Interaction between Human Serum Albumin and the Felbamate Metabolites 4-Hydroxy-5-phenyl-[1,3]oxazinan-2-one and 2-Phenylpropenal

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Felbamate is an anti-epileptic drug associated with hepatotoxicity and aplastic anemia. These toxicities are believed to be mediated by the formation of the reactive species 2-phenylpropenal. 4-Hydroxy-5-phenyl-[1,3]oxazinan-2-one is a metabolic precursor for 2-phenylpropenal. 4-Hydroxy-5-phenyl-[1,3]oxazinan-2-one exists in equilibrium with 3-oxo-2-phenylpropyl carbamate, which can undergo β-elimination to form 2-phenylpropenal. The work presented here investigates the interaction between 4-hydroxy-5-phenyl-[1,3]oxazinan-2-one and human serum albumin (HSA). HSA (40 mg/mL) was found to decrease the half-life of 4-hydroxy-5-phenyl-[1,3]oxazinan-2-one from 4.57 ± 0.44 h to 1.07 ± 0.10 h at pH 7.4. This decrease in the half-life of 4-hydroxy-5-phenyl-[1,3]oxazinan-2-one was due to increased β-elimination of 3-oxo-2-phenylpropyl carbamate, presumably through HSA-mediated general base catalysis. The k_{cat} for HSA-catalyzed decomposition of 4-hydroxy-5-phenyl-[1,3]oxazinan-2-one was determined to be 12.04 min^{-1} M^{-1}. Competitive binding assays using warfarin and ibuprofen showed that HSA-catalyzed decomposition of 4-hydroxy-5-phenyl-[1,3]oxazinan-2-one is dependent on the subdomain IIA binding site of HSA. LC/MS/MS analyses of trypsin digests of HSA incubations with either 4-hydroxy-5-phenyl-[1,3]oxazinan-2-one or 2-phenylpropenal identified HSA-2-phenylpropenal adducts formed specifically at residues His-242 and His-247. These HSA-2-phenylpropenal adducts were found to be slowly reversible, with a decrease in alkylation of 74.0 ± 0.6% after extensive dialysis. Interestingly, only the bis-adduct (His-242 and His-247) could be identified after dialysis. These results demonstrate the first direct example of 2-phenylpropenal conjugation to a human protein in vitro and suggest the possibility that HSA may be involved in the development of felbamate toxicity either by antigen formation or as a route of detoxification of 2-phenylpropenal.

Introduction

Idiosyncratic drug reactions are a type of adverse drug reaction characterized by their unpredictability, rare occurrence, delayed onset, and immune involvement (*1*, *2*). These drug toxicities can be severe and are of much concern to both physicians and pharmaceutical companies. Using the anti-epileptic drug felbamate as a model, our laboratory has focused on understanding what factors lead to idiosyncratic drug reactions.

Since its release in 1993, felbamate has been associated with 18 deaths resulting from either hepatotoxicity (*3*) or aplastic anemia (*4*). We have demonstrated that felbamate is metabolized to 3-oxo-2-phenylpropyl carbamate (CBMA, **7**)[1] (Scheme 1). Under physiological conditions, this aldehyde either spontaneously cyclizes to form the oxazinane 4-hydroxy-5-phenyl-[1,3]oxazinan-2-one (**6**) or undergoes a β-elimination to form the α,β-unsaturated aldehyde 2-phenylpropenal (**8**). By our hypothesis, 2-phenylpropenal is believed to mediate felbamate toxicity by depleting reduced glutathione levels and alkylating cellular proteins (*5*−*7*).

Idiosyncratic drug reactions are thought to involve both the bioactivation of a compound to a reactive intermediate and covalent binding of that reactive intermediate with proteins in vivo (*1*, *2*). Until recently, only indirect evidence has demonstrated that 2-phenylpropenal is capable of alkylating cellular proteins. Such evidence has included conjugation of 2-phenylpropenal with biologically important nucleophiles such as glutathione and *N*-acetylcysteine, as well as irreversible inhibition of glutathione-*S*-transferase M1-1 (*6*, *8*). Our current work has focused on demonstrating the ability of 2-phenylpropenal to form conjugates with human proteins.

Previous work in our laboratory to explore the chemistry of the oxazinane presented the possibility that 2-phenylpropenal may alkylate the protein human serum albumin (HSA).[1] In those experiments, it was demonstrated that the half-life of the oxazinane at pH 7.4 is greatly reduced in the presence of HSA when compared to aqueous buffer (*7*). A similar phenomenon had been observed with 4-hydroxycyclophosphamide, an antineoplastic agent that displays very similar chemical

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[1] Abbreviations: CBMA, 3-oxo-2-phenlypropyl carbamate; HSA, human serum albumin; ESI, electrospray ionization.

