Identification of Multiple Glutathione Conjugates of 8-Amino-2-methyl-4-phenyl-1,2,3,4-tetrahydroisoquinoline maleate (Nomifensine) in Liver Microsomes and Hepatocyte Preparations: Evidence of the Bioactivation of Nomifensine

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ABSTRACT:

8-Amino-2-methyl-4-phenyl-1,2,3,4-tetrahydroisoquinoline maleate (nomifensine), an antidepressant drug, was withdrawn from the market because of increased incidence of hemolytic anemia, as well as kidney and liver toxicity. Although the nature of the potentially reactive metabolites formed after nomifensine metabolism remains unknown and no glutathione (GSH) adducts of these nomifensine reactive metabolites have been reported, bioactivation has been postulated as a potential mechanism for the toxicity of nomifensine. This study was conducted to probe the potential bioactivation pathways of nomifensine in human and animal hepatocytes and in liver microsomes using GSH as a trapping agent. Two types of GSH conjugates were characterized by liquid chromatography/tandem mass spectrometry: 1) aniline oxidation followed by GSH conjugation leading to the formation of nomifensine-GSH sulfonamides (M1 and M2); and 2) arene oxidation followed by GSH conjugation yielding a range of arene C-linked GSH adducts (M3–M9). Nine GSH adducts (M1–M9) were identified in liver microsomes of humans, dogs, monkeys, and rats and in human and rat hepatocytes. In dog hepatocyte preparations, six GSH adducts (M1–M6) were identified. The GSH adducts in dog and rat liver microsomes were formed primarily through aniline and arene oxidation, respectively. Both pathways contributed significantly to the formation of the GSH adducts in human and monkey liver microsomes. The bioactivation pathways proposed here account for the formation of the observed GSH conjugates. These investigations have confirmed the aniline and the arene groups in nomifensine as potential toxicophores capable of generating reactive intermediates.

Nomifensine, a tetrahydroisoquinoline derivative (Fig. 1), was marketed as an antidepressant drug. Although its structure is different from the traditional tricyclic and monoamine oxidase inhibitor antidepressants, nomifensine resembles the tricyclic antidepressants in many of its pharmacological effects in animal models of depressive illness (Hoffman, 1977, 1982; Fiedling and Szewczak, 1984; Kostowski et al., 1984). However, it differs from the tricyclic antidepressants in that it strongly inhibits the reuptake of both norepinephrine and dopamine and is a relatively weak inhibitor of serotonin uptake (Baldessarini, 1982). Nomifensine was originally introduced into the European market in 1976 to 1977 and was marketed as Merital (sanofi-aventis, Bridgewater, NJ) in the United States in 1985. Despite the good efficacy and unique pharmacological profile (Brogden et al., 1984; Kinney, 1985), it was withdrawn from use because of adverse events including drug-induced hemolytic anemia, kidney and liver toxicity, abuse potential, and some deaths (Nielsen and Lund, 1981; Kummer et al., 1985; Salama and Muller-Eckhardt, 1985, 1986; Salama et al., 1991; Stonier and Edwards, 2002). Nomifensine induced the production of antibodies and/or autoantibodies directed against drug- and/or metabolite-red blood cell conjugates in patients who developed immune hemolytic anemia while receiving the drug (Salama and Muller-Eckhardt, 1985, 1986; Salama et al., 1991; Stonier and Edwards, 2002). These observations led to the hypothesis that the hemolytic anemia toxicity of nomifensine was associated with the generation of reactive metabolites in vivo. However, the mechanism of this bioactivation and the nature of the reactive intermediates formed after oxidative metabolism of nomifensine remain unknown.

Three potential bioactivation pathways of nomifensine have been proposed to account for its toxicity (Kalgutkar and Soglia, 2005; Obach and Dalvie, 2006). The oxidation of the aniline group in nomifensine has long been speculated to cause its toxicity. Because compounds containing aromatic amines are widely recognized as being capable of producing toxic metabolites (Uetrecht, 1985; Lessard

ABBREVIATIONS: Nomifensine, 8-amino-2-methyl-4-phenyl-1,2,3,4-tetrahydroisoquinoline maleate; GSH, glutathione; LC/MS/MS, liquid chromatography/tandem mass spectrometry; nomifensine dihydroisoquinolinium perchlorate, 8-amino-2-methyl-4-phenyl-3,4-dihydroisoquinolinium perchlorate; UPLC, ultraperformance liquid chromatography; MS, mass spectrometry; CID, collision-induced dissociation.
ride, and

Fig. 1. Structures of nomifensine, hydroxylated nomifensine metabolites identified in human in vivo, and the nomifensine dihydroisoquinolinium ion.

