Quantitation of Human Whole-Body Synthesis-Secretion Rates of DHA and EPA from Circulating Unesterified α-Linolenic Acid at Steady State

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Abstract

The rate at which dietary α-linolenate (ALA) is desaturated and elongated to its longer chain n-3 PUFA in humans is not agreed upon. In this study, we applied a methodology developed in rodents to investigate whole body, presumably hepatic, synthesis-secretion rates of esterified n-3 PUFA from circulating unesterified ALA in two healthy overweight women after ten weeks of low linoleate diet exposure. During continuous *iv* infusion of d5-ALA, 17 arterial blood samples were collected from each subject at −10, 0, 10, 20, 40, 60, 80, 100, 120, 150, 180, 210 min, and at 4, 5, 6, 7, and 8 h after beginning infusion. Plasma esterified d5-n-3 PUFA concentrations were plotted against infusion time and fit to a sigmoidal curve using nonlinear regression. These curves were used to estimate kinetic parameters using a kinetic analysis developed in rodents. Calculated synthesis-secretion rates of esterified eicosapentaenoate (EPA), n-3 docosapentaenoate, docosahexaenoic acid (DHA), tetracosapentaenate, and tetracosahexaenoate from circulating

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unesterified ALA were 2.1 and 2.7; 1.7 and 5.3; 0.47 and 0.27; 0.30 and 0.30; 0.32 and 0.27 mg/day for subjects S01 and S02, respectively. This study provides new estimates of whole body synthesis-secretion rates of esterified longer chain n-3 PUFA from circulating unesterified ALA in human subjects. This method now can be extended to study factors that regulate human whole-body PUFA synthesis-secretion in health and disease.

Keywords

kinetics; omega-3 fatty acid; docosapentaenoic acid; tetracosahexaenoic acid; synthesis; liver; low linoleate diet; mass spectrometry

INTRODUCTION

Docosahexaenoic acid (DHA, 22:6n-3) is the most concentrated omega-3 (n-3) PUFA in the human brain, and accounts for 20% of total fatty acids in ethanolamine glycerophosphatides of brain gray matter (O'Brien & Sampson 1965). DHA and its metabolites regulate many important physiological functions in brain and other tissues, and is considered anti-inflammatory (Brenna & Carlson 2014, Salem 1989, Serhan *et al.* 2015). Mammals cannot synthesize n-3 PUFA *de novo*, but can produce DHA and other n-3, longer chain, highly unsaturated PUFA (HUFA) by desaturation and elongation of α-linolenic acid (ALA, 18:3n-3), a nutritionally essential C18 n-3 PUFA (Crawford & Sinclair 1972, Tinoco *et al.* 1979). Here, HUFA is defined as PUFA with 20 or more carbons, and three or more double bonds (Bibus & Lands 2015). Omega-3 HUFA derived by desaturation and elongation of ALA include eicosapentaenoic acid (EPA, 20:5n-3), docosapentaenoic acid (DPAn-3, 22:5n-3), DHA, tetracosapentaenoic acid (TPAn-3, 24:5n-3), and tetracosahexaenoic acid (THA, 24:6n-3). EPA, although not as concentrated as DHA in brain, is taken up from plasma into the brain at similar rates as DHA (Ouellet *et al.* 2009) and has been reported to be beneficial in treating depression (Hallahan *et al.* 2016), highlighting the importance of n-3 HUFA for neurological function.

The body's capacity for desaturation and elongation of dietary ALA to EPA and DHA has previously been reported to be low in humans when studied with stable isotope tracer techniques with single oral administration of deuterated or ^{13}C-U-labeled PUFA (Brenna 2002, Burdge 2006, Brenna *et al.* 2009, Lin *et al.* 2010). Studies that orally administered tracers in human subjects found that most administered ^{13}C-U-ALA was β-oxidized or lost, and that only a limited amount was converted to longer-chain n-3 PUFA (Vermunt *et al.* 2000, McCloy *et al.* 2004, Pawlosky *et al.* 2001). Following its oral administration in humans, Pawlosky et al. estimated (Pawlosky et al. 2001) that net conversion of d5-ALA was 0.2%, 0.13% and 0.05% of the dose for d5-EPA, d5-DPAn-3, and d5-DHA, respectively. Using compartmental modeling, rates of mass flow for EPA, DPA, and DHA from each immediate precursor were estimated to be 0.021, 0.075, and 0.464 μmol/day, respectively.

However, studies involving oral administration of labeled ALA are limited in their ability to directly measure hepatic synthesis-secretion rates of n-3 HUFA due to the unique absorption pathway of fatty acids (Domenichiello *et al.* 2015). To overcome this limitation, Igarashi et al. (Igarashi *et al.* 2006) developed a method and kinetic analysis in unanesthetized rats that

involves continuous intravenous infusion of labeled ALA, measuring arterial blood levels and/or liver levels of labeled and unlabeled ALA and its desaturated/elongated n-3 HUFA products, and then applying a non-linear kinetic analysis to calculate whole body (largely liver) PUFA synthesis-secretion rates and other kinetic parameters. Domenichiello and Bazinet et al elaborated and widely applied this approach. Using this method, Domenichiello et al (Domenichiello *et al.* 2014, Domenichiello *et al.* 2016, Metherel *et al.* 2018) estimated whole body synthesis-secretion rates of DHA from circulating ALA to be 1.5 and 0.045 μmol/day in rats consuming a high ALA or a low ALA containing diet, respectively.

Compared to oral administration of stable isotopic ALA, the continuous iv infusion method provides direct rates of hepatic synthesis-secretion of n-3 HUFA, and measures HUFA derived from circulating unesterified ALA thus by-passing the loss of tracer in gastrointestinal tract via oral administration. Considering the advantage of the continuous *iv* infusion method, and conflict over whether ALA alone can provide sufficient EPA and DHA to maintain optimal organ function in human (Saunders *et al.* 2013, Brenna 2002, Barcelo-Coblijn & Murphy 2009), the translation of this infusion method developed in unanesthetized rats to the clinic is of great importance. Accordingly, in this pilot study we developed parameters for continuous *iv* infusion of d5-ALA-albumin in two overweight women consuming a well-defined diet, to quantify hepatic synthesis-secretion rates of EPA, DPAn-3, DHA, TPAn-3, and THA from circulating unesterified ALA. Labeled PUFA appearance vs. infusion time data were fit by sigmoidal curves, and kinetic parameters were estimated as described for the rat (Domenichiello et al. 2014, Domenichiello et al. 2016).

