EPA EPSCoR Science and Engineering Environmental Research Project

Biomarkers for Air Pollutants: Development of Hemoglobin Adduct Methodology for Assessment of Exposure to Butadienes and Polycyclic Aromatic Hydrocarbons

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Abstract

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Title: Biomarkers for Air Pollutants: Development of Hemoglobin Adduct Methodology for Assessment of Exposure to Butadienes and Polycyclic Aromatic Hydrocarbons

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Project Summary:

Objectives/Hypothesis: Objectives include development and validation of biomarker methodology that will detect and measure systemic exposures to chloroprene (2-Cl-1,3-butadiene CAS-126-99-8) and polycyclic aromatic hydrocarbons (PAHs). Covalent adducts with the abundant blood protein hemoglobin (Hb) formed by electrophilic pollutant metabolites will serve as a quantitative biomarkers of exposure in ambient air. Further, calculation of second-order kinetic Hb-binding rate constants of electrophiles from these air pollutants will enable estimation of internal dosage. Based on these rate constants, dosimetry values will reflect concentration x time areas (AUC values) derived from measured blood levels of the Hb adducts.

Approach: The experimental approach will extend methods for detection of exposure to carcinogenic electrophiles through measurement of covalent adducts in Hb. Previously published butadiene biomarker methodology will be modified for use with chloroprene. This involves analysis of Hb adduct derivatives following Edman cleavage of globin *N*-terminal valine adducts formed by epoxide metabolites. Quantification of adducts will be accomplished by selected ion monitoring (SIM) gas chromatography/mass spectrometry (GC/MS) using stable isotope internal standardization. Adducts from PAH exposure will be analyzed following acid hydrolysis of labile PAH-Hb carboxylate adducts, and analyzed at specific sites. Nucleophilic residues in Hb, such as cysteine sulfhydryl and histidine imidazole groups, will be examined following proteolytic cleavage of Hb with trypsin. Analysis of cleaved peptides containing adducts will be accomplished by electrospray ionization (ESI) LC/MS and matrix assisted laser desorption ionization time of flight (MALDI-TOF) MS. Tandem MS studies using triple quadrupole or ion trap MS instrumentation will monitor selected ion decomposition pathways to provide additional analytical specificity and sensitivity. High-resolution accurate mass GC/MS will be used as necessary to define adduct identities.

Expected Results: These studies will provide capability to assess potential, alleged, or systemic exposures through implementation of Hb adduct assay methodology for chloroprene, and selected important PAHs. We will determine sensitivity and reproducibility of adduct assay methods, assess biomarker suitability for application to industrial and ambient air toxicant monitoring, and calculate of rate constants for reaction of electrophiles from toxic air pollutants with Hb in vitro and in limited animal studies. The latter constants, combined with adduct levels, enable dosimetry in the form of the blood concentration x time (AUC) products. Such preliminary studies will provide a methodological basis for future public health studies to assess toxicant exposure in air.

Project Rationale

Rationale for project selection

Development of biomarkers of exposure to chemicals with potential adverse public health effects can provide an important means to assess potential public health risks, particularly if the biomarkers relate mechanistically to the adverse health effects. Such indicators provide evidence of actual chemical-biological interaction, and therefore are more directly related to potential disease processes than are measurements derived from the environment alone. EPA recently has articulated interest in elucidating quantitative relationships between concentrations of gaseous pollutants or particulate matter measured at stationary outdoor air monitoring sites and actual personal exposures. As biomarkers can provide means to assess internal exposure, studies that develop and include such internal markers of exposure may be more useful for reduction of uncertainty in risk assessments than environmental measurements alone.

Processes of chemical manufacturing and energy production are of particular economic importance to Kentucky and the nation. Of additional importance is the need to conduct these processes in a manner that protects public health. The chemical monomer chloroprene (CAS# 126-99-8), used in production of neoprene rubber, is under study by the National Toxicology Program (NTP, 1998), which noted its structural similarity with other potentially carcinogenic monomers. As chloroprene recently has been measured in the ambient air of west Louisville (KPPC, 2000), an effective biomarker of chloroprene exposure could contribute to reduction of uncertainty regarding potential human exposure. At the same time Louisville and many other sites currently are working to address newer responsibilities under the Clean Air Amendments, particularly those regulating fine particulates ($PM_{2.5}$). Among the particulates derived from combustion of fossil fuels are certain polycyclic aromatic hydrocarbons (PAHs) that are immunosuppressive carcinogens (Burns et al., 1996). Other monitoring efforts (France, 2000) have indicated the presence in ambient Louisville air of toxic PAHs derived from combustion particulates. Biomarkers of exposure to PAHs will assist in quantification of human exposure for public health efforts. For these reasons this proposal for studies to develop biomarker methodology was deemed important to local public health efforts.

Relationship of project to institutional plans

The objectives of this grant application are rooted within institutional goals of the University of Louisville (UofL) to develop research, and to address community needs through undergraduate, graduate, and professional educational programs. These goals are derived from a stated objective of becoming a “premier, nationally-recognized metropolitan research university.” This role has been put forth in a plan in concurrence with the Kentucky Council on Postsecondary Education (CPE, 2000). As part of this objective UofL Office of Community Relations hosts Partnerships for Urban Development, an effort that includes in its mission statement the objective: “To develop, promote, and implement university/community partnerships that contribute to urban development with emphasis on African Americans and ethnic minorities, economically disadvantaged neighborhoods, the Urban Core, and west Louisville (OCR, 2000).

The Kentucky Institute for the Environment and Sustainable Development (KIESD), under the auspice of the UofL Vice President for Research, actively works to develop community partnerships with positive environmental impact. Within KIESD the UofL Center for Environmental and Occupational Health Sciences (CEOHS) functions to develop research efforts that emphasize interactions between industry and the environment. CEOHS seeks to stimulate interdisciplinary research in environmental health sciences and to provide a thrust toward education and outreach to the public, industry, and local and state government in Kentucky (KIESD, 2000).

CEOHS provides a focus for interaction on environmental health issues through research and lectures involving students and faculty in disciplines of pharmacology and toxicology, biochemistry and molecular biology, and other health sciences. Interdisciplinary research typically involves undergraduate and graduate students working with faculty to accomplish learning objectives and specific aims of research. Such efforts culminate in publication of research findings in refereed journals, in presentations at scientific meetings, and in selected cases, via community outreach efforts.

A major ongoing community effort of the KIESD currently involves air monitoring in west Louisville around an area known as Rubbertown, a complex of petrochemical and synthetic polymer plants interspersed among lower socioeconomic residential neighborhoods. Residents of the west Louisville Rubbertown area have long been concerned about the health effects from these plants. Issues have arisen from residents' sensory perception of the environment of this area, as well as from knowledge of past incidence of cancer arising from exposure to genotoxic chemicals within the plants (Dannaher et al., 1981). Efforts of UofL personnel, including members of KIESD and CEOHS, are directed toward environmental assessment in the Rubbertown area, with objectives of characterizing problems related to environmental contamination and public health, in keeping with community outreach objectives. It is our hope that these proposed studies for biomarker development will significantly expand CEOHS and KIESD efforts by providing methods to assess exposure of chemical workers in west Louisville, and hopefully, to address concerns of potentially exposed residents in the Rubbertown area.

Project Description

Objectives

Currently the west Louisville air-monitoring project includes faculty from the Speed Scientific School (engineering) and School of Medicine in a joint project with community and scientific partners from the Jefferson County Air Quality District, US EPA Region 4, Kentucky Dept. of Environmental Protection, Rubbertown Industries, City of Louisville, and citizens of west Louisville in the West County Task Force. The air-monitoring program provides state-of-the-art sampling for toxic air pollutants using EPA Compendium Method TO-15 (USEPA, 2000a) at a dozen sites in and around the Rubbertown area of western Jefferson County, KY. Begun in July of 1999, the air monitoring project has been organized by the KIESD Director of Research, and is funded in part by the Kentucky Legislature. Results from analyses of air samples are being disseminated via an internet site (KPPC, 2000) (Bruggers, 2000), as well through traditional data exchange mechanisms of the Jefferson County Air Quality District, US EPA Region 4. Toxic Release Inventory (TRI) data from Rubbertown industries suggest (USEPA, 2000b), and preliminary results from local air monitoring efforts demonstrate, that chloroprene (2-Cl-1,3-butadiene) and 1-3-butadiene are toxic air contaminants in industrial and adjacent areas of west Louisville (KPPC, 2000). Emerging reports from limited monitoring using EPA Compendium Method TO-13A (France, 2000) suggest presence of polycyclic aromatic hydrocarbons (PAHs) from combustion sources as part of particulate matter in ambient air of the area.

