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Development and validation of a sensitive and selective UHPLC–MS/MS method for simultaneous determination of both free and total eicosapentaeonic acid and docosahexenoic acid in human plasma

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ABSTRACT

A sensitive, selective, and quantitative method for the simultaneous determination of free and total eicosapentaeonic acid (EPA) and docosahexenoic acid (DHA) has been developed and validated in human plasma using fatty acid free human serum albumin as a surrogate matrix. Clean-up for free EPA and DHA employs a liquid–liquid extraction with hexane to remove plasma interferences and provide for cleaner chromatography. The method for total EPA and DHA requires a digestion of the triglycerides followed by liquid–liquid extraction with hexane. Ultra high performance liquid chromatography (UHPLC) technology on a BEH C18 stationary phase column with 1.7 μm particle size was used for chromatographic separation, coupled to tandem mass spectrometry (UHPLC–MS/MS). The method for free EPA and DHA was validated over the concentration range of 0.05–25 μg/mL, while total EPA and DHA concentration range was 0.5–250 μg/mL. The results from assay validation show that the method is rugged, precise, accurate, and well suited to support pharmacokinetic studies. To our knowledge, this work represents the first UHPLC–MS/MS based method that combines both free and total EPA and DHA with a relatively small sample volume (25 μL aliquot) and a run time of 1.5 min, facilitating automation and high throughput analysis.

1. Introduction

Beginning as early as the 1950s, researchers were examining the effects of fish oil on serum cholesterol concentration in patients with atherosclerosis, and discovered that sardine oil had similar effects and also lowered serum triglyceride concentrations [1]. After epidemiologic studies in the 1970s showed that fish-eating populations suffered less cardiovascular disease, the benefits of omega-3 fatty acids were further investigated [1]. Numerous studies have substantiated the link between certain fish oils and their desirable effects on cardiovascular, inflammatory diseases, hypertriglycerdemia disorders, as well as certain types of cancer [1,2]. Furthermore, dietary intake of omega-3 (ώ3) fatty acids, specifically eicosapentaeoic (EPA) and docosahexaenoic acids (DHA), are believed to be responsible for the benefits. It has also been shown that ώ3 fatty acids are essential nutrients in humans; however there has been much debate and conflicting data to illustrate their absolute effectiveness [3]. Ethyl esters of these fatty acids are able to be purified, concentrated and utilized for clinical therapeutics. Potential mechanisms of action include inhibition of acyl CoA: 1,2-diacylglycerol acyltransferase, increased mitochondrial and peroxisomal β-oxidation and decreased lipogenesis in the liver, and increased plasma lipoprotein lipase activity [4]. Ethyl esters of EPA and DHA may reduce the synthesis of triglycerides (TG) in the liver as these ώ3 fatty acids are poor substrates for the enzyme responsible for TG synthesis, and inhibit esterification of other fatty acids [4]. Even though the ethyl esters of EPA and DHA are generally well tolerated in humans, some patients can develop adverse reactions including: back pain, flu-like symptoms, infections, angina pectoris, dyspepsia, eructation, skin rashes and taste perversion [4]. Therefore, in order to better monitor the clinical efficacy of EPA and DHA therapy a rapid, sensitive, and accurate bioanalytical method for the analysis of EPA and DHA is required.

Published methods for the analysis of EPA and DHA include sample extraction using thin layer chromatography (TLC) [2], derivatization [2,3,5–11], lipase hydrolysis [5,7,9,12,13], liquid–liquid extraction (LLE) [2,3,5–9,11,12,14], solid phase extraction (SPE) [15,16] or a combination of these procedures with either enzymatic, gas chromatography (GC) or mass spectrometric (MS) detection. To date, a liquid chromatography (LC) method has not been presented with retention times of less than 3 min, to allow for high throughput analysis, or sensitivity. Methods with enzymatic detection [2,8,17]

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often require a sample volume of at least 1000 μL of erythrocytes, thus making it difficult for automation and high throughput analysis. Methods with GC detection often require a sample volume of at least 100 μL of plasma and an analysis run time exceeding 30 min, precluding high throughput analysis [2,3,5–12]. MS detection provides increased selectivity and signal to noise ratio, allowing a reduction in sample volume and the analysis time. Methods for the analysis of EPA and DHA using LC–MS/MS detection have been reported with a plasma volume of at least 100 μL and run time of 15 min [7,14–16]. Based on the endogenous levels within subjects, many of these methods include a correction factor, or endogenous baseline subtraction, to account for endogenous levels of EPA and DHA in the matrix being used for the preparation of standards and quality controls.

The development of a method for analysis of EPA and DHA at physiological levels using MS represents a challenge based on the high endogenous levels of these fatty acids, as well as the vast variability between subjects due to dietary intake and genetics. These factors further complicate the ability to accurately measure EPA and DHA concentrations in study subjects. Accurate quantitation requires a series of calibration standards and quality control samples (QCs) to be prepared in an analyte free or minimal analyte matrix. Therefore the traditional control matrices which would be acquired for standard curve preparation would already contain variable and potentially high levels of these endogenous components. Due to this complicating factor, a surrogate matrix (EPA- and DHA-free) was required.

After initial attempts to prepare a solvent-based calibration curve, screening and charcoal stripping plasma, essentially fatty acid free, ~99% (as determined by agarose gel electrophoresis), lyophilized powder human serum albumin (HSA) was selected and authenticated as a suitable surrogate matrix for this assay. All calibration standards and QCs are prepared in this matrix. Additional information could be gained from clinical study samples if the differential contribution between free and total (bound to the TG) EPA and DHA fatty acids could be distinguished. Therefore the goal of this investigation was to optimize and develop a method suitable for both determinations.

