Part 2 Progress Report

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Title: Biomarkers for Air Pollutants: Development of Hemoglobin Adduct Methodology for Assessment of Exposure to Butadienes and Polycyclic Aromatic Hydrocarbons, SEER project of SIP: Experimental Program To Stimulate Competitive Research (EPSCoR) From The Commonwealth Of Kentucky
Investigators: Harrell E. Hurst and Steven R. Myers
Institution: University of Louisville
Research Category: EPSCoR
Project Period: October 1, 2001 - September 30, 2004

Aims of the Project:
We proposed to develop methodology that will measure systemic exposures to chloroprene (2-chloro-1,3-butadiene CAS-126-99-8) and selected polycyclic aromatic hydrocarbons (PAH: fluoranthene, CAS# 205-44-0; benzo(a)pyrene, CAS# 50-32-8). The methods involve detection and measurement of covalent adducts to the abundant blood protein hemoglobin (Hb) as biomarkers of exposure. The postulated adducts are formed by electrophilic epoxide metabolites of these compounds. Analysis involves synthesis of derivatives through Edman cleavage of globin *N*-terminal valine adducts, or hydrolysis of PAH adducts bound at other more labile sites. Quantification of adducts derived from chloroprene is accomplished by selected ion monitoring gas chromatography/mass spectrometry (SIM-GC/MS) using stable isotope internal standards. Adducts from PAH exposure are analyzed after acid hydrolysis of labile PAH-Hb carboxylate adducts. Tandem MS studies using an ion trap instrument has been used to determine selected ion decomposition pathways to understand the chemistry of the products formed.

SEER Component 1:
Development of Methods to detect Hemoglobin (Hb) Adducts of Chloroprene
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Progress Summary: During the second year of this grant, research focused on development of working assays for measurement of Hb adducts. A postdoctoral fellow with expertise in mass spectrometry was been recruited from the University of New Orleans. A synthetic standard of the epoxide of chloroprene, (1-chloroethenyl)oxirane or CEO, provided by the International Institute of Synthetic Rubber Producers' Chloroprene Scientific Oversight Committee through Matthew Himmelstein of DuPont Haskell Laboratory, was used in model in vitro studies. A SIM-GC/MS assay for Hb chloroprene epoxide adducts has been developed that relies on sequential Edman cleavage and trimethylsilylation reactions. Reactions of CEO with N-terminal valines in mouse red cell hemoglobin have been characterized kinetically.

Development of methodology for measurement of chloroprene epoxide adducts to valine proved challenging. Development of this assay has required much more time than originally anticipated, as significant difficulties were encountered. Initial results did not reveal analytical derivatives until suitable conditions were developed for the two sequential reactions involved. These are a modified Edman cleavage of adducted valine from Hb, and trimethylsilylation of the hydroxyl group formed by opening of the epoxide during adduct formation. Reference and internal standards were synthesized and isolated using valine, ^{13}C-labeled valine, and the tripeptide valine-tyrosine-valine. Subsequently, two GC/MS peaks having chemical characteristics of the desired derivatives were observed from reactions with standards and with treated globin. Much effort was spent toward identification of these analytical products, which were proposed to be either stereo- or positional isomers. Unequivocal identification was not possible, but a hypothesis is put forth that the peaks represent stereo isomers resulting from the CEO valine adducts, as detailed below. Additional difficulties encountered include variability in derivative formation and yield. Conditions for reactions have been repeatedly examined in attempts to produce reliable quantification of adduct formation. Low yield of analytical derivatives is anticipated to provide a serious challenge to use of this assay for ambient exposure assessment.

Key Personnel: Changes in personnel involved addition of a post-doctoral fellow, Md. Yeakub Ali, Ph.D. in September, 2002. Dr. Ali has been supported by this work and has contributed to this research since that time. Jian Cai, Ph.D., accepted another position effective February 1, 2003, and has not participated in this research since that date. Part time student workers contributed efforts during the summer, 2003.

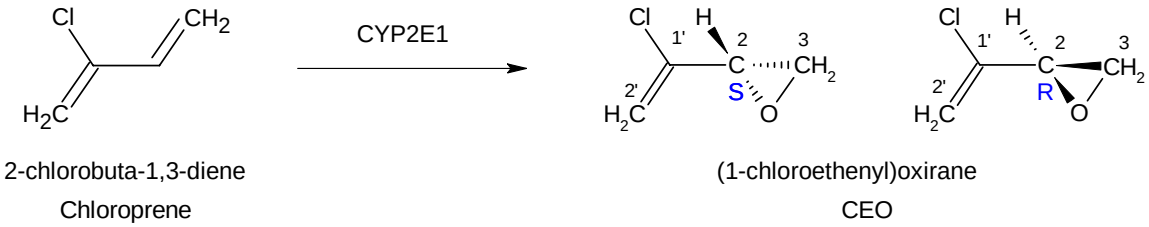
Discussion of Expenditures to Date: Expenditures involving personnel, equipment, and travel are as projected in the original schedule. Expenditures for supplies have lagged behind schedule due to the need to resolve analytical issues (above) prior to performance of in vivo experiments. As a result, no animal experiments have been conducted.

Quality Assurance Statement:

Details included in this report provide potential methods for measurement of hemoglobin adducts as biomarkers of exposure. We have demonstrated in vitro that Hb adducts are formed by reactions of epoxide metabolites of chloroprene and selected polycyclic aromatic hydrocarbons. To be acceptable biomarkers of exposure, the proposed analytical determinations must have defined, adequate specificity, sensitivity, accuracy, and precision to link unequivocal adduct detection with conditions of exposure. The assays rely on the unparalleled specificity of mass spectrometry, which provides the primary methodology for adduct detection. Chromatographic separations and specific chemical reactions, including modified Edman cleavage of amino acids and formation of trimethylsilylether derivatives of hydroxyl functions, provide additional specificity. The sensitivity of each analysis has been determined empirically. However, ultimate suitability of the assays to measure biomarkers of exposure is determined by complex sequential processes derived from the chemical processes of adduct formation and analytical determination. Some of these include the extent and duration of exposure to these toxic air pollutants, the degree of metabolic activation to electrophiles, competition among nucleophilic binding sites for covalent electrophile binding, the nature and stabilities of the adducts, chemistries of sample preparation, and instrumental conditions. Of major importance for success of this project has been discovery of stable adducts which are converted to analytical chemical derivatives, which

are reproducibly and uniquely detectable. Precision has been examined by repeated measures and accuracy has been determined by cross checks using multiple preparations derived from standards prepared gravimetrically. The proposed structures for analyses detailed above will provide unequivocal detection of adducts formed in sufficient quantities for detection. Work remains regarding important assay characteristics of reproducibility and accuracy. We have yet to determine if sensitivity is sufficient for measurement of adducts formed in vivo, as animal studies must be accomplished to determine the extent of adduct formation in vivo.

