Development and Validation of a LC-MS/MS-based Assay for Quantification of Polyunsaturated Fatty Acids from Human Plasma and Red Blood Cells

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INTRODUCTION

Polyunsaturated fatty acids (PUFAs) have essential roles in human physiology. Changes in PUFAs metabolism have important consequences in obesity, type 2 diabetes, insulin resistance, cardiovascular diseases, and also may influence other metabolic pathways[1,2,3]. Considering their importance, accurate quantification of n-3 and n-6 PUFAs is required.

Until recently, PUFAs were quantified using gas chromatography coupled with mass spectrometry (GC-MS)[4] or flame ionization detector (GC-FID)[5], and by liquid chromatography–mass spectrometry (LC-MS) following derivatization[6]. GS-based methods are not especially fast, while chemical derivatization requires additional sample preparation, steps that add increase the complexity of such methods.

OBJECTIVE

We aimed to develop and validate an improved method for the quantification of several PUFAs from human plasma and red blood cells (RBC) using LC-MS/MS. The method requires fewer sample preparation steps and does not require chemical derivatization.

METHOD

Blood samples were collected from a group of overweight children, aged 7-18, (95 males, 99 females) with BMI>+2SD as compared to the World Health Organization reference, and abdominal circumference above the 90th percentile.

RBC were separated from plasma and washed two-times with PBS. The samples were spiked with internal standards and then, total lipids were extracted from plasma and RBC hexane/isopropanol. The extracts were evaporated and reconstituted in 80% methanol. For plasma free PUFAs measurements, a portion of the extracts were transferred to autosampler vials. The rest of extracts were subjected to alkaline hydrolysis for releasing conjugated PUFAs. Finally, the samples were neutralised, evaporated end reconstituted in 80% methanol.

Chromatographic separation was achieved using a reversed phase C18 column with isocratic flow using 90% acetonitrile with ammonium acetate. The mass spectrometer (LCMS-8045, Shimadzu Corporation, Kyoto, Japan) equipped with a ESI source was operated in negative mode. Mass detection was performed in multiple reaction monitoring (MRM) mode, and deuterated internal standards (IS) were used for each targeted compound. The targeted PUFAs were: alfa-linolenic (**ALA**), arachidonic (**ARA**), docosahexaenoic (**DHA**), linoleic (**LA**), and eicosapentaenoic (**EPA**) acids.

The method was validated according to the U.S. Department of Health and Human Services guidelines[7], addressing calibration curve, accuracy, precision, recovery, quality control samples, and sensitivity. Accuracy was evaluated for four different concentrations for each standard (analysed 10-times each). Precision was evaluated by analysing one sample 10 times in the same day (intra-assay precision). One sample was also extracted and analysed 5 times in different days (inter-assay precision). Recovery was estimated using the recovery of IS. For surveying accuracy, quality control samples were used in each analytical run, allowance being set at ±15%.

RESULTS

Multiple reaction monitoring (MRM) method was successfully developed for all PUFAs (Table 1). Where possible, two transitions per compound were used. The **limits of quantification** (LOQ) were situated in the low nanomolar range, excepting linoleic acid, for which the limit was in the high nanomolar range. Elution started at 3.83 min with EPA, while the last was LA at 5.02 min. The HPLC method was ended at 6.5 min (total ion chromatograms showed in Fig. 1).

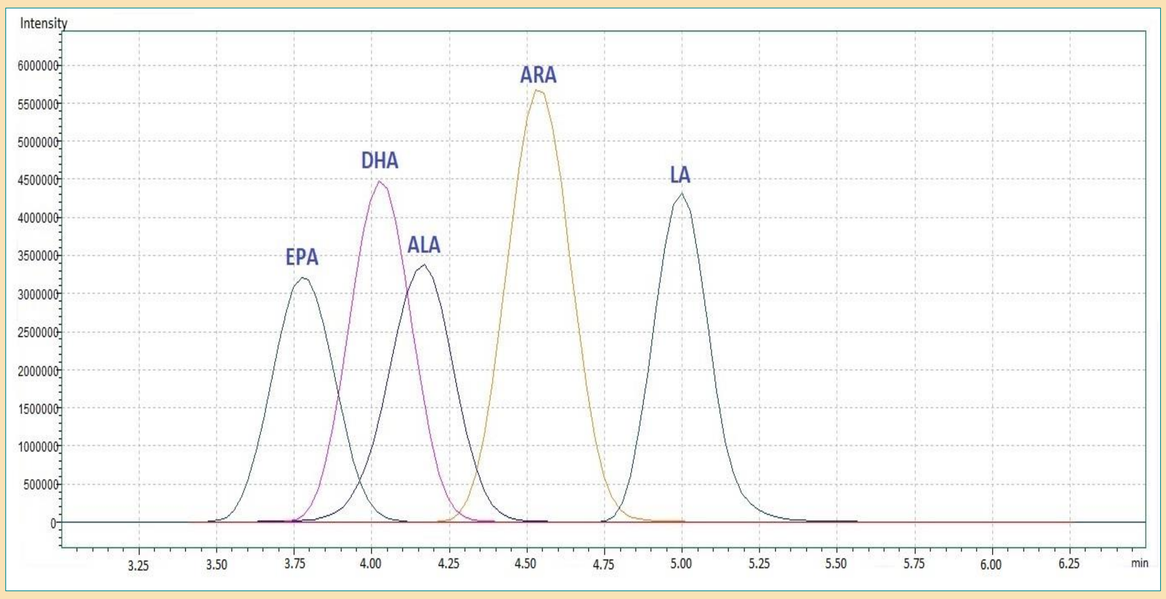
Table 1 – The optimised transitions, retention times (RT) and their relative standard deviation (RSD%), limit of detection (LOD), and limits of quantification (LOQ) for each compound

