PROTEIN ADDUCTS AS MEASURES OF EXPOSURE IN EPIDEMIOLOGICAL RESEARCH

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A dissertation submitted to the faculty of the University of North Carolina at Chapel Hill in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Department of Environmental Sciences and Engineering, Gillings School of Global Public Health.

Chapel Hill
2009

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ABSTRACT

WILLIAM EDWARD FUNK: Protein Adducts as Measures of Exposure in Epidemiological Research
(Under the direction of Stephen M. Rappaport)

Protein adducts can serve as biomarkers of exposure to variety of xenobiotic toxicants and reactive endogenous species. However, despite their utility as exposure biomarkers, protein adducts have rarely been used in epidemiology. One reason for this is the cost and difficulty of obtaining blood samples, which frequently limits the uses of protein adducts as measures of exposure in large-scale studies. In addition, protein adducts are typically low in abundance compared with unmodified proteins, and are thus often difficult to detect in human blood. This research developed two novel methods that address these limitations in current assays of blood-protein adducts. First, to make blood proteins more accessible to epidemiologic studies, a method was developed to measure protein adducts in dried blood spots (DBS). Because DBS are easier to collect and store than conventional venous blood samples, they encourage applications of biomarkers of exposure in population studies. In addition, neonatal DBS which are collected from virtually all live births in the U.S. can be used to investigate chemical exposures in utero. Second, to increase analytical sensitivity for detecting less-abundant adducts, a method was developed to enrich cysteinyl adducts of human serum albumin (HSA). Because the major site of adduction of HSA is the single free sulfhydryl group located at Cys^{34}, the enrichment approach utilized the propensity of mercaptalbumin (i.e., unadducted HSA) to bind with thiol-affinity resins. Furthermore, because the most common adducts of HSA-Cys^{34} are mixed

disulfides formed by reversible reactions between Cys^{34} and serum thiols (notably cysteine), mixed disulfides were liberated via reduction with dithiothreitol (DTT) prior to removing mercaptalbumin with thiol affinity resins. These two methods provide a straightforward approach for obtaining and processing blood specimens so as to promote the use of protein adducts as biomarkers of exposure in epidemiologic investigations.

This dissertation is dedicated to my beloved wife Molly Losh.

ACKNOWLEDGEMENTS

I thank my research advisor Steve Rappaport for his continued guidance, wisdom, and friendship. I am grateful for having had the opportunity to work with him, and for the incredible experience of being involved in such exciting research. Steve taught me the virtue of perseverance. Thanks to my Ph.D. committee members, Dr. Louise Ball, Dr. Avram Gold, Dr. Michael Hudgens, and Dr. Michael Goshe for their support and counsel. Thanks to Dr. Suramya Waidyanatha for all of her valuable insights and ideas, and for teaching me about protein adduct chemistry. I thank Dr. Joachim Pleil for his constant encouragement, gracious support, and friendship, and to the rest of my colleagues and mentors at the US Environmental Protection Agency who made much of this work possible, most notably Dr. Myriam Medina-Vera, Dr. Michael Madden, Scott Clifton, and Matthew Stiegel. Thanks to my UNC lab mates Jon Sobus, Sung Kim, Yu-Sheng Lin, Renan Wu, Berrin Serdar, Alistair Henderson, Russell Bowen, David Kim, and Frank Onyemauwa for their cherished friendships and for making coming to work enjoyable. Thanks to my collaborators at UC Berkeley; specifically Harry Li, Tony Iavarone, Jacques Riby, Maria Demireva, and Professors Evan Williams and Martyn Smith, for providing me with my research home away from home. Thanks to Dr. David Leith and Maryanne Boundy for their unfailing support and understanding during my lengthy transition from graduate student to post-doc.

Most significant, however, I thank my family for the countless ways they supported and encouraged me throughout my graduate work. I thank Molly, for her love and

encouragement; Babette, Cosmo, and Dobro, for always lifting my spirits; and my Mom and Dad, and my mother-in-law Carol, for always believing in me and supporting me along the way.

This work was supported by the National Institute of Environmental Health Sciences through grants P30ES10126, P42ES10126, and U54ES016115.

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1,4-BQ	1,4-benzoquinone
1,4-BQ-Gb	1,4-benzoquinone-globin
2D-gel	two dimensional-gel
ACN	acetonitrile
API	atmospheric pressure ionization
Asp	aspartic acid
BO	benzene oxide
BQ	benzoquinone
°C	degrees centigrade
$CaCl_2$	calcium chloride
CDC	Center for Disease Control and Prevention
CM 10	weak cation exchange ProteinChip array
Cys34	cysteine^{34}
CV	coefficient of variation
Da	dalton
DBS	dried blood spots
DI	deionized
DNA	deoxyribose nucleicacid
DTT	dithiothreitol
EI	electron impact ionization
ESI	electrospray ionization
EtOH	ethanol

FT/ICR	fourier transform / ion cyclotron resonance
g	gravity
Gb	globin
GC/MS	gas chromatography/mass spectrometry
Glu	glutamic acid
GSH	glutathione
h	hour(s)
Hb	hemoglobin
HbA	hemoglobin adult
HbF	hemoglobin fetal
HCl	hydrochloric acid
He	helium
Hg	mercury
His^{143}	$histidine^{143}$
HSA	human serum albumin
$HSA\text{-}Cys^{34}$	$human\ serum\ albumin\text{-}cystein^{34}$
IAA	iodoacedic acid
KDa	kilodalton
LC/MS	liquid chromatography/mass spectrometry
l	liter
LOD	level of detection
M	molar
mA	miliampere

mg	milligram
min	minute(s)
ml	milliliter
mm	milimeter
mmol	milimolar
MRM	multiple reaction monitoring
MS	mass spectrometry
MT	methane sulfonic acid and trifluoroacetic anhydride method
MWCO	molecular weight cutoff
m/z	mass/charge
NaCl	sodium chloride
NC	North Carolina
NICI	negative ion chemical ionization
nm	nanometer
NSQAP	Newborn Screening Quality Assurance Program
PBS	phosphate buffered saline
pmol	picomolar
PPT	precipitation
Q 10	strong anion exchange ProteinChip array
Q-TOF	quadrupole-time of flight
RBC	red blood cell(s)
rpm	rotations per minute
SD	standard deviation

SDS	sodium dodecyl sulfate
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SELDI-TOF	surface enhanced laser desorption ionization – time of flight
Ser^{143}	$serine^{143}$
SIM	selective ion monitoring
S_N1	substitution nucleophilc first order
S_N2	substitution nucleophilc second order
T3	third largest peptide
TCEP	tris(2-carboxyethyl)phosphine
TIC	total ion current
UCB	University of California, Berkeley
µg	microgram
µl	microliter
µM	micromolar
UNC	University of North Carolina
UV-vis	ultraviolet-visible
V	voltage
v/v	volume/volume

1. Background and Significance

1.1. Exposure assessment

1.1.1. Historical background

The science of exposure assessment involves the estimation of parameters to characterize the distributions of environmental and/or biological levels of toxicants across exposed populations, along with statistical evaluation and interpretation of such parameter estimates (Rappaport and Kupper 2008). Quantitative exposure assessment has its roots in industry, where workers in the early 20th century exposed to certain chemical toxicants were deemed at risk for occupational diseases (Rappaport and Kupper 2008). The increased risk in this population motivated governments to set occupational exposure limits to protect workers. In 1946 Threshold Limit Values were established for 150 different chemicals (LaNier 1984). To test for compliance air sampling strategies were developed to measure levels of airborne toxicants within the breathing zones of the workers. However, static air measurements often underestimated worker exposures. This complication led to the use of personal samplers in the 1950s, a method that better reflects the levels of chemical toxicants to which workers are truly exposed (Cherrie 2003).

Since the 1950s, exposure assessment has moved beyond the workplace to environmental settings. With growing concern and awareness of elevated pollution levels, especially within major cities, legislation has been passed to regulate chemical emissions in the outdoor environment. The United States enacted a series of clean air acts starting in 1955 that has resulted in substantial reduction in air pollution, especially during the past three decades

(Elichegaray, Bouallala et al. 2009). Despite these reductions, the state of the science shows that significant health effects remain as evidenced by epidemiological studies that relate exposures to ambient air pollution and non-accidental mortality (Dockery and Pope 1994; Schwartz and Morris 1995; Dominici, Zeger et al. 2000). Thus, ambient air monitoring is critical for air regulatory processes and continues to be an important tool to estimate human exposures to environmental toxicants.

1.1.2. Air measurements versus biomarkers

When inhalation is the primary exposure route for a chemical toxicant, exposure estimates can be derived from measurements of air concentrations (Cherrie 2003). Air measurements can often be made with relative ease and low cost, thus permitting large numbers of measurements to be obtained. However, the large variability in air measurements over-time can bias their use as surrogates of exposure when estimating exposure-response relationship (Lin, Kupper et al. 2005; Scheepers 2008).

One alternative to air measurements is biological monitoring, which involves quantification of exogenous compounds, or their products, within accessible biological matrixes such as blood, breath, and urine (Rothman, Stewart et al. 1995; Mutti 1999). Lin et al. reported that biological measurements typically have smaller intraindividual variability than the corresponding air measurements and thus provide less biasing estimates of true exposure levels (Lin, Kupper et al. 2005). Furthermore, biomarkers also account for all exposure routes (e.g. air, dermal, and ingestion), and capture inter-individual differences in metabolism and uptake, which are missed with air measurements (Rappaport, Symanski et al. 1995; Bartsch 2000; Sabbioni and Jones 2002).

1.1.3. Biomarkers in epidemiological research

The term epidemiology is derived from the Greek word epidemios, and literally means "the study of what is upon the people". Within this framework, environmental epidemiology examines the relationship between levels of environmental exposure with human diseases (Scheepers 2008). One major limitation of epidemiology has been the lack of statistical power to associate exposures with disease outcomes due to exposure misclassification (Benke, Sim et al. 2001). Traditionally, epidemiologists have relied upon surrogate exposure measures, such as self-reported exposures and nonspecific measures such as "…time living within a contaminated environment" (Sim 2002). These exposure estimates can be grossly inaccurate and often fail to reflect the degree and nature of true exposures.

To surmount this limitation, epidemiology has increasingly turned to biomarkers as exposure surrogates (Wild 2009). Biomonitoring provides a more reliable estimate of internal dose, which decreases the likelihood of exposure misclassification (Lin, Kupper et al. 2005). However, biomarkers have limitations in epidemiological research. Because environmental toxicants tend to be chemically reactive species, their short life spans *in vivo* make them difficult to measure directly within biological specimens. As a result, exposures can be underestimated when sampling fails to occur within these narrow measurement windows. This shortcoming has motivated the use of stable adducts of these compounds with abundant blood proteins as exposure biomarkers.

1.2. Protein adducts

1.2.1. Background

Elizabeth and James Miller first observed the formation of addition products between small electrophiles and proteins in the late 1940s (Miller 1947). These investigations demonstrated that when rats were dosed with the hepatic carcinogen, *p*-dimethylaminoazobenzene, metabolites of this chemical would bind tightly to cellular constituents, which the investigators hypothesized to be liver proteins. Although it was not yet recognized at this time that cellular DNA was the target of genotoxic carcinogens, this hallmark study recognized the importance of metabolism in chemical carcinogenesis, and was the first report of protein adducts.

Chemical carcinogenesis is thought to be initiated by modification of DNA by genotoxic carcinogens (Turesky and Vouros 2004). DNA is not, however, the only target site of reactive electrophiles *in vivo*. For example, alkylating agents that can modify DNA share similar reaction kinetics with nucleophilic residues on proteins (Ross 1962). Studies in the 1970s motivated the use of protein adducts as biomarkers of carcinogen dose (Groth and Neumann 1972; Ehrenberg, Hiesche et al. 1974). Protein adducts offer several advantages over DNA adducts as exposure biomarkers. First, proteins are much more abundant than DNA in blood (hemoglobin ~150 mg/ml, serum albumin ~30 mg/ml, DNA ~0.005 mg/ml) (Tornqvist, Fred et al. 2002). Second, unlike DNA adducts, protein adducts are not subject to excision repair processes –this leads to simpler calculations of systemic dose (Skipper and Tannenbaum 1990).

Protein adducts can also be used as biomarkers of noncarcinogenic toxicity (Lopachin and Decaprio 2005) as illustrated by the examples in Table 1.1. Other mechanisms that do not directly involve adduct formation, such as oxidative stress, can also contribute to cellular

damage. Since exposure to electrophilic compounds can lead directly and indirectly to the production of reactive oxygen species, adducts of these species can serve as indirect exposure biomarkers (Fabisiak, Sedlov et al. 2002; Lang, Figueroa et al. 2004). Thus, protein adducts can serve as biomarkers in a host of applications.

Table 1.1. Examples of chemical toxicants that form protein adducts and their organ-specific targets.

Chemical Toxicant	**Organ System Toxicity**
acrolein	neurotoxicity
acrylamide	neurotoxicity
allylamine	cardiotoxicity
2-methylfuran	renal toxicity
N-acetyl-*p*-benzoquinone	hepatotoxocity

Assays of protein adducts have focused upon hemoglobin (Hb) and human serum albumin (HSA), the most abundant protein in blood and plasma, respectively (Tornqvist and Kautiainen 1993). Stable adducts accumulate during the mean residence time of the protein, which allows the integration of exposure over relatively long periods of time. Hemoglobin has a life span of 120 days which is equivalent to the life span of erythrocytes, the cells in which Hb is contained (Ehrenberg, Moustacchi et al. 1983). Consequently, Hb clearance follows zero-order elimination kinetics. In contrast, HSA turnover is independent of the age of the individual protein molecules and follows first-order elimination kinetics with a half-life of 20-25 days (Skipper and Tannenbaum 1990). Figure 1.1 illustrates protein adduct elimination profiles for HSA and Hb

following an acute chemical exposure. Using these elimination parameters, mathematical properties of both proteins as chemical dosimeters have been derived (Ehrenberg, Moustacchi et al. 1983; Sabbioni, Skipper et al. 1987).

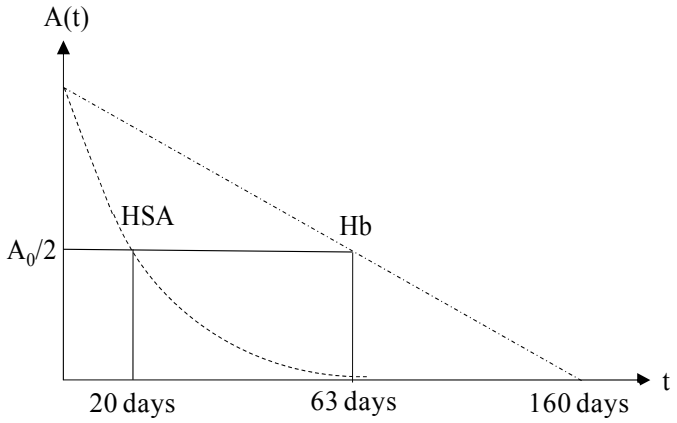

Figure 1.1. Protein adduct elimination profiles for HSA and Hb following an acute chemical exposure. A denotes adduct level, where A_0 is the initial adduct level after an acute exposure, and A(t) is the level of adducts at time t (Adapted from Tornqvist et. al, 1993).

1.2.2. Protein adduct chemistry

Protein adducts are covalent modifications that are formed via reactions between small reactive electrophiles and nucleophilic protein targets. Electrophilic species are characterized as compounds that contain regions of low electron density, whereas nucleophilic targets consist of amino acid residues containing heteroatoms with increased electron density (namely oxygen, nitrogen, and sulfur) (Tornqvist, Fred et al. 2002; Parthasarathi, Subramanian et al. 2004). HSA and Hb contain numerous nucleophilic sites, including the thiols of cysteine residues, carboxylic acid moieties of aspartic acid, glutamic acid, and the *C*-terminus; the amine groups of histidine, lysine, tryptophan, and the *N*-terminus; and the hydroxyl groups of serine and tyrosine (Tornqvist, Fred et al. 2002). Electrophiles can react with nucleophiles by multiple mechanisms,

including S_N1 and S_N2 substitution, 1,4-addition, Schiff-base formation, and radical-mediated reactions (Tornqvist, Fred et al. 2002). Of these, substitution mechanisms are most common. Second-order reaction rates of electrophiles with nucleophilic targets can be estimated using the Swain-Scott empirical relationship

$$\log (k_Y/k_{H_2O}) = sn \qquad \textbf{Eq. 1.1.}$$

where k_Y is the rate constant of reaction between a given electrophile and nucleophile (Y), relative to the electrofile reactivity with water (k_{H_2O}), s is the selectivity constant, and n is the strength of the nucleophile (Swain and Scott 1953). Reaction rates generally increase in line with s, where the reactivity of nucleophilc sites on the protein follows the order O<N<S (Ross 1962). As such, protein sulfhydryls (namely the sulfur atoms of cysteine) tend to be the most nucleophilic sites on proteins. In addition to the physico-chemical nature of the nucleophile, the reactivity of a nucleophilic site is also governed by the microenvironment within the protein. As such, the tertiary structure of the protein, and the proximity with neighboring amino acid residues, can lead to steric shielding and alterations to the electronic properties of the nucleophile, which can in turn alter the reactivity (Lopachin and Decaprio 2005; Stewart, Blindauer et al. 2005).

1.2.3. Protein adducts as exposure biomarkers

Many classes of compounds and/or their reactive metabolites react with proteins to form adducts. These electrophilic groups are summarized in Table 1.2. Many analytical strategies have been employed to detect protein adducts, including laser-induced fluorescence (Weiss 1999; Ozbal, Skipper et al. 2000) and immunochemical approaches (Poirier 1984; Santella 1988; van

Welie, van Dijck et al. 1992). However, the most common analytical approach to detect protein adducts employs mass spectrometry (Rubino, Pitton et al. 2009). Sensitive gas chromatography/mass spectrometry (GC/MS) methods have been developed to identify and quantify specific adducts (Waidyanatha, Lin et al. 1996; Yeowell-O'Connell, Jin et al. 1996; Waidyanatha, Yeowell-O'Connell et al. 1998; Yeowell-O'Connell, Rothman et al. 1998; Rappaport and Yeowell-O'Connell 1999; Waidyanatha, Troester et al. 2002; Aronson, Bartha et al. 2004). Since analytes must be volatile in GC/MS experiments, these methods require that adducts (or modified amino acids) be cleaved and isolated from the protein prior to analysis. In addition, adducts are often derivatized to enhance volatility and sensitivity. For example, the derivatization of quinone adducts using trifluoracetic anhydride to produce trifluorothioacetates, can be detected using negative-ion-chemical-ionization (NICI) mode with GC/MS (Waidyanatha, Yeowell-O'Connell et al. 1998).

Recent advances in liquid chromatography/mass spectrometry (LC/MS) technologies have greatly improved the ability to detect protein adducts of modified proteins, peptides, and amino acids (reviewed in (Rubino, Pitton et al. 2009)). In addition, various ionization techniques, such as electrospray ionization (ESI) and atmospheric pressure ionization (API), coupled with tandem mass spectrometry have enabled researchers to detect and identify protein adducts without the volatility restraints of GC/MS methods (Johnstone and Rose 2001).

Although many assays have been developed to measure protein adducts, one major impediment to the use of protein adducts in large-scale environmental studies remains the cost and difficulty of obtaining blood samples (Funk, Waidyanatha et al. 2008). Traditional protein adduct assays require trained phlebotomists to draw venous blood samples, a procedure that is both invasive and costly. In addition, once blood samples have been obtained, immediate field

processing is often required to separate the red cells (containing Hb) from the plasma (containing HSA), prior to freezing and transporting specimens for analysis. This impediment has motivated the use of dried blood spots for blood collection (Couzin-Frankel 2009).

Table 1.2. Classes of compounds and/or their reactive metabolites that react with proteins through various mechanisms to form adducts.

Electrophilic Agent Class	Examples	Mechanism
Alkylating and arylating	Alkyl halides	Nucleophilic substitution
	Oxiranes	Nucleophilic substitution
	Alkynitrosamides	Nucleophilic substitution
	Alkylnitrosamines	Nucleophilic substitution
	Dialkyl sulfates	Nucleophilic substitution
	Alkyl alkanesulfonates	Nucleophilic substitution
	Quinones	1,4-Addition
Electrophilic nitrogen species	Aromatic amines	Nucleophilic substitution
Carbonyl compounds	Aldehydes	Schiff base
Acylating	Organic acid anhydrides	Nucleophilic substitution or addition
Phosphorylating	Organo phosphorous compounds	Nucleophilic substitution
Metal ions	Beryllium (II)	Multiple mechanisms
Free radicals	Hydroxyl radical	Radical-mediated reactions

1.3. Dried blood spots

1.3.1. Newborn dried blood spots

Guthrie and Susi first used filter paper for the collection and analysis of drops of whole blood to screen newborns for hyperphenylalaninemia, a condition associated with the genetic disease phenylketonuria (Guthrie and Susi 1963). This innovative and simple blood collection technique fostered a national newborn screening program in the United States, where today more than 98% of live births are screened for treatable metabolic diseases (Olshan 2007). Recent

advancements in analytical capabilities, *i.e.* tandem mass spectrometry, have greatly increased the number of disorders that screening laboratories can detect. For example, the State Laboratory of Public Health in North Carolina uses newborn dried blood spots (DBS) collected on Guthrie cards to screen newborns for more than 35 disorders (Table 1.3.).

Table 1.3. Metabolic disorders screened by the State Laboratory Pubic Health in North Carolina as of 2008.

Argininosuccinic aciduria	Carnitine/acylcarnitine translocase deficiency
Citrullinemia	Carnitine palmitoyltransferase II deficiency
Homocystinuria (cystathionine beta synthase)	Medium-chain acyl-CoA dehydrogenase deficiency
Maple syrup urine disease / Branched-chain ketoacid dehydrogenase	Multiple acyl-CoA dehydrogenase deficiency
Phenylketonuria / Hyperphenylalaninemia	Long-chain 3-hydroxyacyl-CoA dehydrogenase deficiency
Tyrosinemia type II	Short-chain acyl-CoA dehydrogenase deficiency
Tyrosinemia type III	Trifunctional protein deficiency
Glutaric acidemia type I	Very long-chain acyl-CoA dehydrogenase deficiency
Multiple carboxylase deficiency	Biotinidase deficiency
3-Hydroxy-3-methylglutaryl-CoA lyase deficiency	Congenital adrenal hyperplasia
Isobutyryl-CoA dehydrogenase deficiency	Galactosemia/ galactose-1-phosphate uridyl transferase deficiency
Isovaleric acidemia / Isovaleryl-CoA dehydrogenase deficiency	Primary congenital hypothyroidism
Beta-ketothiolase (BKT) / Short-chain keto acylthiolase deficiency	Hemoglobin C disease
Methylmalonic aciduria	Hemoglobin E disease
2-Methylbutyryl-CoA dehydrogenase deficiency	Sickle cell disease
3-Methylcrotonyl-CoA carboxylase deficiency	Sickle/hemoglobin C disease
Propionic acidemia	Sickle/hemoglobin E disease

(http://slph.ncpublichealth.com/Newborn/DisordersTested.asp, 2009)

The Newborn Screening Quality Assurance Program (NSQAP) at the Center for Disease Control and Prevention (CDC) has provided technical support and assistance for DBS specimen collection to more than 215 laboratories in 37 countries over the past 25 years (Mei, Alexander et al. 2001). This effort has helped to achieve better consistency in blood collection among laboratories and has worked to assure stringent quality control with the blood collection

products. The Whatman series specimen collection paper used for newborn screening has been rigorously evaluated by the Newborn Screening Quality Assurance Program using an isotope doping method to characterize chromatographic, hematocrit, and blood volume effects (Mei, Alexander et al. 2001). Quality assurance issues concerning collection, transport, and storage of DBS have also been addressed. To assure consistency between lots of specimen collection cards, all lots are routinely evaluated by NSQAP to verify compliance within established guidelines, thus providing consistency in specimen collection between newborn screening laboratories.

1.3.2. Dried blood spots in environmental research

In many states, newborn DBS are stored for extended periods of time in case re-analysis is needed (Couzin-Frankel 2009). These DBS archives create opportunities in molecular epidemiology for retrospective studies. A review article by Mei et al. in 2001 reported more than 160 biomarkers that have been detected in dried blood spots (Mei, Alexander et al. 2001). More recently, researchers have used DBS to screen for genetic abnormalities including cystic fibrosis and hemoglobinopathies (Enevold, Vestergaard et al. 2005; Enevold, Nkya et al. 2007). DBS have also been used in adolescent and adult populations to measure *C*-reactive protein and glycosylated hemoglobin, anti-malarial antibodies, and HbA1c (McDade, Burhop et al. 2004; Corran, Cook et al. 2008; Lomeo, Bolner et al. 2008). In fact, a Pub Med search of "dried blood spots" identified more than 350 peer-reviewed papers that have been published since the 2001 publication of Mei et al.

The recent explosion in the use of DBS to screen human populations stems from several advantages that DBS offer over venous blood collection. Blood collection by heel or finger prick is less invasive than venipuncture. Collection of DBS does not require a trained phlebotomist, which drastically decreases the cost of obtaining blood specimens in population-based studies.

