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High throughput quantitative analysis of polyunsaturated fatty acids in patients with arteriosclerosis disease using LC-MS/MS

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Katsumi Furukawa[1], Yasuhiro Wada[1], Yuka Fujito[2], Kiyomi Arakawa[3], Jun Watanabe[3]

[1] Mitsubishi Chemical Medience Corporation, Tokyo Japan

[2] Shimadzu Analytical & Measuring Center, inc. Kyoto Japan

[3] SHIMADZU CORPORATION, Kyoto, Japan

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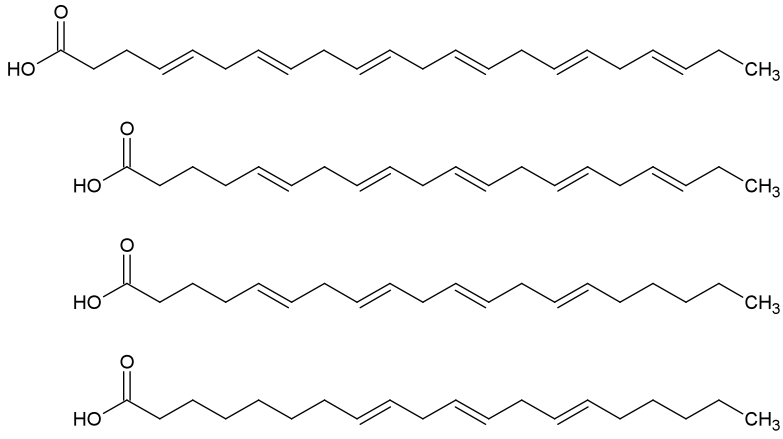
Introduction

The success of using nutritional interventions in inflammatory disease can be measured clinically by the decrease of both the arachidonic acid (AA)/eicosapentaenoic acid (EPA) ratio (the marker of silent inflammation) as well as the AA/dihomogammalinolenic acid (DGLA) ratio (the marker of anti-inflammation). Measuring n-3 long-chain polyunsaturated fatty acids (n-3 LCPUFAs) in clinical samples can be used in the diagnosis of the disease and in defining a strategy for treatment.

A high throughput quantitative LC-MS/MS method was applied to the specific measurement of n-3 LCPUFAs in a patient with arteriosclerosis and the EPA/AA ratio was used as one approach in the diagnosis of thrombus formation.



C22:6n3 DHA (Docosahexaenoic acid)

C20:5n3 EPA (Eicosapentaenoic acid)

C20:4n6 AA (Arachidonic acid)

C20:3n6 DGLA (Dihomo-gamma-linolenic acid)

Fig. 1 Structure of PUFAs

Materials and Methods

Standards of long-chain polyunsaturated fatty acids (PUFAs; DHA, EPA, AA, DGLA) were used to make stock solutions. Several levels of calibrators were made from the stock solutions with hexane as the dilution solvent. Their concentrations were determined by LC-MS/MS using a UFLC HPLC system coupled to a LCMS-8030 triple quadrupole mass spectrometer (Shimadzu Corporation, Japan). Chromatographic separations were carried out using Shim-pack XR-ODSII (2.0mmID x 75 mm; 2.2 um) maintained at 40 °C. DHA, EPA, AA, DGLA were separated using a gradient elution with a flow rate of 0.45mL/min; solvent A, 10mmol/L ammonium formate, and solvent B, acetonitrile.

MRM transitions and responses were automatically optimized for individual compounds in negative ionization electrospray. Serum samples were extracted using hexane solvent extraction.

Standard calibration curves were generated for each fatty acid in serum covering the concentration range of the clinical reference value.

High Speed Mass Spectrometer

Polarity Switching
- 15msec

Scanning Speed
- Max. 15000u/sec

Fig. 2 LCMS-8030 triple quadrupole mass spectrometer

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Results

GC Quantitative analysis

Quantitative analysis of the concentration of a total fatty acid in the biological sample is typically achieved using GC-FID method.

Lipids were extracted from whole blood or serum by using the Folch method and derivatized with methanol (methyl-esterified). Then the fatty acid methyl esters were analyzed by GC-FID. This method involves extensive sample pre-treatment (hydrolysis, extraction and derivatisation) and takes long run time for one analysis (30-40min) so that it should separate various fatty acids with GC.

