DMD Fast Forward. Published on December 2, 2005 as doi:10.1124/dmd.105.007047

SELECTIVE PATHWAYS FOR THE METABOLISM OF PHENCYCLIDINE BY CYTOCHROME P450 2B ENZYMES: IDENTIFICATION OF ELECTROPHILIC METABOLITES, GLUTATHIONE AND *N*-ACETYL CYSTEINE ADDUCTS

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Running Title: Reactive intermediates of PCP and formation of GSH adducts

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Number of text pages: 16

Number of schemes: 1

Number of figures: 8

Number of references: 39

Number of words in abstract: 250

Number of words in introduction: 768

Number of words in discussion: 1079

Abbreviations: P450, cytochrome P450; MBI, mechanism-based inactivator; DLPC, dilauroyl-L-α-phosphotidylcholine; reductase, NADPH-cytochrome P450 reductase; PCP, phencyclidine; GSH, glutathione; *N*AC, *N*-acetyl cysteine; LC-MS, liquid chromatography - mass spectrometry; ESI; electrospray ionization; MS/MS, tandem mass spectrometry; *m/z*, mass to charge ratio; TIC, total ion chromatogram; XIC, extracted ion chromatogram; R_t; retention time.

Abstract

The metabolism of phencyclidine (PCP) has been studied previously in P450-containing microsomal systems. However, the reactive intermediate(s) that covalently binds to the P450 and leads to inactivation or leaves the active site to modify other proteins has not been identified. In this study two electrophilic intermediates of PCP were identified by mass spectrometry and by trapping with reduced glutathione (GSH) or *N*-acetyl cysteine (*N*AC). The tentative structures of these electrophilic intermediates were determined using mass spectrometry. P450s 2B1 and 2B4 formed a metabolite that exhibited an *m/z* of 240 corresponding to the mass of the 2,3-dihydropyridinium species of PCP or its conjugate base the 1,2-dihydropyridine. Chemical reduction of the incubation mixture using $NaBH_4$ resulted in the disappearance of the signal at *m/z* of 240, consistent with reduction of a 2,3-dihydropyridinium species. Furthermore, the reactive metabolite trapped by GSH resulted in an adduct exhibiting an *m/z* of 547 consistent with the mass of the 2,3-dihydropyridinium species of PCP (*m/z* 240) that has reacted with a molecule of GSH (*m/z* 308). However, P450 2B6 formed a different reactive intermediate of PCP that was isolated as a GSH adduct exhibiting an *m/z* of 581 and *N*AC adduct with an *m/z* of 437. LC-MS/MS analysis of these adducts suggested that a di-oxygenated iminium metabolite of PCP could be the reactive intermediate formed by P450 2B6 but not by the other 2B isoforms. These data suggest that P450 2B6 favors oxidation pathways for PCP metabolism that are different from those of P450s 2B1 and 2B4.

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Introduction

Phencyclidine (PCP) was originally developed in 1958 as a human anesthetic but its use in human medicine was discontinued soon after due to its serious psychological side effects (Fauman, et al. 1976). The causes of the long-term psychological effects of PCP have not yet been determined; however, it has been proposed that these side effects could result from the irreversible binding of PCP or its reactive metabolites to critical macromolecules in the brain (Holsztynska and Domino, 1985; Hoag et al., 1987). Studies on PCP metabolism by liver microsomes showed that PCP is metabolized by P450s to give a variety of different products (Figure 1) (Holsztynska and Domino, 1985; Hoag et al., 1987; Osawa and Coon, 1989; Crowley and Hollenberg, 1995; Hiratsuka et al., 1995; Laurenzana and Owens, 1997). However, few studies have reported on the metabolism of PCP by specific P450s using purified enzymes in a reconstituted system. Rat P450 2B1 shares 75-80% amino acid homology with other members of the P450 2B subfamily and has been used as a model for this family (Roberts et al., 1997; Scott, et al., 2001). P450 2B4, the rabbit homologue of P450 2B1 has been used extensively in drug metabolism studies (Osawa and Coon, 1989). P450 2B6 is one of two human 2B isoforms identified to date, and , in addition to being present in the liver, it is also expressed in brain, kidney, intestine, lungs and heart (Gervot et al., 1999, Thum and Borlak, 2000). The role of this isoform in drug metabolism was initially underestimated, but the number of drugs recognized as 2B6 substrates has been constantly increasing (Ekins and Wrighton, 1999; Zukunft et al., 2005). It is now estimated that P450 2B6 is responsible for the metabolism of more than 3% of all clinically used drugs in humans (Rendic, 2002). The other related isoform that belongs to the human 2B subfamily is P450 2B7, a pseudogene expressed in the liver and lung tissues (Willey

et al., 1997); however, to our knowledge the expression of 2B7 at the protein level has not been reported, and the role of this isoform in the metabolism of drugs in humans has not been established. In this study we focused on the role of P450 2B6 in the metabolism of PCP to form reactive metabolites since this isoform has more significance in the metabolism of drugs in humans. In addition, P450 2B6 has been shown to metabolize other drugs such as nicotine to nornicotine (Yamanaka et al., 2005), indicating the significant role that this isoform plays in the metabolism of cyclic amines such as PCP.

PCP has been shown to be a mechanism-based inactivator (MBI) of P450s 2B1, 2B4 and 2B6, and its metabolism leads to modification of the apoprotein of these P450 2B isoforms (Sharma et al., 1997; Jushchyshyn et al., 2003). Several cyclic tertiary amines including the Parkinsonian agent N-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) and the neuroleptic agent 4-(4-chlorophenyl)-1-[4-(4-fluorophenyl)-4-oxobutyl]-4-piperidinol (haloperidol, HP), have been shown to undergo bioactivation pathways that lead to the formation of the nuerotoxic pyridinium metabolites, N-methyl-4-phenylpyridinium (MPP^+) and 4-(4-chlorophenyl)-1-[4-(4-fluorophenyl)-4-oxobutyl] pyridinium (HPP^+), respectively. The formation of these neurotoxic metabolites has been shown to proceed via unstable 2,3-dihydropyridinium intermediates (Castagnoli et al., 1997; Usuki et al., 1996). PCP shares structural features that are similar to those of MPTP and HP; therefore, it could be hypothesized that the piperidine ring moiety of PCP (Figure 1) may undergo similar bioactivation pathways via P450s that lead to the formation of electrophilic 2,3-dihydropyridinium intermediates (Weissman et al., 1985; Castagnoli et al, 1997). These intermediates may then cause covalent modification of amino acid residues in the active sites of P450s or react with other proteins in the vicinity of the P450.

Reactive intermediates can oftentimes be identified using chemical trapping agents such as GSH or *N*AC (Evans et al., 2004; Baillie and Davis, 1993) that form stable adducts which can be characterized using liquid chromatography tandem mass spectrometry (LC-MS/MS). Such studies can provide structural information about the reactive intermediates that are formed during metabolism.

It has previously been shown that the addition of GSH to rabbit microsomes incubated with PCP inhibited metabolism-dependent covalent binding of radiolabeled PCP to microsomal proteins by approximately 50% (Kalir et al. 1983). Therefore, we postulated that GSH may trap reactive intermediates of PCP that are formed during metabolism by P450s. In this study, we have used chemical derivatization and the trapping agents GSH and *N*AC to identify and trap reactive intermediates of PCP that were formed during metabolism by P450's 2B1, 2B4 and 2B6 in the reconstituted system. The putative structures of these intermediates and adducts were determined using electrospray ionization (ESI) MS/MS.

