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Quantification of F-ring isoprostane-like compounds (F_4-neuroprostanes) derived from docosahexaenoic acid in vivo in humans by a stable isotope dilution mass spectrometric assay

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Abstract

Lipid peroxidation has been implicated in the pathophysiological sequelae of human neurodegenerative disorders. It is recognized that quantification of lipid peroxidation is best assessed in vivo by measuring a series of prostaglandin (PG) F_2-like compounds termed F_2-isoprostanes (IsoPs) in tissues in which arachidonic acid is abundant. Unlike other organs, the major polyunsaturated fatty acid (PUFA) in the brain is docosahexaenoic acid (DHA, C22:6 ω-6), and this fatty acid is particularly enriched in neurons. We have previously reported that DHA undergoes oxidation in vitro and in vivo resulting in the formation of a series of F_2-IsoP-like compounds termed F_4-neuroprostanes (F_4-NPs). We recently chemically synthesized one F_4-NP, 17-F_{4c}-NP, converted it to an ^{18}O-labeled derivative, and utilized it as an internal standard to develop an assay to quantify endogenous production of F_4-NPs by gas chromatography (GC)/negative ion chemical ionization (NICI) mass spectrometry (MS). The assay is highly precise and accurate. The lower limit of sensitivity is approximately 10 pg. Levels of F_4-NPs in brain tissue from rodents were 8.7 ± 2.0 ng/g wet weight (mean ± S.D.). Levels of the F_4-NPs in brains from normal humans were found to be 4.9 ± 0.6 ng/g (mean ± S.D.) and were 2.1-fold higher in affected regions of brains from humans with Alzheimer's disease ($P = 0.02$). Thus, this assay provides a sensitive and accurate method to assess oxidation of DHA in animal and human tissues and will allow for the further elucidation of the role of oxidative injury to the central nervous system in association with human neurodegenerative disorders.
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Keywords: Isoprostane; Neuroprostane; Eicosanoid; Lipid

1. Introduction

Excessive production of free radicals derived primarily from molecular oxygen (oxidant stress) has been implicated in the pathogenesis of a wide variety of human disorders [1–4]. The role that oxidative injury plays in neurodegenerative diseases is an area of particularly intense research efforts [5,6]. However, the paucity of accurate and sensitive methods to assess oxidative damage to the central nervous system has hindered progress in this field. Lipid peroxidation is a known consequence of oxidant stress, and levels of various lipid peroxidation products such as malondialdehyde and 4-hydroxynonenal are elevated in several neurodegenerative disorders, including Alzheimer's, Parkinson's, and Huntington's diseases [5–7].

Polyunsaturated fatty acids (PUFAs) are highly susceptible to oxidation and are enriched in brain tissue [8,9]. Previously, we described that a group of prostaglandin (PG)F_2-like compounds, termed F_2-isoprostanes (IsoPs), are formed in significant amounts in vivo as products of the free radical-catalyzed peroxidation of arachidonic acid

Abbreviations: PUFA, polyunsaturated fatty acid; PG, prostaglandin; IsoP, isoprostane; PFB, pentafluorobenzyl; AAPH, 2,2′-azobis-(2-amidinopropane) hydrochloride; BSTFA, *N,O*-bis(trimethylsilyl)-trifluoroacetamide; NP, neuroprostane; GC, gas chromatography; MS, mass spectrometry; NICI, negative ion chemical ionization; SIM, selected ion monitoring

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(C20:4ω-6) [10,11]. Arachidonate is present ubiquitously in tissue phospholipids and other lipid stores and is one of the most abundant fatty acids in most organs in animals and humans [12]. Over the past 10 years, considerable evidence has accumulated showing that the measurement of F_2-IsoPs is arguably the most accurate index of oxidative stress in vivo in a wide variety of tissues and body fluids [13]. Sensitive methods utilizing a variety of mass spectrometric approaches to detect F_2-IsoPs in tissue and fluids have been developed [11,13]. While immunoassays also exist to measure IsoPs, they are considerably less reliable [13].

Unlike other tissues, the brain contains significant amounts of the PUFA, docosahexaenoic acid (DHA; C22:6ω-3) [8,9]. DHA is particularly enriched in neuronal membranes where it accounts for 25–35% of total fatty acids in gray matter. We have previously reported that DHA, like arachidonic acid, can undergo oxidation in vitro and in vivo resulting in formation of a series of F_2-IsoP-like molecules termed F_4-neuroprostanes (NPs) (14). Quantification of these compounds appears to provide a highly sensitive index of oxidative neuronal injury in contrast to the IsoPs, which likely represent a global measure of oxidant status in the central nervous system [15]. Fig. 1 illustrates the structure of F_4-NPs formed from the oxidation of DHA. As is evident, eight regioisomers are formed, each consisting of eight racemic diastereomers for a theoretical total of 128 different compounds.

