DETECTION OF GLUTATHIONE CONJUGATES DERIVED FROM 4-IPOMEANOL METABOLISM IN BILE OF RATS BY LIQUID CHROMATOGRAPHY-TANDEM MASS SPECTROMETRY

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ABSTRACT:

Earlier studies postulated that bioactivation of 4-ipomeanol by cytochrome P450 enzymes may occur through oxidation of its furan ring, following a mechanism similar to the bioactivation of other furan-containing compounds. This would lead to the formation of furan epoxides and α,β-unsaturated di-aldehyde-reactive metabolites that can conjugate with glutathione. These metabolites are thought to be responsible for the cytotoxic and anticancer properties of 4-ipomeanol. We hypothesized that if 4-ipomeanol is metabolized following this pathway, its glutathione conjugates would be isobaric (molecular ion mass = 492 Da) and would be excreted in bile. To investigate this hypothesis, we analyzed by liquid chromatography-tandem mass spectrometry the bile of rats administered d₆/δ₆ 4-ipomeanol (1:1 ratio) intravenously. Hexadeuterated 4-ipomeanol had all deuterium atoms incorporated on its aliphatic chain. Multiple reaction monitoring scans of bile for the mass transition: MH⁺/(MH – 129)⁺, which is characteristic of glutathione conjugates, detected four glutathione conjugates. The observation of the isotope cluster (M + 1)⁺ (d₆/δ₆(MH + 6)⁺ (d₆) in a 1:1 molar ratio confirmed that these conjugates were derived from 4-ipomeanol. Retention of the six deuterium atoms in the glutathione conjugates detected, (MH + 6)⁺, indicates that the bioactivation of 4-ipomeanol took place on the furan ring moiety. Rat hepatic microsomal incubations provided additional evidence. From this study, the mass of the reactive metabolites of 4-ipomeanol can be inferred. The inferred mass (186 Da) matches the mass postulated. A pathway of 4-ipomeanol bioactivation is proposed here. This work represents one step forward to understanding the mechanism of bioactivation of 4-ipomeanol.

4-Ipomeanol [1-(3-furyl)-4-hydroxypentanone] is a natural cytoxin first isolated and purified by Boyd and coworkers in 1972 from Ipomoea batatas (sweet potatoes) infected with the mold Fusarium solani (Boyd and Wilson, 1972; Boyd et al., 1972). Extensive investigations have demonstrated that 4-ipomeanol is a lung-specific toxin in animals, and that its toxicity is initiated through in situ bioactivation catalyzed by cytochrome P450 enzymes (Boyd, 1977; Doster et al., 1978; Buckpitt and Boyd, 1980; Boyd et al., 1982; Durham et al., 1985; Larson and Tjalve, 1988; Gram, 1989). Biodistribution studies of radiolabeled 4-ipomeanol in rats showed that the radioactivity bound to macromolecules of lung and liver could not be removed either by extraction with strong acids or by repeated washes with organic solvents (Boyd and Burka, 1978). Covalently bound radioactive metabolites were found in much higher levels in the lungs compared with nontarget tissues such as the liver. It was also demonstrated that without prior metabolism, 4-ipomeanol was relatively inert and did not bind significantly to tissue components (Boyd and Burka, 1978; Boyd et al., 1978; Buckpitt and Boyd, 1982; Slaughter et al., 1983). These studies suggested that reactive metabolites derived from 4-ipomeanol metabolism bind covalently to proteins, including P450 enzymes. Other studies showed that covalent binding of reactive metabolites of 4-ipomeanol to cellular proteins of the lungs results in pulmonary cytotoxicity, since preclusion of bioactivation resulted in absence of toxicity (Boyd, 1977; Boyd and Burka, 1978; Dutcher and Boyd, 1979). Recently, our group has characterized 4-ipomeanol as a potent mechanism-based inactivator of human hepatic P450 3A4 (Kᵢ = 20 μM) (Alvarez-Diez and Zheng, 2004). We found that inactivation of P450 3A4 by 4-ipomeanol resulted from covalent binding of reactive species of 4-ipomeanol to this enzyme. Previous in vivo studies in rats showed that depletion of endogenous glutathione with diethylmaleate, prior to the administration of radiolabeled 4-ipomeanol, increased toxicity and covalent binding of reactive activity (Boyd et al., 1982; Buckpitt et al., 1982). It was suggested that at least one highly electrophilic metabolite of 4-ipomeanol is formed via cytochrome P450-dependent metabolism, and this metabolite is detoxified by conjugation with the nucleophile glutathione. Toxic doses of 4-ipomeanol depleted pulmonary glutathione. Later, HPLC radiochromatograms of microsomal samples of rat lung and liver incubated with [3H]glutathione and 4-ipomeanol detected two peaks with associated radioactivity (Buckpitt and Boyd, 1980). In this study, similar radiochromatograms were also observed from incubations with [3H]4-ipomeanol and unlabeled glutathione. In 1982, Wolf and colleagues also separated by HPLC two radioactive compounds from incubations of purified P450 4B1 or P450 2B4 of rabbits with

