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New method for GC/FID and GC–C-IRMS analysis of plasma free fatty acid concentration and isotopic enrichment

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ABSTRACT

A simple, direct and accurate method for the determination of concentration and enrichment of free fatty acids (FFAs) in human plasma was developed. The validation and comparison to a conventional method are reported. Three amide derivatives, dimethyl, diethyl and pyrrolidide, were investigated in order to achieve optimal resolution of the individual fatty acids. This method involves the use of dimethylamine/Deoxo-Fluor to derivatize plasma free fatty acids to their dimethylamides. This derivatization method is very mild and efficient, and is selective only towards FFAs so that no separation from a total lipid extract is required. The direct method gave lower concentrations for palmitic acid and stearic acid and increased concentrations for oleic acid and linoleic acid in plasma as compared to methyl ester derivative after thin-layer chromatography. The [^{13}C]palmitate isotope enrichment measured using direct method was significantly higher than that observed with the BF_3/MeOH-TLC method. The present method provided accurate and precise measures of concentration as well as enrichment when analyzed with gas chromatography combustion-isotope ratio-mass spectrometry.

1. Introduction

For many years, our laboratory has been interested in skeletal muscle utilization of lipids [1–4]. A defect in the ability of skeletal muscle to oxidize plasma fatty acids could lead to an increase of intramyocellular lipid, which has been observed in obese as well as type 2 diabetic patients. Fatty acids are an important class of molecules in metabolism [5–8]. Therefore, accurate measurement of plasma free fatty acids (FFAs) has important physiological and clinical implications.

Since the percentage amount of FFAs in human plasma is low (3–10%) compared to fatty acids contained in plasma triacylglycerols, phospholipids or cholesterol esters, any in vitro lipolyic activity would cause an artifactual increase of plasma FFA content [9,10]. Slight amounts of released of fatty acids from any of these ester lipid classes during sample preparation would distort the determined FFA concentration.

Methyl esters are used almost universally for gas chromatographic (GC) analysis of fatty acids [11–13]. The most common procedure used for measuring plasma FFA concentrations and fatty acid enrichment involves the following steps: (a) extraction of lipid from plasma; (b) isolation of FFA from the rest of lipids by thin-layer chromatography (TLC) and/or solid phase extraction (SPE); (c) derivatization of FFA to fatty acid methyl esters (FAME). Boron trifluoride (BF_3) in methanol is the most commonly used reagent for the derivatization step in spite of several disadvantages [14,15]. Drawbacks of this procedure for the estimation of plasma FFA include: (1) a laborious sample preparation with TLC separation of FFAs from the others lipids after the extraction step; (2) the possibility of oxidative deterioration of polyunsaturated lipids during the TLC process; (3) with the purification by SPE, multiple extractions at different pHs are required to obtain the FFA fraction. In addition to this method, there are reports in the literature on direct derivatization methods for the FFAs in plasma extracts under selective conditions [16–18]. However there are some questions concerning the selectivity and accuracy of these methods [9,19].

To overcome these problems, a robust and gentle method for derivatization of fatty acid amides for GC analysis was developed. Amides are more hydrolytically stable and, due to higher polarity elute at higher temperature (Δ+10 to +20 °C) than their corresponding methyl ester. Both of these properties would be advantageous for assessment of short chain fatty acids [20]. Although amide derivatives such as pyrrolidides, picolinyl esters and dimethyloxazolines (DMOX) of fatty acids have been used for mass spectrometric determination of double bond positions in

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polyunsaturated fatty acids, these have not been widely adopted due to lack of a simple, mild and efficient derivatization methodology [21–25] with the exception of picolinyl esters derivatives [26].

We recently developed a very mild and efficient one-step procedure for the conversion of FFAs into their dimethyl, diethyl and pyrrolidide derivatives [27]. This procedure consists of the treatment of fatty acids and amines with bis(2-methoxyethyl)-amino-sulfur trifluoride (Deoxo-Fluor) at 0 °C. Reactions occur in 30 min and products are formed in high yields and purities.

In the current study, we report development of a method for the direct estimation of plasma FFA concentrations as well as their enrichment via derivatization to their dimethylamides. We have applied this method to analysis of plasma fatty acids by GC separation with flame ionization detection (GC–FID) as well with combustion-isotope ratio mass spectrometry (GC–C-IRMS) in ^{13}C-enrichment experiments. The method involves the coupling of the extracted acids with dimethylamine using the Deoxo-Fluor reagent at 0 °C without prior separation of FFA from total lipid extracts (triacylglycerols, phospholipids and cholesterol esters).

