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Exposure Profiling of Reactive Compounds in Complex Mixtures

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Abstract

Humans are constantly exposed to mixtures, such as tobacco smoke, exhaust from diesel, gasoline or new bio-fuels, containing several thousand compounds, including many known human carcinogens. Covalent binding of reactive compounds or their metabolites to DNA and formation of stable adducts is believed to be the causal link between exposure and carcinogenesis. DNA and protein adducts are well established biomarkers for the internal dose of reactive compounds or their metabolites and are an integral part of science-based risk assessment. However, technical limitations have prevented comprehensive detection of a broad spectrum of adducts simultaneously. Therefore, most studies have focused on measurement of abundant individual adducts. These studies have produced valuable insight into the metabolism of individual carcinogens, but they are insufficient for risk assessment of exposure to complex mixtures. To overcome this limitation, we present herein proof-of-principle for comprehensive exposure assessment, using N-terminal valine adduct profiles as a biomarker. The reported method is based on our previously established immunoaffinity liquid chromatography-tandem mass spectrometry (LC-MS/MS) method with modification to enrich all N-terminal valine alkylated peptides. The method was evaluated using alkylated peptide standards and globin reacted *in vitro* with alkylating agents (1,2-epoxy-3-butene, 1,2:3,4-diepoxybutane, propylene oxide, styrene oxide, N-ethyl-N-nitrosourea and methyl methanesulfonate), known to form N-terminal valine adducts. To demonstrate proof-of-principle, the method was successfully applied to globin from mice treated with four model compounds. The results suggest that this novel approach might be suitable for *in vivo* biomonitoring.

Keywords

N-terminal valine adducts; multiple exposure detection; biomonitoring; mixtures; biomarkers

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Introduction

Environmental and occupational exposures have been associated with increased risk for the development of numerous cancers (Davis *et al.* 2010; Swenberg *et al.* 2008). Accurate exposure assessment in humans, however, is challenging because humans are exposed to a variety of mixtures, such as tobacco smoke, exhaust from diesel, gasoline or new bio-fuels. Such exposures contain several thousand compounds, including many known or suspected human carcinogens. Covalent binding of reactive metabolites to DNA and the formation of stable adducts is believed to be the causal link between exposure and carcinogenesis. DNA adducts are well established biomarkers for the internal dose of reactive compounds or their metabolites and are an integral part of science-based risk assessment. Reactive compounds that form DNA adducts often also form protein adducts, such as with albumin and hemoglobin. The corresponding protein adducts are commonly used as surrogate biomarkers for DNA adducts and are well established biomarkers of exposures (Swenberg *et al.* 2008; Törnqvist *et al.* 2002; Wild 2009; Wild and Pisani 1998).

While globin adducts are not causally linked to mutagenesis, they have several advantages over DNA adducts: (i) protein adducts are recognized as good surrogate markers for the internal formation of the activated metabolites, (ii) in molecular epidemiology studies, blood samples are easier to obtain than tissue specimens, (iii) stable hemoglobin (Hb) adducts accumulate over the lifespan of the erythrocytes, which is about 30 days for mice, (iv) they are not removed by enzymatic repair systems like DNA adducts, and (v) due to their stability, protein adducts represent the cumulative exposure prior to sampling, which makes the timing of sample collection less critical. (vi) Lastly, they are excellent biomarkers that integrate *in vivo* metabolism over time and do not require invasive or time sensitive sampling (Törnqvist *et al.* 2002).

Extensive efforts have been made to quantitate adducts in albumin and hemoglobin. Of these, utilization of the modified Edman degradation method for analysis of N-terminal valine adducts is the most common (Boysen *et al.* 2007; Boysen and Hecht 2003; Osterman-Golkar *et al.* 2003; Törnqvist *et al.* 1986; Törnqvist *et al.* 2002). A comprehensive review of mass spectrometry study of protein adducts was recently published by Rubino et al. (Rubino *et al.* 2009). Unfortunately, technical limitations, such as low recovery, sensitivity and the need of adduct specific immunoaffinity chromatography, have prevented comprehensive exposure profiling of complex mixtures (“exposure-omics”), and the majority of studies report measurements of a single adduct or selected few adducts.