Results to Date: CEO is an electrophilic epoxide metabolite of chloroprene that is formed by the Cytochrome P450 isozyme, CYP2E1, as shown in Figure 1.



2-chlorobuta-1,3-diene
Chloroprene

(1-chloroethenyl)oxirane
CEO

Figure 1. Oxidation of chloroprene to epoxide enantionmers.

CEO exists as a pair of enantiomers, or mirror image isomers, given the asymmetric 2-carbon of the substituted oxirane ring, as shown in the 3-D ball and stick models in Figure 2 below. In these models the chlorine is behind the 1´-carbon, using the oxirane structural numbering system published for the homologs, butadiene monoepoxide and isoprene monoepoxide, by Bleasdale et al. (1).

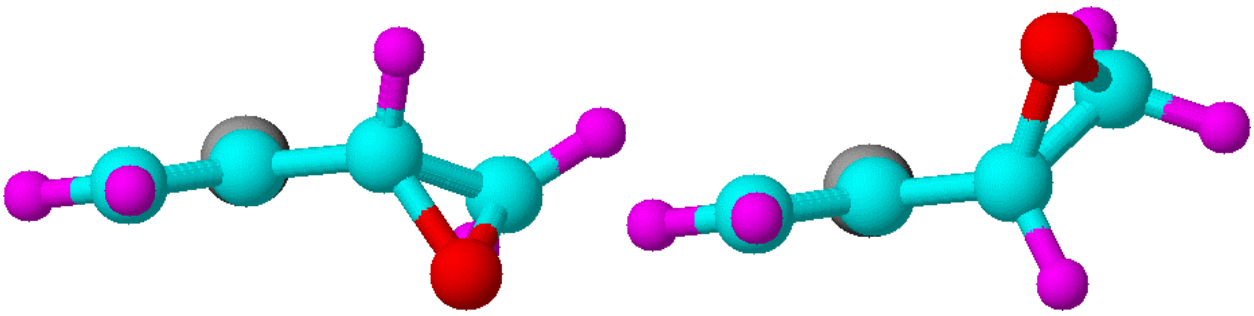


Figure 2. Respective S and R stereochemical forms of (1-chloroethenyl)oxirane.

CEO reacts through opening of the epoxide ring and binds covalently with physiologic nucleophiles to produce adducts with various macromolecules, including nucleic acids (2) and glutathione(3). From analogous reactions that occur with other epoxides of similar compounds, these biochemical reactions are likely involved in the toxicity of chloroprene and its detoxification, respectively. The covalent reaction of CEO with nucleophiles involving epoxide ring opening produces two chemically-possible positional isomeric adducts, as shown in Figure 3, where the nucleophilic macromolecule is a protein such as hemoglobin (Hb) containing valine (Val) as the N-terminal amino acid.

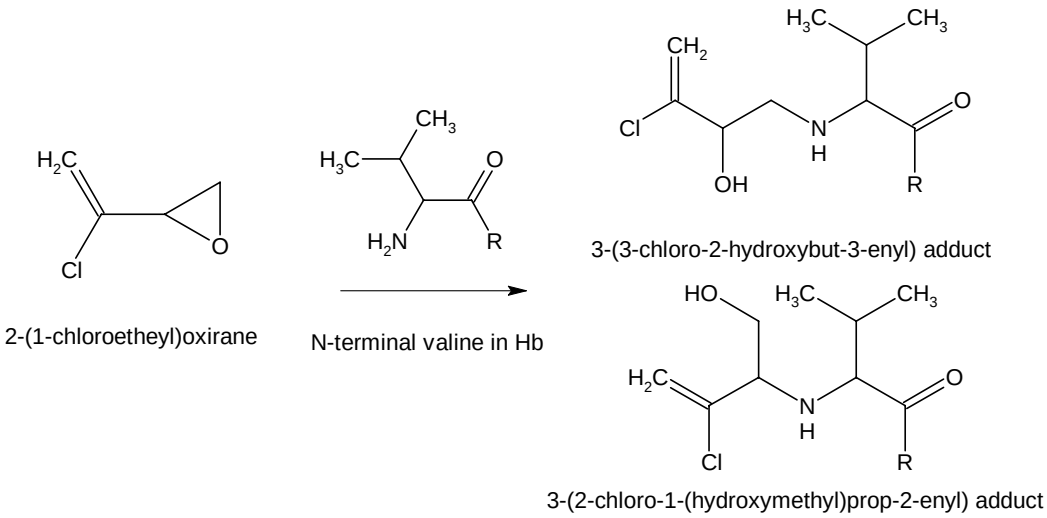


Figure 3. Potential valine adduct regioisomers formed by reaction with CEO.

Both of these positional adduct isomers exist as diastereoisomers, leading to four potential isomeric forms. However other in vitro adduct studies of (1-chloroethenyl)oxirane have indicated predominant products as the diastereomeric pair of adducts formed by attack at the C-3 of (1-chloroethenyl)-oxirane. When the nucleophiles were derived from nucleosides or calf thymus DNA (2) reactions of (1-chloroethenyl)oxirane were stated to be "highly regioselective" for the C-3 carbon (see numbering system in Figure 1 above). In similar studies with glutathione (3) the diastereomeric pair of 3-chloro-2-hydroxybut-3-enyl-adducts (C-3 oxirane ring attachment) were noted to be the major conjugates, while one stereoisomer of the 2-chloro-1-(hydroxymethyl)-prop-2-enyl- adduct (C-2 oxirane ring attachment) was observed as a minor adduct. The latter was indicated to exist as 10% of the abundance of the major conjugates.

Summary of Results: In vitro experiments have been conducted to develop methods to detect these adducts. These involved reactions of CEO with racemic unlabeled valine, $^{13}C_5$-valine, valine-tyrosine-valine (Val-Tyr-Val or VYV), and with hemoglobin in C57Bl/6 mouse red cells. Globin was isolated by precipitation following lysis of the red cells. The model adduct CEO-VYV was synthesized and purified for use as a quantitative reference standard. Weighed aliquots of globin were processed by Edman degradation, followed by trimethylsilyation of the hydroxyl function on the adducts. Aliquots of these extracts were analyzed by SIM-GC/MS analysis. Results indicated two resolved CEO-Hb peaks following SIM-GC/MS analysis Structures were postulated for these adducts. Kinetics of CEO-Hb adduct formation were determined from in vitro reactions of CEO and Hb in mouse red cells.

Methodology is detailed below, with experimental details in smaller font to distinguish methods from results and discussion. Figures present structures and representative chromatographic and mass spectrometric data.

Preparation of CEO-Val, CVO-$^{13}C_5$-Val, and CEO-VYV adducts:
Model adducts were prepared by chemical synthesis and purified by chromatography for use as standards for the Edman degradation and analysis by GC/MS.

