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Production and bioavailability of calcium and magnesium salts of omega-3 fatty acids

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ABSTRACT

In order to provide an alternative to traditional liquid fish oil gelatin capsules, we developed a solid, powdered form of omega-3 fish oil concentrate by forming calcium- and magnesium-fatty acid salts. These salts were produced using a concentrated fish oil ethyl ester that contained in excess of 60% omega-3 fatty acids. The bioavailability of these omega-3 salts was compared with that of fish oil ethyl ester in mice. Animals were given 8 mg of omega-3 fatty acid ethyl ester concentrate (control), calcium- or magnesium-omega-3 salts daily for three weeks. The omega-3 salt products resulted in omega-3 fatty acid content in serum and red blood cell membranes comparable to that produced by the ethyl ester supplementation. In addition, fecal excretion of omega-3 fatty acids was not increased by the presence of calcium or magnesium. In fact, there was a tendency for less omega-3 fatty acids to be excreted.

1. Introduction

Fish oil omega-3 fatty acids, specifically *cis*-5,8,11,14,17-eicosapentaenoic acid (EPA) and *cis*-4,7,10,13,16,19-docosahexaenoic acid (DHA) are known to be beneficial to cardiovascular health (Kris-Etherton et al., 2002; Harris, 2005). Omega-3 fatty acids are suggested to exert various beneficial effects including reduction in blood triacylglycerol levels, prevention of cardiac arrhythmias, stabilization of atherosclerotic plaques, reduction in platelet aggregation, and reduction in blood pressure (Holub, 2002). Despite the strong evidence for the benefit of EPA and DHA in prevention of cardiovascular disease, the average daily consumption of these fatty acids by North Americans is estimated to be between 0.1 and 0.2 g, compared to a suggested daily intake of 0.65 g to confer benefit (Webb, 2005). Since altering dietary patterns of populations is difficult and many people do not like fish, dietary supplementation with EPA and DHA is an important approach to addressing this consumption deficiency.

The omega-3 supplements are primarily sold as soft gelatin capsules. However, there is a growing demand for omega-3 supplements in a tablet format. The use of solid forms may have advantages over the liquid oil capsule products in that there may be increased stability and improved tolerability profile, for example, reduced burp-back. An omega-3 tablet should have a lower manufacturing cost and could be formulated with other healthful dry ingredients such as vitamins and minerals. In addition, a tablet format would represent an alternative to customers who do not like swallowing gelatin capsules or want to avoid animal gelatin because of dietary or religious reasons. BASF has developed a powderized form of omega-3 oil (Dry n-3®) that is used as an ingredient

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in dietary supplements. However, it contains only 15% omega-3 fatty acids and achieving significantly higher concentrations, like those found in liquid capsules, is not feasible because of the volume of binders or fillers required. An alternative approach would be to produce omega-3 fatty acid salts, such as those of calcium and magnesium (Fig. 1), since these are solid at room temperature and could be formulated as tablets with the above listed advantages. An objection to providing omega-3 fatty acids as calcium or magnesium salts is that the absorption of omega-3 fatty acids may be impaired. For example, it is well known that dietary calcium can lower the net absorption of dietary fat by precipitating fat in the digestive tract resulting in increased fat excretion in feces (Gacs & Barltrop, 1977; Denke et al., 1993; Papakonstantinou et al., 2003; Lorenzen et al., 2007). The objective of the work reported here was to produce calcium- and magnesium-fatty acid salts with high EPA/DHA content and to compare their bioequivalence relative to an EPA/DHA ethyl ester concentrate in order to assess their suitability as dietary supplements.

2. Materials and methods

2.1. Materials

Fish oil ethyl ester (4020EE) was produced by Ocean Nutrition Canada Ltd., Mulgrave, NS, Canada. Gas chromatography (GC) standards for fatty acid profile analyses were purchased from Nu-Check Prep, Inc. (Elysian, MN). Nitrogen gas was of USP grade and supplied by Praxair (Dartmouth, NS). Ash content was established by exposing the samples to 900 °C for 5 min. Calcium oxide, magnesium chloride and all other chemicals used in the study were of analytical grade and purchased from Sigma-Aldrich, Ltd. (Oakville, ON).