ABBREVIATIONS: P450, cytochrome P450; LC-MS/MS, liquid chromatography-tandem mass spectrometry; MRM, multiple reaction monitoring; HPLC, high-performance liquid chromatography; CAD, collisionally activated dissociation; L-754,394, N-[2-hydroxy-1-indanyl]-5-[2-[(1,1-dimethylethyl)amino] carbonyl]-4-[[furo[2,3-b]pyridin-5-yl]methyl]piperazin-1-yl]-4-hydroxy-2-[(phenylmethyl)pentanamide.
glutathione and $^3$H-4-ipomeanol in a reconstituted system (Wolf et al., 1982). However, in these studies the structural nature of the glutathione conjugates derived from 4-ipomeanol metabolism was not studied. These studies were performed in vitro, in microsomal incubations; however, glutathione conjugates were not isolated in vivo.

Structure-activity relationship studies of 4-ipomeanol showed that replacement of its furan ring by a phenyl ring diminished the potency in lung toxicity (Boyd and Burkha, 1978), indicating that the furan ring is critical for the cytotoxic effects of 4-ipomeanol. We and others have postulated that the metabolism of 4-ipomeanol by P450 enzymes may occur through oxidation of its furan ring, following a mechanism similar to the bioactivation of other furans (Gram, 1989; Dalvie et al., 2002; Alvarez-Diez and Zheng, 2004). The major pathways reported for the bioactivation of furan and other furan-containing compounds by P450 enzymes lead to the formation of $\alpha,\beta$-unsaturated aldehyde/ketone of di-aldehyde-reactive metabolites (Ravindranath et al., 1984; Ravindranath and Boyd, 1985; Kobayashi et al., 1987; Chen et al., 1995; Khojateh-Bakht et al., 1999; Dalvie et al., 2002). These intermediates are electrophiles that may potentially attack in situ nucleophilic sites of enzymes, inactivating them, or may conjugate with glutathione. Glutathione conjugates are commonly secreted in bile (Gibson and Skett, 1999). We hypothesized that if 4-ipomeanol is bioactivated following this proposed pathway and its reactive metabolites are excreted in the bile as glutathione conjugates, then these glutathione conjugates would be expected to be isobaric, having a molecular mass of 491 Da. The structure of the reactive metabolites of 4-ipomeanol could be inferred by determining the molecular mass of its glutathione conjugates. Liquid chromatography-tandem mass spectrometry (LC-MS/MS) operated using multiple reaction monitoring (MRM) for the ion transitions (MH$^+$)$\rightarrow$(MH$^+ - 129$) $m/z$, which is characteristic of glutathione conjugates (loss of pyroglutamato moiety), has proven to be very sensitive in the detection of glutathione conjugates in complex biological samples, such as bile, and microsomal samples (Yamaguchi et al., 1992; Baillie and Davis, 1993; Jin et al., 1994; Poon et al., 1999; Lee, 2002). In this study, we detected and confirmed that the molecular mass of four glutathione conjugates derived from 4-ipomeanol metabolism matches the predicted mass. We also trapped a reactive metabolite of 4-ipomeanol conjugate with glutathione from 4-ipomeanol metabolism having the proposed molecular mass of 491 Da, which may be potentially excreted in the bile of rats administered with 4-ipomeanol. An equimolar mixture of $d_4$-4-ipomeanol was administered intravenously at a dose of 50 mg/kg in 0.1 M potassium phosphate buffer with traces of methanol to bile duct-cannulated Sprague-Dawley male rats (250–280 g). The bile was collected for 4 h as previously described (Jin et al., 1994). Drug-blank bile samples from these rats had also been collected before the treatment. The samples were centrifuged at 0°C (4000 rpm, 10 min) to remove particulate matter. The resulting supernatants were collected and transferred to LC autosampler vials. A LC-MS/MS scan operated in MRM mode for the ion transitions; however, glutathione conjugates were not isolated in vivo.