Also, DBS can be dried and transported in flat envelopes via standard mail, and long-term storage of DBS requires substantially less space than frozen whole blood or fractionated-blood samples. These advantages have motivated the use of DBS in a large number of population-based surveys (table 1.4.) (McDade, Williams et al. 2007).

Table 1.4. Large population-based surveys conducted using DBS. (Adapted from McDade et al. 2007)

Study	N	Biomarkers Measured	Study Website
Great Smokey Mountains Study	1,071	Androstenedione, DHEA-S, EBV antibodies, estradiol, FSH, LH, testosterone	http://devepi.mc.duke.edu/GSMS.html
Health and Retirement Study	7,000	CRP, HBA1c, total cholesterol, HDL	http://hrsonline.isr.umich.edu
Los Angeles Family and Neighborhood Survey	5,000	CRP, EBV antibodies, HBA1c, total cholesterol, HDL	http://www.lasurvey.rand.org
National Longitudinal Study of Adolescent Health	17,000	CRP, EBV antibodies, HBA1c	http://www.cpc.unc.edu/addhealth
National Social Life, Health, and Aging Study	1,945	CRP, EBV antibodies, HBA1c, hemoglobin	http://www2.norc.org/nshap
Study of the Tsunami Aftermath and Recovery	35,000	CRP, EBV antibodies	http://chd.ucla.edu/STAR/STAR.html

1.3.3. Protein adducts in dried blood spots

The use of DBS to develop assays that measure protein adducts as exposure biomarkers is attractive for large-scale epidemiological studies. Protein adducts in newborn DBS are of particular interest because they afford the opportunity to measure prenatal exposures to chemical toxicants using archived newborn specimens. Since adducts accumulate over the life span of a protein, protein adducts in newborn DBS permit assessment of an integration of exposure over a relatively wide exposure window. For example, the 120-day life span of Hb allows estimation of newborn exposures to adducting species during a substantial span of fetal development [note that

the fetal Hb variant is reported to have a similar half-life as the adult variant] (Brace, Langendorfer et al. 2000).This feature is of particular importance because fetal life and early infancy are developmental periods of heightened susceptibility to environmental hazards (Grandjean, Bellinnger et al. 2007). Factors that put the fetus at higher risk include greater cell proliferation rates, greater number of cells with heightened risk, decreased immunologic competence, and lower capacity to detoxify chemical toxicants and repair DNA (Anderson, Diwan et al. 2000). Consequently, chemical exposures during these sensitive developmental windows can result in increased risk for disease and disabilities in infants and children. Protein adducts in DBS offer valuable opportunities for investigating such chemical exposures *in utero* and their possible links to adverse health outcomes.

1.4. Adducts of HSA-Cys^{34}

1.4.1. Human serum albumin

Human serum albumin (HSA) is the most abundant protein in plasma and represents approximately 60% of total plasma proteins (Kawakami, Kubota et al. 2006). Produced in the liver, HSA is a single-chain biopolymer consisting of 585 amino acid residues that make up three homologous domains (I, II, and III), subdivided into two subdomains (A and B) (Stewart, Blindauer et al. 2005). HSA has a total of 35 cysteines, with 34 of the residues intramolecularly bond as disulfides (Suji and Sivakami 2008). The single remaining free cysteine, Cys^{34}, represents the largest fraction of free thiol in plasma (Fabisiak, Sedlov et al. 2002). Within the tertiary protein structure of HSA, Cys^{34} resides in a unique micro-molecular environment close to three ionizable residues: Asp^{38}, His^{39}, and Tyr^{84} (Figure 1.2.) (Stewart, Blindauer et al. 2005). As a result, Cys^{34} has an unusually low pKa and exists primarily in its more reactive thiolate

form. Despite this fact, crystallographic studies show that Cys^{34} is buried in a crevice approximately 9.5 Å deep, which limits its accessibility (Stewart, Blindauer et al. 2005). Consequently, HSA-Cys^{34} is heterogeneous with respect to redox status, and exists both in the reduced and oxidized forms.

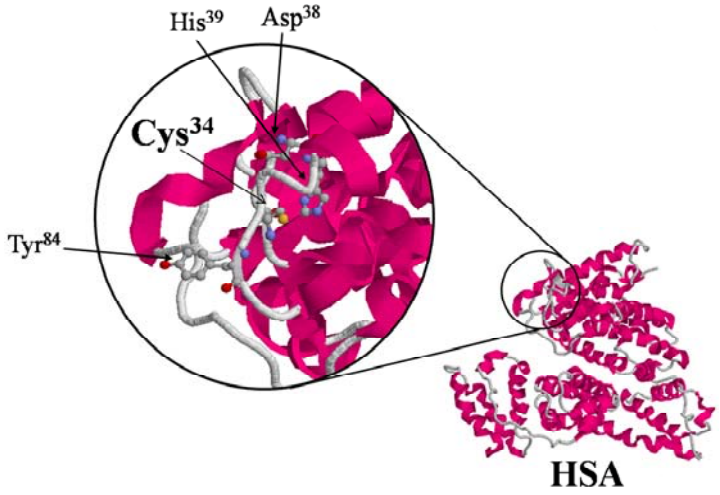

Figure 1.2. The micro-molecular environment of HSA-Cys^{34}. Three ionisable residues: Asp^{38}, His^{39}, and Tyr^{84}, are responsible for Cys^{34}'s unusually low pKa.

1.4.2. Biological role of HSA

HSA has several biological functions, including the maintenance of colloidal osmotic pressure, binding and transport of endogenous compounds, and scavenging of reactive inflammatory mediators (Lang, Figueroa et al. 2004; Ogasawara, Namai et al. 2006; Ogasawara, Mukai et al. 2007; Marjolaine, Rondeau et al. 2008). The Cys^{34} residue of serum albumin is highly conserved across all mammalian species (Stewart, Blindauer et al. 2005), which suggests that Cys^{34} is an essential residue involved in the physiological function of HSA. A variety of compounds have been reported that bind with HSA-Cys^{34}. In the circulating plasma, HSA-Cys^{34}

forms disulfides with free cysteine, glutathione, and homocysteine (Beck, Ambahera et al. 2004). It also transports over 80% of nitric oxide in blood as Cys^{34}-*S*-nitrosothiol (Stamler, Jaraki et al. 1992). In addition, HSA-Cys^{34} is the binding site for clinically important exogenous compounds, such as gold(I) antiarthritic drugs, platinum(II) anticancer drugs, and mercurial drugs (Malik, Otiko et al. 1980; Roberts, Xiao et al. 1996; Ross, Carr et al. 2000; Stewart, Blindauer et al. 2005; Timerbaev, Hartinger et al. 2006). Other drugs that bind with Cys^{34} through disulfide linkages include the antihypertensive drug, Captopril, and the alcohol-abuse drug, Disulfuram (Agarwal, McPherson et al. 1983; Keire, Mariappan et al. 1993).

HSA-Cys^{34} redox state correlates with health status, where a higher degree of oxidation has been reported in cases of hepatic diseases (Watanabe, Matsuzaki et al. 2004), diabetes (Suzuki, Yasuda et al. 1992), renal diseases (Soejima, Matsuzawa et al. 2004), aging (Era, Kuwata et al. 1995), and fatigue (Imai, Hayashi et al. 2002). The major structural change found in higher oxidized HSA is the formation of the mixed disulfides between Cys^{34} and free cysteine (Kawakami, Kubota et al. 2006).

1.4.3. HSA-Cys^{34} adducts as exposure biomarkers

HSA-Cys^{34} is frequently modified by electrophilic xenobiotic species and/or their reactive metabolites (Farmer 1995). As such, the HSA-Cys^{34} adductome has been used extensively in exposure assessment. One of the first applications of protein adducts as exposure biomarkers was as a surrogate to measure exposure to genotoxic chemical carcinogens (Groth and Neumann 1972; Ehrenberg, Hiesche et al. 1974). In these studies, levels of HSA-Cys^{34} modification were employed to quantify *in vivo* carcinogen dose. More recent studies have shown strong correlations between adduct concentrations and occupational and environmental exposures. For example, log-scale scatter plots are provided in Figure 1.3. of HSA-Cys^{34} adducts

of benzene oxide (BO) and 1,4-benzoquinone (1,4-BQ), versus personal benzene exposure for 439 Chinese workers (Yeowell-O'Connell, Rothman et al. 2001; Rappaport, Waidyanatha et al. 2002; Lin, McKelvey et al. 2006). Note that relative adduct concentrations, which are ratios of the individual adduct concentrations to median adduct levels in concurrent controls, were used to account for difference across control groups. With both HSA-Cys^{34}-BO and HSA-Cys^{34}-1,4-BQ, a clear dose-response is observed.

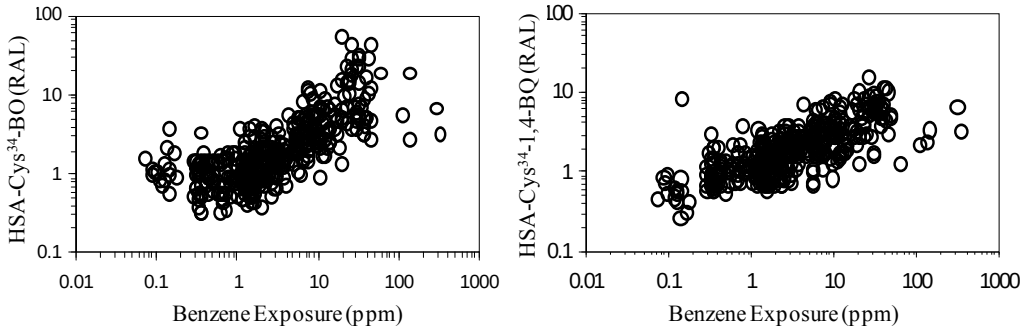

Figure 1.3. HSA-Cys^{34} adducts of benzene oxide (BO) and 1,4-benzoquinone (1,4-BQ), versus personal benzene exposure for 439 Chinese workers. (Figure from Rappaport et. al. The Use of Newborn Blood Spots in Environmental Research: Opportunities and Challenges Meeting, Chapel Hill, NC, Feb 20, 2007)

The utility of HSA cysteinyl adducts as exposure biomarkers is not limited to chemical mutagens and carcinogens. Many other classes of toxicants and/or their electrophilic metabolites covalently modify HSA-Cys^{34} (Lopachin and Decaprio 2005). In fact, it has been hypothesized that adduct formation with protein thiols is a critical initiating step in the mechanism of neurotoxic action (Graham, Amarnath et al. 1995; Lotti 2000). Consequently, HSA-Cys^{34}-bound neurotoxins fall closer to health outcome within the exposure-disease continuum, and therefore

may provide a stronger exposure-disease relationship than those observed with chemical carcinogens.

HSA-Cys^{34} is also modified by scavenging endogenous reactive species. Some notable examples are carbonylation, *S*-nitrosylation, and disulfide formation (Bar-Or, Heyborne et al. 2005; Musante, Bruschi et al. 2006; Musante, Candiano et al. 2007; Perna, Acanfora et al. 2007). Disulfide adducts can undergo further oxidation to form sulfenic, sulfinic, and sulfonic derivatives (Aldini, Regazzoni et al. 2008). All these modifications have been employed as biomarkers of associated physiopatholgical conditions, including oxidative stress, renal disease, growth restriction, and glomerulosclerosis (Aldini, Regazzoni et al. 2008). Given the profusion of chemical species that modify HSA-Cys^{34}, this reactive nucleophilic target is clearly a promising site for surveying modifications as potential biomarkers.

1.5. Specific goals

The primary aim of this study is to extend the use of protein adducts as exposure biomarkers to epidemiological research. Towards this end, the following specific goals are given:

Goal 1: Develop a method to measure protein adducts in DBS. This goal will (1) permit direct measurement of prenatal exposures to chemical toxicants using archived newborn DBS, and (2) will provide a less invasive and less expensive blood collection procedure to make protein adducts assays more applicable to population-based studies. As proof-of-concept that protein adducts can be measured in DBS, benzene oxide adducts of Hb will be measured and compared between newborns and adults. Method validation will be achieved by comparing adult DBS to matching specimens isolated from venous whole blood (Paper 1).

Goal 2: Develop a method to enrich cysteinyl adducts of HSA. The enrichment approach will utilize the propensity of free sulfhydryls in HSA to bind with thiol-affinity resins. HSA will first be reduced to liberate Cys^{34} bound mixed disulfides, and mercaptalbumin will be subsequently removed using thiol-affinity covalent chromatography. Mass spectra will be acquired for intact HSA to assess optimal reduction conditions and the effectiveness of the enrichment procedure. Overall adduct levels will be estimated by measurement of total proteins after enrichment, i.e. after removal of mercaptalbumin, using the Bradford assay and gel electrophoresis (Paper 2)

2. Development of a Method to Measure Protein Adducts in Dried Blood Spots

The goal of this work is to develop a method to measure protein adducts in DBS. The DBS sampling medium offers several advantages over conventional venous blood specimens, including less invasive blood collection, simplified specimen processing and shipment, and most notably, an opportunity to access archived neonatal blood samples which are stored as residual Guthrie cards.

2.1 Isolation of proteins from dried blood spots

2.1.1. Precipitation of proteins using addition of acetone

Obtaining sufficient protein is a major challenge when developing an assay for protein adducts in DBS. This challenge arises from (1) the limited mass of protein contained within a single drop of blood, and (2) the complexity of the whole blood matrix. Given the limited blood volume present in a DBS, high protein recovery is required when isolating proteins for measuring protein adducts. In addition, to be useful for large-scale epidemiological studies, the isolation protocol should be relatively simple and inexpensive. To address these criteria a method was explored to isolate Hb and HSA from DBS using a cosolvency approach.

Many factors affect a protein's solubility. One important parameter is the dielectric constant of the solvent. Polar solvents, such as water, have high dielectric constants. In contrast, organic solvents have relatively low dielectric constants, and often result in protein precipitation (Chin, Wheeler et al. 1994). When two miscible solvents are combined, the resulting dielectric constant is intermediate to those of the individual solvents. Consequently, the dielectric constant

of a mixture can be controlled by the relative ratios of solvents. Proteins in a mixture can then be separated by identifying the solvent combinations that selectively precipitate individual proteins.

Cosolvency experiments were conducted using mixtures of acetone and water. Solvent conditions were explored to separate Hb from HSA because the two proteins make up over 90% of total blood proteins. Initial experiments were conducted using purified Hb and HSA (Sigma-Aldrich, St. Louis, MO) to determine the approximate solvent ratios where each protein precipitated individually. Based on these crude results, twenty solutions were prepared with increasing percentages of acetone, ranging in dielectric constant from approximately 48 to 24. Blood was spotted onto specimen collection cards from a single volunteer via finger lancet and was dried overnight. Single DBS were excised and extracted using 4 ml of deionized water. Extracted blood samples were then concentrated using a speed vacuum system to approximately 200 µl. The concentrated blood samples were added drop-wise to the different solvent mixtures. All protein solutions were incubated for 3 hours at 4°C. Vials were centrifuged at 2,200 x g for 15 minutes to pellet any precipitated proteins and to allow the removal via Pasteur pipet of the supernatant fractions for analysis. The soluble protein fractions were analyzed using UV/vis spectrophotometery, monitoring absorbance at 280 and 523 nm. Solvent blanks were used to subtract out the absorbance due to acetone. Absorbance at 280 nm was used to estimate total protein, whereas absorbance at 523 nm was used for more specific detection of oxyhemoglobin and deoxyhemoglobin. Total protein concentrations were confirmed using the Bradford. Results are shown in Figure 2.1.

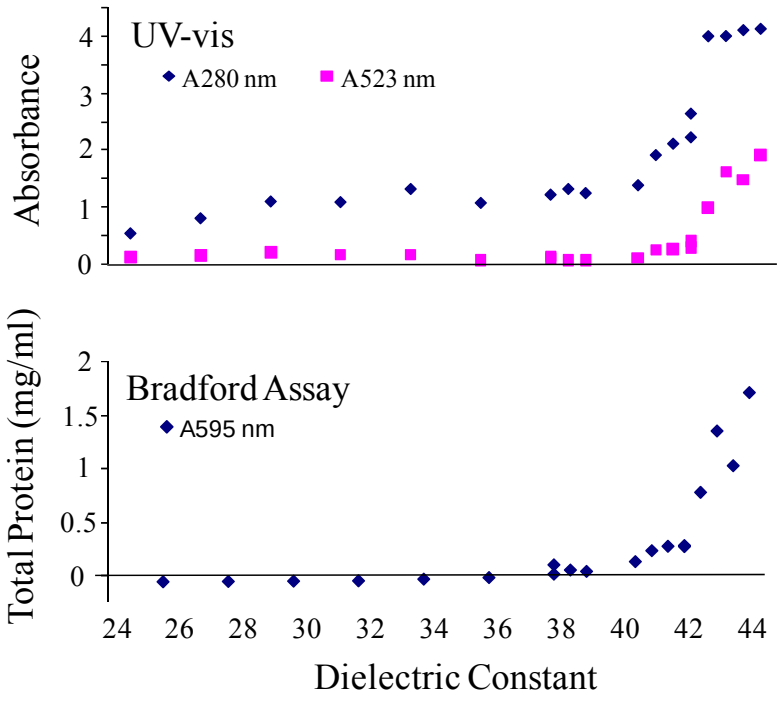

Figure 2.1. DBS protein precipitation using addition of acetone. The upper panel shows UV/vis absorbance for proteins eluted from DBS using various acetone concentrations. Absorbance at 523 nm was used to monitor Hb precipitation, and absorbance at 280 nm was used to track total DBS protein solubility. In the lower panel, the Bradford assay was used for total protein quantitation.

The results in the upper panel of Figure 2.1 were in agreement with the cursory results obtained using isolated Hb and HSA. When examined as single proteins, Hb precipitated when the dielectric constant fell below approximately 40. In contrast, HSA remained soluble until the dielectric constant reached values below approximately 28. Monitoring absorbance at 280 nm, proteins extracted from DBS appeared to precipitate at dielectric constants between 40 and 42, with an increase in precipitation around 28. While such results suggest that the strategy was separating Hb from HSA, results from the more specific Bradford assay indicated that the vast majority of the protein precipitated with a dielectric constant of about 40, suggesting that HSA in

the crude mixture precipitated at substantially higher dielectric constants than when present alone.

It was later confirmed, that the presence of Hb altered HSA's solubility. The non-protein substance that absorbed at 280 nm was present in the supernatant when Hb was precipitated as a single protein. When Hb was removed, and HSA was placed in the Hb supernatant, HSA precipitated under solvent conditions that would otherwise solubilize HSA. This change in solubility may be due to formation of a bilirubin-HSA complex. Bilirubin is formed during hemoglobin decomposition, and is know to bind with HSA with very high affinity (Vlasova, Golovkova et al. 2005). Further studies are required to test this hypothesis.

2.1.2. Isolation of Hb in DBS using addition of ethanol

A method to isolate Hb from DBS was developed using ethanol/water cosolvency. The method was developed using a similar approach to the one described above using acetone/water mixtures. The use of ethanol as a solvent was chosen based on a previous study by Tanaka et al. who used an ethanol/water/chloroform mixture to purify bovine serum albumin that was contaminated with Hb (Tanaka, Sawatani et al. 2001).

Various solvent parameters were investigated using ethanol concentrations ranging from 10 to 65 percent. The experiment was performed twice, with and without 0.6% chloroform. Absorbance was measured at 523 nm to monitor the Hb concentration, and the Bradford assay was used to determine total protein in each sample (Figure 2.2.).

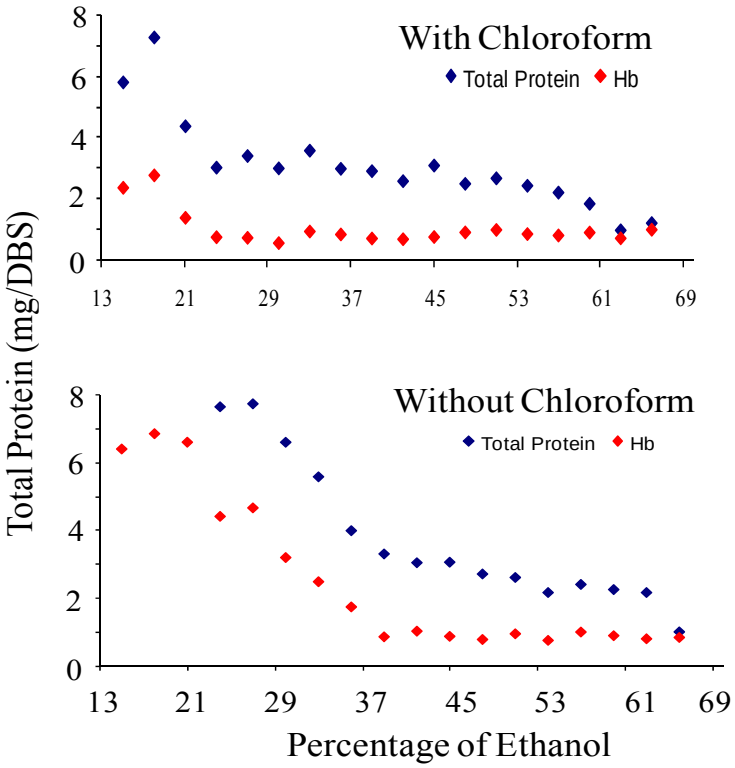

Figure 2.2. DBS precipitation of proteins by addition of ethanol. Total protein precipitation occurred at approximately 21 and 35 percent ethanol, with and without chloroform addition, respectively. HSA precipitated at approximately 61 and 65 percent ethanol, with and without the addition of chloroform, respectively. The range in between these percentages represents conditions where HSA is soluble, and Hb is not.

Although chloroform was observed to shift the solubility of both Hb and HSA, it was excluded from the final protocol because chloroform did not provide any advantage in separating Hb from HSA. In contrast with acetone, the addition of ethanol did not cause HSA to co-precipitate with Hb. Based on experiments performed with single proteins, and with total DBS proteins, 43% ethanol was determined to be the optimal concentration to precipitate Hb while retaining HSA in solution.

2.1.3. Isolation of globin from Hb

After the Hb was isolated from the DBS, a second precipitation step was preformed using acidic acetone at -20°C. This procedure denatures the Hb and separates the globin (Gb) fraction of the protein from the heme fraction via precipitation of the Gb. Details of this procedure are outlined in *Section 3.3.4*. A schematic of the entire DBS Gb isolation method is shown in Figure 2.3.

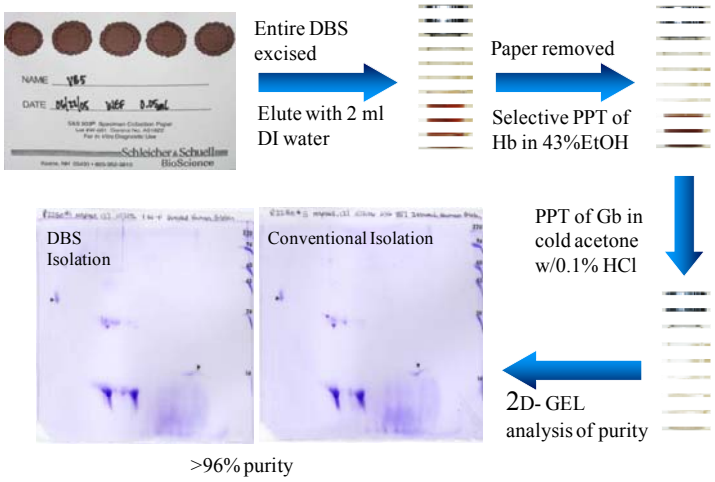

Figure 2.3. A schematic of the DBS Gb isolation method, with two-dimensional electrophoresis gel images comparing the purity of the isolated Gb to our conventional venous blood protocol.

2.1.4. Assessment of Gb purity

To determine the purity of the Gb isolated from DBS, two-dimensional gel electrophoresis was performed using the carrier ampholine method of isoelectric focusing (Kendrick Labs, Madison WI). A detailed description of the procedure is provided in *Section 3.3.5*. Gel images of the isolated Gb are shown in Figure 2.3. A 2D-gel image of the supernatant fraction is shown in Figure 2.4, which is primarily HSA.

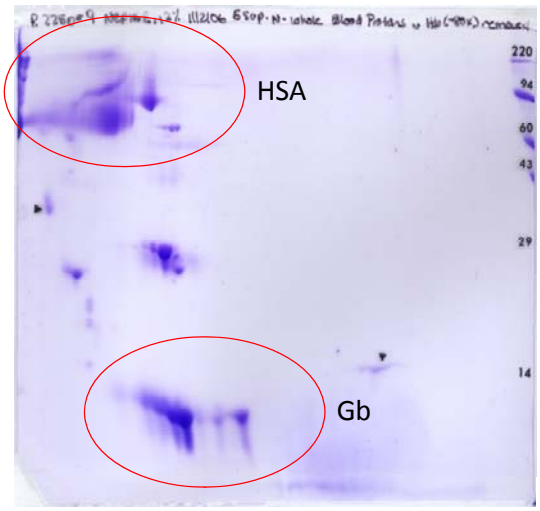

Figure 2.4. Two-dimensional gel electrophoresis image of the supernatant fraction after Hb precipitation.