et al., 1999; Kalgutkar et al., 2005). However, elucidation of nomifensine aniline oxidation products or their glutathione (GSH) adducts has not been described. Reports of metabolite identification in humans in vivo revealed multiple hydroxylated modification at the phenyl ring (Fig. 1, nomifensine hydroxylated metabolites I, II, and III), suggesting that a reactive ortho-quinone (through the oxidation of catechol at the C ring) or a quinone-methide (through the oxidation of phenol at the C ring) intermediate could be formed (Kellner et al., 1977; Heptner et al., 1978; Hornke et al., 1980; Lindberg and Syvälahti, 1986; Kalgutkar and Soglia, 2005). However, no GSH adducts of nomifensine arene oxidative intermediates have been reported. More recently, a nomifensine dihydroisoquinolinium metabolite (Fig. 1) was identified in in vitro samples, including human blood (Obach and Dalvie, 2006). This dihydroisoquinolinium metabolite is electrophilic as evidenced by its reaction with KCN detected by UV spectrometry. Therefore, the dehydrogenation of the N-methyl tetrahydroisoquinoline moiety (B ring) represents another possible route of nomifensine bioactivation that may lead to its toxicity.

The tripeptide GSH, present in all the mammalian tissues, is considered to be a natural reactive chemical defense mechanism. The nucleophilicity of the thiol group of GSH toward electrophiles is further enhanced by GSH S-transferase, which allows it to readily conjugate with certain types of potentially toxic electrophiles. The structures of the resulting conjugates provide insight into the identities of the reactive intermediates from which they were derived. The present studies were conducted to probe possible bioactivation pathways of nomifensine in human and animal hepatocytes and in liver microsomes using GSH as a trapping agent. Metabolites from the incubations of nomifensine with human and animal hepatocytes and in liver microsomes using GSH as a trapping agent. Metabolites from the incubations of nomifensine with human and animal hepatocytes and in liver microsomes were characterized by liquid chromatography/tandem mass spectrometry (LC/MS/MS). Metabolites from incubations of nomifensine dihydroisoquinolinium perchlorate with NADPH and GSH-supplemented human and rat liver microsomes were also identified and compared with those of nomifensine. The bioactivation pathways proposed here account for the formation of the observed GSH conjugates. These findings confirm the existence of reactive metabolites of nomifensine that were trapped by GSH and may help to explain the nomifensine-induced toxicity in humans.

Materials and Methods

Chemicals and Materials. Nomifensine, GSH (reduced), magnesium chloride, and β-nicotinamide adenine dinucleotide 2'-phosphate (reduced, NADPH) were obtained from Sigma-Aldrich (St. Louis, MO). Pooled human, cynomolgus monkey, beagle dog, and Sprague-Dawley rat liver microsomes (protein concentration, 20 mg/ml) were purchased from BD Gentest (Woburn, MA). Freshly isolated beagle dog and Sprague-Dawley rat hepatocytes and cryopreserved human hepatocytes were purchased from CelZDirect (Durham, NC). Nomifensine dihydroisoquinolinium perchlorate was prepared using published methods (Moehrle and Biegholdt, 1988; Venkov and Vodenicharov, 1990). The 2-mM stock solutions of nomifensine and nomifensine dihydroisoquinolinium perchlorate were prepared using 50% acetonitrile/water solution as solvent and were used for all the incubations. All the other reagents and solvents were of the highest quality available.

Incubation of Nomifensine and the Nomifensine Dihydroisoquinolinium Perchlorate with Human, Monkey, Dog, or Rat Liver Microsomes in the Presence of NADPH and GSH. All of the microsomal incubations were conducted at 37°C for 60 min in 100 mM potassium phosphate buffer, pH 7.4, containing MgCl2 (10 mM), liver microsomes (1.0 mg/ml), GSH (10 mM), and test compound (10 μM). After preincubation of the mixture for 5 min, the reactions were initiated by the addition of NADPH (final concentration, 1 mM). Control experiments were conducted in the absence of either NADPH or test compound. Reactions were terminated by the addition of 2 volumes of acetonitrile, followed by vortexing and centrifugation at 14,000 rpm (Eppendorf centrifuge 5810R; Eppendorf AG, Hamburg, Germany) for 10 min to pelletize the precipitated protein. The resulting supernatant was removed and evaporated to dryness under a steady stream of nitrogen. The residue was reconstituted in 20% acetonitrile/water and analyzed by LC/MS/MS.

Incubation of Nomifensine with Human, Dog, or Rat Hepatocytes. All the incubations were conducted at 37°C for 3 h in Williams’ E medium containing nomifensine (40 μM) and hepatocytes (2 million cells/ml). Incubations in the presence of nomifensine at time 0 h or in the absence of nomifensine at 3 h served as negative controls. Reactions were quenched with 2 volumes of acetonitrile, and the samples were prepared by the same methods used in the above liver microsomal studies.

LC/MS/MS Analysis. Metabolite profiling and identification in the microsomal and hepatocyte incubations were achieved by separating the metabolites on an ACQUITY ultra performance liquid chromatography (UPLC) HSS T3 column (2.1 × 100 mm, 1.8 μm, with a VanGuard HSS T3 1.8-μm guard column; Waters, Milford, MA) at room temperature. The mobile phase consisted of water (solvent A, containing 0.1% formic acid) and acetonitrile (solvent B, containing 0.1% formic acid) and was delivered at 0.2 ml/min. The initial composition of solvent B was maintained at 10% for 3 min and then increased in a linear manner to 70% at 13 min and 90% at 16 min. It was then maintained at 90% solvent B for 3 min and finally decreased to 10%. The column was allowed to equilibrate at 10% solvent B for 4 min before the next injection.

