AN ABSTRACT FOR THE DISSERTATION OF

Heather C. Kuiper for the degree of Doctor of Philosophy in Pharmacy presented on August 14, 2009.

Title: Identification and Quantitation of 4-Hydroxy-2-nonenal and 4-Oxo-2-nonenal Metabolites *In Vivo* as Biomarkers of Oxidative Stress.

Abstract Approved:

Dr. Jan Frederik Stevens

Oxidative stress occurs when the balance between reactive oxygen species and antioxidant defense mechanisms in the body becomes tipped heavily in favor of the reactive oxygen species. Reactive oxygen species can be present in excess due to elevated O_2 levels, radiation, infection, smoking, or even excessive exercise. It is also possible that a reduction in antioxidant levels could result in oxidative stress. When this occurs, reactive oxygen species can cause damage by reacting with DNA, proteins, and lipids.

Lipid peroxidation products are breakdown products of polyunsaturated fatty acids, formed under conditions of oxidative stress. These lipid peroxidation products, such as 4-hydroxy-2-nonenal, 4-oxo-2-nonenal, and their metabolites, have been demonstrated to be cytotoxic and genotoxic. They have also been shown to play a role in the development and progression of age related diseases such as Alzheimer's and atherosclerosis. 4-Hydroxy-2-nonenal levels have even been shown to increase with disease progression. Conjugation with glutathione, followed by further metabolism to mercapturic acid conjugates, can mitigate the effects of these lipid peroxidation products

in disease development by facilitating their excretion from the body. The increase of lipid peroxidation products in disease states suggests utility for the mercapturic acid conjugates of these metabolites as biomarkers of oxidative stress *in vivo*.

In order to assess the utility of lipid peroxidation product-mercapturic acid conjugates as biomarker of oxidative stress, we first developed liquid chromatography-tandem mass spectrometry methods by which to analyze the conjugates. Then, using CCl_4 treatment of rats, a widely accepted model of acute oxidative stress, we were able to discover the first *in vivo* evidence for 4-oxo-2-nonenal-mercapturic acid and its phase one metabolites 4-oxo-2-nonen-1-ol-mercapturic acid and 4-oxo-2-nonenoic acid-mercapturic acid. This proved to be non-trivial since 4-hydroxy-2-nonenal-mercapturic acid and 4-oxo-2-nonen-1-ol-mercapturic acid are isomers with similar retention times and fragmentation patterns. The distinction between 4-hydroxy-2-nonenal-mercapturic acid and 4-oxo-2-nonen-1-ol-mercapturic acid is an important one because previous analyses have likely attributed the effects of 4-oxo-2-nonen-1-ol to 4-hydroxy-2-nonenal. These metabolites also form by different pathways, so being able to distinguish between the two could provide insight into the mechanisms of oxidative stress in biological systems. We were also able to show a significant increase in urinary levels of 1,4-dihydroxy-2-nonene-mercapturic acid, 4-hydroxy-2-nonenoic acid lactone-mercapturic acid, 4-oxo-2-nonenal-mercapturic acid, and 4-oxo-2-nonenoic acid-mercapturic acid in the CCl_4 rat model of oxidative stress by semi-quantitative analysis. These results suggest that conjugates of 4-hydroxy-2-nonenal and 4-oxo-2-nonenal metabolites have value as markers of *in vivo* oxidative stress and lipid peroxidation.

The next step was to develop quantitative methods for the analysis of lipid peroxidation product conjugates and to assess their levels in humans. We developed a quantitative method to simultaneously analyze the levels of 4-oxo-2-nonen-1-ol-mercapturic acid, 4-hydroxy-2-nonenal-mercapturic acid, and 1,4-dihydroxy-2-nonene-mercapturic acid in human urine samples utilizing isotope-dilution mass spectrometry. We were also able to detect 4-hydroxy-2-nonenoic acid-mercapturic acid, 4-hydroxy-2-nonenoic acid lactone-mercapturic acid, and 4-oxo-2-nonenoic acid-mercapturic acid with this method. The detection of 4-oxo-2-nonen-1-ol-mercapturic acid and 4-oxo-2-nonenoic acid-mercapturic acid in humans is significant because it demonstrates that 4-

hydroxy-2-nonenal/4-oxo-2-nonenal branching occurs in the breakdown of polyunsaturated fatty acids and suggests that 4-oxo-2-nonen-1-ol may contribute to the harmful effects currently associated with 4-hydroxy-2-nonenal. We were able to show significant decreases in 4-hydroxy-2-nonenal-mercapturic acid, 1,4-dihydroxy-2-nonene-mercapturic acid, and total lipid peroxidation product-mercapturic acid conjugates in a group of seven smokers upon smoking cessation. This data demonstrates the value of 4-hydroxy-2-nonenal and 4-oxo-2-nonenal metabolites as *in vivo* markers of oxidative stress.

Identification and Quantitation of 4-Hydroxy-2-nonenal and 4-Oxo-2-nonenal Metabolites *In Vivo* as Biomarkers of Oxidative Stress

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APPROVED:

Major Professor, representing Pharmacy

Dean of the College of Pharmacy

Dean of the Graduate School

I understand that my dissertation will become part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my dissertation to any reader upon request.

Heather C. Kuiper, Author

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CONTRIBUTION OF AUTHORS

Dr. Jan F. Stevens provided advice and support in all aspects of this dissertation. Dr. Cristobal L. Miranda was responsible for the animal care and treatment, and creatinine analysis in Chapter 3, and the creatinine analysis in Chapter 4. Dr. John D. Sowell developed the HPLC method 2 used in Chapter 3. Dr. Brandi L. Langsdorf provided assistance with standard synthesis for Chapter 4. Dr. John E. Mata, Dr. Carole Jubert, and Dr. Jacqueline Joss designed the human studies in Chapter 4 and carried out the sample collection.

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LIST OF ABBREVIATIONS

AD	Alzheimer's disease
BMI	body mass index
CG	cysteine glycine
DHA	docosahexaenoic acid
DHN	1,4-dihydroxy-2-nonene
DIBAL	diisobutylaluminium hydride
DNPH	2,4-dinitrophenylhydrazine
ELISA	enzyme-linked immunosorbent assay
EPI	enhanced product ion
γ-GT	γ-glutamyl transferase
GC	gas chromatography
GSH	reduced glutathione
GSSG	oxidized glutathione
GST	glutathione *S*-transferase
HHA	4-hydroxy-2-hexenoic acid
HHE	4-hydroxy-2-hexenal
HNA	4-hydroxy-2-nonenoic acid
HNAL	4-hydroxy-2-nonenoic acid lactone
HNE	4-hydroxy-2-nonenal
HPLC	high performance liquid chromatography
HPNE	4-hydroperoxy-2-nonenal
15-HPETE	15-hydroperoxy-5,8,11,13-eicosatetraenoic acid
HPODE	hydroperoxy octadecadienoic acid
9-HPODE	9-hydroperoxy-10,12-octadecadienoic acid
13-HPODE	13-hydroperoxy-9,11-octadecadienoic acid
LC	liquid chromatography
LPO	lipid peroxidation
MA	mercapturic acid or *N*-acetyl-L-cysteine
MAd_3	*N*-(acetyl-d_3)-L-cysteine
MCPBA	3-chloroperoxybenzoic acid
MS	mass spectrometry
NMO	4-methylmorpholine *N*-oxide
OHA	4-oxo-2-hexenoic acid
OHE	4-oxo-2-hexenal
OHO	4-oxo-2-hexenol
ONA	4-oxo-2-nonenoic acid
ONE	4-oxo-2-nonenal
ONO	4-oxo-2-nonen-1-ol
PUFA	polyunsaturated fatty acids
RM	retro-Michael
ROS	reactive oxygen species
SNP	single nucleotide polymorphisms
SRM	selected reaction monitoring

TLC	thin layer chromatography
TOG	tiadiazabicyclo glutathione
TPAP	tetrapropylammonium perruthenate

IDENTIFICATION AND QUANTITATION OF 4-HYDROXY-2-NONENAL AND 4-OXO-2-NONENAL METABOLITES *IN VIVO* AS BIOMARKERS OF OXIDATIVE STRESS

CHAPTER 1

INTRODUCTION

OXIDATIVE STRESS

Oxidative damage is a consequence of aerobic life. Even at atmospheric levels, oxygen is able to exert toxic effects through the production of free radicals and reactive oxygen species (ROS) (1,2). In healthy individuals there is a balance between pro-oxidants and antioxidants which limits the toxic effects of oxygen. In some cases, free radicals and ROS have biologically useful roles so the balance is slightly skewed in favor of ROS. This means that there is always a low-level of oxidative damage in the human body. The free radicals and ROS causing this damage are compounds such as superoxide ($O_2^{\cdot-}$), singlet oxygen ($^1O_2$), hydroxyl radicals ($OH^{\cdot}$), perhydroxy radicals ($HO_2^{\cdot}$), and hydrogen peroxide (H_2O_2). They can be generated by the 'leakage' of electrons from the electron transport chain to O_2 (1,3). Exposure to elevated O_2 levels, radiation, infection, and even excessive exercise can also lead to ROS formation (1). Once formed, ROS cause damage by reacting directly with DNA, proteins, lipids.

The ability of ROS and their degradation products to cause damage to proteins, lipids, and DNA has led to the evolution of antioxidant defense mechanisms to protect against oxidative damage. Enzymatic defenses include those which directly inhibit oxidation, and those that facilitate ancillary reactions. Superoxide is considered to be a major factor in O_2 toxicity and superoxide dismutases defend against it by converting $O_2^{\cdot-}$ into H_2O_2.

$$2\ O_2^{\cdot-} + 2H+ \rightarrow H_2O_2 + O_2$$

Catalases can then convert H_2O_2 to H_2O and O_2.

$$H_2O_2 \rightarrow H_2O + O_2$$

Hydrogen peroxide can also be utilized by glutathione peroxidases to oxidize reduced glutathione (GSH) to oxidized glutathione (GSSG).

$$2H_2O_2 + 2GSH \rightarrow GSSG + 2H_2O$$

These enzymatic mechanisms of $O_2^{\cdot-}$ conversion can limit its ability to cause damage. Many secondary enzymes can provide antioxidant defenses by reacting with and detoxifying ROS. GSH replenishment through glutathione disulfide and glutathione reductase provides the necessary pool of GSH for conjugation and elimination of ROS mediated by glutathione S-transferases (GSTs) (4).

The $OH^{\cdot}$ is a more reactive biological oxidant, forming due to irradiation or from the reaction of H_2O_2 with transition metals such as Fe(II).

$$Fe(II) + H_2O_2 \rightarrow OH^{\cdot} + OH^{-} + Fe(III)$$

Ascorbate and $O_2^{\cdot-}$ can both provide a source of reducing power to convert Fe(III) to Fe(II) for conversion of H_2O_2 to $OH^{\cdot}$.

$$Fe(III) + \text{ascorbate} \rightarrow Fe(II) + \text{semidehydroascorbate}$$

$$Fe(III) + O_2^{\cdot-} \rightarrow Fe(II) + O_2$$

Most transition metals are bound to proteins *in vivo*, preventing them from catalyzing radical reactions. Caeruloplasmin binds copper ions, while ferritin and transferrin bind iron ions (2,5). Haptoglobins and haemopexin are other proteins which limit the availability of pro-oxidants by binding haemoglobin and haem respectively (2). Ascorbate can act as a pro-oxidant and has been shown to induce the decomposition of 13-hydroperoxy-9,11-octadecadienoic acid (13-HPODE), a lipid hydroperoxide, resulting in the formation of lipid peroxidation (LPO) products 4-hydroxy-2-nonenal (HNE) and 4-oxo-2-nonenal (ONE) (6). It has also been demonstrated that ascorbate has antioxidant properties, directly scavenging ROS or through the regeneration of tocopherols (3).

Along with proteins and enzymes, a number of low-molecular weight compounds provide the body with antioxidant defense against free radicals. These include species produced *in vivo*, such as bilirubin, α-ketoglutarate, pyruvate, lipoic acid, ubiquinol, and uric acid, and those obtained through the diet, like ascorbate and tocopherols (2,4). Low-molecular weight antioxidants work by scavenging free radicals and deactivating them, hence stopping the chain reaction. Another mechanism of action is to transport ROS to sites where oxidative events will be less damaging.

Cellular antioxidant mechanisms are an important form of protection against oxidative damage; however, at high levels of ROS formation these mechanisms can become overwhelmed, resulting in oxidative stress. Under conditions of oxidative stress, ROS can react with polyunsaturated fatty acids (PUFAs), causing them to degrade and form electrophilic LPO products, including 4-hydroperoxy-2-nonenal (HPNE), HNE, and ONE (Fig. 1.1). These reactive aldehydes have the ability to react with proteins, peptides, and DNA, causing modifications and disturbing cellular function.

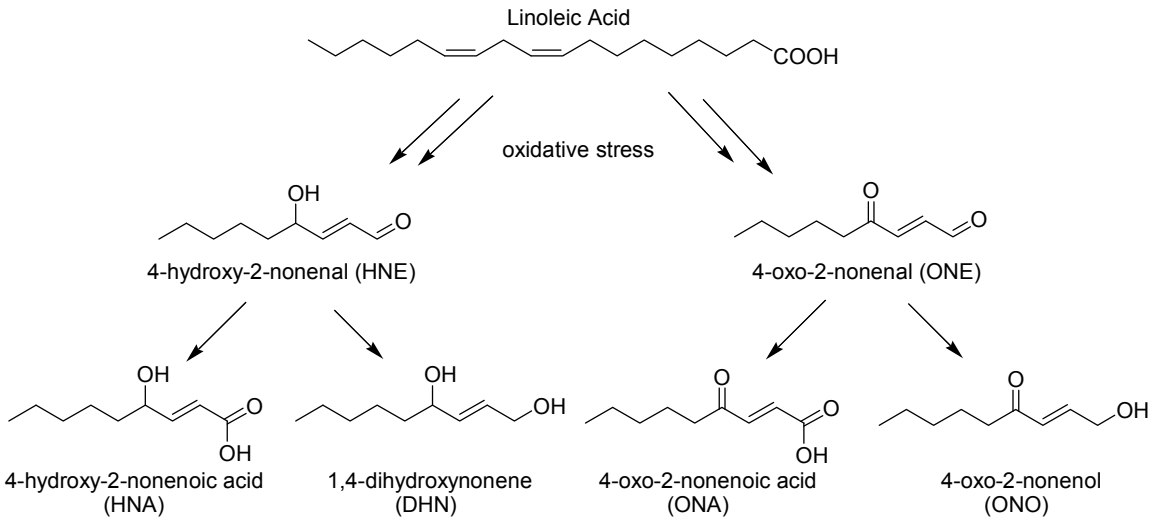

FIGURE 1.1 **Formation of LPO products from linoleic acid.** Linoleic acid breaks down under conditions of oxidative stress to form HNE, ONE, and their phase I metabolites HNA, DHN, ONA, and ONO.

FORMATION OF LIPID PEROXIDATION PRODUCTS

Lipid-based radicals are formed when a free radical abstracts a hydrogen from the bis-allylic methylene functionality of a PUFA. These radicals can undergo double bond rearrangement followed by spontaneous reaction with O_2, and hydrogen abstraction from a nearby PUFA to form lipid hydroperoxides, *e.g.*, 9-hydroperoxy-10,12-octadecadienoic acid (9-HPODE) and 13-HPODE from linoleic acid or 15-hydroperoxy-5,8,11,13-eicosatetraenoic acid (15-HPETE) from arachidonic acid. Subsequently, HPODEs and HPETE are converted into carbon-carbon cleavage products, the mechanisms of which have been demonstrated (7-10). The mechanism of HPNE formation from linoleic acid takes different routes, depending on whether 9- or 13-HPODE is formed initially (Fig. 1.2). The degradation of 13-HPODE occurs via hydrogen abstraction from C-8, radical migration, and oxygenation to give sites of peroxidation at C-10 and C-13. Hock-cleavage will then yield 9-oxononanic acid and HPNE. 9-HPODE breaks down in the opposite manner, first undergoing Hock-cleavage to form 3Z-nonenal. Hydrogen abstraction from C-2, radical migration, and oxygenation provide HPNE.

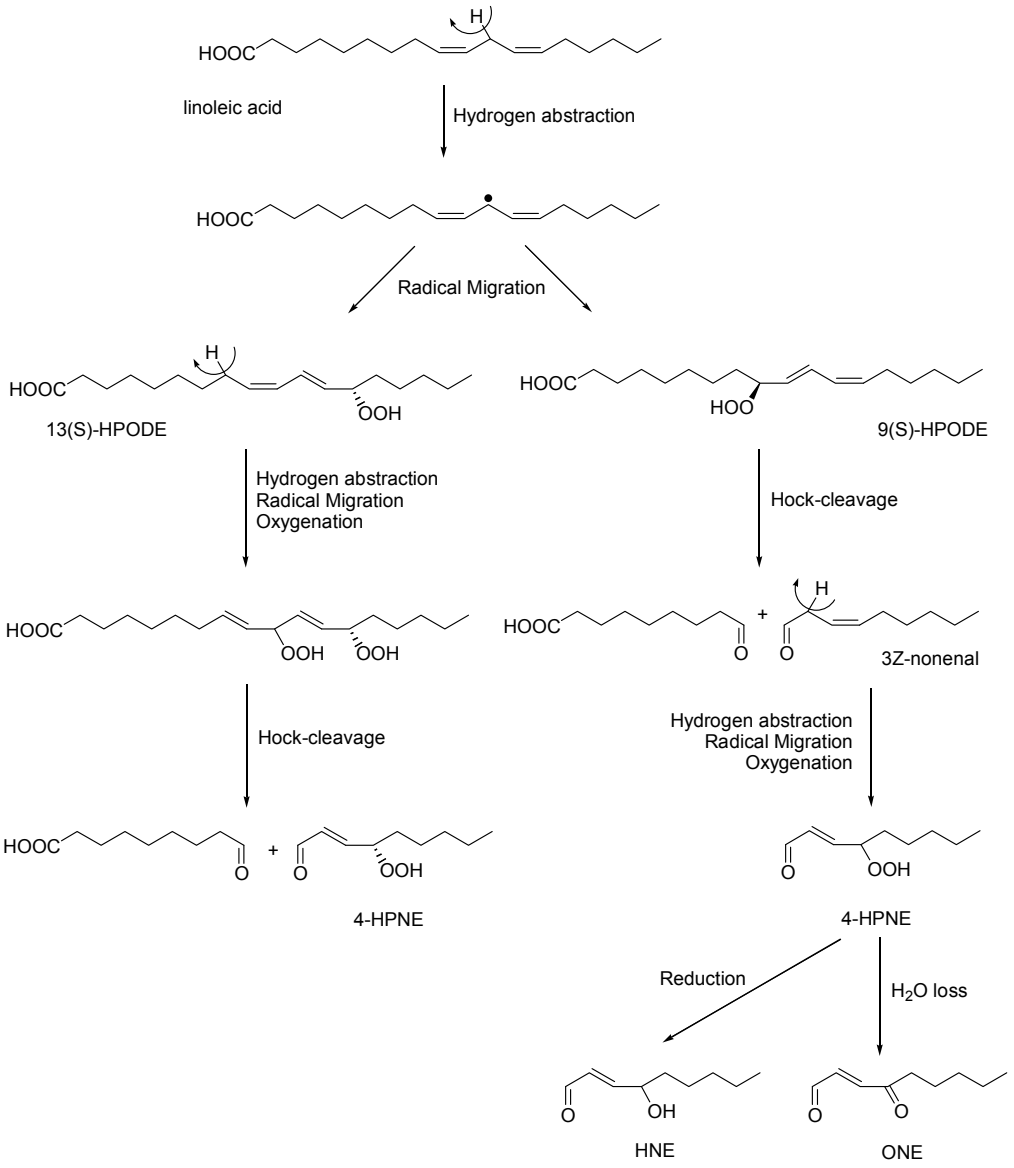

FIGURE 1.2 **Proposed mechanism of formation of HNE and ONE from linoleic acid.**

The dimer pathway of HPNE formation from 15-HPETE has also been elucidated (Fig. 1.3). Addition of a peroxyl radical at the C-11 double bond of 15-HPETE provides the lipid hydroperoxide dimer for which this degradation pathway is named. The carbon centered radical becomes delocalized from C-12 to C-14. If no O_2 is available, homolytic displacement of the peroxide bond will occur, resulting in epoxide and alkoxyl radical formation. If O_2 is present, it will react with the delocalized carbon radical forming a new peroxyl radical. The peroxyl radical can then attack a nearby double bond forming a trimer, or homolytic displacement of the peroxide can facilitate a carbon-carbon bond

cleaving rearrangement resulting in HPNE formation. The reduction of HPNE yields HNE, but H_2O loss could also occur, generating ONE.

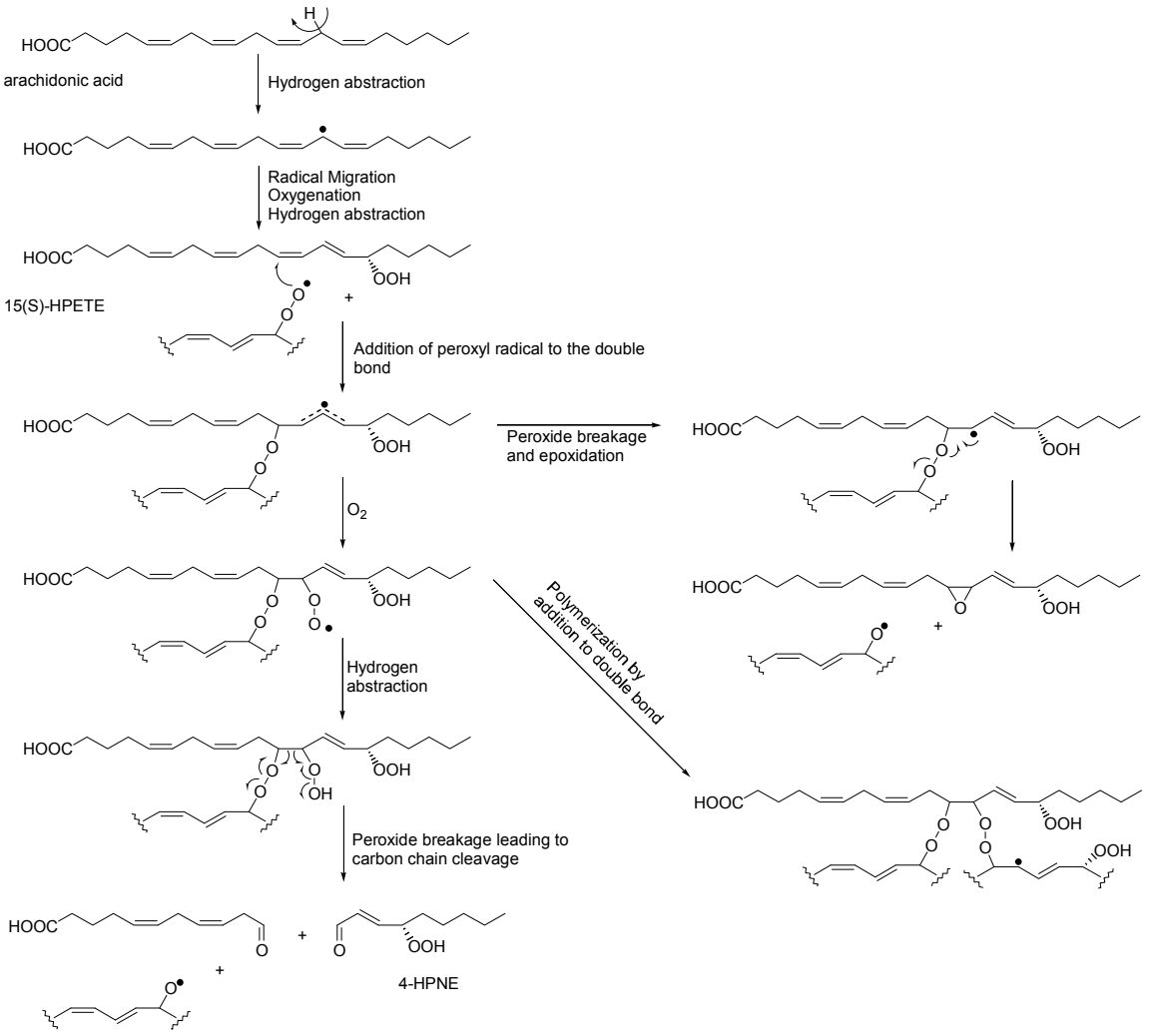

FIGURE 1.3 **Proposed dimer pathway of HNE and ONE formation.**

The LPO products formed under conditions of oxidative stress have been shown to be cytotoxic and genotoxic due to their ability to react with proteins, peptides, and DNA, causing modifications and disturbing cellular function (11,12). This has lead to LPO products being associated with the development and progression of age related diseases such as cardiovascular diseases (13-15), cancer (16), and neurodegenerative diseases (17-19). HNE and ONE react primarily with cysteine, histidine, and lysine via Michael-type additions (20) or in the case of lysine, Schiff base formation (21) (Fig. 1.4).

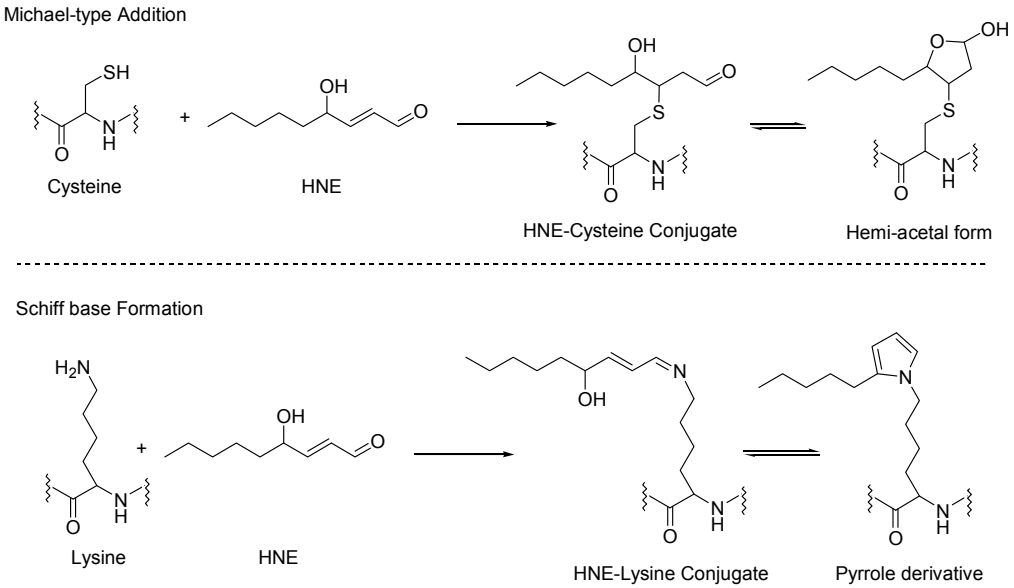

FIGURE 1.4 **HNE conjugate formation with biomolecules.** Michael-type additions can occur with cysteine, histidine, or lysine. Schiff base formation can occur with lysine.

It has also been demonstrated that HNE can crosslink proteins by undergoing both Michael-type addition and Schiff base formation (22) (Fig. 1.5). ONE has been shown to covalently modify DNA through formation of adducts with 2′-deoxyguanosine, 2′-deoxyadenosine, and 2′-deoxycytidine (23-26), while HNE can form adducts with 2′-deoxyguanosine (27) (Fig. 1.6). Consequently, there is considerable interest in mitigating the effects of LPO products.

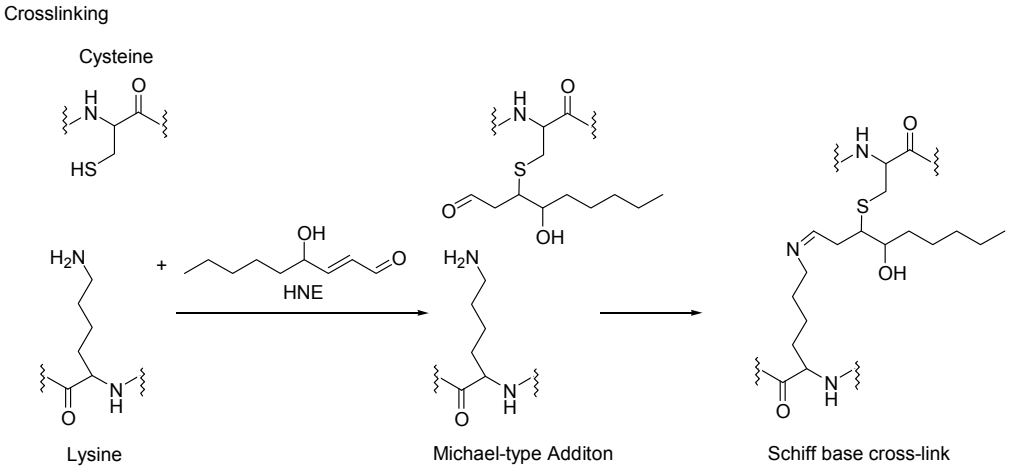

FIGURE 1.5 **Crosslinking of proteins by HNE via Michael-type addition and Schiff base formation.**

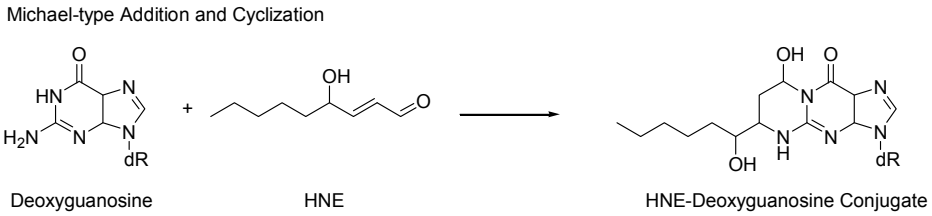

FIGURE 1.6 **DNA adduct formation with HNE.**

METABOLISM AND DETOXIFICATION

Multiple enzymatic pathways are involved in the metabolism of HNE and ONE. Phase I metabolism of HNE is a prominent metabolic pathway. Cytochromes P450 (28) and aldehyde dehydrogenase (29) can catalyze the oxidation of HNE to 4-hydroxy-2-nonenoic acid (HNA), while aldo-keto reductase (30) has been shown to reduce HNE to 1,4-dihydroxy-2-nonene (DHN). Upon conjugation via phase II metabolism, HNA can spontaneously undergo intramolecular condensation to form a lactone, 4-hydroxy-2-nonenoic acid lactone (HNAL) (31). This lactone formation has also been demonstrated to occur from the reduction of HNE-GSH in the hemi-acetal form (32). ONE can be metabolized in similar fashion, forming 4-oxo-2-nonenoic acid (ONA) from aldehyde dehydrogenase mediated oxidation (33) or HNE isomer, 4-oxo-2-nonenol (ONO), via aldo-keto reductase (34,35) mediated reduction. Carbonyl reductase has been demonstrated to reduce ONE at the C-4 position as well, resulting in HNE formation

(36). With the exception of DHN, these phase I metabolites of HNE and ONE maintain their α,β-unsaturation, and are therefore able to form Michael-type conjugates with GSH. Phase II metabolism of HNE and ONE via a 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 glutathione (GSH) with HNE (37), 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) (38). Metabolism by cysteinyl glycinase and *N*-acetyl transferase in the liver and kidneys results in HNE-mercapturic acid (MA) conjugates which can be excreted in the urine. This pathway for HNE removal can initially deplete GSH. However, induced expression of glutamate cysteine ligase, the rate limiting enzyme in GSH synthesis, has been demonstrated in the presence of HNE (39).

Radiolabeled HNE has been used in a number of studies in order to better understand the metabolism of HNE. While radiation hazards and cost are deterrents, using radiolabeled material is advantageous for metabolism studies because the user is able to trace the material as it undergoes biotransformation, and to calculate the recovery of the labeled material. It has been demonstrated that [^{3}H]HNE forms conjugates with GSH and is also found as HNAL-GSH in perfused rat liver and erythrocyte incubations (32). Other incubations of [4-^{3}H]HNE with rat liver slices have demonstrated the phase I and phase II metabolism of HNE, resulting in the formation of HNE-GSH, DHN-GSH, HNE-Cysteine, and HNA (40). It was also shown in this study that after 5 min, 85 % of HNE was consumed, resulting in the formation of oxidation product HNA and conjugate HNE-GSH. Administration of [4-^{3}H]HNE to rats by iv allowed for assessment of *in vivo* HNE metabolism (31). Urinary end products detected were HNE-MA, DHN-MA, HNA-MA, and HNAL-MA. ONE metabolism has also been assessed in the literature (33-36,41).

ANALYTICAL METHODS FOR THE IDENTIFICATION AND MEASUREMENT OF LIPID PEROXIDATION PRODUCTS

The analysis of HNE, ONE, and their metabolites has been carried out by many methods including high performance liquid chromatography (HPLC), thin-layer

chromatography (TLC), gas chromatography-mass spectrometry (GC-MS), enzyme-linked immunosorbent assay (ELISA), and liquid chromatography-mass spectrometry (LC-MS). Our goal was to develop a quantitative method of analysis that can simultaneously detect the LPO-GSH, LPO-CG, and LPO-MA conjugates without derivatization.

Early detection of HNE and other reactive aldehydes was carried out by 2,4-dinitrophenylhydrazine (DNPH) derivatization (12,42). The hydrazone derivatives have a characteristic absorbance maxima at 360-390 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 (42). Direct HPLC determination of HNE at 220 nm has also been demonstrated (43). These HPLC methods only allow for comparison with synthetic standards. In order to obtain additional proof of structure, mass spectrometry should be employed. This need for structural information lead to the development of GC-MS methods for HNE analysis, which involved derivatization of HNE with pentafluorobenzyl-hydroxylamine followed by silylation (44). An enzyme immunoassay to quantitatively assess levels of DHN-MA was recently developed and validated by Guéraud *et al.* (45). LC-MS/MS analyses of (S)-carbidopa derivatized HNE were employed to differentiate between the R- and S- enantiomers (46). HNE-GSH and HNA were also analyzed using this method. Isotope-dilution LC-MS^3 methodology was demonstrated to be useful for the quantitative analysis of DHN-MA and could possibly be utilized to quantify other LPO-MA conjugates (47,48). LC-MS analyses employing selected ion monitoring have also been used to analyze HNE-GSH, HNE, and ONO in mouse liver tissues (49), although separate sample preparation methods were necessary for the conjugated and free metabolites. Like many other researchers, we utilize LC-MS/MS analyses, particularly selected reaction monitoring (SRM) analyses, for the analysis of LPO-GSH, LPO-CG, and LPO-MA conjugates *in vivo* and *in vitro* (50,51).

