Original Article

The bioavailability of eicosapentaenoic acid from reconstituted triglyceride fish oil is higher than that obtained from the triglyceride and monoglyceride forms

Ammar Wakil MRCP[1], Miquel Mir PhD[2], Duane D Mellor BSc[1], Stephen F Mellor BSc[3], Stephen L Atkin PhD[1]

[1]*Hull Royal Infirmary, Michael White Diabetes Centre, Hull, United Kingdom*
[2]*Croda Consumer Care Europe, Mevisa site, Spain*
[3]*Croda Europe Ltd, Cowick Hall, Goole, United Kingdom*

Omega 3 fatty acids have healthcare benefits, but their absorption characteristics are not well defined, particularly for strategies to improve their bioavailability. We performed a double blind study comparing the bioavailability of 20% eicosapentaenoic acid in 4.5 grams of: natural triglyceride, reconstituted triglyceride, enzymatically synthesized triglyceride, monoglyceride and diglyceride. Seven healthy volunteers were given the supplements on five occasions while repeated measurements of eicosapentaenoic acid were taken to calculate the area under the curve for the next 24 hours. There was a significant difference between the mean of calculated area under the curve of eicosapentaenoic acid from reconstituted triglyceride (30.2) and that of the enzymatically synthesized triglyceride (11.9) and monoglyceride (13.4), z=-2.36 and -2.19, respectively, $p<0.05$. In summary, eicosapentaenoic acid bioavailability of chemically reconstituted triglycerides was better than that obtained from enzymatically synthesized triglyceride and monoglyceride.

Key Words: eicosapentaenoic, bioavailability, monoglyceride, diglyceride, triglyceride

INTRODUCTION

Omega–3 long chain polyunsaturated fatty acids (LCPUFAs) have been increasingly recognised to modify inflammatory and autoimmune diseases like atherosclerosis, rheumatoid arthritis, asthma, Alzheimer's disease among other conditions,[1-3] although cardiovascular disease modifying effects are the most well known.[4-6] Recently, the nutritional requirements for n–3 fatty acids has shifted to their adequate intake in order to reduce disease risk rather than to correct or prevent nutritional deficiency.[7] This was illustrated by the JELIS study, in which eicosapentaenoic acid (EPA) added to statins for 4.6 years in hypercholesterolaemic Japanese resulted in 19% relative risk reduction in the rate of major cardiovascular events.[8] Currently, there is an omega–3 prescription medicinal product in the market (i.e. Omacor®/Lovaza®) that is licensed as adjuvant treatment in secondary prevention after myocardial infarction and to reduce serum triglycerides in subjects with hypertriglyceridaemia. EPA and docosahexaenoic acid (DHA) are the most important LCPUFA and can only be obtained from marine rich diets. Their precursor, alpha linolenic acid, (ALA) on the other hand is widely available in plant oil,[9] but the conversion of ALA to DHA and EPA is not efficient.[10] Therefore, the consumption of fish is recommended to achieve adequate intake of EPA and DHA, but the amount of fish required to achieve therapeutic benefits derived from EPA and DHA may be offset by the poor palatability.. In the UK it is recommended to eat two to three portions of fish a week and each portion is thought to provide between 200 and 500 mg of EPA and DHA, respectively. Supplementation with fish oil containing high purity concentration of DHA and EPA with low levels of contaminants, like dioxins or methylmercury, which might be found in oily fish is one method used to raise plasma levels of EPA and DHA.[11] An alternative strategy is to optimise the absorption and bioavailability of EPA and DHA to attain a higher plasma concentration of EPA and DHA.