More recently Rappaport and colleagues reported progress in simultaneous monitoring of multiple adducts on cysteine in human serum albumin (Funk *et al.* 2010; Li *et al.* 2011). Further modifications of the Edman procedure for analysis of N-terminal valine adducts seem promising for future adduct profiling studies (Von Stedingk H. *et al.* 2010; Von Stedingk H. *et al.* 2011). These new approaches are aimed to establish a tool for multi-adduct profiling of reactive, and potentially genotoxic, compounds in mixtures. Such technology is expected to enable determination of the internal dose of numerous carcinogens simultaneously to (a) better understand the effects and fate of individual carcinogens in

mixtures, (b) identify novel, until now, unknown adducts, and (c) investigate potential compound-compound-interactions.

We report herein a novel proof-of-principle for a sensitive and specific method for qualitative profiling of exposure to a broad spectrum of reactive compounds or their metabolites using N-terminal valine adducts of hemoglobin as biomarkers. The reported method is based on our previously established immunoaffinity liquid chromatography-tandem mass spectrometry (LC-MS/MS) method (Boysen *et al.* 2012; Boysen *et al.* 2004; Georgieva *et al.* 2007; Georgieva *et al.* 2010). To enable multi-adduct profiling, the adduct enrichment step has been modified to selectively isolate all alkylated N-terminal peptides of the globin α-chain, independent of adduct structure or chemical properties, prior to analysis by LC-MS/MS (Figure 1).

Materials and Methods

Materials

Trypsin (biotin agarose, from bovine pancreas) was purchased from Sigma-Aldrich (St. Louis, MO). All reagents and solvents used were ACS grade or higher. Amicon 3 filters were obtained from Amicon Inc. (Beverly, MA) and Microspin filter tubes (regenerated cellulose, 0.2 μm) were from Altech Associates Inc. (Dearfield, IL). The non-alkylated (1-11) and methylated (1-11) peptide standards and [$^{13}C_5$] valine labeled non-alkylated (1-11) peptide used for synthesis of internal standards were purchased from Neo-Peptide, a subdivision of Neo-Group, Inc. (Cambridge, MA). The antibodies against the *N*-terminus (depletion) and *C*-terminus (selection) of the mouse α-Hb (1-7) peptides were raised by Open Biosystems Inc. (Huntsville, AL). Immunoaffinity (IA) depletion and selection columns were built using the respective antibody with the highest ELISA titers according to the procedure described in our earlier publication (Boysen, 2004).

Synthesis of standard peptides

The syntheses of analytical standard (AST) and internal standard (IST) peptides were performed by direct reaction of the non-alkylated (1-11) peptide with the reagents of interest in 0.1M NH_4HCO_3 buffer for 72 hours at the optimal molar ratio of 1:50 and pH 6.5. Full scan MS and MS/MS experiments of the products were used to confirm peptide sequence and site of alkylation.

In vitro alkylation of mouse (1-11) peptide or control globin

Globin was extracted from a female 12 week old B6C3F1 mouse as described previously (Mowrer *et al.* 1986). The reactions were performed at a molar ratio of peptide (globin): reagent = 1:1 in 0.1 M NH_4HCO_3 buffer for 72 hours at pH 6.5. Reagents, 1,2-epoxy-3-butene (EB), 1,2:3,4-diepoxybutane (DEB), propylene oxide (PO), styrene oxide (SO), N-ethyl-N-nitrosourea (ENU) and methyl methanesulfonate (MMS), were used alone or in a mixture (Figure 2). After trypsin hydrolysis, the samples were processed over depletion and selection IA columns, and elutes were analyzed as described below.

Animal exposures

Female B6C3F1 mice were exposed in the UAMS Animal Facility to MMS, ENU, EB, and SO. MMS and ENU were given in saline by gavage once a day for 4 days and EB and SO were given once by i.p. injection on the fourth day. Mice were sacrificed on the fifth day and blood and tissues were harvested for further analyses. Globin from 2 samples of highly exposed mice (25 mg/kg body weight MMS and 100 mg/kg body weight ENU, plus 500 μmol/kg body weight both EB and SO) was extracted and analyzed for the expected adducts as described below.