**Scheme 1.** Synthesis of deuterated 4-ipomeanol in two steps.

**Materials and Methods**

**Materials.** Ethyl-$\beta$-oxo-3-furan-propionate, propylene oxide, propylene $d_4$-oxide (98% atom D), and anhydrous ethanol were purchased from Aldrich Chemical Co. (Milwaukee, WI). Pooled human hepatic microsomes were purchased from BD Gentest (Woburn MA). Rat liver microsomes were generously provided by Dr. Robert Schatz, Northeastern University (Boston, MA). $\beta$-Nicotinamide adenine dinucleotide phosphate reduced form (\(\beta\)-NADPH), methyl-glutathione, and hexyl-glutathione were obtained from Sigma-Aldrich (St. Louis, MO).

**Instrument.** A 300-MHz NMR apparatus (Varian Mercury-300; Varian Inc., Palo Alto, CA) was utilized for structure identification. An API 2000 triple-quadrupole mass spectrometer equipped with a heated electrospray source (Applied Biosystems, Foster City, CA) and coupled inline with an Agilent LC system with a 384-well autosampler (Agilent Technologies, Palo Alto, CA) was used for detection of glutathione conjugates and mass analysis. The operating software used for data acquisition and processing, Analyst 2000, was also from Applied Biosystems.

**Synthesis of Deuterated 4-Ipomeanol.** Deuterated 4-ipomeanol was synthesized under anhydrous conditions following a two-step scheme (Scheme 1) previously reported for the synthesis of 4-ipomeanol (Boyd et al., 1972, 1973), which was previously modified by our group (Alvarez-Diez and Zheng, 2004). In this case, propylene oxide was used as deuterating agent. Briefly, ethyl-$\beta$-oxo-3-furan-propionate (18 mmol) was mixed with a 9.0 M excess of $d_4$-propylene oxide in EtOna solution followed by overnight stirring at room temperature. The mixture was brought to pH 7.0 and was extracted with chloroform. A deuterated fural-$\gamma$-lactone intermediate was obtained in 51% yield. In the second step, the resulting deuterated lactone was treated with 10.0 N sulfuric acid, as previously described, giving hexadeuterated 4-ipomeanol in 80% yield. The structure of $d_4$-4-ipomeanol was confirmed by proton nuclear magnetic resonance (1H NMR) analysis (Table 1). 4-Ipomeanol was synthesized as previously reported by us (Alvarez-Diez and Zheng, 2004), and its structure was also confirmed by $^1$H NMR. A 1:1 mixture of $d_4$/$d_5$-4-ipomeanol (10 mM) in 0.1 M potassium phosphate buffer with traces of alcohol was prepared, and its purity was measured by reverse-phase HPLC (Hypersil C18 column; 50 × 2.0 mm, 5 μm; Thermo Hypersil, Keystone Scientific Operations, Bellefonte, PA), using a mass spectrometer detector. The gradient solvent system used to elute the sample was as previously reported for a sample of 4-ipomeanol (Alvarez-Diez and Zheng, 2004). A LC-MS total ion chromatogram was acquired using the following operating conditions: positive ionization mode, ionization voltage, 5.0 kV; orifice potential, 28 V; ion source temperature, 200°C; selected mass range, 120 to 300 Da.

