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The free fractions of circulating docosahexaenoic acid and eicosapentenoic acid as optimal end-point of measure in bioavailability studies on n-3 fatty acids

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ABSTRACT

The high complexity of n-3 fatty acids absorption process, along with the huge amount of endogenous fraction, makes bioavailability studies with these agents very challenging and deserving special consideration. In this paper we report the results of a bioequivalence study between a new formulation of EPA+DHA ethyl esters developed by IBSA Institut Biochimique and reference medicinal product present on the Italian market. Bioequivalence was demonstrated according to the criteria established by the EMA Guideline on the Investigation of Bioequivalence. We found that the free fractions represent a better and more sensitive end-point for bioequivalence investigations on n-3 fatty acids, since: (i) the overall and intra-subject variability of PK parameters was markedly lower compared to the same variability calculated on the total DHA and EPA fractions; (ii) the absorption process was completed within 4 h, and the whole PK profile could be drawn within 12–15 h from drug administration.

1. Introduction

After the original observation that Eskimo populations consuming a diet highly rich in n-3 fatty acids from cold water fish show a low prevalence of coronary heart disease, the hypothesis was raised that marine n-3 fatty acids are able to afford protection against atherosclerosis and thrombosis [1]. Thereafter, a large number of clinical trials and observational studies have been addressed to demonstrate the efficacy of n-3 fatty acids in the prevention of cardiovascular diseases [2,3]. Although some controversy still exists at present concerning the correct place in therapy of n-3 fatty acids, as from 2003 the American Heart Association recommends that adults eat fish twice weekly, or take capsules, in order to consume about 1 g daily of a combination of eicosapentenoic acid (EPA) and docosahexaenoic acid (DHA) [4]. Apart from foods and dietary supplements containing various forms of long-chain omega-3 fatty acids (phospholipids, triglycerides or ethyl esters), popular drug formulations usually contain fixed combinations of the most abundant components of fish oil, namely EPA and DHA as ethyl esters, to a total of 800–880 mg/g in 1-g capsules. One gram daily of these products is indicated for secondary prevention of cardiovascular disease, while doses up to 3 g, in adjunct to diet restrictions, are indicated to reduce triglyceride concentrations in adult patients with severe ($\geq$ 500 mg/dL) hypertriglyceridemia.

Whether they are taken as dietary supplements or as registered drug products, n-3 fatty acids are widespread used at present. In this light, it should be taken into account that the absorption of polyunsaturated fatty acids (PUFA) is a complex process, subject to considerable variability, which may have a direct impact on their clinical effectiveness. After oral intake, fatty acids are dispersed into fine fat droplets in the stomach lumen, and then emulsified by bile acids; at this stage, absorption is highly dependent on the relative availability of pancreatic lipase enzymes, which cleave free fatty acids from their ester bond (or else from triglyceride or phospholipid binding, in the case of dietary origin) thereby favoring their passage into the lymphatic system and blood circulation [5,6]. Calcium ions might also influence absorption, since they can form a complex with free fatty acids and reduce their bioavailability. An important source of circulating EPA and DHA derives from the metabolism of circulating very-low-density lipoproteins and chylomicrons by the action of lipoprotein lipase at the level of capillary beds in the skeletal muscle and adipose tissue, where this enzyme is highly expressed [7]. In the systemic circulation, EPA and DHA are found in two forms: as free fatty acids (Free fraction), which are in equilibrium with the fraction incorporated into triglycerides and phospholipids, in turn bound to plasma lipoproteins (Total fraction). Both the free and total fraction have been taken as index of EPA and DHA bioavailability. Further fate

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of free and total n-3 fatty acid is the incorporation into the plasma membranes of circulating cells or peripheral tissues, with the central nervous system, retina and myocardium cell membranes being highly rich in PUFA [6]. Altogether, total and free EPA and DHA, as well as EPA and DHA incorporated into body cell membranes, constitute the endogenous EPA and DHA fraction, which greatly exceeds the exogenous fraction taken in as food and dietary supplements, or drugs.

