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METABOLISM OF NOMIFENSINE TO A DIHYDROISOQUINOLINIUM ION  
METABOLITE BY HUMAN MYELOPEROXIDASE, HEMOGLOBIN,  
MONOAMINE OXIDASE-A, AND CYTOCHROME P450 ENZYMES

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## ABSTRACT

Nomifensine is an antidepressant agent that was removed from use due to a high incidence of hemolytic anemia. It contains an N-methyl-8-aminotetrahydroisoquinoline ring which has the potential to be oxidized to quaternary dihydroisoquinolinium and isoquinolinium ions, albeit such a transformation had not been previously observed. In this report, we demonstrate the conversion of nomifensine to a dihydroisoquinolinium ion metabolite by several human enzymes. Human liver microsomes supplemented with NADPH generated the dihydroisoquinolinium ion metabolite along with other hydroxylated metabolites, whereas when supplemented with t-butyl peroxide only the dihydroisoquinolinium ion metabolite was observed. Monoamine oxidase A, but not monoamine oxidase B, catalyzed this reaction, as well as human hemoglobin supplemented with H<sub>2</sub>O<sub>2</sub>. Human myeloperoxidase catalyzed this reaction in the presence of H<sub>2</sub>O<sub>2</sub>, and activation of the reaction was observed when incubations were conducted in the presence of acetaminophen at concentrations relevant to those measured in humans. The reaction was also observed in human whole blood. The equilibrium between the dihydroisoquinolinium ion and carbinolamine was shown to have a pK of about 11.7. The dihydroisoquinolinium ion was shown to react with cyanide and borohydride, but not glutathione. These findings suggest that it is possible that the electrophilic nomifensine dihydroisoquinolinium metabolite, which can be generated by several enzymes, could be behind toxic responses to nomifensine such as hemolytic anemia and hepatotoxicity.

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## INTRODUCTION

Nomifensine was an antidepressant drug with a unique receptor binding profile that was introduced in the 1980s (Kinney, 1985). However, it was discontinued from use due to an unacceptable incidence of drug-induced hemolytic anemia (Stonier and Edwards, 2002; Salama and Mueller-Eckhardt, 1985). Its use was also associated with reports of hepatotoxicity (Nielsen and Lund, 1981; Kummer, et al., 1985). Efforts to address the mechanism of nomifensine-induced toxicity have included analysis of sera from patients which showed the presence of antibodies reactive to nomifensine and some of its reported metabolites (Salama and Mueller-Eckhardt, 1985, 1986).

Nomifensine possess an aniline group, which is a substituent in drugs frequently associated with blood and liver toxicities (Kalgutkar, et al., 2005). In an examination of the metabolism of nomifensine in vivo, the observed metabolites included alterations of the phenyl ring, including conjugates suggesting the existence of a catechol intermediate metabolite (Kellner, et al., 1977; Heptner, et al., 1978; Hornke, et al., 1980; Lindberg and Syvalahti, 1986). These represent two possibilities of structural elements in nomifensine that could underlie the toxicity of the drug. We hypothesized that the tetrahydroisoquinoline structure of nomifensine could be the chemical group responsible for toxicity, as metabolism to the dihydroisoquinolinium ion would represent a bioactivation pathway analogous to the formation of other iminium ion metabolites (e.g. MPTP, ticlopidine, mianserin, etc) (Peterson, et al., 1985; Roberts, et al., 1993; Dalvie and O'Connell, 2004). In nomifensine, such an ion would be expected to be

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highly resonance-stabilized with the aniline substituent. Generation and sequestration of such a metabolite within tissues, such as blood cells or hepatocytes could be a possible cause of toxicity. However, in the early reports describing the metabolism of nomifensine in vivo, no observation of a dihydroisoquinolinium ion was made (Kellner, et al., 1977; Heptner, et al., 1978; Hornke, et al., 1980; Lindberg and Syvalahti, 1986). No reports on the metabolism of this compound using in vitro methods have been described.

The objectives of the experiments described herein were to determine whether nomifensine could be metabolized to a dihydroisoquinolinium ion, and if so, what enzymes were capable of catalyzing this reaction. The chemical reactivity of the dihydroisoquinolinium ion was examined. A discussion of this metabolic pathway is offered in the context of its potential involvement in the toxicity of nomifensine. Such knowledge is important for new drug design. The tetrahydroisoquinoline ring system can be a valued component in the development of pharmacophores for receptor ligands, so understanding whether this structural entity can be bioactivated to toxic metabolites, and the mechanism of such a bioactivation pathway, will be of value in designing drugs that contain this ring system but are less likely to undergo similar biotransformation pathways.

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## MATERIALS AND METHODS

*Chemicals and Biological Reagents.* Nomifensine maleate and nomifensine dihydroisoquinolinium perchlorate were prepared using published methods (Venkov and Vodenicharov, 1990; Moehrle and Biegholdt, 1988). Human hemoglobin, human myeloperoxidase, t-butyl peroxide (70%), reduced glutathione, and hydrogen peroxide (30%) were from Sigma (St. Louis, MO). Monoamine oxidases were generously provided by Dr. Dale Edmondson, Emory University. Human liver microsomes, a pool from 60 individual donors, were prepared using standard procedures and maintained in an in-house bank at Pfizer.

*Incubation Procedures.* For human liver microsomes, nomifensine (0.5 – 500  $\mu\text{M}$ ) was incubated with human liver microsomes (0.2 mg/ml) and either NADPH (1.3 mM) or t-butyl peroxide (1.0 mM) in 0.2 ml  $\text{KH}_2\text{PO}_4$ , 25 mM, pH 7.4 containing  $\text{MgCl}_2$  (3.3 mM). Reactions were commenced with the addition of cofactor and conducted at 37°C for 10 min. An initial experiment established linear conditions with respect to protein concentration and time. Reactions were terminated by the addition of four volumes of 0.1 M HCl, and analyzed directly by HPLC-MS as described below. Incubation conditions used to generate nomifensine metabolite profiles were similar to those described above, except that the protein concentration was 1.0 mg/ml, the nomifensine concentration was 10  $\mu\text{M}$ , and the incubation was terminated by addition of an equal volume of  $\text{CH}_3\text{CN}$ . Analysis of these samples was done using HPLC-MS.

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Monoamine oxidase A (0.1 nmol/ml) was incubated with nomifensine (10-1000  $\mu$ M) in 0.2 ml 25 mM potassium phosphate, pH 7.4, at 37°C for 1 hr. An initial experiment established a linear relationship between product generation and incubation time. The incubations were terminated by addition of HCl (0.8 ml of 0.1 M), and analyzed for the dihydroisoquinolinium ion by HPLC-MS.