**Detection of Glutathione Conjugates of 4-Ipomeanol in Bile of Rats by LC-MS/MS.** This study was aimed at detecting glutathione conjugates derived from 4-ipomeanol metabolism having the proposed molecular mass of 491 Da, which may be potentially excreted in the bile of rats administered with 4-ipomeanol. An equimolar mixture of $d_4$/$d_5$-4-ipomeanol was administered intravenously at a dose of 50 mg/kg in 0.1 M potassium phosphate buffer with a trace of methanol to bile duct-cannulated Sprague-Dawley male rats (250–280 g). The bile was collected for 4 h as previously described (Jin et al., 1994). Drug-blank bile samples from these rats had also been collected before the treatment. The samples were centrifuged at 0°C (4000 rpm, 10 min) to remove particulate matter. The resulting supernatants were concentrated, centrifuged at approximately 200 μl using a SpeedVac apparatus, and transferred to LC autosampler vials. A LC-MS/MS scan operated in MRM mode for the ion transitions 492/363 and 498/369 (MH$^+$)$\rightarrow$(MH$^+ - 129$) $m/z$ was acquired. The LC conditions and mass spectrometer parameters used were as follows. Ten to 20-μl samples were injected into a narrow-bore HPLC Prevail C18 column (3 μm, 1 mm i.d. × 15 cm; Alltech Associates, Deerfield, IL). The samples were eluted from the column with a mixture of solvent A (0.05% aqueous trifluoroacetic acid) and solvent B (0.05% trifluoroacetic acid in acetonitrile).
at a flow rate of 50 μL/min. The elution gradient used was 5% solvent B for 10 min, followed by an increase in solvent B from 5 to 90% over the course of 20 min. The flow was directed to the mass spectrometer. The mass spectrometer operating conditions were as follows: positive ionization mode, ionization voltage 5 kV, orifice potential 28 V, ion source temperature 500°C. Collisionally activated dissociation (CAD) of parent ions with voltage 5 kV, orifice potential 28 V, ion source temperature 500°C. Collision-operating conditions were as follows: positive ionization mode, ionization energy was 25 V and the collision gas was maintained at a setting of 2. The mass transitions monitored were 492/363 and 498/369 m/z. The dwell time per transition was 200 ms. Drug-blank bile was also analyzed in a similar manner. The operating potentials used were those that optimized the detection of a mixture of methyl- and hexyl-glutathione conjugates (10 μg/ml of each conjugate in water) and of their (MH−129)+ m/z fragments in LC-MS/MS using MRM and product ion scanning modes. They were determined by manual infusion of the mixture (standard) into the mass spectrometer using a syringe pump.

Trapping the Reactive Metabolites of 4-Ipomeanol with Glutathione in Vitro and Detection of the Conjugates by LC-MS/MS. The aim of this study was to observe whether glutathione conjugates having the postulated mass (491 Da) could also be detected in vitro, in hepatic microsomal samples of rats. Microsomal incubations were prepared following a method previously described by Buckpitt and Boyd (1980) with some modification. Briefly, 400-μl incubation mixtures containing rat hepatic microsomes (25 mg protein/ml), glutathione (1.0 mM), 4-ipo-micronal (0.5 mM), and NADPH (8.0 mM) were prepared in 0.1 M potassium phosphate buffer. Control incubations lacked NADPH. The samples were incubated for 15 min at 37°C in a shaking water bath. The proteins were precipitated by addition of ice-cold ethanol followed by centrifugation for 30 min (3000 rpm, 4°C), and the supernatant was analyzed by LC-MS/MS following a method similar to that reported above. In this case, a single ion transition (492 → 363) was monitored.

Results

Synthesis of Deuterated 4-Ipomeanol. d₆-4-Ipomeanol was synthesized by alkylation of ethyl-β-oxo-3-furan-propionate with d₆-propylene oxide and subsequent acidic hydrolysis and decarboxylation. Its structure was confirmed by ¹H NMR. Spectral data of this compound and of its unlabeled analog (for comparison) are summarized in Table 1. Incorporation of six deuterium atoms in carbons 3, 4, and 5 of 4-ipo-micronal alkyl chain was confirmed by the absence of peaks at 1.78, 3.85, and 1.22 ppm, and by the singlet appearing at 1.90 ppm (Table 1). One major peak (99% of the total area) with a retention time of 1.7 min (capacity factor = 2.4) was observed in the LC-MS total ion chromatogram of a mixture of d₆/d₆-4-Ipomeanol. This peak had the expected m/z ratios for the protonated molecular ions of d₆- and d₆-4-Ipomeanol, 175 and 169 m/z, respectively.