The above framework leads to the notion that the absorption process and bioavailability of n-3 fatty acids present a number of peculiarities, which make bioavailability and bioequivalence (BE) investigations on EPA and DHA especially challenging. In this paper we report the results of a phase I clinical trial carried out in healthy volunteers to compare the plasma concentration-time data after single-dose administration of a new formulation of 1-g EPA+DHA ethyl esters developed by IBSA Institut Biochimique SA, thereafter referred to as omega-3 Test, and reference medicinal product present on the Italian market (omega-3 Reference), aiming to demonstrate the BE between the Test and Reference product. The study protocol was conceived and designed in 2011; the choice of the study design and methods was based on the Guideline on the Investigation of Bioequivalence into effect at that time [8]. In compliance with this guideline, the study was conducted under fasting conditions, since intake of the reference product is authorized irrespective of food intake. At that time we put a special emphasis on the comparison between the data from total and free EPA and DHA fractions. The need of measuring both the total and free fractions was later confirmed by a specific draft guidance released by the FDA [9]. Here we show that the measure of plasma free fraction represents a better and more sensitive endpoint to investigate the absorption process and bioavailability of n-3 fatty acids under fasting conditions.

2. Materials and methods

The present study was a single centre, randomized, single-dose, open-label, 2-way crossover comparative bioavailability study to compare the rate and extent of absorption of a test omega-3 versus a reference omega-3 formulation, under fasting conditions. The study was conducted at inVentiv Health Clinical (former PharmaNet), 2500 rue Einstein, Québec (Québec), Canada, G1P 0A2. The clinical study protocol and informed consent forms were reviewed and approved by an Ethics Committee (IRB Services) and a no-objection letter was received by the Canadian authorities prior to beginning associated study procedures. The study was performed according to the Declaration of Helsinki, Good Clinical Practice (ICH E6), Good Laboratory Practice and local legal requirements.

Seventy-two healthy volunteers of both sexes (32 females and 40 males), aged >18 years, body mass index between 18.5 and 30 (Table 1), with no clinically significant illness and surgery within 4 weeks prior to dosing and no clinically significant history of neurological, endocrinal, cardiovascular, pulmonary, hematological, immunologic, psychiatric, gastrointestinal, renal, hepatic, and metabolic disease, including moderate smokers, were enrolled after they gave their written informed consent to participate to the study.

Table 1
Descriptive statistics of demographic data for subjects included in the pharmacokinetic population ($N=68$).

Parameter	Age (years)	Height (cm)	Weight (kg)	BMI (kg/m^2)
Mean ± SD	44 ± 13	168.0 ± 9.4	72.83 ± 10.93	25.70 ± 2.38
Range	19–72	148.0–187.0	48.20–95.10	20.04–29.90
Median	46	169.5	72.55	25.75

Vital signs measurements, 12-lead electrocardiogram, physical examination, urine drug screen, urine pregnancy test (female subjects), clinical laboratory tests (hematology, biochemistry, urinalysis, Human Immunodeficiency Virus [HIV], Hepatitis C [HCV] antibodies, and Hepatitis B surface antigen [HBSAg]), total docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) plasma concentrations were measured to confirm subjects' eligibility. Females of childbearing potential who were sexually active must have been willing to use acceptable contraceptive methods throughout the study and for 30 days after.

Prior to study start, subjects were randomly assigned to a treatment in accordance with the randomization scheme generated by inVentiv Health Clinical. Subjects were confined to the inVentiv Health Clinical Facility from at least 48 h prior to drug administration until after the 24 h post-dose blood draw, in each period (period 1 and 2).

Under fasting conditions, a single oral dose of either the Test (1000 mg soft capsules, manufactured by IBSA Institut Biochimique SA, Switzerland) or Reference (Seacor, Società Prodotti Antibiotici S.p.A., Italy) omega-3 as a 12×1000 mg capsules (total dose of 12 g) was administered with 300 mL of water. One gram of Test product contains 94.6% of esterified PUFA (of which 46% EPA and 40.7% DHA); 1 g of Reference product contains 95.5% of esterified PUFA (of which 48.4% EPA and 39.9% DHA). Additional water was allowed up to a maximum total volume of 400 mL, in the event that a subject was not able to swallow 12 capsules with the initial volume of water. The total volume of water swallowed for dosing in Period 1 was used for dosing in Period 2. During their stay at the inVentiv Health Clinical facility, subjects were served meals with low content of omega-3 fatty acids. Served meals were identical on Day-1 and on Day 1, and other meals were similar in composition, in each period. Subjects fasted for at least 10 h prior to dosing and were served a controlled meal not less than 4 h post-dose, and at appropriate times thereafter, in each period. Subjects were served standardized post-dose meals, in each period. With the exception of the volume administered at the time of dosing, fluids were not permitted from 1 h before dosing to 1 h after dosing, but water was permitted ad libitum at all other times.

