Published in final edited form as:

Anal Chem. 2013 October 1; 85(19): . doi:10.1021/ac402078p.

Fatty acidomics: global analysis of lipid species containing a carboxyl group with a charge-remote fragmentation assisted approach

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Abstract

Charge-remote fragmentation has been well recognized as an effective approach for dissociation of long aliphatic chains. Herein, we exploited this approach for structural identification of all fatty acids including saturated, unsaturated, and modified ones by using electrospray ionization tandem mass spectrometry (ESI-MS/MS) after one-step derivatization of a charge-carried reagent through an amidation reaction. We tested the approach with different charge-carried reagents with respective to the hydrophobicity, charge strength, and distance from the charge to the carboxyl group. We found all the derivatives with these reagents could yield informative charge-remote fragmentation patterns regardless of the different chemical and physical properties of the reagents and these informative fragmentation patterns all could be effectively used for structural elucidation of lipid species containing a carboxyl group. We further found that the distinguished charge-remote fragmentations of fatty acid isomers enabled us to determine the composition of these isomers without any chromatographic separation. Finally, the abundant fragments yielded from individual derivatized moiety enabled us to sensitively quantify the individual species containing a carboxyl group. The described approach was a great extension to the multidimensional mass spectrometry-based shotgun lipidomics for global analysis of fatty acids including isomers and modifications. We believe that this approach could greatly facilitate the identification of the biochemical mechanisms underlying numerous pathological conditions.

Keywords

Charge-remote fragmentation; fatty acidomics; lipidomics; modified fatty acid; non-esterified fatty acid; shotgun lipidomics

Fatty acid (FA) represents a broad family of lipids containing at least one carboxyl group and a long aliphatic chain¹. Therefore, this family includes, but is not limited to, non-esterified (saturated and unsaturated) and modified FAs. The latter includes all oxidized (e.g., eicosanoids, docosanoids, FAs containing hydroxylated and epoxylated group(s), etc.), nitrosylated, halogenated, and other modified FA species¹. FAs play many essential roles in the biological systems (e.g., providing energy sources, serving as signaling molecules, and being the major structural components in complex lipids of cellular membranes)². Therefore, the analysis of the entire family of FA species, which is referred to as “fatty acidomics” hereafter, is essential, but challenging in lipidomics.

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SUPPORTING INFORMATION AVAILABLE

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The non-esterified FAs vary in chain length, the number of double bonds, the locations of these double bonds on the acyl chains, and with or without branched methyl group(s). The different locations of the double bonds form the isomers of an unsaturated FA for which the chain length and the number of double bonds are identical and of which the importance has been previously discussed^{3, 4}. Modification of these FA species through enzymatic or non-enzymatic processes yields a huge number of modified FA species. A good example is the family of eicosanoids which are produced from the oxidation of twenty-carbon, n-3 or n-6 polyunsaturated fatty acids^{5, 6}.

Identification and quantification of the species present in the individual subclass of this FA family has a long history. GC and HPLC coupled with mass spectrometry have been widely used for this purpose with or without derivatization⁷⁻¹⁵. Methods associated with general derivatization¹⁶⁻¹⁸, *in situ* ozonolysis⁴, charge switch concept¹⁹⁻²¹, differential loss of CO₂ after collision-induced dissociation (CID)³, etc. have been developed for different applications. However, an approach for fatty acidomics is still lacking in lipidomics.

Charge-remote fragmentation has been well recognized as an effective approach for dissociation of long aliphatic chains^{22, 23}. Herein, we exploited this approach for structural identification and quantification of all fatty acids including saturated, unsaturated, and modified ones by using electrospray ionization tandem mass spectrometry after one-step derivatization of a charge-carried reagent through an amidation reaction. We found that the abundant fragments resulting from the derivatized moiety could be employed to sensitively quantify individual FA species. We believe that by using fatty acidomics, identification of the biochemical mechanisms underlying numerous pathological conditions can be greatly accelerated.

