

Detection of Covalent Adducts to Cytochrome P450 3A4 Using Liquid Chromatography Mass Spectrometry

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Protein covalent labeling can be an undesirable property of compounds being studied in drug discovery programs. Identifying such compounds relies on the use of radiolabeled material, which requires an investment in time and resources not typically expended until later in the discovery process. We describe the detection of covalent adducts to cytochrome P450 3A4, the most abundant and important P450 from a human and drug discovery viewpoint, using liquid chromatography mass spectrometry. The technique is illustrated using L-754,394 and 6',7'-dihydroxybergamottin, two known inhibitors of P450 3A4. Mass spectrometry of the intact apoprotein as well as the adducted protein is demonstrated. Such methodology may provide the means for screening compounds for covalent protein binding without the use of a radiolabel. It also provides direct information about mechanism-based inhibitors in terms of extent, stoichiometry, and nature of the adduct(s) (mass shift). This information may provide a means for understanding the mechanism of covalent labeling earlier in a drug discovery environment.

Introduction

Protein covalent binding is an undesirable property of many xenobiotics and has been implicated in the adverse drug reactions associated with such compounds (1, 2). The mechanism is thought to occur through metabolic activation of the compound by the superfamily of cytochrome P450s to a reactive intermediate that can label the P450 or other proteins (3, 4). The current methodology for detecting protein covalent adducts is labor intensive, time-consuming, and relies on the use of radiolabeled compounds (5, 6). Numerous studies with radiolabeled compounds have been reported in the literature definitively showing that metabolic activation leads to covalent labeling of P450s. These studies have also demonstrated specific labeling of individual isoforms (7–16). More recently, liquid chromatography coupled with electrospray mass spectrometry (LC-ESMS) has been employed to detect covalent adducts using unlabeled compounds. Data have been published showing spectra of the intact isoforms of P450 2B1 (17, 18), 2C9 (19), 2E1 (20, 21), 2B6 (22), and 2D6 (23). To date, no data on the mass spectral analysis of covalent adducts to intact P450 3A4, the most abundant and most important isoform from a human and drug discovery viewpoint (24, 25), have been reported. Two reports on mechanism-based inhibitors of P450 3A4 detailed attempts to generate spectra of the intact protein with adduct (26, 27). Even when using a large amount of P450 3A4, no interpretable spectra could be generated. The development of a method for the detection of covalent modification of P450 3A4 using MS may provide for the determination of the site of adduction (protein vs heme), the nature of any adducts

(mass shift), and the extent and stoichiometry, without the need for radiolabeled compounds. Using information from such experiments, chemists could then modify lead compounds to eliminate the propensity for covalent modification of proteins.

This paper describes results from an LC-MS method that, for the first time, provides direct detection of the covalent modification of P450 3A4. The methodology is applicable to P450s in general, and two different examples are shown for compounds that label P450 3A4. The method uses picomole quantities of commercially available P450, making it readily applicable in other drug metabolism/MS laboratories.

Materials and Methods

Chemicals and Reagents. The P450 3A4 mechanism-based inhibitor L-754,394 was synthesized at Merck Research Laboratories (West Point, PA) (28). 6',7'-Dihydroxybergamottin (DHB) was isolated from grapefruit juice concentrate (29). Purified and reconstituted (RECO) cytochrome P450s were purchased from Panvera (Madison, WI) and used as supplied. Human P450 3A4 Supersomes were purchased from Gentest (Woburn, MA). Acetonitrile (ACN) and trifluoroacetic acid (TFA) were from Aldrich (Milwaukee, WI), NADPH was purchased from Fluka (Steinheim, Germany), 2-propanol (IPA) was from Fisher (Fairlawn, NJ), and EDTA was obtained from GibcoBRL (Grand Island, NY).