For hemoglobin and myeloperoxidase, incubations were carried out in optical 96-well plates in a Perkin Elmer plate reader with a 420 nm filter, maintained at 37°C, with readings taken every 24 sec. For incubations with hemoglobin, nomifensine maleate (10 – 1000  $\mu$ M) and H<sub>2</sub>O<sub>2</sub> (0.5 mM) were incubated with human hemoglobin (0.6  $\mu$ M), in 0.25 ml 25 mM KH<sub>2</sub>PO<sub>4</sub>, pH 7.4. For reactions catalyzed by myeloperoxidase, incubations consisted of nomifensine maleate (10 – 1000  $\mu$ M), acetaminophen (44  $\mu$ M), human myeloperoxidase (1.0 U/ml) in 0.25 ml of 200 mM KH<sub>2</sub>PO<sub>4</sub>, pH 7.45. Experiments were also conducted in which the concentration of acetaminophen was varied between 0 and 200  $\mu$ M. Velocity calculations were done using a molar extinction coefficient for nomifensine dihydroisoquinolinium ion of 3153/cm that was determined experimentally.

Nomifensine (10  $\mu$ M) was incubated with whole blood (0.02 ml) added to 0.98 ml isotonic saline at 37°C for 30 min in the presence or absence of H<sub>2</sub>O<sub>2</sub> (0.5 mM). The incubations were terminated with 1 ml CH<sub>3</sub>CN and 0.05 ml were injected on HPLC-MS as described below.

HPLC-MS Conditions. For metabolite profiling, samples were injected onto a Polaris C18 column (4.6 x 250 mm; 5 $\mu$  particle size) equilibrated in 20 mM HOAc,

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adjusted to pH 4.0 with  $\text{NH}_4\text{OH}$  containing 18%  $\text{CH}_3\text{CN}$  at a flow rate of 0.8 ml/min. The  $\text{CH}_3\text{CN}$  was increased to 36% over 20 min, followed by an immediate increase to 86%  $\text{CH}_3\text{CN}$  to wash the column for 5 min. The eluent was introduced into the ionspray source of a Micromass Ultima tandem mass spectrometer operated in the positive ion mode. Settings were as follows: capillary voltage = 0.75 kV, cone voltage = 50V, source temperature = 135°C, desolvation temperature = 350°C, cone gas  $\approx$  180 L/h, desolvation gas  $\approx$  770 L/h, collision energy = 22 eV. Other potentials were set to optimize the signal. Metabolites were identified using precursor and product scanning techniques.

For quantitative analysis of nomifensine dihydroisoquinolinium ion, samples were injected onto a Varian Metasil AQ column (2.0 x 50 mm, 5  $\mu$ ) equilibrated in 20 mM acetic acid, adjusted to pH 4 with  $\text{NH}_4\text{OH}$  containing 15%  $\text{CH}_3\text{CN}$  at a flow rate of 0.5 ml/min. This mobile phase composition was maintained throughout the run, with the analyte eluting at 1.7 min. The effluent was introduced into the ion spray source of the Micromass Ultima mass spectrometer with the same tune file conditions as described above. The analyte was detected with selected reaction monitoring of the transition  $m/z$  237  $\rightarrow$  146. Quantitation was accomplished using a standard curve ranging from 10.0 – 1000 ng/ml.

### *Chemical Reactions and pH Titration of Nomifensine Dihydroisoquinolinium Ion.*

Nomifensine iminium perchlorate (14.8  $\mu\text{M}$ ) was incubated with KCN (0 – 100 mM) in phosphate buffer (900 mM) at pH 7.5. Incubations with reduced glutathione (0 – 100 mM) were conducted at the same concentration of



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nomifensine dihydroisoquinolinium ion, in phosphate buffer at 500 mM, pH 7.5. Incubations (1 ml) were carried out room temperature for 1 h, after which they were read on a Varian Cary 4000 UV/VIS spectrophotometer at 415 nm.

The pH titration was conducted similarly. To solution of nomifensine dihydroisoquinolinium perchlorate (14.8  $\mu\text{M}$ ) was added varying amounts of NaOH (and water to keep the volume addition constant) at ambient temperature, and the pH recorded. The absorbance at 415 nm was recorded.

In the reduction reaction, to an aqueous solution (0.2 ml) of nomifensine dihydroisoquinolinium perchlorate (1 mg/ml) was added 2 mg  $\text{NaBD}_4$ . The reaction was observed to change color from yellow to colorless upon addition of the reducing agent. The reaction was stirred at room temperature for 0.5 h after which 1 mL of water was added followed by extraction into methyl t-butyl ether (3 ml). The solvent was evaporated, the residue was reconstituted in mobile phase and infused into an HPLC-MS.

Data Analysis. Enzyme kinetic data were analyzed using the Enzyme Kinetics module of SigmaPlot (v8, SPSS, Chicago, IL). Data were fit to the following equations:

$$v = \frac{V_{\max} \cdot [S]}{K_M + [S]} \quad \text{or} \quad v = \frac{V_{\max 1} \cdot [S]}{K_{M1} + [S]} + CL_{\text{int}(2)} \cdot [S]$$

## RESULTS

Metabolite Profile of Nomifensine with Peroxidases, Monoamine Oxidases, Cytochrome P450 Enzymes, and Hemoglobin. HPLC-MS profiles of metabolic

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incubations of nomifensine with human liver microsomes, hemoglobin, myeloperoxidase, and monoamine oxidases are shown in Figure 2. Hemoglobin, myeloperoxidase, MAO-A, and human liver microsomes all catalyzed turnover of nomifensine. Four metabolites were observed: nomifensine dihydroisoquinolinium ion (peak 2;  $R_t = 19.3$  min), and three metabolites proposed to arise via monohydroxylation reactions (peaks 3, 4, and 5) because their protonated molecular ions were sixteen mass units greater than nomifensine. The only incubation condition to yield the monohydroxy metabolites was the one containing human liver microsomes supplemented with NADPH, consistent with these metabolites arising via cytochrome P450 mediated catalysis. Human liver microsomes supplemented with NADPH also yielded the dihydroisoquinolinium ion metabolite. Interestingly, when liver microsomes were supplemented with t-butyl peroxide instead of NADPH, only the dihydroisoquinolinium ion metabolite was formed. Hemoglobin, and myeloperoxidase supplemented with  $H_2O_2$  both yielded the dihydroisoquinolinium ion, but no other metabolites. When the myeloperoxidase incubation was also supplemented with acetaminophen, a greater rate of reaction was observed (also see below). Only a trace signal for the dihydroisoquinolinium ion was observed in incubations containing MAO-B, suggesting that this enzyme is not an active catalyst of this reaction. Finally, a trace signal was observed for the ion current at  $m/z$  235 that eluted just after the dihydroisoquinolinium ion, indicative of an isoquinolinium ion metabolite. Interestingly, an N-desmethyl metabolite was not

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observed, despite this type of metabolic reaction being very common for tertiary amines.