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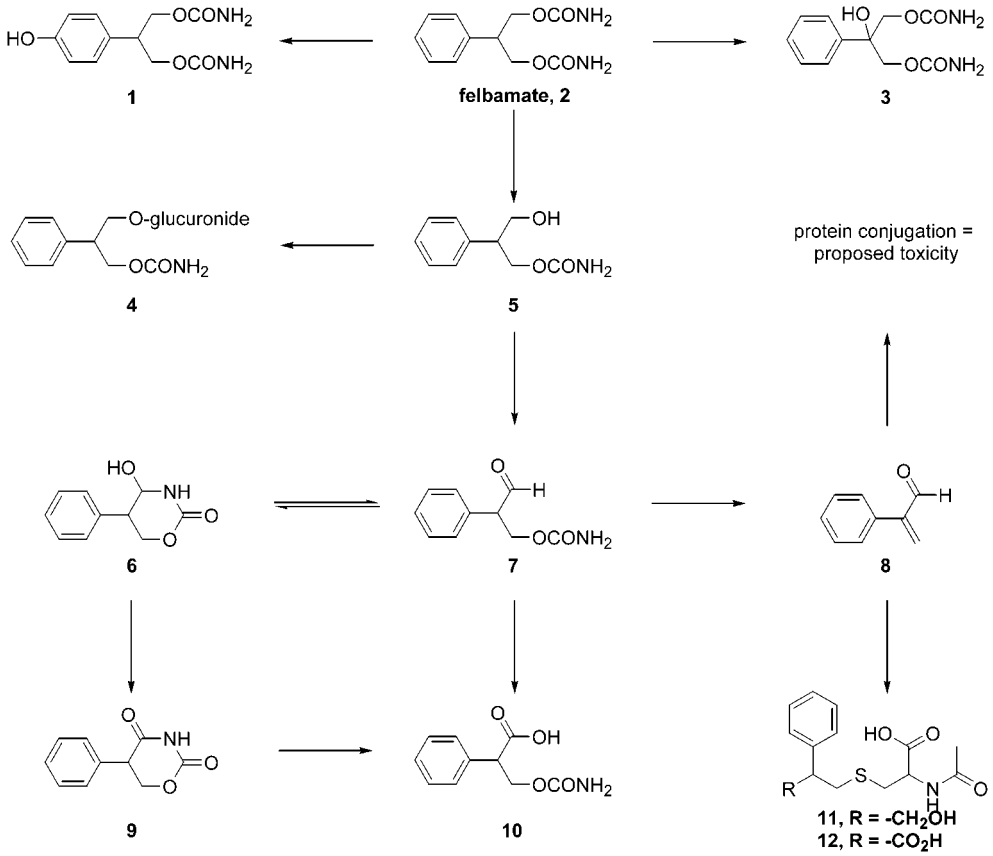
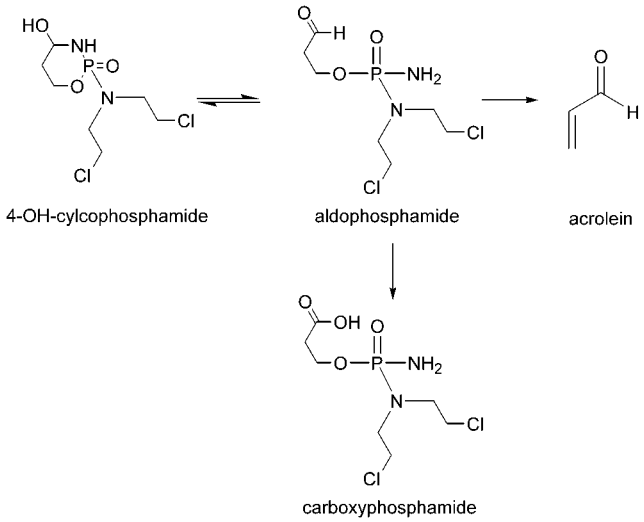
Scheme 1. Metabolism of Felbamate



Scheme 2. Cyclophosphamide Bioactivation



behavior under physiological conditions (Scheme 2). In the case of 4-hydroxycyclophosphamide, the reduction in half-life was due to the HSA-catalyzed β-elimination to form a phosphoramide mustard and acrolein (*9*). During the course of our experiments with the oxazinane, we realized that 2-phenylpropenal which normally appeared in the phosphate buffer incubations was not present in the albumin incubations when analyzed by HPLC/UV detection. We presumed that the missing 2-phenylpropenal was binding to HSA either by hydrophobic binding or covalent modification. On the basis of our observations, we began to investigate the interaction between HSA and the oxazinane.

HSA is the most abundant protein in blood serum (*10*). Much is known about its structure and interaction with both endogenous molecules and xenobiotics. Many drug molecules bind strongly to HSA in at least one of its two hydrophobic pockets known as subdomain IIA (also referred to as Sudlow's Site I) and subdomain IIIA (also referred to as Sudlow's Site II) (*11*). HSA has been shown to display some limited esterase activity with drugs such as aspirin (*12*), penicillins (*13*), trinitrobenzene sulfonate (*14*), and various *p*-nitrophenyl esters (*15*, *16*). HSA is also known to catalyze the β-elimination of certain prostaglandin species (*17*−*19*). Most of these reactions appear to occur within the subdomain IIA binding site and are mediated by the nucleophilic attack or basicity of Lys-199. Further, a result of the hydrolysis reactions is the alkylation of Lys-199.

The purpose of this study was to further characterize the interaction between the oxazinane and HSA. Specificially, we were interested in investigating the mechanism by which HSA accelerates the decompostion of the oxazinane and determining if the resulting 2-phenylpropenal was reacting with HSA.

Experimental Procedures

Chemicals and Instruments. Catalase, γ-globulins, and essentially fatty acid- and globulin-free human serum albumin was purchased from Sigma (St. Louis, MO). HPLC analyses were carried out using either a Waters 2690 Separations Module or a TSP P4000. HPLC-UV detection was achieved using a Waters 486 Tunable detector equipped with a microbore flow cell. LC-MS was carried out using a Finnigan LCQ ion trap mass spectrometer using electrospray ionization (ESI).[1] All experiments were performed in at least triplicate with the exception of the dialysis experiments which were performed in duplicate.

Synthesis of 2-Phenylpropenal. 2-Phenylpropenal was synthesized as previously described (*8*).

Synthesis of 4-Hydroxy-5-phenyl-[1,3]oxazinan-2-one. 4-Hydroxy-5-phenyl-[1,3]oxazinan-2-one, the oxazinane, was synthesized as previously described (*7*).

Stability of 4-Hydroxy-5-phenyl-[1,3]oxazinan-2-one in the Presence of Various Protein Solutions. The oxazinane (100 μM) was incubated at 37 °C in 100 mM potassium phosphate buffer (pH 7.4) containing either human serum albumin, catalase or γ-globulins (40 mg/mL). At each time point, 100 μL of the sample was removed and mixed with 100 μL of 100 μM felbamate as internal standard in 2% acetic acid. Protein was then removed by filtration through a Microcon YM-10 centrifugal filtration device. The concentration of the oxazinane was determined by HPLC using a Waters Symmetry C_{18} column (2.1 × 150 mm) with an isocratic mobile phase of 30% acetonitrile:70% (0.1%) acetic acid. The concentration of the oxazinane was quantified based on a linear calibration curve using felbamate as an internal standard. The half-life was determined using a first-order approximation and plotting ln [oxazinane] versus time.

HSA-Dependent Decompostion of 4-Hydroxy-5-phenyl-[1,3]oxazinan-2-one. The oxazinane (100 μM) was incubated at 37 °C in 100 mM potassium phosphate buffer (pH 7.4) containing human serum albumin (0, 10, 20, 40, or 60 mg/mL). At each time point, 100 μL of the sample was removed and mixed with 100 μL of 100 μM felbamate as internal standard in 2% acetic acid. Sample preparation and quantification of the oxazinane was performed as described above. The k_{cat} value for decomposition of oxazinane by HSA was determined by plotting k versus [HSA]. This is the same method used by Kwon et al. for 4-hydroxycyclophosphamide (*9*).