The protocol was approved for twenty-four subjects. However, potential contamination of the infusate that was prepared in the NIH Clinical Center Pharmacy (Bethesda, MD) (McCarthy 2015) allowed us to complete studies on only two subjects on the same diet. Nevertheless, comparable results on the two subjects that presented in this paper show for the first time that PUFA synthesis-secretion rates from circulating unesterified ALA can be measured in humans, and provide initial estimates and procedures for future reference.

MATERIALS & METHODS

Clinical protocol

This is a substudy of an ongoing clinical protocol (NIAAA Protocol #11-AA-0028) that was approved by the Institutional Review Board of the National Institution of Health Addictions. Its National Clinical Trial identifier is NCT01251887 (ClinicalTrial.gov, accessed June 11, 2018) under the study title as "Dietary Essential Fatty Acid regulation of Omega-3 HUFA Metabolism; Satiety and Body Composition". In the main protocol, healthy overweight premenopausal women volunteers, aged 18–50 yr with body mass index (BMI) of 25–35 kg/m^2, were recruited. Two were chosen and placed on the low linoleate diet for 10 weeks. Each was infused intravenously at a constant rate with d5-ALA-albumin over a period of eight hours. The infusion study was unforeseeably discontinued because the NIH Pharmacy, which was charged with preparing sterile infusate, was closed because of unexpected contamination, and the remaining infusate was discarded (McCarthy 2015).

The two subjects who completed the substudy gave written informed consent and were reimbursed for their participation. Subject S01was aged 28 yr, body weight (BW) 98.2 kg, with BMI 29.9; subject S02 was aged 31 yr, BW 75.8 kg, with BMI 26.5. Each consumed a low-linoleate diet (Table 1) containing one percent of energy (en%) of total calories as linoleic acid (LA), 0.5 en% ALA, 0.04 en% from EPA+DHA and 12.3 en% of monounsaturated and 9.9 en% of the saturated fatty acids for at least 10 weeks prior to testing. Participants were admitted to the NIH Clinical Center Metabolic Unit (Bethesda, MD) at 18:00 the day prior to infusion. After overnight fasting, an antecubital intravenous line was inserted in one arm at 06:30 for infusion and maintaining *iv* fluids. The infusion started at 08:00 and lasted for 8 hours. A clear plexiglas hand-heating box, custom-made by the Department of Chemistry of the University of Vermont (Burlington, VT), was applied to the other arm which was catheterized for removal of arterial blood. Deuterated ALA-albumin infusate was infused into the first arm at a rate of 0.78 mg/kg BW/hr with an Alaris PC 8015 infusion pump (CareFusion, San Diego, CA), equal to 24 mL/min for subject S01 and 18 mL/min for S02. A total of seventeen 3.5 ml blood samples from the arm contralateral to infusion were collected from each subject. One sample was taken 10 min prior to initiation of infusion to verify instrumental baseline. Remaining samples were taken at 0, 10, 20, 40, 60, 80, 100, 120, 150, 180, 210 min, and 4, 5, 6, 7, 8 h after beginning infusion. Both subjects tolerated the infusion without complaint, side effects or an adverse event.

Blood samples were drawn into a 6 mL EDTA-tube at each time point. Plasma was separated shortly after collection by centrifugation at 1,750 g × 15 min at 4 °C in an Allegra X-12R Centrifuge (Beckman Coulter, Brea, CA), frozen over dry ice and stored at −80 °C until chemical analysis.

Chemicals and other materials

Human serum albumin (25%, USP) was purchased from Grifols Therapeutics (Research Triangle Park, NC); methanol and chloroform were purchased from Burdick & Jackson (Muskegon, MI); hexane from EMD Chemicals (Gibbstown, NJ); butylated hydroxytoluene (BHT) from Acros (Geel, Belgium); boron trifluoride (BF_3) in methanol (14% w/w, 0.9 g/L) was from Sigma Chemicals (St. Louis, MO); 23:0 free fatty acid (23:0 FFA), docosatrienoic acid (22:3n-3 FFA), and the GC reference standard GLC–569B containing fatty acid methyl ester (FAME) were purchased from Nu–Chek Prep (Elysian, MN). All chemicals were of analytical grade and used without further purification.

Stable isotope and preparation of infusate

Deuterium-labeled ALA (d5–17, 17, 18, 18, 18-18:3n-3) potassium salt was custom-prepared by Cambridge Isotope Laboratories (Andover, MA), Lot No. PR-24610. Isotope purity was greater than 98%, chemical purity greater than 95%. The preparation of cGMP terminally sterilized infusate was modified according to the report by Wolfe et al (Wolfe *et al.* 1980, Wolfe 1992) and was formulated through binding with human serum albumin in the NIH Clinical Center Pharmacy Department. Briefly, a weighed amount of d5-18:3n-3 potassium salt was dissolved in water-for-injection at 50 °C in a water bath prior to gradually mixing with 25% human albumin solution and then passed through a Millipore

filter. The final concentration of infusate, d5-ALA-albumin solution, was 10 μmol (equivalent to 3.22 mg) of d5-18:3n-3 per mL of sterile solution (PDS Lot No. 136208). Details are described in Diagram 1.

