Available online at www.sciencedirect.com
ScienceDirect
Journal of Chromatography B, 857 (2007) 115–122
JOURNAL OF CHROMATOGRAPHY B
www.elsevier.com/locate/chromb

Quantification of *trans*-4-hydroxy-2-nonenal enantiomers and metabolites by LC–ESI-MS/MS

Ales Honzatko [a], Jiri Brichac [a,1], Matthew J. Picklo [a,b,*]

[a] *Department of Pharmacology, Physiology, and Therapeutics, University of North Dakota, Grand Forks, ND 58203, USA*
[b] *Department of Chemistry, University of North Dakota, Grand Forks, ND 58203, USA*

Received 17 April 2007; accepted 3 July 2007
Available online 10 July 2007

Abstract

Lipid peroxidation is a causal factor in multiple diseases including Alzheimer's disease, atherosclerosis, and alcoholic liver disease. One of the most studied products of lipid peroxidation, *trans*-4-hydroxy-2-nonenal (HNE), has multiple cell signaling and cytotoxic effects. In this work, we developed an LC–MS/MS method for the quantitation of HNE enantiomers, the metabolite *trans*-4-hydroxy-2-nonenoic acid, and HNE-glutathione adducts in a single chromatographic run. In this method, (*R*)-HNE and (*S*)-HNE are derivatized by (*S*)-carbidopa to form diastereomers that are separated by a reversed-phase column. This method was successfully validated and tested using respiring rat brain mitochondria that enantioselectively metabolize HNE. Metabolic profiles of HNE biotransformation, including the enantiomeric disposition of HNE, will provide useful biomarker data regarding lipid peroxidation in disease states.

Keywords: 4-Hydroxynonenal; Chiral separation; Lipid peroxidation; Aldehyde dehydrogenase; Glutathione; Mitochondria; Biomarker

1. Introduction

Lipid peroxidation is a pathologic consequence of oxidative damage to polyunsaturated fatty acids [1–3]. Multiple chemically reactive carbonyl products are formed from lipid peroxidation. Of these products, *trans*-4-hydroxy-2-nonenal (HNE) has gained the most attention with respect to Alzheimer's disease, atherosclerosis, and ethanol intoxication [4–8]. HNE generates adducts with nucleophilic groups including cysteinyl thiols, histidine residues, and primary amines, inhibits multiple enzymes, and has signaling properties [3]. Following administration of HNE in vivo, or incubation of HNE with whole cells or mitochondria, HNE is either metabolized or forms adducts with nucleophilic groups. The majority of HNE is enzymatically metabolized whereas 10% or less covalently modifies nucleophilic groups [9,10].

Given the significance of HNE disposition, a complete understanding of HNE metabolism in normal and disease states is necessary. Major primary pathways of HNE metabolism include oxidation to *trans*-4-hydroxy-2-nonenoic acid (HNEAcid) and conjugation to glutathione (Fig. 1). Glutathione adducts may then undergo subsequent biotransformation by aldehyde dehydrogenases, aldo-keto reductase and mercapturate formation [11–14]. HNE biotransformation may be modulated by the NADH/NAD^+ redox balance and glutathione status [10,15].

The targets of HNE adduction and the pathways of HNE metabolism are influenced by the stereochemistry of HNE [16–18]. HNE possesses a chiral center at C4 (Fig. 1). Hepatic glutathione-(*S*)-transferases preferentially conjugate (*S*)-HNE to GSH to form (*S*)-(4-hydroxy-1-oxononan-3-yl) glutathione (GSHNE) [16,17]. In rat brain mitochondria, (*R*)-HNE is preferentially metabolized over (*S*)-HNE to HNEAcid by aldehyde dehydrogenases (ALDHs) [19,20]. Recent studies indicate that the (*R*)-enantioselectivity of HNE oxidation is governed by the ALDH isozyme and physiologic concentrations of Mg^{2+} ions [21]. The extent to which the stereochemistry of HNE

* Corresponding author at: Department of Pharmacology, Physiology, and Therapeutics, School of Medicine and Health Sciences, University of North Dakota, Grand Forks, ND 58203, USA. Tel.: +1 701 777 2293; fax: +1 701 777 3262.
E-mail address: mpicklo@medicine.nodak.edu (M.J. Picklo).
[1] On leave from the Department of Analytical Chemistry, Charles University, Prague 12843, Czech Republic.

1570-0232/$ – see front matter © 2007 Elsevier B.V. All rights reserved.
doi:10.1016/j.jchromb.2007.07.004

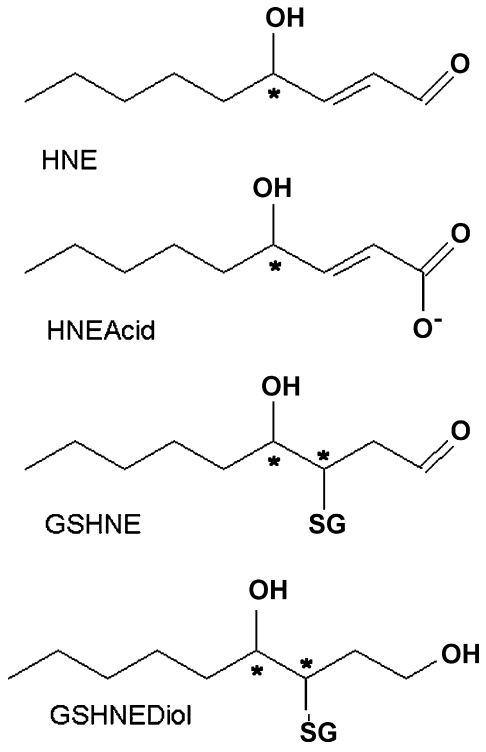

Fig. 1. Chemical structures of HNE and its metabolites, *trans*-4-hydroxy-2-nonenoic acid (HNEAcid), (*S*)-(4-hydroxy-1-oxononan-3-yl)glutathione (GSHNE) and (*S*)-(1,4-dihydroxynonan-3-yl)glutathione (GSHNEDiol). The (*) denotes a chiral center.