Incubations. RECO P450 5× Enzyme Mix {0.5 μM P4503A4, 1 μM P450 reductase, 0.5 μM cytochrome b5, 0.5 μg/μL CHAPS, 0.1 μg/μL liposomes [dilauroyl phosphatidylcholine, dioleoyl phosphatidylcholine, and dilauroyl phosphatidylserine (1:1:1)], 3 mM reduced glutathione, and 50 mM HEPES/KOH (pH 7.4)} and RECO P450 5× Buffer Mix [200 mM HEPES/KOH (pH 7.4), 12 mM reduced glutathione, and 150 mM MgCl₂] were diluted with water to an enzyme concentration of 0.1 μM and a 1× buffer concentration. EDTA was added to a final concentration

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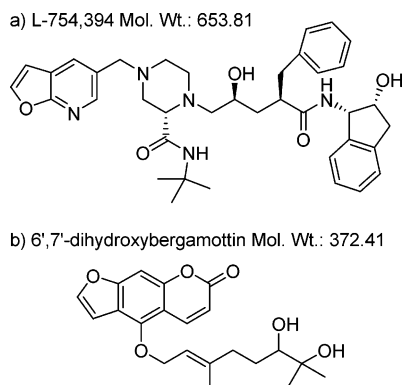


Figure 1. Structures of (a) L-754,394 and (b) DHB.

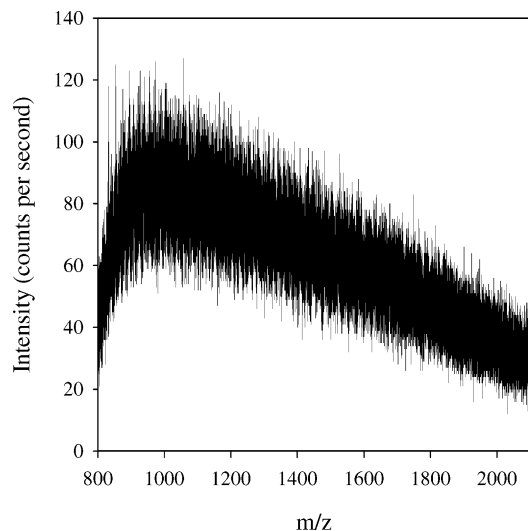


Figure 2. Mass spectrum of P450 3A4 after incubation with L-754,394 and 1 mM NADPH for 60 min using RECO system.

of 260 μ M, and the inhibitor was added at a final concentration of 50 μ M. The mixture was preincubated at 37 $^{\circ}$ C for 3 min prior to the addition of NADPH (100 μ M final concentration). After the addition of NADPH, inactivation of the proteins was allowed to proceed for 2 (L-754,394) or 3 min (DHB), unless otherwise specified. An ice bath was used to quench the incubations. Control incubations, +NADPH - inhibitor or -NADPH + inhibitor, were also generated and analyzed by LC-MS.

Inhibition of P450 3A4 activity was monitored using a standard fluorescence-based assay (30), and depletion of NADPH was followed by monitoring the absorbance at 340 nm. NADPH was not rate limiting in incubations with the inhibitors, as determined by quantifying NADPH depletion as a function of incubation time. The enzyme activity was completely inhibited, as measured by metabolism of a fluorogenic substrate (BFC, 7-benzyloxy-trifluoromethylcoumarin), before the NADPH was depleted, indicating that loss of activity was due to enzyme inhibition and not cofactor depletion. These incubations were analyzed by LC-MS to detect covalent binding, if any.

Gentest 3A4 Supersomes (50 μ L, 40 pm of 3A4) were diluted with phosphate buffer (pH 7.4, 200 μ L); inhibitor (L-754,394) was added to a final concentration of 100 μ M. After the addition of NADPH (250 μ M final concentration), inactivation was allowed to proceed for 3 min. The reaction was stopped by freezing, and samples were kept on ice prior to analysis by LC-LC-MS. Parallel incubations were monitored for enzyme activity using BFC as described above, with complete inhibition being observed.