Chemical analysis

Plasma total lipids were extracted by chloroform: methanol (2:1) according to the method of Folch (Folch *et al.* 1957). Briefly, 200 μl of human plasma was mixed with 1 mL of methanol containing 50 μg of butylated hydroxytoluene and then extracted twice with 2 mL of chloroform. Two internal standards were added to each sample prior to lipid extraction, 0.05 μg of 22:3n-3 for gas chromatography/ mass spectrometry (GC/MS) assay and 5 μg of 23:0 for gas chromatography/ flame ionization detection (GC/FID) assay, respectively. The total lipid extract was dried and hydrolyzed with 1 mL 5% KOH in methanol and derivatized with pentafluorobenzyl bromide and diisopropylamine in acetonitrile (1:10:1000, v/v/v) to form fatty acid pentafluorobenzyl (PFB) esters. A GC/MS negative chemical ionization (NCI) assay was used to quantify deuterated PUFA and unlabeled minor HUFA in total lipids (Pawlosky *et al.* 1992, Lin *et al.* 2005). Fatty acid PFB esters were then transmethylated by 14% of BF_3 in methanol for unlabeled fatty acids. Resulting FAME were detected and quantified using GC/FID (Salem *et al.* 1996, Lin *et al.* 2012).

Unesterified fatty acids were isolated by thin-layer chromatography (TLC) (Domenichiello et al. 2014) from another portion of total lipids extracted from 300 μl of plasma and prepared into PFB esters for quantification of deuterated ALA as describe previously. The same two internal standards as above were added prior to lipid extraction followed TLC separation.

GC/FID and GC/MS

An Agilent 7890A gas chromatograph (Agilent Technologies, Santa Clara, CA) coupled to a flame ionization detector was employed to quantify unlabeled fatty acids. An aliquot of FAME from each sample was injected onto a DB-FFAP fused silica capillary column (Agilent 127-32H2; 15 m × 0.1 mm i.d. × 0.1 μm) through a split/splitless inlet (50:1). The programmed oven temperature and other GC conditions were as previously reported (Lin et al. 2012). GC/MS NCI assay was carried out on an Agilent 6890 GC-5975 inert XL Mass Selective Detector in the negative chemical ionization mode on to a DB-FFAP fused column (30 m × 0.25 mm i.d. × 0.25 μm) and the oven was programmed from 125 °C to 245 °C at 8 °C/min. The M-PFB anion of each analyte was monitored (Lin *et al.* 2011).

Kinetic analysis

Concentrations of deuterated and unlabeled fatty acids (nmol/mL) in plasma samples were quantified by comparing integrated areas of each fatty acid peak in the gas chromatograms with areas of a known amount of corresponding internal standard added to the samples prior to total lipid extraction.

For this kinetic analysis, the plasma input function (into the liver or any other organ that synthesizes and secretes esterified ALA or its elongated products) is plasma steady-state concentration of unesterified labeled ALA, $[d5\text{-}ALA]_{un}$ measured throughout the infusion. Infusing d5-ALA at a constant rate will cause it to achieve a steady-state concentration

rapidly, and to remain as such throughout the infusion. Within the liver, [d5-ALA]$_{un}$ passes through multiple compartments in which it is desaturated and/or elongated (Igarashi et al. 2006). HUFA that are synthesized from d5-ALA are released back into blood, esterified within very low density lipoproteins.

To represent these kinetics, appearance data for the total amount of esterified d5-HUFA$_{i,es}$ (V$_{plasma}$ × [d5-HUFA$_i$]) *vs.* time were fit to a Boltzmann sigmoidal curve using nonlinear regression (GraphPad Prism 6.07, CA) (Domenichiello et al. 2014, Domenichiello et al. 2016), where total plasma volume (V$_{plasma)}$ is 3,000 mL per 70 kg BW (Davies & Morris 1993). The esterified concentration data can be represented by Equation 1, where $C^*_{i,es}$ is defined as V$_{plasma}$*[d5-HUFA]$_{i,es}$ (nmol) for the particular HUFA of interest (i) in the esterified plasma fraction (es), C_{max} is the peak value (top), C_{min} is the minimum value (bottom) of fitting curve, t is time (min), t_{50} is time when C is halfway between C_{max} and C_{min}.

$$C^*_{i,es} = C_{min} + \frac{(C_{max} - C_{min})}{1 + \exp\left(\frac{t_{50} - t}{Slope}\right)} \quad \text{(Eq. 1)}$$

This sigmoidal curve describes the appearance of an esterified labeled HUFA i in plasma during unesterified d5-ALA infusion, and includes a delay followed by rapid rise followed by an approach to a steady-state, when the rate of synthesis-secretion approximates the rate of loss from plasma. The first derivative of the curve described by Eq. 1, defined as S (nmol/min) at any time for a HUFA of interest equals the net rate of change of that labeled HUFA in plasma. This rate equals the synthesis-secretion rate of the esterified HUFA from its precursor, minus its rate of loss from plasma,

$$S = k_{1,i}[d5 - ALA]_{un} - k_{-1,i}[d5 - HUFA]_{i,es} \quad \text{(Eq. 2)}$$

where $k_{1,i}$ (mL/min) is the synthesis-secretion coefficient for esterified HUFAi; [d5-ALA]$_{un}$ (nmol/mL) is the plasma concentration of the infused unesterified d5-ALA; $k_{-1,i}$ (mL/min) is the disappearance coefficient for esterified HUFAi, and [d5-HUFA]$_{i,es}$ (nmol/mL) is the plasma esterified concentration of a d5-labeled HUFAi, that was synthesized from the infused tracer.