All blood samples were drawn into blood collection tubes containing EDTA K3 20, 16, 12, and 0.083 h prior to study drug administration and 1, 2, 3, 3.50, 4, 4.50, 5, 6, 7, 8, 9, 10, 12, 16, 24, and 48 h post-dose, in each period. For the 48 h post-dose time point, a window of ±30 min was allowed for blood collection. The two study periods were separated by a washout period of 28 days.

The following pharmacokinetics (PK) variables were evaluated: area under the plasma concentration $(AUC)_{0-t}$, $(AUC)_{0-inf}$, maximum concentration C_{max}, Residual area, T_{max}, elimination half-life $t_{1/2\ el}$ and elimination rate constant $K_{el,}$ for baseline corrected free and total DHA and baseline corrected free and total EPA; $(AUC)_{0-t}$, C_{max}, and T_{max} for free and total DHA and free and total EPA. Safety and tolerability data were based on adverse events (AEs) and standard laboratory evaluations.

2.1. Blood sample handling and analytics

Blood samples were collected and handled under sodium lamps and kept in ice/water bath until transfer in a freezer. Blood samples were centrifuged at 3000 rpm for at least 10 min at approximately 4 °C. Two aliquots of at least 1.0 mL (when possible) of plasma were dispensed into amber polypropylene tubes (as soon as possible). The aliquots were transferred to a −80 °C freezer, pending transfer to the bioanalytical facility. Free EPA and free DHA plasma concentrations were determined at Algorithme Pharma Inc. (Québec, Canada), while total EPA and total DHA at inVentiv Health Clinical (Québec, Canada),

Canada using validated liquid chromatography LC/MS/MS methods. The analytes free/total EPA and free/total DHA and their internal standards, eicosapentaenoic acid-d5 and docosahexaenoic acid-d5, were extracted from a 0.040 mL aliquot of human EDTA K3 plasma. Calibration curve range used during sample analysis was from 2.00 to 199.99 μg/mL for total EPA, from 10.0 to 1000.0 ng/mL for free EPA, 9.99 to 199.86 μg/mL for total DHA and 42.0 to 2625 ng/mL for free DHA.

In order to measure free EPA/DHA, sample pre-treatment involved the protein precipitation extraction of eicosapentaenoic acid and docosahexaenoic acid from 0.1 mL of human plasma.

In order to measure total EPA/DHA sample pre-treatment involved extraction from human EDTA K3 plasma using a hydrolysis followed by dilutions. The internal standard solution is added to 0.040 mL of plasma and adequately mixed. To the resulting mixture is added the hydrolysis agent (potassium hydroxide). The mix is incubated for 45 min at 60 °C to allow the complete cleavage of the esterified omega 3. After further dilutions, the final extract is injected into the LC/MS/MS and total EPA/DHA are analyzed.

Analysis was performed in compliance with good laboratory practice regulations using fully validated methods.

2.2. Statistical methods

Pharmacokinetic and statistical analyses were performed using Pharsight® Knowledgebase ServerTM (PKS) version 4.0.2 and WinNonlin® 5.3, which were validated for bioequivalence studies by inVentiv Health Clinical. WinNonlin® (version 5.3) was used to calculate PK parameters. For baseline corrected data, using general linear model (GLM) procedures in Statistical analysis system® (SAS® version 9.2 for Windows), analysis of variance (ANOVA) was performed on ln-transformed AUC_{0-t}, AUC_{0-inf}, and C_{max} and untransformed K_{el} and $T_{1/2\ el}$. A non-parametric test (Wilcoxon's Signed-Rank test) was carried out to compare the T_{max} between treatments. For baseline uncorrected data, ANOVA was performed on ln-transformed AUC_{0-t} and C_{max} only. Ratios of least-squares means and 90% geometric confidence intervals were calculated for ln-transformed AUC_{0-t}, AUC_{0-inf} (baseline-corrected data only), and C_{max}. Inter- and intra-subject CVs were also calculated. BE was to be concluded if the 90% geometric confidence intervals of the ratio (A/B) of least-squares means for ln-transformed AUC_{0-t} and C_{max} were within the acceptable range of 80.00% to 125.00% for baseline-corrected free DHA and baseline-corrected free EPA.