Materials and Methods

Materials. Phencyclidine hydrochloride (PCP), nicotinamide adenine dinucleotide phosphate (NADPH), GSH, *N*AC, dilauroyl-L-α-phosphotidylcholine (DLPC), sodium borohydride ($NaBH_4$), and catalase were purchased from Sigma-Aldrich (St Louis, MO). Trifluoroacetic acid (TFA) was purchased from Pierce Chemical (Rockford, IL). PCP authentic standards *cis*-1-(1-phenyl-4-hydroxy-cyclohexyl) piperidine HCl (M1), *trans*-1-(1-phenyl-4-hydroxy-cyclohexyl) piperidine (M2), *cis*-1-(1-phenylcyclohexyl)-4-hydroxypiperidine (M3), *trans*-1-(1-phenylcyclohexyl)-4-hydroxypiperidine (M4), 4-(4'-hydroxy piperidino)-4-phenyl cyclohexanol (M6), 5-[N-(1'-phencyclohexyl)-amino] pentanoic acid HCl (M7) were obtained from the National Institute on Drug Abuse (Research Triangle Park, NC). 1-(1-phenylcyclohexyl)-2,3,4,5-tetrahydropyridinium perchlorate (M5) was a generous gift from Dr. Neal Castagnoli, Jr. (Virginia Polytechnic Institute and State University, Blacksburg, VA).

Enzyme Purification. P450s 2B1 and 2B6 were expressed in *Escherichia coli* MV1304 cells and purified as described by Hanna et al. (Hanna et al., 1998; Hanna et al., 2000) with modifications (Kent et al., 1999). P450 2B4 was expressed and purified according to the methods of Hanna et al. (1998). NADPH-cytochrome P450 reductase (reductase) was expressed in *Escherichia coli* Topp 3 cells and purified according to published protocols (Hanna et al., 1998).

Metabolite identification. Mixtures of P450 2B4 (3 μM), reductase (6 μM) and DLPC (200 μg) were reconstituted at 4^{o} C for 45 minutes at which time catalase (1000 units), 50 mM potassium phosphate buffer (pH 7.4) and excess PCP (0.5 mM) were added to give a final volume of 1.5 mL. This primary mixture was divided into three samples that were pre-incubated at 30^{o} C for 10 minutes. One sample received water only (26 μL) and served as a control, while

the other two samples received NADPH to a final concentration of 0.9 mM. After 40 min of incubation in a water bath, all samples were quenched with 60 μL of 1% TFA/sample and placed on ice. Chemical reduction of the metabolite mixture was performed by treatment of one of the samples that received NADPH with 1 mg of $NaBH_4$ for 10 min at room temperature. All samples were then applied to pre-conditioned 1 mL C-18 solid phase extraction columns (J&W Scientific, Folsom, CA) previously washed with 1 mL methanol and 2 mL water. After the samples were loaded, the columns were washed consecutively with 1 mL water, 2 mL methanol, and 300 μL acetonitrile. The methanol and acetonitrile fractions were collected, combined, and dried under a stream of nitrogen to a final volume of approximately 50 μL. The dried samples were resuspended in 200 μL of solvent A composed of 90% water, 10% methanol and 0.05% TFA for LC-MS analysis. Incubation mixtures with P450s 2B1 and 2B6 were done as described above, except that lipid was omitted from the P450 2B6 reconstitution mixture. The enzymatic activity of the truncated P450 2B6 used in this study has been shown to be higher in the absence of exogenous lipid (Scott et al., 2001)

Trapping of Reactive Intermediates of PCP. P450 2B1 or 2B4 (3 μM) were reconstituted with reductase (6 μM) and DLPC (200 μg) at 4^o C for 45 minutes at which time catalase (2000 units), 50 mM potassium phosphate buffer (pH 7.4) and PCP (0.5 mM) were added to give a final volume of 1 mL. This primary mixture was divided into three samples: control, (-) GSH, and (+) GSH. The control and the (+) GSH samples contained 10 mM GSH, while the (-) GSH sample received an equal volume of water (10 μL). The samples were pre-incubated at 30^o C for 10 minutes, and the metabolism was initiated by the addition of NADPH to a final concentration of 0.9 mM to the (-) and (+) GSH samples while the control sample received water (15 μL). The incubation conditions for P450 2B6 with PCP were as described

above, and lipid was omitted from these mixtures. Incubations of P450 2B6 and 2B4 with PCP were also performed in the presence of 4 mM *N*AC as an alternative trapping agent. The samples were prepared for LC-MS analysis as described above under Metabolite Identification.

LC-MS Analysis. Aliquots (50 μL each) of the previously prepared samples were injected onto a Zorbax Rx-C8 (5 μm, 4.6 x 250 mm) column (Agilent, Palo Alto, CA) and separated by Hewlett Packard 1100 series HPLC system (Hewlett Packard, Palo Alto, CA) using a solvent system composed of solvent A and 90% acetonitrile, 10% methanol and 0.05% TFA (solvent B). A flow rate of 1 mL/min and a linear gradient of 10% to 50% solvent B over 30 minutes, were used to resolve the metabolites and GSH and *N*AC adducts. A series of authentic standards (M1-M7) were resolved using the same mobile phase and gradient. Mass spectrometry was performed using a Finnigan MAT LCQ Classic mass spectrometer with an ESI interface from the liquid chromatography system. The analytes were ionized in positive mode using a capillary voltage of 55 V, a spray voltage set at 4.5 kV, a tube lens offset voltage of 25 V, a capillary temperature of 170^{o} C and an auxiliary gas and sheath gas flow of 30 and 90, respectively. Under similar conditions, the UV spectra and identification of metabolites were done on a triple quadrupole mass spectrometer with an online Surveyor HPLC diode array detector using a ThermoFinnigan TSQ Quantum Ultra AM equipped with an ESI source interfaced to the mass spectrometer. The mass spectrometer and all its peripheral components were controlled by the Xcalibur software. The instrument parameters were optimized using a PCP standard.

LC-MS/MS Analysis. Liquid chromatography was performed as described above. ESI MS/MS studies were performed on the Finnigan LCQ Classic and the TSQ triple quadrupole mass spectrometers using collision induced dissociation (CID) with normalized collision energy

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of 27% applied to precursor ions $[MH]^+$, in the data-dependent scanning mode. The TSQ was used to obtain higher mass sensitivity and peak resolution. Helium was used as the collision gas.

