Enantioselective metabolism of (R)- and (S)-4-hydroxy-2-nonenal in rat.

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Abstract. 4-Hydroxy-2-nonenal (HNE) is an endogenous product of lipid peroxidation, which is believed to play a biological role in the pathogenesis of various diseases. HNE is formed as a racemic mixture of (R)- and (S)- enantiomers. These enantiomers differ in their biological properties. The aim of this study was to investigate separately the *in vivo* metabolism of the two HNE enantiomers in male rats after intravenous administration of the corresponding radiolabeled compounds and to compare the results with those obtained with the racemic mixture. Although the difference in the excretion rates was not statistically significant, the HPLC profiles of urinary metabolites showed qualitative and quantitative differences between the two enantiomers. The level of 3-mercapturic acid-1,4-dihydroxynonane, which is considered as the major urinary metabolite of HNE, was significantly lower in the case of (S)-HNE injected rats. *In vitro* studies using rat liver cytosolic incubations and HNE-glutathione conjugate as substrate were performed to clarify the intermediate pathways involved in their metabolism. Large differences were obtained in the reduction and retro-Michael conversion steps of the metabolism between the conjugates originating from the two enantiomers.

Keywords: Lipid peroxidation, 4-hydroxy-2-nonenal, stereoselectivity, aldo-keto reductase, glutathione conjugation

Abbreviations: DHN: 1,4-dihydroxy-2-nonene; DHN-MA: 3-mercapturic acid-1,4-dihydroxynonane; FAB/MS: Fast Atom Bombardment/Mass Spectrometry; GSH: glutathione; GST: glutathione-S-transferase; HNA: 4-hydroxy-2-nonenoic acid; HNA-lac-MA: lactone form of 3-mercapturic acid-4-hydroxynonanoic acid; HNA-MA: 3-mercapturic acid-4-hydroxynonanoic acid; HNE: 4-hydroxy-2-nonenal; HNE-MA: 3-mercapturic acid-4-hydroxynonanal.

1. Introduction

4-Hydroxy-2-nonenal (HNE) is the major 4-hydroxyalkenal formed during the process of lipid peroxidation and is recognized as being one of the most cytotoxic and genotoxic end products originating from lipid peroxidation of n-6 polyunsaturated fatty acids [11,13]. Moreover, this electrophilic compound has been shown to play a role as a biological signal [10,17,18]. Most of these effects on cellular functions are due to the nucleophilic attack of some protein amino acids, such as cysteine, lysine or histidine, or DNA bases at the C2–C3 double bond of HNE by the mechanism of Michael addition. The carbonyl group can also form Schiff bases with primary amines, such as lysine [11,19].

In vivo and *in vitro* studies on HNE metabolism showed that its biotransformation mainly involves a conjugation step to glutathione (GSH), followed by oxidative or reductive modifications of the HNE part of the adduct, then by the formation of the mercapturic acid conjugate originating from the GSH

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part. All these reactions inactivate HNE, prevent further adduction to cellular biomacromolecules, and make HNE more hydrophilic and easier to excrete. Hence, interest in HNE biotransformations has been increasing during the past ten years.

HNE has a chiral center in C4 and is chemically or biologically produced as a racemic mixture of (R)- and (S)-enantiomers [8]. Enantiomers can differ qualitatively and/or quantitatively in their biological role and also in their metabolic fate. Some authors have reported that (R)- and (S)-HNE have different biological properties. The glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase is selectively inactivated by (S)-HNE, and (S)-HNE is preferentially conjugated to GSH by glutathione-S-transferases (GSTs) as compared to (R)-HNE in guinea-pig and rat cytosolic fractions [15,16]. A recent study with enantiomer (R)-specific monoclonal antibodies revealed that there may be specific targets of each enantiomer in the cells [14]. However, no *in vivo* study has been carried out concerning the enantioselectivity of HNE metabolism. The purpose of this study was to investigate separately the metabolism of the two enantiomers in rats with radiolabeled (R)- and (S)-HNE and to compare the results with those obtained previously with the racemic mixture [1]. Some *in vitro* experiments were also performed to clarify the intermediate pathways involved.