3. Results

Seventy-two subjects were randomized to receive study drugs. Sixty-eight out of 72 subjects completed the study as per protocol and were analyzed in the pharmacokinetics population. All subjects had pre-dose sampling to assess baseline EPA and DHA concentrations, as described in Section 2. All data presented here were corrected for the respective baseline values. The free DHA and EPA concentrations before the administration of Test and Reference products are shown in Table 2.

Free DHA and EPA plasma concentrations showed an increase after dosing that reached a peak 4 h after drug administration. Peak concentrations for both Test and Reference are reported in Table 3. Thereafter, circulating concentrations rapidly decreased, with average $t_{1/2}$ of 0.84 and 1.63 h for DHA and EPA, respectively; plasma DHA and EPA concentrations were back to pre-dosing concentrations within 7–8 h after drug administration (Figs. 1 and 2).

The free fractions of both n-3 fatty acids were a tiny amount compared to total concentrations, with free DHA and EPA being in both cases less than 1% of the respective total fraction. Total DHA and EPA appeared to reach peak plasma concentrations more slowly compared to free fractions; the estimated T_{max} were 8 h and 8.5 h, respectively (Table 4). Likewise, the apparent elimination half-lives were also slower, being 13 and 30 h for DHA and EPA, respectively (Table 4).

Table 2
The free DHA and EPA plasma concentrations before the administration of Test and Reference formulations. Data are expressed as ng/mL.

Relative nominal time (h)	**Omega-3 Test**		**Omega-3 Reference**	
	Free DHA mean ± SD (*n*)	**Free EPA** mean ± SD (*n*)	**Free DHA** mean ± SD (*n*)	**Free EPA** mean ± SD (*n*)
−20	206.8 ± 84.2 (68)	38.9 ± 16.2 (68)	207.9 ± 79.1 (70)	36.8 ± 12.9 (70)
−16	197.1 ± 76.1 (69)	37.9 ± 17.2 (69)	212.0 ± 92.7 (70)	37.9 ± 15.9 (70)
−12	195.0 ± 88.5 (69)	39.2 ± 20.5 (69)	196.8 ± 87.8 (70)	37.4 ± 15.1 (70)
0	497.4 ± 240.8 (69)	93.0 ± 43.8 (69)	458.2 ± 185.1 (70)	83.2 ± 33.4 (70)
Baseline	273.9 ± 101.4 (69)	52.3 ± 20.4 (69)	268.7 ± 89.7 (70)	49.1 ± 16.1 (69)

Table 3
Summary of PK parameters for plasma baseline corrected Free DHA and EPA.

	Free DHA mean ± SD (*n*)		**Free EPA** mean ± SD (*n*)	
	Omega-3 Test	**Omega-3 Reference**	**Omega-3 Test**	**Omega-3 Reference**
AUC_{0-t} (ng h/mL)	1492.9 ± 612.2 (68)	1484.7 ± 656.6 (68)	340.7 ± 146.8 (68)	338.4 ± 126.7 (68)
AUC_{0-inf} (ng h/mL)	1830.2 ± 714.5 (39)	1762.9 ± 774.3 (50)	430.8 ± 268.5 (28)	396.6 ± 166.9 (26)
C_{max} (ng/mL)	527.2 ± 206.4 (68)	505.3 ± 197.4 (68)	111.1 ± 50.1 (68)	106.5 ± 46.6 (68)
T_{max}(h) median (min–max)	4.0 (2.0–5.0)	4.0 (2.0–10.0)	4.0 (2.0–8.0)	4.0 (2.0–10.0)
$t_{1/2\ el}$ (h)	0.8 ± 1.0 (39)	0.9 ± 0.8 (50)	1.6 ± 1.4 (28)	1.6 ± 1.8 (26)