Detection of Glutathione Conjugates of 4-Ipomeanol in Bile of Rats by LC-MS/MS. A mixture of methyl- and hexyl-glutathione conjugates (1:1 molar ratio) was used as standard to determine the parameters that optimize the detection of the ion pairs: MH⁺/MH −

<table>
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<th>H N.</th>
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<td>r; J = 6</td>
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<td>4-CH₉</td>
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<td>d; J = 6</td>
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TABLE 1

1H NMR spectral data

FIG. 1. LC/MS of a mixture of methyl- and hexyl-glutathione conjugates (1:1 molar ratio). These compounds were used as model glutathione conjugates to observe the parameters that optimize their detection. a, neutral loss (129 Da) total ion chromatogram of the mixture. b, mass spectrum of the methyl-glutathione (MH⁺ = 322 m/z)/hexyl-glutathione (MH⁺ = 392 m/z) mixture.

129+ in LC-MS/MS using a MRM scanning mode. The loss of neutral fragments of 129 Da from parent ions is characteristic of glutathione conjugates upon CAD (Jin et al., 1994). The MRM scan of ion pairs, 322/193 m/z (methyl-glutathione) and 392/263 m/z (hexyl-glutathione), was acquired after a mixture of these compounds was injected into the LC-MS/MS system. Figure 1a shows the MRM of these glutathione conjugates. Methyl-glutathione is observed at 3.1 min and hexyl-glutathione at 33.5 min. Both ions pairs were well detected. Methyl- and hexyl-glutathione underwent neutral losses of 129 Da from their molecular ions upon collision in the mass spectrometry system. MRM scans and neutral loss scans of 129 Da gave identical profiles. The mass chromatogram of the sample is observed in Fig. 1b.

Bile samples from rats that were injected with a 50 mg kg⁻¹ mixture of d₆/d₆-4-Ipomeanol (1:1 molar ratio) were analyzed by LC-MS/MS under MRM scanning mode. The PreQ2 voltages and the collision energy used for ion fragmentation, 25 V, were determined from MRM analysis of the alkyl-glutathione sample (standard). The ion pairs monitored were 492/363 and 498/360 m/z. Figure 2, a and b, shows the MRM scans of a bile sample. Four peaks are observed at 5.1, 8.1, 8.5, and 10.3 min in each profile (492/363 m/z and 498/369 m/z), whereas none of these peaks are found in the control bile (Fig. 2c). The peaks observed in the 492/363 m/z profile (named M1−M4) were found to overlay those found in the 498/369 m/z profile (M1′−M4′). We also observe that the ratio of these
makes evident that these metabolites were derived from 4-ipomeanol. In addition, the molecular masses of the glutathione conjugates detected, \((M + 1)^+ = 492\) Da, matches our predictions (see Scheme 2 for details).

Detection of Glutathione Conjugates of 4-Ipomeanol in Vitro by LC-MS/MS. The study was conducted to observe whether glutathione conjugates derived from 4-ipomeanol metabolism having the predicted molecular mass could also be detected by LC-MS/MS (MRM scan) in rat hepatic microsomal incubations. 4-Ipomeanol was incubated with rat liver microsomes in the presence or absence of NADPH. Glutathione was added to the incubation system to trap reactive metabolites of 4-ipomeanol. MRM scans from these microsomal incubations (ion transition 492/363 m/z) were acquired (Fig. 3a). A sharp peak is observed having a retention time similar to that of metabolite M1, observed in the bile sample. As expected, no corresponding peaks were present in the incubation that lacked NADPH (control, Fig. 3b).