To determine if the oxazinane was hydrophobically bound to HSA, a similar set of incubations using HSA (40 mg/mL) was performed under the exact conditions as above, but used 100 μL of 100 μM felbamate in acetonitrile to quench the reaction. Next, 10 μL of 20% acetic acid was added and the samples were centrifuged at 15800*g* for 5 min. The supernatant was removed and analyzed as described above.

Effect of Warfarin and Ibuprofen on the HSA-Dependent Decompostion of 4-Hydroxy-5-phenyl-[1,3]oxazinan-2-one. Ibuprofen or warfarin (600 μM) was incubated at 37 °C in 100 mM potassium phosphate buffer (pH 7.4) containing human serum albumin (40 mg/mL) for 40 min. Next, the oxazinane (100 μM) was added to the incubation. The half-life of the oxazinane under each set of conditions was determined as described above.

Alkylation of HSA. Human serum albumin (4 mg/mL) was incubated in 500 μL of 0.25 M Tris-HCl buffer (pH 7.4) containing 300 μM of either 2-phenylpropenal or the oxazinane at 37 °C. Incubations with 2-phenylpropenal were stopped after 30 min by the addition $NaBH_4$ (10 mg). Incubations with the oxazinane were stopped after 4 h in the same manner. $NaBH_4$ was allowed to react for 30 min, at which point incubations were acidified at about pH 6 with 20% acetic acid (150 μL) then centrifuged for 10 min at 1000*g*, 4 °C. Samples were then filtered through Microcon YM-10 centrifugal filtration devices (Millipore, Bedford, MD). Protein was reconstituted in 500 μL 0.25 M Tris-HCl buffer (pH 8.5) containing 6 M guanidine-HCl, 1.25 mM EDTA, and dithiothreitol (~3 mg) and incubated for 2 h at 37 °C. Iodoacetic acid (10 mg) was added and the samples were incubated for 30 more minutes at 37 °C. Samples were desalted by passage through PD-10 gel filtration cartridges (Amersham-Pharmacia, Piscataway, NJ) equilibrated with 0.4% ammonium bicarbonate. Eluted protein was lyophilized, reconstituted in 5% acetic acid (200 μL), and lyophilized again in preparation for tryptic digest.

Tryptic Digest of HSA. Protein alkylated and denatured as described above was reconstituted in 0.4% ammonium bicarbonate (500 μL) and digested with trypsin (20 μg). The digest was allowed to proceed for 20 h at 37 °C. Samples were then lyophilized, reconstituted in 5% acetic acid (200 μL), lyophilized, and again reconstituted in 5% acetic acid (200 μL). Samples were stored at 4 °C until HPLC analysis. Just prior to analysis, samples were sonicated for 15 min and diluted 1:1 with 5% acetic acid and 5 μL were injected onto a Vydac C_{18} Mass Spec column (1.0 × 250 mm). Separation was achieved using a 3-step gradient using 0.02% TFA (solvent A) and acetonitrile (solvent B). Mobile phase consisted of 100% solvent A from 0 to 5 min, then the concentration of solvent B was increased to 32% over the next 80 min. Over the final 40 min the concentration of solvent B was increased to 55%.

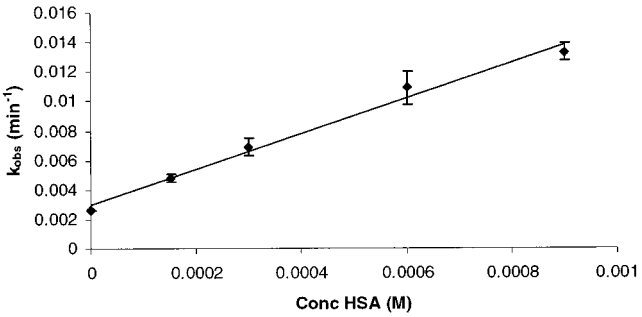


Figure 1. Effect of HSA concentration on the rate constant of decomposition of 4-hydroxy-5-phenyl-[1,3]oxazinan-2-one.

Table 1. Half Life of 4-Hydroxy-5-phenyl-[1,3]oxazinan-2-one under Various Conditions

conditions[a]	$t_{1/2}$ (h)
control	4.57 ± 0.44
γ-globulins (40 mg/mL)	3.04 ± 0.00
catalase (40 mg/mL)	3.15 ± 0.08
HSA (40 mg/mL)[b]	1.07 ± 0.10
HSA (40 mg/mL) + ibuprofen (600 μM)	1.14 ± 0.04
HSA (40 mg/mL) + warfarin (600 μM)	2.05 ± 0.06

[a] All incubations in 100 mM sodium phosphate buffer pH 7.4. [b] 40 mg/mL HSA corresponds to 600 μM.

MS of Peptides. Since 2-phenylpropenal-HSA adducts do not contain fluorophores or other markers through which to be detected, alkylated peptides were identified strictly by searching for *m*/*z*. Peptides were examined for *m*/*z* corresponding to either addition of CBMA via imine formation or addition of 2-phenylpropenal by imine formation or Michael addition. Residues considered as candidates for sites of alkylation included the subdomain IIA and subdomain IIIA residues Lys-190, Lys-195, Lys-199, His-242 His-247, and Tyr-411. Other residues, including Cys-34, Lys-414, Lys-545, His-146, and His-338 were also considered due to reported covalent coupling with other reactive molecules (*13*, *16*, *20*−*23*). For lysine residues, the possibility that trypsin may not cleave the protein if lysine was alkylated was taken into consideration when calculating *m*/*z*. The possibility that some residues contained one or more modifications was also investigated. Only peaks that did not appear in the control samples were considered. Values for LC/MS of tryptic digests were as follows: heated capillary temperature = 190 °C; spray voltage = 4.9 kV; capillary voltage = 30 V; sheath gas flow rate = 45; auxiliary gas flow rate = 30. Data were collected in full scan positive ion mode with a mass range of *m*/*z* 205−2000. Automatic gain control was set at 5 × 10^7 and maximum injection time was set at 300 ms. The number of microscans was set at 3.

