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LC-MS/MS Quantitation of Mercapturic Acid Conjugates of Lipid Peroxidation Products as Markers of Oxidative Stress

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Abstract

Oxidative stress-induced lipid peroxidation (LPO) leads to the formation of cytotoxic and genotoxic 2-alkenals. LPO products such as 4-hydroxy-2(E)-nonenal (HNE) and 4-oxo-2(E)-nonenal (ONE) have been the subject of many studies due to their association with the development of cardiovascular and neurodegenerative diseases, as well as cancer. LPO products are excreted in the urine after conjugation with glutathione (GSH) and subsequent metabolism to mercapturic acid (MA) conjugates. Urinary LPO-MA metabolites are stable end-product metabolites and have gained interest as non-invasive in vivo biomarkers of oxidative stress. This protocol describes a method for the quantitative analysis of LPO-MA metabolites in urine using isotope-dilution liquid chromatography coupled with electrospray tandem mass spectrometry (LC-MS/MS). Included are protocols for preparation of labeled LPO-MA conjugates from unlabeled LPO products and deuterium labeled MA.

Keywords

lipid peroxidation; mercapturic acid; mass spectrometry; oxidative stress

INTRODUCTION

Lipid peroxidation (LPO) products are degradation products of polyunsaturated fatty acids, formed under conditions of oxidative stress. Reactive LPO products, such as 4-hydroxy-2(E)-nonenal (HNE) and 4-oxo-2(E)-nonenal (ONE), exert cytotoxic and genotoxic effects and may contribute to the development and progression of age related and chronic inflammatory diseases. HNE levels have been shown to increase with disease progression. Conjugation with glutathione (GSH), followed by further metabolism to mercapturic acid (MA) conjugates, can mitigate the deleterious effects of these LPO products by facilitating their excretion from the body. The increase of LPO products under conditions of oxidative stress suggests utility for their mercapturic acid conjugates as biomarkers of oxidative stress in vivo. This protocol describes a method for the quantitative analysis of LPO-MA metabolites in urine using isotope-dilution liquid chromatography coupled with electrospray tandem mass spectrometry (LC-MS/MS). Also included in the unit are synthetic preparations of individual LPO products, their LPO-MA conjugates, and deuterium labeled MA (MAd₃) for the preparation of LPO-MAd₃ internal standards (Support Protocols 1 to 5). In addition, details are also provided for the extraction of LPO-MA conjugates from urine

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(Basic Protocol), constructing a calibration curve (Support Protocol 6), and quantification of urinary creatinine (Support Protocol 7).

Basic Protocol LC-MS/MS ANALYSIS AND QUANTITATION OF LPO-MA CONJUGATES

4-Hydroxy-2(E)-nonenal (HNE) is a well established lipid peroxidation (LPO) product that has been shown to contribute to the development and progression of age related diseases such as Alzheimer's and atherosclerosis, in addition to being cytotoxic and genotoxic. HNE is formed from the linoleic acid breakdown product, 4-hydroperoxy-2(E)-nonenal (HPNE) via reduction. 4-Oxo-2(E)-nonenal (ONE) is another HPNE metabolite, formed by a loss of H₂O. HNE and ONE can both undergo phase I metabolism, resulting in the formation of oxidation products: 4-hydroxy-2(E)-nonenoic acid (HNA) and 4-oxo-2(E)-nonenoic acid (ONA), and reduction products: 1,4-dihydroxy-2(E)-nonene (DHN) and 4-oxo-2(E)-nonenol (ONO; Figure 17.14.1). These LPO products are also subject to phase II metabolism, undergoing conjugation with glutathione (GSH) via Michael-type addition mediated by glutathione-S-transferase (GST; Figure 17.14.2). In the liver and kidneys, the GSH adducts are subject to enzymatic cleavage of the glutamyl and glycyl residues, followed by *N*-acetylation of the remaining cysteine residue, to form *N*-acetyl cysteine metabolites, also referred to as mercapturic acid (MA) conjugates. The resulting LPO-MA conjugates can be quantified by isotope-dilution liquid chromatography-tandem mass spectrometry (LC-MS/MS) methods using deuterium-labeled internal standards.

Materials

Urine sample
1 N HCl
Internal standard solutions (see recipe)
Ethyl acetate
Mobile phase solutions A and B (see recipe)
1.5-ml microcentrifuge tubes
Vortex mixer
Pasteur pipettes
13 × 100-mm disposable borosilicate tubes
Zymark TurboVap LV nitrogen evaporation system (Caliper Life Sciences) or equivalent equipment
Small-volume HPLC injection vials with caps (e.g., 12 × 32-mm glass vials with fused glass inserts, 300 µl capacity, and silicone/PTFE septa; MicroSolv Technology Corporation)
High performance liquid chromatography (HPLC) system (Shimadzu Prominence HPLC system or equivalent equipment), including a gradient pump system, degasser, and autosampler
HPLC column: 250 × 2-mm Synergi Max RP C₁₂ column (Phenomenex)
Triple quadrupole or triple quadrupole/linear ion trap mass spectrometer (Applied Biosystems MDS Sciex 4000 QTrap, equipped with a TurboV electrospray source and Analyst 1.4.1 data acquisition software, or equivalent equipment)

Prepare the urine sample

- 1 Thaw a urine sample at room temperature and mix by inversion.

It is recommended that samples are thawed and prepared in batches of ten to thirty in order to limit sample preparation time.

Preferably human or animal urine (minimum volume 400 μ l) is collected over a 24-hour period and an aliquot is processed for analysis. When spot urine from humans is used for comparison, samples should be collected at the same time of the day to minimize variation. Upon collection, urine should either be stored at -80°C or processed immediately.

- 2 Place 200 μ l of urine in a 1.5-ml microcentrifuge tube.
- 3 Acidify the sample to pH 3 by the addition of 20 μ l of 1 N HCl and gentle vortex mixing.

It is recommended that the pH be verified using pH strips (e.g., Baker-pHIX pH 0.0–14, J.T. Baker). Highly concentrated urine samples may require more acid to reach pH 3.

- 4 Add internal standard solutions as follows:

10 μ l of 10 μ M HNE-MAd₃

10 μ l of 10 μ M ONO-MAd₃

5 μ l of 100 μ M DHN-MAd₃

- 5 Vortex the samples gently for 10 sec to mix.

Extract the samples

- 6 Add 700 μ l of ethyl acetate to each sample and shake for 1 min.
- 7 Using a Pasteur pipet, transfer the ethyl acetate (top) layer to a 13 $\times$ 100-mm disposable, borosilicate glass tube.
- 8 Repeat steps 6 and 7, combining the ethyl acetate layers.
- 9 Evaporate the ethyl acetate extracts to dryness under a stream of nitrogen gas using the Zymark TurboVap evaporator with the water bath set at 30 $^{\circ}\text{C}$.
- 10 Resuspend the residue in 100 μ l of 2:8 mobile phase A/mobile phase B and vortex for 10 sec.
- 11 Transfer the sample to a small-volume HPLC injection vial.

