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Identification of 17- α-Ethynylestradiol Modified Active Site Peptides and Glutathione Conjugates Formed During Metabolism and Inactivation of P450s 2B1 and 2B6[1]

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Abstract

The oral contraceptive 17-α-ethynylestradiol (17EE1) is a mechanism-based inactivator of P450s 2B1 and 2B6. Inactivation of P450s 2B1 and 2B6 in the reconstituted system by [^{3}H]17EE resulted in labeling of the P450 apo-protein. Mass spectral analysis of 17EE-inactivated P450 2B1 showed an increase in the mass of the apoprotein by 313 Da, consistent with the mass of 17EE plus one oxygen atom. P450s 2B1 and 2B6 were inactivated with [^{3}H]17EE and digested with CNBr. Separation of the these peptides resulted in the identification of one major labeled peptide for each enzyme. N-terminal sequencing of these peptides yielded the amino acid sequences PYTDAVIHEI (for P450 2B1) and PYTEAV (for P450 2B6) that corresponded to amino acids P_{347}–M_{376} and P_{347}-M_{365} in P450s 2B1 and 2B6, respectively. ESI-LC-MS and MALDI-TOF-MS analysis of the P450 2B1-derived peptide resulted in a mass of 3654 Da consistent with the mass of the P_{347}-M_{376} peptide (3385 Da) plus a 268 Da 17EE-adduct. Chemically reactive intermediates of 17EE that were generated during the metabolism of 17EE by P450s 2B1 and 2B6 were trapped with gluthathione (GSH). ESI-LC-MS/MS analysis of 17EE-GSH conjugates from the incubation mixtures indicated that P450s 2B1 and 2B6 generated different reactive 17EE intermediates that were responsible for the inactivation and protein modification or the formation of GSH conjugates by these two enzymes.

The P450 enzymes belong to a family of microsomal cytochromes that are characterized by their unique heme absorbance spectrum at 450 nm in the reduced, carbon monoxide bound form (1). Mammalian P450s play a pivotal role in the detoxification, metabolism, and clearance of the majority of drugs as well as many carcinogens and pesticides (2). In many instances metabolism of xenobiotics by P450 enzymes results in the production of reactive intermediates. The formation of reactive intermediates that bind to cellular proteins or DNA has been implicated in the toxic or carcinogenic effects of certain environmental pollutants and drugs. Recently, it has also been suggested that protein adduction by reactive intermediates plays a significant role in the mechanisms of some neurotoxic events (3).

The membrane-bound nature of mammalian P450s has made the characterization of their active sites and the elucidation of their three-dimensional structures particularly difficult. Although several crystal structures of modified P450s have been solved (4–8) much of the current

[1]Abbreviations: P450, cytochrome P450; Reductase, NADPH-cytochrome P450 reductase; DLPC, dilauroyl-L-α-phosphatidylcholine; 17EE, 17-α-ethynylestradiol; BSA, bovine serum albumin; 7-EFC, 7-ethoxy-4-trifluoromethyl)coumarin; HFC, 7-hydroxy-4-(trifluoromethyl)coumarin; LC-MS, liquid chromatography-mass spectrometry; ESI, electrospray ionization; MALDI, matrix-assisted laser desorption ionization.

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information concerning the possible functional role of critical amino acid residues involved in substrate binding and catalysis has come only from homology modeling to crystal structures of P450s (9) and from studies with naturally occurring mutants of P450 enzymes or studies with mutants obtained through site-directed mutagenesis (10,11). An additional important chemical tool for the identification of critical active site amino acid residues involved in the binding and/or catalysis of substrates is the use of mechanism-based inactivators (12). Mechanism-based inactivators are substrate-like compounds that undergo catalytic conversion to highly reactive intermediates that covalently bind at the active site and render the enzyme inactive (13). Because of the hydrophobic nature of P450 enzymes and the inherent difficulties encountered upon digestion and manipulation of membrane bound proteins, only a few reports have been published where P450 peptides that were modified by reactive intermediates have been identified. Studies using acetylenic compounds such as 2-ethynylnaphthalene and 9-ethynylphenathrene or secobarbital with the P450 2B family have resulted in the identification of modified active site peptides (12(review),14,15).

Previously, 17EE was shown to be a mechanism-based inactivator for P450s 3A4, 2B1, and 2B6 (16–18). Interestingly, the activity of P450s 2B2 and 2B4, which share a greater than 70% sequence identity with P450 2B1, was only minimally affected by incubations with 17EE in the presence of NADPH (17). Heme destruction was the primary cause for the loss in enzymatic activity of P450 3A4 (18), whereas minimal loss or modification of the heme moiety occurred when P450s 2B1 and 2B6 were inactivated with 17EE. Therefore, a modification of the P450 apoprotein by a reactive 17EE intermediate was believed to be the reason for the loss in enzymatic activity of the 2B enzymes.

The current study demonstrates that a [^{3}H]17EE-derived reactive intermediate bound covalently to P450s 2B1 and 2B6 during the mechanism-based inactivation. LC-MS studies of P450 2B1 inactivated by 17EE revealed an increase in the mass of the P450 2B1 apoprotein by approximately 313 Da, consistent with the addition of 17EE plus one oxygen. Chemical digestion of the [^{3}H]17EE-labeled P450s led to the identification of [^{3}H]17EE modified peptides from P450s 2B1 and 2B6. The overlapping region of the peptides between P450s 2B1 and 2B6 was comprised of a peptide corresponding to P_{347}-M_{365}. Sequence comparisons between P450s 2B1, 2B6, 2B2, and 2B4 in this region suggested that S_{360} may be the most likely residue that was modified by a reactive 17EE intermediate. Sulfhydryl containing compounds such as glutathione (GSH) have been employed to trap reactive electrophiles for subsequent structural analysis of the S-substituted adducts using mass spectrometry (19–21). In this study, GSH was used to trap reactive 17EE intermediates formed by P450s 2B1 and 2B6. The collective MS data suggested that rat and human P450 2B enzymes differ in their metabolism of 17EE and that they release distinct reactive intermediates from their respective active sites.

Experimental Procedures

Materials

Dilauroyl-L-α-phosphatidylcholine (DLPC), NADPH, catalase, and 17-α-ethynylestradiol were purchased form Sigma Chemical Co. (St. Louis, MO). 7-Ethoxy-4-(trifluoromethyl) coumarin (7-EFC) was from Molecular Probes Inc., (Eugene, OR) and 7-hydroxy-4-(trifluoromethyl)coumarin (HFC) was purchased from Enzyme Systems Products (Livermore, CA). Ultima Gold liquid scintillation cocktail was obtained from Packard (Meridian, CT).

Purification of P450 and Reductase

P450 2B1 was purified from microsomes isolated from livers of fasted male Long Evans rats (175–190 g, Harlan, Indianapolis, IN) given 0.1% phenobarbital in the drinking water for 12

days according to the procedure of Saito and Strobel (22). Rat reductase was purified after expression in *Escherichia coli* as previously described (23). P450 2B6 was expressed in *E. coli* MV1304 cells and purified as described by Hanna et al. (24). cDNA of N-terminally truncated P450 2B6 was a generous gift from Dr. James Halpert (University of Texas Medical Center) and was expressed and purified as previously described (24,25). P450s 2B2 and 2B1 were also expressed in *E. coli* MV1304 cells and purified as previously described (25). P450 2B4 was purified from livers of phenobarbital-induced rabbits as described by Coon et al. (26).

