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Short communication

Development and validation of a quantitative LC–tandem MS assay for hexadeca-4,7,10,13-tetraenoic acid in human and mouse plasma

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ABSTRACT

Upon exposure to platinum analogs, mesenchymal stem cells were recently found to excrete minute amounts of specific lipid mediators that induce chemotherapy resistance. One of these lipids is hexadeca-4,7,10,13-tetraenoic acid (FA(16:4)$_{n-3}$). Importantly, FA(16:4)$_{n-3}$ is present in high concentrations in certain fish oils and algae and oral intake of these products also potently induced chemotherapy resistance. These findings suggested that certain foods could negatively affect clinical chemotherapy treatment. In order to allow further study of the relation between FA(16:4)$_{n-3}$ and clinical chemotherapy resistance, a method for the detection and quantification of FA(16:4)$_{n-3}$ in plasma is required. Therefore, a quantification method for FA(16:4)$_{n-3}$ in human and mouse plasma was developed consisting of a liquid–liquid extraction, solid phase clean-up and LC–MS/MS (MRM) analysis. The method was fully validated over a period of three weeks according to the standard protocols and requirements. The linearity range of the method is 1–100 nmol/L ($r^2 > 0.99$) using deuterated FA(16:3)$_{n-3}$ as an internal standard. The limits of quantification and detection are 1.0 nmol/L and 0.8 nmol/L, respectively. Recoveries for spiked concentrations range from 103 to 108%. The intra-day and inter-day mean precision amounts to 98–106% and 100–108%, respectively. No significant loss of FA(16:4)$_{n-3}$ is observed upon storage at −80 °C. The developed assay for the detection and quantification of FA(16:4)$_{n-3}$ in human plasma is robust and reproducible. The validation parameters are within limits of acceptance.

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1. Introduction

The principal treatment for most disseminated cancers is chemotherapy. Unfortunately, the effectiveness of chemotherapy is often momentary due to the onset of resistance. In a recent paper we demonstrated that mesenchymal stem cells, often present in large quantities near developing tumors, secrete minute quantities of specific polyunsaturated fatty acids upon contact with cisplatin and platinum analogous, thereby inducing resistance to a broad spectrum of chemotherapeutic agents [1]. One of these fatty acids was identified as hexadeca-4,7,10,13-tetraenoic acid (FA(16:4)$_{n-3}$) which could be obtained in purified form [2]. A relative increase in FA(16:4)$_{n-3}$ plasma levels was observed in cancer patients treated with cisplatin, whereas in patients receiving non-platinum based therapy no significant increase was detected. Furthermore, oral or intravenous administration of FA(16:4)$_{n-3}$ to tumor-bearing mice shortly before or during chemotherapy induced resistance to cisplatin. Surprisingly, fish oil and algae products were found to contain high quantities of FA(16:4)$_{n-3}$, and when fed to mice, they potently neutralized the effect of cisplatin treatment, suggesting that certain common foods could curtail clinical chemotherapy effectiveness. These findings underscore the importance for a validated assay to detect and quantify FA(16:4)$_{n-3}$ in plasma in order to investigate the effect of clinically administered chemotherapeutics as well as foods on levels of FA(16:4)$_{n-3}$, and to increase our understanding of the relation between FA(16:4)$_{n-3}$ in circulation and (the effectiveness of) chemotherapy. The set-up and validation of such a diagnostic assay are described in this paper.

2. Experimental procedures

2.1. Materials

FA(16:4)$_{n-3}$ was purified from algae according to a method published earlier [2]. FA(16:3-d_6)$_{n-3}$ was from Sigma Aldrich (Zwijndrecht, The Netherlands) and was used as an internal standard (IS). The NH_2-SPE cartridges (WAT054560) and the BEH C18 UPLC column (186002352) were from Waters (Etten-Leur, The