2. Experimental

2.1. Chemicals

Dichloromethane, diisopropylethylamine, hexane, heptane, ethyl acetate (analytical-reagent grade), the Deoxo-Fluor reagent and all the fatty acids (palmitic acid (C16:0), heptanoic acid (C17:0), stearic acid (C18:0), oleic acid (18:1n9), linoleic acid (18:2n6), 1,3-diolein, triolein, 1,2-diolein and cholesteryl pentadecanoate) were obtained from Sigma–Aldrich (St. Louis, MO, USA).

2.2. Apparatus

2.2.1. GC/FID

An Agilent GC-6890 system was equipped with a FID. A H_2 flow rate of 30 mL/min and an airflow rate at 300 mL/min were used. The flow rate of carrier gas (He) was set at 2.5 mL/min. The temperature of the injection port and detector were set at 280 and 300 °C, respectively. The oven temperature was programmed to initiate at 160 °C for 2 min, then the temperature was raised to 200 °C at a rate of 20 °C/min, held there for 4 min, and finally increased to 270 °C at a rate of 5 °C/min and held there for 23 min. The injection volume was 1 μL in the split-less injection mode. A capillary column (SPD-1 fused-silica capillary, 30 m × 0.53 mm I.D., 0.01 μm film thickness; Supelco, Bellefonte, PA, USA) was employed. Only results of major plasma FFAs (C16:0, C16:1, C18:0, C18:1 and C18:2) are reported (Fig. 1).

2.2.2. GC–C-IRMS

A ThermoFinnigan model 2000 Trace GC coupled to a Delta^{plus} XL isotope ratio mass spectrometer with GC combustion III interface was used with a PAL autosampler (CTC Analytics, Zwingen). Carrier gas (He) flow was set at 1.5 mL/min. The temperature of the injection port was set at 280 °C and oven temperature was programmed to initiate at 160 °C for 2 min, then the temperature was raised to 200 °C (at a rate of 20 °C/min) and held for 4 min, and finally increased to 270 °C (at a rate of 5 °C/min) and held there for 23 min. The injection volume was 1 μL in the split-less injection mode using a capillary column (DB-5 fused-silica capillary, 30 m × 0.25 mm I.D., 0.25 μm film thickness; Agilent Technologies, USA). After separation, the fatty acids were converted into CO_2 and H_2O using a combustion oven containing copper, nickel and platinum wires, heated at 940 °C. After water is removed, the CO_2 molecules enter the source of the MS, are ionized and the masses of CO_2 (m/z 44, 45 and 46) are analyzed by magnetic sector mass spectrometry. An external CO_2 reference gas ($\delta^{13}C = -29.10‰$) was used to obtain highly accurate isotopic compositions, or $\delta^{13}C$ values.

2.2.3. GC/EI-MS

The GC (Agilent GC-6890) system was equipped with a mass detector (quadrupole, 5973N) mass detector operating in 70V electron ionization (EI) mode. Carrier gas (He) flow was set at 0.8 mL/min. The temperature of the injection port was set at 250 °C and oven temperature was programmed to initiate at 140 °C for 2 min, the temperature was raised to 200 °C at a rate of 20 °C/min, held for 4 min, increased to 270 °C at a rate of 5 °C/min and held there for 23 min. The injection volume was 1 μL in the split-less injection mode. A capillary column (HP-5 fused-silica capillary, 30 m × 0.25 mm I.D., 0.25 μm film thickness; Supelco, Bellefonte, PA, USA) was employed.

2.3. General derivatization

The FFA (0.039 μmol), derivatizing amine (dimethylamine, diethylamine and pyrrolidides) (30 μL) and diisopropylethylamine (10 μL) were dissolved in CH_2Cl_2 (200 μL), cooled to 0 °C, and Deoxo-Fluor (10 μL) was added dropwise. After 30 min, the reaction was quenched with water and products were extracted into *n*-heptane. The organic layer was then dried in SpeedVac,

