Quantification of Fatty Acid Oxidation Products Using On-line High Performance Liquid Chromatography Tandem Mass Spectrometry

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MATERIALS

The following compounds were purchased from Cayman Chemical Company (www.caymanchem.com)

- (±)-9-hydroxy-10E,12Z-octadecadienoic acid (9-HODE) catalogueue number 38400
- (±)-13-hydroxy-9Z,11E-octadecadienoic acid (13-HODE) catalogue number 38600
- 9-oxo-10E,12Z-octadecadienoic acid (9-oxoODE) catalogue number 38420
- 13-oxo-9Z,11E-octadecadienoic acid (13-oxoODE) catalogue number 38620
- (±)5-hydroxy-6E,8Z,11Z,14Z-eicosatetraenoic acid (5-HETE) catalogue number 34210
- (±)8-hydroxy-5Z,9E,11Z,14Z-eicosatetraenoic acid (8-HETE) catalogue number 34340
- (±)-9-hydroxy-5Z,7E,11Z,14Z-eicosatetraenoic acid (9-HETE) catalogue number 34400
- (±)11-hydroxy-5Z,8Z,12E,14Z-eicosatetraenoic acid (11-HETE) catalogue number 34500
- (±)12-hydroxy-5Z,8Z,10E,14Z-eicosatetraenoic acid (12-HETE) catalogue number 34550
- (±)15-hydroxy-5Z,8Z,11Z,13E-eicosatetraenoic acid (15-HETE) catalogue number 34700
- (±)5(6)-epoxy-8Z,11Z,14Z-eicosatrienoic acid (5,6-EET) catalogue number 50211
- (±)8(9)-epoxy-5Z,11Z,14Z-eicosatrienoic acid (8,9-EET) catalogue number 50351
- (±)11(12)-epoxy-5Z,8Z,14Z-eicosatrienoic acid (11,12-EET) catalogue number 50511
- (±)14(15)-epoxy-5Z,8Z,11Z-eicosatrienoic acid (14,15-EET) catalogue number 50651
- 5-oxo-6E,8Z,11Z,14Z-eicosatetraenoic acid (5-oxoETE) catalogue number 34250
- 12-oxo-5Z,8Z,10E,14Z-eicosatetraenoic acid (12-oxoETE) catalogue number 34580
- 15-oxo-5Z,8Z,11Z,13E-eicosatetraenoic acid (15-oxoETE) catalogue number 34730
- 9α,11α,15S-trihydroxy-5Z,13E-dien-1-oic acid ($PGF_{2\alpha}$) catalogue number 16010
- 9Z,12Z-octadecadienoic acid (linoleic acid, LA) catalogue number 90150
- 5Z,8Z,11Z,14Z-eicosatetraenoic acid (arachidonic acid, AA) catalogue number 90010
- 15S-hydroxy-5Z,8Z,11Z,13E-eicosatetraenoic-5,6,8,9,11,12,14,15-d_8 acid (15S-HETE-d_8) catalogue number 334570
- 9α,11α,15S-trihydroxy-5Z,13E-dien-1-oic-3,3,4,4-d_4 acid ($PGF_{2\alpha}$-d_4) catalogue number 316350
- 2,3-dinor-8-iso Prostaglandin $F_{2\alpha}$(2,3-dinor-8-iso$PGF_{2\alpha}$) catalogue number16290
- 8-iso Prostaglandin $F_{2\alpha}$ (8-iso$PGF_{2\alpha}$) catalogue number16350
- 5-isoprostane $F_{2\alpha}$ (5-i$PF_{2\alpha}$-VI) catalogue number16300
- Prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) catalogue number16010

The following chemicals and solvents were purchased from Fisher Scientific (Pittsburg, PA):

- Methanol (Optima grade) catalogue number A454
- Water (HPLC grade) catalogue number W5
- Acetic Acid (glacial, ACS grade) catalogue number A38
- 2-Propanol (HPLC grade) catalogue number A451
- Hexane (HPLC grade) catalogue number H302
- Sodium Hydroxide (NaOH, ACS grade) catalogue number S318
- Hydrochloric acid (HCl, ACS grade) catalogue numberA144

The following reagents were purchased from Sigma Aldrich (St. Louis, MO):

- Tin (II) chloride dihydrate ($SnCl_2$, 98%, ACS grade) catalogue number 243523
- 2,6-Di-*tert*-butyl-4-methylphenol (BHT, >99%) catalogue number B1378
- Argon (5.0 Ultra High Purity, part number AR5.0UH) was obtained from Praxair Inc. (Cleveland, OH)

- Nitrogen (5.0 Ultra High Purity, part number NI5.0UH) was obtained from Praxair Inc. (Cleveland, OH)