DHA and EPA supplements can be given as free fatty acids, natural and reconstituted triglycerides and ethyl esters. Natural fish oil triglycerides (NTG) correspond to 100% triglycerides whereas chemically reconstituted triglycerides (RTG), as defined in the European Pharmacopeia are a mixture of monoglyceride (MG),[12] diglyceride (DG) and triglycerides with triglyceride being the main component (>60%). Also in NTG, DHA is mainly located in the position sn–2 while EPA being in position sn–1and sn–3 which may have influenced their bioavaiability.[13] In the case of RTG, EPA and DHA are

Corresponding Author: Dr Ammar Wakil, Hull Royal Infirmary, Michael White Diabetes Centre, 220-236 Anlaby Road, Hull, HU3 2RW, United Kingdom.
Tel: 00441482675365; Fax: 00441482675370
Email: ammar.wakil@gmail.com; ammar2001uk@hotmail.com
Manuscript received 20 February 2010. Initial review completed 11 June 2010. Revision accepted 24 August 2010.

randomly distributed in the three positions of the glycerol backbone.

The bioavailability of RTG of LCPUFA is not well studied and their absorption may be altered by their incorporation into different forms of fat molecules. Studies on the different components of RTG in rats showed contradictory results. While one study showed n–3 DHA fatty acid from (DG) and (MG) to be better absorbed than triglyceride and ethyl ester,[14] another one showed no substantial differences in DHA bioavailability between DHA–DG (31.8% 1,2–DG, 67.8% 1,3–DG), DHA triglyceride (94%) and a mixture of DHA–DG and triglyceride (69% triglyceride, 8.2% 1,2–DG, 22.6% 1,3–DG).[15] However, there is a lack of evidence in man. Therefore, in order to study the effect of changing location of omega–3 fatty acids in the glycerol back bone on the bioavailability of EPA and DHA, this study was undertaken to compare the short term bioavailability in healthy humans of different fish oils given as NTG, RTG and the individual components of RTG, i.e. MG, DG and triglycerides (ETG). ETG is produced enzymatically to yield high triglyceride content (Figure 1).

MATERIALS AND METHODS

Subjects and methods

This was a double blind cross-over trial. Exclusion criteria were chronic or recent illnesses, regular concomitant or over the counter medications, allergy to fish, and pregnancy. Ethical approval for the study was obtained from The Hull & East Riding and South Humber Local Research Ethics Committees, UK. Seven healthy volunteers were recruited from an advert in the local paper. All subjects signed a consent form prior to taking part. They received dietary counselling by a dietician to avoid intake of fish or omega 3 fatty acid containing diet one week before and during the course of the trial. Coffee, flax seed and alcohol were avoided a day prior, during and a day after each visit. A 7 day run in period was followed by 5 visits to take the fish oil supplements. There was a washout period of at least 7 days between each fish oil sup-