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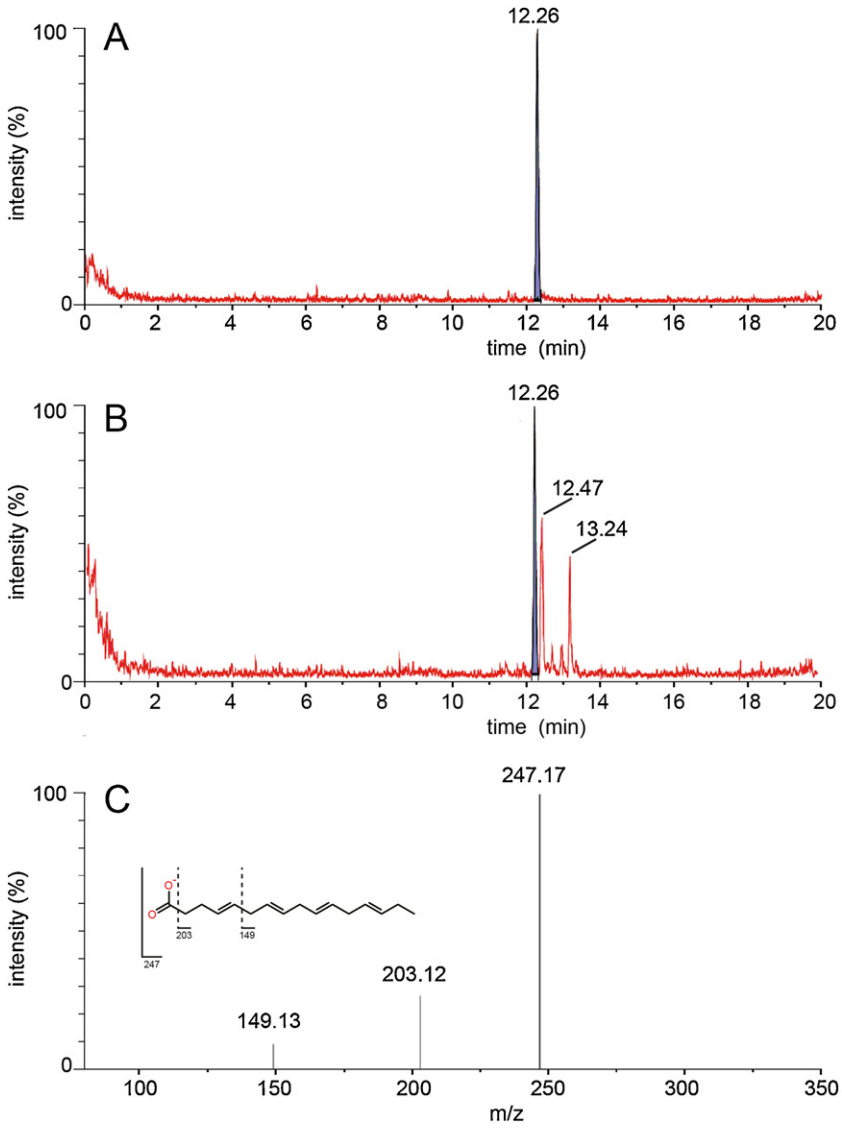

Fig. 1. Detection and quantification of FA(16:4)$_{n-3}$. (A) Chromatogram of 10 nmol/L FA(16:4)$_{n-3}$ in buffer, (B) chromatographic profile of a plasma sample of a healthy human subject for which the FA(16:4)$_{n-3}$ concentration was determined as 3.8 nmol/L, and (C) a daughter scan of FA(16:4)$_{n-3}$ precursor mass 247.2 using negative mode ESI on triple quadrupole mass spectrometer.

Netherlands). All solvents and reagents were from analytical grade. Human plasma of 15 volunteers not undergoing medical treatment was collected and was stored at −20 °C before use. The procedures were approved by the Medical Ethical Committee of our hospital. Mouse plasma was a kind gift from Dr. J. Höppener.

2.2. Column liquid-chromatography and mass spectrometry

The Acquity BEH C18 UPLC column (2.1 × 100 mm, 1.7 μm) was kept at 40 °C in the column-oven present in the Waters Acquity UPLC system (Etten-Leur, The Netherlands). The autosampler temperature was kept at 10 °C. The mobile phase consisted of milli Q water containing 0.1% acetic acid (mobile phase A) or acetonitrile:2-propanol (9:1 v/v, mobile phase B). A 20 min linear gradient of 25–95% B was started upon 10 μL sample injection, after which the system returned to its original situation in 1 min. The flow rate was 0.3 mL/min and total run time was 22 min. The column outlet was coupled to a Waters Xevo triple quadrupole mass spectrometer (Etten-Leur, The Netherlands) equipped with an electrospray ion source operated in the negative mode (see Supplemental information 1 for settings).