Results

Identification of an Electrophilic Metabolite of PCP. LC-MS analysis of incubation mixtures of P450 2B4 with PCP in the presence of NADPH revealed five peaks, M1, M2, M3, M4, and M5 eluting at 11.2, 14.5, 16.2, 17, and 19.6 min in the total ion chromatogram (TIC), respectively (Figure 2B). A new metabolite with an *m/z* of 240 eluted at 18.5 min as shown in Figure 2C. The signal for PCP upon protonation is at *m/z* of 244; therefore, a loss of 4 amu is consistent with the oxidative metabolism of PCP. The online HPLC diode-array UV spectrum of the new metabolite showed a λ_{max} at 234 nm, indicating a shift from the UV spectral characteristics of PCP (λ_{max} 262 nm), and suggesting that this metabolite contains a conjugated double bond system (inset in Figure 2C). This was further confirmed by LC-ESI MS/MS analysis of the metabolite eluting at R_t 18.5 min, which produced characteristic fragments upon CID at *m/z* of 159 and 82, indicative of a structural change on the piperidine moiety of PCP (Figure 3). These data suggested that the 2,3-dihydropyridinium species of PCP was formed, or possibly, the fragmentation pattern could correspond to its protonated conjugate base, the 1,2-dihydropyridine. We have also found that a signal with *m/z* 238 was present at R_t 18.0 min, and the MS/MS fragmentation pattern of this molecular ion suggested that a pyridinium species was also formed (data not shown). These observations are supported by a previous report which showed that the 1-methyl-4-2,3-dihydropyridinium (2,3-$MPDP^+$) species of MPTP undergoes disproportionation to form the neurotoxic pyridinium metabolite MPP^+, and under mass spectral conditions a rearrangement of the 2,3-$MPDP^+$ yields the 1,2-dihydropyridine (Peterson et al., 1985). To further investigate the identity of the new metabolite with *m/z* of 240, we performed chemical reduction of the metabolite mixture using $NaBH_4$. Treatment of the incubation mixture

with $NaBH_4$ resulted in the disappearance of the signal at *m/z* of 240, indicating that the oxidized piperidine moiety of the 2,3-dihydropyridinium species was reduced. Since $NaBH_4$ has been shown to reduce iminium ions (Kalgutkar and Nguyen, 2004), we believe that the metabolite with *m/z* of 240 is the 2,3-dihydropyridinium species of PCP and not the 1,2-dihydropyridine. To confirm the formation of the 2,3-dihydropyridinium species of PCP, we attempted to trap this reactive intermediate with GSH and *N*AC during the P450-catalyzed metabolism.

Trapping Reactive Intermediates of PCP with GSH and *N*AC. The GSH adducts formed during the metabolism of PCP by P450s 2B1 and 2B4 in the presence of 10 mM GSH were analyzed by LC-ESI MS. When GSH was added to the incubation mixtures of P450s 2B1 and 2B4, a new peak was observed at 14.8 min exhibiting an *m/z* of 547 (G1) in the TIC (Figure 4A). The extracted ion chromatogram (XIC) of G1 revealed two peaks with protonated molecular ions $[MH]^+$ at *m/z* of 547 (Figure 4B) that were not present in the samples where NADPH was omitted or the (-) GSH samples (review Figure 2A and B). The addition of GSH to these incubation mixtures resulted in a loss of the 2,3-dihydropyridinium signal at *m/z* of 240. This suggests that GSH trapped the 2,3-dihydropyridinium intermediate during the metabolism of PCP.

The observed *m/z* of 547 corresponds to the mass of one molecule of PCP that had reacted with one molecule of GSH. The two peaks corresponding to G1 were resolved and analyzed by LC-ESI MS/MS. The product ion spectra of both peaks were identical upon CID of the ions at *m/z* of 547, suggesting that the addition of GSH could occur at the C-2 or C-4 position of the 2,3-dihydropyridinium species. The masses of these ions were consistent with the presence of the 2,3-dihydropyridinium species (*m/z* 240), a GSH molecule (*m/z* 308), loss of the thiol group from GSH (*m/z* 275) and fragmentation of the N-C bond between the piperidinyl and

cyclohexyl rings of PCP to give a product ion with an *m/z* of 388, which suggests that GSH was adducted to the piperidine ring of PCP (Figure 5).

In contrast to what was seen with P450s 2B1 and 2B4, no adduct exhibiting an *m/z* of 547 was observed in incubations of P450 2B6 with PCP performed in the presence of GSH. However, GSH adducts of PCP with an *m/z* of 581 were formed during metabolism by P450 2B6. This was not surprising since the signal for the 2,3-dihydropyridinium species (*m/z* of 240) was absent from these incubations (data not shown). Figure 6A shows the TIC of the hydroxylated metabolites extracted from P450 2B6 incubation mixture, and the XIC of two GSH adducts with *m/z* values of 581 (G2) that eluted at approximately 15.6 and 15.8 min are shown in Figure 6B. The *m/z* of G2 is consistent with a di-oxygenated PCP molecule with the addition of one molecule of GSH. This molecular ion was not detected in any of the incubations of PCP with P450s 2B1 or 2B4 in the presence of GSH. These data suggest that human 2B6 forms a reactive intermediate of PCP via a metabolic pathway that is different from that seen with 2B1 and 2B4.

To characterize the G2 adduct formed in the incubation mixtures of P450 2B6, LC ESI-MS/MS analysis was performed. The MS/MS spectrum shown in Figure 7 shows the fragmentation pattern for the G2 adduct at R_t 15.6 min. The second peak at 15.8 min produced identical spectra after the two peaks were resolved, suggesting that isomers of G2 may be formed (data not shown). The product ions were consistent with a dissociation of the glycine residue of GSH (*m/z* 507), a neutral loss of the γ-glutamyl linkage characteristic of GSH adducts (*m/z* 452), dissociation of a H_2O molecule and the γ-glutamyl residue (*m/z* 434), a protonated GSH (*m/z* 308) and its protonated cysteinylglycine moiety (*m/z* 179), and a metabolite of PCP with two oxygens on the piperidine ring (*m/z* 276) (Figure 7). Since G1 and G2 were formed in low

abundance in all cases, additional structural information using NMR to determine the exact position of GSH adduction and hydroxylation has not been possible.

We also were able to trap a reactive intermediate of PCP from incubation mixtures of P450 2B6 using *N*AC as the trapping agent. *N*AC has a simpler structure compared to GSH, making the analysis of MS/MS spectra of the trapped adducts less complicated. In addition, *N*AC has been used successfully for trapping reactive imine intermediates (Skordos et al., 1998*a*). *N*AC adducts of PCP were formed from incubations with 2B6 and observed at R_t 18.9 and 19.13 min with an *m/z* values of 437 (data not shown). Figure 8 shows the LC-ESI MS/MS spectrum of the *N*AC adduct at *m/z* of 437 which eluted approximately at 18.9 min. The second peak eluting at 19.13 min was resolved and produced identical MS/MS spectra. The product ions were consistent with a loss of the phenyl- and cyclohexyl rings of PCP (*m/z* 279), and dissociation of a H_2O molecule from this product ion (*m/z* 261). Together, these data confirm the proposed identity of the GSH adduct of PCP that was obtained from incubations with P450 2B6. We also tested the ability of *N*AC to form adducts with PCP reactive intermediates during metabolism by P450 2B4 and did not detect any *N*AC adducts with *m/z* values of 437. Instead, *N*AC adducts exhibiting an *m/z* of 403 were observed from these incubations and MS/MS analysis of these adducts (data not shown) revealed that *N*AC was adducted to the 2,3-dihydropyridinium species of PCP as was shown with GSH. Because we expected P450 2B1 to form similar *N*AC adducts to those obtained from 2B4 based on what was shown with GSH, these experiments were omitted. These data further confirm that GSH and *N*AC are effective tools for trapping reactive intermediates of PCP during metabolism by P450 2B enzymes.

Discussion

The metabolism of PCP has been studied previously in P450-containing microsomal preparations. PCP has been shown to be an MBI of several P450s in the reconstituted system (Osawa and Coon, 1989; Sharma et al., 1997; Jushchyshyn et al., 2003). Adducts of reactive PCP intermediates to the P450 apoprotein have been observed with P450s 2B1 and 2B4 (Jushchyshyn et al., 2003) suggesting that the reactive intermediates formed may have electrophilic identities; however, information on the identities and structures of the reactive intermediate(s) of PCP responsible for the inactivation of these P450s or modification of other proteins in the vicinity of the P450 is lacking.