Preparation of CEO-Val, CEO-$^{13}C_5$-Val, and CEO-VYV: Dissolve valine (d, l racemic mixture from Sigma Chemical, 10mM, 1.17 mg/ml) in 0.2M phosphate buffer (pH 7.4). Mix 1.3 ml solution with 3.0 µl CEO (final concentration 25mM) in a 1.8 ml vial, incubate at 37°C.

Dissolve $^{13}C_5$-Val (L-valine -U-$^{13}C_5$, 96-98%-^{13}C, Cambridge Isotope Laboratories, 10mM, 1.25 mg/ml) in 0.2M phosphate buffer (pH 7.4). Mix 1.22 ml solution with 2.8 µl CEO (final concentration 25mM) in a 1.8 ml vial, incubate at 37°C. Stop incubation after 50 hours of incubation. Keep the products in refrigerator before purification.

Dissolve VYV (L-Val-Tyr-Val, Sigma 10mM, 3.8 mg/ml) in 0.2M phosphate buffer (pH 7.4). Mix 1.5 ml solution with 3.0 µl CEO (final concentration 25mM) in a 1.8 ml vial, incubate at 37°C for two days. Keep the products in refrigerator before purification.

Purification of CEO-Val and CEO-$^{13}C_5$-Val: Wash column (Strata X column (30mg/1ml) from Phenomenex) with 1 ml 50% MeOH and 1 ml water, load 0.4 ml Val/CVO or d_8-Val/CVO incubation product, wash with 0.5 ml water, elute with 1.2 ml water. Repeat the process till the entire incubation product was processed. SPE fractions were analyzed by electrospray ionization ion trap mass spectrometry Finnigan LCQ Duo) after 1/10 dilution with 50% acetonitrile (ACN) containing 0.5% formic acid. CEO-Val and CEO-$^{13}C_5$-Val were found in 1.2 ml fractions. Small impurity peaks (m/z 118 and 176 in CEO-Val and m/z 123 and 181 in CEO-$^{13}C_5$-Val) were also detected in 1.2 ml fractions. CEO-Val and CEO-$^{13}C_5$-Val are not well retained on Strata X column. Concentrate 1.2 ml fractions with CEO-Val and CEO-$^{13}C_5$-Val by Speedvac. Combine CEO-Val or CEO-$^{13}C_5$-Val fractions together and dry the samples after combination. This yielded white residue in the vials.

Purification of CEO-VYV: Inject aliqots 500µL into semi-preparative Waters HPLC system using 10mm x 25 cm column containing 5µm diameter Ultrasphere-ODS solid phase. Elution is accomplished by using a solvent gradient from 50% water-50% acetonitrile at injection to 100% acetonitrile over 30 minutes. Unreacted VYV eluted between 15 – 17 minutes, while CEO-VYV eluted between 17 – 20 minutes. The latter fraction was collected from each of several separations, and fractions were combined and analyzed by HPLC MS/MS using a ThermoFinnigan LCQ-Duo ion trap mass spectrometer.

CEO-Val, CEO-$^{13}C_5$-Val, and CEO-VYV stock solutions: Dissolve residues from CEO-Val, CEO-$^{13}C_5$-Val, or CEO-VYV fractions in 0.2 ml water.

Analysis of CEO-Val and CEO-$^{13}C_5$-Val by GC/MS:

Modified Edman degradation and trimethylsilylation were used to derivatize the adducted N-terminal amino acid (valine). Samples were analyzed by capillary gas chromatography/mass spectrometry using the HP5890/Micromass AutoSpec GC/MS system.

Modified Edman degradation with trimethylsilylation for GC/MS: Mix 10 µl CEO-Val or CEO-$^{13}C_5$-Val stock solutions, or 10 µl CEO-VYV stock solution, with 1 ml formamide containing 10 µl 1.0M NaOH, and 7 µl neat pentafluorophenylisothiocyanate (PFPITC, Fluka Chemical Co.), set samples on rotator overnight, incubate samples at 50°C for 2 hours, extract twice with 2 ml hexane, dry hexane phase under nitrogen, dissolve residue in 1 ml toluene, wash twice with 2 ml 0.1 M Na_2CO_3, dry toluene phase under nitrogen, incubate with 25 µl ACN and 25 µl *N,O*-bis(trimethylsilyl)-trifluoroacetamide (BSTFA) at 60°C for 1 hour, and inject 1 µl into the GC/MS.

Gas Chromatography/Mass Spectrometry (GC/MS): Compound separation was achieved gas chromatography using a HP5890 GC with capillary column (DB-5MS, 15 m × 0.25 mm I.D. × 0.25 µm film thickness) from J&W Scientific (Folsom, CA). The GC injection port and GC/MS interface temperature were set to 280°C, with constant helium carrier gas pressure at 5 psig. Injections were made in splitless mode with the inlet port purged after 1 min following injection. The GC oven temperature was held initially at 100°C for 1 min, then increased at a rate of 10°C min^{-1} to 230°C, and then at 20°C min^{-1} to 300°C, which was held for 5 min. Components eluting from GC were detected by mass spectrometry using a Micromass AutoSpec with 70eV electron impact ionization, 500 µA Emission current, and operated at1000 resolving power. Full scan mode (m/z 50 – 600) was used to characterize components, or selected ion monitoring (SIM-GC/MS) for more sensitive, selective detection. Masses monitored included m/z 465 for the CEO-valine Edman derivative and m/z 470 for the CEO-$^{13}C_5$-valine derivative.

CEO-Val Edman derivatives, as 1-(3-chloro-2-trimethylsilyloxy-but-3-enyl)-5-isopropyl-3-(pentafluorophenyl)-2-thioxoimidazolidin-4-one or 1-[2-chloro-1-(trimethylsilyloxymethyl)-prop-2-enyl]-5-isopropyl-3-(pentafluorophenyl)-2-thioxoimidazolidin-4-one, were detected following Edman degradation and trimethylsilylation. The unlabeled Edman derivatives have a nominal molecular weight of 500, while the molecular weight of the $^{13}C_5$-derivative is 505. A two minute section of a total ion current chromatogram is shown below in Figure 4, while a representative mass spectrum of the unlabeled derivatives is illustrated in Figure 5.

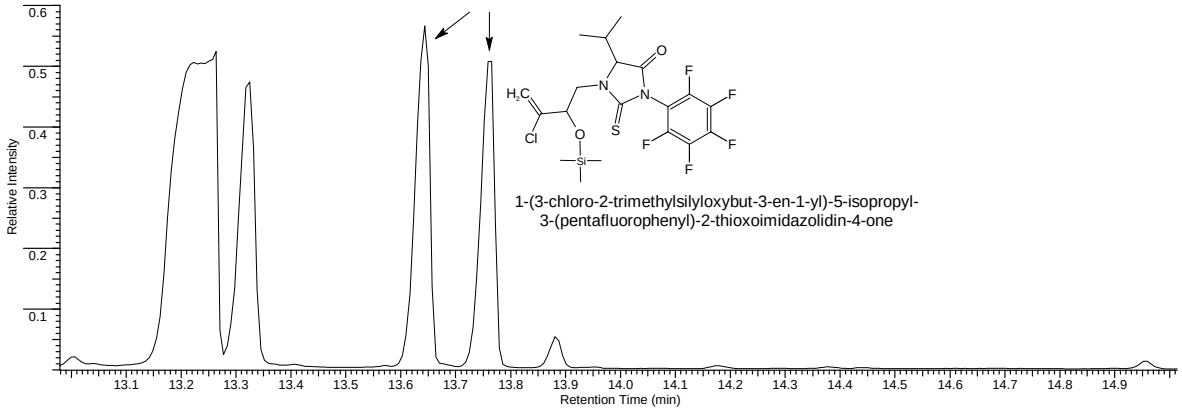


Figure 4. Section of chromatogram showing Edman TMS derivatives of CEO-valine adduct.