BIOMARKERS

Oxidative stress-induced LPO leads to the formation of cytotoxic and genotoxic 2-alkenals, HNE and ONE, as well as their metabolites (11,12). These LPO products have also been shown to contribute to the development and progression of age-related diseases such as Alzheimer's and atherosclerosis (13,17-19) and HNE levels have been demonstrated to increase in these and other disease states (52). Therefore, using HNE and other LPO products as biomarkers of oxidative stress would provide a tool 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. Measurement of HNE-MA, ONE-MA, and their metabolites, however, could provide non-invasive, stable LPO end-products by which to assess oxidative stress *in vivo*. Current methods being used include many drawbacks. The thiobarbituric acid reaction, a colorimetric assay that is widely used to analyze levels of malondialdehyde lacks specificity (53). $F_{2\alpha}$-isoprostane analysis is currently considered the most reliable way to assess oxidative stress *in vivo*; however, $F_{2\alpha}$-isoprostanes are formed only from arachidonic acid degradation. Due to its specificity, $F_{2\alpha}$-isoprostane analysis may not provide a global assessment of oxidative stress (54). The research in this thesis was undertaken in order to develop appropriate analytical methods for the identification and quantitation of HNE-MA, ONE-MA, and their metabolites *in vivo*, with the ultimate goal of utilizing these metabolites as biomarkers of oxidative stress. Using these conjugates to determine levels of oxidative stress prior to the onset of age related diseases could prompt lifestyle changes and lead to a decreased incidence of disease development.

AIM AND OUTLINE OF THE STUDIES

We became interested in LPO products from the detoxification perspective. The original direction of this work was to assess the utility of ascorbic acid as a detoxification agent for HNE. It was already well known that HNE reacted with GSH as a prominent method of removal from the body, and we set out to compare levels of the two conjugates in biological systems. Ascorbyl-HNE conjugate formation turned out to be a minor detoxification pathway (51); however, our interest in GSH conjugates of LPO products

was piqued. The focus of the research shifted to developing LC-MS/MS methods for the quantification of LPO-GSH conjugates in plasma. A timely suggestion of Prof. Donald Reed led to the inclusion of LPO-CG products in the study as well. The preparation and analysis of LPO-GSH and LPO-CG conjugates is discussed in Chapter 2.

As we expanded our studies of phase II metabolites of HNE, we realized it would be beneficial to take a more comprehensive approach and included phase I metabolites as well. About this time, Blair published a review article in which he stated "ONO may contribute to the biological activities that have been ascribed previously to HNE" (55). We decided that we could not accurately assess HNE metabolite levels without ensuring that we were truly analyzing HNE and not ONO. Therefore, the ONE metabolites were included in our studies. As the number of metabolites of interest increased, it was concluded that urinary analyses would provide us with the most comprehensive assessment of oxidative stress in the body, as opposed to the snapshot view plasma analysis provides. Another benefit of analyzing urine is that due to metabolism, only the MA conjugates are present in urine, decreasing the number of analytes to account for. Urinary analyses also allowed for facile sample collection and sample preparation. Semi-quantitative analysis of the LPO-MA conjugates is presented in Chapter 3 along with a discussion of the metabolite synthesis and LC-MS/MS analyses. CCl_4 treatment of rats, a widely accepted animal model of acute oxidative stress, resulted in a significant increase of the urinary levels of DHN-MA, HNA-MA lactone, ONE-MA, and ONA-MA. Our data suggest that conjugates of HNE and ONE metabolites have value as markers of *in vivo* oxidative stress and LPO.

Chapter 4 builds upon Chapter 3, in order to assess the utility of LPO-MA conjugates as biomarkers of oxidative stress. The goal of the research was to develop a method for the simultaneous quantitation of LPO-MA metabolites in the urine of smokers and nonsmokers, since smoking is a well known form of inducing oxidative stress. Quantitative analysis of ONO-MA in vivo was demonstrated for the first time in this work. HNE-MA and DHN-MA were also quantified, with DHN-MA levels corresponding to those already in the literature (56). ONA-MA, HNA-MA, and HNAL-MA were also qualitatively assessed in human urine, demonstrating the branching of HPNE to form HNE and ONE metabolites in humans.

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CHAPTER 2

GENERAL METHODS

The section Synthesis of LPO-GSH and LPO-CG Conjugates was published in Chemical Research in Toxicology
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INTRODUCTION

One of the major obstacles in the study of LPO products is the lack of commercially available standards. It was therefore necessary for us to synthetically prepare the LPO products, their conjugates, and the internal standards used in these studies. This chapter details the synthetic approaches utilized for the method development, identification, and quantitation of HNE and ONE metabolites *in vivo*. The development of sample preparation techniques and evolution of the research are also discussed.

SYNTHESIS OF LIPID PEROXIDATION PRODUCTS

Of the LPO products, HNE has received the most attention in the literature due to its biological activity. Autoxidation of linoleic acid resulted in the production low amounts of HNE, so Esterbauer *et al.* (1,2) developed a synthetic approach to making HNE. They reacted the Grignard of propynal diethylacetal with hexanal, followed by $LiAlH_4$ reduction of the triple bond, to afford HNE diethyl acetal. Hydrolysis under mild acidic conditions resulted in the formation of HNE with a 53% yield (Fig. 2.1, A). A second method for HNE synthesis (2,3) reacted 1,3-bis(methylthio)allyl-lithium with hexanal to form 1,3-bis(methylthio)1-hexene-4-ol. Conversion of this compound by mercuric chloride affords HNE, 47% yield (Fig. 2.1, B). The drawback of this method is that 1,3-bis(methylthio)allyl-lithium is not commercially available. Another preparation of HNE (2,4) involved the conversion of furan to fumaraldehyde monodimethylacetal and its subsequent reaction with n-butyl Grignard to 4-hydroxynonenal dimethylacetal. Saponification with 2.5% H_2SO_4 gave HNE in 71% yield (Fig. 2.1, C). Gardner *et al.* (5) developed an efficient synthesis of HNE, epoxidizing 3(Z)-nonenol with 3-chloroperoxybenzoic acid, oxidizing the alcohol to an aldehyde with periodinane, and opening the expoxide with NaOH to give HNE in 48% yield (Fig. 2.1, D). Our group modified Gardner's method by using a tetrapropylammonium perruthenate (TPAP)/4-methylmorpholine *N*-oxide (NMO) oxidation to obtain more consistent yields (Fig. 2.1, E). A cross-metathesis reaction utilizing a ruthenium catalyst for the coupling of acrolein and octen-3-ol, resulting in HNE in 75% yield has also been reported (6) (Fig. 2.1, F). A one-pot synthesis of HNE was presented by Sugamoto *et al.* (7), using a cobalt (II)

porphyrin-catalyzed reduction-oxygenation of 2,4-nonadienal to afford HNE in 58% yield (Fig. 2.1, G). Using Horner-Wardsworth-Emmons chemistry, HNE and ONE have been prepared from hexanoyl chloride and triethyl phosphonoacetate in 96% and 92% yields respectively (8) (Fig. 2.1, H). ONE synthesis has also been described from 2-pentylfuran by Zhang *et al.* (9) as an oxidation followed by isomerization with a 68% yield (Fig. 2.1, I). Oxidation and tautomerization of 2-pentylfuran were employed by Annangudi *et al.* (10) to give ONA with a 95% yield (Fig. 2.1, J). HNA preparation was demonstrated by reduction of γ-nonalactone with diisobutylaluminium hydride (DIBAL) in 80-85% yield (11) (Fig. 2.1, K). While important to LPO research, synthetic routes to LPO products other than HNE are not particularly prominent in the literature.

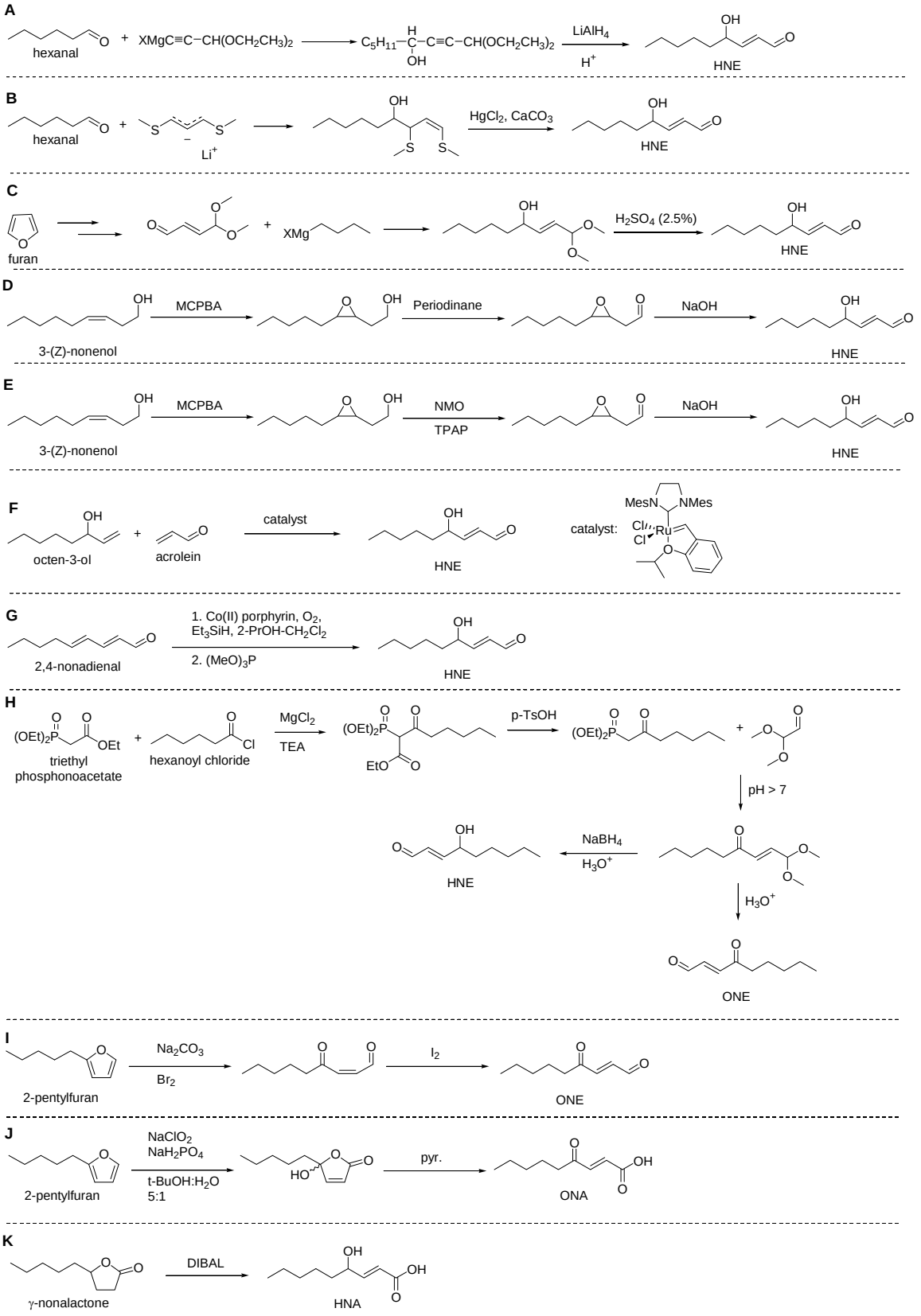

FIGURE 2.1 **Synthetic approaches to HNE, ONE, ONA, and HNA formation.**

SYNTHESIS OF HNE AND ONE METABOLITES

The LPO products were synthesized as shown in Figure 2.2. Initial work focused only on HNE and other metabolites were added to the repertoire as the research progressed.

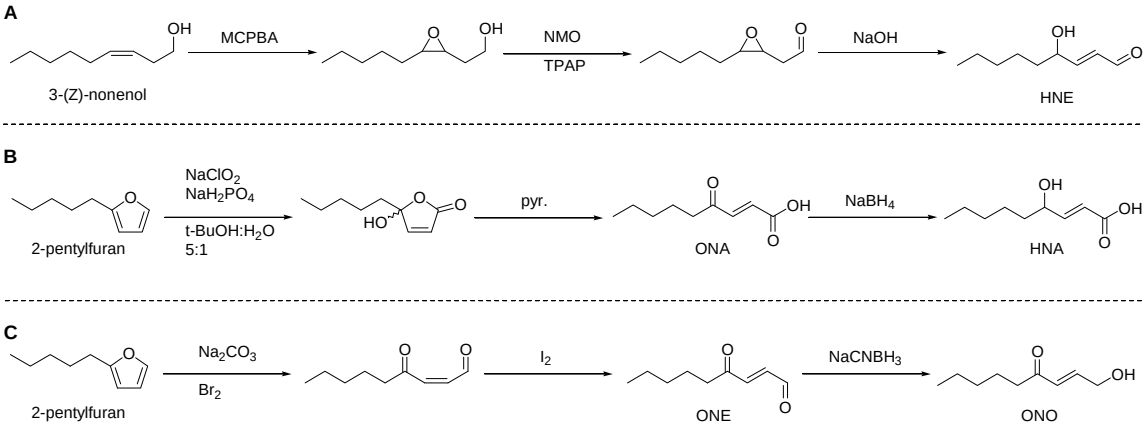

FIGURE 2.2 **Synthetic routes to HNE, ONA, HNA, ONE, and ONO.**

HNE was synthesized from 3-(*Z*)-nonenol following a method adapted from Gardner *et al.* (5) (Fig. 2.2, A). Briefly, 3-(*Z*)-nonenol (2 mmol) was dissolved in 8 ml of CH_2Cl_2, and 3-chloroperoxybenzoic acid (MCPBA) (2 mmol) was added. The reaction mixture was stirred for 1 h at room temperature and, after the addition of 8 ml of 10% aq $NaHCO_3$, stirred for 45 min. The reaction mixture was washed with water and dried with 2 g of powdered molecular sieves. NMO (3 mmol) was added and the mixture was stirred under argon for 30 min. After addition of TPAP (0.1 mmol), the mixture was stirred for 1 h under argon, filtered through silica gel and rinsed with ethyl ether. Next, 16 ml of 1.3 M sodium hydroxide was added to the filtrate and the solution was stirred vigorously for 15 min. The reaction mixture was washed with water, dried with anhydrous $MgSO_4$, filtered, and concentrated *in vacuo*. The yield was 35 %. Our adaptations to the method of Gardner *et al.* (5) gave a slightly lower yield; however, we found that the changes resulted in more consistent yields. Data are ^{1}H NMR (400 MHz, $CDCl_3$) δ 9.63 (d, J = 8 Hz, 1H), 6.86 (dd, J = 5, 16 Hz, 1H), 6.35 (ddd, J = 2, 8, 16 Hz, 1H), 4.48 (m, 1H), 1.76 (d, J = 5 Hz, 2H), 1.43 (m, 7H), 0.94 (t, J = 6 Hz, 3H) (Fig. 2.3). ^{13}C NMR (100 MHz, $CDCl_3$) δ 14.0, 22.6, 24.9, 31.7, 36.5, 71.1, 130.6, 159.0, 193.6

(Fig. 2.4). ^{1}H-^{1}H COSY NMR (400 MHz, $CDCl_3$) (Fig. 2.5). ^{1}H-^{13}H HSQC NMR (400 MHz, $CDCl_3$) (Fig. 2.6).

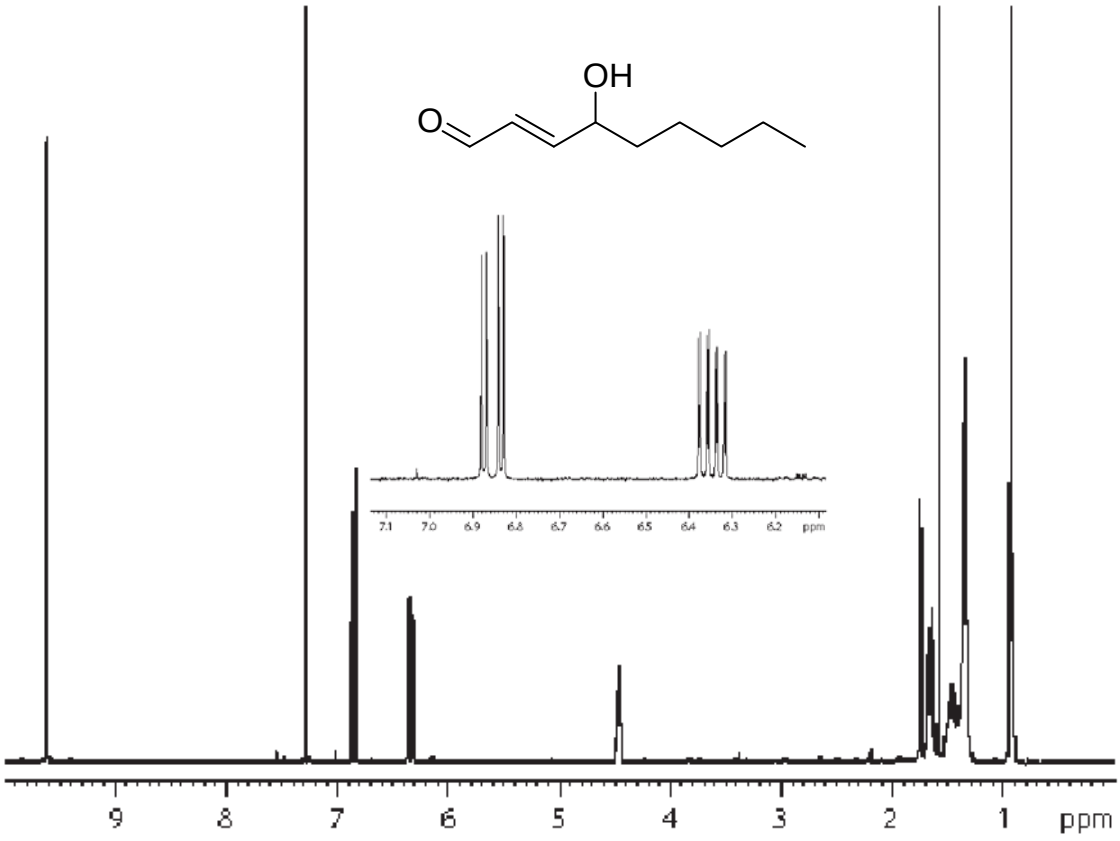

FIGURE 2.3 **400 MHz ^{1}H NMR of HNE in $CDCl_3$**

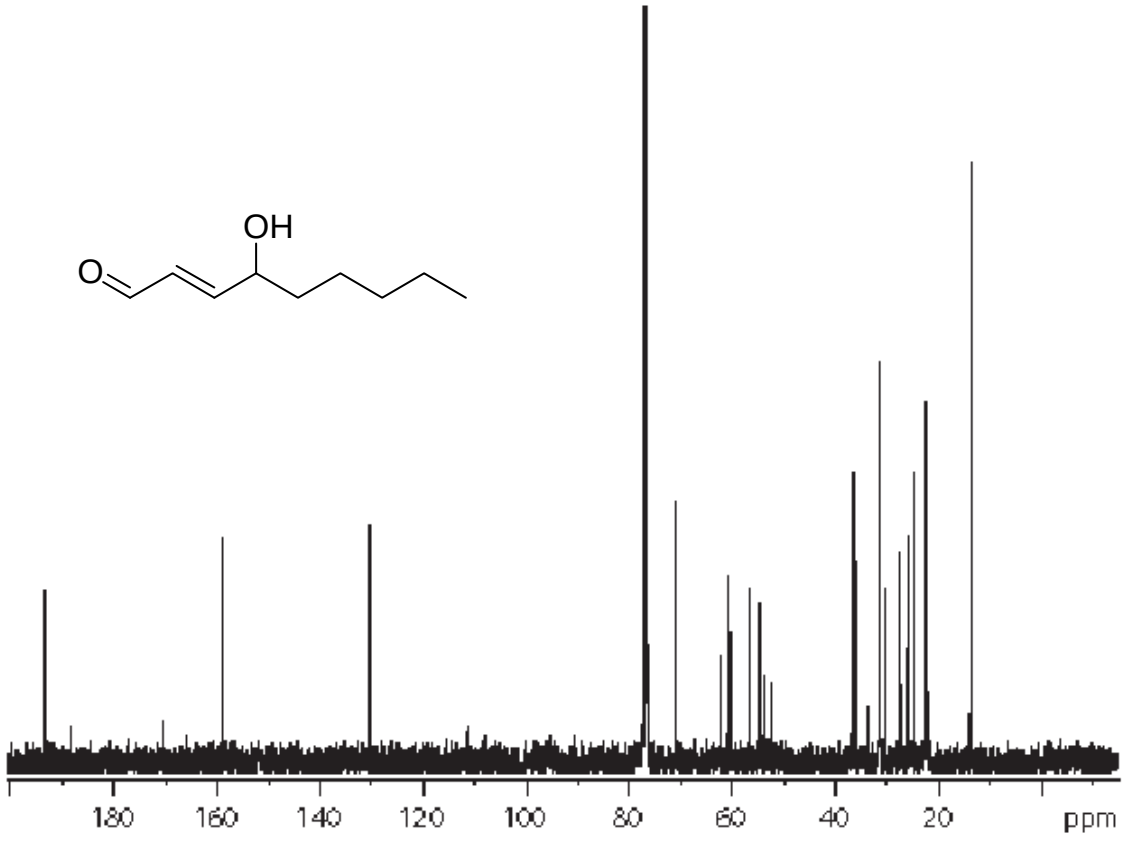

FIGURE 2.4 **100 MHz ^{13}C NMR of HNE in $CDCl_3$**

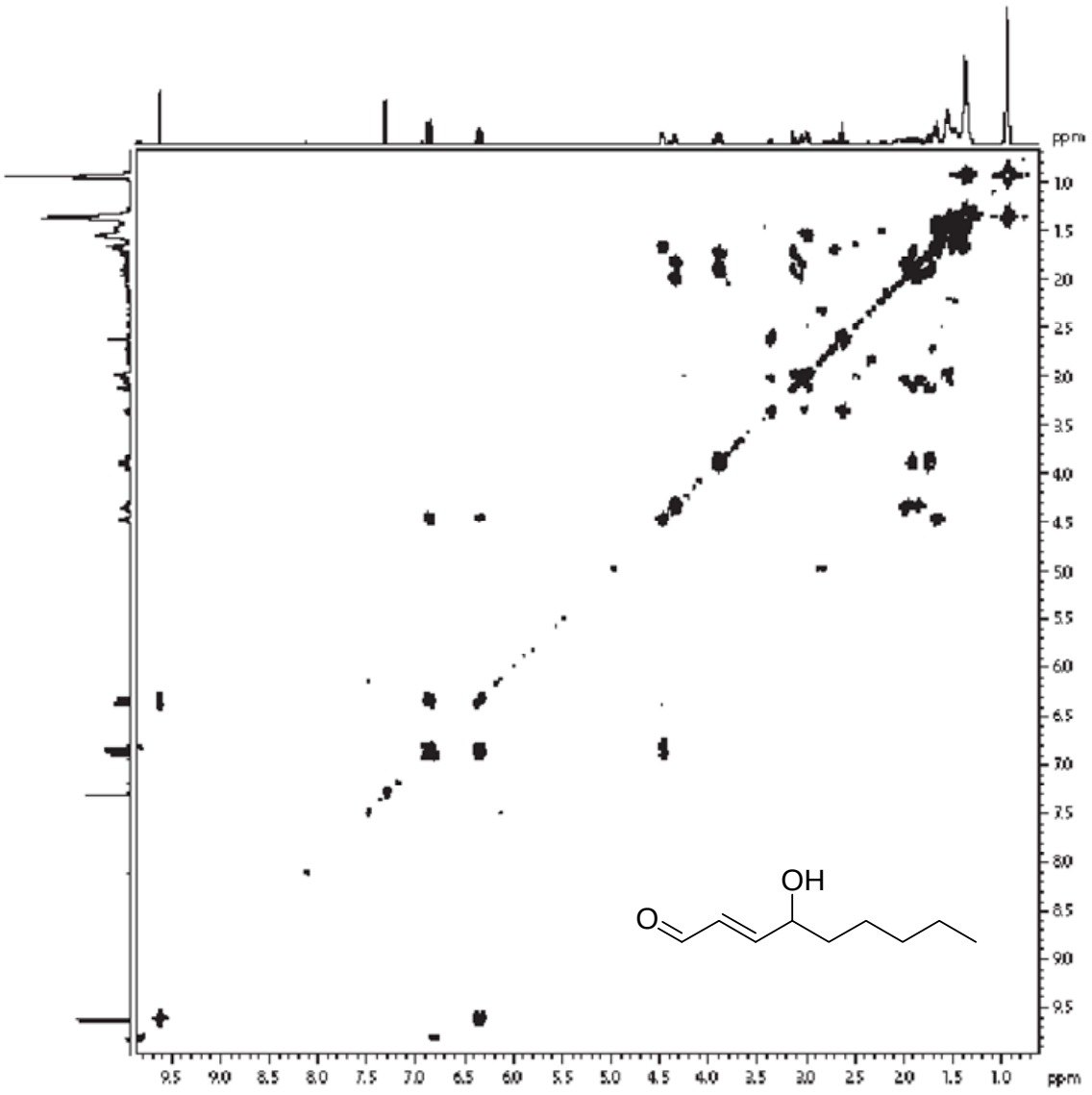

FIGURE 2.5 **400 MHz ^{1}H-^{1}H COSY NMR of HNE in $CDCl_3$**

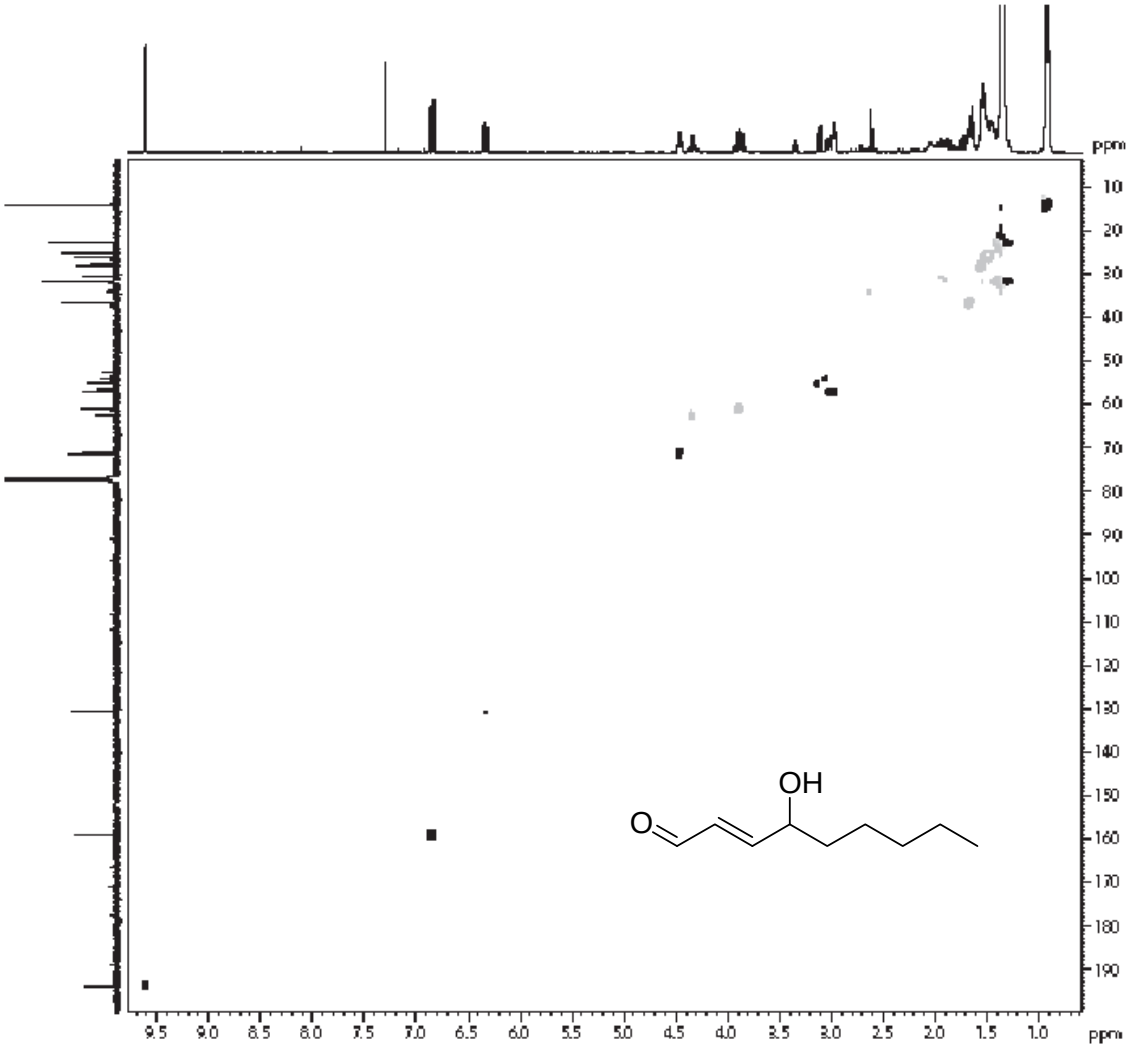

FIGURE 2.6 **400 MHz ^{1}H-^{13}C HSQC NMR of HNE in $CDCl_3$**

ONA was synthesized from 2-pentylfuran, following the method of Annangudi *et al.* (10) with slight modification of the sample purification (Fig. 2.2, B). To a stirred solution of 5:1 t-BuOH-H_2O was added 2-pentylfuran (2 mmol), KH_2PO_4 (3 mmol), and $NaClO_2$ (6 mmol). The mixture was stirred at 4 ^{o}C for 1.5 h. The solvent was removed by rotary evaporator, and the residue was extracted with $CHCl_3$. It was necessary to add 5 ml of H_2O to separate the layers. Upon extraction, the combined organic layers were washed with brine, dried with $MgSO_4$, and filtered and concentrated *in vacuo*. The residue was taken up in tetrahydrofuran-acetone-H_2O (5/4/1, v/v/v, 40 ml), 200 µl of pyridine was added, and the reaction was stirred at room temperature for 2 h. The solvent

was then removed by rotary evaporation and the residue was brought up in ether, followed by extraction with H_2O (pH 10, 1.3 M NaOH). Following extraction, the H_2O layers were acidified with 1 N HCl to pH 2 and extracted with ether. The combined ether layers were dried with $MgSO_4$, filtered, and concentrated *in vacuo*. Data are ^{1}H NMR (400 MHz, $CDCl_3$) 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) (Fig. 2.7).

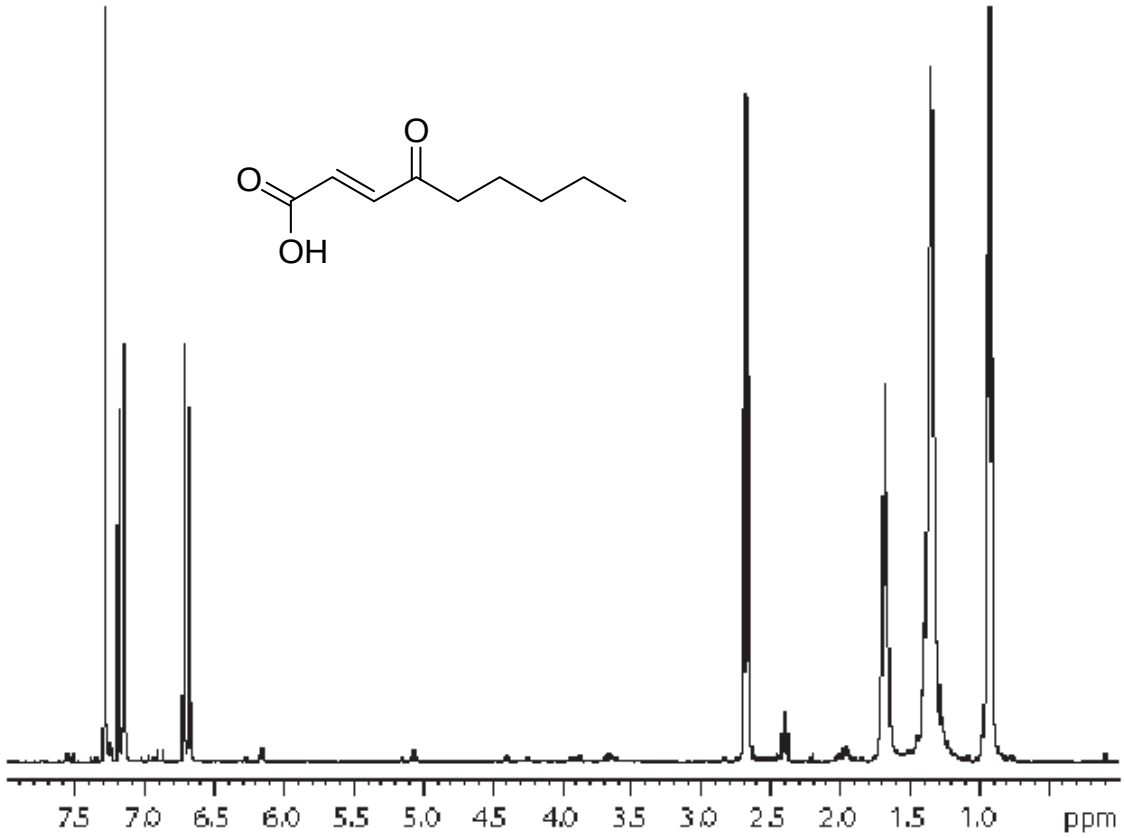

FIGURE 2.7 **400 MHz ^{1}H NMR of ONA in $CDCl_3$**

HNA was prepared by addition of 0.1 mmol of sodium borohydride to a stirred solution of ONA (0.05 mmol) in 5 ml of ethanol. After 45 min at room temperature, the reaction mixture was acidified to pH 3 with 1 N HCl. The mixture was then extracted with ethyl ether. The organic layer was dried with anhydrous $MgSO_4$, filtered, and concentrated *in vacuo* to give HNA (Fig. 2.2, B). Data are ^{1}H NMR (400 MHz, $CDCl_3$) 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) (Fig. 2.8).