As butadienes have been shown to be genotoxic and are carcinogenic in laboratory animals following inhalation exposure at low to mid parts per million (ppm) levels (Melnick et al., 1992); (Melnick et al., 1993; Melnick et al., 1995; Ward et al., 1995) (Osterman-Golkar et al., 1996a; Sorsa et al., 1996a; Melnick et al., 1999), their presence in urban air is basis for local concern. Certain particulate matter components, such as the PAH benzo(a)pyrene, are known carcinogens. Therefore need exists for local development, implementation, and application of methodology for biomarkers of internal exposure assessment for these unsaturated organic compounds, which are metabolized to reactive, electrophilic epoxides. Development of such biomarkers will help reduce uncertainties of presumed or significant exposures to these potential carcinogens, and will complement ongoing ambient air monitoring. Additionally, certain of the biomarkers may be useful for local industrial hygiene applications within plants that utilize these monomers but do not have capabilities for biological exposure assessment.

Objectives and Hypotheses: The objectives of the proposed studies involve development and validation of biomarkers of exposure to chloroprene and selected PAHs such as benzo(a)pyrene (BaP). As these airborne compounds are inhaled in contaminated air, blood proteins are among the earliest proteins exposed. Beyond initial exposure, blood provides the medium for internal distribution of metabolites of these toxicants following metabolism in the liver and other sites. As these chemicals possess unsaturated sites subject to bioactivation through oxidative metabolism to reactive electrophilic epoxides, proteins containing nucleophilic functional groups will react to form chemical adducts with these reactive unsaturated compounds or their epoxides. Among these nucleophilic sites are sulfhydryl and primary amino groups of the protective protein glutathione, as well as more abundant proteins such as hemoglobin (Hb). Indeed, epoxides metabolites of 1,3-butadiene (Kohn et al., 1993; Melnick et al., 1995; Osterman-Golkar et al., 1996a) and several PAHs (Day et al., 1990) have been shown to react with Hb, providing a preferred basis for detection of exposure. For dose monitoring of exposure to electrophilic compounds, Hb is preferred for several reasons. Among these are its accessibility in large amounts, availability of methods for

chemical identification, and well-determined life span due to absence of repair (Tornqvist et al., 1995). Analysis of Hb adducts has been developed as a biomarker of exposure to butadiene (Neumann et al., 1995; Osterman-Golkar et al., 1991; Osterman-Golkar et al., 1993; Osterman-Golkar et al., 1996b; Richardson et al., 1996; Sorsa et al., 1996b; Sun et al., 1989), providing a precedent for development of a similar system for chloroprene and PAHs. With this knowledge we propose as a major objective to test the **hypothesis: Analysis of hemoglobin adducts can provide a sensitive, specific biomarkers of exposure to airborne carcinogens, including chloroprene and PAHs such as benzo(a)pyrene to assess local industrial and ambient air exposures.**

In previously-cited studies analysis of butadiene Hb adducts was accomplished using combined gas chromatography/mass spectrometry (GC/MS), which provides for efficient sample presentation and mixture resolution by capillary gas chromatography, and for sensitive compound detection using the unmatched specificity of mass spectrometry. Recent studies (Osterman-Golkar et al., 1998) have reported detection of as little as 20 pmol epoxybutene-Hb adduct per gram of globin following experimental studies involving mice exposed to 10 ppm in air. Beyond detection alone, estimation of recent systemic exposure is possible following determination of kinetic constants for reactions of activated metabolites with Hb and appropriate Hb adduct quantification. This approach has been used for assessment of systemic exposure to similar toxic unsaturated compounds, including acrylamide (Calleman et al., 1994; Bergmark, 1997), benzene (Lindstrom et al., 1998; Yeowell-O'Connell et al., 1998; Troester et al., 2000), ethylene oxide (Tornqvist et al., 1986) (Walker et al., 1992), and isoprene (Sun et al., 1989; Melnick et al., 1996a; Tareke et al., 1998). Recent research has indicated similar aspects of chloroprene and butadiene with respect to potential carcinogenicity (Tice et al., 1988; Dong et al., 1989; Melnick et al., 1996b; Sills et al., 1999), but no biomarker for chloroprene exposure has been published.

In addition to the butadienes and other volatile organics, the hydrocarbon particulates, generally found in areas of high industrialization, also are extremely important air pollutants. Among these are polycyclic aromatic hydrocarbons (PAHs). These compounds include of some of the most toxic as well as carcinogenic compounds known to man, and clearly have important impact on human health (Pitot et al., 1996). Particulate materials are causative agents involved in bronchitis and cardiopulmonary disease (Watkinson et al., 1998; Kodavanti et al., 2000a; Kodavanti et al., 2000b), and may contain PAHs implicated in a wide variety of cancers. Therefore, the development of sensitive biomarkers for exposure to this series of compounds would provide a vehicle by which we can assess exposure to these compounds prior to onset of disease.

Until recently, evidence for the reaction of metabolically activated PAH or of synthetic epoxides with Hb was confined to the analogous recovery of BaP tetrols following treatment of the protein with acid (Taghizadeh et al., 1994). This approach has been derived from similar work with nucleic acid adducts, as generation of tetrol PAH derivatives from adducted DNA as proof of prior existence of adducts is based on the known susceptibility of the N-guanosine adducts of BaP-diol-epoxides to hydrolysis (Naylor et al., 1990; Fernandes et al., 1998; Jerina et al., 1991). Questions of whether such derivatives arose from covalent or non-covalent binding have been discussed (Tannenbaum et al., 1994). However, red blood cells isolated from normal human blood donors are unlikely to be contaminated with BaP tetrols or shorter-lived non-hemoglobin adducts. Thus isolation and identification of tetrols from human Hb have provided convincing evidence for the formation of adducts with this protein.

Development and implementation of such Hb adduct biomarkers for toxic compounds in Louisville air pollution are an important objective for local risk assessment. As feasibility is dependent on

suitable sensitivity and reproducibility of the assays, determinations of specificity, sensitivity, and reproducibility of potential biomarkers for exposures to chloroprene and PAHs are primary experimental objectives of this proposal. Successful development will allow use of Hb adducts as a molecular dosimeter for purported exposures as a complement to environmental monitoring. Rather than dependence on inferred exposures and conservative worst case estimates, measurement of Hb adducts in blood can provide specific, definitive evidence for risk assessment studies in the form of internal concentration-time products (Ehrenberg et al., 1983; Calleman et al., 1994; Bergmark, 1997; Lindstrom et al., 1998). The form of the exposure estimate is similar to a blood level area under time curve (AUC), a standard measure of experimental drug and toxicant systemic exposure. In providing this data quantification of Hb adducts of chloroprene and PAHs will indicate significant internal exposure and will dramatically reduce uncertainty for risk characterization in prospective studies of air pollutants from industrial and combustion sources.