*Kinetics of Nomifensine Dihydroisoquinolinium Ion Formation.* In order to gain a more quantitative understanding of the activities of these enzymes to catalyze the dehydrogenation of nomifensine to the dihydroisoquinolinium ion, the enzyme kinetics were measured. These are summarized in Table 1. For liver microsomes, the kinetic data suggested two distinguishable activities when supplemented with either NADPH or t-BuOOH: a low  $K_M$  activity of 26 or 11  $\mu\text{M}$  along with a high  $K_M$  activity which did not approach saturation. This observation could be due to two or more P450 enzymes present in liver microsomes that can catalyze this reaction. Interestingly, hemoglobin also demonstrated this biphasic enzyme kinetic behavior, despite being a pure protein. Myeloperoxidase and MAO-A demonstrated simple enzyme kinetic behavior. The  $K_M$  value for MAO-A was considerably higher ( $>1$  mM) than for the other enzymes/proteins.

Adjusting the enzyme kinetic values to intrinsic clearance values normalized on a per nmol protein basis allows for comparison of the nomifensine dehydrogenation activities of these enzymes to each other. The rank order of intrinsic clearances was myeloperoxidase  $>$  hemoglobin  $>$  cytochrome P450  $>$  MAO-A.

Myeloperoxidase has been shown to be stimulated by the presence of various phenolic compounds (Subrahmanyam, et al., 1991). The nomifensine dehydrogenase activity of myeloperoxidase was readily observed when in the presence of acetaminophen, a phenolic drug, and the enzyme kinetics were

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measured in the presence of 6.7  $\mu\text{g/ml}$  acetaminophen which is similar to unbound plasma concentrations observed in vivo after typical doses of this drug. The relationship between myeloperoxidase catalyzed nomifensine dehydrogenase activity and acetaminophen concentration, at nomifensine concentrations of 100  $\mu\text{M}$  (approximately equal to  $K_M$ ) and 1 mM (approximately 10X  $K_M$ ) is shown in Figure 3. Maximal stimulation of the activity was observed at 50  $\mu\text{g/ml}$ .

*Chemical Reactivity of Nomifensine Dihydroisoquinolinium Ion.* In aqueous solution, alicyclic iminium ions are in equilibrium with carbinolamines. With increasing pH, the carbinolamine form predominates. The effect of pH on the iminium ion – carbinolamine equilibrium was examined by measurement of the absorbance of nomifensine dihydroisoquinolinium perchlorate at 415 nm (an absorbance maximum peak of the conjugated iminium-aniline system). The inflection point was at a pH of about 11.7, indicating that the iminium form predominates at physiological pH (Figure 4). Stabilization of the iminium form can be derived from the resonance stabilization imparted by the aniline ring. Other iminium ion – carbinolamine equilibria can occur at lower pH values when the iminium ion does not possess any special stabilization, such as that of the iminium ion metabolite of nicotine (Brandange and Lindblom, 1979).

To probe its potential as a reactive electrophile, nomifensine dihydroisoquinolinium ion was tested for reaction with cyanide and glutathione at physiological pH. Incubations with increasing concentration of cyanide demonstrated a decrease in absorbance at 415 nm, with a 50/50 ratio of iminium

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ion and adduct (presumed to be the  $\alpha$ -cyanoamine) occurring at about 10 mM KCN (Figure 4). Attempts to isolate the cyanide adduct product resulted in reversal of the reaction to the dihydroisoquinolinium ion. Nomifensine dihydroisoquinolinium ion did not show any detectable reaction with GSH. Reaction between nomifensine dihydroisoquinolinium and NaBD<sub>4</sub> resulted in the generation of deuterated nomifensine that was one mass unit greater than nomifensine when analyzed by mass spectrometry ( $m/z$  240 vs 239).

### *Conversion of Nomifensine to Nomifensine Dihydroisoquinolinium Ion by Blood.*

While it is of interest that nomifensine can be oxidized to the dihydroisoquinolinium ion by hemoglobin and myeloperoxidase when supplemented with H<sub>2</sub>O<sub>2</sub>, potential relevance in vivo would require that this conversion can occur in intact blood cells without high concentrations of peroxide. Upon incubation of nomifensine with whole blood diluted in isotonic saline, formation of the dihydroisoquinolinium ion was observed in the presence or absence of hydrogen peroxide (Figure 5).

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## DISCUSSION

Despite its unique profile as an effective antidepressant, nomifensine was removed from use due to an unacceptable incidence rate of hemolytic anemia (Stonier and Edwards, 2002). Generation of this adverse effect could arise via the generation of a reactive metabolite, but proof of such an intermediate for nomifensine has not been reported, despite demonstration of nomifensine antibodies in patients (Salama and Mueller-Eckhardt, 1985, 1986). Nomifensine is an aromatic amine, and compounds containing aromatic amines such as dapsone have been shown to be associated with blood and liver toxicities (Kalgutkar, et al., 2005; Bluhm, et al., 1999). Also, a methyl catechol metabolite of nomifensine was demonstrated in humans suggesting the possible presence of a catechol intermediate metabolite that could be the precursor of an electrophilic ortho-quinone. In the results described in the present report, the generation of a dihydroisoquinolinium ion metabolite from nomifensine was demonstrated. Previous studies have shown that tetrahydropyridine analogs including tetrahydroisoquinolines and beta carboline derivatives have been found to inhibit mitochondrial respiration and complex I (Morikawa et. al. 1998; Mateeva et. al. 2005). Tetrahydroisoquinoline and tetrahydro- $\beta$ -carboline derivatives are also activated to the respective N-methyl-isoquinolinium or N-methyl- $\beta$ -carbolinium metabolites (Castagnoli, et al., 1997) by two metabolic steps, which involve N-methylation by a non-specific N-methyltransferase and subsequent oxidation by MAO (Naoi, 1994). The nomifensine dihydroisoquinolinium ion metabolite offers another possibility that could lead to an understanding of the toxicity of

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nomifensine, and possibly design of an alternate agent that possesses the desired pharmacological effects while lacking the toxic effects.

The reactivity of nomifensine dihydroisoquinolinium ion as an electrophile was probed using model nucleophiles. The pH dependence of the equilibrium between iminium ion and carbinolamine provides insight into the reactivity of the  $\alpha$ -carbon, when considering hydroxide ion serving as the nucleophile. The pK of this equilibrium was approximately 11.7. This value is considerably greater than that previously measured for nicotine  $\Delta 1'(5')$  iminium ion (pK  $\approx$  8.4; Brandange and Lindblom, 1979). It is proposed that the greater pK for nomifensine dihydroisoquinolinium ion vs nicotine iminium ion is due to the conjugation of the iminium double bond to the aromatic ring as well as the aniline nitrogen for nomifensine. Thus, the iminium ion for nomifensine can be resonance stabilized (Figure 6), while for nicotine iminium ion this is not possible. This is further supported by the observation that while a cyano adduct for nomifensine dihydroisoquinolinium ion can be generated, it cannot be isolated, whereas 5'-cyanonicotine is a stable, isolable product from nicotine  $\Delta 1'(5')$  iminium ion (Peterson, et al., 1987). Whether nomifensine antibodies arise from an immune response to an immunogen that is generated from a dihydroisoquinolinium ion – protein adduct is not known.