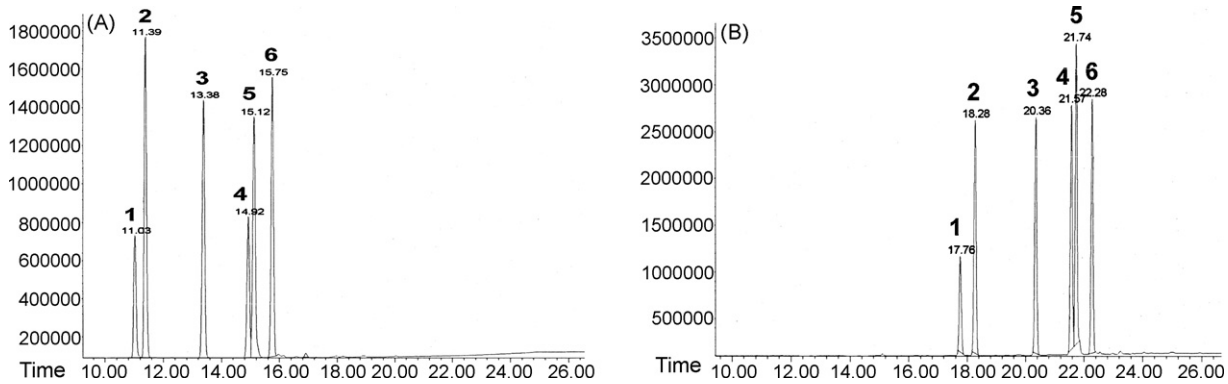

Fig. 1. GC–FID chromatograms of the reaction products of (1) C16:1 (palmitoleic acid), (2) C16:0 (palmitic acid), (3) C17:0 (heptadecanoic acid), (4) C18:2 (linoleic acid), (5) C18:1 (oleic acid) and (6) C18:0 (stearic acid) with BF_3/MeOH and dimethylamine respectively. Conditions: GC–FID (Agilent GC-6890) the flow rate of carrier gas (He) was set at 2.5 mL/min. The oven temperature was programmed to initiate at 160 °C and held for 2 min. The temperature was raised to 200 °C at a rate of 20 °C and held for 4 min, and finally increased to 270 °C at a rate of 5 °C and held for 23 min. Column: SPD-1 fused-silica capillary column (30 m × 0.53 mm I.D., 0.10 μm film thickness; Supelco, Bellefonte, CA, USA). "A" methyl ester derivatives; "B" dimethylamide derivatives.

reconstituted in 500 μL of heptane and transferred to glass autosampler vial.

2.4. TLC/BF_3 method

This method has been previously described in detail [15,28]. Briefly, C17:0 internal standard was added to plasma or water blanks, lipids were extracted with Dole reagent [isopropanol–heptane–hydrochloric acid (1 M) (40:10:1, v/v/v)] dried and FFA were separated by TLC on silica gel plates using a heptane–ether–acetic acid [60:40:3] solvent system [29]. FFAs were visualized by spraying with 0.01% rhodamine 6G. TLC scrapings containing FFA were extracted with chloroform–methanol 3:1, dried and FAME were prepared by reaction with 14% BF_3 in methanol.

2.5. Specificity of derivatization to FFAs

Fatty acid amide synthesis was specific to FFA. Fatty acid esters (triacylglycerols, phospholipids, etc.) present in biological samples are not hydrolyzed in the presence of Deoxo-Fluor reagent [27]. To test the selectivity of this method towards FFA, we conducted the following experiment: A standard palmitic acid (C16:0, 10 μg, 0.039 μmol) was mixed with the internal standard heptadecanoic acid (C17:0, 10 μg, 0.037 μmol) along with three acylglycerols 1,3-diolein, triolein and 1,2-diolein. We used 0.113 μmol of triacylglycerol equivalent to 0.339 μmol of fatty acid, and 0.171 μmol of each diacylglycerol, equivalent to 0.342 μmol of fatty acid. Therefore, the total amount of fatty acid from acylglycerols was 1.023 mmol, compared to 0.039 μmoles of the palmitate. Treatment with the reaction conditions described above gave C16:0 and C17:0 dimethylamide peaks upon analysis with GC–FID whereas no peak was detected for C18:1 (Fig. 2). This result, clearly demonstrates our method is very mild and does not cause hydrolysis of esters (triacylglycerols, diacylglycerols, etc.).

2.6. Validation of the analytical method for GC–FID

For measures of linearity, repeatability and limits of detection and quantification, standard solutions (1.0 mg/mL) of C16:0, C16:1, C18:0, C18:1 and C18:2 were diluted separately with heptane to various concentrations (1–100 μg). Each was mixed separately with 10 μg of internal standard (C17:0). After their derivatization to dimethylamide derivatives, 1.0 μL of each mixture was subjected to GC analysis. Each concentration and recovery of each standard solution was determined in triplicate. Peak areas obtained from above analyses were analyzed with Eq. (1) to determine the internal response factor (IRF). Inter-day precision was estimated from the analysis of freshly prepared control samples (concentrations ranging from 6.20 to 350 μmol/L) on 6 separate days.

$$\text{internal response factor} = \frac{\text{area}_{IS} \times \text{amount}_{SC}}{\text{amount}_{SC} \times \text{area}_{IS}} \quad (1)$$

IS is the internal standard and SC is the specific compound of interest.

The assay precision (coefficient of variation, C.V., in %) was assessed by expressing the standard deviation of the repeated measurements as a percentage of the mean value. Recovery of added fatty acids was estimated by adding a known quantity of FFA in a human plasma sample and calculating each percentage of recovery.