The first derivative for the appearance curve (Eq. 2) was determined for each esterified PUFAi as a function of time, using curve fitting. The peak first derivative is at the inflection point of the plot of $C^*_{i,es}$ vs. time, and is defined as S$_{max,i}$ (nmol/min). As S$_{max,i}$ ignores uptake of synthesized labeled unesterified HUFA from blood at the time, it is less than the true rate of syntheisis, $k_{1,I}$ [d5-ALA]$_{un}$, as described by the inequality of Eq.3.

$$S_{max, i} \leq k_{1, i}[d5 - ALA]_{un} \quad (\text{Eq. 3})$$

Rearrangement gives an estimate of $k_{1,i}$, where the approximation represents the inequality.

$$k_{1, i} \approx \frac{S_{max, i}}{[d5 - ALA]_{un}} \quad (\text{Eq. 4})$$

Multiplying $k_{1,i}$ by the plasma concentration of unesterified unlabeled ALA, $[ALA]_{un}$, gives the synthesis-secretion rate ($J_{syn,est}$ nmol/min) of esterified PUFA i,

$$J_{syn, est, i} = k_{1, i}[ALA]_{un} \approx \frac{S_{max, i}}{[d5 - ALA]_{un}}[ALA]_{un} \quad (\text{Eq. 5})$$

Plasma turnover (F_i, min^{-1}) and half-life ($t_{1/2,i}$, min) of esterified HUFA i then can be estimated as follows.

$$F_i = \frac{J_{syn}}{V_{plasma}[HUFAi]_{est}} \quad (\text{Eq. 6})$$

$$t_{1/2, i} = \frac{0.693}{F_i} \quad (\text{Eq. 7})$$

RESULTS

Plasma fatty acid concentrations in total lipids

Table 2 summarizes plasma unlabeled fatty acid concentration profiles for the two subjects during the infusion period, presented as mean (± SD) nmol per mL of plasma for 17 samples from each subject. The similar plasma concentrations of 18:1n-9 (896 vs. 1,011 nmol/mL) and of 18:2n-6 (1,166 vs 1,340 nmol/mL), respectively, support the fact that both subjects were on the same diet. Only trace amounts of n-3 HUFAs were found in the free fatty acid fraction.

Concentrations of deuterated n-3 HUFA

For each subject, plasma concentrations of unesterified d5-18:3n-3 are presented as a function of infusion time in Figure 1. The concentrations reached steady state about 10 min after infusion began, and averaged 1.2 and 1.7 nmol/mL for S01 and S02, respectively. Steady-state levels were maintained until the end of infusion at 8 h.

The major labeled esterified desaturation-elongation products in plasma total lipids were d5-18:3n-3, d5-20:5n-3, d5-22:5n-3, d5-22:6n-3, d5-24:5n-3, d5-24:6n-3. The amounts ([d5-n-3 HUFA] × V_{plasma}) vs. time data (Figure 2) fit well to a sigmoidal curve which plateaued within 300 min after for both subjects (Figs. 2a & 2b). Plasma EPA rose throughout the infusion, reaching an inflection point at approximately 5 h, at 818 nmol for S01 and 504 nmol for S02, then leveling off. The amount of d5-20:5n-3 in S01 and S02 in plasma at the end of infusion was 1185 and 716 nmol, respectively.

Like d5-EPA, d5-22:5n-3 (DPA) and d5-22:6n-3 (DHA) were first detected at 40 min. Both d5-22:5n-3 and d5-22:6n-3 increased for up to 4 h and then leveled off. After 7 h, total esterified d5-22:5n-3 in plasma was 580 nmol for S01, 920 nmol for S02, while the amount of d5-22:6n-3 was 148 nmol for S01 and 44 nmol for S02.

D5-24:5n-3 and d5-24:6n-3 were detected in the first hour of infusion at a similar magnitude compared to d5-22:6n-3 in both subjects. The total amount of d5-24:5n-3 was similar to d5-24:6n-3 in S01 at 7 h, 66.2 nmol vs. 65.6 nmol, while d5-24:5n-3 was greater than d5-24:6n-3, 38.7 vs. 14.9 nmol, respectively.

First derivative of nonlinear fit of deuterated esterified n-3 HUFA concentrations

The first derivatives (S, nmol/min) (Eq. 2) of the best-fit sigmoidal curves of esterified amount vs. infusion time are presented in Figure 3. The peaks of these curves ($S_{max,i}$) represent the best estimate for synthesis-secretion rate for the deuterated HUFA, i (Eq. 3). For d5-20:5n-3, S_{max} occurred at about 200 min after the beginning of infusion and was 3.23 nmol/min in S01 and 2.29 nmol/min in S02 (Fig. 3a). For d5-22:5n-3, S_{max} occurred earlier in S01 (2.43 nmol/min at 94 min) compared to S02 (4.14 nmol/min at 156 min) (Fig. 3b). For d5-22:6n-3 (Fig. 3c), S_{max} for S01 was higher compared to S02 (0.67 nmol/min vs. 0.21 nmol/min, respectively). S_{max} for d5-22:6n-3 occurred at a similar time for both subjects, at 120 min of infusion. For both subjects, d5-24:5n-3 (Fig. 3d) and d5-24:6n-3 (Fig. 3e) presented similar magnitudes of S_{max} at similar times to d5-22:5n-3 and d5-22:6n-3, respectively.

Synthesis-secretion coefficients and rates

S_{max} and synthesis-secretion parameters (see Methods) derived are presented in Table 3. The synthesis-secretion coefficient for EPA, $k_{1,EPA}$, was 1.91 mL/min for S01 and 1.29 ml/min for S02. $k_{1,DPA}$ was 1.44 mL/min and 2.32 mL/min for S01 and S02, $k_{1,DHA}$ was 0.39 and 0.12 mL/min, $k_{1,TPA}$ was 0.23 and 0.12 mL/min, and $k_{1,THA}$ was 0.24 and 0.10 mL/min for S01 and S02, respectively.

Multiplying $k_{1,i}$ by the plasma concentration of unesterified unlabeled ALA, 2.52 nmol/mL for S01 and 4.74 nmol/ml for S02, gave whole-body synthesis-secretion rates $J_{syn,i}$ for a given esterified HUFA$_i$ (Eq.5). $J_{syn,EPA}$ equaled 4.81 nmol/min for S01 and 6.09 nmol/min in S02. $J_{syn,DPAn-3}$ was equal to 3.62 and 11.0 nmol/min in S01 and S02, respectively. In both subjects, $J_{syn,EPA}$ and $J_{syn,DPAn-3}$ were higher than $J_{syn,DHA}$, which equaled 0.99 and 0.56 nmol/min for S01 and S02, respectively. $J_{syn,THA}$ and $J_{syn,TPAn-3}$ were close in both subjects, 0.5–0.6 nmol/min.