In this report, the conversion of nomifensine to nomifensine dihydroisoquinolinium ion was observed. However, in previous examinations of the metabolism of nomifensine in humans, neither the dihydroisoquinolinium ion nor any downstream products (such as the lactam) were reported (Heptner, et

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al., 1978; Hornke, et al., 1980; Lindberg and Syvalahti, 1986). But in these earlier studies, a quantitative description of the metabolite profile was not obtained, so the dihydroisoquinolinium ion or any downstream metabolites may have been missed. Alternately, due to its existence as a charged species, it is possible that nomifensine dihydroisoquinolinium ion could be generated and trapped within the cell or subcellular organelles. Since nomifensine dihydroisoquinolinium ion can be generated by hemoglobin and MAO-A, it could also be trapped in erythrocytes or mitochondria, respectively, and elicit toxicity. Demonstration of this conversion in whole red blood cells, even in the absence of  $H_2O_2$ , further supports this hypothesis.

Several enzymes catalyze the oxidation of nomifensine to nomifensine dihydroisoquinolinium ion. The heme-containing enzymes cytochrome P450, myeloperoxidase, and hemoglobin all catalyze the reaction, likely all with a peroxidase-type mechanism. Human liver microsomes generated several metabolites, including the dihydroisoquinolinium ion, when supplemented with NADPH, but only the dihydroisoquinolinium ion was generated when supplementation was done with t-butyl peroxide. The latter cosubstrate is known to support P450 catalyzed peroxidation reactions. MAO enzymes have a different mechanism, so the conversion of nomifensine to nomifensine dihydroisoquinolinium ion can arise by different oxidation mechanisms. Hemoglobin catalyzed drug oxidations have been reported previously, but these are generally considered rare (Mieyal, et al., 1976; Starke, et al., 1984; Giardina, et al., 1995).



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Myeloperoxidase catalyzed oxidations have been implicated in the toxicity of other drugs such as clozapine (Ip and Uetrecht, 2006; Hofstra and Uetrecht, 1993). The conversion of nomifensine to the dihydroisoquinolinium ion by myeloperoxidase was stimulated by acetaminophen. A great deal of experimental data has been generated in support of acetaminophen bioactivation to reactive intermediates that cause hepatotoxicity. However, to our knowledge, investigation of acetaminophen as an agent that could activate the process of myeloperoxidase-generated reactive intermediates has not been shown. Activation occurred at acetaminophen concentrations that are relevant to unbound circulating levels attained after standard dosing of acetaminophen for analgesia (Figure 3). This finding opens the possibility of the involvement of acetaminophen as an indirect contributor to toxicities of other drugs that involve myeloperoxidase catalyzed processes. In a previous investigation, stimulation of myeloperoxidase by phenols had been demonstrated (Subrahmanyam, et al., 1991). Furthermore, stimulation of the conversion of oxyhemoglobin to methemoglobin and peroxide by phenols has been known for many years (Wallace and Caughey, 1975). Whether this mechanism is also the one underlying the stimulation of myeloperoxidase by acetaminophen, and whether acetaminophen could also stimulate nomifensine oxidation catalyzed by hemoglobin represent areas in need of further research.

Since several enzymes can catalyze this reaction, it is challenging to deconvolute the contributions that each could have to conversion in vivo. Enzyme kinetic data were gathered for this reaction for the different enzymes, in

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a preliminary effort to determine this. In consideration of the total quantity of hemoglobin in the body relative to the other proteins, it could be proposed that hemoglobin would be the main protein involved in this transformation in vivo. In a typical 70 kg human, estimates of the total amount of blood hemoglobin, hepatic cytochrome P450, and neutrophil myeloperoxidase are approximately 11000, 19, and 2.6  $\mu\text{mol}$ , respectively. Combination of these values with the intrinsic clearance values in Table 1 suggests that the contribution of hemoglobin to this reaction would far outweigh the contributions of the other enzymes. However, it must also be considered that the measurement of the enzyme kinetics for hemoglobin and myeloperoxidase in this report were made at a high concentration of hydrogen peroxide. While the identities of several enzymes that can catalyze this reaction have been made, further work is required to delineate the contributions of each of these.

In conclusion, the conversion of the tetrahydroisoquinoline containing drug nomifensine to a dihydroisoquinolinium ion metabolite has been demonstrated. Several enzymes and proteins can catalyze this reaction. Nomifensine dihydroisoquinolinium ion possesses electrophilic reactivity. Thus, these investigations have identified the N-alkyl tetrahydroisoquinoline ring moiety as a potential toxicophore capable of generating dihydroisoquinolinium ion metabolites. Further investigation of the metabolism and reactivity of this structure type is ongoing.

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References:

Bluhm RE; Adedoyin A; McCarver DG; Branch RA (1999) Development of dapsone toxicity in patients with inflammatory dermatosis: activity of acetylation and hydroxylation of dapsone as risk factors. *Clin. Pharmacol. Ther.* 65: 598-605.

Brandange S. and Lindblom L. (1979) Synthesis, structure and stability of nicotine  $\Delta^1(5')$  iminium ion, an intermediary metabolite of nicotine. *Acta Chem. Scand., Ser. B: B33:* 187-191.

Castagnoli N Jr, Rimoldi JM, Bloomquist J, and Castagnoli KP (1997) Potential metabolic bioactivation pathways involving cyclic tertiary amines and azaarenes. *Chem Res Toxicol* 10: 924-940.

Dalvie, DK and O'Connell, TM (2004) Characterization of novel dihydrothienopyridinium and thienopyridinium metabolites of ticlopidine in vitro: Role of peroxidases, cytochromes P450, and monoamine oxidases. *Drug Metab. Dispos.* 32: 49-57.

Esclade L, Guillochon D, Thomas D. (1986) Aromatic hydroxylations and peroxidations by hemoglobin systems. *Xenobiotica* 16: 615-24.

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Giardina B, Messina I, Scatena R, Castagnola M (1995) The multiple functions of hemoglobin. *Crit. Rev. Biochem. Mol. Biol.* 30: 165-196.

Heptner, W.; Hornke, I.; Cavagna, F.; Fehlhaber, H. W.; Rupp, W.; Neubauer, H. P. (1978) Metabolism of nomifensine in man and animal species. *Arzneimittel-Forschung* 28: 58-64.

Hofstra, A.H. and Uetrecht, J.P. (1993) Myeloperoxidase -mediated activation of xenobiotics by human leukocytes. *Toxicology* 82: 221-42.

Hornke, I.; Fehlhaber, H. W.; Girg, M.; Jantz, H. (1980) Metabolism of nomifensine (Alival, Merital): isolation and identification of the conjugates of nomifensine-14C from human urine. *British Journal of Clinical Pharmacology* 9: 255-64.