Previously, we had developed an assay to quantify F_4-NPs utilizing a modification of the method we employ to measure F_2-IsoPs [14,29]. While this approach appears to produce reasonably reproducible results, assay variability was often greater than ±15% depending on the biological sample analyzed. This is likely attributable to several causes. The first is that the F_2-IsoP, $[^2H_4]$15-F_{2t}-IsoP (8-iso-PGF$_{2}\alpha$), is utilized as an internal standard to quantify NPs. While this IsoP is structurally similar to NPs, the former is more polar and likely has different extraction and chromatographic characteristics compared to NPs, potentially reducing the accuracy of the method to quantify NPs from sample to sample. In addition, measurement of NPs utilizing the assay previously developed is based on the quantification of the peak areas of multiple compounds eluting from the gas chromatograph

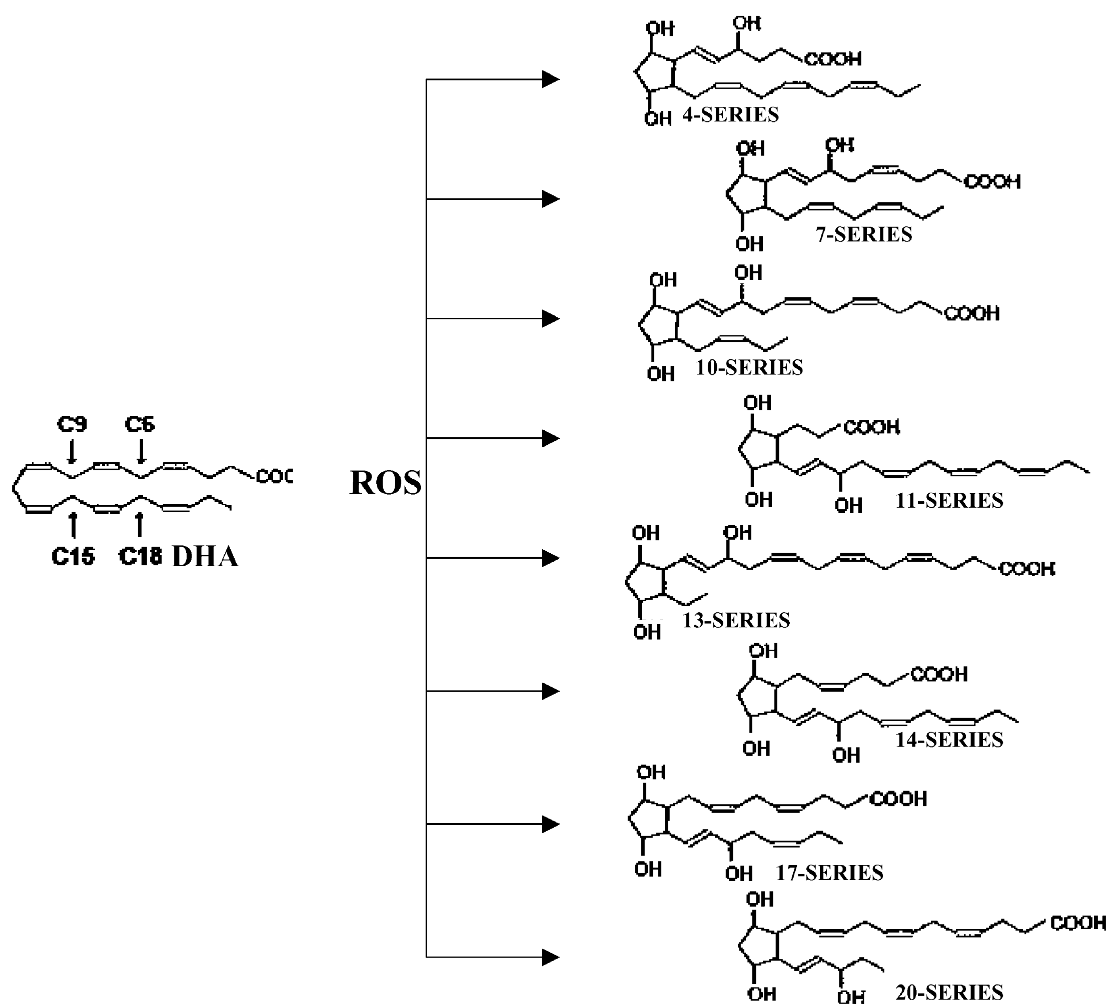

Fig. 1. F_4-NPs formed via the peroxidation of DHA by reactive oxygen species (ROS). Eight regioisomers are formed, each consisting of eight racemic diastereomers. For simplicity, stereochemistry is not shown.

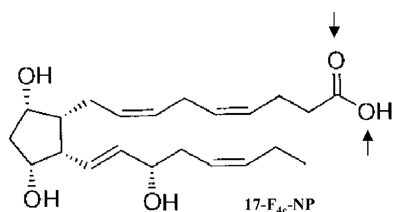

Fig. 2. Structure of the 17-series NP, 17-F_{4c}-NP, that was synthesized and converted to an ^{18}O-labeled internal standard for use in this assay. Arrows indicate ^{18}O atoms. Stereochemistry at each of the chiral carbon atoms is as noted.

over approximately a 1.5–2 min interval, resulting in the potential for co-eluting compounds that are not NPs to interfere with NP quantification [14]. Thus, we sought to develop a new assay for NPs that, like the old assay, allows for the simultaneous processing of a number of samples.