2.2. Synthesis of calcium salt from 4020EE concentrate

The fatty acid content of the fish oil ethyl ester concentrate (4020EE) used as starting material is shown in Table 1. A suspension of 16.7 g (50 mmol) of 4020EE and 1.40 g (25 mmol) of calcium oxide was heated under stirring to reach 120 °C and then 7 mL (390 mmol) of distilled water were added dropwise. The reaction mixture was refluxed at 120–130 °C for 5 h, then cooled to room temperature and lyophilized.

2.3. Synthesis of magnesium salt from 4020EE concentrate

A mixture of 4020EE (50 g, 0.15 mol), NaOH (5.9 g, 0.1475 mol) in 8.9 g of water was heated to 60–65 °C and then ethanol (95%, 8.6 mL) was added. The reaction was refluxed for 1 h under nitrogen, cooled to 50 °C and an aqueous solution of $MgCl_2{\cdot}6H_2O$ (6.61 g, 32.5 mmol) was added. The reaction was then refluxed at 100–105 °C for 5 h. After cooling to 4 °C the liquid was decanted, and the solid material was pulverized, washed repeatedly with water and dried by lyophilization.

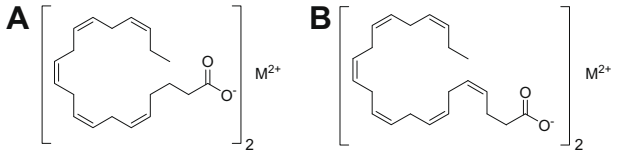

Fig. 1 – Structures representing calcium or magnesium salts of EPA and DHA, respectively (M^{2+} = Ca^{2+} or Mg^{2+}).

Table 1 – Fatty acid profile 4020EE ethyl ester by gas chromatography.

Fatty acid profile	[%][a]	Fatty acid profile	[%][a]
16:0	0.33	20:5n3	44.42
16:1	0.39	22:0	0.47
18:0	1.10	22:1n11	2.61
18:1n9 (Oleic)	1.84	22:1n9	0.74
18:1n7	0.70	22:1n7	0.41
18:4n3	0.36	21:5n3	2.67
20:0	0.48	22:4n6	0.61
20:1n9	2.05	22:5n6	1.03
20:1n7	1.02	22:5n3	8.97
20:3n6	0.35	22:6n3	23.69
20:4n6	2.74	24:1n9	0.89
20:4n3	1.82		
Unknowns%			0.36
Saturates%			2.38
Monounsaturates%			10.62
Total Omega-3			81.92
Total Omega-6			4.73
Remaining polyunsaturates			0.00
Polyunsaturates%			86.64
Total			99.64

a [%] = Peak area of a single peak/total peak area × 100. The values are averages of two independent runs.

2.4. Determination of the fatty acid profiles

The calcium- and magnesium-omega-3 salts were converted to methyl esters for fatty acid analysis by GC. Briefly, the salts were saponified with NaOH in methanolic solution at 100 °C for 7 min followed by methylation with a boron trichloride-methanol solution at 100 °C for 30 min. The fatty acid methyl esters were extracted with tetrahydrofuran (THF) and washed with a 20% NaCl solution. An adaptation of the standard method of analysis for EPA and DHA was employed (Council of Europe, 2006).

2.5. Determination of calcium and magnesium content by inductively coupled plasma – optical emission (ICP-OE) spectrometry

The omega-3 salt samples were digested with nitric acid, followed by microwave-assisted extraction at high temperature and pressure in sealed Teflon vessels. The samples were appropriately diluted before analysis. The analyses were performed by a contract laboratory (Research and Productivity Council, Fredericton, NB).

2.6. Bioavailability study

Calcium, magnesium, EPA and DHA contents of the ethyl ester concentrate, calcium and magnesium omega-3 fatty salts

Table 2 – Omega-3 content of 40:20 ethyl ester, Ca-omega-3 salt and Mg-omega-3 salt.

