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Quantitation of total fatty acids in plasma and serum by GC-NCI-MS

E. Kish-Trier [a,*], E.L. Schwarz [a], M. Pasquali [a,b], T. Yuzyuk [a,b]

[a] ARUP Institute for Clinical and Experimental Pathology, Salt Lake City, UT, United States
[b] Department of Pathology, University of Utah, Salt Lake City, UT, United States

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ABSTRACT

Polyunsaturated fatty acids (PUFAs), including essential omega-3 and omega-6 fatty acids, play important roles in diverse physiological and pathological processes. Diligent monitoring of PUFAs is recommended for individuals with increased risk of developing essential fatty acid deficiency (EFAD), including premature and very low birth weight infants, patients on prolonged parenteral nutrition, and those with dietary restrictions, for example due to inborn errors of metabolism. Here, we present a gas chromatography-negative chemical ionization-mass spectrometry (GC-NCI-MS) method for the quantitation of total levels of twenty-two fatty acids (C12-C22) in serum/plasma, including omega-3 and omage-6 PUFAs. Hydrolysis was used to release esterified fatty acids, which were analyzed by GC-NCI-MS as pentafluorobenzyl esters in selected-ion monitoring (SIM) mode. The calibration curves for all analytes had consistent slopes with R^2 of $\geqslant$0.990. Intra- and inter-assay precision CVs were $\leqslant$9.0% and $\leqslant$13.2%, respectively. Samples were found to be stable for 24 h at room temperature, at least 7 days at 4 °C, at least 75 days at −20 °C, and for three freeze/thaw cycles. No matrix effects or interferences were observed. This method offers improvements over published studies including smaller sample volume, inclusion of additional internal standards, analysis in a single injection, and use of methane reagent gas. This method could be used in a clinical laboratory setting for the diagnosis of EFAD, evaluation of nutritional status, and diet monitoring.

1. Introduction

Fatty acids (FAs), as major constituents of lipids, are involved in diverse aspects of eukaryotic cellular function such as energy storage, membrane structure and dynamics, and signal transduction [1]. The length of the alkyl chain, degree of its saturation, and position of the double bond (ω3, ω6, ω7 or ω9) confer the biological activities unique to the various FAs. Almost all polyunsaturated FAs (PUFAs) can be synthesized by the human body with the exceptions of omega-3 alpha-linolenic acid (C18:3ω3) and omega-6 linoleic acid (C18:2ω6). These can only be obtained from diet and are precursors to multiple long-chain omega-3 and omega-6 PUFAs, such as docosahexaenoic acid (DHA, C22:6ω3) and arachidonic acid (ARA, C20:4ω6), which are critical to normal growth, neurological development, vision, immune response and inflammation. The rate of conversion of alpha-linolenic and linoleic acids to long-chain, omega-3 and omega-6 PUFAs is slow and can be further limited by enzymatic insufficiency due, for example, to prematurity or severe liver damage [2–5]. Deficiency in long-chain omega-3 and omega-6 PUFAs, referred to as essential fatty acid deficiency (EFAD), can result in dermatitis, poor wound healing, infection, decreased growth, impaired learning, and infertility [6,5].

Given the clinical implications in human health, FA analysis has been an area of interest for many years [7,8]. LC-MS/MS methods provide considerable sensitivity and selectivity [9,10], however, the clinically validated methods for FA quantitation are predominantly GC–MS-based [11,12]. GC provides more resolving power than LC and is well suited to profiling FAs, many of which are positional isomers. In order to decrease polarity and increase volatility, FAs are derivatized to form methyl esters (FAMEs) or pentafluorobenzyl esters (PFB-FAs) [13,8]. Ionization of FAMEs is achieved

Abbreviations: ACN, acetonitrile; AMR, analytical measurement range; CI, chemical ionization; DHA, docosahexaenoic acid; DIPEA, N,N-diisopropylethylamine; DPA, docosapentaenoic acid; EFAD, essential fatty acid deficiency; EI, electron ionization; EPA, eicosapentaenoic acid; FA, fatty acid; FAME, fatty acid methyl ester; GC, gas chromatography; HCl, hydrochloric acid; IS, internal standard(s); LC, liquid chromatography; LNC, low normal control; LOD, limit of detection; LOQ, limit of quantitation; MS, mass spectrometry; NaOH, sodium hydroxide; NC, normal control; NCI, negative chemical ionization; PFB, pentafluorobenzyl; PUFA, polyunsaturated fatty acid; RT, room temperature; SD, standard deviation; SIM, selected ion monitoring; TT, triene/tetraene.

* Corresponding author.
E-mail address: erik.kishtrier@aruplab.com (E. Kish-Trier).

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by electron ionization (EI), whereas PFB-FAs are ionized by chemical ionization (CI). EI induces considerable fragmentation of FAMEs [13], whereas negative CI (NCI) of PFB-FAs results in little or no fragmentation, which is advantageous for quantitation [14].

Only a fraction of FAs exist in a free form, the majority are bound by proteins or esterified in higher order lipid structures, such as triacylglycerols and phospholipids [15]. Quantitation of total (free and esterified) FAs in plasma or serum is clinically useful for the diagnosis of EFAD, as well as evaluating nutritional status. Monitoring FA levels is important in individuals with increased risk of developing EFAD due to prematurity, prolonged parenteral nutrition, malabsorption or severe liver damage, as well as in patients with genetic disorders, such as cystic fibrosis and those on dietary restrictions due to inborn errors of metabolism.

Numerous methods for the quantitation of FAs in various matrices have been published, yet few include clinical validation data or reference range information. Here, we describe the development and comprehensive validation of a GC-NCI-MS method for the quantitation of total levels of 22 FAs (C12-C22) in plasma and serum. This robust and reliable method is well suited for clinical laboratory use in the diagnosis of EFAD, as well as in evaluation of nutritional status and diet monitoring.