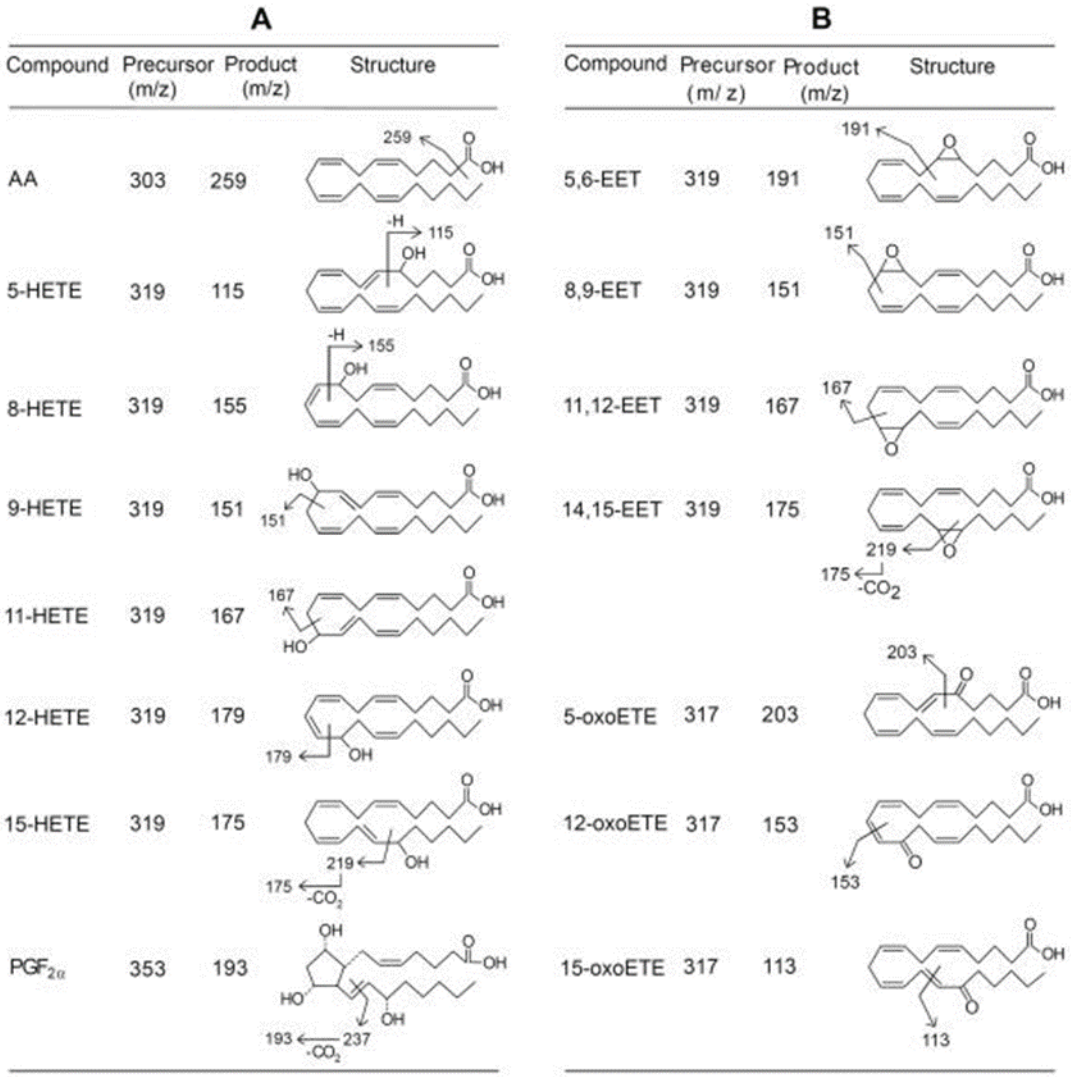

Figure 1 Precursor ion →product ion transitions and suggested structures of the major product ion derived from each eicosanoid. ESI negative-ion full scan was used for identification of precursor ions for each of the analytes and ESI negative-ion product scan was used for identification the specific product ions from each of the precursors. Suggested structures consistent with the product ions produced are depicted for each analyte.

INSTRUMENTATION

An AB SCIEX API 4000 triple quadrupole mass spectrometer interfaced to a Shimadzu HPLC system through an electrospray ionization source controlled by a PC is used for single column developmental work. Alternatively, for high-throughput dual high pressure binary gradient elution (Fig. 2) we used a Shimadzu Prominence HPLC system (Shimadzu Scientific Instruments, Columbia, MD) composed of four Shimadzu LC-20AD pumps, a Shimadzu DGU-20A5 vacuum degasser and a SIL-HTC autosampler with three, 2-position, 6 port, high pressure switching valves attached. For higher sensitivity AB SCIEX (Framingham, MA) API 5000 mass spectrometer in the negative ESI mode. A centrifuge capable of spinning sample tubes at a relative centrifugal force of 2000 × g, and a vortex mixer (Fisher Scientific MaxiMix catalogue number 12-815-50 or equivalent). Organic solutions are measured using a syringe (Gastight, Hamilton Company series 1700) or a bottle top dispenser (Fisher Scientific, catalogue number 03-692-178). Plasma, urine and other aqueous solutions are measured using positive displacement pipettors (Fisher Scientific catalogue number 21-377-328).