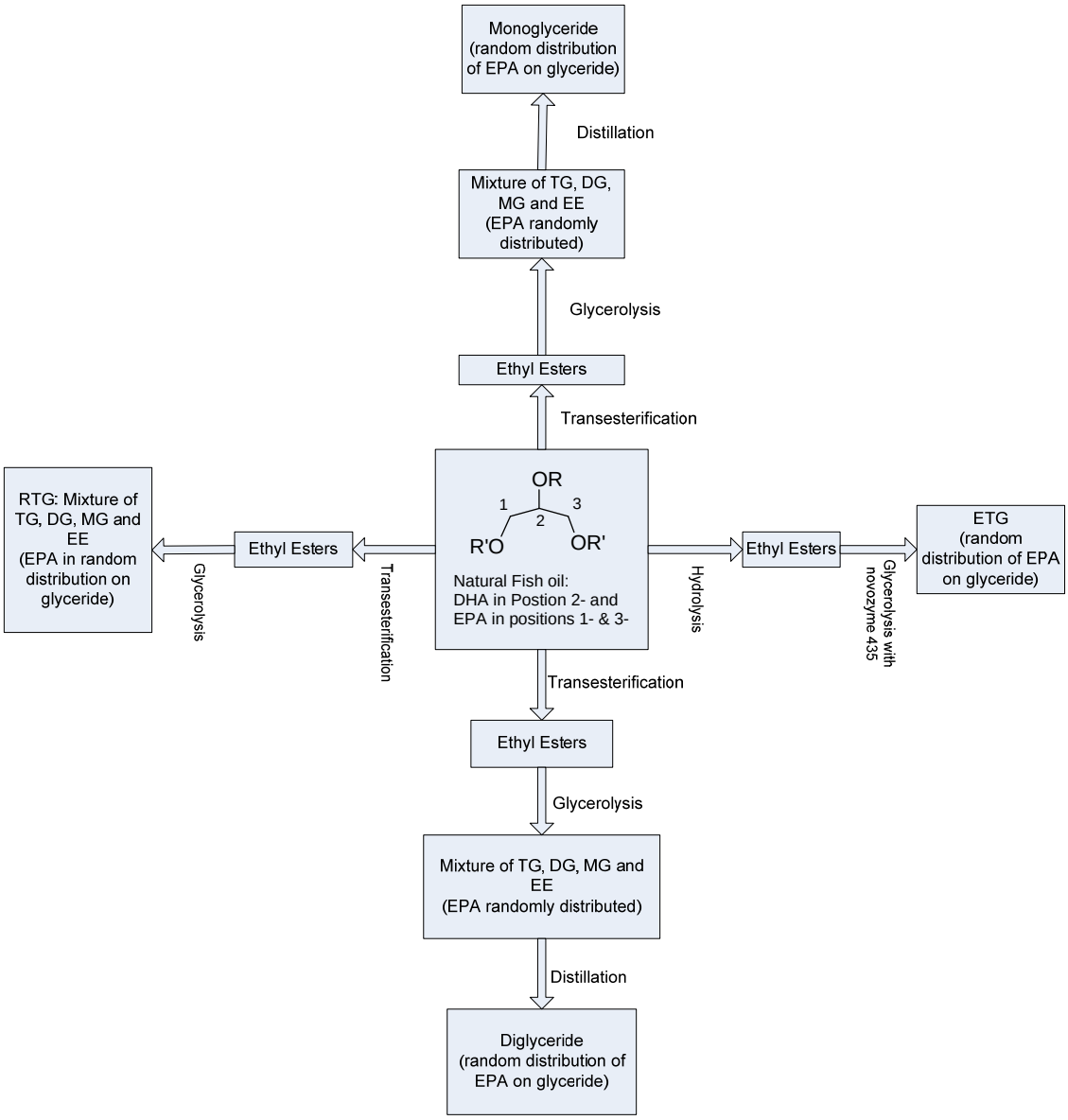

Figure 1. The process of conversion of natural fish oil to the 4 fish oil supplements. NTG: natural triglyceride, RTG: reconstituted triglyceride, DG: diglyceride, ETG: triglyceride and MG: monoglyceride.

plement. Subjects were asked to fast for 10 hours prior to each visit. At the start of each visit a polyethylene catheter was inserted and baseline blood samples taken for fatty acids analysis. This was followed by the intake of a random fish oil supplement and repeated blood sampling at times 2, 4, 6, 8 and 24 hours. A fish-free, low fat lunch was provided after blood sampling at 4 hours after baseline. Subjects were free to eat another meal after the 8 hour blood sample provided they adhered to the above instructions. Subjects fasted for 10 hours prior to the final 24 hour blood sample. Anthropometric measures, blood pressure and pulse rate were taken at the first visit. Samples were separated by centrifugation at 2000 g for 15 min at 4°C, and the aliquots stored at –80°C within 1 hour of collection, before shipping for batch analysis for fatty acids in total serum lipids.