alters physiologic processes is becoming clearer. Studies by Hashimoto et al. [18] demonstrate that (*R*)-HNE protein adducts localize differently to cells in vivo than (*S*)-HNE protein adducts. Recent studies by Coleman et al. [22] demonstrate that (*S*)-HNE may be a more potent agonist for the peroxisome proliferator-activated receptor (PPAR) β/δ.

Multiple methods have been developed to measure the content of HNE or its metabolites, but have a number of disadvantages. HNE can be derivatized with reagents such as 5,5-dimethyl-1,3-cyclohexanedione or dinitrophenylhydrazine followed by analysis with LC with UV, fluorescence, or MS/MS detection [23–25]. However, these methods do not allow quantification of HNE enantiomers or HNE metabolites in the same analytical run. An LC–MS/MS method is reported for analysis of GSHNE and mercapturate conjugates of HNE but lacked the incorporation of an internal standards [26]. GC–MS methods can be used for separation of HNE enantiomers and HNEAcid but requires significant sample preparation and may not be suitable for the measurement of GSHNE adducts [27]. Direct LC separation of HNE enantiomers using a chiral stationary phase has been described using UV detection [28]. It is possible to couple this form of direct LC separation to MS/MS detection, since MS/MS detection of racemic HNE using positive ionization has been described [29].

Recently, a facile indirect separation method for analysis of HNE enantiomers was developed [19]. In this method, HNE is derivatized with (*S*)-carbidopa to form diastereomers that then are separated using a reversed-phase, C18 column. However, owing to the lack of a suitable internal standard, this particular method with UV detection was suitable only for determination of enantiomeric ratios. In this work, we expanded this method of reversed-phase separation of HNE-(*S*)-carbidopa diastereomers to yield an LC–MS/MS method for the separation and quantitation of the HNE enantiomers from biological matrix. This method provides for sensitive quantification of HNE enantiomers owing to the use of ESI in negative mode. In addition to quantifying the individual HNE enantiomers, we are able to measure during the same chromatographic run the HNE metabolites, HNEAcid, GSHNE, and the reduced GSHNE metabolite (*S*)-(1,4-dihydroxynonan-3-yl) glutathione (GSHNEDiol) [19]. This method provides for the facile analysis of multiple endpoints related to HNE stereochemistry and metabolism.

2. Experimental methods

2.1. Chemicals and reagents

All chemicals were of analytical or HPLC grade. Racemic HNE dimethylacetal was prepared according to Chandra and Srivastava [30]. d_{11}-HNE was purchased from CDN ISOTOPES (Pointe-Claire, Quebec, Canada). In this molecule, the hydrogen atoms on the aliphatic tail (C5-C9) were replaced with deuterium atoms. HNEAcid, d_{11}-HNEAcid, (*S*)-(4-hydroxy-1-oxononan-3-yl)glutathione (GSHNE), d_{11}-GSHNE, (*S*)-(1,4-dihydroxynonan-3-yl)glutathione (GSHNEDiol) and d_{11}-GSHNEDiol were synthesized by this laboratory as described previously [10,19]. (*S*)-Carbidopa (optical purity 99.9+%) and other chemicals were purchased from Sigma (St. Louis, MO, USA). Preparation and separation of HNE enantiomers were done in our lab according to the procedure described previously by Allevi and colleagues with modifications described in our previous work [19,31].

2.2. LC–MS/MS analysis of HNE-(S)-carbidopa diastereomers and HNE metabolites

The LC–MS/MS instrument consisted of an Agilent 1100 series HPLC system coupled to an API 3000 triple quadrupole mass spectrometer Perkin-Elmer/Sciex (Concord, Ontario, Canada). For separation, a Beta Basic 18 reversed-phase column (50 mm × 2.1 mm I.D.; Thermo-Hypersil Keystone) was used. The mobile phase consisted of phase A: 100% methanol and phase B: 10 μM ammonium acetate buffer pH 3.5. The pH was adjusted with glacial acetic acid. A linear gradient was applied (0–2 min 30% A, 3 min 60% A, 12–17 min 80% A) and the flow rate was set at 200 μL/min. Turbo ionspray was used at a temperature of 500 °C with nitrogen as a nebulizer gas at a flow rate of 12 L/min. Samples were analyzed in negative ion mode with an ion spray voltage of −4200 kV.

