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HUMAN ABSORPTION OF FISH OIL FATTY ACIDS AS TRIACYLGLYCEROLS, FREE ACIDS, OR ETHYL ESTERS

Larry D. Lawson and Bronwyn G. Hughes

Murdock Pharmaceuticals, Springville, Utah 84663

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Summary. The transient rise in plasma triacylglycerol fatty acids after single-dose ingestion of fish oil as triacylglycerols, free acids, or ethyl esters with linseed oil as an absorption standard was used to determine the relative absorption of fish oil fatty acids in eight men. As free acids, the fish oil fatty acids were well absorbed (≥95%). As triacylglycerols, eicosapentaenoic acid (1.00 g) and docosahexaenoic acid (0.67 g) were absorbed only 68% and 57% as well as the free acids. The ethyl esters were absorbed only 20% and 21% as well as the free acids. The incomplete absorption of eicosapentaenoic and docosahexaenoic acids from fish oil triacylglycerols correlates well with known in vitro pancreatic lipase activity.

Fish oil is currently being extensively studied in the treatment of cardiovascular and inflammatory diseases. The components of fish oil considered responsible for its therapeutic actions are eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), which are major fatty acids in many cold water marine fish. To elucidate the specific actions of EPA and DHA, several clinical studies have been conducted with 70-95% EPA or DHA preparations purified from fish oil as ethyl esters (1-3). Fish oil in its natural triacylglycerol form, and to a lesser extent, fish oil ethyl esters are widely marketed at drug and health food stores. Although numerous studies have shown that fish oil is at least partly absorbed (1-3), no studies have reported the degree of absorption of fish oil or its ethyl esters in man even though it has been known since 1965 (4,5) that fish oils containing EPA and DHA are poor substrates for porcine pancreatic lipase. Furthermore, a recent study on the rat thoracic lymph duct content showed that only half as much of the fatty acids from fish oil was found in the lymph when compared to the fatty acids from corn oil (6). Fatty acid ethyl esters have also been shown to have impaired absorption in rats (7). During the preparation of this manuscript a report was published which demonstrated that the free acid of pure EPA was markedly better absorbed in man than the ethyl ester (8).

The purpose of this study was to assess the human absorption of the fatty acids of fish oil when ingested as triacylglycerols, ethyl esters, or

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free acids. The relative degree of absorption was determined from the transient increase of fatty acids in plasma triacylglycerol following single-dose ingestion compared to the increase of an absorption standard, alpha-linolenic acid (ALA), co-ingested with the fish oils as linseed oil. Linseed oil was chosen because triacylglycerol ALA does not resist porcine pancreatic lipase hydrolysis (9), and ALA is found in only small amounts (1-2%) in plasma triacylglycerol. To the best of our knowledge this is the first report of the determination of human absorption of fish oil as triacylglycerols, free fatty acids, or ethyl esters.

MATERIALS AND METHODS

Protocol. Eight male volunteers, ages 25-41 (mean = 32), who had not ingested fish or fish oil for at least two days prior to the study were randomly given 5.0 g of fish oil (containing 1.00 g EPA and 0.67 g DHA) triacylglycerols, free acids, or ethyl esters and 2.0 g of linseed oil as triacylglycerols. The oils were taken at 8:00 a.m. in gelatin capsules after an overnight fast and consumed along with a low fat breakfast consisting of a Carnation Instant Breakfast, skim milk and fruit, followed by a lunch of the same items. Blood samples were taken from the antecubital vein immediately prior to consuming the oils and at 2,3,4,5,6,7 and 8 h after consumption. One and two weeks later the same protocol was followed while rotating the fish oil form consumed.

In a second study designed to compare the absorption of ALA as triacylglycerol with linoleic acid as triacylglycerol, 5 volunteers were given 11 g of safflower oil containing 8.3 g of linoleic acid, in addition to the 2.0 g of linseed oil, containing 1.04 g of ALA.