Fish oil

The fish oil supplements were provided by Croda Europe Ltd, Goole, UK, shipped in dark containers in amber vials frozen at 0–4ºC and ready to be thawed before consumption. Each vial had 4.5 grams of fish oil. The investigators, participants and analysing lab were blinded to the type of fish oil supplements throughout the trial. Blinding of fish oil supplements was provided by Croda and the decoding was done before data analysis. The supplements involved in the trial consisted of NTG – sardine and anchovy oil and four other molecular species obtained by modification of this batch of oil. The fatty acid composition of the five species is therefore similar across the product range under test and is detailed in Table 1. RTG were obtained by glycerolysis and also served as the feed to obtain MG and DG by thin film distillation under high vacuum. Finally, ETGs were obtained by enzymatic glycerolysis. It is worth emphasizing that the chemically RTG are a mixture of mono–, di– and triglycerides where the triglyceride is the main component, whereas the enzymatically synthesized ETG are triglycerides with almost no MG or DG (Table 2). The distribution of the molecular species across the range of supplements was established by gel permeation chromatography.

EPA analysis

EPA analysis was performed by Nutrasource Diagnostics Inc, University of Guelph Research Park, Ontario, Canada. Serum was withdrawn into a 13×100 mm open top test tube. Water was added to make up a volume of 200 µl and 25–50 µg of 17:0 PC was added as internal standard. Then 2 ml of chloroform/methanol and vortex was added for 15 seconds[16,17] and 200 µl water and slightly vortex sample was added for approximately 3 seconds. The sample was then centrifuged for 5 minutes at 3000 rpm. Most of the lower Chloroform phase was removed with a pipette into a 4 ml glass vial before drying down using nitrogen. Fatty Acid methyl esters were prepared as described before and before being analysed by Varian Model 3400 using a 60 m×0.32 mm ID×0.15 micron film thickness DB-23 column.[18,19]

Statistical analysis

Area under the curve ($AUC_{0\text{-}24h}$) was used to determine the bioavailability of EPA from the different fish oil supplements. The mean AUC for EPA for each fish oil supplement was calculated using the linear trapezoid method, baseline levels were normalised to 0. Since the subject number was small (n=7), potentially jeopardising the strong assumption of normality required for a parametric test, a non-parametric statistical test, the Wilcoxon signed rank test, was used to compare the AUCs of RTG and NTG versus other fish oil supplements using SPSS software version 15.

RESULTS

The clinical characteristics of the seven healthy volunteers are summarised in Table 3. The mean age, blood pressure, BMI and waist circumference were 33 years, 123/76 mm-Hg, 23 and 76 cm, respectively. Figure 2 shows the mean concentration of EPA in mg/100 ml of serum plotted against the 24 hours for the 5 different fish oil supplements. We found a significant difference between the calculated mean AUC of EPA from RTG

Table 1. Typical fatty acid composition in fish oils provided by Croda Europe Ltd

Fatty acid	Percentage
14:0	7.3
16:0	17.2
16:1(n–7)	8.6
16:2(n–4)	1.8
16:3(n–4)	2.3
16:4(n–1)	2.4
18:0	3.4
18:1(n–9)	9.6
18:1(n–7)	2.9
18:3(n–3)	0.8
18:4(n–3)	2.3
20:4(n–6)	1.1
20:4(n–3)	0.7
20:5(n–3)	19.8
22:1	1.6
22:5(n–3)	1.8
22:6(n–3)	7.9

Table 2. Molecular species in the different fish oils

Fish oil	TG(%)	DG(%)	MG(%)	FFA(%)	EE(%)
NTG	99.0	-	-	1.0	-
RTG	61.2	32.9	2.8	-	3.1
MG	-	5.7	94.3	-	-
DG	10.0	90.0	-	-	-
ETG	96.8	2.8	0.1	-	0.3

NTG: natural triglyceride, RTG: reconstituted triglyceride, DG: diglyceride, ETG: triglyceride and MG: monoglyceride.

Table 3. Subjects' characteristics

Total number (n= 7)	
Female/male	4/3
Mean age in years (SD)	33 (8.4)
Mean systolic blood pressure in mm-Hg (SD)	123 (7)
Mean diastolic blood pressure in mm-Hg (SD)	76 (3)
Mean BMI in $kg/m^{2,}$ (SD)	23.2 (3)
Mean waist circumference in cm (SD)	78.6 (6)

mm Hg: millimetre of mercury, BMI: body mass index, Kg: Kilogram and m2: square metre.