Samples can be stored overnight at -20°C or analyzed immediately. If the samples are frozen overnight, ensure they are completely thawed prior to analysis.

Set up the LC-MS/MS system

- 12 Set up an HPLC system with two pumps for gradient separation, a degasser, and autosampler. Set the autosampler to deliver a 10- μ l volume.
- 13 Equilibrate the system with 20% mobile phase B for 20 min prior to analysis and set up the gradient file as follows:
 - a. Using a linear gradient, bring the mobile phase from 20% B to 50% B over 10 min.
 - b. Over the next 2 min, bring the mobile phase up to 90% B.

- c. Maintain the concentration at 90% B for 7 min.
 - d. Return to 20% B over 1 min.
 - e. Equilibrate at 20% B for 5 min.
 - f. Maintain the flow rate at 0.2 ml/min for all steps.
- 14** Set up a mass spectrometer with an electrospray source set to negative ionization mode. Using Analyst software, set the instrument parameters as follows:
- Ion-spray voltage: -4500 V
 - Declustering potential: 40 V
 - Source temperature: 400 °C
 - Source gas: nitrogen
 - Curtain gas: nitrogen
 - Collision gas: nitrogen
- 15** Set the instrument to run in selected reaction monitoring (SRM) mode and set the transitions as shown in Table 17.14.1.
- 16** Inject the samples onto the LC-MS/MS system for analysis.
- It is recommended to inject a blank sample prior to starting sample analysis. This allows the user to verify the column is in good condition and that the instruments are running correctly. Include the synthetic standards (Support Protocols 1 to 5) and/or calibration curve (Support Protocol 6) in this analysis. Analysis should appear similar to that shown in Figure 17.14.3.*

Perform data analysis

- 17** Determine the analyte and internal standard peak areas using Analyst 1.4.1.
- Peaks can be compared to the retention times listed in Table 17.14.1 and shown in Figure 17.14.3.*
- 18** Generate standard curves for HNE-MA, ONO-MA, and DHN-MA using concentration injected (nM) on the x-axis and analyte peak area (A) divided by internal standard peak area (IS) on the y-axis.
- Calibration curves are constructed by linear regression of the data points: $y = mx + b$, where $y = A/IS$, x = sample concentration, m = slope, and b = intercept.
- 19** Calculate the sample concentrations (nM) from the calibration curve: $x = (y - b) / m$.
- 20** Adjust the sample concentrations for reduction of sample volume during work-up (i.e., the residue from 200 µl extracted urine is reconstituted in 100 µl of HPLC solvent; hence the adjustment factor is 2). Thus, the calculated sample concentration divided by 2 gives the endogenous urine concentration.
- 21** Use creatinine levels (mg/liter; Support Protocol 7) to standardize all samples.
- 22** To obtain analyte concentrations in mg/g creatinine from the endogenous analyte concentration in µM, multiply the µM concentration by the analyte's molecular weight (mol. wt.) and divided by the creatinine concentration:
- $$\text{analyte conc (}\mu\text{M)} \times \text{analyte mol. wt.} = \text{analyte conc (}\mu\text{g/liter)},$$

analyte conc ($\mu\text{g/liter}$)/creatinine conc (mg/liter) = milligram analyte/gram creatinine

Support Protocol 1 SYNTHESIS OF ONA

This protocol describes the synthesis of the ONA standard for the analysis of LPO-MA conjugates. It is adapted from the method of Annangudi et al. (2005).

Materials

t-Butanol
2-pentylfuran
 KH_2PO_4
 NaClO_2
Chloroform
Brine
 MgSO_4 (anhydrous)
Tetrahydrofuran
Acetone
Pyridine
Ethyl ether
1.3 M NaOH
1 N HCl
25-ml round-bottom flasks
Stir plate
Separatory funnel
Filter apparatus
Rotary evaporator
NMR

Synthesis

1. Prepare a solution of 5:1 t-butanol- H_2O (10 ml) in a 25-ml round-bottom flask and start the solution stirring at 4 °C.
2. Add 2-pentylfuran (2 mmol), KH_2PO_4 (3 mmol), and NaClO_2 (6 mmol) to the t-butanol- H_2O solution.
3. Let the reaction stir at 4 °C for 1.5 hr.
4. Remove the solvent on a rotary evaporator.
5. Extract the residue with chloroform (three times, 5 ml each).
Add 5ml of H_2O in order to separate the layers.
6. Combine the organic layers and wash them with brine (two times, 15 ml each).
7. Dry the organic layers with anhydrous MgSO_4 , filter off the MgSO_4 .

8. Concentrate the reaction mixture on a rotary evaporator.
9. Dissolve the residue in tetrahydrofuran/acetone/H₂O (5/4/1, v/v/v, 40 ml).
10. Add 200 µl of pyridine and stir the reaction at room temperature for 2 hr.
11. Remove the solvent on a rotary evaporator.
12. Dissolve the residue in 5 ml ethyl ether.
13. Extract (three times, 5 ml each) with H₂O adjusted to pH 10 with 1.3 M NaOH.
14. Keep the H₂O (bottom) layers and acidify them with 1 N HCl to pH 2.
15. Extract the acidic H₂O layers with ethyl ether (three times, 15 ml each), keeping the organic (top) layers.
16. Dry the organic layers with anhydrous MgSO₄, filter of the MgSO₄.
17. Concentrate the reaction mixture on a rotary evaporator.

The end product is an oil and it can be stored up to 6 months at -20 °C.

18. Verify the product identity and purity by ¹H NMR.

Data are ¹H NMR (400 MHz, CDCl₃): δ 7.18 (d, J= 16 Hz, 1H), 6.71 (d, J= 16 Hz, 1H), 2.68 (t, J= 7 Hz, 2H), 1.69 (m, 1H), 1.31 (m, 5H), 0.94 (t, J = 7, 3H).

Support Protocol 2 SYNTHESIS OF HNA

This protocol describes the synthesis of the HNA standard for the analysis of LPO-MA conjugates (Kuiper et al., 2008).