2. Materials and methods

2.1. Materials

GSH, NADPH, phenobarbital and organic solvents were obtained from Sigma-Aldrich (Saint-Quentin-Fallavier, France). *Pseudomonas fluorescens* lipase was purchased from Fluka (Saint-Quentin-Fallavier, France). HNE diethylacetal was synthesized as described by Esterbauer and Weger [12]. [4-^{3}H]HNE diethyl acetal was synthesized at CEA, Service des Molécules Marquées CEN (Saclay, France), according to the method developed in our laboratory, with a specific activity of 222 GBq/mmol [7]. HNE was prepared from [4-^{3}H]HNE diethyl acetal as previously described [1]. Radiolabeled (R)- and (S)-HNE enantiomers were separated using the method of Allevi et al. [6]. HNE-GSH conjugate was synthesized as previously described [4] and the different radiolabeled stereoisomers were separated by HPLC as previously described [4] using a two step gradient with mobile phase A containing 15% acetonitrile and 85% acetic acid 1%, delivered for 40 min, and mobile phase B containing 40% acetonitrile and 60% water, delivered for 35 min.

2.2. Animals

Male Wistar rats (180–220 g) were weakly anesthetized with diethyl ether and received 500 μL of Ringer's solution containing either 1.45 mg [4-^{3}H](S)-HNE ($n = 3$) or 1.8 mg [4-^{3}H](R)-HNE ($n = 3$) or 1.6 mg [4-^{3}H]HNE ($n = 3$) into the penis vein (final specific activity: 0.60 MBq/mg). Animals were placed in individual metabolic cages and urine was collected for 2 days. Experiments were performed according to institutional guidelines governing animal experimentation.

2.3. Analysis

Urinary excretion of radioactivity was estimated and urinary metabolites were analyzed as previously described [1]. Peak identification was carried out using Fast Atom Bombardment mass spectrometry (FAB/MS) as previously described [1].

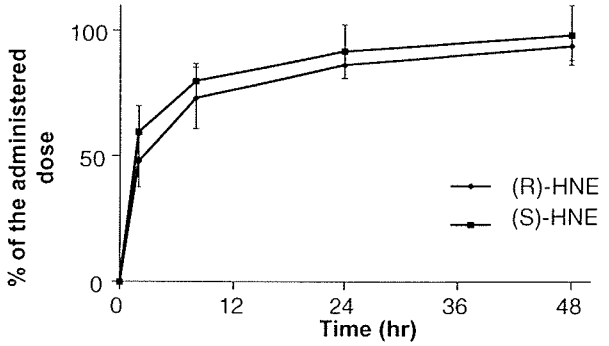


Fig. 1. Cumulative urinary excretion of radioactivity following intravenous administration of (R)- or (S)-[H^3]HNE. Values are means ± SD, $n = 3$. Aliquots of urine were counted for radioactivity in a Packard 2200CA scintillation counter (Perkin Elmer, Boston, MA) with Ultimagold (Perkin Elmer) as scintillation cocktail.

Rat liver cytosolic fractions were prepared and cytosolic incubations were performed as previously described using 2.5 mg of cytosolic proteins [4]. Some incubations were supplemented with 0.5 μmol NADPH as enzyme cofactor or with 1 mM phenobarbital (final concentration) as enzyme inhibitor. HNE-GSH metabolites were analyzed using radio-HPLC as previously described [4].

3. Results

3.1. Urinary excretion of radioactivity

Cumulative urinary excretion of radioactivity following intravenous administration of (R)- or (S)-[^{3}H]-HNE showed that urine is the almost exclusive route of excretion of HNE (Fig. 1). The urinary excretion was rapid and extensive since 48% (± 10.3) and 59.6% (± 10.2) of the administered radioactivity was excreted within the first two hours, for (R)- and (S)-[^{3}H]-HNE, respectively. After 48 hours, the urinary excretion amounted to 93.8% (± 5.6) and 98.2% (± 11.9), for (R)- and (S)-[^{3}H]-HNE, respectively. The excretion of radioactivity originating from (S)-HNE was slightly faster and excreted in a slightly higher quantity than radioactivity originating from the other enantiomer. However, these differences were not statistically significant.