The final method involves two aliquots from study samples processed in parallel. In the first, the extraction of free EPA and DHA is performed in plasma at acidic pH (pH 4.0) with LLE using hexane. In a second aliquot, the fatty acids are digested off the TG backbone using acetonitrile and hydrochloric acid (6 N) at 104 °C for 45 min, followed by addition of methanol and sodium hydroxide (10 N) at 104 °C for 45 min. The digested samples are then acidified (pH 2.5) and extraction of all resulting total fatty acids is then performed with hexane. It is important to note that while both extraction procedures were optimized for EPA and DHA, all of the other endogenous fatty acids are processed simultaneously. However, the selectivity of the mass spectral method monitors for only the desired analytes, EPA and DHA, without contribution from the other fatty acids.

The relatively small plasma volume (25 μL ×2) used here allows sample extraction in the 96-well format increasing throughput analysis, as compared to previously validated methods. Moreover, the relatively short run time of 1.5 min allows for the analysis of about 300 samples per day.

2. Experimental

2.1. Chemicals and reagents

Ammonium acetate, and formic acid were purchased from EMD (Gibbstown, NJ, USA). [$^2H_5$]-EPA, DHA and [$^2H_5$]-DHA were purchased from Cayman Chemical Corporation (Ann Arbor, MI, USA). EPA, DHA, fatty acid free human serum albumin, hydrochloric acid (HCl) and sodium hydroxide (NaOH) were purchased from Sigma–Aldrich (St. Louis, MO, USA). HPLC grade acetonitrile, hexane, dimethylformamide, isopropanol, and methanol were purchased from Burdick and Jackson (Muskegon, MI, USA). Human plasma was obtained from Bioreclamation Inc. (East Meadow, NY, USA) and screened for minimal EPA and DHA contribution prior to use.

2.2. Equipment

An Eppendorf 5810R centrifuge with a rotor capacity for four 96-well plates (Brinkmann Instrument, Westbury, NY, USA), and a Mettler UMX2 balance (Hightown, NJ, USA) were used. A Hamilton Mircrolab STAR liquid handler (Reno, NV, USA) was used for plasma transfer and a TomTec Quadra 3 SPE (Hamden, CT, USA) was used for liquid transfer. Arctic White LLC 96-well round 2 mL polypropylene plates and ArctiSeal silicone mats with PTFE film (Bethlehem, PA, USA) were used for the LLE extractions from either plasma (free analysis) or digested plasma (total analysis). Micronic 1.4 mL tubes and TPE push caps, fitted into CoMo rack with lid (McMurray, PA, USA), were used for the digestion procedure for total analysis. One milliliter silanized glass vials along with 96-well plate covers (Blue CapMat with Pre-Cut T/S Septa) from MicroLiter Analytical Supplies (Suwanee, GA, USA) were used for sample introduction to the UHPLC–MS/MS. An ACQUITY^{TM} UPLC integrated system from Waters (Milford, MA, USA), consisting of an sample manager combined with a sample organizer capable of holding ten 96-deep well plates, and a binary solvent manager were used. A triple quadrupole mass spectrometer API 4000 (Applied Biosystems/MDS-Sciex, Concord, Ontario, Canada) was used.

2.3. Preparation of calibration standards and quality control (QC) samples

Stock solutions of EPA and DHA were individually prepared in dimethylformamide (DMF) at final concentrations of 20 mg/mL. Both stable label internal standards, [$^2H_5$]-EPA and [$^2H_5$]-DHA, were received at concentrations of 500 μg/mL in acetonitrile and were diluted to a working concentration of 500 ng/mL in acetonitrile. All stock solutions and working solutions were stored at −20 °C until use. For determination of free EPA and DHA, stock solutions were used to prepare working solutions for calibration standards (WS) and QC (WQ) containing EPA and DHA at a concentration of 1 mg/mL in 50/50 (v/v) acetonitrile/water. For determination of total EPA and DHA the stock solutions in DMF were spiked directly into fatty acid free serum albumin for preparation of calibration standards or serum and/or plasma for QC samples.

The WS or stocks were used to make calibration standards in fatty acid free serum albumin at 25, 10, 5, 2, 0.5, 0.25, 0.125 and 0.05 μg/mL for analysis of free EPA and DHA, and 250, 100, 50, 20, 5, 2.5, 1.25, and 0.5 μg/mL for analysis of total EPA and DHA using a serial dilution procedure. The WQ or stocks were used to make QC samples, in fatty acid free serum albumin at 25, 20, 2.5, 0.2 and 0.05 μg/mL for the analysis of free EPA and DHA, and 250, 200, 25, 2 and 0.5 μg/mL for the analysis of total EPA and DHA. The WQ or stocks were also used to make QC samples in human plasma. However, these concentrations were prepared at 25, 20, 2.5, 0.2 and 0.05 μg/mL for the analysis of free EPA and DHA, and 250, 200, 25, 2 and 0.5 μg/mL for the analysis of total EPA and DHA above the level of endogenous compounds already present in the plasma. The endogenous levels within blank plasma were quantified, and standard addition calculations were used to determine the nominal concentration of the plasma QC samples. QC samples were divided into 0.5 mL aliquots and frozen at −20 °C or extracted

immediately. In the first validation run, fresh QC samples prepared in serum albumin or plasma were analyzed against fresh calibration standards prepared in serum albumin. For each subsequent validation run, frozen replicate aliquots of the QC samples prepared in serum albumin and plasma were thawed at room temperature and analyzed against a freshly prepared standard curve prepared in serum albumin.