2.3. Sample preparation and clean-up

50 μL of plasma, 40 μL IS (78.0 nmol/L) and 120 μL methanol were added to a glass tube. The tube was vortex-mixed for 20 s and 400 μL chloroform was added followed by vortex-mixing for 20 s. After the addition of 150 μL milli-Q water the tube is vortex-mixed again for 20 s. After 10 min on ice, the tubes were centrifuged at 8000 × *g* for 5 min and the upper (aqueous) phase was aspirated off. The remaining lower phase containing the lipid fraction was evaporated to dryness under a gentle stream of nitrogen at 30 °C, dissolved in 100 μL chloroform and subjected to normal phase SPE clean-up.

SPE columns were preconditioned with 2 × 2 mL hexane. The chloroform–lipid extract was transferred to the top of the column and allowed to penetrate the column. 2 × 2 mL chloroform:2-propanol (2:1) was added in order to wash the column and 2 × 2 mL diethyl ether:acetic acid (49:1) was used to elute the fatty acids.

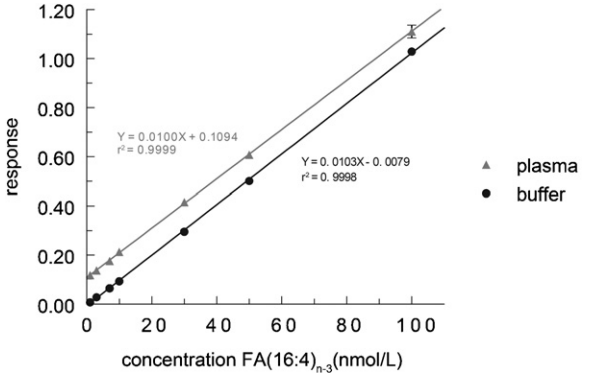

Fig. 2. Calibration curves for $FA(16:4)_{n-3}$ in PBS buffer and $FA(16:4)_{n-3}$ spiked in plasma.

After solvent evaporation, the dried extract was reconstituted in 100 μL acetonitrile, transferred to a sample vial and subjected to LC–MS/MS analysis.

For the preparation of the calibration curve in plasma, 50 μL of the standard calibration solution of the $FA(16:4)_{n-3}$ (0–200 nmol/L) was added to each vial, containing 50 μL plasma, resulting in final concentration of 0–100 nmol/L, which were then subjected to above-mentioned extraction procedure and analysis. Determination of the fundamental parameters of the bioanalytical method validation was performed according to the guidelines of the US Food and Drug Administration [3]. The analyte response at the Lower Limit of Quantification (LLOQ) was defined as 5 times the blank response and the Limit of Detection (LOD) was defined as 3 times the blank response. A detailed implementation is described and graphically shown in Supplemental information 2.

3. Results

In Fig. 1A, a typical chromatographic profile for buffer containing 10 nmol/L $FA(16:4)_{n-3}$ is shown. In Fig. 1B the chromatogram of a human plasma sample containing 3.8 nmol/L $FA(16:4)_{n-3}$ (m/z 247.2) is shown. The retention time of $FA(16:4)_{n-3}$ was 12.3 min, the retention time of the IS 13.1 min (not shown). The ions formed during fragmentation of the parent ion 247.2 were typically 203.2 and a minor amount of 149.2 (see Fig. 1C). Therefore, the parent/daughter transition 247.2 → 203.2 was chosen as the MRM channel setting for $FA(16:4)_{n-3}$. Similarly, MRM channel settings for FA (16:3-d_6)$_{n-3}$ were the parent/daughter transition 255.4 → 211.3. No significant interfering peaks were observed at the retention times in the MRM channels of the analyte or the IS in plasma or PBS samples.

Over the validation period of 21 days, 9 calibrations were performed in a human plasma pool prepared from the plasma of three healthy donors. The calibrations consisted of 7 data points over a range of 1–100 nmol/L. The regression equation for the calibration, where regression coefficients are expressed as mean ± standard deviation (SD), was $y = [0.0100\ (0.0001)]x + [0.1094\ (0.0018)]$. In order to assess the matrix effect, 6 similar calibrations were performed in PBS resulting in the regression equation $y = [0.0103\ (0.0001)]x–[0.0079\ (0.0029)]$. Both calibrations had a r^2 of 0.99 or better. Calibration curves are shown in Fig. 2. Additional statistical data are shown in Supplemental information 3.