In this study, a new metabolite of PCP shown to be the 2,3-dihydropyridinium species was detected in incubation mixtures of P450s 2B1 and 2B4 with PCP. Adducts derived from reactive intermediates of PCP were formed using reconstituted systems containing purified P450s 2B1, 2B4 and 2B6 and identified using the trapping agents GSH and *N*AC. An earlier study by Ward et al. (1982) suggested that M5 of PCP is the species responsible for the inactivation of P450s and for binding to macromolecules. However, M5 requires further metabolism by the P450s to cause inactivation, as indicated by the absolute requirement for NADPH to observe any loss in activity (Hoag et al., 1987; Crowley and Hollenberg, 1995; Jushchyshyn et al., unpublished results). Therefore, M5 does not appear to be the ultimate reactive species but may actually serve as a precursor to the ultimate P450-derived reactive intermediate(s).

The LC-ESI MS/MS data reported here indicate that two types of intermediate electrophiles react with GSH and *N*AC during the metabolism of PCP. To our knowledge, GSH

and *N*AC adducts resulting from the metabolism of PCP by P450s in the reconstituted system have not previously been reported. The reactive intermediate of PCP trapped by GSH may have been formed via an α-carbon oxidation of the piperidine ring of PCP by P450s 2B1 and 2B4 to generate M5, that could then rearrange to the enamine (Sayre et al. 1991). Alternatively, direct oxidation of PCP could occur to form the enamine similar to what Skordos et al. (1998*b*) have shown with 3-methylindole. This enamine could then undergo further oxidation by the P450, which would result in the formation of the electrophilic 2,3-dihydropyridinium species that has been postulated by Castagnoli et al. (1997). This species is a good Michael acceptor and would serve as a target for nucleophilic attack by the GSH or *N*AC thiol group. This intermediate may alternatively react with nucleophiles in the P450 active site or with other proteins in the vicinity of the P450s. Similar dehydrogenation reactions carried out by P450s have been shown to occur without the need for oxygenation of the substrate (Skiles and Yost, 1996; Guengerich FP, 2001; Muenier et al., 2004). In addition, other cyclic tertiary amines have been shown to undergo similar bio-activation pathways to yield the 2,3-dihydropyridinium intermediate (Castagnoli et al., 1997; Usuki et al., 1996; Dalvie and O'Connell, 2004; Kalgutkar and Nguyen, 2004). A tentative pathway for the formation of PCP reactive intermediate *in vitro* and a likely mechanism leading to the formation of GSH adducts of PCP is presented in Scheme 1A.

Although human P450 2B6 metabolizes many of the same substrates as the rat and rabbit 2B isoforms to give the same products, in this case a different reactive PCP intermediate was identified from incubation mixtures of the human enzyme. The tentative structure of this intermediate was determined using LC-ESI MS/MS analysis of the adducts formed by reaction with GSH or *N*AC. The data indicate that a di-oxygenated iminium metabolite was generated that could then react with GSH to form G2 having an *m/z* of 581, as shown in Scheme 1B. The

formation of a di-oxygenated species was further confirmed by detecting an *N*AC adduct with an *m/z* of 437. Therefore, it appears that P450 2B6 favors another pathway where the 4-hydroxylated enamine could undergo a second hydroxylation at C-3 to generate an iminium ion. Alternatively, the 4-hydroxylated enamine may undergo further oxygenation to form a 2,3-epoxide. This epoxide intermediate may then undergo ring opening to give a hydroxyl at C-3 leading to an iminium ion intermediate as has been shown with 3-methylindole (Skordos et al., 1998*b*). In both cases the iminium ion intermediate could serve as an electrophilic center for GSH addition and lead to formation of the adduct shown (Scheme 1B).

A previous study by Samuel et al. (2003), on the metabolism of several Merck compounds showed that GSH adducts were formed by rat liver microsomal preparations that had different masses from those formed by the human liver microsomal preparations. We report here similar observations where different reactive intermediates of PCP and their corresponding GSH and *N*AC adducts were formed by the 2B P450s from rats (2B1), rabbits (2B4) and humans (2B6).

Reactive intermediates of PCP could arise from the oxidation of any of the ring carbons of the piperidine, cyclohexyl and phenyl rings of PCP to generate partially oxidized species or from the addition of one or more oxygen atoms to generate hydroxylated PCP metabolites. Our data show that the piperidine ring of PCP is the major site for P450-mediated metabolism, and that the oxidation of this moiety leads to the generation of reactive metabolites that may be involved in modification of proteins including the P450. Several piperidine-derived metabolites have previously been identified in studies of PCP biotransformation, indicating that this ring is an important site of metabolism by P450s. Also a limited number of metabolites have been shown to result from the metabolism of the cyclohexyl ring, while the metabolism of the phenyl

ring appears to be much less likely as indicated by the absence of any metabolites in which the phenyl ring has been modified (Holsztynska and Domino, 1985; Sayre et al., 1997). Thus, we believe that the new metabolites and adducts seen in this study are due to metabolic activation of the piperidine moiety by P450 2B isoforms leading to the formation of the reactive intermediates as proposed in Scheme 1.

This study provides information on the structures of the chemically reactive intermediates of PCP that may form covalent adducts with amino acid residues in the active site of P450 during metabolism, or with other proteins in the vicinity of the enzyme. Our data also provide evidence for the identities of some proposed reactive intermediates of PCP and shed light on the mechanisms by which P450 2B enzymes activate PCP leading to the formation of reactive intermediates.

Acknowledgements

We are grateful to Dr. James Halpert for his kind gift of the P450 2B6 cDNA and to Hsia-lien Lin for expression and purification of reductase. The authors also thank Dr. Kathleen Noon for helpful discussions about the mass spectral data.

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Footnotes

This work was supported in part by NIH grant CA-16954.

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Figure Legends

Fig. 1. *Structures of PCP and its metabolites.*

Fig. 2. *LC-ESI MS chromatograms of PCP metabolites*. The sample was incubated in a reconstituted mixture containing P450 2B4 and 0.5 mM PCP for 40 min. Incubation mixtures of P450 2B1 with PCP gave similar results. (A) TIC of a control sample showing the elution of PCP at approximately R_t 21.5 min with an *m/z* of 244, (B) TIC of a sample incubated with NADPH showing PCP eluting at R_t 21.5 min and other peaks corresponding to PCP metabolites and (C) XIC of the 2,3-dihydropyridinium metabolite of PCP with a R_t at approximately 18.5 min and an *m/z* of 240. The inset represents the diode-array UV spectra of the 2,3-dihydropyridinium species (solid line) with a λ_{max} at 234 nm and PCP (broken lines) with a λ_{max} at 262.

Fig. 3. *LC-ESI MS/MS spectrum of the 2,3-dihydropyridinium of PCP eluting at R_t 18.5 min with an m/z of 240*. This sample was obtained from incubation of the reconstituted system containing P450 2B4 with 0.5 mM PCP and 0.9 mM NADPH. The metabolites were extracted and analyzed by LC-ESI MS/MS as described in *Materials and Methods*. The *m/z* of 240 could also correspond to the conjugate base 1,2-dihydropyridine of PCP as shown.