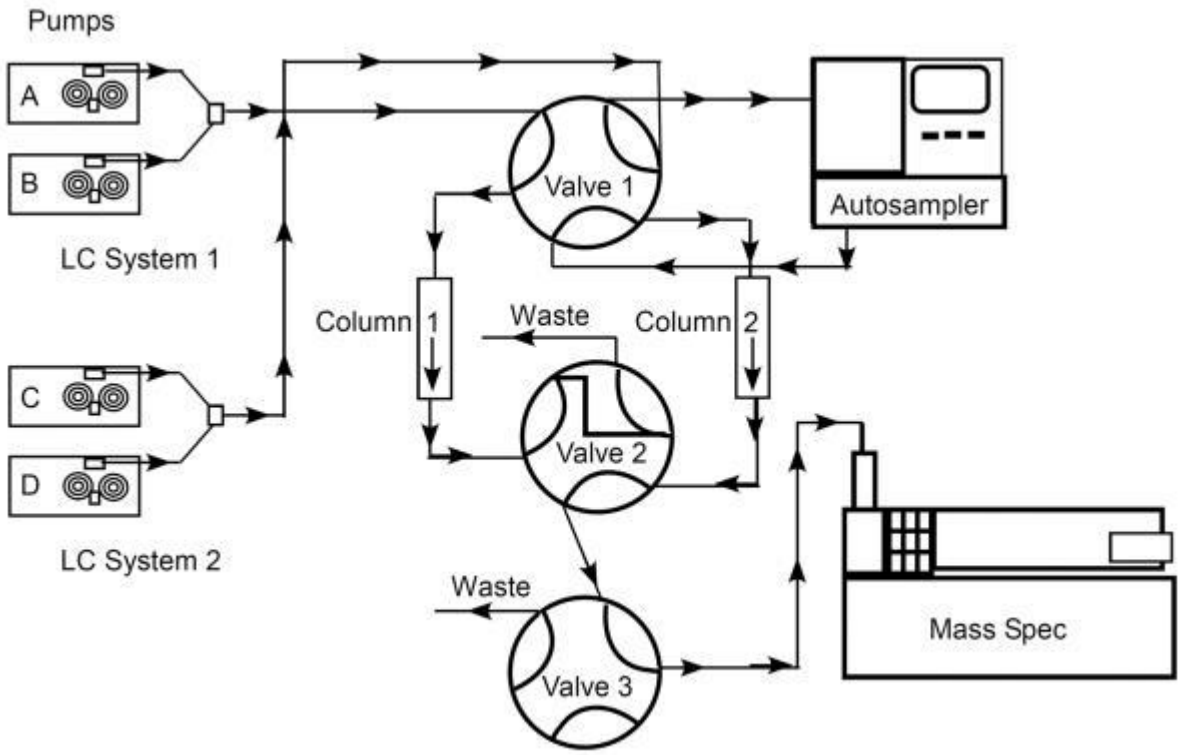

Figure 2 Dual High Pressure Mixed Binary Gradient Elution HPLC System. The plumbing schematic for the HPLC system used for high-throughput chromatography is shown. The valves are configured so that column 2 is in-line with the mass spectrometer while valve 3 serves as a bypass valve to waste, preventing effluent from reaching the mass spectrometer during any period of non-data collection.

PROTOCOL

Preparation of Standard Solutions for Plasma and Urine Samples

Stock solutions of all the standards were prepared in 85% methanol. Glass containers were flushed with argon and kept at −20°C. Our data indicate that the standards solutions are chemically stable for at least six months under these conditions. Working solutions were prepared monthly by diluting the stock solutions into 85% methanol. To prepare the standard curves, 10 µl of internal standard solution containing 20 ng/µl $PGF_{2\alpha}$-d_4 and 4 ng/µl 15(S)-HETE-d_8 was added into 200 µl of each dilution of the standard solutions. The entire process is detailed below:

1. Aliquot 200µl of plasma (or urine) sample into a clean 12 × 75mm borosilicate glass test tube
2. Add 10µl of internal standard mixture (20 ng/µl $PGF_{2\alpha}$-d_4 and 4 ng/µl 15(S)-HETE-d_8)
3. Add 1.0ml 10% v/v acetic acid in water/2-propanol/hexane (2/20/30, v/v/v)
4. Vortex briefly to mix
5. Add 2.0mL Hexane
6. Cap tube with polypropylene stopper (Fisher Scientific catalogue #14-376-77)
7. Vortex mix for three (3) minutes
8. Centrifuge sample at room temperature 2000 × g for five (5) minutes
9. Remove upper hexane layer with a glass Pasteur pipette; place in clean 12 × 75mm test tube
10. Evaporate hexane extract under N_2 flow.
    a. For unesterified fatty acid analysis: proceed with step 26.
11. For total fatty acids profile; suspend pellet in 1.0ml of 2-propanol
12. Add 1.0ml of aqueous 2M NaOH (saturated with Argon)
13. Vortex briefly to mix
14. Blanket sample with Argon gas
15. Cap tube immediately with polypropylene stopper (Fisher Scientific catalogue #14-376-77)
16. Place in 60°C water bath for 60min to hydrolyse lipids
17. Cool to room temperature