3.2. Analysis of the different HNE urinary metabolites

Radio-HPLC metabolic profiles of urine samples obtained from 0 to 2 hours after (S)-, (R)- or racemic [^{3}H]HNE administration displayed quantitative differences, the profile obtained after racemic HNE corresponding to an average between the two profiles of the enantiomers (Fig. 2). The four peaks corresponding to less polar metabolites were identified by FAB/MS as 3-mercapturic acid-HNE (HNE-MA), 3-mercapturic acid-4-hydroxynonanoic acid (HNA-MA) and its lactone form (HNA-lac-MA), and 3-mercapturic acid-1,4-dihydroxynonane (DHN-MA), which are the mercapturic derivatives of HNE, its oxidized and reduced forms, respectively (Fig. 3). Polar metabolites originate mostly from ω-oxidation of HNA-MA [3]. HNA-lac-MA comes from the oxidation and further metabolism of HNE-GSH which is in a hemiacetal form and HNA-MA comes from the hydrolysis of the HNA-lac-conjugates which can occur at physiological pH [4,5]. The main difference between (R)- and (S)-HNE urinary metabolic profiles was

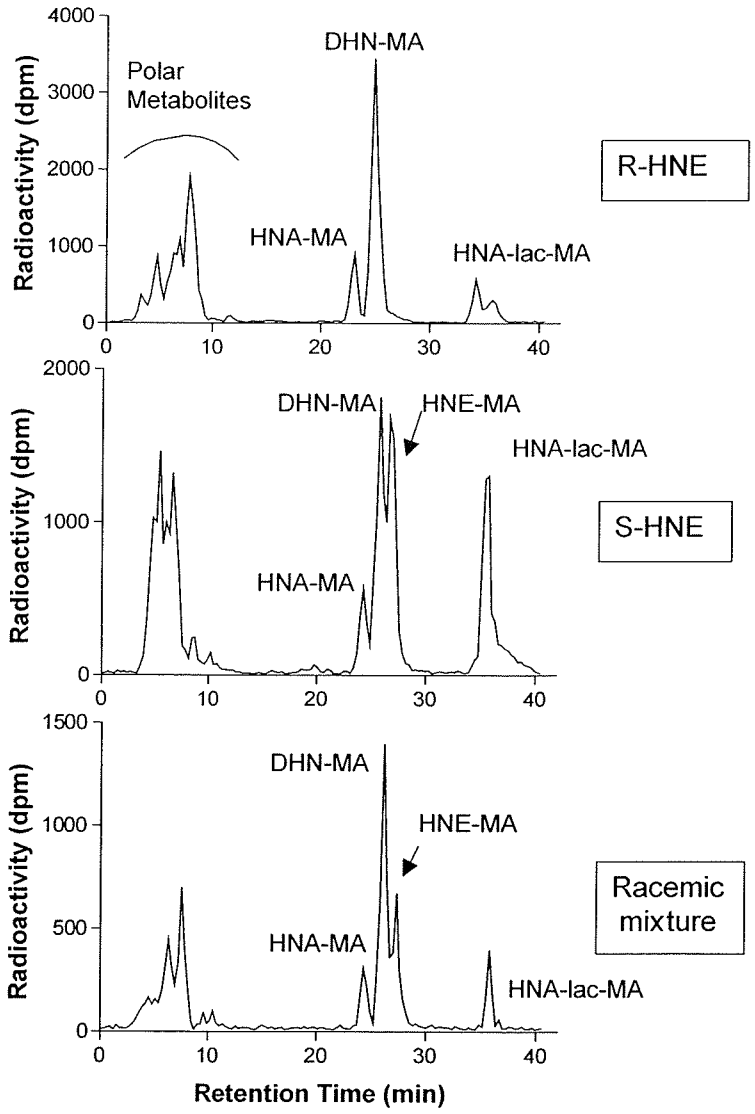


Fig. 2. Radio-HPLC metabolic profiles of [^{3}H]HNE after intravenous administration (0–2 h urine samples). These are typical HPLC profiles coming from a single rat urine sample. HPLC separations were performed on an analytical reversed phase column and radioactivity detection was carried out "on line" as described before [1]. Chromatographic conditions were as follows: solvents were delivered at a flow rate of 0.8 ml/min: 0–18 min:100% A (90% 20 mM ammonium acetate in water, adjusted to pH 4.5 with acetic acid, 10% acetonitrile); 18–19 min: linear gradient from 100%A to 100% B; 19–60 min : 100%B (80% aqueous buffer as described above and 20% acetonitrile).