Immunoaffinity enrichment and purification of *in vitro* reaction mixtures or globin samples from mice—Globin was first trypsinized as described previously (Boysen *et al.* 2004). After filtration on Amicon 3 columns and concentration in a speed-vac to about 0.3 mL, samples were loaded to the depletion IA columns, containing antibodies specific to the N-termini of trypsinized α-globin, in 0.5 mL PBS and incubated for 2 hrs. Then the samples from the depletion columns were slowly dripped to selection columns, containing antibodies specific to the C-termini of trypsinized α-globin, (rinsed 2 times with 0.5 mL PBS) incubated for an additional 2 hrs and washed five times with 2 mL water. The alkylated peptides were eluted in 3 mL freshly prepared 5% formic acid (FA) followed by drying overnight in a speed vac. Samples were dissolved in 150 μL water and filtered on Microspin filters (2 times 50 μL HPLC water washes), dried in a speed vac and stored at −20°C until MS analysis. Between sample analyses, both sets of depletion and selection IA columns were regenerated with 2 times 3 mL 5% FA, 2 times 5 mL water, 2 times 5 mL PBS and stored in PBS at 4°C.

Tandem Mass Spectrometry Analysis—The qualitative analysis of the adducted N-terminal Val (1–7) peptides by LC–ESI–MS/MS was performed with an Acquity UPLC (Waters) coupled to a TSQ-Quantum Ultra triple quad mass analyzer (ThermoScientific). An Acquity UPLC BEH column C18 1.7μm 2.1 × 150 mm was operated with a linear gradient of 0.1% FA to 70% acetonitrile-0.1% FA in 10 min, at a flow rate of 0.4 mL/min. The retention times of N-terminal Val adducts were determined with synthesized peptide standards. The peptides were detected in single reaction monitoring (SRM) mode, monitoring the transition of the doubly charged precursor ions to the a1-or y2-fragment listed in **Table 1**. The MS conditions were as follows: column temperature 60°C, collision energy 20 eV. The IA purified samples were reconstituted in 20 μl of water and 1 to 2 μl were injected. Characterization of mouse SO-Val (1-7) was performed by monitoring the product ions derived from double charged precursor ion $[M+2H]^{2+}$ m/z 434.4 at collision energies ranging from 10 to 40 eV.

Results and Discussion

We report herein a modified version of our successfully established LC-MS/MS method for quantitation of BD-derived N-terminal valine adducts that allows simultaneous analysis of multiple adducts (Boysen *et al.* 2007; Boysen *et al.* 2012; Boysen *et al.* 2004; Georgieva *et al.* 2007; Georgieva *et al.* 2010). To enable adduct profiling, the adduct enrichment step has been modified to enable enrichment of all alkylated N-terminal peptides (Figure 1).

Therefore, two sets of IA columns containing antibodies specific to N- or C-terminus of the target peptide (α-chain peptide 1-7) were prepared to generate depletion and selection IA chromatography columns, respectively. In the first enrichment step, non-alkylated peptides are depleted with depletion IA columns containing antibodies specifically raised against the N-terminus of the non-alkylated peptide. The unbound alkylated peptides, not retained on the depletion columns, are directly loaded onto the selection IA columns. Subsequently, the peptides of interest, the alkylated N-terminal peptides, are retained on the selection IA columns containing antibodies raised against the C-terminus. It was hypothesized that alkylation on the opposite N-terminus should not affect peptide enrichment. After washing with water, alkylated peptides are eluted and analyzed by LC-MS/MS. An advantage of this novel design is that peptides and antibodies are commercially available by various vendors. Therefore it is not necessary to synthesize standard peptides to raise adduct specific antibodies, a practice in our previous studies (Boysen *et al.* 2004). Prior to purification of real samples, IA columns were tested for recovery by loading AST and IST and performing the procedure for separation and enrichment (data not shown).