Ip, J. and Uetrecht, J. P. (2006) In vitro and animal models of drug - induced blood dyscrasias. *Env. Tox. Pharmacol.* 21: 135-140.

Jonen, H. G.; Kahl, R.; Kahl, G. F. (1976) Enhancement of microsomal aniline and acetanilide hydroxylation by hemoglobin. *Xenobiotica* 6: 307-20.

Kalgutkar, A.S., Gardner, I., Obach, R.S., Shaffer, C.L., Callegari, E., Henne, K.R., Mutlib, A.E., Dalvie, D.K., Lee, J.S., Nakai, Y., O'Donnell, J.P., Boer, J.,

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and Harriman, S.P. (2005) A comprehensive listing of bioactivation pathways of organic functional groups. *Curr. Drug Metab.* 6: 161-225.

Kinney J L (1985) Nomifensine maleate: a new second-generation antidepressant. *Clin. Pharm.* 4: 625-636.

Kellner, H. M.; Baeder, C.; Christ, O.; Heptner, W.; Hornke, I.; Ings, Robert M. J. (1977) Kinetics and metabolism of nomifensine in animals. *Br. J. Clin. Pharmacol.* 4(Suppl. 2), 109S-116S

Kummer H, Marti F, Wegmann W (1985) Granulomatous hepatitis caused by nomifensine. *J. Suisse Med.* 115: 1674-1678.

Lindberg RLP and Syvalahti EKG. (1986) Metabolism of nomifensine after oral and intravenous administration. *Clin. Pharmacol. Ther.* 39: 378-383.

Mateeva, NN, Winfield, LL, and Redda KK. (2005) The chemistry and pharmacology of tetrahydropyridines. *Cur. Med. Chem.* 12: 551-571.

Mieyal JJ, Ackerman RS, Blumer JL, Freeman LS (1976) Characterization of enzyme like activity of human hemoglobin. *J. Biol. Chem.* 251: 3436-3441.

Moehrle, H and Biegholdt, M. (1988) Carbinolamine equivalents in the 8-aminotetrahydroisoquinoline series. *Arch. Pharm.* 321: 287-91.

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Morikawa N, Naoi, M., Maruyama, W., Ohta, S, Kotake, Y., Kawai, H., Niwa, T., Dostert, P., and Mizuno, Y. (1998) Effects of various tetrahydroisoquinoline derivatives on mitochondrial respiration and the electron transfer complexes. *J. Neural Transm.* 105: 677-688.

Naoi M, Maruyama W, Niwa T and Nagatsu T (1994) Novel toxins and Parkinson's disease: N-methylation and oxidation as metabolic bioactivation of neurotoxin. *J Neural Transm (Suppl.)* 41:197-205.

Nielsen J L and Lund N O (1981) Drug fever due to nomifensine treatment in patients with endogenous depression. *Int. Pharmacopsych.* 16: 66-68.

Peterson, L A.; Caldera, P S.; Trevor, A; Chiba, K; Castagnoli, N., Jr. (1985) Studies on the 1-methyl-4-phenyl-2,3-dihydropyridinium species 2,3-MPDP<sup>+</sup>, the monoamine oxidase catalyzed oxidation product of the nitrostriatal toxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP). *J. Med. Chem.* 28: 1432-1436.

Peterson, L A.; Trevor, A; Castagnoli, N, Jr. (1987) Stereochemical studies on the cytochrome P-450 catalyzed oxidation of (S)-nicotine to the (S)-nicotine  $\Delta^1(5')$ -iminium species. *J. Med. Chem.* 30: 249-254.

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Roberts, P.; Kitteringham, N. R.; Park, B. K. (1993) Elucidation of the structural requirements for the bioactivation of mianserin in vitro. *J. Pharm. Pharmacol.* 45: 663-665.

Salama A and Mueller-Eckhardt C (1985) The role of metabolite-specific antibodies in nomifensine-dependent immune hemolytic anemia. *N. Eng. J. Med.* 313: 469-474.

Salama A, Mueller-Eckhardt C (1986) Two types of nomifensine-induced immune haemolytic anaemias: drug-dependent sensitization and/or autoimmunization. *Br J Haematol* 64:613-620.

Starke, DW.; Blisard, KS.; Mielal, JJ. (1984) Substrate specificity of the monooxygenase activity of hemoglobin. *Mol. Pharmacol.* 25: 467-475.

Stonier PD and Edwards JG (2002) Nomifensine and haemolytic anemia. In: *Pharmacovigilance* (Ed. Mann RD and Andrews EB) Wiley, New York, pp. 155-166.

Subrahmanyam VV, Kolachana P, and Smith MT. (1991) Metabolism of hydroquinone by human myeloperoxidase : mechanisms of stimulation by other phenolic compounds. *Arch. Biochem. Biophys.* 286: 76-84.



DMD #10173

Venkov, A. and Vodenicharov, D. (1990) A new synthesis of 1,2,3,4-tetrahydro-2-methyl-4-phenylisoquinolines. *Synthesis* 253-255.

Wallace WJ and Caughey WS (1975) Mechanism for the autooxidation of hemoglobin by phenols, nitrite and 'oxidant' drugs. Peroxide formation by one electron donation to bound dioxygen. *Biochem. Biophys. Res. Comm.* 62: 561-567.

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Footnote to Title:

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### Figure Legends.

Figure 1. Structures of nomifensine, nomifensine dihydroisoquinolinium ion, and nomifensine isoquinolinium ion.

Figure 2. HPLC-MS chromatograms of nomifensine incubation mixtures. Peak 1: nomifensine; peak 2: nomifensine dihydroisoquinolinium ion; peaks 3, 4, and 5 are putative hydroxy metabolites possessing molecular ion 16 mass units greater than nomifensine.

Figure 3. Effect of acetaminophen on the myeloperoxidase catalyzed conversion of nomifensine to nomifensine dihydroisoquinolinium ion.

Figure 4. Relationship between nomifensine dihydroisoquinolinium ion and adducts at the alpha carbon. Panel A: pH titration curve for dihydroisoquinolinium ion and  $\alpha$ -carbinolamine. Nomifensine dihydroisoquinolinium perchlorate (14.8  $\mu$ M) in aqueous solution was adjusted with NaOH and the absorbance at 415 nm was recorded at each pH. Panel B: Equilibrium between dihydroisoquinolinium ion and proposed  $\alpha$ -cyanoamine (filled triangles) and dihydroisoquinolinium ion and possible glutathione adduct (open squares). To nomifensine dihydroisoquinolinium perchlorate (14.8  $\mu$ M) in aqueous solution was added varying concentrations of KCN or GSH at pH 7.5. Incubations were allowed to stand for 1 h, followed by recording the absorbance at 415 nm.