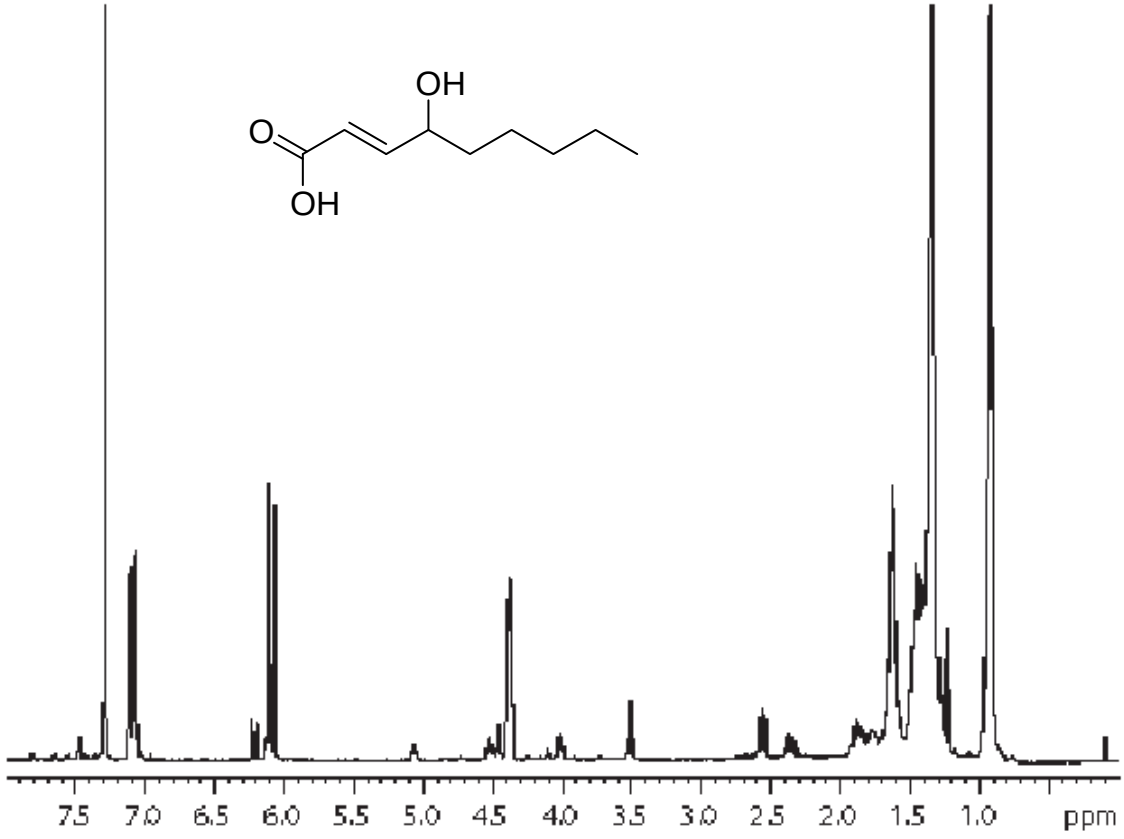

FIGURE 2.8 **400 MHz ^{1}H NMR of HNA in $CDCl_3$**

ONE was synthesized from 2-pentylfuran following the method of Zhang *et al.* (9) (Fig. 2.2, C). To a stirred solution of acetone-H_2O (85/15, v/v, 200 ml) was added 2-pentylfuran (20 mmol) and 10 g of anhydrous Na_2CO_3. The reaction was cooled to -15 ^{o}C and a solution of bromine (20 mmol) in acetone-H_2O (4/1, v/v, 30 ml) was added dropwise over 30 min. The reaction was allowed to slowly return to room temperature, with continuous stirring for 2 h. The reaction was filtered to remove Na_2CO_3 and extracted with ether. The combined either layers were washed with brine, dried with $MgSO_4$ and filtered. Iodine was added and the reaction was stirred at room temperature for 4 h, washed with saturated NaS_2O_3, washed with brine, dried with $MgSO_4$, filtered, and concentrated *in vacuo*. The material was purified on a silica column with hexane-

ether 10:1 as the eluting solvent. Data are ^{1}H NMR (400 MHz, $CDCl_3$) δ 9.82 (d, J = 7 Hz, 1H), 6.91 (d, J = 16 Hz, 1H), 6.81 (dd, J = 7, 16 Hz, 1H), 5.45 (t, J = 7 Hz, 2H), 1.70 (m, 2H), 1.36 (m, 4H), 0.94 (t, J = 7, 3H) (Fig. 2.9).

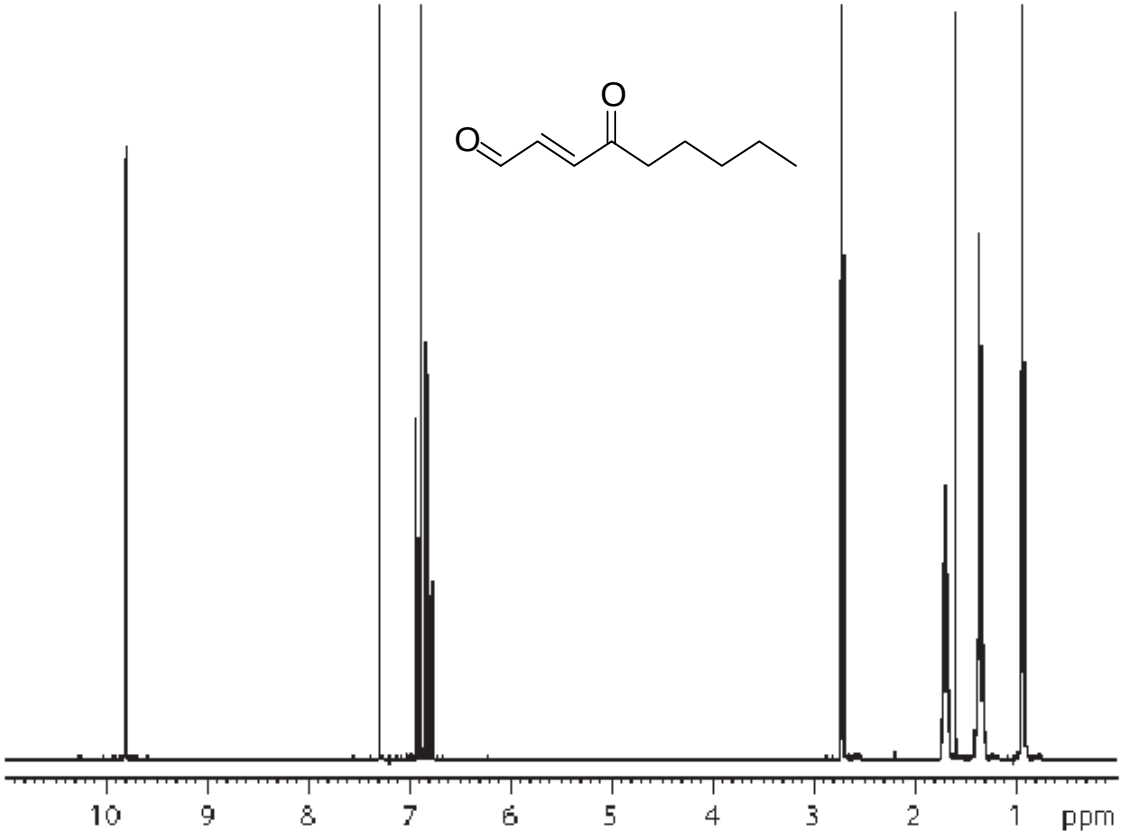

FIGURE 2.9 **400 MHz ^{1}H NMR of ONE in $CDCl_3$**

ONO was prepared by addition of 17.05 ml of phosphate buffer (0.1 M, pH 3) and 950 µl of a 50 mM sodium cyanoborohydride solution in 1 N NaOH to a stirred solution of ONE (0.01 mmol) in 1 ml of methanol. After stirring for 15 h at room temperature, the reaction mixture was extracted with ethyl acetate and the organic layer was concentrated *in vacuo* to yield ONO (Fig. 2.2, C). Data are ^{1}H 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, 3H) (Fig. 2.10).

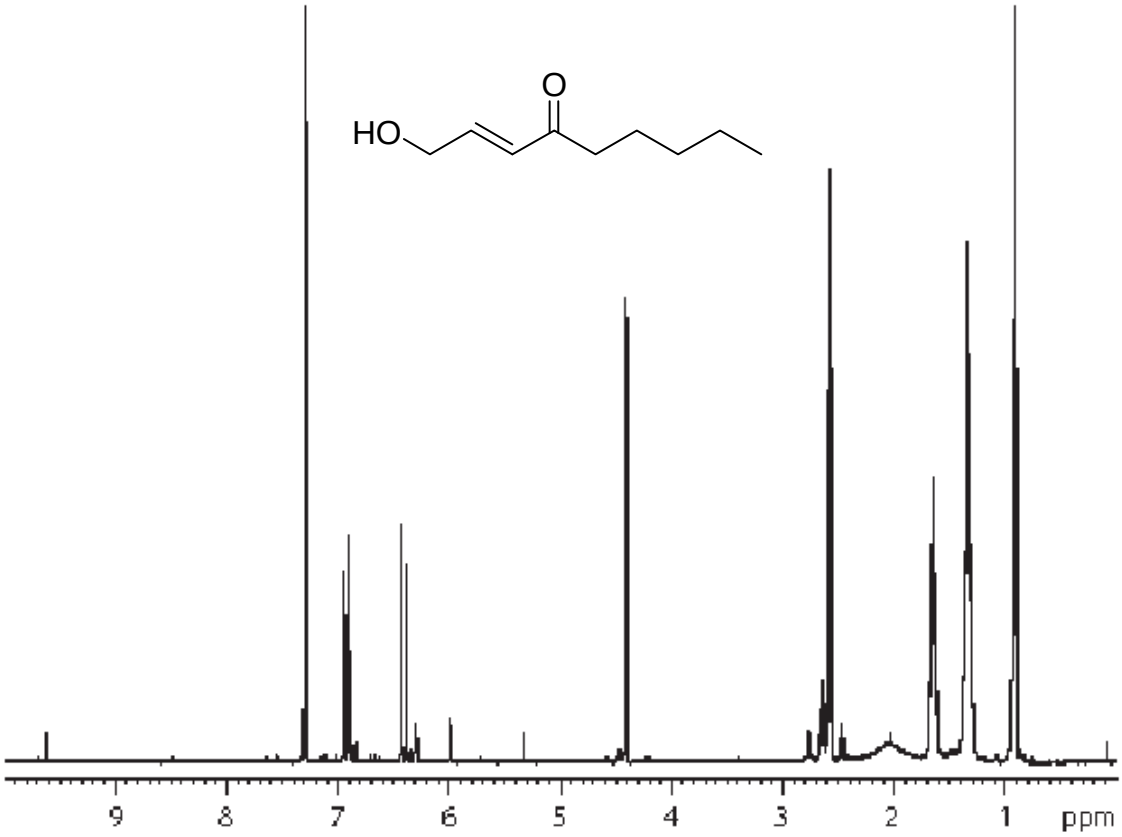

FIGURE 2.10 **300 MHz ^{1}H NMR of ONO in $CDCl_3$**

SYNTHESIS OF LPO-GSH AND LPO-CG CONJUGATES

The majority of the research discussed in this thesis relates to the LPO-MA conjugates. The work on these conjugates evolved from the study of LPO-GSH conjugates, specifically HNE-GSH. Initial synthetic attempts were made by combining 0.32 mmol GSH in 0.5 M, pH 8.2 phosphate buffer with 0.32 mmol HNE in ethanol and stirring the reaction mixture at 37 ^{o}C for 2.5 h (Fig. 2.11).

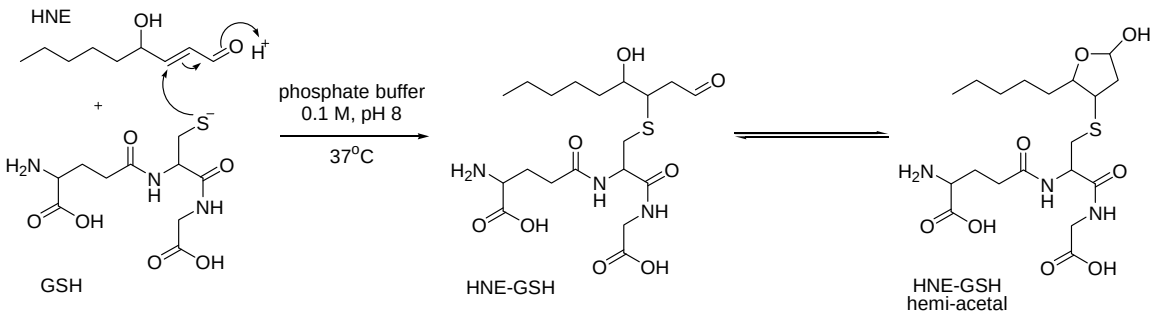

FIGURE 2.11 **Synthesis of HNE-GSH.**

The reaction mixture was then acidified to pH 3 with formic acid and purified by preparative HPLC. Purification was carried out on an Econosil C_{18} 10U 22 x 250 mm column (Alltech Associates, Deerfield, IL) with a flow rate of 11.2 ml/min. Solvent A consisted of MilliQ H_2O containing 0.1 % trifluoroacetic acid and solvent B was acetonitrile. A linear gradient from 5 % B to 100 % B was carried out over 30 min and UV detection at 215 nm was used. Fractions were collected, analyzed by LC-MS, and those found to contain HNE-GSH were lyophilized and redissolved in ethanol for use as standards. This method of purification proved to be time consuming and provided inconsistent separation results. Eventually, it was concluded that preparative HPLC did not provide satisfactory sample clean up, and a small-scale preparation was undertaken instead. Briefly, 100 µl of a 1 mM solution of LPO product in ethanol was combined with 100 µl of a 10 mM solution of GSH or CG in 0.1 M, pH 8 phosphate buffer, 400 µl of H_2O, and 400 µl of 0.1 M, pH 8 phosphate buffer. The reaction mixture was stirred at 37 ^oC for 2 h and acidified to pH 3 with 1 N HCl. Sample purification was carried out using Strata-X solid phase extraction (SPE) columns (60 mg, Phenomenex, Torrance, CA). Columns were preconditioned with 1.2 ml of acetonitrile containing 0.1 % formic acid and equilibrated with 1.2 ml of H_2O containing 0.1 % formic acid. Samples were then loaded onto the column, washed with 1.2 ml aqueous 0.1 % formic acid, and eluted with 1 ml of 1:1 acetonitrile-H_2O containing 0.1 % formic acid. Sample concentration was determined based on the assumption that the LPO product had been completely consumed in the reaction.

This procedure was utilized for the synthesis of GSH and CG conjugates with HNE, HNA, ONE, ONE, and ONA. The reactions of HNA with GSH or CG also resulted in spontaneous H_2O loss and lactone formation. The MA conjugates were synthesized in the same manner as the GSH and CG conjugates, however, extraction was used instead of SPE for sample purification. Briefly, a 20 mM solution of MA was prepared in 0.1 M phosphate buffer, pH 8. To 50 µl of this solution was added 450 µl of the same phosphate buffer and 400 µl of water. A 1 mM solution of the LPO product of interest was made up in ethanol and 100 µl was added to the MA solution. The reaction was stirred at 37 ^{o}C for 2 h and then acidified to pH 3 with 1 N HCl. It was then extracted with ethyl acetate, 3 x 1 ml, evaporated under nitrogen using a Zymark TurboVap LV (Caliper Life Sciences, Hopkinton, MA), and reconstituted in 1 ml of 2:8 acetonitrile-H_2O containing 0.1 % formic acid.

Because DHN has no α,β-unsaturation with which a Michael-type adduct can form, the DHN-GSH, DHN-CG, and DHN-MA adducts were made by first synthesizing the appropriate HNE conjugate as described above. The aldehyde moiety was then reduced with 10 µl of a 5 M sodium borohydride solution in 1 N NaOH (13) (Fig. 2.12). The reduction mixture was stirred at room temperature for 30 min then acidified to pH 3 with 1 N HCl. Sample purification was carried out using SPE for the DHN-GSH and DHN-CG or extraction for DHN-MA.

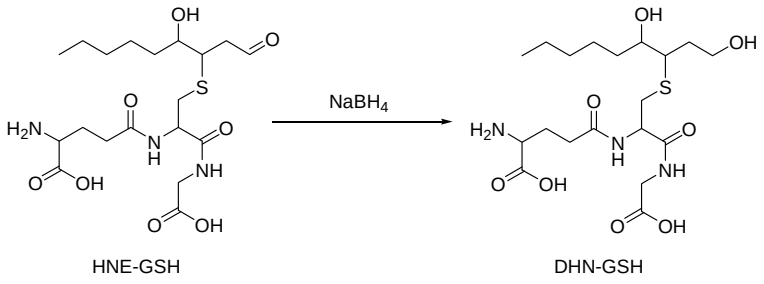

FIGURE 2.12 **Reduction of HNE-GSH to DHN-GSH.**

These conjugates were then analyzed by LC-MS/MS. A Shimadzu Prominence HPLC system (Shimadzu, Columbia, MD) consisting of four LC-20AD pumps, a DQU-

$20A_5$ degasser, and an SIL-HTc autosampler equipped with switching valves was used for the chromatography. The HPLC column was a 250 x 2 mm Synergi Max RP C_{12} column (Phenomenex, Torrance, CA). The mobile phase consisted of Solvent A, 0.1 % (v/v) formic acid in water, and Solvent B, acetonitrile containing 0.1 % (v/v) formic acid. The flow rate was 0.2 ml/min. Separations were carried out using a linear solvent gradient from 20 to 50 % B in 10 min, a linear gradient from 50 to 90 % B over the next 2 min, held constant at 90 % B for 7 min, returned to 20 % B after 1 min, and equilibrated at 20 % B for 5 min. This method was later adapted to include the conjugates of HNE with ascorbic acid and used for THP-1 cell analyses (12). The MS and MS/MS analyses (Fig. 2.13-2.24) were carried out using an Applied Biosystems MDS Sciex hybrid triple quadrupole/linear ion trap mass spectrometer (4000 QTrap) equipped with a TurboV electrospray source (Concord, Canada). The TurboV source was maintained at 400 ^{o}C. The ion-spray voltage was -4500 V and the declustering potential was 40 V. Nitrogen was used as the source gas, curtain gas, and collision gas. Analyses were performed in negative ion mode, and the SRM transitions, collision energies, and retention times shown in Table 2.1.

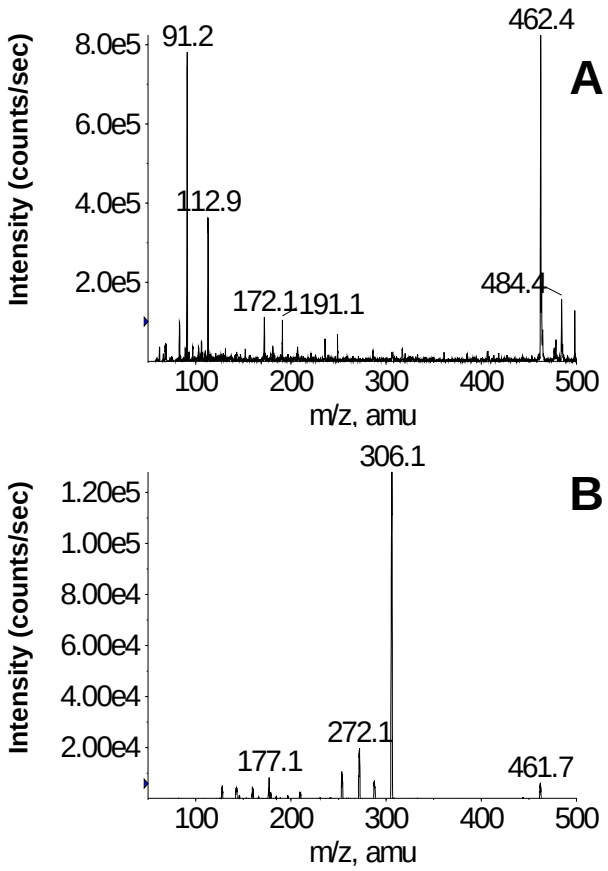

FIGURE 2.13 **Mass Spectra for HNE-GSH.** (A) Q1 spectrum (B) MS/MS spectrum of *m/z* 462.

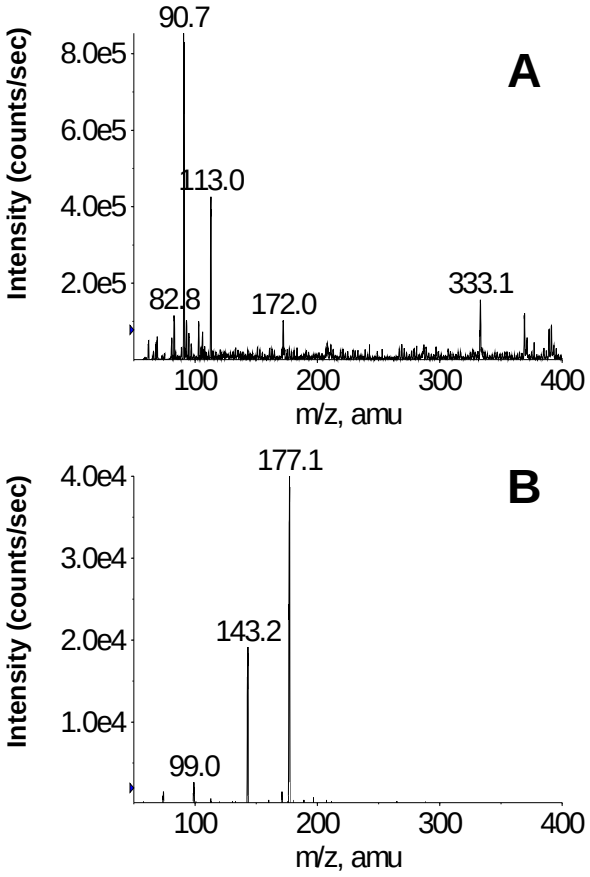

FIGURE 2.14 **Mass Spectra for HNE-CG.** (A) Q1 spectrum (B) MS/MS spectrum of *m/z* 333.

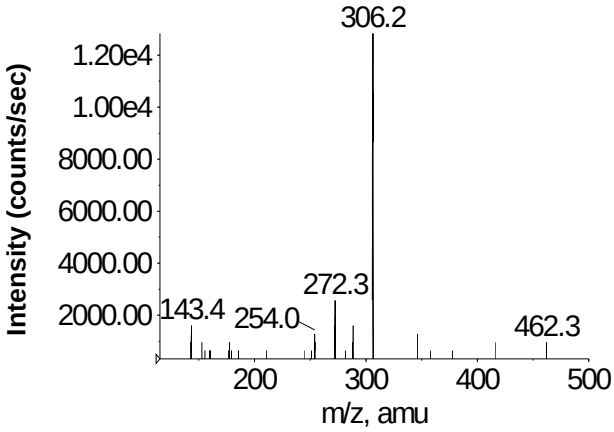

FIGURE 2.15 **Mass Spectrum for ONO-GSH.** MS/MS spectrum of *m/z* 462.

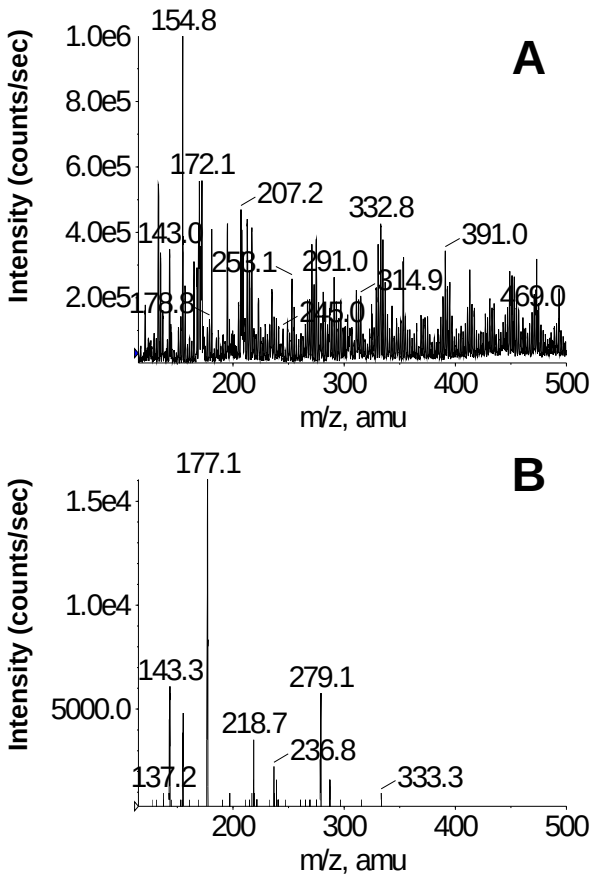

FIGURE 2.16 **Mass Spectra for ONO-CG.** (A) Q1 spectrum (B) MS/MS spectrum of *m/z* 333.

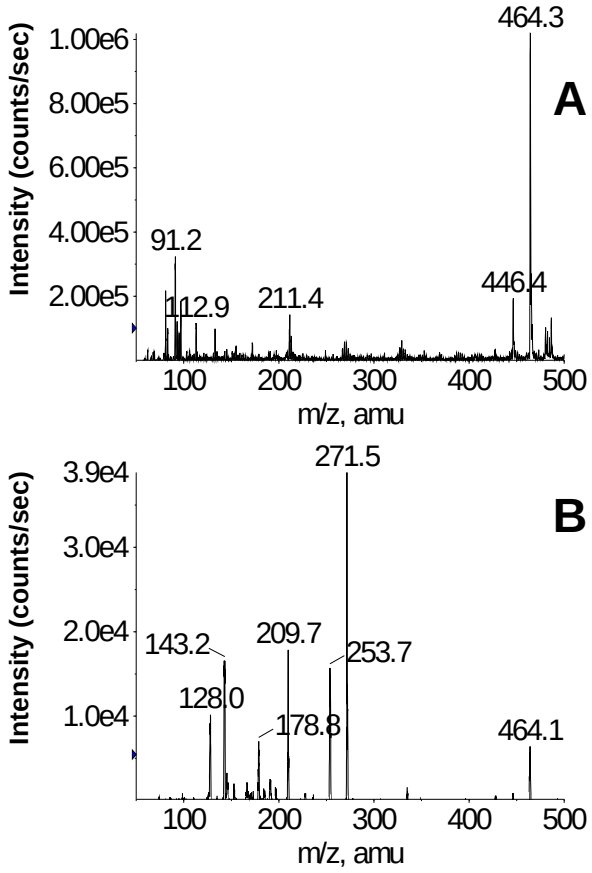

FIGURE 2.17 **Mass Spectra for DHN-GSH.** (A) Q1 spectrum (B) MS/MS spectrum of *m/z* 464.

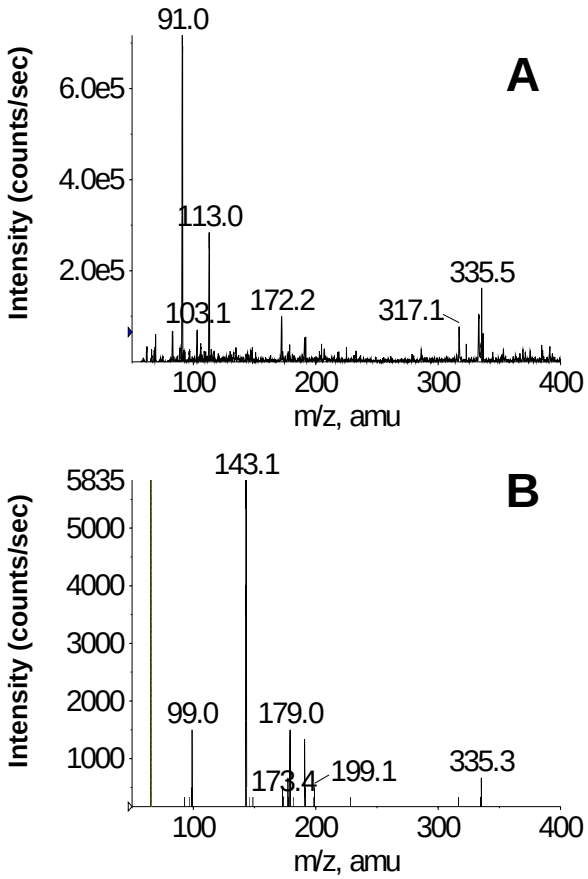

FIGURE 2.18 **Mass Spectra for DHN-CG.** (A) Q1 spectrum (B) MS/MS spectrum of *m/z* 335.

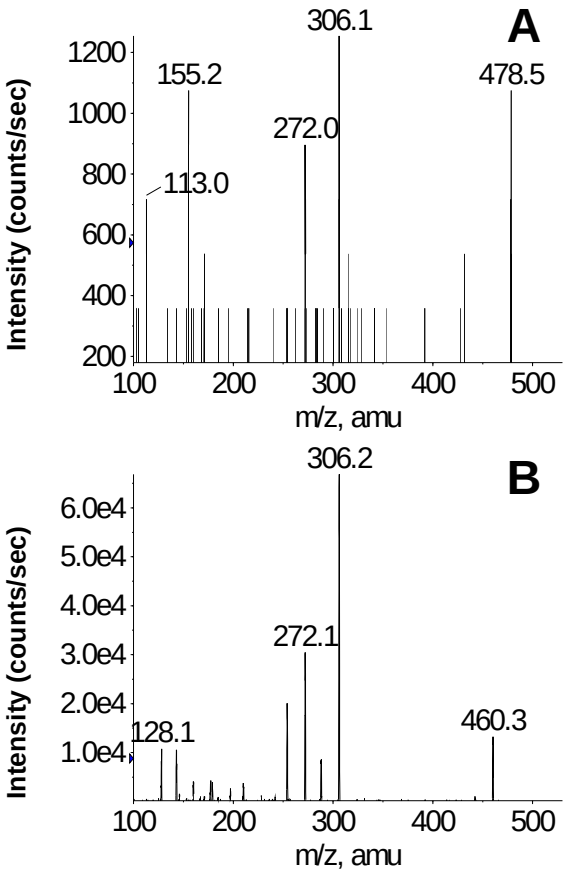

FIGURE 2.19 **Mass Spectra for HNA-GSH and HNAL-GSH.** (A) MS/MS spectrum of *m/z* 478 (B) MS/MS spectrum of *m/z* 460.

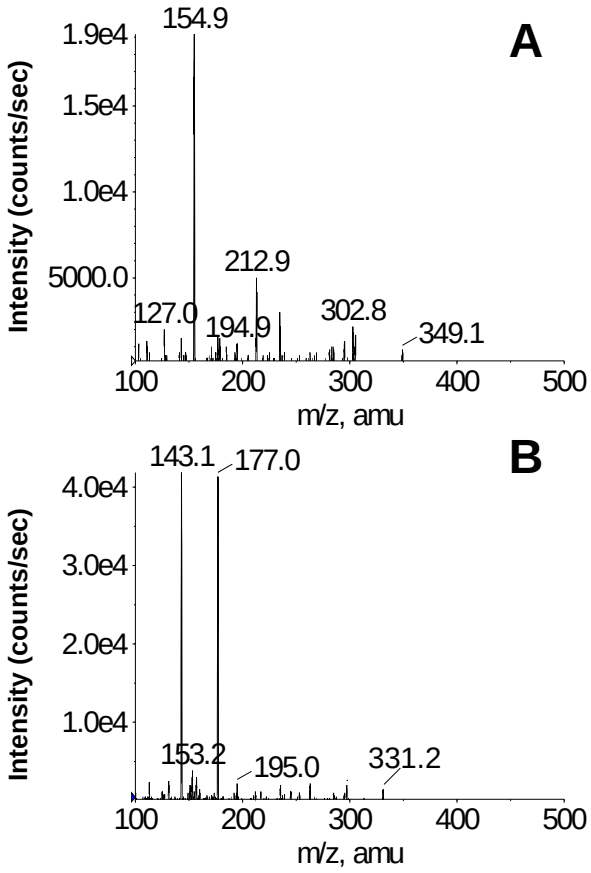

FIGURE 2.20 **Mass Spectra for HNA-CG and HNAL-CG.** (A) MS/MS spectrum of *m/z* 349 (B) MS/MS spectrum of *m/z* 331.

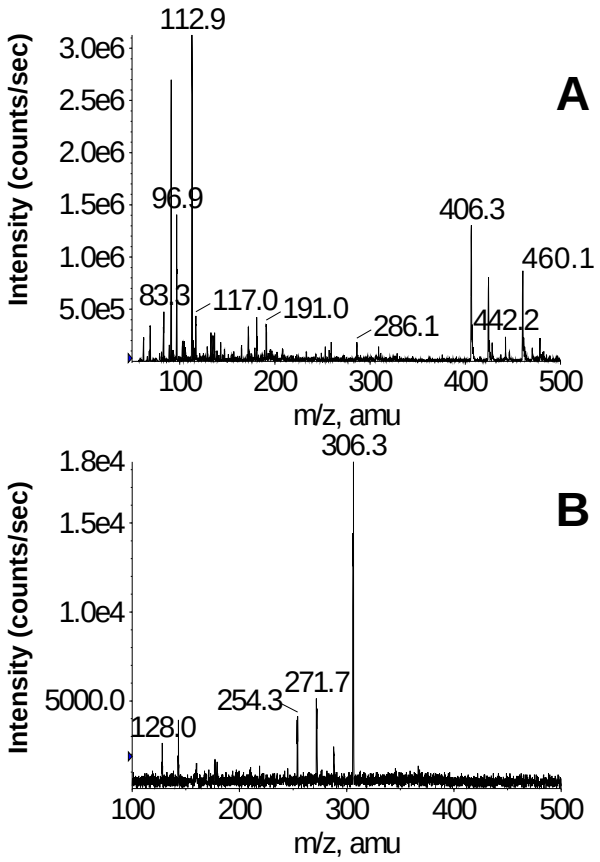

FIGURE 2.21 **Mass Spectra for ONE-GSH.** (A) Q1 spectrum (B) MS/MS spectrum of *m/z* 460.

