Detection of Glutathione Conjugates Derived from 4-Ipomeanol Metabolism in Bile of Rats by Liquid Chromatography-Tandem Mass Spectrometry (LC-MS/MS)

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Running title:

Glutathione Conjugates of 4-Ipomeanol

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Abbreviations:

API, atmospheric pressure ionization; GST, glutathione transferases; LC-MS/MS, liquid chromatography tandem mass spectrometry; MRM, multiple reaction monitoring; m/z, mass to charge ratio; β-NADPH, β-nicotinamide adenine dinucleotide phosphate reduced form; NMR, nuclear magnetic resonance.
Abstract

Earlier studies postulated that bioactivation of 4-ipomeanol by P450 enzymes may occur through oxidation of its furan ring, following a similar mechanism to the bioactivation of other furan-containing compounds. This would lead to the formation of furan epoxides and \( \alpha,\beta \)-unsaturated di-aldehyde reactive metabolites that can conjugate with glutathione. These metabolites are thought to be responsible for the cytotoxic and anti-cancer 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 LC-MS/MS bile of rats administered \( d_0/d_6 \) 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 4 glutathione conjugates. The observation of the isotope cluster \((M+1)^+ (d_0) / (MH+6)^+ (d_6)\) in a 1:1 molar ratio confirmed that these conjugates were derived from 4-ipomeanol. Retention of the 6 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 with the mass postulated. A pathway of 4-ipomeanol bioactivation is proposed here. This work represents one step forward into understanding the mechanism of bioactivation of 4-ipomeanol.
Introduction

4-Ipomeanol, [1-(3-furyl)-4-hydroxypentanone], is a natural cytotoxin first isolated and purified by Boyd et al. in 1972 from *Ipomoea batatas* (sweet potatoes) infected with the mold *Fusarium solani* (Boyd and Wilson, 1972a; Boyd et al., 1972b). 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 (Doster et al., 1978; Durham et al., 1985; Gram, 1989; Boyd, 1977; Larson et al., 1988; Boyd et al., 1982; Buckpitt et al., 1980). Biodistribution studies of radiolabelled 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, 1978a). Covalently bound radioactive metabolites were found in much higher levels in the lungs compared to non-target 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 et al., 1978a; Buckpitt et al., 1982a; Boyd et al., 1978b; Slaugther 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 et al., 1978a; Dutcher et al., 1979). Recently, our group has characterized 4-ipomeanol as a potent mechanism-based inactivator of human hepatic P450 3A4 ($K_i = 20 \mu M$) (Alvarez-Diez et al., 2004). We found that inactivation of
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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 radiolabelled 4-ipomeanol, increased toxicity and covalent binding of radioactivity (Boyd et al., 1982; Buckpitt et al., 1982b). 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 $^3$H-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 $^3$H-4-ipomeanol and unlabelled glutathione. In 1982, Wolf et al. also separated by HPLC two radioactive compounds from incubations of purified P450 4B1 or P450 2B4 of rabbits with 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, 1978) indicating that the furan ring is critical for the cytotoxic effects of 4-ipomeanol. We and
other 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; Alvarez-Diez and Zheng, 2004; Dalvie et al., 2002). The major pathways reported for the bioactivation of furan and other furan-containing compounds by P450 enzymes lead to the formation of α,β-unsaturated aldehyde/ketone of dialdehyde reactive metabolites (Dalvie et al., 2002; Kobayashi et al., 1987; Chen et al., 1995; Khojateh-Bakht et al., 1999; Ravindranath et al., 1984; Ravindranath et al., 1985). These intermediates are electrophiles which may potentially attack in situ nucleophilic sites of enzymes, inactivating them, or conjugate with glutathione. Glutathione conjugates are commonly secreted in bile (Gibson et al., 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 expect 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)+ → (MH+129) m/z, which is characteristic of glutathione conjugates (loss of pyroglutamate moiety), has proven to be very sensitive in the detection of glutathione conjugates in complex biological samples, such as bile, and microsomal samples (Baillie and Davis, 1999; Yamaguchi et al., 1992; Poon et al., 1999; Lee, 2002; Jin et al., 1994). In this study we detected and confirmed that the molecular mass of 4 glutathione conjugates derived from 4-ipomeanol metabolism matches with predicted mass. We also trapped a reactive metabolite of 4-ipomeanol conjugate with glutathione in hepatic microsomal
incubations of rat. Its molecular mass was also confirmed by LC-MS/MS. The stable
isotope technique using deuterated 4-ipomeanol permitted us to ascertain that the
glutathione conjugates detected in rat bile derive from metabolism of 4-ipomeanol.