Mass spectrometric (MS) analyses were performed on a Thermo LTQ Orbitrap mass spectrometer (Thermo Fisher Scientific, Waltham, MA), which was interfaced to a Waters ACQUITY UPLC system with a UV detector. MS analyses experiments were conducted using a standard electrospray ionization source operating in positive ionization mode. Source operating conditions were 4.0 kV spray voltage, 275°C heated capillary temperature, 14 V capillary voltage, and sheath and auxiliary gas flow at 40 and 10 arbitrary units of the manufacturer, respectively. Data were centroided. The full-scan and MS/MS product ion scan mass spectra acquired at a resolving power of 30,000 (at m/z...
400) and 15,000, respectively. The product ion scan activation parameters used an isolation width of 2 Da (for fragments with m/z values that were 1 Da different from the intended fragment, the isolation width was set to 1 Da), normalized collision energy of 35%, and an activation time of 30 ms. Calibration of the instrument was performed using the standard LTQ calibration mixture with caffeine, the peptide MRFA, and Ultramark 1621 (Alfa Aesar, Ward Hill, MA) dissolved in an acetonitrile/methanol/water solution containing 1% acetic acid. The predicted chemical formulas and calculated accurate mass were obtained from Chemdraw Ultra 8.0 (CambridgeSoft Corporation, Cambridge, MA) based on the proposed fragmentation pathways and the putative fragment structures. The data were filtered in Qual Browser (Thermo Fisher Scientific) based on accurate mass thresholds. In addition, the Qual Browser chemical formula calculator was used to provide chemical formulas and experimental error values for molecular ions and product ions of nomifensine and its metabolites. Fragmentations were proposed based on plausible protonation sites, subsequent isomerization and even electron species, as well as bond saturation. Comparison between the parent and metabolite product ion spectra further aided in the identification of metabolite structures and site(s) of modifications in the parent molecule.

Results

Metabolite Profiles in Liver Microsomes. The structures of the metabolites of nomifensine and nomifensine dihydroisoquinolinium perchlorate in liver microsomal incubations were characterized with respect to mass, chemical formula, and fragmentation pattern on LC/MS/MS as described in the experimental procedures.

After a 60-min incubation of nomifensine in liver microsomes in the presence of NADPH at 37°C, several Phase I metabolites of nomifensine, including multiple hydroxylated metabolites, an N-de-
methylated metabolite, the dihydroisoquinolinium ion and its down-
stream products, such as lactam metabolites, were identified in all the
species tested (data not shown). In addition, in the presence of
NADPH and GSH, nine new metabolites (M1–M9) were detected in
the liver microsomal incubations across species. These metabolites
were not found in the control experiments that were conducted in the
absence of either NADPH or nomifensine. Figure 2 illustrates com-
parative LC/MS chromatograms of the GSH conjugates of the nomi-
fensine oxidative metabolites from an interspecies metabolism study.
The profiles of these new metabolites show qualitative similarities
among the different species. The UV spectra of the GSH conjugate
profiles (Fig. 3) suggest the quantitative differences in the relative
abundance of these metabolites in each species.

The samples were analyzed using UPLC/Fourier transform MS to
obtain accurate mass measurement and high-resolution product ion
mass spectra for each of the selected metabolite peaks. Table 1
summarizes the LC retention times (Rt), accurate masses, chemical
formulas, and postulated molecular structures of these new metabo-
lites identified in liver microsomes. In full-scan positive ion mode,
metabolites M1 through M9 predominantly formed doubly protonated
ions [M + 2H]2+ at m/z 280.6, whereas their corresponding singly
charged [M + H]+ counterparts, at m/z 560, were much less abundant.

After a 1-h incubation of nomifensine dihydroisoquinolinium per-
chlorate in human and rat liver microsomes in the presence of
NADPH and GSH, multiple hydroxylated metabolites and the down-
stream products of dihydroisoquinolinium ion (e.g., lactam metabo-
lites) were identified (data not shown). In addition, a very minor
metabolite, M10, was detected. This metabolite was not found in the
control experiments. In full-scan positive ion mode, metabolite M10
forms a doubly charged ion at m/z 279.6.