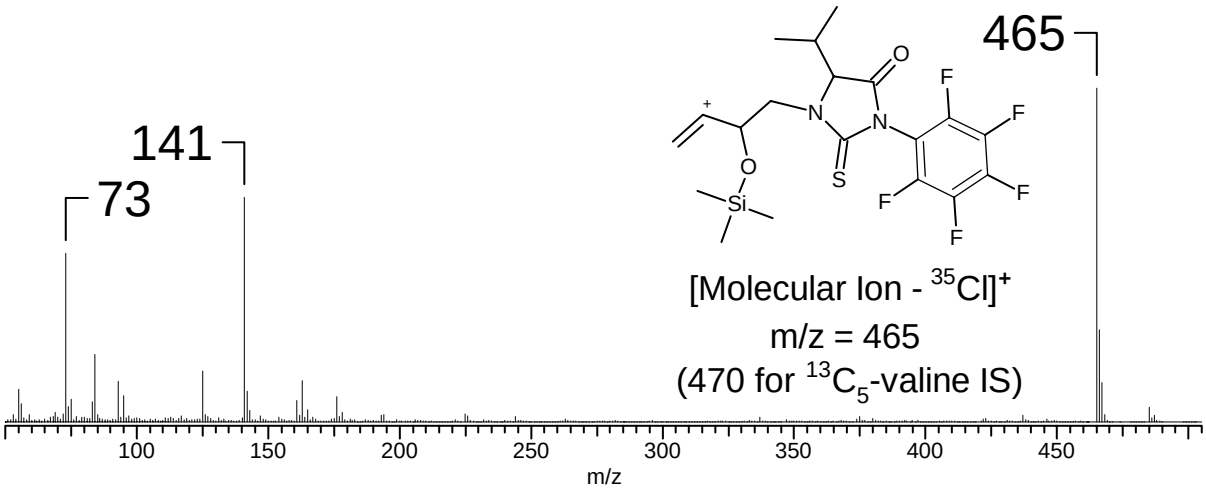


Figure 5. Electron impact mass spectrum of Edman TMS derivative of CEO-valine.

The chromatogram clearly illustrates formation of two compounds, which are postulated to be diastereomers (see below) of the structure included in the chromatogram. The mass spectrum is dominated by the $[M-35]^+$ ion at m/z 465 which results from facile elimination of chloride anion (Cl^-) from the molecule. A lesser abundant ion exists at m/z 485, which is attributable to loss of a methyl group from the molecule. Other abundant ions are m/z 73, attributable to $(CH_3)_3Si^+$, and m/z 141, likely $(CH_3)_3Si\text{-}O\text{-}C_4H_4^+$.

Total ion current and selected mass (m/z 465, 470) chromatograms are shown in Figure 6. Mass spectra obtained from both peaks in a mixture of unlabeled and $^{13}C_5$-labeled adducts are shown below in Figure 7.

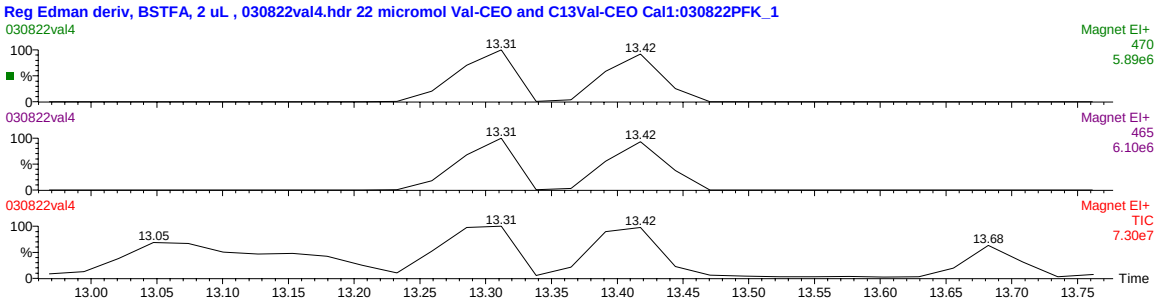


Figure 6. Total ion current, m/z 465 , and 470 mass chromatograms of CEO-valine adducts.

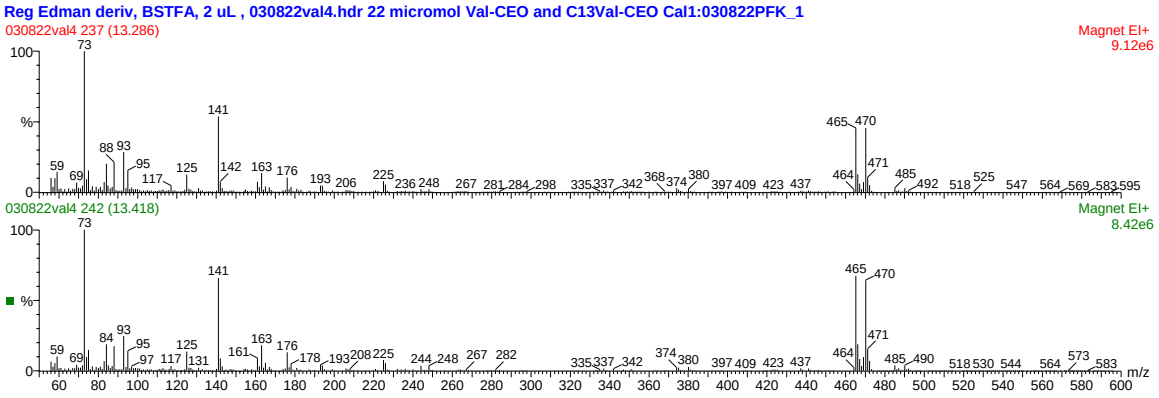


Figure 7. Mass spectra of peaks at 13.3 min (top) and 13.4 min.