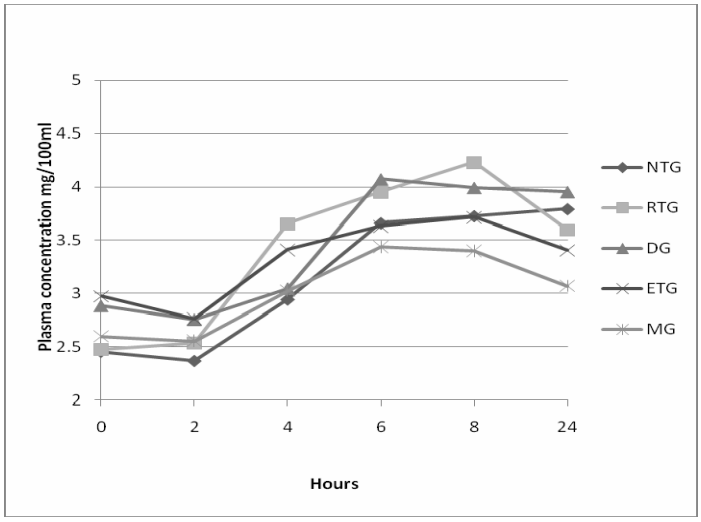

Figure 2. The mean serum concentration in mg/100 ml of different fish oils EPA plotted against time. NTG: natural triglyceride, RTG: reconstituted triglyceride, DG: Diglyceride, ETG: triglyceride and MG: monoglyceride.

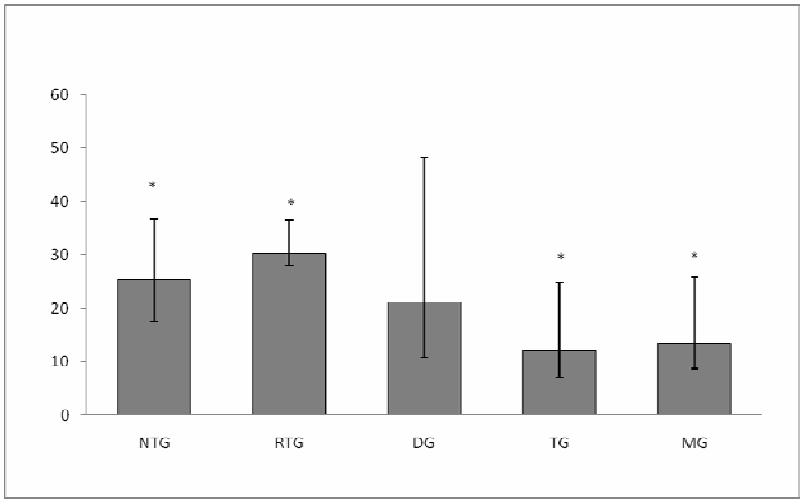

Figure 3. Mean and standard deviation of $AUC_{0\text{-}24h}$ of the EPA from different fish oil supplements. * denotes a significant difference between NTG and RTG on the one hand and TG and MG on the other. NTG: natural triglyceride, RTG: reconstituted triglyceride, DG: diglyceride, ETG: triglyceride and MG: monoglyceride.

(mean=30.2, SD=6.2) and that of the ETG (mean=11.9, SD=12.8) and MG (mean=13.4, SD=12.5), z = –2.36 and –2.19, respectively, $p<0.05$. There was no significant difference in the mean AUC of RTG when compared with those obtained from DG and NTG. The mean AUC of EPA taken from NTG (mean=25.3, SD=11.2) was significantly higher than that obtained from ETG, z = –2.19, $p<0.05$, as seen in Figure 3.

DISCUSSION

These data show that the highest mean area under the concentration curve for EPA was from RTG. Furthermore, when this was compared to the other mean AUCs we found it to be significantly higher than MG and ETG. There was no significant difference in the mean AUC of RTG when compared with those obtained from DG and NTG. To the best of our knowledge this is the first report in humans on the short-term bioavailability of the different components, i.e. MG, DG and ETG, of reconstituted triglycerides.