Figure 5. HPLC-MS chromatograms of nomifensine incubated with human blood. Peak 1: nomifensine; peak 2: nomifensine dihydroisoquinolinium ion. Nomifensine was incubated with human blood (2%) in saline at 37°C for 0.5 h.

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Figure 6. Nomifensine oxidation to nomifensine dihydroisoquinolinium ion along with various equilibrium phenomena. Oxidation to the iminium ion could occur via  $\alpha$ -carbon hydroxylation to the carbinolamine which will be in equilibrium with the iminium ion. The iminium ion can be in equilibrium with nucleophile adducts. In both of these cases, at physiological pH, the iminium ion is favored. Alternately, it is also possible but less likely that the iminium ion could be generated by an initial N-hydroxylation of the aniline and dehydration to the imine, which would be in equilibrium with the iminium ion.

TABLE 1.

Enzyme kinetic parameters for the metabolism of nomifensine to nomifensine dihydroisoquinolinium ion by human enzymes (Mean +/- SD).

Enzyme/System	Kinetic Model	$K_{M(1)}^a$	$V_{max(1)}^b$	$CL_{int(2)}^c$	$CL_{int(total)}^c$	$r^2$
Liver Microsomes + NADPH	Two Enzyme	$26 \pm 8$	$1.8 \pm 0.2$	0.0028	0.072	0.993
Liver Microsomes + t-BuOOH	Two Enzyme	$11 \pm 2$	$3.3 \pm 0.3$	0.0015	0.30	0.990
Hemoglobin	Two Enzyme	$21 \pm 2$	$6.7 \pm 0.2$	0.0039	0.32	0.996
Myeloperoxidase + APAP	Single Enzyme	$109 \pm 8$	$187 \pm 5$	N/A	1.7	0.993
Monoamine Oxidase A	Single Enzyme	$1130 \pm 360$	$7.7 \pm 2.2$	N/A	0.0068	0.998

<sup>a</sup>Units for  $K_M$  values are  $\mu M$

<sup>b</sup>Units: nmol/min/nmol enzyme (For liver microsomes, a value of 0.3 nmol total P450/mg protein was used.)