Here the top spectrum was taken from the peak at 13.3 min, while the bottom spectrum came from the 13.4 min peak. These figures illustrate the great similarity in chemistry of the two separable derivatives. These also show that the differences in isotopes in the labeled and unlabeled valine analogs are evident only at the high m/z peaks. Exact structural assignment is not possible from this data as the chemical formula ($C_{19}H_{22}ClF_5N_2O_2SSi$) is consistent with two possible regioisomers (positional isomers), 1-(3-chloro-2-trimethylsilyloxybut-3-en-1-yl)-5-isopropyl-3-pentafluorophenyl-2-thioxoimidazolidin-4-one (as drawn, Figure 4), or 1-[2-chloro-1-(trimethylsilyloxymethyl)prop-2-en-1-yl]-5-isopropyl-3-pentafluorophenyl-2-thioxoimidazolidin-4-one (not shown) in which attachment is at the 2-carbon of CEO. Based on the almost identical spectra obtained in the GC/MS studies, on MS/MS studies of model compounds in the ion trap mass spectrometer, and on similar reactions with physiological nucleophiles and CEO in published studies (2,3), we propose these peaks to be stereo isomers of 1-(3-chloro-2-trimethylsilyloxybut-3-en-1-yl)-5-isopropyl-3-pentafluorophenyl-2-thioxoimidazolidin-4-one as shown in the Figure 4. If regioisomers were present in similar abundance, it is likely that their mass spectra would exhibit greater difference, and more chromatographic peaks should be observed due to the combination of regio- and stereo isomers. This assignment is consistent with observations of Munter et al. who observed regioselective attack at C-3 of (1-chloroethenyl)oxirane in adducts with DNA (2) and glutathione (3).

Standard response curve for analysis of CEO-VYV by GC/MS:

CEO-VYV was analyzed as a quantitative reference along with CEO-$^{13}C_5$-valine as internal standard. Chromatographic peak areas of selected ions were used as a selective response for quantitation, and peak area ratios (area m/z 465 / area m/z 470) were plotted versus amount ratios of CEO-VYV / CEO-$^{13}C_5$-valine in a standard response curve. A representative curve is shown in Figure 8.

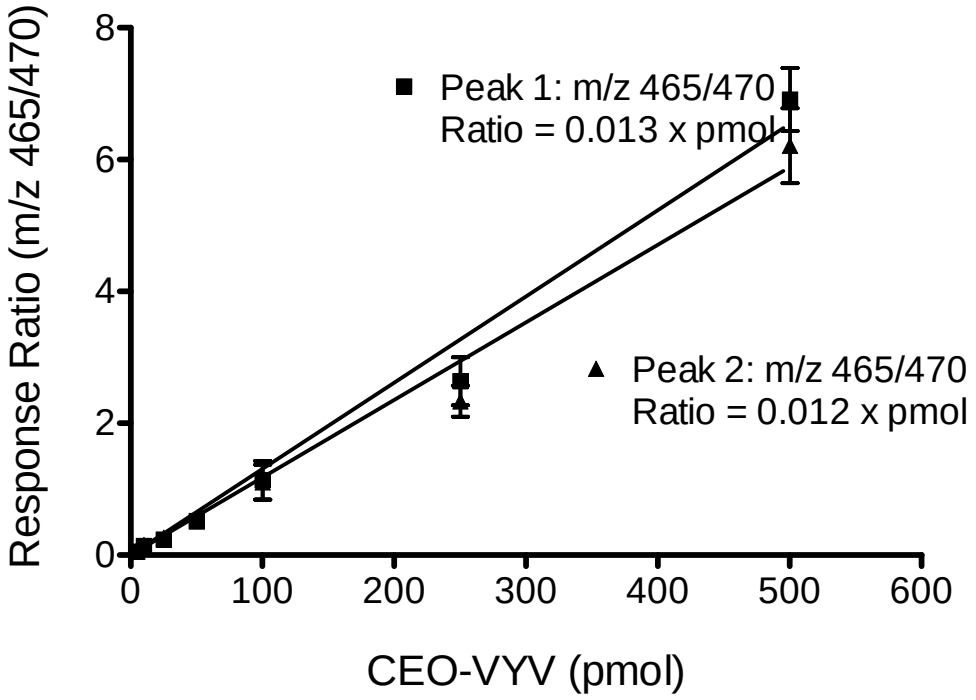


Figure 8. Standard response curve for peaks 1 and 2 in CEO-VYV standards.

In-Vitro Exposure Methods:
Packed red cells (125 µL) from C57Bl/6 mice were diluted in phosphate-saline (2.0 mL) at pH 7.4 in a 25 mL vial. chloroprene epoxide (CEO) was injected as vapor (25 or 100 ppm) into a series of vials. The vials were incubated with a gentle shaking at 37ºC for 1, 2, 4, 6, 8, 18, 24, 48 or 72 hours. Vials were removed after each incubation period, and globin immediately was treated with 5 volumes of ice-cold 1% acidified Acetone in HCl. The globin precipitates were washed twice with 3 volumes of pure acetone to removed residual acid, dried under N_2, and were stored at –20ºC. A duplicate operation was performed with a sample blank. A calibration curve with 8 points from 0 to 500 picomole range was generated using weighed CEO-VYV to standardize unknown quantities of CEO-globin in exposed blood.

In Vitro Adduct Levels:
Plots of CEO-globin versus time of CEO exposure for two isomers at low and high level are shown in Figures 9 and 10. These illustrate chemical kinetics of CEO-globin formation with exposure, and show an exponentially-declining rate of formation as CEO-globin of increases with time to plateau levels that are isomer specific for at both low and high exposure levels. This indicates that CEO is a reactive electrophile that binds with hemoglobin to form N-terminal

CEO-valine adducts within 24 hours of exposure. As noted in Table 1 the rate of formation of isomer 2 is more than 4 times higher than isomer 1. From data at low and high level exposure, the reaction of CEO and hemoglobin can be described as pseudo 1st order with half life of about 7 hours.

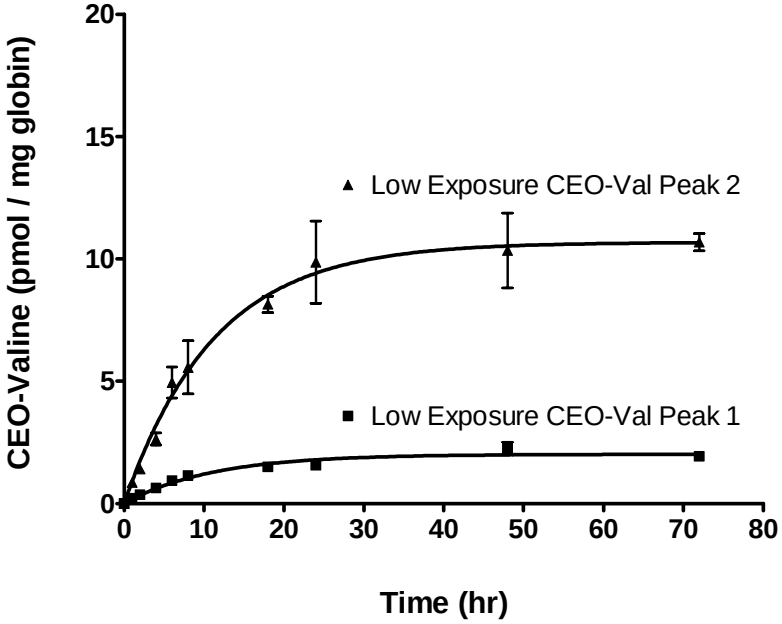


Figure 9. Formation of CEO-valine Hb adducts in mouse red cells after 25 ppm vapor exposure.