Enzyme Activity Assays and Inactivation

Purified P450 2B1 and reductase were reconstituted with lipid for 45 min at 4 C. Incubation mixtures contained 0.5 M P450 2B1 or 0.67 M P450 2B6, 1 M reductase, 200 g DLPC/mL, 110 units catalase/mL, 17EE or DMSO in 50 mM potassium phosphate buffer (pH 7.4). After equilibrating the reaction mixture at 30 °C for 3 min, the reactions were initiated by adding NADPH to a final concentration of 1.2 mM (primary reaction mixture). At the indicated times, duplicate 10 μL samples (5 pmol P450 2B1 in the reconstituted system) of the primary reaction mixture were removed and mixed with 990 μL of a secondary reaction containing 0.2 mM NADPH, 100 μM 7-EFC and 40 μg BSA/mL in 50 mM potassium phosphate buffer (pH 7.4), and incubated at 30 °C for 5 min. For the P450 2B6 assays, duplicate 12 μL samples (8 pmol P450 2B6 in the reconstituted system) of the primary reaction mixture were mixed with 988 μL of the secondary reaction mixture and incubated for 10 min at 30 °C. Enzyme activity was stopped by adding ice-cold acetonitrile to a final concentration of 25%. The 7-EFC-*O*-deethylation activity was measured spectrofluorometrically as described by Buters et al. (27). Fluorescence of the samples was measured directly at room temperature on a RF5301 spectrofluorometer (Shimadzu Scientific Instruments, Inc., Columbia, MD) with excitation at 410 nm and emission at 510 nm.

ESI-LC-MS Analysis of P450 2B1 and P450 2B6

LC-MS analysis of P450 2B1 was performed with a HP 1100 auto sampler and dual pump system (Agilent Technologies, Schaumburg, IL) in conjunction with an ion trap mass spectrometer (LCQ, Thermo Electron Corporation, San Jose, CA) essentially as previously described (28). The LCQ was optimized for P450 analysis using a 50 μg/mL solution of horse heart myoglobin. The sheath gas was set at 80 (arbitrary units), the auxiliary gas was set at 15 (arbitrary units), the spray voltage was 5 kV, and the capillary temperature was 200 °C. P450 2B1 was incubated with 17EE, with and without 1 mM NADPH, for 10 min at 30 °C. Samples contained 1 μM P450 2B1, 1 μM reductase, 80 μg DLPC/mL, 5 μg catalase/mL, and 40 μM 17EE in 50 mM potassium phosphate buffer (pH 7.4). The samples were injected onto a 2.1 mm ×150 mm C8 column (5 μm Grace Vydac Advances, Hesperia, CA; 50 pmol P450 2B1 on-column) equilibrated with 40 % B (B: CH_3CN, 0.1% TFA; A: 0.1% TFA in H_2O). The column was washed for 10 min at 0.3 mL/min at initial conditions. The LC eluent was then directed into the LCQ mass spectrometer (Thermo Electron Corporation, San Jose, CA) and the reconstitution mixture components were separated by applying a linear gradient to 90% B over the course of 15 min. The column was washed for an additional 30–40 min at 90% B to remove the remaining lipid. The protein mass spectra were de-convoluted using the Thermoquest Bioexplore 1.0 software package to give the masses associated with each protein envelope. The retention times for the components of the reconstituted system were: 4 min for clipped reductase, 10 min for reductase, 16 min for P450 2B1, and 18 min for DLPC (under these conditions heme eluted from the column while the LC flow was diverted away from the mass spectrometer).

HPLC Separation of P450s

P450 2B1 or P450 2B6 were reconstituted with reductase and lipid for 45 min at 4 °C. The reconstituted sample contained 1 μM P450, 1 μM reductase, 50 μg DLPC, 50 μg catalase, 1 mM GSH, 40 μM 17EE (supplemented with trace amounts of with [^{3}H]17EE), and 1.2 mM NADPH in a total volume of 2–4 mL of 50 mM potassium phosphate buffer (pH 7.4). Control samples received water instead of NADPH. The samples were inactivated for 30 min at 30 ° C. Aliquots were removed at 0 and 30 min to evaluate the loss in 7EFC activity as described above. The remaining sample (2–4 nmol P450) was concentrated to 100 μL in a Centricon-50 micro-concentrator and washed twice with 500 μL 50 mM potassium phosphate buffer (pH 7.4). The sample was injected onto a Poros R1 column (3.9 mm × 15 mm, PerSeptive Biosystems Inc., Framingham, MA) equilibrated with 30% B (B: 95% CH_3CN, 0.1% TFA; A: 0.1% TFA in H_2O) at 3 mL/min. The column was washed for 2 min at initial conditions. The concentration of B was raised to 65% over the next 15 min, followed by a linear increase to 100% B over 3 min. The column was washed with 100% B for 2 min and then brought back to initial conditions. The column eluent was monitored at 220, 280, and 405 nm and fractions were collected every 0.5 min.

Digestion of P450s and Separation of Peptides

The P450-containing fractions were pooled and concentrated on a speed-vac to approximately 300 μL. TFA was added to a final concentration of 70%. Samples received 30 – 40 μL of a 40 mg/100 μL CNBr stock dissolved in 70% TFA. The solutions were purged with N_2, sealed and incubated at room temperature in the dark for 20 h. The majority of the TFA was removed from the digest under a gentle stream of N_2 in a fume hood. The remaining sample was dried in a speed-vac. The resulting peptides were dissolved in 100 μL of 70% TFA. The peptides obtained after digesting with CNBr were resolved on a C4 column (protein and peptide, Grace Vydac, Hesperia, CA) equilibrated with 10% B (B: 95% CH_3CN, 0.08% TFA; A: 0.1% TFA in H_2O) at 1 mL/min. The concentration of B was increased to 80% over 45 min and then to 100% in 5 min. The column was washed with 100% B for 5 min before re-equilibration with 10% B. The column eluent was monitored at 220 and 280 nm and one mL fractions were collected. For ESI-MS or MALDI-MS analysis, the P450 2B1 samples were reconstituted and inactivated with 17EE as described above. A fraction of the sample (20%) also contained trace amounts of [^{3}H]17EE. HPLC separation, digestion with CNBr, and HPLC separation of the resulting peptides was carried out separately for the radio-labeled and the non-labeled samples as described above. The peptide fraction from the non-radioactive digest that corresponded to the major radioactive peptide fraction in the radiolabeled digest was collected and analyzed by ESI-MS and MALDI-MS at the University of Michigan Protein Core Facility. The radiolabeled peptide fraction was subjected to *N*-terminal amino acid sequencing.