Materials

ONA (Support Protocol 1)
Ethanol
Sodium borohydride
1 N HCl
Ethyl ether
MgSO₄
25-ml round-bottom flasks
Stir plate
Separatory funnel
Filter apparatus
Rotary evaporator
NMR
Additional reagents and equipment for preparing a solution of ONA (Support Protocol 1)

Synthesis

1. Prepare a solution of ONA (0.05 mmol; see Support Protocol 1) in 5 ml of ethanol in a 25-ml round-bottom flask.

2. Add 0.1 mmol of solid sodium borohydride to the ONA solution and stir at room temperature.
3. After 45 min, acidify the reaction mixture to pH 3 with 1 N HCl.
4. Extract with ethyl ether (three times, 5 ml each), keeping the organic layers (top).
Addition of 10 ml of H₂O will make the layers easier to distinguish.
5. Dry the organic layers with anhydrous MgSO₄, filter off the MgSO₄.
6. Concentrate the reaction mixture on a rotary evaporator.
The end product is an oil and it can be stored up to 6 months at −20 °C.
7. Verify the product identity and purity by ¹H NMR.

Data are ¹H NMR (400 MHz, CDCl₃): δ 7.10 (dd, J = 5, 16 Hz, 1H), 6.10 (d, J = 16 Hz, 1H), 4.39 (dd, J = 5, 11 Hz, 1H), 1.65 (m, 2H), 1.58–1.22 (m, 7H), 0.93 (t, J = 6, 3H).

Support Protocol 3 SYNTHESIS OF ONO

This protocol describes the synthesis of the ONO standard for the analysis of LPO-MA conjugates (Kuiper et al., 2008).

Materials

LPO product ONE (Cayman Chemical)
Methanol
Sodium phosphate buffer, 0.1 M, pH 3 (see recipe)
50 mM sodium cyanoborohydride in 1 N NaOH
Ethyl acetate
50-ml round-bottom flasks
Stir plate
Separatory funnel
Filter apparatus
Rotary evaporator
NMR

Synthesis

1. Prepare a solution of ONE (0.01 mmol) in 1 ml of methanol in a 50-ml round-bottom flask.
2. Add 17.05 ml of sodium phosphate buffer (0.1 M, pH 3) and 950 µl of a 50 mM sodium cyanoborohydride solution prepared in 1 N NaOH
3. Stir the reaction mixture for 15 hr at room temperature.
4. Extract with ethyl acetate (three times, 20 ml each), keeping the organic (top) layer.
5. Concentrate the organic layer on a rotary evaporator.
The end product is an oil and can be stored up to 6 months at −20 °C.
6. Verify the product identity and purity by ¹H NMR.

Data are ^1H NMR (400 MHz, CDCl_3): δ 6.93 (dt, $J = 4, 16$ Hz, 1H), 6.42 (dt, $J = 2, 16$ Hz, 1H), 4.42 (dd, $J = 2, 4$ Hz, 2H), 2.59 (t, $J = 7$ Hz, 2H), 1.66 (m, 2H), 1.37 (m, 5H), 0.93 (t, $J = 7, 3$ Hz).

Support Protocol 4 SYNTHESIS OF MAd_3

This protocol describes the synthesis of the MAd_3 standard for the preparation of the internal standard LPO- MAd_3 conjugates. It is adapted from the method of Slatter et al. (1991).

Materials

1.5 M NaOH
Cystine
[$^2\text{H}_6$]Acetic anhydride
1,4-Dithiothreitol
Ethyl ether
Liquid nitrogen
Methanol
1 N HCl
Ethyl acetate
25-ml round-bottom flasks
Stir plate
Ice bath
Rotary evaporator
Lyophilizer
52 $\times$ 2.5-cm Sephadex LH-20 column
Separatory funnel
High performance liquid chromatography (HPLC) system (Shimadzu Prominence HPLC system or equivalent equipment), including a gradient pump system, degasser, and autosampler
Triple quadrupole or triple quadrupole/linear ion trap mass spectrometer (Applied Biosystems MDS Sciex 4000 QTrap, equipped with a TurboV electrospray source and Analyst 1.4.1 data acquisition software, or equivalent equipment)

Synthesis

- 1 Prepare 13 ml of a 1.5 M NaOH solution in a 25-ml round-bottom flask and stir the solution while cooling it in an ice bath.
- 2 Add 5.3 mmol (1.27 g) of cystine.
- 3 Add 1 ml [$^2\text{H}_6$]acetic anhydride (10.6 mmol) dropwise over 20 min.
- 4 Remove the ice bath and allow the reaction to continue stirring at room temperature for 1 hr.

- 5 Add 10.6 mmol (1.65 g) of 1,4-dithiothreitol and continue stirring at room temperature for 1 hr more.
- 6 Concentrate the reaction mixture on a rotary evaporator.
According to Slatter et al. (1991), a gum is obtained at this stage. In our hands, the reaction volume was reduced by a few milliliters and we proceeded with step 7 starting from the remaining aqueous reaction mixture.
- 7 Wash the remaining material with ethyl ether (three times, 10 ml each).
- 8 Freeze with liquid nitrogen and lyophilize.

Purify the reaction mixture

- 9 Dissolve the reaction mixture in 10 ml of methanol and purify on a 52×2.5 -cm Sephadex LH-20 column using methanol as the eluting solvent.
- 10 Verify fraction purity by LC-MS in negative ion mode.
- 11 Combine fractions showing a peak at m/z 165 and remove the methanol on a rotary evaporator.
- 12 Further purify reaction mixture by adding H_2O to dissolve, then acidifying to pH 3 with 1 N HCl.
- 13 Extract with ethyl acetate (three times, 10 ml each), keeping the organic (top) layer.
This step should ensure the removal of any cystine or cysteine present.
- 14 Concentrate the organic layer on a rotary evaporator.
The product will be white crystals and can be stored up to 6 months at $-20^\circ C$.
- 15 Verify purity by LC-MS analysis of m/z 165 in negative mode.
Material should be free of cystine, cysteine, and unlabeled MA.

Support Protocol 5 SYNTHESIS OF LPO-MA AND LPO-MAd₃ CONJUGATES

This protocol describes the synthesis of the LPO-MA and LPO-MAd₃ conjugates for use as standards and internal standards (IS), respectively, for LC-MS/MS analyses (Kuiper et al., 2008, 2010).

Materials

MA (Sigma-Aldrich) and MAd₃ (Support Protocol 4)
Sodium phosphate buffer, 0.1 M, pH 8 (see recipe)
LPO products HNE and ONE (Cayman Chemical), ONA (Support Protocol 1), HNA (Support Protocol 2), and ONO (Support Protocol 3)
Ethanol
5 M Sodium borohydride in 1 N NaOH
1 N HCl
Ethyl acetate
2:8 mobile phase A/mobile phase B
1.5-ml microcentrifuge tubes
Stir plate with warming

Zymark TurboVap LV nitrogen evaporation system (Caliper Life Sciences)

1. Dissolve MA or MAD₃ in 0.1 M sodium phosphate buffer, pH 8, to form a 20 mM solution.
2. Prepare a 1.0 mM solution of the LPO product of interest in ethanol.