MS/MS Experiments. Values for MS/MS experiments of tryptic digests were as follows: heated capillary temperature = 190 °C; spray voltage = 4.9 kV; capillary voltage = 30 V; sheath gas flow rate = 45; auxiliary gas flow rate = 30. The parent ion was set at either *m*/*z* 742 or 787. Collision energy was set at 40%, isolation width was set at 1.5 amu, automatic gain control was set at 2 × 10^7 and maximum injection time was set at 500 ms. The number of microscans was set at 1.

Dialysis of Alkylated HSA. Dialysis experiments were performed in order to investigate the reversibility of 2-phenyl-

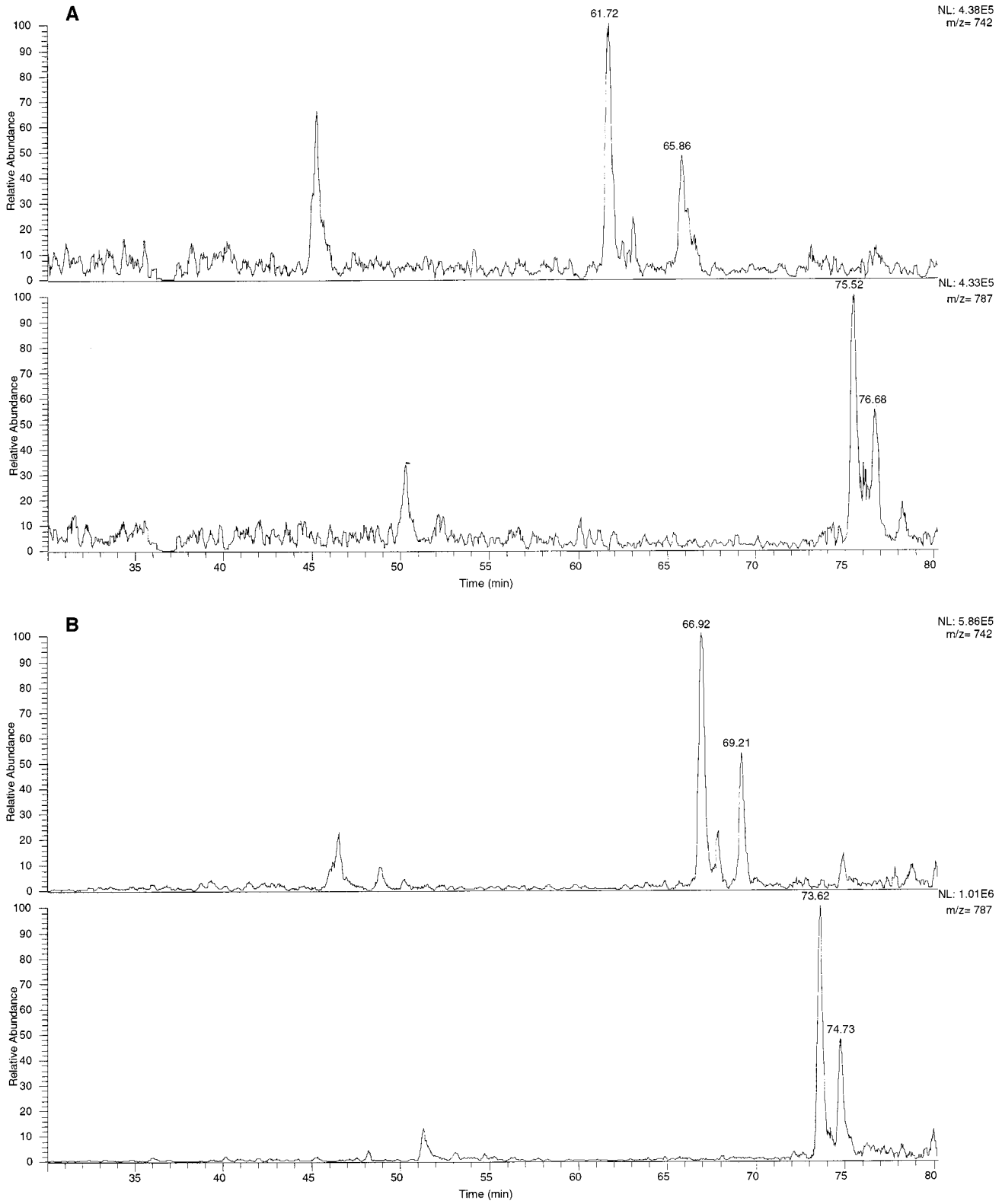


Figure 2. (A) LC/MS analysis of peptide digest of HSA incubated with 2-phenylpropenal. (Top) LC chromatograph with mass filter *m*/*z* 742 showing peak associated with alkylation at His-242 at $RT = 61.72$ min and peak associated with alkylation at His-247 at $RT = 65.86$ min. (Bottom) LC chromatograph with mass filter *m*/*z* 787 showing peaks associated with the dialkylated peptide at $RT = 75.52$ and 76.68 min. (B) LC/MS analysis of peptide digest of HSA incubated with the oxazinane. (Top) LC chromatograph with mass filter *m*/*z* 742 showing peak associated with alkylation at His-242 at $RT = 66.92$ min and peak associated with alkylation at His-247 at $RT = 69.21$ min. (Bottom) LC chromatograph with mass filter *m*/*z* 787 showing peaks associated with the dialkylated peptide at $RT = 73.62$ and 74.73 min. All peaks were confirmed by MS/MS.

propenal-HSA adducts. Human serum albumin was alkylated by 2-phenylpropenal as described in "Alkylation of HSA"; however, before adducts were trapped by the addition of $NaBH_4$, samples were placed into a 0.1−0.5 mL 10 000 MWCO Slide-A-Lyzer dialysis cassette (Pierce, Rockford, IL) and dialyzed against 1 L of 100 mM potassium phosphate buffer (pH 7.4). The dialysis buffer was changed once daily for 3 days. On the fourth day, the protein was removed from the dialysis cassette

and carried through the rest of the alkylation and digest procedure. The percent of alkylated peptide was determined with and without dialysis by the following method. In each sample, the areas of the peaks for the mono- and dialkylated peptides were added together and divided by the sum of the areas of the peaks for the monoalkylated, dialkylated, and native peptide of the same sequence. This ratio in dialyzed protein samples was compared to the same ratio in undialyzed protein samples to give a "percent change in alkylation" value. Dialyzed and undialyzed incubations were performed in duplicate.