The matrix effect, calculated using the equation from Midttun et al. [4], amounts to 99.6 ± 4.9% and can thus be considered negligible. Therefore, further calibrations were conducted in PBS buffer. The endogenous level of $FA(16:4)_{n-3}$ in the plasma of the 15 healthy subjects was determined (see Fig. 3). The median concentration was 6.6 nmol/L and the calculated mean concentration was 8.3 ± 7.1 nmol/L.

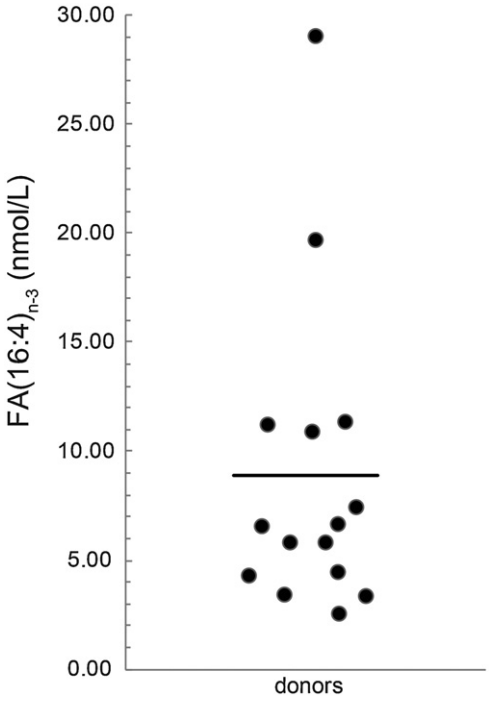

Fig. 3. Scatter plot of $FA(16:4)_{n-3}$ reference values determined in plasma of healthy human subjects. The horizontal line depicts the average endogenous level.

Mean extraction efficiencies ± CV ($n = 3$) for QC concentrations 5, 20 and 80 nmol/L were 107.3 (3.4), 92.4 (1.5) and 109.8 (3.0)%, respectively. The intra-batch precisions ($n = 9$) for the same QC concentrations were 11.0, 3.5 and 5.1%, respectively. The inter-batch precisions ($n = 27$) were 9.1, 3.8 and 5.4%, respectively.

Accuracy ($n = 10$) was determined for the QC with the lowest concentration, 5 nmol/L (QC1) and for the QC with the highest concentration, 80 nmol/L (QC3). The CV values were 7.7% and 1.8%, respectively. The LLOQ, amounted to 1.0 nmol/L (CV 16%). The LOD was estimated to be 0.8 nmol/L.

Stability of $FA(16:4)_{n-3}$ was determined and the results can be found in Table 1. No decrease in concentration was observed when samples after clean-up were left on a lab-table at ambient temperature for 24 h. The loss in analyte after three freeze–thaw cycles was negligible for both the QC1 and QC3 samples. No change in the concentration of $FA(16:4)_{n-3}$ was observed upon storage of donor plasma samples for 1 month at −80 °C.

Finally, we assessed the applicability of the current method for studies executed with animal models such as mice. For this, FDA guidelines were followed and the accuracy and precision of the analysis method were determined (see Table 2). The endogenous concentration $FA(16:4)_{n-3}$ in the mouse plasma was 2.82 nmol/L. and the results of the accuracy and precision determination were within limits of acceptance. Together, these findings show that $FA(16:4)_{n-3}$ can be detected and quantified in both human and mouse plasma.

4. Discussion

In this study, the set-up and validation of a reverse-phase UPLC method for the determination and quantification of $FA(16:4)_{n-3}$ in human and mouse plasma are presented. Due to its advantages over alternative techniques, including the high sensitivity and selectivity [5], UPLC–MS/MS (MRM) was used for $FA(16:4)_{n-3}$ quantification. Since isotopically labeled $FA(16:4)_{n-3}$ is not commercially available, the closely related $FA(16:3)_{n-3}$ was used as an internal standard to allow correction for loss during isolation and clean-up.