It is recommended that all conjugation reactions be run simultaneously in order to save time.

HNE is the starting LPO product for preparation of 1,4-dihydroxynonane (DHN) conjugates. DHN lacks an α , β -unsaturated aldehyde moiety with which a Michael-type adduct can form and conjugates must therefore be prepared by reduction of HNE-MA(MAD₃) (steps 4a and 4b).

3. In a 1.5-ml microcentrifuge tube, combine 50 μ l of the MA or MAD₃ solution, 450 μ l sodium phosphate buffer (0.1 M, pH 8), 400 μ l of water, and 100 μ l of LPO product solution.
4. Stir the reaction at 37°C for 2 hr.
 - a. Remove the reaction from heat and continue stirring until the reaction reaches room temperature.
 - b. Add 10 μ l of a 5 M sodium borohydride solution in 1 N NaOH and continue stirring for 30 min.

Steps 4a and 4b are only used in the preparation of DHN-MA and DHN-MAD₃. For all other conjugates, stir for 2 hr, and then acidify.
5. After 2 hr, acidify the reaction mixture to pH 3 with 1 N HCl.
6. Extract the reaction three times with ethyl acetate as in steps 6–8 of the Basic Protocol.
7. Combine the ethyl acetate layers and evaporate them under nitrogen.
8. Reconstitute the residue in 1.0 ml of 2:8 mobile phase A/mobile phase B, resulting in a concentration of 100 μ M.

Support Protocol 6 CONSTRUCTING A CALIBRATION CURVE

This protocol describes the preparation of a calibration curve for the quantitative analysis of the LPO-MA conjugates (Kuiper et al., 2010).

Materials

HNE-MA, ONO-MA, and DHN-MA (as prepared in Support Protocol 5)

HNE-MAD₃, ONO-MAD₃, and DHN-MAD₃ (as prepared in Support Protocol 5)

2:8 Mobile phase A/Mobile phase B

Small-volume HPLC autosampler vials with 300 μ l glass inserts and caps

1. In the HPLC autosampler vials, prepare an eight-point calibration curve (10 nM to 5 μ M) using HNE-MA, ONO-MA, DHN-MA, and their corresponding MAD₃ internal standards to a total volume of 100 μ l.
2. Add internal standards to each sample as follows:

10 μ l of 10 μ M HNE-MAD₃

10 µl of 10 µM ONO-MAd₃

5 µl of 100 µM DHN-MAd₃

3. Add HNE-MA, ONO-MA, and DHN-MA according to Table 17.14.2.
4. Use 2:8 Mobile phase A/Mobile phase B to bring the total volume to 100 µl as shown in Table 17.14.2.

Calibration curve generation is described in step 18 of the Basic Protocol.

Support Protocol 7 QUANTITATION OF URINARY CREATININE

When spot urine is used for quantification of LPO product metabolites, it is recommended to correct the calculated analyte concentrations for variability in urinary output based on the level of creatinine in the urine. This protocol describes the quantification of creatinine for use in Step 21 of the Basic Protocol.

Materials

Urine sample (100 µl aliquot of the sample used for the Basic Protocol)

Creatinine Assay Kit (Cayman Chemical or equivalent)

Spectrophotometer reading at 500 nm (SpectraMax 190 or equivalent)

1. Perform measurement and quantification of the urine according to the creatinine assay kit manufacturer's directions.
2. Convert the concentration from mg/dl to mg/liter for further calculations.

REAGENTS AND SOLUTIONS

Use Milli-Q purified water or equivalent in all recipes and protocol steps. For common stock solutions, see APPENDIX 2A; for suppliers, see SUPPLIERS APPENDIX.

Internal standard solutions

HNE-MAd₃: Take 100 µl of the 100-µM solution (Support Protocol 5) and add 900 µl of 2:8 mobile phase A/mobile phase B to make a 10-µM solution. Store up to 6 months at -20 °C.

ONO-MAd₃: Take 100 µl of the 100-µM solution (Support Protocol 5) and add 900 µl of 2:8 mobile phase A/mobile phase B to make a 10-µM solution. Store up to 6 months at -20 °C.

DHN-MAd₃: Use the 100-µM solution prepared in Support Protocol 5. Store up to 6 months at -20 °C.

Mobile phase solutions A and B

Mobile phase solution A: Add 200 µl of formic acid (0.1% v/v) to 200 ml of water and mix. Make fresh daily.

Mobile phase solution B: Add 200 µl of formic acid (0.1% v/v) to 200 ml of acetonitrile and mix. Make fresh daily.

Sodium phosphate buffer, 0.1 M, pH 3

Dissolve 145 µl of H₃PO₄ (85 wt % in H₂O) and 2.73 g NaH₂PO₄·2H₂O in 200 ml of water and using a pH meter, adjust the pH to 3 with H₃PO₄ while stirring. Store up to 1 year at 4 °C.

Sodium phosphate buffer, 0.1 M, pH 8

Dissolve 0.21 g $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$ and 3.32 g $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$ in 200 ml of water and using a pH meter, adjust the pH to 8 with 1 M NaOH while stirring. Store up to 1 year at 4°C.

COMMENTARY

Background Information

Under conditions of oxidative stress, reactive oxygen species (ROS) can react with polyunsaturated fatty acids (PUFAs), causing them to degrade and form electrophilic LPO products, such as HNE and ONE (Fig. 17.14.1). These reactive aldehydes have the ability to react with proteins, peptides, and DNA, causing covalent modifications of these biomolecules and altered cellular function (Benedetti et al., 1980; Esterbauer et al., 1991; LoPachin et al., 2009). This has led to LPO products being associated with the development and progression of age related diseases such as cardiovascular diseases (Rahman et al., 2002; Spiteller, 2007; Facchinetti et al., 2007), cancer (Barbin, 1998), and neurodegenerative diseases (Butterfield and Sultana, 2007; Picklo and Montine, 2007; Lovell and Markesbery, 2007). HNE and ONE react primarily with cysteine, histidine, and lysine via Michael-type additions (Doorn and Petersen, 2002; LoPachin et al., 2009) or in the case of lysine, Schiff base formation (Sayre et al., 1993). It has also been demonstrated that HNE can crosslink proteins by undergoing both Michael-type addition and Schiff base formation (Cohn et al., 1996). ONE has been shown to covalently modify DNA through formation of adducts with 2'-deoxyguanosine, 2'-deoxyadenosine, and 2'-deoxycytidine (Rindgen et al., 1999, 2000; Lee et al., 2000; Pollack et al., 2003), while HNE can form adducts with 2'-deoxyguanosine (Winter et al., 1986). Consequently, there is considerable interest in mitigating the effects of LPO products.

