In Vitro Metabolism of Oxymetazoline: Evidence for Bioactivation to a Reactive Metabolite

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Non-standard abbreviations: LC/MS/MS, liquid chromatography/tandem mass spectrometry; HPLC, high-performance liquid chromatography; [M+H]⁺, protonated molecular ion; m/z, mass-to-charge; NMR, nuclear magnetic resonance spectroscopy; ROESY, Rotating Frame Nuclear Overhauser Effect; P450, cytochrome P450; NADPH, reduced form of β-nicotinamide adenine dinucleotide phosphate; S9, post-mitochondrial supernatant fraction from homogenized tissue; BHT, 2,6-di-t-butylhydroxytoluene; DMSO, dimethylsulfoxide
Abstract

Oxymetazoline has been widely used as a nonprescription nasal vasoconstrictor for more than 40 years; however, its metabolic pathway has not been investigated. This study describes the in vitro metabolism of oxymetazoline in human, rat and rabbit liver S9 fractions and their microsomes supplemented with NADPH. The metabolites of oxymetazoline identified by LC/UV/MS/MS included M1 (monohydroxylation of the t-butyl group), M2 (oxidative dehydrogenation of the imidazoline to an imidazole moiety), M3 (monohydroxylation of M2), M4 (dihydroxylation of oxymetazoline) and M5 (dihydroxylation of M2). Screening with 9 human expressed P450s identified CYP2C19 as the single CYP isoform catalyzing the formation of M1, M2 and M3. Glutathione conjugates of oxymetazoline (M6) and M2 (M7) were identified in the liver S9 fractions, indicating the capability of oxymetazoline to undergo bioactivation to reactive intermediate species. M6 and M7 were not detected in those liver S9 incubations without NADPH. Cysteine conjugates (M8 and M9) derived from glutathione conjugates and hydroxylated glutathione conjugates (M10 and M11) were also identified. The reactive intermediate of oxymetazoline was trapped with glutathione and N-acetylcysteine and identified by LC/MS/MS. M6 was isolated and identified by 1D/2D NMR as the glutathione conjugate of a p-quinone methide. We have shown the tendency of oxymetazoline to form p-quinone methide species via a bioactivation mechanism involving a CYP2C19-catalyzed two electron oxidation. Nevertheless, we conclude that the formation of this reactive species might not be a safety concern for oxymetazoline nasal products due to the typical low dose and brief dosage regimen limited to nasal delivery.
Introduction

Oxymetazoline, [6-tert-butyl-3-(2-imidazolin-2-ylmethyl)-2,4-dimethylphenol], Fig. 1, is a vasoconstrictor that acts directly on nasal membranes and has been available as a nonprescription intranasal drug in the United States for more than 40 years, approved for the relief of nasal congestion due to common colds and allergic rhinitis. Excessive doses of nonprescription oxymetazoline nasal products have been associated with cardiovascular and/or central nervous system adverse events, indicating systemic absorption from the nasal mucosa (Glazener et al., 1983; Soderman et al., 1984; Thrush, 1995), but with no reports so far of serious toxicity. The intranasal route of drug administration provides direct access by drugs to the systemic circulation, prompting a rapid onset of action and absorption while avoiding gastrointestinal first-pass metabolism usually at a fraction of the oral dose, thus allowing a drug to undergo hepatic metabolism before elimination (Bommer, 2006). No human metabolism, pharmacokinetic or toxicological data have been reported for oxymetazoline so far. An in vitro phase II O-glucuronide metabolite catalyzed by UGT1A9 has been identified by LC/MS/MS in human liver microsomes and has been characterized by 1D and 2D NMR (Mahajan et al., 2010).

In the years since the USFDA approval of oxymetazoline, both the pharmaceutical industry and the USFDA have become increasingly aware of the formation and identification of reactive drug metabolites due to their potential to elicit tissue injury. Researchers have identified numerous functional groups on drugs that can be bioactivated to reactive intermediates (Kalgutkar et al., 2005), including p-alkyl substituted phenols as found in oxymetazoline (Bolton et al., 1990; Thompson et al., 1990; Thompson et al., 1992; Thompson et al., 1995; Yan et al., 2005). The p-alkyl phenolic structure of oxymetazoline suggests that it might be bioactivated to
a toxic $p$-quinone methide electrophilic intermediate during P450-dependent metabolism, as has been seen with other $p$-alkyl phenols such as BHT, eugenol, $p$-cresol (Bolton et al., 1990; Thompson et al., 1990; Thompson et al., 1992; Thompson et al., 1995; Monks and Jones, 2002; Yan et al., 2005) and a reactive metabolite of troglitazone (Kassahun et al., 2001).

In light of these concerns, the objective of the current study was to investigate the in vitro metabolism of oxymetazoline, including its bioactivation potential, using human, rat and rabbit liver S9 fractions and their microsomes; to identify the formation of a reactive species by trapping it with nucleophiles such as glutathione and $N$-acetylcysteine; to characterize the glutathione conjugate of oxymetazoline by 1D- and 2D NMR in order to identify the putative reactive intermediate; and to identify the specific human P450 isoforms involved in the oxidation of oxymetazoline. The identification of the glutathione conjugates of oxymetazoline demonstrates its tendency to form a $p$-quinone methide intermediate (Thompson et al., 1995).
Materials and Methods

Reagents. Oxymetazoline, alamethicin, NADPH, reduced glutathione and dimethyl sulfoxide were all purchased from Sigma-Aldrich (St. Louis, MO). All chemicals and reagents were obtained at the highest purity available. Pooled male human, male Sprague-Dawley rat and male New Zealand rabbit liver S9 fractions as well as pooled human, rat and rabbit liver microsomes were obtained from XenoTech, LLC (Kansas City, KS). Human recombinant cDNA-expressed CYP1A2, CYP2B6, CYP2C8, CYP2C9, CYP2C18, CYP2C19, CYP2D6, CYP2E1 and CYP3A4 Supersomes® were purchased from BD Gentest (Woburn, MA). The protein content of all Supersomes® was 5 mg/mL. The liver S9 fractions, microsomes and expressed P450 Supersomes® were stored at - 80°C according to the supplier’s instructions, and were used shortly after receipt.