Table 1
Summary of the validation results, mean value (CV).

Precision					
Intra-batch precision ($n=9$)			Inter-batch precision ($n=27$)		
Low, 5 nmol/L	Medium, 20 nmol/L	High, 80 nmol/L	Low, 5 nmol/L	Medium, 20 nmol/L	High, 80 nmol/L
5.0 (11.2)	20.0 (3.5)	80.4 (5.1)	5.0 (9.1)	19.7 (3.8)	78.8 (5.4)

Accuracy ($n=10$)	
Low, 5 nmol/L	High, 80 nmol/L
4.4 (7.7)	82.8 (1.8)

Recovery ($n=3$)		
Low, 5 nmol/L	Medium, 20 nmol/L	High, 80 nmol/L
5.0 (2.0)	17.9 (3.3)	84.4 (1.7)

Ambient temperature stability ($n=3$)	
Low, 5 nmol/L	High, 80 nmol/L
4.9 (7.0)	79.7 (1.9)

Freeze/thaw stability ($n=3$)	
Low, 5 nmol/L	High, 80 nmol/L
5.3 (8.0)	78.2 (3.7)

Storage stability ($n=3$)			
Donor	4	5	11
Day 0	11.3 (3.5)	29.1 (1.9)	5.9 (2.9)
Day 30	11.4 (4.3)	29.3 (1.5)	5.8 (8.9)

Table 2
The accuracy and precision of the current analysis method applied to the determination of FA(16:4)$_{n-3}$ in mouse plasma, mean value (CV).

Intra-batch precision ($n=9$)		
Low, 5 nmol/L	Medium, 20 nmol/L	High, 80 nmol/L
5.2 (5.6)	17.5 (6.0)	74.8 (2.2)

Accuracy ($n=10$)	
Low, 5 nmol/L	High, 80 nmol/L
5.2 (6.2)	75.0 (1.2)

The extraction of FA(16:4)$_{n-3}$ was efficient as all spiked analyte was recovered and no matrix effect was observed after introduction of an orthogonal SPE sample cleanup step. The linearity range was verified at 1–100 nmol/L and the method was validated using QC samples ranging from 5 to 80 nmol/L. The LLOQ and LOD of the method was determined at 1.0 and 0.8 nmol/L, respectively. These values are well below the lowest concentration of 2.6 nmol/L established for healthy human subjects. All CV values were within accepted limits [3]. During chemotherapy treatment, the FA(16:4)$_{n-3}$ concentration increased 3–5 times compared to levels observed prior to therapy [1]. Consequently, the established linearity range and LLOQ allow the quantification of FA(16:4)$_{n-3}$ in human plasma in relation to chemotherapeutical efficacy and resistance development in cancer patients.

The LC–MS/MS analysis consisted of a 20 min linear gradient. The reason for this is 2-fold: (1) effective separation is established preventing interference from other lipids, e.g. fatty acids sharing a similar parent/daughter transition (see Fig. 1B). (2) The method could allow the simultaneous analysis of other lipids that mediate chemotherapy resistance such as 12-keto-5,8,10-heptadecatrienoic acid (KHT) [1]. Unfortunately however, KHT is not commercially or academically available to our knowledge thus preventing inclusion in the current method at present.

In order to assess the applicability of the current method for animal models such as mice, the analysis method was extended for the quantification of FA(16:4)$_{n-3}$ in mouse plasma. The results showed that the approach can be applied to the determination of FA(16:4)$_{n-3}$ in both mouse and human plasma.

In conclusion, the presented method is proven to be selective, accurate, sensitive and applicable to human and mouse plasma. Numerous applications emerge, including clinical monitoring of the development of chemotherapy resistance in relation to the levels of FA(16:4)$_{n-3}$, and studies on the effect of therapeutics or diets on the concentration of FA(16:4)$_{n-3}$ in plasma and its relation to the effectiveness of chemotherapy. Moreover, the stability of endogenous FA(16:4)$_{n-3}$ stored at −80 °C allows for analysis of retrospective patient cohorts. Finally, the method described here will serve as a starting point toward the detection of FA(16:4)$_{n-3}$ in complex lipid mixtures and other foods taken by patients undergoing chemotherapy or cancer patients.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jchromb.2013.01.012.

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