The half-lives of circulating esterified EPA, DPAn-3, TPAn-3, and THA were approximately 3 to 8 days, while the esterified plasma DHA half-life ranged from 129 to 182 days in the two subjects.

DISCUSSION

In this paper, we translated a stable isotope tracer technique and kinetic analysis developed in unanesthetized rats (Domenichiello et al. 2014), to directly estimate whole body synthesis-secretion rates of esterified n-3 HUFA from circulating unesterified ALA in two healthy overweight woment after ten weeks on a low linoleate diet. In each subject, we continuously infused d5-18:3n-3-albumin solution intravenously into one arm for 8 h, to rapidly establish a steady-state plasma concentration, and followed the appearance of esterified desaturated-elongated n-3 d5-HUFA products in plasma as a function of time by repeated sampling of blood from the other heated arm.

Steady-state plasma concentrations of esterified HUFA, including d5-EPA, d5-DPAn-3, d5–DHA, d5-TPAn-3, and d5-THA, were achieved in about 5 hr, while a steady-state concentration of infused unesterified d5-ALA was reached within 15 min after starting the infusion. As in rats, appearance curves for synthesized d5-n-3 HUFA in study subjects were sigmoidal; amounts (concentration $\times$ plasma volume) initially rose slowly, then more rapidly, and eventually tended to level off as synthesis approximated loss from plasma. We estimated whole body synthesis-secretion rates as 1.4 μmol (0.47 mg)/day and 0.80 μmol (0.27 mg)/day for DHA, and as 6.9 μmol (2.1 mg)/day and 8.8 μmol (2.7 mg)/day for EPA in subjects S01 and S02, respectively (Table 3).

The infusion technique is minimally invasive and anesthesia free, allowing us to directly estimate esterified HUFA synthesis-secretion rates from plasma unesterified ALA. Compared to rats, the large blood volume and relative ease of implanting catheters in humans allowed for a sufficient amount of blood to be drawn without disturbing *in vivo* metabolism or hemodynamics. In rat studies, due to possible blood coagulation in narrow catheters, heparin maybe necessary to maintain catheter patency (Domenichiello et al. 2014). Heparin can activate lipoprotein lipase and thus affect *in vivo* lipid metabolism and lipoprotein secretion (Domenichiello et al. 2014, Domenichiello et al. 2015), but heparin is not necessary during infusion in humans with wider catheters. Appearance concentrations for esterified d5-HUFA that were synthesized and secreted from d5-ALA were fit well to Boltzman sigmoidal curves. The r^2 of the goodness of fit ranged from 0.950 to 0.997 for all d5-labeled n-3 HUFAs in each subject, except for d5-THA in subject S02 (0.915), indicating the kinetic analysis appropriately described the data.

The low linoleate diet contained 1 en% of total calories as LA and 0.5 en% ALA, compared to 8.9 en% as LA and 1.1 en% as ALA in dietary intake in United States (Hibbeln *et al.* 2006). Thus, the estimates of synthesis-secretion rates for circulating unesterified ALA might be expected to be in a higher range because they would be minimally suppressed by endogenous ALA derived from diet. Their calculated synthesis-secretion rates of DHA from circulating ALA were 1.43 and 0.80 μmol per day, respectively. These rates are 200 times greater than rates reported in adult rats, which weigh about 0.5 kg (Domenichiello et al.

2014, Domenichiello et al. 2016). Therefore, per kg weight, the synthesis-secretion rates are of the same order of magnitude, although rat oxidative metabolism is about 2.5 times human oxidative metabolism (Sokoloff 1978). The diet of the two subjects contained EPA and DHA (0.04 en%), while the reported rodent diets were free of the n-3 HUFA. Previous studies have shown that DHA can downregulate enzymes involved in its own synthesis which may lead to a decreased synthesis rate (Lin et al. 2011). Therefore, the synthesis-secretion rates estimated in this report likely underestimate the capacity for humans to synthesize n-3 HUFA, including DHA. Though, this requirement is not agreed upon, estimates for the requirement for human brain DHA range from 2.4–3.8 mg/day (Umhau *et al.* 2009). Though participants in this study appear to synthesize DHA at a lower rate than required by the brain, the subjects consumed about 100 mg/day as EPA and DHA (~ 60% as DHA). Therefore, synthesis of DHA was likely inhibited by dietary intake of EPA and DHA and did not make a major contribution to maintaining brain DHA. It would be important to use this steady-state infusion method to measure DHA synthesis-secretion in vegans and vegetarians, to determine if DHA synthesis-secretion is increased to meet the body demands with a low or absent EPA/DHA containing diet.

We estimated synthesis-secretion rates of DHA and other n-3 HUFAs from circulating unesterified ALA. It is likely, based on animal studies, that other circulating and tissue pools can be desaturated/elongated to HUFA. Igarashi et al (Igarashi *et al.* 2007) proposed using a λ factor to account for dilution of the hepatic precursor labeled ALA-CoA pool by unlabeled recycling ALA. λ equals the ratio of steady-state liver labeled ALA-CoA to plasma unesterified labeled ALA, and was estimated as 0.35 in rats fed an n-3 adequate diet (Igarashi et al. 2007). This means that synthesis rates of unesterified DHA from circulating unesterified ALA may be underestimated due to dilution by about 3-fold in rats (Igarashi et al. 2007) or by 10 to 20-fold as estimated by Domenichiello et al (Domenichiello et al. 2014). A similar correction factor λ may operate in humans to increase estimated DHA synthesis rates. Additionally, because Equation 5 is an inequality, synthesis rates are likely underestimated in this paper. Finally, underestimation also may occur because unesterified n-3 PUFAs in the synthesis pathway between ALA and DHA may be reincorporated and elongated to esterified DHA, and we have not taken this into account. In addition to the liver, as other tissues (e.g. skin, (Moore *et al.* 1995)) can synthesize but not secrete DHA from ALA, which would further increase the estimated whole body synthesis rate of DHA. Therefore, the calculated DHA synthesis rates in this study, 1.4–0.80 μmol (0.47–0.27 mg) per day, is likely underestimated for multiple reasons.