2.4. Sample preparation for the analysis of free EPA and DHA

Hexane (0.5 mL) was added to each well of the 2 mL ArcticWhite 96-well polypropylene plate. The plate was sealed with the ArctiSeal mat and vortex-mixed in an inverted position for approximately 3 min. Subsequently, the hexane was discarded and the plate was left to dry in a chemical hood. This wash step was used to remove any plastic residue from the plates and plate seals. Serum albumin or plasma samples (25 μL) were transferred to the washed 96-well plate using a Hamilton STAR liquid handler. A 50 μL aliquot of internal standard solution (500 ng/mL of [$^2H_5$]-EPA, and [$^2H_5$]-DHA in acetonitrile) was added to all tubes with the exception of the blanks, which instead received 50 μL of acetonitrile. A 100 μL aliquot of 0.1% formic acid and then added to all wells, followed by caps and vortex-mixed for approximately 1 min. After mixing, 600 μL of hexane was added to all wells to extract EPA, DHA and their internal standards. After vortex-mixing and centrifugation, 250 μL of the hexane layer was transferred to a 2 mL polypropylene 96-well plate containing silanized 1 mL glass-inserts using a TomTec liquid handler and evaporated under a stream of nitrogen at 45 °C. The extract then was reconstituted in 250 μL of 50/50 (v/v) acetonitrile/water.

Free extraction efficiency for EPA was determined to be approximately 81% and 88% in serum albumin and human plasma, respectively. For DHA, the extraction efficiency was determined to be approximately 83% and 93% for serum albumin and human plasma, respectively.

2.5. Sample preparation for the analysis of total EPA and DHA

Hexane (0.5 mL) was added to each well of the 2 mL ArcticWhite 96-well polypropylene plate. The plate was sealed with the ArctiSeal mat and vortex-mixed in an inverted position for approximately 3 min. Subsequently, the hexane was discarded and the plate was left to dry in a chemical hood. This wash step was used to remove any plastic residue from the plates and plate seals. Serum albumin or plasma samples (25 μL) were transferred to a plate containing micronic 1.4 mL tubes using a Hamilton STAR liquid handler. A 150 μL aliquot of internal standard solution (500 ng/mL of [$^2H_5$]-EPA, and [$^2H_5$]-DHA in acetonitrile) was added to all tubes with the exception of the blanks, which instead received 150 μL of acetonitrile. Following on the procedure from Lagerstedt et al. [5], the plate was capped and vortex-mixed for approximately 1 min. After mixing 200 μL of 90/10 (v/v) acetontrile/6 N HCl was added to all tubes, capped, covered with the CoMo rack lid, and the plate was vortex-mixed for approximately 1 min. The plate was then incubated at approximately 104 °C for approximately 45 min. After allowing the plate to cool, it was centrifuged for approximately 5 min at approximately 3220 × *g*, followed by the addition of 200 μL of 90/10 (v/v) methanol/10 N NaOH. The plate was capped, covered with the CoMo rack lid, and vortex-mixed for approximately 1 min. The plate was then incubated at approximately 104 °C for approximately 45 min. After allowing the plate to cool, it was centrifuged for approximately 5 min at approximately 3220 × *g*, and 200 μL of supernatant was transfer to the ArcticWhite 96-well plate followed by the addition of 100 μL 6 N HCl. After mixing, 600 μL of hexane was added to all wells to extract EPA, DHA and their internal standards. After vortex-mixing and centrifugation, 200 μL of the hexane layer was transferred to a 2 mL polypropylene 96-well plate containing silanized 1 mL glass-inserts using a TomTec liquid handler and evaporated under a stream of nitrogen at 45 °C. The extract then was reconstituted in 300 μL of 50/50 (v/v) acetonitrile/water.

Total extraction efficiency for EPA was determined to be approximately 79% and 84% in serum albumin and human plasma, respectively. For DHA, the extraction efficiency was determined to be approximately 84% and 89% for serum albumin and human plasma, respectively. A commercially available (Supelco), menhaden fish oil triglyceride standard was also used to assess total extraction recovery. This solution was spiked into serum albumin and then extracted to determine efficiency of the hydrolysis reaction. The recovery from this triglyceride reference standard was 75% and 94% for EPA and DHA, respectively.

2.6. Chromatographic conditions for EPA and DHA

The analytical column used was a BEH C18, 2.1 mm × 50 mm with 1.7 μm particle size, from Waters. The column temperature was maintained at 60 °C and the sample compartment was maintained at room temperature. Mobile phase A consisted of 2 mM ammonium acetate, pH 4 and mobile phase B was acetonitrile. The UHPLC system was held at 60% B for 0.4 min followed by a linear gradient from 60% B to 90% B for 0.8 min. The conditions were then was held at 90% B until 1.45 min to remove late eluting substances from the column, after which the system was returned to initial conditions. The total run time including the sample load was approximately 1.5 min and the flow rate was maintained constant at 1 mL/min throughout the run. A typical injection volume of 2 μL in a 10 μL loop (partial loop injection mode) was used. Injection carry-over was assessed with each analytical run by inclusion of a HLQ sample followed by an injection of a blank. No significant carry-over was noted with any analytical run.

2.7. Mass spectrometric conditions

An API 4000 with a TurboIonspray interface (TIS) was operated in the negative ionization mode. The instrument was optimized for EPA, DHA, [$^2H_5$]-EPA, and [$^2H_5$]-DHA by infusing a 10 ng/mL solution of purified stocks in acetonitrile/water (50/50, v/v) at 500 μL/min through an Agilent pump 1100 series (Palo Alto, CA, USA) directly connected to the mass spectrometer. The MRM transitions of *m/z* 301–257, *m/z* 327–283, *m/z* 306–262, and *m/z* 332–288 were chosen for EPA, DHA, [$^2H_5$]-EPA, and [$^2H_5$]-DHA, respectively. The optimized mass spectrometric conditions for EPA and DHA included the following conditions: TIS source temperature, 700 °C; TIS voltage, −4500 V; curtain gas, 20 psi (nitrogen); nebulizing gas (GS1), 60 psi (zero air); TIS gas (GS2), 70 psi (zero air); collision energy for EPA and DHA, were −18 eV; declustering potential −60 eV. However the collision energy was detuned to achieve linearity across the calibration range. The collision energy values used in this study were −13 eV for EPA and −11 eV for DHA.