The purity of the isolated globin, assessed by two dimensional gel electrophoresis, was determined by laser densitometry to be ~96%. The purity of HSA in the supernatant fraction was 46.6%. Because HSA represents 12% of the total protein in whole blood, this indicates that HSA was substantially purified in the supernatant fraction, although the supernatant still contained other blood proteins (~34.6% from Hb). The percentage of ethanol could be increased during the cosolvency precipitation to achieve greater HSA purity, but this would be at the expense of reducing the purity of Hb.

2.1.5. Qunatitiation of isolated Gb from adult and newborn DBS

Gb was isolated from 50 adult DBS and 52 newborn DBS using the procedures detailed in *sections 2.1.2 and 2.1.3*, for precipitation of Hb and Gb, respectively. The adult DBS were prepared using archived venous blood that was previously stored at -80°C. Each adult DBS was prepared using 50 µl aliquots of whole blood. The newborn DBS came from heel sticks and were received within 30 days of blood collection from the North Carolina Laboratory of Public Health (Raleigh, NC). Each newborn DBS consisted of a single spot excised from the specimen

collection cards which was then individually sealed in a plastic bag and stored at room temperature until extracted. Globin mass isolated for each DBS specimen was determined using the Bradford method.

The mean Gb isolated from adult and newborn DBS was 5.0 mg (SD = 0.9) and 9.4 mg (SD = 2.4), respectively. These results are consistent with data from previous studies, in which newborns had higher Hb concentrations than adults (Kates and Kates 2007).

The mass of Gb required for protein adduct assays is less than 5 mg of isolated protein (Waidyanatha, Yeowell-O'Connell et al. 1998; Rappaport, Waidyanatha et al. 2002). Based on the mass of Gb isolated from the newborn and adult DBS, and given the relatively high purity of the protein (determined via two-dimensional gel electrophoresis), DBS appear to be a suitable medium for obtaining blood specimens for assaying Hb adducts.

2.2 1,4-benzoquinone adducts in dried blood spots

2.2.1. 1,4-benzoquinone adducts of Hb in adult and newborn DBS

As proof-of-concept that protein adducts can be measured in DBS, 1,4-benzoquinone adducts of Gb (1,4-BQ-Gb) were measured in 50 adult and 51 newborn DBS specimens. A method to measure mono-*S*-substituted cysteinyl adducts of 1,4-benzoquinone (1,4-BQ) was developed in our laboratory and is described in detail elsewhere (Waidyanatha, Yeowell-O'Connell et al. 1998). In short, 3 mg of Gb was spiked with 5 µg of isotopically labeled internal standard ([$^2H_3$]1,4-BQ-Gb). Ascorbic acid was added to reduce 1,4-BQ adducts to hydroquinone, and trifluoroacetic anhydride and methanesulfonic acid were reacted with the protein to cleave cysteinyl protein adducts and convert them to volatile trifluorothioacetates**.** The derivatized analytes were solvent exchanged into hexane and were concentrated to a final volume of

approximately 200 µl under a gentle stream of nitrogen. Quantitative analysis was performed using GC/MS in chemical ionization-negative ion mode.

Levels of 1,4-BQ-Gb quantified in the 51 newborn and 50 adult DBS are shown in Figure 2.5. The estimated mean level of 1,4-BQ-Gb in the newborn and adult DBS was 3.93 pmol/mg Gb (SD 1.41) and 1.84 pmol/mg Gb (SD 0.63), respectively. Estimated adduct levels were not assumed to be distributed normally and were compared between groups using the nonparametric Wilcoxon rank-sum test. 1,4-BQ-Gb levels were significantly different between the two groups ($p < 0.005$), with newborn DBS having approximately two times higher adduct levels than adult DBS.

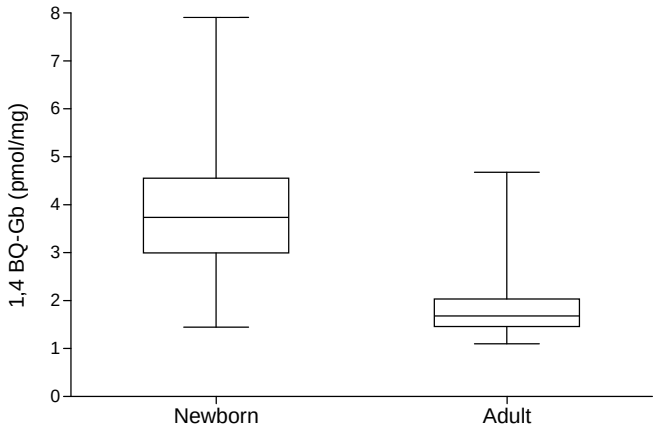

Figure 2.5. 1,4-BQ-Gb levels in newborn and adult DBS.

With our conventional Gb isolation procedure (isolated from venous blood), Hb samples are dialyzed against deionized water prior to Gb precipitation. This step is not included in the DBS Gb isolation protocol because smaller soluble compounds are likely to be removed when the Hb is precipitated, and the supernatant is decanted. However, a possible explanation for the elevated adduct levels observed in the newborn population could be that additional adducts are

formed with small thiol sources, such as glutathione. These may be present in the DBS samples since the DBS protocol did not include a dialysis step. This hypothesis is tested in *Section 2.2.2*.

2.2.2. Reassessment of 1,4-BQ-Gb following dialysis

Globin isolated from 48 newborn and 22 adult DBS was dialyzed to remove any quinone-adducted small thiols. Aqueous Gb samples were transferred to 12-14 KDa molecular weight cut off (MWCO) dialysis tubing and were dialyzed against 4 liters of deionized water at 4°C. The water bath was changed 3 times during the 12 hour-hour assay. Dialyzed Gb was transferred to pre-weighed 4 ml glass vials and was dried using a speed vacuum system and weighed. Three mg portions of Gb were re-assayed for 1,4-BQ-Gb using the MT assay described above.

The mean level of 1,4-BQ-Gb in the newborn DBS was 1.39 pmol/mg Gb (SD 0.60) and the mean level of 1,4-BQ-Gb in the adult DBS was 0.68 pmol/mg Gb (SD 0.15). These results are summarized in Figure 2.6 and are compared to those from the undialyzed specimens. As before, adduct concentrations were compared between groups using the nonparametric Wilcoxon rank-sum test. Consistent with undialyzed Gb adduct levels, 1,4-BQ-Gb was approximately two times higher in the newborn DBS samples. The mean adduct levels were significantly different between the two populations ($p < 0.05$). Interestingly however, adducts levels were significantly lower in both post-dialyzed groups. This result was surprising, given that the adult Gb had previously been dialyzed.

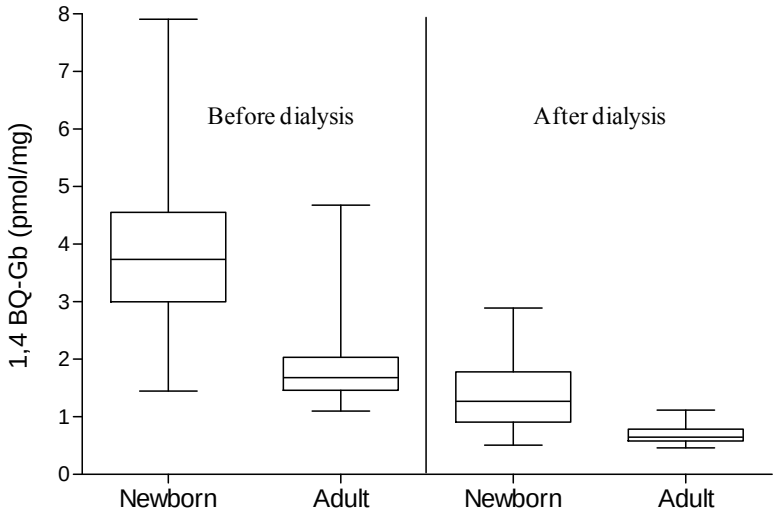

Figure 2.6. 1,4-BQ-Gb levels in newborn and adult DBS, before and after dialysis.

2.2.3. Reaction of newborn and adult Gb with 1,4-BQ

Newborn DBS had consistently higher 1,4-BQ-Gb concentrations than the adult DBS for both the dialyzed and undialyzed Gb specimens. A possible explanation for the elevated adduct levels in the newborn DBS is that 65-85% of newborn Hb is comprised of the HbF variant, whereas the dominant Hb variant in adults is HbA (Cochran-Black, Cowan et al. 2001). Both protein variants contain four subunits; however, HbA has two α-subunits and two β-subunits, whereas the HbF contains two α-subunits and two γ-subunits. In both the β- and γ-subunits Cys^{93} is conserved, and is the site of 1,4-BQ modification. However, in the γ-subunit His^{143} is replaced with Ser^{143}. This amino acid substitution impedes 2,3-bisphosphoglycerate binding in the HbF variant. Because 2,3-bisphosphoglycerate is a competitive inhibitor of oxygen binding, this change results in higher oxygen affinity in fetal blood compared to adults (Orzalesi and Hay 1972). Due to the close proximity of $His^{143\gamma}$ and $Cys^{93\gamma}$ (Figure 2.7), we hypothesize that this

amino acid substitution could also cause structural changes that may provide better access to $Cys^{93\gamma}$ in the fetal variant, thus increasing Cys^{93} activity. If so, such a mechanism could act as a defensive mechanism to scavenge reactive electrophilic species more efficiently during fetal development.

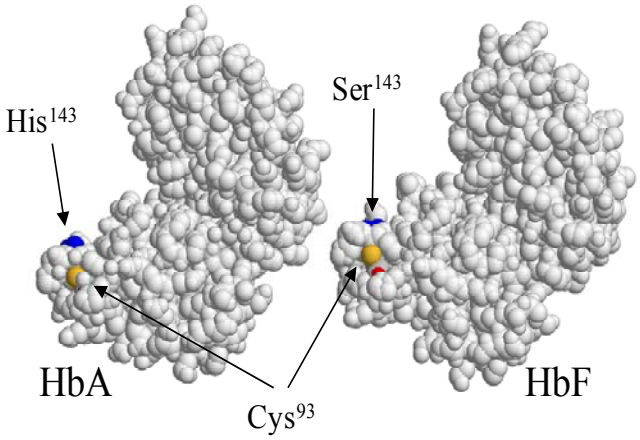

Figure 2.7. Three dimensional space-filling structures of the adult and fetal variants of Hb, showing the greater accessibility of Cys^{93} in the HbF variant. (http://www.rcsb.org/pdb/home/home.do)

To test whether the HbF variant was more reactive than the HbA variant, blood was obtained from a single newborn and the mother at birth. Hb was isolated from the blood and the proteins were reacted with 1,4-BQ. Whole umbilical cord and matching maternal blood collected by venipuncture were obtained from UNC Hospitals. The umbilical cord blood (42 ml) was collected at birth, placed in citrate, stored a 4ºC until received by our lab 13 hours later. The maternal blood (14 ml) was collected one hour prior to receipt by our lab.

Both specimens were immediately centrifuged at 2,200 x g for 15 minutes. The upper plasma layer, containing HSA, was removed by Pasteur pipet. The red blood cell fraction was

washed three times with 0.9% sodium chloride solution. The plasma and red blood cell fractions were divided; half the specimens were frozen at -20˚C and half were stored overnight at 4˚C.

The red blood cells were diluted with an equal volume of deionized water and stored at 4˚C overnight to lyse the red blood cells. The lysate was centrifuged at 30,000 x g for 45 minutes at 4˚C to pellet the cell membranes. The supernatant fractions were decanted and dialyzed using 6-8 KDa MWCO dialysis tubing overnight, with 3 changes of water (4 liters each). Hemoglobin concentrations were determined in each of the samples using the Bradford assay. Concentrations of the maternal Hb and fetal Hb were determined to be 111.3 mg/ml and 107.5 mg/ml, respectively.

One μM 1,4-BQ was reacted with the maternal and fetal Hb, using a 221-fold molar excess of Hb. Because newborn and maternal blood share a common HSA variant, maternal and fetal HSA were also reacted with 1,4-BQ to serve as positive controls. The reactions proceeded for 1 hour at 37°C. Based on previous 1,4-BQ kinetic experiments performed in our laboratory, the reactions were assumed to have gone to completion. The solutions were dried using a speed vacuum system. One mg aliquots of HSA and 3 mg aliquots of Hb were assayed in duplicate for 1,4-BQ cysteinyl adducts using the MT assay (*Section 2.2.1.*).

Mass spectrometry response ratios, relative to the isotopically labeled internal standards, are provided in Table 2.1. Relative responses were compared across groups to assess if the HbF variant was more reactive than the HbA adult variant. To account for differences observed with the adult and newborn DBS, the HbF variant would need to be approximately twice as reactive as the HbA variant to be detected.

Table 2.1. Reaction of adult and fetal Hb variants with 1,4-BQ. Results are provided as NCI-GC/MS relative ion response ratios between the analytes and the internal standards.

Reaction status	Sample ID	Protein mass (mg)	Relative response (RR)	RR/protein mass
Unreacted	MaternalHSA	1	0.108	0.108
	MaternalHSA	1	0.109	0.109
	FetalHSA	1	0.073	0.073
	FetalHSA	1	0.092	0.092
Reacted with 1,4-BQ	MaternalHSA	1	20.86	20.86
	MaternalHSA	1	22.49	22.49
	FetalHSA	1	16.02	16.02
	FetalHSA	1	15.09	15.09
Unreacted	MaternalHb	3	0.321	0.107
	MaternalHb	3	0.272	0.091
	FetalHb	3	0.303	0.101
	FetalHb	3	0.310	0.103
Reacted with 1,4-BQ	MaternalHb	3	0.678	0.226
	MaternalHb	3	0.745	0.249
	FetalHb	3	0.768	0.256
	FetalHb	3	0.912	0.304

1,4-BQ adduct levels, before and after reaction with 1,4-BQ, are displayed in Figure 2.8, with standard deviations noted within each group. These results include Hb and HSA from both cord blood and matching maternal blood specimens. HSA was significantly more reactive than Hb in both the fetal and maternal samples. As a result, 1,4-BQ-HSA concentrations are plotted as natural logarithms. Although statistical conclusions are limited given the extremely small sample size, the reactivity of the two Hb variants do not appear to be sufficiently different to explain the differences observed previously between the newborn and adult DBS.

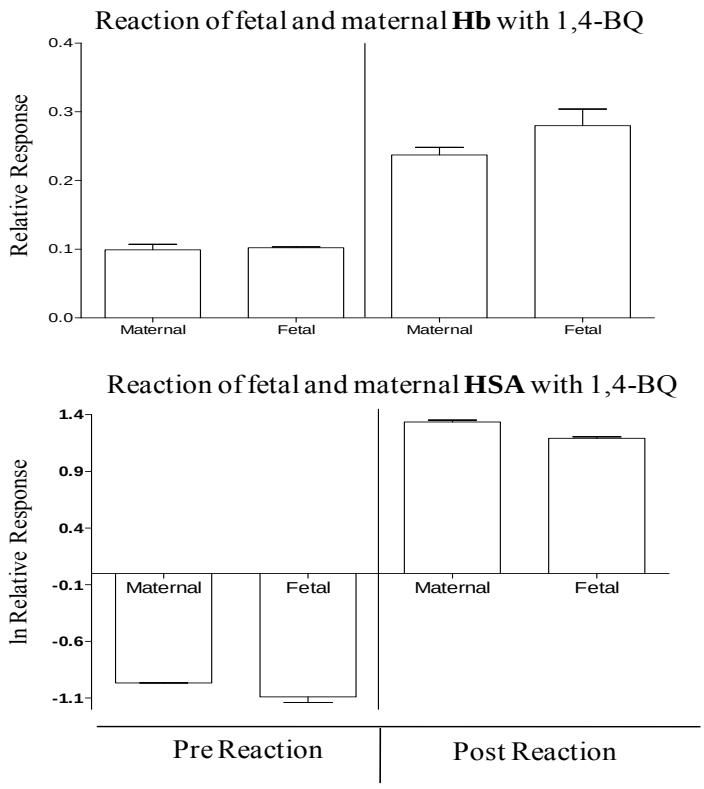

Figure 2.8. Measurement of 1,4-BQ adducts of fetal and adult blood proteins before and after reaction with 1,4-BQ.

2.2.4. Assessment of the stability of 1,4-BQ-Gb in DBS stored at room temperature for up to 1 year

The stability of 1,4-BQ-Gb in DBS was assessed in DBS stored at room temperature for up to one year. Fresh blood was collected from an adult volunteer by venipuncture in heparin vacutainer tubes. The blood was immediately applied to specimen collection paper in 60 µl aliquots. At time intervals of 0, 0.5, 1, 3, 6, and 12 months, DBS were excised and extracted in 2 ml of deionized water using increasing numbers of DBS (3 DBS for times 0, 0.5, and 1 month; 4 DBS for times 3 and 6 months; and 6 DBS for the specimens stored for 12 months) to recover adequate protein for adduct measurement. The samples were agitated at 160 rpm for 90 minutes

and the specimen collection paper was removed using forceps. All samples were stored at -80˚C until assayed for 1,4-BQ-Gb.

The efficiency of recovering proteins from DBS stored at room temperature significantly declined after 6 months of storage (Figure 2.9). This effect may have been exacerbated by heparin which had been used as an anticoagulant in the blood used to prepare the DBS. Dried blood spots collected by finger lancet and stored at room temperature were extracted after storage for 7 to 10 months. A significant decline was also observed in these samples, though the effects appeared less extreme than for DBS prepared from the blood containing heparin. This observation is encouraging because aged DBS are more likely to have been collected by finger or heel lancet, and would therefore be devoid of blood anticoagulants such as heparin. Further studies are required to explore the effects of storage and protein recovery.

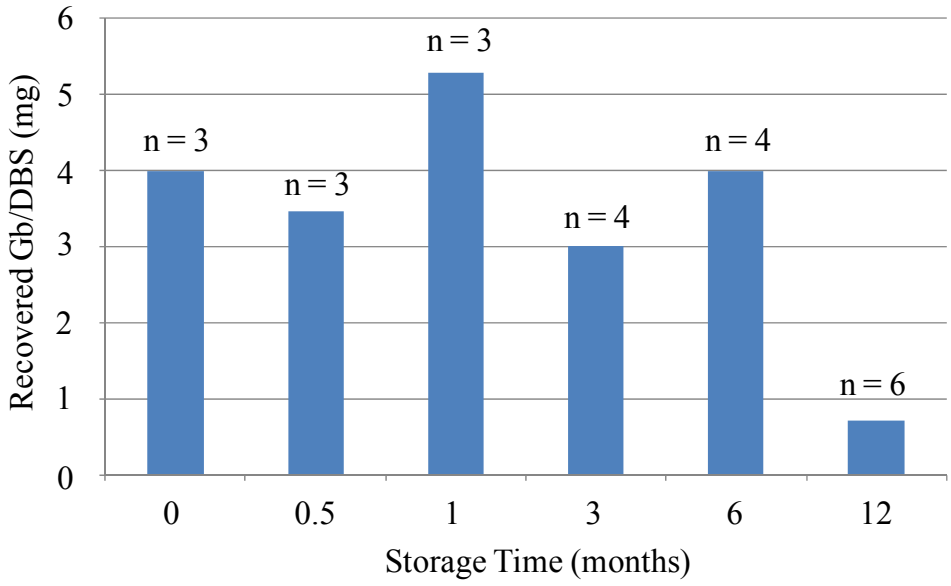

Figure 2.9. Recovery of blood proteins from DBS after extended storage at room temperature. Note that DBS specimens were pooled at each time point.

1,4-BQ-Gb was measured in DBS to assess the stability of these 1,4-BQ adducts during storage (Figure 2.10). Samples were analyzed using the MT assay at each time point using 3 mg of Gb. Surprisingly, adduct levels increased with greater storage times; with a significant rise in adduct levels between 0 and 0.5 months. Mean adult and newborn DBS 1,4-BQ-Gb levels (presented in *section 2.3.1)* are also shown for comparison with the archived samples in Figure 2.10.

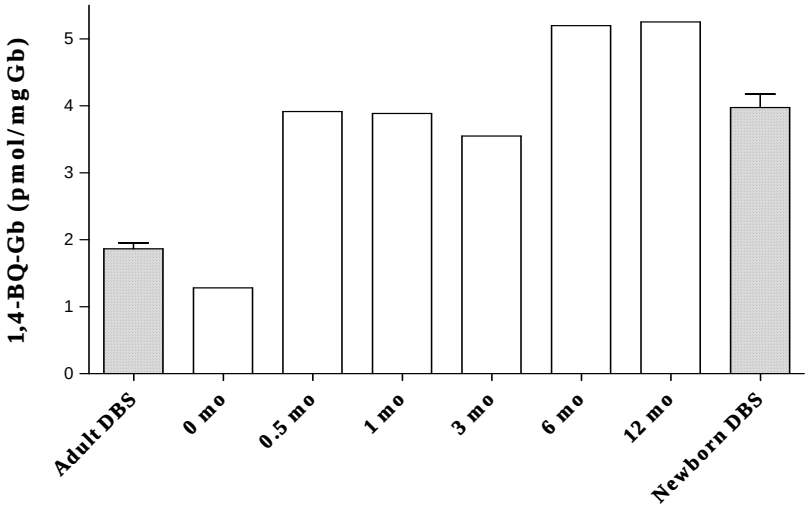

Figure 2.10. Stability of 1,4-BQ adducts of globin in DBS stored at room temperature for up to one year.

All adult DBS samples from *section 2.2.2.* were eluted 18-36 hours after being dried on the specimen collection paper. In contrast, newborn DBS specimens were extracted between 7 and 30 days after blood collection. Based on these results, adduct levels appeared to increase with storage time, and observed differences in adduct levels between adult and newborn DBS could simply point to the difference in storage time.

2.2.5. Conclusions

A method has been developed to extract Hb from DBS using the addition of ethanol to selectively precipitate the Hb from the DBS whole blood matrix. This method is relatively simple and inexpensive, and thus is applicable to molecular epidemiological studies. Using this protocol, Hb can be recovered with high yield in DBS stored for up to 6 months at room temperature. The purity of the isolated protein was determined to be suitable for protein adduct assays.

1,4-BQ adducts of Gb in DBS significantly increased when stored at room temperature. Reasons for the increase in adducts during storage may be related to quinone-specific redox properties. Further investigations are required to explore this phenomenon.

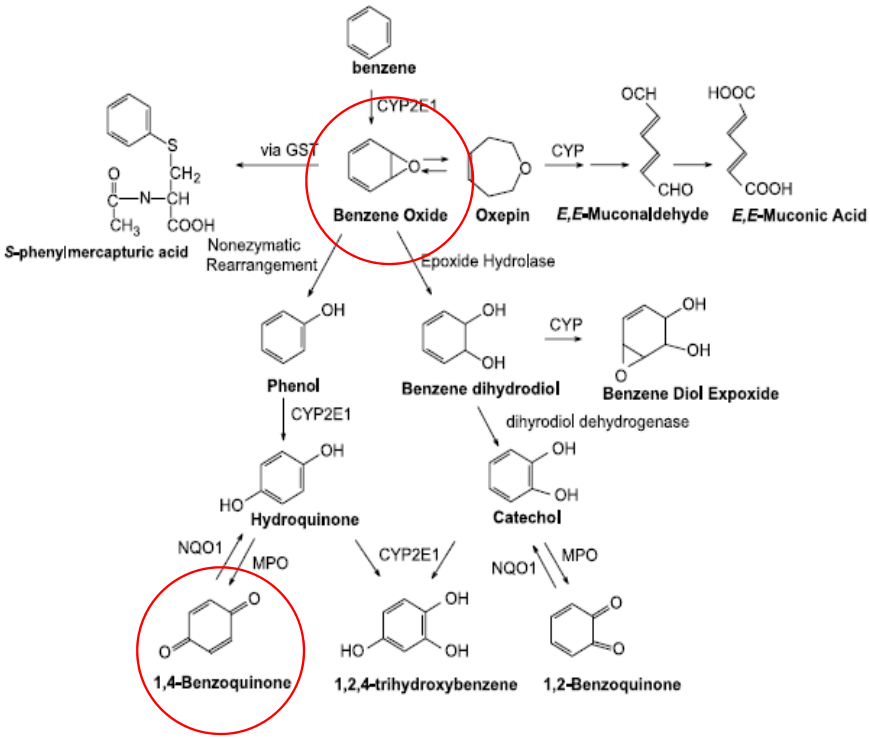

Figure 2.11. Schematic of the metabolism of benzene, highlighting the reactive metabolites BO and 1,4-BQ

To demonstrate that proteins can be isolated from DBS and used in protein adduct assays as exposure biomarkers, an alternative adduct, benzene oxide (BO), was selected for monitoring. Like 1,4-BQ, BO is a reactive metabolite of benzene (Figure 2.11), and its adducts have been detected in both Hb and HSA (Rappaport, McDonald et al. 1996; Yeowell-O'Connell, McDonald et al. 1996; Troester, Lindstrom et al. 2000). Exposure to benzene is among the few established environmental causes of leukemia in adults (Savitz and Andrews 1996; Sonawane, Bayliss et al. 2000; Vermeulen, Li et al. 2004). The mechanism by which benzene and its reactive intermediates induce leukemia is not fully understood. However, BO, benzoquinones, and muconaldehydes are all electrophilic metabolites of benzene that modify DNA and proteins, and are suspected to play a causal role in benzene-induced leukemia (Sonawane, Bayliss et al. 2000). A method for measuring BO cysteinyl adducts of Gb in DBS is presented in *Section 3.*

3. Paper I. Hemoglobin adducts of benzene oxide in neonatal and adult dried blood spots [Funk, W.E., Waidyanatha, S., Chaing, S.H., Rappaport, S.M. (2008) Cancer Epidemiology Biomarkers and Prevention, 17(8) 1896-1901]

3.1. Abstract

Adducts of reactive chemicals with Hb or HSA can be used as biomarkers of internal doses of carcinogens. Because dried blood spots are easier to collect and store than conventional venous blood samples, they encourage applications of biomarkers of exposure in large epidemiologic studies. In addition, neonatal dried blood spot can be used to investigate chemical exposures *in utero*. Here, we report a simple method to isolate Hb from dried blood spot with high recovery and purity using the addition of ethanol to aqueous dried blood spot extracts. To prove the concept that dried blood spot–derived proteins can be used to assay for adducts, we measured Hb adducts of benzene oxide, a reactive metabolite of the ubiquitous air pollutant benzene in nine neonatal and nine adult dried blood spots (from volunteer subjects), using a gas chromatography–mass spectrometry method that we had previously developed. For comparison, benzene oxide–Hb adducts were measured in the same nine adult subjects using Hb that had been isolated and purified using our conventional method for venous blood. The geometric mean of benzene oxide–Hb levels in all dried blood spot samples ranged from 27.7 to 33.1 pmol/g globin. Neither of the comparisons of mean (logged) benzene oxide–Hb levels between sources (adult conventional versus adult dried blood spot and adult dried blood spot versus newborn dried blood spot) showed a significant difference. Based upon the estimated variance of the

benzene oxide–Hb levels, we had 80% power to detect a 1.7-fold difference in geometric mean levels of benzene oxide–Hb in our sample of nine subjects.