HPLC. Incubations with RECO or Supersomes were separated for mass spectral analysis using two-dimensional chromatography. The first dimension used an Applied Biosystems (Foster City, CA) POROS R2/10 4.6 mm \times 10 mm column, and

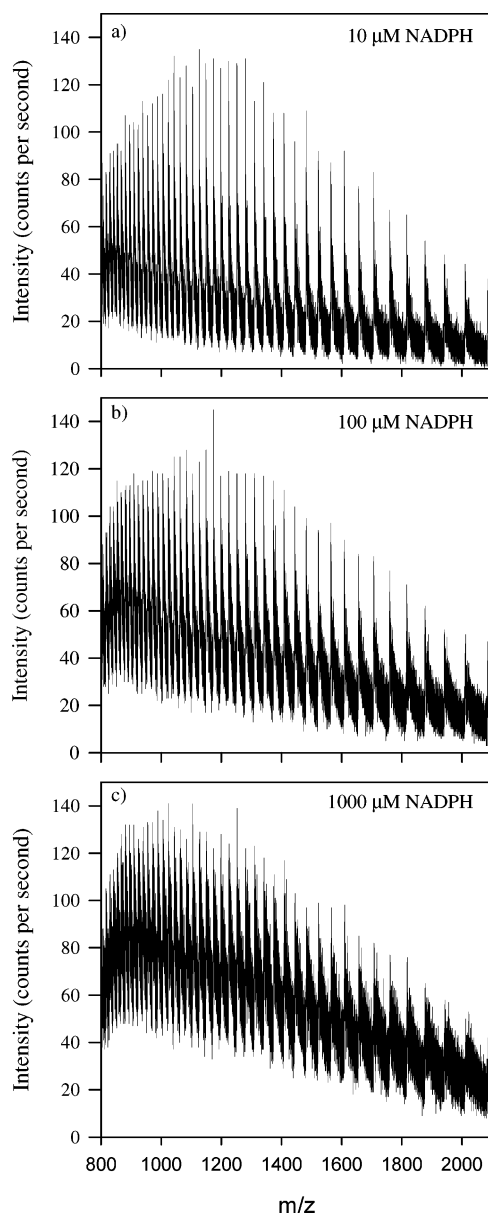


Figure 3. Mass spectrum of P450 3A4 from RECO system after 15 min of incubation with (a) 10 μ M NADPH, (b) 100 μ M NADPH, and (c) 1000 μ M NADPH.

the second dimension used a Phenomenex (Torrance, CA) Jupiter 50 mm \times 1 mm C4 column. Typically, the incubation mixture was injected onto the POROS column and the peaks corresponding to P450 3A4, P450 reductase, and cytochrome b5 were collected using a fraction collector. The HPLC used was a Waters (Milford, MA) Alliance 2795 equipped with a 996 photodiode array detector and Waters Fraction Collector II. The pump was operated at 1 mL/min and a linear gradient of 30% B for 3 min, 30–50% B in 5 min, hold at 50% B for 1.5 min, 50–95% B in 2.5 min, and hold 95% B for 6 min, where A was 0.1% TFA and B was 0.1% TFA in ACN.

The second dimension used an Agilent 1100 capillary HPLC system with online photodiode array detection. The autosampler was bypassed in favor of a 500 μ L injection loop. The proteins were eluted using a gradient of (A) 0.05% TFA in 10% ACN vs (B) 0.05% TFA in 50% ACN and 50% IPA. The gradient timetable was as follows: 25–60% B in 5 min, 60–72% B in 7 min, 72–95% B in 1 min, followed by holding the gradient at 95% B for 4 min, for a total run time of 17 min.

ES-MS. The Agilent HPLC was interfaced to a Micromass QTOF Ultima (Manchester, United Kingdom) quadrupole time-of-flight mass spectrometer. The instrument was tuned to a