ESI-LC-MS Analysis of GSH-conjugates

P450s 2B1 or 2B6 (1 μM) were reconstituted with reductase and incubated with 17EE as described above for the enzyme activity assays except that no lipid was added to the P450 2B6 incubation mixtures. The primary reaction mixture contained catalase (110 units), 10 mM GSH, 1 mM ascorbate, 10 μM 17EE and 100 mM potassium phosphate buffer (pH 7.5) in a total volume of 1 mL. Each of the reconstitution mixtures was divided into two samples and incubated at 30 ºC for 5 min. One sample received 1 mM NADPH and the control sample received an equivalent volume of water. The mixtures were incubated for 30 min at 30 ºC (60 min for P450 2B6). GSH-conjugates were isolated after adding 60 μL 10% TFA to each 1 mL of sample and then applying the sample to a 1 mL Accubond solid-phase extraction ODS C-18 cartridge previously washed with 1 mL of methanol followed by 2 mL of water. After the samples were loaded, the cartridges were washed sequentially with 2 mL of water, 2 mL of methanol, and 0.3 mL of acetonitrile. The organic phases were pooled and dried under N_2 gas.

The dried samples were re-suspended in 100 μL of 50% CH_3CN, 0.1% acetic acid. Samples (50 μL) were analyzed on a C18 reverse phase column (Luna, 3 micron, 100 × 4.6 mm, Phenomenex, Torrance, CA) using a gradient of 20 – 30% B (A: 0.1% acetic acid in water, B: 0.1% acetic acid in CH_3CN) in 5 min followed by a linear increase to 40% B by 15 min and to 90% B by 30 min at a flow of 0.3 mL/min. The column effluent was directed into a LCQ mass analyzer. The ESI conditions were: Sheath gas 90 arbitrary units; auxiliary gas 30 arbitrary units; capillary temperature 170 ºC; capillary voltage 4.5 kV; spray voltage 30 V, and 25% collision energy. Data was acquired in positive mode using the Excalibur software package (Thermo Electron Corporation, San Jose, CA) via full scan (*m/z* 150 – 1000) and data-dependent scanning (*m/z* 50–1000 of the first and second most intense ions).

Results

ESI-LC-MS Analysis of P450 2B1

P450 2B1 was incubated with 17EE either in the presence or absence of NADPH and analyzed by LC-MS. Figure 1 shows the de-convoluted spectrum of P450 2B1 incubated with 17EE and NADPH. The two peaks correspond to the non-adducted apo-P450 with a mass of 55895 ± 2 Da and the modified apo-P450 with a mass of 56208 ± 5 Da. The change in mass of approximately 313 Da is consistent with a modification of the apoprotein by one molecule of 17EE plus one oxygen atom. The inset to Figure 1 depicts the de-convoluted spectrum of a control sample of P450 2B1 incubated with 17EE in the absence of NADPH. The mass obtained for the unmodified P450 2B1 was 55894 ± 2 Da which was within 0.01% of the theoretical mass of 55899 Da calculated from the protein database. 17EE-adducted P450 2B6 was considerably more difficult to analyze than P450 2B1. Even though the unmodified enzyme could be readily observed and the correct theoretical mass could be obtained from the de-convoluted spectrum, attempts to reproducibly identify an adducted apo-P450 2B6 product with a mass increase consistent with a 17EE modification analogous to that of P450 2B1 were not successful (data not shown).

HPLC Separation of P450s

P450 2B1 and 2B6 in the reconstituted system were incubated with [^{3}H]17EE with and without NADPH at 30 °C. These conditions resulted in a loss of approximately 84–90% and 76–80% of the 7-EFC-*O*-deethylation activity of P450 2B1 and 2B6, respectively. Separation of the components in the reconstituted system by reverse-phase chromatography and liquid scintillation counting of a portion of each 1 mL fraction showed that the majority of the radioactivity eluted at the solvent front when samples were incubated without NADPH (P450 2B1, data not shown and P450 2B6, Fig. 2A). Virtually no labeling of any of the protein components in the reconstituted system was observed in the absence of NADPH. The HPLC profile of a P450 2B6 sample incubated with [^{3}H]17EE in the presence of NADPH is shown in Figure 2 B. Radioactivity can again be seen eluting near the solvent front and presumably represents 17EE and metabolites of 17EE. When metabolism of 17EE was allowed to proceed, the majority of the protein-associated counts were observed in the P450 2B1-containing fractions between 12.5 min and 14 min. This observation was consistent with the above protein mass spectrometry results demonstrating the covalent attachment of a reactive 17EE intermediate to the apoprotein. Similarly, P450 2B6 incubated with [^{3}H]17EE and NADPH co-eluted between 13 and 14 min with the radiolabel bound to the apo-P450 protein (Fig. 2B). This result indicated that a reactive 17EE intermediate was also covalently bound to the P450 2B6 apoprotein. These data further demonstrated that the adducted protein could be eluted off the HPLC column successfully but suggested that the inconsistent ESI-LC-MS data obtained for the adducted enzyme were due to an inability of the modified human enzyme to properly vaporize or ionize in the mass spectrometer. Less than 10% of the injected radioactivity was associated with the catalase eluting at 8.5 min or reductase eluting between 9–10 min.

Digestion of P450s and Separation of Peptides

The pooled, radiolabeled P450 2B1 or 2B6 fractions were digested using CNBr. The resulting peptides were separated on a C4 reverse-phase column and analyzed for radioactivity. Representative HPLC profiles obtained with 17EE-inactivated and CNBr-digested P450 2B1 and P450 2B6 are shown in Figures 3 A and B, respectively. The majority of the counts from the P450 2B1 digests co-eluted with a well-separated peptide at 23 min (Fig. 3A). A second peptide eluting at 26 min also contained approximately 5% of the radioactivity followed by the undigested apoprotein eluting at approximately 36 min. The fractions containing the labeled peptides were collected and applied to a Prosorb membrane for *N*-terminal Edman sequencing. The sequencing results (shown in Table 1 in the Supplemental Material) indicate that the primary sequence corresponds to that of the P450 2B1 CNBr peptide P_{347}-M_{376}. The same sequence was obtained from three different P450 2B1 samples that were digested with CNBr. MS analysis was used to confirm the identity of this peptide. The 17EE-modified peptide was subjected to ESI–MS and MALDI-MS. Table 1 shows that the mass of the 17EE-labeled peptide was 3654.6 Da as determined by both ESI and MALDI MS. The only P450 2B1 CNBr-derived peptide that could be obtained with a similar theoretical mass was that corresponding to the sequence P_{347}-M_{376}. The theoretical mass of this unmodified peptide containing the expected homoserine lactone C-terminus would be 3385.8 Da. To confirm that the peptide with a mass of 3654.6 Da arose from a modification of the peptide with a mass of 3385.8 Da, the remainder of the sample was subjected to treatment with base (pH 9 with NH_4OH with incubation at room temperature for 2 h). Under these conditions, the homoserine lactone would be converted to the homoserine resulting in a peptide with a mass of 3670.4 Da. In addition, base treatment also resulted in the appearance of a second peptide that was 268.7 Da smaller with a mass of 3401.7 Da. This mass would be consistent with the theoretical mass of the P_{347}-M_{376} peptide that contained a homoserine at the C-terminus after the loss of an ester-linked adduct. The difference in the observed masses and the theoretical masses under both conditions was approximately 268 Da. This mass is approximately 44 Da lower than the mass increase observed by LC-MS of the intact protein after inactivation with 17EE. The strongly acidic conditions of the CNBr digestion procedure may have resulted in a chemical conversion of the adduct to one with a mass 44 Da lower than expected. This could occur from the loss of CO_2.