MATERIALS AND METHODS

Materials

All FAs were purchased from either Nu-Chek Prep, Inc. (Elysian, MN) or Cayman Chemical (Ann Arbor, MI). All the FAs were used without further purification. Internal standard 7,7,8,8-d₄-Palmitic acid used as an internal standard for quantification of non-esterified FAs was obtained from Cambridge Isotope Laboratories, Inc. (Tewksbury, MA). N-(4-aminomethylphenyl)pyridinium (AMPP), N-(benzenemethylamine)-2,4,6-trimethylpyridinium (BMA-TMP), N-(4-aminomethylbenzyl)-2,4,6-trimethylpyridinium (AMB-TMP), and 4-aminomethyl-1-methylpyridin-1-ium (AMMP) used as derivative reagents were synthesized as described in Supporting Information in details. Other chemicals were purchased from Sigma-Aldrich (St. Louis, MO).

Preparation of FA Mixtures

The stock solutions of the FAs were prepared in CHCl₃/MeOH (1:1, v/v). The concentration of each stock solution was determined based on its weight. The mixtures of FA isomers were prepared from individual isomer stock solutions.

Human plasma lipid sample preparation

This study was approved by the Institutional Review Board of Sanford-Burnham Medical Research Institute and was conducted according to the principles expressed in the Declaration of Helsinki. Fasting blood samples were collected from healthy individuals and cold stored using a standardized procedure as previously described²⁴.

Individual plasma sample (~ 100 µL) was accurately transferred into a disposable glass culture test tube. An internal standard (d₄-16:0 FA, 200 nmol/mL plasma) for quantification

of FA species was added to the tube prior to lipid extraction. Lipid extraction was performed by using a modified Bligh and Dyer procedure as previously described⁹. Each lipid extract was resuspended into a volume of 1500 μL of chloroform/methanol (1:1, v/v) per mL of original plasma, flushed with nitrogen, capped, and stored at $-20\text{ }^{\circ}\text{C}$.

Derivatization of carboxyl-containing species

Lipid extract, equivalent to 0.05 to 0.1 mg of the original sample protein content or approximately 10 μL of original plasma sample, was used for the derivatization of FAs. After evaporation of the solvent in a lipid solution under a N_2 stream, 10 μL of ice-cold acetonitrile- N,N -dimethylformamide (4/1, v/v) was added into a conical tube containing the lipid residue at $0\text{ }^{\circ}\text{C}$. Then 10 μL of ice-cold 640 mM (3-(dimethylamino)propyl)ethyl carbodiimide hydrochloride aqueous solution was added. The tubes were briefly vortexed with a mixer, and then 10 μL of ice-cold 10 mM N -hydroxybenzotriazole and 10 μL of 30 mM AMPP (or other derivatizing reagents), both in acetonitrile, were added. The tubes were thoroughly vortexed, filled with nitrogen, capped, and incubated at $70\text{ }^{\circ}\text{C}$ for 90 min. After incubation, the derivatives were extracted with 1.5/1.5/1.5 mL of $\text{CHCl}_3/\text{MeOH}/\text{water}$. The bottom layer was collected and the solvents were evaporated under a N_2 stream. The residue was resuspended into 100 μL of $\text{CHCl}_3/\text{MeOH}$ (1/1, v/v) for MS analysis.

MS Analysis of Derivatized FAs

MS and tandem MS analyses of derivatized FAs in the positive ion mode were performed by using a TSQ Vantage mass spectrometer (Thermo Fisher Scientific, San Jose, CA) equipped with an automated nanospray apparatus (Advion Bioscience, Ithaca, NY) as previously described^{25, 26}. Alternatively, MS and tandem MS analyses of derivatized FAs in the positive ion mode were also performed by using a Q-Exactive mass spectrometer (Thermo Fisher Scientific, San Jose, CA) equipped with an automated nanospray apparatus (Advion Bioscience, Ithaca, NY) as previously described²⁷.

Individual FA derivative solutions were diluted to approximately 1 pmol/ μL (i.e., 1 μM) in $\text{CHCl}_3/\text{MeOH}/\text{isopropanol}$ (1:2:4, v/v/v) before direct infusion. The total concentration of each FA mixture was diluted to be less than 50 pmol/ μL or to the concentration as indicated to avoid possible lipid aggregation. Product ion analysis of a diluted lipid solution was performed by fixing collision gas pressure at 1 mT or as indicated while varying collision energies to achieve optimal conditions (i.e., approximately 40% abundance of the molecular ion relative to the base fragment ion). Identical CID conditions were always employed for analysis of FA isomers. The collision energy for precursor-ion scanning (PIS) analysis for quantification of FA species was determined through step variation of collision energy (i.e., 1 eV each) with collision gas pressure of 0.7 mT to avoid peak tailing as previously described²⁸. The collision energy which minimally affected the peak intensities of FA species in PIS was selected for the quantification purpose.

