TG relative to the maximal rise in α-linolenic acid, and found a nearly 100% absorption of EPA and DHA as FFA, whereas only 68% and 20% of EPA as natural TG or EE, respectively, were absorbed. Other data point to an equal absorption of n-3 FA, in the form of either TG and EE [11,12]. Thus Luley et al. [11] compared the absorption of EPA and DHA from natural fish oil TG with that from EE at two different levels of EPA and DHA (54% and 35%) and found no difference in absorption. Nordoy et al. compared the content of n-3 FA in chylomicron TG and the increase in chylomicron TG after a test meal that included a large dose of n-3 FA provided either as rTG or as EE. These authors found a similar absorption of n-3 FA irrespective of whether the supplement was rTG or EE, despite a lower rate of hydrolyses by intestinal lipase of fatty acids from ethyl esters than from triacylglycerols in vitro [12]. The same was demonstrated by Krokan et al. [13] who found that the absorptions of EPA and DHA from synthetic EEs rich in EPA and DHA were fully comparable to that of natural triacylglycerols containing smaller amounts of these FA. In a study by Hansen et al. volunteers were given EPA and DHA either as natural TG or as EE at a dose of approximately 3.5 g/day for 7 weeks. They found equal incorporations of EPA and DHA into plasma phospholipids from the two formulations. The incorporation of EPA into plasma cholesterol esters from EE was, however, significantly lower than that from natural TG [14].

Apart from a study comparing microencapsulated fish-oil-enriched foods with n-3 PUFAs in fish oil capsules [18], and studies on the bioavailability of preparations of isolated n-3 PUFA, this issue has been dealt rather scarcely in the scientific literature. Harris et al. [19] compared the relative effects of n-3 fatty acids in triglyceride and methyl ester forms in patients with type IV hyperlipidemia and found them equally effective hypotriglyceridemic agents. They also compared the rate and extent of enrichment of blood cell membranes and plasma phospholipids with EPA and DHA from either 2 servings of oily fish per week or from 1–2 capsules/d of ethyl esters. They found that equal

amounts of EPA and DHA from oily fish on a weekly basis or from fish-oil capsules on a daily basis were equally effective at enriching blood lipids with n-3 fatty acids [20].

Taking the mean of the increase in EPA plus DHA in all three plasma lipid classes (grand total) for the two natural fish oils (fish body oil and CLO) as unity (100%, Fig. 1), the mean relative bioavailability, unadjusted for dosage, of EPA plus DHA from EE was 73%, from FFA 91% and from rTG 124%. Adjusted for dosage, the results were 76%, 86% and 134%, respectively. This finding of an equal or lower bioavailability of EPA plus DHA from FFA compared with natural TGs is in contrast to the findings of Lawson and Hughes [10], from an 8 h absorption study, and those of Beckermann et al. [21], using a 1-week crossover design. Both studies included 8 volunteers and both found an increased bioavailability of FFA compared with natural TG. However, comparing FFA, TG and EE in a 24 h absorption study in 8 volunteers, El Boustani et al. [9] found an equal absorption of EPA from FFA and natural TG. All three studies demonstrated a reduced bioavailability of EE preparations when compared with natural TGs. In contrast to these studies in humans, de Schrijver et al. [22] found a decreased assimilation of fish oil-derived FFA in rats, compared with natural fish oil. The explanation for the differences in FFA absorption in the aforementioned studies could be due to interactions between the n-3 FA preparations and diet. FFA form insoluble salts, e.g. with Ca (Ca-soaps), which influence their assimilation. The results regarding FFA bioavailability preparations do not allow definitive conclusions to be drawn, although they indicate that FFA preparations have a bioavailability comparable to that of natural TGs in humans.

Our findings of a lower bioavailability of EPA and DHA from EE than from FFA or TG are in accord with the demonstration that pancreatic lipase hydrolyses EEs to a lesser degree than TG [9,10] and at a slower rate [13]. They are also in accord with some studies examining the bioavailability of n-3 EE [9,10,14,21] but not with the findings of other studies [11–13]. The relationship between the intake of capsules and meals could offer an explanation for these discrepancies. Thus, in the study by Nordoy et al. [12], who found an equally good absorption of n-3 PUFA from EE and TG, the n-3 FA were given as part of a lipid-rich meal. Also, Lawson and Hughes [23] found that the absorptions of EPA and DHA from EE were increased substantially by co-ingestion with a high-fat meal. The assimilation of EEs may thus be enhanced when given as part of a (lipid-rich) meal. In the present study, the volunteers were recommended but not instructed to take the capsules at 'meal times', and not specifying lipid-rich meals. This may suggest a reason for the lower EE bioavailability observed.