Discussion

For the first time, we report the detection of glutathione conjugates of 4-ipomeanol in vivo, in rat bile. Four glutathione conjugates with \(MH^+ = 492\) m/z were observed in the MRM scan, whereas none were found in control bile samples. Four corresponding hexadeuterated glutathione conjugates with \(MH^+ = 498\) were also observed. The ratio of the \(d_6\) to \(d_0\)-glutathione conjugates observed is approximately 1:1, which matches the ratio of the \(d_0/d_6\)-4-ipomeanol mixture administered to the rats. The protonated molecular ions, 492 and 498 m/z, exactly fit the molecular mass of postulated glutathione conjugates derived from \(d_0\) and \(d_6\)-ipomeanol metabolism. The microsomal study provides additional evidence for the formation of glutathione conjugates derived from 4-ipomeanol. Since the mass of the deprotonated glutathione moiety is 306 Da, the mass of reactive metabolites will therefore be 186 Da (492–306 Da).

The stable tracer \(d_6\)-4-ipomeanol allowed us to track the fate of 4-ipomeanol disposition in vivo and to confirm that the glutathione conjugates observed by LC-MS/MS derive from this parent compound. 4-Ipomeanol was labeled with six deuterium atoms in its aliphatic chain. Retention of the six deuterium atoms in the glutathione conjugates detected in bile (\(MH^+ = 6\)) indicates that the bioactivation catalyzed by cytochrome P450 takes place on the furan ring moiety instead of on the aliphatic chain. This further supports the mechanism for the bioactivation of 4-ipomeanol proposed in Scheme 2.

Previous studies on the metabolism of other furan-containing compounds have shown that the furan ring is responsible for their bioactivation (for review see Dalvie et al., 2002). It has been reported that their major pathway of bioactivation involves ring opening, producing \(\alpha,\beta\)-unsaturated dicarbonyl metabolites (Ravindranath et al., 1984; Ravindranath and Boyd, 1985; Chen et al., 1995; Khojateh-Bakht et al., 1999; Dalvie et al., 2002). For example, bioactivation of 3-methylfuran by P450 enzymes produces unsaturated dialdehyde metabolites, 4-oxo-2-pentenal and 2-methylbutene-1,4 dial (Ravindranath et al., 1984; Ravindranath and Boyd, 1985). It was postulated that this reactive metabolite may be formed by direct furan ring opening or via an epoxide intermediate. Furan undergoes metabolic activation similar to \(cis\)-2-butene-1,4-dial (Chen et al., 1995). A furanepoxide intermediate was postulated to be the reactive metabolite of L-754,394, a potent mechanism-based inhibitor of P450 3A4 (Sahali-Sahly et al., 1996).

In this study we detected in vitro and in vivo glutathione conjugates, derived from 4-ipomeanol metabolism, that have the predicted masses (isobaric). Our data also indicate that bioactivation occurred in the furan ring moiety. This further supports the bioactivation pathway previously proposed for 4-ipomeanol. We propose here that two furanepoxide regioisomers, 1 and 2, and an \(\alpha,\beta\)-unsaturated di-aldehyde, 3 (Scheme 2) may be formed from P-450-mediated bioactiva-
tion of 4-ipomeanol. The epoxides and the unsaturated di-aldehyde are electrophilic intermediates that could conjugate with the nucleophile glutathione through a substitution nucleophilic bimolecular reaction or by 1,4-addition Michael addition to the double bond, respectively. This would result in four glutathione conjugates (4–7). These four conjugates may also result from conjugation of epoxides 1 and 2 with glutathione. Although glutathione could also attack carbons 3 and 5 of the furan epoxide ring producing two potential thio-hemiacetals, these structures are likely unstable. All four glutathione conjugates proposed have a molecular mass of (MH)$^+ = 492$ Da, which matches with our findings.

Dalvie et al. (2002) reviews the multiple glutathione conjugate structures (cyclic and acyclic) identified or postulated for alkyl furan compounds. We monitored by LC-MS/MS the mass transitions corresponding to potentially equivalent glutathione conjugate structures; however, only glutathione conjugates having masses of 492 Da were observed. Although dehydration of the proposed structures 4 and 7 would seem to be reasonable since the resulting furan structures would be more stable, we did not detect by LC-MS/MS the mass transitions of the dehydrated conjugates (474 → 345 m/z). These data, taken together, concur with previous studies in microsomes that suggested that the furan ring in the glutathione conjugates of 4-ipomeanol is no longer intact (Buckpitt and Boyd, 1980). Two radiolabeled peaks were separated by anion-exchange HPLC in rat microsomal samples of liver and lung incubated with radiolabeled 4-ipomeanol and glutathione. These peaks, unlike 4-ipomeanol, lacked ultraviolet absorbance at 254 nm. In addition, the 1-keto moiety of these glutathione conjugates was not reduced to alcohol (since their chromatographic profile was different from the profile observed for glutathione conjugates derived from 1,4-ipomeadiol), which suggested that their furan rings were no longer intact.