For optimization of MS/MS parameters, approximately 0.5 μM concentrations of each analyte were prepared in methanol:ammonium acetate buffer (50:50, v/v). Each scan time for individual multiple reaction monitoring (MRM) mode, expressed as parent ion/daughter ion, was set to 150 ms. The established MRM parameters and collision energies are summa-

Table 1
MS/MS conditions for determination of HNE enantiomers after derivatization with (*S*)-carbidopa and HNE metabolites

Analyte	Parent ion/daughter ion		Collision energy (V)	
	MRM 1	MRM 2	MRM 1	MRM 2
(*R,S*)-HNE-(*S*)-carbidopa	363.3/241.3	363.3/319.3	−18	−12
(*R,S*)-d_{11}-HNE-(*S*)-carbidopa	374.3/252.3	374.3/330.3	−22	−12
HNEAcid	171.1/99.1	171.1/152.9	−20	−16
d_{11}-HNEAcid	182.1/138.3	171.1/109.4	−16	−10
GSHNE	462.2/306.2	462.6/272.2	−26	−28
d_{11}-GSHNE	473.3/306.2	473.3/272.4	−30	−28
GSHNEDiol	464.3/272.2	464.3/254.1	−30	−30
d_{11}-GSHNEDiol	475.3/272.2	475.3/254.1	−30	−30

rized in Table 1. All these combinations of parent and daughter ions were used for quantification (MRM1). For quality confirmation we used one other daughter ion in MRM mode (MRM2). The ions for GSHNE and GSHNEDiol are identical to those reported by Aldini et al. [14]. The linearity of each calibration curve was determined by plotting the peak area ratio of analyte to internal standard versus the nominal concentration of the analyte. The limit of quantification (LOQ) was determined using the equation $LOQ = 10\sigma/S$, where σ is the standard deviation of responses and S is the slope of the calibration curve. S and σ were determined from the calibration curve of the analyte in the low concentration range close to LOQ. The standard deviation σ was determined as the residual standard deviation of a regression line.

2.3. Measurement of enantioselective metabolism of HNE by rat brain mitochondria

All experimental protocols were in accordance with the NIH guidelines for use of live animals and were approved by the University of North Dakota Institutional Animal Care and Use Committee. Sprague-Dawley rats (male, 250 g) were purchased from Charles River Laboratories (Wilmington, MA, USA). Rats were deeply anesthetized with ketamine (100 mg/kg) and xylazine (13 mg/kg), the brain was removed, and mitochondria were isolated as described previously [10].

For HNE metabolism studies, brain mitochondria (0.4 mg/mL final concentration) were placed into respiration buffer (40 mM KCl, 50 mM mannitol, 50 mM sucrose, 1 mM EGTA, 10 mM HEPES free acid, 5 mM $MgCl_2$, and 10 mM K_2HPO_4, pH 7.2) in 1.5 mL microfuge tubes at 37 °C [10]. Racemic HNE was added with final concentration of 10, 20, 40 μM. After 3 min 50 μL of 3% (w/w) phosphoric acid was added, that decreased the final pH to approximately 3, and tubes were immediately chilled and stored at −80 °C.

2.4. Sample preparation for indirect separation of HNE-(S)-carbidopa diastereoisomers and HNE metabolites

For LC–MS/MS analysis, 25 μL of sample was mixed with 25 μL of internal standard mixture followed by addition of 6.6 μL 0.1 M KOH and 10 μL of 50 mM (*S*)-carbidopa on ice. The internal standard mixture contained 20 μM d_{11}-HNE, 5.0 μM d_{11}-HNEAcid, 0.1 μM d_{11}-GSHNE and 5.0 nM d_{11}-GSHNEDiol in 25% (v/v) methanol. Calibration curves were measured in the ranges of 1–40 μM for HNE-(*S*)-carbidopa, 0.2–20 μM for HNEAcid, 5–500 nM for GSHNE and 0.5–50 nM for GSHNEDiol. Increasing the pH of the (*S*)-carbidopa derivatization reaction resulted in a shorter time for derivatization, but the determination of HNEAcid was confounded at a pH above 5.0. For this reason, the pH was adjusted with KOH precisely to pH 5.0. Following derivatization, 200 μL of 25% (v/v) methanol was added and the sample was centrifuged at 10,600 × *g* for 10 min. Typically, 20 μL of supernatant was injected into the LC–MS/MS system.

2.5. Method validation

Calibration standards in respiration buffer containing mitochondria were prepared from the stock solution of standards in ethanol by sequential dilution with respiration buffer containing mitochondria (0.4 mg/mL) and 10% (v/v) of phosphoric acid (3%, w/w). Intra-day precision and accuracy of the assays performed in replicates ($n = 6$) were tested by using four different concentrations of individual analytes. Inter-day precision and accuracy of the assays were determined from the results of six replicate assays on six different days. Precision was evaluated by the relative standard deviation (R.S.D.). Accuracy was estimated based on the mean percentage error (bias) of measured concentration to the actual concentration. Recovery was tested at four concentration levels by adding known amounts of the studied compounds to the respiration buffer without mitochondria. The peak area ratios of analyte and internal standard were calculated. Recoveries were evaluated by comparing the calculated concentration with assay value for known amounts prepared and assayed in respiration buffer.

2.6. LC–MS/MS analysis of HNE enantiomers by direct separation

For direct chromatographic resolution of HNE enantiomers, a chiral column Chiralpak AD-RH (150 mm × 2.1 mm I.D., Chiral Technologies, Exton, PA, USA) was used. For quantitation, the same MS system was employed except that LC–MS/MS was performed in positive mode [29]. Preliminary experiments in our laboratory showed that HNE detection was poor using negative mode. The mobile phase consisted of phase A: 100% methanol and phase B: 0.1% acetic acid. An isocratic mode was applied

Table 2
MS/MS conditions for determination of HNE enantiomers using chiral column Chiralpak AD-RH

Analyte	Parent ion/daughter ion		Collision energy (V)[a]
	MRM 1	MRM 2	
(*R*,*S*)-HNE	157.2/97.1	157.2/121.1	15
(*R*,*S*)-d_{11}-HNE	168.3/149.1	168.3/150.3	15

[a] A collision energy of 15 V was used for both MRM1 and MRM2.