Currently, there is a growing interest of 4-ipomeanol as a potential pro-drug for P450-
directed gene therapy of liver and brain cancers (Mohr et al., 2000; Rainov et al., 1998).
Successful targeting of brain tumors transfected with P450 4B1 has been reported in
animal studies (Rainov et al., 1998). Our findings help to further support the proposed
pathway of bioactivation of 4-ipomeanol by P450 enzymes.
Materials and Methods

Materials
Ethyl-β-oxo-3-furan-propionate, propylene oxide, propylene $d_6$-oxide (98% atom D) and anhydrous ethanol were purchased from Aldrich Chemical Co. Inc. (Milwaukee WI). Pooled human hepatic microsomes were purchased from Gentest Co. (Woburn MA). Rat liver microsomes were generously provided by Dr. Robert Schatz, Northeastern University (Boston, MA). β-Nicotinamide adenine dinucleotide phosphate reduced form (β-NADPH), methyl-glutathione, and hexyl-glutathione were obtained from Sigma Chemical Co. (St. Louis, MO).

Instruments
A 300-MHz NMR (Variant Mercury-300, Variant, Palo Alto, CA) was utilized for structure identification. 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, 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 Inc.

Synthesis of deuterated 4-ipomeanol
Deuterated 4-ipomeanol was synthesized under anhydrous conditions following a 2-step scheme previously reported for the synthesis of 4-ipomeanol (Boyd et al., 1972b; Boyd et al., 1973) which was previously modified by our group (Alvarez-Diez and Zheng, 2004). In this case, $d_6$-propylene oxide was used as deuterating agent. Briefly, ethyl-β-oxo-3-furan-propionate (18 mmols) was mixed with a 9.0 molar excess of $d_6$-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 furyl-γ-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 $d_6$-4-ipomeanol was confirmed by proton nuclear magnetic resonance ($^1$H NMR) analysis (Table 1). 4-ipomeanol was synthesized as previously reported by us (in press) and its structure was also confirmed by $^1$H NMR. A 1:1 mixture of $d_0$/-$d_6$-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 C$_8$ column; 50 x 2.0 mm, 5 $\mu$m, Keystone Scientific, 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, 2004). A LC-MS total ion chromatogram (TIC) was acquired using the following operating conditions: positive ionization mode, ionization voltage, 5.0 kV; orifice potential, 28 volts; 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 that may be potentially excreted in the bile of rats administered with 4-ipomeanol. An equimolar mixture of $d_0$-$d_6$-4-ipomeanol was administered intravenously at a dose of 50 mg/Kg of in 0.1 M potassium phosphate buffer with trace of methanol to bile-duct cannulated Spague-Dawley male rats (250-280 g). The bile was collected for 4 hours as previously described (Jin L, 1994). Drug-blank bile samples from these rats had also been collected before the treatment. The samples were centrifuged at 0 °C (4,000 rpm, 10 minutes) in order to
remove particulate matter. The resulting supernatants were collected, concentrated to 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+/ (MH+ -129)] m/z was acquired. The LC conditions and mass spectrometer parameters used were as follows. Ten to twenty microliters samples were injected into a narrow-bore HPLC Prevail C18 column (3 µm, 1 mm i.d. x 15 cm; Alltech). The samples were eluted from the column with a mixture of solvent A (0.05 % aqueous TFA) and solvent B (0.05 % TFA in acetonitrile) at a flow rate of 50 µl/min. The elution gradient used was 5 % solvent B for 10 minutes, followed by an increase in solvent of B from 5 to 90 % over the course of 20 minutes. The flow was directed to the mass spectrometer. The mass spectrometer operating conditions were as follows: positive ionization mode, ionization voltage 5kV, orifice potential 28 volts, ion source temperature 500 0C. Collisionally activated dissociation (CAD) of parent ions with m/z of 492 and 498 Da was performed using N2 as target gas in Q2 (collision cell). The collision energy (CE) was 25 volts 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