Approach

Several research groups have developed methods for sensitive and specific measurement of Hb adducts since initial studies determined alkylation of Hb with ethylene oxide (Osterman-Golkar et al., 1976). Such methods have been applied as biomarkers following inhalation studies involving exposure of experimental animals to ppm levels of 1,3-butadiene (CAS# 106-99-0) (Osterman-Golkar et al., 1998; Perez et al., 1997; Richardson et al., 1996) and isoprene (CAS# 78-79-5) (Sun et al., 1989; Bond et al., 1991; Tareke et al., 1998). Review of the limited scientific literature on chloroprene (CAS# 126-99-8) has not revealed such studies or Hb adduct assay procedures developed for exposure assessment, despite potential toxicity of chloroprene and structural features in common with butadiene. As previously mentioned, chloroprene and butadiene are of interest due to documented presence in of these toxic monomers in ambient air samples taken in west Louisville. Given the similarities in structure, an approach analogous to studies done with butadiene will be investigated in the current proposal. Our approach will be to develop biomarker assays in proposed studies through analogy with published work, with modifications as necessary to address differences in toxicant chemistry and potential exposure situations.

Published studies of Hb binding have been conducted for certain PAHs (Pastorelli et al., 1999) (Taghizadeh et al., 1994; Tannenbaum, 1990), although the chemistry of this family of compounds is much more complex than that of butadienes. Selection of compounds for these studies is based on emerging data from environmental assessment studies being conducted in the west Louisville area. Recently detected PAHs are of interest (France, 2000), including acenaphthene (CAS# 83-32-9), dibenzofuran (CAS# 271-89-6), fluorine (CAS# 86-73-7), phenanthrene (CAS# 85-01-8), and fluoranthene (CAS# 205-44-0), as is the classic PAH carcinogen benzo(a)pyrene (BaP, CAS# 50-32-8). This series of PAHs includes some representative compounds found at higher concentrations in the west Louisville Rubbertown area. These compounds have been clearly established as carcinogens (Hopkins et al., 1966; Buu-Hoi et al., 1968; Coombs et al., 1976; Danz et al., 1988; Hecht et al., 1995) in rodent model systems , and are potential causative agents in a variety of respiratory diseases such as asthma and bronchitis. Other PAHs may be added for study as air-monitoring studies proceed.

Materials
1,3-Butadiene (99+ %), 1,3-butadiene-d_6 (98atom %), 1,3-butadiene monoepoxide (98 %), and 1,3-butadiene diepoxide (97%) will be obtained from Aldrich Chemical. Chloroprene will be obtained from staff of Dupont Dow Elastomers, who will also provide information regarding its safe handling, metabolism, and methods for synthesis of epoxide metabolites (Lynch, 2000). Acenapthene, dibenzofuran, fluorene, phenanthrene, and fluoranthene and their corresponding epoxide and diol epoxide derivatives will be purchased from the National Cancer Institute Chemical Carcinogen Repository maintained by the Midwest Research Institute, or synthesized by published methods, such as peroxidation with peroxybenzoic acid (Morrison et al., 1973).. Derivatization agents for GC/MS, such as BSTFA (*N*,*O*-bis[trimethylsilyl]-trifluoroacetamide) will be obtained from Pierce chemical company. These studies may use other specialized reagents too numerous to mention here; these will be obtained in appropriate purities for from various lab suppliers.

Synthesis of globin adduct standards
Quantitative adduct standards and internal standards will be synthesized using modifications of techniques previously described (Cai et al., 1995; Cai et al., 1999). Internal standards will use d_8-valine, or model adducts prepared from the tripeptide ValGlyGly as described by Perez and coworkers (Perez et al., 1997).

Animals
C57BL/6 male mice will be used as an animal model for generation of Hb adducts in vivo and as a means to assess exposure dosimetry. The C57BL/6 mouse meets criteria as a model for controlled binding experiments, including similarity to human hemoglobin. The C57BL/6 mouse expresses only a single Hb (Popp, 1973) as does the human population. C57BL/6 mice have been used in investigations of chemical carcinogens and other related topics, including detailed studies of the metabolism, macromolecular binding, and excretion of carcinogens. The hemoglobin amino acid sequence of C57BL/6 has been fully characterized, differing from human hemoglobin in the alpha chain by only 15 amino acids out of 143 residues, yielding a homology of 90%. Therefore, use of the C57BL/6 mouse for studies of hemoglobin adduct formation should correlate closely with formation of adducts in human hemoglobin (Shapiro et al., 1980).

Sources and storage of erythrocytes
Hemoglobin will be obtained from packed red blood cells obtained from Harlan Bioproducts for Science, Inc., from waste human blood specimens obtained under waste protocols from nearby hospital clinical laboratories, or from fresh blood drawn from C57BL/6 mice obtained through normal laboratory animal supply vendors for the UofL Research Resources Center.

Determination of hemoglobin concentration by spectrophotometry
The concentration of Hb in samples will be determined by a spectrophotometric technique using Drabkin's solution to form cyanomethemoglobin (van Kampen et al., 1965). Absorbance values for hemoglobin are determined at a wavelength of 540 nm using an extinction coefficient of 11.0 mM^{-1}. Concentration of hemoglobin in chromatographic fractions will be determined by measurement of the absorbance at 415 nm using the oxyhemoglobin extinction coefficient of 125 mM^{-1}.

Determination of free sulfhydryl groups in hemoglobin
Free sulfhydryl groups in Hb are determined spectrophotometrically based on formation of 4-thiopyridone in the reaction of 4,4'-dithiodipyridine with Hb sulfhydryl groups. Control and experimental samples are diluted 1:150 with 100 mM sodium phosphate, pH 7.15, containing 1mM

EDTA. To a 3 ml solution of the diluted Hb sample, 200 µl of a 1 mM solution of 4,4'-dithiodipyridine are added. Absorbance at 324 nm is determined after a 40-minute incubation in the dark at room temperature. Corrections are made for background and absorptions of Hb and 4,4'-dithiodipyridine. The reactive sulfhydryl concentration is directly proportional to the absorbance at 324 nm.

Elimination of reactive electrophilic products from blood in vitro
Epoxides of butadiene and chloroprene will be determined from in vitro incubation studies as previously described by Nieusma and coworkers (Nieusma et al., 1997). Briefly, assays for total butadiene monoepoxide is conducted by headspace analysis of in vitro incubates, internal standardization with 2-butanol, and gas chromatographic (GC) analysis on a cross-linked 5%-phenyl methyl silicone capillary GC column (0.25 mm x 30 m), such as DB-5 (J&W Scientific). Detection will be accomplished by mass spectrometry using an HP 5973 mass selective detector quadrupole MS in positive ion, electron impact (EI) ionization full scan mode. Appropriate masses for selected ion monitoring (SIM) GC/MS, as necessary, will be determined from full scan mass spectra.

Production of adducts in blood
Production of adducts in blood will be accomplished in vitro using anticoagulated blood from mice or human waste samples through modification of the procedure described by Lindstrom and coworkers (Lindstrom et al., 1998; Nieusma et al., 1997). Initial in vitro experiments will involve use of isolated erythrocytes exposed in buffer to separate solutions of butadiene, chloroprene, or PAH epoxides at relatively high concentrations to produce adequate levels of adducts. In this procedure erythrocytes pelleted at 3000 *x* g from one ml of freshly obtained blood are suspended in 2 ml PBS and treated with 350 nmol of each epoxide added in 35 µl of dry tetrahydrofuran. The mixture is incubated at 37°C with agitation, and adducts are analyzed as detailed below.

Male C57BL/6 mice (wt 20-25 g) will be pretreated intraperitoneally with chloroprene or PAHs in doses ranging from 1 to 1000 µmol/kg body weight. At selected time intervals, red cells are obtained as previously described from animals anesthetized with carbon dioxide. Isolation of the Hb and globin will take place as described, and the PAH adducts will be analyzed and quantified by various chromatographic techniques including HPLC and GC/MS. We will analyze the decay over time of Hb - PAH adducts by assessing losses in vivo. Additionally, to address stability of adducts formed with Hb and PAHs, we will analyze Hb - PAH adduct levels in stored globin samples at various times after isolation of the protein from mice.