(65% A) and the flow rate was set at 100 μL/min. Turbo ionspray was used at a temperature 500 °C with a nebulizer gas flow rate of 12 L/min. Samples were analyzed in positive ion mode with an ion spray voltage of 5500 kV. The ions monitored and the collision energies utilized are provided in Table 2.

2.7. Comparison of direct and indirect separation methods for analysis of mitochondrial metabolism.

Mitochondria were incubated with racemic HNE for 3 min. Following termination of the reaction, the d_{11}-HNE internal standard (40 μM) was added and samples were divided in two portions. One part was used for derivatization with (*S*)-carbidopa in which 20 μL of the sample was mixed with 5 μL of 0.1 M KOH and 20 μL of 50 mM (*S*)-carbidopa. After 30 min on ice, 150 μL of 50% methanol was added and 5 μL used for reversed-phase LC with ESI-MS/MS detection in negative mode. The second portion was mixed with acetonitrile (1:1) and 150 μL was injected onto the HPLC using a reversed phase column and UV detection for preparation of HNE and d_{11}-HNE. Approximately 2 mL was collected and 70 μL was analyzed by chiral phase separation (Chiralpak AD-RH) and detection with ESI-MS/MS in positive mode. The preparation of HNE prior to using the Chiralpak AD-RH column was performed to prolong the lifetime of the column. For the direct and indirect separations methods, calibration curves were made in the same way as the samples, in the range of 40–80 μM racemic HNE, and with 40 μM d_{11}-HNE as an internal standard. All samples were prepared in two independent days in triplicate. The use of a 5 μL injection volume for the indirect method of HNE enantiomer determination allowed for the HNE enantiomer content in the samples and standards to fall within the amounts of HNE enantiomers used for method validation (see above and Table 4). The dilution of HNE enantiomers during processing of samples for direct determination of HNE enantiomeric content allowed for the HNE enantiomer content in the samples and standards to fall within the calibration curves derived for use of the Chiral AD-RH column (see above and Fig. 5).

3. Results and discussion

3.1. Indirect separation of HNE and quantitation by MS-MS detection

We showed previously that HNE enantiomers are easily derivatized with (*S*)-carbidopa and the resulting diastereoisomers are separated by reverse-phase chromatography. The

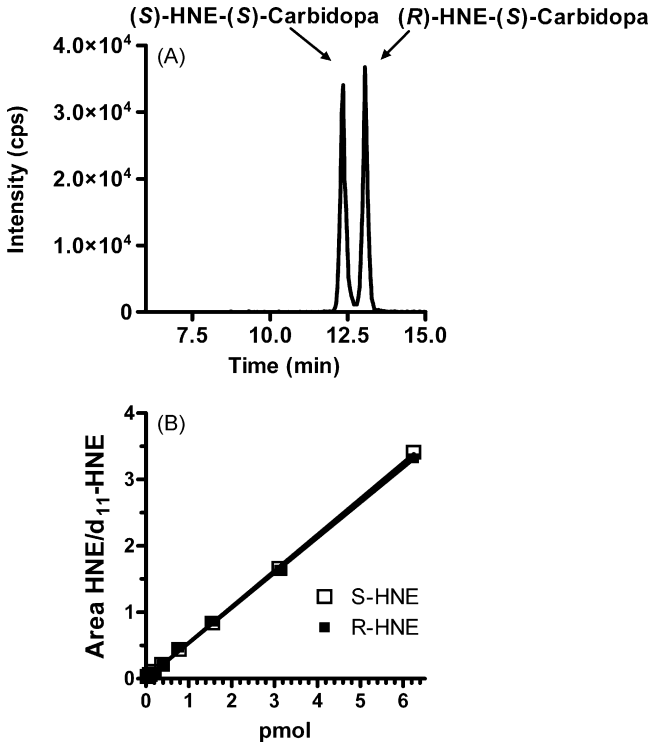

Fig. 2. LC–MS/MS detection and quantification of derivatized HNE enantiomers following (*S*)-carbidopa derivatization. (A) Chromatogram of derivatized HNE enantiomers (180 fmol each) detected by MS/MS in MRM mode using negative ionization. (B) Calibration curves were calculated as the ratio of response of the analyte divided by response of its d_{11}-form. For (*R*)-HNE the slope of the regression line 0.528 ± 0.004, the *y* intercept = 0.010 ± 0.001, and $r^2 = 0.999$. For (*S*)-HNE the slope of the regression line 0.054 ± 0.003, the *y* intercept = -0.003 ± 0.009, and $r^2 = 0.999$.

chromatographic parameters described presently were optimized for a smaller column (Beta-Basic C18; 50 mm × 2.1 mm I.D) more suitable for LC–MS/MS. As a mobile phase, a combination of methanol–ammonium acetate buffer was used to improve sensitivity and various gradients were tested to achieve good resolution and symmetric peak shapes of analytes as well as a short run time.