Determination of the Composition of Individual FA Isomers

Product-ion ESI mass spectra of an isomeric mixture and its corresponding individual isomers were acquired under identical experimental conditions in the profile mode and were processed by averaging and smoothing. Relative intensity of individual fragment ions detected by these product-ion ESI mass spectra were determined by normalizing the absolute counts of each individual fragment ion to the absolute counts of the molecular ion or the base peak fragment ion in the same product ion mass spectrum. Multiple linear regression analysis was performed in the MATLAB numerical computing environment (MathWorks, Natick, MA). In the analysis, the normalized fragment ions resulting from the ion of an isomeric mixture were used as the responses and the corresponding fragment ions separately resulting from the individual isomeric ions were used as the predictors according

to the linear model described by Formulas 1 and 2. Multiple linear regression analysis was accomplished to achieve total least square. Herein, we assumed a linear contribution of individual fragment ions resulting from individual isomers to the corresponding fragment ions resulting from the isomeric mixture as follows:

$$I_i = \sum x_j I_{ji} \quad (i=1, 2, 3, \dots, n, j=1, 2, 3, \dots, m, \text{ where } n > m) \quad (1)$$

and

$$\sum x_j = 1 \quad (2)$$

where i is a specific fragment ion present in the mass spectrum of the isomeric mixture, j is one of the isomers yielding the same molecular ion, I_i is the normalized intensity of a specific fragment ion i from an isomer mixture, I_{ji} is the normalized intensity of a specific fragment ion i present in the mass spectrum of an isomer j , and x_j is the composition of isomer j present in the isomeric mixture (normalized using Formula 2). This model was extensively tested and validated with a variety of known mixtures (see below).

RESULTS AND DISCUSSION

The Effects of Charge Environment on Charge-Remote Fragmentation for Structural Elucidation of FA Species

We hypothesized that environmental changes of the fixed charge derivatized with FA could affect the fragmentation of the linked aliphatic chain and we could optimize the charge environment for best structural elucidation of FA species. As proof of concept, we prepared a few reagents containing different sizes of conjugated ring systems, different locations of the charge in the conjugated systems, and different modifications of the conjugated systems as well as a reagent with additional distance of the charge to the carboxyl group (Figure S1). We derivatized a series of different FA species (Figure S1) and tested the effects of the charge environment on charge-remote fragmentation. When we derivatized a FA species with these reagents, we found that all derivatized FA ions except the one with AMB-TMP yielded an essentially identical fragmentation pattern in the region of aliphatic chain of a FA (Insets of Figure 1A to 1C) regardless of the changes in the conjugated charge systems. The assignment of these fragment ions corresponding to the aliphatic chain was exemplified in Figure S2. It should be noted that the fragment ions yielded from the individually derivatized reagent were very abundant and different from each other (Figure 1). The one carried the charge distant away from carboxyl group and not conjugated with the benzyl ring (i.e., AMB-TMP) yielded very significantly different product-ion mass spectra of derivatized FA species from others (Figure 1D, compared to Figure 1A to 1C). These results indicated that the distance between the conjugated charge system and the carboxyl moiety dramatically affected the charge-remote fragmentation whereas the location of the original charge did not play a major role as long as this location was within a conjugated system.

Since the fragmentation patterns yielded from the AMB-TMP-derivatized FA ions were too complicated and the information about the location of double bonds was not straightforward in comparison to those resulted from other derivatives, examination of the utilization of this derivative was not further explored. We decided utilizing AMPP as a representative of the derivative reagents for the rest of the current study after determining the derivatization yields (AMPP ~ BMA-TMP > AMMP > 90%), extraction recoveries (similar for all the derivatives), and ionization efficiencies (AMPP ~ BMA-TMP > AMMP) of the derivatives with the reagents of AMPP, AMMP, and BMA-TMP, as well as comparing with other studies in the literature²⁰.