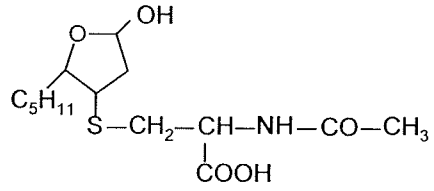
in the respective proportions of DHN-MA, HNE-MA and HNA-lac-MA. The main metabolite of (R)-HNE was DHN-MA while HNE-MA was absent and the oxidized mercapturic derivatives were relatively minor peaks. The various less polar metabolites of (S)-HNE were much more equally represented with a rather high proportion of HNE-MA.

The cumulative excretion of the four metabolites obtained 48 hours after (R)- or (S)-HNE administration is shown in Table 1. The results obtained in the 0–2 hour urinary metabolic profiles were confirmed. While the total proportion, i.e. about 40% of the administered dose, of the four metabolites remained

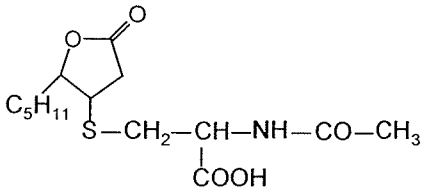
Table 1
Urinary metabolites of R- and S-HNE in 0–48 hr urine samples

	HNA-MA	DHN-MA	HNE-MA	HNA-lac-MA
(R)-HNE	9.2 ± 1.2	27.3 ± 6.6	ND	5.3 ± 2.0
(S)-HNE	6.5 ± 1.2	9.8 ± 0.4	13.3 ± 1.2	11.6 ± 1.5

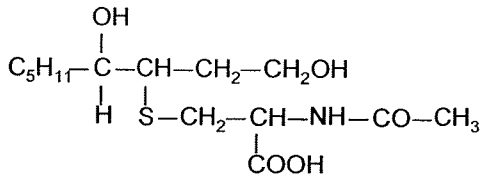
Values are expressed as percentage of total radioactivity and are means ± SD, $n = 3$. ND < 1% of total radioactivity in urine. HNA-MA, 3-mercapturic acid-4-hydroxynonanoic acid; DHN-MA, 3-mercapturic acid-1,4-dihydroxynonane; HNE-MA, 3-mercapturic acid-4-hydroxynonanal and HNA-lac-MA, HNA-MA lactone form.



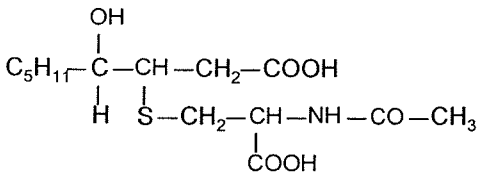
HNE-MA (3-mercapturic acid-4-hydroxynonanal)



HNA-lac-MA (lactone form of HNA-MA)



DHN-MA (3-mercapturic acid-1,4-dihydroxynonane)



HNA-MA (3-mercapturic acid-4-hydroxynonanoic acid)

Fig. 3. Non polar HNE metabolites.

the same for both enantiomers, DHN-MA was shown to be the major metabolite of (R)-HNE while HNE-MA was not detected. For (S)-HNE, the major metabolite was HNE-MA but the other metabolites were also present.