NADPH-Supplemented Liver S9 Fractions and Microsomal Incubations. To 640 µL of 0.1 mM potassium phosphate buffer (pH 7.4) containing 3 mM magnesium chloride was added 250 µL of human, rat or rabbit liver S9 fraction (5 mg protein/mL) and 10 µL of 50 µM oxymetazoline in the incubation tubes. Each tube was pre-incubated for 3 min at 37°C in a reciprocal shaking bath to which 100 µL of 2 mM NADPH was added to initiate the reaction, which took place under ambient oxygen conditions. The final incubation volume of each tube was 1000 µL. The reaction was terminated at 1 h by the addition of 2 volumes of room temperature acetonitrile to precipitate proteins, and the resulting mixture was chilled for 30 min at 4°C, followed by centrifugation at 3000g to pelletize the proteins. The supernatant was collected and dried under a stream of nitrogen in a Turbo Vap® LV drier (Zymark, Holliston, MA). The dried residue was reconstituted in 200 µL of mobile phase [85:15 v/v, 10 mM
ammonium formate (pH 4.0):acetonitrile] in preparation for LC/MS/MS analysis. Negative control incubations of oxymetazoline without NADPH, drug or protein were run simultaneously without the appearance of oxymetazoline metabolites. LC/MS/MS analyses were performed immediately following reconstituting the dried residue in mobile phase and after 10 days storage at -20°C; comparisons showed no differences in the relative abundances of [M+H]+ and product ions, thus confirming metabolite stability. The incubation reactions with human, rat and rabbit liver microsomes were performed in a manner analogous to those with the S9 fractions, substituting liver microsomes (1 mg protein/mL) for liver S9 fractions.

An ion trap LC/MS/MS system consisting of an Agilent 1100 HPLC (Agilent Technologies, Palo Alto, CA) with a Symmetry C18 column (5 µ, 2.1 × 150 mm, Waters Corporation, Milford, MA) coupled to a Finnigan LCQ Deca XPPLUS ion trap mass spectrometer (Thermo Electron Corporation, San Jose, CA) was utilized to identify oxymetazoline metabolites. A 20 µL aliquot of each reconstituted sample in mobile phase was injected into the equilibrated HPLC. A mobile phase gradient was used, with 10 mM ammonium formate (pH 4.0) as solvent A, and acetonitrile as solvent B. The initial mobile phase composition was 85:15 A/B (v/v) and was transitioned by linear gradient to 20:80 A/B in 30 min, at a constant flow rate of 0.400 mL/min. The eluent from the HPLC column was first passed through a UV detector set at 254 nm and was then introduced directly into the mass spectrometer via electrospray ionization in positive ion mode. Ionization was assisted by sheath and auxiliary gas (nitrogen) set at 60 and 40 psi, respectively. The electrospray voltage was 5 kV and the heated ion transfer capillary settings were 300°C and 30 V. Relative collision energies of 25 to 30% were used when operating in the MS/MS mode of the ion trap. Full scan and MS/MS spectra were
acquired.

**Trapping Reactive Metabolites of Oxymetazoline with Glutathione or N-Acetylcysteine-Supplemented S9 Fractions.** A 10 µL aliquot of 50 µM oxymetazoline was added to 250 µL of liver S9 fractions (5 mg protein/mL) of various species with the same conditions as those used in the liver S9 fraction incubations, except that the incubation medium was supplemented with 100 µL of 2 mM glutathione as a trapping agent. The trapping experiments with N-acetylcysteine and rabbit liver S9 fractions were performed in an analogous manner, substituting 100 µL of 3 mM N-acetylcysteine for glutathione. Control reactions omitting drug, protein, NADPH, or trapping agent were incubated simultaneously and analyses of the conjugates formed was performed using the LC/MS/MS method described for the S9 fractions.

**Biosynthesis and NMR Identification of Oxymetazoline-Glutathione Conjugate.** A 50 µL aliquot of 100 µM oxymetazoline and 2500 µL of rabbit liver S9 fraction (10 mg protein/mL) were added to 1450 µL of 0.1 M potassium phosphate buffer (pH 7.4) containing 3 mM magnesium chloride. Tubes containing the reaction mixtures were pre-incubated for 3 min at 37°C and reactions were initiated by addition of 500 µL aliquots each of 4 mM NADPH and 4 mM glutathione. The resulting solutions were incubated for 3 h at 37°C and were processed similarly to the liver S9 fractions. The dried supernatants were reconstituted in mobile phase and 100 µL aliquots were injected into the HPLC and analyzed with the same LC mobile phase gradient as that used with the S9 fractions. The semi-purified fractions containing the glutathione conjugate were collected between 4.0 and 5.0 min with a time-based fraction collection system. The semi-purified collected fractions were pooled, dried in the Turbo Vap® LV drier at room temperature, reconstituted with 2 mL of mobile phase and re-injected into the
LC to obtain the purest conjugate. The UV peak at 254 nm eluting at 4.2 min contained the glutathione conjugate and was collected after each purification cycle, after which the fractions were pooled and dried as previously described. The dried residue was reconstituted in mobile phase and a purity check was performed by LC/UV at 254 nm. The final fraction containing purified oxymetazoline glutathione conjugate was dried in the Turbo Vap® LV drier at room temperature and the powder obtained was used for NMR analyses. The LC/MS/MS method previously described was used to confirm the identity of the oxymetazoline glutathione conjugate.

$^1$H-NMR spectra were acquired on a 600 MHz Varian Inova NMR Spectrometer (Varian Inc., Palo Alto, CA) fitted with a Varian triple resonance 5 mm cold probe. Each sample was dissolved in DMSO-d$_6$ at 298.1° K and was then transferred into a 5 mm DMSO-matched Shigemi tube. Chemical shifts are expressed in ppm relative to tetramethylsilane as the internal standard. ROESY experiments were recorded with mixing times of 500 and 750 ms, using a spin-lock field of 4 kHz. A total of 64 complex points were recorded in F1 scan mode, with 64 scans acquired per increment. Solvent suppression was achieved by appending the WET pulse-gradient train to the beginning of the ROESY pulse sequence.