Recently, one of us developed a facile method to synthesize F-ring NPs [16]. The first of these compounds synthesized is shown in Fig. 2. Based upon the nomenclature, we have previously published (which is analogous to that nomenclature used to name F_2-IsoPs) and which has been accepted by the Eicosanoid Nomenclature Committee sanctioned by the Joint Committee on Biological Nomenclature of IUPAC, this molecule is termed 17-F_{4c}-NP, in that it contains a hydroxyl group at carbon 17, an F-type prostanoid ring, four double bonds, and *cis* sidechain stereochemistry [17]. In addition, the stereochemistry at each of the chiral carbon atoms is as noted in Fig. 2. The synthesis of this molecule was, in part, undertaken to enable the development of a mass spectrometric assay based on stable isotope dilution methodology. The method of assay that has been developed is described herein.

2. Experimental

2.1. Materials

Docosahexaenoic acid, pentafluorobenzyl (PFB) bromide and diisopropylethylamine were obtained from Sigma (St. Louis, MO). Dimethylformamide, and undecane were obtained from Aldrich (Milwaukee, WI). 2,2′-Azobis-(2-amidinopropane) hydrochloride (AAPH) was obtained from Eastman Kodak Co. (Rochester, NY) and *N,O*-bis(trimethylsilyl)-trifluoroacetamide (BSTFA) was obtained from Supelco Inc. (Bellefonte, PA). C18 and silica Sep-Paks were purchased from Waters Associates (Milford, MA). TLC was performed on silica gel 60ALK6D plates (Whatman International Ltd., Maidstone, UK). Docosahexaenoic acid was from Nu-Chek Prep Inc. (Elysian, MN).

2.2. Mass spectrometry

Gas chromatography (GC)/negative ion chemical ionization (NICI) mass spectrometry (MS) was performed using a Hewlett-Packard HP5989A GC/MS instrument interfaced with an IBM Pentium III computer system. GC was performed using a 15 m, 0.25 mm diameter, 0.25 μm film thickness, DB1701 fused silica capillary column (Fisons, Folsom, CA). The column temperature was programmed from 190 to 300 °C at 15 °C/min. Methane was used as the carrier gas at a flow rate of 1 ml/min. Ion source temperature was 250 °C, electron energy was 70 eV, and filament current was 0.25 mA. For analysis, compounds were dissolved in 10 μl of undecane which was dried over a bed of calcium hydride.

2.3. Statistical analyses

Data were analyzed using GraphPad Prizm software. Statistical analysis was done using Student's *t*-test, and *P* values were considered significant if <0.05.

2.4. Oxidation of DHA

Five milligrams of fresh DHA was dissolved in 100 μl ethanol and added immediately to 4.9 ml of phosphate-buffered saline solution (pH 7.4) containing 10 mM AAPH. The DHA oxidation reaction mixture was incubated in a shaking water bath at 37 °C for varying amounts of time, after which it was placed immediately at −80 °C until further processed.

2.5. Preparation of $^{18}O_2$-labeled 17-F_{4c}-NP

Unlabeled 17-F_{4c}-NP was chemically synthesized by one of us (J.C.) as reported [16]. The structure of 17-F_{4c}-NP was confirmed using MS and nuclear magnetic resonance. Unlabeled 17-F_{4c}-NP was subsequently converted to an $^{18}O_2$-labeled derivative for use as an internal standard by the method of Murphy and Clay involving successive steps of methylation and alkaline hydrolysis with $Li^{18}OH$ [18]. This yielded an internal standard with an unlabeled blank of 15 parts per 1000 when analyzed by GC/NICI MS. [$^{18}O_1$]17-F_{4c}-NP was present at 190 parts per 1000. The fraction of labeled molecules remained unaltered when the labeled standard was subjected to the full assay procedure. The $^{18}O_2$-labeled 17-series F_4-NP was standardized by GC/MS in comparison to deuteriated 15-F_{2t}-IsoP (8-iso $PGF_{2}\alpha$) that had been accurately quantified by weight. The ionization efficiencies of these two compounds during NICI MS are very similar with the $M - 181$ [$M - CH_2C_6F_5$] ion for 15-F_{2t}-IsoP and 17-F_{4c}-NP comprising greater than 95% of the fragment ions.

2.6. Purification and analysis of F_4-NPs from tissue

Fig. 3 summarizes the purification and derivatization scheme for the analysis of F_4-NPs. The assay can be utilized to quantify either F_4-NPs in the free form in a biological fluid or compounds that are present esterified in tissue lipids and are then hydrolyzed. The majority of analyses