The elution profile and the corresponding radioactivity trace for P450 2B6 digested with CNBr are shown in Figure 3B. The majority of the radiolabel was observed in the flow through. The majority of the peptide-associated radioactivity eluted between 24–25 min. When these fractions were subjected to *N*-terminal Edman sequencing a sequence corresponding to P_{347}-M_{365} was also obtained (Table 1, Supplemental Material). MALDI-MS analysis of the peptide derived from the CNBR digest of 17EE-labeled P450 2B6 showed the presence of the unmodified peptide P_{347}-M_{365} with an expected mass of 2238 Da (data not shown). This peptide is smaller than the P450 2B1 peptide because it contains a methionine residue at position 365. This result facilitated the assignment of the 17EE-modified peptide region to the 19 amino acids between residues 347–365.

Sequence comparison of residues P_{347} – M_{376} in P450 2B1 with P450s 2B6, 2B4, and 2B2

We have previously reported that P450 2B2 was not inactivated and P450 2B4 was not significantly inactivated by 17EE (17). Therefore, we have compared the amino acid sequences for P450s 2B1, 2B6, 2B2 and 2B4 between residues 347 and 376 (Table 2). Only those residues in P450s 2B6, 2B2, and 2B4 that differ from the residues in P450 2B1 are shown. Interestingly, S_{360} is the only residue that is identical in both P450s 2B1 and 2B6 (the isozymes that were inactivated by 17EE) and could form an ester linkage with a reactive intermediate of 17EE. For P450s 2B2 and 2B4 (the isozymes that were not inactivated by 17EE) the amino acid in position 360 is a glycine or alanine residue, respectively. S_{360} is also near two important

residues, V_{363} and V_{367}, in the substrate recognition sequence 5 (29). V_{363} has previously been shown to play a role in the differential hydroxylation of adrostenedione and testosterone and in metabolite switching to the *N*-deethylation pathway during the metabolism of lidocaine (30,25).

ESI-LC-MS/MS analysis of glutathione conjugates obtained from incubating P450s 2B1 and 2B6 with 17EE in the presence of GSH

One of the major 17EE-SG conjugates having a molecular ion at *m/z* 620 was observed in P450 2B1 samples incubated with 17EE, NADPH, and GSH (Fig. 4A). This adduct eluted at 20 min (inset to Fig. 4A) and was not observed in control samples (data not shown). The mass of this conjugate was consistent with the addition of glutathione, one oxygen, and two hydrogens to the 17EE molecule (MH^+ + 323). MS/MS analysis of the *m/z* 620 ion revealed the following daughter ions: *m/z* 602 (loss of H_2O), *m/z* 545 (loss of glycyl moiety), *m/z* 527 (loss of H_2O and glycyl moieties), *m/z* 473 (loss of γ-glutamyl + NH_3), *m/z* 455 (loss of γ-glutamyl, NH_3 and H_2O), *m/z* 308 (loss of 17EE + O) and *m/z* 253 (loss of glutathione + C_2OH_2 + H_2O) (Fig. 4B). The latter two ions became more prominent with increasing collision energy. A structure consistent with this fragmentation pattern is shown in Fig. 4C. A similar fragmentation result could also be obtained if the initial metabolism of 17EE involved epoxidation of the A ring followed by GSH adduction. In addition, a minor peak with a corresponding MH^+ of 618 was seen in all 2B1 samples. This ion eluted at 17.5 min and represented approximately 10% of the amount of the *m/z* 620 ion (assuming similar ionization; data not shown).

P450 2B6 samples that were incubated in the presence of 17EE, NADPH and GSH displayed two major 17EE-GS conjugates that eluted as a split peak between 16.8 – 17.3 min (Figure 5A). These conjugates were only seen in samples that had been incubated with NADPH (data not shown). The molecular ions that were associated with both peaks had a *m/z* 618 (inset to Fig. 5A). The mass of this GS conjugate was 2 mass units lower than that observed with P450 2B1. This difference is best explained by a hydroxylation and GSH addition to A ring of 17EE. MS/MS analysis resulted in the following daughter ions for both of these GSH conjugates: *m/z* 600 (loss of H_2O), *m/z* 543 (loss of glycyl moiety), *m/z* 489 (loss of γ-glutamyl moiety), *m/z* 472 (loss of γ-glutamyl + NH_3), and *m/z* 343 (loss of most of the GSH molecule with retention of the sulfur atom on the steroid), (Fig. 5B and 5C). Similar molecular ions could theoretically be obtained if GSH reacted with an ortho quinone on the A ring of 17EE, resulting from oxidation at the 2- or 4- position of the A ring to give a dihydrodiol. One of the possible structures, namely the 2,3-OH-1-SG conjugate is shown in Fig. 5D, although a 3,4-OH-1-SG, or a 2,3-OH-4-SG conjugate structure can not be determined without NMR analysis. Assignment of the ion with a *m/z* 343 was confirmed on a Q-Tof mass spectrometer (data not shown). In contrast to P450 2B1, where a small amount of the 618 ion was generated in addition to the major 620 ion, P450 2B6 produced primarily the 618 doublet as the major ions. When a more concentrated sample from a P450 2B6 reaction mixture was analyzed, two additional peaks with a *m/z* of 618 and 620 were also seen (data not shown).

Discussion

Hepatic as well as extra-hepatic P450s such as P450s 3A4, 2C9, 2C19, 1A1 and 1A2 have been found to metabolize 17EE primarily to 2-hydroxy 17EE and 4-hydroxy 17EE (16,31). The metabolism of 17EE by human P450 enzymes such as P450s 3A4 and 2B6 results in the production of reactive intermediates that are able to alkylate the P450 heme or modify the apo-protein (16–18). This observation not only raises a potential concern about drug interactions with other therapeutics that are also metabolized by these enzymes but the ensuing reactive 17EE intermediates may also lead to toxic or carcinogenic effects (3). A previous study demonstrated that in the presence of 17EE and NADPH, a loss of approximately 70% of the

enzymatic activity of P450s 2B1 and 2B6 occurred (17). Because the same samples did not show a comparable loss in the ability of the 2B P450s to bind CO, it was proposed that this loss in enzymatic activity was primarily due to the formation of an adduct with the apo-P450 by a reactive intermediate. This report now provides direct evidence for the formation of such apoprotein adducts.

When P450 2B1 was inactivated with 17EE in the presence of NADPH, the increase in the mass of the apoP450 was consistent with the addition of 17EE plus one oxygen atom. Similar analytical procedures using human P450 2B6 were unable to determine a definitive mass increase because the error in the mass assignment of the adducted protein was too high after de-convolution. In general, we and others have found that mass assignments using LC-MS analysis of adducted human P450s has been difficult and often exhibit unacceptably large errors. Perhaps the adducted enzyme has a greater tendency to aggregate and is poorly ionized. The results with the 17EE-adducted P450 2B6 are similar to our previous studies where a bergamottin-adducted P450 2B6 could be detected, but due to the significant error in the mass assignment it was not possible to accurately determine the number of oxygen atoms attached to the reactive intermediate (32). Others have also observed that inactivated apo-P450 3A4 was difficult to analyze by a variety of mass spectrometry techniques (33). Despite these difficulties with mass spectral approaches, confirmation of adduct formation with apoP450 was obtained using HPLC analysis of P450s 2B1 and 2B6 incubated with [^{3}H]17EE and NADPH. In both instances, the radioactivity was associated with the fraction(s) containing the P450.