Multiple enzymatic pathways are involved in the metabolism of HNE and ONE. Phase I metabolism of HNE is a prominent metabolic pathway. Cytochromes P450 (Amunom et al., 2007) and aldehyde dehydrogenase (Mitchell and Petersen, 1987) can catalyze the oxidation of HNE to HNA, while aldo-keto reductase (Srivastava et al., 2000) has been shown to reduce HNE to DHN. Upon conjugation via phase II metabolism, HNA adducts can spontaneously undergo intramolecular condensation to form the corresponding γ -lactones (Alary et al., 1995). HNA lactone has also been reported to arise from the oxidation of HNE-GSH in the hemi-acetal form (Boon et al., 1999). ONE can be metabolized in similar fashion, forming ONA from aldehyde dehydrogenase-mediated oxidation (Doorn et al., 2006) or the HNE isomer, ONO, via aldo-keto reductase- (Doorn et al., 2003; Jian et al., 2005) mediated reduction. Carbonyl reductase has been demonstrated to reduce ONE at the C-4 position as well, resulting in HNE formation (Doorn et al., 2004). With the exception of DHN, these phase I metabolites of HNE and ONE retain their α , β -unsaturated carbonyl moiety, and are therefore able to form Michael-type conjugates with GSH. Phase II metabolism of HNE and ONE via Michael-type addition with GSH is mediated by GSTs. It has been demonstrated that GST 8-8 has a high specific activity for conjugation of GSH with HNE (Jensson et al., 1986), suggesting a major biological function of GSTs for the conjugation and deactivation of reactive electrophilic products. It has further been demonstrated that γ -glutamyl transferase (γ -GT) metabolizes HNE-GSH to HNE-cysteine glycine (CG; Enoiu et al., 2002). Metabolism by cysteinyl glycinase and *N*-acetyl transferase in the liver and kidneys results in HNE-MA conjugates which are excreted in the urine. This pathway for HNE removal can initially deplete GSH. HNE also plays a role in the recovery of depleted GSH levels by inducing the expression of glutamate cysteine ligase, the rate limiting enzyme in GSH synthesis (Iles and Liu, 2005).

Early detection of HNE and other reactive aldehydes was carried out by 2,4-dinitrophenylhydrazine (DNPH) derivatization (Esterbauer and Zollner, 1989; Esterbauer et al., 1991). The hydrazone derivatives have characteristic absorbance maxima at 360 to 390 nm and are easily detected by TLC or HPLC. Peak identification using this method was carried out by comparison to synthetic standards. The main drawback of DNPH derivatization is the lack of specificity, since DNPH will react with all carbonyls. Another derivatization method for HPLC analysis is the reaction of aldehydes with 1,3-cyclohexanedione to form fluorescent decahydroacridine derivatives (Esterbauer and Zollner, 1989). Direct HPLC determination of HNE at 220 nm has also been reported (Lang et al., 1985). These HPLC methods rely on retention time comparison with synthetic standards. Chromatography coupled with mass spectrometry provides a higher confidence level for structure identity and greater analytical selectivity and sensitivity. GC-MS methods for HNE analysis require derivatization, e.g., with pentafluorobenzyl-hydroxylamine followed by silylation (Van Kuijk et al., 1986). An enzyme immunoassay to quantitatively assess levels of DHN-MA was recently developed and validated by Guéraud et al. (2006). LC-MS/MS analyses of (S)-carbidopa derivatized HNE were employed to differentiate between the *R*- and *S*- enantiomers (Honzatko et al., 2007). HNE-GSH and HNA were also analyzed using this method. Isotope-dilution LC-MS³ methodology was demonstrated to be useful for the quantitative analysis of DHN-MA and could possibly be utilized to quantify other LPO-MA conjugates (Alary et al., 1998; Rathahao et al., 2005; Blair, 2010). LC-MS methods employing selected ion monitoring have also been used to analyze HNE-GSH, HNE, and ONO in mouse liver tissues (Warneke et al., 2008), although separate sample preparation methods were necessary for the conjugated and free metabolites. This protocol describes a quantitative LC-MS/MS method for the analysis of LPO-MA conjugates in urine. We have used this method for analysis of LPO-MA conjugates in an animal model of acute oxidative stress and in a smoking cessation study (Kuiper et al., 2008, 2010).

Oxidative stress-induced LPO leads to the formation of cytotoxic and genotoxic 2-alkenals, HNE and ONE, as well as their metabolites (Benedetti et al., 1980; Esterbauer et al., 1991). These LPO products have also been shown to contribute to the development and progression of age-related diseases such as Alzheimer's and atherosclerosis (Spiteller, 2007; Butterfield and Sultana, 2007; Picklo and Montine, 2007; Lovell and Markesbery, 2007; Poli et al., 2008). Therefore, HNE and other LPO products show promise as biomarkers of oxidative stress and as diagnostic tools for assessing disease risk and/or development. In some cases, however, even these secondary products of oxidative stress such as HNE and ONE may not be suitable biomarkers due to high reactivity. By contrast, HNE-MA, ONE-MA, and their metabolites are stable end-product metabolites and more suitable to assess oxidative stress in vivo.

Existing methods for assessing in vivo oxidative stress are not without drawbacks. The thiobarbituric acid reaction, a colorimetric assay that is widely used to analyze levels of malondialdehyde lacks specificity (Knight et al., 1988). F_{2α}-isoprostane analysis is currently considered the most reliable way to assess oxidative stress in vivo; however, F_{2α}-isoprostanes are formed only from arachidonic acid degradation. Due to its specificity, F_{2α}-isoprostane analysis may not provide a global assessment of oxidative stress (Guichardant and Lagarde, 2009). This protocol discusses appropriate analytical methods for the identification and quantitation of HNE-MA, ONE-MA, and their metabolites as in vivo biomarkers of oxidative stress.

Critical Parameters and Troubleshooting

Prior to analysis of LPO-MA conjugates, there are several important issues to consider. This protocol discusses the quantitative analysis of HNE-MA, ONO-MA, and DHN-MA. HNA-MA, HNAL-MA, and ONA-MA can all be detected in human urine as well, but have not

been quantitatively analyzed. The endogenous amounts of ONA-MA, estimated at < 10 nM, were too small to quantify by isotope-dilution LC-MS/MS with satisfactory precision and accuracy. HNA-MA and HNAL-MA presented a different challenge. Due to the spontaneous conversion of HNA-MA into HNAL-MA under aqueous conditions, we have thus far been unable to obtain homogenous synthetic standards of either material for use in our calibration curves.

It should be noted that while we have previously demonstrated the presence of ONE-MA in rat urine (Kuiper et al., 2008), it was not detectable in our human urine samples. Its absence in human urine is likely due to preferential phase I metabolism of ONE or ONE-GSH resulting in metabolites ONO-MA and ONA-MA.