Elucidation of FA Structures after Derivatization

Product-ion mass spectral analyses of the $[FA+AMPP]^+$ ions by ESI-MS/MS demonstrated informative fragment ions for structural determination (Figures 1B and 2; Figures S3-S7). Obviously, in combination with the m/z of the molecular ion, the number of carbons and the number of double bonds, as well as the location(s) of these double bonds could be readily identified. Generally, the product-ion mass spectra of saturated FA species after AMPP derivatization displayed a series of low abundance fragment ions between which the mass difference is 14 Da, representing the sequential loss of a methylene group (Figure 2A). An abundant, common fragment ion at m/z 239 in the product-ion mass spectra of derivatized, saturated FA species was present and corresponded to the loss of alkanes containing $(N - 3)$ carbon atoms from the aliphatic chain of FA species (N is the total number of carbon atoms in FA species), yielding a conjugated stable fragment ion with carbonyl group (i.e., $CH_2=CH-CO-NH-AMPP^+$). In the product-ion mass spectra, the most abundant fragment ions were those yielded from the derivative reagent with or without the nitrogen of the amide bond (i.e., m/z 169 and 183) (Figure 2A).

In contrast to the sequential loss of 14 Da from the saturated FA chains (Figure 2A), the product-ion mass spectra of the $[FA+AMPP]^+$ of 18:1 FA isomers showed not only identical and abundant fragment ions resulted from the derivatized group including ions at m/z 169.1 and 183.1, but also very distinct and informative signatures in the region between m/z 190 and 420 (Figure 2B-2D). The abundant fragment ions yielded from the derivatized group could be used for effectively determining the presence of carboxyl moiety through PIS of these ions and for sensitively determining the concentration of the derivatized FA species in comparison to the selected internal standard(s) (see below). Those distinct signatures provided wealthy information about the locations of double bonds, thereby allowing us not only to determine the structure of an isomer, but also to determine the composition of these isomers in a particular mixture (see below). Similarly, distinct and informative product-ion mass spectra of the isomers of all examined FAs were also demonstrated (e.g., Figures S3 and S4 for 18:3 and 20:3 FA, respectively).

Similar to the structural elucidation of FA isomers, modified FA isomers could also be equally identified from the product-ion mass spectra of their derivatives. For example, the product-ion mass spectra of the $[FA+AMPP]^+$ of nitrosylated (i.e., 9- and 10- NO_2) 18:1 FA isomers (Figure S5) showed distinguished fragmentation patterns that structural and compositional determination of these isomers alone or in their mixtures was without any difficulties. Similarly, product-ion mass spectral analyses of derivatized eicosanoid isomers demonstrated very distinct and informative fragmentation patterns (Figure S6 showed the product-ion mass spectra of representative hydroxyeicosatetraenoic acid (HETE) isomers, Figure S7A and S7B demonstrated the product-ion mass spectra of representative diHETE isomers, and Figure S7C and S7D were the product-ion mass spectra of representative epoxyeicosatetraenoic acid (EET) isomers). Again, the product-ion mass spectra of the corresponding isomers demonstrated very distinguished fragmentation patterns so that structural and compositional determination of those groups of isomers alone or in their mixtures could readily be achieved.

In the study, we further extended this fatty acidomics approach for structural determination of all other species or isomers containing a carboxyl group including branched fatty acids (e.g., phytanic acid (Figure S8A)), ring-containing species (e.g., retinoic acid, Figure S8B), and bile acids (Figure S8C and S8D). It was clearly demonstrated that the methyl group(s) (except the α -methyl) of all the isoprenoid fatty acids could readily be determined (see molecular structure and fragmentation in Figure S8A). The signatures of the fragment ions corresponding to the sterol rings of two bile acids (chenodeoxycholic (3 α ,7 α -dihydroxy-5 α -cholanic) and deoxycholic (3 α ,12 α -dihydroxy-5 α -cholanic) acids) due to the position

difference of a hydroxyl group were significantly different (Insets of Figure S8C and S8D). These differences allowed us to readily determine the isomers alone or in their mixtures. Accordingly, the structures of the fatty acid species as examined could readily be determined by the fatty acidomics approach in combination with the m/z of the molecular ion.