**Incubation of Oxymetazoline with Expressed Human P450 Isoforms.** Human recombinant cDNA-expressed CYP1A2, CYP2B6, CYP2C8, CYP2C9, CYP2C18, CYP2C19, CYP2D6, CYP2E1, and CYP3A4 Supersomes® (125 pmole protein/mL) in 0.1 M potassium phosphate buffer (pH 7.4) containing 3 mM magnesium chloride were each mixed with 10 µL of 50 µM oxymetazoline, and pre-incubated for 3 min at 37°C in a reciprocal shaking water bath, after which 100 µL of 2 mM NADPH was added to initiate the reaction. The final volume of each
incubation was 1000 µL. The reactions were processed as previously described for S9 fractions in preparation for LC/MS/MS analyses.
Results

Identification of Oxymetazoline Metabolites from S9 Fractions. The rabbit was selected as the primary species for the study of liver S9 fractions because these fractions contained the largest number of metabolites and the lowest percentage of unchanged drug recovered. By LC/UV analyses of the solutions in which oxymetazoline was incubated with NADPH-supplemented rabbit liver S9 fraction, four major metabolites (M1, M2, M6 and M7), and seven minor metabolites of oxymetazoline (M3-M5, M8-M11) were detected and identified, as shown in the LC-UV chromatogram at 254 nm (Fig. 2). LC/MS/MS analyses of rabbit and rat liver S9 incubations confirmed the presence of metabolite [M+H]+ ions at m/z 277 (M1), 259 (M2), 275 (M3), 293 (M4) and 291 (M5) (Table 1); human liver S9 fraction yielded metabolite [M+H]+ ions representing M1, M2, and M3. Liver S9 fraction incubations from all species also showed peaks representing conjugate [M+H]+ ions at m/z 566 (M6) and 564 (M7), tentatively identified as glutathione conjugates. Additional peaks representing M8 at m/z 380 and M9 at m/z 378 were identified as the cysteine conjugates, resulting from dipeptidase hydrolysis of the glutathione conjugates of oxymetazoline and M2, respectively. The monohydroxylated glutathione conjugates (M10 and M11) derived from M1 and M3, respectively, were also detected. The metabolites were identified based on differences in amu from the [M+H]+ ion at m/z 261 which represented oxymetazoline. Table 1 summarizes the S9 metabolites and their retention times (tR), as identified by LC/MS/MS analyses of reaction mixtures from the different species. Unchanged oxymetazoline was the largest component recovered from 1 h incubations containing liver S9 fractions of the different species, based on the UV response at 254 nm. Recoveries of unchanged oxymetazoline ranged from approximately 90% for human liver S9, 80% for rat liver
S9 and 35% for rabbit liver S9 fractions. Our proposed human metabolic pathways for oxymetazoline are shown in Fig. 3.

MS/MS analyses of the [M+H]$^+$ ion at $m/z$ 261, representing oxymetazoline, yielded product ions at $m/z$ 243 (loss of H$_2$O), $m/z$ 205 [loss of 56 amu (C$_4$H$_8$, t-butyl)], $m/z$ 191 [loss of 70 amu from cleavage of the imidazoline ring (C$_3$H$_6$N$_2$)] and $m/z$ 177 [loss of 84 amu from cleavage of the methylimidazoline ring (C$_4$H$_8$N$_2$)] (Table 1). The MS/MS spectrum of the [M+H]$^+$ ion at $m/z$ 261 and the proposed fragmentation pattern of oxymetazoline are shown in Fig. 4. The appearance of product ions at $m/z$ 205, 191 and 177 aided the identification of the various metabolites.

The monohydroxylated metabolite M1 was represented by a [M+H]$^+$ ion at $m/z$ 277, 16 amu larger than the [M+H]$^+$ ion of oxymetazoline. Three possibilities for monohydroxylation exist: hydroxylation of the t-butyl moiety, hydroxylation of an aromatic ring methyl group, or hydroxylation of the imidazoline ring. MS/MS analyses of the [M+H]$^+$ ion at $m/z$ 277 indicated product ions at $m/z$ 259 and 205 (Fig. 5A) (Table 1). The loss of C$_4$H$_8$O from the [M+H]$^+$ ion generated a product ion at $m/z$ 205, indicating that the hydroxyl group was attached to the t-butyl group and not to either a ring methyl group or the imidazoline ring. If the hydroxyl group were attached to a ring methyl group, a product ion (base peak) at $m/z$ 259 would have been observed, resulting from the loss of water from a hydroxybenzyl group (Ramanathan et al., 2000; Vacher et al., 2007). The product ion at $m/z$ 259 resulted from the loss of water. Furthermore, monohydroxylation of the imidazoline moiety to a hydroxyimidazoline intermediate and subsequent dehydration would have resulted in the formation of the more stable imidazole moiety as observed with M2 (4’,5’-dehydro-oxymetazoline) (Acheampong et al., 1996; He et al., 1996).
2000). Therefore, we can conclude that monohydroxylation selectively occurred on the \( t \)-butyl group.

**M2** nearly co-eluted with oxymetazoline with a \([M+H]^+\) ion at \( m/z \) 259, two hydrogen atoms less than the \([M+H]^+\) ion of oxymetazoline. MS/MS analyses of the \([M+H]^+\) ion at \( m/z \) 259 resulted in the generation of product ions at \( m/z \) 241, 203, 177 and 81 (Fig. 5B) (Table 1). The product ion at \( m/z \) 177 resulted from cleavage of the methylimidazole moiety, indicating that the aromatic moiety was unchanged and that the imidazoline ring had been dehydrogenated to an imidazole group. The appearance of a product ion at \( m/z \) 81 resulted from cleavage of the methylimidazole moiety. Furthermore, the product ions at \( m/z \) 241 and 203 were two hydrogen atoms less than the corresponding ions at \( m/z \) 243 and 205 of oxymetazoline, indicating the loss of water (18 amu) and of the \( t \)-butyl group (56 amu), respectively. From these results, we propose that the imidazoline ring was metabolized to a putative hydroxyimidazoline intermediate which then spontaneously eliminated water to the more stable imidazole ring (Acheampong et al., 1996; He et al., 2000).