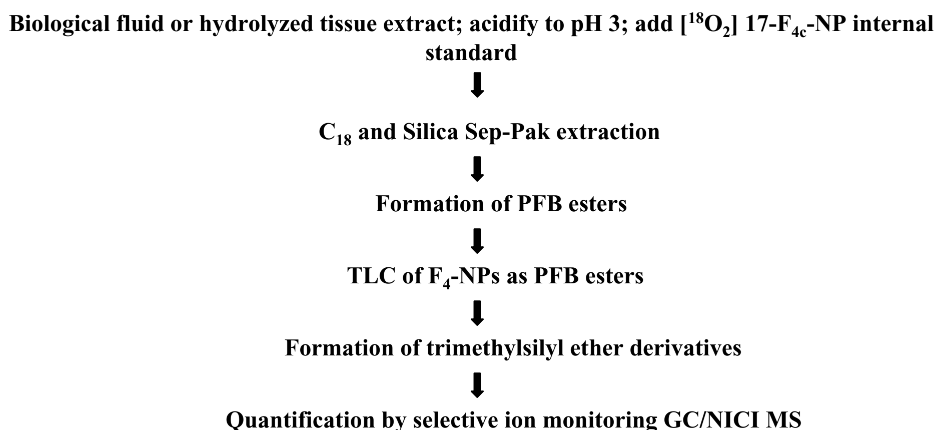

Fig. 3. Scheme for purification, derivatization, and analysis of F_4-NPs.

that we have performed are in brain and thus the workup of F_4-NPs from this tissue is utilized to summarize the assay method. However, this method can be applied to any DHA-containing tissue (e.g. liver, heart, or other tissues) or fluid (cerebrospinal fluid, plasma, or urine).

A sample of tissue (e.g. brain; 75–300 mg) is homogenized in 5 ml ice-cold chloroform:methanol (2:1, v/v) containing BHT (0.005%) to prevent autooxidation and placed at room temperature with occasional shaking for 1 h. Two milliliters of 0.9% NaCl is then added, the sample is then vigorously shaken and centrifuged, and the aqueous and protein layers removed. The sample is dried under a stream of nitrogen, and resuspended in 2 ml methanol containing BHT. Esterified F_4-NPs in phospholipids are hydrolyzed using chemical saponification by adding 2 ml 15% aqueous potassium hydroxide. The sample is acidified to pH 3 with 1 M HCl and diluted to 25 ml with pH 3 H_2O. One nannogram of the $[^{18}O_2]$17-F_{4c}-NP standard is then added to mixture. For the measurement of free F_4-NPs in a biological fluid, the extraction and hydrolysis steps are omitted and the sample is simply acidified, diluted, and the internal standard added as noted.

The mixture is subsequently vortexed and applied to a C18 Sep-Pak column preconditioned with 5 ml methanol and 5 ml of water (pH 3). The column is washed sequentially with 10 ml of water (pH 3) and 10 ml heptane. F_4-NPs are eluted with 10 ml ethyl acetate/heptane (50:50, v/v). The ethyl acetate/heptane eluate from the C18 Sep-Pak is then dried over anhydrous Na_2SO_4 and applied to a silica Sep-Pak preconditioned with ethyl acetate. The column is washed with 5 ml of ethyl acetate/heptane (75:25, v/v) and F_4-NPs are eluted with 5 ml ethyl acetate/methanol (50:50, v/v). The ethyl acetate/methanol eluate is evaporated under a stream of nitrogen. F_4-NPs are converted to pentafluorobenzyl esters by treatment with a mixture of 40 μl of 10% pentafluorobenzyl bromide in acetonitrile and 20 μl of 10% *N*,*N*-diisopropylethylamine in acetonitrile at 40 °C for 20 min. The reagents are dried under nitrogen and the residue subjected to TLC using a solvent system of chloroform/ethanol (90:10, v/v). The TLC is run to 15 cm. Approximately 2–5 mg of the methyl ester of 17-F_{4c}-NP is chromatographed on a separate lane and visualized by spraying with a 10% solution of phosphomolybdic acid in ethanol followed by heating. Compounds migrating in the region from immediately below to 2 cm above the methyl ester of 17-F_{4c}-NP are scraped and extracted from the silica gel with 1 ml ethyl acetate. This TLC cut is much narrower than that reported previously, and allows a more selective isolation of NP isomers co-eluting with the $[^{18}O_2]$17-F_{4c}-NP internal standard than the previous assay. The ethyl acetate is dried under nitrogen and F_4-NPs are converted to trimethylsilyl ether derivatives by adding 20 μl BSTFA and 10 μl dimethylformamide and incubating at 40 °C for 5 min. The reagents are dried under nitrogen and F_4-NPs are redissolved in 10 μl of undecane for analysis by GC/MS.

The major ion generated in the NICI mass spectrum of the pentafluorobenzyl ester, tris-trimethylsilyl ether derivative of F_4-NP is *m/z* 593, which represents the $M - 181$ ($M - CH_2C_6F_5$) carboxylate anion. The $^{18}O_2$-labeled internal standard generates an analogous ion at *m/z* 597. Quantification of endogenous F_4-NPs is accomplished by selected ion monitoring (SIM) comparing the ratios of the integrated areas of the appropriate peaks of *m/z* 593 SIM chromatogram to that of large peak in the *m/z* 597 SIM chromatogram. The recovery of 17-F_{4c}-NP through the assay is approximately 20–30%.