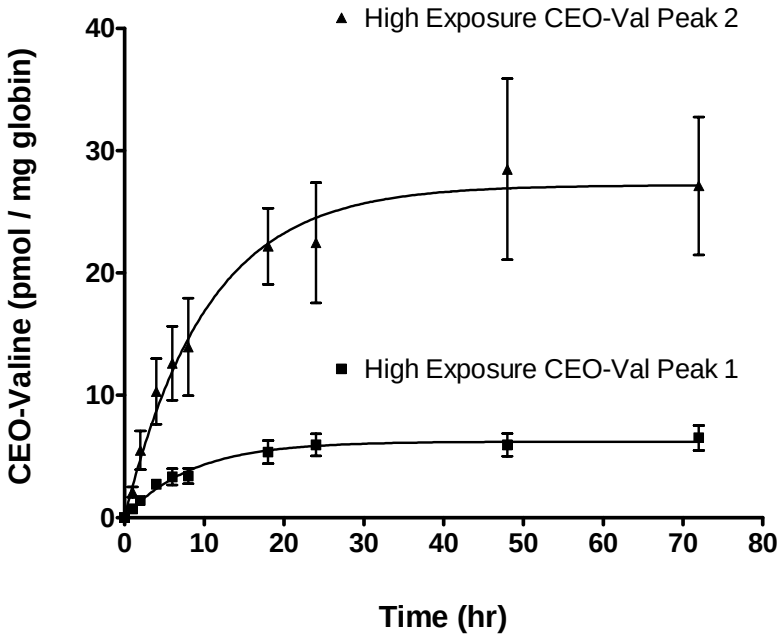


Figure 10. Formation of CEO-valine adducts in mouse red cells after 100 ppm vapor exposure.

Table 1. Parameters for formation of CEO-valine in mouse red cell Hb exposed to CEO in vitro.

Parameter	25 ppm CEO Peak 1	100 ppm CEO Peak 1	25 ppm CEO Peak 2	100 ppm CEO Peak 2
CEO-Val Maximum (/globin)	2.0 pmol/mg	6.2 pmol/mg	11 pmol/mg	27 pmol/mg
Rate Constant	0.09 hr^{-1}	0.12 hr^{-1}	0.09 hr^{-1}	0.10 hr^{-1}
Halflife	7.6 hr	5.8 hr	7.8 hr	7.2 hr
R^2 of Nonlinear Fit	0.95	0.84	0.95	0.74

Attempts to confirm the structure of CEO-valine adducts:

Several approaches were used in attempts to confirm the identities of the two peaks tentatively identified as diastereoisomers of 1-(3-chloro-2-trimethylsilyloxybut-3-en-1-yl)-5-isopropyl-3-pentafluorophenyl-2-thioxoimidazolidin-4-one. These involved the synthesized reference standard CEO-VYV as analyzed using the ion trap system, shown in Figures 11 and 12. Note the essentially identical Electrospray (ESI) MS (Figure 11) and MS/MS spectra (Figure 12). The virtually identical spectra support the hypothesis that the two resolved compounds are diastereoisomers.

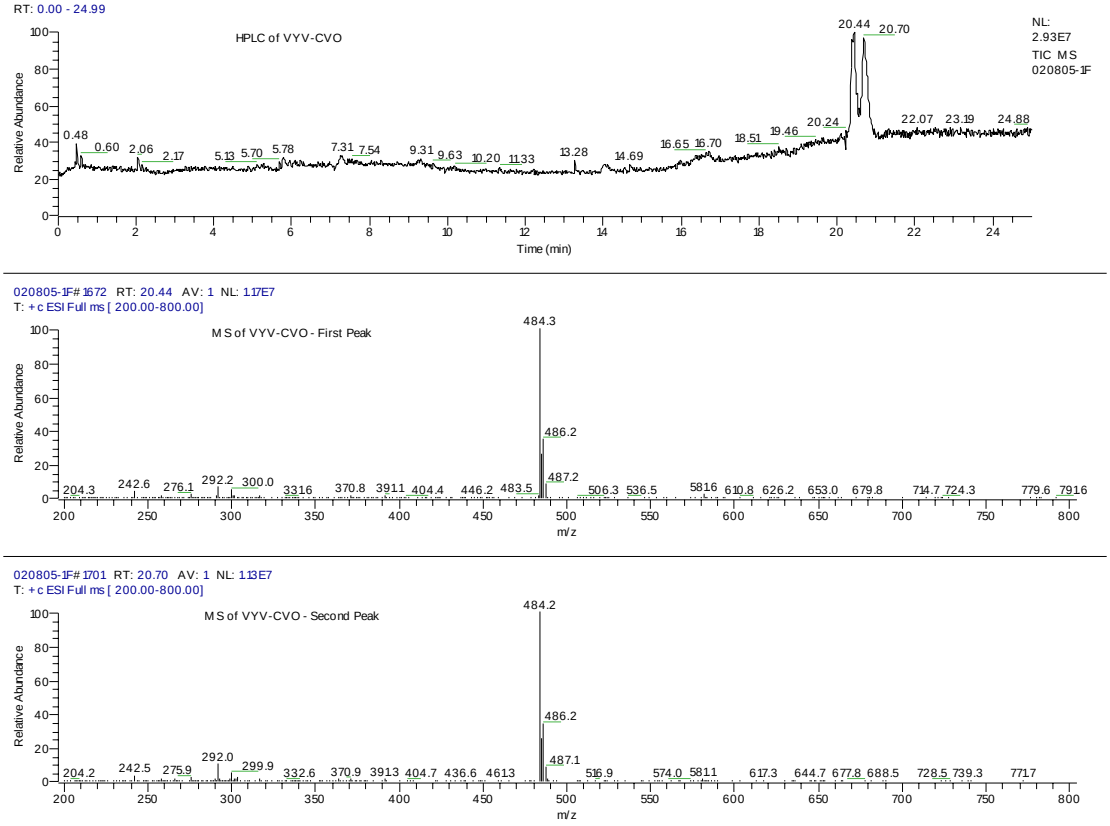


Figure 11. HPLC/ESI-MS spectra of of apparent CEO-VYV diastereoisomers.