    a. At this point samples may be stored refrigerated overnight if necessary
18. Acidify sample by adding 1.2 ml of 2.0 M HCl in water
19. Add 2.0mL Hexane
20. Cap tube with polypropylene stopper (Fisher Scientific catalogue #14-376-77)
21. Vortex mix for three (3) minutes
22. Centrifuge sample at room temperature 2000 × g for five (5) minutes
23. Remove upper hexane layer with a glass Pasteur pipette; place in clean 12 × 75mm test tube
24. Repeat steps 19 through 23 and combine both hexane layers
25. Evaporate hexane extract under N_2 flow
26. Vortex pellet into 100 µl of 85% v/v methanol in water
27. Store under Argon at −20°C until analysis by LC-MS/MS
28. Centrifuge at 2000 × g immediately before analysis
    a. For oxidized fatty acids and their precursor compounds in urine, the same protocol is used as described for plasma above except that a more sensitive mass spectrometer is needed for the analysis

LC-MS/MS analysis

A 40µl sample (in 85% methanol) was injected onto a reverse-phase C18 HPLC column (XPERTEX, 2.1 × 250mm, 5 µm particle, P.J. Cobert Associates, St. Louise, Missouri) at flow rate of 0.2 ml/min. Solvent A was water (plus 0.2% v/v acetic acid) and solvent B was methanol (plus 0.2% v/v acetic acid). The column was equilibrated with 85% B. The separation was performed starting at 85% B for 10 min, then using a gradient to 100% B over two min, followed by 100% B for 10 min and then 100% A for 10 min. HPLC column effluent was introduced into an AB SCIEX API 4000 triple quadrupole mass spectrometer. The source of the mass spectrometer was configured with the electrospray needle voltage set at −4.2 kV, and a Turbo ion spray temperature of 350 °C. Nitrogen gas was used for the nebulizer, curtain and collision gas. The gas flow rates, collision energies, declustering potentials, entrance potentials, focusing potentials, and collision cell exit potentials were adjusted to give optimal signal response from the direct infusion of a dilute solution of each standard in aqueous methanol. Analyses were performed using electrospray ionization in negative-ion mode with MRM of precursor and characteristic product ions specific for each analyte monitored for 100 milliseconds per transition. The mass-to-charge ratio (m/z) transitions monitored are shown in Fig. 1. The MRM transitions for two internal standards was m/z 327 → 182 for 15(S)-HETE-d_8 and m/z 357 → 197 for $PGF_{2\alpha}$-d_4. The internal standard $PGF_{2\alpha}$-$d_4$was used for quantification of $PGF_{2\alpha}$ and 15(S)-HETE-d_8 was used for quantification of EETs, HETEs, HODEs, oxoETEs, oxoODEs, linoleic acid, and arachidonic acid. In cases where greater sensitivity was needed for detection, such as in measuring urine F_2isoprostanes, an AB SCIEX API 5000 triple quadrupole mass spectrometer was used.

CALCULATIONS AND EXPECTED RESULTS

A typical LC-MS/MS chromatogram for analysis of 15 different fatty acid derivatives including two internal standards using MRM mode is shown in **Fig 3**. Although the retention time and the precursor ions for 9-HODE and 13-HODE are nearly the same, they can still be quantified using specific product ions (m/z 171 for 9-HODE and m/z 195 for 13-HODE).