2. Materials and methods

2.1. Reagents, chemicals, and tubes

10 N sodium hydroxide (NaOH) and concentrated hydrochloric acid (HCl) were purchased from VWR. N,N-diisopropylethylamine (DIPEA) and Pentafluorobenzyl bromide (PFB-Br) were obtained from Sigma-Aldrich. Organic solvents were HPLC grade or higher and water was Type I. Twenty-two FA standards and twelve internal standards (IS) were obtained commercially (Tables S.1 and S.2). Delipidated human serum was purchased from Golden West Biologicals (#MSG3000) or SeraCare Life Sciences (#22011). SeraCare Life Sciences serum was used in stearic acid validation experiments. All glassware used in this procedure was pre-treated with CONTRAD 70 (Decon Labs) to decrease background levels of FAs. Hydrolyses were carried out in Chomsystems tubes (#2010).

2.2. Calibrators and controls

Six, non-zero, calibrators were prepared in ethanol by combining twenty-two FA standards at concentrations spanning the analytical measurement range (AMR; Table S.1). A mix of twelve stable-isotope-labeled IS was prepared in ethanol. FAs, for which corresponding IS were not available, were assigned IS with similar chemistry (Table S.1). Calibration curve slope, y-intercept, and R^2 were monitored over twelve runs to evaluate reproducibility of calibration. A Normal Control (NC), prepared from serum of normal healthy donors after $\geqslant$12 h fasting, and a Low Normal Control (LNC), obtained by a twofold dilution with delipidated serum, were used in all validation runs to evaluate acceptability. Single use aliquots of calibrators, IS mix, NC, and LNC were stored at −80 °C until use.

2.3. Samples

Reference ranges were established using plasma and serum samples submitted to our laboratory and reported as normal on routine biochemical genetic tests (n = 239). In addition, specimens collected after $\geqslant$12 h of fasting from healthy children between 6 months and 17 years of age (n = 59) and from healthy adults (n = 8) were also included in this study. A total of 306 plasma/serum samples were analyzed to establish reference ranges in three age groups: <1 month (n = 57), 1 $\leqslant$ 12 months (n = 120), >1 year (n = 129). Reference intervals were determined using EP evaluator (Data Innovations) by non-parametric analysis and represent the central 95% (2.5–97.5%) of the population. A non-parametric Mann–Whitney test (2-tailed) was used to test differences in FA concentrations between various age groups. P-value <0.05 was considered to be significant. All samples were de-identified and used according to protocols approved by the IRB of the University of Utah. The specimens were stored at −80 °C until analysis.

2.4. Sample preparation

Calibrators, controls, and patient samples were extracted and derivatized according to a procedure modified from Lagerstedt et al. [11]. Briefly, 50 µl of sample was combined with 50 µl IS mix in a glass tube and hydrolyzed in the presence of 1 ml acetonitrile (ACN):6 N HCl (9:1). 1 ml MeOH:10 N NaOH (9:1) was then added and the sample was hydrolyzed again. Both hydrolyses were carried out at 100 °C for 45 min to release esterified FAs. 180 µl of 6 N HCl and 3 ml of hexane were then added to the samples, followed by vortexing for 2 min at 1200 rpm and centrifugation at room temperature (RT) for 4 min at 1200 × *g* to separate phases. The organic layer was transferred to a new glass tube and dried down under nitrogen at 37 °C. Samples were derivatized with a mix of 100 µl 10% PFB-Br and 100 µl 10% DIPEA, both in ACN, for 30 min at RT. After incubation, 20 µl 6 N HCl and 1 ml of hexane were added to each tube. Tubes were vortexed and 150 µl of the upper organic layer was transferred to an autosampler vial.

2.5. GC–MS conditions

Analysis was conducted with an Agilent Technologies 5977A/7890B GC–MS using helium carrier gas. 2 µl of extracted sample were delivered by split injection (220:1) onto the first of two ZB-1 ms (15 m × 0.25 mm I.D. × 0.25 µm film) columns (Phenomenex #7Eg-G001-11) connected in tandem by a purged ultimate union. Separation of FAs was achieved by temperature gradient. The oven temperature program started at 150 °C, increased to 200 °C in two minutes, followed by a ramp to 310 °C in 22 min. Columns were back-flushed post-run with 5 void volumes at 325 °C to remove non-volatile compounds. GC parameters were as follows: inlet temperature (300 °C), pulsed split mode, column 1 flow (1.2 ml/min), column 2 flow (1.24 ml/min), and MS transfer line (315 °C). FAs were ionized using NCI with methane and detected by a single quadrupole in selected-ion monitoring (SIM) mode. MS parameters were as follows: source temperature (240 °C), quadrupole temperature (150 °C), and methane flow (40%, 0.2 ml/min). Dwell times ranged from 60 to 150 ms, depending on peak width and number of analytes in the SIM group. The GC–MS system was controlled by a Agilent MassHunter Workstation.

2.6. Data analysis

Data were batch processed with Agilent MassHunter Quantitation. Peaks were integrated with the MQ4 algorithm, followed by visual inspection and manual adjustment, if required. Separate linear calibration curves were fitted for each analyte using 1/x weighting. The response of each analyte was normalized using IS. Analytes were reported in whole numbers. Quantitation of the 22 FAs permitted calculation of aggregate values (*i.e.*, total omega-3 and omega-6) in addition to the triene/tetraene (TT) ratio (see Section 3.7). These calculations were carried out in Microsoft Excel. Results, expressed as a percentage of total, were calculated by

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