Note also that ONE-MA and HNAL-MA are isobaric and co-elute under the conditions described. Separation and analysis of these metabolites can be carried out by LC-MS/MS using a method described by Kuiper et al. 2008.

When preparing the urine samples for analysis, be sure to acidify. The LPO-MA conjugates will remain in the water layer if the sample is not acidic.

Anticipated Results

HNE-MA, ONO-MA, and DHN-MA are found in humans at low mg/g creatinine concentrations (Alary et al., 1998; Kuiper et al., 2010) and have been found in rats at the low end of the human range (Alary et al., 1998; Rathahao et al., 2005; Guéraud et al., 2006; Mally et al., 2007). The concentrations will vary between subjects based on type and level of oxidative stress. Typical human levels are 0.17 to 12.19 mg/g creatinine (7.4 to 225 nM) HNE-MA, 0.05 to 2.26 mg/g creatinine (1.7 to 177 nM) ONO-MA, and 0.22 to 17.90 mg/g creatinine (6.6 to 316 nM) DHN-MA. The limits of quantitation are 5 nM for HNE-MA, 0.5 nM for ONO-MA, and 10 nM for DHN-MA. When the samples undergo a volume concentration step as in this protocol, the limit of quantitation will change accordingly. A 2× sample volume concentration would cause the limits of quantitation to become 2.5 nM, 0.25 nM, and 5 nM for HNE-MA, ONO-MA, and DHN-MA respectively.

Time Considerations

The synthesis of each LPO product takes a different amount of time. ONA preparation takes ~ 7 hr, HNA requires ~2 hr, ONO preparation takes ~17 hr, and MAD₃ takes ~ 3 days. These products can all be prepared ahead of time and stored at -20°C for up to 6 months. The synthesis of LPO-MA standards and LPO-MAD₃ internal standards takes ~ 4 or 5 hr. Urine preparation for 20 samples takes ~ 3 or 4 hr. LC-MS/MS analysis takes 26 min per sample. When calculating analysis time, sample blanks, standards, and calibration curve samples need to be counted too. The standards can be frozen prior to use and the urine samples can be stored for about 1 week at -20°C if necessary, allowing each step to be carried out on different days.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Literature Cited

- Alary J, Bravais F, Cravedi JP, Debrauwer L, Rao D, Bories G. Mercapturic acid conjugates as urinary end metabolites of the lipid peroxidation product 4-hydroxy-2-nonenal in the rat. *Chem Res Toxicol.* 1995; 8:34–39. [PubMed: 7703364]
- Alary J, Debrauwer L, Fernandez Y, Cravedi JP, Rao D, Bories G. 1,4-Dihydroxynonene mercapturic acid, the major end metabolite of exogenous 4-hydroxy-2-nonenal, is a physiological component of rat and human urine. *Chem Res Toxicol.* 1998; 11:130–135. [PubMed: 9511904]
- Amunom I, Stephens LJ, Tamasi V, Cai J, Pierce WMJ, Conklin DJ, Bhatnagar A, Srivastava S, Martin MV, Guengerich FP, Prough RA. Cytochromes P450 catalyze oxidation of α , β -unsaturated aldehydes. *Arch Biochem Biophys.* 2007; 464:187–196. [PubMed: 17599801]
- Annangudi SP, Sun M, Salomon RG. An Efficient Synthesis of 4-Oxoalkenoic Acids from 2-Alkylfurans. *Synlett.* 2005; 9:1468–1470. Key reference for the synthesis of ONA. A modification of this method is described in Support Protocol 1.
- Barbin A. Formation of DNA etheno adducts in rodents and humans and their role in carcinogenesis. *Acta Biochim Pol.* 1998; 45:145–161. [PubMed: 9701507]
- Benedetti A, Comporti M, Esterbauer H. Identification of 4-hydroxynonenal as a cytotoxic product originating from the peroxidation of liver microsomal lipids. *Biochim Biophys Acta.* 1980; 620:281–296. [PubMed: 6254573]
- Blair IA. Analysis of endogenous glutathione-adducts and their metabolites. *Biomed Chromatogr.* 2010; 24:29–38. [PubMed: 20017120]
- Boon PJ, Marinho HS, Oosting R, Mulder GJ. Glutathione conjugation of 4-hydroxy-trans-2,3-nonenal in the rat in vivo, the isolated perfused liver and erythrocytes. *Toxicol Appl Pharmacol.* 1999; 159:214–223. [PubMed: 10486308]
- Butterfield DA, Sultana R. Redox proteomics identification of oxidatively modified brain proteins in Alzheimer's disease and mild cognitive impairment: insights into the progression of this dementing disorder. *J Alzheimers Dis.* 2007; 12:61–72. [PubMed: 17851195]
- Cohn JA, Tsai L, Friguet B, Szweda LI. Chemical characterization of a protein-4-hydroxy-2-nonenal cross-link: immunochemical detection in mitochondria exposed to oxidative stress. *Arch Biochem Biophys.* 1996; 328:158–164. [PubMed: 8638925]
- Doorn JA, Petersen DR. Covalent modification of amino acid nucleophiles by the lipid peroxidation products 4-hydroxy-2-nonenal and 4-oxo-2-nonenal. *Chem Res Toxicol.* 2002; 15:1445–1450. [PubMed: 12437335]
- Doorn JA, Srivastava SK, Petersen DR. Aldose reductase catalyzes reduction of the lipid peroxidation product 4-oxonon-2-enal. *Chem Res Toxicol.* 2003; 16:1418–1423. [PubMed: 14615967]
- Doorn JA, Maser E, Blum A, Claffey DJ, Petersen DR. Human carbonyl reductase catalyzes reduction of 4-oxonon-2-enal. *Biochemistry.* 2004; 43:13106–13114. [PubMed: 15476404]
- Doorn JA, Hurley TD, Petersen DR. Inhibition of human mitochondrial aldehyde dehydrogenase by 4-hydroxynon-2-enal and 4-oxonon-2-enal. *Chem Res Toxicol.* 2006; 19:102–110. [PubMed: 16411662]
- Enoiu M, Herber R, Wennig R, Marson C, Bodaud H, Leroy P, Mitrea N, Siest G, Wellman M. γ -Glutamyltranspeptidase-dependent metabolism of 4-hydroxynonenal-glutathione conjugate. *Arch Biochem Biophys.* 2002; 397:18–27. [PubMed: 11747306]
- Esterbauer H, Zollner H. Methods for determination of aldehydic lipid peroxidation products. *Free Radic Biol Med.* 1989; 7:197–203. [PubMed: 2680787]
- Esterbauer H, Schaur RJ, Zollner H. Chemistry and biochemistry of 4-hydroxynonenal, malonaldehyde and related aldehydes. *Free Radic Biol Med.* 1991; 11:81–128. [PubMed: 1937131]
- Facchinetti F, Amadei F, Geppetti P, Tarantini F, Di Serio C, Dragotto A, Gigli PM, Catinella S, Civelli M, Patacchini R. α , β -unsaturated aldehydes in cigarette smoke release inflammatory mediators from human macrophages. *Am J Respir Cell Mol Biol.* 2007; 37:617–623. [PubMed: 17600310]