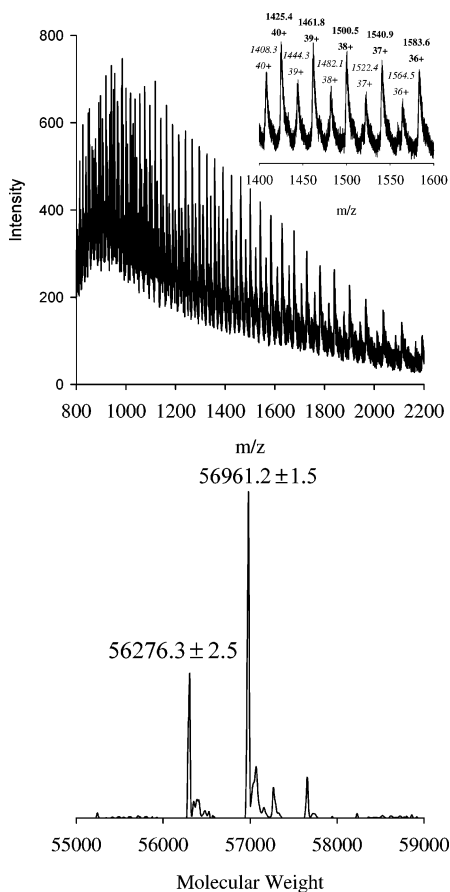


Figure 4. Mass spectrum (top) and deconvolved spectrum (bottom) of P450 3A4 (100 pm, RECO system) after 2 min of incubation with L-754,394 and NADPH. The inset shows expansion of the ion envelope for m/z 1400–1600 with labeled ion series (italics, apoprotein; bold, adducted protein).

resolution of 10000 (full-width at half-height) and calibrated using NaI clusters. Data acquisition (continuum mode) was over the mass range of 800 to 2300, with a spectrum being acquired every 2.5 s. Data acquisition and processing were carried out using MassLynx 3.5. Spectra were background subtracted (polynomial order 5, below curve % 35, tolerance 0.01) and a mass range of 1200–1600 was used for deconvolution. MaxEnt 1 was used for spectral deconvolution using continuum data. The output range was 55000–59000 Da using a resolution of 0.1 Da/channel. A Uniform Gaussian model with a width at half-height of 0.6–1 Da was used throughout. The MaxEnt process was permitted to continue iterating until convergence.

Results and Discussion

In this study, we employed L-754,394 (28) and DHB (29) as model compounds for covalent labeling of recombinant P450 3A4 (Figure 1). For L-754,394, it has been previously shown that it labels a protein corresponding to P450 3A4 using radioactive compound and HPLC with radiometric detection of the labeled protein (26, 31). For DHB, it is postulated that this compound is a mechanism-based inhibitor of P450 3A4, although no direct experimental evidence of this is available (no radiolabeled compound was available) (27, 29, 32).

Initial studies with these mechanism-based inhibitors of P450 3A4 involved optimizing incubation conditions (time and NADPH concentration) to give protein mass spectra with high signal-to-noise ratios to enable detection of adduct ions. Standard incubation conditions (60 min, 1 mM NADPH) resulted in mass spectra with low

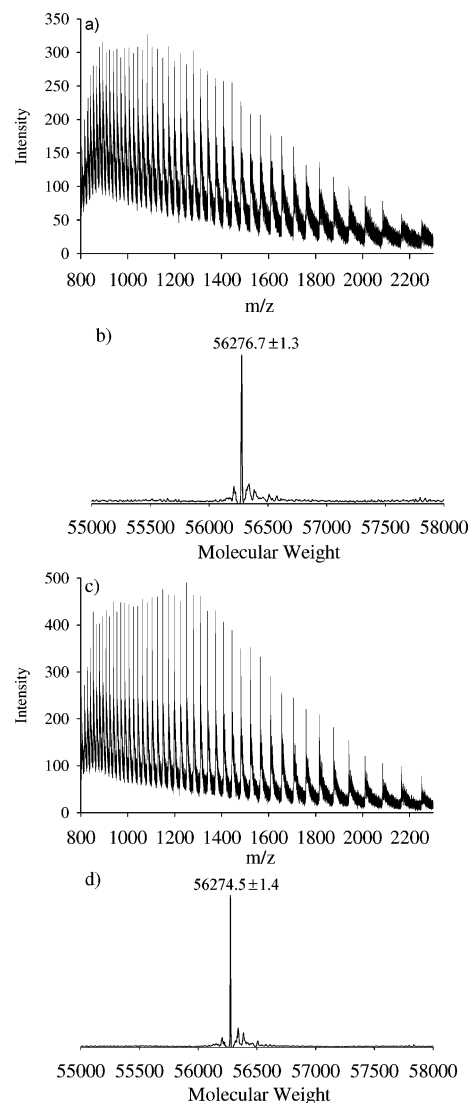


Figure 5. Mass spectra and deconvolved spectra of P450 3A4 from control 2 min RECO system incubations (a,b) +L-754,394, -NADPH and (c,d) -L-754,394, +NADPH.