3. Results

3.1. Assay results

A representative SIM chromatogram obtained from the analysis of basal ganglia brain tissue from a normal mouse is shown in Fig. 4. At the bottom is the *m/z* 597 SIM chromatogram representing the $[^{18}O_2]$17-F_{4c}-NP internal standard. In the upper *m/z* 593 chromatogram, several peaks are present that represent putative NP regioisomers eluting from

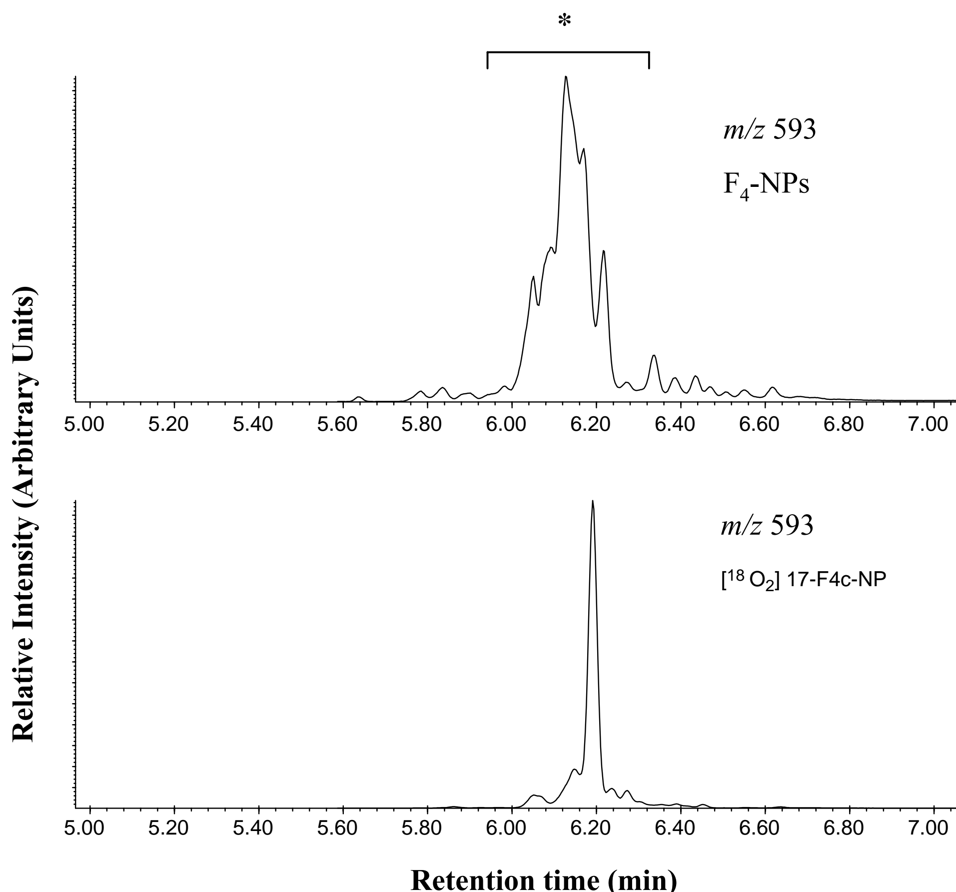

Fig. 4. Analysis of F_4-NPs using GC/MS in basal ganglia brain tissue from a normal mouse.

the GC over a 30 s interval. The identification of all of the compounds represented in these peaks as F_4-NPs was confirmed using a variety of chemical and mass spectrometric approaches [14]. First, no peaks were present when *m/z* 592 was monitored, indicating that the *m/z* 593 peaks were not natural isotope peaks of compounds generating an ion of less than 593 Da. When the compounds were analyzed as $[^2H_9]$trimethylsilyl ether derivatives, the *m/z* 593 peaks all shifted up 27 Da, indicating the compounds all have three hydroxyl groups. When the compounds were analyzed following catalytic hydrogenation, there was a disappearance of the *m/z* 593 peaks and the appearance of new intense peaks 8 Da higher at *m/z* 601 Da. No peaks were detected at *m/z* 599 or 603, indicating that all of the compounds contained four double bonds. Collectively, these results indicated that the compounds represented by the *m/z* 593 peaks contain the same functional groups and number of double bonds expected for F_4-NPs. Finally, we have also previously provided evidence for the identification of these compounds as F_4-NPs employing GC/electron ionization mass spectrometry [14].

For quantification purposes, we measure NPs that comprise the majority of compounds and elute from 6.0 to 6.4 min. These are denoted by the starred (*) bar above the chromatographic peaks. The area of the entire set of peaks beneath the starred (*) bar is integrated and compared to the area of the internal standard peak. As is evident, the major chromatographic peaks elute before the 17-F_{4c}-NP internal standard and endogenous 17-F_{4c}-NP, while present, is likely a less abundant component of F_4-NPs generated in vivo. This would be predicted based upon DHA peroxidation mechanisms that we have studied and which are discussed below. Further separation of various NP isomers is not possible using the chromatographic methods employed in this assay. The amount of F_4-NP represented by the starred (*) peak areas in the *m/z* 593 chromatogram (Fig. 4) is 9.39 ng/g wet brain tissue.