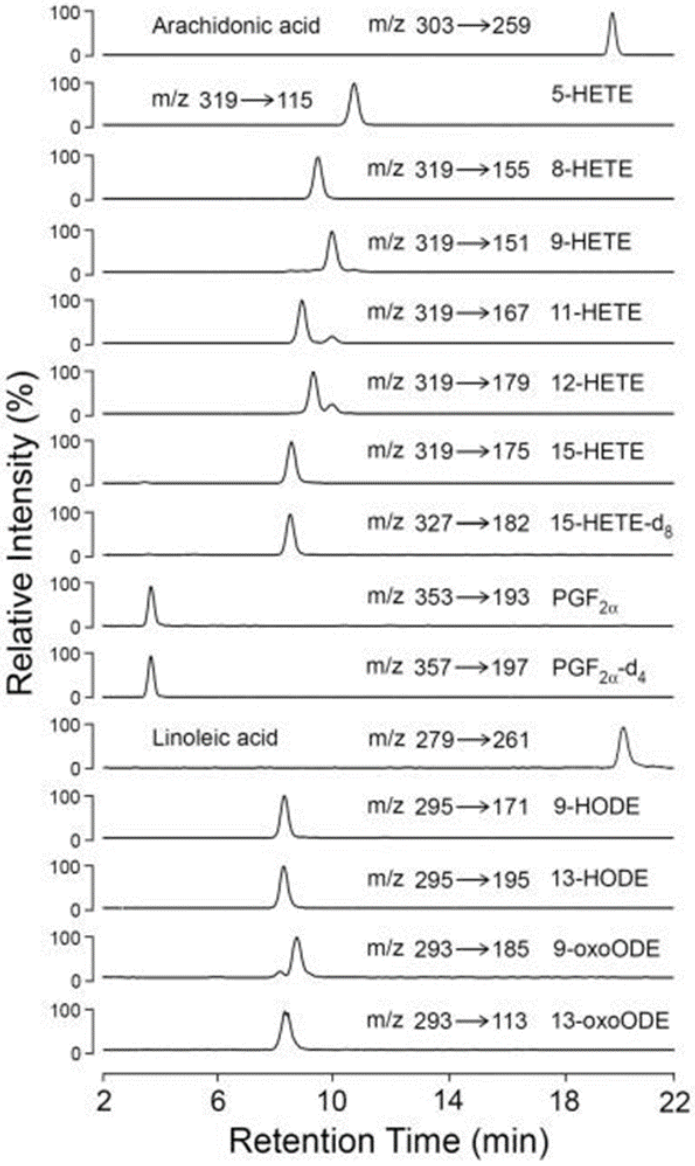

Figure 3 Negative ion LC-MS/MS chromatography of selected un-oxidized fatty acids and oxidized fatty acid standards

Calibration curves and limit of detection (LOD)

Calibration curves for all the fatty acid derivatives monitored, relative to their selected internal standard, are shown in **Fig. 4**. Serial dilutions of authentic fatty acid derivatives including arachidonic acid, linoleic acid and their oxidation products (HETEs, EETs, oxoETEs, HODEs and oxoODEs) in an 85% methanol solution demonstrate a linear response over a wide range of the standard concentrations throughout the assay range (Fig 4 A-C). Analyte recovery was quite high with more than 95% for arachidonic acid and linoleic acid, and more than 85% for their oxidation products except for $PGF_{2\alpha}$ which had only 10% recovery. Use of the stable isotope synthetic internal standard for $PGF_{2\alpha}$ allows us to account for the reduced lipid extraction characteristics of this oxidized lipid species. **Fig. 5** shows the chromatography and calibration curve of authentic $PGF_{2\alpha}$ relative to its internal standard $PGF_{2\alpha}$-d_4. Because biomarkers of oxidation accumulate only to trace amounts in biological samples, sensitivity is critical. We therefore determined the limit of detection (LOD) and limit of quantitation (LOQ) of LC-MS/MS for the species being monitored. The LOD was expressed as the lowest amount of analyte on column generating a signal-to-noise ratio of at least three. The LOQ was examined by reducing the concentration of standard solution gradually and is expressed as the lowest concentration yielding a signal-to-noise ratio of at least 10. As shown

in **Table 1**, the most readily detected analyte is 11-HETE, with LOD<2.6pg and LOQ<0.09 ng/ml. The LOD and LOQ provided are instrument specific. The instrument selected for these analyses is relatively old, and not of the highest sensitivity. Even with the abundance of the molecular species being monitored in plasma or urine this protocol allows for ready detection and quantification even with almost decade old triple quadrupole mass spectrometers.

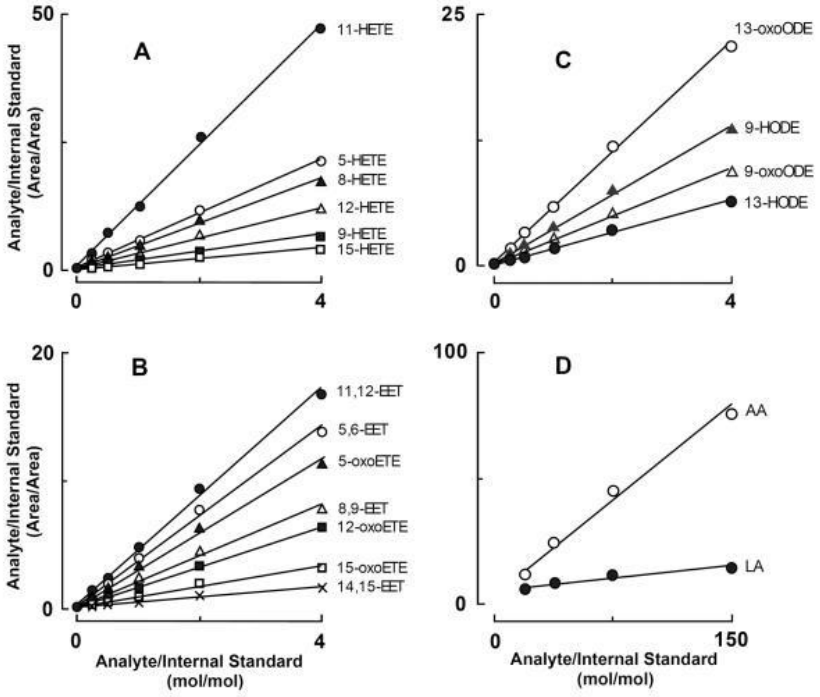

Figure 4 Internal standard calibration curves for the un-oxidized fatty acids and oxidized fatty acids analysed using LC-MS/MS. 15(s)-HETE-d8 (12 fmol on column) was used as internal standard for preparing these standard calibration curves. Calibration range: 0 – 4 mol/mol, Panel A) 15(S)-HETE-d8 for HETEs, Panel B) EETs, oxoEETs, Panel C) HODEs, and oxoODEs, Panel D) 18 – 150 mol/mol 15(S)-HETE-d8 for linoleic acid (LA) and arachidonic acid (AA).