2.8. Data analysis

MS data were acquired and processed (integrated) using the proprietary software application Analyst™ (Version 1.4.2, Applied Biosystems/MDS-Sciex, Canada). Calibration plots of analyte/internal standard peak area ratio versus EPA and DHA concentrations were constructed and a weighted $1/x^2$ linear regression was used for both analytes. Concentrations of EPA and DHA in QC samples were determined from the appropriate calibration line, and used to calculate the bias and precision of the method with an in-house LIMS (Study Management System, SMS2000, version 2.1, GlaxoSmithKline).

3. Results and discussion

3.1. Challenges during method development

The objective was to develop a rugged, sensitive, and relatively high throughput UHPLC–MS/MS method allowing simultaneous determination of EPA and DHA (free and total) with a run time of less than 2 min. The first obstacle to overcome was the variable and elevated endogenous levels of poly unsaturated fatty acids (PUFAs) in plasma. Secondly, existing assays for DHA and EPA involve chemical derivatization to form methyl esters followed by quantification using GC–MS with run times often exceeding 5 min. And finally, in performing a validation with a surrogate matrix, a direct comparison of QC results from serum albumin and plasma was completed to ensure validity of the method. The approaches used to resolve these challenges are discussed in detail below.

3.2. Elevated and variable endogenous levels of PUFA

Upon initiating development for a human method, it was quickly noted the endogenous levels of EPA and DHA were much higher than the expected assay LLOQ (approximately 0.4/30 and 0.6/10 μg/mL for free/total EPA and DHA, respectively) and up to a 100-fold difference in concentrations was noted between subjects. Based on these observations screening innumerable lots of human plasma for the lowest endogenous levels was not viable, therefore we sought to identify an appropriate surrogate matrix.

Initial attempts in identifying an appropriate surrogate matrix were to prepare the calibration standards and QC samples in acetonitrile/water and compare those to QC samples prepared in human plasma. As one might expect, linearity and reproducibility were acceptable for both analytes, but accuracy values from plasma QCs were approximately 40% above theoretical values for EPA when quantified against the acetontrile/water curve. Due to the lack of matrix effects, as indicated through the infusion experiment, the source of this error is unknown. Therefore this approach was deemed inappropriate.

Using charcoal, attempts were made to strip the fatty acids from the human plasma prior to use; both activated carbon SPE cartridges and bulk carbon were investigated. Due to the volume of the SPE cartridges and the volume of plasma that could be cleaned at any given batch volume, this approach was not viable. Alternatively, activated carbon was mixed at a ratio of 1.5 g of carbon to every 10 mLs of plasma and placed in a glass bottle and sealed. The glass bottle was cleaned with hexane prior to addition of plasma and allowed to dry. The plasma/carbon mixture was vortex mixed for 1 h. The plasma was then filtered using a 0.2 μm filtering apparatus. In the end, this approach was found to remove nearly all of the endogenous free DHA and EPA from the plasma, but unfortunately had no effect on the total levels of the fatty acids still bound to the TG backbone.

Fatty acid free human serum albumin (HSA) was then investigated for its applicability as a surrogate matrix. It was prepared from essentially globulin free albumin and received as a lyophilized powder which was is then reconstituted in deionized water at 40 mg/mL. This concentration is consistent with normal serum albumin concentrations which range from 3.5 to 5 g/dL [18]. Based on positive preliminary data, human serum albumin was used for all method development and validation going forward; in the end its use allowed for a method with acceptable accuracy and precision values across all calibration ranges.

3.3. Development of a LC–mass-spectrometer suitable assay

Method development for the isolation of free EPA and DHA investigated solid-phase (SPE) and liquid–liquid extraction (LLE). After several optimization experiments an LLE with hexane after acidification of the plasma samples was decided upon due to analyte recovery, ease of automation, and speed associated with this technique.

Most of the literature citations for isolation and detection of total EPA and DHA involve chemical derivatization to form methyl-esters followed by quantification, utilizing GC–MS. Following the procedure reported by Lagerstedt et al. [5] for digestion of the TGs to release the bound fatty acids from the triglyceride backbone, a LC friendly, MS based assay without chemical derivatization was developed. This method consisted of an acidic extraction, followed by basic digestion at 104 °C to hydrolyze the poly-unsaturated fatty acids into their chain form. Following digestion, samples were acidified and clean-up was performed using LLE with hexane, thereby enabling detection using a purified extract. Utilizing a commercially available Menhaden fish oil triglyceride reference standard, digestion/extraction efficiency for the total assay was approximately 97% and 75% for DHA and EPA, respectively (data not shown).

The UHPLC technology, on a BEH C18 stationary phase column with 1.7 μm particle size, allowed resolution between EPA and DHA with a run time of 1.5 min. The same UHPLC conditions were used for both the free and the total assay. During method development a sensitivity gain of approximately 4-fold was observed by changing the acid modifier from formic to acetic acid in the mobile phase. A peak-to-peak resolution of approximately 2.9 was achieved. The retention factor (k) values were approximately 3.3 and 4.6 for EPA and DHA, respectively. The peak width values at the base of the peaks were approximately 2.1 s for both EPA and DHA.

While utilizing UHPLC separation to increase efficiency of this method, care was taken to ensure that there were no co-eluting peaks, or other endogenous components that would potentially interfere with accurate quantitation of EPA and DHA. In order to evaluate these phenomena, the slope to the gradient was decreased and the run time was extended to 60 min. Peak area ratios were monitored and compared between the 1.5 and 60 min gradient for the serum albumin and plasma QC samples, along with incurred samples. Virtually no effect on the respective response factors were noted for these various analytes.