This study was aimed to observe whether glutathione conjugates having the postulated mass (491 Da) could be also detected in vitro, in hepatic microsomal samples of rats. Microsomal incubations were prepared following a method previously described by Buckpitt et al. (Buckpitt, 1980) with some modification. Briefly, 400 µl incubation mixtures containing rat hepatic microsomes (25 mg protein/ml), glutathione (1.0 mM), 4-ipomeanol (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 minutes at 37°C in a shaking water bath. The proteins were precipitated by addition of ice-cold ethanol followed by centrifugation for 30 minutes (3000 rpm, 4°C), and the supernatant analyzed by LC-MS/MS following a similar method as that reported above. In this case, a single ion transition (492 → 363) was monitored.

Results

Synthesis of deuterated 4-ipomeanol
d$_6$-4-Ipomeanol was synthesized by alkylation of ethyl-β-oxo-3-furan-propionate with d$_6$-propylene oxide and subsequent acidic hydrolysis and decarboxylation. Its structure was confirmed by $^1$H NMR. Spectral data of this compound and of its unlabelled analog (for comparison) are summarized in Table 1. Incorporation of six deuterium atoms in carbons 3, 4 and 5 of 4-ipomeanol 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 minutes (capacity factor = 2.4) was observed in the LC-MS total ion chromatogram of a mixture of d$_0$/d$_6$-4-ipomeanol. This peak had the expected m/z ratios for the protonated molecular ions of d$_6$- and d$_0$-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: \( \text{MH}^+ / (\text{MH}-129)^+ \) 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, 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 minutes and hexyl-glutathione at 33.5 min. Both ions pairs were well detected. Methyl- and hexyl-glutathione underwent neutral losses of 129 daltons 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 Figure 1b.

Bile samples from rats that were injected with a 50 mg kg\(^{-1}\) mixture of \( d_0 / d_6 \)-4-ipomeanol (1:1 molar ratio) were analyzed by LC-MS/MS under MRM scanning mode. The Pre-Q2 voltages and the collision energy used for ion fragmentation, 25 volts, were determined from MRM analysis of the alkyl-glutathione sample (standard). The ion pairs monitored were 492/363 and 498/360 m/z. Figure 2a and 2b 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) while none of these peaks are found in the control bile (Figure 2c). The peaks observed in the 492/363 m/z profile (named M1 to M4) were found to overlay those found in 498/369 m/z profile (M1’ to M4’). We also observe that the ratio of these profiles is approximately 1:1 ratio, which is similar to the ratio of the \( d_0 \)-
4-ipomeanol/d₆-4-ipomeanol mixture administered to the rats. The observation of the isotope cluster of \((M+1)^+ / (M+7)^+\) in a 1:1 ratio, 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 with 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 in order to trap reactive metabolites of 4-ipomeanol. MRM scans from these microsomal incubations (ion transition 492/363 m/z) were acquired (Figure 3a). A sharp peak is observed having a similar retention time as metabolite M1, observed in the bile sample. As expected, no corresponding peaks were present in the incubation that lacked NADPH (control, Figure 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, while none were found in control bile samples. Four corresponding hexadeuterated glutathione conjugates with MH⁺ of 498 were also observed. The ratio of the d₀- to d₆- glutathione conjugates observed is 1:1 approximately, which matches the ratio of the d₀/d₆-4-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, therefore the mass of reactive metabolites will be 186 Da (492-306 Da).