The retention time of (*S*)-HNE-(*S*)-carbidopa and (*R*)-HNE-(*S*)-carbidopa were 12.3 and 13.1 min, respectively (Fig. 2A). Calibration curves demonstrated excellent linearity using eight different amounts of enantiomers, 50 fmol to 6.25 pmol (Fig. 2B). For both enantiomers, an LOQ of 12 fmols was determined. Racemic d_{11}-HNE was included as an internal standard for (*R*)-HNE and (*S*)-HNE, respectively. There was no difference in derivatization with (*S*)-carbidopa with the d_{11}-(*R*)-HNE or d_{11}-(*S*)-HNE.

3.2. Detection and quantitation of HNE enantiomers and metabolites of HNE in a single analysis

We combined our analysis of HNE enantiomers following (*S*)-carbidopa derivatization with that of the LC–MS/MS methods we used previously for the determination of the HNE metabolites HNEAcid, GSHNE, and GSHNEDiol [10,19]. Combining these methods would allow for the determination of

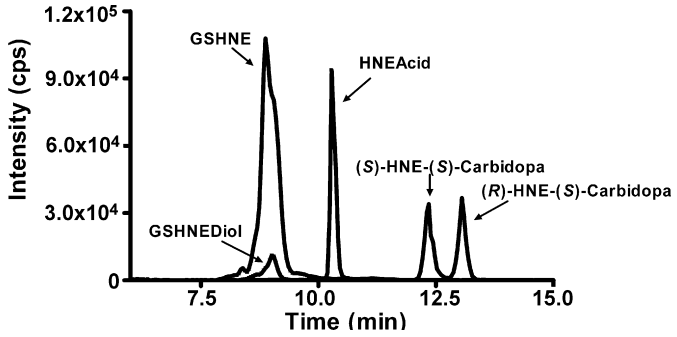

Fig. 3. Separation of derivatized HNE enantiomers and HNE metabolites by reversed-phase chromatography followed by MS/MS detection with electrospray ionization in negative mode. A representative chromatogram is shown of a mixture of derivatized racemic HNE (8 pmol), and its metabolites HNEAcid (20 pmol), GSHNE (500 fmol) and GSHNE (100 fmol). The sample volume was 20 μL. The MRM1 ions used for detection of the individual analytes are given in Table 1.

multiple data parameters (quantitation of HNE enantiomers and HNE metabolites) from a single preparation of biological sample, a consideration useful for small amount of experimental samples or human samples. A representative chromatogram of an LC–MS/MS run for determination of the above five analytes is shown in Fig. 3. All five analytes had their corresponding d_{11}-internal standards included in the analysis. The MRM1 ions used for detection are given in Table 1. Although GSHNE and GSHNEDiol coeluted, different ions were used for detection. Under the described conditions, GSHNE was not derivatized by (*S*)-carbidopa although GSHNE does possess an aldehyde (not shown). This may be a result of cyclic hemiacetal formation between the C4 hydroxyl group and the aldehyde at C1 of GSHNE [32]. Table 3 provides the retention times and LOQs for the analytes. Although the LOQ for HNEAcid was approximately 600 fmol, low LOQs for the derivatized HNE diastereomers (12 fmol) and GSHNE (15 fmol) were obtained. Similar values for GSHNE were obtained by Volkel et al. [26].

Table 3
Retention times (RT) and limit of quantification (LOQ) for individual analytes using C18 reversed-phase column

Analyte	RT (min)	LOQ (fmol)
(*S*)-HNE-(*S*)-carbidopa	12.32	12
(*S*)-d_{11}-HNE-(*S*)-carbidopa	12.22	
(*R*)-HNE-(*S*)-carbidopa	13.05	12
(*R*)-d_{11}-HNE-(*S*)-carbidopa	12.87	
HNEAcid	10.49	598
d_{11}-HNEAcid	10.38	
GSHNE	8.74	18
d_{11}-GSHNE	8.66	
GSHNEDiol	8.92	138
d_{11}-GSHNEDiol	8.80	

3.3. Method validation for the combined method

To test the specificity and validity of this method, individual samples of rat brain mitochondria in respiration buffer were prepared that did not contain the analytes or internal standards. No significant interference was observed in the chromatograms at retention times of the analytes and internal standards for untreated samples. The intra- and inter-day precision (R.S.D.) calculated following replicate analysis of HNE enantiomers, HNEAcid, GSHNE and GSHNEDiol in diluted acidified matrix (respiration buffer containing mitochondria) were <8.6% for all analytes in the studied range of concentrations (Table 4). The intra- and inter-day bias assessed during the replicate analysis for all analytes varied between −3.5% and 10.7% (Table 4). The mean overall absolute recovery was 98.7% for (*S*)-HNE,

Table 4
Precision and accuracy parameters of combined HNE enantiomers and HNE metabolite analysis

Concentration (μM)	Intra-day						Inter-day					
	R.S.D. (%)			Bias (%)			R.S.D. (%)			Bias (%)		
(*S*/*R*)-HNE, HNEAcid	(*S*)-HNE	(*R*)-HNE	HNEAcid	(*S*)-HNE	(*R*)-HNE	HNEAcid	(*S*)-HNE	(*R*)-HNE	HNEAcid	(*S*)-HNE	(*R*)-HNE	HNEAcid
2.00	4.70	3.90	3.39	6.00	7.00	0.00	1.39	5.67	7.00	−3.50	3.00	−1.00
5.00	6.90	6.40	2.47	5.40	4.40	−0.80	3.73	3.30	2.52	4.80	5.00	0.00
10.00	3.90	3.80	4.21	4.50	4.00	5.00	4.93	4.38	4.16	3.40	2.40	3.60
20.00	5.20	5.10	2.94	−1.50	1.35	−0.15	2.26	1.52	4.19	−0.95	0.75	−1.30