We compared our direct FFA determination method with TLC/BF_3 method for plasma FFA determination. Plasma was prepared from the blood of one adult male volunteer as described above, transferred to separate storage tubes, and frozen. On 3 successive days, one of these tubes was thawed, and three replicate aliquots of 250 μL of this plasma were transferred to glass tubes. Internal standard (C17:0, 10 μg) was added to all the samples. The first groups of three replicates were subjected to FFAs determination by the published procedure with extraction, TLC purification and BF_3/MeOH derivatization. The second group of aliquots was prepared using the dimethylamide derivatization described above following isolation of the FFAs by TLC. A third group of aliquots was prepared and analyzed according to the above direct procedure (that is extraction and derivatization of fatty acids into fatty acid dimethylamides). Analyses of FFA methyl esters as well as FFA dimethylamides were determined by GC–FID as described in the experimental section.

Analysis of variance (SAS 9.1 for Windows, Cary, NC) was used to determine whether there were differences in the determination of plasma FFA concentrations after TLC isolation with dimethylamide and BF_3/MeOH methods and by direct dimethylamide method. To test for differences, we used Scheffe's multiple-comparison procedure. Values of $p < 0.05$ were considered statistically significant.

2.7. Isotope infusion

We also determined the appropriateness of this method to measure ^{13}C enrichment of plasma palmitate during a euglycemic-hyperinsulinemic clamp (same samples as described in Table 4). After taking blood for baseline determinations, an intravenous priming dose of 0.085 mg/kg of NaH_3CO_3 was given. Then, a constant rate continuous infusion of [^{13}C]palmitate was begun (0.011 μmol kg^{-1} body wt. min^{-1}) and continued during the entire period via a calibrated infusion pump (IVAC 560 pump; IVCA, San Diego, CA). The palmitate tracer (60 mg of the potassium salt of [^{13}C]palmitate, 99% enrich; Cambridge Isotope Laboratories, Andover, MA) was dissolved in heated sterile water and passed through a 0.2 μm filter into 25% warm human serum albumin (250 mL) to make a 0.670 mmol/L solution (mean ± SD, 0.668 ± 0.016 mmol/L).

2.8. Blood specimens

Volunteers were recruited and medically screened. Participants were of stable weight, in good general health and with normal values for hematological, renal, thyroid and hepatic function. The University of Pittsburgh Institutional Review Board approved the investigation and all volunteers gave informed written consent. Plasma was collected under standardized conditions in a 10-mL tube-containing potassium EDTA. The uncoagulated blood was then put on ice immediately and centrifuged at 2000 × *g* at 4 °C for 10 min. Plasma was separated from the cells, divided into two

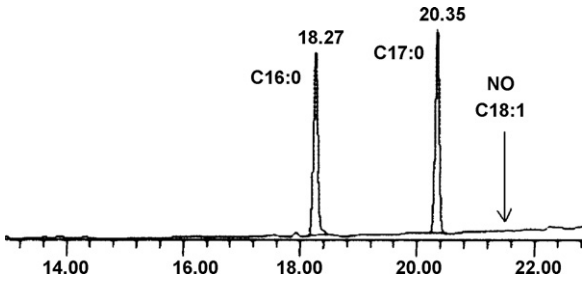

Fig. 2. GC–FID chromatograms of a reaction products with dimethylamine/Deoxo-Fluor for concentrations (0.0391 and 0.0371 μmol) of C16:0, (2) C17:0 0.113 μmol of triolein (C18:1n9), and 0.342 μmol of diolein, and shows no peak for (3) C18:1 at 21.74 min retention time (indicated by arrow above).

Table 1
Comparison of retention times (min) of FAME and FFA amides derivatives

Derivatives	Retention time for C16:1 (min)	Retention time for C16:0 (min)	Retention time for C18:2 (min)	Retention time for C18:1 (min)	Retention time for C18:0 (min)
Methyl ester	11.03	11.39	14.92	15.12	15.75
Dimethylamide	17.76	18.28	21.57	21.74	22.28
Diethylamide	19.98	20.46	23.49	23.64	24.15
Pyrrolidide	23.79	24.21	27.57	27.76	28.49

1.5 mL aliquots, which were frozen within 3 h at −80 °C until analyzed.

2.9. Sample preparation

Aliquots (250 μL) of plasma were spiked with 0.0371 μmol of heptadecanoic acid as internal standard. The samples were then processed according to the following procedure.