Fig. 4. *LC-ESI MS chromatogram of PCP metabolites and GSH adducts*. The sample was incubated in a reconstituted incubation mixture containing P450 2B4, 0.5 mM PCP, and 10 mM GSH for 40 min. Incubation mixtures of P450 2B1 with PCP and GSH gave qualitatively similar results. (A) TIC of a sample incubated with NADPH showing PCP eluting at approximately 21.5

min and other peaks corresponding to hydroxylated metabolites of PCP (M1-M5) and GSH adducts (G1) indicated by the arrow, and (B) XIC of GSH adducts eluting at approximately 14.6 and 14.8 min with an *m/z* of 547.

Fig. 5. *LC-ESI MS/MS spectrum of the resolved G1 adduct of PCP eluting at 14.6 min with an m/z of 547.* This sample was obtained from incubation of the reconstituted system containing P450 2B4 with 0.5 mM PCP and 10 mM GSH. The metabolites and GSH adduct(s) were extracted and analyzed by LC- ESI MS/MS as described in *Materials and Methods.* Background subtraction was done to reveal low abundance ions. The calculated average mass of this adduct is 547.6 Da. The exact site of adduct formation by the thiol group of GSH on one of the carbons of the piperidine ring is hypothetical and could not be determined from these data.

Fig. 6. *LC-ESI MS chromatogram of a GSH adduct of PCP formed during metabolism by P450 2B6.* A reconstituted mixture containing P450 2B6 was incubated with 0.5 mM PCP in the presence of 10 mM GSH and the metabolites separated as described in *Materials and Methods*. (A) TIC showing PCP eluting at 21.5 min and other peaks corresponding to hydroxylated metabolites M1 through M4, and (B) XIC of the GSH adducts (G2) eluting at approximately 15.6 and 15.8 min and exhibiting *m/z* values of 581.

Fig. 7. *LC-ESI MS/MS spectrum of the G2 adduct of PCP eluting at approximately 15.6 min with an m/z of 581*. The MS/MS conditions are described under *Materials and Methods.* This sample was obtained from an incubation of the reconstituted system containing P450 2B6 with PCP (0.5 mM) and 10 mM GSH. The calculated average mass for this adduct is 581 Da.

Fig. 8. *LC-ESI MS/MS spectrum of the NAC adduct of PCP eluting at 18.9 min with an m/z of 437*. This sample was obtained from a reaction mixture of P450 2B6 incubated in the presence of PCP and 4 mM *N*AC as described under *Materials and Methods*.

Scheme 1. *Proposed metabolic pathways for the metabolism of PCP and the formation of reactive intermediates trapped by reaction with GSH or NAC.*

Scheme 1

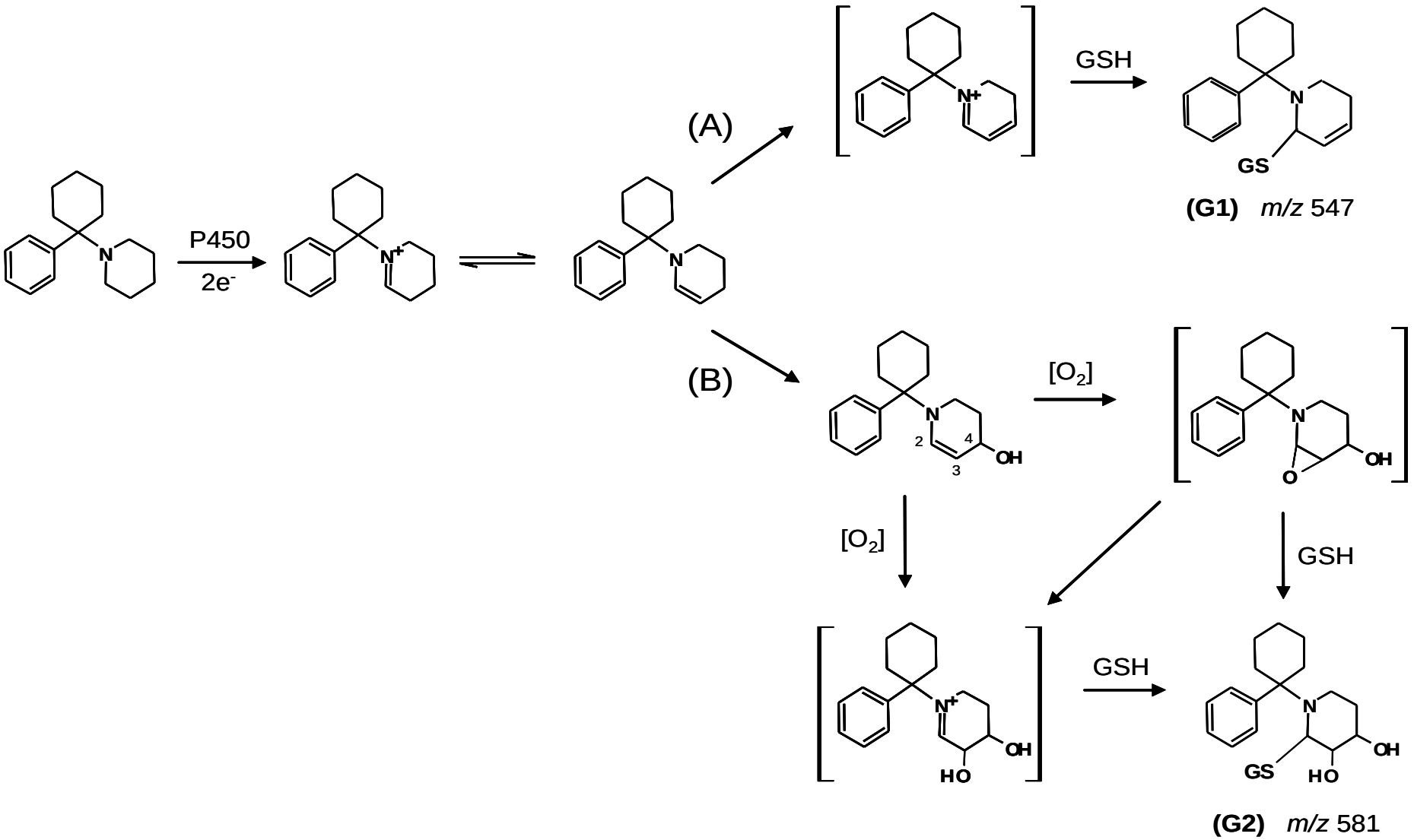


Figure 1

PCP

(M1/M2)

(M3/M4)

(M5)

(M6)

(M7)