**Metabolite Profiles in Hepatocytes.** Incubation of nomifensine in
cryopreserved human hepatocytes and freshly isolated dog and rat

![Fig. 3. Comparative LC/UV chromatogram of the GSH conjugates of the nomifensine oxidative metabolites from the incubations of nomifensine with liver microsomes of humans, dogs, monkeys, and rats in the presence of NADPH and GSH at 37°C for 1 h. (Drug concentration in the incubations is 100 μM. λmax = 232 nm, which was determined by PDA total scan.) The rank order of the relative contribution of aniline oxidation versus arene oxidation to the formation of the GSH adducts in liver microsomes of the species tested (estimated by the ratio of the sum of UV peak areas of M1 and M2 to the sum of UV peak areas of M3 to M9 in each species) is dog (2.8) > human (0.91) > monkey (0.6) > rat (0.16).](image-url)
nomifensine, including multiple hydroxylated metabolites, an N-de-
methylated metabolite, the dihydroisoquinolinium ion, N-glucu-
onides of the parent and the hydroxylated metabolites, and 0-meth-
ylated products of the hydroxylated metabolites (data not shown). In
addition, the nine GSH adducts, M1 to M9, were found in both human
and rat hepatocytes. In dog hepatocytes, only six GSH conjugates, M1
to M6, were detected. These metabolites were not found in the control
experiments. Figure 4 illustrates comparative LC/MS chromatograms
of the GSH conjugates of the nomifensine oxidative metabolites in
hepatocytes from an interspecies metabolism study.

Nomifensine. The product ion mass spectra of metabolites are
frequently compared with those of the parent compound to determine
the sites of modification. Figure 5 shows the product ion mass
spectrum of nomifensine. Table 2 summarizes the accurate mass
measurements and the chemical formulas of the first-generation prod-
uct ions of the nomifensine molecular ion at m/z 239, which has not
been described before. Figure 6 shows the proposed plausible frag-
mentation pathways of nomifensine molecular ion at m/z 239. The
characteristic product ion at m/z 196 in the collision-induced dissoci-
ation (CID) mass spectrum of the nomifensine molecular ion is
formed through a retro Diels-Alder type of reaction (Fig. 6, pathway
a). The cross-ring cleavage at N(2)-(C(1) and N(2)-C(3) bonds gener-
ates ions at m/z 210 and m/z 208 (Fig. 6, pathway b). Further
fragmentation of the ions at m/z 196, 210, and 208 leads to prominent
loss of a C6H6 moiety, giving rise to second-generation product ions
at m/z 118, 132, and 130, respectively. The cleavage of the bond
between C(4) and the phenyl ring (C ring) leads to the formation of an
ion at m/z 161 (Fig. 6, pathway c), which, after a loss of CH3-NH (29
Da) or C6H3N (41 Da) moiety, generates ions at m/z 132 and 120,
respectively. These characteristic fragmentation patterns were applied
toward the structural identification of nomifensine metabolites.

M1. Accurate mass measurement of the doubly charged ion at R of
8.52 min (M1) provided the chemical formula of [C26H35N5O7S]2+
(Table 1), suggesting the introduction of a GS moiety (305 Da) and an
oxygen atom (16 Da) to the nomifensine molecule. Figure 7 shows the
product ion mass spectrum of M1. CID of the doubly charged mol-
ecular ion at m/z 280.6 revealed a series of abundant first-generation
product ions at m/z 145, 181, 223, 238, 239, 247, 256, 287, 322, and
323 (Fig. 7A). Table 3 summarizes the accurate mass measurements
and chemical formulas of these product ions. Accurate mass measure-
ment showed the chemical formula of the ion at m/z 239 as
[C16H18N2]1+ (Table 3). Further fragmentation of this ion led to the
formation of the second-generation product ions at m/z 210, 208, 196,
161, 132, 130, 120, and 118 (Fig. 7B), which are consistent with those
found with nomifensine (Figs. 5 and 6). This information suggests that
the nomifensine molecular ion is regenerated in the ion source after a
loss of 322 Da and one charge from the doubly protonated molecular
ion at m/z 280.6. The proposed fragmentation pathways were given in
Fig. 8. Accurate mass measurement of the singly charged ion at m/z

<table>
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<th>Compound</th>
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<th>Chemical Formula</th>
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<th>Calculated Mass</th>
<th>Mass Error</th>
<th>ΔM</th>
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<td>239.1543</td>
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<tr>
<td>M1</td>
<td>8.52</td>
<td>[C26H35N5O7S]+</td>
<td>280.6127</td>
<td>280.6126</td>
<td>1.4</td>
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322 provided the chemical formula of $[\text{C}_{10}\text{H}_{16}\text{O}_{7}\text{N}_{3}\text{S}]^+$ (Table 3), suggesting a GSH sulfinic acid residue (GS$^-$), which was generated through the loss of 239 Da and one charge (Fig. 8) from the doubly charged ion at $m/z$ 280.6. CID of the ion at $m/z$ 322 yielded second-generation product ions at $m/z$ 256, 247, and 181 (Fig. 7C) that reflect components of the modified tripeptide moiety. It is likely that the two first-generation even-electron fragment ions, $m/z$ 239 and 322, were formed through the heterolytic cleavage of the S-N bond, with the retention of one charge in each fragment (Fig. 8, pathway a).