In rat microsomal incubations, we observed only one glutathione conjugate derived from 4-ipomeanol metabolism with mass of 492 Da. Earlier studies showed two radiolabeled conjugates in rat microsomal incubations (Buckpitt and Boyd, 1980). The discrepancies in the results obtained are very unlikely due to ionization problems in the mass spectrometry, since sensitive detection of glutathione conjugates was achieved, especially in the in vitro study. If two conjugates were to be formed in microsomal incubations, their ionization patterns would be expected to be similar, as shown in the bile. Buckpitt and Boyd (1980) added cystolic fraction (containing glutathione transferases) to the microsomal incubations with 4-ipomeanol. We did not. The glutathione transferases prominent in the cytosol often facilitate glutathione conjugation (Gibson and Skett, 1999). It is possible that only one conjugate may be formed spontaneously in the absence of cystolic fractions. Although it was claimed that two conjugates were also formed when a heated cystolic fraction was added, the glutathione 5-transferase activity was not measured; thus, there is not clear evidence of its complete inactivation. Another possible explanation for the different result obtained is that one of the two radiolabeled compounds observed may be the result of radiolabeling a cytosolic compound. No structural information of the radiolabeled peaks was provided, only their chromatographic profile and the fact that they lack ultraviolet absorbance.

We and others were able to easily synthesize reactive metabolites of 3-methylfuran conjugated with the nucleophilic agent disemicarbazone and used them as model compounds of 4-ipomeanol metabolism (Ravindranath et al., 1984; unpublished report). However, to date, it has not been possible to synthesize reactive metabolites of 4-ipomeanol using similar or other synthetic approaches. It is reasoned that the presence of a ketone group, an electron-withdrawing group, in the aliphatic chain of 4-ipomeanol as compared with the electron-donating aliphatic chain of 3-methylfuran may be responsible for the synthetic difficulties encountered. We could not determine which isomers are responsible for the four peaks observed in the MRM scans since the synthetic metabolites are not yet available. The amount of glutathione conjugates observed, although sufficient for good detection, was well below the amount required for peak collection for NMR analysis and structural identification. LC-MS operated in tandem, in conjunction with the stable isotope technique, is a highly sensitive tool for detection of very small amounts of metabolites (nanogram to picogram levels) (Poon et al., 1999). Since the study published by Buckpitt and Boyd (1980), no further work on identification of reactive metabolites of 4-ipomeanol has been published.
although the metabolism of this compound is still of great interest to toxicologists. We believe that the lack of sufficiently sensitive analytical instrumentation limited further work. Despite the absence of NMR spectra, our mass spectrometry data provide the following evidence showing formation of glutathione conjugates derived from 4-ipomeanol metabolism. First, the molecular ions monitored matched the expected molecular weights of the conjugates. Second, the characteristic neutral loss of 129 Da for glutathione conjugates was observed. Third, the isotope abundance ratio of deuterated to nondeuterated peaks was consistent with that of 4-ipomeanol given to the rats.

In conclusion, LC-MS/MS using MRMs (a selective scanning mode), along with the stable isotope technique, made it possible to detect glutathione conjugates derived from 4-ipomeanol. Four glutathione conjugates were detected in bile samples obtained from rats administered 4-ipomeanol. One glutathione conjugate was also found in rat liver microsomal incubations in the presence of NADPH. All glutathione conjugates have a molecular mass (MH)⁺ of 492 Da, as predicted. This infers the formation of an α,β-unsaturated di-aldehyde-reactive metabolite (186 Da) and two furan epoxides (186 Da). This work represents one step forward in understanding the bioactivation of 4-ipomeanol by P450 enzymes.

References


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