Subject S02 produced more d5-22:5n-3 than d5-20:5n-3. However, despite this greater amount of d5-22:5n-3, subject S02 produced relatively less d5-22:6n-3 compared to S01. This is consistent with the unlabeled, endogenous HUFA concentrations in plasma total lipids (Table 2). The ratio of DPAn-3 to EPA in S01 is 0.66 compared to 1.43 in S02, while the ratio of DHA to DPAn-3 in S01 is 9.4 compared to 2.5 in S02. It is possible that subject S02 had genetic fatty acid desaturase (*FADS)* polymorphisms that could affect HUFA appearance in the plasma. For instance, it has been reported that single nucleotide polymorphisms (SNP) in the *FADS* gene cluster results in differences in serum phospholipid PUFAs (Gillingham *et al.* 2013, Hong *et al.* 2013). It is possible that subject S01 carries GG group in *FADS* SNP, while subject S02 T-allele group. Future studies could utilize the

steady-state infusion method to measure the effect of genetic variants of FADS and fatty acid elongase (ELOVL) genes on n-3 HUFA synthesis-secretion rates (Steer *et al.* 2012).

The daily synthesis-secretion rates of n-3 HUFA were estimated from an 8 h infusion started in the morning. If the rates changed throughout the day, or the esterified n-3 HUFA possiblely continues to rise after finishing infusion of d5-ALA-albumin, this would not be noted in our estimates. Additionally, we measured synthesis-secretion of HUFA and not synthesis of HUFA. Therefore, conditions that impact HUFA synthesizing tissues from secreting these fatty acids will also lead to underestimates of the synthesis rate, such as liver disease. To further consider our method and kinetic analysis, it would be worthwhile to conduct other approaches to interpret these experimental data, such as multi-compartmental modeling analysis (Pawlosky et al. 2001, Wastney *et al.* 1996). Two or more approaches could provide multi-dimensional understanding of PUFA metabolism. Furthermore, looking into lipid classes and molecular species using liquid chromatography mass spectrometry might further elucidate PUFA metabolism.

In summary, this study establishes a proof-of-concept method and kinetic analysis in human subjects to estimate whole body, presumably hepatic, synthesis-secretion rates of n-3 HUFA from circulating unesterified ALA. These now could be extended for measuring the whole body synthesis-secretion and turnover of other PUFAs directly or from other precursors in a variety of physiological or pathophysiological states, and in relation to different diets.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Abbreviations

ALA	α-linolenic acid
DHA	docosahexaenoic acid
DPAn-3	n-3 docosapentaenoic acid
EPA	eicosapentaenoic acid
ELOVL	fatty acid elongase

FADS	fatty acid desaturase
FAME	fatty acid methyl ester
FFA	free fatty acid
GC/FID	gas chromatography/flame ionization detection
GC/MS NCI	gas chromatography/mass spectrometry negative chemical ionization
HUFA	long chain, highly unsaturated fatty acid (20 C, 3 double bonds)
LA	linoleic acid
SNP	single nucleotide polymorphism
THA	tetracosahexaenoic acid
TLC	thin-layer chromatography
TPAn-3	tetracosapentaenoic acid
WFI	water-for-injection