Precision and accuracy

Precision and accuracy of the intraday assay were assessed using standards dissolved in PBS buffer that were assayed in replicates (n=4) for known concentrations of analytes. All samples were analysed on the same day and their back-calculated concentrations were determined from the internal calibration curves prepared the same day. The interday assay precision and accuracy were assessed by assaying known concentrations of analytes in replicates (n=3) over three different days. The precision was expressed as the coefficient of variation (%) and the accuracy as the percentage bias (%). For the intraday and interday assay (Table 2) the CV% and % bias for almost all of the analytes are substantially less than 10%, and typically in the 3–5% range.

LC-MS/MS analysis of oxidised fatty acids in plasma

This LC-MS/MS method can be used to identify and quantify fatty acid derivatives in biological matrices like plasma, serum or other tissues. The levels of some oxidized and un-oxidized fatty acids in human plasma are shown in **Table 3**.

Table 1. Limit of Detection (LOD) and Limit of Quantitation (LOQ)

Analyte	Transition (m/z)	[a]LOD (pg)	[b]LOQ (ng/ml)
Arachidonic	303 → 259	91	3.1
5-HETE	319 → 115	4.8	0.16
8-HETE	319 → 155	4.8	0.1
9-HETE	319 → 151	9.6	0.3
11-HETE	319 → 167	2.6	0.09
12-HETE	319 → 179	3.2	0.11
15-HETE	319 → 175	13	0.45
5,6-EET	319 → 191	16	0.54
8,9-EET	319 → 151	19	0.64
11,12-EET	319 → 167	16	0.54
14,15-EET	319 → 175	26	0.9
5-oxoETE	317 → 203	32	1.1
12-oxoETE	317 → 153	32	1.1
15-oxoETE	317 → 113	64	2.2
PGF2α	353 → 193	7.1	0.24
Linoleic acid	279 → 261	617	21
9-HODE	295 → 171	15	0.5
13-HODE	295 → 195	30	1
9-oxoODE	293 → 185	47	1.6
13-oxoODE	293 → 113	29	1

[a]LOD was defined as a peak whose signal-to-noise ratio is at 3:1.
[b]LOQ was defined as a peak whose signal-to-noise ratio is at 10:1

TABLE 2. Intraday and Interday Assay Precision and Accuracy

		Concentration (ng/ml)							
		Intraday Assay (n=4)				Interday Assay (n=9)			
Analytes	Added	Mean	SD	CV (%)	Bias (%)	Mean	SD	CV (%)	Bias (%)
Arachidonic	300	313	10	3.3	4.4	280	9.5	3.4	−6.8
5-HETE	15	5.5	1.4	9.3	2.8	15	1.2	7.7	−0.1
8-HETE	15	16	1.3	8.3	6.4	15.6	1.2	7.8	4
9-HETE	15	15.5	2.1	14	3.4	15	1.6	10.9	0.1
11-HETE	15	15	0.6	4.3	0.2	14.7	0.8	5.4	−2.2
12-HETE	15	15.4	0.5	3.4	2.7	14.9	0.8	5.3	−3.9
15-HETE	15	15.2	1.2	7.7	1.2	15	0.8	5.6	−0.1
PGF2α	5	5.5	0.6	10	9.6	5.4	0.6	10.6	7.8
Linoleic acid	450	473	16	3.3	5	431	23	5.3	−4.2
9-HODE	15	15.2	1.1	7.2	1.3	14.9	0.8	5.5	−0.5
13-HODE	15	15.3	0.6	3.8	1.7	15.1	0.6	4	0.8

TABLE 3. Levels of Selected Oxidized Fatty Acids and Their Precursors in Human Plasma from Healthy Volunteers

	ng/ml plasma	
Fatty acids	Mean (n=15)	SD
Arachidonic acid	638	228
5-HETE	2.9	1.4
8-HETE	2.5	1.1
9-HETE	3.3	1.3
11-HETE	3.2	1.1
12-HETE	12	3.5
15-HETE	4.8	2
PGF2α	4.1	1
Linoleic acid	2124	946
9-HODE	16	5
13-HODE	47	20

LC-MS/MS analysis of F2-isoprostanes in urine

Determination of isoprostanes in urine provides a superior non-invasive methodology to assay the index of oxidative stress. Since urine contains markedly less arachidonic acid (and linoleic acid) and other interfering compounds than plasma or serum, these samples are much less prone to the artificial generation of interfering oxidized fatty acids.