Quantitation of FA Species Including Isomers from Charge-Remote Fragmentation

Unlike the LC-MS approach(es), shotgun lipidomics does not directly analyze the separate isomers of a fatty acid. The mass levels of individual FA isomers are determined through two-step processing. First, the mass levels of the FA are measured through comparing the FA ion intensity with that of the selected internal standard after derivatization with AMPP determined by using either ESI-MS or combination with tandem MS in the PIS mode. Then, the composition of the isomers of the FA is determined based on its fragmentation pattern (i.e., isomer mixture) in comparison to those of the authentic individual isomers through multiple linear regression analysis as described under MATERIALS AND METHODS.

To determine the possibility of ESI-MS analysis for quantitation of FA species after AMPP derivatization, ESI-MS analysis of FA mixtures at concentration of approximately 1 pmol/ μ l for each was performed. The mass spectra of these mixtures displayed nearly equal intense ion peaks (Figure 3A). From the testing with a variety of different mixtures, we did not find any significant differences of the ionization efficiencies and/or derivative efficiencies between the different derivatized FA species within experimental errors. The observation of identical ionization efficiency of derivatized FA species was consistent with those findings obtained with other polar lipids that the polar head group plays the major role in lipid ionization in ESI-MS and different molecular species of a polar lipid class possess essentially identical ionization efficiency after correction for differential ^{13}C isotope distribution in the low concentration region²⁹. We believe that the variation of those intensity differences between the derivatized FA species was likely due to the mass differences of individual species in the mixture since we did not find any tendency of variation with the chain length and the number of double bonds. It was unfortunate that many unknown peaks were present in the mass spectra of the derivatized FA species (Figure 3A) and those peaks became very intense when the concentration of the FA mixtures was lower. Therefore, we explored the possibility of tandem MS for quantification of derivatized FA species.

To determine the enhanced sensitivity and specificity of detection for these AMPP-derivatized FA ions, MS/MS analysis of PIS169 and PIS183 (see above) was performed. We found that both PIS169 and PIS183 analyses yielded sensitive and specific detection of AMPP-derivatized FA ions. Considering its sensitivity and stability over PIS169, we selected PIS183 for the quantification purpose whereas PIS169 was only used to confirm the presence of the detected FA ions. In the study, however, we found that the ions corresponding to the unsaturated FA species showed enhanced signals relative to those of saturated FA species (Figure 3B) and that this enhancement did not depend on the number of double bond(s) as well as the concentration of the mixtures (Figure 3C and 3D). This enhancement factor was determined as 1.27 based on the peak intensity ratios of unsaturated FA species to the selected internal standard (i.e., $\text{d}_4\text{-16:0 FA}$) under various concentrations (Figure 3C and 3D).

We determined the linearity of quantitation by spiking a different amount of internal standard to the nearly equimolar mixtures of other FA species and measuring the peak intensity ratios relative to the peak intensity of the spiked internal standard (Figure 3D). It should be emphasized that we spiked a minimal amount of internal standard to achieve the intensity of internal standard greater than 5% of the base peak to minimize any error

propagation. The linearity of peak intensity ratios of individual FA species to the standard after ^{13}C de-isotoping as previously described²⁸ vs. their corresponding molar ratios in the mixtures was analyzed by linear regression of log plots as discussed previously³⁰ (Figure 3D). An essentially identical linear correlation was well obtained for all the saturated FA species examined whereas a different correlation was obtained from all unsaturated FA species of which an essentially identical linear correlation was also observed (Figure 3D). The slope of a linear regression between the expected and determined relative peak intensity ratios of a FA species to the internal standard could also determine the enhancement factor for unsaturated FA species in fatty acidomics by using PIS183 and comparing to the saturated FA species as reference(s).

Both dilution experiment and linear regression analysis also implicated the limit of quantification (i.e., 1.0 fmol/ μL), reproducibility (intraday CV < 5.6% and interday CV < 9.1%), linear dynamic range (i.e., over 2500 fold), etc. for quantification of AMPP-derivatized FA species by using PIS183 and an internal standard. The largest standard deviation for quantitative analysis of FA species was unsurprisingly at the lowest concentration (i.e., 0.1 fmol/ μL of FA mixture) (Figure 3C and 3D) at which the effect of background noise was apparent.