The method was evaluated using alkylated peptide standards and globin reacted *in vitro* with a few selected model alkylating agents. For this investigation, EB, DEB, PO, SO, ENU and MMS were chosen because they are known to form N-terminal valine adducts and represent a variety of compound classes (Kautiainen *et al.* 2000; Osterman-Golkar *et al.* 2003; Swenberg *et al.* 2001; Zhang *et al.* 2005). For the standards synthesis, optimal conditions for reactions of epoxides with peptide standards were determined experimentally (data not shown). It was found that reactions at pH 6.5 with a 1:50 molar ratio mainly produced the single valine alkylated peptide standards. The site of alkylation was confirmed by LC-MS and MS/MS analysis (Figure 3). The MS/MS spectrum suggests single alkylation at the N-terminal valine based on observed b- and y-fragments (Figure 3). Fragments indicative of alkylation at the lys^7 or lys^{11} were not observed. The N-terminal valine group is the primary site of alkylation in reactions carried out at pH <7 because at higher pH all three NH_2-goups are available to attack the epoxide ring. At pH<7 the ε-NH of lys^7 and lys^{11} are protonated and are much less reactive. Therefore, all subsequent peptide reactions were carried out at pH 6.5 to specifically alkylate NH_2-group of the N-terminal valine. These conditions were chosen to maximize standard synthesis. Under physiologic conditions the N-terminal valine has been shown to be the primary site of alkylation in the 1-7 peptide (Basile *et al.* 2001; Basile *et al.* 2002). In addition, for reactions with EB and SO, the expected positional (regional) isomers and diastereomers were resolved as separate peaks (Figures 4 and 5). This simple synthesis approach has been found suitable for synthesis of all peptide standards of the selected model compounds. This synthesis approach is more convenient compared to the previous strategy of synthesizing the alkylated valine and subsequent attachment to the 2-11 peptide as described by Jayaraj et al. (Jayaraj *et al.* 2003).

As a first step of method development, the overall recovery was determined by using SO-Val (1-11) peptide standard. The mean recovery of the SO-Val was 72.5 ± 23.7% (n=15) based on the peak area of peptide standard hydrolyzed and injected directly compared to the same amount hydrolyzed and processed over depletion and selection IA columns. Blank samples consisting of water, processed over both IA columns did not contain any detectable adducts.

These results suggest that the modified adduct enrichment procedure effectively isolates alkylated *N*-terminal peptides.

The investigation was expanded to analyses of 1-11 peptides that had been reacted with the selected model compounds, known to form N-terminal valine adducts, individually or as a mixture (Figure 2). Except for ENU, all other reactions of epoxide with the mouse peptide produced the corresponding alkylated peptide standards (data not shown). Surprisingly, the reaction of 1-11 mouse peptide with ENU did not result in the expected ethyl-Val (Et-Val). Instead after trypsin hydrolysis an ion with m/z of 790.4 was observed and further investigated by LC-MS/MS. The MS/MS spectrum suggests it is a carbamoylated-adduct (ENU-Val) (Figure 6 and Supplemental Figure S1). The formation of an ENU-Val adduct has been previously reported for methylnitrosourea (Zhang *et al.* 2005) and suggests that all nitrosourea derivatives may in fact form this type of adduct. Further validation and structural characterization of this specific adduct is under way.

After synthesis and analyses of various model adduct standards, we extended our effort to analysis of *in vitro* alkylated globin. Therefore, control mouse globin was treated with several epoxides individually or as a mixture as described in Materials and Methods. The treated globin was analyzed and the expected adducts were readily detected (Figure 4). As with the peptide reaction, ENU did not produce detectable amounts of Et-Val. Instead a clear peak corresponding to the ENU-Val adduct was observed, demonstrating that it is also formed in globin. As with *in vitro* reacted peptides, HB- and SO-Val were detected as individually resolved peaks for corresponding isomers.

Subsequently, to demonstrate that the novel method is suitable for *in vivo* biomonitoring, we analyzed globin from two B6C3F1 mice that had been treated with MMS, ENU, EB and SO. Figure 5 shows the representative extracted ion chromatograms corresponding to the model adducts. Me-Val, ENU-Val, HB-Val, *pyr*-Val and SO-Val were clearly detected. Similar to *in vitro* results, positional isomers and diastereomers of SO-Val and HB-Val were resolved as separate peaks. No peaks were observed for HP-Val since the mice were not treated with PO. Surprisingly, no clear peaks were found for THB-Val, the main BD derived adduct. This is probably due to the fact that EB was given by i.p. injection and the time was insufficient to form significant amounts of EB-diol available for adduct formation. Interestingly, the ENU-Val-adducts from ENU were clearly detected, demonstrating its formation *in vivo*. The expected Et-Val adducts were not observed, suggesting that they either do not form or form at undetectable amounts. Overall, the results suggest that this novel approach might be suitable for *in vivo* biomonitoring and future studies to investigate physiologic exposure and dose-response are ongoing.

Unfortunately, we also observed some non-alkylated peptide, suggesting that the depletion step is not complete. Further computational investigation demonstrates that about 99.99% of non-alkylated peptides had been removed. However, the huge excess of non-alkylated peptides still leads to clearly detectable peaks. At this time it is not apparent whether further increase in depletion efficiency is needed, since the adducts of interest were clearly detected in both *in vitro* treated globin and *in vivo* exposed mice.