Figure 2

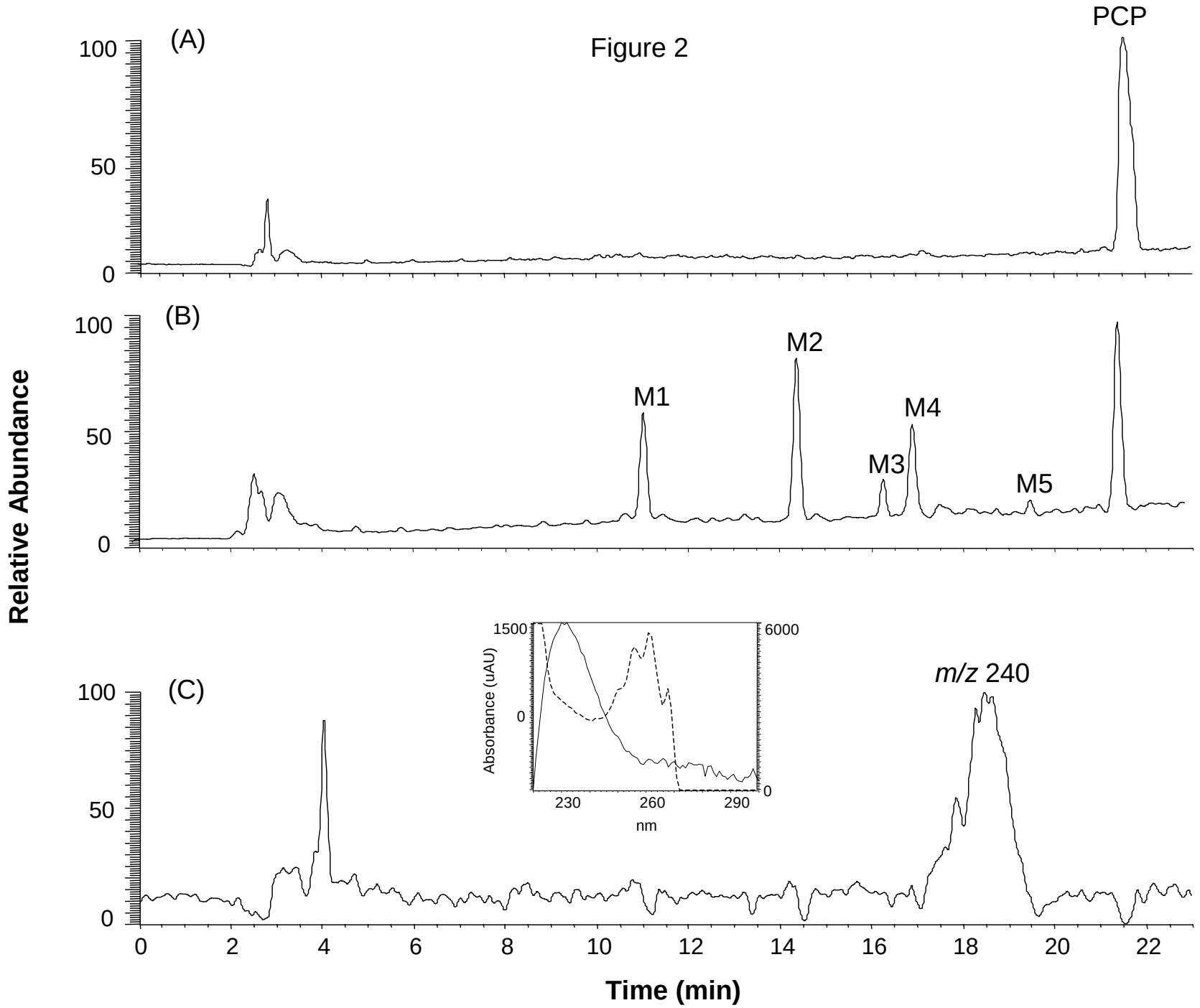


Figure 3

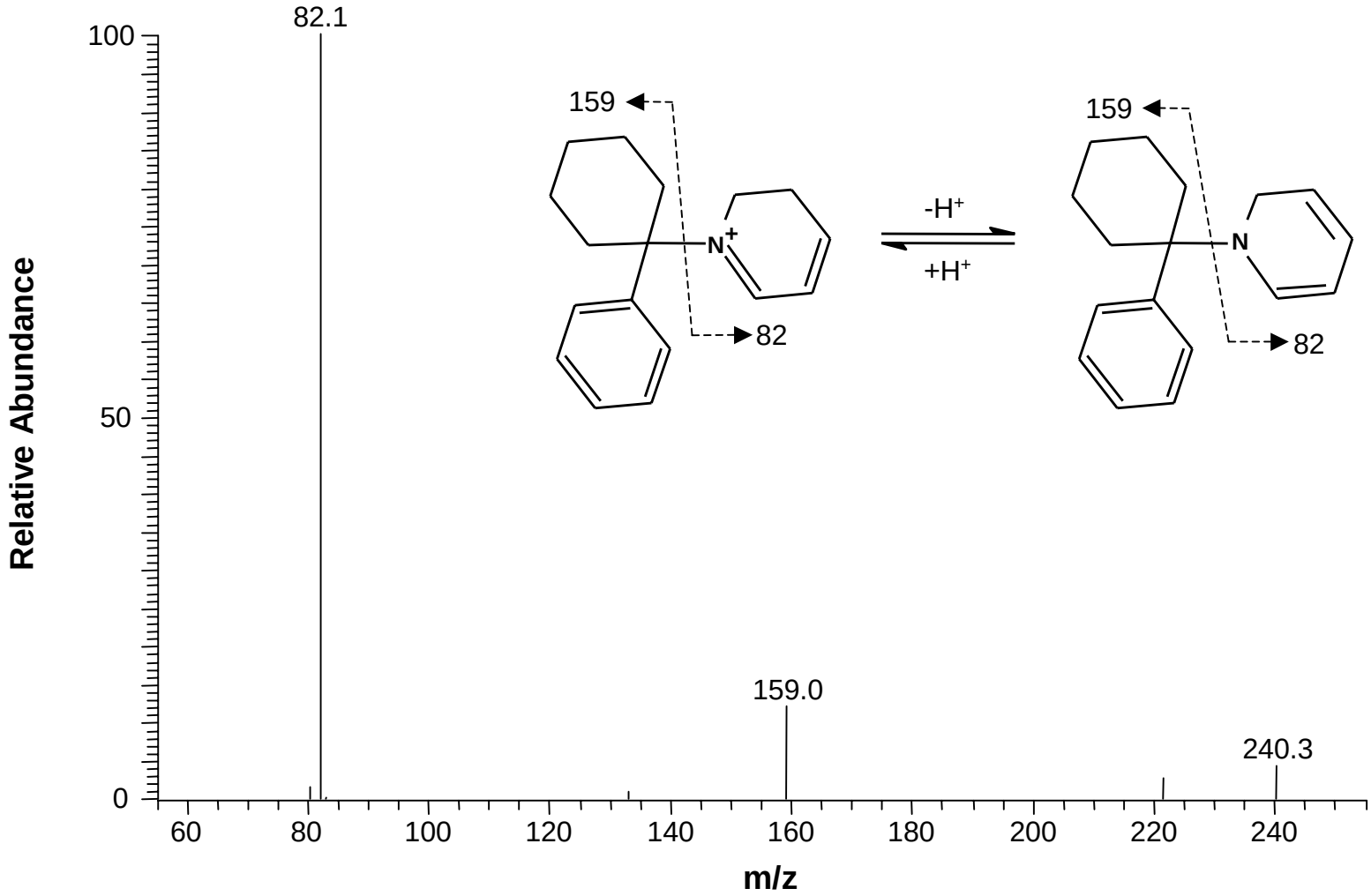
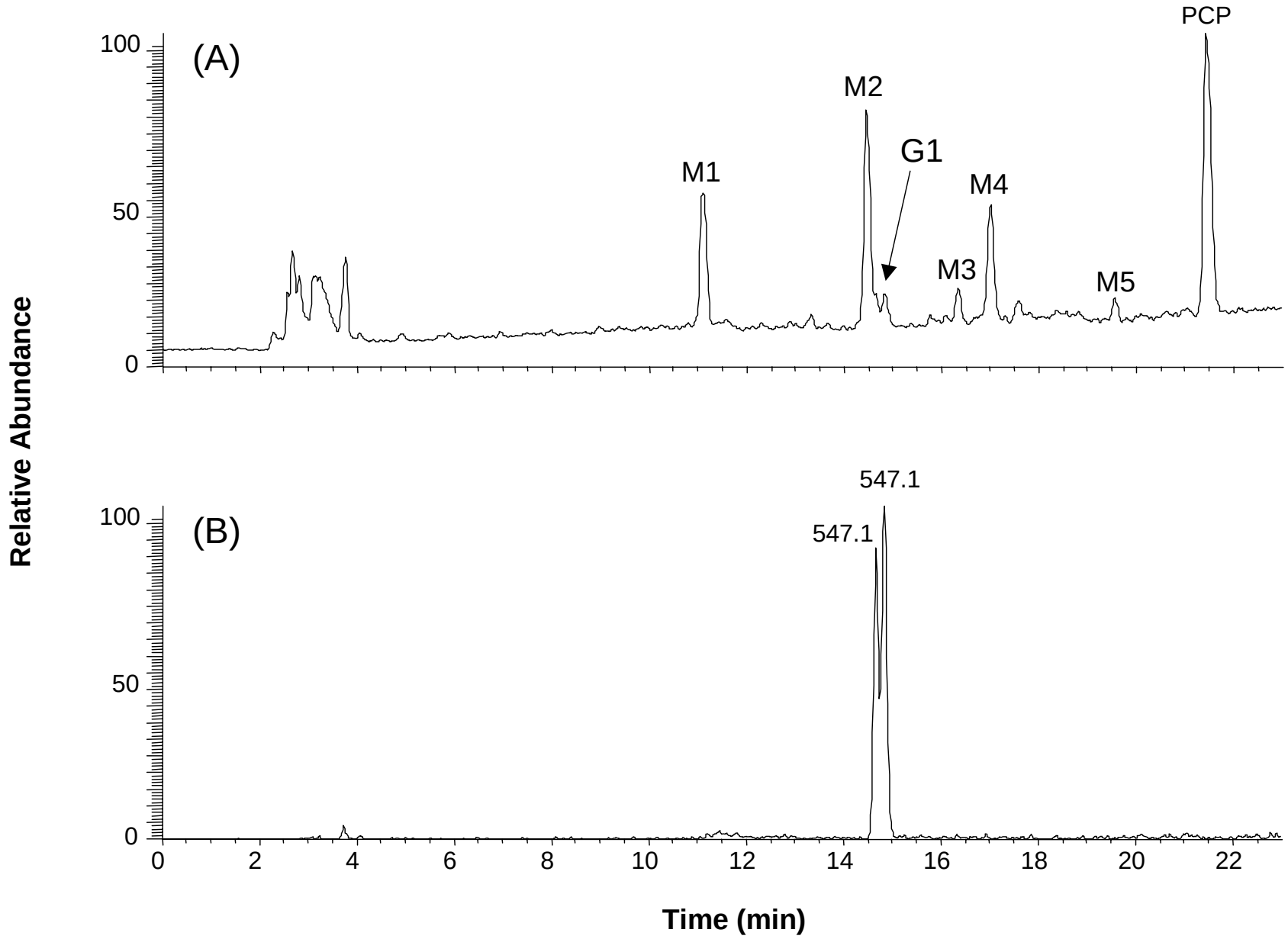