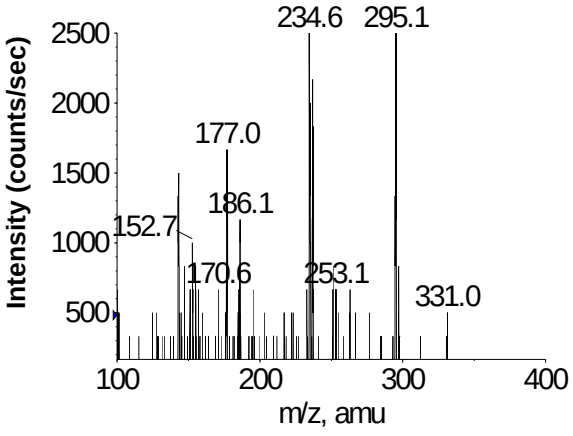

FIGURE 2.22 **Mass Spectrum for ONE-CG.** MS/MS spectrum of *m/z* 331.

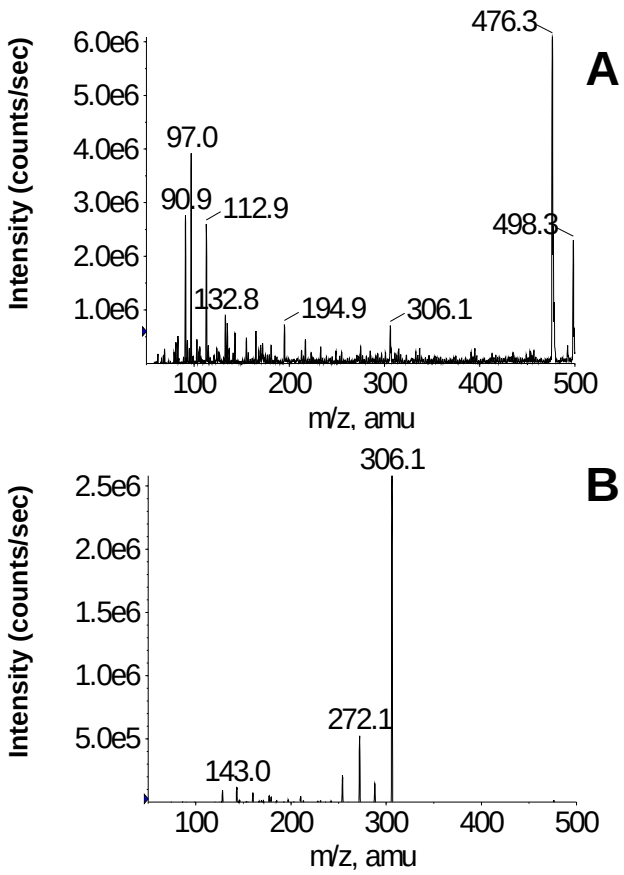

FIGURE 2.23 **Mass Spectra for ONA-GSH.** (A) Q1 spectrum (B) MS/MS spectrum of *m/z* 476.

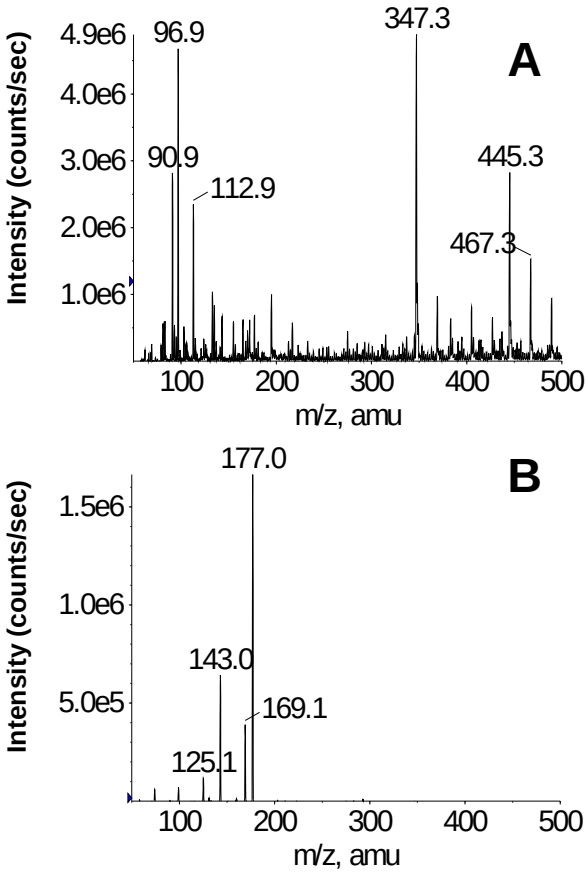

FIGURE 2.24 **Mass Spectra for ONA-CG.** (A) Q1 spectrum (B) MS/MS spectrum of *m/z* 347.

Table 2.1 LC-MS/MS properties of LPO-GSH and LPO-CG metabolites.

Analyte	MW	SRM transition	Collision energy (eV)	Retention time (min.)
HNE-GSH and ONO-GSH	463	462 → 306	25	6.5, 6.7, 7.0
		462 → 272	25	7.2, 7.5
		462 → 143	25	
HNE-CG and ONO-CG	334	333 → 177	25	5.5
		333 → 143	25	6.0, 6.3
DHN-GSH	465	464 → 272	30	6.4, 6.6
		464 → 143	30	
DHN-CG	336	335 → 179	25	5.8
		335 → 143	25	
HNA-GSH	479	478 → 306	25	7.5, 7.8
		478 → 272	25	
HNAL-GSH	461	460 → 306	25	8.8, 9.0, 9.2
		460 → 272	25	
HNA-CG	350	349 → 213	25	7.9, 8.1
		349 → 155	25	
HNAL-CG	332	331 → 177	25	7.8
		331 → 143	25	
ONE-GSH	461	460 → 306	25	8.7
		460 → 272	25	
ONE-CG	332	331 → 177	25	7.7
		331 → 162	25	
ONA-GSH	477	476 → 306	25	7.8, 8.0
		476 → 272	25	
ONA-CG	348	347 → 177	25	6.7, 6.9
		347 → 169	25	
		347 → 143	25	

IDENTIFICATION OF AN APPROPRIATE INTERNAL STANDARD

Several attempts were made to identify an internal standard that was suitable for use with GSH, CysGly, and MA conjugates with LPO products. Octenal-GSH was the first internal standard used in these experiments (Fig. 2.25).

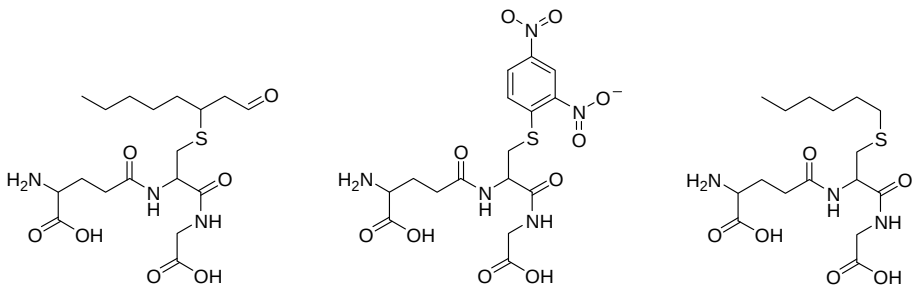

FIGURE 2.25 **Internal standards.**

It was prepared by reacting 0.32 mmol of octenal with 0.325 mmol GSH in 0.1 M, pH 7.4 phosphate buffer and 2 ml of acetonitrile. The reaction was stirred at 37 ^{o}C for 24 h and then acidified to pH 3 with formic acid. Purification was carried out by preparative HPLC as described above for HNE-GSH. Utilization of octenal-GSH for plasma preparation gave inconsistent results however, and it was concluded that octenal-GSH did not behave in a similar enough manner to HNE-GSH to be useful for our analyses. The next internal standard we considered was S-(2,4)-dinitrobenzene (CDNB)-GSH (Fig. 2.25). This standard was obtained by reacting 10 mg of GSH and 6.6 mg CDNB in 500 µl of 0.1 M, pH 7.4 phosphate buffer for 40 min at 37 ^{o}C. Upon biological sample preparation, the suitability of CDNB-GSH as an internal standard was called into question. Another potential internal standard, S-hexyl-GSH was analyzed (Fig. 2.25), and a comparison of calibration curves and plasma samples was carried out using CDNB-GSH and S-hexyl-GSH. From this comparative analysis, it was determined that S-hexyl-GSH behaved most similarly to HNE-GSH and was the most suitable internal standard.

Shortly after determining that S-hexyl-GSH was an appropriate internal standard for HNE-GSH analyses, we decided to add DHN-GSH, and the CG and MA conjugates of both HNE and DHN to our repertoire. DHN, being a diol has no α,β-unsaturation by which to react with GSH, CG, or MA. Therefore, it is necessary to synthesize HNE conjugates and reduce the aldehyde with $NaBH_4$ to obtain the DHN conjugates. This reduction step provides the advantage that $NaBD_4$ can be used instead of $NaBH_4$ resulting in the addition of a deuterium label to the DHN conjugates at the C-1 position (Fig. 2.26).

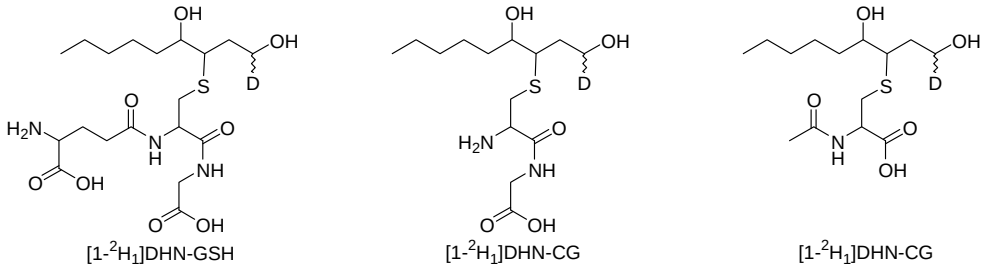

FIGURE 2.26 **Deuterium labeled DHN-GSH, DHN-CG, and DHN-MA for use as internal standards.**

This labeled material is an even better internal standard than the S-hexyl-GSH, since it has the same properties as the DHN conjugates, and behaves the same way during sample preparation, chromatography, and ionization. The deuterium label also provided appropriate internal standards for each of the GSH, CG, and MA conjugates, whereas S-hexyl-GSH worked well only as an internal standard for the GSH conjugates. This realization led to the search for other methods by which to incorporate deuterium labeling into our internal standards. The next step in the development of internal standards was to prepare [9-$^2H_3$]HNE and [9-$^2H_3$]DHN adducts using [9-$^2H_3$]HNE purchased from Cayman Chemical (14). This provided us with internal standards for six of our LPO product metabolites (Fig. 2.27). The [9-$^2H_3$]HNE-MA was also utilized as the internal standard for the semi-quantitative work described in Chapter 3.

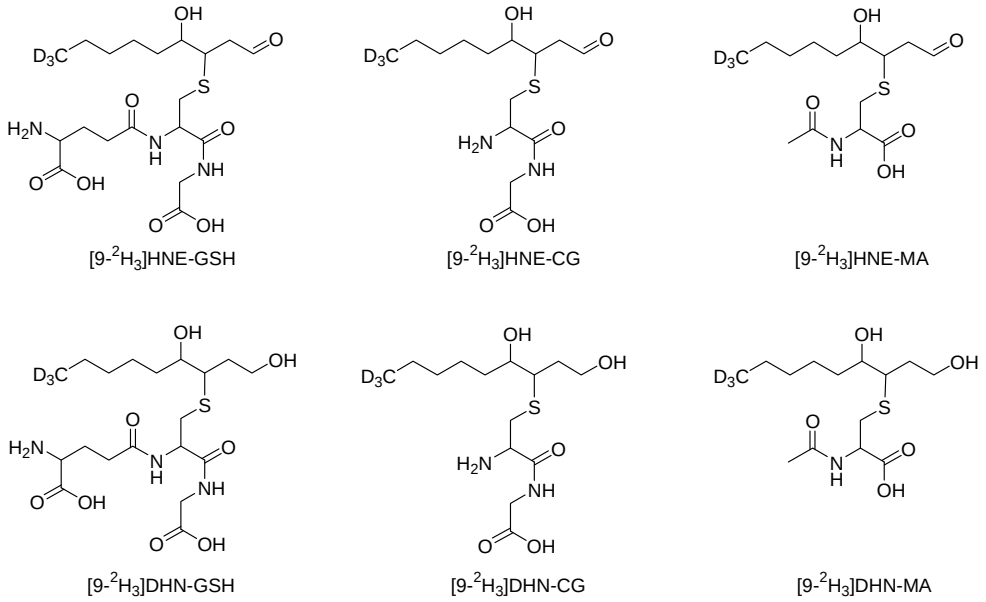

FIGURE 2.27 **Deuterium labeled HNE and DHN metabolites for use as internal standards.**

These internal standards were promising, but could not be easily adapted to our expanding pool of LPO product metabolites. We became interested in the oxidation product of HNE, HNA, as well as ONE and its phase I metabolites as we were conducting the research described in Chapter 3. Therefore, it became necessary to have labeled internal standards for each metabolite in order to perform absolute quantitation. Our focus also shifted to the urinary metabolite MA conjugates at this time, leading us to investigate methods by which to label the MA portion of the conjugate. A convenient method for synthesis of *N*-(acetyl-d_3)-L-cysteine (MAd_3) was described by Slatter *et al.* (15) (Fig. 2.28).

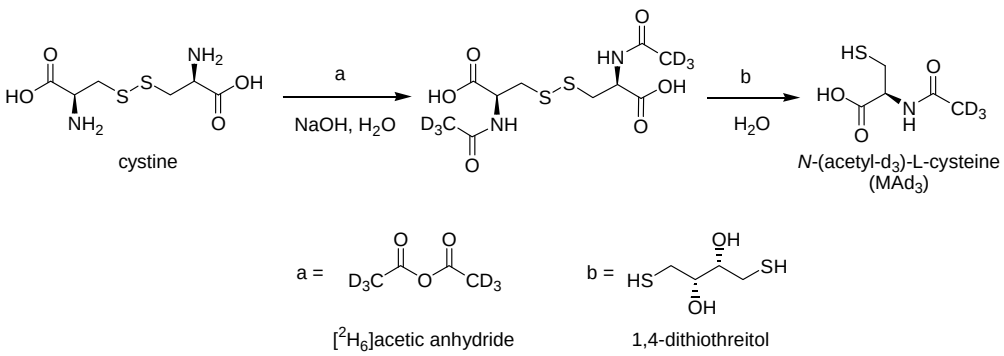

FIGURE 2.28 **Synthetic route to *N*-(acetyl-d_3)-L-cysteine.**

Briefly, cystine (5.3 mmol) was added to 13 ml of a 1.5 M NaOH solution and the mixture was cooled in an ice bath with stirring. [$^2H_6$]Acetic anhydride (10.6 mmol) was added dropwise over 20 min, the ice bath was removed, and the reaction continued stirring at room temperature for 1 h. 1,4-Dithiothreitol (10.6 mmol) was added and the reaction continued stirring at room temperature for 1 h, after which it was concentrated *in vacuo*, washed with ether, frozen, and lyophilized. The crystals were purified on a 52 x 2.5 cm Sephadex LH-20 column using methanol as the eluting solvent. The MAd_3 was further purified by acidification to pH 3 with 1 M HCl, then extracted with ethyl acetate. The organic layer was concentrated *in vacuo*. MAd_3 was used in place of MA for LPO-MA adduct formation (14) for use as internal standards. This material provided us with convenient internal standards for use with each of our LPO products of interest and is further utilized in the research described in Chapter 4. The LC-MS/MS properties of all internal standards tested are shown in Table 2.2.

Table 2.2 LC-MS/MS properties of internal standards.

Analyte	MW	SRM transition	Mode	Collision energy (eV)	Retention time (min.)
Octenal-GSH	433	434 → 287	Positive	25	7.5
		434 → 306		25	
		418 → 272		25	
CDNB-GSH	473	474 → 457	Positive	25	6.9
		474 → 345		25	
S-hexyl-GSH	391	392 → 317	Positive	25	9.7
		392 → 246		25	
		392 → 160		25	
[1-$^2H_1$]DHN-GSH	466	465 → 306	Negative	25	6.2, 6.4, 6.5
		465 → 272		25	
		465 → 143		25	
[1-$^2H_1$]DHN-CG	337	336 → 177	Negative	25	5.8
		336 → 143		25	
[1-$^2H_1$]DHN-MA	322	321 → 192	Negative	25	9.5
		321 → 174		25	
		321 → 143		25	
[9-$^2H_3$]HNE-GSH	466	465 → 306	Negative	25	6.4, 6.6, 6.9
		465 → 272		25	
[9-$^2H_3$]HNE-CG	337	336 → 177	Negative	25	5.4, 5.6
		336 → 143		25	
[9-$^2H_3$]HNE-MA	322	321 → 192	Negative	25	10.1, 10.4
		321 → 174		25	
		321 → 143		25	
[9-$^2H_3$]DHN-GSH	468	467 → 272	Negative	30	6.2, 6.4, 6.6
		467 → 143		30	
[9-$^2H_3$]DHN-CG	339	338 → 179	Negative	30	5.9
		338 → 143		30	
[9-$^2H_3$]DHN-MA	324	323 → 194	Negative	30	9.5
		323 → 143		30	

PLASMA SAMPLE PREPARATION

Initial attempts at plasma preparation were made following the procedure of Völkel *et al.* (16), precipitating out the proteins using an equal volume of ice-cold ethanol

followed by centrifugation. The supernatant was then removed for analysis. We found that this method produced inconsistent results by LC-MS/MS analysis, so ultrafiltration was employed as another means by which to remove proteins from the sample, but no improvements were seen. We also tried solid phase extraction (SPE) as a sample purification method, but found that the results were no better than those obtained by simple protein precipitation. Due to the added labor and expense involved with SPE, this method was abandoned for biological samples. SPE continues to be utilized for purification of LPO-GSH and LPO-CG synthetic standards. Protein precipitation with acetonitrile containing 0.1 % formic acid was also explored and is the most reliable method we have found for plasma preparation of GSH and CG conjugates. The limitation of this method is that samples are diluted in the process, making it more difficult to detect metabolites present at low concentrations. Extraction with ethyl acetate has been the most effective plasma preparation technique and allows for sample concentration. This method only allows analysis of the LPO-MA conjugates, however, because the LPO-GSH and LPO-CG conjugates remain in the water layer. Under acidic conditions, the amino group in GSH and CG carries a charge. The charge causes GSH and CG conjugates to be soluble in water, whereas MA is not charged at low pH and its conjugates can be extracted with organic solvent. Interestingly, only HNE-MA and DHN-MA are found in plasma after extraction.

DERIVATIZATION ATTEMPTS TO DISTINGUISH BETWEEN ISOMERS HNE-MA AND ONO-MA

In a recent review (17), Ian Blair stated that "HNE and ONO are isomeric. Therefore, all studies, which have assumed endogenous HNE is responsible for modifying proteins and GSH, will need to be re-evaluated to determine whether ONO also plays a role in causing such modifications. It is also likely that ONO may contribute to the biological activities that have been ascribed previously to HNE." Initial chromatographic attempts to separate isomeric HNE-MA and ONO-MA proved unsuccessful. HNE-MA and ONO-MA are both present as multiple diastereomers in biological samples, making it difficult to achieve baseline separation. Selective reduction of the HNE-MA metabolite to DHN-MA was attempted as a means to distinguish

between them. This was done by reduction of synthetic HNE-MA and ONO-MA with $NaCNBD_3$ (Fig. 2.29).

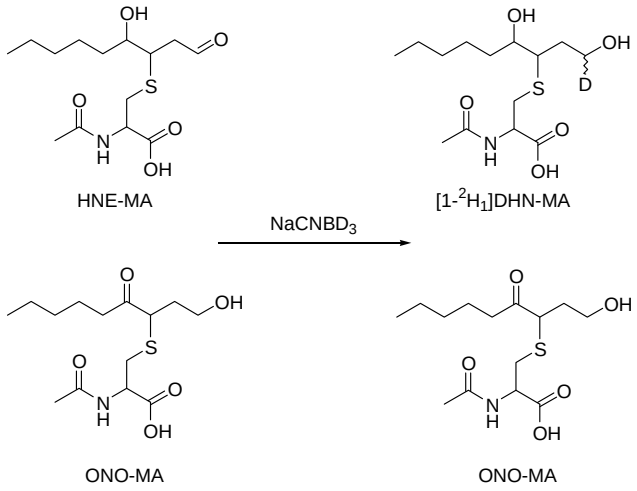

FIGURE 2.29 **Selective reduction of HNE-MA to [1-$^2H_1$]DHN-MA.** The ketone of ONO-MA will not be reduced by $NaCNBD_3$.

A 1 μM solution (1 ml in 0.1 M, pH 3 phosphate buffer) containing HNE-MA or ONO-MA was reduced with 10 μl of 5 M $NaCNBD_3$ in 30 min at room temperature. Samples were then acidified to pH 3 with 1 N HCl, extracted with ethyl acetate (3 x 1 ml), evaporated under N_2, and reconstituted in 2:8 acetonitrile-H_2O containing 0.1 % formic acid for LC-MS/MS analysis. Urine samples were prepared in a similar manner, beginning with the addition of [9-$^2H_3$]HNE-MA and [9-$^2H_3$]DHN-MA as internal standards. The urine was then acidified to pH 3 with 1 N HCl, extracted with ethyl acetate (3 x 1 ml), evaporated under N_2, dissolved in 0.1 M, pH 3 phosphate buffer, and reduced with 10 μl of 5 M $NaCNBD_3$ in 30 min at room temperature. Samples were then acidified to pH 3 with 1 N HCl, extracted with ethyl acetate (3 x 1 ml), evaporated under N_2, and reconstituted in 2:8 acetonitrile-H_2O containing 0.1 % formic acid for LC-MS/MS analysis. The reduction process was effective in synthetic standards, reducing HNE-MA to [1-$^2H_1$]DHN-MA while not affecting the ONO-MA. However, in urine samples, the selective reduction proved more difficult. Even with extraction prior to sample reduction, it was not possible to reduce the HNE-MA without also reducing the

ONO-MA. Other compounds in the urine made it necessary to add too great an excess of $NaCNBD_3$ to achieve selective reduction.

Derivatization methods were also attempted to separate the compounds. Oximes were synthesized by reacting HNE-MA or ONO-MA, 100 μM in acetonitrile, with hydroxylamine (2 mg) and O-methyl hydroxylamine (2.5 mg) in methanol (Fig. 2.30).

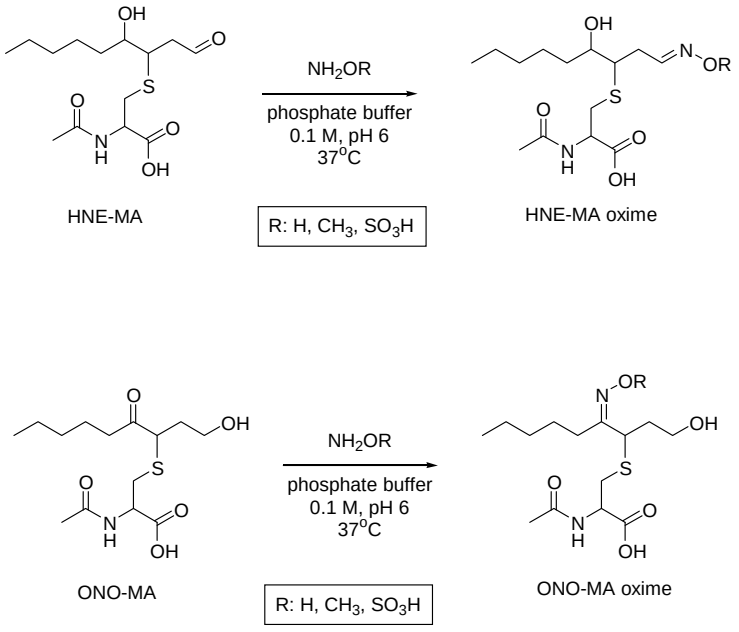

FIGURE 2.30 **Derivatization of HNE-MA and ONO-MA to form oximes**. The derivatization is a means by which to differentiate between these isomeric metabolites.

The reaction was stirred in 0.1 M, pH 6 phosphate buffer at 37 ^{o}C for 1 h. Samples were then acidified to pH 3 with 1 N HCl, extracted with ethyl acetate (3 x 1 ml), evaporated under N_2, and redissolved in 2:8 acetonitrile-H_2O containing 0.1 % formic acid for LC-MS/MS analysis. The goal of these derivatizations was to obtain enough structural difference between the HNE-MA oxime and the ONO-MA oxime that we could distinguish between the two by LC-MS/MS analyses. Unfortunately, the differences in structure were not great enough to allow for chromatographic separation. MS/MS analyses also demonstrated that there were no differences in fragmentation pattern. Further derivatizations were attempted by the same method, using hydroxylamine-O-sulfonic acid as the derivatizing agent. The goal of these experiments was to alter the

chromatography or fragmentation with a larger derivatizing group; however, we were still unable to differentiate between isobaric HNE-MA and ONO-MA using this method.

It was eventually determined that biological samples contained a peak at 9.7 min which did not correspond to the synthetic HNE-MA. Further analysis of this peak as discussed in Chapter 3, led to the first *in vivo* detection of ONO-MA.

FREE LIPID PEROXIDATION PRODUCTS

In order to have a complete understanding of HNE and ONE metabolism, it is necessary to analyze both free and conjugated LPO products. Ideally, all products would be measured simultaneously; however, a method for this analysis does not currently exist. It is possible to analyze HNA, ONA (Table 2.3), and all LPO-MA conjugates in the same LC-MS/MS run. HNE, ONO, ONE, and DHN are not readily ionizable and require derivatization prior to analysis and must therefore be analyzed separately. It is also necessary to utilize multiple derivatization techniques for these LPO products since ONE contains an aldehyde and a ketone, while HNE and ONO both contain an alcohol and a carbonyl, and DHN contains only alcohols.

Table 2.3 LC-MS/MS properties of free LPO products.

Analyte	MW	SRM transition	Collision energy (eV)	Retention time (min.)
ONA	170	169 → 125	25	14.9
		169 → 71	25	
HNA	172	171 → 127	25	12.6
		171 → 125	25	
		171 → 99	25	
ONE di(methyloxime)	212	213 → 151	25	17.5
		213 → 139	25	
		213 → 125	25	
HNE methyloxime	185	186 → 99	25	15.8, 15.9
		186 → 84	25	
		186 → 71	25	
ONO methyloxime	185	186 → 126	25	15.6, 15.7
		186 → 112	25	
		186 → 99	25	
DHN *N,N*-dimethyl glycine di-ester	330	331 → 228	25	2.9
		331 → 104	25	
DHN N,N-dimethyl glycine mono-ester	245	245 → 104	25	4.2
		245 → 58	25	

Oxime Formation

Utilizing the same oxime chemistry that had been attempted for distinction between HNE-MA and ONO-MA provided a convenient way to make free ONE, HNE, and ONO ionizable for LC-MS/MS analysis (Fig. 2.31, Table 2.3). The methyl-oximes are made by combining 50 μL of 10 mM NH_2OCH_3 in MeOH, 100 μL of 1 mM ONO, ONE, or HNE in EtOH, and 850 μL of 0.1 M phosphate buffer, pH 6 and stirring at 37 ^oC for 1 h. The samples are then acidified to pH 3 with 1 N HCl and analyzed by LC-MS/MS. It is important to note that ONE derivatization will result in the formation of the mono and dioximes. Both the syn and anti isomers are formed; however, using our HPLC method, these isomers coelute and can therefore be analyzed simultaneously.

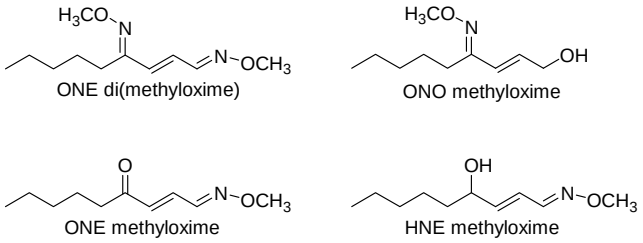

FIGURE 2.31 **ONE, ONO, and HNE methyloxime derivatives.**

Synthesis and Derivatization of DHN

The analysis of free DHN first required the synthesis of DHN (Fig. 2.32).

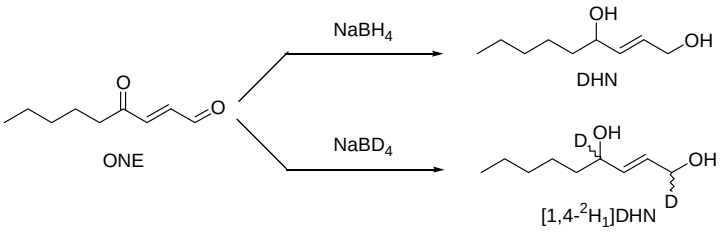

FIGURE 2.32 **Synthetic routes to free DHN and labeled internal standard [1,4-$^2H_1$]DHN.**

To 250 μmol of ONE in 1 ml MeOH, was added 2 mmol $NaBH_4$ or $NaBD_4$ in 0.5 mL 1.3 N NaOH and 0.5 ml H_2O. Five milliliters of 0.1 M, pH 8 phosphate buffer and 1 ml of H_2O were added and the mixture was stirred at room temperature for 2 h. The reaction mixture was acidified to pH 3 with 1 N HCl and extracted with ethyl acetate. The organic layers were dried with $MgSO_4$, filtered, and dried on a rotary evaporator. Data for DHN are ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.83 (dt, J = 5, 16 Hz, 1H), 5.73 (dd, J = 6, 16 Hz, 1H), 4.15-4.09 (m, 3H), 1.52-1.41 (m, 8H), 0.91 (t, J = 6 Hz, 3H) (Fig. 2.33). Data for [1,4-$^2H_1$]DHN are ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.79 (dd, J = 5, 16 Hz, 1H), 5.69 (d, J = 16 Hz, 1H), 4.11-4.07 (m, 1H), 1.54-1.25 (m, 8H), 0.90 (t, J = 7, 3H) (Fig. 2.34).