Purification of hemoglobin from erythrocytes
Methodology involves isolation of globin from washed, lysed erythrocytes obtained from anticoagulated blood as described in detail by previous publications from this lab (Cai et al., 1995; Cai et al., 1999). Briefly, erythrocytes from one ml of freshly obtained blood are washed twice with phosphate-buffered saline (PBS) with pelleting at 3000 x g and re-suspension in 2 ml PBS. The cells are then isolated by centrifugation and lysed at 4°C in 1 volume of double distilled water. The cell debris is removed by centrifugation at 10,000 x g for 10 min. The lysate is cooled at 4°C and added drop wise to 300 ml of rapidly stirred 0.015% HCI/acetone maintained at -10°C. The precipitated globin is pelleted by centrifugation, dried overnight at 25°C under nitrogen, and stored at -70 °C. Prior to analyses of acid-labile adducts, globin is dissolved in H_20, and washed with 4 x 1 volume ethyl acetate followed by 4 x 1 volume H_20-saturated 1-butanol to remove non-covalently bound compounds.

Measurement of adducts in globin

For analysis of N-terminal valine adducts, globin aliquots are weighed, and then subjected to modified Edman degradation (Tornqvist et al., 1986) to remove and derivatize the *N*-terminal valine for capillary GC/MS using SIM (Watson, 1990). Known (butadiene) and structurally inferred (chloroprene) ions will be analyzed during initial development of these assays. The chemistry involves reaction of the adducted *N*-terminal amino acid of globin (valine) with pentafluorophenyl isothiocyanate (PFPITC), as shown in Figure 1, below.

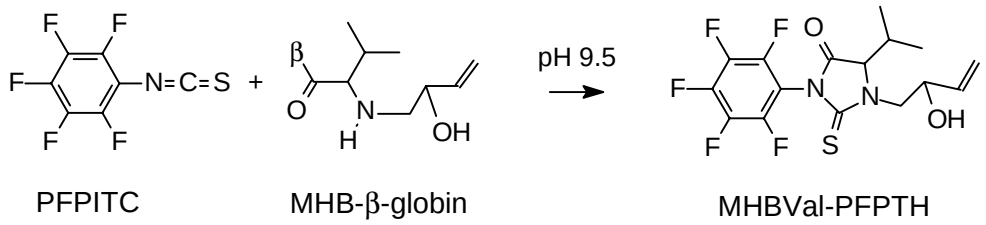

Figure 1. Edman cleavage of MHB-adducted Hb producing volatile derivative for GC/MS

The reaction gives a volatile pentafluorophenyl thiohydantoin (PFPTH) derivative that includes the substituted terminal amino acid; the chemistry of the Hb adduct determines the nature of the substituent. Shown above is the monohydroxybutadiene derivative (MHBVal-PFPTH) formed from reaction of epoxybutadiene with the terminal valine of Hb. Given the chemistry of 1-3-butadiene and chloroprene (2-Cl-1,3-butadiene) and potential epoxides metabolites, several adducts are possible, and diastereomers have been noted (Richardson et al., 1996). Ionic masses definitive for each PFPTH derivative and ionization technique will be used for SIM MS detection in negative ion chemical ionization (NICI) mode, as this mode has proven to be most sensitive (Tornqvist et al., 1986). Studies with butadiene (Osterman-Golkar et al., 1991) monitored ions at mass/charge (m/z) 318 (M^--HF-CH2=CH-CHO) and 374 (M^--HF) for detection and quantification of PFPTH derivatives of the *N*-terminal valine 1,2-epoxy-3-butene Hb adduct in NICI MS ionization mode, and revealed this adduct to be stable. Similarly Richardson and coworkers (Richardson et al., 1996) noted ions used for NICI SIM GC/MS for MHBVal-PFPTH as m/z 374, 323, and 318. These researchers observed a 3:1 ratio of carbon-1 over carbon-2 attachment following opening of the monoepoxide of butadiene. Samples of adducts containing reactive (protic) functional groups will converted to trimethylsilyl derivatives using BSTFA or may use other chromatographic derivatization reagents as necessary for efficient separation and to produce useable chromatographic peaks.

Quantitative determinations will involve comparison of selected ion peak area ratios obtained from GC/MS analyses against standard curves produced with synthetic standards. Nominal mass resolution GC/MS studies will use Hewlett Packard capillary GC and quadrupole MS systems. Detection limits and reproducibility will be evaluated. A promising alternative for investigation involves analogous detection of Hb adducts for the epoxybutanediol metabolite, which were found to be about 70-fold higher than the epoxybutene adduct in exposed human subjects (Perez et al., 1997). Alternative MS procedures will include daughter ion analysis (Osterman-Golkar et al., 1998) by tandem ESI LC/MS using a Micromass triple quadrupole MS or Finnigan ion trap MS systems. Tryptic digest analysis of peptide adducts will employ a Micromass MALDI-TOF MS. High-resolution accurate-mass SIM GC/MS will utilize a VG double-focusing magnetic sector instrument. These additional MS techniques will be used as needed to address experimental requirements for additional sensitivity and specificity.

Analysis of globin adducts by proteolytic digestion and MS
Additional approaches of possible utility include analysis of adducts at internal amino acids of Hb through enzymatic cleavage to peptides. Analysis is by electrospray ionization (ESI) or matrix assisted laser desorption ionization time of flight (MALDI-TOF) MS following HPLC separation of peptides (Banks, 1997; Yates, III et al., 1997; McCormack et al., 1997). This methodology will allow mass spectral characterization of adducts through shifts in normal masses of fragments from Hb peptides formed by proteolytic (e.g., trypsin) digestion of Hb. Analysis of cleaved PAHs will be accomplished by HPLC, utilizing Waters solvent delivery systems, photodiode array detector or fluorescence detector, with selective fraction collection. A Synchropak CM-300 column uses a sodium acetate gradient (pH 6.4, 50 mM - 250 mM) to elute the Hb fractions. Additionally, samples will also be analyzed by reverse phase HPLC using a 25 cm x 4.6 mm Vydac C4 column in which samples and adducts will be eluted from the column with gradients of acetonitrile and water containing 0.1 % trifluoroacetic acid. Fractions will be collected and individually analyzed for the presence of adducts.

Kinetic methodology for tissue dosimetry using globin adducts
The kinetic basis for use of protein adducts to provide systemic dosimetry of electrophiles originated from the work of Ehrenberg and coworkers (Ehrenberg et al., 1974; Ehrenberg et al., 1983; Ehrenberg et al., 1996), and has been used for exposure assessment of various electrophilic compounds such as acrylamide (Calleman et al., 1992; Bergmark et al., 1993; Calleman et al., 1994) and benzene oxide (Lindstrom et al., 1998), among others.

As an overview of this approach, consider that [E] represents the blood concentration of the electrophile E for which dosimetry is desired. The goal is to obtain the systemic (blood) dose D_E of E integrated over time t during which the electrophile disappears exponentially by all processes as described by the first-order rate constant k_e having units of reciprocal time (time^{-1}). The theoretical dose D_E is given by Equation (1):

$$(1) \quad D_E = \int_0^t [E](t)dt = \frac{[E]_0}{k_e} \cdot (1 - e^{-k_e t}) \qquad (1)$$

In this equation $[E]_0$ is the level of E at $t = 0$, and $(1- e^{-ke\,t})$ represents the fraction of $[E]_0$ remaining at time t. However, in ambient exposures, $[E]_0$ is unknown and is the object of the experimental study. This integral describes the product of the concentration [E] over time, or area under the concentration x time curve (AUC).

Within blood E can react with any suitable nucleophile, for example a nucleophilic site in Hb. This can be represented by adduct concentration as [E-Hb], and Hb concentration as [Hb]. Typically in hemoglobin this nucleophilic site can be amino acids cysteine or the valine *N*-terminal primary amine. For reaction of toxic diol epoxides metabolites of PAHs, carboxyl groups in acidic amino acids are typical target sites (Taghizadeh et al., 1994).