3.3. *Liver cytosolic metabolism of HNE-GSH originating from (R)- or (S)-HNE*

In order to clarify the intermediate pathways involved in the biotransformation of HNE into the different urinary metabolites obtained above, incubation of HNE-GSH, which is known to be the primary and major metabolite of HNE [4], were performed with liver cytosolic fractions. Moreover, HNE-GSH is the precursor of all urinary mercapturic acid conjugates of HNE [5], which represent the major part of urinary metabolites. The results are presented in Table 2. Metabolites were formerly identified using mass-spectrometry analysis [4]. HNE-GSH originating from (S)-HNE was quantitatively more metabolized than HNE-GSH originating from the other enantiomer and gave more metabolites originating from a retro-Michael reaction (unconjugated metabolites) but gave no reduced metabolite (DHN-GSH). HNE-GSH originating from (R)-HNE was metabolized mostly as HNA-lac-GSH and also as DHN-GSH, but in a smaller proportion. When NADPH was added to the incubation, the formation of DHN-GSH amounted to 30% (± 3.2) with HNE-GSH originating from (R)-HNE but was unchanged in the case of

Table 2
Metabolites of HNE-GSH in rat liver cytosolic incubations

	HNE-GSH synthesized from (R)-HNE	HNE-GSH synthesized from (S)-HNE
DHN-GSH	13.5 ± 1.5	ND
HNE-GSH	32.6 ± 1.5	11.4 ± 2
HNA-lactone-GSH	26.8 ± 5.3	21 ± 9
HNA	2.2 ± 0.8	5.1 ± 0.8
DHN	4.3 ± 0.7	3.9 ± 0.8
HNE	2.9 ± 0.5	15 ± 2.3
HNA-lactone	10.5 ± 1.3	9.8 ± 2.1

Values are expressed as percentage of total radioactivity and are means ± SD, $n = 3$. ND < 1% of total radioactivity. GSH, glutathione; DHN, 1,4-dihydroxy-2-nonene; HNE, 4-hydroxy-2-nonenal; HNA, 4-hydroxy-2-nonenoic acid.

(S)-HNE deriving HNE-GSH. In contrast, when incubations were added with phenobarbital, reduction of HNE-GSH was inhibited.

4. Discussion

The difference in the biotransformation/excretion rate between (R)-HNE and (S)-HNE was not statistically significant probably due to the too low number of animals analyzed. However, the results showed a tendency in which (S)-HNE was slightly faster excreted than the other enantiomer. This faster excretion of (S)-HNE can be explained by a preferential biotransformation of this enantiomer. Hiratsuka et al. [15] showed that the *in vitro* conjugation of (S)-HNE to glutathione occurred at a higher rate (× 2.5) for the (S)- than for the (R)-enantiomer when the two enantiomers were incubated with rat liver dialyzed cytosolic fraction. These authors suggested that the rGST A4-4, which represents a minor but highly HNE specific enzyme, is most likely to be the isoform involved in this (S)-selective conjugation of HNE enantiomers. More recently, these authors [16] showed that all major GST isoforms isolated from guinea-pig or rat liver also catalyzed the (S)-preferential conjugation of the HNE enantiomers.

Moreover, in the present study, when the HNE-GSH conjugates arising from either (S)- or (R)-HNE were incubated with rat liver cytosolic fraction, our results showed that the deconjugation of the adduct was 5 times more efficient in the case of the conjugate coming from the (S)-HNE than with the one coming from the other enantiomer. This retro-Michael reaction has previously been shown to be GST-catalyzed [4] and the (S)-preferential GSH conjugation of HNE by GSTs could explain the (S)-preferential reverse reaction.