	4020EE	Ca-Omega-3 salt	Mg-Omega-3 salt
EPA (mg/g)	388	310	344
DHA (mg/g)	189	149	172
Total n-3 (mg/g)[a]	661	526	598
Ca (wt%)	–	11.7	–
Mg (wt%)	–	–	2.7
Yield	–	96%	81%

a Sum of 16:4ω3, 18:3ω3, 18:4ω3, 20:4ω3, 20:5ω3 (EPA), 21:5ω3, 22:5ω3 (docosapentaenoic acid, DPA), and 22:6ω3 (DHA).

used in this study are outlined in Table 2. The study was carried out in the research facilities of KGK Synergize (London, ON) in full compliance with regulations of the Canadian Council on Animal Care. C57BL/6 mice were divided into 3 groups containing 10 animals in each group. After acclimation the treatment groups received the omega-3 preparations daily by oral gavage for 3 weeks, at a dose of 8 mg/day. The calcium- and magnesium-omega-3 salts were suspended in glycerol by warming to 37 °C and then sonicating for 5–10 min. The control group received 4020EE concentrate in glycerol. Fecal samples were collected weekly and pooled for each group. At the end of the study, blood was collected by heart puncture, and blood serum and red blood cells were isolated. Blood serum, red blood cells and fecal samples were analyzed for omega-3 fatty acid content.

3. Results and discussion

The fatty acid profile of the fish oil ethyl ester (4020EE) used as starting material for production of calcium- and magnesium-omega-3 salts was determined by GC and reported in Table 1. The oil contained 388 and 189 mg/g of EPA and DHA, respectively (Table 2). Incorporation of EPA and DHA into calcium and magnesium salts was roughly proportional to that in the starting oil suggesting that neither omega-3 fatty acid was preferred over the other. Both calcium and magnesium salts were prepared in the form of a white, free flowing powders in a 96% and 81% yield, respectively, and were insoluble in water. Contrary to the magnesium salt, the calcium salt was insoluble in methanol but very soluble in tetrahydrofuran. Although calcium and magnesium belong to the group of alkali earth metals and the chemistry of these two metals has a large degree of similarity there are also striking differences that may underlie the differing physical properties. For instance, they are different in their preferred coordination geometry (Pavlov et al., 1998). The structures of calcium and magnesium salts of EPA and DHA are depicted in Fig. 1a and b, respectively. The actual calcium or magnesium salts made from 4020EE concentrate are mixtures containing a large number of different molecules based on various combinations of the fatty acids in the starting material.

Calcium salts of fatty acids derived from various oils, including fish oil, are typically produced through hydrolysis with hydrated calcium oxide (Bondioli et al., 1993; Strohmaier et al., 2006). Although the hydrolysis of 4020EE ethyl ester concentrate was easily achieved with hydrated calcium oxide (Fig. 2a), the reaction failed with hydrated magnesium oxide. The difference in reactivity could be attributed to the difference in pK_B (the negative logarithm of the base dissociation constant; −2.37 for calcium hydroxide versus −0.85 for magnesium hydroxide) and/or to the difference in pK_{sp} (the negative logarithm of solubility product equilibrium constant; 5.26 for calcium hydroxide versus 10.76 for magnesium hydroxide). This observation prompted us to look for an alternative process to produce the magnesium-fatty acid salt. Interestingly, there are reports from literature describing the production of magnesium salts of fatty acids involving the use of hydrated magnesium oxide (Cinco, 1977; Mueller, 1977; Kabashima et al., 1999). However, fatty acids rather than esters were used in these examples and it may be that hydrated magnesium oxide is not a strong enough agent to achieve direct hydrolysis of the fatty acid ethyl esters, particularly those of EPA and DHA. The reactivity of carboxyl groups of EPA and DHA is restricted due to the steric hindrance generated by the presence of multiple double bonds (all of them present in a *cis* framework) leading to the curvature of these molecules. A two step procedure based on the generation of sodium or potassium salts, followed by cation exchange with hydrated magnesium chloride (Fig. 2b), gave a process that resulted in an 81% yield. The use of magnesium chloride is also very advantageous due to its water solubility and high specific magnesium content.