Concentration (nM)		Intra-day				Inter-day			
		R.S.D.(%)		Bias (%)		R.S.D.(%)		Bias (%)	
GSHNE	GSHNEDiol	GSHNE	GSHNEDiol	GSHNE	GSHNEDiol	GSHNE	GSHNEDiol	GSHNE	GSHNEDiol
50.00	10.00	6.25	5.96	9.00	10.10	8.00	8.61	7.80	10.70
125.00	25.00	3.17	3.90	−1.12	−3.60	4.08	3.33	−1.76	6.96
250.00	50.00	2.40	7.21	4.32	−4.44	4.62	4.32	−3.76	4.20
500.00	100.00	4.77	5.23	6.26	1.13	3.22	3.38	0.98	4.57

All analytes were diluted to the given concentrations in acidified matrix (respiration buffer containing mitochondria, 0.4 mg/mL) and prepared for LC–MS/MS analysis. Each matrix sample contained all five analytes. Data were calculated from six individual replicates for each point for intra-day R.S.D. and were calculated six different days for inter-day R.S.D.

97.5% for (*R*)-HNE, 94.5% for HNEAcid, 94.7% for GSHNE and 95.2% for GSHNEDiol. Our studies were not specifically designed to determine the absolute lower limits of quantitation (LLOQ's) for these analytes in this mitochondrial, biological matrix but rather for the determination of analytes in metabolic assays. Nonetheless, based upon the concentrations used for the validation parameters the LLOQ's for these analytes were at least 3.8 pmols for HNEAcid, (*R*)-HNE, and (*S*)-HNE, 94 fmols for GSHNE, and 19 fmols for GSHNEDiol. Likely, much smaller LLOQ's for HNEAcid and the individual HNE enantiomers in biological matrix can be obtained.

3.4. Application of the combined method to metabolism experiments

In these experiments, we examined the applicability of the combined method for biological experiments. Owing to our previous characterization of HNE metabolism by rat brain mitochondria, we utilized this combined method for analysis of HNE metabolism in the same system. Whole, respiring rat brain mitochondria were incubated with increasing concentrations of racemic HNE for 3 min followed by analysis of HNE enantiomers content and HNE metabolites. Our results demonstrate that consumption of (*R*)-HNE occurred at a higher rate than (*S*)-HNE (Fig. 4A). These data also show that consumption of (*S*)-HNE saturated at a lower concentration than that of (*R*)-HNE. As previously observed, the formation of HNEAcid occurred at a much higher (over 20-fold) rate than that of GSH adducts (Figs. 4B and C). The results confirm our previous data showing the enantioselective consumption of (*R*)-HNE over (*S*)-HNE and that HNEAcid formation is a major route of HNE detoxification versus GSH adduction [10,19].

3.5. Direct separation of HNE enantiomers and detection by MS/MS

Given that the enantiomers of HNE can be directly separated using chiral phase chromatography, we defined the LC–MS/MS parameters for analysis of the HNE enantiomers following direct separation using the Chiralpack AD-RH column that contains tris-(3,5-dimethylphenylcarbamate) derivatized amylose on 5 μm silica gel as the chiral stationary phase. LC–MS/MS was performed with electrospray ionization in positive mode since minimal signal was detected using ionization in negative mode. Chromatographically, the retention times of (*S*)-HNE and (*R*)-HNE were 25.0 and 28.5 min, respectively. The peaks were not fully separated with fronting of the (*S*)-HNE peak (Fig. 5A). For generation of calibration curves, racemic d_{11}-HNE was included as an internal standard for (*R*)-HNE and (*S*)-HNE, respectively. Acetic acid was used to increase the ionization. A gradient was not applied owing to the long equilibration time necessary with this column. A representative calibration curve is shown in Fig. 5B. Calibration curves were linear for the six concentration of enantiomers (1–200 pmol) tested. LOQs were determined as 3.6 and 1.4 pmol for (*R*)-HNE and (*S*)-HNE, respectively. These values correspond to 180 fmol/μL and 70 fmol/μL as we utilized a 20 μL injection volume. These LOQs are similar to those