3.4. Validation of conjugate matrix method with direct comparison to plasma

Upon completion of method development (conjugate matrix selection and digestion optimization) a two-tiered validation was completed to ensure that serum albumin can be utilized as an appropriate surrogate matrix. This validation consisted of standard calibration curves prepared in only serum albumin, with preparation of quality control samples in both serum albumin and human plasma (containing endogenous levels of EPA and DHA). The endogenous levels were quantified and the theoretical values were determined based on standard addition calculations. Figs. 1 and 2 illustrate the level of endogenous interference in a blank serum, plasma blank, and lower limit of quantification (LLOQ) sample from both the free and total assay.

As illustrated in Fig. 3, the slopes of the calculated line equations between the surrogate matrix and plasma are similar, signifying that the ionization and extraction efficiency of both matrices are comparable. Based on acceptance of the QC samples, illustrated in Table 1, standard additional concentrations above endogenous levels are accurate.

3.5. Selectivity and linearity

The characteristic precursor $[M-H]^-$ to product ion transitions, m/z 301–257, m/z 328–283, m/z 306–262, and m/z 322–288 are consistent with the structures of the EPA, DHA, [$^2H_5$]-EPA,

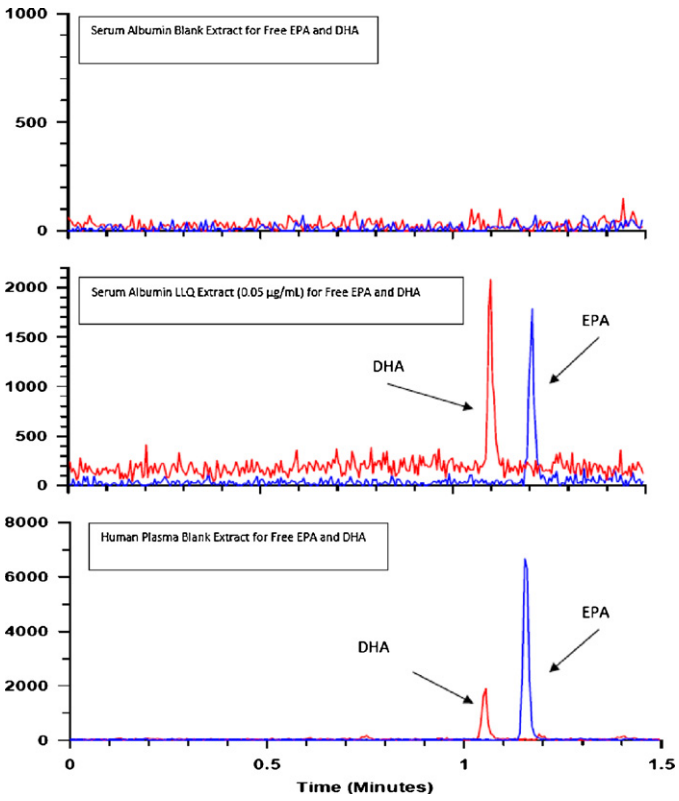

Fig. 1. Endogenous contribution and LLOQ chromatograms for free assay.

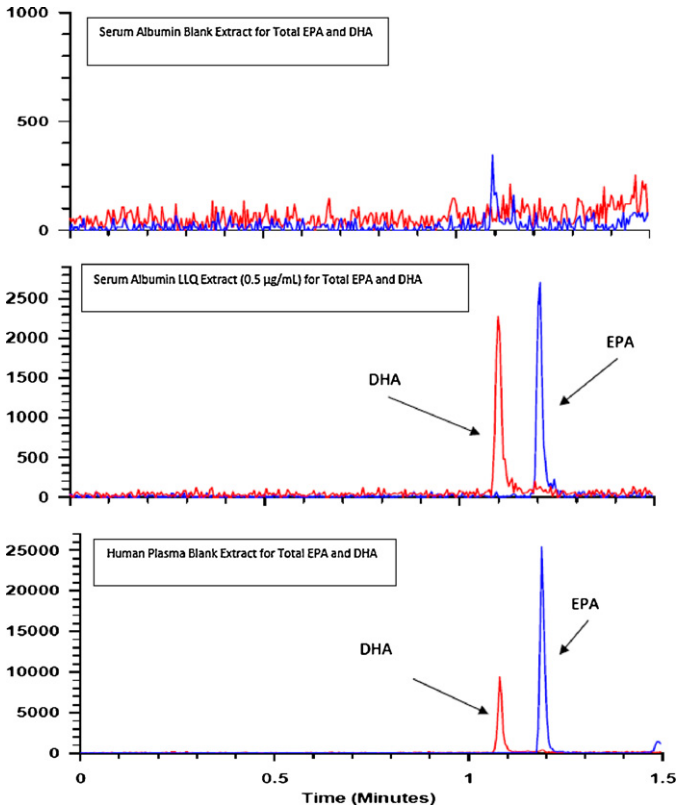

Fig. 2. Endogenous contribution and LLOQ chromatograms for total assay.

and [$^2H_5$]-DHA, respectively. The selectivity of the method was established by the analysis of samples of control human blood from 6 individual volunteers for both the free and total assay. UHPLC–MS/MS chromatograms of the blanks and QC samples were visually examined and compared for chromatographic integrity and potential interferences. Representative UHPLC–MS/MS chromatograms for EPA and DHA at the limit of quantification (LOQ) are shown in Figs. 1 and 2. No unexpected interferences at the retention times of EPA, DHA, or the internal standards were observed. Post-column infusion experiments were also performed in order to investigate potential ion suppression effects from endogenous plasma interferences on the MRM transition of the studied transitions (data not shown). For this purpose, a control treated plasma sample was extracted according to the validated method and injected into the UHPLC system as described previously. A continuous post-column infusion at 20 μL/mL of a solution containing 10 ng/mL EPA and DHA was used. No evidence of ion suppression or enhancement was observed at the retention times of the investigated compounds.