As shown in Equation (2), in vitro studies allow determination of the second-order rate constant $k_{Hb\text{-}E}$, with units of (L (g Hb)$^{-1}$ h^{-1}), that characterizes alkylation of the nucleophilic site. In this case [Hb] and [E-Hb] are measured in vitro, $[E]_0$ is known, and the first-order rate constant k_e is determined through incubation of the reactive electrophile E with blood while monitoring and exponentially fitting disappearance of [E].

$$(2) \quad k_{Hb-E} = \frac{[E-Hb]}{[Hb] \cdot [E_0]} \cdot k_e \qquad (2)$$

For dosimetry following exposure the systemic dose to blood is obtained from Equation (3), which will have units of concentration x time and is the AUC for [*E*] as described in Equation (1).

$$(3) \quad D_E = \frac{1}{k_{E-Y}} \cdot \frac{[E - Hb]}{[Hb]}$$

The average concentration of electrophile in blood, [*E*], can be estimated from the systemic dose using Equation (4).

$$(4) \quad [E] = \frac{2 \cdot D_E}{t_{er}}$$

This takes into account the lifetime of the erythrocyte (t_{er}), which varies among species. In the case of humans t_{er} is about 120 days (Guyton, 1977), while in the mouse the erythrocyte lifespan is 40 days (Osterman-Golkar et al., 1976).

Kinetic studies will be conducted in vitro as detailed above to determine second order reaction constants for butadienes. These will enable calculation of blood concentration AUC values from measured adduct levels using the Hb adduct toxicant dosimetry method (Bergmark, 1997) (Calleman et al., 1994; Ehrenberg et al., 1983; Lindstrom et al., 1998; Perez et al., 1997). After reaction constants are produced in vitro, additional in vivo studies will compare intraperitoneal or intravenous dosage with exposure estimates determined from blood levels of Hb adducts as a means to validate the exposure assessment methodology.

Expected Results

Upon completion of the proposed project, we expect to have capabilities to assess exposure to certain toxic unsaturated organic air pollutants that have been documented in ambient air samples taken in west Louisville, KY, or may be of interest for industrial hygiene at facilities using the monomers. In early, published studies (Osterman-Golkar et al., 1991), NICI mode SIM GC/MS analysis enabled measurement of Hb adducts following animal exposures at 250 ppm butadiene. These high experimental levels of butadiene gave globin adduct concentrations of the order of 500 pmol/g after only 12 days exposure. A more recent report (Richardson et al., 1996) indicated lower limits of detection at about 10 pmol/g using the procedure to study Hb adduct levels following intraperitoneal injection of butadiene monoepoxide. These results may forecast limits for chloroprene detection at low ppb levels in ambient air in west Louisville, but the methodology should enable detection of exposures to lower ppm levels as may exist in industrial situations (Osterman-Golkar et al., 1996b; Fajen et al., 1990).

In vitro kinetic studies will provide second-order rate constants following erythrocyte exposure to chloroprene and other adducts that will be useful for calculation of dosimetry at levels where adducts are detected. For reaction of epoxybutene with the *N*-terminal valine of rat Hb, Osterman-Golkar and coworkers (Osterman-Golkar et al., 1998) measured a second order rate constant, k_{val} = 0.4×10^{-4} L (g globin)$^{-1}$ h^{-1}. This result provides a basis for checking results obtained with butadiene in the proposed study. Upon validation of methodology, kinetic rate constants can be used for exposure assessment as previously noted.

At present the biochemistry of chloroprene-protein interactions is only presumed by analogy. However, the proposed studies will add significant information to the limited data generally available on biomarkers for chloroprene. Specific ions for Hb adducts from chloroprene or metabolites will be determined from analogous chemistry considering the substitution of chlorine

for hydrogen at the 2-carbon of butadiene. This represents a net increase in mass of analogous butadiene products by 34 (or 36 for ^{37}Cl isotope) mass units. Detection limits for Hb adducts arising from chloroprene and other carcinogenic air pollutants will be determined as a function of the study. As no studies of Hb biomarkers are evident in the open literature for chloroprene, publication of this preliminary work will provide a basis for future studies that address risk assessment for this monomer.

Other planned studies will determine practical limits for detection of adducts from PAHs. Depending of exposure levels it is reasonable to expect detection of PAH epoxides and diol epoxides that alkylate carboxylate groups to form similarly labile esters. As part of method development, we will determine the capillary GC-MS detection limits for the diols and tetrols derived from hydrolysis of the PAH-Hb esters. Studies of PAH binding to internal cysteines and other amino acids of Hb will reveal and localize binding. Development of methods for specific measurement of adducts at specific sites will provide the basis that leads to methods for exposure dosimetry. As additional data become available from west Louisville air monitoring studies, we will broadened the scope of study to encompass a variety of structures, including acenaphthene, dibenzofuran, fluorene, phenanthrene, and fluoranthene and their corresponding epoxide and diol epoxide derivatives.

In summary, we expect to develop specific methods for monitoring hemoglobin adducts that can be applied toward detection of exposures and assessment of dosimetry of electrophilic, genotoxic chemicals and metabolites that alkylate proteins. Ideally these proposed biomarkers could be used to determine the extent of human exposure in and around the economically important polymer plants and areas of west Louisville, where questions have arisen of potential health effects of air pollution. Prior to such use, careful studies must develop procedures and protocols for such application. Questions of what protein adducts to study, how they should be analyzed, and what is their sensitivity for biological monitoring of industrial and ambient residential air pollutant levels must be answered. Then appropriate application of such analyses will reduce uncertainties in assessment of presumed or actual risks arising from toxic air pollutants in the industrial area that is western Louisville, KY.

General Project Information

Laboratory Resources.
The Therapeutics and Toxicology Laboratory (TTL) occupies approximately 1,200 ft^2 of laboratory space in the Dept. Pharmacology & Toxicology, University of Louisville School of Medicine. Adjacent rooms offer about 300 ft^2 of faculty and staff office space. The lab is equipped with 5 fume hoods, bench space, and storage facilities. Sample refrigerators as well as -20°C and -80°C freezers are within or near the laboratory on the same floor. TTL equipment available to this project includes electronic balances, low-speed centrifuges, liquid/liquid and solid-phase extraction apparatus for sample preparation, and equipment for chemical synthesis of standards and analytical derivatives. A walk-in cold room and freezer are available nearby.

Analytical Instrumentation.
Within the lab five gas chromatographs and four HPLC systems enable compound separation with various means of detection. Three Hewlett Packard quadrupole GC/MS systems enable low resolution scanning of spectra or SIM MS detection. With these, spectral libraries allow computerized library searches of approximately 250,000 spectra. A VG high-resolution double-focusing magnetic MS enables accurate mass spectral analysis of gas, liquid, or solid samples.

Ionization techniques among these systems include electron impact; chemical ionization with methane, isobutane, and ammonia reagent gases; and fast-atom bombardment. Located on the same floor within the Department of Pharmacology and Toxicology are instruments for tandem MS studies of proteins, including a Micromass triple quadrupole MS with electrospray ionization and atmospheric chemical ionization for liquid chromatography/MS, a Finnigan ion trap MS with electrospray ionization, and Micromass matrix-assisted laser desorption ionization time of flight (MALDI-TOF) MS. These instruments enable detailed characterization of peptide chains and proteins through mass analyses of amino acid sequences and adduct substituent groups.

Computer Facilities.
Excellent computer facilities are available. Pentium PCs and specific Windows9x software control lab analytical instrumentation, while Pentium PCs are used by Drs. Cai, Hurst and Myers and for data analysis, graphics, and word processing. Most computers are linked by 10 Mb/s Ethernet and TCP/IP protocol to the UofL wide area network and Unix server systems for WWW & email access and transfer of data files.

Animal Facilities.
The University of Louisville has an AALAC-accredited Research Resources Center whose primary purpose is the maintenance of laboratory animals for research. These state-of-the-art facilities have continually received the highest rating following accreditation inspection and will be used to house mice for the studies described in this application. Treated mice will be maintained in isolator cages separate from other mice.

Personnel.
Harrell E. Hurst, Ph.D., Professor and TTL Director, will provide overall project supervision, reporting of results, and liaison with KIESD air monitoring studies. Dr. Hurst will direct the studies of adducts from butadienes.