Accurate mass measurement of ions at $m/z$ 238 and 323 indicated chemical formulas of $[\text{C}_{16}\text{H}_{18}\text{N}_{2}]^+$ and $[\text{C}_{10}\text{H}_{17}\text{O}_{7}\text{N}_{3}\text{S}]^+$, respectively (Fig. 7, D and E; Table 3). This represents one hydrogen atom less than $m/z$ 239 and one hydrogen atom more than $m/z$ 322, respectively, suggesting odd-electron ions. This supports a likely scenario that the ion pair at $m/z$ 238 and 323 is formed through the homolytic cleavage of the S-N bond with the retention of one proton in each fragment (Fig. 8, pathway b). The putative structures of the second-generation product ions yielded through the CID of $m/z$ 323 and 238 were also listed in Fig. 8. The ions at $m/z$ 287 and $m/z$ 145 are formed through the S-C bond cleavage in the GSH sulfinic acid residue (Fig. 8, pathway c versus a and b). Therefore, the mass spectra of M1 suggest the formation of a GSH-based sulfonamide conjugate with the GS moiety binding to the aniline group of nomifensine (Fig. 8).

M2. Similar to that of M1, accurate mass measurement of the ion at $R_t$ of 8.35 min (M2) provided the chemical formula of $[\text{C}_{26}\text{H}_{35}\text{N}_{5}\text{O}_{7}\text{S}]^+$ (Table 1), suggesting the introduction of a GS moiety and one oxygen atom to the nomifensine molecule. CID of the molecular ion of M2 at $m/z$ 280.6 revealed very similar fragmentation patterns to those found in M1 (Supplemental Fig. S1; Table S1). Nomifensine is a racemic mixture with one chiral center at C-4 in the molecule. Extra chiral centers are introduced into the molecule when the nomifensine oxidative metabolite reacts with the optically pure
enantiomer of GSH molecule. An extra chiral center may also be introduced into the molecule via the formation of sulfinamide bond. This may lead to the formation of diastereomeric products. Therefore, M1 and M2 are likely diastereomers with a GS moiety attached to the nitrogen atom of the aniline group.

M5. Accurate mass measurement indicated that M5 (R_t of 6.39 min) was formed by the oxidation of nomifensine followed by GSH conjugation (Table 1). Figure 9 shows the product ion mass spectrum of M5. Table 4 lists the accurate mass measurements and chemical formulas of the product ions of the doubly protonated molecular ion of M5 at m/z 280.6. On CID, a series of singly charged and structurally informative first-generation product ions were observed indicative of glycyl (m/z 485 and 243), γ-glutamyl (m/z 130 and 431), and cysteinyl (such as m/z 328, 311, 287, 285) components of the GSH conjugate (Fig. 9A). Figure 10 described the plausible fragmentation pathways. Accurate mass measurement of the ion at m/z 287 and 285 provided the chemical formulas of \([\text{C}_{16}\text{H}_{19}\text{ON}_{2}\text{S}]^{+}\) and \([\text{C}_{16}\text{H}_{17}\text{ON}_{2}\text{S}]^{+}\), respectively, suggesting the addition of one S and one O atom to nomifensine and the C-linked GSH adduct at aromatic ring (Tables 2 and 4). The four first-generation product ions at m/z 249, 243, 211.6, and 197.6 are doubly charged (Figs. 9A and 10). Therefore, the MS spectra of M5 suggest a GS moiety added to the aromatic ring through C-S bond (Ballard et al., 1991; Baillie and Davis, 1993). CID of the critical ion at m/z 285 gives three abundant ions at m/z 254, 242, and 209 (Fig. 9B) corresponding to the neutral loss of CH_3NH_2 (31 Da), CH_2=NH (43 Da), and C_2H_3N_2O (76 Da) moieties (accurate mass data not shown), respectively. However, this information could not help to determine which aromatic ring has been modified.

M3, M4, M6, M7, M8, and M9. Accurate mass measurement of the ion at R_t of 8.13 min (M3), 7.72 min (M4), 6.00 min (M6), 5.60 min (M7), 5.27 min (M8), and 4.60 min (M9) provided the chemical formula of \([\text{C}_{26}\text{H}_{35}\text{N}_{5}\text{O}_{7}\text{S}]^{2+}\) (Table 1) like that of M5, and therefore

![Fig. 5. Product ion mass spectrum obtained by CID of the molecular ion of nomifensine at m/z 239 in positive-ion mode (see Table 2).](image-url)

**TABLE 2**

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suggested the introduction of a GS moiety and one oxygen atom to the nomifensine molecule. CID of the molecular ion of M6 at m/z 280.6 and the second-generation product ion spectrum of M6 at m/z 285 revealed very similar fragmentation patterns to those observed in M5 (Supplemental Fig. S2; Table S2). Therefore, M6 and M5 are likely diastereomers. For similar reasons, M3 and M4 are likely diastereomers. Whereas the first-generation product ion spectra of M3 and M4 at m/z 280.6 are similar to those seen in M5 and M6, the CID of the fragment ions at m/z 285 shows subtle changes (Supplemental Figs. S3 and S4; Table S3 and S4), suggesting regioisomers of M5 and M6. The first-generation product ion spectra of M7, M8, and M9 at m/z 280.6 are very similar to those found in M5, M6, M3, and M4; the CID of the fragment ion m/z 285 is either identical or shows subtle changes (Supplemental Figs. S5, S6, and S7; Table S5, S6, and S7), suggesting regio- and/or diastereomeric isomers.