2.10. Extraction of plasma-FFAs

Lipids were extracted from plasma using the Dole method [29]. Briefly, the solvent was prepared by mixing isopropanol–heptane–hydrochloric acid (1 M) (40:10:1, v/v/v) and was thoroughly stirred before use. During the extraction procedure, lipids were protected against oxidation by adding 0.05 mg mL^{-1} butylated hydroxytoluene (BHT) to the solvent. Human plasma (250 μL) was mixed with 10 μL of the internal standard heptadecanoic acid (10 μg) in *n*-heptane, in a 13-mL Pyrex glass tube (Corning, NY, USA) with a PTFE lined screw cap. Extraction solvent (2.5 mL) was added, the tubes were thoroughly vortexed for 40 min and allowed to incubate at room temperature for 10 min. Heptane (4.0 mL) and water (2.0 mL) were added and the tubes were thoroughly vortexed for 5 min. The tube was centrifuged at 1000 × *g* for 10 min at 4 °C. The upper phase (heptane) was transferred into a fresh 13 mm × 100 mm screw top tube and dried in a SpeedVac centrifugal concentrator (Savant, Farmingdale, NY). The dried sample was then used for derivatization.

2.11. Derivatization method

Briefly, the dried sample from above was diluted with CH_2Cl_2 (200 μL), and diisopropylethylamine and dimethylamine (10 and 30 μL, respectively) were added. The mixture was cooled to 0 °C, and bis(2-methoxyethyl)amino-sulfur trifluoride (Deoxo-Fluor) (10 μL) was added. The resulting mixture was vortexed for 5 s. After 5 min at 0 °C, the tubes were warmed to room temperature and kept there for 15 min. Water (2 mL) and heptane (4 mL) were added, the tubes were vortexed for 10 min. The organic (upper) layer was collected and dried using a SpeedVac. The residue was resuspended in heptane (500 μL), which was transferred to an autosampler vial, from which 1 μL was injected into the GC–FID.

3. Results and discussion

3.1. GC Separation of fatty acids

To determine which of the fatty acid amide derivatives was most suitable for quantifying FFA as well as for closest resemblance to FAME, we prepared dimethyl, diethyl and pyrrolidide derivatives of C16:0, C16:1, C18:0, C18:1 and C18:2.

As shown in Fig. 1, the separation followed the typical trend for the linear alkanes, with retention time proportional to chain length. The elution was linear with the number of carbons for saturated amides. Under identical chromatographic conditions, the five major FFAs in human plasma eluted as methyl esters, dimethylamides, diethylamides and pyrrolidides in 16, 23, 25 and 29 min, respectively (Table 1). The separations between the methyl esters,

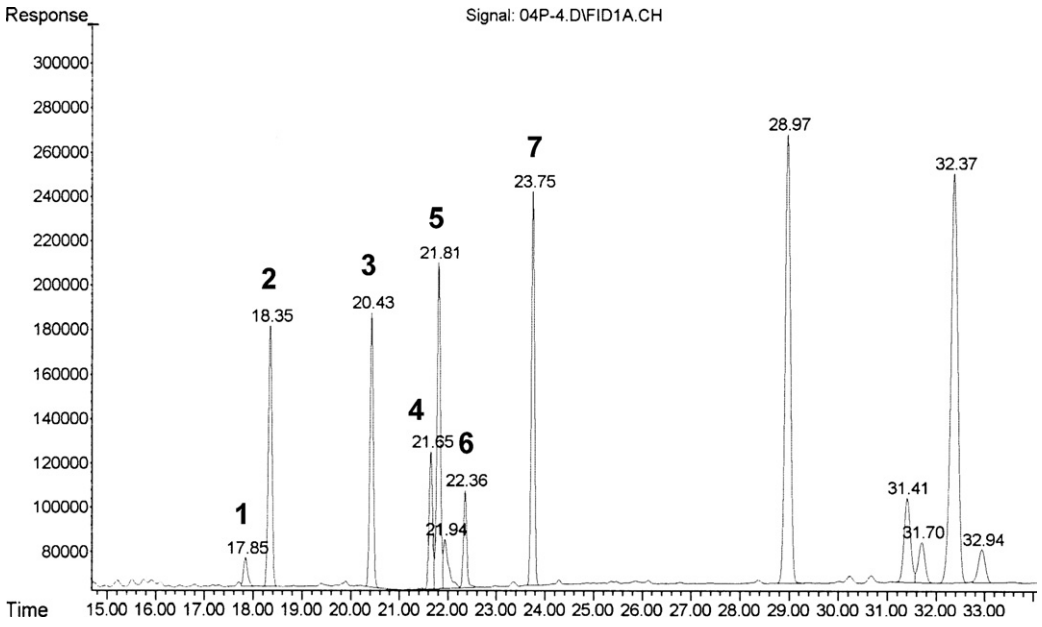

Fig. 3. GC–FID chromatogram of a plasma sample. (1) C16:1, (2) C16:0, (3) C17:0, (4) C18:2, (5) C18:1, (6) C18:0 and (7) reagent peak.