3.2. Introduction

Biomarkers of internal dose can be more accurate and precise surrogates for carcinogen exposures than environmental measurements per se (Lin, Kupper et al. 2005). However, because chemical carcinogens are usually reactive electrophiles with very short life spans in vivo (e.g., alkylating and acylating agents, aldehydes, alkylnitrosamines, dialkylsulfates, oxiranes, quinones, reactive oxygen, and nitrogen species; (Miller and Miller 1966), it is rarely possible to measure them in target tissues. This has motivated the use of adducts of these electrophiles with abundant blood proteins, particularly Hb and HSA, as measures of carcinogen dose (Groth and Neumann 1972; Ehrenberg, Hiesche et al. 1974). Electrophiles enter the blood from absorption in the lungs or gut (e.g., inhalation of ethylene oxide) or, more typically, via the metabolism of procarcinogens in the liver or other tissues (e.g., production of benzene oxide by cytochrome P450 metabolism of benzene). Once in the blood, electrophiles react at varying rates with all available nucleophiles to form adducts by numerous mechanisms (Tornqvist, Fred et al. 2002). Hb and human serum albumin contain a myriad of nucleophilic sites, namely the free thiol groups of Cys; amine groups of His, Trp, Lys, and the N-termini; hydroxyl groups of Ser and Tyr; and the carboxylic acid groups of Asp, Glu, and the C-termini. Because protein adducts are not repaired and are much more abundant than DNA adducts in blood (1 ml of blood contains about 150 mg Hb, 30 mg of human serum albumin, and 0.003-0.008 mg of DNA; (Tornqvist, Fred et al. 2002)), they are potentially more useful measures of internal dose than DNA adducts, which have paradoxically received far more attention in this regard. Indeed, the kinetics of

production and elimination of Hb and human serum albumin adducts are sufficiently simple to permit straightforward estimates of systemic doses of carcinogens over the mean residence time of these proteins (28 days for human serum albumin and 63 days for Hb in humans; (Ehrenberg, Moustacchi et al. 1983; Granath, Ehrenberg et al. 1992; Rappaport, Waidyanatha et al. 2002)).

Levels of targeted Hb and/or human serum albumin adducts have been investigated in human blood for several environmental toxicants that are either electrophilic carcinogens or their precursors, that is, ethylene oxide, benzene, 1,3-butadiene, acrylamide, aflatoxin B1, a variety of aromatic amines, and polycyclic aromatic hydrocarbons (reviewed in (Tornqvist, Fred et al. 2002)). However, the need to obtain venous blood samples has limited the utility of protein adducts (and other blood-based biomarkers) as measures of exposure in large epidemiologic studies. This has motivated investigators to use dried blood spots that can be obtained by a simple skin prick as sources of blood biomarkers. Guthrie and Susi (Guthrie and Susi 1963) first used dried blood spots in the 1960s to screen newborn populations for hyperphenylalanine associated with the genetic disease phenylketonuria. Neonatal dried blood spots offer valuable opportunities for investigating chemical exposures in utero and their possible links to childhood cancers. Immunoassays have been applied to measure a variety of biomarkers in dried blood spots from adult populations, for example, folate (O'Broin and Gunter 1999), transferrin receptor (McDade and Shell-Duncan 2002; Shell-Duncan and McDade 2004), immunoglobulin E (Tanner and McDade 2007), Epstein-Barr virus antibodies (McDade, Stallings et al. 2000), leptin (Miller, Sharrock et al. 2006), and C-reactive protein (McDade, Burhop et al. 2004). Thus, dried blood spots can potentially be used in both prospective and retrospective studies to process large numbers of blood specimens from human subjects.

A single dried blood spot contains about 50 μL of human blood (O'Broin and Gunter 1999). Assuming a protein concentration of 192mg/mL (Longe and Blanchfield 2002), one dried blood spot should contain about 9.6 mg of protein, of which there should be about 7.7 mg of Hb (80% of total protein) and 1.2mg human serum albumin (12% of total protein). Because most current assays for protein adducts typically require between 1 and 10 mg of globin (from Hb) or human serum albumin, a single dried blood spot should contain sufficient quantities of these proteins to measure adducts. Here, we describe experiments to isolate Hb in high purity from a single dried blood spot, to purify the resulting globin, and to measure cysteinyl adducts of benzene oxide (benzene oxide–Hb) in these proteins. We previously detected benzene oxide–Hb in globin isolated from conventional venous blood samples in both benzene-exposed and control subjects and showed that levels of benzene oxide–Hb increased with the level of benzene exposure (Yeowell-O'Connell, Rothman et al. 2001). Detection of benzene oxide– Hb in control subjects points to production of adducts from environmental exposures to benzene and/or dietary and endogenous sources of benzene oxide or other precursor molecules that produce the same adduct (Yeowell-O'Connell, Rothman et al. 1998). In the current study, we show that benzene oxide– Hb is present at comparable levels in adult globin, isolated either from dried blood spots or from conventional RBCs, and in globin obtained from neonatal dried blood spots.

3.3. Methods and Methods

3.3.1. Chemicals

S-phenylcysteine and [$^2H_5$]S-phenylcysteine were kindly provided by Dr. A. Gold of the Chemistry Core of the Superfund Basic Research Program at the University of North Carolina, Chapel Hill. Hydrochloric acid (concentrated), acetone (nanograde), hexanes

(pesticide grade), and ethanol (100%) were purchased from Fisher Scientific. Methanesulfonic acid was obtained from Fluka Chemical Company. Trifluoroaceticanhydride was from Pierce and was distilled once before use. Human Hb was purchased from Sigma Chemical Company. ProteinSaver 903 specimen collection cards were purchased from Whatman. Safety lancets for preparation of fresh adult dried blood spots were obtained from Fisher Scientific. Bio-Rad protein assay reagents were purchased from Bio-Rad Laboratories.

3.3.2. Dried blood spots

Nine newborn dried blood spots, collected via heel lancet, were obtained from the North Carolina Laboratory of Public Health. These single dried blood spots, which were collected on Whatman ProteinSaver 903 specimen collection cards, were obtained within 30 days of collection. Nine adult dried blood spots were prepared using archived frozen whole blood, randomly selected from 191 adult volunteer subjects collected in the state of North Carolina in 1998 and stored at -80ºC (Lin, McKelvey et al. 2006). After thawing to room temperature, 50 µL aliquots of these blood specimens were spotted on specimen collection cards to produce adult dried blood spot; these dried blood spots were stored at room temperature for 2 weeks before processing. Five of the nine adult dried blood spots were spotted in duplicate to assess assay precision. Fresh adult dried blood spots, required for experiments to develop the method, were prepared on specimen collection cards with blood obtained by finger lancet from laboratory volunteer subjects.

3.3.3. Precipitation of Hb via addition of ethanol

To determine optimal conditions for precipitating Hb, while leaving other blood proteins in solution, dried blood spot specimens were precipitated from varying mixtures of ethanol or water. Eighteen individual dried blood spots from a single volunteer subject were dried overnight

and then excised with scissors and extracted with 2 ml of deionized water by agitation on a shaker table (Lab-Line 4626, Barnstead) at 160 rpm for 90 min. The specimen collection paper was removed using forceps, and the eluted blood was concentrated to ~200 µL using a speed vacuum system (Savant SC110, ThermoFisher Scientific). To determine the optimum concentration of ethanol or water to precipitate Hb, the eluted blood was added Drop-wise to aqueous solutions containing between 15% and 66% ethanol in increments of 3% ethanol. All samples were incubated at 4ºC for 1 h and were centrifuged at 30,000 x g to pellet the precipitated protein. The supernatant fraction, containing soluble proteins, was decanted and discarded. Total protein concentration was measured in the precipitated protein fraction using a variation of the Bradford assay (Bio-Rad Protein Assay, Bio-Rad Laboratories) based upon absorbance at 595 nm. Hb concentrations were estimated by selective absorbance at 523 nm.

3.3.4. Isolation of globin from dried blood spots and RBCs

Dried blood spots were excised from specimen collection cards with scissors and placed in 4-mL glass vials with Teflon-lined caps. Deionized water (2 ml) was added, and the vials were briefly agitated with a vortex mixer and then gently mixed on a rotary shaker (Lab- Line 4626, Barnstead) at 160 rpm for 90 min. The specimen collection paper was removed with forceps, and the eluted blood was dried with a speed vacuum system. After dissolving the dried proteins in 570 µL of deionized water, 430 µL of ethanol (43% v/v, which was found to be optimal in the preliminary experiment described above) was added drop-wise to selectively precipitate Hb. Samples were incubated at 4°C for 1 h, transferred to 1.5-mL plastic vials, and centrifuged at 30,000 x g to pellet the Hb. The precipitated Hb was reconstituted in 200 µl of deionized water and then added drop-wise to preweighed 4-ml glass vials containing 2 ml of 0.1% HCl in acetone (20ºC) to precipitate globin. Vials were incubated for 4 h at 20ºC, centrifuged, and the

supernatant was decanted. The globin was then washed thrice with ice-cold acetone and was dried in a vacuum oven overnight at 37ºC and 15 mmol/l Hg. The amount of isolated globin was determined gravimetrically.

Globin had been isolated from RBCs from the same nine subjects who provided dried blood spots according to our conventional method (Yeowell-O'Connell, McDonald et al. 1996). Portions of these conventional globin specimens were processed in parallel with globin derived from dried blood spots.

3.3.5. Analysis of globin purity from dried blood spots

Two-dimensional electrophoresis was done using the carrier ampholine method of isoelectric focusing by Kendrick Laboratories. Nonequilibrium pH gradient electrophoresis was carried out in a glass tube with an inner diameter of 2.0 mm at 200 V for 13 h using 1.5% (pH 3.5-10) and 0.25% (pH 8 to 10.5) ampholines (GE Healthcare). Tropomyosin (1 Ag; molecular weight, 33,000; pI 5.2) and 1 Ag of lysozyme (molecular weight, 14,000; pI 11.5) were added as internal markers. After equilibrium in SDS sample buffer (10% glycerol; 50 mmol/l dichlorodiphenyltrichloroethane; 2.3% SDS; 0.0625 mol/L Tris; pH 6.8), each tube gel was sealed to the top of a stacking gel overlying a 12% acrylamide slab gel (0.75 mmol/l thick), and SDS slab gel electrophoresis was carried out for 4 h at 15 mA per gel. The following proteins were added as molecular weight standards: myosin (molecular weight, 220,000), phosphorylase A (molecular weight, 94,000), catalase (molecular weight, 60,000), actin (molecular weight, 43,000) carbonic anhydrase (molecular weight, 29,000), and lysozyme (molecular weight, 14,000; Sigma Chemical Co). Gels were scanned with a laser densitometer (model PDSI, Molecular Dynamics Inc.), which had been checked for linearity with a calibrated neutral density filter set (Melles Griot). The images were analyzed using Progenesis Discovery

software (version 2005, Nonlinear Dynamics). The general method of computerized analysis included automatic spot finding, background subtraction (Progenesis mathematical model), quantification, and matching with detailed manual checking. Purity of the globin derived from dried blood spot was assessed by comparing two-dimensional gels of globin from dried blood spot with globin derived from RBCs, assuming the latter to be 100% pure.

3.3.6. Analysis of benzene oxide-Hb (MT assay)

The cysteinyl adduct of benzene oxide–Hb was assayed using the procedure of Yeowell-O'Connell et al. (Yeowell-O'Connell, McDonald et al. 1996) with minor modifications. After protein isolation, 4.5 mg of globin (from either a dried blood spot or a conventional RBC specimen) and 1 pmol of [$^2H_5$]S-phenylcysteine (internal standard) were placed in 4-ml glass vials and dried in a vacuum oven (70-80ºC; 15 mmol/l Hg). Trifluoroaceticanhydride (750 µl) and methanesulfonic acid (20 µl) were added, and the vials were capped tightly with Teflon-lined caps. This reaction cleaves the cysteinyl adduct of benzene oxide and converts it to the volatile derivative phenyltrifluorothioacetate. The reaction mixture was heated at 100ºC for 40 min, cooled to room temperature, and the excess trifluoroaceticanhydride was removed under a stream of nitrogen. Hexane (1 ml) was added, and the organic extract (containing phenyltrifluorothioacetate) was washed with 1 ml of 0.1 mol/L Tris buffer (pH 7.3) and twice with 1 ml of deionized water. The hexane layer was then transferred to 1.5-ml high recovery autosampler vials (Agilent Technologies) and was reduced to a final volume of about 50 µl under nitrogen for analysis of phenyltrifluorothioacetate by gas chromatography–mass spectrometry in negative ion chemical ionization mode. A Hewlett Packard 5890 series II plus gas chromatograph and a Hewlett-Packard 5989B MS engine were used with a DB-5 fused silica capillary column (60 m; 0.25-mm internal diameter; 0.25-µm phase thickness; J and W

Scientific, Inc.) operating a helium carrier gas at a flow rate of 1 ml/min. The injection port and source temperatures were 250ºC and 100ºC, respectively. The oven temperature was held at 50ºC for 3 min and then ramped at 5ºC/min to 140ºC. Late-eluting compounds were removed by increasing the oven temperature to 250ºC at 50ºC/min, where it was held for 10 min. Injections (3 μl) were made in the splitless mode. Ions of phenyltrifluorothioacetate (m/z 206) and [$^2H_5$]phenyltrifluorothioacetate (m/z 211) were monitored using selective ion monitoring. To estimate the precision associated with the gas chromatography–mass spectrometry of the benzene oxide–Hb analyte, triplicate injections were done for four dried blood spots (three adult and one newborn), and duplicate injections were done for one adult dried blood spot.

3.3.7. Statistical analyses

Adduct data were transformed to natural logarithms before statistical analyses to satisfy underlying assumptions about homogeneity of variance and large sample size. Sources of random variation of adduct levels, from errors associated with the assay and gas chromatography– mass spectrometry injections, were estimated using a nested random-effects model where injections were nested within assays and assays within dried blood spot specimens (Proc NESTED of SAS). The corresponding coefficients of variation (CV) were estimated as follows: $CV_{assay} = [\exp(\hat{\sigma}_a^2) - 1]^{1/2}$, $CV_{injections} = [\exp(\hat{\sigma}_i^2) - 1]^{1/2}$ and $CV_{method} = [\exp(\hat{\sigma}_a^2 + \hat{\sigma}_i^2) - 1]^{1/2}$, where $\hat{\sigma}_a^2$ and $\hat{\sigma}_i^2$ represent the estimated variance components (of logged data) for assays and injections, respectively, from the random-effects model (Yeowell-O'Connell, Rothman et al. 2001). Comparisons of mean (logged) adduct levels between sources of nine blood specimens, namely, adult conventional versus adult dried blood spot and adult dried blood spot versus newborn dried blood spot, used two-tailed Student's t tests with unequal variances and a significance level of $\alpha = 0.05$. Pearson's correlation coefficient was estimated for (logged)

adduct levels from adult-conventional and adult–dried blood spot specimens. Blood specimens with multiple assays and/or injections were aggregated (logged levels: first by injection and then by assay) before testing of mean values and estimation of r. Statistical analyses used SAS software (version 9.1 for Windows, SAS System Software) for random-effects models and Microsoft Excel for t tests and correlation.

3.4. Results

3.4.1. Hb Precipitation with the addition of ethanol

As shown in Fig. 3.1., Hb in blood from extracted dried blood spots began to precipitate at an ethanol concentration of ~20% and reached a plateau at ~37%. Human serum albumin and most of the other plasma proteins remained soluble until an ethanol concentration of ~47% was reached. Because Hb and total protein were measured using different methods (Bio-Rad assay for total protein and absorbance at 523 nm for Hb), and because a combination of Hb and human serum albumin was used as a standard in the Bio-Rad assay to approximate all of the proteins in blood, total precipitated protein was normalized to the Hb measurements. This adjustment was based on two-dimensional gel electrophoresis results (described hereafter), which showed that, after precipitation with 43% ethanol, Hb represented 96% of the total protein. Based on these results, an ethanol percentage of 43% was chosen to optimize Hb recovery from the dried blood spot while preventing other proteins from precipitating.

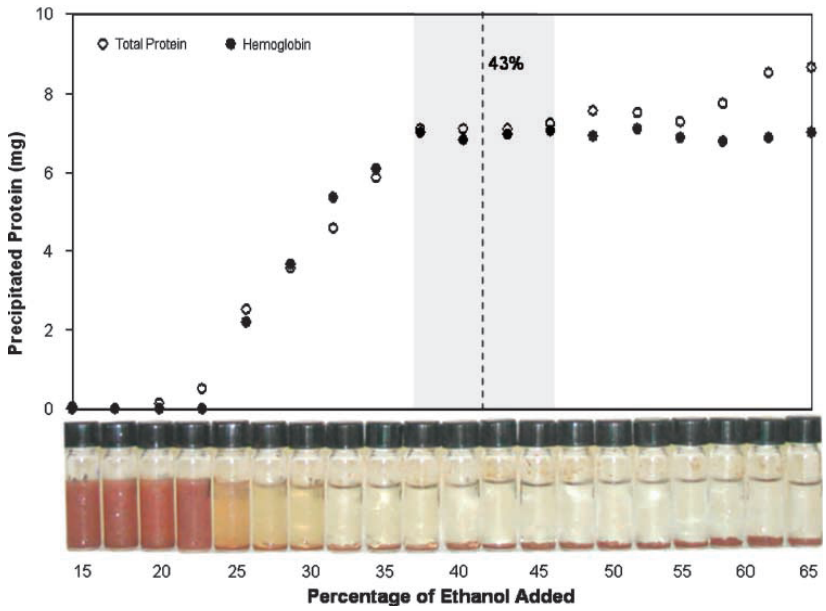

Figure 3.1. Selective precipitation of Hb from dried blood spots by addition of ethanol. Total protein measurements were normalized to Hb levels using results from the two-dimensional gel electrophoresis analysis, which showed that dried blood spot–derived globin was 96%pure when Hb was precipitated with 43%ethanol.

3.4.2. Globin isolation from dried blood spots

The estimated mean globins isolated from adult and newborn dried blood spots were 6.4 mg (SD, 0.96) and 7.2mg (SD, 2.20) of globin, respectively. This corresponds to a recovery of >80% based on previously reported Hb levels in whole blood (adults, 140 mg/mL; newborns, 193 mg/mL; (Kates and Kates 2007)). The purity of the isolated globin, assessed by two dimensional gel electrophoresis, was determined by laser densitometry to be ~96%.

3.4.3. *Precision of benzene oxide-Hb measurements in dried blood spots*

The estimated components of variance of (logged) benzene oxide–Hb levels for assays and gas chromatography–mass spectrometry injections were $\hat{\sigma}_a^2$ = 1.62 x 10^{-2} and $\hat{\sigma}_i^2$ = 7.25 x 10^{-3}. The corresponding CV values are as follows: CV_{assay} = 0.128, $CV_{injection}$ = 0.085, and CV_{method} = 0.154. Using conventional samples of globin derived from RBCs, we

previously reported values of CV_{assay} = 0.28 and $CV_{injection}$ = 0.10 (Yeowell-O'Connell, Rothman et al. 2001).

3.4.4. *Comparisons of benzene oxide-Hb levels across sources of specimens*

Table 3.1 lists the estimated means, SDs, and ranges of logged benzene oxide–Hb levels and the corresponding geometric mean benzene oxide–Hb levels for samples of nine globin specimens from different sources. The estimated statistics were very similar across sources. The geometric mean benzene oxide–Hb levels ranged from 27.7 to 33.1 pmol/g globin. Neither of the comparisons of mean (logged) benzene oxide–Hb levels between sources (adult conventional versus adult dried blood spot and adult dried blood spot versus newborn dried blood spot) was significant at $\alpha = 0.05$. Figure 2 shows a scatter plot of the (logged) benzene oxide–Hb levels in globin derived from adult conventional RBCs and dried blood spots in single specimens of blood obtained from the same subjects. The Pearson r for the nine data pairs was 0.732.

Table 3.1. Summary statistics of benzene oxide-Hb levels in samples of globin isolated from different sources (n = 9 specimens per source)

Source of globin	Estimated mean of logged BO-Hb levels	Estimated SD of logged BO-Hb levels	Range of logged BO-Hb levels	Estimated geometric mean BO-Hb level (pmol/g globin)
Adult DBS	3.32	0.326	2.98-3.83	27.7
Newborn DBS	3.47	0.449	3.02-4.48	32.1
Adult conventional	3.50	0.351	2.99-3.94	33.1

Abbreviations: BO, benzene oxide; DBS, dried blood spot.

3.5. Discussion

Although blood protein adducts of targeted electrophiles have been measured in humans exposed to prominent genotoxins (see review by Tornqvist et al.; (Tornqvist, Fred et al. 2002)), all previous investigations used venous blood samples. The purpose of this investigation was to prove the concept that protein adducts can be measured in dried blood spot-derived globin from both newborn and adult subjects. We focused upon one adduct, namely, benzene oxide–Hb, that we had previously measured in globin isolated from benzene-exposed workers and control subjects from Shanghai, China (Yeowell-O'Connell, Rothman et al. 2001).

An important innovation of our assay involves the isolation of relatively pure Hb from a dried blood spot based upon precipitation in 43% ethanol. Conventional Hb adduct assays have relied on first separating RBCs (which are composed almost exclusively of Hb) from the plasma by centrifugation. This is not possible with dried blood spots because RBCs are lysed during the blood-drying process, giving rise to a more complex whole blood matrix. Precipitating Hb from dried blood spots via addition of ethanol provides a relatively simple and inexpensive means of obtaining globin for use in molecular epidemiologic studies. Although our focus here concerned isolation of Hb, it should be noted that, after precipitation of Hb, the supernatant fraction could also be used to provide additional proteins, notably human serum albumin.

We successfully measured benzene oxide–Hb in all dried blood spot specimens from nine newborns and nine adults in North Carolina and also in globin isolated from conventional RBCs for the same adult subjects. No significant differences in mean (logged) benzene oxide–Hb concentrations were detected between samples of globin isolated from the

different sources (adult dried blood spot versus newborn dried blood spot and adult dried blood spot versus adult conventional). Because these t tests were conducted with the logged adduct levels, in a natural scale, they tested for differences between geometric mean benzene oxide–Hb concentrations in the different sources of Hb. The lack of significant differences between geometric mean levels indicates that the typical blood concentrations of benzene oxide–Hb were comparable across sources. This indicates that the typical benzene oxide– Hb level in adult dried blood spots was similar to that measured in conventionally isolated adult globin and that the typical benzene oxide–Hb level in a North Carolina newborn was similar to that measured in a North Carolina adult. Given a pooled estimate of the variance of 0.144 for logged benzene oxide Hb concentrations, our tests (with nine specimens per group) had a power to detect a 1.7-fold difference in geometric mean values.