This report further describes the *N*-terminal sequence identification of peptides derived from CNBr digests of the 17EE-modified, purified P450s. The peptides that were modified by a reactive 17EE intermediate corresponded to P_{347}-M_{376} in P450 2B1 and P_{347}-M_{365} in P450 2B6. While S_{360} seems to be a likely candidate for modification based on sequence alignments with P450s 2B2 and 2B4 which are not inactivated by 17EE and do not contain S_{360}, *N*-terminal sequencing did not identify an adduct to S_{360} in P450 2B1. If this adduct were base labile, as was seen after base treatment of the modified CNBr peptide derived from a P450 2B1 digest, it would not be expected to survive the initial chemical coupling steps required for Edman degradation. Although the major radiolabeled peptide from digested, inactivated P450 2B1 was well resolved on reverse-phase HPLC, the CNBr digest of inactivated P450 2B6 lacked the same extent of chromatographic separation. Therefore, we focused on the P450 2B1 fractions, using ESI-LC-MS and MALDI-MS to confirm the identity of the peptide collected in the radioactivity-containing fractions. In contrast to the 313 Da mass shift that was seen with the whole protein prior to digestion, the mass increase in the adducted peptide was approximately 268 Da. Presumably this difference in mass was due to the harsher conditions employed during the digestion with CNBr which involved exposing the 17EE-adducted protein to 70% trifluoroacetic acid for 20 h. The acidic conditions required during the CNBr digestion procedure generally result in peptides containing a C-terminal methionine lactone. Incubations with base will convert this lactone to the homoserine. Furthermore, basic incubations would also facilitate removal of an ester linked adduct, the latter being expected to result from a nucleophilic attack by S_{360}. A 16 Da increase in mass from 3654.4 to 3670.4 was observed when the 17EE-adducted P450 2B1 peptide was incubated under basic conditions and was presumably due to conversion to the homoserine. The additional new mass of 3401.7 Da that was also observed was similar to the theoretical mass of the peptide spanning P_{347}-Homoserine$_{376}$ following hydrolysis of the ester linked 17EE adduct (loss of 268.7).

We have previously reported that P450 3A4 was inactivated by a reactive metabolite of 17EE and that the inactivation was due to a combination of heme destruction and adduction of a reactive intermediate to the apoprotein. GC-MS analysis of the metabolites of 17EE generated by P450 3A4 indicated that a metabolite with a mass of 312 Da had been generated. This same metabolite had previously been observed with P450s 2B1 and 2B6, the 2B isofoms that were

inactivated by 17EE, but not in incubation mixtures from P450s 2B2 or 2B4. Glutathione has been used extensively to trap reactive intermediates, greatly facilitating the elucidation of the molecular structures of these intermediates (19–21). We have also employed this approach in an attempt to capture some of the reactive intermediates that were generated by P450s 2B1 and 2B6 during the metabolism of 17EE. Incubations with P450 2B1 led to the isolation of a GS-17EE conjugate with a *m/z* of 620 (MH^+ + 323). Tandem mass spectrometry resulted in a fragmentation pattern that could be accommodated by the structure shown in Fig. 4C. The GSH conjugate proposed in Fig. 4 is consistent with previous observations with glutathione conjugates where the fragment at *m/z* 308 is usually indicative of an adjacent methylene group. The work of Ortiz de Montellano (34,35) describes the metabolism of small acetylenic compounds by P450 enzymes and proposes that adduct formation to the protein occurs through the terminal ethynyl carbon (34,35). The assignment of the exact positions of oxidation and conjugation with GSH through the terminal or the internal ethynyl carbon could not be verified by the mass spectrometry techniques used here. Ring expansion of the D ring by a homoannulation mechanism has also been described previously (36,37). Studies by Sisenwine et al. suggested that D-homoannulation may be a major metabolic route in the metabolism of norgestrel in female rhesus monkeys as well (38,39). The metabolic scheme for such a ring expansion involves an initial epoxidation of the ethynyl bond at the internal carbon. The resultant intermediate then undergoes a rearrangement involving ring expansion to an aldehyde that could potentially be further oxidized to the corresponding carboxylic acid (36). A metabolite corresponding to this 17-formyl-D-homosteroid has been observed in incubation mixtures from P450 2B and 3A enzymes that were inactivated by 17EE (17,18). Alternatively such a reactive intermediate could also react with either the heme or the apoprotein.

The two major GSH conjugates that were observed from P450 2B6 incubation mixtures both had *m/z* values of 618, identical MS/MS patterns and, eluted very close to each other (16.9 and 17.5 min), appearing almost as doublets. Similar molecular ions and fragmentation patterns would be expected if the GSH conjugate arose from conjugation with an ortho quinone on the A ring (subsequent to hydroxylation and further oxidation). Metabolites consistent with 2- and 4-hydroxylation of the A ring of 17EE aromatic ring have been observed in humans (16,31, 40). Previous studies in microsomes from phenobarbital-treated rats resulted in the identification of 2- and 4-hydroxylation products on the A ring of 17EE. These studies also indicated some loss of the [^{3}H] label from the 2- and 4-H positions when the incubations were performed in the presence of GSH (41). These closely eluting GSH conjugated species may be the result of alternate isomeric conformations, or of two species adducted on different positions of the A ring. In addition to the two major 17EE-GS conjugates, a minor conjugate (comprising approximately 10% of the 618 ions) with the same *m/z* of 620 as that seen with P450 2B1 was observed. It is interesting to note that the rate of inactivation of P450 2B6 by 17EE is approximately 10-fold slower compared to P450 2B1. Therefore, P450 2B6 may primarily metabolize 17EE to the dihydrodiol and form a reactive intermediate that can be trapped by GSH to form the 618 ion. The reactive intermediate that results in the formation of the 618 ion may not be involved in the inactivation of the P450s and either loses water and re-aromatizes or becomes conjugated to GSH if the trapping reagent is present. Studies by Lin et al.[2] with 17EE and P450 3A5 indicated that this enzyme was inactivated by 17EE only in the presence of cytochrome b_5 but that the GS-17EE-conjugate with a *m/z* of 618 could be formed in the presence or absence of b_5. In contrast, oxidation at the ethynyl moiety results in a reactive 17EE intermediate that is responsible for the inactivation of both enzymes by binding to the apoprotein. This intermediate can also be trapped by GSH but leads to the 620 ion that was observed with both enzymes. P450 2B1 is inactivated more rapidly because it generates this intermediate more readily.

[2]Hsia-lien Lin and Paul F. Hollenberg (unpublished results).