We did not have data on the position of the LCPUFA (including EPA) in the glyceride molecule in the different supplements though the process of manufacture was likely to place them randomly. The higher bioavailability of RTG compared with ETG may be due to the RTG components (DG and MG) facilitating the intestinal phase digestion and acting as emulsifying agents in the stomach and thereby increasing the absorption and bioavailability from RTG.[20,21] On the other hand, in the case of NTG versus ETG, since both forms are almost 100% glycerides and the only difference is that EPA is mainly located in positions sn–1and 3 in NTG whereas it is randomly distributed in all three glycerol positions in the ETG, this implies that short-term bioavailability of EPA in the positions sn–1 and 3 of the glycerol backbone is higher than in sn–2. This is possibly related to the accessibility of sn–1 and 3 fatty acids to pancreatic lipase compared to the inaccessibility of sn–2 fatty acids, although others have

shown that after quick absorption of LCPUFA in the sn–2 position of TG in the first few hours, the overall 24 hour lymphatic appearance in the rats was not affected by the intramolecular position of the LCPUFA.[22-24] It remains unclear why RTG absorption was preferentially better than the MG but not the DG, since both MG and DG should have more or less the same random distribution of LCPUFA in the triglyceride molecule as they were subjected to a similar conversion process (Figure 1). This study also confirms that serum EPA concentration supplemented as triglyceride reaches a peak a few hours after supplementation[21,25] and concurs with other reports showing that EPA from reconstituted triglycerides are not less absorbed than those taken from natural fish oil.[26]

It is well known that prolonged intervention with fish oil (EPA and DHA) lowers postprandial triglyceride levels, while an acute intake of EPA with a mixed fat meal is not associated with a change in postprandial triglyceridaemia. This study was designed to compare the bioavailability of EPA from different fish oils formulas prepared and given as a single test dose in the fasting state and is not comparable.[27] Furthermore, a previous study reported the percentage of EPA fatty acid in chylomicrons after 6 hours of fish oil ingestion to be comparable to the EPA in total lipids taken after 24 hours of the same EPA rich test meal; 19.7 vs. 22.6.[28] Therefore we used the EPA in serum lipids instead of EPA of chylomicrons TG, the latter being more precise in the first few hours when a fat rich meal is given, which was applicable in the study here.

The limitation of our study was that the concentration of EPA and DHA used were lower than in other nutritional products, where higher concentrations of EPA/DHA are usually incorporated in the supplement, though the weight of the administered supplements (4.5 grams) meant that the total quantity was comparable. This concentration level was needed to have a direct comparison with the natural fish oil and thus establish the effect of the location of the omega–3 fatty acids on the glycerol backbone on its bioavailability. Another limitation of this study was the lack of data on the position of the LCPUFA in the triglyceride in the different supplements, though this was likely to be random, that also made interpretation of some of the data difficult.

This novel data is the first report in humans on the short-term bioavailability of the different components of MG, DG and ETG, of RTG. The results show that the EPA bioavailability over a 24 hour period from chemically RTG i.e. the mixture of MG, DG and ETG where the triglyceride is the main component, was better than that obtained from ETG and MG alone, but was not significantly different from that of DG and NTG. These data may have important implications for the optimisation of LCPUFA absorption for their potential health benefits.

AUTHOR DISCLOSURES

The University of Hull has received unrestricted grant from Croda Europe Ltd in support of conducting this research. Both Miquel Mir and Steve Mellor are affiliated to Croda and have helped in writing the manuscript but their contribution was limited to describing technical aspects of the fish oil supplements development used in the study and provided by Croda. Ammar Wakil, Duane Mellor and Stephen Atkin have no conflict of interest to declare.