**M3**, the monohydroxylated metabolite of 4′,5′-dehydro-oxymetazoline, was represented by a \([M+H]^+\) ion at \( m/z \) 275, two hydrogen atoms less than the \([M+H]^+\) ion of **M1**. MS/MS analyses of the \([M+H]^+\) ion at \( m/z \) 275 indicated product ions at \( m/z \) 257, 203 and 81 (Table 1). The product ion pattern of **M3** was similar to that of **M1**, except that ions at \( m/z \) 257 and 203 were two hydrogen atoms less than the corresponding ions of **M1**, indicating an oxidative dehydrogenation of the imidazoline ring to form the imidazole group. The product ion at \( m/z \) 203 resulted from the loss of \( C_4H_8O \) (72 amu), and supported the presence of a monohydroxylated \( t \)-butyl group. The appearance of a product ion at \( m/z \) 81 resulted from
cleavage of the methylimidazole group as with M2.

M4, the second-generation dihydroxylated metabolite of oxymetazoline, displayed a $[\text{M}+\text{H}]^+$ ion at $m/z$ 293, 32 amu larger than the $[\text{M}+\text{H}]^+$ ion of oxymetazoline. MS/MS analyses of the $[\text{M}+\text{H}]^+$ ion at $m/z$ 293 displayed product ions at $m/z$ 275, 245, and 221 (Fig. 5C) (Table 1), indicative of monohydroxylation of the $t$-butyl group and monohydroxylation of either the o- or p-methyl groups on the aromatic ring to form a hydroxybenzyl metabolite. The characteristic loss of C$_4$H$_8$O (72 amu) from the $[\text{M}+\text{H}]^+$ ion generated a product ion at $m/z$ 221, which corroborated the hydroxylation of the $t$-butyl group. The product ion (base peak) at $m/z$ 275 was generated by the loss of water (18 amu) from the hydroxybenzyl group to generate a “$p$-quinonemethide-like” product ion C$_{16}$H$_{23}$N$_2$O$_2^+$ (Ramanathan et al., 2000; Vacher et al., 2007) (Fig. 5C). The product ion at $m/z$ 245 (C$_{15}$H$_{21}$N$_2$O$^+$) resulted from the loss of CH$_2$O (30 amu) from the product ion at $m/z$ 275, supporting the presence of a hydroxybenzyl group. MS/MS analyses were insufficient to determine which o- or p-methyl group was monohydroxylated.

M5, the second-generation dihydroxylated metabolite of M2, displayed the $[\text{M}+\text{H}]^+$ ion at $m/z$ 291, two hydrogen atoms less than the $[\text{M}+\text{H}]^+$ ion of M4. MS/MS analysis of the $[\text{M}+\text{H}]^+$ ion at $m/z$ 291 displayed product ions at $m/z$ 273 (base peak), 243 and 219, all of which were two hydrogen atoms less than the corresponding product ions of M4 (Table 1).

M6, the glutathione conjugate of oxymetazoline, showed a $[\text{M}+\text{H}]^+$ ion at $m/z$ 566, corresponding to the addition of 305 amu (one molecule of glutathione) to the $[\text{M}+\text{H}]^+$ ion of oxymetazoline. MS/MS analyses of the $[\text{M}+\text{H}]^+$ ion at $m/z$ 566 yielded product ions at $m/z$ 548, 473, 437, 380, 293 and 259 (Fig. 6A) (Table 1), which are characteristic product ions for a glutathione conjugate and which result from the sequential fragmentation of the glutathione
moiety (Mutlib et al., 2000; Chen et al., 2002; Mutlib et al., 2002). The proposed fragmentation pattern of M6 is shown in Fig. 6A. The major product ion at m/z 437 resulted from the loss of pyroglutamic acid (129 amu) from the [M+H]+ ion at m/z 566, indicative of a glutathione moiety (Mutlib et al., 2000; Chen et al., 2002; Mutlib et al., 2002). The loss of water (18 amu) from the [M+H]+ ion at m/z 566 produced a product ion at m/z 548, which further produced a product ion at m/z 473, consistent with the loss of glycine (75 amu) (Mutlib et al., 2000). The product ion at m/z 380 resulted from the loss of 93 amu from m/z 473. The other glutathione-related product ions were at m/z 293 and m/z 259, consistent with cleavage of the cysteiny1 C-S bond (87 amu) from m/z 380 (cysteinate ion) with retention of sulfur on the aromatic ring of oxymetazoline (Chen et al., 2002) and with the loss of H2S (32 amu) from the fragment at m/z 293 respectively, to yield the product ion at m/z 259; thus confirming M6 to be the glutathione conjugate of oxymetazoline. Evidence for the formation of the glutathione conjugate of M2 (M7) resulted from the appearance of the [M+H]+ ion at m/z 564, two hydrogen atoms less than the [M+H]+ ion of M6. MS/MS analyses of the [M+H]+ ion at m/z 564 yielded product ions at m/z 546, 471, 435, 378, 291 and 257 (Fig. 6B) (Table 1), all of which are characteristic product ions for a glutathione conjugate, as observed for M6, as a result of the sequential fragmentation of the glutathionyl moiety to form the product ion at m/z 257. The product ion at m/z 435 resulted from the characteristic loss of pyroglutamic acid (129 amu) from m/z 564. The product ions were two hydrogen atoms less than the corresponding product ions for M6, indicating the presence of an imidazole ring.

M8, the cysteine conjugate of oxymetazoline, showed a [M+H]+ ion at m/z 380, which is 119 amu greater than the [M+H]+ ion of oxymetazoline suggesting the addition of one molecule
of cysteine. MS/MS analyses of the [M+H]^+ ion at m/z 380 resulted in the generation of product ions at m/z 293 and 259 (Table 1). The product ion at m/z 293 resulted from cleavage of the cysteinyl C-S bond from the [M+H]^+ ion with the retention of sulfur on the aromatic ring of oxymetazoline, followed by the subsequent loss of H₂S from m/z 293 to form the product ion at m/z 259, confirming M₈ to be the cysteine conjugate of oxymetazoline, which results from the dipeptidase hydrolysis of the glutathione conjugate (Mutlib et al., 1999). M₉, the cysteine conjugate of M₂, produced a [M+H]^+ ion at m/z 378, which is two hydrogen atoms less than the [M+H]^+ ion of M₈. MS/MS analyses demonstrated product ions at m/z 291 and 257 (Table 1), an ion pattern similar to that observed for M₈, except that the product ions were two hydrogen atoms less than the corresponding ions for M₈, confirming M₉ to be the cysteine conjugate of M₂.