Conclusion

A proof of principle of a novel LC-MS/MS method for analysis of a broad spectrum of N-terminal valine adducts is presented. This method advances exposure monitoring by establishing methodology for qualitative profiling of exposures to complex mixtures and could lead to unprecedented insights into the biological importance of individual carcinogens in mixtures. The adduct-profiling method allows the monitoring of a variety of known endogenous alkylating agents in response to, and in addition to, environmental and occupational exposures. Here, we examined acute exposure to show proof-of-principle, and application to low and chronic exposures are needed to complete method validation. The long term goal of our research is to advance current adduct analysis from targeted to untargeted technology for human bio-monitoring.

ACKNOWLEDGEMENTS

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Abbreviations

BD	1,3-butadiene
DEB	1,2:3,4-diepoxybutane
EB	1,2 epoxy-3-butene
EB-diol	3,4-epoxy-1,2-butanediol
ENU	N-ethyl-N-nitrosourea
ENU-Val	carbamoylated-valine
Et-Val	ethyl-valine
FA	formic acid
HB-Val	N-(2-hydroxy-3-buten-1-yl)-valine
Hb	hemoglobin
HP-Val	1-hydroxy (or 2-hydroxy)-propyl- valine
IA	immunoaffinity
LC-MS/MS	Liquid Chromatography-Tandem Mass Spectrometry
Me-Val	methyl-valine
MMS	methyl-methanesulfonate
H_2N-Val	non-alkylated-valine
PO	propylene oxide
pyr-Val	N,N-(2,3-dihydroxy-1,4-butadiyl)-valine
SO	styrene oxide

SO-Val	1-phenyl-2-hydroxyethyl-valine, or 2-phenyl-2-hydroxyethyl-valine
THB-Val	2,3,4-trihydroxybutyl-valine

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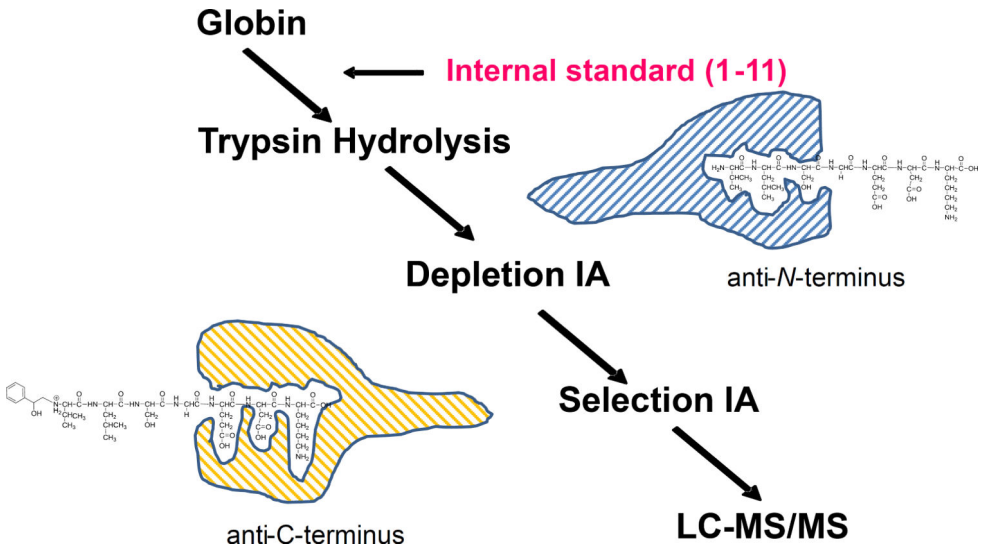

Figure 1.

Scheme of adduct profiling assay. The alkylated N-terminal peptides of the α-chain are isolated from trypsin hydrolyzed globin by series of depletion and selection IA chromatography, prior to analysis by LC-MS/MS as described in Materials and Methods.