REFERENCES

1. Calder PC. n–3 Polyunsaturated fatty acids, inflammation, and inflammatory diseases. Am J Clin Nutr. 2006;83:S1505-19.
2. Cleland LG, Caughey GE, James MJ, Proudman SM. Reduction of cardiovascular risk factors with longterm fish oil treatment in early rheumatoid arthritis. J Rheumatol. 2006; 33:1973-9.
3. Connor WE. Importance of n-3 fatty acids in health and disease. Am J Clin Nutr. 2000;71:S171-5.
4. Din JN, Newby DE, Flapan AD. Omega 3 fatty acids and cardiovascular disease--fishing for a natural treatment. BMJ. 2004;328:30-5.
5. Akabas SR, Deckelbaum RJ. Preface. Am J Clin Nutr. 2006; 83:S1451.
6. Breslow JL. n-3 Fatty acids and cardiovascular disease. Am J Clin Nutr. 2006;83:S1477-82.
7. Gebauer SK, Psota TL, Harris WS, Kris-Etherton PM. n-3 Fatty acid dietary recommendations and food sources to achieve essentiality and cardiovascular benefits. Am J Clin Nutr. 2006;83:S1526-35.
8. Yokoyama M, Origasa H, Matsuzaki M, Matsuzawa Y, Saito Y, Ishikawa Y et al. Effects of eicosapentaenoic acid on major coronary events in hypercholesterolaemic patients (JELIS): a randomised open-label, blinded endpoint analysis. Lancet. 2007;369:1090-8.
9. Kurowska EM, Dresser GK, Deutsch L, Vachon D, Khalil W. Bioavailability of omega–3 essential fatty acids from perilla seed oil. Prostaglandins Leukot Essent Fatty Acids. 2003;68:207-12.
10. Bezard J, Blond JP, Bernard A, Clouet P. The metabolism and availability of essential fatty acids in animal and human tissues. Reprod Nutr Dev. 1994;34:539-68.
11. Domingo JL, Bocio A, Falco G, Llobet JM. Benefits and risks of fish consumption Part I. A quantitative analysis of the intake of omega-3 fatty acids and chemical contaminants. Toxicology. 2007;230:219-26.
12. European Pharmacopoeia Commission CoE. European Pharmacopoeia 6th edn. Strasbourg, France; 2008.
13. Sadou H, Leger CL, Descomps B, Barjon JN, Monnier L, Crastes de Paulet A. Differential incorporation of fish-oil eicosapentaenoate and docosahexaenoate into lipids of lipoprotein fractions as related to their glyceryl esterification: a short-term (postprandial) and long-term study in healthy humans. Am J Clin Nutr. 1995;62:1193-200.
14. Banno F, Doisaki S, Shimizu N, Fujimoto K. Lymphatic absorption of docosahexaenoic acid given as monoglyceride, diglyceride, triglyceride, and ethyl ester in rats. J Nutr Sci Vitaminol (Tokyo). 2002;48:30-5.
15. Tamai T, Murota I, Maruyama K, Baba T, Toyama T, Watanabe N et al. Effects of supplemented diacylglycerol rich in docosahexaenoic acid on serum triacylglycerol in a diet-induced hyperlipidemic model of rats are essentially equivalent to those of triacylglycerol rich in docosahexaenoic acid. Biol Pharm Bul. 2007;30: 2381-8.
16. Folch J, Lees M, Sloane Stanley GH. A simple method for the isolation and purification of total lipides from animal tissues. J Biol Chem. 1957;226:497-509.
17. Holub BJ, Skeaff CM. Nutritional regulation of cellular phosphatidylinositol. Methods Enzymol. 1987; 141: 234-44.
18. Morrison WR, Smith LM. Preparation of Fatty Acid Methyl Esters and Dimethylacetals from Lipids with Boron Fluoride--Methanol. J Lipid Res. 1964;5:600-8.