So far, only one member of the P450 2B family has been crystallized. P450 2B4 has been crystallized in both an open and a substrate-bound or closed conformation (7,42). Two residues, I_{363} and V_{367} that are located in the K helix and are components of the SRS 5 region are found in close contact (less that 5 Å) with the 4-(4-chlorophenyl)imidazole inhibitor incorporated into the closed conformation of the crystal. A functional importance for these two residues has been proposed based on their roles in steroid metabolism (30). Sequence alignments for P450s 2B1, 2B6, 2B4, and 2B2 between P_{347} and the amino acid residue at position 376 show significant conservation across the four enzymes. The single nucleophilic residue that is identical for P450s 2B1 and 2B6 but different for P450s 2B4 and 2B2, is S_{360}. Residue G_{360} in P450 2B4 is located at the C-terminal end of the K helix and is thought to belong to SRS 5. It is conceivable that this residue, particularly when present as a serine, may have an important functional role in the metabolism of larger molecules such as steroids. Although 17EE was metabolized by P450 2B4, this enzyme (as well as P450 2B2) was not inactivated, presumably because the reactive 17EE intermediate responsible for inactivation could not be generated. This observation suggests that S_{360} in P450s 2B1 and 2B6 may not only be a target for a reactive 17EE intermediate but may also play a role in substrate orientation or activation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

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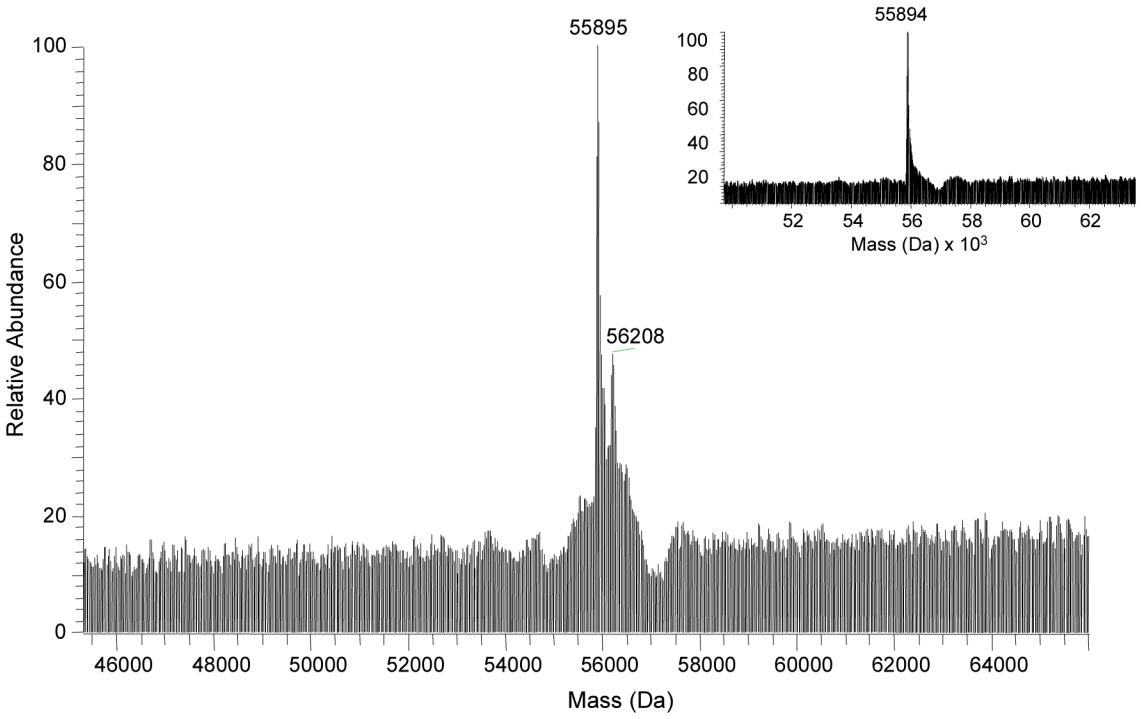

Figure 1.

ESI-LC-MS analysis of 17EE-inactivated P450 2B1. P450 2B1 in the reconstituted system was incubated with 17EE in the presence or absence of NADPH and subjected to LC-MS analysis as described in Experimental Procedures. The de-convoluted spectra of apo-P450 2B1 and 17EE-adducted apo-P450 2B1 (55,895 Da, and 56,208 Da, respectively) are shown. The inset shows the mass obtained for a control sample incubated with 17EE but without NADPH (55,894 Da). The data shown are representative examples from three separate analyses.

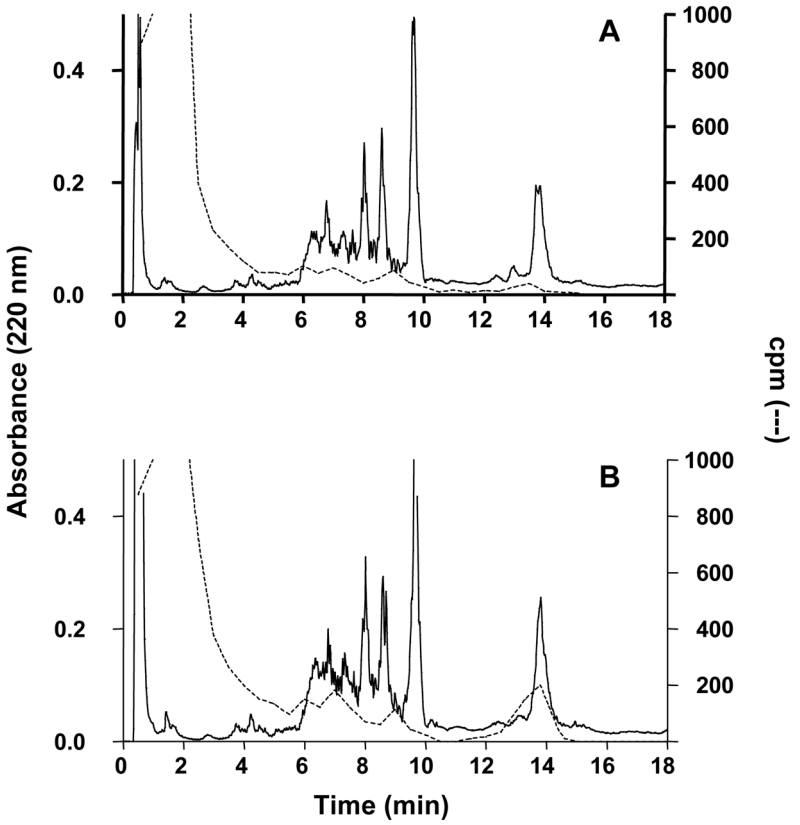

Figure 2.

HPLC separation of the components of the reconstituted system containing P450 2B6. P450 2B6 in the reconstituted system was incubated with [^{3}H]17EE in the presence or absence of NADPH as described in Experimental Procedures. A) – NADPH, B) + NADPH. The elution of catalase (8.5 min), reductase (9.5–10 min), and P450 2B6 (13–14 min) was monitored at 220 nm (—). Radioactivity was monitored by liquid scintillation counting of 10% of each 1 mL fraction (-----).