M₁₀, the glutathione conjugate of M₁, showed a [M+H]^+ ion at m/z 582, which is 305 amu more than the [M+H]^+ ion of M₁, indicating the addition of one molecule of glutathione to M₁. MS/MS analyses of the [M+H]^+ ion at m/z 582 resulted in the generation of product ions at m/z 564, 453, 435, 309 and 275 (Table 1), all of which are characteristic product ions of a glutathione conjugate, as observed for M₆ from the sequential fragmentation of the glutathionyl moiety to form the product ion at m/z 275. The product ion at m/z 453 resulted from the characteristic loss of pyroglutamic acid (129 amu) from m/z 582. Furthermore, product ions at m/z 564, 453, 309 and 275 were all 16 amu more than product ions at m/z 548, 437, 293 and 259 obtained with M₆, suggestive of a glutathione conjugate of M₁. MS/MS analyses were insufficient to identify the site of monohydroxylation.

M₁₁, the glutathione conjugate of M₃, produced a [M+H]^+ ion at m/z 580, which is two...
hydrogen atoms less than the [M+H]+ ion of M10. MS/MS analyses of the [M+H]+ ion at m/z 580 produced product ions at m/z 562, 451, 433, 307 and 273 (Table 1). The product ion at m/z 451 resulted from the characteristic loss of pyroglutamic acid (129 amu) from m/z 580. A product ion pattern similar to that shown for M10 was observed for M11 except that product ions were two hydrogen atoms less than the corresponding ions for M10 and thus confirming M11 to be the glutathione conjugate of M3.

**Oxymetazoline Metabolism by Liver Microsomes.** When oxymetazoline was incubated with human, rat or rabbit liver microsomes supplemented with NADPH, LC/MS/MS analyses of the rabbit liver microsome incubations indicated the presence of M1, M2, M3, M4, and M5; rat liver microsome incubations generated metabolites M1, M2, M3, M4; and human liver microsomes generated metabolites M1, M2, and M3. Unchanged oxymetazoline was observed in liver microsomes of each species as the [M+H]+ ion at m/z 261.

**Trapping of Reactive Metabolites of Oxymetazoline with Glutathione and N-Acetylcysteine Supplemented S9 Fractions.** Supplemental glutathione was used as a nucleophile to ensure trapping of all of the reactive intermediates of oxymetazoline in liver S9 fractions. LC/MS/MS analyses of the incubations indicated glutathione conjugates M6 and M7, as previously detected in the liver S9 fractions not supplemented with glutathione. Additionally, the glutathione conjugates of M1 and M3 were also detected. MS/MS analyses were performed for each of the glutathione conjugate [M+H]+ ions to confirm their identities. To confirm that this bioactivation pathway is NADPH dependent, negative control liver S9 fraction incubations of oxymetazoline were performed in the absence of NADPH. No glutathione conjugates were detected in these incubations, demonstrating that oxymetazoline and its metabolites (M1, M2 and M3) were
bioactivated to reactive intermediates in the presence of NADPH, which were subsequently trapped with glutathione. Thus, we propose that the reactive intermediate was a \( p \)-quinone methide which could undergo nucleophilic substitution by glutathione (Thompson et al., 1995) (Fig. 7).

When \( N \)-acetylcysteine was used to trap the reactive intermediates of oxymetazoline in the rabbit liver S9 fraction incubations, \( N \)-acetylcysteine conjugates of oxymetazoline and \( M_2 \) were detected, displaying \([M+H]^+\) ions at \( m/z \) 422 and 420 respectively, corresponding to the addition of 161 amu (representing one acetylcysteine molecule) to both oxymetazoline and \( M_2 \) (data not shown). MS/MS analyses of the \([M+H]^+\) ion at \( m/z \) 422 indicated product ions at \( m/z \) 380 corresponds to the loss of an acetyl group, at \( m/z \) 293 corresponding to cleavage of the cysteinyl C-S bond from \( m/z \) 380 with the retention of sulfur on the aromatic ring of oxymetazoline, and the loss of \( \text{H}_2\text{S} \) from \( m/z \) 293 to generate the product ion at \( m/z \) 259 (Chen et al., 2002). MS/MS analyses of the \([M+H]^+\) ion at \( m/z \) 420 exhibited product ions at \( m/z \) 378, 291 and 257, which were two hydrogen atoms less than the corresponding product ions of the \( N \)-acetylcysteine conjugate of oxymetazoline and indicated the formation of the \( N \)-acetylcysteine conjugate of \( M_2 \). Neither of the \( N \)-acetylcysteine conjugates were detected in those incubations without \( N \)-acetylcysteine and NADPH. These results with the addition of \( N \)-acetylcysteine further confirm that oxymetazoline and \( M_2 \) are bioactivated to reactive intermediates in the presence of NADPH via the proposed formation of \( p \)-quinone methide reactive intermediates, which are similarly trapped by \( N \)-acetylcysteine. These findings further confirm that glutathione and \( N \)-acetylcysteine are effective agents for trapping the reactive intermediates of oxymetazoline in the liver S9 fractions, via the proposed formation of a reactive intermediate.
Characterization of the Glutathione Conjugate of Oxymetazoline by NMR. The proposed structure of M6 as a glutathione conjugate of a p-quinone methide was unambiguously characterized by 1D- and 2D-NMR using the biosynthesized M6. Since it was possible that nucleophilic attack by glutathione could occur at either the o- or p-methylene group of the quinone methide intermediate to form o- or p-glutathione conjugates of oxymetazoline, the chemical shifts and integrations of the relevant protons in the 1H-NMR (Table 2) and cross peak interactions of adjacent protons in the 2D ROESY spectrum were utilized to determine the position of the glutathione attachment. The 1H-NMR spectrum of biosynthesized M6 showed that the 4-methyl protons (H_B) of oxymetazoline at 2.107 ppm (Fig. 8A) had disappeared and reappeared as 4-S-benzylic protons (H_B) at 3.689 ppm (Fig. 8B). The chemical shift of the 2-methyl protons (H_C) of M6 was observed at 2.070 ppm, similar to the shift of 2.181 ppm of the 2-methyl protons (H_C) of oxymetazoline. Also, the methyl protons of the t-butyl group (H_A) were observed at 1.317 ppm, similar to 1.347 ppm of oxymetazoline. The replacement of the 4-methyl group with the S-benzylic protons (H_B) indicated that glutathione was conjugated at the 4-methyl group. The bridge methylene protons (H_E) of oxymetazoline were observed at 3.858 ppm, and the imidazoline ring protons (H_D) at 3.774 ppm, but could not be observed in the NMR spectrum of M6 because of their overlapping chemical shifts with the other protons of glutathione.