Materials. Efamol brand (Guildford, Surrey, U.K.) fish oil in gelatin capsules was used throughout the study. The oil was extracted from a mixture of cold water marine fish, sardine being the most dominant. We found that this fish oil had nearly the identical fatty acid composition as well as stereospecific structure as MaxEPA (Seven Seas, Marfleet, U.K.). The fatty acid composition of the fish oil is given in Table 2. Linseed oil containing 52% ALA was obtained from Flora (Vancouver, BC) and put into gelatin capsules.

Synthesis of fish oil free acids and ethyl esters. The free acids of fish oil were prepared by saponification with 0.3 N NaOH in 90% methanol as described by Kates (10). Extraction with hexane removed all traces of unreacted triacylglycerol. The free acids were washed with 2% $NaHCO_3$ and water until the water gave a neutral pH. Solvent was removed in a rotary evaporator until a constant weight was achieved.

The ethyl esters were prepared by refluxing 60 g fish oil in one liter of 4% (v/v) concentrated H_2SO_4 in ethanol for 90 min, followed by extraction with hexane and washing with 2% $NaHCO_3$ and water until a neutral pH was obtained. Purity of the ethyl esters and the free acids was estimated to be 99% or better as determined by thin-layer chromatography. The free acids and ethyl esters were placed in gelatin capsules immediately before consumption.

Plasma triacylglycerol analysis. After removal of blood samples into tubes containing Na_2EDTA, plasma was isolated by centrifugation at 2500 rpm for 15 min at 4°C. Total lipids from 1 ml plasma were extracted with 10 ml chloroform/methanol (2:1, v/v) containing 0.005% butylated hydroxytoluene and 1 ml of 0.9% saline. 1.00 mg of trigamma-linolenin (Nu-Chek-Prep, Elysian, MN) was added as an internal standard. After re-extraction of the aqueous

layer with 7 ml of prepared lower phase, the total lipid extract was concentrated and separated into lipid classes by thin-layer chromatography on silica gel 60A plates (Whatman, Hillsboro, OR) developed in hexane/ether/acetic acid (80:20:1, v/v/v). After visualization with 2',7'-dichlorofluorescein spray, the triacylglycerol fraction was removed and directly esterified to methyl esters with 3.0 ml of benzene/14% BF_3 in methanol (1:1, v/v) by heating at 90° C for 30 min. Fatty acid methyl esters were analyzed on a Hewlett-Packard 5880A gas chromatograph using a 30 m x 0.32 mm Supelcowax 10 bonded phase carbowax capillary column (Supelco, Bellefonte, PA) at a split ratio of 30 and a column flow rate of 2.4 ml helium/min.

Determination of relative absorption. The transient increase in plasma triacylglycerol fatty acids as a measure of fatty acid absorption in man has been shown to compare accurately with traditional fecal fat analysis studies (11, 12). We have found that use of a co-ingested, well-absorbed standard, such as linseed oil ALA significantly improves the precision and accuracy of the method. The relative absorption of a fatty acid was calculated from the maximum, weight-adjusted increase of that fatty acid in the plasma triacylglycerol fraction relative to the maximum, weight-adjusted increase of the absorption standard, ALA, as follows:

$$\text{Relative absorption of a fatty acid} = \frac{(C^{FA}_{Max} - C^{FA}_{o}) \div W^{FA}}{(C^{ALA}_{Max} - C^{ALA}_{o}) \div W^{ALA}}$$

C^{FA}_{Max} = maximum concentration attained by a given fatty acid

C^{FA}_{o} = fasting concentration of a given fatty acid

W^{FA} = weight of the fatty acid ingested

It is important to note that this method of estimating absorption is valid only if the fatty acid studied achieves maximum concentration at the same time as the absorption standard. If a fatty acid is more slowly absorbed than the absorption standard, the attained maximum concentration will be lower than that of the standard. Because of endogenous contributions to the plasma triacylglycerol fraction during fat absorption (13), this method is most accurate for determining the absorption of fatty acids which are normally low in plasma, especially when ingested in small amounts. For example, we found that ingestion of 1.2 g of linoleic acid gave a relative absorption value of 2.5, whereas ingestion of 8.7 g gave a more realistic absorption value of 1.02.