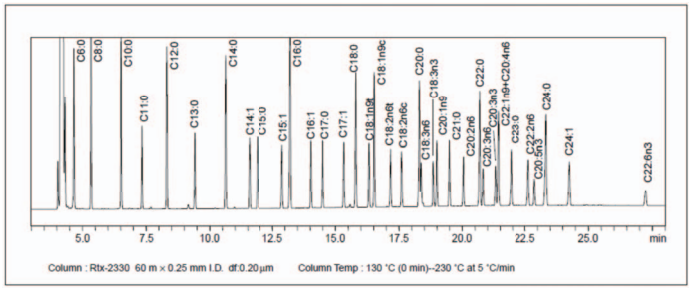

Fig. 3 Analysis of FAMEs using a GC method

LC-MS/MS method development

To help accelerate sample throughput in a clinical setting an optimized MRM method was developed using a high speed data acquisition LC/MS/MS triple quadrupole system. Responses to EPA, AA, DHA, DGLA were optimized for negative ion MRM detection to achieve LOQ at a therapeutic limit of 20ng/mL with a linear calibration range from 20ng/mL to 10ug/mL and a sample analysis time of 6 minutes.

Because the reference values of four fatty acids quantitated in serum or heparin plasma with GC method are respectively DGLA 10.9-43.5 ug/mL, AA 85.1-207.8 ug/mL, EPA 11.6-107.2 ug/mL, DHA 48.6-152.4 ug/mL, EPA/AA ratio 0.11-0.50, this assay method of PUFAs using LC-MS/MS has proven useful enough in the analysis of standard.

Analytical Conditions for LC-MS/MS

HPLC: UFLC system

Column: Shim-pack XR-ODSⅡ 75mm x 2 mmI.D., 2.2 um
Mobile phase: A: 10mM Ammonium Acetate - Water,
B: Acetonitrile
Flow rate: 0.45 mL/min
Gradient program: B conc.70%(0 min) - 90%(3 min) - 100%(3.01-4.0 min) - 70%(4.01-6.0 min)
Column temperature: 40 °C

MS: LCMS-8030 triple quadrupole mass spectrometer

Ionization: ESI, Negative
Ion spray voltage: -3.5 kV
MRM transition:

	Quantitative ion	CE (V)	Qualitative ion	CE (V)
C20:3n6	305.10>59.05	22	305.10>261.20	16
C20:4n6	303.10>259.25	13	303.10>59.15	22
C20:5n3	301.10>257.25	11	301.10>59.10	23
C22:6n3	327.10>283.20	11	327.10>59.05	26

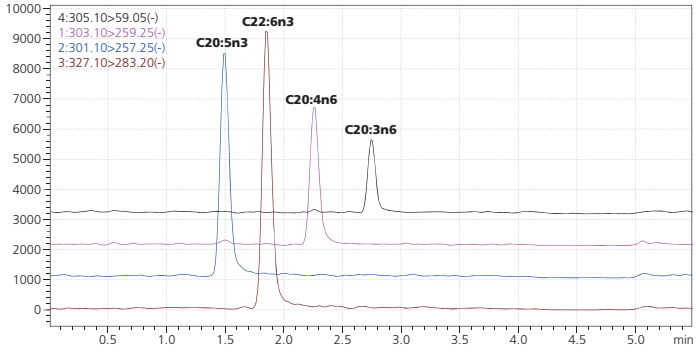

Fig. 4 PUFAs' analysis using LC-MS/MS
(100ppb mixture of 4 PUFAs)