IMPORTANT CAVEATS

Sample collection. For sample collection it is important to minimize any extraneous oxidation that may occur after its initial collection. Storage conditions must be carefully chosen to exclude air which can be a primary source of contaminating oxidant. It is important to blanket biological tissues and fluids (like serum or plasma) with an inert atmosphere such as nitrogen or, preferably, with heavier than air argon gas to displace the oxygen around the sample. Ideally, antioxidants are included in the liquid portion of the sample. Commonly used antioxidants are free radical scavenging compounds like fat soluble butylated hydroxytoluene (BHT) and divalent metal ion scavengers. The choice of divalent metal ion scavengers is critical to avoid extraneous oxidation; pentacoordinate chelators like diethylenetriaminepentaacetic acid (DTPA), which envelope divalent ions and do not leave open coordination sites available where catalysis can occur, are the reagents of choice for this purpose. Tetra-coordinated divalent metal ion species in complexes with chelators like ethylenediaminetetraacetic acid (EDTA) and ethyleneglycoltetraacetic acid (EGTA) have open sites that can promote oxidation, and even paradoxically enhance the redox potential of the active site on the divalent metal ion. In addition, sample storage at low temperatures is crucial. Many laboratories use ultralow (−70°C to −80°C) freezers for this purpose. Experience has shown that lipids in biological samples survive long term storage better at these lower temperatures. It is also important to limit the number of freeze thaw cycles a sample will be exposed to during its lifetime. Smaller volume, one-time use, aliquots are preferred for storage, since oxidized lipid recovery is degraded in biological samples through multiple freeze thaw cycles. Indeed, it is wise to aliquot a biological specimen into multiple vials destined for lipid analysis, overlay the samples with argon, and then "snap freeze" these portions in liquid nitrogen immediately after collection and processing.

Fatty acid Extraction

Antioxidants in sample preservation. To quantify total free oxidized and un-oxidized fatty acids, lipids in the sample are hydrolysed to their free fatty acid components with base at elevated temperature before extraction. Antioxidants need to be included in the hydrolysis medium; these can be BHT and DTPA as described above. Sometimes it is helpful to also include a mild reducing agent like tin chloride ($SnCl_2$), dithionate or borohydrides (sodium borohydride and sodium cyanoborohydride for example). If using a mild reducing agent, we prefer tin chloride since all of the others have partial solubility in isopropyl alcohol and may carry through the extraction procedure, and both interfere with ionization efficiency and, over time, contaminate the mass spectrometer source (i.e. tin chloride does not partition much into the hexane layer of the Hara extraction).

With reducing reagents, labile species like lipid hydroperoxides and cyclic endoperoxides are reduced to their corresponding hydroxyl compounds. Obviously, any assay that requires separate determination of the hydroperoxide or endoperoxide oxidation products must avoid the use of reducing agents like tin chloride. It should be noted that tin chloride reduction is the classic way to produce prostaglandin D_2 from PGH_2, raising the possibility of artificial elevation in F_2-Isoprostane levels in plasma with addition of tin chloride. However, in our hands, control studies have shown that addition of tin chloride to plasma does not increase measured F_2-Isoprostane levels, presumably because the level of hydroperoxide present in plasma is exceedingly low relative to the F_2-Isoprostane level.

To analyse isoprostanes in plasma it has been shown by others that one should first add reducing agent (to eliminate unstable hydroperoxides and cyclic endoperoxides) and then hydrolyse for optimal results. In our opinion, analyses of urine F_2-isoprostane is the superior method for global oxidative stress measures since there is very little precursor arachidonate or hydroperoxide/endoperoxide present, and artifactual formation of the oxidized lipid during prolonged storage does not occur. In plasma matrix, we have found improved recovery of some un-oxidized fatty acids (arachidonic and linoleic acid) with shorter hydrolysis times (30 minutes) at higher temperatures (60°C), whereas hydrolysis for $PGF_{2\alpha}$ is optimal at longer times (60 minutes) at lower temperatures (45°C). The choice of base used for saponification and its concentration are also important. Generally, sodium

hydroxide is used, which is easily neutralized with acid to form a water-soluble salt. Optimization of the conditions of hydrolysis for the analyte of interest needs to be done using authentic standards.

As mentioned above, antioxidants may need to be incorporated into one or both phases of the extraction solvents. Years of experience have shown us that it is inefficient to include a derivatization step for any compound unless the derivatives are absolutely necessary to provide the sensitivity for the detection of the analytes. Most modern mass spectrometers have the sensitivity to measure compounds down to the femtomole and even attomole range. Careful choice of sample amounts and their dilutions coupled with the resolution and concentrative properties of high performance liquid chromatography should allow for easy detection of most oxidized fatty acid derivatives and their parent compounds.

Fatty Acid Analysis. Which fatty acids to monitor? The last step is the separation and quantitation of the oxidized fatty acid derivatives and their parent compounds from the sample matrix. Which fatty acid and oxidized fatty acid species to monitor is largely dependent on the sample matrix and scientific question? While cyclooxygenase (COX) derived oxidized species like $PGF_{2\alpha}$ and lipoxygenase products like HETEs and HODEs predominate in plasma, non-enzymatic free radical-derived oxidized species like the F_2-isoprostanes predominate in urine. As far as biological mechanisms and outcomes are concerned, there may be times when it is desirable to monitor both groups of products in either matrix. As a global measure of oxidative stress in a patient it is most desirable to use a non-invasive assay such as urinalysis of oxidized lipid species. The invasive plasma (or serum) matrix determination of oxidized lipid species is useful for the evaluation of COX pathway activity, as in inflammatory disease states like arthritis and atherosclerosis. In urine the stable end products of the free radical oxidized fatty acids, such as 2,3-dinor-8-isoprostaglandin $F_{2\alpha}$, predominate.