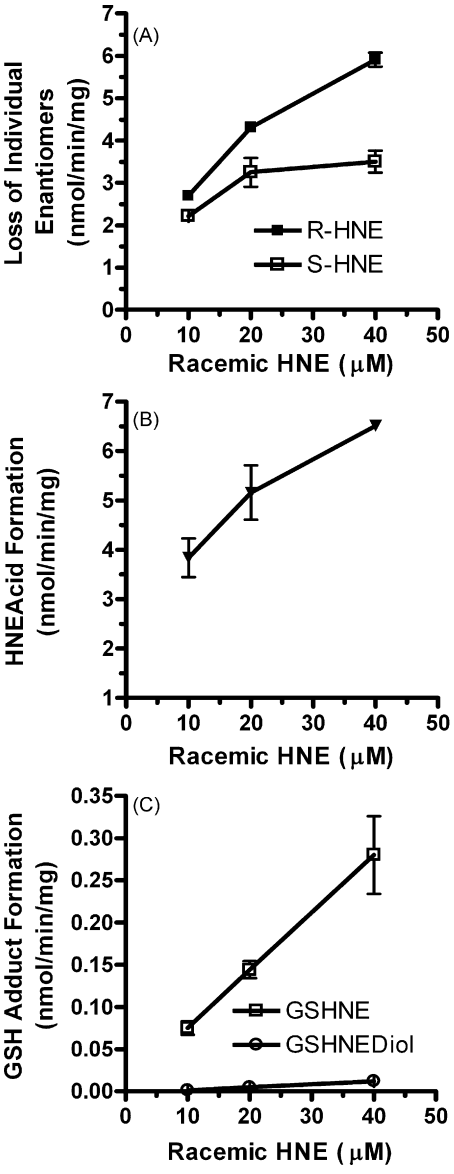

Fig. 4. Enantioselective metabolism of racemic HNE by rat brain mitochondria. Mitochondria were incubated with increasing concentrations of racemic HNE. Samples were taken after 3 min for measurement of enantiomeric content of HNE (A) and measurements of the metabolites HNEAcid (B) and GSH adducts (C). Results are expressed as the means ± S.D. ($n = 3$) for each individual racemic HNE concentration used.

obtained by Gioacchini et al. [29] for detection of racemic HNE by infusion MS with ESI in positive mode.

In order to compare the direct and indirect methods for analyses of biological samples, we measured the consumption of racemic HNE (80 μM) by rat brain mitochondria followed by HNE enantiomeric analyses by both methods. The indirect and direct methods provided nearly identical results (Table 5) and indicate that the presence of biological matrix does not confound the use of the indirect versus the direct method for determination of the HNE enantiomers.

A comparison of the direct separation method followed by ESI-MS/MS in positive mode to separation of the (*S*)-carbidopa diastereomers followed by ESI-MS/MS in negative

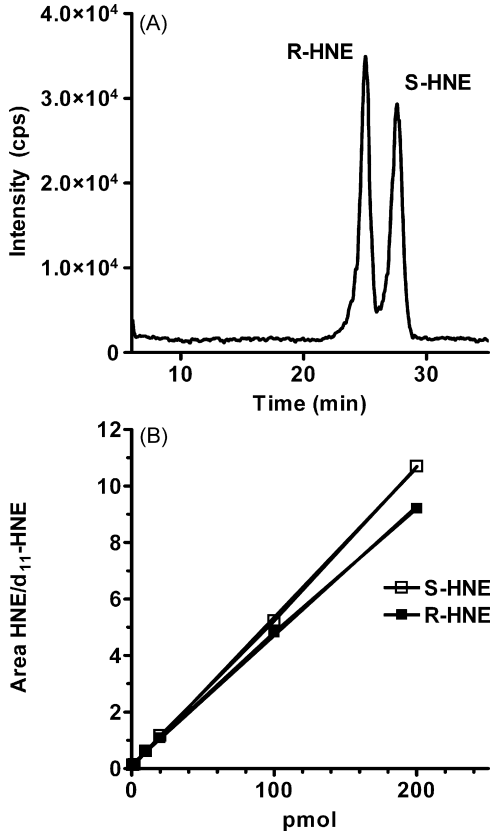

Fig. 5. Separation and quantitation of (*R*)-HNE and (*S*)-HNE by chiral phase chromatography followed by MS/MS. (A) Chromatogram HNE enantiomers (100 pmols each) detected by MS/MS in MRM mode using positive ionization. (B) Calibration curves were calculated as the ratio of response of the analyte divided by response of its d_{11}-form. For (*R*)-HNE the slope of the regression line 0.053 ± 0.001, the *y* intercept $= 0.058 \pm 0.038$, and $r^2 = 0.999$. For (*S*)-HNE the slope of the regression line 0.046 ± 0.001, the *y* intercept $= 0.136 \pm 0.042$, and $r^2 = 0.999$.

mode demonstrates that the method utilizing (*S*)-carbidopa derivatization has multiple advantages. The chromatographic separation of the (*S*)-carbidopa derivatized enantiomers provided a shorter retention time and better resolution than the direct separation method. Furthermore, following LC separation, detection of the derivatized enantiomers by ESI-MS/MS in negative mode (LOQ's of 12 fmols for the enantiomers), likely owing to the incorporation of a polar carboxylic group, provided more than 100-fold greater sensitivity than detection of the native enantiomers (LOQ's of 3.6 pmol for (*R*)-HNE and 1.4 pmol for (*S*)-HNE) with ESI-MS/MS in positive mode.

Table 5
Comparison of direct vs. indirect separation methods for biological samples

Method	HNE measured (μM)		(*S*)-HNE (%)	
	Direct	Indirect	Direct	Indirect
Day 1	64.9 ± 1.2	62.6 ± 0.46	55.0 ± 0.7	54.1 ± 1.5
Day 2	68.1 ± 0.3	67.4 ± 1.5	55.5 ± 1.1	55.0 ± 0.3

Rat brain mitochondria were incubated with 80 μM HNE. Samples were taken after 3 min after the addition of HNE for measurement of concentration and enantiomeric ratio of HNE using the direct or indirect methods. All samples were performed on two separate days in triplicate. The concentration of HNE found and the ratio of enantiomers (expressed as percentages of recovery of (*S*)-HNE are provided. Data are the mean ± S.D. ($n = 3$).

4. Conclusion

In summary, we developed and validated a novel LC–MS/MS method for the quantification of HNE enantiomers and metabolites of HNE. By obtaining more detailed metabolite profile, we gain a better understanding of HNE biotransformation, its regulation, and the changes that occur in disease states. Metabolic profiles of HNE may provide useful biomarkers for study of lipid peroxidation in disease.