In the bioavailability study, mice received 8 mg/day of 4020EE or salt product. Thus mice in the control (4020EE), calcium- and magnesium-omega-3 groups received 5.3, 4.2, and 4.8 mg omega-3 fatty acids daily, respectively. This dose range

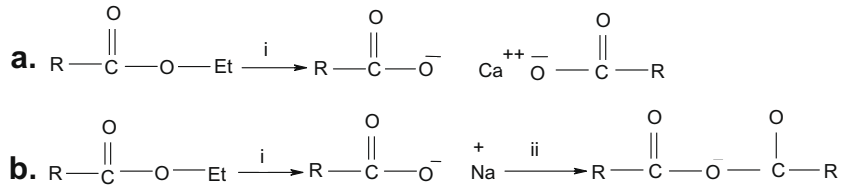

Fig. 2 – Reaction schemes for the preparation of calcium and magnesium salts of omega-3 fatty acid concentrate. (a) The use of calcium oxide for the preparation of the calcium-omega-3 fatty acid salt: R = hydrocarbon chain (40% EPA residue, 20% DHA residue); i = $CaO/H_20/N_2$, 120–130 °C, 5 h. (b) A two step procedure for the preparation of the magnesium-omega-3 fatty acid salt: R = hydrocarbon chain (40% EPA residue, 20% DHA residue); i = aq. $NaOH/EtOH/N_2$, 60–65 °C, 1 h; ii = $MgCl_2{\cdot}6H_2O/H_20/N_2$, 100–105 °C, 5 h. Most of the calcium and magnesium salt molecules formed will be asymmetric, i.e. the two hydrocarbon entities (R) will not be equivalent.

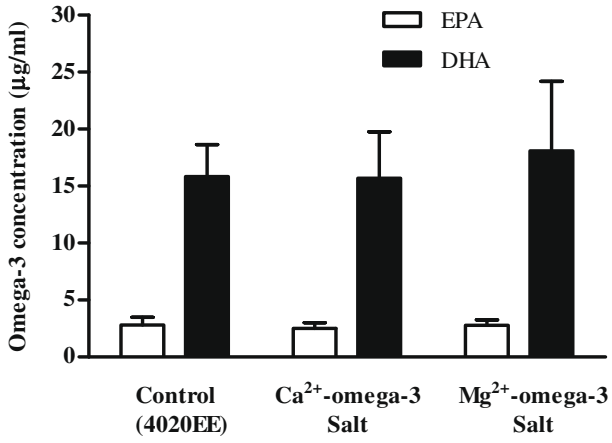

Fig. 3 – Concentration of EPA and DHA in serum. Mice received by oral gavage 4020EE control, calcium-omega-3 salt (Ca-omega-3 salt), or magnesium-omega-3 salt (Mg-omega-3 salt) 8 mg/day for 3 weeks. Mice in the control (4020EE), calcium- and magnesium-omega-3 groups received 5.3, 4.2, and 4.8 mg omega-3 fatty acids daily, respectively. Results are the mean ± standard deviation, $n = 10$.

approximates a 1-gram per day dose in humans using typical scaling assumptions. Blood serum levels of EPA and DHA reflect the sum of these fatty acids in cholesterol esters, phospholipids, and triacylglycerols. Calcium- or magnesium-omega-3 salt supplementation resulted in serum EPA and DHA content that were equivalent to each other and to that seen in the 4020EE supplemented group (Fig. 3). The levels of EPA and DHA in red blood cell membranes are shown in Fig. 4. Calcium-omega-3 salt supplementation resulted in the incorporation of EPA and DHA into red blood cells to a similar extent as that seen in the control group. However, magnesium-omega-3 salt supplementation resulted in a slightly lower degree of EPA and DHA in red blood cells ($p < 0.05$). Given that the actual omega-3 fatty acid dose in the calcium and magnesium salt groups was lower than that in the control group, these results suggest that the provision of omega-3 fatty acids as a calcium or magnesium salts results in levels of EPA and DHA in serum and red blood cell membranes similar to those produced by ethyl esters in the liquid form.

The principle concern with providing omega-3 fatty acids as salts was the potential for the fatty acids to remain bound to the calcium or magnesium. For example, it was previously shown that dietary calcium fortification increases fecal saturated fat content through fatty acid salt formation in the digestive tract (Denke et al., 1993). It has also been reported that calcium phosphate binds bile acids and disrupts formation of lipid micelles necessary for transport of fatty acids into the bloodstream (Van der Meer et al., 1990). Interestingly, in the present study, there was a slight tendency for the Ca- and Mg-omega-3 salt products to lower fecal excretion of EPA and DHA compared to the ethyl ester oil, but only in the magnesium-omega-3 salt group this was statistically significant (Fig. 5). Since the feces was collected weekly and pooled and the animals were not housed in metabolic cages, these findings must be regarded as preliminary and will need to be confirmed by a more comprehensive investigation. Indeed, the degree of absorption of fatty acids is highly dependent on the amount of co-ingested fats and other food components such that a subsequent study should investigate the omega-3 salts when added directly into the animal diet.