We further examined the effects of other co-existing lipids present in lipid extracts of biological samples on quantitative analysis of FA molecular species by fatty acidomics. For this purpose, we spiked various amounts of 20:4 FA to mouse liver lipid extracts (5.9 pmol/ μL). Fatty acidomics analyses of these mixtures after proper dilution for minimizing potential aggregation detected a well linear correlation of the spiked amount of 20:4 FA with that determined by the method (correlation coefficient $r^2 = 0.9994$) by applying a correct factor for enhancement of unsaturation.

The second key component of quantitation in fatty acidomics was to determine the composition of isomers of a FA. In fatty acidomics, this composition was determined from the signature of the fragment ions yielded from the isomeric mixture (e.g., those highlighted with the boxes in Figure 4). We found that the composition could accurately be determined through multiple linear regression analysis. In the process, we used the normalized fragment ions resulted from the ion of an isomeric mixture as the responses and the corresponding normalized fragment ions separately detected from individual isomeric ions as the predictors according to Formulas 1 and 2 to achieve maximal coefficients of determination (r^2). As proof of concept, a variety of isomeric mixtures of 18:1 FA were prepared and the product-ion mass spectra of these mixtures (Figure 4) as well as product-ion mass spectra of individual 18:1 FA isomers (Figure 2B to 2D) were acquired under identical CID experimental conditions. The aforementioned model was tested with a combination of three sets of ion peak intensities and two normalization conditions. The ion sets included (1) the fragment ions at m/z 196, 211, 225, 239, 253, 267, 281, 295, 309, and 323, representing the sequential loss of individual methylene groups in the region after charge-remote fragmentation; (2) the ions after dropping off the ions at m/z 211, 267, and 295 from set 1, which generally represented the lowest intensities among the ion set; and (3) the three ions at m/z 253, 295, and 323, which separately characterized the double bond positions of 18:1 FA isomers (Figure 2). Two normalized conditions included either to the molecular ion or to the most intense fragment ion resulted from the derivatized reagent (i.e., m/z 183). Ion sets normalized to the most abundant fragment peak gave more matched results to the theoretical compositions of the different 18:1 FA mixtures. Small variation of these three different data sets normalized to the fragment base peak (< 7%) was obtained from multiple linear regression analysis (i.e., means $\pm$ SD of n-7/n-9/n-12 18:1 FA isomers: $0.343 \pm 0.003/0.330 \pm 0.015/0.327 \pm 0.011$; $0.203 \pm 0.014/0.419 \pm 0.008/0.378 \pm 0.006$; $0.063 \pm 0.004/0.639 \pm 0.011/0.298 \pm 0.015$; $0.078 \pm 0.001/0.340 \pm 0.022/0.582 \pm 0.023$) with all

correlation coefficients (r^2) of > 0.99 . These ratios were well comparable to the experimentally prepared ones of the isomers (i.e., n-7/n-9/n-12 18:1 FA isomers: 0.33/0.33/0.33; 0.20/0.40/0.40; 0.06/0.60/0.34; 0.06/0.34/0.60, respectively). These results strongly support the method for quantification of isomeric composition and the data processing model described in the section of METHODS.

A few points related to the quantitative analysis by using the fatty acidomics were worthy of noting. First, separate internal standards for saturated and unsaturated FA species, respectively, are recommended for accuracy and mutual validation of quantification of FA species. Second, we tested the approach with both unit-resolution and high-resolution mass spectrometers and essentially identical results from both instruments were obtained. Third, product-ion mass spectra of individual FA isomers and their mixtures could be acquired separately, but under identical MS conditions, particularly those for CID. Fourth, it would be better to use a high-resolution mass spectrometer for product-ion MS analysis of the composition of FA isomers present in biological samples to reduce any interference of other molecular ion(s) present in the region. Alternatively, multidimensional MS as previously described^{26, 29} could be used for quantitative analysis of the FA isomeric compositions through neutral loss monitoring of relevant fragment ions. Fifth, appropriate internal standard(s) (the stable isotope-labeled one(s) if available) should always be used for quantification of all the modified FA species including nitrosylated, hydroxylated, halogenated, branched ones. Sixth, pre-fractionation by using chromatographic separation or other techniques should be considered for enrichment of very low abundance modified FA species prior to or after derivatization if necessary. To this end, it is intriguing to recognize that a cation exchange column could be employed for enrichment of all derivatized FA species since the lipids carrying a cation present in biological samples are very rare and thus, the derivatized FA species in this case are unique.