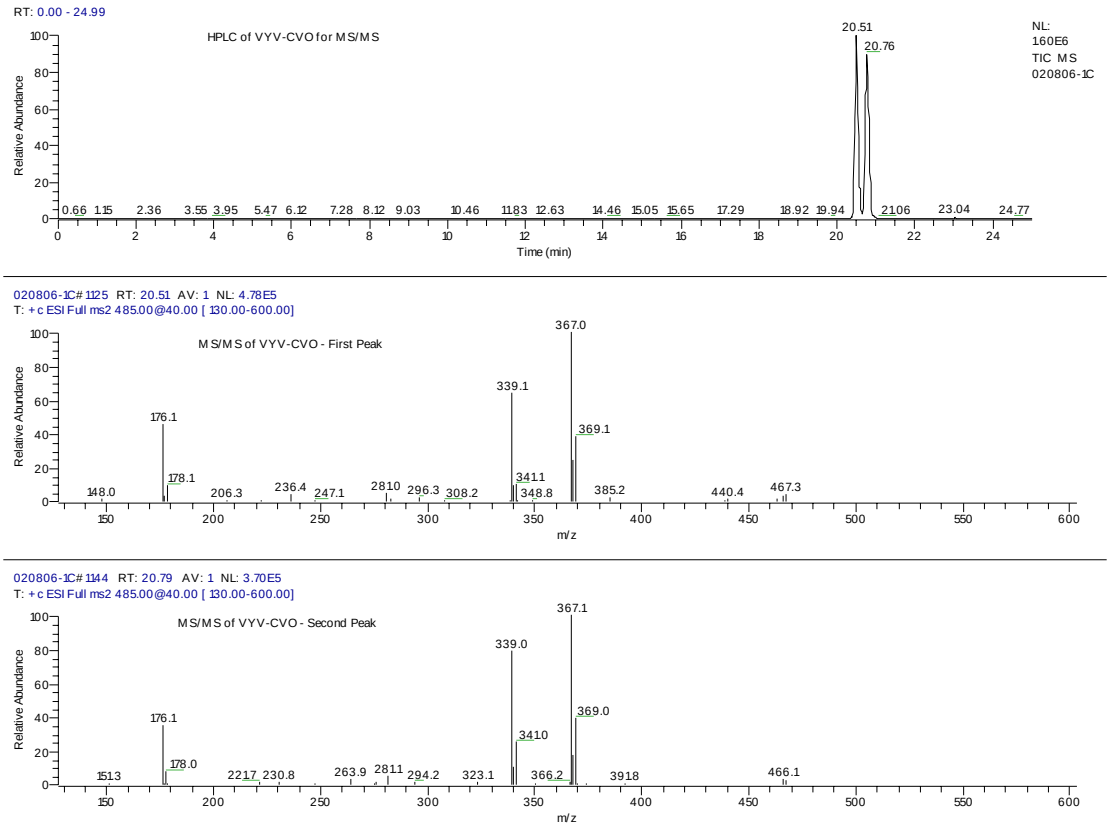


Figure 12. HPLC/ESI-MS/MS of apparent CEO-VYV diastereoisomers.

HPLC/Electrospray/MS/MS of CEO-VYV: VYV-CEO stock solution was diluted 1/10 with 0.5% formic acid and 10 µl was injected to a Spectra System 4000 HPLC with Spectra System AS3000 auto sampler. The separation column was Waters Spherisorb 5 µm ODS2 (4.6×250mm). A gradient started from 0% Solvent B (95% ACN with 0.05% TFA) and increased to 50% Solvent B in 15 minutes and then Solvent B was maintained at 50% for 10 minutes (Solvent A was 5% ACN with 0.05% TFA). The flow rate was set at 0.5 ml/min. Under these conditions, two peaks at Rt 20.4 and 20.7 min were detected.

Attempts at NMR Analysis of CEO-VYV Sample: CEO-VYV was provided to Dr. Andrew Lane of the James Graham Brown Cancer Center, University of Louisville, in hope of determining structure of the compound. As analyzed NMR spectra did not reveal the position of conjugation of CEO and the tripeptide VYV, given the lack of adequate signal from the secondary amino hydrogen, as detailed below.

NMR Analysis of CEO-VYV Sample: Sample was presented as a dried powder after HPLC purification. MW = 483. Sample (0.26 mg) was dissolved in 0.35 mL 0.1 M HCl containing 10% D_2O in a 5 mm Shigemi tube. 0.1 mM DSS was added as a shift reference. Concentration = 1.53 mM (nominal). NMR spectra were recorded at 10 °C on a Varian Inova600 NMR spectrometer with 14.1 Tesla superconducting magnet.
Experiments carried out:
1. 1D Watergate with 5 s recycle time (with and without flipback)
2. Watergate TOCSY, 20 ms mixing time
3. Watergate TOCSY, 50 ms mixing tome
4. Watergate ROESY, 200 ms mixing time
5. Watergate ROESY, 500 ms mixing time
6. DQCOSY
7. Sample lyophilized and redissolved in dry d6 acetone.

Results.
1. 1D spectra in water showed the presence of 4 H_N peaks from peptide hydrogens.
However, the integrals indicated that compared with the tyrosine side-chains protons (2,6 and 3,5), there are actually only 2 NH protons. Each H_N comprises two sets of doublets, with an 8.5 Hz coupling to CαH for Y (restricted rotation about $\phi \approx +120°$). Each doublet integrated to 0.5 protons. This indicates the presence of two independent compounds in the sample, and in essentially equal concentration.

2. The TOCSY spectrum showed that the H_N peaks belong to a tyrosine residue and a valine residue. Thus the NH (secondary amine) peak must be exchanging rapidly with the solvent, as it was not observed. The peak was also not observed in acetone. The TOCSY spectrum made unambiguous assignment of the spin systems possible.

3. Sequence-specific assignments were made using the ROESY spectra. Once the amino acid residues had been assigned (and each one shows 2 sets of peaks), the remaining resonances could be attributed to the N-terminal modification.

4. The CEO moiety also consists of two sets of peaks in an AMX-like spin system (i.e. XCH-CH_2Y). Based on models of the possible structures, the ROESY cross-peak intensities indicate that one set belongs to structure A and the other probably structure B. Absolute proof needs to find correlations to the secondary amine proton, which seems to be very labile. Alternatives would be to try HMBC spectra in D_2O using a 3 mm probe.
Molecules A and B are in equal amounts (within ca. 10% error). Chemical shift assignments are given in Table 2.