Results

HSA-Catalyzed Decomposition of the Oxazinane. HSA was found to decrease the half-life of 4-hydroxy-5-phenyl-[1,3]oxazinan-2-one at pH 7.4. At the physiological concentration of HSA (40 mg/mL) the half-life of the oxazinane is reduced from 4.57 ± 0.44 h to 1.07 ± 0.10 h. Because the half-life of the oxazinane decreased linearly as the concentration of HSA was increased, a k_{cat} for HSA on disappearance of the oxazinane could be determined by plotting k vs [HSA] (Figure 1). Based on this method, HSA was found to have a k_{cat} of 12.04 min^{-1} M^{-1}.

To ensure that the observed decrease in the oxazinane half-life was truly a result of its accelerated decomposition and not a result of its time-dependent hydrophobic binding to HSA, we repeated the above experiment, but this time stopped the reaction with acetonitrile. Acetonitrile should disrupt any hydrophobic binding of the oxazinane to HSA. If the oxazinane demonstrated time-dependent hydrophobic binding to HSA, we would expect to observe no decrease in its half-life. Results from these experiments showed that the half-life of the oxazinane was 0.73 ± 0.11 h, which is similar to our previous HSA incubations. On the basis of these results, it seems that the disappearance of the oxazinane is due to decomposition rather than hydrophobic binding. Further, 2-phenylpropenal produced in the incubations may be covalently binding to HSA.

In an effort to demonstrate that the HSA-catalyzed decomposition of the oxazinane is a phenomenon specific to HSA, incubations with the control proteins γ-globulins and catalase were performed. In each of these cases, the half-life of the oxazinane was 3 h (Table 1). Only HSA was capable of dramatically decreasing the half-life.

Determination of the Active Site on HSA for Decomposition of the Oxazinane. To determine what location on HSA is responsible for the decomposition of the oxazinane, incubations with warfarin, a subdomain IIA binding compound (*24*), and ibuprofen, a subdomain IIIA binding compound (*25*), were performed (Table 1). The half-life of the oxazinane in the presence of HSA and warfarin was 2.05 ± 0.06 h. In the presence of HSA and ibuprofen, the half-life of the oxazinane was determined to be 1.14 ± 0.04 h. The half-life in incubations without either drug was 1.07 ± 0.10 h. On the basis of the increased half-life in the warfarin incubations, it appears that subdomain IIA is involved in the HSA-catalyzed decomposition of the oxazinane.

LC/MS of Tryptic Digest Of Oxazinane and HSA Incubations. Four peaks were found that contained a modification upon incubation with either the oxazinane or 2-phenylpropenal (Figure 2). For each of these, the triply charged ion was the most abundant by ESI-MS. Two peaks with *m*/*z* 742 were identified which is consistent with alkylation of the peptide VHTECCHGDLLECADDR (residues 241−257) via Michael addition of 2-phenylpropenal at one residue. Two peaks were observed with *m*/*z* 787 which is consistent with alkylation of the same peptide at two residues.

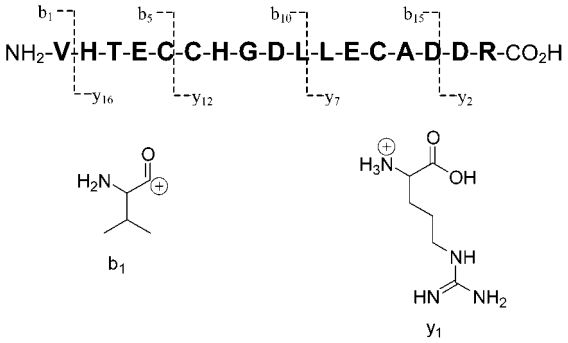


Figure 3. Nomenclature for *y* and *b* series ions resulting from MS/MS fragmentation of the peptide containing HSA residues 241−257.

MS/MS of Alkylated Peptides. MS/MS experiments performed for both *m*/*z* 742 and 787 confirmed that 2-phenylpropenal modifies the peptide VHTECCHGDLLECADDR. For each set of MS/MS experiments, many of the corresponding *y* and *b* fragmentation ions were identified (Figure 4). Of these fragmentation ions, the *b* series was the most useful in discerning residues alkylated by 2-phenylpropenal. For the first peak with *m*/*z* 742, the b_2, b_9^{2+}, b_{10}^{2+}, b_{12}^{2+}, b_{15}^{2+}, and b_{16}^{2+} ions have a *m*/*z* $(134 + b_n^{z+})/z$ where b_n^{z+} is the analogous b ion of the native peptide with charge *z*. The only way the b_2 ion would have an increased *m*/*z* is through modification of the His-242 residue. Therefore, the first monoalkylated peptide was assigned the sequence VHTECCHGDLLECADDR, with alkylation at His-242. For the second peak with *m*/*z* 742, the *m*/*z* of the b_2 ion is 237.2 which corresponds to no alkylation at His-242. The b_9^{2+}, b_{15}^{2+} ions both have a *m*/*z* $(134 + b_n^{z+})/z$ where b_n^{z+} is the analogous b ion of the native peptide with charge *z*. On the basis of this, one can assume that alkylation is at the His-247 residue. This assumption can be made because the cysteine residues have been modified by iodoacetic acid and no other residues from 241 to 249 contain sites nucleophilic enough to react with 2-phenylpropenal. This assumption is also supported by the fact that the y_{10} ion is the same *m*/*z* as in the native peptide while greater y ions show an increase in *m*/*z* of 134. Both peaks with *m*/*z* 787 gave the same MS/MS spectrum. For each peak, the b_7, b_9^{2+}, b_{10}, b_{12}^{2+}, b_{15}^{2+}, and b_{16}^{2+} ions have a *m*/*z* $(268 + b_n^{z+})/z$ when compared to the analogous b ion of the native peptide. Although the b_2 ion was not visible, it is again reasonable to conclude that the increase in the *m*/*z* for b_7 was due to alkylation at His-242 and His-247 since residues 241−247 contain no other nucleophilic residues. The appearance of two peaks with *m*/*z* 787 is likely due to the formation of regioisomers or diastereomers. These possibilities arise because the imidazole moiety can undergo alkylation at the 1- or 3-positions and the 2-position of 2-phenylpropenal becomes chiral as a result of the Michael reaction. Indeed, each peak may represent collections of isomers associated with alkylation at His-242 and His-247.