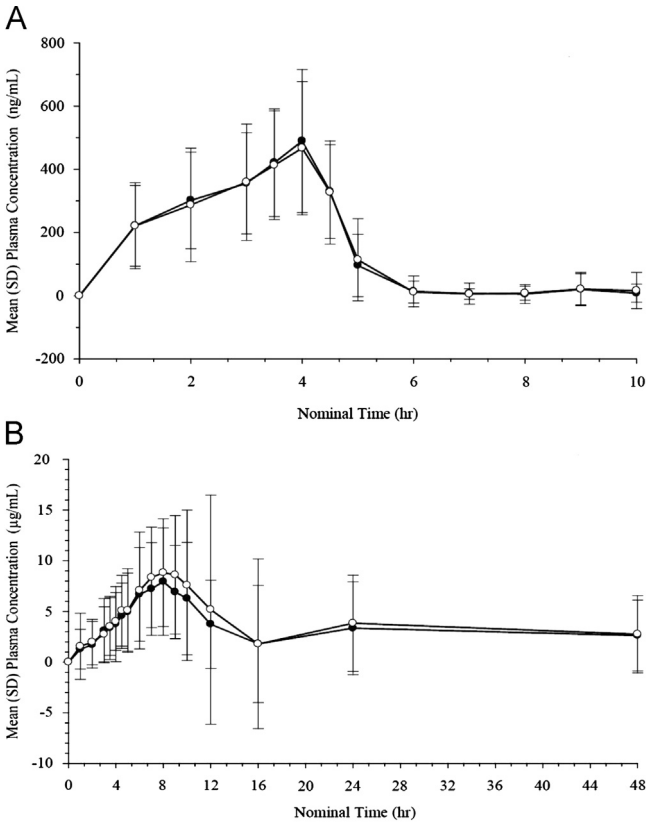

Fig. 1. Baseline-corrected free (panel A) and total (panel B) plasmatic concentrations of DHA. Values are expressed as ng/mL (free DHA) or μg/mL (total DHA), the means ± SD of 68 replicates per group. Omega-3 Test = full circles; omega-3 Reference = empty circles.

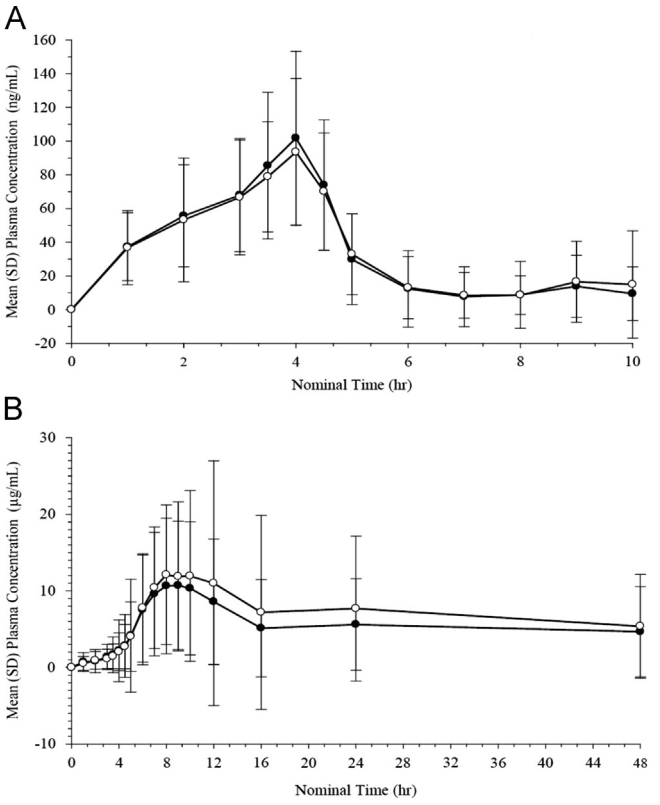

Fig. 2. Baseline-corrected free (panel A) and total (panel B) plasmatic concentrations of EPA. Values are expressed as ng/mL (free EPA) or μg/mL (total EPA), the means ± SD of 68 replicates per group. Omega-3 Test = full circles; omega-3 Reference = empty circles.

Table 5 shows that overall variability of free n-3 fatty acids PK parameters was far lower compared to that of the respective total fractions. In particular, intra-subject variability of the AUC_{0-t} was about one third compared to that of total fractions for both DHA and EPA, whereas C_{max} were about 40% lower compared to total EPA and DHA. Because of such huge difference in intra-subject variability, BE between Test and Reference product was demonstrated if free DHA and EPA were taken as end-point of measure to test bioavailability, whereas criteria to demonstrate BE were not met if the calculation was made on the total n-3 fatty acid fraction, in spite of the enrolment of a quite large study population.