Identification and Quantitation of Non-esterified FA Species in Human Plasma Lipid Extracts

To demonstrate the capability and utility of the current approach for fatty acidomics, we determined the levels of non-esterified FA (NEFA) species including isomers present in human plasma. These NEFA species varied over a very broad range of concentrations and many isomeric species are present in very low abundance. Figure 5A displayed a representative PIS183.1 mass spectrum of an AMPP-derivatized human plasma lipid extract, which showed a few with very abundant ion peaks at m/z 423.2, 427.2, 447.2, 449.2, and 451.2, corresponding to derivatized 16:0, d₄-16:0, 18:2, 18:1, and 18:0 FAs, respectively. These were identified by both PIS and product ion analyses as described above. Close examination of the low abundance region (Insets a and b, Figure 5A) revealed the presence of other numerous NEFA species in low abundance. The presence of isomeric FA species corresponding to individual unsaturated NEFA species was identified and quantified through analysis of the fragmentation patterns as described above and the results were tabulated (Table S1). A representative product ion analysis of 18:3 FA isomers was exemplified (Figure 5B) from a low abundance precursor ion (as indicated with an arrow in Figure 5A). The presence of the characteristic fragment at m/z 347.2 in combination with other fragment ions demonstrated the presence of n-6 18:3 FA isomer. The product ion mass spectrum of derivatized 18:3 FA ion also clearly indicates that the n-3 18:3 FA isomer is more abundant than n-6 18:3 FA isomer (Figure 5B, compared to Figure S3), which contradicts the results from one study³¹, but is consistent with the others^{7, 32}.

It was unexpected that the levels of human plasma NEFA species from three healthy individuals examined as 514.49 ± 6.23 , 470.16 ± 2.73 , and 224.95 ± 10.04 nmol/mL in total (Table S1). However, these values were well within the range of human plasma NEFA levels which are varied from ~ 200 to ~ 550 nmol/mL^{7, 31-33} determined by other analytical

methods. Moreover, it was also demonstrated that more NEFA species present in human plasma were identified and quantified by using the current approach (Table S1) than those previously reported by using other methods^{7, 31, 32}. These results indicate that the accuracy of the current approach is comparable to the other methods and may be more advanced in cost effectiveness and sensitivity.

SUMMARY

In the current study, a fatty acidomics approach was developed. It was found that compared with a proper internal standard and by using PIS analysis, the distinguished charge-remote fragmentations of the derivatized FA isomers can be used to determine the composition of the FA isomers and sensitively quantify the individual derivatized FA species. Taken together, we believe that as a powerful addition to lipidomics, fatty acidomics could greatly accelerate the identification of the biochemical mechanisms underlying numerous pathological conditions.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was partly supported by National Institute on Aging Grant R01 AG31675, National Institute of General Medical Sciences Grant R01 GM105724, and intramural institutional research funds. XH has financial relationship with LipoSpectrum LLC. Special thanks to Ms. Imee Tiu for her editorial assistance.

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Figure 1.

Product-ion mass spectra of arachidonic acid after derivatized with a variety of charge-carried reagents. Product-ion MS analyses of arachidonic acid which was derivatized with N-(benzenemethylamine)-2,4,6-trimethylpyridinium (BMA-TMP) (Panel A), N-(4-aminomethylphenyl)pyridinium (AMPP) (Panel B), 4-aminomethyl-1-methylpyridin-1-ium (AMMP) (Panel C), and N-(4-aminomethylbenzyl)-2,4,6-trimethylpyridinium (AMB-TMP) (Panel D) were performed at collision energy of 35 eV for AMMP and 40 eV for others as described under “MATERIALS AND METHODS”. The insets of Panels A to C displayed the identical fragmentation patterns in the mass region corresponding to the FA chain in which the m/z of the molecular ion (M^+) was arbitrarily set as zero and other fragment ions separately indicated with the losses of the masses from the molecular ion.