Our ability to measure benzene oxide–Hb in dried blood spots proves the concept that a single dried blood spot provides suitable Hb for determinations of a prominent adduct in human populations. This conclusion is reinforced by the high pairwise correlation of benzene oxide–Hb levels measured in globin from dried blood spot and conventional RBCs from the same nine adult subjects (Fig. 3.2). Because dried blood spots can be collected much more simply and with greater subject acceptance than venous blood samples, this opens the door to the use of protein adducts as biomarkers of human exposure to benzene and to a host of genotoxic and carcinogenic substances in either adults or newborns. With the relentless improvements in analytic sensitivity that we are witnessing, it is reasonable to expect that it will soon be possible to conduct assays of numerous protein adducts with a small portion of a dried blood spot, rather than an entire dried blood spot as reported here.

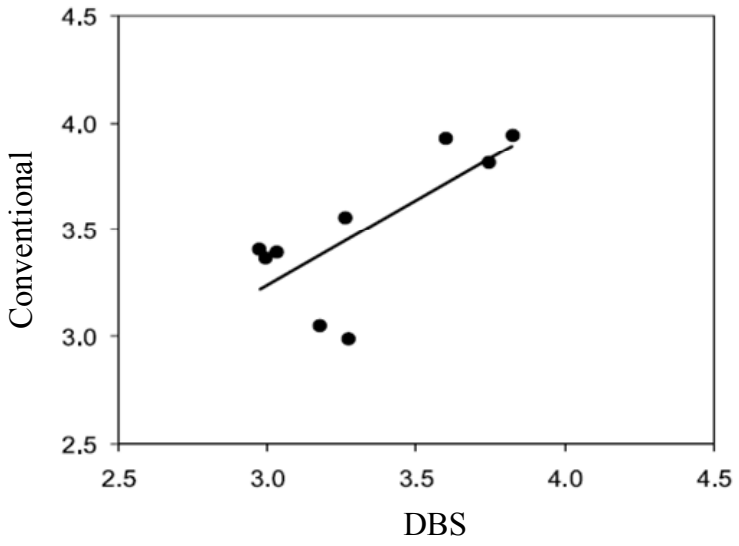

Figure 3.2. Scatter plot of logged levels of benzene oxide-Hb adducts measured in globin isolated from dried blood spots and from conventional RBCs from the same nine adult blood specimens (r = 0.732).

Given the relatively long residence time of 63 days for chemically stable Hb adducts in human blood (Tornqvist, Fred et al. 2002), Hb adducts provide rather steady measures of human exposure either in utero or in adult populations. Such steady biomarkers provide less biasing surrogates of the true long-term exposure levels that give rise to human diseases and are therefore preferable to short-term biomarkers such as urinary metabolites, whose levels vary greatly from day to day (Rappaport and Kupper 2008).

It is also interesting to note that the geometric mean concentration of benzene oxide–Hb, which in our adult subjects (dried blood spot and conventional globin; n = 18) was $e^{(3.32+3.50)/2} = e^{3.41} = 30.3$ pmol/g globin, is quite similar to the median value of 37.1 pmol/g globin reported in 44 control subjects from Shanghai, China (Yeowell-O'Connell, Rothman et al. 2001). This suggests that the magnitudes of environmental, dietary, and endogenous

sources of the precursors of benzene oxide–Hb, which include environmental benzene and other (unknown) contributors, are similar indifferent parts of the world.

4. Development of a Method to Enrich Cysteinyl Adducts of Human Serum Albumin

In this study we report a novel method to enrich and detect cysteinyl adducts of HSA. The enrichment approach utilizes the propensity of free sulfhydryls in HSA to bind with thiol-affinity resins. The majority of HSA-Cys^{34} in freshly isolated plasma is unmodified. Furthermore, within the oxidized fraction, the majority of HSA is bound reversibly with mixed disulfides. HSA-Cys^{34}-mixed disulfides can be liberated via reduction with dithiothreitol (DTT), and mercaptalbumin can subsequently be removed using thiol-affinity covalent chromatography. A schematic of the enrichment approach is provided in Figure 4.1.

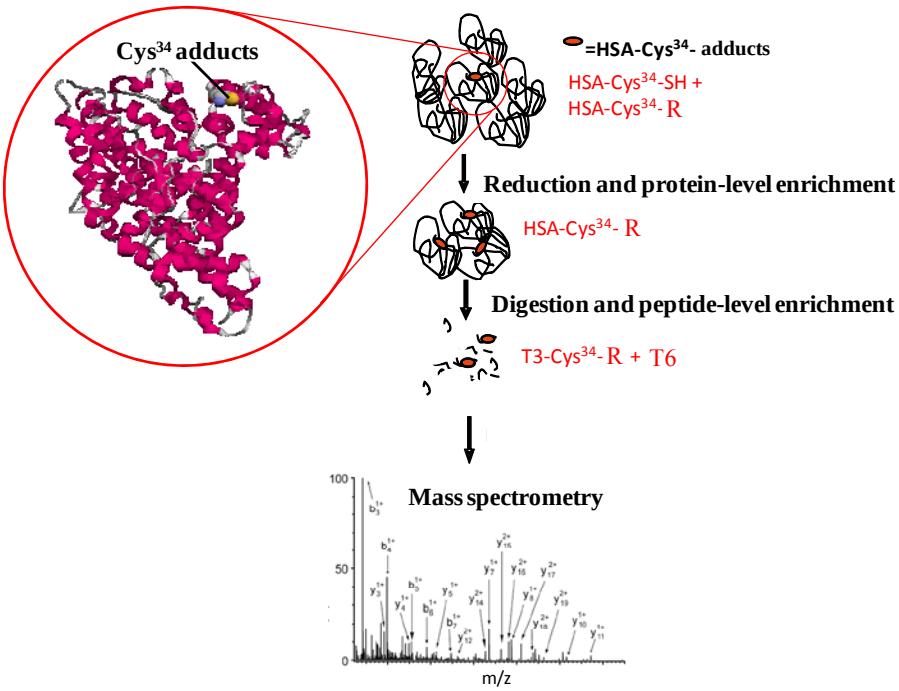

Figure 4.1. HSA-Cys^{34} adduct enrichment scheme. HSA-Cys^{34}-SH represents mercaptalbumin, and HSA-Cys^{34}-R represents a pool of irreversible Cys^{34} adducts.

4.1. Initial Experiments

4.1.1. Protein-level enrichment

Five hundred mg of commercial HSA was added to 7.5 ml of 0.1 M sodium phosphate buffer with 0.3 M NaCl, pH 6.7. One and a half ml aliquots, each containing 100 mg of protein, were reacted with 25 mM DTT in phosphate buffer to liberate the Cys^{34} bound mixed disulfides. Samples were agitated at 160 rpm at room temperature for 1 hour on a shaker table, and excess DTT was removed by extensive dialysis against DI water.

Fifteen g of freeze-dried Activated Thiol Sepharose 4B was washed with 3 liters of DI water for 15 minutes on a sintered glass filter. A slurry was prepared using 75% settled resin to 25% 0.1 M sodium phosphate binding buffer with 0.3 M NaCl (pH 7.4). The resin and the

binding buffer were degassed by bubbling in nitrogen to remove dissolved oxygen. Approximately 4.5 ml of resin were added to 6 ml glass columns connected to a vacuum manifold (Figure 4.2.). Binding buffer was added slowly under a light vacuum to pack the columns.

Figure 4.2. Thiol-affinity covalent chromatography manifold. HSA samples are loaded onto the Sepharose 4B resin on top of the manifold and the enriched fraction is collected under light vacuum inside the manifold.

Reduced HSA was added to the thiol-affinity resin. In addition, non-reduced HSA was added to 2 columns to assess the effectiveness of the protein reduction step. The protein solutions were passed drop-wise through the columns and the flow-through fractions were reloaded two additional times providing a one hour total residence time with the resin. The flow-through fractions were recovered and the columns were washed with 2-ml aliquots of binding buffer until absorbances at 280 and 343 nm reached baseline. Absorbance at 280 nm is a non-specific absorbance that was used to monitor protein, and the absorbance at 343 nm is the absorbance

maximum for 2-thiopyridone, which is released as HSA binds to the thiol-affinity resin. The flow-through fractions, and all wash steps, were combined and reduced in volume using a speed vacuum system. All samples were dialyzed against deionized water to remove buffer salts and the 2-thiopyridone.

A second enrichment was preformed off-column because the amount of HSA removed by the thiol-affinity resin was less than expected (discussed below). The resin was removed from the 6-ml glass columns as a slurry in phosphate buffer and was transferred to 25 ml glass vials. The enriched protein was reloaded onto the resin and was agitated at 200 rpm for 24 hours. To remove the unbound protein, the resin was transferred back to the vacuum manifold columns and eluted as described above. All samples were concentrated using a speed vacuum system and dialyzed against deionized water. Protein quantitation, before and after the enrichment experiments, was performed using the Bradford assay.

Results from the protein-level enrichment experiments are shown in Figure 4.3. Based on total protein mass, before and after enrichment, the second enrichment was more effective in removing unmodified HSA than the first. The increase in the incubation time resulted in an approximate two fold higher binding with the resin. When the protein was reduced prior to enrichment, 25-35 percent higher removal efficiency was observed.

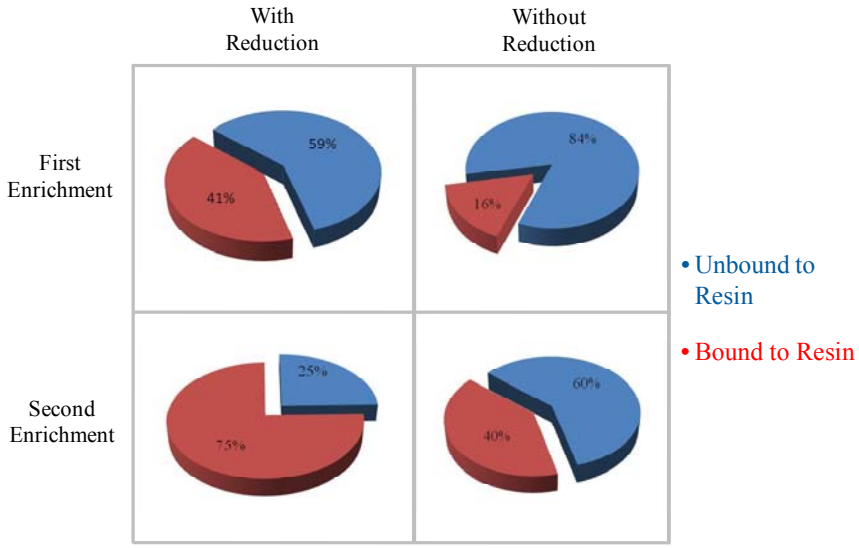

Figure 4.3. Percentage of HSA removed by the Sepharose 4B resin, with and without protein reduction with DTT.

Additional experiments which increased contact between the protein and the resin to 48 and 72 hours did not result in greater removal of HSA. These results suggest that 24 hours of contact with the resin is sufficient for removing the mercaptalbumin.

4.1.2. Peptide-level enrichment

HSA enriched at the protein-level was enzymatically digested using trypsin, and further enriched at the peptide-level. The protein-level enriched samples were treated with 8 mM DTT in a 0.2 M ammonium bicarbonate buffer with 8 M urea, pH 8.5, to denature and reduce the intramolecular disulfides. Solutions were incubated at room temperature for 30 minutes, and were then diluted with additional 0.2 M ammonium bicarbonate buffer to dilute the DTT and urea to appropriate concentrations for enzymatic digestion. Protein digestion was performed using 10:1 HSA to trypsin for 24 hours at 37˚C.

After digestion, tryptic peptides were transferred to 2 KDa MWCO size exclusion spin columns. The spin columns were used to remove DTT, to buffer exchange to the Sepharose 6B

buffer, and to remove low molecular weight interfering peptides. Tryptic digests of HSA theoretically contain 79 peptides. The peptide of interest, containing the Cys^{34} modifications, resides on the third largest peptide (T3, MW= 2433 Da). Consequently, size exclusion can be used to remove many of the smaller peptides. The twelve largest peptides are shown in table 4.1. Since 2 KDa MWCO spin columns are not expected to precisely discriminate at their exact mass cutoff, peptides greater than 1500 Da are included in the table. Peptides containing cysteine residues were removed prior to the size exclusion step (described below) by mixing with thiol-affinity resins. Peptides containing Cys residues are highlighted in red in Table 4.1. Note that of the 79 trypic peptides, only the adducted T3-Cys^{34} peptides and T6 peptide should remain after this second step of the enrichment using thiol-affinity resins, since these are the only larger peptides that do not have free thiols.

Table 4.1. Peptide masses and amino acid sequences of the twelve largest tryptic peptides. Peptides containing free Cys residues are highlighted in red (Note that the T3 peptide is not labeled in red because unadducted Cys^{34}-peptides have been removed at this stage). Peptides in red can be removed using thiol-affinity resins.

Peptide	Mass (Da)	Peptide Sequence
T1	2917	SHCIAEVENDEMPADLPSLAADFVESK
T2	2593	LVRPEVDVMCTAFHDNEETFLK
T3	2433	ALVLIAFAQYLQQC^{34}PFEDHVK
T4	2404	MPCAEDYLSVVLNQLCVLHEK
T5	2203	EFNAETFTFHADICTLSEK
T6	2045	VFDEFKPLVEEPQNLIK
T7	1916	VHTECCHGDLLECADDR
T8	1854	RPCFSALEVDETYVPK
T9	1743	HPYFYAPELLFFAK
T10	1624	DVFLGMFLYETAR
T11	1601	QNCELFEQLGEYK
T12	1512	VPQVSTPTLVEVSR

Sepharose 6B resin was prepared using the procedure outlined above with the Sepharose 4B resin. Sepharose 6B has the same 2-pyridyl disulfide active group as Sepharose 4B, however the 6B resin contains a shorter spacer arm that is more appropriate for smaller molecular weight species (Figure 4.4.). Tryptic peptides were incubated with the resin for 24 hours at room temperature. The samples were agitated at 200 rpm to prevent settling of the resin. Following the peptide-level enrichment, non-bound peptides were recovered using the procedure outlined above in *Section 4.4.1*.

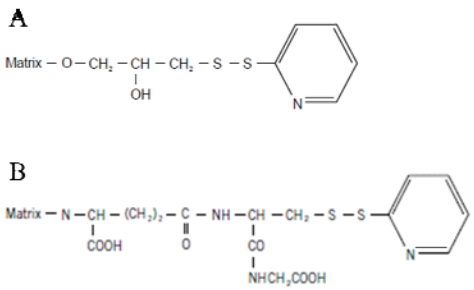

Figure 4.4. Chemical moieties for (A) Sepharose 6B and (B) Sepharose 4B. Note that the 2-pyridyl disulfide active group on the distal end of each of the resins is identical.

4.1.3. Mass spectrometry

The fully-enriched protein samples were analyzed at the University of California, Berkeley (UCB). Two mass spectrometry platforms were used to obtain initial spectra; surface-enhanced laser desorption ionization/time-of-flight MS (SELDI-TOF MS) and Fourier transform/ion cyclotron resonance MS (FT/ICR MS).

SELDI-TOF MS. Weak cation exchange (CM 10) and strong anion exchange (Q 10) ProteinChip arrays (Ciphergen Biosystems, Fremont, CA) were used to capture fractions of adduct-enriched peptides. ProteinChip surfaces were loaded using approximately 10 µg of peptides per chip. Samples were dissolved in 100 mM ammonium acetate, pH 4.5, for the CM 10 chip arrays, and 100 mM sodium acetate, pH 8, for the Q 10 chip arrays. After incubation for 1 hour in a humidity chamber, the chip arrays were rinsed 3 times with binding buffer and twice with deionized water to remove unbound peptides. Sinapinic acid, prepared in 50% acetonitrile and 50% trifluoroacidic acid, was added to each sample to act as an energy absorbing matrix. Samples were analyzed using a PBSII SELDI-TOF mass spectrometer (Ciphergen Biosystems, Fremont, CA). The resulting spectra are provided in figure 4.5.

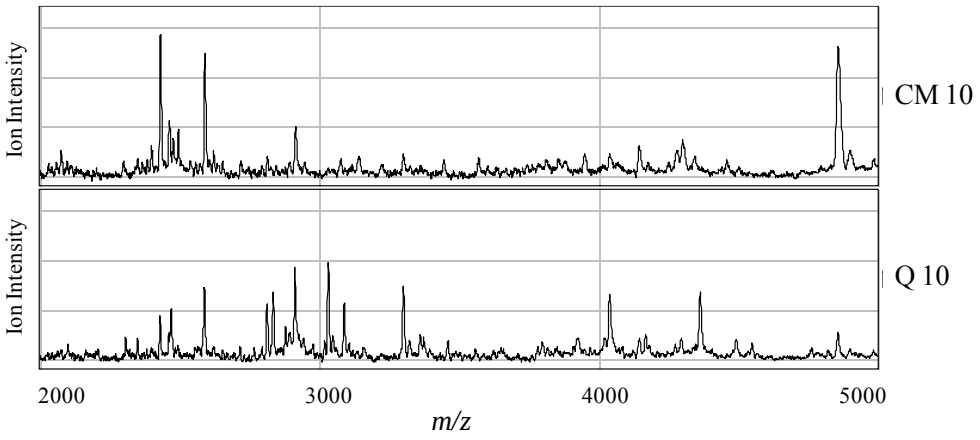

Figure 4.5. SELDI-TOF mass spectra of fully-enriched peptide samples. The CM 10 ProteinChip surface has weak cation exchange properties and the Q 10 ProteinChip array uses strong anion exchange chemistry.

FT/ICR MS. Nanoelectrospray FT/ICR MS was employed to acquire high-resolution mass spectra. To reduce the salt concentrations in the peptide solutions, several solvent systems were explored. Peptides dissolved in 20 fold reverse-osmosis water yielded the highest quality spectra, and are provided in Figure 4.6. A pattern of masses differing by 22 Da was observed in the spectra, which is indicative of sodium adduction. Significant signal loss was encountered when C18 ZipTips (Millipore, Billerica, MA) were used to remove excess salt from the peptide samples.

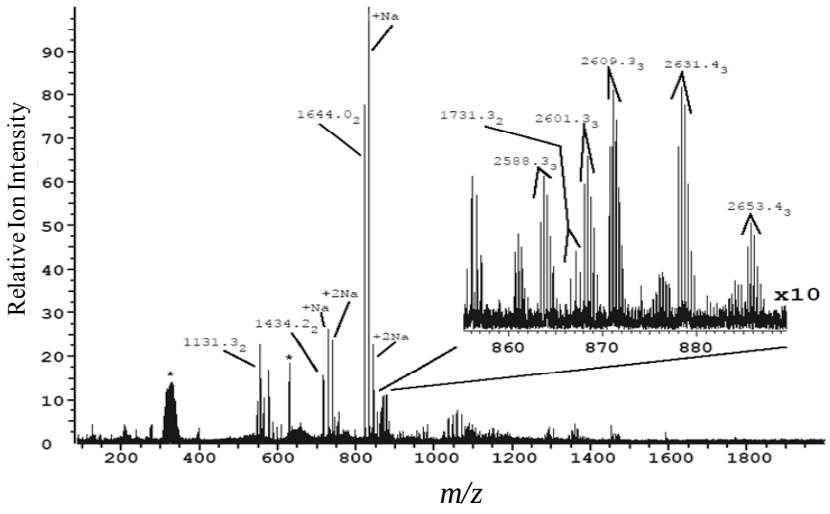

Figure 4.6. FT-ICR spectrum of the fully-enriched peptide sample, with the mass region of greatest interest expanded. The expanded mass range contains peptides with masses between ~2400 and 2700 Da, which corresponds with the mass of the T3 peptide (2433 Da) plus the anticipated adduct masses.

4.1.4. Conclusions

In both the SELDI-TOF MS and FT/ICR MS experiments quality spectra were obtained with a large number of peaks in the 2.5 - 4.0 KDa mass range of interest (T3 MW 2433.26 + adducts). Substantial differences in the spectra were observed between the two mass spectrometry techniques. This finding was anticipated given the differences between the mass spectrometry platforms. For instance, the ProteinChip arrays used in SELDI-TOF experiments are designed to capture specific fractions of the total peptides. While this aids in increasing analytical sensitivity, spectra will contain different peptides depending on the chip array chosen for the analysis.

These initial results were encouraging, given this first attempt at enriching HSA-Cys^{34} adducts. However, experiments are required to (1) quantify the degree of protein adduct enrichment, and (2) verify that the ions detected in these initial spectra were T3-Cys^{34} peptides.

To achieve the first goal, HSA was tagged with a specific Cys^{34} adduct, and the level of the modification was monitored throughout the enrichment process. This experiment is detailed in *Section 4.2*. A method to verify that MS peaks are truly derived from T3-Cys^{34} peptides is described in *Section 4.3.1*.

4.2. Assessment of adduct enrichment using BO modified HSA

4.2.1. Modification of HSA with BO

The degree of adduct enrichment achieved at both the protein and peptide levels was assessed using HSA modified with BO. Concentrations of HSA-Cys^{34}-BO were quantified using the NCI-GC/MS method described in *Section 3.3.6* (MT assay). When adducts are cleaved from the protein during the MT assay, the sulfur atom of HSA-Cys^{34} is transferred to the analyte. As a result, this method confirms that adducts were bound to Cys. Because Cys^{34} represents the only free thiol in intact HSA, this procedure is specific to HSA-Cys^{34} adducts, and will be used to determine adduct concentrations before and after the enrichment process.

One hundred mg of commercial HSA was reacted with 0.35 M BO at 37°C for 10 hours in 0.1 M phosphate buffer, pH 8. Extensive dialysis was performed to remove unreacted BO. Aliquots were removed in triplicate and spiked with isotopically labeled *S*-phenyl cysteine as an internal standard. The level of BO modification achieved by the reaction was determined using the MT assay. Dilutions of the BO modified HSA were prepared with unmodified HSA to achieve a starting adduct concentration that provided a strong ion signal using a minimal amount of the protein.

Adduct concentrations were determined to be 1.15 μmol BO/g HSA. This degree of modification is equivalent to ~1 BO modification in every 13 HSA molecules. Based on the ion

response from this sample, 10-fold dilutions were prepared and used as the starting protein in the enrichment experiment. This dilution factor was used because it provided an approximate S/N of 10:1 when 100 µl of the sample was spiked onto a 1-mg HSA matrix. This procedure would allow the level of BO-HSA adducts to be assessed throughout the experiment using relatively small aliquots of HSA at each step.

4.2.2. Enrichment of BO adducts of HSA

Enrichment was performed using the general approach outlined in *Sections 4.1.1 and 4.1.2*, with significant modifications to minimize sample loss, prevent protein/peptide precipitation, and increase the degree of enrichment.

One hundred mg of diluted HSA-Cys^{34}-BO was reduced with 10 mM DTT at room temperature for 1 hour in triplicate. The solutions were transferred to pre-conditioned 10 KDa MWCO spin columns to remove excess DTT and to liberate small thiols. Spin columns were used in this step, in place of dialysis, to minimize the time between reduction and reaction with the thiol-affinity resin. This modification was made to minimize the HSA auto-oxidation that can rapidly occur post-reduction (Ogasawara, Mukai et al. 2007). The samples were buffer-exchanged into degassed phosphate binding buffer to prepare the samples for thiol-affinity enrichment.

Reduced HSA was transferred to degassed Activated Thiol Sepharose 4B resin (prepared as described in *section 4.1.1*). Vials were capped with nitrogen and sealed with parafilm. The samples were incubated for 16 hours (incubation time that was determined to be optimal for HSA binding in previous experiments) on a shaker table at 160 rpm. Samples were eluted from the resin on a single Millipore disposable 22 µm filter. The resin was rinsed in batches using 80 ml of total binding buffer under gentle vacuum. All samples were transferred to fresh 50 ml

polypropylene tubes and were frozen at -80ºC. The bulk volumes were lyophilized until dry, and were redissolved in a buffer containing 0.2 M tris-HCl, 8 M urea, pH 7.4, for enzymatic digestion. The mean protein recovered in each of the three samples, measured with the Bradford assay, was 7.9 mg.

Peptide-level enrichment was performed using 6 mg of each sample. This step reserved ample protein for assessment of enrichment at the protein-level. After 8 mM TCEP was added to the sample buffer, the solutions were heated at 50º C for 15 minutes, to reduce the intramolecular disulfide bridges. Each sample was diluted with 0.2 M tris buffer to dilute the urea and TCEP concentrations prior to enzymatic digestion. Trypsin was added with a 10:1 substrate to enzyme ratio and the samples were incubated at 37º C for 3 hours. Ten KDa MWCO spin columns were used to remove large miscleavages, undigested protein, and trypsin prior to dialysis. Extensive dialysis was preformed against 4 liters of 100 mM phosphate buffer, 0.3 M NaCl, pH 7.4, using 2 KDa MWCO dialysis tubing. The buffer was changed three times over a period of 20 hours.