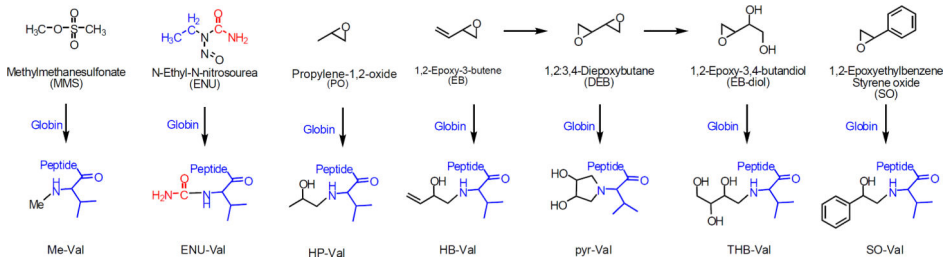

Figure 2.
Overview of selected model compounds and their corresponding N-terminal valine adducts.

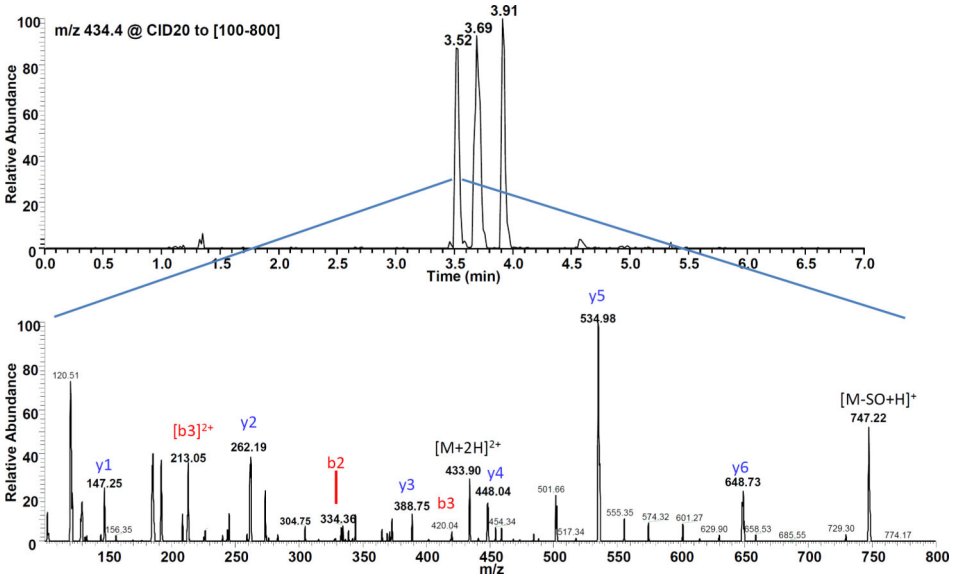

Figure 3.

Characterization of mouse SO-Val (1-11) standard. Mouse 1-11 peptide standard was reacted with SO in 0.1 M NH_4HCO_3 at pH 6.5 for 72 h. MS/MS fragmentation suggests single alkylation at the N-terminal valine.

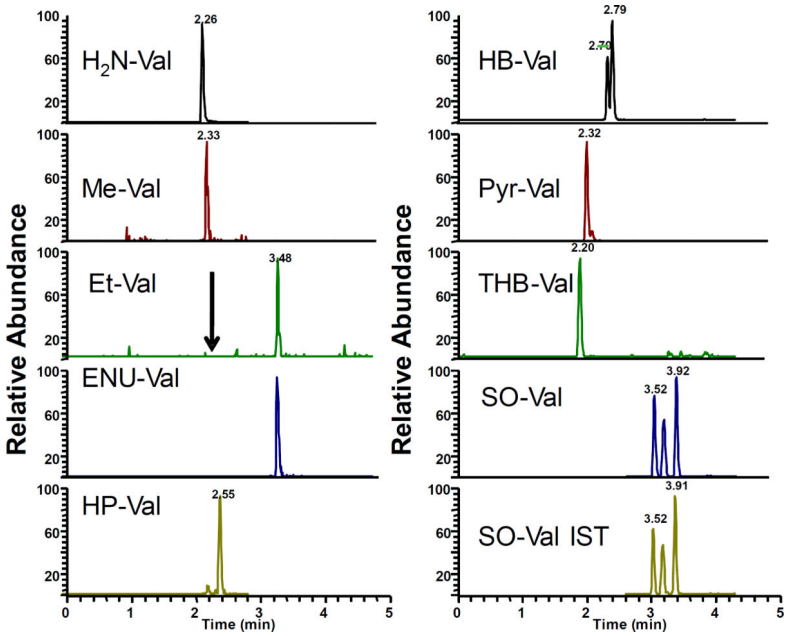

Figure 4.