3.2. Assay parameters and validation

Quantificaton of F_4-NPs is based on the intensity of the starred (*) peaks shown in Fig. 4. The lower limits of detection (signal to noise ratio of approximately 4:1) of F_4-NPs is in the range of 10 pg. Several procedures were performed to establish the accuracy of this assay. Initially, a standard curve was constructed by adding varying amounts of unlabeled 17-F_{4c}-NP to a fixed quantity of 1 ng of $[^{18}O_2]$17-F_{4c}-NP and the measured ratio of *m/z* 593–597 to the expected ratio compared (Fig. 5A). This standard curve was found to be linear over a 100-fold concentration range. A larger linear range was arbitrarily not tested using purified NP.

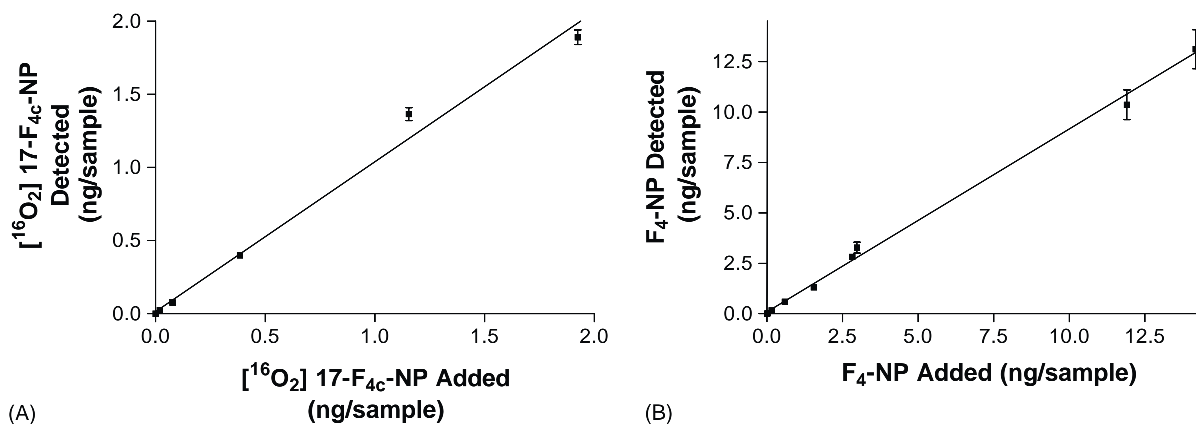

Fig. 5. Standard curves for the analysis of F_4-NPs by GC/MS. Increasing amounts of (A) $[^{16}O_2]$17-F_{4c}-NP, and (B) F_4-NPs generated in vitro from the peroxidation of DHA were added to 1 ng of $[^{18}O_2]$17-F_{4c}-NPs and assayed for F_4-NPs. The amount of either $[^{16}O_2]$17-F_{4c}-NP or F_4-NPs formed from the peroxidation of DHA that was added to the internal standard is plotted on the *x*-axis against the amount of F_4-NP detected using the assay is shown on the *y*-axis. The data are expressed as mean ± S.E., $n = 3$ replicate aliquots per condition. The linear equations describing the standard curves are (A) $y = 1.02x + 0.03$; $r^2 = 0.99$ and (B) $y = 0.92x + 0.08$; $r^2 = 0.99$.

Since this assay is utilized to measure a complex mixture of F_4-NPs, a second standard curve was constructed by adding increasing amounts of F_4-NPs generated from the peroxidation of DHA in vitro to a known amount of $[^{18}O_2]$17-F_{4c}-NP (between 0.5 and 2 ng, depending on the amount of F_4-NPs generated from DHA that were added). Again the measured ratio of *m/z* 593–597 to the expected ratio was compared (Fig. 5B). The standard curve was found to be linear over a 1000-fold concentration range.

Experiments were then carried out to establish the precision and accuracy of the assay. Precision was first measured by analyzing four aliquots of F_4-NPs generated from the oxidation of DHA in vitro. The mean of three replicate measurements of the ratio of *m/z* 593–597 was found to be ±3% at an anaylate level of 0.32 ng. Additionally, assay precision was determined using F_4-NPs isolated from mouse brain tissue and the mean of four replicates was found to be ±8.9% (see section 3.7 below). By comparison, utilizing the previous assay methods that we have reported [14], precision quantifying F_4-NPs in the same mouse brain tissue was ±19.5%.

Accuracy was then assessed using F_4-NP aliquots derived from the DHA oxidized in vitro. For this, 0.5 ng of $[^{16}O_2]$17-F_{4c}-NP was added to approximately 0.5 ng of F_4-NPs derived from the oxidation of DHA and 0.5 ng $[^{18}O_2]$17-F_{4c}-NP. The amount of endogenous F_4-NPs measured was subtracted from the total measured and the accuracy of the assay to quantify the added 0.5 ng of $[^{16}O_2]$17-F_{4c}-NP was calculated. The accuracy was found to be 97%. It should be noted that the assay described herein measures only part of NPs that are formed from the oxidation of DHA in vitro and in vivo, since a portion of the compounds are excluded during TLC purification. This does not detract, in any way, from the utility of this method to accurately assess NP formation and DHA oxidation in vitro and in vivo. Similar methods to quantify IsoPs that measure several isomers as opposed to the myriad of compounds that are formed has proven invaluable to advancing our understanding of arachidonate oxidation [10,11].