The oxidation products from arachidonic acid can be found in human urine whereas the linoleate derivatives are typically present at insignificant amounts, often approximating analytical noise levels. The two-step free radical oxidation products from arachidonic acid, the isoprostanes and $PGF_{2\alpha,}$ are the most abundant and most persistent (stable). The 2,3–dinor-8-isoprostaglandin $F_{2\alpha,}$ a metaobolite of 8-isoprostaglandin $F_{2\alpha}$, and 5-isoprostglandin $F_{2\alpha}$ predominate in urine from humans. In fact, stability studies with prolonged incubations of urine at 37° C reveal that addition of an antioxidant cocktail as a sample preservative may not even be necessary. Urine specimens have proven to be stable at room temperature with respect to almost all oxidized fatty acid species examined. The stability has been observed for a number of hours, and even many days in specific instances; as expected, inclusion of the antioxidant mixtures mentioned above markedly increases this stability. F_2-isoprostanes at room temperature are stable for days. What is important is to obtain a clean catch of urine that is free of cell debris or bacteria. Most samples analysed from males are of this nature; however those from females can sometimes contain cellular material rich in the precursor arachidonic acid. This interference can mostly be eliminated from all samples by briefly spinning them in a centrifuge before aliquoting for storage. Visible hematuria and urinary tract infections may present samples that yield abnormally high levels of oxidation products. Control studies have found that spot urine collection provides levels of F_2-isoprostanes on par with those obtained from a 24-hour collection. The dilution levels of these oxidized species from urine are corrected with respect to the urine creatinine level and are expressed as mass of oxidized or un-oxidized fatty acid to mass of creatinine. A clean catch spot urine analysis of oxidized fatty acids like F_2-isoprostanes can provide an immediate non-invasive global systemic index of oxidative stress.

Standards

The standards themselves are carefully chosen, based on their chemical structure, to avoid deuterium exchange with the solvents. When possible, the concentrations of the stable isotope labelled internal standards are verified against primary standards, typically from the linearity plots of their unlabelled isotopologues. Care must be taken to include an amount of stable isotope labelled unsaturated parent fatty acid (arachidonic or linoleic acid) in the general concentration range of endogenous fatty acid derivative levels. If oxidation is observed in this internal standard channel for a particular sample; it could be re-prepared and re-run. At worst, if all samples show some degree of artificial oxidation, the extraction method itself may need to be modified by incorporating either better or higher concentrations of antioxidants. Use of distinct stable isotope labelled precursor to monitor for potential artificial oxidation (by monitoring of potential MRM of heavy isotope labelled isotopologues) is critical for assay development to ensure the assay is not artificially generating the oxidation products monitored in the endogenous sample. Absolute quantitation of the contribution from artifactual oxidation products to samples should be less than 10% (maximum) of the amount measured.

QC Standards for Fatty Acid Analysis Performance

It is helpful to first run a solution containing both the analytes and internal standards in the same solvent mixture used for the sample dissolution but not in the sample matrix (i.e. in a simple solvent system). This standard is used primarily to assess the suitability of the chromatographic system to perform the assay. If peaks are poorly resolved, out of sequence, or absent, the run can be stopped immediately and the chromatographic issues addressed without losing a precious sample. The sample matrix itself presents a formidable challenge to the chromatography system as well as the mass spectrometer. It is wise to prepare a significant volume of control standard consisting of analytes spiked into the matrix of interest at physiologically relevant concentrations (typically at the midpoint of the compound's standard curve). Several of these control standards are processed in parallel with the samples (at least one for every five to 10 samples). Minimally, this check standard is analysed first and a blank is run immediately thereafter. These high-throughput analyses demand a robust HPLC system with an autosampler allowing for the storage of a run-full of samples (up to 100 per day) under refrigeration.

ABREVIATIONS

AA	arachidonic acid
BHT	butylated hydroxytoluene
DTPA	diethylenetriaminepentaacetic acid
ETEs eicosatrienoic acids	EETs, epoxy-eicosatrienoic acids
ESI	electrospray ionization
ESI^-	electrospray negative ionization
$PGF_{2\alpha}$	9α,11α,15S-trihydroxy-5Z,13E-$d_8$acid-1-oic acid
15(S)-HETE-d_8	15S-hydroxy-5Z,8Z,11Z,13E-eicosatetraenoic-5,6,8,9,11,12,14,15-$d_8$acid
H(P)ETE	hydroxy-eicosatetraenoic acid and hydroperoxy-eicosatetraenoic acid
HPLC	high performance liquid chromatography
H(P)ODE	hydroxy-octadecadienoic acid and hydroperoxy-octadecadienoic acid
LC-MS/MS	liquid chromatography with electrospray ionization on-line tandem mass spectrometry
LA	linoleic acid
LOD	limit of detection
MS	mass spectrometry
MRM	multiple-reaction monitoring
oxoETEs	oxo-eicosatetraenoic acids
oxoODEs	oxo-octadecadienoic acids
$PGF_{2\alpha}$-d_4	9α,11α,15S-trihydroxy-5Z,13E-dien-1-oic-3,3,4,4-$d_4$acid