The linearity of the method was evaluated by analyzing eight calibration standards in duplicate over the nominal concentration ranges of 0.05–25 μg/mL for free EPA/DHA and 0.5–250 μg/mL for total EPA/DHA. The correlation coefficients obtained using $1/x^2$ weighted linear regression were better than 0.9967 and 0.9960 for free EPA and DHA, respectively and 0.9932 and 0.9972 for total EPA and DHA, respectively.

3.6. Bias and precision

At all QC concentrations examined, the bias and precision values were less than 15% over all six validation analytical runs (see

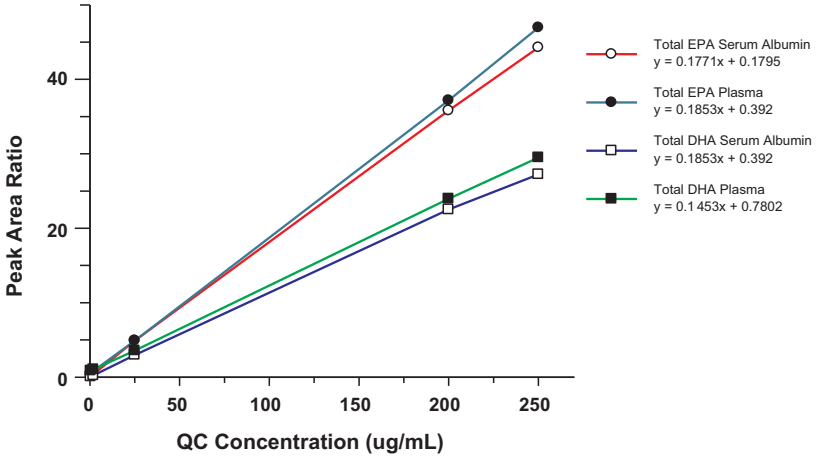

Fig. 3. Comparison of slopes for plasma and serum albumin for total assay.

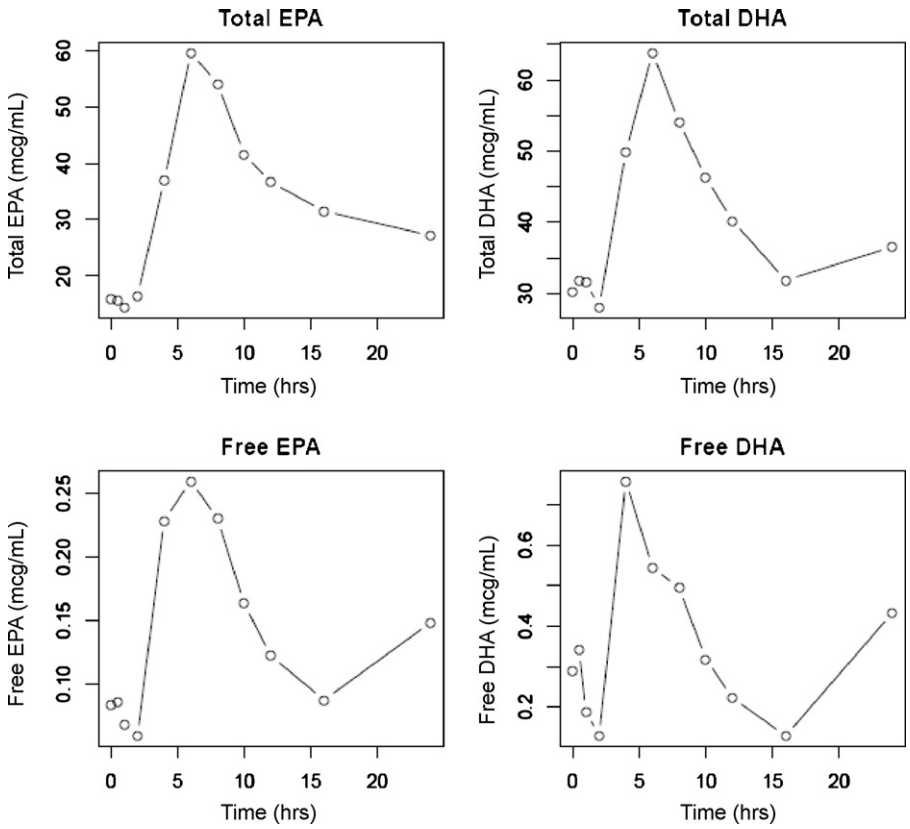

Fig. 4. Typical PK profiles for free/total EPA/DHA in human plasma from a recent clinical study.

Table 1). The nominal concentration for the plasma QCs were calculated based on the standard addition method after quantifying the endogenous contribution. As defined by the lower and upper validation sample concentrations possessing acceptable accuracy and precision, the validated range of this method based on 25 μL of EDTA human plasma is 0.05–25 μg/mL and 0.5–250 μg/mL for free EPA/DHA and total EPA/DHA, respectively.

3.7. Stability of EPA and DHA in biological matrix

The stability of EPA/DHA in spiked human serum albumin and plasma samples stored at room temperature were assessed at 0.2/20 μg/mL for the free assay and 2/200 μg/mL for the total assay (in replicates of six) by comparing the mean concentrations of samples extracted after storage for 24 h against those of the sam-

Table 1
Bias, precision and mean QC sample concentrations for free and total EPA and DHA in human plasma and serum albumin.