Figure 4



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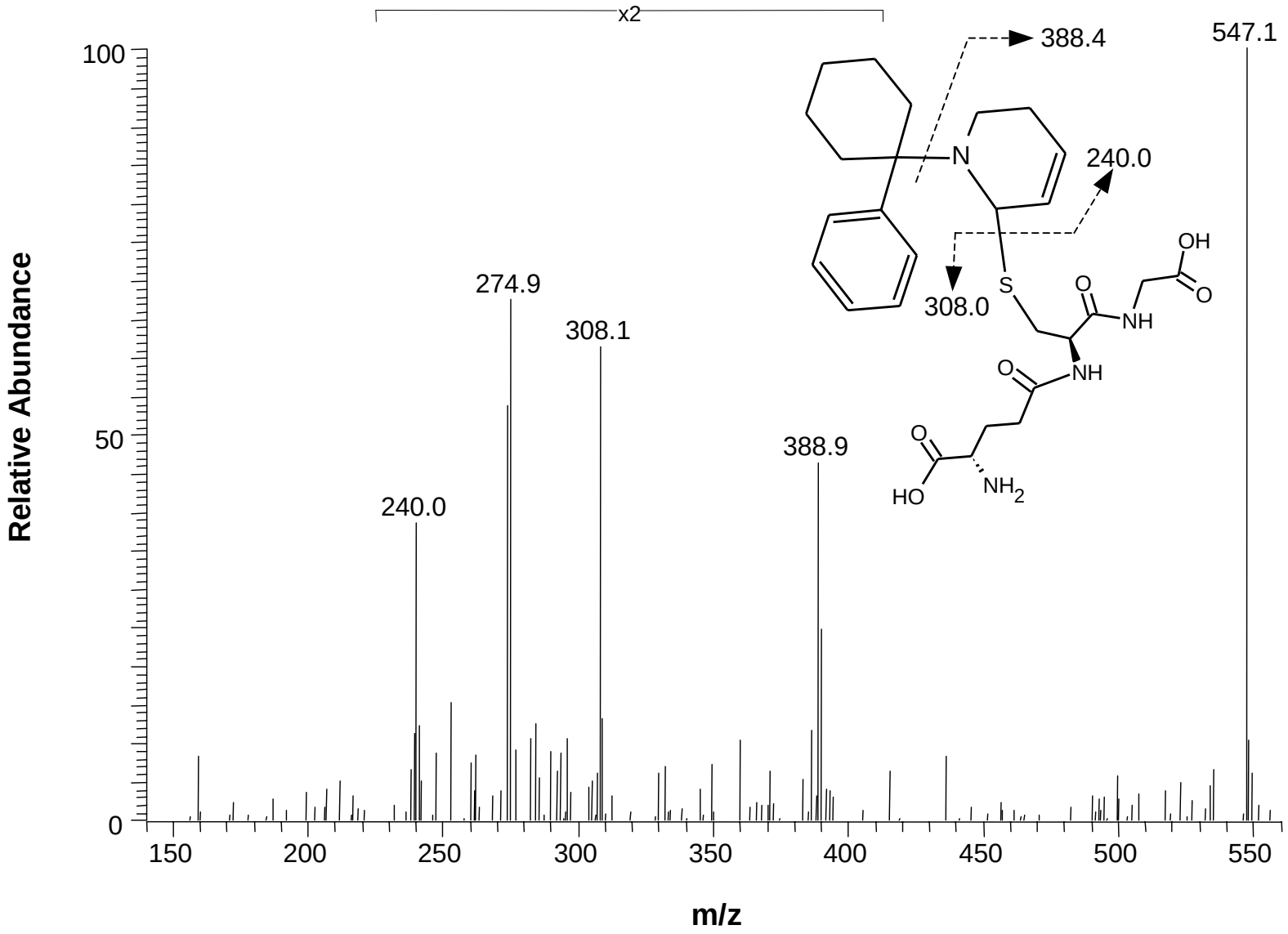