Extracted ion chromatograms of mouse globin treated *in vitro* with a mixture of EB, DEB, PO and SO, MMS and ENU. The treated globin was processed as described in Materials and Methods and analyzed by LC-MS/MS. [$^{13}C_5$]SO-Val was utilized as internal standard.

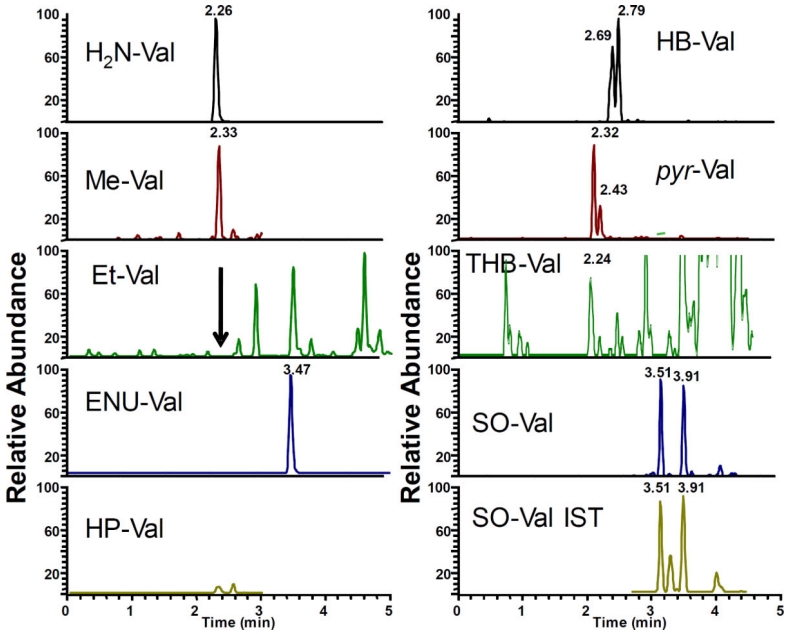

Figure 5.

Representative extracted ion-chromatogram of exposed mouse globin. B6C3F1 mice were treated with EB, SO, MMS and ENU and globin was isolated and analyzed as described in Materials and Methods. [$^{13}C_5$]SO-Val was utilized as internal standard.

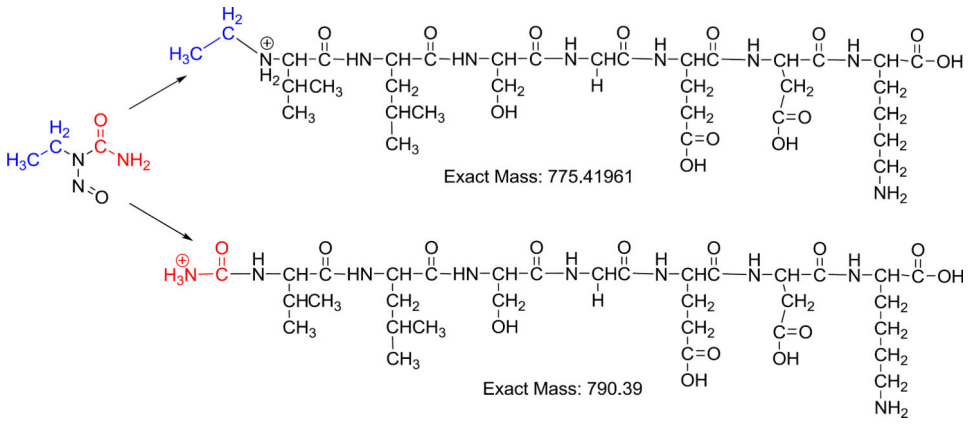

Figure 6.
Proposed formation of the ENU-derived carbamoyl adduct (ENU-Val).

Table 1

Selected SRM transitions

Adduct	Ion transition[a]
H_2N-Val	374 → 262[b]
Me-Val	381 → 262[b]
Et-Val	396 → 116
ENU-Val	790 → 535[c]
HP-Val	403 → 130
HB-Val	409 → 142
pyr-Val	417 → 158
THB-Val	426 → 176
SO-Val	434 → 192

[a] doubly charged precursor ion to a1-fragment

[b] doubly charged precursor ion to y2-fragment

[c] singly charged precursor ion to y5-fragment