**M10.** In the incubations of nomifensine dihydroisoquinolinium perchlorate with NADPH and GSH-supplemented human and rat liver microsomes, M10 was identified as a very minor metabolite. Accurate mass measurement provided the chemical formula of [C_{26}H_{33}N_{5}O_{7}S]^{2-} (measured mass, 279.6050 Da; calculated mass 279.6045 Da; mass error, 2.0 ppm), indicating that M10 was formed by the incorporation of a GS moiety and one oxygen atom to the nomifensine dihydroisoquinolinium ion. Figure 11 illustrates the product ion mass spectrum of M10. Table 5 lists the accurate mass measurements and chemical formulas of the product ions of the molecular ion at m/z 279.6. Similar to the first-generation product ion mass spectra of M3 through M9, on CID of the doubly charged ion at m/z 279.6, a series of singly charged first-generation product ions were observed indicative of the γ-glutamyl (m/z 130 and 429), cysteinyl (m/z 326, 309, 283, and 285), and glycyl (m/z 242) components of the GSH conjugate (Fig. 11). Figure 12 describes the plausible fragmentation pathways. Accurate mass measurement of the ions at m/z 285 and 283 provided the chemical formula of [C_{16}H_{17}ON_{2}S]^{+} and [C_{16}H_{15}ON_{2}S]^{+}, respectively, suggesting the addition of one S and one O atom to nomifensine dihydroisoquinolinium ion and a C-linked GSH adducts at aromatic ring (Table 5). The four first-generation product ions at m/z 284, 242, 210.6, and 196.6 are doubly charged (Figs. 11 and 12; Table 5). Therefore, the MS spectra of M10 suggested a GS moiety added to the aromatic ring through a C-S bond. The CID of the critical ion at m/z 283 could not help to determine which aromatic ring was modified. In the incubations of nomifensine dihydroisoquinolinium perchlorate with NADPH- and GSH-supplemented human and rat liver microsomes, no M1 to M9 metabolites were detected.

**Discussion**

Two types of GSH conjugates of the potentially reactive metabolites of nomifensine were identified by LC/MS/MS. Figure 13 illustrates the proposed bioactivation pathways to form these GSH conjugates of the oxidative nomifensine intermediates and the oxidative nomifensine dihydroisoquinolinium intermediate. The product ion mass spectra of M1 and M2 indicate the regeneration of the nomifensine molecular ion (m/z 239) and an ion at m/z 322 with the elemental composition of [C_{16}H_{30}O_{7}N_{2}S]^{+} in the ion source. Uetrecht (1985) reported that the reaction of GSH with synthetic standard of procainamide nitrosamine yielded procainamide-GSH sulfinamide conjugate. The product ion spectra of the singly charged molecular ion also showed a fragment ion at m/z 322. By comparison with synthetic standards, it was determined that on hydrolysis of this conjugate, two products, procainamide and GSH sulfinic acid, were...
formed (Calam and Waley, 1962; Uetrecht, 1985). Thus, it was believed that the ion at \( m/z \) 322 in the mass spectra of the procainamide-GSH sulfinamide conjugate was generated from the sulfinic acid residue. For similar reasons, the mass spectra of M1 and M2 in our studies suggest the formation of nomifensine-GSH sulfinamides. Studies have shown that the obligatory step in the bioactivation of all the anilines involves \( N \)-hydroxylation on the primary amine nitrogen leading to the formation of the \( N \)-hydroxylamine intermediate. It is also known that \( N \)-hydroxylamine is readily converted nonenzymatically to the nitroso derivative, which reacts with GSH to form an initial unstable mercaptal derivative that rearranges to GSH-based sulfinamide conjugate (Uetrecht, 1985). It is likely that the primary aniline group in nomifensine was metabolized initially in liver microsomes to the \( N \)-hydroxylamine and then underwent a further two-electron oxidation to the more reactive nitrosoamine intermediate (Fig. 13, pathway a). GS attack on the nitrosoamine led to the formation of the final metabolites M1 and M2.