	Free EPA in serum albumin					Free EPA in human plasma				
Concentration (μg/mL)	0.05	0.125	2.5	20	25	0.1248	0.2748	0.25748	20.0748	25.0748
Mean	0.0495	0.2077	2.5783	19.9776	24.5992	0.1246	0.2826	2.5824	19.6675	25.2989
Average bias (%)	−0.9	3.8	3.1	−0.1	−1.6	−0.1	2.9	0.3	−2.0	0.9
Between-run CV (%)	2.1	0.9	1.0	3.7	2.3	7.4	5.8	4.8	2.9	5.2
	Free DHA in serum albumin					Free DHA in human plasma				
Concentration (μg/mL)	0.05	0.125	2.5	20	25	0.3251	0.4751	2.7751	20.2751	25.2751
Mean	0.0492	0.2165	2.6309	20.0519	24.4267	0.3197	0.4798	2.7779	19.7912	25.3265
Average bias (%)	−1.7	8.2	5.2	0.3	−2.3	−1.7	1.0	0.1	−2.4	0.2
Between-run CV (%)	0.8	0.6	3.4	4.0	4.6	5.8	6.6	6.3	4.5	4.5
	Total EPA in serum albumin					Total EPA in human plasma				
Concentration (μg/mL)	0.5	1.25	25	200	250	2.983	4.483	27.483	202.483	252.483
Mean	0.498	2.036	26.449	186.27	230.809	3.061	4.258	25.111	196.905	246.341
Average bias (%)	−0.4	1.8	5.8	−6.9	−7.7	2.6	−5.0	−8.6	−2.8	−2.4
Between-run CV (%)	4.7	1.0	3.6	2.6	2.4	8	9.2	5.8	4.6	4.6
	Total DHA in serum albumin					Total DHA in human plasma				
Concentration (μg/mL)	0.5	1.25	25	200	250	7.34	8.84	31.84	206.84	256.84
Mean	0.534	2.026	25.32	188.735	234.58	7.452	8.924	30.726	206.943	255.007
Average bias (%)	6.7	1.3	1.3	−5.6	−6.2	1.5	1.0	−3.5	0.0	−0.7
Between-run CV (%)	1.0	4.0	2.6	4.3	5.1	4.6	6.6	4.3	4.8	4.7

Table 2
Maximum bias observed (%) during stability assessment.

	24 h ambient temperature	3-Cycle freeze–thaw	Process extract
Free DHA plasma	12.8	−5.6	−1.9
Free DHA serum albumin	11.4	6.5	7.1
Free EPA plasma	9.7	4.4	5.3
Free EPA serum albumin	5.2	4.3	8.9
Total DHA plasma	−5.6	−7.4	−9.9
Total DHA serum albumin	−7.6	−8.0	−8.1
Total EPA plasma	−4.9	8.4	6.8
Total EPA serum albumin	−9.8	−6.6	−8.2

Values calculated versus measured values on day 0.

ples extracted immediately upon thawing. The difference between the stored QC samples and the reference QC samples and CV (%) between replicates was less than 15%, which indicates EPA and DHA are stable in human serum albumin and plasma after storage at room temperature for at least 24 h. Table 2 illustrates the maximum bias observed during assessment of stability in biological matrix.

3.8. Stability of free and total EPA and DHA during freeze–thaw cycles

The stability of EPA/DHA in human serum albumin and plasma after three freeze–thaw cycles from −20 °C to room temperature were assessed at 0.2/20 μg/mL for the free assay and 20/200 μg/mL for the total assay (in replicates of six) by comparing the mean concentrations against those of the freshly prepared spiked samples. The difference between the freeze–thaw samples and the freshly spiked serum albumin or plasma expressed as percent difference and CV (%) between replicates was less than 15% which indicates that both EPA and DHA are stable in human serum albumin and plasma after at least three freeze–thaw cycles from −20 °C to room temperature. Table 2 illustrates the maximum bias observed during assessment of stability in biological matrix.

3.9. Matrix dilution

The ability to dilute samples containing EPA/DHA at concentrations above the HLQ was demonstrated by performing 6 replicate 10-fold dilutions of human serum albumin and plasma samples spiked at 200 μg/mL for the free assay and 1000 μg/mL for the total assay. In all cases the samples were diluted with human serum albumin. Concentrations of EPA and DHA in these matrix dilution samples were determined and corrected for the dilution factor. The bias and within-run precision values were less than 15% indicating that a 10-fold dilution of human serum albumin and plasma samples, using human serum albumin as the diluent containing EPA and DHA above the HLQ is valid.

3.10. Stability in processed samples

The stability of EPA and DHA in processed samples from the free assay of human serum albumin and plasma was assessed by re-injecting validation run 1 after storage at room temperature for 72 h, against freshly prepared calibration standards. The accuracy, precision and sensitivity of these samples were found to be acceptable on re-injection, indicating that the processed samples for the free assay were stable when stored at room temperature for at least 72 h. The stability of EPA and DHA in processed samples from the total assay of human serum albumin and plasma was assessed by re-injecting validation run 2 after storage at room temperature for 24 h, against freshly prepared calibration standards. The accuracy, precision and sensitivity of these samples were found to be acceptable on re-injection, indicating that the processed samples for the total assay were stable when stored at room temperature for at least 24 h. Table 2 illustrates the maximum bias observed during assessment of stability in biological matrix.

3.11. Accuracy determination using standard addition method

To ensure the accuracy of results from incurred study samples as determined by the standards and QC from an *in vitro* preparation, potential interferences from metabolites or endogenous fatty acids, protein binding, and matrix effects, are unknown and need to be evaluated. Additionally, this method utilized a surrogate matrix for preparation of calibration standards and quality control samples. Therefore, it is necessary to confirm the accuracy of the data for EPA and DHA in incurred samples using an additional method; where a standard addition method was used [19]. The concentrations of EPA and DHA from a pooled incurred sample were determined using the validated method (referred to as 'actual concentration'). Then known quantities of EPA and DHA were added *ex vivo* to this pooled sample to create a "calibration line" as outlined in Section 2.3 for both free and total EPA and DHA. These spiked "standards" were extracted and analyzed using the validated method. The analyte/internal standard peak area ratios were plotted against the added concentrations of EPA and DHA using GraphPad Prisma 5 software and linear regression was performed with $1/x^2$ weighting. The standard addition lines then were extrapolated to the x-intercept, where this value should represent the actual concentration of EPA and DHA in the pooled incurred sample. When comparing the extrapolated value (x-intercept) with the actual measured concentration for free and total EPA and DHA, the percent difference was less than 10% in all cases. The excellent agreement between the extrapolated and actual concentrations shows that the validated method using human serum albumin as the surrogate matrix is accurate for determination EPA and DHA concentrations in incurred samples.