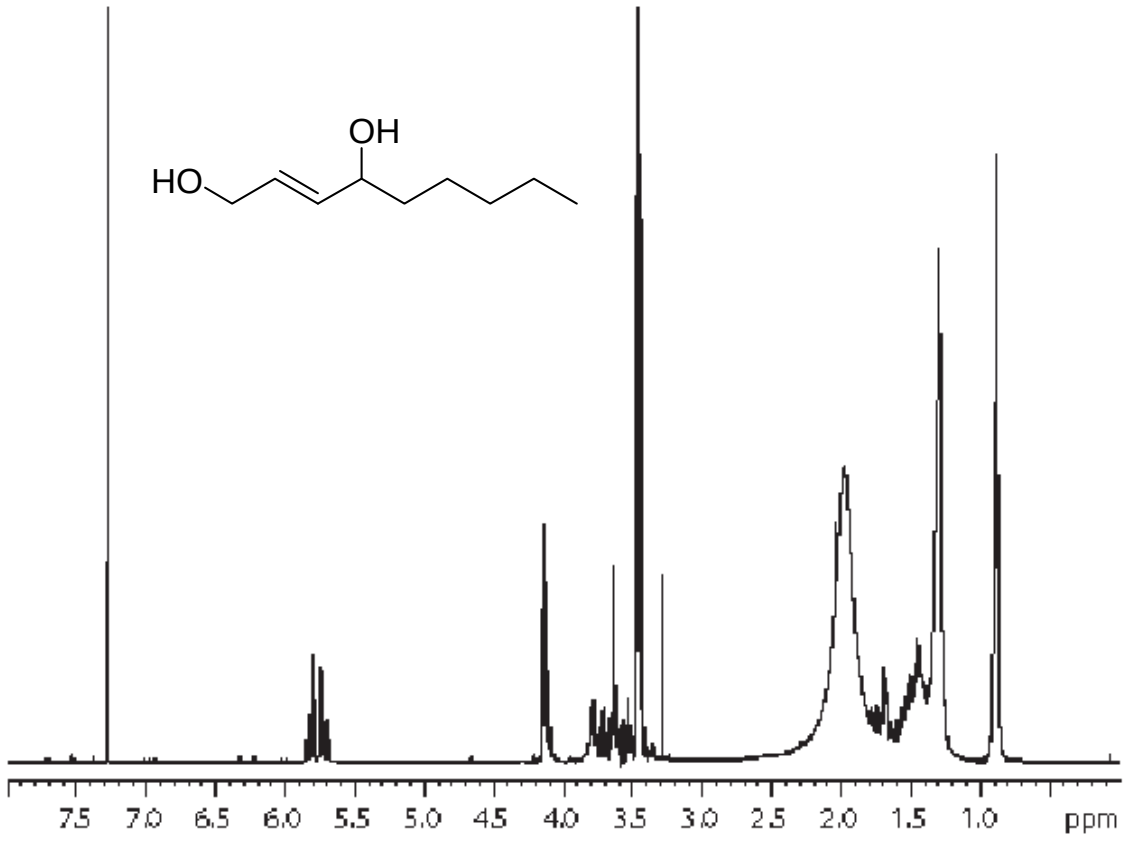

FIGURE 2.33 **400 MHz ^{1}H NMR of DHN in $CDCl_3$**

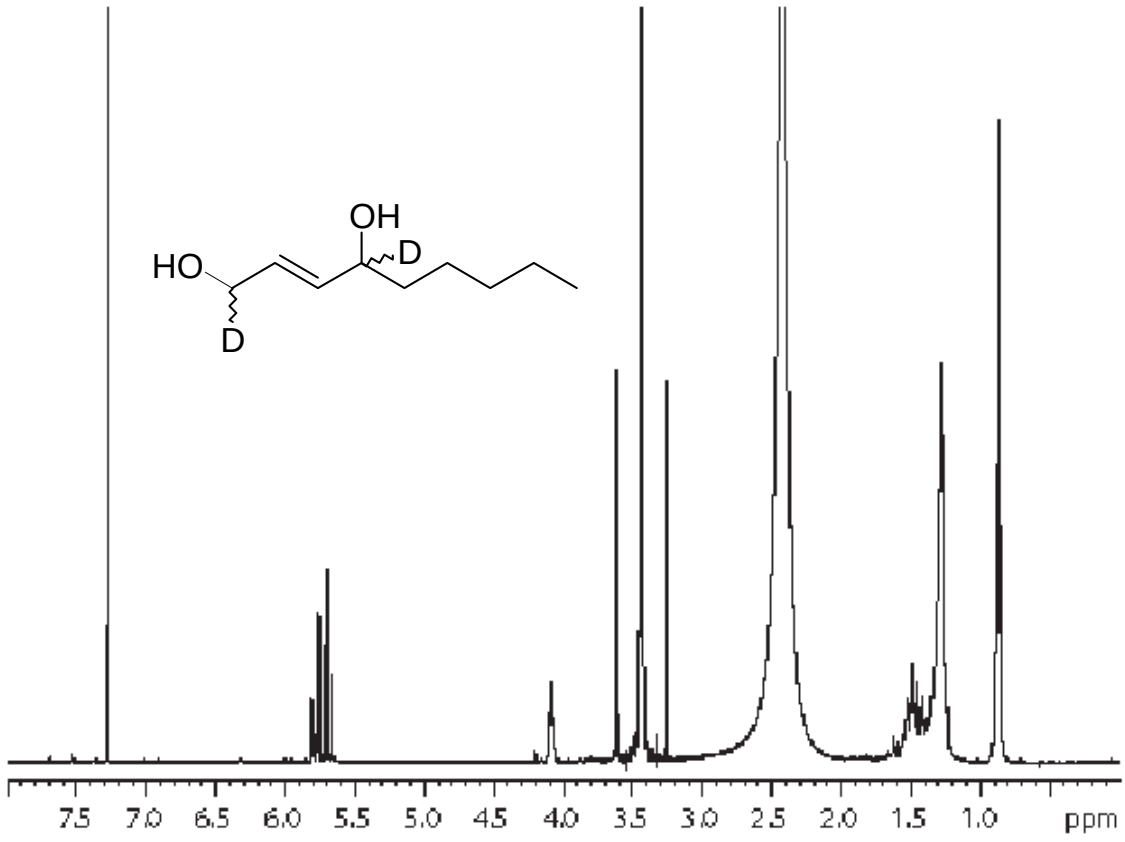

FIGURE 2.34 **400 MHz ^{1}H NMR of [1,4-$^{2}H_1$]DHN in $CDCl_3$**

Derivatization was carried out by preparation of the dimethylglycine ester of DHN (Fig. 2.35). Dimethylgycine (0.1 mmol), and 1,1'-carbonyl diimidazole (0.1 mmol) were combined in CH_2Cl_2 and stirred at room temperature overnight, following the method of Johnson (18). The crude imidazolide (200 µl) was reacted with 100 µl of a 1 mM DHN or [1,4-$^{2}H_1$]DHN solution in DCM, along with 1 µl of triethylamine. The reaction mixture was stirred at 65^{o}C for 10 min, evaporated under N_2, and dissolved in 1:1 acetonitrile-H_2O containing 0.1% formic acid for LC-MS analysis (Table 2.3).

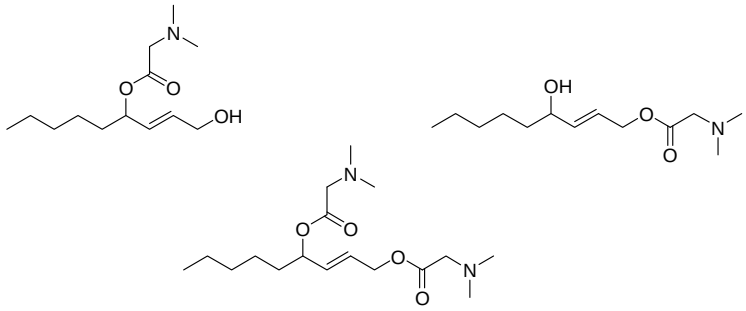

FIGURE 2.35 ***N,N*-dimethylglycine esters of DHN.**

Synthesis of 1,4-Nonanediol

γ-Nonalactone (10 mmol) was reduced with $LiAlH_4$ (11 mmol) in dry ether to yield 1,4-nonanediol (19) (Fig. 2.36). Data are ^1H NMR (300 MHz, $CDCl_3$) δ 3.69-3.59 (m, 2H), 3.28 (m, 1H), 1.63-1.58 (m, 2H), 1.49-1.43 (m, 4H), 1.30-1.28 (m, 6H), 0.89-0.87 (m, 3H) (Fig. 2.37).

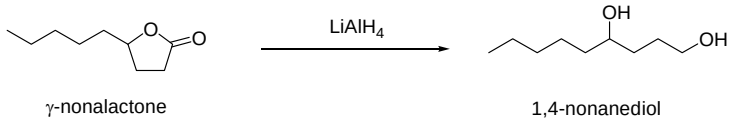

FIGURE 2.36 **Synthetic route to 1,4-nonanediol.**

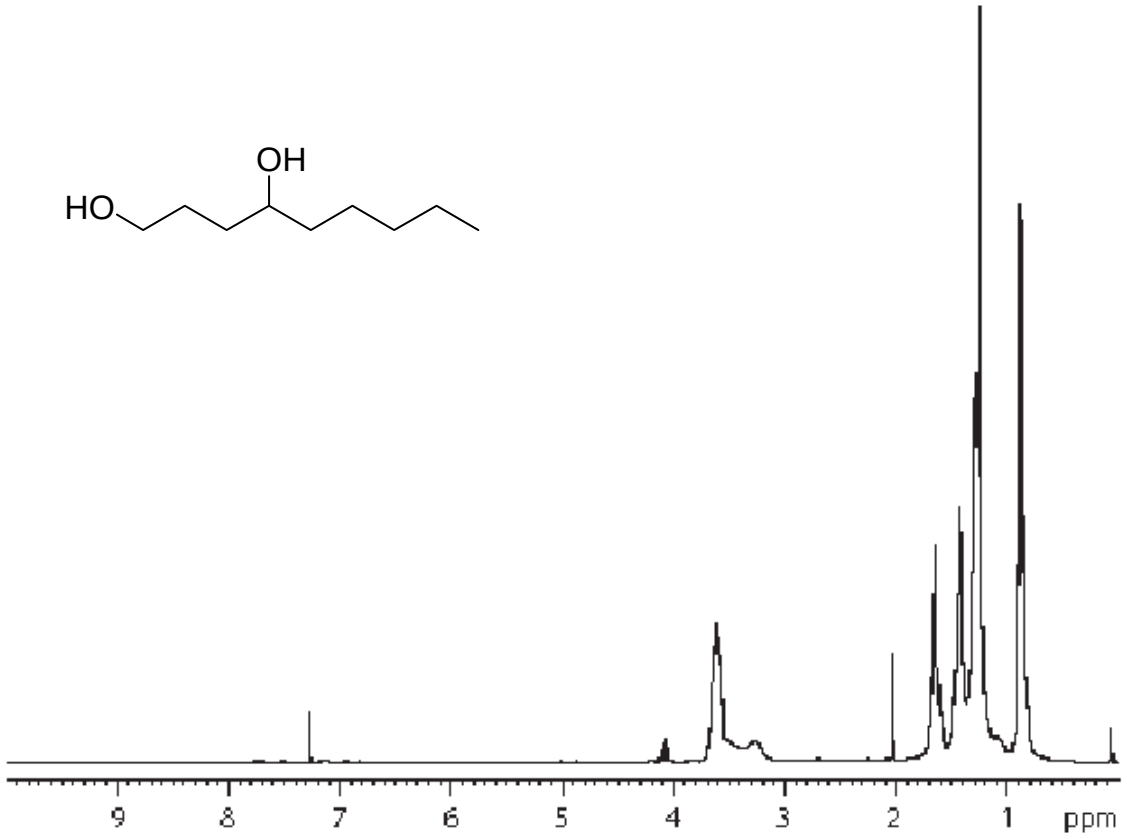

FIGURE 2.37 **300 MHz ^{1}H NMR of NDO in $CDCl_3$**

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CHAPTER 3

MERCAPTURIC ACID CONJUGATES OF 4-HYDROXY-2-NONENAL AND 4-OXO-2-NONENAL METABOLITES ARE *IN VIVO* MARKERS OF OXIDATIVE STRESS

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ABSTRACT

Oxidative stress-induced lipid peroxidation leads to the formation of cytotoxic and genotoxic 2-alkenals, such as HNE and ONE. Lipid-derived reactive aldehydes are subject to phase-2 metabolism and are predominantly found as MA conjugates in urine. This study shows evidence for the *in vivo* formation of ONE and its phase-1 metabolites, ONO and ONA. We have detected the MA conjugates of HNE, DHN, HNA, the lactone of HNA, ONE, ONO, and ONA, in rat urine by liquid chromatography-tandem mass spectrometry comparison with synthetic standards prepared in our laboratory. CCl_4 treatment of rats, a widely accepted animal model of acute oxidative stress, resulted in a significant increase of the urinary levels of DHN-MA, HNA-MA lactone, ONE-MA, and ONA-MA. Our data suggest that conjugates of HNE and ONE metabolites have value as markers of *in vivo* oxidative stress and lipid peroxidation.

INTRODUCTION

LPO products are breakdown products of fatty acids, formed under conditions of oxidative stress. HNE is a well established LPO product that has been shown to contribute to the development and progression of age-related diseases such as Alzheimer and atherosclerosis (1-4) in addition to being cytotoxic and genotoxic (5,6). The mechanism of formation for HNE from linoleic acid via HPNE has been previously demonstrated (7). Once HNE is formed, it can be further metabolized by cytochrome P450, aldehyde dehydrogenase, aldo-keto reductase, and conjugated by GST. Certain isoforms of murine and human P450s (8) and aldehyde dehydrogenase (9) can catalyze the oxidation of HNE to HNA. When conjugated, HNA can undergo spontaneous intramolecular condensation, resulting in lactone formation (10). Aldo-keto reductase 1B1 has been shown to reduce HNE to form DHN (11). GSH can form conjugates with HNE and other LPO products (6), via a Michael-type addition mediated by GST (12-14). The GSH can then be further metabolized in the liver and in the kidney to form MA, resulting in the conjugates shown in Fig. 3.1. A number of studies have examined HNE and its metabolites *in vivo* (10,15-18), and have demonstrated the formation of the MA conjugates, HNE-MA, DHN-MA, HNA-MA and HNA-MA lactone *in vivo* (10). In

addition, histidine-DHN and histidine-HNA have been found in the urine of obese Zucker rats, a model of metabolic syndrome (19).

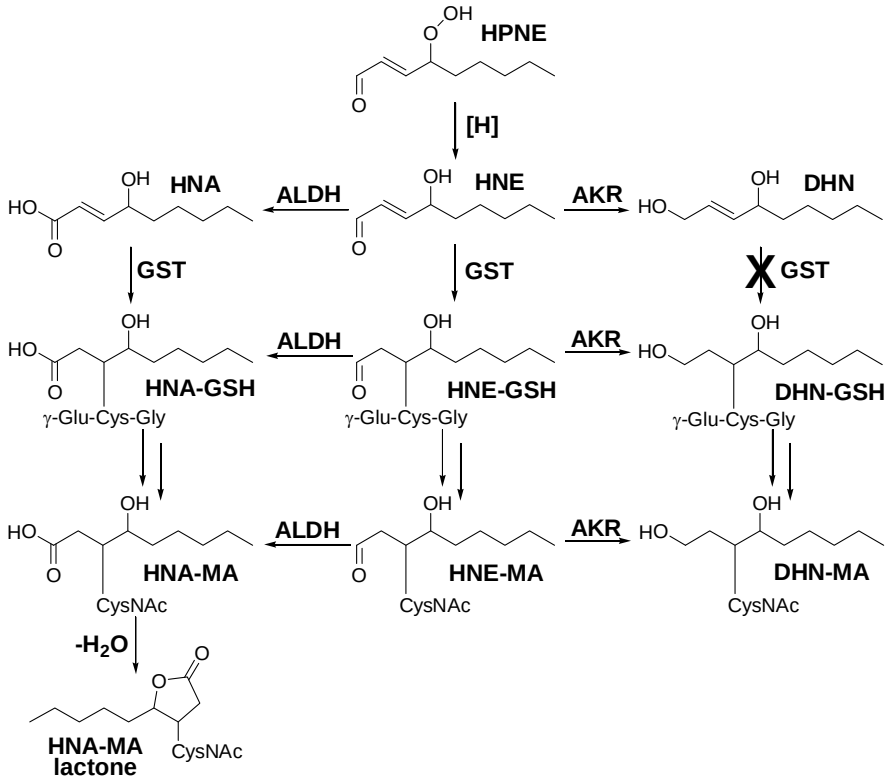

FIGURE 3.1 **Formation of LPO-MA conjugates from HNE.** Under conditions of oxidative stress, linoleic and arachadonic acids can be oxidized to form HPNE. HPNE can then be reduced to HNE and further metabolized by aldehyde dehydrogenase (ALDH), aldo-keto reductase (AKR), and GST. These enzymes cause oxidization, reduction, or GSH conjugation, respectively. Once LPO-GSH conjugates have formed, the GSH is further metabolized to MA. The LPO-MA conjugates are analyzed in this study. It is important to note that once HNE has been reduced to DHN, it will no longer form a Michael-type conjugate with GSH due to the lack of α,β-unsaturation. Also, HNA-MA is subject to intramolecular condensation, resulting in the formation of a lactone.

Not only can HPNE break down into HNE, but it can also form ONE as shown *in vitro* (20) and in cultured cells (21). ONE can be reduced at the C-4 position by carbonyl reductase to form HNE (22), but it can also be reduced at the C-1 position by aldo-keto reductase to form ONO (21,23,24) or oxidized by aldehyde dehydrogenase (human aldehyde dehydrogenase 2) to form ONA (25). Again, GST can mediate conjugate formation, resulting in metabolism to the MA conjugates shown in Fig. 3.2. In view of

these findings, ONE metabolites are expected to be formed *in vivo* as end products of LPO but their presence *in vivo* has not been demonstrated.

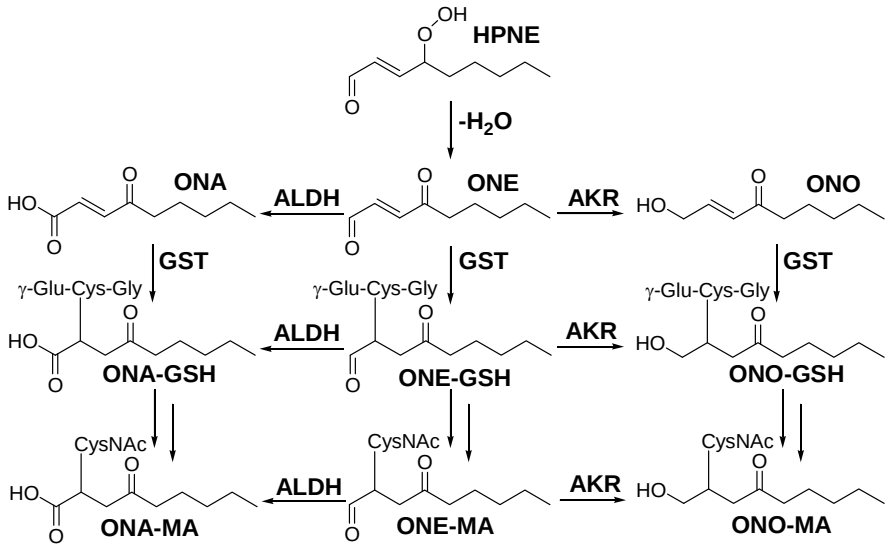

FIGURE 3.2 **Formation of LPO-MA conjugates from ONE.** HPNE can eliminate a water molecule, resulting in the formation of ONE. Similarly to HNE, ONE undergoes oxidation by aldehyde dehydrogenase (ALDH), reduction by aldo-keto reductase (AKR), and GSH conjugation by GST. Unlike HNE, ONE has two possible sites of conjugate formation, at the C-2 and C-3 positions. Thus, after reduction, the ONO formed retains its α,β-unsaturation, allowing it to form GSH conjugates via this route. The MA conjugates were analyzed in this study and are shown here as the C-2 conjugates.

Here we report that phase-2 metabolites of ONE, ONA, and ONO are formed after an acute oxidative stress insult in rats. Rats were exposed to CCl_4, an established model of *in vivo* oxidative stress (26). MA conjugates of HNE and ONE metabolites were detected in the urine by LC-MS/MS comparison with synthetic standards. Our results demonstrate, for the first time, that ONE metabolites, in addition to HNE metabolites, are formed *in vivo* as products of LPO. Currently, F_2-isoprostanes are considered to be the most reliable marker of *in vivo* oxidative stress and LPO (27). The CCl_4 induced formation of the HPNE-derived LPO product conjugates shows potential for these metabolites as additional or alternative markers of oxidative stress in animals and in humans.

EXPERIMENTAL PROCEDURES

Reagents

[9-$^{2}H_3$]-4-Hydroxy-2-nonenal was purchased from Cayman Chemical Co. (Ann Arbor, MI). [^{2}H]Chloroform was purchased from Cambridge Isotope Laboratories (Andover, MA). HPLC-grade formic acid (0.1%) in water was purchased from Honeywell Burdick and Jackson (Muskegon, MI). MCPBA was purchased from TCI America (Portland, OR). All other chemicals were purchased from Sigma-Aldrich.

Synthesis of HNE and ONE Metabolites

HNE—HNE was synthesized from 3-(*Z*)-nonenol following a method adapted from Gardner *et al.* (28). Briefly, 3-(*Z*)-nonenol (2 mmol) was dissolved in 8 ml of CH_2Cl_2, and 3-chloroperoxybenzoic acid (2 mmol) was added. The reaction mixture was stirred for 1 h at room temperature and, after the addition of 8 ml of 10% aq $NaHCO_3$, stirred for 45 min. The reaction mixture was washed with water and dried with 2 g of powdered molecular sieves. 4-NMO (3 mmol) was added and the mixture was stirred under argon for 30 min. After addition of TPAP (0.1 mmol), the mixture was stirred for 1 h under argon, filtered through silica gel and rinsed with ethyl ether. Next, 16 ml of 1.3 M sodium hydroxide was added to the filtrate and the solution was stirred vigorously for 15 min. The reaction mixture was washed with water, dried with anhydrous $MgSO_4$, filtered, and concentrated *in vacuo*. The yield was 35%. Our adaptations to the method of Gardner *et al.* (28) gave a slightly lower yield; however, we found that the changes resulted in more consistent yields. Data are ^{1}H NMR (400 MHz, $CDCl_3$) δ 9.63 (d, J = 8 Hz, 1H), 6.86 (dd, J = 5, 16 Hz, 1H), 6.35 (ddd, J = 2, 8, 16 Hz, 1H), 4.48 (m, 1H), 1.76 (d, J = 5 Hz, 2H), 1.43 (m, 7H), 0.94 (t, J = 6 Hz, 3H).

ONA—ONA was synthesized from 2-pentylfuran, following the method of Annangudi *et al.* (29) Data are ^{1}H NMR (400 MHz, $CDCl_3$) 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).

HNA—To a stirred solution of ONA (0.05 mmol) in 5 ml of ethanol was added 0.1 mmol of sodium borohydride. After 45 min at room temperature, the reaction mixture was acidified to pH 3 with 1 N HCl. The mixture was then extracted with ethyl ether.

The organic layer was dried with anhydrous $MgSO_4$, filtered, and concentrated *in vacuo*. Data are ^{1}H NMR (400 MHz, $CDCl_3$) 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).

ONE—ONE was synthesized from 2-pentylfuran following the method of Zhang *et al.* (30). Data are ^{1}H NMR (400 MHz, $CDCl_3$) δ 9.82 (d, J = 7 Hz, 1H), 6.91 (d, J = 16 Hz, 1H), 6.81 (dd, J = 7, 16 Hz, 1H), 5.45 (t, J = 7 Hz, 2H), 1.70 (m, 2H), 1.36 (m, 4H), 0.94 (t, J = 7, 3H).

ONO—To a stirred solution of ONE (0.01 mmol) in 1 ml of methanol were added 17.05 ml of phosphate buffer (0.1 M, pH 3) and 950 μl of a 50 mM sodium cyanoborohydride solution in 1 N NaOH. After stirring for 15 h at room temperature, the reaction mixture was extracted with ethyl acetate and the organic layer was concentrated *in vacuo*. Data are ^{1}H 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, 3H).

LPO-MA Adducts—All adducts were synthesized in the same manner and characterized by LC-electrospray ionization-MS/MS (see Table 1). A 20 mM solution of MA was prepared in 0.1 M phosphate buffer, pH 8. To 50 μl of this solution was added 450 μl of the same phosphate buffer and 400 μl of water. A 1 mM solution of the LPO product of interest was made up in ethanol and 100 μl was added to the MA solution. The reaction was stirred at 37 ^{o}C for 2 h and then acidified to pH 3 with 1 N HCl. It was then extracted with ethyl acetate, 3 x 1 ml, evaporated under nitrogen using a Zymark TurboVap LV (Caliper Life Sciences, Hopkinton, MA), and reconstituted in 1 ml of 20:80 acetonitrile:water containing 0.1 % formic acid.

DHN-MA—Because DHN has no α,β-unsaturation with which a Michael-type adduct can form, the DHN-MA adduct was made by first synthesizing HNE-MA as described above. The aldehyde moiety was then reduced with 10 μl of a 5 M sodium borohydride solution in 1 N NaOH (31). The reduction mixture was stirred at room temperature for 30 min then acidified to pH 3 with 1 N HCl. It was then extracted with ethyl acetate, 3 x 1 ml, evaporated under nitrogen, and reconstituted in 1 ml of 20:80 acetonitrile:water containing 0.1 % formic acid.

Animal Treatment

The experimental protocol for the animal studies was approved by the Institutional Animal Care and Use Committee at Oregon State University. Male F344 rats (Harlan, Indianapolis, IN), weighing 280-320 g, were housed in individual plastic cages covered with Hepa filter and allowed free access to standard animal chow and water *ad libitum*. After 1 week of acclimatization, the rats were transferred to metabolism cages. Six animals were divided into two groups of three, with one group receiving an intraperitoneal dose of 1 ml/kg CCl_4 (dissolved in corn oil), and the other group (control) receiving the vehicle alone. The CCl_4 dose of 1 ml/kg was chosen on the basis of literature reports (32,33). Urine was collected from the rats during a 24 h period after treatment.

Urine Samples

A volume of 0.5 ml of rat urine was acidified with 225 μl of 1 N HCl to pH 3. To the urine was added 5 μl of a 1 μM solution of [9-$^2H_3$]HNE-MA as an internal standard. The samples were extracted with ethyl acetate, 3 x 1 ml. The ethyl acetate layers were combined and evaporated under nitrogen. Samples were then reconstituted in 200 μl of 20:80 acetonitrile:water containing 0.1% formic acid.

Urinary creatinine was measured using a Creatinine Assay Kit, catalog no. 500701 (Cayman Chemical). The assay was performed according to the manufacturer's directions. There was no significant difference between treated and control creatinine levels ($p = 0.9$).

HPLC

A Shimadzu Prominence HPLC system (Shimadzu, Columbia, MD) consisting of four LC-20AD pumps, a DQU-20A_5 degasser, and an SIL-HTc autosampler equipped with switching valves was used for all chromatography. Two chromatographic systems were used, and unless otherwise stated, all data presented were recorded with system 1. For system 1, the HPLC column was a 250 x 2 mm Synergi Max RP C_{12} column (Phenomenex, Torrance, CA). The mobile phase consisted of Solvent A, 0.1 % (v/v) formic acid in water, and Solvent B, acetonitrile containing 0.1 % (v/v) formic acid. The

flow rate was 0.2 ml/min. Separations were carried out using a linear solvent gradient from 20 to 50 % B in 10 min, a linear gradient from 50 to 90 % B over the next 2 min, held constant at 90 % B for 7 min, returned to 20 % B after 1 min, and equilibrated at 20 % B for 5 min. System 2 consisted of a 50 x 2.1 mm Inertsil ODS-3 column (Varian, Lake Forest, CA). The mobile phase consisted of Solvent A, 0.1 % (v/v) formic acid in water, and Solvent B, methanol. The flow rate was 0.3 ml/min, and separations were carried out using a linear solvent gradient from 45 to 90 % B over 5 min, held constant at 90 % B for 1 min, then returned to 45 % B and equilibrated for 3 min.

Mass Spectrometry

An Applied Biosystems MDS Sciex hybrid triple quadrupole/linear ion trap mass spectrometer (4000 QTrap) equipped with a TurboV electrospray source (Concord) was used for these analyses. The TurboV source was maintained at 400 ^{o}C. The ion-spray voltage was -4500 V and the declustering potential was 40 V. Nitrogen was used as the source gas, curtain gas, and collision gas. Various scanning techniques, all run in negative ion mode, were used for the characterization and detection of LPO-MA products, including Q1, product ion scanning, and SRM. All SRM transitions and collision energies are shown in Table 3.1.

Table 3.1 LC-MS/MS properties of ONE and HNE metabolites detected in the urine of rats.

Analyte	MW	SRM transition	Collision energy (eV)	Retention time (min.)	Figure trace	*p* value[a]
ONE-MA	317	316 → 162	25	3.3[b]	5B	0.0017
HNE-MA	319	318 → 189	25	8.9, 9.3	3A	
		318 → 171	25		3A	0.18
		318 → 162	25		3A	
ONO-MA	319	318 → 189	25	8.9, 9.3, 9.7	3B	
		318 → 171	25		3B	
		318 → 162	25		3B	0.10
DHN-MA	321	320 → 191	30	8.3	6A	0.020
		320 → 143	30		6A	0.0055
ONA-MA	333	332 → 169	25	10.3	6C	0.010
		332 → 162	25		6C	0.0079
		332 → 84	25		6C	0.0083
HNA-MA	335	334 → 162	25	10.1	6E	0.30
HNA-MA lactone	317	316 → 162	25	3.9[b]	5A	0.096
		316 → 143	25		5A	0.048

[a] *p* values indicate statistical differences between CCl_4-dosed rats and controls.
[b] Recorded using chromatographic system 2.

Data Analysis

Peak area analysis was performed using Analyst 1.4.1 (Applied Biosystems). Analyte peak areas were normalized for the internal standard (IS) peak area, a 2.5-fold sample concentration, and for the creatinine concentration in mg/ml. Thus, all data are represented as analyte peak area/(IS peak area × 2.5 × mg/ml creatinine). For these analyses, peak 3 (9.7 min, Fig. 3.3B) from the *m/z* 318 → 162 SRM transition was used to quantify ONO-MA, and peak 2 (9.3 min, Fig. 3.3A) from the *m/z* 318 → 171 SRM transition was used to quantify HNE-MA. Statistical comparisons were performed using GraphPad (San Diego, CA). Data are shown as mean ± S.E.

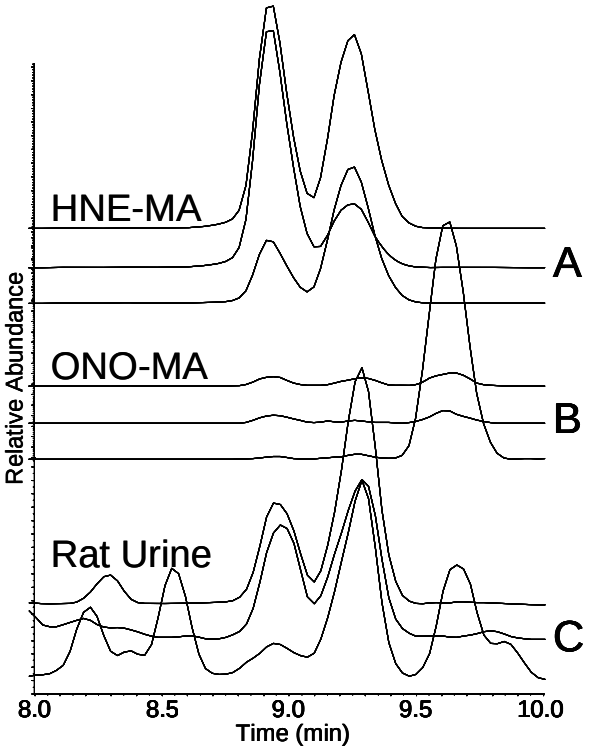

FIGURE 3.3 **SRM chromatograms for the isobaric compounds HNE-MA and ONO-MA.** A, standard reaction mixture of HNE-MA; transitions shown are from top to bottom: *m/z* 318 → 171, *m/z* 318 → 189, and *m/z* 318 → 162. B, standard reaction mixture of ONO-MA; transitions shown are from top to bottom: *m/z* 318 → 171, *m/z* 318 → 189, and *m/z* 318 → 162. C, rat urine sample; transitions shown are from top to bottom: *m/z* 318 → 171, *m/z* 318 → 189, and *m/z* 318 → 162. The HNE-MA gives only two peaks (8.9 and 9.3 min), whereas the ONO-MA gives three (8.9, 9.3 and 9.7 min). The standards confirm that the third peak contains only ONO-MA. This third peak was used to represent ONO-MA for comparison between oxidatively stressed rats and control rats.

RESULTS

Detection of the MA Conjugate of ONO

Using SRM transitions for the MA conjugate of HNE, three chromatographic peaks (8.9, 9.3, and 9.7 min, Fig. 3.3C) were observed in the urine of CCl_4-dosed rats, of which only the first two corresponded with the two chromatographic peaks produced by a synthetic sample of HNE-MA (Fig. 3.3A). The product ion spectra of the chromatographically resolved HNE-MA isomers (first two peaks at 8.9 and 9.3 min.) showed β-elimination fragment ions observed at *m/z* 189 and *m/z* 171 (Fig. 3.4).

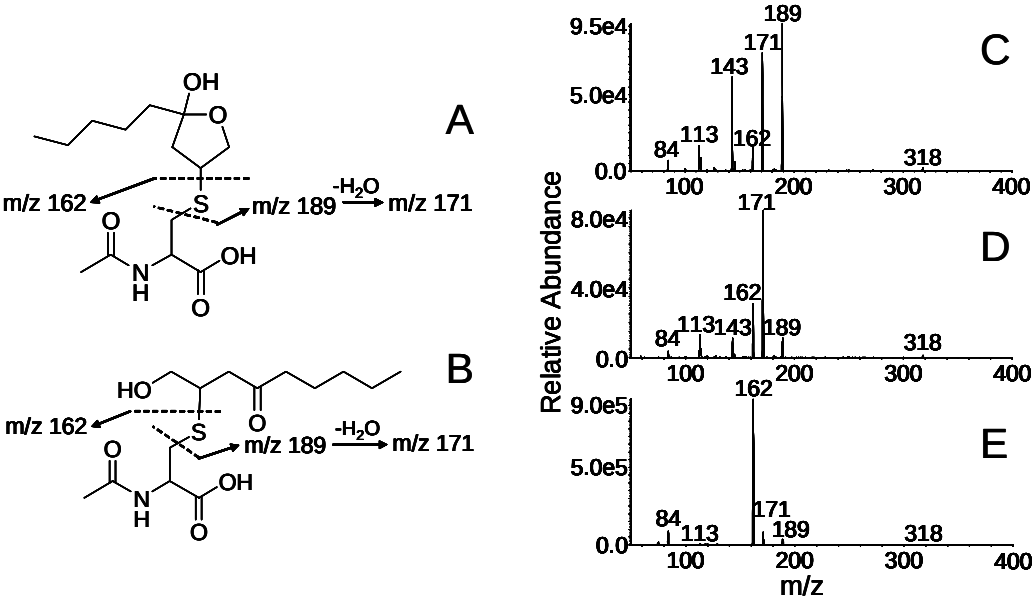

FIGURE 3.4 **Fragmentation patterns for each chromatographic peak of ONO-MA.** A, proposed mass fragments of the hemiketal form of ONO-MA with *m/z* 189 (β-elimination), *m/z* 171 (β-elimination followed by H_2O loss), and *m/z* 162 (mercapturate formed upon RM cleavage). B, the preferred open chain form of ONO-MA produces mass fragments with the same *m/z* values as the cyclic form, but the mercapturate fragment with *m/z* 162 is expected to predominate due to RM cleavage. C, MS/MS spectrum for the first minor peak of a standard solution of ONO-MA. The fragments with *m/z* 189 and *m/z* 171 are the most prominent. D, MS/MS spectrum recorded for the second minor ONO-MA peak, showing the *m/z* 171 fragment as the most abundant ion. E, MS/MS spectrum for the unique third and major peak of ONO-MA. The mercapturate peak at *m/z* 162 represents the most abundant fragment ion, which is suggestive of RM cleavage of the open chain form.

The third chromatographic peak at 9.7 min (urine), absent from the SRM chromatogram of HNE-MA, showed the greatest signal for the *m/z* 318 → 162 SRM transition, indicating release of mercapturate (*m/z* 162) from the molecular anion. This suggests that it favored retro-Michael (RM) cleavage over β-elimination upon collision-induced dissociation, presumably because it prefers the formation of an enolate (thus setting the stage for RM) over formation of a cyclic hemiacetal or hemiketal (more resistant to RM). Because hemiketal or hemiacetal formation is less favorable for ketones than for aldehydes (34), we hypothesized that the third peak represented the MA conjugate of ONO, which is isobaric with HNE-MA. Synthetic ONO-MA indeed showed the expected retention time (9.7 min, Fig. 3.3B) and MS/MS spectrum (major fragment with

m/z 162), which was taken as evidence for the excretion of ONO-MA in the urine (Fig. 3.3). ONO metabolites have not previously been reported as *in vivo* products of oxidative stress.