Sepharose 6B resin was prepared as described in *section 4.1.2*. Peptide samples were removed from the dialysis tubing and were transferred directly to the Sepharose 6B resin. The reaction vials were sealed with parafilm and were agitated at 160 rpm for 16 hours at room temperature. Enriched peptides were eluted from the resin using disposable Millipore 22 μm filters attached to a gentle vacuum. All samples were dialyzed against 4 x 4 liters of deionized water, and the final peptide concentrations were estimated with the Bradford assay. The Bradford assay was used only to obtain approximate peptide concentrations because the dye reagent used in the Bradford assay primarily reacts with arginine residues and less so with histidine, lysine, tyrosine, tryptophan, and phenylalanine. As a result, the assay response will differ depending on the amino acid sequence of the peptides.

The degree of adduct enrichment at the protein- and peptide-levels were assessed using the MT assay (detailed in *section 3.3.6*). One hundred µg of each sample was spiked into a 1 mg HSA matrix. Twenty-five pmol of [$^2H_5$]S-phenylcysteine was added to each sample as an internal standard. All samples were assayed in duplicate.

4.2.3. Results and conclusions

At the protein level, the degree of enrichment was anticipated to be approximately twelve-fold based on the total protein mass before and after enrichment. However, the true enrichment was determined to be 2.5-fold, based on HSA-Cys^{34}-BO quantitation with the MT assay. These results indicate that, if the thiol-affinity reaction was complete, there was ~40% oxidized HSA remaining after the protein reduction step. These results are inconsistent with our previous results (*Section 4.1.1*), which indicated the oxidized fraction was approximately 25% of the total protein after reduction with DTT.

At the peptide-level, the HSA-Cys^{34}-BO response was below the level of detection (LOD). The amount of peptide used in the assay was increased 10-fold to account for a possible under-estimation in peptide quantitation. The response from this sample was still beneath the LOD. These results suggest that either the T3 peptide was lost during the peptide-level enrichment process, or the BO adduct was modified or lost during processing.

4.3. Peptide-Level Enrichment

Experiments were performed at the University of California, Berkeley (UCB) in the School of Public Health and the Department of Chemistry to investigate the loss of the T3 peptides in the fully-enriched samples. In *section 4.3.1*, a ESI-MS/MS method is developed to monitor T3 and T3 modified peptides. In addition, experiments were performed to optimize the

conditions for removing small interfering peptides using size exclusion spin columns, and to determine how effectively the Sepharose 6B resins remove thiol containing peptides.

4.3.1. Acquisition of T3 mass spectra

An ESI-MS/MS method was developed to detect T3 and modified T3 peptides, based on the method of Aldini et al. (Aldini, Regazzoni et al. 2008). Spectra were acquired using a triple quadrupole TSQ Quantum Ultra mass spectrometer equipped with a nanospray ionization source (Thermo Fisher Scientific, Waltham, MA). Peptides were dissolved in 50% methanol/50% water/0.1% formic acid (v/v/v) in 2 μM concentrations. Peptides were introduced into the mass spectrometer by static infusion. The nanospray ion source parameters were as follows: spray voltage 800 V, capillary temperature 260ºC, tube lens offset 207 V. MS/MS mass spectra were recorded in the positive ion mode with collision pressure set at 1.5 mTorr and collision energy set at 31 V. Data acquisition time was 1 minute. The MS/MS data were interpreted by manually comparing the acquired spectra with theoretical m/z values of the corresponding T3 peptide fragment ions generated from Protein Prospector v 5.3.0 (UCSF, http://prospector.ucsf.edu/prospector/mshome.htm).

Product-ion scan mode was used to detect fragment ions that were specific to the T3 peptide. Fragment ions b3, b4, and y7 were identified and used as signature fragments to confirm adducts were T3 in origin. These product ions were chosen based on their relative abundance and frequency of detection. In addition, none of the selected peptide sequence tags contain the Cys^{34} moiety. As such, they are common product ions to all T3 peptides, regardless of T3-Cys^{34} modifications. A spectrum of an ESI-MS/MS product ion scan is provided in Figure 4.7. This MS procedure is a general approach that can be used to detect HSA-Cys^{34} adducts with high analytical sensitivity.

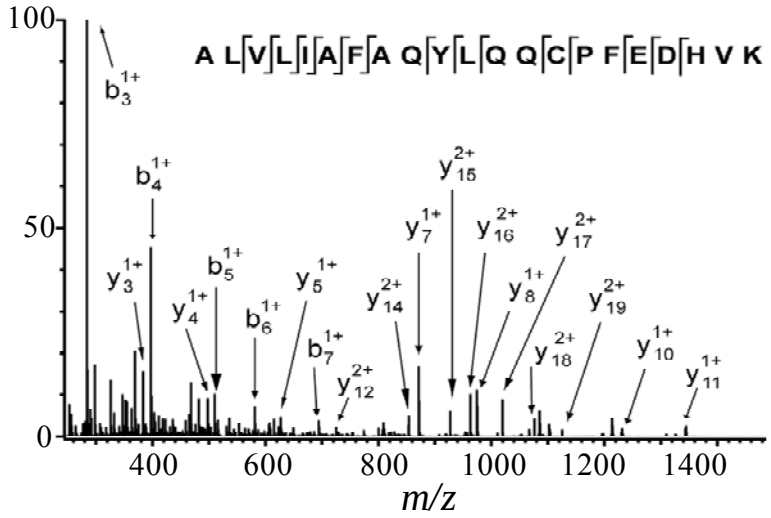

Figure 4.7. MS/MS product-ion scan of the T3 peptide. Fragment ions b3, b4, and y7 were identified as peptide sequence tags.

4.3.2. Evaluation of size exclusion-spin columns for removing small peptides

Experiments were performed to determine the efficiency of removing low molecular weight peptides using Amicon Ultra-4 centrifugal filter devices (Millipore, Billerica, MA). This step replaces the lengthy dialysis procedure used in previous experiments. Theoretically HSA produces 79 peptides when enzymatically digested using trypsin. Cys^{34} resides on the third largest peptide (MW 2433 + modification mass). As a result, spin filters were proposed as a simple means to remove low molecular weight peptides prior to treatment with thiol-affinity resins. This step is important because many of the smaller peptides do not possess Cys residues, and therefore cannot be removed via thiol-affinity covalent chromatography.

Fifteen-ml spin columns were employed with both 2 KDa and 3KDa MWCO capacities. In preliminary studies the 2 KDa MWCO did not remove as much peptide mass as expected. The 3 KDa columns were used to determine if more peptides could be removed, without loss of the

T3 peptide (2433 Da). The spin filters were pre-conditioned with deionized water to remove filter membrane preservatives. After digesting 0.8 mg of HSA with trypsin, according to the conditions detailed in *Section 4.1.2*, the digest was divided and transferred to the spin filters in 400-µl volumes. The volumes were diluted to 15 ml with deionized water, and were centrifuged at 2400 rpm until the retained volumes were reduced to 400 µl. Ten µl aliquots were removed from each sample; the procedure was repeated three times with aliquots removed after each spin.

An additional experiment was performed in parallel using the synthetic T3 peptide to determine if any of the peptide was lost by passage through the size exclusion filters. T3 peptide was dissolved in methanol, and subsequently diluted with deionized water to a final concentration of 30 µg/ml. Two KDa and a 3KDa MWCO spin filters were pre-conditioned with deionized water, and 0.3 mg of T3 peptide was added to each. The filters were centrifuged at 2400 rpm until the retained volumes were approximately 400 µl. Ten-µl aliquots were removed from both the retained and the flow-through fractions for analysis. Relative peptide masses were determined using a nanodrop spectrophotometer measuring absorbance at 220 nm (peptide bond absorbance maximum). Each sample was assayed using 2-µl aliquots in triplicate.

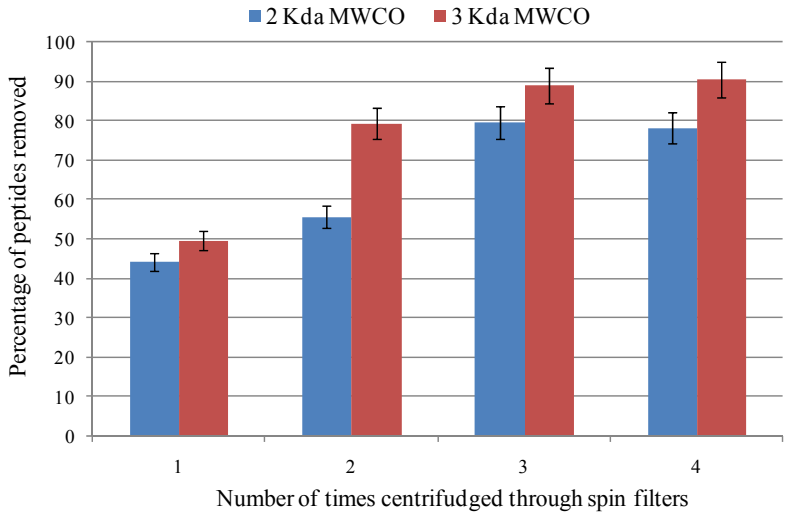

Figure 4.8. Comparison of the percentage of HSA tryptic peptides removed using 2 KDa versus 3 KDa MWCO spin filters.

Results from the 2 KDa versus 3 KDa MWCO spin filter experiment are shown in Figure 4.8. The 3 KDa spin columns removed approximately 10% more mass than the 2 KDa MWCO columns. There was no significant difference between processing the samples three times versus four times. This latter finding suggests that centrifuging the samples three times was adequate to remove lower molecular weight peptides. The 2 KDa MWCO spin filters removed 78 to 80 percent of the peptide mass. Although some of the sample may be loss during processing, this percentage is surprisingly close to the 22% of theoretical mass of tryptic peptides that are above 2 KDa.

When the synthetic T3 peptide (2433 Da) was centrifuged with the 2 KDa MWCO filter using the same conditions described above, the absorbance in the flow-through fraction was below the level of detection. However, when the T3 peptide was processed with the 3 KDa MWCO filters, approximately 6% of the mass passed through the filter membrane into the flow-

through fraction. These results are consistent with the results above, demonstrating that T3 peptide loss occurs when using the higher molecular weight filters. Thus, the optimal procedure involved the use of 2 KDa MWCO spin filters and processing the sample three times.

4.3.3. Evaluation of peptide removal via Sepharose 6B resin

The peptide removal efficiency was evaluated using Sepharose 6B resin. Five mg of commercial HSA was enzymatically digested with trypsin using the previously described protocol (*Section 4.1.2*). The resulting tryptic digest was split, and half of the sample was incubated with the thiol-affinity resin. Prior to enrichment, a 10 KDa MWCO spin filter was used to buffer exchange the peptides into the Sepharose 6B binding buffer. Smaller peptides were not removed using the 2 KDa MWCO filters to better evaluate the capacity of the resin to remove Cys containing peptides. Peptides were loaded onto washed and hydrated Sepharose 6B resin and incubated at room temperature for 1 hour. Note that sulfhydryl containing peptides were found to react significantly faster than the intact protein with the thiol-affinity resins, and therefore required substantially less incubation time. Aliquots of both the unenriched tryptic peptides, and the peptide-level enriched sample, were captured on a C18 ZipTips (Millipore, Billerica, MA), and were eluted with 70% acetonitrile. Mass spectra were acquired using a quadrupole time-of-flight (Q-TOF) mass spectrometer equipped with a Z-spray ion source (Q-Tof Premier, Waters, Milford, MA). Ions were formed by nanoelectrospray ionization (nanoESI) from emitters made from borosilicate capillary tubes (1.0 mm o.d./0.78 mm i.d, Sutter Instruments, Novato, CA). These capillaries were pulled to a tip with an inner diameter of approximately 5-20 μm using a Flaming/Brown micropipette puller (Model P-87, Sutter). The spray was initiated by applying a potential of 0.5 to 1 kV to a platinum wire (0.127 mm diameter, Aldrich, Milwaukee, WI) which was inserted into the nano ESI emitter to within approximately 2 mm of the tip. The flow rates

were approximately 50 to 200 nl/min. No back pressure was used. The instrument parameters were as follows: sample cone voltage 30 V, extraction cone voltage 3.0 V, ion guide voltage 3.0 V, source block temperature 80 ºC, accelerating voltage into the argon-filled cell 4 V, and first pumping stage pressure 1.5 mbar. The distribution of positively charged sodium formate cluster ions formed by positive ion mode electrospray ionization, each consisting of a number of sodium formate units in addition to a sodium cation, was used to provide a wide range of masses of known value for external mass calibration (Zhou and Hamburger 1996). Mass spectra were processed using MassLynx software (version 4.1, Waters). The resulting spectra are provided in Figure 4.9.

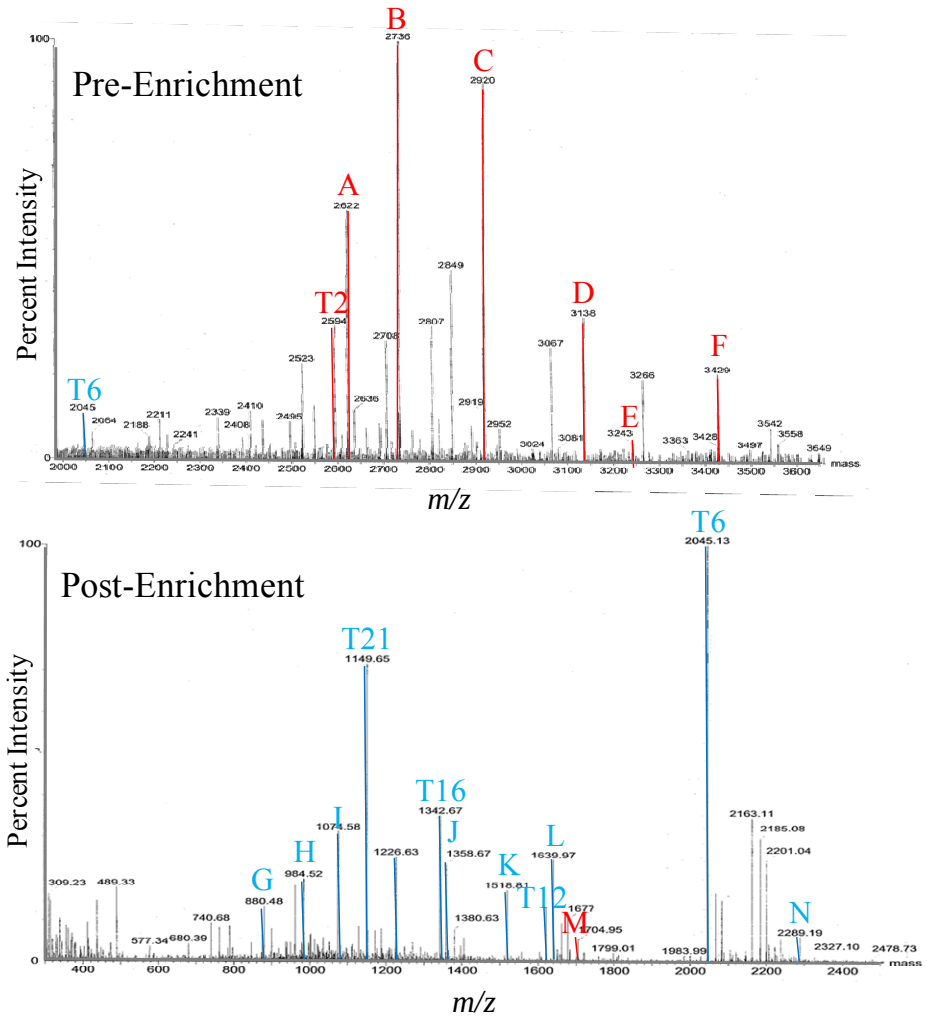

Figure 4.9. QTOF mass spectra of HSA digests, before and after peptide-level enrichment. Peptides identified as tryptic peptides are labeled as T peptides. Letters A through N correspond with tryptic peptide fragments. All peptides containing Cys residues are shown in red, and all peptides that do not contain Cys are shown in blue.

Theoretical maps of tryptic peptides, which included peptides from up to five miscleavages, were generated using ProteinProspector (version 4.27.2 basic, The Regents of the University of California). The ProteinProspector generated peptides included trypsin autolysis products. Tryptic peptides, identified on the basis of theoretical masses, are labeled in the spectra as T2, T6, T16 and T21. Peptides identified as miscleaved fragments are labeled A through N.

All peptides and peptide fragments containing Cys residues are shown in red, whereas peptides without Cys are labeled in blue. Amino acid sequences are provided for each peptide in Table 4.2.

Table 4.2. Masses and amino acid sequences of probable tryptic peptides and tryptic peptide fragments that were identified using ProteinProspector, version 4.27.2 basic.

Peptide ID	m/z	Amino acid sequence
T2	2594	LVRPEVDVMCTAFHDNEETFLK
T6	2045	VFDEFKPLVEEPQNLIK
T16	1343	AVMDDFAAFVEK
T21	1150	LVNEVTEFAK
A	2622	EQLKAVMDDFAAFVEKCCKADDK
B	2736	LVRPEVDVVMCTAFHDNEETFLKK
C	2920	ATKEQLKAVMDDFAAFVEKCCKADDK
D	3138	HPEAKRMPCAEDYLSVVLNQLCVLHEK
E	3243	AVMDDFAAFVEKCCKADDKETCFAEEGKK
F	3429	HPYFAPELLFFAKRYKAAFTECCQAADK
G	880	AEFAEVSK
H	985	TYETTLEK
I	1075	LDELRDEGK
J	1343	AVMDDFAAFVEK
K	1519	LDELRDEGKASSAK
L	1640	KVPQVSTPTLVEVSR
M	1705	RYKAAFTECCQAADK
N	2289	QTALVELVKHKPKATKEQLK

In the pre-enriched peptides two tryptic peptides were identified, T6 and T2. In addition, six miscleaved peptides were identified. All of the identified peptides in the pre-enriched sample contain Cys residues, with the exception of T6. After enrichment, three tryptic peptides were identified in the spectrum; T6, T12, and T21. In addition, eight miscleaved peptides were identified. With exception of one minor peptide (M), none of the identified peptides in the post-

enriched samples contained Cys residues. These results provide conclusive evidence that the thiol-affinity resins are highly effective in removing peptides containing free sulfhydryls, while permitting peptides without Cys to be recovered. It is notable that while T6 is a very minor peak in the pre-enriched sample, it becomes the ion with the greatest intensity in the post-enriched spectrum. Since the T6 peptide is the only non-Cys containing peptide that is above 2 KDa in mass (the mass cutoff for the spin filters), it was anticipated to be the most abundant interfering peptide after treatment with thiol-affinity resins and removal of small peptides.

4.3.4. Non-selective T3 peptide binding to Sepharose 6B resin

Experiments were performed to determine if peptides were lost during incubation with the Sepharose 6B resin due to non-specific binding. Two different synthetic peptides were used in this experiment; the HSA T3 tryptic peptide (MW 2433.3), and a tryptic 14-mer from the β-subunit of Hb (MW 1420.7 Da). Because both peptides contained sulfhydryl gro ups and should specifically bind to the thiol-affinity resin, the peptides were partially modified with iodoacetamide (IAA) to assess non-specific binding. In this experiment, the unmodified peptides should form disulfides with the thiol-affinity resins, whereas the IAA modified proteins should be recovered in the flow-through fraction. Reactions were performed in PBS using 25 mM IAA for 90 minutes at 37ºC. The degree of IAA modification was assessed using the ESI-MS/MS method described in *section 4.3.1*. Based on these results, the modified peptides were combined with unmodified peptides to create a solution with an approximate 70:30 ratio, respectively. The peptide mixtures were then reacted with Sepharose 6B resin using the previously described conditions. Mass spectra were acquired pre- and post-enrichment using peptides collected on ZipTips via direct infusion (ESI-MS/MS conditions detailed in *Section 4.3.1*.).

Spectra of the Hb 14-mer peptide are provided before and after reaction with the thiol-affinity resin in Figure 4.10. In panel A, a full scan spectrum of the 14-mer is displayed pre-enrichment. The triply and doubly charged ions are labeled for both the modified peptide (494.30 Da and 740.58 Da) and the unmodified peptide (474.63 Da and 711.53 Da), respectively. A full-scan spectrum is provided after incubation with the thiol-affinity resin in panel B. The unmodified 14-mer was well resolved in the pre-enriched sample. In contrast, after enrichment the unmodified peptide was not detected. Panels C and D show the modified peptide before and after enrichment in selective ion monitoring mode (SIM). These spectra are provided to illustrate the high recovery of the adducted peptide after reaction with the resin. Total ion current (TIC) plots are shown above the spectra, where the loss in total ion intensity is approximately 25% after enrichment.

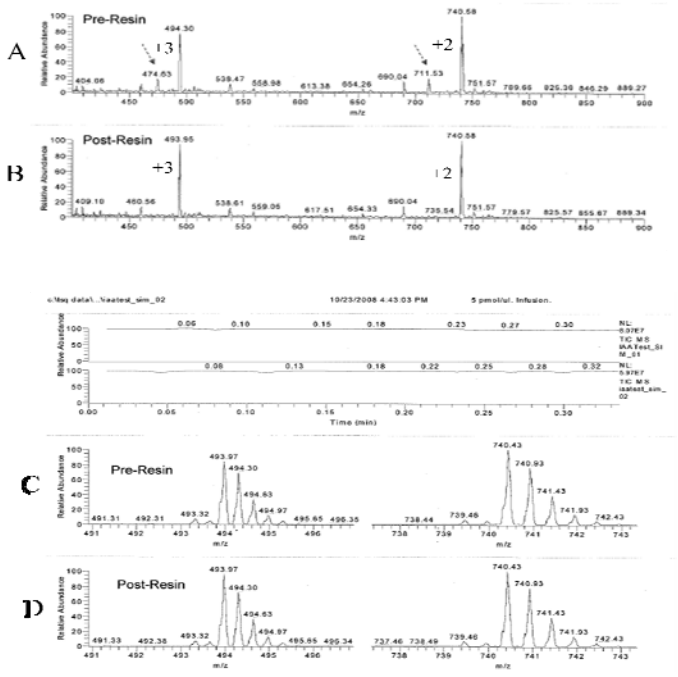

Figure 4.10. Spectra of the hemoglobin 14-mer peptide before and after enrichment with the thiol-affinity resin. Panels A and B provide full-scan spectra of the modified and unmodified peptide, where the unmodified peptide is completely removed in the pot-enriched spectrum. Panels C and D are SIM spectra of the modified peptide that demonstrate the peptide is recovered from the resin with high yield.

Unlike the Hb 14-mer peptide, the modified HSA T3 peptide was almost completely lost during incubation with the Sepharose 6B resin due to non-selective binding, and was not detected in the post-enriched spectrum. To determine whether the adducted peptide could be recovered from the resin, several elution conditions were explored. In each case, the fully-modified IAA-T3 peptide was incubated with Sepharose 6B. Post-reaction, the resin was washed using a series of extraction solutions with various percentages of acetonitrile (ACN). A SIM spectrum of the fully-modified IAA-T3 peptide is shown in Figure 4.11 before reaction with the resin.

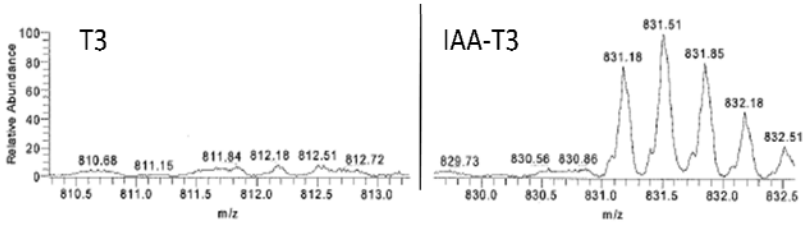

Figure 4.11. SIM spectra of the predominant +3 ions of the unmodified and modified T3 peptides before incubation with Sepharose 6B.

After incubation with the resin, the modified T3 peptide was recovered with high yield using 70% ACN. Because the T3 peptide is an unusually hydrophobic peptide (discussed below), we hypothesize that the T3 peptide was non-selectively lost on the Sepharose 6B resin through van der Waals interactions. Spectra are provided in Figure 4.12 before and after enrichment, using 70% ACN to elute the peptide after incubation. Although recovery of the T3 peptide requires an additional elution step due to the non-specific binding to the resin, this non-selective interaction may provide additional enrichment by further isolating the T3 peptide from less hydrophobic peptides with do not interact with the resin.

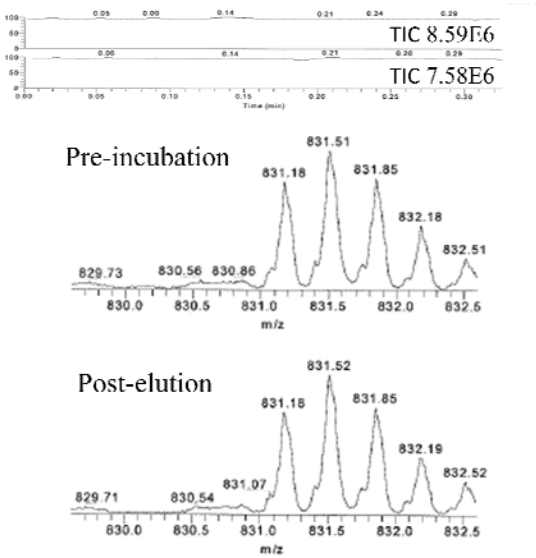

Figure 4.12. SIM spectra of the fully-modified IAA-T3 peptide before and after reaction with Sepharose 6B resin. After incubation with the resin, the peptide was eluted using 70% ACN.