<sup>c</sup>Units: ml/min/nmol

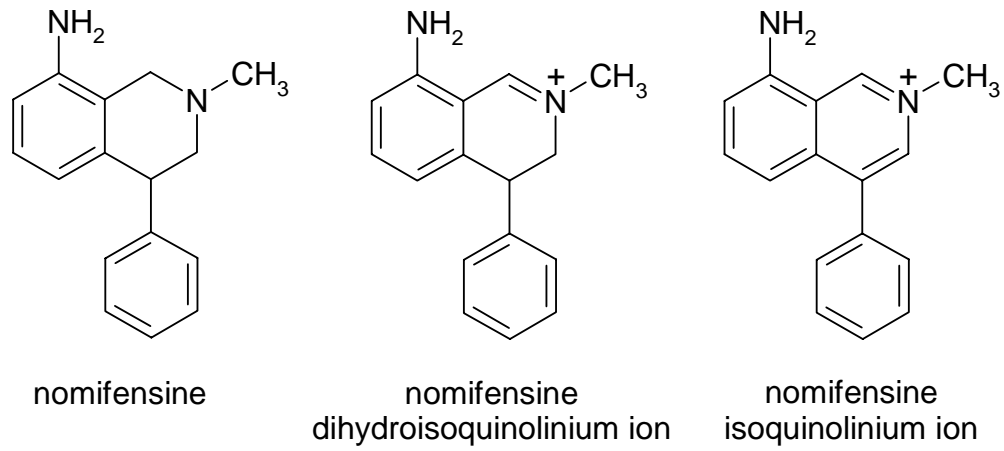


Figure 1

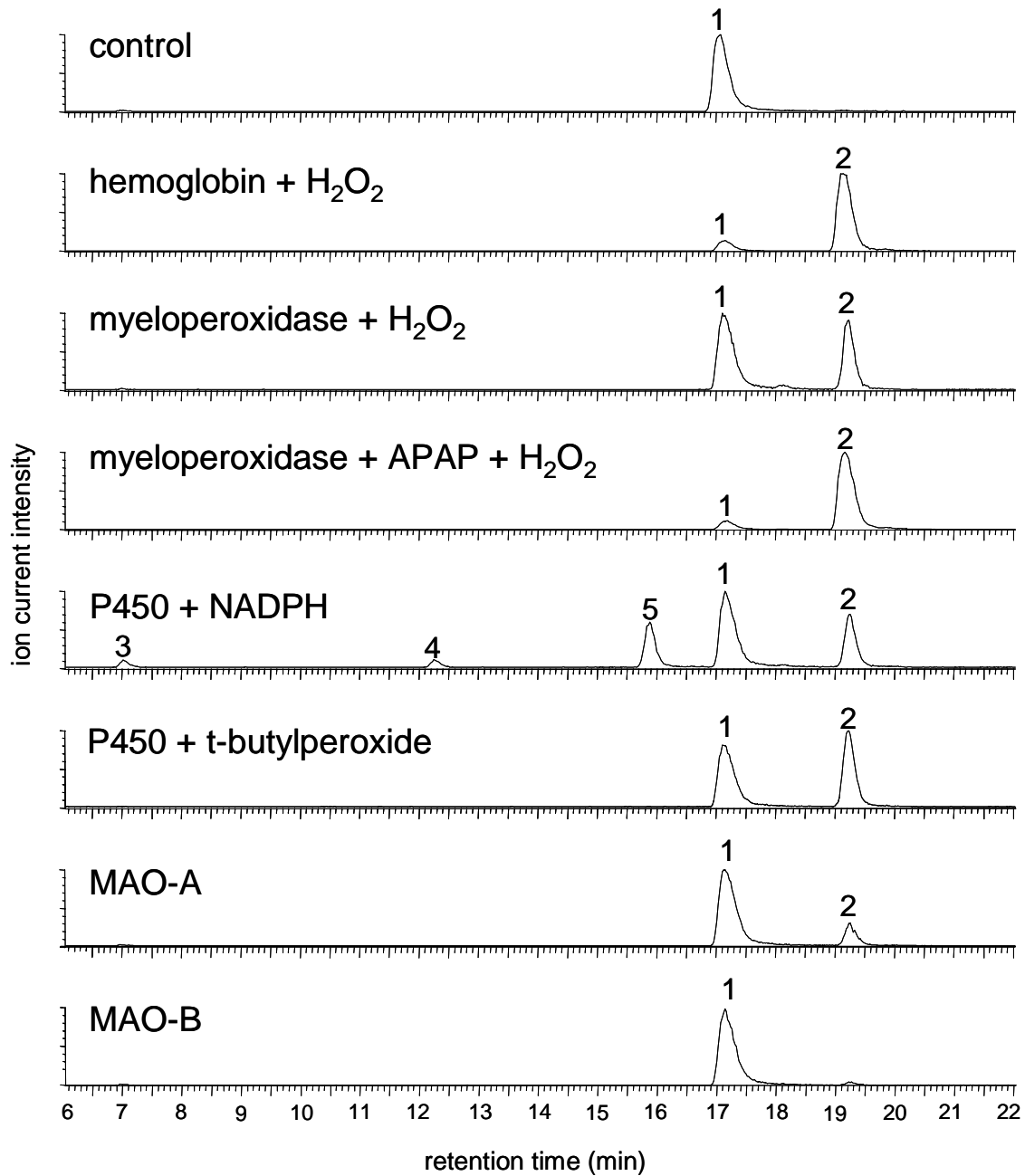


Figure 2

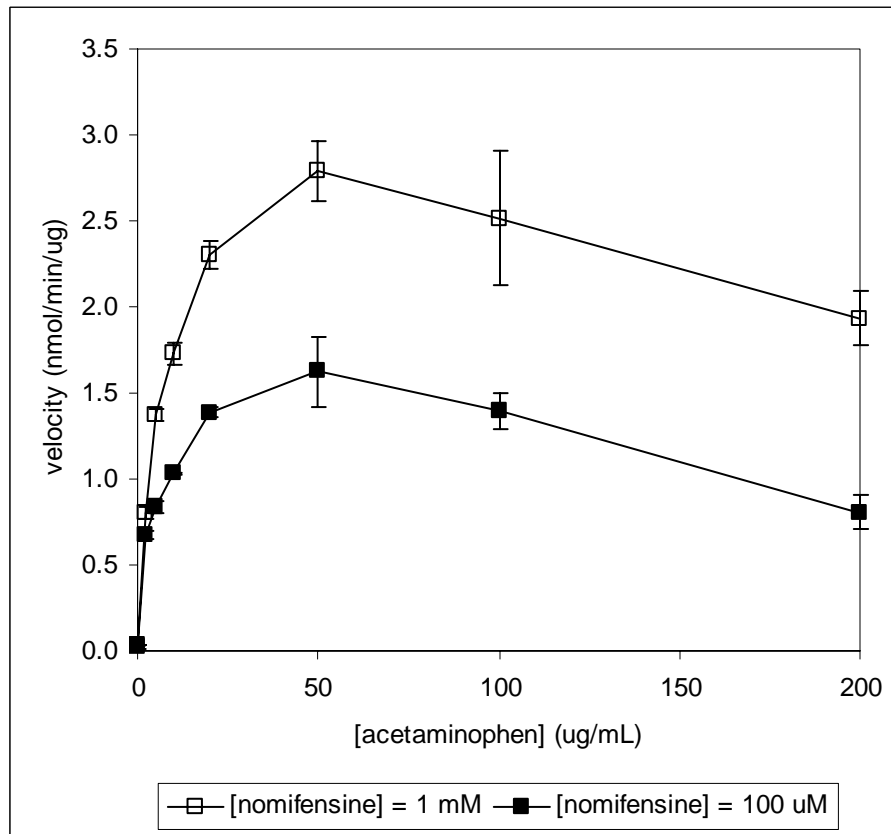


Figure 3



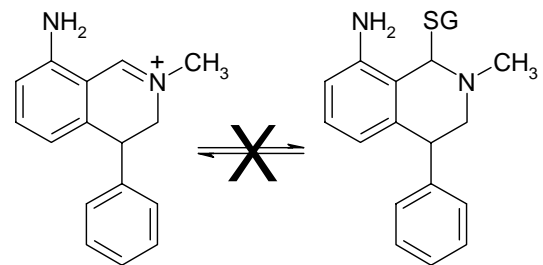
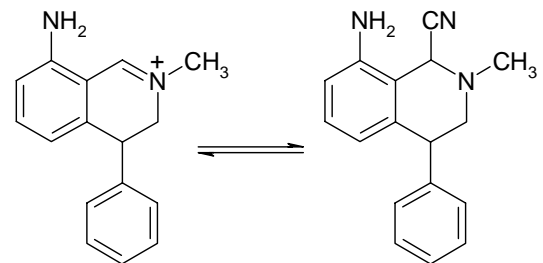
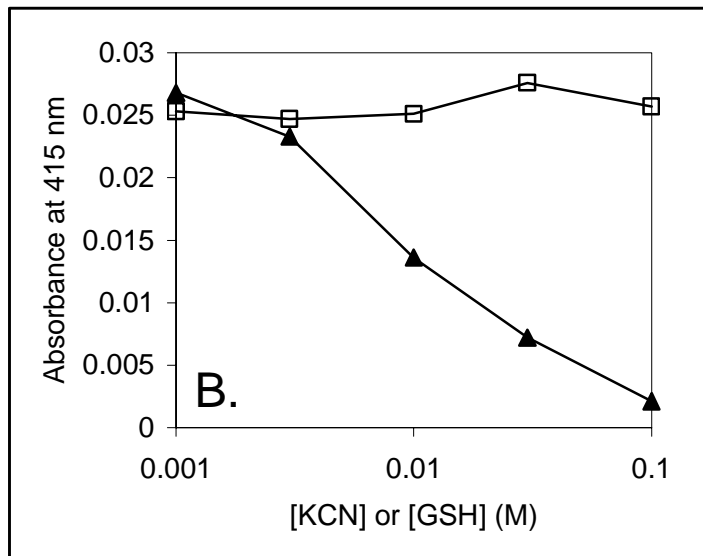
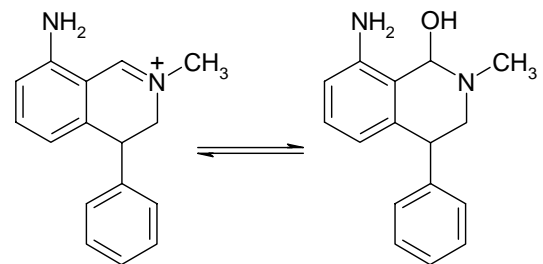
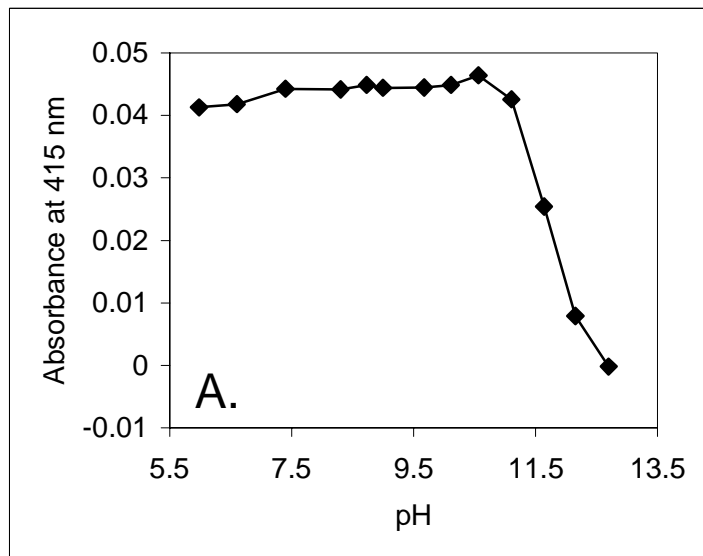