Distinction between the Isobaric metabolites, HNA-MA Lactone and ONE-MA

When first analyzed using chromatographic system 1, it was not possible to distinguish between the isobaric metabolites HNA-MA lactone and ONE-MA. Both compounds produced the same mercapturate fragment (*m/z* 162), and they co-eluted. A second chromatographic system was developed in order to separate the compounds (system 2). The now-resolved HNA-MA lactone differed from ONE-MA by the production of an additional fragment ion with *m/z* 143, which allowed the distinction between the two conjugates under the new chromatographic conditions (Fig. 3.5). Both MA conjugates were detected in rat urine (Fig. 3.5C).

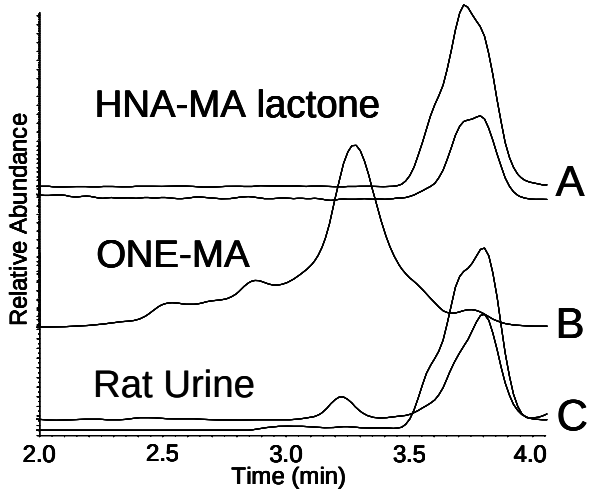

FIGURE 3.5 **SRM chromatograms of the isobaric compounds ONE-MA and HNA-MA lactone.** A, standard reaction mixture of HNA-MA, showing the lactone form and SRM transitions from top to bottom: *m/z* 316 → 143 and *m/z* 316 → 162. B, standard reaction mixture of ONE-MA, the predominant SRM transition for this compound is *m/z* 316 → 162. C, rat urine sample; transitions shown from top to bottom: *m/z* 316 → 162 and *m/z* 316 → 143. These compounds were separated using chromatographic system 2.

Detection of DHN-MA, ONA-MA and HNA-MA in Rat Urine

The remaining three conjugates were readily distinguished based on their chromatographic behavior and MS/MS fragmentation (Fig. 3.6). DHN-MA eluted at 8.3 min, and its major fragment ions were observed with *m/z* 191 and *m/z* 143, both β-elimination fragments. ONA-MA eluted at 10.3 min and yielded fragment ions with *m/z* 169, *m/z* 162 (RM cleavage), and *m/z* 84. HNA-MA eluted at 10.1 min and produced a fragment ion with *m/z* 162, formed by RM cleavage. LC-MS/MS comparison of the synthetic conjugates with the biological samples provided confirmation of the identity of each conjugate.

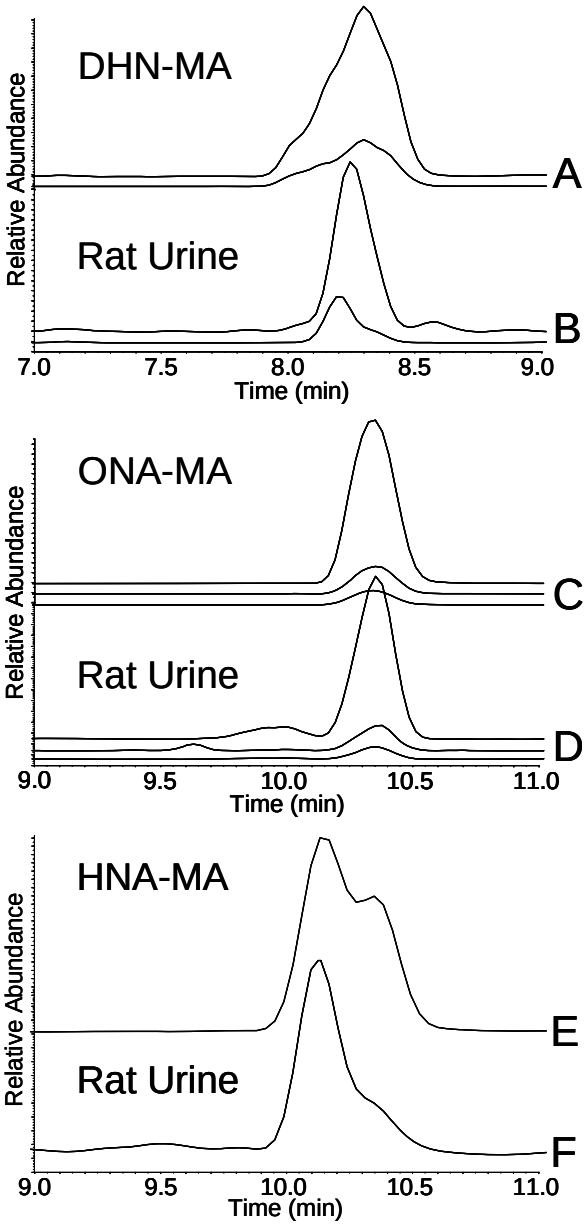

FIGURE 3.6 **SRM chromatograms for DHN-MA, ONA-MA, and HNA-MA.** A, standard reaction mixture of DHN-MA; transitions are from top to bottom: *m/z* 321 → 191 and *m/z* 321 → 143. B, rat urine sample; transitions shown are from top to bottom: *m/z* 321 → 191 and *m/z* 321 → 143. C, standard reaction mixture of ONA-MA; transitions are from top to bottom: *m/z* 332 → 162, *m/z* 332 → 169 and *m/z* 332 → 84. D, rat urine sample; transitions shown are from top to bottom: *m/z* 332 → 162, *m/z* 332 → 169 and *m/z* 332 → 84. E, standard reaction mixture of HNA-MA, transition *m/z* 334 → 162. F, rat urine sample, transition *m/z* 334 → 162.

Comparison of CCl_4-dosed Rats with Control Animals

CCl_4-dosing caused significant increases in the levels of ONE-MA, DHN-MA, ONA-MA, and HNA-MA lactone compared with the control animals (Fig. 3.7). Urinary concentrations of ONO-MA, HNE-MA, and HNA-MA were elevated in the CCl_4-treated rats, but the increases were not significant at the $p < 0.05$ level. Statistical comparisons were performed using the Student's *t*-test and *p* values for each conjugate are shown in Table 3.1. All calculations included normalization for creatinine levels to account for variation in urine concentration between rats. There was no difference in the creatinine levels between the treatment and control groups ($p = 0.9$).

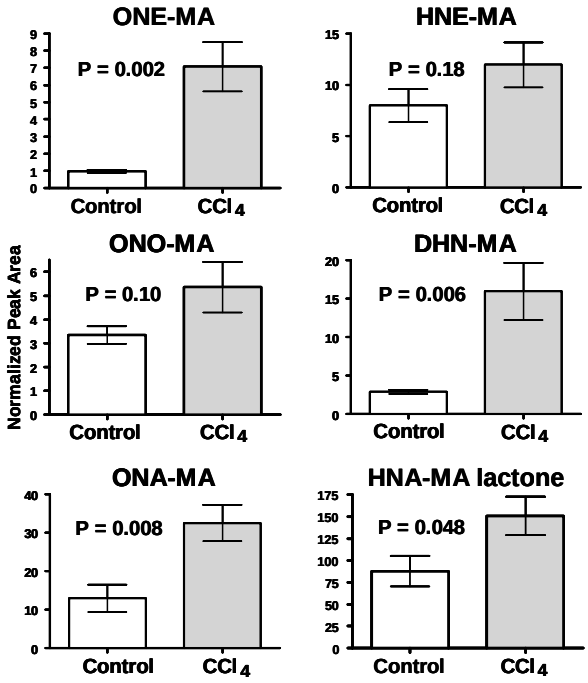

FIGURE 3.7 **Comparison of HPNE metabolites in control rats and rats oxidatively stressed with CCl_4.** ONE-MA, DHN-MA, ONA-MA, and HNA-MA lactone all show a significant increase in CCl_4-dosed rats with *p* values of 0.002, 0.006, 0.008, and 0.048, respectively. HNE-MA, ONO-MA, and HNA-MA did not increase significantly in CCl_4-dosed rats. Data are shown as mean ± S.E.

DISCUSSION

Lipid hydroperoxides, specifically hydroperoxy octadecadienoic acids (HPODEs), may be formed enzymatically through the action of lipoxygenases (35,36) or non-enzymatically through the reaction of linoleic acid with reactive oxygen species (37). Degradation of lipid hydroperoxides yields a multitude of reactive aldehydes (38), but only HNE and, more recently, ONE have been studied extensively with regard to their detrimental effects in biological systems. HNE and ONE have been shown to cause adverse effects because of their ability to covalently bind to 2'-deoxyguanosine (39-42), to cause protein cross-linking (43), and to induce aggregation of low-density lipoproteins (44). ONE shows greater toxicity in cultured cells, presumably due to its reactivity as a bifunctional electrophile (45). Furthermore, aldo-keto reductase-mediated reduction of the aldehyde functionality renders HNE inactive as a Michael acceptor whereas ONE retains its electrophilicity after conversion into ONO, a regioisomer of HNE (Fig. 3.8). The formation of ONO as a reactive metabolite of ONE has received little or no attention in the literature. Moreover, it was suggested by Blair that "ONO may contribute to the biological activities that have been ascribed previously to HNE" (24). Phase-2 metabolites of ONO and HNE are difficult to distinguish from one another by LC-MS/MS due to similar chromatographic behavior, lack of mass difference, and similar MS/MS spectra. We prepared the MA adducts of HNE and ONO by chemical methods and demonstrated that at least one (set of) ONO-MA isomer can be distinguished from HNE-MA and detected in rat urine on the basis of chromatographic retention times and MS/MS fragmentation (Fig. 3.3). The *in vivo* formation of ONO-MA has not previously been reported in the literature.

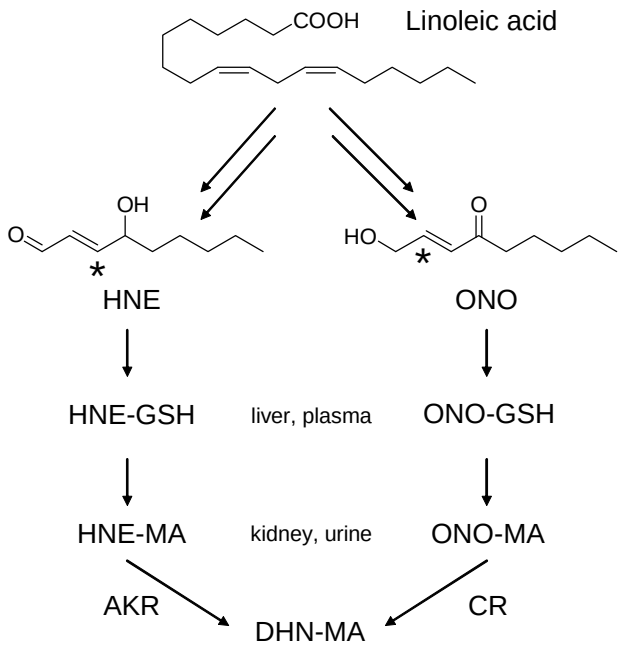

FIGURE 3.8 **LPO-induced degradation of linoleic acid and conversion into the isobaric metabolites, HNE-MA and ONO-MA.** DHN-MA can be formed by aldehyde reduction of HNE-MA or ketone reduction of ONO-MA. The *asterisk* indicates the electrophilic position. CR, carbonyl reductase; AKR, aldo-keto reductase.

Thiadiazabicyclo-ONE-glutathione (TOG) has recently been identified as a major phase-2 metabolite of ONE in cultured, immortalized endothelial (EA.hy 926) cells (46). Because the γ-glutamic acid residue of the GSH-ONE adduct participates in cyclization, the bicyclic TOG metabolite cannot be further metabolized to a MA adduct of ONE or its derivatives. Our data indicate that ONE is produced *in vivo* and undergoes phase-2 metabolism to form ONE-MA (Fig. 3.5), which would compete with TOG formation. Furthermore, our data also indicate that ONE-MA is metabolized to the corresponding carboxylic acid, ONA-MA, based on LC-MS/MS comparison with a synthetic sample of ONA-MA (Fig. 3.6). At present, it is not known whether the oxidation of the aldehyde functionality precedes or follows the conjugation reaction because ONA retains its ability to form a conjugate with GSH. Like ONO-MA, ONE-MA and ONA-MA have not previously been reported as *in vivo* metabolites.

To determine whether the formation of HNE and ONE increases under conditions of oxidative stress *in vivo*, we measured the MA conjugates of these LPO products in the

urine of rats exposed to CCl_4 and in the urine of control animals. CCl_4 is known for its ability to cause LPO in the liver through the formation of the trichloromethyl radical ($CCl_3^{\bullet}$) via cytochrome P450-mediated homolytic cleavage of the carbon-chlorine bond (47-49). The $CCl_3^{\bullet}$ radical can form chloroform by abstracting a hydrogen from the bisallylic methylene functionality in polyunsaturated fatty acids, thereby forming lipid-based radicals that spontaneously react with molecular oxygen to form regioisomeric lipid hydroperoxides, *e.g.*, 9-HPODE and 13-HPODE from linoleic acid. Alternatively or additionally, $CCl_3^{\bullet}$ may directly react with molecular oxygen to give trichloromethyl peroxy radicals ($CCl_3OO^{\bullet}$), a reactive species considered by some authors to produce HPODEs (47-49). Subsequently, these HPODEs are converted into carbon-carbon cleavage products, such as HPNE, HNE and ONE (50). Regardless of the precise mechanism of CCl_4-induced LPO, acute CCl_4 poisoning is an accepted model of *in vivo* oxidative stress and LPO. For instance, HNE-deoxyguanosine adducts have been shown to have significantly increased levels in F344 rats treated with CCl_4 as compared with control animals (39,42).

In our studies the urinary levels of the HNE metabolites DHN-MA and HNA-MA lactone and the levels of the ONE metabolites ONE-MA and ONA-MA were significantly higher in the CCl_4-treated rats compared to the control animals (Fig. 3.7). The urinary levels of HNE-MA and ONO-MA were higher in the CCl_4-treated rats, but the difference was not statistically significant at $p < 0.05$ ($p = 0.18$ and $p = 0.10$, respectively). This is an unexpected finding, because HNE is generally considered to be a marker of LPO and *in vivo* oxidative stress. It is conceivable that the lack of statistical difference is due to inter-individual variation in the metabolism of HNE conjugates to form DHN and HNA conjugates. Similarly, ONO-MA may be converted into DHN-MA by carbonyl reductase (22). Our semiquantitative data (normalized peak areas), however, suggest that oxidation of the aldehyde functionality in the HNE and ONE conjugates is the preferred metabolic pathway because HNA-MA lactone and ONA-MA are among the most abundant end products of HPNE metabolism (Fig. 3.7).

The arachidonic acid-derived $F_{2\alpha}$-isoprostanes are generally considered to be the most reliable markers of *in vivo* oxidative stress. The value of $F_{2\alpha}$-isoprostanes as oxidative stress markers in humans is exemplified by studies of smokers (51,52), patients

with renal failure (53), patients with coronary artery disease (54), and patients with lupus erythematosus (55). Kadiiska *et al*. (56) observed a significant increase of the urinary concentrations of $F_{2\alpha}$-isoprostanes in rats injected intraperitoneally with 120 or 1200 mg/kg of CCl_4 compared to control animals. Sicilia *et al.* (57) reported elevated $F_{2\alpha}$-isoprostanes levels in liver and kidney tissue of rats after an oral CCl_4 dose of 1 ml/kg. However, these authors observed no statistical difference between the urinary levels of $F_{2\alpha}$-isoprostanes obtained from treated and control animals in the same study (57), which they attributed to kinetic differences between oral gavage and the i.p administration used by Kadiiska *et al.* (56). Our findings indicate that the end products of HPNE metabolism are elevated in urine obtained from CCl_4-treated rats, which holds promise for these metabolites as additional or alternative markers of oxidative stress in animals and humans.

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CHAPTER 4

QUANTITATION OF MERCAPTURIC ACID CONJUGATES OF 4-HYDROXY-2-NONENAL AND 4-OXO-2-NONENAL METABOLITES IN A SMOKING CESSATION STUDY

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ABSTRACT

The breakdown of polyunsaturated fatty acids (PUFAs) under conditions of oxidative stress results in the formation of lipid peroxidation (LPO) products. These LPO products such as 4-hydroxy-2-nonenal (HNE) and 4-oxo-2-nonenal (ONE) can contribute to the development of cardiovascular and neurodegenerative diseases and cancer. Conjugation with glutathione, followed by further metabolism to mercapturic acid (MA) conjugates, can mitigate the effects of these LPO products in disease development by facilitating their excretion from the body. We have developed a quantitative method to simultaneously assess levels of 4-oxo-2-nonen-1-ol (ONO)-MA, HNE-MA, and 1,4-dihydroxy-2-nonene (DHN)-MA in human urine samples utilizing isotope-dilution mass spectrometry. We are also able to detect 4-hydroxy-2-nonenoic acid (HNA)-MA, 4-hydroxy-2-nonenoic acid lactone (HNAL)-MA, and 4-oxo-2-nonenoic acid (ONA)-MA with this method. The detection of ONO-MA and ONA-MA in humans is significant because it demonstrates that HNE/ONE branching occurs in the breakdown of PUFAs and suggests that ONO may contribute to the harmful effects currently associated with HNE. We were able to show significant decreases in HNE-MA, DHN-MA, and total LPO-MA in a group of seven smokers upon smoking cessation. These data demonstrate the value of HNE and ONE metabolites as *in vivo* markers of oxidative stress.

INTRODUCTION

Oxidative degradation of polyunsaturated fatty acids (PUFAs) occurs under conditions of oxidative stress when the cellular antioxidant defense mechanisms are overwhelmed, leading to the formation of electrophilic lipid peroxidation (LPO) products. 4-Hydroxy-2-nonenal (HNE) and 4-oxo-2-nonenal (ONE) are two of the most thoroughly studied LPO products. These reactive aldehydes have been shown to be cytotoxic and genotoxic (1,2), as well as to contribute to the development and progression of cancer (3), cardiovascular diseases such as atherosclerosis and chronic obstructive pulmonary disease (4-6), and neurodegenerative diseases like Alzheimer's (7-9). In biological systems, HNE and ONE undergo phase I metabolism, resulting in their respective oxidation products 4-hydroxy-2-nonenoic acid (HNA) (10) and 4-oxo-2-nonenoic acid (ONA) (11) or reduction products 1,4-dihydroxy-2-nonene (DHN) (12)

and 4-oxo-2-nonen-1-ol (ONO) (13-15) (Fig. 4.1). HNE, ONE, and their phase I metabolites have also been shown to undergo phase II metabolism, forming Michael-type conjugates with glutathione (GSH) (2), a reaction mediated by glutathione *S*-transferase (GST) (16-18). Upon conjugation, HNA can form a lactone (HNAL) via spontaneous intramolecular condensation (19). Further metabolism of these LPO-GSH conjugates in the liver and kidney results in LPO-mercapturic acid (MA) conjugates which are excreted in urine.

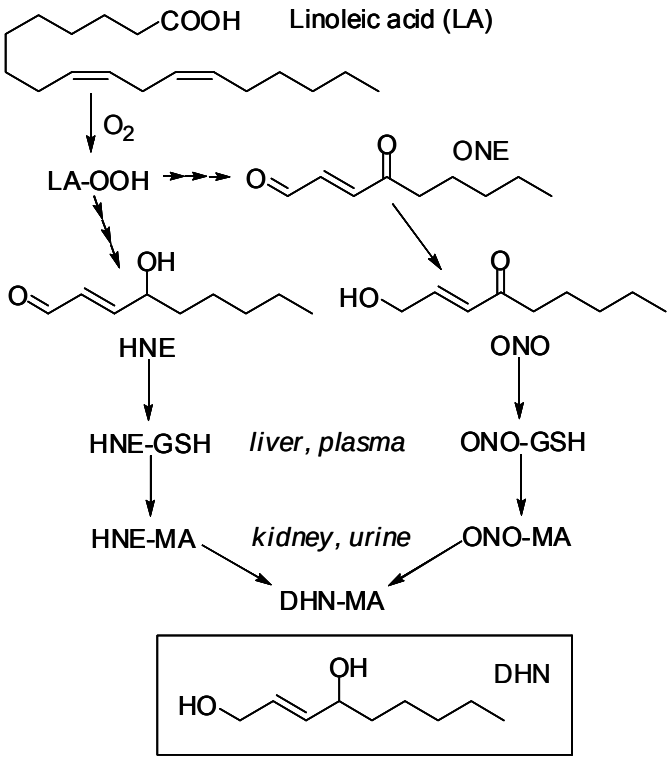

FIGURE 4.1 **LPO-induced degradation of linoleic acid.** HNE and ONE undergo phase I and phase II metabolism, resulting in the excretion of MA conjugates including HNE-MA, ONO-MA, and DHN-MA. DHN-MA may originate from DHN-GSH and possibly ONO-GSH, but it is shown as a metabolite of HNE-MA and ONO-MA for simplicity.

We have previously reported that HNE and ONE metabolite levels are significantly increased in rats after an acute oxidative stress insult (20). In that study we were able to differentiate between HNE-MA and its isomer ONO-MA which had not been previously demonstrated. This is an important distinction because previous analyses

have likely attributed the effects of ONO to HNE. These metabolites also form by different pathways, so being able to distinguish between the two could provide insight into the mechanisms of oxidative stress in biological systems. Previous studies have focused on the quantitation of DHN-MA (21-24).

Here we report the quantitation of HNE-MA and ONO-MA, as well as DHN-MA in human urine. The phase I metabolites of HNE-MA and ONE-MA represent biologically relevant pathways for the elimination of these LPO products in a rat model of oxidative stress (20). We have detected HNE-MA, DHN-MA, HNA-MA, HNAL-MA, ONO-MA, and ONA-MA in human samples and are able to quantitate the HNE-MA, ONO-MA, and DHN-MA metabolites in smokers. Twelve weeks of smoking cessation resulted in a significant decrease in the levels of urinary HNE-MA, DHN-MA, and overall LPO-MA. These results demonstrate the potential utility of these metabolites as non-invasive diagnostic tools for assessing oxidative stress *in vivo*.

EXPERIMENTAL PROCEDURES

Reagents

[^{2}H]Chloroform was purchased from Cambridge Isotope Laboratories (Andover, MA). HPLC-grade formic acid (0.1 %) in water was purchased from Honeywell Burdick and Jackson (Muskegon, MI). 3-Chloroperoxybenzoic acid and dithiothreitol were purchased from TCI America (Portland, OR). HNE-MA (1 mg in 100 μl ethanol) was purchased from Cayman Chemical (Ann Arbor, MI). Cotinine was purchased from Alfa Aesar (Ward Hill, MA) and cotinine-d_3 (99 atom % D, 1 mg/ml in methanol) was from Sigma-Aldrich (St. Louis, MO). All other chemicals were purchased from Sigma-Aldrich (St. Louis, MO).

Synthesis

LPO products—HNE, HNA, ONE, ONO, and ONA and their MA conjugates were prepared and chemically characterized as described in our previous work (20).

Deuterium labeled MA—*N*-(acetyl-d_3)-L-cysteine (MAd_3) was prepared using the method of Slatter et al. (25). Briefly, cystine (5.3 mmol) was added to 13 ml of a 1.5 M NaOH solution and the mixture was cooled in an ice bath with stirring. [$^2H_6$]Acetic

anhydride (10.6 mmol) was added dropwise over 20 min, the ice bath was removed, and the reaction continued stirring at room temperature for 1 h. 1,4-Dithiothreitol (10.6 mmol) was added, and the reaction mixture was stirred continuously at room temperature for 1 h, concentrated *in vacuo*, washed with ether, frozen, and lyophilized. Purification was performed on a 52 × 2.5 cm Sephadex LH-20 column using methanol as the eluting solvent. Fraction purity was verified by LC-MS analysis in negative ion mode. Only fractions containing a peak at m/z 165 (MAd_3) were carried forward. Further purification was carried out by acidification to pH 3 with 1 M HCl and extraction with ethyl acetate in order to remove any remaining cystine or cysteine. The organic layer was concentrated *in vacuo*. The resulting white residue was characterized by LC-MS/MS analysis and found to be free of cystine, cysteine, and unlabeled mercapturic acid.

Preparation of LPO-MAd_3 Conjugates—MAd_3 was used in place of MA for LPO-MA adduct formation (20) for use as internal standards. Briefly, a 20 mM solution of MAd_3 was prepared in 0.1 M sodium phosphate buffer, pH 8. To 50 μl of this solution was added 450 μl of the same phosphate buffer and 400 μl of water. A 1.0 mM solution of the LPO product of interest was made up in ethanol and 100 μl was added to the MAd_3 solution (10-fold molar excess). The reaction was stirred at 37 ^{o}C for 2 h and then acidified to pH 3 with 1 N HCl. It was then extracted with ethyl acetate, 3 × 1 ml, evaporated under nitrogen using a Zymark TurboVap LV (Caliper Life Sciences, Hopkinton, MA), and reconstituted in 1.0 ml of 2:8 acetonitrile-H_2O containing 0.1 % formic acid, yielding a nominal LPO-MAd_3 concentration of 100 μM. LC-MS/MS analyses were used to verify conjugate formation.

Sample Collection

This study protocol #4312 was approved by Oregon State University's and Samaritan Health Systems' Institutional Review Boards. Participants were recruited by newspaper advertisements in the Corvallis, Oregon area. Participants who responded were enrolled because they met our study participation criteria: age 18-65 years, healthy, current smoker motivated to quit for one arm of the study, nonsmoker with minimal exposure to second-hand smoke in the control group, BMI < 35 kg/m^2, subjects may not be taking any prescription, no over-the-counter or herbal medications that induce or

inhibit the liver enzymes involved in drug metabolism (CYP P450 3A4, 2D6), no known active liver disease (hepatitis, cirrhosis), no excessive alcohol use defined as > 1 drink per day for women and > 2 drinks per day for men. Nonsmokers were enrolled into the study and matched by age and BMI to one of the smokers already enrolled in the study (Table 4.1). Each subject signed an informed consent statement and completed a questionnaire that provided the following information: age, sex, weight, height, history of tobacco use, and health status prior to enrollment in the study.

Urine samples were collected from smokers and nonsmokers at Good Samaritan Hospital (Corvallis, OR). On the day of the study, 23 smokers and 23 nonsmokers provided at least 10 ml of clean catch urine. A second urine sample was collected at least 12 weeks after smoking cessation from the seven smokers who successfully quit smoking and their nonsmoking counterparts. Upon collection, samples were frozen and stored at -80 ^{o}C until analysis. Smoking cessation was carried out using either Chantix® (varenicline tartrate, which blocks nicotine receptors in the brain), Zyban® (bupropion hydrochloride, presumably acting by modulation of noradrenergic and dopaminergic receptors in the brain), or quitting 'cold turkey'. Support was provided during the cessation process through a smoking cessation class and phone calls. Self reporting and cotinine levels were used to verify the success of smoking cessation.

TABLE 4.1
Study participant characteristics.

Parameter	All enrolled Participants		Cessation Study[b]	
	Nonsmokers (n = 23)	Smokers (n = 23)	Nonsmokers (n = 7)	Smokers (n = 7)
Gender[a]	10 Male, 13 Female	9 Male, 14 Female	4 Male, 3 Female	4 Male, 3 Female
Age (years)[a]	42.7 ± 12.0	42.5 ± 10.8	37.4 ± 10.7	38.7 ± 8.6
BMI (kg/m^2)[a]	26.8 ± 4.5	27.0 ± 4.6	25.9 ± 4.1	26.0 ± 4.1
Years Smoked	0	19.5 ± 12.5	0	16.2 ± 7.5

[a] No significant difference between the two groups.
[b] Nonsmokers were matched by age and BMI to one of the smokers.

Urine Samples

A volume of 0.2 ml of human urine was acidified with 20 μl of 1 N HCl to pH 3. To the urine was added 5 μl of a 100 μM solution of DHN-MAd_3, 10 μl of a 10 μM solution of HNE-MAd_3, and 10 μl of a 10 μM solution of ONO-MAd_3 as internal standards. The samples were extracted with ethyl acetate (2 × 700 μl). The ethyl acetate layers were combined and evaporated under nitrogen. Samples were then reconstituted in 100 μl of 2:8 acetonitrile:water containing 0.1 % formic acid and analyzed by LC-MS/MS.

Urinary creatinine was measured using a Creatinine Assay Kit, catalog no. 500701 (Cayman Chemical). The assay was performed according to the manufacturer's directions. There was no significant difference between smoker and non-smoker creatinine levels ($p = 0.37$).

Urinary cotinine was analyzed by LC-MS/MS. To a volume of 0.2 ml of human urine was added 5 μl of 10 μM cotinine-d_3 as an internal standard. Proteins were precipitated by the addition of 795 μl of MeCN containing 0.1 % formic acid and centrifugation. The supernatant was analyzed by LC-MS/MS.

Calibration Curves

A calibration curve was constructed from standard solutions of HNE-MA, DHN-MA, and ONO-MA in 2:8 acetontrile:water (both with 0.1 % formic acid). The ONO-MA concentrations ranged from 10 nM to 1.0 μM and included six points, while the DHN-MA and HNE-MA concentrations ranged from 50 nM to 5.0 μM and included seven points. HNE-MAd_3 (10 μl of a 10 μM solution), DHN-MAd_3 (5 μl of a 100 μM solution), and ONO-MAd_3 (10 μl of a 10 μM solution) were added as internal standards. The final volume of each standard solution was 100 μl.

A second calibration curve was prepared for the analysis of urinary cotinine. The curve, prepared in ethanol, included seven points with concentrations ranging from 0.1 nM to 1 μM. Cotinine-d_3 (5 μl of 10 μM) was used as the internal standard.

Standard Addition Curves

The standard addition curves were prepared by adding synthetic HNE-MA, DHN-MA, or ONO-MA and the corresponding internal standard to urine samples. One milliliter aliquots of urine were used for each point on the curve. The urine was acidified to pH 3 with 1 N HCl and spiked with varying concentrations of HNE-MA (0.5-5.0 μM), DHN-MA (0.1-5.0 μM), or ONO-MA (0.25-1.0 μM) and a fixed amount of internal standard, i.e., HNE-MAd_3 (20 μl of a 10 μM solution), DHN-MAd_3 (10 μl of a 100 μM solution), or ONO-MAd_3 (20 μl of a 10 μM solution). Samples were then vortex mixed and extracted with ethyl acetate (2 × 2 ml). The combined ethyl acetate layers were evaporated under N_2 and reconstituted in 200 μl of 2:8 acetonitrile:water containing 0.1 % formic acid.

HPLC

A Shimadzu Prominence HPLC system (Shimadzu, Columbia, MD) consisting of four LC-20AD pumps, a DQU-20A_5 degasser, and an SIL-HTc autosampler, equipped with switching valves, was used for all chromatography. For LPO product analyses, the HPLC column used was a 250 × 2 mm Synergi Max RP C_{12} column (Phenomenex, Torrance, CA). The mobile phase consisted of Solvent A, 0.1 % (v/v) formic acid in water, and Solvent B, acetonitrile containing 0.1 % (v/v) formic acid. The flow rate was 0.2 ml/min. A linear solvent gradient was used, running from 20 to 50 % B in 10 min, 50 to 90 % B over the next 2 min, held constant at 90 % B for 7 min, returned to 20 % B over 1 min, and equilibrated at 20 % B for 5 min. For analysis of cotinine, the HPLC column used was a 150 × 2 mm Synergi Hydro RP C_{18} column (Phenomenex). The mobile phase consisted of Solvent A, 0.1 % (v/v) formic acid in water, and Solvent B, acetonitrile containing 0.1 % (v/v) formic acid. The flow rate was 0.2 ml/min. Separations were carried out by isocratic elution at 5 % B with a run time of 5 min. Cotinine eluted at 2.33 minutes.

Mass Spectrometry

An Applied Biosystems MDS Sciex hybrid triple quadrupole/linear ion trap mass spectrometer (4000 QTrap) equipped with a TurboV electrospray source (Concord,

Canada) was used for these analyses. The TurboV source was maintained at 400 °C. The ionspray voltage was 4500 V and the declustering potential was 40 V. Nitrogen was used as the source gas, curtain gas, and collision gas. Various scanning techniques, all run in negative ion mode, were used for the characterization and detection of LPO-MA products, including Q1, product ion scanning, and selected reaction monitoring (SRM). All SRM transitions and collision energies for the LPO-MA conjugates are shown in Table 4.2. SRM in positive ion mode was used for the quantitation of cotinine. The transitions used were m/z 177 → 80 as the quantifier and m/z 177 → 98 as the qualifier.

TABLE 4.2
LC-MS/MS properties of ONE and HNE metabolites detected in the urine of human smokers and nonsmokers.

Analyte	MW	SRM Transition	Collision energy (eV)	Retention Time (min.)	Figure	Concentration (nM)[#]
ONE-MA	317	316 → 162	25	Peak not found		
ONE-MA-d_3	320	319 → 165	25	Peak not found		
HNE-MA	319	318 → 189*	25	10.5, 10.8	2	7.4-225
		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*	25	11.3	2	1.7-177
ONO-MA-d_3	322	321 → 165	25	11.3		
DHN-MA	321	320 → 191*	25	9.8	2, 3	6.6-316
	321	320 → 143	25	9.8		
DHN-MA-d_3	324	323 → 191	25	9.8		
	324	323 → 143	25	9.8		
ONA-MA	333	332 → 169	25	12.2	2	
	333	332 → 162	25	12.2		
ONA-MA-d_3	336	335 → 169	25	12.2		
	336	335 → 162	25	12.2		
HNA-MA	335	334 → 162	25	10.2	2	
	335	334 → 143	25	10.2		
HNA-MA-d_3	338	337 → 165	25	10.2		
	338	337 → 143	25	10.2		
HNAL-MA	317	316 → 162	25	13.2-13.6	2	
	317	316 → 143	25	13.2-13.6		
HNAL-MA-d_3	320	319 → 165	25	13.2-13.6		
	320	319 → 143	25	13.2-13.6		

*Quantifying transition. Other transitions used as qualifiers.
[#]Concentration is shown as a range encompassing the levels found for all study participants.