4.3.5. T3 peptide loss through precipitation

In addition to losing the T3 peptide during incubation with the Sepharose 6B resin, substantial loss was encountered during other stages of the enrichment protocol. Precipitation was visibly observed under several conditions, especially preceding the reaction with the Sepharose 6B resin. When tryptic digests were used, as opposed to the synthetic T3 peptide alone, loss of the T3 peptide was confirmed using ESI-MS/MS (method detailed in *Section 4.3.1*.). In many experiments the precipitate appeared as tiny fibers. Once the fibers were observed, the precipitate could not be resolubilzed, even with solvent conditions that dissolved the T3 peptide prior to precipitation. This observation led us to question whether the T3 peptide could be oligermerizing as amyloid-type fibrils. The HSA T3 peptide is unique in that 12 of the 21 amino acid residues are hydrophobic (Figure 4.13.). In addition to having almost 60%

hydrophobic residues, modifications on Cys^{34} can contribute to the hydrophobicity of T3, depending on the physicochemical nature of the adduct. Other peptides with excessive hydrophobic sequences have been observed to form unique aggregates that can result in irreversible precipitation. This phenomenon has been extensively studied in association with the deposition of amyloid fibrils in neurodegenerative diseases, including Alzheimer's disease (Tjernberg, Lilliehook et al. 1997; Thirumalai, Klimov et al. 2003; Britschgi, Olin et al. 2009). Although low resolution structures of such aggregates have been determined, little is known at the molecular level about the oligomerization process (Harper and Lansbury 1997; Rochet and Lansbury 2000). However, the large number of consecutive hydrophobic residues on T3 is consistent with amyloidogenic fibril moieties (Kelly 1998; Rochet and Lansbury 2000; Britschgi, Olin et al. 2009). Because the likelihood of amyloid-type aggregation, and the morphology of the formed oligomers, depends on peptide concentration, temperature, pH, and salt concentration (Thirumalai, Klimov et al. 2003), conditions were explored using the T3 peptide that avoid peptide loss due to precipitation.

^{21}ALVLIAFAQYLQQC^{34}PFEDHVK^{41}

Figure 4.13. Amino acid sequence of the HSA tryptic T3 peptide. Residues in red have hydrophobic R groups. The hydrophobicity of Cys^{34} is dependent on the physicochemical nature of the modification.

Several conditions were found that readily dissolved the T3 peptide. The dried T3 peptide could be solubilized by first dissolving the peptide in methanol. Once the peptide was completely dissolved, water could be added to a final concentration of approximately 60% water. In addition, the T3 peptide was soluble in acetonitrile (ACN). These conditions were compatible

downstream of the enrichment process, when analysis of the peptides was made via direct infusion; However, these conditions were not compatible with the LC-MS conditions, which required an initial lower percentage of organic solvant. To address this limitation, suitable aqueous conditions were explored by adjusting the acidity of the Sepharose 6B buffer. Under highly acidic conditions (pH 3), the synthetic peptide remained in solution. However, regardless of the conditions used to solubilize the T3 peptide (on its own or within HSA tryptic digests), loss of signal was observed overtime that was generally unpredictable.

4.4. Conclusions

In the initial studies outlined in *Section 4.1*, we provide an overview of our novel approach for enriching and detecting protein adducts of HSA-Cys^{34}. Following on these initial experiments, a specific GC/MS approach was used to quantify the degree of enrichment using HSA modified with BO (see *Section 4.2*). These results indicated that adducts were enriched approximately 2.5 fold at the protein-level; however, the T3 peptide adducts were lost during the peptide enrichment phase. To explore this loss of T3 peptide adducts, targeted experiments were performed using IAA modified HSA. A general mass spectrometry approach was developed to track the enrichment process, that is applicable to any stable HSA-Cys^{34} modification. These experiments were detailed in *Section 4.3*.

Based on these results, our method provides a general approach to detect stable modifications on HSA-Cys^{34} with high analytical sensitivity. Although enrichment at both the protein and peptide levels theoretically provides the greatest degree of enrichment, the peptide-level enrichment procedure was inconsistent, often resulting in significant analyte loss. Further

studies are required to fully characterize the stability of the T3 peptide under the peptide-level enrichment conditions.

Additional experiments were performed to optimize the protein-level enrichment procedure. Some of the conditions that were optimized include (1) conditions for reducing HSA, (2) the required amount of Sepharose 4B, and (3) the duration of contact time needed for HSA and the thiol-affinity resin. Lengthy dialysis steps were replaced with spin filter procedures to increase sample throughput, and the entire procedure was scaled down to minimize the amount of sample required for the assay. In the initial enrichment experiments several weeks were required to fully enrich a single sample of 100 mg of HSA. In our final procedure, HSA-Cys^{34} adducts are enriched and analyzed in two days, and require less than 0.5 mg of starting protein. Our final protein adduct enrichment method is presented in *Section 5*.

5. Paper II. Enrichment of Cysteinyl Adducts of Human Serum Albumin as Biomarkers of Exposure [Funk, W., Li, H, Lavarone, A., Williams, E., Riby, J., Rappaport, S., *Biochemical Journal* (Submitted)]

5.1 Abstract

Protein adducts can serve as biomarkers of exposure to a host of xenobiotic toxicants and reactive endogenous molecular species. In this study, we report a novel method to enrich cysteinyl adducts of human serum albumin (HSA), which provides greater analytical sensitivity to detect adducts of low abundance. Because the major site of adduction of HSA is the single free sulfhydryl group located at Cys^{34}, the enrichment approach takes advantage of the propensity of mercaptalbumin (i.e., unadducted HSA) to bind with thiol-affinity resins. Furthermore, because the most common adducts of HSA-Cys^{34} are mixed disulfides formed by reversible reactions between Cys^{34} and serum thiols (notably cysteine), mixed disulfides were liberated via reduction with dithiothreitol (DTT) prior to reacting mercaptalbumin with thiol affinity resins. Reduction with DTT was judged to be more effective than that with *tris(*2-carboxyethyl)phosphine (TCEP) in preliminary experiments. Mass spectrometry employing electrospray ionization was used to detect mercaptalbumin and HSA-Cys^{34} modifications before and after treatment of HSA with thiol-affinity resins. Prior to reduction of mixed thiols, differences in the adduct content were detected across samples of freshly isolated HSA, archived HSA (stored at -80^{o}C for 11 y), and commercial HSA. Cysteinylated and glycosylated adducts were present in all types of HSA with relative abundances decreasing in the order commercial HSA > archived HSA > fresh HSA. After thiol reduction and treatment with thiol-affinity resin

the mass corresponding to mercaptalbumin was no longer observed in mass spectra of HSA samples. The HSA content of pre- and post-enrichment HSA samples was quantified by measuring total proteins with the Bradford assay and gel electrophoresis. After removal of mercaptalbumin, the protein content of post-enrichment samples reflected the quantity of adducted HSA-Cys^{34} in those samples. The ratio of the amount of HSA adducts to the amount of HSA prior to enrichment was 0.029 mg adducts/mg HSA in fresh HSA and was 0.323 mg adducts/mg HSA in archived HSA. The elevated adduct levels in the archived samples could be due in part to differences in specimen preparation and storage, rather than to differences in levels of circulating electrophiles. Interestingly, archived specimens pooled from nonsmoking subjects had marginally greater adduct levels than those pooled from smoking subjects ($p = 0.057$, $n = 14$). These results indicate that thiol-affinity resins provide a straightforward means for removing mercaptalbumin from HSA samples in studies to characterize protein adducts of reactive chemicals in the blood.

5.2 Introduction

It has been suggested that biomarkers of exposure should be used in studies of the effects of toxic chemicals on human health (Wild 2009). Biomonitoring can also provide a more precise estimate of internal dose, which decreases increases the power to detect relationships between adverse health effects and causal agents (Lin, Kupper et al. 2005). However, because environmental toxicants are often chemically reactive electrophiles, their short life spans *in vivo* make them difficult to measure directly in biological media. This has motivated the use of stable adducts of these compounds, and/or their metabolites, with abundant blood proteins, notably hemoglobin and human serum albumin (HSA), as exposure biomarkers (reviewed by Tornqvist

et al., (Tornqvist, Fred et al. 2002); and Rubino et al., (Rubino, Pitton et al. 2009)). Stable adducts accumulate over the mean residence time of a protein and thereby reflect the integral of the blood level of the reactive species over 28 d for human serum albumin (HSA) and 60 d for hemoglobin.

Human serum albumin contains 35 cysteine residues, 34 of which are bound as intramolecular disulfides. Although the remaining cysteine, Cys^{34}, has the only free sulfhydryl group in HSA, it represents the largest fraction of thiols in serum (Fabisiak, Sedlov et al. 2002), where it has been estimated to account for approximately 80% of the antioxidant capacity (Ogasawara, Mukai et al. 2007; Marjolaine, Rondeau et al. 2008). Within the tertiary protein structure of HSA, Cys^{34} resides in a unique microenvironment close to three ionizable residues: Asp^{38}, His^{39}, and Asp^{38} (Stewart, Blindauer et al. 2005). As a result, Cys^{34} has an unusually low pKa (<6.7 compared to about 8.0 - 8.5 for thiols in most other proteins and peptides) and exists primarily in the highly nucleophilic thiolate form (Stewart, Blindauer et al. 2005). Examples of the many chemical species that form adducts with Cys^{34} include oxirane and quinone metabolites of benzene, naphthalene, and pentachlorophenol (Yeowell-O'Connell, Rothman et al. 1998; Waidyanatha, Rothman et al. 2004; Waidyanatha, Sangaiah et al. 2005) (Waidyanatha, Lin et al. 1996; Tsai, Lin et al. 2002), nitrogen mustards (Noort, Hulst et al. 2002), 4-hydroxy-*trans*-2-nonenal, 2-propenal, malondialdehyde, glyoxal, and other products of lipid peroxidation (Aldini, Dalle-Donne et al. 2006; Aldini, Gamberoni et al. 2006; Aldini, Regazzoni et al. 2008), metal ions such as Ag^{+}, Hg^{2+}, and Au^{+}, and a host of drugs including auranofin, D-penicillamine, ethacrynate, and cisplatin (reviewed by (Kragh-Hansen, Chuang et al. 2002)). Yet, although some HSA-Cys^{34} adducts have been detected in studies of humans and animals, the analytical matrix is complex and the levels of measured individual adducts have been very low (less than

one in a million unadducted protein molecules (Rubino, Pitton et al. 2009)). The combination of matrix effects plus low abundance has made it difficult to simultaneously measure numerous HSA adducts in samples of human serum and to identify unknown adducts.

Here we report a novel method to selectively enrich Cys^{34} adducts in fresh or archived samples of HSA. The enrichment approach takes advantage of the propensity of HSA with unmodified Cys^{34} (mercaptalbumin) to bind with thiol-affinity resins (Haugen 1989). Because adducted HSA is comprised primarily of small mixed-disulfides that exhibit reversible binding with HSA-Cys^{34}, disulfide adducts were reduced to mercaptalbumin by dithiothreitol (DTT) prior to treatment with thiol-affinity resins. Using electrospray ionization (ESI) mass spectrometry, we characterized intact HSA from various sources and investigated the effects of reduction of mixed disulfides on the enrichment process. Enrichment was assessed both by mass spectrometry of the intact proteins and also by quantifying the amounts of HSA via the Bradford assay and gel electrophoresis before and after use of thiol-affinity resins.

5.3. Experimental Section

5.3.1. Chemicals

Dithiothreitol (DTT)**,** *tris(*2-carboxyethyl)phosphine (TCEP), iodoacetamide (IAA), phosphate buffered saline, Activated Thiol Sepharose 4B, sequence grade trypsin, sodium chloride, calcium chloride, ammonium sulfate, nitrogen, BioRad reagent dye, sodium dodecyl sulfate, BioSafe Coomassie G-250 dye (Bio-Rad, Hercules, CA), ammonium sulfate, Tris base, ProteasMAX, acetonitrile (Fisher Optima grade, 99.9%) and formic acid (Pierce, 1 ml ampoules, 99+%) were purchased from Fisher Scientific (Pittsburgh, PA). Commercial HSA was from

Sigma (St. Louis, MO). Water was purified to a resistivity of 18.2 MΩ·cm (at 25 °C) using a Milli-Q Gradient ultrapure water purification system (Millipore, Billerica, MA).

5.3.2. Isolation of HSA

Four ml of whole blood were obtained from a healthy Asian volunteer subject (age = 34 y) by venipuncture in heparin. The blood was immediately centrifuged for 15 minutes at 3000 x g. The plasma layer was transferred to a 15-ml centrifuge tube, and saturated ammonium sulfate was added drop-wise to a final concentration of 60% ammonium sulfate. The plasma was vortexed briefly and centrifuged for 30 minutes at 3000 x g to pellet the precipitated protein. The supernatant, containing HSA, was transferred to 10-kDa molecular weight cutoff (MWCO) spin columns (Amicon Ultra-4, Millipore, Billerica, MA) and the protein was desalted using 5 x 15-ml volumes of deionized water. The isolated HSA was immediately aliquoted and frozen at -80°C. Archived HSA from 40 volunteer subjects (pooled and balanced from males/females, smokers/nonsmokers, and black/white subjects) had previously been isolated according to a similar protocol (Lin, McKelvey et al. 2006), which included dialysis to remove small molecules, lyophilization to constant weight, and dissolving the purified HSA in deionized water at 50 mg/ml. Archived samples were stored at -80^{o}C prior to processing in the current study.

5.3.3. *MS characterization of HSA*

The heterogeneity of HSA with respect to Cys^{34}-adduct status was evaluated in freshly isolated and archived HSA (described above), and commercial HSA from Sigma-Aldrich. Protein samples were analyzed using an Agilent 1200 series liquid chromatograph (LC; Santa Clara, CA) that was connected in-line with an LTQ Orbitrap XL hybrid mass spectrometer equipped with an Ion Max electrospray ionization source (ESI; Thermo Fisher Scientific,

Waltham, MA). The LC was equipped with C8 guard (Poroshell 300SB-C8, 5 μm, 12.5 × 2.1-mm, Agilent) and analytical (75 × 0.5-m) columns and a 100-μl sample loop. Solvent A was 0.1% formic acid/99.9% water and solvent B was 0.1% formic acid/99.9% acetonitrile (v/v). For each sample, approximately 50 to 100 pmol of protein analyte was injected onto the column using an Agilent 1200 autosampler. Following sample injection, analyte trapping was performed for 5 min with 99.5% A at a flow rate of 90 μl/min. The elution program consisted of a linear gradient from 25% to 95% B over 34 min, isocratic conditions at 95% B for 5 min, a linear gradient to 0.5% B over 1 min, and then isocratic conditions at 0.5% B for 14 min, at a flow rate of 90 μl/min. The column and sample compartments were maintained at 35 ºC and 10 ºC, respectively. Solvent (Milli-Q water) blanks were run between samples, and the autosampler injection needle was rinsed with Milli-Q water after each sample injection, to avoid cross-contamination between samples. The mass spectrometer ESI source parameters were as follows: ion transfer capillary temperature 275 ºC, normalized sheath gas (nitrogen) flow rate 25%, ESI voltage 2.0 kV, ion transfer capillary voltage 33 V, and tube lens voltage 125 V. Mass spectra were recorded in the positive ion mode over the range m/z = 500 to 2000 using the Orbitrap mass analyzer, in profile format, with full MS automatic gain control target settings of 3 × 104 and 5 × 105 charges for the linear ion trap and the Orbitrap, respectively, and an Orbitrap resolution setting of 6 × 104 (at m/z = 400, FWHM). Raw mass spectra were processed using Xcalibur software (version 4.1, Thermo) and measured charge state distributions were deconvoluted using ProMass software (version 2.5 SR-1, Novatia, Monmouth Junction, NJ), using the default "large protein" parameters and a background subtraction factor of 1.5. Week-to-week reproducibility of the measured masses of intact, adducted HSA proteins was within ± 3 Da.

5.3.4. Reduction of HSA mixed disulfides

The efficiency of reducing small mixed disulfides bound to HSA-Cys^{34} was investigated with both DTT and TCEP. One-mg portions of commercial HSA were treated with one of 11 different concentrations of DTT or TCEP that ranged from equimolar to 270-fold molar excess, with reaction times ranging from 5 to 60 minutes. All experiments were conducted at room temperature in phosphate buffer, pH 7.4. Following protein reduction, samples were reacted with iodoacetamide (IAA), and the degree of reduction was assessed by monitoring the number of IAA additions (+57 Da per modification) above the nominal mass of HSA (66,436±3 Da) (Beck, Ambahera et al. 2004; Kleinova, Belgacem et al. 2005). Reduction status was assessed before and after reduction using a LTQ Orbitrap XL hybrid mass spectrometer under the same MS conditions described above. Reduction conditions were considered optimal when they were stringent enough to reduce the Cys^{34}-mixed disulfides while also preserving the intramolecular disulfide linkages.

For enrichment experiments, 2-mg portions of fresh HSA (n = 6) and 0.5-mg portions of archived HSA (n = 16) were reduced using a 2.7-fold molar excess of DTT to release Cys^{34}-bound mixed disulfides in 1-ml and 0.5-ml volumes, respectively. This concentration of DTT had been found to be optimal in preliminary experiments with commercial HSA (described above).

5.3.5. Using IAA-modified HSA as a positive control

IAA was reacted with HSA to create a Cys^{34} adduct that would serve as a positive control for the enrichment experiments. Commercial HSA was treated with a 2.7 molar excess of DTT and was reacted with 150 mM IAA in phosphate buffer, pH 7.4, at 37°C for 1 h. After removing excess IAA and buffer salts with a 10-kDa MWCO spin column, the fully-modified HSA was

diluted with freshly isolated HSA to produce a starting solution containing 0.047 mg of the IAA-modified HSA per mg of HSA.

5.3.6. Using thiol-affinity resins to remove mercaptalbumin from HSA

A slurry containing 75 mg or 250 mg (dry mass) of Activated Thiol Sepharose 4B hydrated resin in degassed 4B binding buffer (100 mM Tris-HCl, 0.5 M NaCl, pH 7.4) was prepared in a ratio of 75% settled medium to 25% buffer for the archived and fresh HSA samples, respectively. The slurry was transferred to a 1.5-ml polypropylene spin tube containing cellulose acetate membranes with a 0.22-µm pore size (Pierce Spin Cups, Thermo Scientific, Pittsburgh, PA). The reduced HSA starting solution, containing either fresh or archived HSA was added to the resin, mixed by vortexing, capped with nitrogen, and sealed with Parafilm®. After incubating at room temperature for 16 h on a rotary suspension mixer, the unbound proteins were removed from the thiol-affinity medium by centrifuging at 7000 rpm for 5 minutes. The flow-through fraction containing the HSA-Cys^{34} adducts was recovered.

5.3.7. Quantitation of total proteins and HSA

Mass spectrometry employing electrospray ionization was used to detect mercaptalbumin and HSA-Cys^{34} modifications before and after treatment of HSA with thiol-affinity resins. The total amounts of proteins in all pre- and post-enrichment samples were determined in duplicate with the Bradford assay according to the standard microtiter plate protocol from the manufacturer (Bio-Rad, Hercules, CA) using commercial HSA for the standard curve. The purity of the isolated HSA was determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Aliquots containing 10 µg of total protein in 10 µl of deionized water were mixed with 10 µl of 2X Laemmli sample buffer (Bio-Rad, Hercules, CA) containing 10% SDS and 5% BME and boiled for 10 min. The denatured samples and prestained molecular

weight markers (Precision-Plus Protein Standards, Bio-Rad) were loaded on 4 – 20% polyacrylamide gradient gels (NuSep, Lawrenceville, GA) and separated at 100 V for one hour. The gels were stained with Bio-Safe Coomassie G-250 dye (Bio-Rad) and the relative concentrations of the various protein bands were determined by densitometry using an AlphaImager HP (Alpha Innotech, San Leandro, CA**)**. The purity of HSA was expressed as the percentage of the sum of all protein bands.

5.3.8. Estimation of adduct levels in HSA samples

The HSA content of samples before and after removal of mercaptalbumin by thiol-affinity resins was quantified by measuring total proteins with the Bradford assay and gel electrophoresis (described above). After removal of mercaptalbumin, the protein content of post-enrichment samples reflected the quantity of adducted HSA-Cys^{34}. Proportions of HSA adducts were therefore estimated as ratios of mean quantities of HSA after thiol-affinity treatment to those observed before treatment (mg adducts/mg HSA). For freshly-isolated HSA that had been spiked with IAA-modified HSA (positive control), this ratio was adjusted by subtracting the proportion of IAA adducts that had been added (0.047 mg IAA adducts/mg HSA).

5.4. Results and discussion

5.4.1. Heterogeneity of HSA

Human serum albumin is heterogeneous with respect to the thiol redox state, and exists in both the reduced and oxidized forms. The degree of HSA oxidation *in vivo* has been reported to increase with oxidative stress and various disease conditions, suggesting that HSA functions as an important scavenger of reactive systemic oxidants (Kawakami, Kubota et al. 2006; Turell, Carballal et al. 2009). However, after collection of blood specimens, Cys^{34} can be further

oxidized during sample storage and processing. In particular, oxidation of Cys^{34} has been observed to initiate addition reactions with cysteine, and mixed disulfides (Sengupta, Chen et al. 2001; Beck, Ambahera et al. 2004; Kawakami, Kubota et al. 2006; Ogasawara, Namai et al. 2006).

In this study, the degree of HSA-Cys^{34} modification was examined for three different HSA preparations: freshly isolated HSA, archived HSA, and commercial HSA. As shown in Figure 1, ESI mass spectrometry of the intact proteins points to different adduct profiles for these three sources of HSA. The spectrum of freshly isolated HSA (Figure 1A) was dominated by the mass corresponding to mercaptalbumin (66,436 Da), with a few lower abundance masses also observed, including one at 66,556 Da representing HSA+Cys and another at 66,599 Da, corresponding to HSA+Gluc (the glycosylated product). [Since the primary site for nonenzymatic glycosylation is Lys (Shaklai, Garlick et al. 1984; Kisugi, Kouzuma et al. 2007), glycosylation should not preclude modifications of Cys^{34}]. Commercial HSA contained the largest fraction of HSA+Cys, which was twice as abundant as mercaptalbumin (Figure 2B). The ESI mass spectrum of commercial HSA also showed lower abundant masses at 66,473 Da, consistent with NO modification, and at 66,512 Da (modification unknown). In the archived HSA spectrum, mercaptalbumin was the most abundant species (Figure 1C). However, prominent peaks corresponding to cysteinylation and glycosylation were also observed at higher levels than observed in the fresh HSA sample.

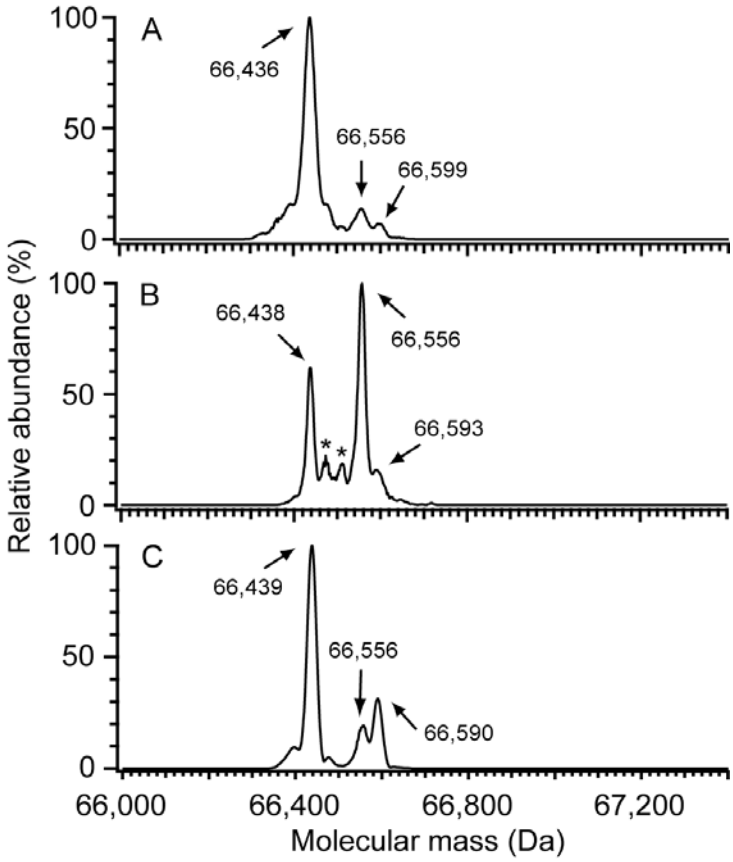

Figure 5.1. Deconvoluted ESI mass spectra showing heterogeneity of HSA from various sources. (A) Freshly isolated HSA; (B) commercial HSA, and (C) HSA stored at -80°C for 11 years. Mercaptalbumin (66,436 Da) is the most abundant species in fresh HSA (A), with minor peaks at 66,556 Da and 66,599 Da, corresponding to cysteinylation and glycosylation, respectively. Commercial HSA (B) was the most highly oxidized of the three HSA preparations. The most abundant modification was from cysteinylated (66,556 Da), with peaks at 66,473 and 66,593 Da consistent with the addition of NO and glucose, respectively. Archived HSA (C) displayed elevated cysteinylation (66,556 Da) and glycosylation (66,590 Da), and also contained an unknown abundant modification at 66,496 (+60) Da. The modification at 66,496 Da appears to be an irreversible modification at Cys^{34}. The asterisks in (B) denote masses of 66,473 Da and 66,512 Da.