Acknowledgments

We gratefully acknowledge the support of NIH grants P20 RR 17699-01 and AA 15145-01. We thank Laura J. Leiphon and Tonya C. Murphy for the preparation of mitochondria.

References

[1] K. Uchida, Prog. Lipid. Res. 42 (2003) 318.
[2] N. Zarkovic, Mol. Aspects Med. 24 (2003) 281.
[3] H. Esterbauer, R. Schaur, H. Zollner, Free Radic. Biol. Med. 11 (1991) 81.
[4] M.J. Picklo, T.J. Montine, V. Amarnath, M.D. Neely, Toxicol. Appl. Pharmacol. 184 (2002) 187.
[5] J.J. Chen, S. Schenker, G.I. Henderson, Hepatology 25 (1997) 142.
[6] J. Chen, N.C. Robinson, S. Schenker, T.A. Frosto, G.I. Henderson, Hepatology 29 (1999) 1792.
[7] K. Uchida, S. Toyokuni, K. Nishikawa, S. Kawakishi, H. Oda, H. Hiai, E.S. Stadtman, Biochemistry 33 (1994) 12487.
[8] J.C. Vallve, K. Uliaque, J. Girona, A. Cabre, J. Ribalta, M. Heras, L. Masana, Atherosclerosis 164 (2002) 45.
[9] A. Laurent, E. Perdu-Durand, J. Alary, L. Debrauwer, J.P. Cravedi, Toxicol. Lett. 114 (2000) 203.
[10] M.J. Meyer, D.M. Mosely, V. Amaranth, M.J.S. Picklo, Chem. Res. Toxicol. 17 (2004) 1272.
[11] J. Alary, F. Gueraud, J.P. Cravedi, Mol. Aspects Med. 24 (2003) 177.
[12] J. Alary, L. Debrauwer, Y. Fernandez, A. Paris, J.P. Cravedi, L. Dolo, D. Rao, G. Bories, Chem. Res. Toxicol. 11 (1998) 1368.
[13] J. Alary, F. Bravais, J.P. Cravedi, L. Debrauwer, D. Rao, G. Bories, Chem. Res. Toxicol. 8 (1995) 34.
[14] G. Aldini, P. Granata, M. Orioli, E. Santaniello, M. Carini, J. Mass Spectrom. 38 (2003) 1160.
[15] P.J. Boon, H.S. Marinho, R. Oosting, G.J. Mulder, Toxicol. Appl. Pharmacol. 159 (1999) 214.
[16] A. Hiratsuka, K. Hirose, H. Saito, T. Watabe, Biochem. J. 349 (2000) 729.
[17] A. Hiratsuka, K. Tobita, H. Saito, Y. Sakamoto, H. Nakano, K. Ogura, T. Nishiyama, T. Watabe, Biochem. J. 355 (2001) 237.
[18] M. Hashimoto, T. Sibata, H. Wasada, S. Toyokuni, K. Uchida, J. Biol. Chem. 278 (2003) 5044.
[19] A. Honzatko, J. Brichac, T.C. Murphy, A. Reberg, A. Kubatova, I.P. Smoliakova, M.J. Picklo Sr., Free Radic. Biol. Med. 39 (2005) 913.
[20] J. Brichac, A. Honzatko, M.J. Picklo, J. Chromatogr. A 1149 (2) (2007) 305–311.
[21] J. Brichac, K.K. Ho, A. Honzatko, R. Wang, X. Lu, H. Weiner, M. Picklo, Chem. Res. Toxicol. 20 (6) (2007) 887–895.
[22] J.D. Coleman, K.S. Prabhu, J.T. Thompson, P.S. Reddy, J.M. Peters, B.R. Peterson, C.C. Reddy, J.P. Vanden Heuvel, Free Radic. Biol. Med. 42 (2007) 1155.
[23] T.I. Williams, M.A. Lovell, B.C. Lynn, Anal. Chem. 77 (2005) 3383.
[24] R. Andreoli, P. Manini, M. Corradi, A. Mutti, W.M. Niessen, Rapid Commun. Mass Spectrom. 17 (2003) 637.

[25] T.I. Williams, B.C. Lynn, W.R. Markesbery, M.A. Lovell, Neurobiol. Aging (2005).
[26] W. Volkel, R. Alvarez-Sanchez, I. Weick, A. Mally, W. Dekant, A. Pahler, Free Radic. Biol. Med. 38 (2005) 1526.
[27] G. Bringmann, M. Gassen, S. Schneider, J. Chromatogr. A 670 (1994) 153.
[28] C. Schneider, N.A. Porter, A.R. Brash, Chem. Res. Toxicol. 17 (2004) 937.
[29] A.M. Gioacchini, N. Calonghi, C. Boga, C. Cappadone, L. Masotti, A. Roda, P. Traldi, Rapid Commun. Mass Spectrom. 13 (1999) 1573.
[30] A. Chandra, S.K. Srivastava, Lipids 32 (1997) 779.
[31] P. Allevi, M. Anastasia, F. Cajone, P. Ciuffreda, A. Sanvito, J. Org. Chem. 58 (1993) 5000.
[32] V. Amarnath, W.M. Valentine, T.J. Montine, W.H. Patterson, K. Amarnath, C.N. Bassett, D.G. Graham, Chem. Res. Toxicol. 11 (1998) 317.