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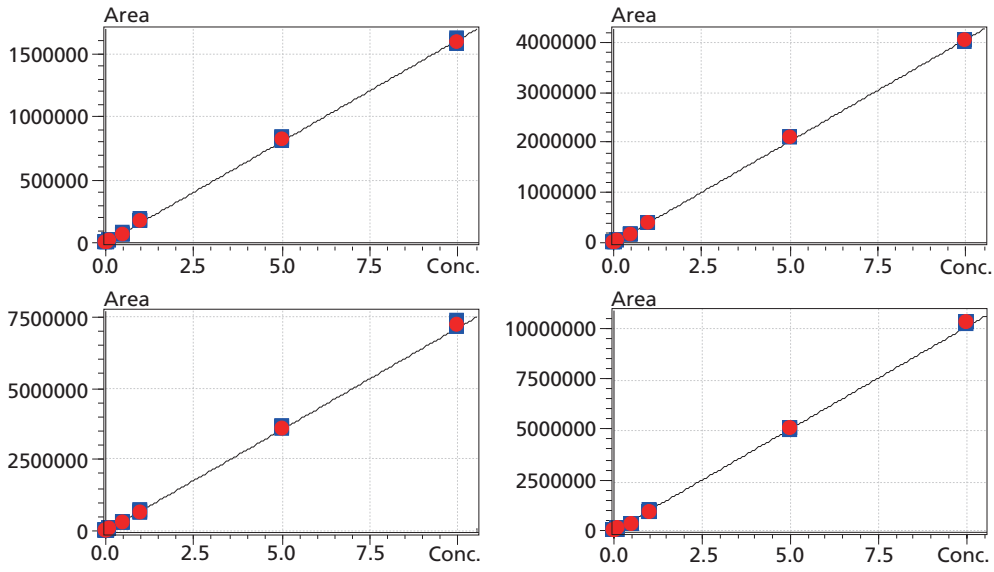

Fig. 5 Calibration curve of PUFAs

Quantitative Analysis of PUFAs in serum

N-3 fatty acids were treated from clinical samples (whole blood or serum) using alkaline water and/or hexane and following further solvent dilution injected onto the LC-MS/MS system. This method was able to omit some sample preparation processes which were used in GC method.

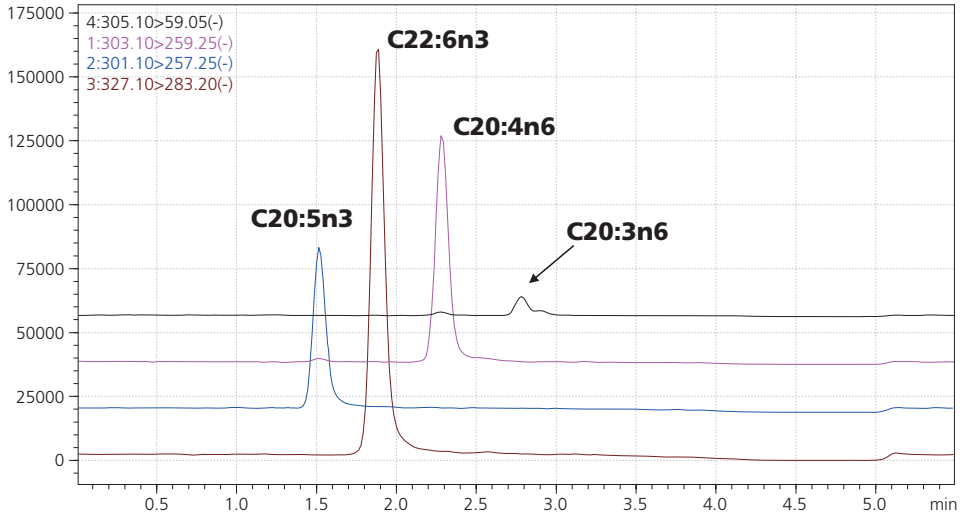

Fig. 6 The analysis of PUFAs in serum using LC-MS/MS

Table 1 The calculated results of PUFAs in serum using LC-MS/MS and GC

	LC-MS/MS results			
	sample diluted (x10)	%RSD (n=6)	calculated conc. in sample	LCMS result / GC result
C20:3n6	0.280 ug/mL	1.54%	2.80 ug/mL	1.08
C20:4n6	1.580 ug/mL	0.60%	15.8 ug/mL	1.05
C20:5n3	0.679 ug/mL	1.00%	6.79 ug/mL	1.03
C22:6n3	1.162 ug/mL	0.58%	11.6 ug/mL	1.22

These results indicated that this LC-MS/MS method was not influenced by sample matrix in serum so much and had excellent reproducibility and was verified by comparison with the conventional GC method.

Conclusions

- High throughput LC-MS/MS method for long-chain polyunsaturated fatty acids (PUFAs; DHA, EPA, AA, DGLA) was developed.
- The method was able to assay the reference value for the PUFAs.
- The method was verified by comparision with the GC method which is conventional for PUFA's quantitative analysis.

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SHIMADZU CORPORATION. International Marketing Division
3. Kanda-Nishikicho 1-chome, Chiyoda-ku, Tokyo 101-8448, Japan
Phone: 81(3)3219-5641 Fax. 81(3)3219-5710
URL http://www.shimadzu.com