Ethyl esters constitute a major category of the fish oil omega-3 concentrate market and constitute the only FDA-approved omega-3 fatty acid prescription drug (Lovaza®). The results of this study suggest the exciting potential for omega-3 salts as an alternative to the traditional liquid format of fish oil ethyl ester concentrates. In addition, the salts could be formulated with dry ingredients to produce a supplement

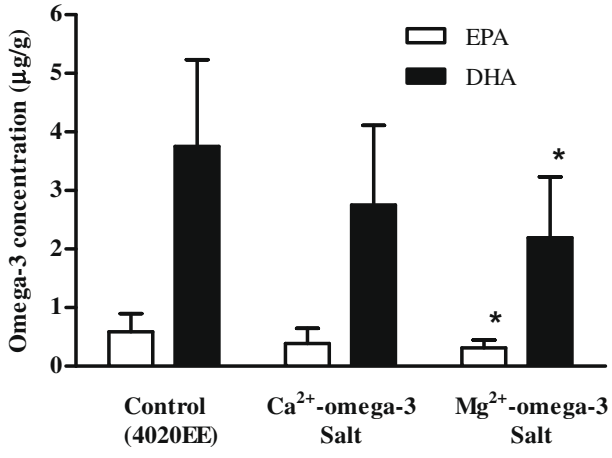

Fig. 4 – Concentration of EPA and DHA in RBC. Mice received by oral gavage 4020EE control, calcium-omega-3 salt (Ca-omega-3 salt), or magnesium-omega-3 salt (Mg-omega-3 salt) 8 mg/day for 3 weeks as described in Fig. 3. Results are the mean ± standard deviation, $n = 10$. *Significantly lower than concentration in the control group, $p < 0.05$.

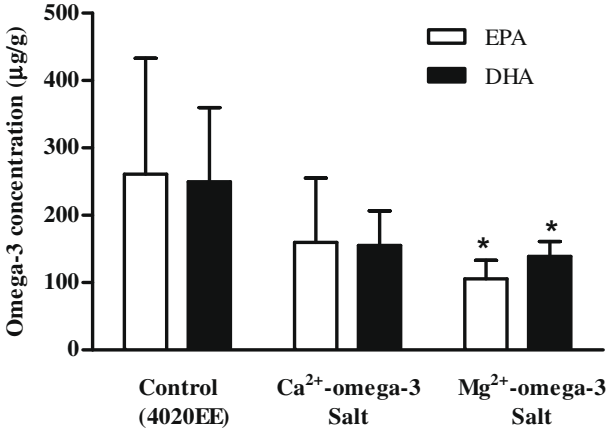

Fig. 5 – Concentration of EPA/DHA in fecal samples. Mice received by oral gavage 4020EE control, calcium-omega-3 salt (Ca-omega-3 salt), or magnesium-omega-3 salt (Mg-omega-3 salt) 8 mg/day for 3 weeks as described in Fig. 3. Results are the mean ± standard deviation, $n = 10$. *Significantly lower than concentration in the control group, $p < 0.05$.

that could deliver significant levels of minerals and/or vitamins in addition to EPA and DHA.

In conclusion, we have developed robust methodologies for producing calcium and magnesium salts from fish oil omega-3 concentrates in the form of free flowing white powders. A proof-of-concept animal study indicates that these omega-3 salts are bioequivalent to the corresponding ethyl esters.

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Appendix. List of abbreviations

DHA, *cis*-4,7,10,13,16,19-docosahexaenoic acid; EPA, *cis*-5,8,11,14,17-eicosapentaenoic acid; GC, gas chromatography; RDI, recommended daily intake; THF, tetrahydrofuran; 4020EE, fish oil ethyl ester containing minimum 40% EPA and 20% DHA.

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