19. Stark KD, Holub BJ. Differential eicosapentaenoic acid elevations and altered cardiovascular disease risk factor responses after supplementation with docosahexaenoic acid in postmenopausal women receiving and not receiving hormone replacement therapy. Am J Clin Nutr. 2004;79:765-73.
20. Ramirez M, Amate L, Gil A. Absorption and distribution of dietary fatty acids from different sources. Early Hum Dev. 2001;65(Suppl):S95-101.
21. Garaiova I, Guschina I, Plummer S, Tang J, Wang D, Plummer N. A randomised cross-over trial in healthy adults indicating improved absorption of omega-3 fatty acids by pre-emulsification. Nutr J. 2007;6:4.
22. Mu H, Porsgaard T. The metabolism of structured triacylglycerols. Prog Lipid Res. 2005;44:430-48.
23. Bottino NR, Vandenburg GA, Reiser R. Resistance of certain long-chain polyunsaturated fatty acids of marine oils to pancreatic lipase hydrolysis. Lipids. 1967;2:489-93.
24. Ikeda I, Sasaki E, Yasunami H, Nomiyama S, Nakayama M, Sugano M et al. Digestion and lymphatic transport of eicosapentaenoic and docosahexaenoic acids given in the form of triacylglycerol, free acid and ethyl ester in rats. Biochim Biophys Acta. 1995;1259:297-304.
25. Hall WL, Sanders KA, Sanders TA, Chowienczyk PJ. A high-fat meal enriched with eicosapentaenoic acid reduces postprandial arterial stiffness measured by digital volume pulse analysis in healthy men. J Nutr. 2008;138:287-91.
26. Dyerberg J, Madsen P, Moller J, Aardestrup I, Schmidt EB. Bioavailability of n-3 Fatty Acid Formulation. London: Bi & Gi Publishers; 1995.
27. Hansen J-B, Grimsgaard S, Nilsen H, Nordøy A, Bønaa K. Effects of highly purified eicosapentaenoic acid and docosahexaenoic acid on fatty acid absorption, incorporation into serum phospholipids and postprandial triglyceridemia. Lipids. 1998;33:131-8.
28. Nordoy A, Barstad L, Connor W, Hatcher L. Absorption of the n-3 eicosapentaenoic and docosahexaenoic acids as ethyl esters and triglycerides by humans. Am J Clin Nutr. 1991; 53:1185-90.

Original Article

The bioavailability of eicosapentaenoic acid from reconstituted triglyceride fish oil is higher than that obtained from the triglyceride and monoglyceride forms

Ammar Wakil MRCP[1], Miquel Mir PhD[2], Duane D Mellor BSc[1], Stephen F Mellor BSc[3], Stephen L Atkin PhD[1]

[1]*Hull Royal Infirmary, Michael White Diabetes Centre, Hull, United Kingdom*
[2]*Croda Consumer Care Europe, Mevisa site, Spain*
[3]*Croda Europe Ltd, Cowick Hall, Goole, United Kingdom*

重組甘油三酯魚油的 EPA 生物利用率優於甘油三酯及單甘油酯的形式

ω-3 脂肪酸對於健康有益，然而有關其吸收特性卻無明確的了解，尤其不清楚如何改善它們的生物可利用性。我們進行一個雙盲試驗來比較不同形式的 4.5 克油脂其內含的 20%二十碳五烯酸(EPA)之生物利用率，包括：天然形式甘油三酯、重組甘油三酯、酵素合成甘油三酯、單甘油酯及双甘油酯。七個健康自願者皆分五次給予油脂補充品，每次攝入油脂後的 24 小時內，重複測量血清 EPA，並計算曲線下面積。結果發現，攝取重組甘油三酯(30.2)的 EPA 平均曲線下面積與攝取酵素合成甘油三酯(11.9)或單甘油酯(13.4)有顯著差異，z=-2.36 與-2.19，$p<0.05$。總之，當 EPA 以重組甘油三酯形式呈現，其生物利用率優於酵素合成甘油三酯或單甘油酯的形式。

關鍵字：二十碳五烯酸、生物利用率、單甘油酯、双甘油酯、甘油三酯