Table 2
Linearity, precision, accuracy, limit of detection and quantification, and recovery of five major FFAs

Validation parameters	Analyte (C16:0)	Analyte (C16:1)	Analyte (C18:0)	Analyte (C18:1)	Analyte (C18:2)
Linearity interval (μmol/L)	0–250 (r^2 = 0.985)	0–50 (r^2 = 0.988)	0–100 (r^2 = 0.990)	0–350 (r^2 = 0.997)	0–50 (r^2 = 0.971)
Inter-day repeatability RSD, %	3.4% (31.3)	2.7% (6.25)	3.9% (12.5)	2.5% (43.75)	2.4% (6.25)
concentration (μmol/L)	3.6% (62.5)	2.6% (12.5)	4.0% (25)	2.6% (87.5)	2.6% (12.5)
	3.1% (125)	2.4% (25)	4.2% (50)	3.1% (175)	2.3% (25)
	3.7% (250)	2.8% (50)	3.9% (100)	2.9% (350)	2.0% (50)
Limit of detection (pmol)	(1.95)	(2.09)	(1.69)	(1.84)	(2.21)
Limit of quantification (pmol)	(2.93)	(3.15)	(2.64)	(2.73)	(2.86)
Recovery: added known quantity in	35.7 (96.2)	8.9 (99.2)	35.7 (96.2)	35.7 (96.2)	35.7 (96.2)
(μmol/L) (and recovery in %)	71.4 (96.4)	17.9 (96.6)	71.4 (96.4)	71.4 (96.4)	71.4 (96.4)
	142.9 (96.5)	35.7 (103.1)	142.9 (96.5)	142.9 (96.5)	142.9 (96.5)
	285.7 (97.0)	71.4 (99.0)	285.7 (97.0)	285.7 (97.0)	285.7 (97.0)

Accuracy and precision of the dimethylamide (direct) method at different FFA concentrations on three successive triplicate standards. Recovery studies were conducted by adding known quantities of FFA to a human plasma sample.

dimethylamides and diethylamides of oleic (C18:1) and linoleic (C18:2) acids were approximately the same. Dimethylamides of fatty acids elute from the GC column first due to its lower molecular weight among the amide derivatives of FFAs. Diethylamide derivatives of FFAs eluted from the GC column approximately two minutes later due to one extra carbon atom. Due to interference of the reagent (diethylamine) with C18:1, this derivative was discarded. Furthermore, the pyrrolidide derivatization was eliminated due to the long retention time and they show overlapping peaks with fatty acid esters (triacylglycerols/phospholipids). Therefore, dimethylamide derivatization was selected for direct analysis of FFA in plasma. Representative chromatograms of the five major plasma standard FFA methyl ester derivatives and dimethylamide derivatives are shown in Fig. 1A and B. All other components in the lipid extract elute from the GC column well after elution of the FFA and hence do not interfere with the FFA quantification. Therefore, it is important to include a temperature program (time and temperature ramp) to remove all remaining components of the total lipid extract to eliminate possible interference for the next sample (Fig. 3; components eluting after 25 min).

3.2. Limit of detection and quantification

The limit of detection (LOD) and the limit of quantification (LOQ) corresponding to signal-to-noise values of 3 and 10 (according to the IUPAC rules and Miller and Miller suggestion [30,31]), respectively (in the range of 1.69–3.15 pmol) were evaluated for the FFAs. The data in Table 2 demonstrating linearity, repeatability, low limit of quantification and nearly 100% recovery of added FFA to a plasma sample support the suitability of the present method for its application in physiological studies.

3.3. Specificity of dimethylamide direct method to FFAs

We also performed an experiment in which cholesteryl pentadecanoate was added to each of three replicate plasma specimens before determination of FFA by our direct method. We did not detect any peak for pentadecanoic acid (the product of hydrolysis of cholesteryl pentadecanoate is pentadecanoic acid) in the GC–FID chromatogram (data not shown). This result, along with our results from the experiments with triacylglycerol and diacylglycerols clearly demonstrates our method is very mild and does not cause hydrolysis of lipid esters in plasma (Fig. 2).