It has been found that the nomifensine iminium form, as opposed to its \( \alpha \)-carbinolamine form, predominates at physiological pH because of the resonance stabilization imparted by the aniline group (Obach...
and Dalvie, 2006). At physiological pH, the nomifensine dihydroisoquinolinium ion reacted with the strong nucleophile KCN (presumed to form the \(\text{CN}^-\)-cyanoamine) that were detected only by UV spectrometry. Obach and Dalvie (2006) reported that attempts to isolate this cyanide adduct resulted in the regeneration of nomifensine dihydroisoquinolinium ion, indicating a labile bond between the CN group and the nomifensine molecule. They also reported that there was no reaction between GSH and the nomifensine dihydroisoquinolinium ion under chemical conditions (Obach and Dalvie, 2006). Our studies showed that on CID of the molecular ions of M1 and M2, the nomifensine molecular ion could be regenerated easily in the ion source of the mass spectrometer, suggesting the relatively labile bond between the modified GS moiety and nomifensine. In the absence of NMR spectra to confirm the exact location of the GS moiety in M1 and M2, one speculation is that the GS moiety might add to the nomifensine oxidative intermediate via a dehydrogenation pathway, in which the GS moiety attacks the \(\alpha\)-position (C-1) of the oxidative dihydroisoquinolinium metabolite (Fig. 14, oxidative nomifensine dihydroisoquinolinium metabolite and its resonance structure IV). In the incubations of the nomifensine dihydroisoquinolinium perchlorate with liver microsomes supplemented with NADPH and GSH, only M10 was observed. No M1 through M9 metabolites were detected. The mass spectra of M10 showed that the GS moiety is attached to the aromatic ring of the oxidative dihydroisoquinolinium intermediate through C-S bond (Fig. 13, pathway b). These findings collectively indicated that the formation of M1 and M2 did not result from the GS attack on the \(\alpha\)-carbon of the oxidative dihydroisoquinolinium intermediate to yield a putative structure V (Fig. 14), which could be generated via a dehydrogenation pathway. Therefore, the initial step in the formation of M1 and M2 must be through the aniline oxidation pathway to form an \(N\)-hydroxylamine intermediate (Fig. 13, pathway a), and the GS moiety in M1 and M2 is bond to the parent molecule at the aniline group.

It is well known that aniline-containing drugs and environmental toxins can display tumorigenic, mutagenic, and cytotoxic results via metabolic activation. The identification of nomifensine/GSH-based sulfinamide conjugates in this study clearly shows the modification of the aniline group in nomifensine by mammalian liver microsomes and hepatocytes. Additional mechanisms of activation of the formed \(N\)-hydroxylamine, such as the sulfate and/or acetyl conjugations, may also exist in vivo. It was found that the sulfonyloxy group and/or acetoxy group in the \(N\)-O-sulfate and/or \(N\)-O-acetyl conjugates of \(N\)-hydroxylamines are very good leaving groups that may be eliminated to generate a highly reactive nitrenium or carbo-

<table>
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<tr>
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<th>Calculated Mass Da</th>
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FIG. 8. Proposed fragmentation pathways of the doubly protonated molecular ion \([M + 2H]^{+}\) of M1 (\(R_t = 8.52\) min) after CID (see Figs. 5–7; Tables 2 and 3).

TABLE 3

List of accurate masses and chemical formulas of the product ions obtained by CID of the molecular ion of M1 (\(R_t = 8.52\) min) at m/z 280.6 (see Figs. 2, 7A, and 8).
nium ion. Thus, in this case, the nitrenium or carbonium ion may be the ultimate reactive intermediate involved in the covalent modification of the macromolecules and may lead to the toxicity seen (Kal-gutkar et al., 2005). The aniline group may undergo $N$-hydroxylation followed by $N$-glucuronidation. The resultant $N$-glucuronides can be hydrolyzed in urine under acidic conditions or by $/$H$_9252$-glucuronidase in intestinal microflora to the corresponding $N$-hydroxylamines and cause bladder and colon cancer (Parkinson, 2001).

The mass spectra of GSH conjugates M3 through M9 suggest the addition of a GS moiety to the arene in the nomifensine molecule (Fig. 13, pathway $c$). The mass spectra also indicate the introduction of only one oxygen atom into the nomifensine molecule; thus, these nomifensine GSH adducts were not formed through catechol metabolites. They are likely formed through arene oxide intermediates, such as epoxides, benzoquinone imines (A ring), or a quinine-methide intermediate (C ring). It is well known that when arene oxides escape enzymatic destruct-
FIG. 10. Proposed fragmentation pathways of the doubly protonated molecular ion \([M + 2H]^2+\) of M5 \((R_t = 6.39 \text{ min})\) after CID (see Fig. 9; Table 4).