The stable tracer $d_6$-4-ipomeanol allowed us to track the fate of 4-ipomeanol disposition \textit{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 (Dalvie et al., 2002; Chen et al., 1995; Khojateh-Bakht et al., 1999; Ravindranath et al., 1984; Ravindranath et al., 1985). For example, bioactivation of 3-methylfuran by P450 enzymes produces an unsaturated dialdehyde metabolites, 4-oxo-2-pentenal and 2-methylbutene-1,4 dial (Ravindranath et al., 1984; Ravindranath et al., 1985). It was postulated that this reactive metabolite may be formed by direct furan ring opening or via an epoxide intermediate. Furan undergoes similar metabolic activation to \textit{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 which 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 regio-isomers, 1 and 2, and an α,β-unsaturated di-aldehyde, 3, (Scheme 2) may be formed from P-450 mediated bioactivation of 4-ipomeanol. The epoxides and the unsaturated di-aldehyde are electrophilic intermediates which could conjugate with the nucleophile glutathione through a S_N2 reaction or by 1,4-addition Michael addition to the double bond, respectively. This would result in 4 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 conjugates 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 radiolabelled peaks were separated by anion-exchange HPLC in rat microsomal samples of liver and lung incubated with radiolabelled 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 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 radiolabelled 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 expect to be similar as shown in the bile. Boyd et al. added cytosolic fraction (containing glutathione transferases (GST)) 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
cytosolic fractions. Although it was claimed that two conjugates were also formed when a heated cytosolic fraction was added, the GST activity was not measured so there is not clear evidence of its complete inactivation. Another possible explanation for the different result obtained is that one of the two radiolabelled compounds observed may be the result of radiolabelling a cytosolic compound. No structural information of the radiolabelled 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 and 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 to 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 amount of metabolites (nanogram to picogram levels) (Poon et. al., 1999). Since the study published by Buckpitt et al. in 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 provides the following evidences showing formation of glutathione conjugates derived from 4-ipomeanol metabolism. First, the molecular ions monitored matched with 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 non-deuterated peaks was consistent with that of 4-ipomeanol given to the rats.

In conclusion, LC-MS/MS using MRM, (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 with 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 into understanding the bioactivation of 4-ipomeanol by P450 enzymes.
References


Legend to the Figures

Figure 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 (TIC) of the mixture. b. Mass spectrum of the methyl-glutathione (MH⁺ = 322 m/z) / hexyl-glutathione (MH⁺ = 392 m/z) mixture.

Figure 2. LC/MRM ion chromatograms of a rat bile dosed with 50 mg Kg⁻¹ of a mixture of d₀-4-ipomeanol / d₆-4-ipomeanol (molar ratio ~ 1:1). The bile specimen was collected between 0 and 4 hours post-dosing. a. MRM scan acquired using the mass transition 492 → 363 m/z. This profile depicts the compounds in the sample having protonated molecular ions (MH⁺) with mass of 492 Da which eliminated 129 Da upon CAD (characteristic fragment of glutathione conjugates); Retention time of glutathione conjugates of 4-ipomeanol referred to as metabolites M₁ to M₄ are indicated. b. MRM scan acquired using the mass transition 498 → 369 m/z. c. LC/MRM ion chromatogram of a drug-blank bile sample from a rat (control) for the two mass transitions indicated. Solvent front (S).

Figure 3a. LC/MRM ion chromatogram of rat hepatic microsomal samples incubated with 4-ipomeanol for 15 minutes in the presence of NADPH. The MRM scan was acquired by using the mass transition m/z 492→363 or [(MH)⁺ → (MH-129)⁺]. The retention time of a metabolite conjugated with glutathione, referred as metabolite M₁, is indicated. b. Control. LC/MRM ion chromatogram of rat hepatic microsomal samples incubated with 4-ipomeanol in the absence of NADPH.

Scheme I. Synthesis of deuterated 4-ipomeanol in two steps.

Scheme II. Proposed metabolic pathway of 4-ipomeanol bioactivation by cytochrome P450 monooxygenases. Three reactive metabolites are proposed, two furanepoxide regio-isomers (structures 1 and 2), and an α,β-unsaturated di-aldehyde metabolite (structure 3). Conjugation of these reactive metabolites with glutathione (GSH) would result in glutathione conjugates 4 to 7. These four conjugates are isobaric [(MH)⁺ = 492 Da]. See text for details.
Table 1. $^1$H NMR Spectral data

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(J) Coupling constant, (s) singlet, (d) doublet, (t) triplet, (m) multiplet.
Figure 1.
Figure 2.
Figure 3.

(a) Intensity vs. Time for M1.

(b) Intensity vs. Time for another event.

Figure 3.
Scheme I.
Scheme II.