Steven R. Myers, Ph.D., Associate Professor and Director of the KIESD, will direct studies with polycyclic aromatic hydrocarbons, provide biochemical and HPLC analyses, implement animal adduct studies, provide access to human blood samples through human studies protocols approved by the UofL Human Studies Committee, and act as general liaison to KIESD.

Jian, Cai, Ph.D., Technical Director of the Mass Spectrometry Core Laboratory, will coordinate analyses proposed in these studies, oversee daily operation of mass spectral instrumentation, and provide chemical synthetic support.

A postdoctoral fellow to be named will implement the studies under direction of Drs. Hurst, Myers, and Cai.

One or more graduate students will participate directly in these studies, with intent of development of a Ph.D. dissertation research project.

Project Timeline.
Year one:

- Acquisition of materials
- Hiring of the postdoctoral fellow
- Chemical synthesis of standards
- Initial in vitro studies to form adducts

Year two:

Continue in vitro adduct production and kinetic studies
Initiate in vivo studies of adduct production and analysis in mice

Project reports will be provided on annual basis or as required by granting agency.

Dissemination of Results
Research results will be provided to local investigators through CEOHS seminars and nationally through peer reviewed journals and presentation at national meetings, such as the annual meetings of the Society of Toxicology or American Society for Mass Spectrometry.

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Resumes

Biographical Sketch

NAME	POSITION TITLE		
Harrell E. Hurst, Ph.D.	Professor		
EDUCATION/TRAINING			
INSTITUTION AND LOCATION	DEGREE	YEAR(s)	FIELD OF STUDY
University of Kentucky	B.S.	1972	Chemistry
University of Kentucky	M.S.	1976	Toxicology
University of Kentucky	Ph.D.	1978	Toxicology

RESEARCH AND PROFESSIONAL EXPERIENCE:

Academic Appointments:

1978 - 1980	Instructor, Department of Pharmacology and Toxicology, University of Louisville School of Medicine
1980 - 1984	Assistant Professor, Department of Pharmacology and Toxicology, University of Louisville School of Medicine
1984 - 1991	Associate Professor, Department of Pharmacology and Toxicology, University of Louisville School of Medicine
1991 - Present	Professor, Department of Pharmacology and Toxicology, University of Louisville School of Medicine

Specialized Training:

April 6-12, 1980	Basic Workshop on Pharmacokinetics, St. Augustine, FL, Edward R. Garrett, Ph.D. Graduate Research Professor, University of Florida

Nov. 13-15, 1990	Workshop, Physiologically Based Pharmacokinetic Models - Principles and Implementation, Chemical Industry Institute Toxicology, Research Triangle Park, NC
February 23, 1992	Implementing PBPK-Interactive Computer Session, Continuing Education Course, Society of Toxicology Annual Meeting, Seattle, WA
August 3-21, 1992	International Workshop on Physiologically Based Pharmacokinetic Modeling and Risk Assessment, Colorado State University, Fort Collins, CO
March 13, 1994	Molecular Biomarkers in Toxicology, Continuing Education Course, Society of Toxicology Annual Meeting, Dallas, TX

Professional Societies:

1977 - Present	American Chemical Society
1980 - Present	American Society for Mass Spectrometry
1981 - Present	Sigma Xi
1983 - Present	Ohio Valley Regional Chapter, Society of Toxicology, 1986: Chapter President
1986 - Present	Society of Toxicology
1989 - Present	International Society for the Study of Xenobiotics

Biographical Sketch
Harrell E. Hurst
Page 2: Selected Publications:

Hurst, H.E. and Dorough, H.W.: A convenient method for synthesis of ^{14}C-carbonyl methylcarbamates. *J. Labelled Compd. Radiopharm.* 14: 11-16 (1978).

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Tsueda, K., Mosca, P.J., Heine, M.F., Loyd, G.E., Durkis, D.A.E., Malkani, A.L., and Hurst, H.E.: Mood during continuous epidural infusion of morphine or fentanyl. *Anesthesiol.* 88(4): 885-891 (1998).

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Biographical Sketch

NAME	POSITION TITLE
Steven R. Myers, Ph.D.	Associate Professor

EDUCATION/TRAINING

INSTITUTION AND LOCATION	DEGREE	YEAR(s)	FIELD OF STUDY
University of Kentucky, Lexington, KY	B.S.	1979	BIOLOGY/CHEMISTRY
Marshall University, Huntington, WVa	M.S.	1982	BIOMEDICAL SCI.
University of Kentucky, Lexington, KY	PH.D.	1986	PHARMACOLOGY

PROFESSIONAL EXPERIENCE:

Assistant Research Professor, University of Kentucky College of Medicine,
Department of Pharmacology, 1988 - 1991
Assistant Professor, University of Louisville School of Medicine,
Department of Pharmacology and Toxicology, 1991-1996
Associate Professor, University of Louisville School of Medicine,
Department of Pharmacology and Toxicology, 1997 - present

MEMBERSHIPS:

American Association for Cancer Research, 1990 - present
Society of Toxicology, 1991 - present
Sigma Xi, 1991 - present
International Society for the Study of Xenobiotics, 1991 - present
Ohio Valley Society of Toxicology, 1991 - present
American Society for Pharmacology and Experimental Therapeutics, 1993 - present
Society of Polycyclic Aromatic Compounds, 1991 - present
American Society for Mass Spectrometry, 2000 - present

PUBLICATIONS

Myers, S.R. and Flesher, J.W. Metabolism of the potent carcinogen 3-methylcholanthrene in preparations of human bone marrow. *Drug Metab Disposition* 18(3):664-669, 1990.

Flesher, J.W., Myers, S.R., and Stansbury, K.H. The site of substitution of the methyl group in the bioalkylation of benzo(a)pyrene. *Carcinogenesis* 11(3):493-496, 1990.

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Myers, S.R. and Flesher, J.W. Bioalkylation of benz(a)anthracene, 7-methylbenz(a)anthracene, and 12-methylbenz(a)anthracene in rat lung cytosol preparations. *Biochem Pharmacol* 41:(11):1683-1689, 1991.

Myers, S.R. and Pinorini, M.T. Reaction of Benzo(a)pyrene -7,8-diol-9,10-epoxide with human hemoglobin and the chromatographic resolution of the covalent adducts. *Polycyclic Aromatic Compounds* 6:143-150, 1994.

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Myers, S.R., Spinnato, J.A., Pinorini, M.T., Cook, C., Boles, C.B., and Rodgers, G.C. Characterization of 4-aminobiphenyl hemoglobin adduct in maternal and fetal blood samples. *J Toxicol Environ Health* 47:101-114, 1996.

Myers, S.R., Pinorini-Godly, M.T., and Spinnato, J.A., HPLC and GC/MS determination of 4-aminobiphenyl hemoglobin adducts in fetuses exposed to the tobacco smoke carcinogen in utero. *Toxicology* 107:209-217, 1996.

Binkova, B., Lewtas, J., Miskova, I., Rossner, P., Cerna, M., Mrackova, G., Peterkova, M., Mumford, J., Myers, S.R., and Sram, R. Biomarker studies in Northern Bohemia. *Environ. Health Perspect.* 104:591-597, 1996.

Cerna M., Pastorkova, A., Myers, S.R., Rossner, P., and Binkova, B. The use of a urine mutagenivity assay in the monitoring of environmental exposurte to genotoxins. *Mutation Res.* 391:99-110, 1997

Kemper RA; Elfarra AA; Myers SR, Metabolism of 3-butene-1,2-diol in B6C3F1 mice. Evidence for involvement of alcohol dehydrogenase and cytochrome p450. *Drug Metab Dispos* 26: 914-20, 1998.

Myers, S.R., Pinorini-Godly, M.T., Reddy, T.V., Daniel, F.B., and Reddy, G. *Gas chromatographic and mass spectrometric determination of hemoglobin adducts of 1,3 dinitrobenzene and 1,3,5-trinitrobenzene in shrew (cryptotis parva). Int. J. Toxicol* 18: 317-325, 1999.