Reversibility of Alkylation by 2-Phenylpropenal. On the basis of dialysis experiments, the alkylation of HSA by 2-phenylpropenal is reversible on the scale of several days. After 4 days of dialysis, the percent change

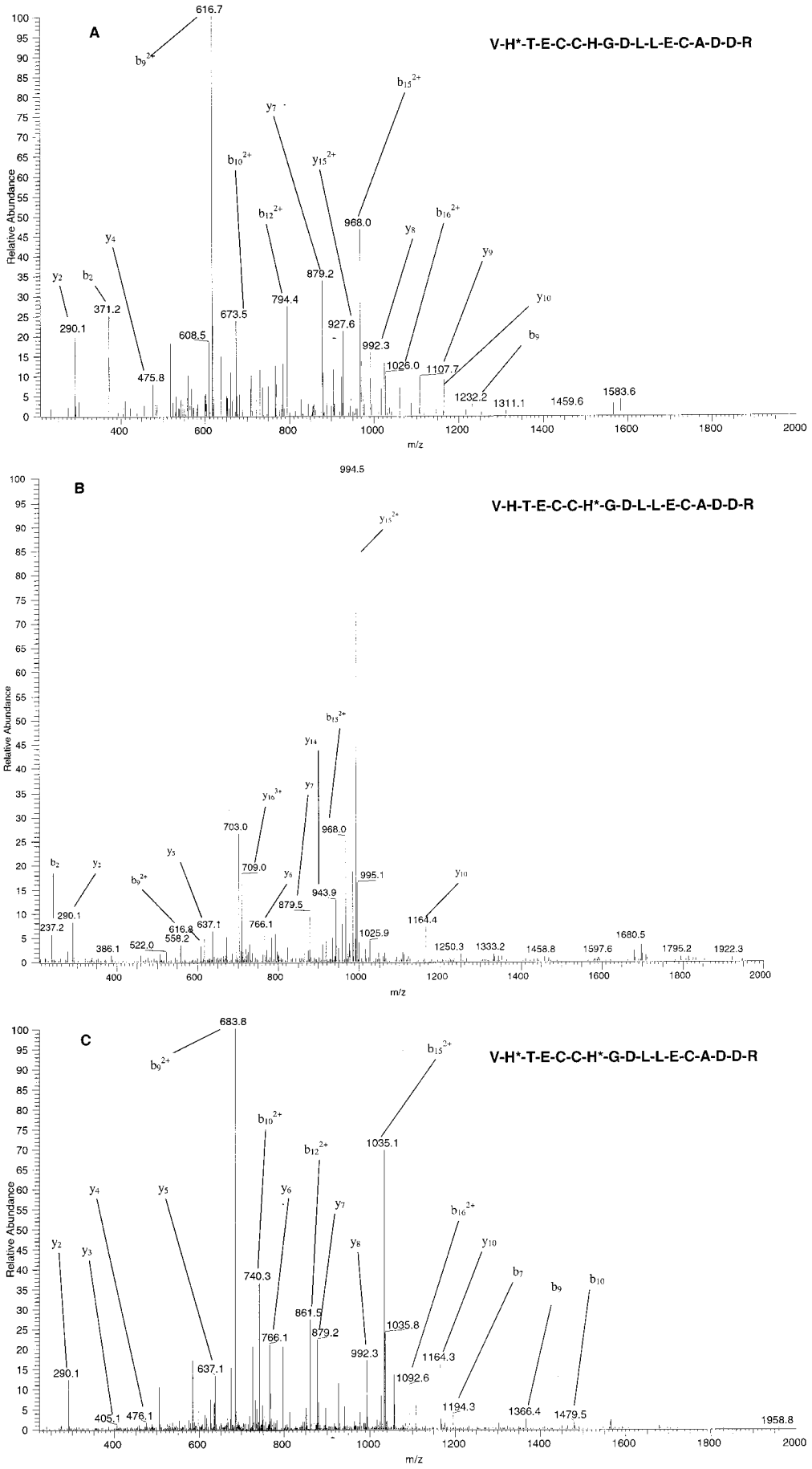


Figure 4. Results of MS/MS experiments for (A) alkylation at His-242, (B) alkylation at His-247, and (C) alkylation at both His-242 and His-247. The *y* and *b* fragmentation ions are labeled accordingly.