Data Analysis

Peak area analysis was performed using Analyst 1.4.1 (Applied Biosystems). Analyte peak areas were normalized for the internal standard peak area, a 2-fold sample

concentration, and for the creatinine concentration in mg/ml. Thus, all data are represented as mg/g creatinine unless otherwise stated. The standard addition curve samples were concentrated 5-fold during sample preparation and this was taken into account for the calculation of endogenous metabolite levels using these curves. LPO-MA is the sum of HNE-MA, DHN-MA, and ONO-MA concentrations represented as mg/g creatinine. Statistical comparisons were performed with GraphPad (San Diego, CA) using a paired or unpaired Student's *t* test as appropriate. Data are shown either as a range of concentrations or as mean ± S.D.

RESULTS

Quantitation of LPO products in Human Urine

We have developed an LC-MS/MS method for the simultaneous quantitation of HNE-MA, ONO-MA, and DHN-MA in human urine. Previous human studies have focused only on the quantitation of DHN-MA (22). A study in rats by Mally et al. (26) quantified both HNE-MA and DHN-MA, however they did not account for ONO-MA in their analysis.

Quantitation of HNE-MA

A calibration curve was constructed, using standard solutions containing varying concentrations of HNE-MA and a fixed concentration of HNE-MAd_3 (1.0 μM) as the internal standard. We also prepared a standard addition curve in order to assess the accuracy of our method and to investigate the possibility of matrix effects. Urine aliquots were spiked with a fixed amount of internal standard (10 μl of a 10 μM solution) and varying amounts (0.5-5.0 μM) of HNE-MA. Both curves were analyzed by LC-MS/MS (Fig. 4.2A). Extrapolation of the standard addition curve ($R^2 \geq 0.998$) to y = 0 gave a sample concentration of 910 nM HNE-MA. However, it was also necessary to account for concentration (5×) of the sample during preparation. Thus, the calculated 910 nM concentration divided by 5 gave the endogenous urinary HNE-MA concentration of 182 nM. This urinary concentration, calculated by the standard addition method, corresponded to a concentration of 203 ± 4.5 nM HNE-MA in aliquots (n = 3) of the same urine sample calculated using the calibration curve ($R^2 \geq 0.998$, Fig. 4.2A). The

lower limit of quantitation was determined to be 5 nM (S/N = 10) for these analyses, allowing for detection of 2.5 nM concentrations of HNE-MA since smoker and nonsmoker samples were concentrated 2× during sample preparation.

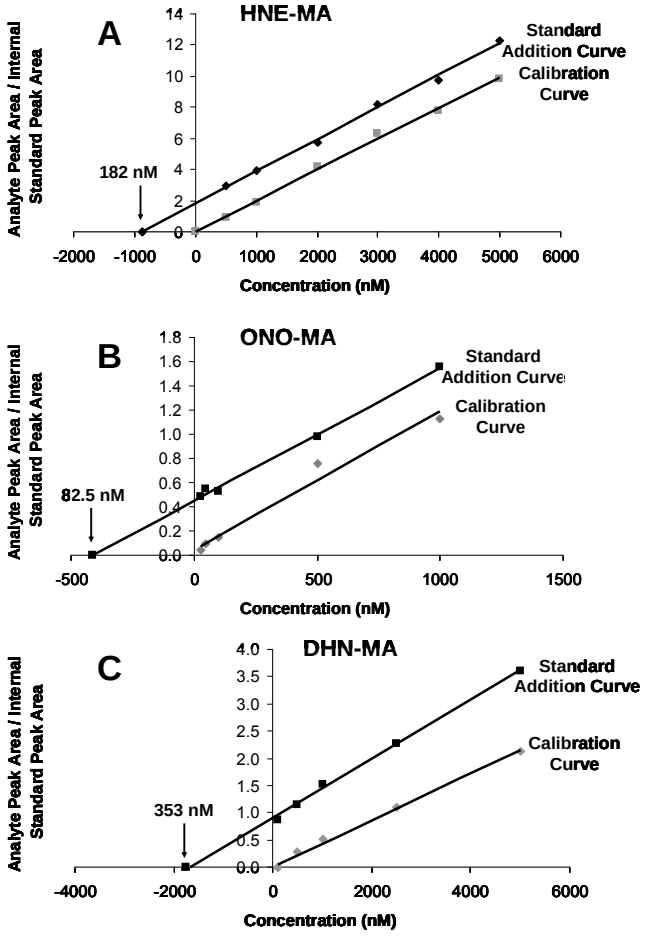

FIGURE 4.2 **Calibration curve and standard addition curve plots for HNE-MA (A), ONO-MA (B), and DHN-MA (C).** The calibration curves were derived from the analysis of synthetic standards ranging in concentration from 0.5 to 5.0 μM for HNE-MA, 0.25 to 1.0 μM for ONO-MA, and 0.1 to 5.0 μM for DHN-MA. All curves were constructed using a fixed concentration of internal standard (1.0 μM HNE-MAd_3, 1.0 μM ONO-MAd_3, and 5.0 μM DHN-MAd_3). The standard addition curves were prepared by spiking 1.0 ml aliquots of urine with a fixed amount of internal standard and known amounts of LPO-MA conjugate. Samples were concentrated five times during preparation. Extrapolation of the standard addition curve to y = 0 and division by 5 to account for concentration of the sample during preparation, gave endogenous urinary concentrations of 182 nM HNE-MA, 82.5 nM of ONO-MA, and 353 nM of DHN-MA.

Validation of HNE-MA Standard

A HNE-MA standard (1 mg in 100 μl ethanol) was purchased from Cayman Chemical for use to validate the concentration of our synthetically prepared material. In order to do this, a calibration curve was prepared using varying amounts (0.5-5.0 μM) of the Cayman HNE-MA standard and a fixed amount of HNE-MAd_3 (10 μl of a 10 μM solution). This curve was analyzed by LC-MS/MS and compared to the calibration curve prepared using HNE-MA synthesized in our lab. The curve prepared from HNE-MA prepared in our lab had a slope of 0.0020 and $R^2 \geq 0.997$, while the Cayman HNE-MA resulted in a curve with a slope of 0.0023 and $R^2 \geq 0.994$, demonstrating that our method of HNE-MA synthesis provided comparable results to those obtained with the commercial material.

Quantitation of ONO-MA

Varying concentrations of ONO-MA and a fixed concentration of internal standard ONO-MAd_3 (1.0 μM) were used to prepare a calibration curve. A standard addition curve was also prepared by spiking urine aliquots with a fixed amount of internal standard (10 μl of a 10 μM solution) and varying concentrations of ONO-MA (0.25-1.0 μM). Both curves were analyzed by LC-MS/MS (Fig. 4.2B). Extrapolation of the standard addition curve ($R^2 \geq 0.998$) to y = 0 (calculated concentration 412.5 nM) and accounting for sample concentration (5×) gave an endogenous ONO-MA concentration of 82.5 nM. This urinary concentration, calculated by the standard addition method, corresponded to a concentration of 67.1 ± 7.9 nM ONO-MA in aliquots (n = 3) of the same urine sample analyzed using the calibration curve ($R^2 \geq 0.975$, Fig. 4.2B). The lower limit of quantitation was determined to be 0.5 nM (S/N = 10) for these analyses, allowing for detection of 0.25 nM concentrations of ONO-MA since smoker and nonsmoker samples were concentrated 2× during sample preparation.

Quantitation of DHN-MA

A calibration curve was constructed, using varying amounts of DHN-MA and a fixed amount of DHN-MAd_3 (5.0 μl of a 100 μM solution) as the internal standard. We also prepared a standard addition curve, spiking urine aliquots with a fixed amount of internal

standard (5.0 μl of a 100 μM solution) and varying concentrations of DHN-MA (0.1-5.0 μM). Both curves were analyzed by LC-MS/MS (Fig. 4.2C). Extrapolation of the standard addition curve ($R^2 \geq 0.998$) to y = 0 (calculated concentration 1765 nM) and accounting for sample concentration (5×) gave an endogenous DHN-MA concentration of 353 nM. This urinary concentration, calculated by the standard addition method, corresponded to a concentration of 457 nM DHN-MA in an aliquot (n = 1) of the same urine sample analyzed using the calibration curve ($R^2 \geq 0.996$, Fig. 4.2C). The lower limit of quantitation was determined to be 10 nM (S/N = 10) for these analyses, allowing for detection of 5 nM concentrations of DHN-MA since samples were concentrated 2× during sample preparation.

Stability of Standards

The LPO-MA conjugate solutions used in this study were generally found to be stable over a period of six months when stored at -20 ^{o}C. HNA-MA is the one exception since it spontaneously converts to HNAL-MA and should be prepared fresh every few weeks. We found it best to assess the standards and internal standards by LC-MS/MS each time a batch of samples was run to ensure that the levels remained consistent over time, preparing new standards from the LPO product and MA if necessary.

LPO products in human urine

The LPO products, HNE-MA, DHN-MA, HNA-MA, HNAL-MA, ONO-MA, and ONA-MA were all detected in human urine samples (Fig. 4.3). We were not able to quantify HNA-MA, HNAL-MA, and ONA-MA in human urine. 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.

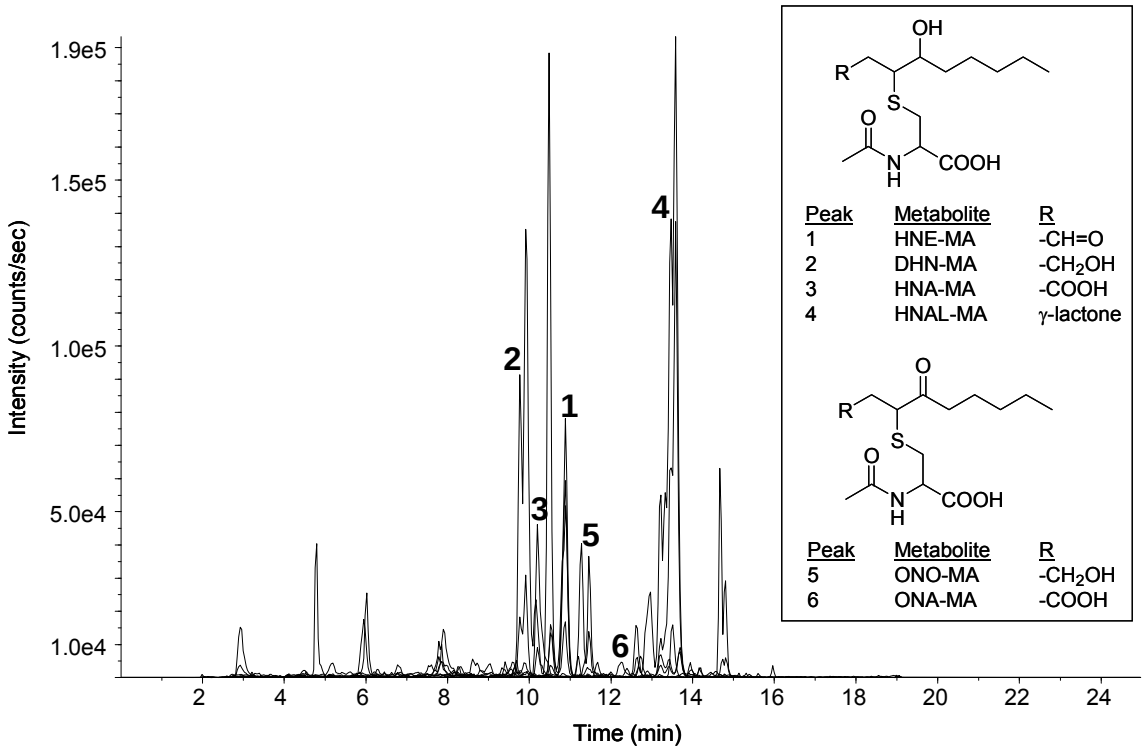

FIGURE 4.3 **LC-SRM chromatogram of a human urine sample.** Key to chromatographic peaks: (1) HNE-MA *m/z* 318 → 189 and *m/z* 318 → 171; (2) DHN-MA *m/z* 320 → 191 and *m/z* 320 → 143; (3) HNA-MA *m/z* 334 → 162; (4) HNAL-MA *m/z* 316 → 162 and *m/z* 316 → 143; (5) ONO-MA *m/z* 318 → 162; (6) ONA-MA *m/z* 332 → 169 and *m/z* 332 → 162. ONE-MA was not detected.

ONE-MA

It should be noted that while we have previously demonstrated the presence of ONE-MA in rat urine (20), 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. ONE is also able to covalently modify cysteine, histidine, and lysine residues in proteins (27,28), making it undetectable by our methods. Moreover, the GSH conjugate of ONE retains its ability to undergo Schiff base formation. It is also possible that thiadiazabicyclo-ONE-glutathione (TOG), a metabolite of ONE in cultured endothelial (EA.hy 926) cells (29), is formed *in vivo*. The formation of TOG involves cyclization of the γ-glutamic acid residue of ONE-GSH, preventing further metabolism of the GSH moiety to form ONE-MA. TOG formation is likely a

minor pathway, however, since we are able to quantify ONO-MA levels and to detect ONA-MA in the human urine samples.

Metabolite confirmation

Enhanced product ion (EPI) scanning, along with the comparison of biological samples to synthetic standards prepared in our lab, was used to ensure correct metabolite identification. EPI was performed by selecting a m/z of interest in Q1, inducing fragmentation in Q2, then utilizing the linear ion trap mode to trap these fragments in Q3, followed by Q3 scanning in quadrupole mode. This technique allows for the sensitive MS/MS comparison of analytes in synthetic and biological samples. Figure 4.4 shows EPI spectra in negative ion mode for synthetic and biological DHN-MA, which eluted at 9.8 min (Fig. 4.4A, 4.4C). Both of these samples demonstrate that DHN-MA produces fragments with *m/z* 191 and *m/z* 143, both β-elimination fragments and *m/z* 173, formed via a McLafferty rearrangement (Fig. 4.4B, 4.4D). Similar experiments were performed for HNE-MA and ONO-MA in our previous work (20).

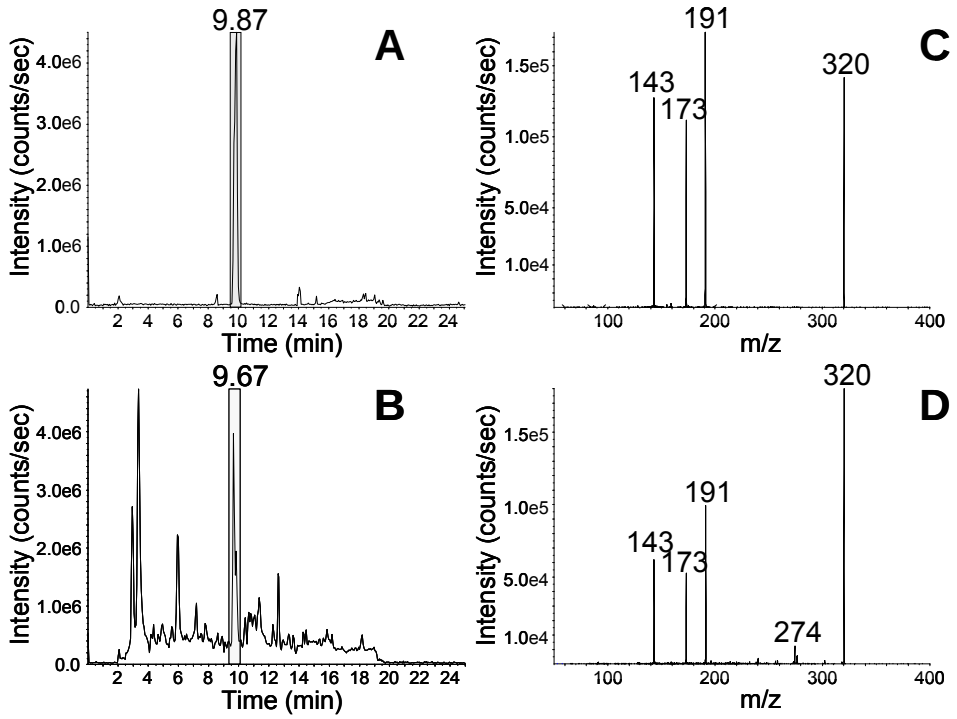

FIGURE 4.4 **LC-EPI chromatograms of a DHN-MA synthetic standard and a human urine sample.** (A) Negative ion electrospray EPI scanning of m/z 320 of a standard reaction mixture of DHN-MA. (B) Negative ion electrospray EPI scanning of m/z 320 of a human urine sample. (C) The EPI spectrum of synthetic DHN-MA shows fragments with *m/z* 191, *m/z* 143, and *m/z* 173. (D) The EPI spectrum of endogenous DHN-MA in a human urine sample shows the same fragments as the synthetic DHN-MA sample, with *m/z* 191, *m/z* 143, and *m/z* 173.

Smoking Cessation

Smoking cessation caused significant decreases in the urinary levels of HNE-MA, DHN-MA, and LPO-MA present in a group of seven human subjects (Fig. 4.5). There was no significant change in any of these metabolites over the same time period in nonsmoking subjects (paired Student's *t* test, $p > 0.05$). We also did not find any significant differences in metabolite levels between the smoker and nonsmoker groups prior to or following cessation (unpaired Student's *t* test, $p > 0.05$). Neither did we find any significant correlation between LPO-MA and age ($p = 0.88$) or BMI ($p = 0.35$). All calculations included normalization for creatinine levels to account for variation in urine concentration between individuals. There was no significant difference in the urinary creatinine levels before and after smoking cessation ($p = 0.45$).

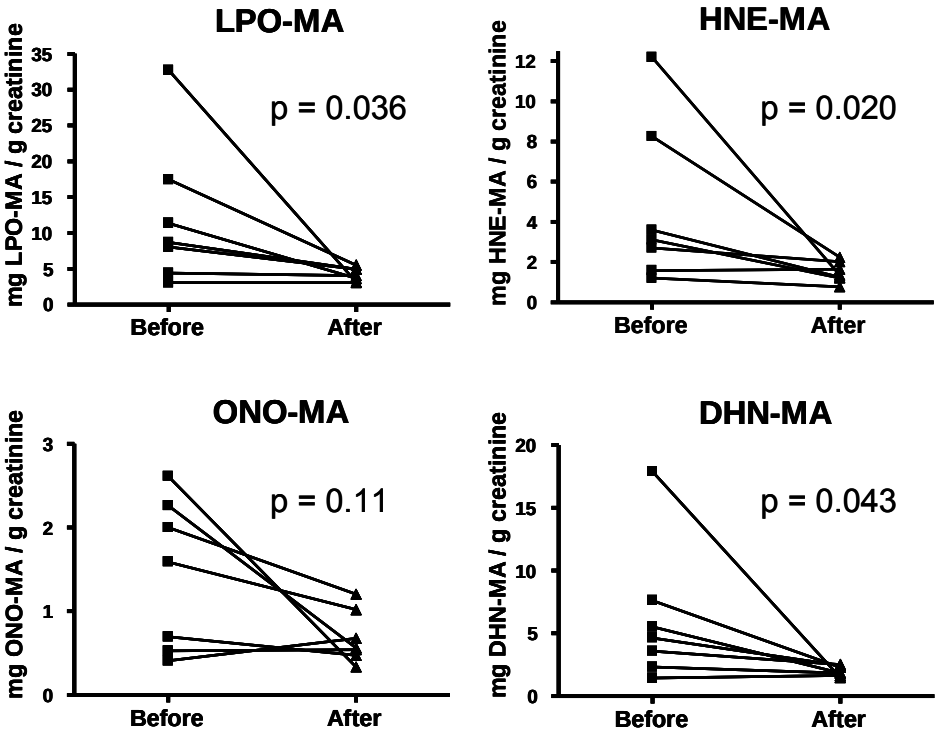

FIGURE 4.5 **Comparison of LPO metabolites in smokers before and after smoking cessation.** HNE-MA, DHN-MA, and LPO-MA were significantly decreased after 12 weeks of smoking cessation in humans with p values of 0.020, 0.043, and 0.036 respectively. LPO-MA is the sum of HNE-MA, DHN-MA and ONO-MA. Data were analyzed on a logarithmic scale.

DISCUSSION

A semi-quantitative method for analysis of HNE-MA and ONE-MA metabolites in the urine of oxidatively stressed rats was previously reported (20). While this method allowed for the simultaneous analysis of multiple LPO-MA conjugates, the data was not quantitative. Appropriate internal standards for each of our analytes of interest are necessary in order to perform absolute quantitation. We first synthesized MAd_3 following the method of Slatter et al. (25). MAd_3 conjugates of HNE, DHN, HNA, ONE, ONO, and ONA, were then prepared as described by Kuiper et al. (20). Quantitation of endogenous LPO-MA conjugates was subsequently achieved by isotope-dilution LC-MS/MS using SRM. We now demonstrate, for the first time, the quantitative

determination of ONO-MA in addition to HNE-MA and DHN-MA *in vivo* at low mg/g creatinine levels.

In smokers and nonsmokers, we found the urinary levels of ONO-MA to be in the range 0.05-2.26 mg/g creatinine (1.7-177 nM). HNE-MA was present in the range of 0.17-12.19 mg/g creatinine (7.4-225 nM) and DHN-MA at levels of 0.22-17.90 mg/g creatinine (6.6-316 nM). Low LPO-MA conjugate levels in a subgroup of the smokers resulted in the lack of statistical difference between the smoker and nonsmoker groups prior to smoking cessation. Alary et al. (22) also assessed DHN-MA in humans and found production of 5 μg/24 h in seven healthy human volunteers, which corresponds to 2.7 ng/ml (8.4 nM). These levels are comparable to the low levels of DHN-MA in our study. In a study of the urinary excretion of LPO-MA conjugates in rats, Mally et al. (26) measured 113.8 ± 36.8 pmol/mg creatinine for HNE-MA (36 μg/g creatinine) and 1.19 ± 0.33 nmol/mg creatinine for DHN-MA (382 μg/g creatinine). Rathahao et al. (21) and Guéraud et al. (23) found urinary production of DHN-MA in rats to be in the range of 45-230 ng/24 h (equivalent to 8.8-45 nM, assuming a urine production of 16 ml/24 h), with the higher concentrations appearing in $BrCCl_3$ stressed animals. Alary et al. (22) also analyzed rat urine and found DHN-MA production of 10 ng/24 h or 0.8 ng/ml (2.5 nM). Urinary levels of DHN-MA in lean and Zucker obese rats (1.1 μM and 2.9 μM, resp.), reported by Orioli et al. (24), however, are much higher than the other reported values. With the exception of the levels reported by Orioli et al. (24), urinary levels of HNE-MA and DHN-MA in rats seem to be lower than or at the low end of the range of human levels determined in Alary's study (22) and in our present study. Since the urinary levels of LPO-MA conjugates in rats tend to fall within the same range as the levels we found in human samples, our quantitation method should be applicable to animal investigations as well.

Like HNE and ONE, $F_{2\alpha}$-isoprostanes are formed from lipid hydroperoxides via radical-mediated pathways. $F_{2\alpha}$-isoprostanes are generally considered to be the most reliable markers of *in vivo* oxidative stress (30,31). Smoking cessation has been shown to result in significant decreases of urinary $F_{2\alpha}$-isoprostane levels after one or two weeks (32,33). The study conducted by Chehne et al. (33) demonstrated that the decrease of urinary $F_{2\alpha}$-isoprostane levels upon smoking cessation was similar between patients

having clinically manifested atherosclerosis with or without hypercholesterolemia and/or hypertension, indicating that cigarette smoke is a major contributor to *in vivo* oxidative stress compared to other risk factors of atherosclerosis. Similar to urinary $F_{2\alpha}$-isoprostane levels, our study of apparently healthy participants showed significant decreases in the levels of MA conjugates of HNE and DHN in the urine upon smoking cessation, reflecting a similar pathway of formation via lipid hydroperoxides.

The LPO metabolites of our study differ from the $F_{2\alpha}$-isoprostanes in that they are also products of phase I and phase II metabolism. Thus, the levels of MA conjugates of HNE, DHN, and ONO reflect both formation of HNE and ONE and their subsequent metabolism. Expression levels of GSTs may therefore co-determine urinary levels of HNE-MA, DHN-MA, and ONO-MA. GSTP1, a GST isoenzyme involved in HNE conjugation (34,35), was induced in lung tissue of smokers whereas other GSTs, GSTA2 and GSTM1, showed no difference in expression levels between smokers and nonsmokers (36). On the other hand, genetic polymorphism of GST may affect gene expression if the mutation is located in the promoter region. Qian et al. (37) studied single nucleotide polymorphisms (SNPs) of GSTA4, another GST that accepts HNE as a substrate (38). Qian et al. (37) found that the presence of genotypes TA and AA at locus –1718 of GSTA4 was associated with a 37 % significantly decreased risk of lung cancer compared to the TT genotype. The authors suggested that the TA and AA genotypes, with the SNP in the promoter region, may have increased GSTA4 expression and thus greater capacity to detoxify HNE as compared to the TT genotype. Dwivedi et al. (39) determined that GSTA4 null mice have higher levels of hepatic HNE after CCl_4 treatment than wild-type mice, indicating reduced HNE conjugation in GSTA4 null mice. In the study by Qian et al. (40), the TT genotype had a prevalence of 77 % in lung cancer patients (n = 500) and 68 % in cancer-free control subjects (n = 517). The common occurrence of the TT genotype, presumably having reduced GSTA4 expression and reduced capacity to conjugate HNE, may explain the low urinary levels of LPO-MA (< 7 mg/g creatinine) we found in 9 out of 23 smokers.

The low LPO-MA excretion in nine smokers may also be due to smoking-induced phase I metabolism, resulting in enhanced conversion of HNE and ONE into DHN which is not a GST substrate. Aldo-keto reductase 1B10 (AKR1B10) is known to reduce HNE

to DHN and to reduce ONE to ONO (41). Its up-regulation in smokers, shown by Fukumoto et al. (42) and Nagaraj et al. (43), would direct the metabolism of HNE to DHN, resulting in decreased formation of GST-mediated metabolites and MA conjugates (Scheme 1).

We developed a method for the accurate quantitation of ONO-MA, HNE-MA, and DHN-MA in human urine by isotope-dilution LC-MS/MS. We also detected HNA-MA, HNAL-MA, and ONA-MA. The significance of the *in vivo* detection of ONO-MA and ONA-MA is that these conjugates represent HNE/ONE branching in the breakdown of lipid hydroperoxides as shown in Scheme 1, suggesting that ONO may contribute to the deleterious effects previously ascribed to HNE. Our findings also show that LPO-MA conjugates are elevated in urine obtained from smokers and decrease significantly following smoking cessation, demonstrating the utility of these metabolites as markers of *in vivo* oxidative stress.

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CHAPTER 5

LIPID PEROXIDATION PRODUCTS DERIVED FROM ω-3 POLYUNSATURATED FATTY ACIDS

INTRODUCTION

The LPO products detailed thus far are derived from ω-6 PUFAs under conditions of oxidative stress. Similar six carbon breakdown products can be generated from ω-3 PUFAs (Fig. 5.1).

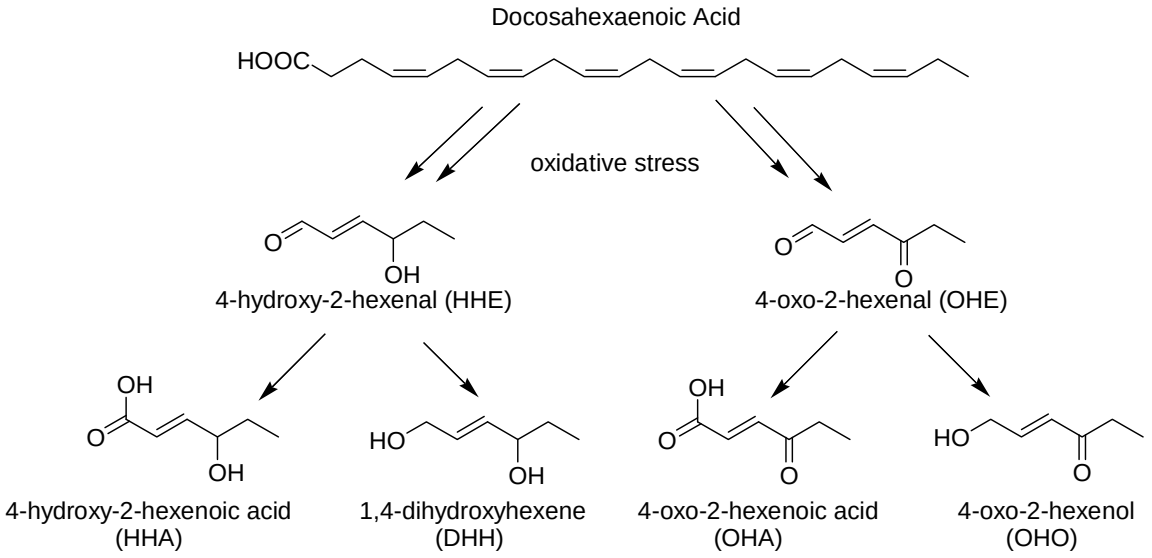

FIGURE 5.1 **Formation of LPO products from DHA.** DHA breaks down under conditions of oxidative stress to form HHE, OHE, and their phase I metabolites HHA, DHH, OHA, and OHO.

Van Kuijk *et al.* (1) demonstrated the specific formation of 4-hydroxy-2-hexenal (HHE) from docosahexaenoic acid (DHA). In a metabolism study by Winter *et al.* (2), rats were dosed with radiolabeled HHE and HHE-MA was detected in their urine. Multiple studies have assessed a panel of PUFA degradation products, including HHE, HNE and other hydroxy-alkenals (3-6). 4-Hydroxy-2-hexenoic acid (HHA) was found to be a prominent ω-3 PUFA metabolite in two of these studies (4,5). Although 4-oxo-2-hexenal (OHE) and its metabolites have received less attention than their hydroxy counterparts, Kasai *et al.* (7) found OHE to be mutagenic in the Salmonella mutagenicity assay. Maekawa *et al.* (8) also characterized a OHE and its 2′-deoxyguanosine adduct. Most LPO product research has focused on metabolites of the ω-6 pathway and their utility as biomarkers of oxidative stress. However, it is also likely that these ω-3 PUFA derived LPO products have relevance as biomarkers of oxidative stress. Due to the high concentration of DHA present in brain (9,10), it is possible that these LPO products could be even more relevant

as biomarkers of Alzheimer's disease (AD). It has been demonstrated that DHA is highly susceptible to oxidation due to its six double bonds (9), therefore increasing the likelihood of LPO product formation in the oxygen rich brain (11). Long *et al.* (12) have even determined that HHE is a neurotoxic aldehyde formed when DHA is oxidized.

AD is the most common form of dementia in older adults and is currently the fourth leading cause of death in the United States (11,13). Millions of people worldwide are affected by AD and as life expectancy increases, the number of people affected by AD will continue to increase (14). AD is characterized by severe neurodegeneration due to the formation of amyloid-β plaques and hyperphosphorylated tau protein in the brain (11,14). It has been suggested that these pathological alterations are caused by mitochondrial dysfunction, increased apoptosis (15), and oxidative stress (16,17). At this point in time, there is not a valid method for diagnosis of AD prior to the onset of dementia (15). Therefore, developing biomarkers to aid in early diagnosis is of the utmost importance. Early detection will not only allow for early treatment, but could decrease the psychological and financial burden on AD patients and their families.

We began the process of investigating LPO products formed from DHA and their conjugation reactions with GSH and MA as possible biomarkers of AD and oxidative stress respectively. OHE, 4-oxo-2-hexenol (OHO), and 4-oxo-2-hexenoic acid (OHA) were synthesized in our laboratory (Fig. 5.2), as were their GSH and MA conjugates of OHE and OHO.

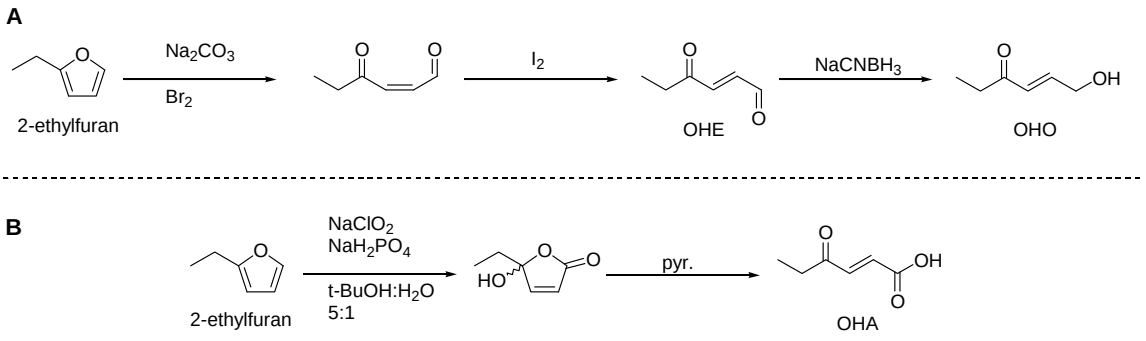

FIGURE 5.2 **Synthetic routes to OHE, OHO, and OHA.** OHE was synthesized from 2-ethylfuran by oxidation and isomerization. OHO is prepared by reduction of OHE. OHA is synthesized from 2-ethylfuran by oxidation and tautomerization.