Statistical analysis was performed using paired Student's t-tests.

Triacylglycerol stereospecific structure analysis. The stereospecific structure of fish oil and linseed oil was determined by the method of Kuksis (14) and as recently reported from this laboratory (9). Briefly, random diacylglycerols were generated by ethyl magnesium bromide action. The 1,2;2,3-diacylglycerols were separated from 1,3-diacylglycerols by thin-layer chromatography on boric acid treated plates and converted to 1,2;2,3-phosphatidylcholines, by the addition of phosphorus oxychloride and dry choline chloride. The 2-position of 1,2-phosphatidylcholine was hydrolyzed by bee venom pancreatic lipase A_2 (Sigma, St. Louis, MO), with the resulting 1-lysophosphatidylcholine being purified by thin-layer chromatography. The fatty acid composition of the 1-lysophosphatidylcholine was used for the composition of the 1-position. The composition of the 2-position was determined as 4 x 1,2;2,3-diacylglycerol composition minus 3 x triacylglycerol composition. Since EPA and DHA are somewhat resistant to porcine pancreatic lipase (4), lipase generation of 2-monoacylglycerols, the

by a slower lipoprotein lipase clearance. Maximal values were most commonly attained at five hours independent of the fish oil form ingested with an overall average of 5.7 h. The maximum values for ALA were always achieved at the same time as the fish oil fatty acids. Therefore, the maximal increase of a fatty acid in the plasma triacylglycerol relative to the increase of ALA can be used as an accurate measure of relative absorption. The absolute concentration of a fatty acid in plasma triacylglycerol at maximum and basal levels varied considerably from person to person and from day to day for the same person with coefficients of variation (C.V.) of 30-50%, but this variation was markedly reduced to 10-20% by comparing increases to the increase of the absorption standard. Interestingly, a non-ingested fatty acid, arachidonic acid, was also found to vary in a parallel manner with the ingested fatty acids, but its amount of increase (60-90%) was independent of the form of fish oil. In agreement, Emken (13) has shown with deuterium labeled triolein that at least 40% of the increase of oleic acid in plasma triacylglycerol comes from endogenous sources, such as adipose tissue. This may account for the large amount of variation (C.V. = 60-80%) seen by other investigators (8, 16) who have tried to determine the relative absorption of a fatty acid by comparing it to the concentration of arachidonic acid in plasma triacylglycerol.

The relative uptake into plasma triacylglycerol of the major fatty acids unique to fish oil is given in Table 1. The free acid form of all the fatty acids except docosenoic acid (22:1) was absorbed as well as the ALA in triacylglycerol form. Free fatty acids would be expected to be nearly completely absorbed since they are major hydrolysis products of pancreatic

Table 1. Relative Absorption of Fish Oil Fatty Acids by Eight Men After Ingestion of Fish Oil as Free Acids, Triacylglycerols, or Ethyl Esters

	Fatty Acid					
	20:5n-3 (EPA)	22:6n-3 (DHA)	18:4n-3	20:1	22:1	22:5n-3
Free acid	1.01±0.09[a]	1.07±0.13[a]	0.97±0.13	0.95±0.17	0.63±.24[c]	1.16±0.24[b]
Triacylglycerol	0.69±0.13	0.61±0.10[c]	0.91±0.17[c]	0.86±0.24[d]	0.54±.16[d]	0.95±0.10[c]
Ethyl ester	0.20±.04[a]	0.22±0.10[a]	0.44±0.11[a,c]	0.34±0.12[a,c]	0.06±0.03[a,c]	0.26±0.06[a]
Position of first double bond	5	4	6	9	11	7

Values represent mean ± S.D. of the maximal increase of each fatty acid in plasma triacylglycerol relative to the maximal increase in ALA.