4. Discussion and conclusion

In this study we have tested BE between an EPA + DHA ethyl ester 1-g Test formulation and 1-g capsules of the Reference present on the Italian market. Both the free and total fractions of EPA and DHA have been estimated on the same plasma samples, and a comparison between the kinetic profiles of the free versus the total fractions of both n-3 fatty acids could be carried out. Free DHA and EPA showed a relatively rapid increase in plasma concentrations, reaching a peak 4 h after drug administration; thereafter a rapid decrease ensued, and plasma concentrations were back to pre-dosing concentrations within 8 h from administration. The whole process was completed before plasma concentrations of the total fractions reached the peak of plasma concentrations. It is quite obvious that the apparent elimination phase following the peak of free DHA and EPA plasma concentrations does not reflect a real body clearance process but rather the rapid redistribution of EPA and DHA free fatty acids into the triglyceride and phospholipidic components of total fraction. The latter in turn undergo systemic distribution and incorporation into body cell membranes, and/or catabolism [6], both processes accounting for longer elimination half-lives compared to those of free fractions. Taken together, the above considerations lead to the concept that the different biochemical forms of DHA and EPA are present in a dynamic equilibrium, which can be described as follows:

free DHA and EPA ↔ total DHA and EPA ↔ DHA and EPA integrated into cell membranes

Although the draft FDA guideline on omega-3-acid ethyl esters [9] suggests that, if the bioavailability study is conducted under fasting conditions, BE should be based on total EPA and DHA concentrations, here we show that the use of free EPA and DHA concentrations as end-point of measure brings about some interesting advantages. First, free EPA and DHA are more sensitive to early changes in circulating n-3 fatty acid concentrations associated to EPA and DHA intake. The absorption process, if assessed using free DHA and EPA concentrations as index, is completed within 4 h, and the whole PK profile can be drawn within 12–15 h from drug administration see also [10]; thus, effective comparisons among study drugs in BE trials can be obtained with a relatively low number of blood samplings. Second and more important, overall and intra-subject variability of PK parameters calculated on the free DHA and EPA fractions are markedly lower compared to variability calculated on the total DHA and EPA fractions in the same study population. In the present study this fact caused that the enrolment of 72 healthy volunteers provided sufficient power to demonstrate BE between study drugs if statistical analysis was performed on the free fraction dataset, whereas – because of a 3-fold higher intra-subject $AUC_{0-\infty}$ variability as calculated on

Table 4
Summary of PK parameters for plasma baseline corrected Total DHA and EPA.

	Total DHA mean ± SD (*n*)		**Total EPA** mean ± SD (*n*)	
	Omega-3 Test	**Omega-3 Reference**	**Omega-3 Test**	**Omega-3 Reference**
AUC_{0-t} (μg h/mL)	149.3 ± 131.5 (67)	170.5 ± 186.5 (67)	258.9 ± 254.6 (67)	325.9 ± 382.7 (67)
AUC_{0-inf} (μg h/mL)	207.3 ± 245.5 (17)	235.4 ± 346.2 (23)	705.9 ± 1215.4 (20)	878.9 ± 1204.4 (25)
C_{max} (μg/mL)	12.3 ± 8.6 (67)	14.1 ± 10.7 (67)	13.4 ± 9.6 (67)	16.4 ± 15.5 (67)
T_{max} (h) median (min–max)	8.0 (3.0–47.8)	8.0 (1.0–48.3)	9.0 (6.0–47.8)	8.0 (1.0–47.9)
$t_{1/2\ el}$ (h)	14.9 ± 18.4 (17)	11.1 ± 9.0 (23)	26.9 ± 29.5 (20)	32.5 ± 28.4 (25)

Table 5
Intra- and inter-subject coefficients of variations: comparisons between the total and free fractions of EPA and DHA. The relevant ratios of 90% confidence intervals are also reported.

		CV intra-subject (%)	CV inter-subject (%)	90% Geometric confidence interval		
				Ratio (%)	Lower (%)	Upper (%)
AUC_{0-t}	Free EPA	29.41	27.89	100.06	92.15	108.66
	Total EPA	90.29	90.33	88.21	70.61	110.21
C_{max}	Free EPA	30.32	35.27	103.83	95.38	113.02
	Total EPA	48.45	55.29	86.85	76.08	99.14
AUC_{0-t}	Free DHA	25.48	43.59	102.32	95.24	109.94
	Total DHA	75.61	59.05	88.52	78.64	99.63
C_{max}	Free DHA	23.11	37.74	104.84	98.21	111.91
	Total DHA	42.82	27.44	88.52	78.64	99.63

the total DHA and EPA fractions – we failed to obtain the same result when statistical analysis was performed on the total fraction dataset, despite such large sample size.