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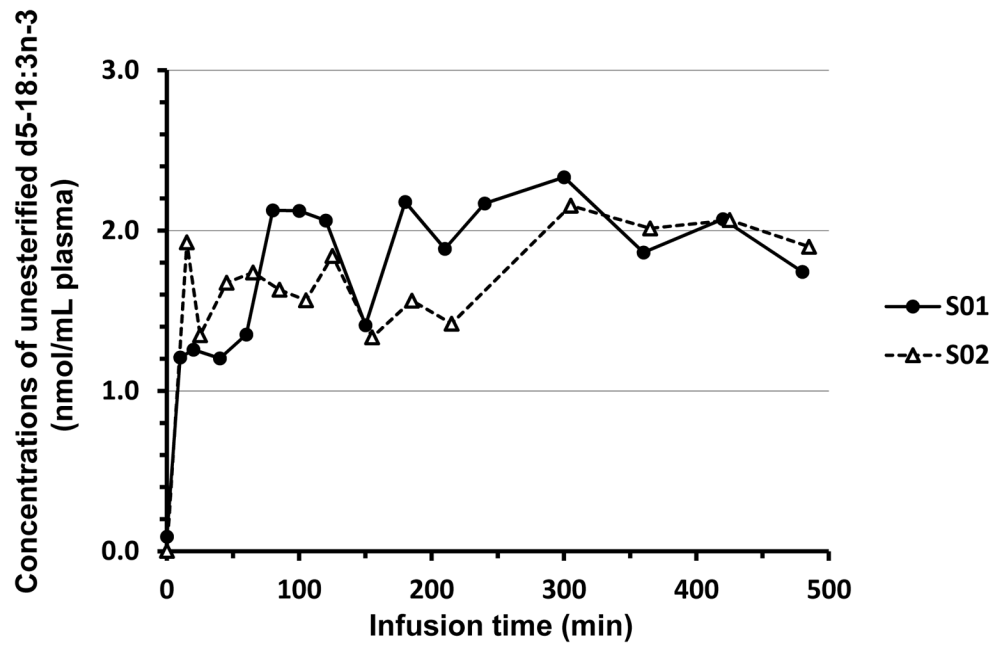
Figure 1.
Time-courses of experimental plasma concentrations (nmol/mL) of unesterified d5-18:3n-3 as a function of infusion time (min) for two women. The plasma concentration of d5-18:3n-3 of S02 at 6 h was 4.37 nmol/mL but is not presented.
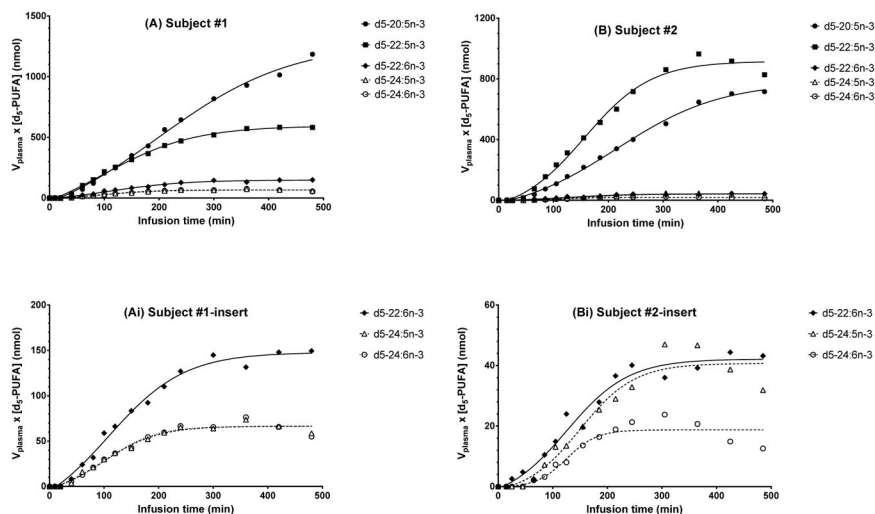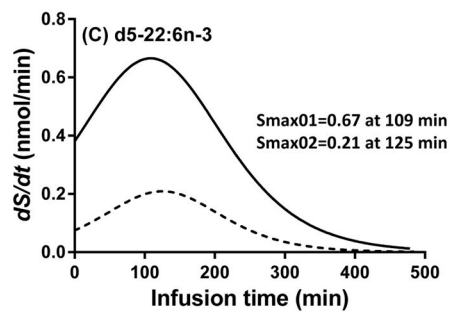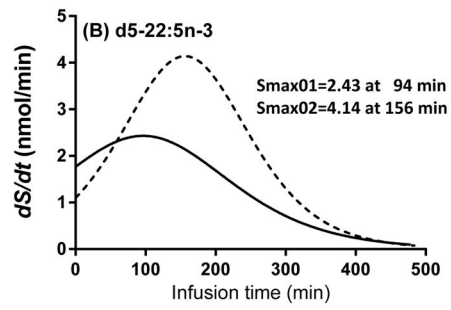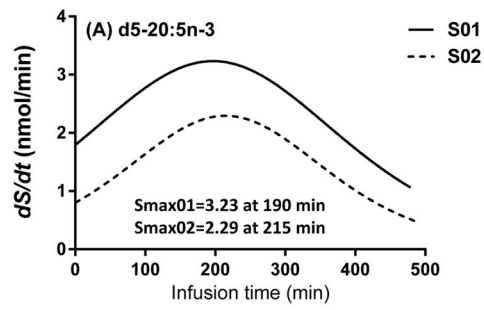
Figure 2.
Best-fit curves for the experimentally determined total amount of d5-n-3 PUFAs ([d5-PUFA]*V_{plasma}, nmol) synthesized from d5-18:3n-3 as a function of infusion time (min). Fit is to nonlinear Boltzmann Sigmoidal equation. Symbols expressed as total amount in plasma (nmol). (**A**) subject S01 d5-PUFA, (**Ai**) insert for subject S01 for d5-22:6n-3, d5-24:5n-3, and d5-24:6n-3; (**B**) subject S02 d5-PUFA, (**Bi**) insert for subject S02.

(A) d5-20:5n-3

Smax01=3.23 at 190 min
Smax02=2.29 at 215 min

(B) d5-22:5n-3

Smax01=2.43 at   94 min
Smax02=4.14 at 156 min

(C) d5-22:6n-3

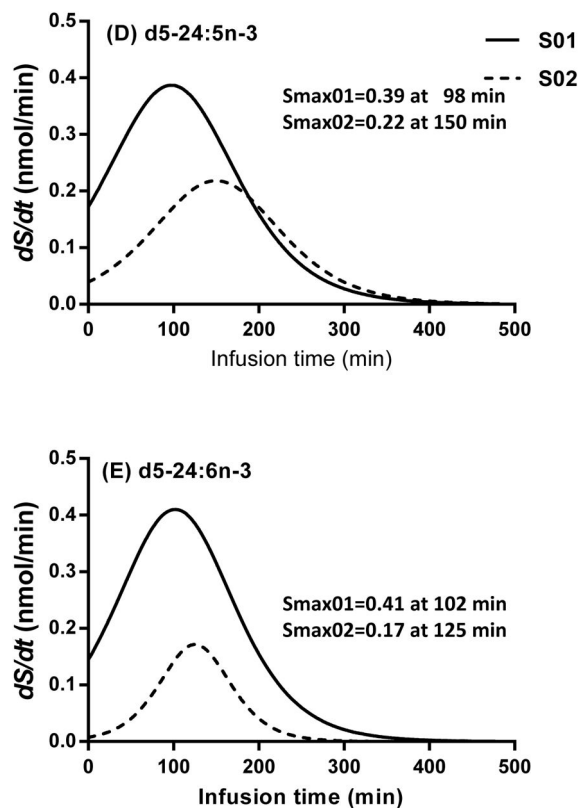Smax01=0.67 at 109 min
Smax02=0.21 at 125 min

Figure 3.
First derivatives (nmol/min) of nonlinear sigmoidal equations for the d5-n-3 metabolites synthesized from d5-18:3n-3. Values were expressed as the first derivatives multiplied by total plasma volume of each subject as a function of an infusion time over 8 h. (**3A**) d5-20:5n-3, (**3B**) d5-22:5n-3, (**3C**) d5-22:6n-3, (**3D**) d5-24:5n-3, and (**3E**) d5-24:6n-3.