3.3. Formation of F_4-NPs from the oxidation of DHA in vitro

In order to demonstrate the ability of this assay to detect increases in F_4-NP levels with increased oxidative stress, the time course of F_4-NP formation during DHA oxidation was assessed. DHA was oxidized as described, and aliquots of the reaction mixture were taken at time 0, 3 and 8 h and measured for F_4-NPs. The results are shown in Fig. 6. As is evident, F_4-NPs increased over 6-fold after 8 h of oxidation.

3.4. Levels of F_4-NPs in brain of mice

Mouse models provide an important tool to study the impact of various genotypic and phenotypic alterations associated with certain human neurodegenerative disorders [5,15].

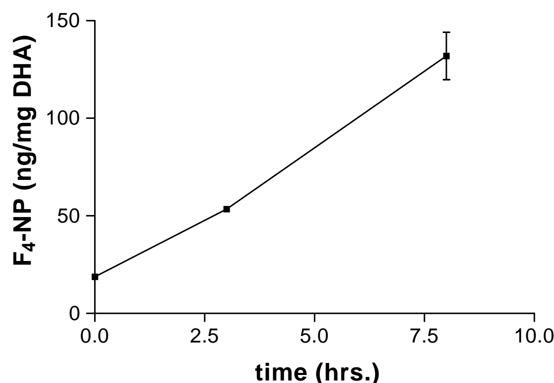

Fig. 6. Time course of formation of F_4-NPs during peroxidation of DHA in vitro using AAPH. The data are expressed as mean ± S.E., $n = 3$ replicate aliquots per condition.

In order to examine the role of lipid peroxidation, and in particular NP formation, on neurological dysfunction in mice, it is necessary to be able to accurately quantify these compounds in brain tissue and establish normal values. Young (<1 month old) C57Bl/6 mice were sacrificed by isoflurane intoxication and cervical dislocation, and cerebral hemispheres were removed with the cerebellum dissected away. F_4-NPs were then quantified as described. MS tracings obtained were virtually identical to that obtained for human brain (vide infra, data not shown). The average F_4-NP content of these mouse cerebral hemispheres was 8.7 ± 2.0 ng/g wet tissue weight ($n = 4$).

3.5. Levels of F_4-NPs in brain samples from normal humans and Alzheimer's disease patients

A number of studies have reported increased oxidative stress in certain brain regions of patients with Alzheimer's disease [5–7]. We have previously found that levels of F_4-NPs are increased in hippocampal and temporal lobe brain tissue and cerebrospinal fluid from Alzheimer's disease patients [14,19,20]. For those studies, we utilized an assay employing an IsoP internal standard. For the reasons cited previously, this assay is probably not ideal. In order to demonstrate the ability of the new assay reported herein to detect increases in F_4-NPs in Alzheimer's disease patients, levels were determined in brain samples from Alzheimer's disease patients and normal humans who died of causes unrelated to neurodegenerative diseases. For each analysis, a 250 mg section of temporal lobe tissue was dissected from post-mortem brains of patients with or without a definitive diagnosis of Alzheimer's disease. Tissue was harvested and flash frozen in liquid N_2 within 5 h of death. These tissue samples were subsequently assayed for F_4-NPs. The results are shown in Fig. 7. Temporal lobe tissue from Alzheimer's disease patients contained 2.1 fold higher F_4-NP levels (10.4 $\pm$ 2.8 ng/g wet tissue weight) than did control tissue (4.9 $\pm$ 0.6 ng/g, $P = 0.02$, $n = 4$ brain samples for both groups).

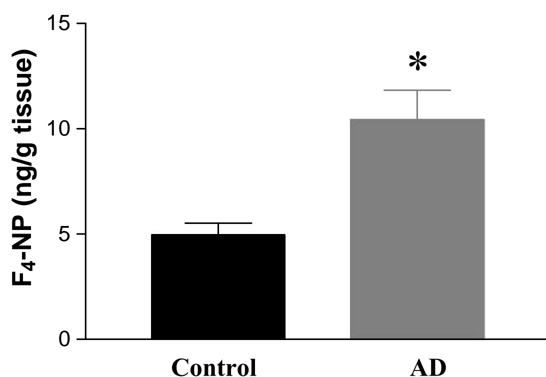

Fig. 7. F_4-NP levels in post-mortem temporal lobe tissue from Alzheimer's disease patients vs. controls. The data are expressed as means $\pm$ S.E., $n = 4$ separate brain samples per condition. $^*P = 0.02$.

