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Determination of sex-based differences in serum γ-linoleic acid and dihomo-γ-linoleic acid using gas chromatography–mass spectrometry

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ABSTRACT

Because serum unsaturated fatty acids can provide useful information on disease diagnosis, the simultaneous determination of several fatty acids in small volumes of human serum would be beneficial for clinical applications. In the present study, serum fatty acids were extracted with *n*-heptane/chloroform from 10 μL of serum collected from 26 healthy Japanese subjects (11 men, ages 23–37 years; 15 women, ages 18–37 years) after deproteinization with perchloric acid, derivatization to their methyl ester using *p*-toluenesulfonic acid as an acid catalyst, and subsequent separation and measurement by gas chromatography–mass spectrometry (GC–MS) in the selected ion monitoring mode. Nine types of fatty acids (palmitoleic acid [PLA], oleic acid [OA], lenoleic acid [LA], γ-linolenic acid [GLA], α-linolenic acid [ALA], dihomo-GLA [DGLA], arachidonic acid [AA], eicosapentaenoic acid [EPA], and docosahexaenoic acid [DHA]) were analyzed in the serum within 35 min by GC–MS. The concentrations of these fatty acids in serum ranged from 3.64 ± 0.38 μM (GLA) to 413 ± 26.3 μM (LA). Among these nine fatty acids, GLA and DGLA levels were significantly lower in women than in men ($p = 0.0034$ and 0.0012, respectively), suggesting that there may be sex-based differences in the biosynthetic production or metabolic processes of GLA and DGLA in humans.

1. Introduction

Omega-3 (ω-3) fatty acids, particularly long-chain ω-3 polyunsaturated fatty acids (PUFAs), docosahexaenoic acid (DHA, C22:6, ω-3), and eicosapentaenoic acid (EPA, C20:5, ω-3), are biologically important nutrients that affect eicosanoid metabolism, membrane properties, gene expression, and other biological processes [1,2]. DHA and EPA are essential for living animals and are acquired through ingestion of a fish-based diet or by biosynthesis from shorter, less-unsaturated ω-3 fatty acids, primarily α-linolenic acid (ALA, C18:3), by desaturation and elongation reactions in vivo [3,4]. Additionally, lenoleic acid (LA, C18:2, ω-6), which is consumed in a standard daily diet, is converted to γ-linolenic acid (GLA, C18:3, ω-6) by desaturase, and GLA is then elongated to form dihomo-GLA (DGLA, C20:3, ω-6) [3]. DGLA can also be converted to arachidonic acid (AA, C20:4, ω-6) by desaturase [3]. Interestingly, ω-3 fatty acids compete with ω-6 (and also ω-9) fatty acids for the enzymes responsible for desaturation and elongation and for incorporation into membrane phospholipids.

The concentrations of fatty acids in serum have been reported to be altered in various diseases, including diabetes [5] and depressive disorder [6]. In particular, PUFA levels have been found to be correlated with symptoms or severity of neurological disorders, such as Alzheimer's-type dementia [7] and schizophrenia [8]. Additionally, the beneficial effects of fatty acid supplementation have been studied in patients with neurological disorders, such as depression [9,10], bipolar disorders [11], and chronic epilepsy [12], in several

Abbreviations: AA, arachidonic acid; ALA, α-linolenic acid; BMI, body mass index; CH_3CN, acetonitrile; DGLA, dihomo-GLA; DHA, docosahexaenoic acid; DMF, dimethylformamide; EPA, eicosapentaenoic acid; FAME, fatty acid methyl ester; GC, gas chromatography; GLA, γ-linolenic acid; HPLC, high-performance liquid chromatography; IS, internal standard; LA, lenoleic acid; LOD, limit of detection; LOQ, limit of quantification; MeOH, methanol; MS, mass spectrometry; OA, oleic acid; PLA, palmitoleic acid; PUFA, polyunsaturated fatty acid; RME, relative mean error; RSD, relative standard deviation.

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small clinical trials. Therefore, determination of the levels of fatty acids in biosamples such as serum or plasma may be relevant for the diagnosis of psychiatric diseases.

To date, several studies have described the detection and quantification of fatty acids in human samples, such as plasma, erythrocyte membrane lipids, and adipose tissue, using high-performance liquid chromatography (HPLC), including precolumn derivatization with fluorescence moieties [13–18]. Recently, we reported the determination of fatty acids in human serum by HPLC-fluorescence detection after precolumn derivatization with a fluorescent regent, 4-*N*,*N*-dimethylaminosulfonyl-7-*N*-(2-aminoethyl) amino-2,1,3-benzoxadiazole [19]. However, in this previous study, only five types of fatty acids could be identified using this HPLC-fluorescence detection method [19]. On the other hand, gas chromatography (GC) is traditionally used for the analysis of fatty acids, commonly separated as their methyl ester derivatives [20–23]. Generally, GC is thought to provide high resolution and rapid analysis as compared with HPLC and is suited for the simultaneous separation of hydrophobic fatty acids. In addition, mass spectrometry (MS) is usually coupled to GC as the detection system and increases the detection selectivity and sensitivity for fatty acids.

Thus, in this study, we examined several types of fatty acids in human serum using GC–MS in order to detect and quantify more fatty acids in serum samples than detected in our previous work [19]. Based on these results, we also discuss sex-based differences in fatty acid concentrations in the serum of healthy subjects.