3.4. Comparison of FFA determination procedures

We compared the determination of FFA concentration using BF_3/MeOH and dimethylamide derivatization after isolation of the FFA by TLC with the direct, dimethylamide derivatization procedure. The concentrations found for each fatty acid methyl ester and dimethylamide derivatives were nearly identical following TLC (Table 3). However, the direct method gave lower concentrations for C16:0 and C18:0 and increased concentrations for C18:1 and C18:2 in plasma as compared to either derivative after TLC. Concentration differences observed for FFA were significant by Scheffe's multiple-comparison procedure ($p < 0.05$) only for C16:0, C18:0, C18:1, and C18:2 (Table 3). This result is similar to that reported by Patterson et al. who described that TLC/BF_3 causes an increase in concentration of C16:0 and C18:0 (Patterson et al. plasma FFA using TLC gave: C16:0 +13%, C16:1 −3%, not significant, C18:0 +38%, C18:1 −3% NS, C18:2 −18%. Our results: using TLC gave: C16:0 +16%; C16:1 not significant, C18:0 +14% C18:1 −13%, C18:2 −14%) compared with a direct methylation with iodomethane followed by SPE [28].

The comparison of change during a hyperinsulinemic-euglycemic clamp in five major plasma FFA concentrations level were observed by both the dimethylamide direct method and BF_3/MeOH/TLC. Blood samples were collected at baseline, −30, −15, 0, 150, 165, 180, 270, 285 and 300 min of insulin infusion for measurements of plasma FFA (Table 4). Table 4 shows that the total amounts of FFAs were approximately the same, but the measured concentration of each individual fatty acid varied between methods. Most importantly, as this was a study examining palmitate kinetics, the C16:0 concentrations were consistently higher over all timepoints when determined with the BF_3/MeOH-TLC

Table 3
Comparison of plasma FFA concentrations determined after TLC isolation with BF_3/MeOH and dimethylamide and by direct dimethylamide method

Free fatty acid	BF_3/MeOH-TLC (μM)	Dimethylamide-TLC (μM)	Dimethylamide (μM)
C16:0	74 ± 2[a]	73 ± 4[a]	63 ± 1[b]
C16:1	7 ± 4[a]	7 ± 6[a]	7 ± 2[a]
C18:0	25 ± 5[a]	25 ± 3[a]	22 ± 1[b]
C18:1	77 ± 6[a]	76 ± 4[a]	87 ± 2[b]
C18:2	49 ± 3[a]	49 ± 5[a]	57 ± 2[b]

Values shown are mean ± SD for FFA concentrations in plasma on three successive triplicates. Means with different superscripts are significantly different ($p < 0.05$).

Table 4
Comparison of plasma FFA concentrations determined by dimethylamide direct method and with previously published method (BF_3/MeOH-TLC)

Time (min)	Dimethylamide						BF_3/MeOH-TLC					
	C16:0 (μM)	C16:1 (μM)	C18:0 (μM)	C18:1 (μM)	C18:2 (μM)	Total FFA (μM)	C16:0 (μM)	C16:1 (μM)	C18:0 (μM)	C18:1 (μM)	C18:2 (μM)	Total FFA (μM)
BK	130	20	38	146	69	403	149	20	46	144	74	433
−30	127	20	38	145	69	399	146	19	47	145	75	431
−15	127	19	38	145	68	396	150	18	46	144	72	430
0	134	20	33	139	67	393	151	18	46	136	68	419
150	32	6	9	22	15	83	41	5	11	21	15	93
165	32	5	9	22	15	82	41	5	11	20	15	92
180	29	5	9	22	15	80	38	5	11	21	15	89
270	22	3	7	10	7	49	30	3	7	8	8	55
285	21	3	6	9	7	46	30	3	8	9	8	57
300	22	3	6	10	7	48	29	3	6	8	7	54

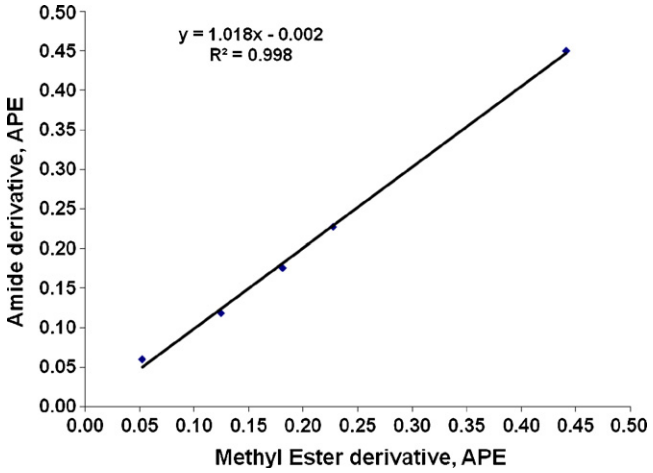

Fig. 4. Comparison of dimethylamide and methyl ester derivative for [^{13}C]palmitate enrichment.

method, as described above for plasma samples and by Patterson et al. [28].