The position of the glutathione attachment of M6 was further confirmed by examination of the 2D-ROESY spectrum of the glutathione conjugate of oxymetazoline for differences in the cross peak interactions of the 2- and 4-methyl protons, as compared to those of oxymetazoline.
Although the 2D-ROESY spectrum of oxymetazoline exhibited several cross peak interactions, we identified a strong key cross peak interaction between the H_B protons of the 4-methyl group of oxymetazoline at 2.107 ppm and the single aromatic proton (H_F) at 6.898 ppm, which could further determine that glutathione had been conjugated with the 4-methyl group of oxymetazoline. The bridge methylene protons (H_E) exhibited cross peak interactions with both the 2-methyl and 4-methyl protons of oxymetazoline, and thus were not distinctive. As predicted, a characteristic cross peak interaction was observed between the methylene protons of the 4-S-benzyl group (H_B) at 3.689 ppm and the aromatic H_F proton at 6.918 ppm of M6 (Fig. 9). Therefore, ^1H-NMR data indicating the deshielding effect of a sulfur atom on the chemical shift differences between H_B and H_C protons and differences in the proton integration of these same protons, as well as the 2D-ROESY data plot which demonstrated cross peak interactions between H_B and H_F, together indicated that the glutathionyl moiety was conjugated with the 4-methyl group (Fig. 9). Furthermore, 1,4-quinone methides are more electrophilic than 1,2-quinone methides (Bolton and Thompson 1991; Bolton et al., 1992; Krol and Bolton, 1997).

**Identification of the Human-Expressed P450 Enzymes.** When oxymetazoline was incubated with a panel of human cDNA-expressed P450 enzymes (CYP1A2, CYP2B6, CYP2C8, CYP2C9, CYP2C18, CYP2C19, CYP2E1, CYP2D6 and CYP3A4), CYP2C19 was identified as the only enzyme catalyzing the formation of M1, M2 and M3.

When oxymetazoline was incubated with cDNA-expressed CYP2C19 supplemented with NADPH and glutathione, LC/MS/MS analyses revealed the formation of the glutathione conjugates M6 and M7. Furthermore, no glutathione conjugates were detected when any of the other P450 enzymes were similarly incubated. To further confirm this bioactivation pathway,
DNA-expressed CYP2C19 incubations of oxymetazoline were performed in the absence of NADPH. No glutathione conjugates were detected in the incubations. The formation of glutathione conjugates suggests the involvement of CYP2C19 in the bioactivation of oxymetazoline to a reactive intermediate.
Discussion

The metabolic disposition of oxymetazoline in humans had not previously been studied. In this study, we have examined the in vitro metabolism of oxymetazoline by pooled human, rat or rabbit liver microsomes and the liver S9 fractions from these same animal species, supplemented with NADPH, in an attempt to elucidate the metabolic pathways leading to the formation of reactive intermediates. The metabolites were identified from the comparison of their [M+H]+ ions, product ion patterns resulting from MS/MS analyses of the metabolite [M+H]+ ions, and comparisons of these patterns to the characteristic product ions of oxymetazoline. In addition, the glutathione conjugates of oxymetazoline and its oxidative metabolites were identified from the comparison of characteristic product ion patterns of glutathione conjugates. The observation that the glutathione conjugates M6 and M7 were two of the four major metabolites formed in the liver S9 fractions indicated the likelihood that oxymetazoline and its direct oxidation metabolites undergo oxidation to putative p-quinone methides. Interestingly, oxymetazoline was more efficiently metabolized by rabbit liver S9 fractions (~65 %) than by rat (~20 %) or human liver S9 fractions (~10 %). The metabolites common to the liver S9 fractions of these animal species were produced by monohydroxylation of the t-butyl group of oxymetazoline (M1), 4′,5′-dehydro-oxymetazoline (M2), monohydroxylation of M2 (M3) and the glutathione conjugates of oxymetazoline (M6) and of M2 (M7). The rabbit S9 liver fractions exhibited a markedly different metabolite profile producing, in addition to the four major and seven minor metabolites observed in the HPLC-UV chromatogram, other second-generation trace metabolites which were not detected in the UV chromatogram but which were identified by extracted ion MS/MS analyses of the theoretical [M+H]+ ions of the glutathione conjugates of other oxidative
metabolites and their hydrolytic products, such as cysteinylglycinates and cysteinates. These analyses of S9 fractions demonstrated interspecies similarities in the metabolism of oxymetazoline in liver S9 fractions. Analysis of the LC-UV peak areas for rabbit liver S9 fractions indicated that the recovery of the glutathione conjugates (M6 and M7) accounted for approximately 10 and 9%, respectively, when compared to the unmetabolized oxymetazoline levels at 60 min incubation.

A proposed metabolic scheme for oxymetazoline in human liver S9 is shown in Fig. 3. The selective monohydroxylation of the t-butyl group observed with oxymetazoline had previously been observed with the food preservative BHT (Bolton et al., 1990), with the H1-receptor antagonists terfenadine and ebastine (Jones et al., 1998; Hashizume et al., 2002), and with the isopropyl group of the anti-inflammatory drug ibuprofen (Hamman et al., 1997). The putative hydroxyimidazoline intermediate (HMA) (Fig. 3) resulting from the hydroxylation of imidazoline ring of oxymetazoline was not detected because it would likely eliminate water to produce the resonance-stabilized imidazole metabolite (4',5'-dehydro-oxymetazoline; M2). Similar mechanisms have been reported for the other imidazoline-containing drugs, brimonidine (Acheampong et al., 1996) and monoxidine (He et al., 2000). On the other hand, M3 could be formed either from M2 by subsequent hydroxylation of the t-butyl group or from hydroxylation of the imidazoline ring of M1 via the putative hydroxyimidazoline intermediate (HM_B) (Fig. 3). The second-generation dihydroxylated metabolites resulting from monohydroxylation of the t-butyl group and monohydroxylation of one of the aromatic methyl groups to form a hydroxybenzyl metabolite were identified in the rat and rabbit liver S9 fractions, but were not observed in the human liver S9 fractions. The human and rat liver microsomes produced fewer
oxidative metabolites as compared to their respective liver S9 fractions; on the other hand, rabbit liver microsomes generated similar numbers of oxidative metabolites (M1-M5), detected in the S9 fractions.