2. Materials and methods

2.1. Chemicals

Palmitoleic acid (PLA, C16:1, ω-7), oleic acid (OA, C18:1, ω-9), LA, ALA, GLA, DGLA, AA, EPA, DHA, heptadecanoic acid (C17:0), which was used as internal standard (IS), and Supelco37 component FAME mix were purchased from Sigma–Aldrich (St. Louis, MO, USA). Dimethylformamide (DMF) and perchloric acid (60%) were purchased from Nacalai Tesque, Inc. (Kyoto, Japan). Chloroform, *n*-heptane, *n*-hexane, and *p*-toluenesulfonic acid were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Water was purified using the Milli-Q Autopure system (Yamato Co., Ltd, Tokyo, Japan). HPLC-grade acetonitrile (CH_3CN) and methanol (MeOH) were purchased from Kanto Kagaku Kogyo (Tokyo, Japan). All chemicals were of analytical grade.

2.2. Human serum

Human serum was obtained from 26 healthy Japanese subjects. This study was approved by the Ethics Committee of the Faculty of Pharmaceutical Science, Toho University (No. 24-6 and 26-1). Age- and sex-matched healthy volunteers (11 men, ages 26.8 ± 6.70 years; 15 women, 26.4 ± 7.60 years [mean ± SD]) were recruited after providing written informed consent. Approximately 5.0 mL blood was drawn from the arm vein of each volunteer into VENOJECT II tubes (VP-AS109 K; Terumo, Tokyo, Japan) between 11:00 and 13:00 before consuming lunch. Blood was transferred to a shaded container and allowed to stand for 30 min at room temperature. Samples were then centrifuged at 1200 × *g* for 15 min, and the isolated sera were stored at –80 °C until analysis.

2.3. Fatty acid extraction and derivatization

The extraction procedure for serum fatty acids and subsequent methyl esterification were performed according to previously published reports with minor modifications [7,19]. Ten microliters of human serum was mixed with 50 μM heptadecanoic acid (10 μL) as an IS and CH_3CN (10 μL). Then, 30 μL perchloric acid (60%) was added for deproteinization, and 800 μL chloroform, 100 μL *n*-heptane, and 100 μL H_2O were added for the extraction of fatty acids. After the solution was centrifuged at 3000 × *g* for 5 min, the organic layer (400 μL) was transferred to a tube and evaporated under reduced pressure without heating for 22 min. Following the addition of 10 mM *p*-toluenesulfonic acid (PTSA) in MeOH (200 μL) to the dried residue and incubation at 62 °C for 2 h for methyl esterification, H_2O (200 μL) and *n*-hexane (200 μL) were sequentially added, mixed for 2 min, and centrifuged at 4000 × *g* for 5 min. The hexane layer (150 μL), which included fatty acid methyl esters (FAMEs), was obtained. Then, 10 μL sample solution was subjected to GC–MS analysis.

Table 1
SIM conditions for GC–MS analysis of fatty acid methyl esters and an internal standard.

Fatty acid methyl esters	Quantification ion (*m*/*z*)	Confirmation ion (*m*/*z*)
PLA, OA	74	55
LA	67	55
GLA, ALA, DGLA, AA	79	67
EPA	79	91
DHA	79	67
IS	74	87

2.4. GC–MS

GC–MS analysis was performed using GC coupled to a mass spectrometer (QP2010; SHIMADZU, Japan). Separation of the FAMEs was performed on a polar capillary column (DB-23; J&W Scientific, Folsom, UK) with 60 m × 0.25 mm × 0.25 μm film thickness. The carrier gas was helium. The temperature program was optimized to separate each FAME quickly as follows: initial temperature 50 °C, held for 2 min, increased at 10 °C/min to 200 °C, and kept at 200 °C for 10 min. Finally, the temperature was increased at 10 °C/min to 220 °C for another 15 min. The mass spectrometer was operated in the electron impact ionization mode at 70 eV. Both the interface and ionization source were kept at 230 °C. The purchased FAME mixture, which was diluted 100 times with *n*-hexane, was analyzed first. Subsequently, based on their order of elution and the retention times, each fatty acid was confirmed without decomposition. The fatty acids in serum sample were determined by their retention times and the *m*/*z* values of their molecular ions by comparison with the chromatograms and mass spectra analyzed beforehand. Measurements were then performed in the SIM mode using the two most intense fragment ions for quantification and confirmation (Table 1). Stock solutions of the nine fatty acids and heptadecanoic acid (IS) were prepared at 30 and 15 mM in CH_3CN:DMF (1:1) and stored at –20 °C. Working solutions were made up with CH_3CN at concentrations ranging from 0.5 to 800 μM according to the concentration of each fatty acid in the serum samples. The peak area ratio of the IS was then plotted against each concentration ($n = 4$).

2.5. Recovery

Serum samples (10 μL) were spiked with a known amount (10 μL) of each standard at four different concentrations and were treated in a manner similar to that described above. A calibration curve was constructed by plotting the peak area ratio to the IS of each spiked sample against the added concentration, and a working curve was constructed by plotting the peak area of the IS against the concentration. Precision was expressed as the relative standard deviation (RSD, %), and the accuracy was expressed as the relative mean error (RME, %). The equations used to calculate RSD and RME were as follows: RSD (%) = (standard deviation/mean) × 100 and