Figure 6

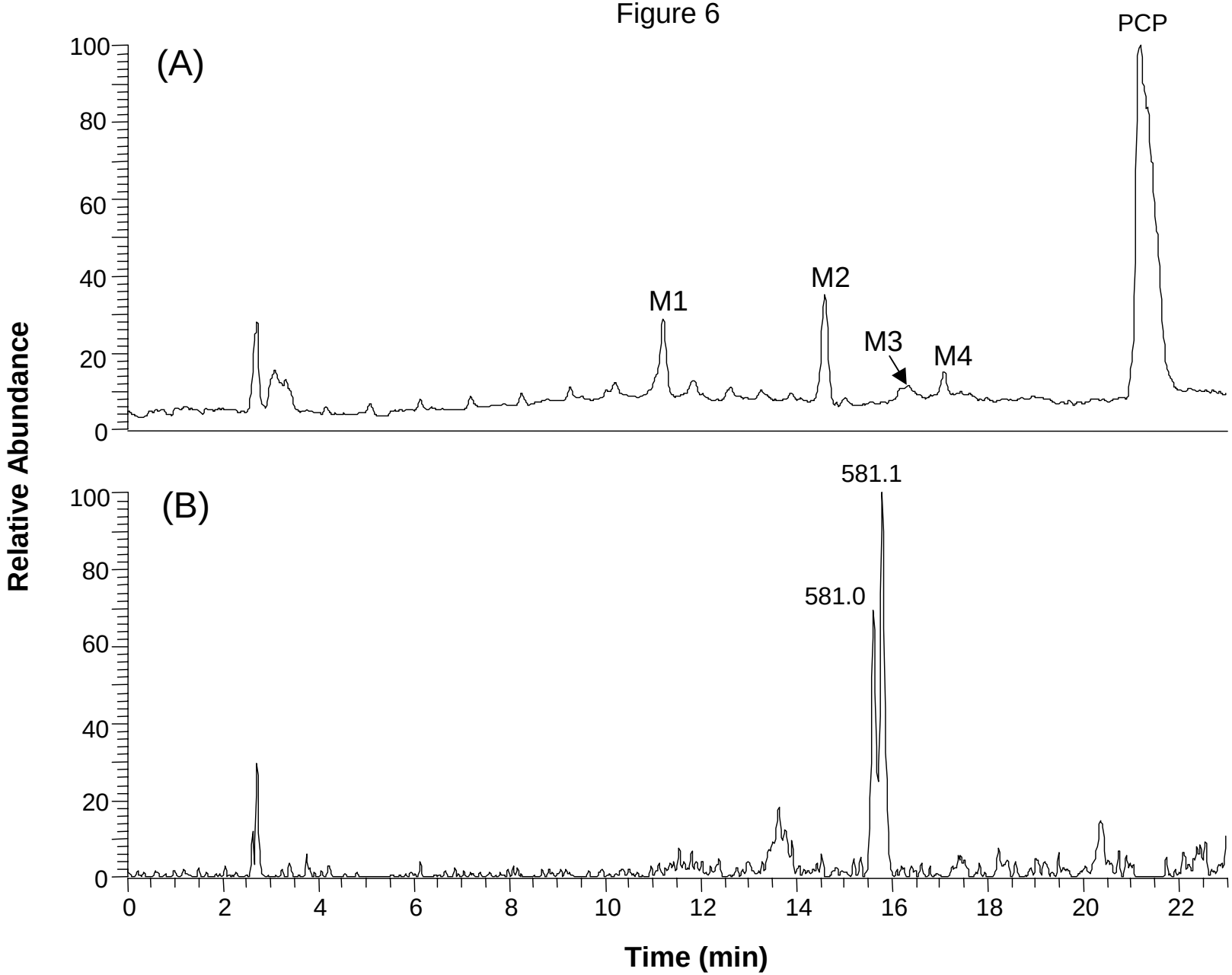

Figure 7

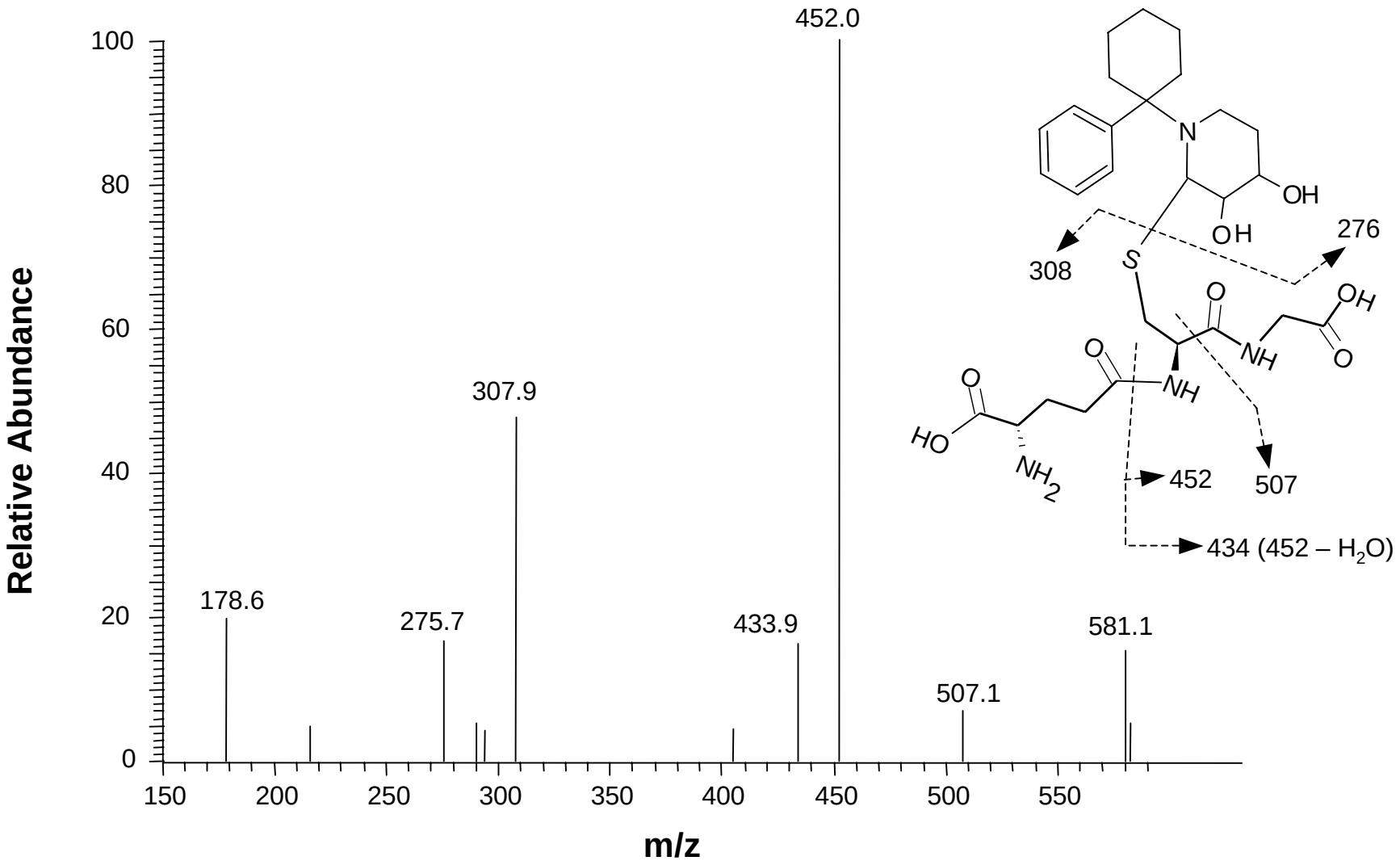

Figure 8