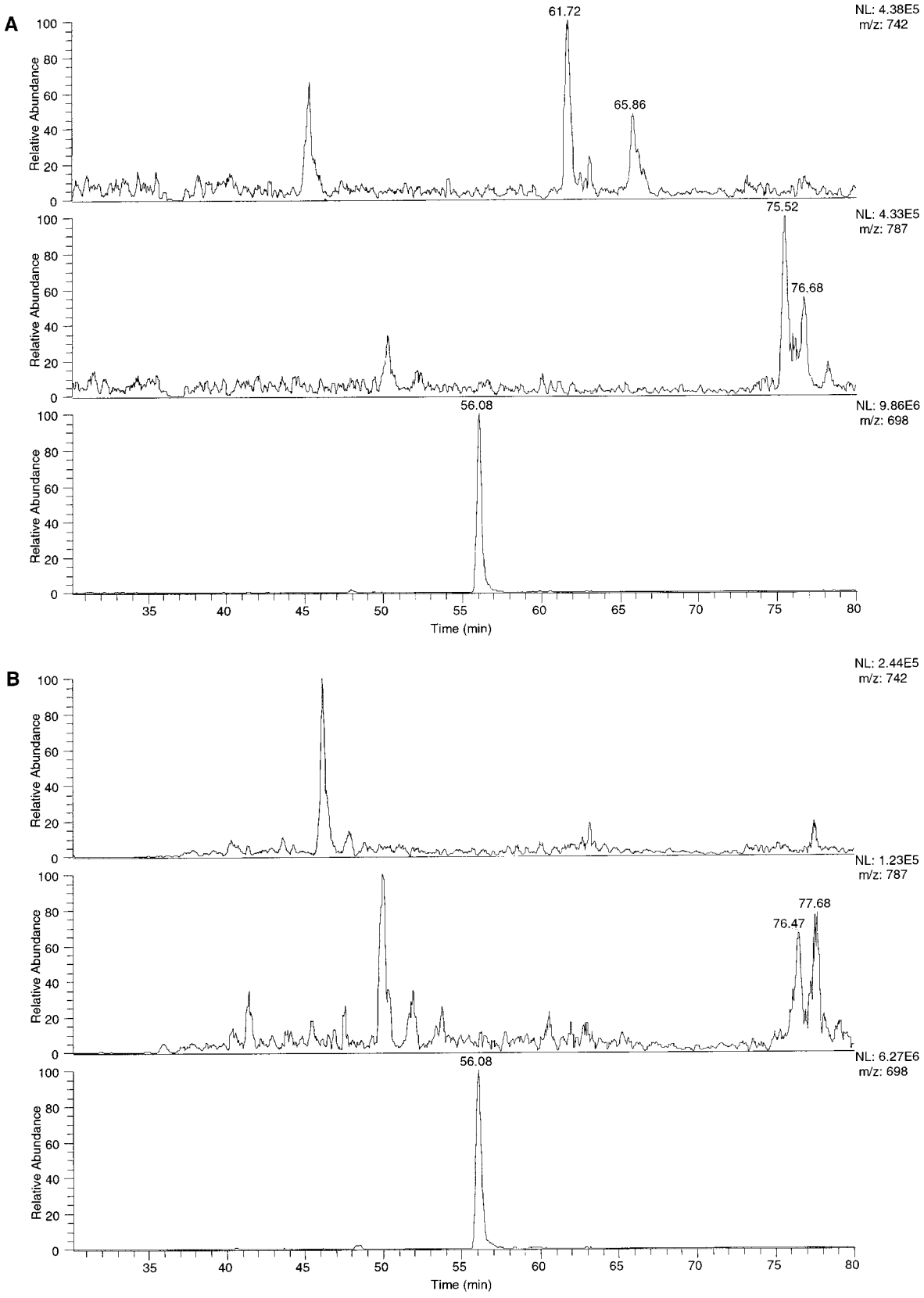
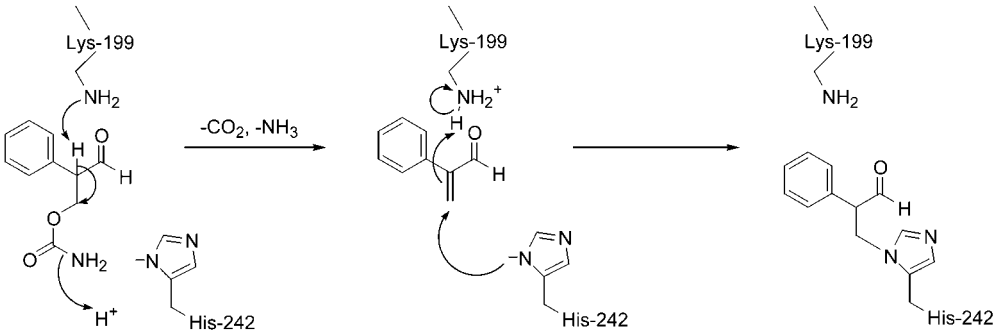


Figure 5. LC/MS analysis of peptide digests of HSA incubated with 2-phenylpropenal showing the effect of dialysis. Each sample was incubated with 2-phenylpropenal for 30 min. (A) Sample denatured and digested without dialysis. (Top) LC chromatograph with mass filter *m*/*z* 742 showing peak associated with alkylation at His-242 at $RT = 61.72$ min and peak associated with alkylation at His-247 at $RT = 65.86$ min. (Middle) LC chromatograph with mass filter *m*/*z* 787 showing peaks associated with the dialkylated peptide at $RT = 75.52$ and 76.68 min. (Bottom) LC chromatograph with mass filter *m*/*z* 698 showing peak associated with native peptide of same sequence. (B) Sample denatured and digested after after dialysis in 100 mM phosphate buffer (pH 7.4) which was changed daily for 4 days. (Top) LC chromatograph with mass filter *m*/*z* 742 showing no peaks observed for either monoalkylated peptide. (Middle) LC chromatograph with mass filter *m*/*z* 787 showing peaks associated with the dialkylated peptide at $RT = 76.47$ and 77.68 min. (Bottom) LC chromatograph with mass filter *m*/*z* 698 showing peak associated with native peptide of same sequence.

Scheme 3. Proposed Mechanism for HSA-Catalyzed Decomposition of 4-Hydroxy-5-phenyl-[1,3]oxazinan-2-one



in alkylated peptide was 74.0 ± 0.6%. While this is a rough estimate for the extent of dialysis, it does provide some information about the reversibility of HSA alkylation by 2-phenylpropenal. The fact that some alkylation was still observed after dialysis means that the reverse reaction must occur at a relatively slow rate. It is interesting to note that, after dialysis, only dialkylated peptide was detectable. No monoalkylated peptide was observed (Figure 5).

Discussion

Human serum albumin has been shown to catalyze the disappearance of the 4-hydroxy-5-phenyl-[1,3]oxazinan-2-one metabolite of felbamate under physiological conditions. In the presence of HSA, the rate of disappearance of the oxazinane is the same as in a buffer solution at a pH ≥10. This phenomenon appears to be selective for HSA since other proteins that were screened, catalase and γ-globulins, did not affect the half-life of the oxazinane as dramatically as HSA.

The HSA-catalyzed disappearance of the oxazinane appears to be due to decomposition rather than simply hydrophobic binding to HSA. The k_{cat} for the decompostion of the oxazinane by human serum albumin was calculated to be 12.04 min^{-1} M^{-1}. This k_{cat} is an order of magnitude lower than the k_{cat} for the HSA-catalyzed decomposition of the structurally similar molecule 4-hydroxycyclophosphamide (k_{cat} = 285 min^{-1} M^{-1}) (*9*) and about the same value as for the HSA-catalyzed β-elimination of various prostaglandins (k_{cat} ranges from ∼1 to 21 min^{-1} M^{-1} as calculated using data reported in ref *17*).

Although the k_{cat} for the decomposition of the oxazinane seems to be small, this interaction between HSA and the oxazinane may have some significance in vivo. Our kinetics experiments attempted to mimic physiological conditions in which the concentration of HSA is in excess of the oxazinane. Because the physiological concentration of HSA is so high in plasma (600 μM) and the concentration of the oxazinane is relatively low (<50 μM), the effect on the half-life of the oxazinane is quite dramatic. Under physiological conditions, the half-life of the oxazinane is reduced by over 75%. As a consequence, HSA may contribute considerably to the metabolism of the oxazinane in vivo.