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S.R. Myers, J.A. Spinnato and M.T. Pinorini-Godly. Tobacco Smoke Hemoglobin Adducts in Maternal and Fetal Blood *International Journal of Polycyclic Aromatic Compounds*, 17(3):151-166, 2000.

S.R. Myers, Lynda L. Song, Dan Lantvit, Ronald A. Lubet, Vernon Steele, Gary J. Kelloff[4], Richard C. Moon[3], and John Pezzuto[2,3]. Hemoglobin adducts of the carcinogen 7,12-dimethylbenz(a)anthracene :implications for chemopreventive studies. *International Journal of Polycyclic Aromatic Compounds*, in Press, 2000.

J. Lewtas, and R. Williams, SR Myers, and S. Wise, *Sources of Human Exposure to Airborne PAH. International Journal of Polycyclic Aromatic Compounds*, in press, 2000.

Biographical Sketch

NAME	POSITION TITLE		
Jian Cai	Technical Director for Mass Spectrometry		
EDUCATION/TRAINING			
INSTITUTION AND LOCATION	DEGREE	YEAR(s)	FIELD OF STUDY

Shanghai College of Chemical Industry, Shanghai, PRC	B.S.	1978-1981	Analytical Chemistry
Shanghai Institute of Pharmaceutical Industry, Shanghai, PRC	M.S.	1983-1986	Pharmaceutical Chemistry
Dept. Pharmacology & Toxicology, University of Louisville, Louisville, KY	Ph.D	1993-1999	Pharmacology

RESEARCH AND PROFESSIONAL EXPERIENCE:

Professional Experience:

1981 – 1983	Research worker, Shanghai Institute of Chemical Industry
1986 – 1991	Assistant Researcher, Shanghai Institute of Pharmaceutical Industry
1991 – 1993	Research Associate, Dept. Pharmacology and Toxicology, University of Louisville School of Medicine, Louisville, KY
1999 – 2000	Postdoctoral fellow, Dept. Pharmacology and Toxicology, University of Louisville School of Medicine, Louisville, KY
2000 – present	Technical Director for Mass Spectrometry, Dept. Pharmacology and Toxicology, University of Louisville School of Medicine, Louisville, KY

Research Interest: Application of mass spectrometry and gas chromatography techniques to biomedicine, structure elucidation of organic compounds through spectrometry, and organic synthesis

Awards:

1981	Excellent Student, Shanghai College of Chemical Industry
1981	Excellent Student of Shanghai, Shanghai
1982	Model Worker, Shanghai Institute of Chemical Industry
1998	Second Place Award, Student Poster Competition, Ohio Valley Society of Toxicology Regional Meeting
1998	K.C.Huang Outstanding Student Award, Dept. Pharmacology and Toxicology, University of Louisville School of Medicine

Publications:

Cai,J. and Yi, D.N. Improvement of carbon detector. (Chinese) *Anal. Instrument* 2: 73-76 (1988)

Cai, J. Application of capillary GC in pharmaceutical analysis in biological samples. (Chinese) *Chinese J. Pharm. Anal.* 11: 121-124 (1991).

Cai, J., Myers, S.R., Hurst, H.E.: Measurement of the hemoglobin N-(2-oxoethyl)valine adduct in ethyl carbamate-treated mice, *Toxicol. Appl. Pharmacol*. 131(1): 73-79 (1995).

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Itemized Budget for EPA KY EPSCoR Grant Application								
Biomarkers for Air Pollutants: Development of Hemoglobin Adduct Methodology								
Harrell E. Hurst, PI			Year 1		KY+UofL	Year 2	(3%CoLA)	KY+UofL
Personnel	Salary	%FTE	Cost	Federal	CostShare	Cost	Federal	CostShare
Prin. Invest.	$84,966	30%	$25,490					
Co-PI	$67,381	30%	$20,214					
Res.Scientist	$36,000	50%	$18,000					
Subtotal			$63,704					
Postdoctoral *	$28,500	100%	$28,500					
Total Personnel Costs			**$92,204**	$9,220	$82,984	$94,970	$8,547	$86,423
Fringe Benefit								
Rate	24%		$15,289					
Rate*	17%		$4,845					
Total FB			**$20,134**	$2,013	$18,121	$20,738	$1,866	$18,872
Travel								
State meetings			$1,000					
Scientific meeting			$1,000					
Total Travel Costs			**$2,000**	$2,000	$0	$2,060	$0	$2,060
Equipment								
Glove box			$4,500					
Personal computer			$2,000					
Total Equipment Costs			**$6,500**	$0	$6,500	$0	$0	$0
Supplies								
MS supplies			$8,000					
Stable isotopes			$5,000					
GC supplies			$6,000					
HPLC supplies			$7,000					
Reagents, solvents			$5,000					
Glassware, disposibles			$5,000					
Chemical syn. Supplies			$5,000					
In vitro biological supplies			$2,000					
Animals			$3,000					
Total Supply Costs			**$46,000**	$46,000	$0	$47,380	$47,380	$0
Contractual costs								
MS instrument use			$8,000					
Instrument maintenance			$8,000					
Mouse housing perdiem			$1,000					
Total contractual costs			**$17,000**	$17,000	$0	$17,510	$17,510	$0
Other costs								
Office supplies			$1,500					
Upgrade software			$1,000					
Total other costs			**$2,500**	$134	$2,366	$2,575	$611	$1,964
Total Direct Costs			**$186,338**	$76,368	$109,970	**$185,233**	$75,915	$109,318
Indirect Costs Base ->				$76,368	$103,470		$75,915	$109,318
Indirect Costs	44%			$33,602	$45,527	$81,503	$33,402	$48,100
Total Requested from EPA				**$109,970**			**$109,317**	
Total Cost Share					$155,497			$157,419
Total Project Cost/yr					$265,467			$266,736
Total Project Cost								$532,203

Budget Justification

Personnel
Harrell E. Hurst, Ph.D., Principal Investigator, will direct the studies of adducts from butadienes, provide overall project supervision, and report results. Funds for salary release at 30% full time equivalent are provided in the budget.

Steven R. Myers, Ph.D., Co-Principal Investigator, will direct studies with polycyclic aromatic hydrocarbons, provide biochemical and HPLC analyses, implement animal adduct studies, and conduct in vitro studies with human blood samples. Funds for salary release at 30% full time equivalent are provided in the budget.

Jian, Cai, Ph.D., Technical Director of the Mass Spectrometry Core Laboratory, will provide support for necessary chemical synthesis, coordinate analyses proposed in these studies, and oversee operation of mass spectral instrumentation. As many mass spectral techniques are planned and Dr. Cai will be a key participant, funding at 50% of full time salary is provided in the budget.

A postdoctoral fellow to be named will provide technical assistance with proposed studies under direction of Drs. Hurst, Myers, and Cai. The fellow will manage data and be fully involved with all studies, and 100% full time salary is requested. As this individual will participate in a variety of complex research activities and will bear responsibilities for implementing studies, we will seek an individual with at least one year of postdoctoral experience for full time involvement in the study.

Travel
The budget includes travel and per diem costs for trips to meet with the KY Council on Environmental Quality and KY EPSCoR project sponsors, and to present research findings at an annual scientific meeting each year.

Equipment
Funds are requested in the initial year for equipment. The request includes funds for purchase of a glove box to isolate carcinogenic or other hazardous chemicals from researchers. As potential for exposure is great when compounds are weighed, the model requested is large enough to contain and operate an analytical balance. Funds are requested to purchase a personal computer for use in management of data from the study.

Supplies
Mass spectrometer supplies include expendable items for a high-resolution magnetic sector instrument and three quadrupole mass spectrometers, such as electron multipliers, ionization source filaments and ion focus elements, vacuum gauge tubes, vacuum fittings, and capillary transfer lines. Vacuum pump fluids will be required for mechanical rough pumps and for high vacuum diffusion pumps. Gasses for chemical ionization is included.