3.12. Application of validated method to clinical study

Based on the before mentioned information and statistics, this method was validated to internationally accepted criteria of robustness, selectivity, and stability; and was therefore deemed appropriate to support clinical studies where the levels of EPA and DHA were to be monitored. The most recent application of this method for clinical study support involved analysis of greater than 2800 distinct samples which were analyzed for a total of 11,556 analytical results (free- and total-EPA and DHA), over 40 analytical batches of samples; a breakdown of the quality control statistics are outlined in Table 3. Over the course of this entire study, only 14 samples (representation of 0.12% of total study samples) required repeat analysis because the measured concentration was incongruous with the anticipated drug plasma concentration versus time profile. This low, required repeat percentage value, further signifies

Table 3
Bias, precision and mean study QC sample concentrations for free and total EPA and DHA in serum albumin from a recent clinical study.

	Total EPA in serum albumin			Total DHA in serum albumin		
Concentration (μg/mL)	2	25	200	2	25	200
Mean	2.04	24.82	1356.14	2.06	26.16	1479.11
Average bias (%)	1.9	−0.7	578.1	3.2	4.7	639.6
n	96	96	96	96	96	96
Between-run CV (%)	3.5	1.4	4.1	4.8	4.9	6.3
	Free EPA in serum albumin			Free DHA in serum albumin		
Concentration (μg/mL)	0.2	2.5	20	0.2	2.5	20
Mean	0.203	2.489	18.791	0.198	2.477	18.979
Average bias (%)	1.4	−0.4	−6	−0.7	−0.8	−5.1
n	106	106	106	106	106	106
Between-run CV (%)	4.4	1.2	3.3	3.1	2.8	2.7

the validity of the analytical method. A representative PK profile for all monitored analytes is represented in Fig. 4.

3.13. Incurred sample reproducibility

As mandated by various regulatory authorities, methods must be investigated for incurred sample reproducibility (ISR), where a certain percentage of study samples are reanalyzed using the method some time period after the original analysis. The time interval between the original and reanalysis should be within acceptable stability limits for the analytes. Selection of the incurred samples should provide representation across subjects, dose cohorts and PK time points, and re-analyzed as complete PK profiles when possible. To be compliant with departmental standard operating procedures (SOP), approximately 10% of the total study samples or a maximum of 200 samples (excluding controls and placebos) were selected.

The evaluation of bioanalytical methods through the reanalysis of incurred samples can be taken as one additional measure of assay reproducibility. This reanalysis expands upon the data collected during the validation of the method, since incurred samples may contain elements that are not present in the standards and quality control samples used during validation experiments. Fig. 5 shows a representative plot of the ISR results for total DHA. ISR results for total EPA as well as free EPA and DHA were all within the acceptable limits for ISR (data not shown) indicating assay reproducibility, stability and ruggedness.

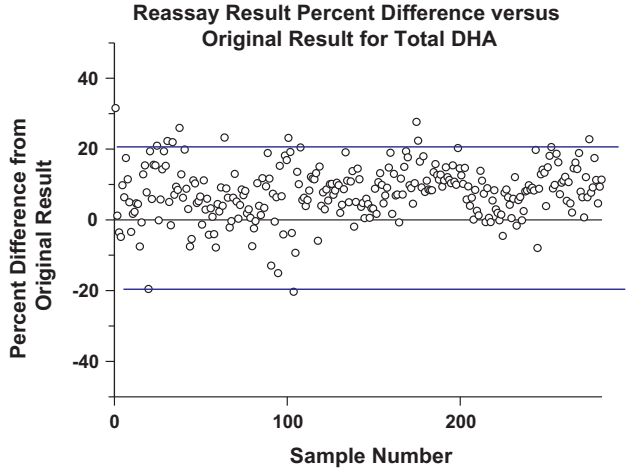

Fig. 5. Representative incurred sample reproducibility graph for total DHA.

4. Conclusion

A semi-automated sample preparation method in 96-well plate format for determination of free and total EPA and DHA in human plasma was developed, using human serum albumin as a surrogate matrix. A surrogate matrix for calibration standard and QC preparation was required due to the endogenous and variable levels of these fatty acids in donor lots which are typically employed. Substantial cost savings will be realized by performing this assay in-house. In re-developing and validating this assay using UHPLC–MS/MS, result turn-around-time has also been reduced over other traditional assays. The method for free EPA/DHA was validated over the concentration range of 0.05–25 μg/mL, while total EPA/DHA was validated from 0.5 to 250 μg/mL. Validation consisted of standard curves being prepared in human serum albumin with quality control samples being prepared in both human serum albumin and plasma to confirm the feasibility of using serum albumin as a conjugate matrix. Statistical analysis on the plasma QC and stability samples were performed using the standard addition method. Blank plasma was extracted along with plasma spiked with EPA or DHA at the predetermined concentration levels. The endogenous level of either EPA or DHA was added to the known spiked level for determination of the standard addition theoretical level.

While the free method utilizes LLE purification, the total method employs a simple digestion to hydrolyze the PUFAs followed by a LLE clean-up with hexane. UHPLC technology with a 1.7 μm particle size column was used for chromatographic separation which was then coupled to tandem mass spectrometry (UHPLC–MS/MS). The results from assay validation show that the method is rugged, precise, accurate, and well suited to support PK studies. The relatively small sample volume (25 μL) and a run time of 1.5 min facilitate automation and allow for high throughput analysis.

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