Screening with human recombinant cDNA-expressed CYP1A2, CYP2B6, CYP2C8, CYP2C9, CYP2C18, CYP2C19, CYP2D6, CYP2E1 and CYP3A4 Supersomes® identified CYP2C19 as the only isoform tested which catalyzed the oxidation of oxymetazoline. Since no human therapeutic or toxic plasma concentrations of oxymetazoline have been reported, the 50 µM substrate concentration used in the screening study to predict the relative contribution of each isoform to the total oxymetazoline metabolism was at least 130-fold greater than the usual therapeutic intranasal dose of 400 nM. At this concentration of oxymetazoline, no detectable oxidative metabolites were produced by the other CYPs, suggesting that the other enzymes are unlikely to participate in the clinically relevant in vitro oxidation. Our studies have also confirmed that CYP2C19 can catalyze the bioactivation of oxymetazoline to a reactive intermediate.

The glutathione conjugates of oxymetazoline and their hydrolytic metabolites (cysteinates and cysteinylglycinates) were identified in NADPH- and glutathione-supplemented liver S9 incubations, by the characteristic MS/MS product ion pattern reported for these conjugates. The presence of glutathione-conjugated metabolites in the incubations indicated the formation of a reactive electrophilic intermediate that is known to undergo non-enzymatic nucleophilic addition of glutathione (Thompson et al., 1995). To confirm this bioactivation pathway, liver S9 incubations of oxymetazoline were performed in the absence of NADPH. No glutathione conjugates were detected in these incubations. As a result, the identification and
characterization of glutathione conjugates is a widely accepted indirect approach for the
detection of electrophilic reactive intermediates formed during the metabolism of alkyl phenol
xenobiotics (Thompson et al., 1995; Bolton et al., 1997; Monks and Jones, 2002; Yan et al.,
2005). Further confirmation of the presence of a reactive intermediate formed from
oxymetazoline utilized trapping as the N-acetylcysteine conjugate, which was subsequently
identified by LC/MS/MS.

1D- and 2D NMR structural analyses of the oxymetazoline glutathione conjugate (M6)
demonstrated that the thiol group of glutathione is attached to the 4-benzylic group of the alleged
p-quinone methide rather than to a ring carbon, which is the expected product from the putative
p-quinone methide intermediate and not from an epoxide pathway. Epoxide metabolites would
yield a glutathione conjugate attached at a ring carbon rather than at the 4-benzylic carbon. We
propose that the putative reactive intermediate is a p-quinone methide species, since it was
effectively trapped with glutathione or N-acetylcysteine. Our proposed quinone methide
pathway is illustrated in Fig. 7. We postulate that the formation of the putative p-quinone
methide involves successive one-electron oxidation steps by P450 as suggested by Koymans et
al. (1993) and Thompson et al. (1995), or alternatively, hydroxylation of the 4-methyl group
generating a 4-hydroxybenzyl group which can undergo dehydration to form a p-quinone
methide species. The electrophilic p-quinone methide subsequently undergoes a non-enzymatic
nucleophilic addition by glutathione at the 4-benzylic carbon. Considering that p-quinone
methide is abundant in liver S9 incubations of oxymetazoline for the three animal species, the
toxicological significance of this reactive intermediate cannot be completely overlooked. As
Michael acceptors, quinone methides can alkylate cellular nucleophiles such as proteins and
other macromolecules. Their rate of formation and reactivity can influence their toxicity (Bolton et al., 1990; Thompson et al., 1990; Thompson et al., 1992; Thompson et al., 1995; Monks and Jones, 2002). Bolton et al. (1990) have observed that bulky alkyl substituents ortho to the phenolic group affected the rate of reaction of the $p$-quinone methide with nucleophiles, and that hindered phenols, which have $o$-methyl or $o$-$t$-butyl groups adjacent to the phenoxy group, help to shield the quinoid oxygen from solvent interactions, increasing the stability of the $p$-quinone methide and allowing time for its diffusion away from the site of formation and from interaction with vital cellular macromolecules, thus reducing its toxicity potential (Bolton et al., 1995). In contrast, unhindered $p$-alkyl phenols will form reactive $p$-quinone methides that are more hepatotoxic and cytotoxic by the same mechanism (Bolton et al., 1995; Yan et al., 2005). Thus, we attribute the reduction or the absence of toxicity potential of oxymetazoline to steric hindrance by the $o$-$t$-butyl and the $o$-methyl groups, which reduce its toxicity potential by the same mechanism (Bolton et al., 1995; Yan et al., 2005). We did not determine whether covalent binding of the intermediate to proteins occurred and, if so, what levels of binding were reached.

In conclusion, oxymetazoline has demonstrated the likelihood to be bioactivated to a reactive intermediate species, as identified from its corresponding glutathione conjugates, in incubations with liver S9 fractions supplemented with NADPH. Consequently, a bioactivation pathway is proposed. We rationalize that this bioactivation pathway is unlikely to be a safety concern with oxymetazoline nasal products. Other factors contributing to the margin of safety are the brief intranasal dosage regimen, for a few days at low doses. Uetrecht (2001 and 2008) has observed that drug-induced toxicity due to formation of reactive intermediates is seldom associated with drugs administered at doses of 10 mg or less. In addition, we have reported the
previously unknown in vitro metabolite profile of oxymetazoline in human, rabbit and rat liver microsomes and their liver S9 fractions, and we have identified CYP2C19 as the enzyme that contributes to the hepatic disposition following intranasal administration. The margin of clinical safety for nonprescription oxymetazoline products, despite bioactivation to a $p$-quinone methide species, provides another reminder that toxicity is often multifactorial and bioactivation does not always lead to toxicity.
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Authorship Contributions

Participated in research design: Mahajan, Uttamsingh, Daniels, Gan, LeDuc, and Williams

Conducted experiments: Mahajan

Contributed new reagents or analytic tools: Mahajan

Performed data analysis: Mahajan, Uttamsingh, Daniels, Gan, LeDuc, and Williams

Wrote or contributed to the writing of the manuscript: Mahajan, Uttamsingh, Daniels, Gan, LeDuc, and Williams

Other: Williams (Research Advisor)
References


Footnotes

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Figure Legends

Figure 1. Structure of oxymetazoline.