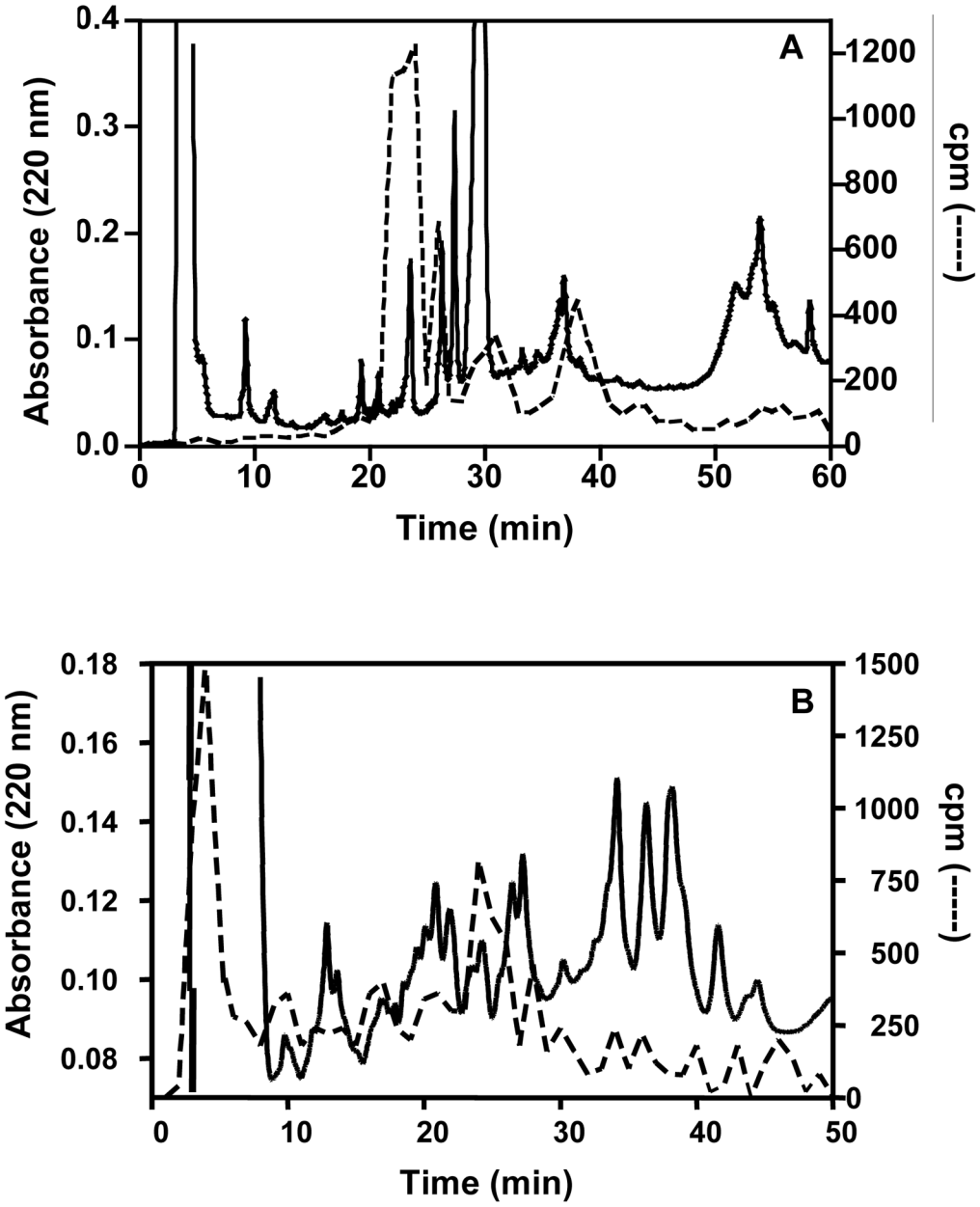

Figure 3.

HPLC reverse-phase separation of a peptide mixture generated by digesting 17EE-inactivated P450s 2B1 or 2B6 with CNBr. CNBr digestion conditions are described in the Experimental Procedures. A) HPLC-purified, [^{3}H]17EE-labeled P450 2B1 digested with CNBr, B) HPLC-purified, [^{3}H]17EE-labeled P450 2B6 digested with CNBr. The elution of the peptides was monitored at 220 nm (—) and by liquid scintillation counting of 10% of each 1 mL fraction (-----).

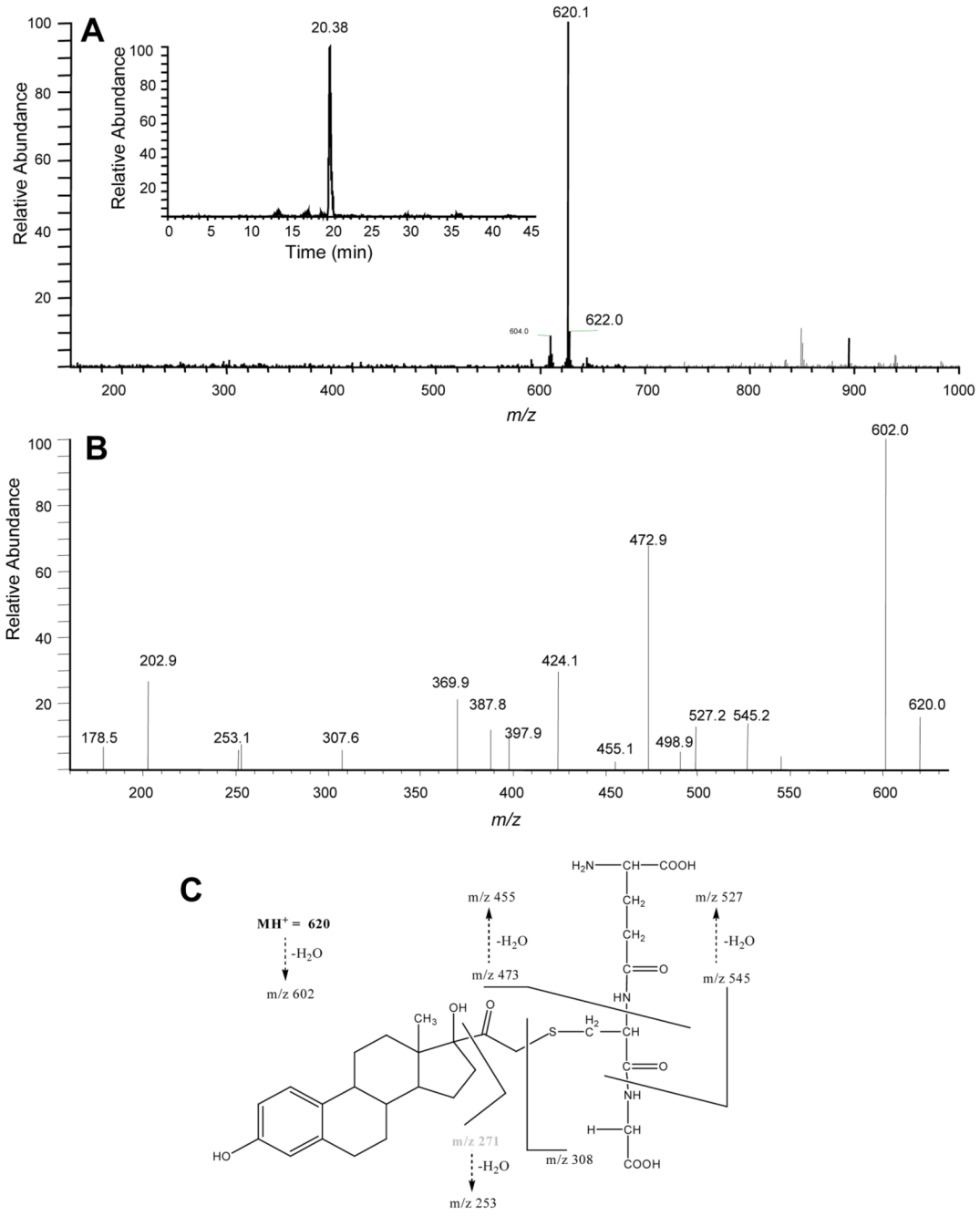

Figure 4.