Figure 2.

Product-ion mass spectra of a representative saturated fatty acid 20:0 and 18:1 fatty acid isomers after derivatized with AMPP. Derivatization of fatty acids with AMPP and product-ion MS analyses of derivatized 20:0 FA (Panel A) as well as n-7 18:1 (Panel B), n-9 18:1 (Panel C), and n-12 18:1 (Panel D) FA isomers at collision energy of 50 eV for 20:0 FA and 40 eV for all of 18:1 FA isomers were performed as described under “MATERIALS AND METHODS”. The majority of the abundant fragment ions after charge-remote fragmentation with AMPP was assigned and illustrated in the corresponding molecular structures.

Figure 3.

Relationship of relative peak intensity with concentration and correlation of the determined peak intensity ratio from mass spectra with the expected molar ratio in the sample. Positive-ion ESI-MS analysis (Panel A) and precursor-ion scanning (PIS) of m/z 183.1 (Panel B) of a FA mixture at 1 μ M and collision energy of 69 eV with collision gas pressure at 0.7 mT (in order to avoid peak tailing in the PIS spectra) were performed as described under MATERIALS AND METHODS. The ion peak intensity ratio of individual derivatized FA species relative to d_4 -16:0 FA ion peak was determined from the PIS183.1 spectra after correction for ^{13}C isotope effects as previously described²⁸. An independent relationship of the ratios relative to d_4 -16:0 FA ion peak with concentration (ranging from 0.1 to 1000 nM) from representative FA species (18:3, solid diamond; 20:3, solid square; 22:6, solid circle; 14:0, open diamond; 17:0, open circle; and 22:0, open square) was demonstrated in Panel C. This result well indicated the limit of quantification of the method for individual FA species. The correlation of molecular ion intensity ratio of representative FA species (as aforementioned) relative to d_4 -16:0 FA ion with the molar ratio in the prepared mixture solutions was demonstrated in Panel D with linear regression trend lines of $\log(y)=0.9814\log(x)+0.0281$ for saturated FA species (Broken line) and $\log(y)=1.0182\log(x)+0.0877$ for unsaturated FA species (Solid line). The correlation coefficients (r^2) of the linear plots for all examined individual FA species were > 0.99 . Data are presented as the means $\pm$ SD from at least four separate sample preparations.

Figure 4.

Product-ion mass spectra of representative mixtures of 18:1 fatty acid isomers after derivatized with AMPP. Preparation of isomeric 18:1 FA mixtures and their derivatization with AMPP as well as product-ion MS analyses of derivatized mixtures of n-7/n-9/n-12 18:1 FA isomers in 0.33:0.33:0.33 (Panel A), 0.2:0.4:0.4 (Panel B), 0.06:0.60:0.34 (Panel C), and 0.06:0.34:0.60 (Panel D) theoretical ratios at collision energy of 40 eV were performed as described under “MATERIALS AND METHODS”. The signatures highlighted with the broken lined boxes were used to determine the composition of 18:1 FA isomers in the mixtures through multiple linear regression analysis of these signatures as the responses with the fragmentation patterns of individual 18:1 FA isomers (demonstrated in Figure 2B to 2C) as the predictors.

Figure 5.

Representative mass spectral analysis of derivatized non-esterified fatty acid species present in a human plasma lipid extract. A lipid extract of human plasma from a healthy individual was prepared by a modified Bligh and Dyer procedure and a portion of the lipid extract (~1/10) was derivatized with AMPP as described under “MATERIALS AND METHODS”. Precursor-ion scans of 183.1 (Panel A) and 169.1 (not shown) were performed to identify the molecular ions corresponding to FA species. Fragmentation patterns of the ions corresponding to unsaturated FA species were acquired by using a Q-Executive mass spectrometer for analysis of isomeric species composition as described under “MATERIALS AND METHODS”. An example of the product ion analysis of a low abundance ion (as indicated with an arrow in Figure 5A) corresponding to derivatized 18:3 FA was given (Panel B). This demonstrated the presence of low abundance of n-6 18:3 FA isomer (i.e., the presence of a characteristic fragment at m/z 347.2 indicated by an asterisk, Figure 5B inset).