5.4.2. Reduction of HSA-Cys^{34} mixed disulfides

The enrichment of HSA adducts of reactive electrophiles can be maximized by reducing mixed thiol disulfides to mercaptalbumin prior to treatment with thiol-affinity resins. We explored the reduction of mixed thiol disulfides using DTT and TCEP at room temperature with

1-mg specimens of commercial HSA. Eleven different concentrations of DTT or TCEP were tested that ranged from a molar equivalent of reducing agent to 270-fold molar excess, with reaction times ranging from 5 to 60 minutes. These results are shown in Figure 2. The reaction time did not have a significant effect in any of the experiments using TCEP or DTT (data not shown)

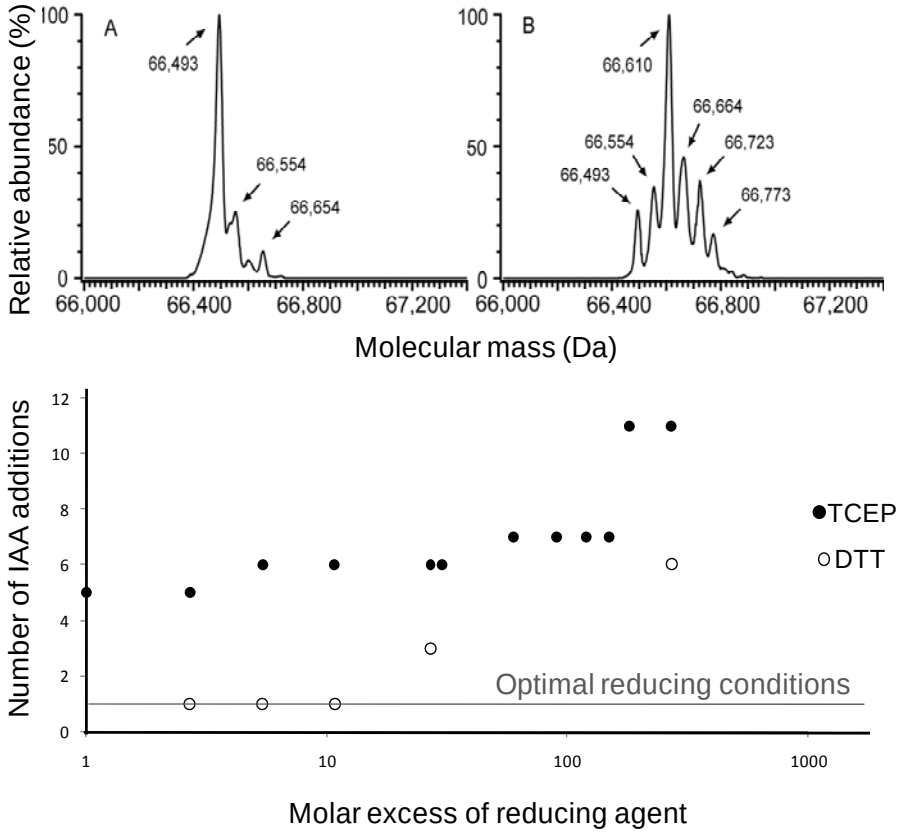

Figure 5.2. Reduction of commercial HSA using TCEP and DTT. In the bottom panel, optimal reduction conditions are labeled corresponding to the addition of one IAA molecule. In the upper panel, deconvoluted ESI mass spectra illustrate (A) optimal conditions using 2.7 molar excess DTT and (B) more stringent reduction conditions using 270 molar excess DTT. In spectrum A, peaks at 66,493 and 66,654 Da correspond to HSA-Cys^{34}-IAA and HSA-Cys^{34}-IAA+Gluc, respectively. The peak at 66,554 Da is thought to represent a pool of irreversible adducts. In spectrum B, the series of ~57 Da mass additions indicate reduction of intermolecular disulfide linkages, with the addition of 2 IAA molecules representing a broken disulfide.

When commercial HSA was treated with TCEP at concentrations ranging from molar equivalence to a 270-fold molar excess, between two and five intramolecular disulfides were cleaved, corresponding to 5 – 11 IAA modifications, respectively. In contrast, no intramolecular disulfides were broken with DTT at concentrations between 2.7- and 10.8-fold molar excess. As illustrated in Figure 2A, incubation of commercial HSA with a 2.7-fold molar excess of DTT effectively reduced Cys^{34}-mixed disulfides, while preserving the intramolecular disulfide linkages. The most intense peak in the deconvoluted ESI mass spectrum was at 66,493 Da, corresponding to HSA with a single IAA modification. A second abundant peak was observed at 66,554 Da. Although this latter modification (a mass addition of 118 Da) is consistent with the addition of a second IAA molecule, this peak was also observed in spectra from HSA that had not been treated with IAA. In addition, for IAA to modify a second cysteine on HSA, an intramolecular disulfide linkage would have to be broken. This acetylation pattern would result in a mass at 66,611 Da (plus two IAA molecules) in addition to the observed peak at 66,554 Da, because a single reduced intramolecular disulfide would result in two free thiols. Thus, the evidence supports the conclusion that Cys^{34} was the only cysteine residue reduced under these conditions. Other masses in this spectrum occurred at 66,654 Da, which is consistent with glycosylated HSA-Cys^{34}-IAA, and a minor peak was observed between 66,554 Da and 66,654 Da, which is consistent with glycosylated mercaptalbumin. The latter suggests that a small fraction of HSA was not modified with IAA.

At a 27-fold molar excess of DTT, commercial HSA was modified with two additional IAA molecules, indicating one broken intramolecular disulfide. As shown in Figure 2B, two additional intramolecular disulfides were reduced when DTT concentrations reached 270-fold

excess. Under these conditions, a series of peaks were observed with mass differences of approximately 57 Da, representing additions of 1 to 6 IAA molecules.

Based on these results, a 2.7 fold molar excess of DTT was selected for reducing mixed disulfides without disrupting the intramolecular disulfide bridges. Reactions were performed at room temperature for 5 minutes. These reduction conditions are consistent with previous studies (Aldini, Vistoli et al. 2008).

5.4.3. Qualitative Assessment of HSA-Cys^{34} Adduct Enrichment

Figure 3 shows the deconvoluted ESI mass spectra of HSA before and after enrichment of the Cys^{34} adducts with thiol-affinity resins. Three prominent peaks were observed in the pre-enrichment spectrum at 66,436, 66,556, and 66,599 Da, corresponding to HSA, HSA+Cys and HSA+Gluc, respectively, consistent with previous findings (Beck, Ambahera et al. 2004; Bar-Or, Bar-Or et al. 2005). After enrichment, three abundant peaks were observed in the spectrum at 66,493, 66,545, and 66,705 Da. The first peak at 66,493 (+57) Da corresponds to HSA-Cys^{34}-IAA (positive control), which was not resolved in the pre-enrichment spectrum, where the starting concentration of HSA-Cys^{34}-IAA was 0.047 mg/mg HSA. The most intense peak in the post-enrichment spectrum (Figure 3B) was at 66,545 Da, which corresponds to a mass addition of 109 Da. In the three-dimensional structure of HSA, the Cys^{34} residue resides in a cleft that is about 10 Å deep (He and Carter, 1992), which can sterically limit access to this residue by large electrophiles. It is therefore reasonable to expect Cys^{34} adducts that are of limited mass, i.e., 109 Da.

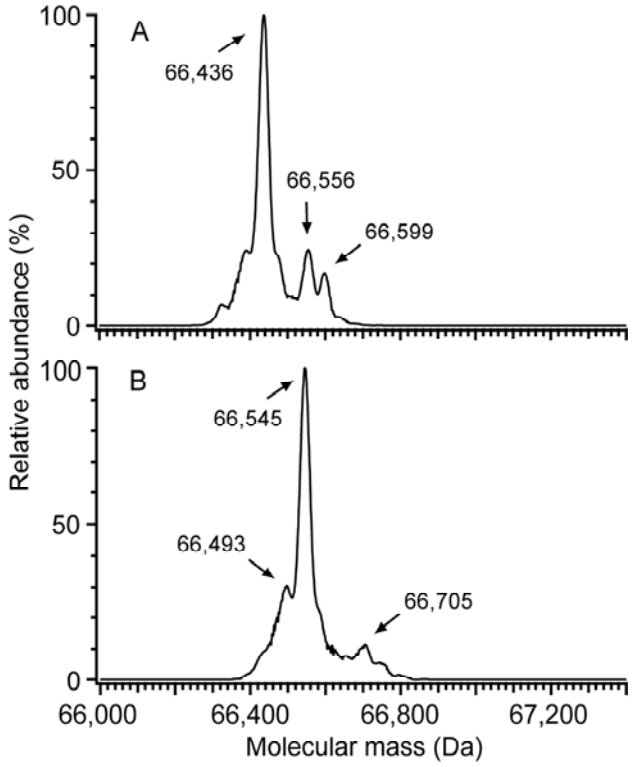

Figure 5.3. Deconvoluted ESI mass spectra of fresh HSA (A) before and (B) after treatment with thiol-affinity resins (0.047 mg/mg HSA-Cys^{34}-IAA was added prior to enrichment as a positive control). In the pre-enrichment sample,(A), peaks were observed at 66,436, 66,556, and 66,599 Da, corresponding to mercaptalbumin, cysteinylated HSA, and glycosylated HSA, respectively. After enrichment, (B), a peak was observed at 66,493 Da for the HSA -Cys^{34}-IAA positive control, which was not detected before enrichment. Peaks at 66,545 and 66,705 Da are thought to represent a pool of Cys^{34} and glycosylated Cys^{34} irreversible adducts, respectively. Note that no peak corresponding to unmodified HSA was observed after treatment with thiol-affinity resins.

A peak at 66,705 (66,545 + 160) Da was also observed in the post-enrichment spectrum. This probably represents the corresponding pool of *N*-linked glycosylated HSA-Cys^{34} adducts. Because all of the ions resolved in the post-enriched spectrum were adducted HSA-Cys^{34} species, it appears that treatment of HSA with thiol affinity resins effectively removed mercaptalbumin.

5.4.4. Quantitative assessment of HSA-Cys^{34} before and after treatment with thiol-affinity resins

The amount of HSA in each sample was calculated using the Bradford assay to measure total protein, and SDS-PAGE to determine the purity of HSA in each sample. SDS-PAGE images are shown pre- and post-enrichment in Figure 4. Before treatment with thiol-affinity resins, the fresh HSA sample contained 70.6% HSA, and the archived HSA had a mean purity of 46.9% (SD 6.8). After treatment, the mean purity was 67.6% (SD 4.0) and 68.4% (SD 9.7) for the fresh and archived HSA, respectively. The archived HSA may have been altered during the isolation process (where dialysis and lyophilization were used) or may have degraded over time (11 y at -80^{o}C), thus resulting in the observed lower HSA purity. Further work will be needed to determine the effects of HSA isolation and storage on levels of HSA-Cys^{34} adducts. After enrichment, the purity of the fresh and archived HSA specimens were similar, suggesting that the thiol-affinity resin removed the lower molecular weight contaminants from the archived HSA.

One fresh HSA specimen (sample 1.5 in Figure 4), and two of the pooled archived samples (one of the duplicate samples of samples 6 and 9 in Figure 4), were excluded from analysis due to low volumetric and protein recoveries after treatment with thiol-affinity resins. The estimated mean adduct level for fresh HSA was 0.076 mg (SD 0.010) after enrichment. When the amount of HSA-Cys^{34}-IAA positive control was subtracted from these values (0.047 mg), the mean adduct level was 0.029 mg. The estimated mean adduct level in the archived specimens was 0.323 mg (SD 0.059), which was much higher than in freshly isolated HSA. Adduct levels in the archived samples were marginally higher in nonsmoking subjects (mean = 0.349 mg/mg HSA) compared to smoking subjects (mean = 0.289 mg/mg HSA) (p = 0.057). Since we expected to observe higher adduct levels in smoking subjects compared to control

subjects, interpretation of the apparent increase in adduct levels among nonsmokers must await information about the identities of the particular adducts involved. No statistical differences were observed in adduct levels between either male and female subjects or black and white subjects ($p > 0.10$).

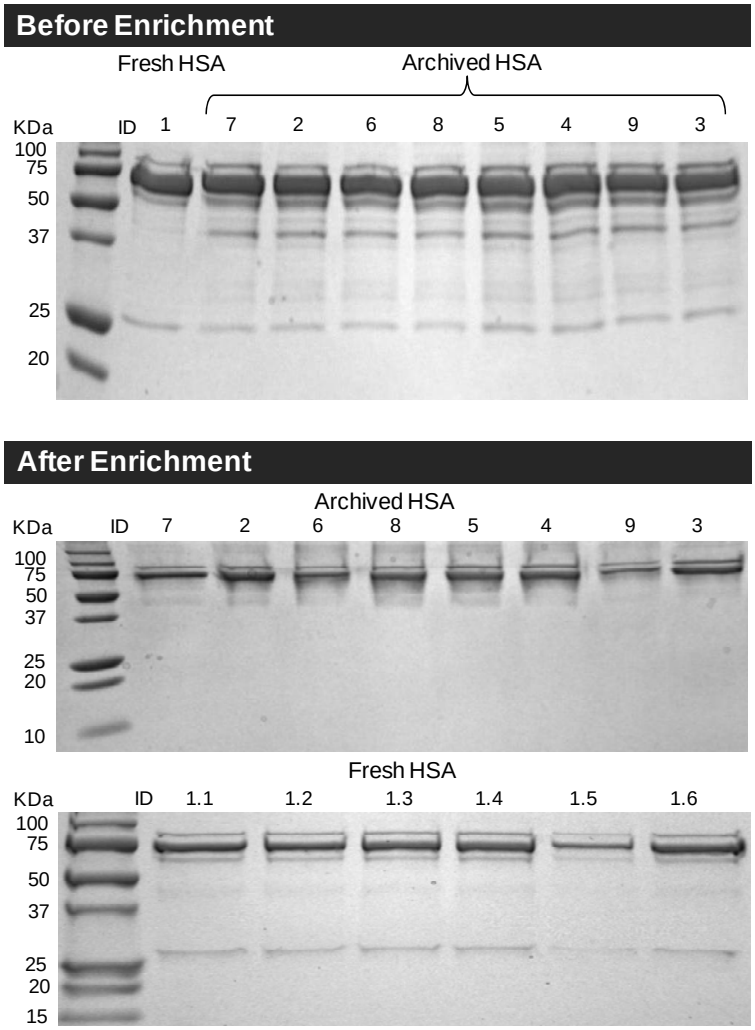

Figure 5.4. SDS-PAGE images of HSA before and after enrichment. Before Enrichment (upper panel): fresh HSA is labeled as ID 1 and pooled archived HSA is labeled with IDs 2 - 9. After Enrichment (lower panel): pooled archived HSA is labeled with IDs 2 - 9 and fresh HSA samples are labeled with IDs 1.1 - 1.6.

5.5. Conclusions

Protein adducts can serve as biomarkers of exposure to a host of xenobiotic toxicants, and reactive endogenous species. However, because the levels of protein adducts are small compared to the unmodified proteins, they are difficult to detect and identify without prior enrichment. In this study, we present a novel method to enrich cysteinyl adducts of HSA by removing mercaptalbumin (i.e., unadducted HSA) with thiol-affinity resins. This approach should allow many adducts of reactive electrophiles to be measured at the HSA-Cys^{34} locus. Since this is the most nucleophilic reaction site in serum, the method should have general applicability for measuring adducts of systemic importance, at least for relatively small adducting species.

Prior to adduct enrichment, HSA was reduced to release Cys^{34}-bound mixed disulfides. Of the many reducing conditions explored with DTT and TCEP, a 2.7-fold molar excess of DTT was determined to be optimal for releasing small thiols, while preserving the intramolecular disulfide linkages in HSA. As seen in Figure 2A, after HSA was reduced with DTT, the bulk of HSA was mercaptalbumin. Prior to reduction, the most abundant modification on HSA-Cys^{34} was cysteinylation. Although cysteinylation was relatively minor in freshly isolated HSA (the 66,556 Da component in Figure 1A), higher levels of cysteinylated HSA was observed in the archived samples (66,556 Da; Figure 1C). As such, it is important to reduce HSA prior to treatment with thiol-affinity resins to achieve maximum enrichment of non-disulfide adducts.

Table 5.1. Amounts of Human Serum Albumin (HSA) Measured after Enrichment in Fresh and Archived Samples. (HSA-Cys^{34} adducts are represented by the amounts of HSA after enrichment by treatment with thiol-affinity resins).

Subject or Pool	Type of HSA	Smoker	Gender	Race	HSA. (mg)	Adducts (mg/mg HSA)[a]
1	Fresh	No	M	A	1.41	0.079 [b]
1	Fresh	No	M	A	1.41	0.065 [b]
1	Fresh	No	M	A	1.41	0.071 [b]
1	Fresh	No	M	A	1.41	0.074 [b]
1	Fresh	No	M	A	1.41	0.091 [b]
2	Archived	No	F	B	0.262	0.357
2	Archived	No	F	B	0.262	0.295
3	Archived	No	F	W	0.212	0.304
3	Archived	No	F	W	0.212	0.278
4	Archived	No	M	W	0.214	0.331
4	Archived	No	M	W	0.214	0.346
5	Archived	No	M	W	0.184	0.460
5	Archived	No	M	W	0.184	0.419
6	Archived	Yes	F	B	0.274	0.296
7	Archived	Yes	F	W	0.264	0.259
7	Archived	Yes	F	W	0.264	0.315
8	Archived	Yes	M	B	0.261	0.305
8	Archived	Yes	M	B	0.261	0.321
9	Archived	Yes	M	W	0.208	0.237

Legend: M=male, F=female, A=Asian, B=black, W=white.
[a] HSA measured after treatment with thiol-affinity resins.
[b] Includes 0.047 mg IAA-HSA/mg HSA (positive control).

Using mass spectrometry, we demonstrated that treatment of HSA with thiol-affinity resins effectively removed mercaptalbumin from the samples (Figure 3). Based upon measurements of total proteins before and after treatment, we observed about 10 times higher levels of non-disulfide adducts in archived specimens of HSA compared to fresh HSA (Table 1). This points to the possibility that HSA-Cys^{34} had been modified during either isolation or storage (11 y at -80^{o}C), or both. Because archived specimens will often be used to characterize adduct levels associated with chemical exposures, it is important to determine the exact nature of modifications to HSA-Cys^{34} that occur during isolation and storage.

6. Discussion and Conclusions

6.1. *Summary of scientific contributions*

Two novel methods were developed in this dissertation research. The first is a method to measure protein adducts in DBS which acts to extend the use of protein adducts as exposure measures to large-scale epidemiological studies. In addition, measurement of protein adducts in DBS opens the possibility to access exposures during fetal growth, a critical stage of human development that is highly sensitive to chemical assault. Fetal exposures can be monitored by utilizing vast archives of residual newborn DBS that exist in the US and around the world.

The second method developed in this thesis is a procedure to enrich protein adducts, which provides greater analytical sensitivity to detect lower abundant adducts. This method is a general approach which enriches cysteinyl adducts of HSA, thus providing a deeper view into the HSA-Cys^{34} adductome. Because chronic exposure to environmental toxicants tend to be low, relative to workplace exposures, this approach aims to extend protein adducts to environmental epidemiology as biomarkers of exposure.

6.2. Suggestions for future research

6.2.1. Enrichment of protein adducts in DBS

This work is presented as two distinct analytical methods, though it is important to note that the two procedures are highly complementary. While we chose to isolate Hb in DBS for adduct analysis, DBS could also be used to obtain HSA. A single DBS contains approximately 1 mg of HSA, which is greater than twice the amount of protein required in our adduct enrichment

procedure. HSA purified from DBS specimens could be enriched for HSA-Cys^{34} adducts, and used to achieve higher analytical sensitivity to detect exposures to chemical toxicants.

To extend the adduct enrichment protocol to DBS, a method must first be developed to recover HSA from DBS that will provide both high protein recovery and purity. Our current DBS protein isolation protocol could be altered to optimize for HSA purity by using higher ethanol concentrations during precipitation. However, it is unlikely adequate protein purity can be achieved using this single step, given the large abundance of Hb in the DBS matrix. In addition, a HSA isolation method would need to be robust with respect to DBS storage. In the current DBS procedure, total protein recovery rapidly declined in specimens that were stored for greater than 6 months. This limitation would limit the use of DBS in studies using archived specimens. This is especially restricting in cases involving newborn DBS, which may have been stored for years under less than ideal conditions.

Once a procedure has been developed to isolate HSA from DBS that circumvents the current protein isolation limitations, DBS repositories can be exploited to examine links between specific prenatal exposures and childhood diseases.

6.2.2. Protein adductiomics: an 'omics' approach to biomarker discovery

Protein adducts can serve as biomarkers of exposure to a wide array of xenobiotic toxicants and reactive endogenous species. However, despite their widespread application in occupational and environmental toxicology, more global analytical approaches have been largely neglected for the discovery of new biomarkers. Systems biology techniques have been utilized in genomics, proteomics, and metabolomics to globally profile genes and gene mutations, protein expression, and metabolites, respectively (MacGregor 2004; Baak and Mohan 2007; Tan, Lambros et al. 2007; Zhang, Wei et al. 2007). However, despite these large initiatives, the

protein adductome has yet to be exploited in this manner. Because the HSA-Cys^{34} adductome is comprised extensively of reactive electrophiles, which are compounds that have been implicated with many adverse health endpoints, its utility as a unique biological entity for 'omics' experiments deserves attention.

Many assays have been developed to measure specific protein modifications as exposure biomarkers. These methods typically focus on the measurement of single adducts, or at best, small numbers of adducts at a given time. Adducts that are monitored in these assays are chosen *a priori*, based on suspected exposures to given toxicants. Because of sensitivity constraints, these assays typically mandate the use of selective ion monitoring. As such, the analytical approaches used tend to be highly specific to the analytes of interest. Although these assays can be highly sensitive, they do not lend themselves well to discovery-type experiments, where the identity of the modification is unknown.

Examples of more general approaches to measure protein adducts are very limited. Noort et al, reported a generic mass spectrometry approach to verify exposure to organophosphates, based on pepsin digestion of butyrylcholinesterase to a common precursor peptide (Noort, Fidder et al. 2006). Michael adducts of reactive carbonyls on HSA-Cys^{34} have also been monitored using neutral loss scans (Beck, Ambahera et al. 2004; Aldini, Gamberoni et al. 2006; Aldini, Regazzoni et al. 2008). A similar approach has been reported by Orioli et al. to measure peptides with histidine residues in the urine of genetically obese rats (Orioli, Aldini et al. 2007). Aldini et al. investigated a precursor ion scan strategy to detect HSA-Cys^{34} modifications on the tryptic/chymotryptic peptide ^{31}LQQCPF^{36} (Aldini, Orioli et al. 2007). Although the study by Aldini et al. is perhaps the best example of how an 'omics' approach could be used to measure protein adducts, the authors did not report levels of any adducts in unmodified HSA. So, despite

these recent efforts measuring protein/peptide modifications using more general strategies, true systems biology approaches for discovering candidate biomarkers are still pending.

In this dissertation a method is presented for enriching cysteinyl adducts of HSA (*Section 5*). In addition, a non-adduct specific MS approach is described for monitoring HSA-Cys^{34} modifications with high analytical sensitivity (*Section 4.3.1*). These methods are suitable for true omics investigations, and can be used in discovery experiments to identify new exposure biomarkers. Adducts can be profiled using a multi-stage mass spectrometry approach. In the first stage, adduct-enriched HSA specimens can be enzymatically digested using trypsin, and mass spectra can be acquired using high resolution ESI-LC-MS/MS (e.g. Orbitrap or FT-ICR MS) in full-scan mode to identify candidate peptides. The peptides can be further characterized in product-ion scan mode, using fragment ions b3, b4, and y7 as signature fragments (described in *Section 4.3.1*) to confirm adducts reside on the T3 peptide. Accurate masses can be then generated to determine the elemental composition of the unknown modifications, and adducts can be proposed based on probable molecular structures. Identities of the adducts can be confirmed using synthesized T3 adducted peptides; matching retention times, masses, and MS fragmentation patterns. This global approach profiling the Cys^{34} adductome has significant potential in generating novel biomarkers of exposure, and identifying causal agents associated with human diseases.

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