[a] Significantly different from the triacylglycerol form, $p<0.005$.
[b] Significantly different from the triacylglycerol form, $p<0.05$.
[c] Significantly different from 20:5n-3, $p<0.005$.
[d] Significantly different from 20:5n-3, $p<0.05$.

most common method of 2-position composition determination, could not be used. The 3-position composition was determined by subtracting the 1- and 2-position compositions from 3 x triacylglycerol composition.

RESULTS

Relative absorption of linseed oil ALA. The absorption of ALA from linseed oil was compared to the absorption of linoleic acid from safflower oil in 5 volunteers. The maximal, weight-adjusted, increases of ALA and linoleic acid were found to be equal; the linoleic acid/ALA ratio equaled 1.02 ± 0.06 S.D. Since linoleic acid in triacylglycerol form has been shown to be at least 95% absorbed in normal adult men (15), the ALA in linseed oil can also be considered to be at least 95% absorbed and is, therefore, a useful standard against which to compare the absorption of other fatty acids.

Relative absorption of fish oil fatty acids. The time course for the appearance of EPA in plasma triacylglycerol relative to the maximal rise in ALA is shown in Figure 1 for all three fish oil forms. A nearly identical pattern was also found for DHA (not shown). After a lag time of about three hours, there was a sharp increase until maximal values were reached, followed

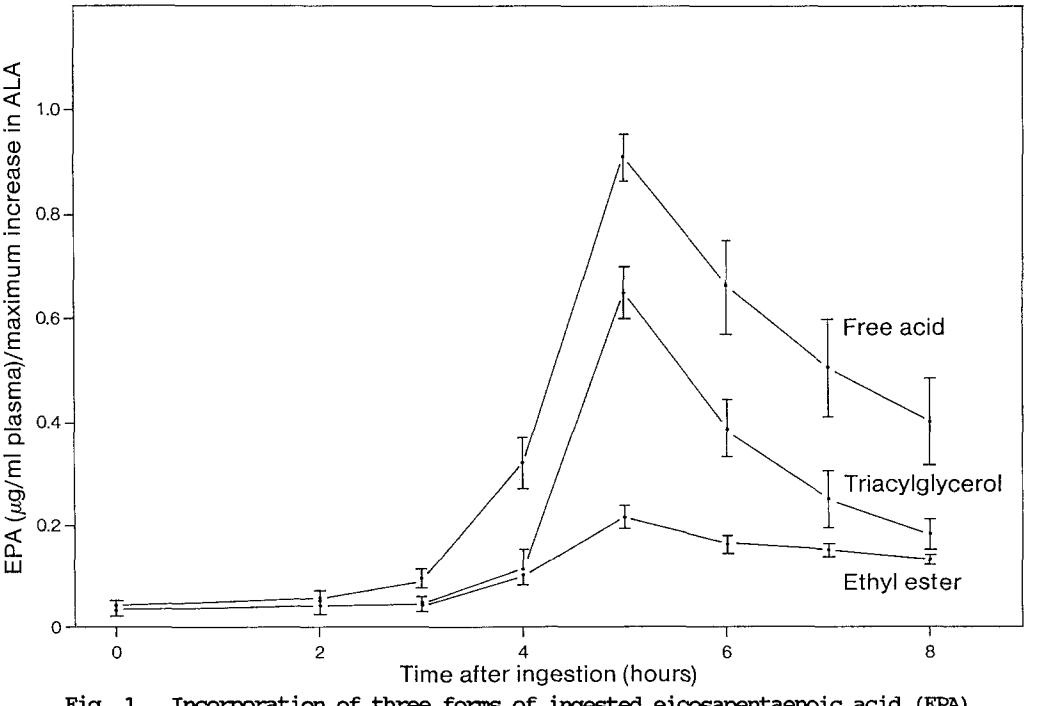

Fig. 1. Incorporation of three forms of ingested eicosapentaenoic acid (EPA) into human plasma triacylglycerol (TG), relative to the maximum incorporation of alpha-linolenic acid (ALA). At time zero a 5.0 g dose of fish oil as free acids, triacylglycerols or ethyl esters containing 1.00 g EPA and a 2.0 g dose of linseed oil containing 1.04 g ALA were given to male volunteers. Points represent the mean ± S.E.M. (n = 5) for those who had maximum incorporation values at 5 h.