- Guéraud F, Peiro G, Bernard H, Alary J, Créminon C, Debrauwer L, Rathahao E, Drumare MF, Canlet C, Wal JM, Bories G. Enzyme immunoassay for a urinary metabolite of 4-hydroxynonenal as a marker of lipid peroxidation. *Free Radic Biol Med*. 2006; 40:54–62. [PubMed: 16337879]
- Guichardant M, Lagarde M. Hydroxy-alkenals from the peroxidation of n-3 and n-6 fatty acids and urinary metabolites. *Eur J Lipid Sci Technol*. 2009; 111:75–82.
- Honzatko A, Brichac J, Picklo MJ. Quantification of trans-4-hydroxy-2-nonenal enantiomers and metabolites by LC-ESI-MS/MS. *J Chromatogr B Analyt Technol Biomed Life Sci*. 2007; 857:115–122.
- Iles KE, Liu RM. Mechanisms of glutamate cysteine ligase (GCL) induction by 4-hydroxynonenal. *Free Radic Biol Med*. 2005; 38:547–556. [PubMed: 15683710]
- Jensson H, Guthenberg C, Alin P, Mannervik B. Rat glutathione transferase 8–8, an enzyme efficiently detoxifying 4-hydroxyalk-2-enals. *FEBS Lett*. 1986; 203:207–209. [PubMed: 3732510]
- Jian W, Arora JS, Oe T, Shuvaev VV, Blair IA. Induction of endothelial cell apoptosis by lipid hydroperoxide-derived bifunctional electrophiles. *Free Radic Biol Med*. 2005; 39:1162–1176. [PubMed: 16214032]
- Knight JA, Pieper RK, McClellan L. Specificity of the thiobarbituric acid reaction: its use in studies of lipid peroxidation. *Clin Chem*. 1988; 34:2433–2438. [PubMed: 3197281]
- Kuiper HC, Miranda CL, Sowell JD, Stevens JF. Mercapturic acid conjugates of 4-hydroxy-2-nonenal and 4-oxo-2-nonenal metabolites are in vivo markers of oxidative stress. *J Biol Chem*. 2008; 283:17131–17138. Key reference for the distinction between isomeric LPO-MA conjugates HNE-MA and ONO-MA. One of the methods on which the Basic Protocol and Support Protocols 1 to 5 and 7 are based. [PubMed: 18442969]
- Kuiper HC, Langsdorf BL, Miranda CL, Joss J, Jubert C, Mata JE, Stevens JF. Quantitation of mercapturic acid conjugates of 4-hydroxy-2-nonenal and 4-oxo-2-nonenal metabolites in a smoking cessation study. *Free Radic Biol Med*. 2010; 48:65–72. Key reference for the quantitation of LPO-MA conjugates using isotope-dilution mass spectrometry. One of the methods on which the Basic Protocol and Support Protocols 1 to 7 are based. [PubMed: 19819328]
- Lang J, Celotto C, Esterbauer H. Quantitative determination of the lipid peroxidation product 4-hydroxynonenal by high-performance liquid chromatography. *Anal Biochem*. 1985; 150:369–378. [PubMed: 3841466]
- Lee SH, Rindgen D, Bible RH Jr, Hajdu E, Blair IA. Characterization of 4-oxo-2-nonenal as a novel product of lipid peroxidation. *Chem Res Toxicol*. 2000; 13:565–574. [PubMed: 10898588]
- LoPachin RM, Gavin T, Petersen DR, Barber DS. Molecular mechanisms of 4-hydroxy-2-nonenal and acrolein toxicity: nucleophilic targets and adduct formation. *Chem Res Toxicol*. 2009; 22:1499–508. [PubMed: 19610654]
- Lovell MA, Markesbery WR. Oxidative DNA damage in mild cognitive impairment and late-stage Alzheimer's disease. *Nucleic Acids Res*. 2007; 35:7497–7504. [PubMed: 17947327]
- Mally A, Amberg A, Hard GC, Dekant W. Are 4-hydroxy-2(E)-nonenal derived mercapturic acids and (1)H NMR metabonomics potential biomarkers of chemically induced oxidative stress in the kidney? *Toxicology*. 2007; 230:244–255. [PubMed: 17184893]
- Mitchell DY, Petersen DR. The oxidation of alpha-beta unsaturated aldehydic products of lipid peroxidation by rat liver aldehyde dehydrogenases. *Toxicol Appl Pharmacol*. 1987; 87:403–410. [PubMed: 3564015]
- Picklo MJS, Montine TJ. Mitochondrial effects of lipid-derived neurotoxins. *J Alzheimers Dis*. 2007; 12:185–193. [PubMed: 17917163]
- Poli G, Schaur RJ, Siems WG, Leonarduzzi G. 4-Hydroxynonenal: a membrane lipid oxidation product of medicinal interest. *Med Res Rev*. 2008; 28:569–631. [PubMed: 18058921]
- Pollack M, Oe T, Lee SH, Silva Elipse MV, Arison BH, Blair IA. Characterization of 2'-deoxycytidine adducts derived from 4-oxo-2-nonenal, a novel lipid peroxidation product. *Chem Res Toxicol*. 2003; 16:893–900. [PubMed: 12870892]
- Rahman I, van Schadewijk AA, Crowther AJ, Hiemstra PS, Stolk J, MacNee W, De Boer WI. 4-Hydroxy-2-nonenal, a specific lipid peroxidation product, is elevated in lungs of patients with chronic obstructive pulmonary disease. *Am J Respir Crit Care Med*. 2002; 166:490–495. [PubMed: 12186826]