3.5. Validation of the analytical method for isotope enrichment by GC–C-IRMS

We added known amounts of [^{13}C]palmitate to unlabeled palmitate to produce an enrichment standard curve (0%, 0.05%, 0.10%, 0.15%, and 0.20%, 0.40%). The measured enrichment using the dimethylamide derivative was nearly identical to the expected enrichment ($y = 1.0277x + 0.0093$; $r^2 = 0.997$). Comparison of the calculated vs. measured enrichment showed a mean difference of 0.002% APE (in the range of 0.05–0.40 APE). This demonstrated that even low enrichments could be accurately determined ±0.002% APE. Moreover, the isotope enrichment determined using our new method gave nearly identical results to that obtained using the standard method of conversion to methyl esters with BF_3/MeOH with a slope near unity and an intercept of 0 (Fig. 4).

Based on the measurement of quality control samples, we determined that the within-day precision ($n = 3$) for GC–C-IRMS analyses was at least ±0.002% APE; the between-day precision ($n = 5$, 4-month period) was at least ±0.01% APE.

The palmitate isotope enrichment measured using the current method was consistently higher across all timepoints than that observed with the BF_3/MeOH-TLC method (Table 5). This finding would be expected based on the increased concentration of palmitate with the BF_3/MeOH-TLC procedure (Table 4). This same phenomenon, higher concentration and lower enrichment observed with the BF_3/MeOH-TLC procedure, was also observed by Patterson et al. [28,31].

3.6. GC–MS analysis of enrichment

We compared our dimethylamide derivative method with the methyl ester method for the [^{13}C]palmitate using GC–EI-MS. The percentage of $M^{\bullet+}$ in the case of methyl ester derivative was ≥98%, as expected. In the case of dimethylamide, $M^{\bullet+}$ was ≥84% along with M^{1-} of 15%. This finding is not surprising given that nitrogen-containing fatty acid derivatives are susceptible to removal of hydrogen atoms from random positions on the hydrocarbon chain during the electron ionization process [32]. Although amide derivatives are ideal for mass spectrometric analysis of double bond position [21–26,33] they are not suitable for analysis of enrichment by GC–EI-MS.

Table 5
Comparison of plasma C16 enrichment determined by dimethylamide direct method with previously published method (BF_3/MeOH-TLC)

Time (min)	Dimethylamide				BF_3/MeOH-TLC			
	R $^{13}C/^{12}C$	d $^{13}C/^{12}C$ (per mil) vs. PDB	AT% $^{13}C/^{12}C$ (%)	APE%	R $^{13}C/^{12}C$	d $^{13}C/^{12}C$ (per mil) vs. PDB	AT% $^{13}C/^{12}C$ (%)	APE%
BK	0.0111	−15.71	1.0940		0.0109	−22.49	1.0811	
−30	0.0154	367.25	1.5132	0.419	0.0150	336.65	1.4798	0.399
−15	0.0156	386.68	1.5343	0.440	0.0152	350.76	1.4952	0.414
0	0.0158	403.06	1.5522	0.458	0.0152	351.78	1.4963	0.415
150	0.0258	1292.02	2.5109	1.417	0.0221	965.02	2.1604	1.079
165	0.0260	1310.36	2.5305	1.437	0.0216	917.93	2.1097	1.029
180	0.0237	1104.94	2.3107	1.217	0.0214	906.74	2.0977	1.017
270	0.0255	1265.08	2.4821	1.388	0.0203	806.29	1.9894	0.908
285	0.0261	1321.17	2.5420	1.448	0.0204	814.40	1.9981	0.917
300	0.0257	1288.26	2.5069	1.413	0.0201	793.05	1.9751	0.894

4. Conclusions

In this report, we describe a new, improved, simple and efficient procedure for measuring plasma FFA concentrations by quantitative GC–FID and fatty acid [^{13}C]palmitate isotopic enrichment by GC–C-IRMS. This method involves the use of dimethylamine/Deoxo-Fluor to derivatize plasma FFAs to their dimethylamides. This new method is specific for FFAs without contributions from esterified (triacylglycerols, diacylglycerols, phospholipids and cholesterol ester) fatty acids, thus no separation step (TLC or SPE) is required.

Our derivatization and GC method reported here have a number of advantages over existing methods and are suitable for analysis of large numbers of plasma samples from *in vivo* metabolic studies. Compared to other methods, our method is equally sensitive (limit of detection <2 pmol) and reproducible (C.V. < 5%) but significantly more rapid. The greatly reduced time for analysis makes our method more suitable for analysis of large samples numbers.

In summary, we report an optimized method for GC/FID quantitation and GC–C-IRMS enrichment of individual FFAs in human plasma. Our GC method separates and accurately quantifies the five major fatty acids present in human plasma (i.e., those that contribute more than 2% of total lipid) as well as enrichment with GC–C-IRMS with a high degree of sensitivity, reproducibility and enrichment values.

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