Table 2. Triacylglycerol Stereospecific Structure and Fatty Acid Composition of Efamol Fish Oil (Mole%)

	Fatty Acid[a]								
Position	16:0	16:1 (n-9)	18:1[d]	18:4 (n-3)	20:1[d]	20:5 (n-3)	22:1[e]	22:5 (n-3)	22:6 (n-3)
All	13.9	8.1	11.7	4.0	4.9	18.0	3.6	2.2	10.8
1[b]	18	8	22	4	6.5	15	4	1	2
1[c]	(43)	(35)	(62)	(33)	(44)	(27)	(36)	(11)	(5)
2	16	6	9	5	4.5	18	2	4	15
2	(38)	(24)	(26)	(42)	(29)	(33)	(19)	(63)	(42)
3	8	10	4	3	4	21	5	2	20
3	(20)	(41)	(12)	(26)	(27)	(39)	(45)	(26)	(53)

Position 1 = lysophosphatidylcholine; position 2 = 1,2;2,3-diacylglycerol x 4 minus triacylglycerol x 3; position 3 = triacylglycerol x 3 minus positions 1 and 2.

[a] Values are means of four experiments and include only the major fatty acids.
[b] Mole percent of a position (e.g., 15% of position 1 is 20:5n-3).
[c] Mole percent of a fatty acid (e.g., 27% of 20:5n-3 is in position 1).
[d] Includes n-7 and n-9 isomers, n-9 being dominant.
[e] Includes n-9 and n-11 isomers, n-11 being dominant.

lipase action on triacylglycerols and are directly absorbed. When ingested in triacylglycerol form, only EPA, DHA and 22:5n-3 were absorbed significantly less efficiently than when ingested as free acids. EPA, ingested in triacylglycerol form, was absorbed slightly, but significantly, better than DHA. In triacylglycerol form, 18:4n-3, 20:1 and 22:1 were absorbed equally as well as free acids. The ethyl esters of all the fatty acids were poorly absorbed compared to either the free acids or the triacylglycerols. The ethyl ester of 18:4n-3 was more efficiently absorbed than the ethyl esters of EPA and DHA, perhaps because of its shorter chain length. No ethyl esters (less than 0.05% compared to triacylglycerols) were found in the plasma of people fed the ethyl esters.

Triacylglycerol stereospecific structure. The fatty acid composition of the fish oil and its stereospecific structure are given in Table 2. EPA was approximately evenly distributed among all three positions while DHA was found almost exclusively in the 2- and 3-positions. The ALA in linseed oil was found to be distributed similarly to the EPA in fish oil: 25% in position 1, 30% in position 2, and 45% in position 3 (not shown).

DISCUSSION

The results clearly indicate that fish oil ethyl esters are poorly absorbed in man and that even the triacylglycerols are not completely absorbed. The incomplete absorption of ethyl esters appears to be a