Ingredients for 1000 mL

1. D5-linolenic potassium: 3.22 g

2. Water-for-Injection (WFI): 100 mL

3. Human serum albumin 25%: 900 mL

Procedures of preparation

1. Place 900 mL of WFI in a 2L beaker in water bath at 50°C

2. Add the weighed D5-linolenic acid (29.8 g), mix for 10 min

3. Gradually add albumin 8100 mL (50 to 100 mL each time)

4. Mix well

5. Filter into a sterile 13 L beaker, use a Millipak-100, 0.2 micron filter

6. Aseptically aliquot 42.5 g to 50 mL clean, sterile glass vials

7. Store vials at a refrigerator (2°C to 8°C) after candling and inspection

Diagram 1.
Preparation of D_5-linolenic acid albumin solution (3.22 mg/mL)

Table 1

Significant fatty acids in the study diet

Fatty acids	18:2n-6	18:3n-3	20:4n-6	EPA+DHA [1]	Mono-unsaturated	Saturated
Fatty acids expressed in percent of total daily energy (en%) [2]						
1	0.5	0.04	0.04	**12.3**	9.9
Fatty acids expressed in weight % of total fatty acids (wt%) [3]						
3	1.7	0.13	0.13	**41**	33

[1] EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid. Daily intake of EPA+DHA is equivalent to 100 mg.

[2] Total daily fat on the diets was 30 percent of energy consumed.

[3] A 2000 kilocalorie diet was employed for the calculation of fatty acid composition and amount in the diet. Thus, one percent of energy is equivalent to 2.2 g of fatty acids.

Table 2

Fatty acid concentrations in human plasma total lipids (nmol/mL)

Fatty acid	S01 MEAN		SD	S02 MEAN		SD
14:0	43.9	±	4.7	36.8	±	3.5
16:0	1265	±	111	1152	±	95
18:0	367	±	36	374	±	23
20:0	15.8	±	3.3	8.7	±	1.8
22:0	30.5	±	6.8	17.4	±	3.7
24:0	21.5	±	4.9	15.2	±	3.0
16:1n-7	153	±	15	91.9	±	6.0
18:1n-9	896	±	93	1011	±	106
18:1n-7	98.0	±	7.3	104	±	10.4
20:1n-9	7.8	±	0.6	9.3	±	1.0
24:1n-9	48.1	±	10.3	21.8	±	4.7
18:2n-6	1166	±	122	1340	±	112
18:3n-6	15.3	±	2.0	12.3	±	1.3
20:2n-6	8.6	±	3.1	13.6	±	1.1
20:3n-6	98.2	±	3.8	79.1	±	2.7
20:4n-6	492	±	33	268	±	10.9
22:4n-6	9.6	±	0.5	8.5	±	0.5
22:5n-6	16.8	±	4.2	8.0	±	0.9
18:3n-3	24.3	±	2.1	33.5	±	2.4
20:5n-3	16.5	±	1.3	14.2	±	0.8
22:5n-3	10.9	±	3.1	20.3	±	1.0
22:6n-3	99.0	±	6.0	51.2	±	2.6
24:5n-3	2.1	±	0.1	2.0	±	0.1
24:6n-3	2.8	±	0.2	1.5	±	0.1

SUMMARY

Fatty acid	S01				S02			
	MEAN		SD		MEAN		SD	
AA/20:3n-6 *	5.0	±	0.2		3.4	±	0.1	
DPAn-3/EPA *	0.7	±	0.1		1.4	±	0.1	
DHA/DPAn-3 *	9.4	±	1.2		2.5	±	0.1	
Saturates	1744	±	154		1604	±	123	
MUFA	1203	±	122		1238	±	126	
n-6 PUFA	1807	±	159		1729	±	126	
n-3 PUFA	617	±	38		363	±	13.9	
n-6 HUFA	156	±	11		123	±	5.9	
n-3 HUFA	131	±	9.4		89	±	4.3	

Data are mean ± SD for samples collected at 17 time points in each subject.

*
indicates the ratio of fatty acid concentrations.

Table 3

Synthesis-secretion parameters of d5–n-3 PUFAs estimated from circulating unesterified d5–18:3n-3

n-3 HUFA	Subject	Time at S_{max} (min)	S_{max} (nmol/min)	k_1 (mL/min)	J_{syn} (nmol/min)	Daily J_{syn} (μmol/d)	Daily J_{syn} (mg/d)	F (min^{-1})	$t_{1/2}$ (day)
EPA	S01	190	3.23	1.91	4.81	6.93	2.12	7.7E-05	6.2
	S02	215	2.29	1.29	6.09	8.77	2.68	1.5E-04	3.3
DPAn-3	S01	94	2.43	1.44	3.62	5.21	1.74	8.8E-05	5.2
	S02	156	4.14	2.32	11.0	15.8	5.29	1.9E-04	2.6
DHA	S01	109	0.67	0.39	0.99	1.43	0.47	2.6E-06	182
	S02	125	0.21	0.12	0.56	0.80	0.27	3.7E-06	129
TPAn-3	S01	98	0.39	0.23	0.58	0.83	0.30	7.4E-05	6.5
	S02	150	0.22	0.12	0.58	0.84	0.30	1.0E-04	4.8
THA	S01	102	0.41	0.24	0.61	0.88	0.32	5.8E-05	8.2
	S02	125	0.17	0.10	0.46	0.66	0.27	1.0E-04	4.8

[1] S01, subject 1; S02, subject 2.

[2] HUFA: long chain, highly unsaturated fatty acids with ≥ 20 carbon chains and ≥ 3 C-C double bonds.

[3] EPA, eicosapentaenoic acid; DPA, docosapentaenoic acid; DHA, docosahexaenoic acid; TPAn-3, tetracosapentaenoic acid; THA, tetracosahexaenoic acid

[4] S_{max}, maximal slope of first derivative of plasma total amount time-course curves of newly synthesized d5-n-3 HUFA.

[5] k_1, the synthesis-secretion coefficient of specific d5- n-3 HUFA.

[6] J_{syn}, whole body synthesis-secretion rates of n-3 HUFA from circulating unesterified 18:3n-3. Calculated by multiplying k_1 by unesterified ALA concentration, 2.52 nmol/mL for S01 and 4.74 nmol/mL for S02.

[7] F, turnover of esterified n-3 HUFA, calculated by dividing J_{syn} (nmol/min) by amount of HUFA in plasma total lipids (nmol) (Table 2)

[8] $t_{1/2}$, half-life of esterified n-3 HUFA derived from unesterified ALA in plasma