Figure 2. Representative expanded LC-UV chromatogram of oxymetazoline incubations in rabbit liver S9 fractions in the presence (A) and absence (B) of NADPH at 254 nm.

Figure 3. The proposed metabolic pathways of oxymetazoline following incubations in human liver S9 fraction.

Figure 4. MS/MS spectrum of \([\text{M+H}]^+\) ion at \(m/z\) 261 for pure oxymetazoline.

Figure 5. MS/MS spectrum of \([\text{M+H}]^+\) ion at \(m/z\) 277 for M1 (A) MS/MS spectrum of \([\text{M+H}]^+\) ion at \(m/z\) 259 for M2 (B) MS/MS spectrum of \([\text{M+H}]^+\) ion at \(m/z\) 293 for M4 (C) from the oxymetazoline incubations containing rabbit liver S9 fraction supplemented with NADPH.

Figure 6. Representative MS/MS spectrum of \([\text{M+H}]^+\) ion at \(m/z\) 566 for M6 (A) MS/MS spectrum of \([\text{M+H}]^+\) ion at \(m/z\) 564 for M7 (B) from the oxymetazoline incubations containing rabbit liver S9 fraction supplemented with NADPH.

Figure 7. The proposed mechanism for the formation of glutathione conjugate of oxymetazoline via 1,4-quinone methide in the incubations containing NADPH and glutathione.

Figure 8: \(^1\text{H-NMR}\) spectrum of oxymetazoline in DMSO-\(d_6\) (A) \(^1\text{H-NMR}\) spectrum of the glutathione conjugate of oxymetazoline (M6) in DMSO-\(d_6\) (B). Spectra were acquired on a 600 MHz Varian Inova NMR spectrometer. The protons \(H_D\) and \(H_E\) were not clearly observed in the NMR spectrum of M6 due to overlapping with the glutathione protons between 3-4 ppm.

Figure 9. 2D-ROESY NMR spectrum and structure of the oxymetazoline glutathione conjugate (M6) showing the key 2D-NMR cross peak proton interactions between \(H_B\) and \(H_F\). Samples of the glutathione conjugate of oxymetazoline (M6) were dissolved in DMSO-\(d_6\). The 2D-ROESY
NMR spectra were acquired on a 600 MHz Varian Inova NMR spectrometer at 298.1 °K.
**Table 1:** Oxymetazoline metabolites identified in the different S9 fractions

<table>
<thead>
<tr>
<th></th>
<th>t&lt;sub&gt;R&lt;/sub&gt; (min)</th>
<th>[M+H]&lt;sup&gt;+&lt;/sup&gt;</th>
<th>Product Ions (m/z)</th>
<th>Human Liver S9</th>
<th>Rat Liver S9</th>
<th>Rabbit Liver S9</th>
</tr>
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<tbody>
<tr>
<td>Parent</td>
<td>12.2</td>
<td>261</td>
<td>243, 205, 191, 177</td>
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<td></td>
</tr>
<tr>
<td>M1</td>
<td>4.95</td>
<td>277</td>
<td>259, 205</td>
<td>√</td>
<td>√</td>
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</tr>
<tr>
<td>M2</td>
<td>12.36</td>
<td>259</td>
<td>241, 203, 177, 81</td>
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<td>√</td>
<td>√</td>
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<tr>
<td>M3</td>
<td>5.64</td>
<td>275</td>
<td>257, 203, 81</td>
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<td>√</td>
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<tr>
<td>M4</td>
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<td>293</td>
<td>275, 245, 221</td>
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<td>√</td>
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<tr>
<td>M5</td>
<td>2.77</td>
<td>291</td>
<td>273, 243, 219</td>
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<td>√</td>
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<tr>
<td>M6</td>
<td>4.22</td>
<td>566</td>
<td>548, 473, 437, 380, 293, 259</td>
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<td>√</td>
<td>√</td>
</tr>
<tr>
<td>M7</td>
<td>4.65</td>
<td>564</td>
<td>546, 471, 435, 378, 291, 257</td>
<td>√</td>
<td>√</td>
<td>√</td>
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<tr>
<td>M8</td>
<td>3.17</td>
<td>380</td>
<td>293, 259</td>
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<td>M9</td>
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<td>M10</td>
<td>3.86</td>
<td>582</td>
<td>564, 453, 435, 309, 275</td>
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<tr>
<td>M11</td>
<td>6.34</td>
<td>580</td>
<td>562, 451, 433, 307, 273</td>
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</table>
Table 2. Comparison of the chemical shift of protons of oxymetazoline and glutathione conjugate of oxymetazoline (M6)

<table>
<thead>
<tr>
<th>Relevant Proton</th>
<th>Oxymetazoline</th>
<th>Oxymetazoline Glutathione Conjugate</th>
</tr>
</thead>
<tbody>
<tr>
<td>HA</td>
<td>1.347 (9 Hs)</td>
<td>1.317 (9 Hs)</td>
</tr>
<tr>
<td>HB</td>
<td>2.107 (3 Hs)</td>
<td>3.689 (2 Hs)</td>
</tr>
<tr>
<td>HC</td>
<td>2.181 (3 Hs)</td>
<td>2.070 (3 Hs)</td>
</tr>
<tr>
<td>HD</td>
<td>3.774 (4 Hs)</td>
<td>nd</td>
</tr>
<tr>
<td>HE</td>
<td>3.858 (2 Hs)</td>
<td>nd</td>
</tr>
<tr>
<td>HF</td>
<td>6.898 (1 H)</td>
<td>6.918 (1 H)</td>
</tr>
</tbody>
</table>

nd: the chemical shifts for protons H_D and H_E could not be clearly identified in the spectrum due to their overlapping with the glutathione protons between 3-4 ppm.
Figure 5
Figure 6
Figure 7
Figure 8

A

B

ppm

ppm
Figure 9

[Chemical structure diagram with annotations:]

- **Glutathione** connected with an S bond.
- H$_B$ at 3.689 ppm.
- H$_F$ at 6.918 ppm.
- H$_C$ at 2.070 ppm.
- ROESY interaction consistent with structure proposed.