signal-to-noise ratios and no clear ion envelope as typified by Figure 2. The degradation of P450s, as evidenced by mass spectral appearance, as a function of incubation time, NADPH concentration, and inhibitor is speculated to be a result of reactive oxygen species (26, 33). In support of this, degradation of P450 3A4 mass spectral signal-to-noise, in the absence of inhibitor, was observed as a function of NADPH concentration, with reduced S/N at higher NADPH concentrations (Figure 3). As a result of these observations, short incubation times (2–5 min) and a lower NADPH concentration (100 μ M) were used in these studies. The optimized conditions resulted in good quality mass spectra (see below), permitting the detection of the apoprotein and covalently labeled protein without the need for scavengers of reactive oxygen species such as catalase. The requirement for short incubation times and reduced concentrations of NADPH may explain why previous attempts at detecting adducts to P450 3A4 have been unsuccessful.

Incubations were prepared at least in triplicate to assess the accuracy and reproducibility of the measurement of the protein molecular mass and that of the adduct. The expected molecular masses for recombinant P450 3A4 and P450 reductase from the RECO system

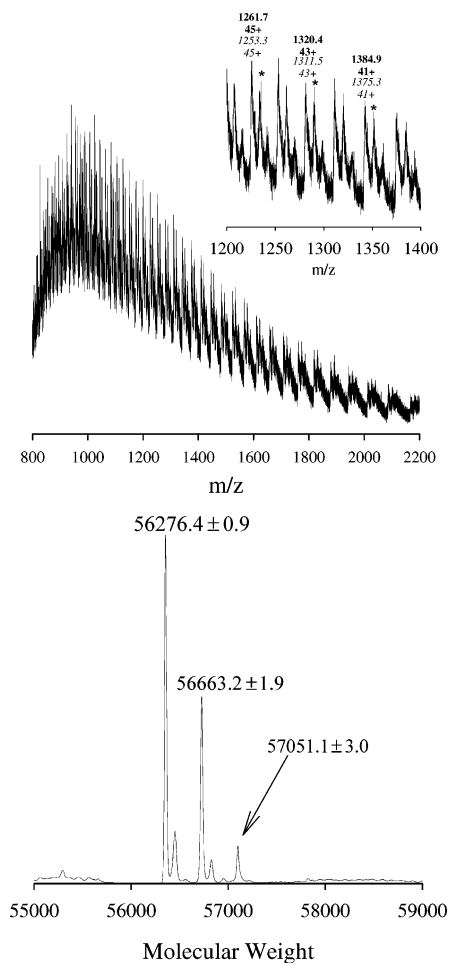


Figure 6. P450 3A4 (100 pm, RECO system) after a 3 min incubation with DHB and NADPH. Top, mass spectrum; bottom, deconvoluted spectrum. The inset shows expansion of m/z range 1200–1400 with labeled ion series (italics, apoprotein; bold, adducted protein, marked with *).

are 56277 and 76688 Da, respectively (34), and 57229 Da for the Gentest Supersomes recombinant P450 3A4 (35). The RECO recombinant P450 3A4 has a modified N-terminal required for high yield expression in *Escherichia coli*. All data presented below are consistent with these values giving mass accuracies ranging from 0.0005 to 0.016%.

Incubation of L-754,394 with a RECO P450 system resulted in the detection of the apoprotein and apoprotein plus 684.9 ± 2.9 Da (Figure 4). This mass shift corresponds to the mass of the inhibitor plus two oxygen atoms. Previously, several pathways have been proposed for the metabolic activation of L-754,394 to reactive metabolites that are implicated in protein labeling (26, 31). This method and the data that it generates provide new insights into the mechanism of inhibition and labeling. While the exact mechanism cannot be elucidated, certain ones potentially can be ruled out and others investigated with more confidence. For example, oxidation of the fused furanopyridine to an epoxide, followed by nucleophilic attack from the protein, seems less likely given the mass shift of the adduct. The error in the mass measurement suggests a double oxidation or an oxidation plus water as possibilities. Alternatively, oxidation of the protein could explain the second oxygen, but given that the apoprotein is not shifted by 16 amu, this seems less likely. Definitive proof of the mechanism