On the basis of experiments performed with warfarin and ibuprofen, it seems that the subdomain IIA binding site of HSA is involved in the decomposition of the oxazinane. Warfarin is known to specifically bind to subdomain IIA of HSA with a high affinity (K_A = 3.3 × 10^5 M^{-1}) (*24*), whereas ibuprofen is known to specifically bind to subdomain IIIA (K_A = 2.7 × 10^6 M^{-1}) (*25*). Half-life experiments revealed that only warfarin appears to compete with the oxazinane for binding to HSA. Warfarin had a significant effect on the disappearance of the oxazinane, indicating that subdomain IIA is important. As mentioned earlier, subdomain IIA is known to be involved in the hydrolysis of several small molecules such as aspirin, penicillins, and trinitrobenzene sulfonate through nucleophilic attack by Lys-199. In these examples, Lys-199 is covalently modified at the ε-NH_2. While 2-phenylpropenal does not alkylate Lys-199, Lys-199 may act as the basic residue that catalyzes the β-elimination and leads to the decomposition of the oxazinane. The same idea has been proposed for the HSA-mediated β-elimination of several prostaglandins (*19*).

In addition to the disappearance of the oxazinane, the covalent binding of 2-phenylpropenal to HSA was also observed. The major sites of alkylation as determined by the methods described in this paper are residues His-242 and His-247. The same alkylation pattern was observed when HSA was incubated with either the oxazinane or with 2-phenylpropenal itself. This finding is rather remarkable considering the high electrophilicity normally associated with 2-phenylpropenal (*8*). On the basis of other examples in the literature, one may expect a molecule as reactive as 2-phenylpropenal to alkylate other solvent-accessible nucleophilic residues such as Cys-34 or Lys-199 (*22*, *12*−*14*); however, no evidence of these possibilities could be found.

The apparent selectivity of the two alkylation sites is very interesting. On the basis of the crystal structure of HSA, these two residues lie just across the hydrophobic pocket of subdomain IIA from Lys-199 (*23*, *26*). In fact, the nucleophilicity of Lys-199 is attributed to hydrogen bonding between the ε-NH_2 of Lys-199 and the imidazole of His-242. Using this structural information and the results of the HSA incubations with the oxazinane, one could propose a mechanism for the HSA-catalyzed decomposition of the oxazinane (Scheme 3). In this mechanism, the oxazinane binds to HSA as CBMA in subdomain IIA in the vicinity of Lys-199. Lys-199 then acts as a general base to deprotonate CBMA at the benzyllic position. The 2-phenylpropenal that is generated by the subsequent β-elimination can then alkylate the neighboring His-242 or His-247 residues.

There is some difficulty in directly relating the alkylation of HSA to the accelerated decomposition of the oxazinane. Data reported from our in vitro experiments are from incubations in which a 5-fold excess of alkylating agent was used. Evidence of alkylation was observed in incubations that used lower concentrations of alkylating

agent (down to ~0.5 M equiv, data not shown); however, characterization of the peaks becomes difficult at these lower concentrations. When alkylation experiments were performed at the same conditions as the half-life experiments, evidence of alkylation could not be detected; however, this is likely due to the low proportion of alkylated protein to native protein expected at these conditions and does not invalidate our hypothesis. Evidence supporting our hypothesis includes the fact that the 2-phenylpropenal peak that is normally detected by UV−vis in assays performed in buffered solutions is not detected in the presence of HSA and the fact that at higher concentrations of oxazinane, selective alkylation of HSA is apparent.

Whether or not alkylation of HSA by 2-phenylpropenal occurs under in vivo conditions is currently unknown. In vivo, HSA has a half-life of approximately 20 days (*10*) and would be exposed to constant low levels of the oxazinane in patients undergoing felbamate therapy. Under such conditions, it is not unlikely that low levels of HSA-2-phenylpropenal adducts, similar to those observed in vitro, would exist. This is the subject of current investigations.

The alkylation of HSA by 2-phenylpropenal is significant for several reasons. Most importantly, this is the first direct example of 2-phenylpropenal alkylating a human protein. Indirect evidence has included the formation of glutathione conjugates both in vitro and in vivo, and the irreversible inhibition of glutathione-*S*-transferase M1-1 (*6*, *8*). In addition, these results point to HSA as a possible antigen for the immune-mediation believed to be involved in felbamate toxicity. Anti-HSA antibodies have been observed in patients suffering from drug allergies to other drugs such as penicillin (*27*) and nitrofurantoin (*28*). To date, patients on felbamate therapy have not been screened for anti-HSA antibodies.

Dialysis experiments show that the alkylation of HSA by 2-phenylpropenal is reversible; however, given that even after 4 days of dialysis some alkylated peptide is still present, the release of 2-phenylpropenal is likely to be very slow. The physiological significance of this involves several possibilities. The fact that this covalent modification appears to be slowly reversible means that these HSA-2-phenylpropenal adducts may be stable enough to survive protein processing and result in antigen presentation. Another possibility is that alkylation of HSA may be a detoxifiying mechanism, much like glutathione conjugation, through which excess 2-phenylpropenal is removed, preventing a more general toxicity from occurring. If this is the case, concomitant use of drugs that bind to subdomain IIA of HSA may increase the risk of a patient developing a felbamate idiosyncratic drug reaction. Conversely, it is possible that alkylation of HSA would affect its ability to bind certain highly HSA-bound drugs, thereby affecting the "free" fraction of such drugs and their pharmacokinetic behaviors. Finally, the fact that this alkylation is reversible presents the possibility that HSA could serve to carry and deliver 2-phenylpropenal formed in the liver to distal sites, such as hemopoietic tissue, thereby contributing to the development of aplastic anemia.

In summary, we have demonstrated that HSA is capable of catalyzing the decomposition of the felbamate metabolite, 4-hydroxy-5-phenyl-[1,3]oxazinan-2-one. This decomposition results in the release of the reactive species, 2-phenylpropenal, which can alkylate HSA at the His-242 and His-247 residues in subdomain IIA of HSA. A model in which HSA serves to accelerate the formation of 2-phenylpropenal has also been proposed. Whether or not this alkylation is of significance in vivo is not known; however, this is the first direct example of 2-phenylpropenal conjugating a human protein. Currently, work is in progress to determine whether HSA in patients undergoing felbamate therapy is alkylated by 2-phenylpropenal. HSA adducts of other small molecules such as aspirin and penicillin have been observed in vivo (*12*, *20*, *27*). The fact that the oxazinane is found in measurable amounts in patient urine (*7*) supports the idea that HSA-2-phenylpropenal adducts may be observed in vivo.

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