- Rathahao E, Peiro G, Martins N, Alary J, Guéraud F, Debrauwer L. Liquid chromatography-multistage tandem mass spectrometry for the quantification of dihydroxynonene mercapturic acid (DHN-MA), a urinary end-metabolite of 4-hydroxynonenal. *Anal Bioanal Chem.* 2005; 381:1532–1539. [PubMed: 15770473]
- Rindgen D, Nakajima M, Wehrli S, Xu K, Blair IA. Covalent modifications to 2'-deoxyguanosine by 4-oxo-2-nonenal, a novel product of lipid peroxidation. *Chem Res Toxicol.* 1999; 12:1195–1204. [PubMed: 10604869]
- Rindgen D, Lee SH, Nakajima M, Blair IA. Formation of a substituted 1,N(6)-etheno-2'-deoxyadenosine adduct by lipid hydroperoxide-mediated generation of 4-oxo-2-nonenal. *Chem Res Toxicol.* 2000; 13:846–852. [PubMed: 10995257]
- Sayre LM, Arora PK, Iyer RS, Salomon RG. Pyrrole formation from 4-hydroxynonenal and primary amines. *Chem Res Toxicol.* 1993; 6:19–22. [PubMed: 8448343]
- Slatter JG, Rashed MS, Pearson PG, Han DH, Baillie TA. Biotransformation of methyl isocyanate in the rat. Evidence for glutathione conjugation as a major pathway of metabolism and implications for isocyanate-mediated toxicities. *Chem Res Toxicol.* 1991; 4:157–161. Key reference for the synthesis of MAD₃. A modification of this method is described in Support Protocol 4. [PubMed: 1782345]
- Spiteller G. The important role of lipid peroxidation processes in aging and age dependent diseases. *Mol Biotechnol.* 2007; 37:5–12. [PubMed: 17914157]
- Srivastava S, Dixit BL, Cai J, Sharma S, Hurst HE, Bhatnagar A, Srivastava SK. Metabolism of lipid peroxidation product, 4-hydroxynonenal (HNE) in rat erythrocytes: role of aldose reductase. *Free Radic Biol Med.* 2000; 29:642–651. [PubMed: 11033416]
- Van Kuijk FJ, Thomas DW, Stephens RJ, Dratz EA. Occurrence of 4-hydroxyalkenals in rat tissues determined as pentafluorobenzyl oxime derivatives by gas chromatography-mass spectrometry. *Biochem Bioph Res Co.* 1986; 139:144–149.
- Warnke MM, Wanigasekara E, Singhal SS, Singhal J, Awasthi S, Armstrong DW. The determination of glutathione-4-hydroxynonenal (GSHNE), E-4-hydroxynonenal (HNE), and E-1-hydroxynon-2-en-4-one (HNO) in mouse liver tissue by LC-ESI-MS. *Anal Bioanal Chem.* 2008; 392:1325–1333. [PubMed: 18815773]
- Winter CK, Segall HJ, Haddon WF. Formation of cyclic adducts of deoxyguanosine with the aldehydes trans-4-hydroxy-2-hexenal and trans-4-hydroxy-2-nonenal in vitro. *Cancer Res.* 1986; 46:5682–5686. [PubMed: 3756915]

Figure 17.14.1.

Formation of LPO products from linoleic acid. Under conditions of oxidative stress, linoleic acid is oxidized to form intermediary hydroperoxy octadecadienoic acids (HPODEs) that further degrade into HNE and ONE. Both 2-alkenals are converted by phase I enzymes into HNA, DHN, ONA, and ONO.

Figure 17.14.2.
HNE conjugate formation with cysteine via Michael-type addition.

Figure 17.14.3.

LC-MS/MS-SRM chromatogram of LPO-MA conjugates detected in human urine. SRM transitions are: (1) HNE-MA m/z 318 $\rightarrow$ 189 and m/z 318 $\rightarrow$ 171; (2) DHN-MA m/z 320 $\rightarrow$ 191 and m/z 320 $\rightarrow$ 143; (3) HNA-MA m/z 334 $\rightarrow$ 162 and m/z 334 $\rightarrow$ 143; (4) HNAL-MA m/z 316 $\rightarrow$ 162 and m/z 316 $\rightarrow$ 143; (5) ONO-MA m/z 318 $\rightarrow$ 162; (6) ONA-MA m/z 332 $\rightarrow$ 169 and m/z 332 $\rightarrow$ 162.

Table 17.14.1

LC-MS/MS properties of LPO-MA metabolites.

Analyte	Mol. wt.	SRM transition	Collision energy (eV)	Retention time (min.)
ONE-MA	317	316 → 162	25	13.3
ONE-MA-d ₃	320	319 → 165	25	13.3
HNE-MA	319	318 → 189 ^a	25	10.5, 10.8
		318 → 171	25	10.5, 10.8
HNE-MA-d ₃	322	321 → 189	25	10.5, 10.8
		321 → 171	25	10.5, 10.8
ONO-MA	319	318 → 162 ^a	25	11.3
ONO-MA-d ₃	322	321 → 165	25	11.3
DHN-MA	321	320 → 191 ^a	25	9.8
		320 → 143	25	9.8
DHN-MA-d ₃	324	323 → 191	25	9.8
		323 → 143	25	9.8
ONA-MA	333	332 → 169	25	12.2
		332 → 162	25	12.2
ONA-MA-d ₃	336	335 → 169	25	12.2
		335 → 162	25	12.2
HNA-MA	335	334 → 162	25	10.2
		334 → 143	25	10.2
HNA-MA-d ₃	338	337 → 165	25	10.2
		337 → 143	25	10.2
HNAL-MA	317	316 → 162	25	13.2–13.6
		316 → 143	25	13.2–13.6
HNAL-MA-d ₃	320	319 → 165	25	13.2–13.6
		319 → 143	25	13.2–13.6

^aQuantifying transition. Other transitions used as qualifiers.

Table 17.14.2

Calibration Curve Set Up.

Concentration	HNE-MA	ONO-MA	DHN-MA	IS ^a	2:8 mobile phase A/mobile phase B
10 nM	-----	10 µl of a 100 nM solution	-----	25 µl	65 µl
50 nM	5 µl of a 1 µM solution	5 µl of a 1 µM solution	5 µl of a 1 µM solution	25 µl	60 µl
0.10 µM	10 µl of a 1 µM solution	10 µl of a 1 µM solution	10 µl of a 1 µM solution	25 µl	45 µl
0.25 µM	25 µl of a 1 µM solution	25 µl of a 1 µM solution	25 µl of a 1 µM solution	25 µl	0 µl
0.50 µM	5 µl of a 10 µM solution	5 µl of a 10 µM solution	5 µl of a 10 µM solution	25 µl	60 µl
1.0 µM	10 µl of a 10 µM solution	10 µl of a 10 µM solution	10 µl of a 10 µM solution	25 µl	45 µl
2.5 µM	25 µl of a 10 µM solution	-----	25 µl of a 10 µM solution	25 µl	25 µl
5.0 µM	5 µl of a 100 µM solution	-----	5 µl of a 100 µM solution	25 µl	65 µl

^a25 µl of IS consists of 10 µl of 10 µM HNE-MAd3, 10 µl of 10 µM ONO-MAd3, and 5 µl of 100 µM DHN-MAd3 as described in step 2 from Support Protocol 6.