Table 2. The chemical shift assignments (wrt DSS, 283 K)

Residue	HN	Hα	Hβ	Me/ø
V1a	-	3.70	2.20	0.95, 1.08
V1b	-	3.76	2.25	0.95, 1.07
Y2a	9.01	4.85	3.12, 2.83	7.16, 6.81
Y2b	9.04	4.85	3.12, 2.85	7.16, 6.81
V3a	8.56	4.21	2.15	0.94, 0.96
V3b	8.54	4.21	2.15	0.94, 0.96
	CH	CH_2		
CEOa	4.50	3.0, 2.52		
CEOb	4.50	2.84, 2.6		

Attempts at chemical modification of CEO-VYV for structural definition: Attempts were made to chemically modify CEO-VYV to provide additional structural information. This approach involved use of oxidation reagents and catalysts in the hope of oxidizing the 3-chloro-2-hydroxy-3-buten-1-yl hydroxyl group to a ketone. If accomplished this would eliminate the asymmetric center of this structure, cause the diastereoisomers to coalesce, if present, and eliminate chromatographically resolution. Alternatively, regioisomers would remain distinct, different compounds, and likely distinguishable by chromatographic or mass spectral properties. Two reagent systems were used in attempts. These included pyridinium chlorochromate (PCC) (4) (5) and palladium acetate (6). Neither catalyst gave definitive, unambiguous results. PCC appeared to destroy the structure such that essential structural information was lost. Palladium acetate appeared less destructive, but did not result in definitive structural assignment.

a) Oxidation of CEO-Val / CEO-$^{13}C_5$-val by PDA:
Step 1: 10 µL Val-CEO (0.5 mg/mL in aq) and 10 µL ^{13}Cval-CEO (0.5 mg/mL in aq) were added to 0.3 mL DMF (N, N-dimethyl formamide). 1.5 mg palladium acetate (trimer) and 2.0 mg sodium acetate were also added to the solution. The sample was incubated for 2 hrs at room temperature with constant shaking.

Step 2: After completion of oxidation, the sample was treated with 1.0 mL formamide, 10 µL NaOH and 10 µL PFPITC and was shaken overnight at room temperature and then incubated for 2 hours at 50°C.

Step 3: After completion of Edman degradation, the derivative was extracted 2 times with 2 mL diethyl ether and then dried under N_2 gas. The residue was dissolved in 1.0 mL toluene and then washed twice with 2.0 mL 0.1M Na_2CO_3 and was dried under N_2. The Edman derivative was dissolved in 50 µL acetonitrile and was ready for GC-MS analysis.

Step 4: The sample was dried again under N_2 and was dissolved in 25 µL acetonitrile and treated with 25 µL BSTFA and incubated at 60°C for 1 hour. The trimethylsilyl (TMS) derivative of the Edman product was ready for GC-MS analysis.

b) Oxidation of Edman derivative by PDA:

Step 1: 10 µL Val-CEO (0.5 mg/mL) and 10 µL ^{13}Cval-CEO (0.5 mg/mL) were added to 1.0 mL formamide, 10 µL 1M NaOH and 10 µL PFPITC. All samples were shaken overnight (about 16 hours) at room temperature (25°C) and then incubated for 2 hours at 50°C.

Step 2: After completion of Edman degradation, the derivative was extracted 2 times with 2 mL diethyl ether and then dried under N_2 gas. The residue was dissolved in 1.0 mL toluene and then washed twice with 2.0 mL 0.1M Na_2CO_3 and was dried under N_2. The Edman derivative was dissolved in 50 µL acetonitrile and was ready for GC-MS analysis.

Step 3: The derivative was dried again under N_2 and dissolved in 0.3 mL DMF (N, N-dimethyl formamide). The sample was treated with 1.0 mg Palladium acetate (trimer), 2.0 mg Sodium acetate and 20 µL of deionized water and then incubated for 2 hours with constant shaking at room temperature for oxidation of Edman derivative.

Step 4: After completion of oxidation, the sample was extracted with ether and washed with Na_2CO_3 to remove residual oxidizing agent and dried by N_2 as mention in step 2 of this method. The Edman derivative was dissolved in 50 µL acetonitrile and was ready for GC-MS analysis

Step 5: The sample was dried again and dissolved in 25 µL acetonitrile and treated with 25 µL BSTFA and incubated at 60°C for 1 hour. The trimethylsilyl (TMS) derivative of the Edman derivative was ready for GC-MS analysis.

Oxidation of Edman derivative by PCC:

Step 1: Edman derivatives was produced by following the procedures in step 1 and step 2 in PDA method b). The Edman derivative was dissolved in 50 µL acetonitrile and was ready for GC-MS analysis.

Step 2: The sample was dried again under N_2 and dissolved in 0.3 mL DMF (N, N-dimethyl formamide). The sample was treated with 100 µL PCC (0.5 mg/mL in CH_2Cl_2), 2.0 mg Sodium acetate and 20 µL deionized water and then incubated for 2 hours with constant shaking at room temperature for oxidation of Edman derivative.

Step 3: After completion of oxidation, the product was extracted by ether and washed by Na_2CO_3 to remove residual oxidizing agent and dried N_2, by following step 2 and was ready for GC-MS analysis.

Step 4: The dried sample was dissolved in 25 µL acetonitrile and treated with 25 µL BSTFA and incubated at 60°C for 1 hour. The trimethylsilyle (TMS) derivative of the Edman derivative was ready for GC-MS analysis.

Conclusions:

1. Chloroprene epoxide (CEO) reacts with Hb protein in red cells to form globin N-terminal valine-(3-chloro-2-hydroxy-3-buten-1-yl) adducts.

2. The two evident valine adducts appear to be stereoisomers with attachment at the terminal (C-3) carbon, as opposed to regio (positional) isomers at C-2 or C-3 of (1-chloroethenyl)oxirane. It has not yet been possible to determine the specific stereoisomeric configuration of the isomers obtained from the racemic CEO reagent.

3. When hemoglobin in red cells reacts in vitro with racemic *R*,*S*-CEO, the second eluting stereoisomer is favored in a ratio of about 5:1.

4. CEO reaction in vitro with excess Hb in red cells appears to be 1st order with half life of about 7 hours. Reactions in vitro are essentially (~90%) complete within 24 hours.

Planned Future Activities:

Efforts will continue toward refinement of GC/MS and other MS techniques for quantification of CEO adducts in mouse globin following exposure to CEO. An objective will be to maximize detection of trace amounts of the globin adducts that result from exposure to CEO. Following refinement of the assay for these adducts, mice will be treated with chloroprene to examine suitability of the mass spectrometric assay(s) as a biomarker of chloroprene exposure.

Publications from Project:

Results of this work have been presented at the following sites:

Hurst, H.E., Biomarkers for air pollutants: Development of hemoglobin adduct methodology for exposure assessment, *9th Annual Kentucky EPSCoR Conference* Lexington, KY, May 12, 2003.

Ali, M.Y. and Hurst, H.E. Development of a GC/MS method to determine hemoglobin *N*-valine adducts from (1-chloroethenyl)oxirane, a chloroprene metabolite. *51st ASMS Conference on Mass Spectrometry and Allied Topics*, Montreal, Canada, June 11, 2003. A published abstract from this presentation has been included separately.

Ali, M.Y. and Hurst, H.E. Development of a GC/MS method to determine hemoglobin *N*-valine adducts from (1-chloroethenyl)oxirane, a chloroprene metabolite. *2nd Annual James Graham Brown Cancer Center Retreat*, Louisville, KY, September, 17, 2003.

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