Compound	Transition (m/z)	RT (min)	RSD% for RT	LOD* (nmol/L)	LOQ** (nmol/L)
ALA	277.25→259.15	4.17	1.1	3.59	17.96
ARA	303.3→259.35, 303.3→205.35	4.6	0.5	3.28	13.14
DHA	327.3→283.35, 327.3→229.35	4.08	0.6	0.82	2.47
EPA	301.25→257.3, 301.25→203.3	3.83	0.5	2.02	6.12
LA	279.2→261.25	5.07	0.5	10.7	285.26
ALA D14	291.3→271.4	4.07	0.5	N/A	N/A
ARA D8	311.25→267.4, 311.25→212.4	4.53	0.33	N/A	N/A
DHA D5	332.25→288.4, 332.25→234.4	4.05	0.55	N/A	N/A
EPA D5	306.25→264.4, 306.25→208.4	3.81	0.5	N/A	N/A
LA D4	283.25→265.4	5.02	0.35	N/A	N/A

* LOD - lowest concentration of the calibration standard detected with a signal-to-noise (S/N) ratio ≥ 3:1
** LOQ was established as the lowest concentration of the calibration standard detected with a S/N ratio ≥ 10:1

All acceptance criteria found in the validation guidelines were met. **Calibration curves** showed a very good concentration / response correlation, R-squared ≥ 0.995 for all calibrants. Accuracy and precision results were al within the 15% set allowance limit. For a**ccuracy,** the relative standard deviation (RSD%) ranged from 4.9 and 6.2%, while for **precision** RSD ranged from 8% for FFA to 13% for plasma conjugated fatty acids and RBC. **Recovery** reproducibility ranged from 3.3 to 11%.

Figure 1 - The overlaid total ion chromatograms of the targeted fatty acids



Measurements did not follow a normal distribution. The results, expressed as minimum, 1st quartile, median, 3rd quartile and maximum, are showed in Table 2.

Table 2 - Descriptive statistics of quantification results. Data is expressed in µmol/L

PUFAs	Form	Minimum	Q1	Median	Q3	Maximum
ALA	plasma free	0.50	2.24	3.79	6.75	23.16
	plasma conj.	0.31	10.27	16.76	25.64	62.81
	RBC	0.23	0.98	1.42	2.00	8.70
ARA	plasma free	3.18	5.38	7.18	9.90	32.24
	plasma conj.	186.15	316.02	377.40	470.76	868.71
	RBC	98.70	261.80	355.80	430.70	988.60
DHA	plasma free	1.52	4.68	6.29	9.55	42.36
	plasma conj.	92.61	207.04	272.25	372.70	698.39
	RBC	26.19	111.44	160.94	228.31	1080.29
EPA	plasma free	0.07	0.17	0.24	0.32	2.88
	plasma conj.	2.09	4.97	7.30	10.53	75.66
	RBC	0.61	1.92	2.87	4.04	44.69
LA	plasma free	25.58	92.17	140.71	217.16	601.06
	plasma conj.	507.90	959.60	1208.10	1433.90	2550.40
	RBC	53.64	149.25	210.79	285.54	566.69

DISCUSSION

The hexane/isopropanol lipid extraction was adapted after a previously described method[8]. By comparing it to the Bligh–Dyer method, we found that hexane/isopropanol was more efficient and also less labour-intensive.

Even though chemical derivatization provides better sensitivity, in our sample matrix, PUFAs can be reliably quantified without it. For all PUFAs excepting LA, the LOQs were situated in low nanomolar range. LOQ for LA's was in high nanomolar range but not considered a problem because its minimum observed level was in micromolar range. Negative ESI responses were probably enhanced by using ammonium acetate in the mobile phase, providing enhanced sensitivity and specificity.

CONCLUSIONS

- Since derivatization was not employed, the sample preparation protocol was less time-consuming and labour-intensive.
- The LC-MS/MS proved significantly faster than previously published GC-based methods – 6.5 min. compared to approximately 20 min[4,5]
- The proposed method offers a fast, sensitive, and reliable quantification of selected omega 3 and 6 fatty acids in human plasma and red blood cells.

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