Analysis of GSH conjugates derived from P450 2B1 samples incubated with 17EE in the presence of NADPH and GSH. P450 2B1 was reconstituted with reductase and incubated with 17EE and GSH as described in Experimental Procedures. (A) Mass spectrum of the molecular ion eluting at 20.4 min and extracted ion chromatogram of the molecular $[M+H]^+$ ion with a *m/z* of 620 (Inset), (B) MS/MS spectrum for *m/z* 620, (C) Theoretical structure of the GS-17EE conjugate based on MS/MS fragmentation. The data shown are representative of at least three separate analyses.

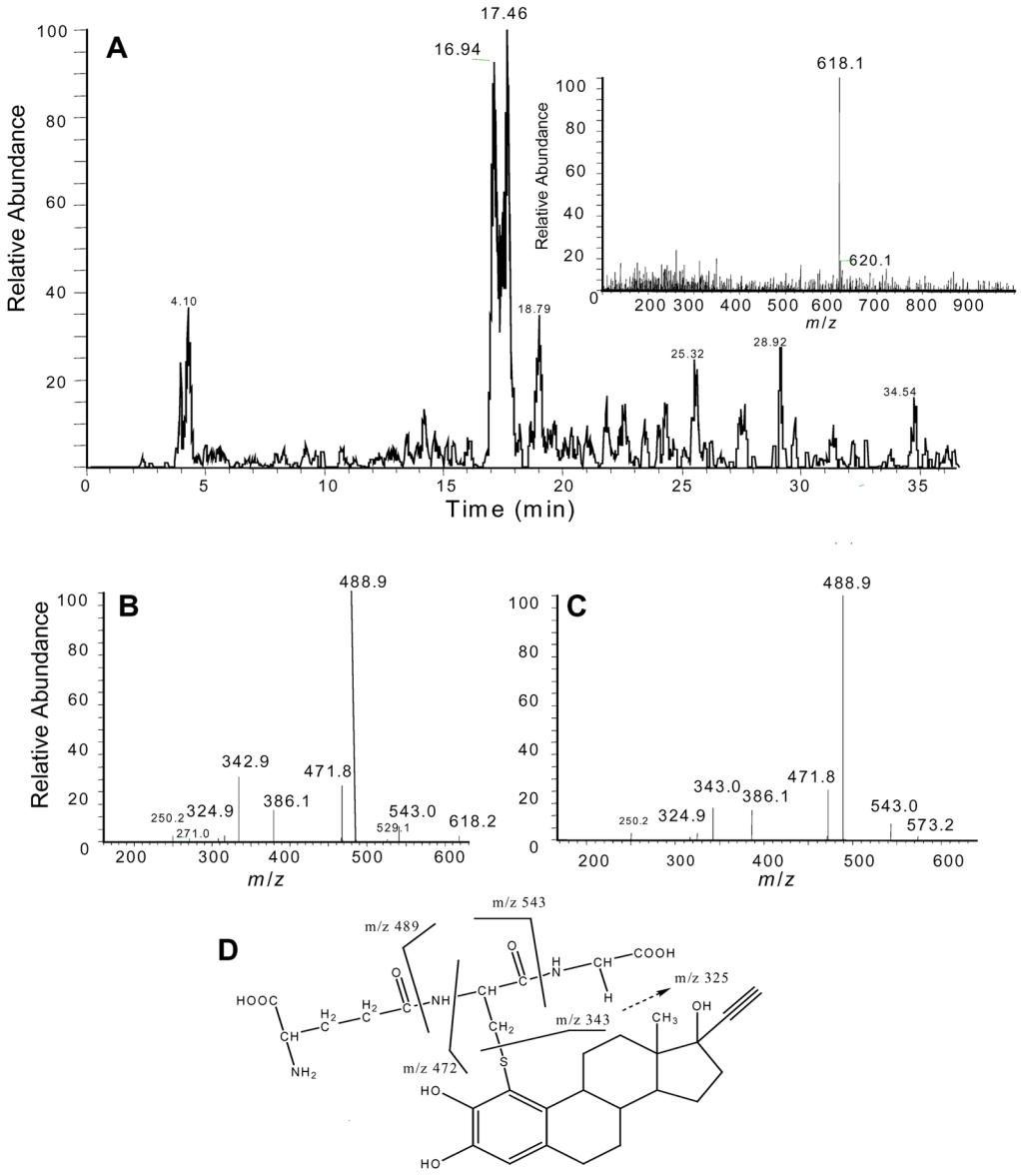

Figure 5.

Analysis of GSH conjugates derived from P450 2B6 samples incubated with 17EE in the presence of NADPH and GSH. P450 2B6 was reconstituted with reductase and incubated with 17EE and GSH as described in Experimental Procedures. (A) Extracted ion chromatogram of the molecular $[M+H]^+$ ions with a *m/z* of 618 and mass spectrum of the molecular ion(s) eluting at 16.9 and 17.5 min (Inset), (B) MS/MS spectrum of the ion eluting at 16.9 min, (C) MS/MS spectrum of the ion eluting at 17.5 min, (D) One of the possible theoretical structures of this GS-17EE conjugates. The data shown is representative of at least three separate analyses.

Table 1

Mass analysis of the 17EE-labeled peptide derived from CNBr-digested P450 2B1[a].

Mass analysis Method	Theoretical mass (Da)[b]	Observed mass (Da)	Difference (Da)[c]
ESI	3385.8	3654.6	268.8
MALDI (lactone)	3385.8	3654.4	268.6
MALDI (homoserine)	3404.8	3670.4 + 3401.7	265.6

[a] P450 2B1 was inactivated with 17EE and treated with CNBr as described in Experimental Procedures.

[b] The theoretical masses listed correspond to those of the native, unadducted peptides calculated using MacPromass (15).

[c] The mass difference between the observed mass and the theoretical mass of the peptide.

Table 2

Sequence homology between P450s 2B1, 2B6, 2B4 and 2B2 for the peptide spanning the amino acid sequence P_{347} – M_{376}[a].

P450	Amino acid sequence																													
2B1	$_{347}$P	Y	T	D	A	V	I	H	E	I	Q	R	F	S	D	L	V	P	I	G	V	P	H	R	V	T	K	D	T	M_{376}
2B6				E				Y									L		M					I			Q	H		S
2B4													L	G			I		F					T						Q
2B2														A			A				L									

[a]Amino acid sequences were obtained from drnelson.utmem.edu/homepage. Only the residues in P450s 2B6, 2B4, and 2B2 that are different from those in P450 2B1 in this region are shown. The potentially modified serine residue in P450 2B1 at position 360 is indicated in bold.