Our study also address whether the stereochemistry of EPA and DHA in acylglycerol molecule influenced n-3 PUFA assimilation, as some authors have raised the question of whether structural differences in TG influence their bioavailability [8,24]. Thus, Greenland Eskimos obtain their n-3 PUFA mainly from marine mammal TGs and the long-chained n-3 PUFA of these TGs are located in the sn-1/3 positions, as opposed to fish oils, in which n-3 PUFA are found mainly in the sn-2 position. Lingual and pancreatic lipases stereo-specifically liberate fatty acids from the 1 and 3 positions of triglycerides, leaving the 2-monoglyceride intact [25,26]. Consequently the n-3 PUFAs are absorbed either as sn-2-monoglycerides or as FFA, depending on the molecular structure of dietary triglycerides. This question was addressed in the present study by using a randomized rTG, thus enabling us to study both the effects of concentrated fish oil preparations and stereoisometry. In the re-esterification process, which adds, on average, one extra n-3 FA to each TG molecule, the positioning of EPA and DHA in the glyceride molecule are at random. Thus the sn-1/3 and sn-2 positions are equally esterified by EPA and DHA in

the glycerol molecule. Our results show that the randomization of EPA and DHA in acylglycerols has no negative bearing on the bioavailability of long-chain n-3 PUFAs in humans. These findings correspond to those of de Schrijver et al. [22] in rats. In a 24-day study, these authors found similar bioavailability of natural fish oil and randomized fish oil compared with soya bean oil, whereas the absorption of FFA derived from fish oil was moderately lower when compared with the other fish oil supplements [22]. Christensen et al. reached the same conclusion on comparing the absorption patterns over 24 h in rats fed either fish oil or seal oil. They found an initially better absorption of n-3 FA from fish oil than from seal oil, but the overall effects of the structure of the dietary triacylglycerols on n-3 PUFA assimilation were negligible [27]. The finding that the molecular structure of glycerides does not influence FA assimilation is consistent with the fact that, although marine mammals obtain their n-3 FA from fish with the n-3 PUFA predominantly in the sn-2 position, they store these fatty acids in the sn-1/3 position. The fact that organ fatty acid stereochemistry is independent of the structure of the dietary acylglycerols has been further documented by Christensen and Høy [28], who found no difference in organ lipid content and composition of n-3 PUFAs between two groups of rats fed either seal oil or fish oil for 17 days.

Interestingly, we found an even higher index of bioavailability (124%) of rTG than of natural TG (fish body oil and CLO), although it should be stressed that this difference was only significant when compared with CLO. A tentative explanation for this finding could be that besides TG, rTG also contains both diglycerides and monoglycerides. The European Pharmacopoeia define a "re-esterified triglyceride" with a triglyceride level $> 50\%$. The levels of triglycerides in the present and in comparable products are about 55–60%, diglycerides about 38–42% and monoglycerides 1–3%. This facilitates the normal acylglyceride absorption conditions in the intestine. Monoglycerides and diglycerides may facilitate micellar formation, in which the sn-2-monoglycerides from dietary TGs are absorbed and preserved by reacylation of TG in the enterocyte, and subsequently secreted as lymphatic chylomicrons. Lawson and Hughes [23] found that absorption of fish oil FA from EE was increased substantially by co-ingestion with a high-fat meal. Also, Silverman et al. [29] found that the relative absorption of EPA from tuna fish meat was significantly higher than from fish oil. The findings by Nordoy et al. [12] that n-3 FA as rTG and EE was equally well absorbed could likewise be due to the fact that they were given as part of a lipid-rich meal. This again emphasizes the effect of meal conditions on n-3 FA absorption.

In conclusion, our results demonstrate that bioavailability may differ between the commonly used types of concentrated fish oil preparations. rTG has superior bioavailability, whereas EEs may have a lower bioavailability. FFA has medium bioavailability, which is not different from that of natural fish oil TG. Our study further documents that the stereoisomery of n-3 PUFA in the glyceride molecule does not influence the assimilation of EPA plus DHA in humans. It should, however, be stressed that these conclusions are based on a relatively short-term (2 week) study at a fixed daily n-3 FA dose of approximately 3.5 g; it cannot be ignored, even if it seems unlikely that a different period of supplementation or a different dose may have led to different results.

Conflicts of interest

JD is scientific and medical advisor for a fish-oil manufacturing company (Napro Pharma AS, Norway). PM, JMM, IA, and EBS declare no conflicts of interest.

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