universal phenomenon since all the fatty acids examined in fish oil gave low values, indicating that fatty acid ethyl esters are poor substrates for pancreatic lipase. Pancreatic lipase also appears to have reduced activity toward triacylglycerols containing EPA and DHA. In vitro pancreatic lipase studies (17) have shown reduced lipase activity for trioctadecenoins in which the double bond is in the third to sixth positions from the carboxyl-end. These results have been confirmed for the polyunsaturated fatty acids of black currant seed oil in which 6,9,12-18:3 and 6,9,12,15-18:4, but not 9,12,15-18:3 were discriminated against by porcine pancreatic lipase (9). Since the first double bonds of DHA and EPA are in positions 4 and 5, respectively, reduced pancreatic lipase activity toward DHA and EPA appears to be a plausible explanation for their reduced absorption. Further evidence for double bond position as a factor in reduced absorption is the fact that 7,10,13,16,19-22:5n-3 has a more distal first double bond than EPA or DHA, and was also better absorbed than EPA or DHA. Bottino et al. (5) likewise found that EPA and DHA of whale and menhaden oils, but not 22:5n-3, resisted porcine pancreatic lipase hydrolysis. The slightly, but significantly, lower absorption of DHA than EPA can also be accounted for by the fact that the double bonds of DHA are closer to the carboxyl-end than those of EPA. An alternate explanation for the better absorption of EPA over DHA could be simply that EPA is more concentrated in the 2-position, which is not hydrolyzed by pancreatic lipase; however, as shown in Table 2, less EPA (33%) was found in the 2-position than DHA (42%). Furthermore, the resistance of EPA and DHA to porcine pancreatic lipase hydrolysis appears to be independent of their triacylglycerol position (5).

Since the free acids of EPA and DHA from fish oil were absorbed five-fold more efficiently than the ethyl esters, investigations of the metabolic effects of concentrated preparations of EPA and DHA would be far more revealing when administered in the free acid form rather than as ethyl esters. The mildly irritating effects of free fatty acids on the esophagus are easily avoided by the use of gelatin capsules, which are also necessary to prevent auto-oxidation of the fish oil.

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REFERENCES

1. Terano, T., Hirai, A., Hamazaki, T., Kobayashi, S., Fujita, T.,Tamura, Y., and Kumagai, A. (1983) Atherosclerosis 46, 321-331.
2. Payan, D.G., Wong, M.Y.S., Chernov-Rogan, T., Valone, F.H., Pickett, W.C., Blake, V.A., Gold, W.M., and Goetzl, E.J. (1986) J. Clin. Immun. 6, 402-410.

3. von Schacky, C., and Weber, P.C. (1985) J. Clin. Invest. 76, 2446-2450.
4. Brockerhoff, H. (1965) Arch. Biochem. Biophys. 111, 586-592.
5. Bottino, N.R., Vandenberg, G.S., and Reiser, R. (1967) Lipids 2, 489-493.
6. Chen, I.S., Hotta, S.S., Ikeda, I., Cassidy, M.M., Sheppard, A.J., and Vahouny, G.V. (1987) J. Nutr. 117, 1676-1680.
7. Lawson, L.D., Hill, E.G., and Holman, R.T. (1985) Lipids 20, 262-267.
8. El Boustani, S., Colette, C., Monnier, L., Descamps, B. Crastes de Paulet, A., and Mendy, F. (1987) Lipids 22, 711-714.
9. Lawson, L.D., and Hughes, B.G. (1988) Lipids 23, in press.
10. Kates, M. (1986) Techniques of Lipidology, p.125. Elsevier, New York.
11. Goldstein, R., Blondheim, O., Levy, E., Stankiewicz, H., and Freier, S. (1983) Am. J. Clin. Nutr. 38, 763-768.
12. Fallstrom, S.P., Nygren, G.O., and Olegard, R. (1977) Acta Paediatr. Scand. 66, 111-116.
13. Emken, E.A. (1986) In Fat Absorption (A. Kuksis, Ed.), Vol. I, pp. 159-176. CRC Press, Boca Raton, FL.
14. Kuksis, A. (1984) Lipid Research Methodology 10, 77-131.
15. Olubajo, D., Marshall, M.W., Judd, J.T., and Adkins, J.T. (1986) Nutr. Res. 6, 931-955.
16. Stone, K.J., Willis, M.H., Kirtland, S.J., Kernoff, P.B.A., and McNicol, G.P. (1979) Lipids 14, 174-180.
17. Heimermann, W.H., Holman, R.T., Gordon, D.T., Kowalyshyn, D.E., and Jensen, R.G. (1973) Lipids 8, 45-47.