DHN-MA is the major urinary metabolite of racemic HNE in the rat after intravenous or intraperitoneal administration [1,9]. This compound is also present at a basal level in the urine of humans and rats [2]. In the present study, we showed that there was (R)-preferential formation of DHN-MA *in vivo*. This compound results from the conjugation of HNE with GSH as described above, followed by the reduction of the aldehyde function of the conjugate and the formation of the mercapturic acid conjugate originating from the GSH part of the adduct, the last step occurring mainly in the kidney. The reduction step is important in the biotransformation of HNE because it prevents any dissociation of the Michael adducts of HNE with GSH but also with protein amino acids [4]. This reaction is most likely to be catalyzed by a member of the aldo-keto reductase (AKR) family [4]. We showed here that the HNE-GSH adduct incubated with liver cytosolic fractions gave DHN-GSH only when the adduct came from (R)-HNE. The fact that only the proportion of DHN-GSH coming from (R)-HNE-GSH was modified when NADPH (the cofactor of the AKR enzymes) was added to the incubation indicates that this selective reduction should

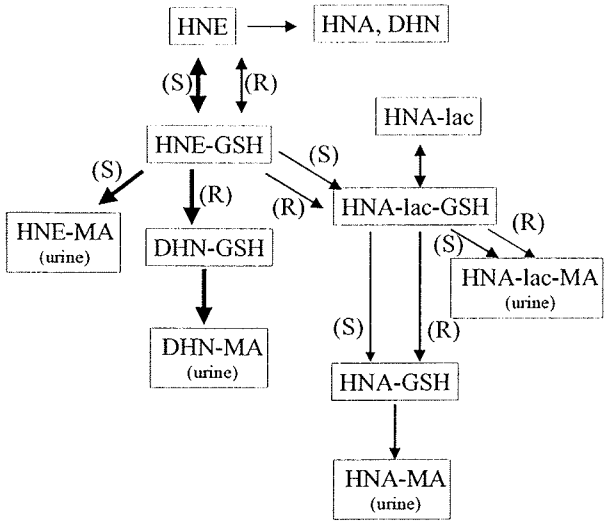


Fig. 4. Proposed metabolic pathways of (R)- and (S)-HNE GSH, glutathione; DHN, 1,4-dihydroxy-2-nonene; HNE, 4-hydroxy-2-nonenal; HNA, 4-hydroxy-2-nonenoic acid; HNA-MA, 3-mercapturic acid-4-hydroxynonanoic acid; DHN-MA, 3-mercapturic acid-1,4-dihydroxynonane; HNE-MA, 3-mercapturic acid-4-hydroxynonanal and HNA-lac-MA, HNA-MA lactone form.

actually be catalyzed by a member of this family of enzymes. Moreover, the use of phenobarbital, which is a potent inhibitor of AKR enzymes, inhibited the reduction of (R)-HNE-GSH. Hiratsuka et al. [15] showed that the reduction of HNE, catalyzed by alcohol dehydrogenase, was (R)-selective. However, this enzyme, which is not an AKR enzyme, is not involved in the reduction of the HNE-GSH adduct in rat liver or kidney [4].

Yet, *in vivo*, we obtained about 10% of DHN-MA with radiolabeled (S)-HNE. The methodology used to obtain the two racemates ensures the purity of (R)-HNE because this enantiomer is selectively acetylated by the lipase of *Pseudomonas fluorescens* [6] and then separated from the non acetylated HNE. In contrast, this acetylation step may not be complete and may leave some unreacted (R)-HNE in the non acetylated HNE fraction. This unreacted (R)-HNE may contaminate (S)-HNE. In the *in vitro* experiments, HNE-GSH isomers were further purified by HPLC [4]. In other respects, the reduction step may also be catalyzed by enzymes that are not present in liver cytosol and which may exhibit less (R)-selectivity toward HNE. So DHN-MA, considered to be the major urinary metabolite of HNE in the rat, comes rather from the (R)-HNE enantiomer. NMR analysis of DHN-MA isolated from rat urine (data not shown) indicated the presence of two diastereoisomers, the most abundant being identical to the DHN-MA standard arising from (R)-HNE (Guéraud et al., in press).

In conclusion, various enzymes involved in HNE biotransformation show enantioselectivity (Fig. 4). The (S)-preferential conjugation of HNE with GSH by GST has been well investigated. We showed here that the reduction of the conjugate, which may be an important reaction for the biological activity of HNE, was enantioselective. However, the enzyme(s) involved in this enantioselective reaction still needs to be further characterized.

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