4. Discussion

Oxidative stress has gained increasing attention as one mechanism that contributes to the development and progression of important neurodegenerative diseases [5–7], creating a need for accurate methods to assess free-radical mediated damage to neural tissue. The quantification of lipid peroxidation provides as attractive means of assessing such damage, as the brain is rich in highly-oxidizable lipids. Nonetheless, a reliable method to quantify lipid peroxidation in vivo has been elusive. Measuring lipid peroxidation products such as malondialdehyde and 4-hydroxynonenal has provided some insights into the role of oxidant stress in central nervous system dysfunction, but the quantification of these products in complex biological fluids and tissues is often unreliable [5–7,21]. The measurement of F_2-IsoPs, a family of PG-like molecules formed from the free radical-mediated peroxidation of arachidonic acid, has emerged as the gold-standard method for quantification of lipid peroxidation in various tissues in which arachidonate is one of the most abundant PUFAs present [13].

Unlike other organs, the primary PUFA in the brain is DHA and we have previously shown that oxidation of DHA generates a series of F_2-IsoP-like compounds termed F_4-NPs [14]. We have reported that NPs appear to provide a sensitive index of oxidative neuronal injury owing to the fact that DHA is concentrated in neurons [19]. Indeed, F_4-NP levels have been found to be elevated in human neurodegenerative diseases such as Alzheimer's diseases [19], and multiple mouse models of neurologic disease, including viral encephalitis [22], apoE-null genotype [15], and endotoxin-induced cerebral inflammation [23].

The previous assay we had developed for NPs was reasonably accurate although the reliability varied depending on the type of biological sample analyzed. This diminished our enthusiasm regarding the widespread applicability of the method. The significant variability of the former assay is likely due to two factors; the use of an F_2-IsoP, $[^2H_4]$15-F_{2t}-IsoP, rather than an F_4-NP as an internal standard, and the wide TLC cut employed which results in the potential for co-eluting compounds that are not NPs to interfere with NP quantification [14,29].

The development of a facile chemical synthesis for one NP, 17-F_{4c}-NP, has allowed us to develop a new assay for the F_4-NPs utilizing this compound as an ^{18}O-labeled internal standard [16]. The assay is both highly sensitive and accurate. As illustrated in Fig. 4, the starred (*) group of peaks in the *m/z* 593 chromatogram eluting over approximately 20–25 s are utilized to quantify the F_4-NPs. We have shown that the *m/z* 593 peaks eluting over this time frame contain three hydroxyl groups and four double bonds, and have previously demonstrated that these compounds contain a prostanoid F-ring, indicating that they are indeed F_4-NPs [14]. The substitution of the $[^{18}O_2]$17-F_{4c}-NP for the $[^2H_4]$15-F_{2t}-IsoP standard allows a significantly narrower TLC region to be analyzed for F_4-NPs that coelute with the

internal standard. This yields a GC/MS chromatogram containing far fewer NP isomers and results in enhanced assay robustness, as demonstrated by the 2.2-fold improvement in assay precision versus the old method. Using the chromatographic approaches of this assay, it is not possible to further separate the F_4-NPs shown in Fig. 4. However, the fact that several NP isomers are quantified by the method described herein does not detract from the importance or reliability of this assay since it is as precise, accurate, and sensitive as other assays that we and others have developed to quantify other eicosanoids [24–27].

While it may appear attractive to attempt to quantify NP isomers using simpler approaches than those outlined herein employing, for example liquid chromatography MS or immunoassay methods, as has been done for F_2-IsoPs, these methods have potentially significant shortcomings. Immunoassays for lipid peroxidation products such as IsoPs are highly inaccurate due to cross-reaction of the polyclonal antibiodies utilized with other compounds in biological fluids [13]. In addition, we have attempted liquid chromatography/tandem MS analysis of F_4-NPs in brain samples but have, to date, been unsuccessful in our approaches possibly because this detection method is less sensitive than GC/MS (unpublished data). Nonetheless, liquid chromatography/MS has been successfully utilized to quantify IsoPs in human urine and plasma [28].

As noted, 17-F_{4c}-NP appears to be formed in less abundance than other NPs. We have recently explored the peroxidation of DHA in vitro in detail and have found that this can be explained by the fact that the precursor hydroperoxyl radical involved in the formation of 17-F_{4c}-NP, 13-hydroperoxy-DHA, preferentially undergoes alternative oxidation reactions to form other complex peroxidation products at the expense of NPs (unpublished data).

Utilizing this assay, we have determined that NPs are readily detectable in animal and human brain tissue. In addition, we have shown that levels of these compounds are significantly increased in brains from Alzheimer's disease patients compared to control patients. These observations are highly relevant because they suggest that this method may be useful to quantify the impact of various therapeutic interventions on the extent of oxidant stress in association with human neurodegenative disorders and animal models of these diseases.

Although GC/MS assays for eicosanoids are generally not as efficient as other methods of analysis (i.e. immunoassays), approximately 15–20 samples can be processed and analyzed for F_4-NPs per day using this method. In this regard, it is as equally facile as GC/MS assays for the quantification of other prostanoids. This issue is of major importance because it allows for reasonable sample throughput.

In summary, we have reported methods to accurately quantify F_4-NPs that are produced in vivo utilizing a chemically synthesized ^{18}O-labeled 17-F_{4c}-NP internal standard. It is anticipated that this mass spectrometric assay will prove to be a valuable analytical tool to further explore the role of oxidant stress and NP formation in the pathophysiology of human neurodegenerative diseases.

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