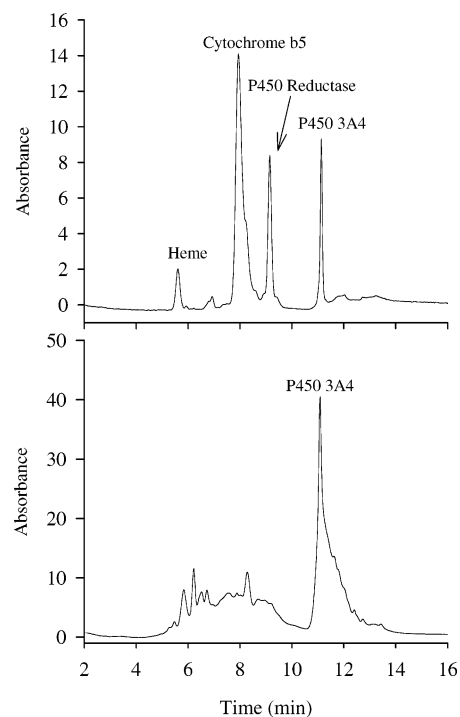


Figure 7. Comparison of the LC-UV chromatograms for the separation of the RECO incubation mixture (top) and the Supersome incubation mixture (bottom).

will require complete structural characterization of the peptide–inhibitor adduct. Control incubations carried out using only NADPH or L-754,394 did not show modification of the P450 reductase in the incubation was identical to that of the controls, and the molecular mass observed was as expected (data not shown). The fact that the P450 reductase was unchanged also reveals information about the compound. Once L-754,394 is metabolically activated, it only reacts with the P450 3A4 protein, indicating it does not have time to diffuse from the active site and label other proteins. Therefore, one can speculate on the relative reactivity of metabolites based on their propensity to label proteins other than P450 3A4. The literature value for the partition coefficient (moles product formed per moles enzyme inactivated) for L-754,394 is 1.35, consistent with a highly reactive intermediate (9, 26).

DHB is a component of grapefruit juice that has been implicated in many drug–drug interactions (27, 29, 32). The exact mechanism of inactivation has not been elucidated, although adduction to heme has been ruled out and indirect evidence suggests that inactivation is through modification of the apoenzyme (27, 32). LC-MS analysis of P450 3A4 after incubation with DHB resulted in the deconvoluted spectrum shown in Figure 6. The apoprotein is present, as well as a peak corresponding to the apoprotein plus 386.8 ± 2.1 Da. A second adduct is present as well, showing a mass shift of 387.9 ± 3.5 relative to the first adduct. Control experiments were identical to those observed for L-754,394 indicating that metabolic activation is required for protein labeling and that covalent labeling is occurring since no adducts are detected when DHB is incubated in the absence of NADPH (data not shown). These mass shifts can be rationalized by the mass of DHB plus one oxygen atom or the mass of two oxygen and two DHB molecules. The mass spectrum of the P450 3A4 is shown in the top panel