SYNTHESIS OF OHE, OHO, AND OHA

OHE was synthesized from 2-ethylfuran following the method of Zhang *et al.* (18) (Fig. 5.2, A). To a stirred solution of acetone-H_2O (85/15, v/v, 200 ml) was added 2-pentylfuran (20 mmol) and 10 g of anhydrous Na_2CO_3. The reaction was cooled to -15 ^oC and a solution of bromine (20 mmol) in acetone-H_2O (4/1, v/v, 30 ml) was added dropwise over 30 min. The reaction was allowed to slowly return to room temperature, with continuous stirring for 2 h. The reaction was filtered to remove Na_2CO_3 and extracted with ether. The combined either layers were washed with brine, dried with $MgSO_4$ and filtered. Iodine was added and the reaction was stirred at room temperature for 4 h, washed with saturated NaS_2O_3, washed with brine, dried with $MgSO_4$, filtered, and concentrated in vacuo. The material was purified on a silica column with hexane-ether 10:1 as the eluting solvent. Data are ^1H NMR (400 MHz, $CDCl_3$) δ 9.80 (d, J = 7 Hz, 1H), 6.91 (d, J = 16 Hz, 1H), 6.80 (dd, J = 7, 16 Hz, 1H), 2.75 (q, J = 7 Hz, 2H), 1.18 (t, J = 7, 3H) (Fig. 5.3).

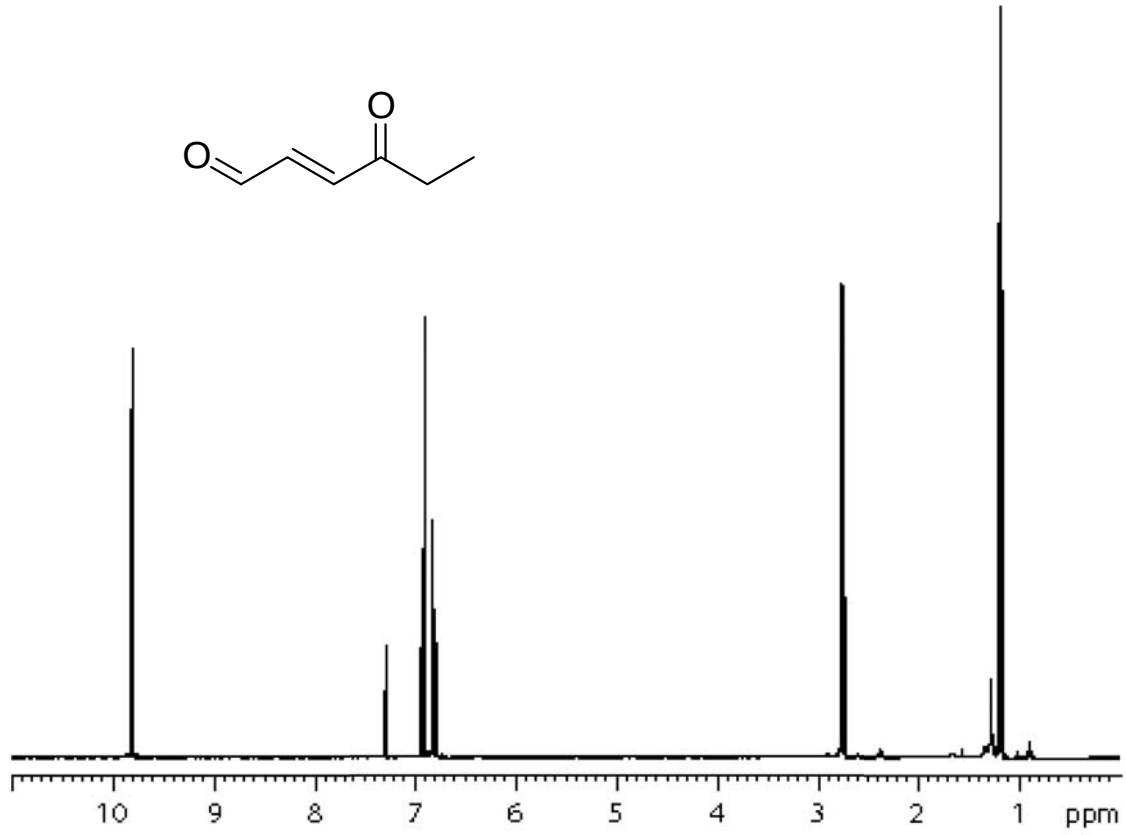

FIGURE 5.3 **400 MHz ^{1}H NMR of OHE in $CDCl_3$**

OHO was synthesized from OHE using the reduction procedure for ONO formation from Kuiper *et al*. (19) (Fig. 5.2, A). To a stirred solution of OHE (1 mmol) in 5 ml of methanol were added 29 ml of phosphate buffer (0.1 M, pH 3) and 280 μl of a 5 M sodium cyanoborohydride solution in 1 N NaOH. After stirring for 15 h at room temperature, the reaction mixture was extracted with ethyl acetate and the organic layer was concentrated *in vacuo* to yield OHO. Data are ^{1}H NMR (300 MHz, $CDCl_3$) δ 6.89 (d, J = 16 Hz, 1H), 6.36 (d, J = 16 Hz, 1H), 4.35 (br s, 2H), 2.47 (q, J = 7 Hz, 2H), 1.13-1.03 (br t, 3H).

OHA was synthesized from 2-ethylfuran, following the method of Annangudi *et al.* (20) with slight adaptation to the sample purification (Fig. 5.2, B). To a stirred solution of 5:1 t-BuOH-H_2O was added 2-pentylfuran (2 mmol), KH_2PO_4 (3 mmol), and $NaClO_2$ (6 mmol). The mixture was stirred at 4^{o}C for 1.5 h. The solvent was removed by rotary evaporation, and the residue was extracted with $CHCl_3$. It was necessary to add

5 ml of H_2O to separate the layers. Upon extraction, the combined organic layers were washed with brine, dried with $MgSO_4$, and filtered and concentrated *in vacuo*. The residue was taken up in tetrahydrofuran-acetone-H_2O (5/4/1, v/v/v, 40 ml), 200 µl of pyridine was added, and the reaction was stirred at room temperature for 2 h. The solvent was then removed by rotary evaporation and the residue was brought up in ether, followed by extraction with H_2O (pH 10, 1.3 M NaOH). Following extraction, the H_2O layers were acidified with 1 N HCl to pH 2 and extracted with ether. The combined ether layers were dried with $MgSO_4$, filtered, and concentrated *in vacuo*. Data are ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.13 (d, J = 16 Hz, 1H), 6.68 (d, J = 16 Hz, 1H), 2.70 (q, J = 7 Hz, 2H), 1.14 (t, J = 7 Hz, 3H) (Fig. 5.4). ^{13}C NMR (75 MHz, $CDCl_3$) δ 7.6, 35.0, 130.0, 140.6, 170.0, 200.4 (Fig. 5.5).

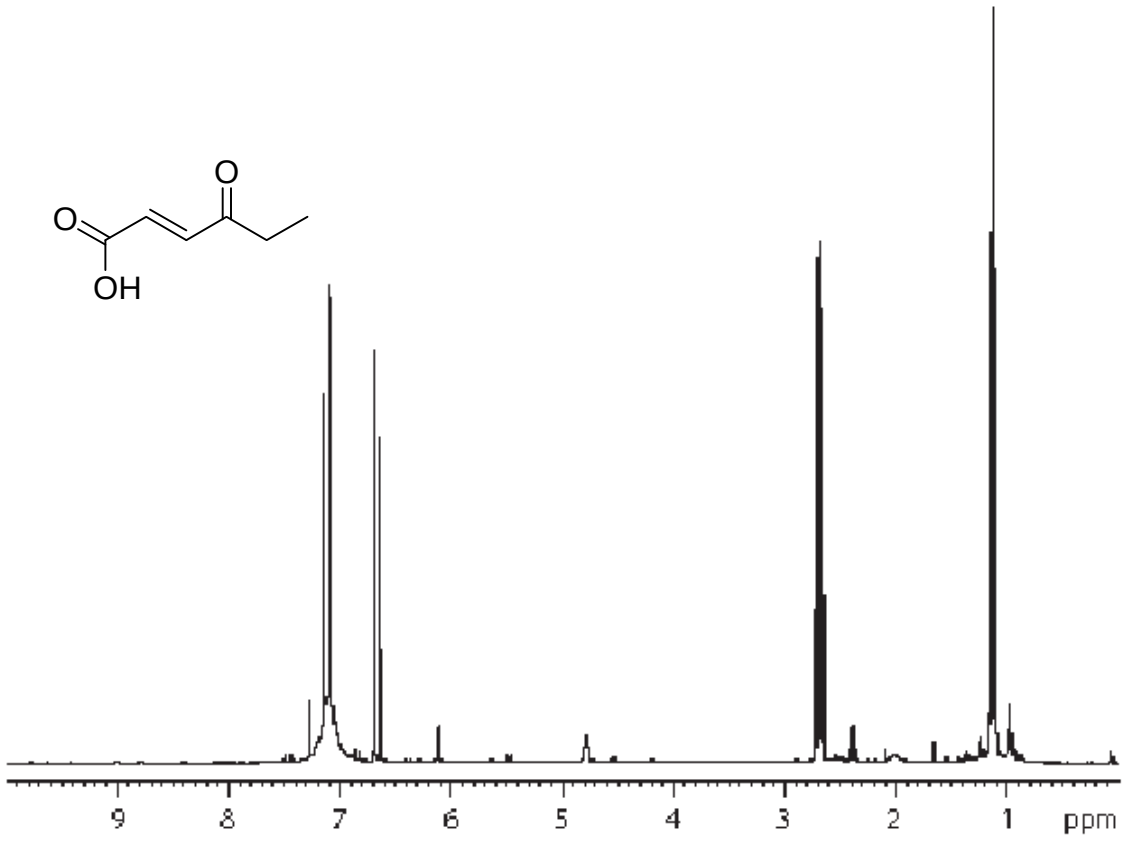

FIGURE 5.4 **300 MHz ^{1}H NMR of OHA in $CDCl_3$**

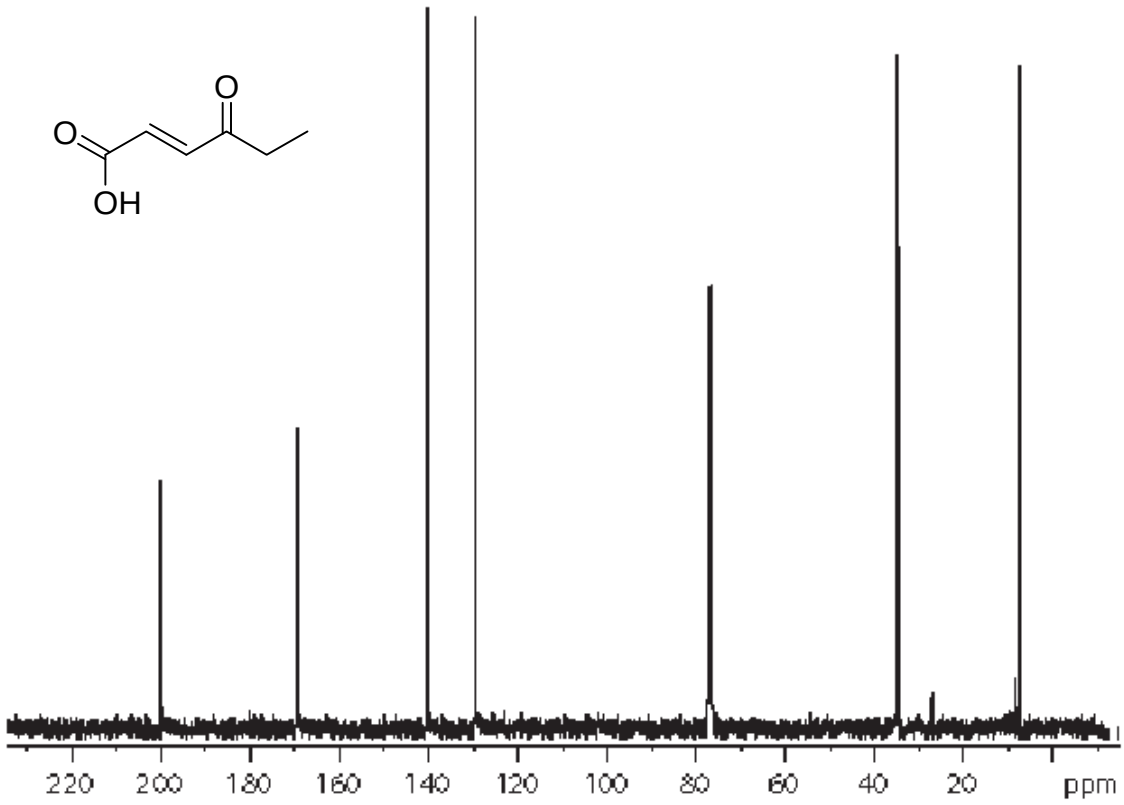

FIGURE 5.5 **75 MHz ^{13}C NMR of OHA in $CDCl_3$**

LPO-GSH and LPO-MA adducts were synthesized in the manner detailed in Chapter 2.

ANALYTICAL METHODS AND BIOLOGICAL SAMPLES

LC-MS and LC-MS/MS analyses were carried out as described in Chapter 3 (method 1) and Chapter 4 (LPO-MA method). SRM transitions, collision energies, and retention times are shown in Table 5.1 and spectra are Figures 5.6-5.9.

Table 5.1 LC-MS/MS properties of OHE metabolites

Analyte	MW	SRM transition	Collision energy (eV)	Retention time (min.)
OHE-GSH	419	418 → 382	25	3.1
		418 → 306	25	
		418 → 272	25	
OHO-GSH	421	420 → 306	25	3.0
		420 → 272	25	
		420 → 143	25	
OHE-MA	275	274 → 162	25	Multiple peaks from 5.5-7.7
		274 → 84	25	
OHO-MA	277	276 → 162	25	5.3
		276 → 147	25	
		276 → 129	25	

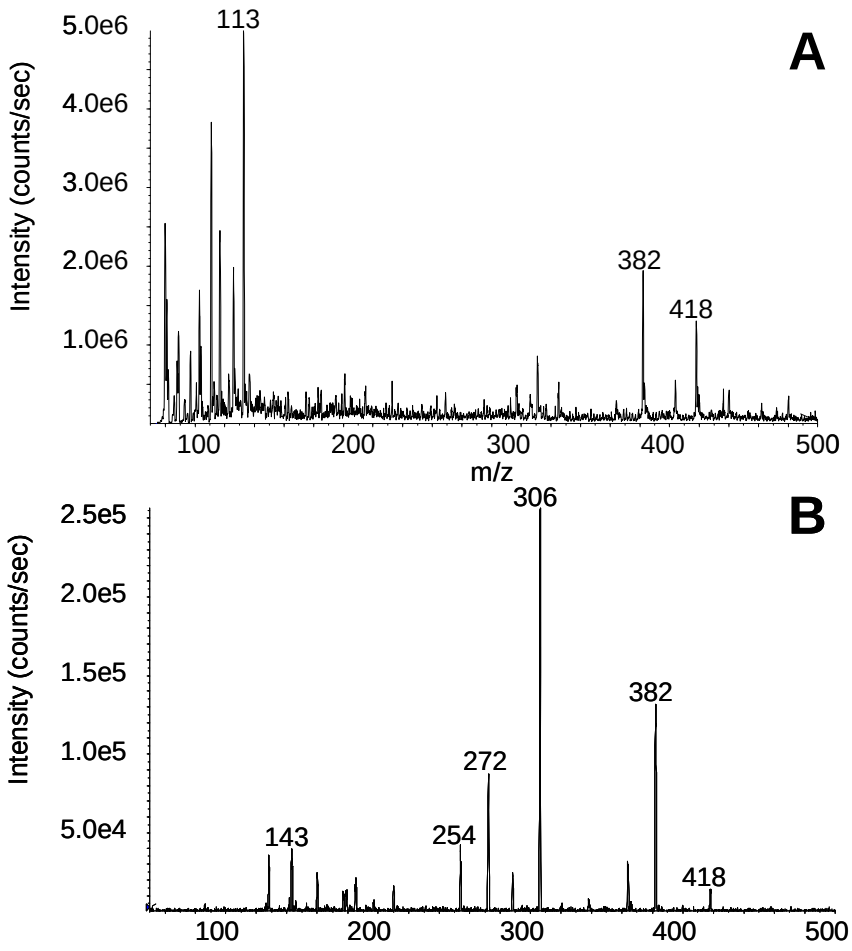

FIGURE 5.6 **Mass Spectra for OHE-GSH.** (A) Q1 spectrum (B) MS/MS spectrum of *m/z* 418.

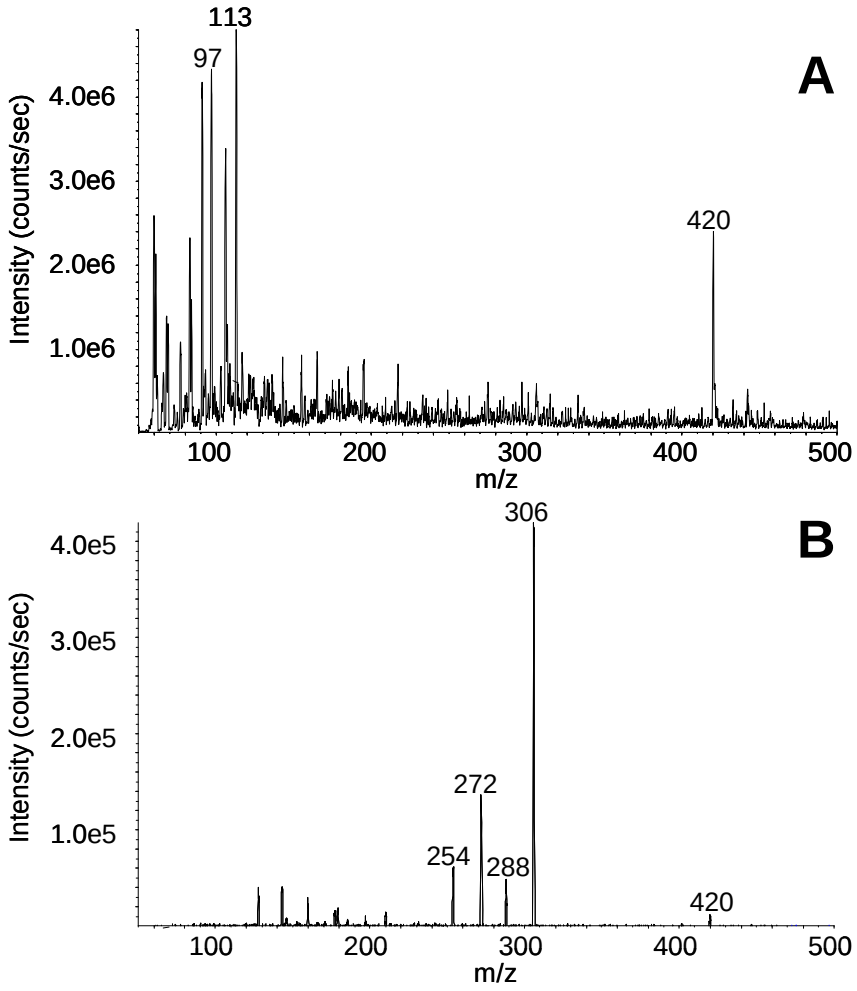

FIGURE 5.7 **Mass Spectra for OHO-GSH.** (A) Q1 spectrum (B) MS/MS spectrum of *m/z* 420.

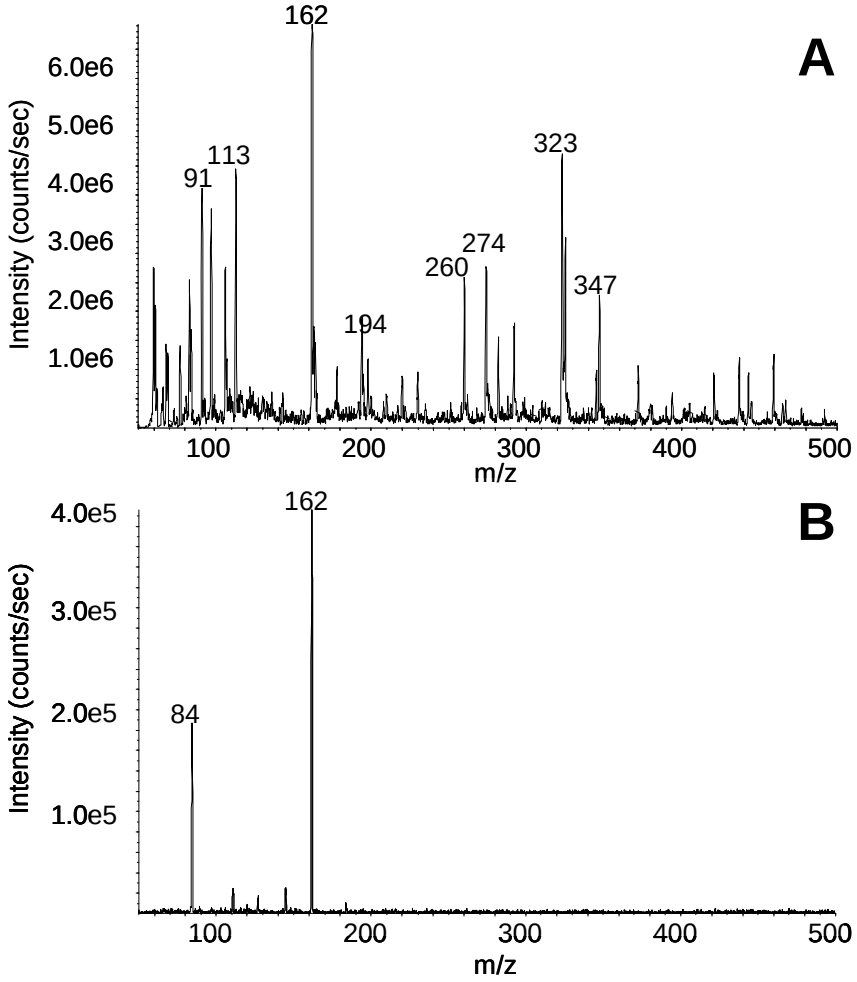

FIGURE 5.8 **Mass Spectra for OHE-MA.** (A) Q1 spectrum (B) MS/MS spectrum of *m/z* 274.

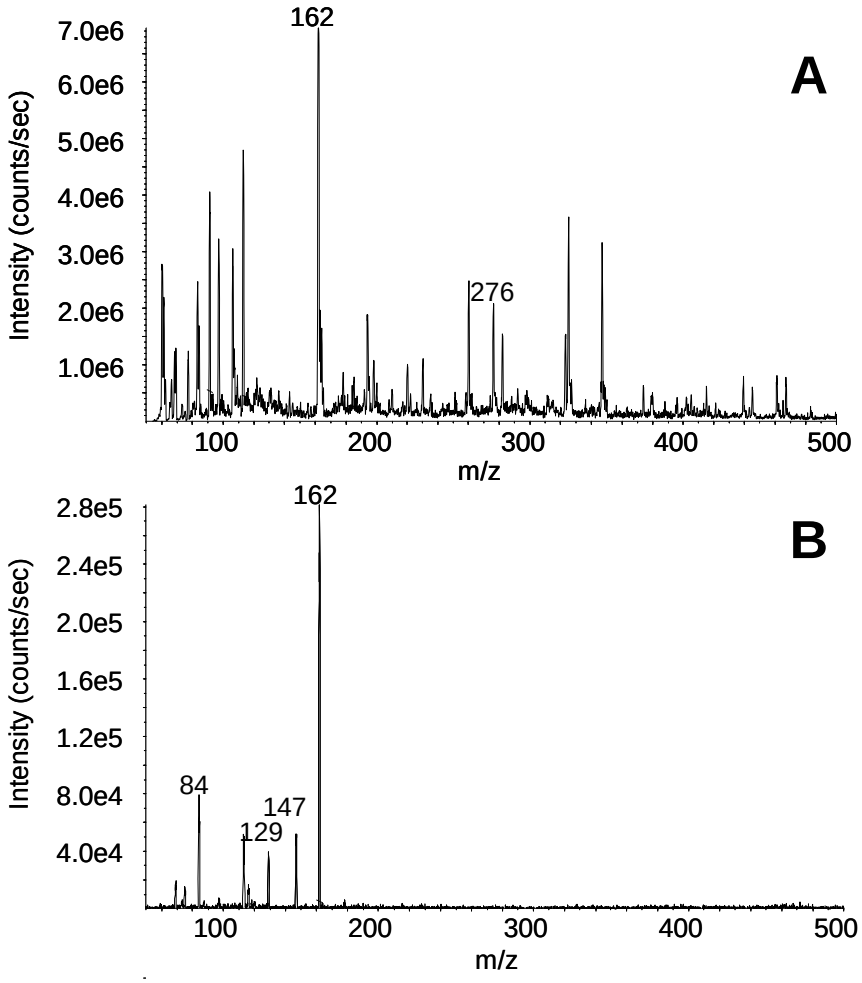

FIGURE 5.9 **Mass Spectra for OHO-MA.** (A) Q1 spectrum (B) MS/MS spectrum of *m/z* 276.

Due to time constraints, this was as far as the research progressed. The goal was to complete the synthesis of all six LPO products (Fig. 5.1), as well as their GSH and MA conjugates. We then planned to analyze urine samples to assess the level of these LPO-MA conjugates compared to the ω-6 derived LPO-MA. More importantly, we planned to analyze cerebral spinal fluid samples from AD patients and non-AD patients for these metabolites. This study would have allowed us to assess whether or not our LPO-GSH conjugates play an important role in detoxification of LPO products in the brain of AD patients and whether or not they show potential as biomarkers for detection of AD.

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CHAPTER 6

CONCLUSION

SUMMARY OF THE RESEARCH

The goal of this work was to evaluate the diagnostic value of LPO product metabolites as biomarkers of oxidative stress. The significance of the research is that reliable assessment of oxidative stress *in vivo* may prompt early therapeutic intervention or lifestyle changes before the onset of age-related disease. We determined the levels of LPO product metabolites in two biological models of oxidative stress: rats dosed with CCl_4 and cigarette smoking. LPO products HNE, DHN, HNA, ONE, ONO, and ONA as well as their GSH, CG, and MA conjugates were synthetically prepared in our laboratory, to be used as standards in our LC-MS/MS assays. A comparison of metabolite levels in the two *in vivo* models of oxidative stress has demonstrated that LPO metabolites are present at significantly higher levels under conditions of oxidative stress.

In order to accurately quantify these compounds, appropriate internal standards for each metabolite were needed. Multiple compounds were assessed as possible internal standards, resulting in the conclusion that using isotopomers of our metabolites was preferable to using compounds that were just structurally similar. Preparing labeled versions of each LPO product, however, would have been work intensive and time consuming. Conveniently, at this time we were having difficulty obtaining consistent results from plasma sample preparation methods and decided to focus on analysis of urinary metabolites instead. Only MA conjugates are present in the urine, limiting the number of metabolites for analysis. This also simplified matters regarding our internal standard choice. We were able to prepare deuterium labeled MA and react it with each LPO product, thereby obtaining all the internal standards necessary for quantitation.

LC-MS/MS conditions for the simultaneous analysis of these conjugates have also been established. This method development was not trivial due to the fact that HNE-MA and ONO-MA are isobaric. Many different chromatographic conditions were used in attempt to achieve separation between these conjugates; however, due to their multiple isomers, only limited separation was accomplished by HPLC. Conveniently, enough separation was possible when SRM analyses were performed, the transition *m/z* 318 → 162 contained a peak that was present in only the ONO-MA standard and not the HNE-MA standard. Indeed, analysis of a urine sample by EPI scanning demonstrated that this peak predominately contained the *m/z* 162 fragment that corresponds to the retro-Michael

cleavage product of ONO-MA. The preferred conformation of HNE-MA is a hemi-acetal, preventing it from undergoing retro-Michael and limiting its ability to form a fragment with *m/z* 162. The metabolites ONE-MA and HNAL-MA are also isobaric. They have enough structural differences, however, that simply developing a second chromatographic method provided a means to distinguish between them.

Once analytical methodology was developed for the LPO-MA conjugates, we proceeded to assess their presence in biological samples. In a study of CCl_4 dosed rats, we were able to distinguish between HNE-MA and its isomer ONO-MA. This is the first demonstration of the *in vivo* formation of ONO-MA. This research also allowed us to provide the first *in vivo* evidence for ONE-MA and ONA-MA. CCl_4 treatment is a model of oxidative stress, allowing us to determine what happens to HNE, ONE, and their metabolites *in vivo* under conditions of oxidative stress. We found that urinary levels of DHN-MA, HNAL-MA, ONE-MA, and ONA-MA were significantly higher in the CCl_4 dosed rats than in the control animals. This semi-quantitative data suggests that the preferred metabolic pathway for HNE and ONE metabolism includes oxidation and conjugate formation, because HNAL-MA and ONA-MA were found to be the most abundant metabolites. The preferred order for the oxidation and conjugation reactions has yet to be elucidated.

Following our semi-quantitative analyses, we set out to perform absolute quantitation of the LPO-MA metabolites. Deuterium labeled MA (MAd_3) was synthesized and reacted with each LPO product of interest for use as internal standards. This material allowed for successful quantitation of HNE-MA, ONO-MA, and DHN-MA by isotope-dilution LC-MS/MS. Human smoking cessation samples were then analyzed to assess absolute levels of HNE-MA, ONO-MA, and DHN-MA in smokers before and after cessation. We were able to demonstrate quantitative determination of ONO-MA for the first time *in vivo*, in addition to providing quantitative determination of HNE-MA and DHN-MA. The HNE-MA and DHN-MA metabolites have previously been quantified; however, most of these measurements were performed using rat samples. DHN-MA is the only LPO-MA conjugate previously quantified in humans. Our method has the added benefit of simultaneous quantitation of multiple LPO-MA conjugates, giving a better overall picture of LPO metabolism in human systems. We were also able to provide

qualitative assessment of HNA-MA, HNAL-MA, and ONA-MA in human urine. Our findings demonstrated that the LPO-MA conjugate levels in smoker urine decrease significantly following 12 weeks of smoking cessation, demonstrating the utility of these metabolites as biomarkers of oxidative stress.

ONE-MA could not be detected in the human urine samples. Because we were previously able to detect ONE-MA in rat urine samples, it is likely that metabolism of ONE-MA to its reduction product ONO-MA, oxidation product ONA-MA, or both is predominately occurring. Indeed, we were able to quantify ONO-MA in human urine, and qualitatively assess ONA-MA. The ONA-MA was present in concentrations < 10 nM, values too low for precise and accurate quantitation using isotope-dilution LC-MS/MS. The presence of ONO-MA and ONA-MA suggests that in humans, HPNE can be both reduced to form HNE, or undergo H_2O loss to form ONE; and that both pathways are important for the metabolism and excretion of LPO products.

While the ω-3 PUFA derived LPO products discussed in Chapter 5 were not assessed in biological samples due to time constraints, they do represent an important pathway of LPO product formation and metabolism. In conjunction with the ω-6 PUFA derived LPO products that are the focus of this thesis; these six carbon metabolites have potential utility as biomarkers of oxidative stress.

The overall purpose of this research to develop synthetic and analytical methods for the assessment of LPO product metabolites in biological samples as biomarkers of oxidative stress has been realized. The presence of HNE-MA isomer ONO-MA was demonstrated for the first time *in vivo* along with ONE-MA and ONA-MA. In human urine samples, ONO-MA was quantified for the first time, using a method that allowed for the simultaneous quantitation of HNE-MA and DHN-MA. In rats dosed with CCl_4 to induce oxidative stress, higher levels of LPO-MA metabolites were present than in the control animals. In human smokers, LPO-MA levels were demonstrated to decrease following smoking cessation. Taken together, this work demonstrates that levels of LPO-MA conjugates are increased *in vivo* under conditions of oxidative stress and have utility as biomarkers of oxidative stress.

BIOMARKING OXIDATIVE STRESS: QUO VADIS?

LPO-MA conjugates are elevated under conditions of oxidative stress which demonstrates that these compounds have value as biomarkers of oxidative stress *in vivo*; however, this research also opens the door to many new questions.

The LPO-MA conjugates are products of phase I and phase II metabolism. This means that any changes in activity of LPO metabolizing enzymes or GSH levels will have an effect on the levels of LPO-MA conjugates excreted in the urine. This raises the question whether LPO-MA conjugates are a measure of LPO product formation or an index of the physiological response to oxidative stress. As discussed in Chapter 4, it is possible for GST expression levels to be affected by oxidative stress. In smokers, GSTP1 is induced in lung tissue while other GSTs are expressed no differently than in nonsmokers. It has also been suggested that genetic polymorphisms in the promoter region can lead to reduced GSTA4 expression and therefore reduced capacity to conjugate HNE.

Utilization of CCl_4 as a source of oxidative stress induction can lead to impaired liver function. It is conceivable that CCl_4 induced liver damage decreases GST activity, possibly resulting in lower levels of LPO-GSH conjugates than with normal liver function. Further exploration of the effects of different types of oxidative stress on phase II metabolism could provide increased insight as to the way levels of LPO-MA conjugates change in response to differing sources of oxidative stress. For example, running a marathon is one way to induce an acute oxidative stress insult. Higher levels of LPO-MA conjugates could be obtained in the urine of marathon runners than would be observed for chronic smokers. This does not mean that the marathon runners are less healthy than the smokers; it may very well be a reflection of decreased GST activity caused by smoking. This type of information is crucial to gaining a thorough understanding of the meaning behind LPO-MA conjugate levels.

Therefore, we propose a multiplex biomarker assay of LPO-MA conjugates in combination with stable endproducts of LPO, e.g., F_2-isoprostanes or hydroxy octadecadienoic acids, in order to be able to distinguish between oxidative stress-induced formation of LPO products and LPO product metabolism. High levels of both types of biomarkers would indicate elevated LPO product formation but also adequate

elimination. In contrast, high levels of F_2-isoprostanes but low levels of LPO-MA conjugates would suggest a condition of increased oxidative stress with inadequate detoxification capacity which would place such individuals at risk for developing age-related disease. Such a proposed multiplex biomarker assay would therefore provide a means to determine which way the 'oxidative stress' balance is likely to swing for each individual.

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