Figure 4

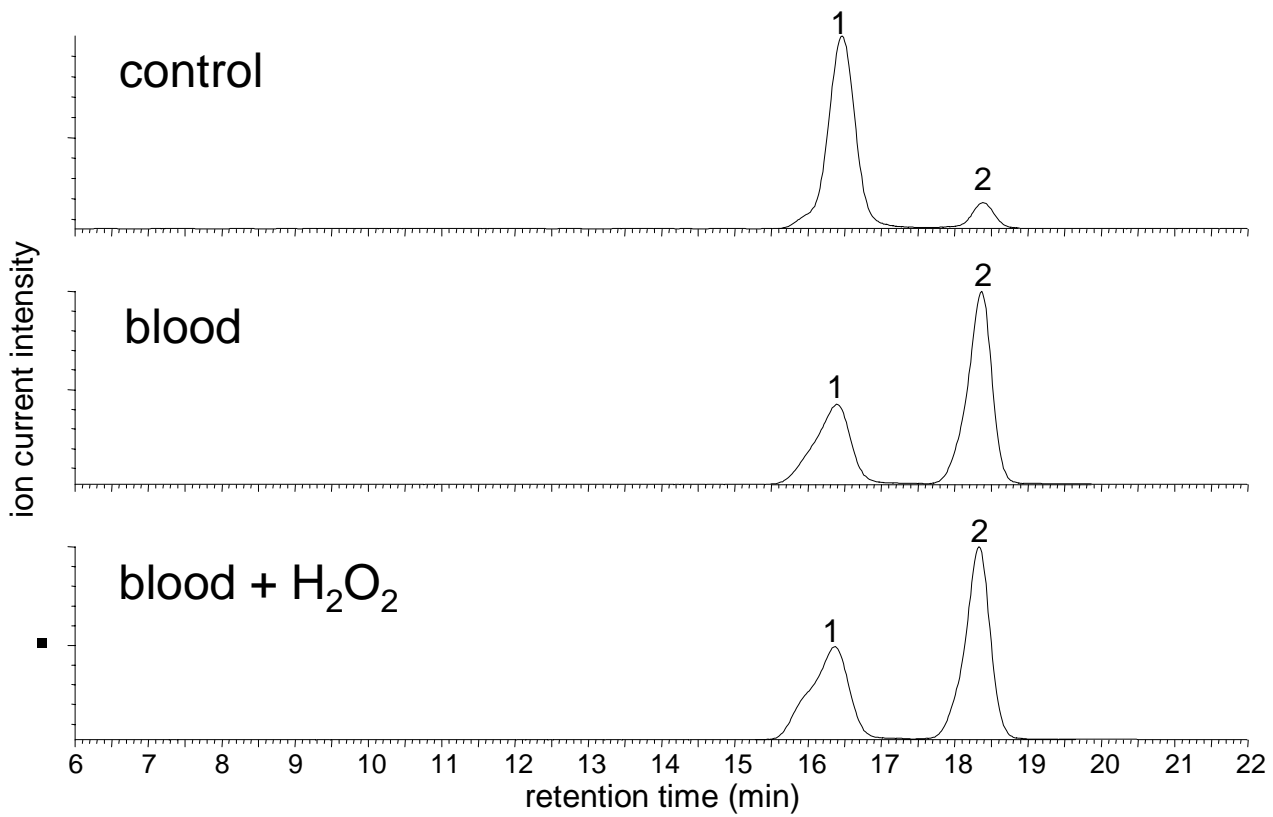


Figure 5

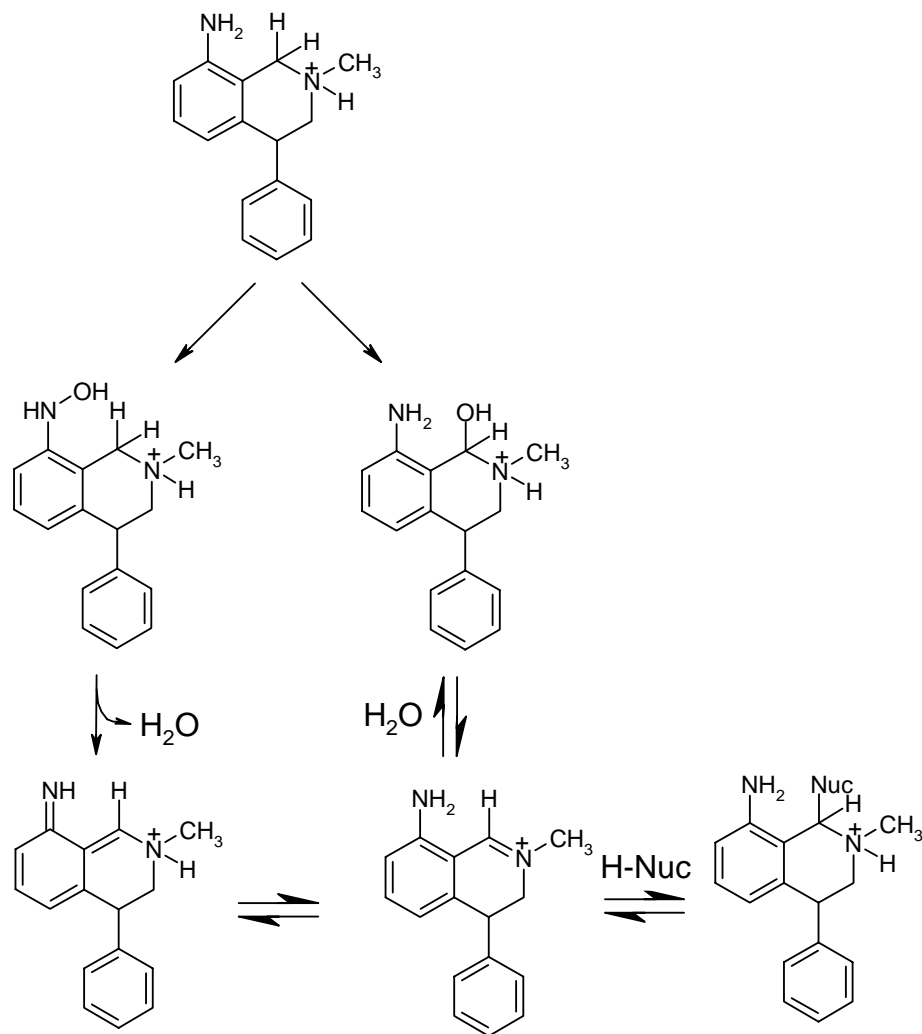


Figure 6