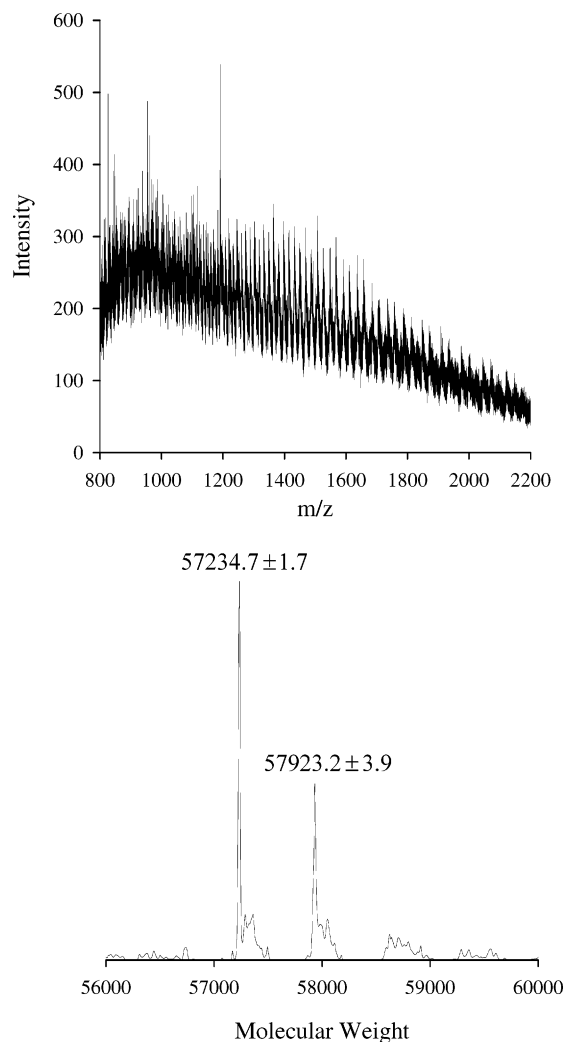


Figure 8. Mass spectrum (top) and deconvolution (bottom) for the analysis of P450 3A4 from Supersomes after 2 min of incubation with L-754,394 and NADPH (40 μ M injected).

of Figure 6. A magnification of the ion envelope reveals the appearance of the main P450 ion series, as well as the two series for the mono- and diadducts. The experiment was repeated several times ($n = 4$) to verify the reproducibility of the observed peaks.

Activation of the furan of DHB to an epoxide, followed by nucleophilic attack by the protein, could explain such an adduct. Furanocoumarins have been proposed to inactivate P450s through such a mechanism (36, 37). The fact that two adducts are detected could be used to support the binding of more than one ligand in the P450 active site. It has been postulated that P450s, and P450 3A4 in particular, can bind more than one substrate (38–40), and this is supported by the crystal structure of P450 3A4 (41). In their report, the authors' state that, "the relatively large size of the active site cavity near the catalytic center of the enzyme may contribute to the heterotropic cooperativity by facilitating alternative binding modes for multiple substrate molecules." In another published report of the P450 3A4 crystal structure (42), a small active site was found where "an unexpected peripheral binding site is identified, located above a phenylalanine cluster, which may be involved in the initial recognition of substrates or allosteric effectors." Alternatively, the adduct could be nonspecific binding of a reactive species outside of the active site, as postulated

for P450 2C9 and tienilic acid (19). However, given the high concentration of reduced glutathione used in these experiments, the latter explanation seems less likely as the GSH would act as a scavenger for electrophilic species. Regardless of the exact mechanism, the data provide direct experimental evidence that P450 3A4 is covalently modified by DHB, consistent with the indirect evidence previously reported (27, 32). No modification of P450 reductase in the incubation or P450 3A4 in the control incubations was observed.

To determine whether or not the detection of an adduct could be carried out in a more complex system, experiments using Supersomes were carried out. These samples are microsomes prepared from a cell line that overexpresses P450 3A4 and thus contain many other proteins. A comparison of the separation of the components of the RECO system vs the Supersomes is shown in Figure 7. It is apparent that the Supersome sample is a more complex system and more similar to, the microsomal system commonly used for in vitro covalent binding studies with radiolabeled compounds (2). Nonetheless, it is still possible to detect the intact P450 3A4 in this complex system, even when injecting 40 μ M of P450 3A4 (Figure 8). The deconvoluted spectrum shows the same adduct peak (protein + L-754,394 + 2O) as detected when using the RECO system.

Conclusions

The data presented here show, for the first time, direct mass spectral evidence of compounds that are metabolically activated and covalently bind to P450 3A4. The data provide insight into the reactive metabolites that covalently label P450 3A4, providing stoichiometry and mass of metabolite data. Experiments such as these are anticipated to assist our understanding of mechanism-based inhibitors in a drug discovery environment. The method uses picomole quantities of commercially available P450, making it readily applicable in other drug metabolism/MS laboratories. In the future, peptide mapping of the digested protein may also permit the identification of active site residues or contact sites for protein–inhibitor interactions.

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Supporting Information Available: LC-UV and MS chromatograms and deconvoluted spectra for Figure 3a–c, LC-UV and MS chromatograms for Figures 4–6 and 8, and a raw spectrum and expansion of the spectrum for Figures 4, 6, and 8. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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