Isotopes include ^{13}C-, or ^{2}H-labeled standards of butadiene, butadiene monoepoxide, diepoxy butadiene, valine, and the tripeptide valine-glycine-glycine.

Gas and high performance liquid chromatographic supplies consist of capillary columns for each GC instrument. High-purity helium is required as carrier gas for each. Oxygen and hydrocarbon gas scrubbers, and adsorptive traps for split injector vents will be required to prevent release of carcinogenic materials into the laboratory. Vespel-graphite ferrules are required for each column, as are deactivated injection port liners and septa. HPLC supplies consist of 4 mm, 2mm, and 1 mm inner diameter columns of differing stationary phases for isolation of globin, adducted peptides

from tryptic digestion, and synthetic standard materials. High-pressure pump seals, check valves, low dead volume tubing, fittings, injector components, and detector lamps for spectrophotometric detectors have been included.

Reagents include BSTFA, PFPITC, reagents for methylation and acetylation, and chemical standards for chloroprene, butadiene, benzo(a)pyrene, acenaphthene, dibenzofuran, fluorene, phenanthrene, and fluoranthene. Metabolites of these materials, involving epoxides and dihydrodiols, have been considered. High-purity solvents, such as methanol, isopropanol , acetonitrile, toluene, methylene chloride, hexane, butyl acetate, and salts for buffers are necessary.

Glassware includes general lab ware, disposable glass concentration tubes, autosampler tubes, tubes for frozen sample storage, and specialized glassware for chemical synthesis and product recovery. Variable pipettes, with disposable tips, for volumetric measurement, microliter syringes, and disposable gloves for chemical hygiene and are included in this budget category.

In vitro biological supplies include enzymes for proteolytic digestion of globin samples, cofactors, and isolated packed erythrocytes. Membranes and disposable appliances for purification of peptide-adduct products by dialysis are included here.

Animals include approximately C57BL/6 mice purchased from a general lab animal supplier each year. The budget includes approximately 125 mice in each of the two years.

Contractual Costs
Support is included for use of electrospray ionization triple quadrupole and matrix-assisted laser ionization mass spectrometers, which are not within the investigators' laboratory. Use of these instruments, which are heavily used in a proteomics support core facility managed by another departmental faculty member, will require fees for use.

A contingency fee has been included for non-routine repairs of any of the essential chromatographic or mass spectrometric instrumentation used in this project.

Mouse housing per diem charges have been budgeted for the UofL Research Resources Center, which manages all experimental animal use.

Other Costs
These include moderate allocations for office supplies and upgrade of software necessary to manage data and report research findings.

Second year
Expenses in the second year have been increased by three percent over the first year's budget. This increment will accommodate salary increases, increases in supplies due to inflation, and other, potential unanticipated price increases.

Indirect costs of 44% apply on all items except equipment.

Quality Assurance Statement

Criteria for acceptable data quality

Hypotheses put forth in this proposal center on objectives of developing methods for measurement of hemoglobin adducts as biomarkers of exposure. These 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 and adequate specificity, sensitivity, accuracy, and precision to link unequivocal adduct detection with conditions of exposure. As proposed the assays will 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, digestion of protein by trypsin or other enzymes at specific amino acids, as well as formation of chromatographic derivatives, will provide additional specificity. The sensitivity of each analysis must be determined empirically, as this important parameter results from complex sequential processes derived from the chemical processes of adduct formation and analyses. 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 binding, the nature and stabilities of the adducts, chemistries of sample preparation, and instrumental conditions. Of major importance for success of this project is discovery of analytically accessible, stable adducts which can be converted to chemical derivatives that are reproducibly and uniquely detectable. With preliminary candidate structures for analyses, we will determine sensitivity, as well as the important assay characteristics of reproducibility and accuracy. Precision will be examined by repeated measures, while determination of accuracy will depend on cross checks using multiple preparations derived from standards prepared gravimetrically.

Study design

All experiments will follow a similar design. Study of each compound will begin with acquisition or synthesis of the epoxides(s) for the compounds of interest, as these are the postulated reactive compounds formed by metabolism. Specific epoxides structures will be based on compound metabolic studies that are published, otherwise available from industrial sources, or postulated by analogy. In vitro studies will involve incubation of red blood cells with the epoxides, and candidate adducts will be analyzed by methods previously described. For each assay figures of merit mentioned in the preceding paragraph will be determined. As analytical methods emerge, animal experiments involving single intraperitoneal injection of epoxides and then parent compounds, which will confirm in vivo formation of adducts postulated through in vitro experiments. Subsequent in vitro studies measuring disappearance of electrophiles and appearance of adduct will be necessary to define second order pharmacokinetic rate constants. As previously detailed these rate constants will be used to infer systemic exposure from blood levels of adducts.

Sample handling procedures

All chemicals will be handled carefully with due respect to their toxic nature and potential for chemical and biochemical reaction. Information available from material safety data sheets will be fully considered. Information regarding potential chemical hazards will be sought from the open literature, from the commercial sources of the chemicals, and through consultation with the University of Louisville Department of Environmental Health and Safety and other sources.

Unique numbers that establish date of collection, source, and linkage to laboratory notebook details regarding each experiment will identify all samples. Blood samples will be handled with regard for potential biochemical degradation, and will be stored in most stable form, such as precipitated

globin dried under nitrogen. Isolated proteins will be maintained frozen at minus 70°C. Blood samples will be regarded as potential sources of viruses, and will be handled with biohazard precautions. Animals will be maintained as stipulated by the University of Louisville Research Resources Center. Treatment and sampling will be accomplished according to standard procedures established by the Institutional Animal Care and Use Committee of the University.

Analytical instrument calibration

Mass spectrometers will be calibrated using standard tuning procedures recommended by the manufacturer. As an example, electron ionization GC/MS systems will be tuned with computer-automated (Autotune) procedures using perfluorotributylamine as the tuning compound for mass axis and ion intensity. For full scan spectra that require library searchable spectra of standard ion abundances across mass axis, BFB (bromofluorobenzene) will be used to examine, document, and tune for standard mass intensities. Similar procedures using comparisons with accepted standards or published spectra will be used for other mass spectral techniques. Quantitative chromatographic and mass spectrometric analyses will utilize internal standards, and stable isotope analogs will be used whenever possible in the latter assays. Instrument responses will be recorded as response ratios that compare peak areas of analytes and corresponding internal standards. Quantification will be determined by comparison of response ratios of samples to those of standards.

Procedures for data reduction and reporting, with statistical methods

All biomarker assays will be developed and reported with consideration for the primary objectives of evaluation of specificity, sensitivity, accuracy, and precision for each determination. Signal to noise ratios of three to one will be absolute minimum for detection, while assay precision will be defined and included in calculations for limits of quantification of each assay. All significant quantitative measurements will be conducted in triplicate, with reporting of the mean $\pm$ standard deviation for each. For comparisons between two mean values, the Student's t-test will be used. Among multiple means, analysis of variance (ANOVA) will be used unless specific procedural issues dictate use of nonparametric tests. Computerized statistical testing will rely on commercially available software, such as the SigmaStat program (SPSS, Inc.). Fitting of rate constants to data for compound disappearance or appearance of adduct will rely on nonlinear least squares software such as SigmaPlot (SPSS, Inc.), Scientist (MicroMath, Inc.), or other nonlinear fitting routines as are generally available in commercial software products.

Qualitative or quantitative procedures used to evaluate success of project

The project will be judged successful through development of specific, reproducible methodology for analytical measurement of covalent hemoglobin adducts produced in vivo following administration of each chemical. A measure of success with each will depend on limits of sensitivity for each assay and the ability to measure adducts following defined in vitro and in vivo exposures of these toxic air pollutants. As the studies proposed do not include animal inhalation exposures due to absence of available inhalation facilities, it will not be possible to relate adduct levels to ambient exposure levels in these initial experiments. Extension of studies to include this important information will await future studies. For chloroprene such studies might be possible in the future through use of inhalation facilities made available by industry.