Especially if one looks at total DHA and EPA PK parameters (both AUCs and C_{max}), ethyl ester formulations of these fatty acids should be considered as highly variable drug products. Such variability is accounted for by dynamic equilibrium among different biochemical forms, and the presence of a huge endogenous fraction, as well as by the complex absorption process. Unlike dietary lipids, which are hydrolyzed mostly by colipase-dependent pancreatic lipase, absorption of PUFA ethyl esters also requires carboxyl ester lipase (also referred to as bile salt-dependent lipase). The activity of these pancreatic enzymes is stimulated by dietary fat; thus, absorption of PUFA ethyl esters is increased in a dose-dependent fashion by the amount of fat present in meals [5]. Based on these considerations, the draft FDA guideline on omega-3-acid ethyl esters suggests that BE studies are conducted in fasting conditions as well as after intake of high-fat meals, and indeed studies in fed conditions certainly increase n-3 fatty acid esters absorption. However, these trials are potentially biased by a number of drawbacks. First, the administration of a high-fat meal to the study population could not correspond to the use of n-3 fatty acids in the clinical routine. In fact, at least concerning the hypertriglyceridemic indication, these patients are strongly recommended to adopt dietary fat restrictions; therefore, the profile of absorption of these drugs in the clinical practice might significantly differ from that observed in the framework of BE studies. There might also be a negative drawback on standardization of the study conditions, since high-fat meals may contain some amounts of PUFA that, even though very limited, would not be included in the assessment of baseline endogenous DHA and EPA concentrations. An alternative approach to overcome the problem of scarce DHA and EPA ethyl ester absorption would be that adopted in this study, i.e. to administer drug dosages higher than those approved in the SmPC; this is admitted by the EMA guideline [8] in the case of drugs that are endogenous substances, provided that such increased dosages do not raise safety issues into the study population.

In the present study we have demonstrated the BE between the study drug by IBSA Institut Biochemique and a reference compound present on the Italian market, according to the criteria of the EMA guideline for BE studies [8]. Two previous trials were addressed to demonstrate BE between the above products, both missing the target. In a first attempt, a standard single-dose crossover design was deemed unsuitable because of high variability of n-3 fatty acids and the influence of huge endogenous fraction. Whole blood levels of DHA and EPA were therefore compared after long-term (4 weeks) treatments in a randomized, parallel-group design. Twenty-four volunteers per group received 3 g daily of Test of Reference drug; baseline levels of circulating DHA and EPA were assessed weekly, and a time-course from 0 to 8 h was carried out after the last dosing. Data adjustments were conducted to subtract baseline levels to each subject, as well as to correct for the different DHA/EPA titration between study drugs. BE demonstration was not fully achieved, with the huge inter-subject variability being considered a major confound in the study. Data correction for the different DHA/EPA titration was also criticized [11]. The following trial had a classical randomized crossover design, with 8-week washout between treatments. A dose higher than those indicated in the clinical practice, 12-g was administered as a single dose. Well aware of the relevance of high drug variability in this setting, the investigators adopted quite an original approach; out of an initial population of 50 volunteers, 10 subjects were selected for having very low circulating DHA and EPA levels at baseline. While this approach significantly reduced the overall variability, and enabled to demonstrate BE between study drugs, the sample size of the trial fell below the minimal

acceptable number of subjects suggested by the EMA guideline, which is 12, thereby failing to accomplish all of the regulatory criteria for BE demonstration [12].

In the latter study, the total fraction of DHA and EPA plasma levels was taken as end-point of measure. Interestingly, in both the above discussed trials the measurement of DHA and EPA free fractions was not considered in the study design.

In conclusion, in the framework of a standard BE study, we took the opportunity to compare two different end-points of measure, namely the free and total plasma fractions of EPA and DHA, which can be both taken as index of EPA and DHA bioavailability. We clearly showed that the measure of plasma free fraction represents a better and more sensitive end-point to investigate the absorption process and bioavailability of n-3 fatty acids under the experimental conditions adopted here. We believe that the present findings may provide a useful contribution to the current regulatory debate in the field of omega-3 fatty acid BE studies.

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