FIG. 11. Product ion mass spectrum obtained by CID of the molecular ion of metabolite M10 at \(m/z\) 279.6 from NADPH-supplemented liver microsomal incubations containing nomifensine (10 \(\mu M\)) and GSH (10 mM) (see Table 5).
were observed in the hepatocyte preparations of all the species tested, group. Our results indicated that both arene and aniline oxidations
animals showed that the primary metabolic pathway was direct
diates. In vivo metabolism study on nomifensine in humans and
sponding arenol, leading to the detoxication of the epoxide interme-
However, the epoxide intermediates may undergo hydration catalyzed
fensine GSH adducts are likely formed through the arene epoxidation.
may catalyze the detoxication reactions or other reactions that com-
pathways to form these nomifensine metabolites.
ion, toxicity may result. The ongoing effort to determine the exact
positions of the GS and hydroxyl groups at the nomifensine arene by
NMR spectra would bring even greater clarity to the detailed metabolic
Hepatocyte preparations contain multiple metabolic enzymes that
may catalyze the detoxication reactions or other reactions that com-
pete with the bioactivation pathways for the specific functional
groups; therefore, the results in hepatocytes should be more relevant
to the toxicity observed in vivo. For example, some of the nomi-
fensine GSH adducts are likely formed through the arene epoxidation.
reactions for aniline
similar in liver microsomes of humans, dogs, monkeys, and rats and
tively low. Finally, the GSH conjugate profiles are qualitatively
linium intermediate toward the nucleophilic GSH molecule is rela-
tively low. Finally, the GSH conjugate profiles are qualitatively
suggested that the formation of these nomifensine conjugates
does not occur through a dihydroisoquinolinium intermediate; there-
fore, the reactivity of the oxidative nomifensine dihydroisoquo-
sulinium perchlorate with NADPH- and
oxidations followed by GSH conjugation yielded another type of GSH
conjugate (M3, M4, M5, M6, M7, M8, and M9), with a GS moiety
added to the nomifensine arene through a C-S bond. Incubation of
nomifensine dihydroisoquinolinium perchlorate with NADPH- and
GSH-supplemented rat and human liver microsomes yielded none of
the GSH conjugates that were identified in nomifensine (M1–M9),
suggesting that the formation of these nomifensine conjugates
does not occur through a dihydroisoquinolinium intermediate; there-
fore, the reactivity of the oxidative nomifensine dihydroisoquinol-
linium intermediate toward the nucleophilic GSH molecule is rela-
tively low. Finally, the GSH conjugate profiles are qualitatively
similar in liver microsomes of humans, dogs, monkeys, and rats and
in human and rat hepatocytes. In dog hepatocyte preparations, six
GSH adducts were identified. The GSH adducts in dog and rat liver
suggested that even in the presence of multiple metabolic enzymes,
the nomifensine reactive intermediates were formed and were not
completely detoxicated. Therefore, these reactive metabolites are
likely responsible for the toxic effect of nomifensine observed in vivo.

Although similar GSH adducts were detected in liver microsomes
of all the species, the GSH adducts generated in dogs were primarily
through aniline oxidation pathway, and the arene oxidation yielded
most of the GSH adducts in rats (Fig. 3). In humans and monkeys,
both aniline and arene oxidation pathways contributed significantly
to the formation of the GSH conjugates. The quantitative difference in
GSH conjugate profiles of nomifensine (aniline oxidation versus
arene oxidation) in liver microsomes among different species suggests
it may show different toxicity profiles at certain dose levels among
these species.

In conclusion, incubation of nomifensine with human and animal
hepatocyte preparations and NADPH- and GSH-supplemented liver
microsomes resulted in the formation of multiple GSH conjugates that
were characterized by LC/MS/MS. The results revealed two types of
GS conjugates to nomifensine oxidative metabolites. First, aniline
oxidation followed by GSH conjugation leads to the formation of
nomifensine/GSH-based sulfamides (M1 and M2), with a GS moi-
eity bond to the nomifensine molecule at aniline group. Second, arene
oxidations followed by GSH conjugation yielded another type of GSH
conjugate (M3, M4, M5, M6, M7, M8, and M9), with a GS moiety
added to the nomifensine arene through a C-S bond. Incubation of
nomifensine dihydroisoquinolinium perchlorate with NADPH- and
GSH-supplemented rat and human liver microsomes yielded none of
the GSH conjugates that were identified in nomifensine (M1–M9),
suggesting that the formation of these nomifensine conjugates
does not occur through a dihydroisoquinolinium intermediate; there-
fore, the reactivity of the oxidative nomifensine dihydroisoquinol-
linium intermediate toward the nucleophilic GSH molecule is rela-
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similar in liver microsomes of humans, dogs, monkeys, and rats and
in human and rat hepatocytes. In dog hepatocyte preparations, six
GSH adducts were identified. The GSH adducts in dog and rat liver

<table>
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<tr>
<th>Fragment Ion</th>
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**TABLE 5**

List of accurate masses and chemical formulas of the product ions obtained by CID of the molecular ion of M10 at m/z 279.6 (see Figs. 11 and 12).
FIG. 13. Proposed bioactivation pathways to form GSH conjugates of the oxidative nomifensine intermediates (M1–M9) and GSH conjugate of the oxidative nomifensine dihydroisoquinolinium intermediate (M10).

FIG. 14. Formation of the putative GSH conjugate (V) of the oxidative nomifensine intermediates through dehydrogenation pathway.
microsomes were generated primarily through aniline and arene oxidation, respectively. Both aniline and arene oxidation pathways contributed significantly to the formation of the GSH adducts in human and monkey liver microsomes. Thus, these investigations have confirmed that the aniline and the arene rings in nomifensine are capable of generating reactive intermediates and, because of this, are potential toxicophores.

Acknowledgments. We thank Drs. Chuncang Gu, Minli Zhang, and Scott W. Grimm for valuable comments on the work. We also thank Sara Schock and Xin (Cindy) Shen for counting the viable hepatocytes.

References